Chapter 4

Tumor eradication by p53-specific CD8+ T cells is accompanied by destruction the hematopoietic compartment
Chapter 4.1

Generation of a p53 TCR-tg mouse model

Marjolein M. Lauwen, Suzanne van Duikeren, Sandra A. Bres, Linda de Quartel, Cornelis J.M. Melief, Sjoerd H. van der Burg and Rienk Offringa

Introduction to chapter 4.2
Introduction

Mutation of the tumor suppressor p53 occurs in a majority of human cancers and is an important step in cancer initiation. Frequently these mutations result in an accumulation of p53, whereas normal somatic cells express only low steady state levels of p53. Antibody responses and T-helper cell responses against p53 can often be found in cancer patients (1-3) indicating the immunogenic potential of mutated p53 presented on cancer cells. Several reports have described the existence of p53 specific cytotoxic T lymphocyte (CTL) in healthy donors (4-9). 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 (10, 11). Important insights on the availability of the CD8+ T-cell repertoire in cancer patients have been obtained by using HLA-A2 transgenic mice. The endogenous p53 specific CTL repertoire in p53 +/- HLA-A2-tg mice is blunted as a result of negative selection of self-reactive T cells (12). Consequently the CTL repertoire in p53 +/- HLA-A2-tg mice only recognizes several subdominant epitopes and with lower avidity than CTL found in p53 +/- HLA-A2-tg mice suggesting that the p53 CTL repertoire in patients is largely absent and should therefore be reconstituted. Current efforts focus on improving the function of infused p53 TCR transduced T cells (13-16). The original TCR that is used in these studies was originally obtained from a p53 +/- mouse expressing the human class I HLA-A2 (12). This approach allowed the isolation of high avidity CD8 co-receptor independent CTL recognizing human p53 by circumventing major self-tolerance effects. Ten years ago we published that a wild-type (wt) p53 specific CTL clone (1H11) obtained from a p53 +/- mouse exerted marked anti-tumor activity in the absence of demonstrable toxicity. This clone can be easily expanded in vitro by repeated antigen stimulation to achieve high numbers of p53 CTL for adoptive T-cell therapy in tumor bearing mice. However for further translational studies on the feasibility of immunotherapy by p53 CTL we also wished to have a naive p53 CTL population. Therefore, we generated a p53 specific TCR transgenic mouse, by introducing the TCR from the 1H11 p53 CTL clone (17) into a p53 +/- B6 background. In this way we created p53 TCR-tg mice either with or without self-tolerance for p53. In this chapter we describe the T-cell characteristics in this p53 TCR transgenic mouse (p53 TCR-tg).

Materials and methods

p53 specific CTL clone. The p53 specific CTL clone (1H11) was isolated from p53/- C57BL/6 (B6) mice as described previously (17). The TCR specifically recognizes the 9-mer peptide AIYKKSQHM (p53158-166) in the context of H-2Kb.

Generation of p53 TCR transgenic mice. Rearranged TCR V(D)J α and V(D)J β regions from CTL clone 1H11 were separately amplified by RT-PCR. TCR usage was determined by PCR with V gene subfamily-specific primers (previously described in 18). The identity of the amplified TCR Vα10 and Vβ6 chains was confirmed by sequence analysis of cloned anchor products. Subsequently these TCR chains were cloned into the CD2 mini gene cassette (19). TCRα chain and TCRβ chain constructs were co-microinjected into B6 oocytes. TCR transgenic mice were genotyped by PCR for the presence of the CD2 cassette (forward primer GGTGTGGACTCCACCAGTCTCTCCTCGAGCTCACGAGTACTTC) in combination with the
TCRα (reverse primer GGAAAAGCTACACTGGAGCTCTGCGTTCTC) and TCRβ (reverse primer GATGCCACCATCACCATGTGTGGTCTCTAC) chain (Invitrogen). Founder mice carrying both TCR constructs were backcrossed into p53+/+ B6 and p53 -/- B6 background. p53 genotyping was described previously (20). Expression of transgenic TCR was confirmed by flow cytometric analysis of TCR Vβ6 expression.

Animals. p53+/+ B6 (wild-type), p53-/- and p53 TCR transgenic mice (all C57BL/6 Kh H-2b background) were bred in our own facilities (Leiden, Netherlands). All experiments were performed in accordance with experimental guidelines and were approved in advance by an animal ethical committee. Single cell suspensions were made of thymus, spleen and blood. Splenocytes and blood were depleted of erythrocytes.

FACS analysis. Direct fluorescent labeling of tissues was performed according to standard procedures (BD Pharmingen). Intracellular IFN-γ production was analyzed after 18 hour stimulation with p53158-166 peptide and Brefeldin A, and further performed according to manufacturer’s protocols (cytometer, BD Pharmingen). Antibodies used; FITC-labeled CD4 (RM4-5), PE-labeled Vβ6 (RR4-7) PerCP-conjugated CD8α (53-6.7) and APC-conjugated IFN-γ (XMG1.2) (all antibodies purchased from BD Pharmingen). Results were analyzed by using standard Cell Quest software (BD).
Results and discussion

We generated wt p53^{158-166} T-cell receptor transgenic mice (TCR-tg) and crossed it on a p53 deficient background (p53 /-) to generate p53 +/- TCR-tg, p53 /- TCR-tg and p53 /- TCR-tg mice (Figure 1). Transgenic mice expressing p53 (p53 +/- and p53 /-) show a significant population of CD4-CD8- double negative (DN) cells in the thymus when compared to p53 +/- B6 (wt) and p53 /- TCR-tg mice (Figure 3A, upper panel). As a result the frequency of CD8+ CD4+ double positive (DP) cells in p53 +/- TCR-tg, p53 /- TCR-tg is decreased in comparison to p53 +/- B6 and p53 /- TCR-tg mice. This disturbed thymocyte differentiation is thought to be the result of negative selection due to the early expression of the TCR already in the DN stage (21, 22). This is a classical feature seen in other TCR transgenic models, such as the HY TCR-tg model, in which central tolerance negatively selects self-reactive T cells in the thymus (21). The massive apoptosis resulting from negative selection in p53 +/- TCR-tg and p53 /- TCR-tg mice leads to a drastic reduction in thymic cell numbers (Figure 2). p53 /- TCR-tg mice show a normal CD4+CD8+ thymocyte differentiation with a clearly identified DP population and a small DN population (Figure 3A). Moreover, thymic
cell numbers in p53 -/- TCR-tg mice are comparable to non-transgenic age matched control mice (Figure 2). Thymocyte development in non-transgenic p53 -/- control mice results in a normal distribution of CD4-CD8- DN, CD4+CD8+ DP and single positive (SP) cells (data not shown).

Negative selection of T cells in the thymus of p53 +/+ TCR-tg and p53 +/- TCR-tg mice results in the absence of CD8high cells which is present in the p53 -/- TCR-tg littermates and p53 +/+ B6 control mice (Figure 3A, indicated with 1). However in p53 +/- TCR-tg and p53 +/+ TCR-tg mice a clear CD8low population appears in the periphery, that is absent in all control mice (Figure 3A, indicated with 2). Similarly a CD8low population appears in male HY transgenic mice, but not in female HY TCR-tg mice (21). The frequency of TCR expressing cells is comparable in all littermates, in p53 +/- TCR-tg, p53 +/- TCR-tg and p53 -/- TCR-tg mice (Figure 3B-D). The CD8low T cells have a similar frequency of transgenic TCR expression level as CD8high cells in p53 +/- TCR-tg littermates (Figure 3B-D). Furthermore, the level of TCR expression of all T-cell subsets is comparable in all littermates.

The effect of central tolerance in our transgenic mouse model is such that the CD8+ cells in the periphery of p53 +/- TCR-tg or p53 +/- TCR-tg mice do not respond to peptide pulsed target cells or p53 positive tumor cell in vitro (Figure 4) in contrast to CD8+ T cells from p53 -/- TCR-tg mice. In vitro stimulation of p53 TCR-tg cells shows that only cells from p53 -/- TCR-tg mice respond effectively by IFN-γ production. Recognition by p53 -/- TCR-tg cells of target cells depends CD8 co-receptor expression since CD4+ T cells also express the transgenic TCR (Figure 3) but do not react to target cells.
References

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p53 specific T-cell therapy of hematologic tumors
Chapter 4.2

Tumor eradication by p53-specific CD8+ T cells is accompanied by destruction of the hematopoietic compartment

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Submitted
Abstract

The p53 oncoprotein has been proposed as an attractive target for adoptive T-cell therapy of cancer. However, feasibility and safety of this approach have not been addressed in a clinically relevant model. We show that infusion of mice with high affinity p53-specific CD8+ T cells can result in a potent CTL response, which causes rapid death due to the destruction of the hematopoietic system. Importantly, no signs of immune pathology are observed in non-hematopoietic tissues. Accordingly, combination of T cell infusion with the transfer of stem cells that do not present the p53 CTL epitope allows for efficient tumor eradication without toxicity. Our data demonstrate the importance of testing immunotherapies with auto-reactive TCRs in pre-clinical models, and suggest that tumor targeting through p53-specific CTLs may be a valuable alternative to current allogeneic transplant regimens, provided that the selectivity of these CTLs for the hematopoietic compartment is conserved between mouse and man.
Introduction

T-cell mediated targeting of the majority of cancers relies on recognition of tumor-associated auto-antigens (1). Expression of these antigens in normal somatic tissues, including the thymus (2, 3), may incapacitate the best part of the T-cell immune repertoire, leaving us with the low affinity T-cell subset that escaped deletion. It is conceivable, and in fact demonstrated in a pre-clinical mouse model (4), that such low affinity T cells are compromised in their capacity to eliminate tumors, in spite of their presence in high numbers. Because the endogenous T-cell repertoire against tumor associated auto-antigens may lack the potential, even when optimally manipulated, for therapeutic efficacy against cancer, efforts have been geared towards replenishment of this repertoire with genetically engineered T cells expressing high affinity T-cell receptors (TCRs) against the antigens of choice. A recent study in melanoma patients has demonstrated the feasibility of this approach for reconstructing the T-cell repertoire through transduction of autologous lymphocytes with the genes encoding a TCR that targets the melanoma/melanocyte antigen MART-1 (5).

The ultimate aim of TCR gene therapy is to generate a powerful T-cell response against one or more tumor-associated T-cell epitopes that leaves the tumor with very little chance for escape. This implies that the engineered T-cell response should be capable of eliminating any cell expressing the target antigens concerned, including normal tissues. In the case of the lineage-specific antigens expressed by melanoma and prostate cancer, collateral damage to the corresponding normal tissues may be an acceptable price for therapy. However, if the target antigens of choice are also expressed by vital normal tissues, therapeutic anti-cancer efficacy may be associated with life-threatening auto-immune pathology. In the latter case, large differences in expression level between cancer and normal tissues could offer a window of opportunity for selective cancer targeting. The tumor suppressor protein p53 is an antigen for which such differences are found. Due to mutations that are causally related to oncogenesis, p53 is over-expressed in approximately 50% of human cancers. In contrast, the steady state expression levels of wild-type p53 in normal tissues are very low. Peptides spanning the mutated p53 sequences would constitute true tumor-specific target antigens. However, the p53 mutations observed in human cancers are highly diverse, limiting their use as targets to patient-tailored approaches. The use of p53 as a general tumor antigen therefore relies on the targeting of peptides derived from the wild-type sequence, and on the difference in their presentation by malignant versus normal tissues (6).

The potential of tumor targeting through p53-specific CD8+ cytotoxic T lymphocytes (CTLs) has been demonstrated in two different model systems. Theobald and coworkers have isolated high affinity CTLs specific for a wild-type human p53-derived peptide in the context of HLA-A2, which were generated in HLA-A2-transgenice mice (7). A series of studies have shown that retroviral transduction of the TCR from these CTLs into human lymphocytes yields T cells capable of targeting HLA-A2.1-positive, p53-overexpressing tumors (8-12), providing an incentive for clinical studies with this TCR. In addition, we have previously described a murine model involving high affinity CTLs specific for a wild-type murine p53 peptide in the context of H-2Kb. We demonstrated that infusion of these CTLs into tumor-bearing p53+/+ mice resulted in eradication of tumors in the absence of detectable autoimmune pathology in normal somatic tissues (13, 14), supporting the concept of selective tumor targeting through p53. Notably, both CTLs were isolated from mice that lacked the target antigen concerned. The human p53/HLA-A2.1 specific CTLs were directed against a sequence that is not conserved between mouse and human p53, whereas the murine p53/H-2Kb-specific
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CTLs were isolated from p53-/- mice (7, 13). The failure, in both systems, to isolate high affinity CTLs against ‘self’ p53 epitopes suggested that the expression of low levels of wild-type p53 was sufficient to cause deletion of their precursors in the thymus (6). Nevertheless, wild-type p53 expression apparently did not sensitize normal somatic cells for recognition by high affinity p53-specific CTLs (7-13). In view of this paradox, we investigated the anti-tumor efficacy and toxicity of p53-specific TCR-transgenic and TCR-modified T cells in a clinically relevant mouse model. The results of our study point at the complications associated with adoptive transfer of p53-specific CTLs, but also identify a window of opportunity for tumor targeting through p53.

Materials and methods

Generation of p53 TCR transgenic mice. The p53 specific CTL clone (1H11) was isolated from p53-/- C57BL/6 (B6) mice as described previously (13). The TCR specifically recognizes the 9-mer peptide A1YKKSQHM (p53\(^{158-166}\)) in the context of H-2Kb. Rearranged TCR V(D)\(\alpha\) and V(D)\(\beta\) regions from CTL clone 1H11 were separately amplified by anchored RT-PCR, subsequent to identification of the TCR chains by PCR with V gene subfamily-specific primers (15). The identity of the cloned TCR V\(\alpha\)10 and V\(\beta\)6 chains was confirmed by sequence analysis. These TCR chains were cloned into the CD2 mini gene cassette (16). TCR\(\alpha\) chain and TCR\(\beta\) chain constructs were co-microinjected into B6 oocytes. TCR transgenic mice were genotyped by PCR for the presence of the CD2 cassette (forward primer GGTGTGGACTCCACCAGTCTCACTTC) in combination with the TCR\(\alpha\) (reverse primer GGAAAAGCTACACTGGAGCTCTGCGTTCTC) and TCR\(\beta\) (reverse primer GATGCCACCATCACCATGTGTGTCCTCTAC) chain (Invitrogen). Founder mice carrying both TCR constructs were backcrossed into p53+/+ and p53 -/- B6 background. P53 genotyping was described previously (17). Expression of transgenic TCR was confirmed by flow cytometric analysis for V\(\beta\)6.

Animals, cell preparation and analyses. Wild-type (p53+/+), CD90.1+ congenic, p53-/- and p53 TCR transgenic mice (all C57BL/6 Kh H-2b background) were bred in our own facilities (Leiden, Netherlands). Congenic CD45.1+ mice were purchased from Taconic Farms. Bm1 mice, expressing the H2-K\(^{bm1}\) molecule, were purchased from The Jackson Laboratories. All mouse experiments were approved in advance by the Leiden University ethical committee. Single cell suspensions were made of thymus, spleen and blood by mechanical disruption. Splenocytes and blood were depleted of erythrocytes. For analysis of bone marrow, right and left femur and tibia were flushed with PBS using a syringe. Direct fluorescent labeling of tissues was performed according to standard procedures (BD Pharmingen). For quantification of cell numbers standard amounts of unlabeled beads (BD Calibrite, BD Pharmingen) were added to the sample. Intracellular IFN-\(\gamma\) production was analyzed after 18 hour stimulation with p53\(^{158-166}\) peptide and Brefeldin A, and further performed according to manufacturer’s protocols (cytofix/cytoperm, BD Pharmingen). Carboxy Fluoroscein Succinimidyl Ester (CFSE) was purchased from Molecular Probes. Antibodies used; FITC-labeled V\(\beta\)3 (KJ25)+V\(\beta\)8 (MR5-2)+V\(\beta\)11 (RR3-15)+V\(\beta\)13 (MR12-3)+V\(\beta\)14 (14-2) (together V\(\beta\) mix), CD3e (145- 2C11), CD4 (RM4-5), PE-labeled CD8b2 (53-5.8), V\(\beta\)6 (RR4-7), CD117 (c-Kit, 2B8), CD4 (RM4-5), PerCP-conjugated CD8\(\alpha\) (53-6.7), CD90.1 (HIS51), CD45.1 (A20), APC-conjugated CD4 (RM4-5), IFN-\(\gamma\) (XMG1.2), CD90.2 (53-2.1), CD90.1 (HIS51), CD45.1 (A20), B220 (CD45R, RA3-6B2) (all antibodies purchased from BD Pharmingen). Results were analyzed with standard Cell Quest software (Becton Dickinson). For determination of colony forming units of granulocyte-
macrophage progenitors (CFU-GM) analysis, femur and tibia were flushed with PBS using a syringe and $5 \times 10^6$ bone marrow cells were cultured as described previously (18). Clusters (colonies) of 20 or more cells were scored using an inverse light microscope.

**Adoptive T-cell transfer, vaccination and stem cell transfer.** CD8+ T lymphocytes were isolated from p53 TCR-tg spleens by immunomagnetic labeling (BD Pharmingen). Mice received $10^9$ p53 TCR-tg splenocytes cells (containing approximately $0.5 \times 10^6$ CD8+ cells; (Figures 1 and 2) or $1 \times 10^6$ CD8+ enriched p53 TCR-tg cells (Figures 4 and 5) i.v. together with a s.c. vaccination of 100 μg 30-mer p53\(^{142-171}\) peptide (bearing the p53\(^{158-166}\) epitope, manufactured in our own facilities as described previously, 19), 50 μg CpG (Dimitri Filippov, Department of Chemistry, Leiden University) and 50 μg α-CD40 antibody (FGK-45). In the case of stem cell transfer, CD90.1+ p53+/+ recipient mice received 9.5 GY total body irradiation. One day later, $5 \times 10^6$ CD3+-depleted (Miltenyi Biotech) bone marrow cells from p53+/+ B6, p53 -/- B6 or p53 +/+ Bm1 donor mice were infused.

**Adoptive transfer of p53 TCR retrovirally transduced cells.** Optimized p53 TCR (13) and OVA-specific OT-1 TCR (20) were produced by GeneArt (GeneArt GmbH) and cloned into the retroviral vector pMX to create pMX-p53-Iαopt-IRES-p53-Iβopt (p53 TCR) and pMX-OT-Iαopt-IRES-OT-Iβopt (OVA TCR). Retroviral supernatants were obtained by transfection of Phoenix-E packaging cells with the indicated retroviral vectors using the FuGene 6 transfection reagent (Roche). Donor splenocytes (p53 +/+ and p53 -/-, both CD45.2+) were separated over a ficoll gradient to remove dead cells, after which they were co-cultured with the retroviral supernatant as described previously (21). CD45.1+ recipient mice received lymphodepleting treatment prior to donor lymphocyte infusion, consisting of 2 mg cyclophosphamide (Baxter) i.p. at days –5 and –4, and 2 mg of fludarabine (Schering Plough) i.p. at days –3, –2 and –1. At day 0, these mice were infused with $2 \times 10^5$ transduced splenocytes and vaccinated with 100 μg peptide, 20 μg CpG and 50 μg α-CD40. Infusion of p53 TCR transduced cells or mock transduced cells were combined with a vaccination containing 100 μg p53\(^{142-171}\) peptide. Infusion of OT-1 TCR transduced cells was combined with a vaccination containing 100 μg ovalbumin\(^{241-270}\) peptide.

**Tumor protection experiments.** Prior to adoptive transfer p53+/+ CD90.1+ mice received lymphodepleting treatment as described above. CD8+ purified p53/- TCR-tg T cells were infused on day 0 together with a s.c. vaccination and/or a s.c. injection of 200.000 EL-4 lymphoma cells (14) in the contra-lateral flank. At day 2 mice received p53+/+ B6 or p53 -/- B6 bone marrow cells prepared as described above. Mice were examined for body weight and tumor size every 3 days. Mice were sacrificed by cervical dislocation when tumor size reached a maximal volume of 100 mm\(^2\)

**ELISA assays.** Bone marrow cultures (see above) of p53+/+ B6 or p53+/+ Bm1 mice were seeded at a concentration of $5 \times 10^4$ cells/well in a 96-well U-bottom plate (Costar) and pulsed for one hour with increasing amounts of p53\(^{158-166}\) peptide up to 6 μg/ml. Subsequently cells were thoroughly washed and co-cultured with $5 \times 10^4$ p53 -/- TCR-tg splenocytes. After 18 hours at 37°C, supernatant was harvested and IFN-γ production was measured by sandwich ELISA as described previously (22).
In vivo cytotoxicity assay. Mice (CD45.1+) were infused with naïve TCR-tg cells isolated from CD45.2+, p53-/- donor mice and received a vaccination. A control group did not receive a vaccination. Three days after vaccination, a 1:1 mixture of CFSE labeled p53+/+ CD45.2 + (CFSE high) and p53-/- CD45.2 + (CFSE low) splenocytes was injected as described previously (23). On day 7 spleens were harvested and the presence of CSFE-labeled target cells was determined by flow cytometry.

Statistical analysis. Statistical significance of bar graphs was determined by using Student’s T-test. Significance of survival curves was determined using a Log Rank test. All analyses were performed using GraphPad software.

Immunohistochemistry. Tissue cryosections (4 µM) were fixed for 10 minutes in ethanol-acetone and were subsequently incubated with biotin-conjugated anti-B220 antibody (RA3 6B2, BD Pharmingen), secondary horseradish peroxidase (HRP) antibody (StreptABC complex, DAKO) followed by incubation with diaminobenzidine and finally stained with hematoxylin. Colon and liver sections were directly stained with hematoxylin.

Results
Validation of pre-clinical model for p53-specific CD8+ T cell transfer
To perform an in depth analysis of the potential and pitfalls of p53-targeted cancer immunotherapy, we generated transgenic mice expressing the TCR alpha and beta chains of a CD8+ CTL clone that was previously shown to eliminate tumors through recognition of a murine wild-type p53 peptide (p53¹⁵⁸⁻¹⁶⁶) (13). In view of the fact that this CTL clone was isolated from p53-/- mice, and previously published data suggested that the p53-specific CTL repertoire may be blunted by negative selection in the thymus (7), we crossed these TCR-tg mice into a p53-/- background. Evaluation of the repertoire of thymic T-cell precursors on basis of CD4 and CD8 markers showed impaired thymocyte differentiation in the TCR-tg mice with p53+/+ and p53+/- genotypes, as revealed by a predominant population of double-negative thymocytes (24, 25) (Supplemental Figure 1), and decreased cellularity (Supplemental Figure 2), pointing at thymic deletion of the T-cell progenitors expressing the transgene-encoded p53-specific TCR. A prominent CD8hi, single-positive population was only detected in the spleen of p53-/- mice (Supplemental Figure 1). These CD8+ T cells express high, transgene-encoded Vβ6-levels (data not shown) and are capable of secreting IFN-γ when cultured in vitro in the presence of synthetic p53¹⁵⁸⁻¹⁶⁶ peptide or tumor cells (Supplemental Figure 2). Our data provide the first direct evidence that, as previously suspected, the p53-specific CD8+ T-cell response is blunted by self-tolerance through thymic deletion. Furthermore, our results validate this p53¹⁵⁸⁻¹⁶⁶-specific TCR as a pre-clinical model for testing the feasibility and safety of tumor targeting through replenishment of the p53-specific T-cell repertoire.

Potent p53-specific CTL immunity causes hematopoietic ablation.
To test whether reconstitution of the p53-specific CD8+ T-cell repertoire could result in efficient CTL immunity against p53, we adoptively transferred splenocytes from p53-/- TCR-tg donors to p53+/+ non-transgenic recipients. This resulted in expansion and activation of the p53-specific CD8+ T cells, provided that the recipient mice also received p53-specific vaccination (Figure 1; Supplemental Figure 3). In contrast, infusion of splenocytes from
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p53+/+ TCR-tg or p53+/- TCR-tg donors did not result in detectable p53-specific CD8+ T-cell responses (Figure 1), in accordance with our observation that the transgene-encoded p53-specific CD8+ T-cell repertoire only emerges in the periphery of p53-/- TCR-tg mice (Supplemental Figure 1). Strikingly, expansion of the p53-/- TCR-tg CD8+ T cells coincided with a loss of bodyweight and severe reduction in host blood cell counts (Figure 2 A, B). Onset of hematopoietic ablation was observed at day 6 after adoptive transfer, while weight loss became apparent after 9 days. This toxicity eventually resulted in death of the mice. Detailed examination of the hematopoietic compartment of these mice revealed a severe decline in T- and B- lymphocytes (Figure 2B-G), erythrocytes, hemoglobin levels and platelets (data not shown). A similar decline was observed in the hematopoietic stem cells (Figure 2H-I), while histological analysis revealed a disturbed splenic anatomy (Figure 2J). This elimination of the entire hematopoietic system and concurrent weight loss and mortality was only observed when infused splenocytes were derived from p53-/- TCR-tg donors and when recipients were vaccinated, indicating that it was caused by the expanded p53-specific CD8+ T-cell population. Remarkably, histological analysis of the affected mice did not reveal detectable immune pathology in any of the non-hematopoietic tissues examined (Figure 2K, L), suggesting that toxicity of the p53-specific CTLs was limited to the hematopoietic compartment of the recipient.

Figure 1. Expansion of p53-specific CTL from p53-/- TCR-tg donors in p53 +/- recipients.

A) CD4 and CD8 staining of splenocytes of p53+/+ TCR-tg, p53+/- TCR-tg or p53-/- TCR-tg CD90.2+ donor mice. B) These splenocyte populations were each infused into p53+/+ non-transgenic CD90.1+ recipients. Where indicated, recipient mice received a single s.c. dose of p53-specific peptide vaccine at the day of infusion. The expansion of donor-derived T cells expressing the transgenic TCR (CD90.2+ TCR Vβ6+) was examined by flow cytometric analysis of the splenocytes at day 7 after infusion. Representative examples of 6 mice per group are shown.
Figure 2. Expansion of p53-specific TCR -tg CD8+ T-cells in p53+/+ recipients results in weight loss and hematopoietic ablation.

Groups (n=4) of p53+/+, CD90.1+ non-transgenic mice were infused with splenocytes isolated from p53-/- TCR-tg, CD90.2+ donor mice. Where indicated (filled bars), recipient mice received a single s.c. dose of p53-specific peptide vaccine at the day of infusion. A) Relative total bodyweight at day 10 after infusion as compared to starting weight. B to I) Relative numbers of hematopoietic cells at day 10. Peripheral blood cell counts: total lymphocytes (B), B220+ B cells (C), recipient-type CD90.1+ T cells (D). Bone marrow cell counts: total cells (E), B220+ B cells (F), recipient-type CD90.1+ T cells (G), CD117+ (c-Kit+) stem cells (H), colony-forming units in the presence of GM-CSF (I). Figures show mean values and SEM for 4 mice per group. Results were statistically significant between vaccinated and non-vaccinated groups in all cases (p<0.05 *, p<0.01**, p< 0.001*** as determined by Student’s T-test.

J-L) Immunohistochemical analysis of spleen (J), colon (K) and liver (L) sections from p53+/+ mice that received TCR-tg T cells in combination with vaccination or without vaccination. Spleen sections were stained for B220+ B cells and counterstained with hematoxylin. Colon and liver sections were stained with hematoxylin (similar stainings were performed for sections of ileum; not shown).
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no vaccination + vaccination

J) spleen

K) colon

L) liver
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Functionality of genetically engineered p53-specific CTLs

Reconstitution of the self-reactive T-cell repertoire through retroviral transduction of TCR genes into autologous T cells is currently being considered as a means to overcome the absence of high-affinity T cells targeting tumor-associated auto-antigens (5, 21, 26). The feasibility of this approach for p53-specific TCRs was tested in our preclinical model by retroviral transduction of the alpha and beta chains of the p53\(^{158-166}\)-specific TCR into lymphocytes of p53\(^+/+\) and p53\(-/-\) origin. Infusion of p53\(^{158-166}\) TCR-transduced p53\(-/-\) lymphocytes in combination with vaccination of the p53\(^+/+\) recipients resulted in an expansion of the donor type CD45.2\(^+\) T cells. This expansion was accompanied by deletion of host type CD45.1\(^+\) lymphocytes, weight loss and mortality (Figure 3). When p53\(^+/+\) lymphocytes were transduced with the p53\(^{158-166}\) TCR, transgene expression was similar (Supplemental Figure 4). However, upon infusion, the p53\(^{158-166}\) TCR-transduced p53\(^+/+\) lymphocytes failed to expand. In line with this, only minor host lymphocyte depletion and weight loss were observed (Figure 3). The failure of p53\(^+/+\) lymphocytes cells to expand upon infusion was a specific effect of introduction of the p53\(^{158-166}\) TCR, because the p53\(-/-\) and p53\(^+/+\) lymphocytes expanded comparably upon modification with a control ovalbumin-specific OT-I TCR (Supplemental Figure 4) (20). In mice that received OT-I TCR-modified T cells, expansion was not accompanied by weight loss (data not shown), in line with the fact that the TCR concerned targets an antigen that is not expressed in the host. These observations support the notion that infusion of p53-specific CD8\(^+\) T cells can cause ablation of the p53\(^+/+\) hematopoietic system. Furthermore, our data argue that fratricide among p53-

Figure 3. Functionality of genetically engineered p53-specific CTLs.

CD45.1\(^+\) recipient mice were infused with p53\(-/-\) CD45.2\(^+\) (open bars, n=5) or p53 \(^+/+\) CD45.2\(^+\) splenocytes (filled bars, n=5) that were retrovirally transduced with genes encoding the p53-specific TCR. A control group received mock transduced p53\(-/-\) splenocytes (grey bars, n=3). All recipient mice received a single s.c. dose of p53-specific peptide vaccine at the day of infusion. This experiment was performed twice with similar outcome. A) Expansion of infused T cells as reflected by the percentage of CD45.2\(^+\) donor-type lymphocytes in the peripheral blood on day 11 after infusion. B) Depletion of recipient lymphocyte compartment as reflected by numbers of CD45.1\(^+\) recipient-type lymphocytes in peripheral blood on day 11 after infusion. Unlabeled beads were used for calibration to determine lymphocyte numbers in relation to blood volume. Lymphocyte counts are depicted as percentage of counts detected in mice receiving mock transduced cells. C) Relative total bodyweight as compared to starting weight. All bars show mean values and SEM. Where indicated results were statistically significant between mice receiving p53 TCR-transduced splenocytes as compared to mice receiving mock-transduced splenocytes (p<0.05 *, p< 0.001***), as determined by Student’s T-test.
specific TCR-transduced autologous T cells occurs when the T cells themselves present the p53 antigen. Although such fratricide prevents ablation of the host hematopoietic system, it will likely also compromise the anti-tumor effect of adoptive therapies targeting p53.

**Stem cell transfer allows for selective tumor targeting in the absence of toxicity**

Adoptive immunotherapy of leukemia, as currently applied in the clinic, involves lymphoablative treatment followed by MHC-mismatched stem cell transplantation. This treatment is generally combined with donor lymphocyte infusion (DLI) with the aim of eliciting a graft versus leukemia (GVL) reaction that is capable of eradicating the remaining leukemia cells and preventing relapse. Unfortunately, GVL-reactions are frequently associated with cytotoxic impact of the infused lymphocytes against non-hematopoietic tissues, resulting in graft-versus-host disease (GVHD). This treatment-associated toxicity may be reduced by focusing the donor lymphocyte response on antigens that are exclusively expressed in the hematopoietic compartment (27). Our data indicated that the immunopathology caused by adoptively transferred p53-specific CD8+ T cells was similarly restricted to the hematopoietic compartment (Figure 2), suggesting that p53 could serve as target antigen for non-toxic targeting of leukemia when applied in combination with protective stem cell transfer.
We first tested whether toxicity of adoptive transfer could be prevented by providing the recipient mice with a hematopoietic system that was less sensitive to the cytolytic action of the p53-specific CTLs. We generated bone marrow chimeras by transplanting hematopoietic stem cells of p53+/+ B6, p53-/- B6 or p53+/+ Bm1 origin into lethally irradiated p53+/+ B6 mice (Figure 4A). We used hematopoietic stem cells from Bm1 mice, because the variant H-2Kb (Kbm1) molecule expressed by these mice differs in peptide-binding from its wild-type counterpart (28). Accordingly, p53+/+ Bm1 cells are recognized less efficiently by p53 TCR-tg cells than their B6 counterparts (Supplemental Figure 5). In line with our intend, we found reconstitution with stem cells of either p53-/- B6 or p53+/+ Bm1 origin to prevent the weight loss and mortality that was otherwise associated with infusion of p53-specific peptide vaccine (where indicated). At the same day, the mice were challenged s.c., the contra lateral flank, with a tumorigenic dose of EL-4 lymphoma cells. Depletion of the recipient hematopoietic compartment by the p53-specific CTL response was countered by infusing the mice, 2 days later, with bone marrow-derived stem cells (BM) of indicated origin. Mice received bone marrow from p53+/+ B6 (black circle, n=9) or p53-/- B6 (white circle, n=10) origin. Control group (white triangle, n=10) received p53-/- B6 bone marrow but no vaccination. B) Relative total bodyweight after infusion as compared to starting weight. †: mice died as a result of hematopoietic ablation. Mice received stem cells from p53+/+ B6 (black circle, n=9) or p53-/- B6 (white circle, n=10). Control group (white triangle, n=10) received p53-/- B6 bone marrow but no vaccination. C) Tumor development over time. Numbers indicate tumor incidence in group that received p53-/- B6 BM and vaccination (white circle, 0/9) and control group (black circle, 8/10). Tumor incidence in group receiving stem cells from p53+/+ B6 origin (white triangle) is not shown because mice died of hematopoietic ablation before tumors became apparent. D) Overall survival of mice over time (* Log-rank test p=0.013).

Figure 5. Eradication of hematological tumors through p53-targeted adoptive immunotherapy.

A) Experimental procedure: mice were subjected to lymphodepletion (LD) for 5 days before being infused (AT = adoptive transfer) with p53-/- TCR-tg CD8+ T cells in combination with a single s.c. dose of p53-specific peptide vaccine (where indicated). At the same day, the mice were challenged s.c., the contra lateral flank, with a tumorigenic dose of EL-4 lymphoma cells. Depletion of the recipient hematopoietic compartment by the p53-specific CTL response was countered by infusing the mice, 2 days later, with bone marrow-derived stem cells (BM) of indicated origin. Mice received bone marrow from p53+/+ B6 (black circle, n=9) or p53 -/- B6 (white circle, n=10) origin. Control group (white triangle, n=10) received p53-/- B6 bone marrow but no vaccination. B) Relative total bodyweight after infusion as compared to starting weight. †: mice died as a result of hematopoietic ablation. Mice received stem cells from p53+/+ B6 (black circle, n=9) or p53 -/- B6 (white circle, n=10). Control group (white triangle, n=10) received p53-/- B6 bone marrow but no vaccination. C) Tumor development over time. Numbers indicate tumor incidence in group that received p53 -/- B6 BM and vaccination (white circle, 0/9) and control group (black circle, 8/10). Tumor incidence in group receiving stem cells from p53+/+ B6 origin (white triangle) is not shown because mice died of hematopoietic ablation before tumors became apparent. D) Overall survival of mice over time (* Log-rank test p=0.013).
TCR-tg T cells (Figure 5B, C). These results support our notion that toxicity of the infused p53-specific CTLs is limited to the host hematopoietic compartment.

Subsequently, we tested the anti-leukemic efficacy of adoptively transferred p53-specific CTLs when combined with protective stem cell transfer. As a conditioning regimen for effective adoptive T-cell therapy, we first treated the mice with the lymphocyte ablative drugs fludarabine and cyclophosphamide, in a manner similar to clinical therapies using adoptive T-cell therapy (5). Subsequently, the mice received bone marrow stem cells from either p53-/- B6 or p53+/+ B6 mice, p53-/- TCR-tg T cells and a tumorigenic dose of lymphoma cells (Figure 6A). Mice that received p53-/- TCR-tg T cells in combination with p53+/+ stem cells exhibited severe weight loss and mortality, in a manner observed in previous experiments. In contrast, recipients of p53-specific T cells and p53-/- stem cells did not show life threatening weight loss and, moreover, displayed tumor-free survival (Figure 6B, C). Our data show that the infused p53-specific CTLs promoted survival of the mice by eradicating the leukemia cells, provided that the recipients were reconstituted with hematopoietic stem cells that did not present the p53 target peptide. Importantly, the tumoricidal action of the infused p53-specific CTLs depended on concomitant p53-specific vaccination of the recipient mice, in that the majority of non-vaccinated mice displayed progressive tumor growth (Figure 6B, C).

Discussion

Generation of tumor-reactive T lymphocytes by transfer of specific TCR genes into autologous lymphocytes was recently shown to be a promising strategy for overcoming the failure of the endogenous T-cell repertoire against cancer (5). Prior work has established several strategies for obtaining high affinity TCR specific tumor-associated self-antigens (29). Importantly, when such tumor-associated self-antigens are targeted by an infused high affinity T-cell compartment it will be essential to assess the potential toxicity of this approach. TCRs targeting p53 in the context of common HLA class I molecules have been considered prime candidates for TCR gene therapy, because p53 is mutated and over-expressed in approximately half of all human cancers. Furthermore, the p53-specific CD8+ T-cell repertoire may be blunted by central tolerance (8, 9), which could render vaccination-based approaches by themselves ineffective. Our present studies with a TCR targeting an immunodominant, MHC class I-restricted peptide derived from murine wild-type p53 show that thymic deletion indeed prevents high affinity p53-specific CTL from emerging in the periphery (Supplementary Figure 1), and furthermore demonstrate that reconstruction of the p53-specific CTL repertoire through infusion of genetically engineered T cells can effectively be applied for tumor targeting in vivo (Figure 5). Our studies also highlight two potential complications of this approach. First, the ablation of the host hematopoietic system by the adoptively transferred p53-specific CTLs, resulting in mortality of the recipient mice (Figures 2 and 3). Secondly, the failure to generate potent p53-specific CTL responses through TCR gene transfer into autologous, p53+/+ lymphocytes (Figure 3). Both problems are related to the sensitivity of the host hematopoietic compartment to the TCR concerned. Importantly, we show that these hurdles can be circumvented by using stem cells and lymphocytes that are not efficiently targeted by the p53-specific CTLs, such as obtained from p53-/- mice or MHC-mismatched Bm1 donor mice (Figures 3 and 4). Under these conditions, a window of opportunity can be created for p53-specific targeting of tumors in the absence of life-threatening toxicity.
The main basis for this window of opportunity is the confinement of the pathological impact of p53-specific CTLs to the hematopoietic compartment, which leaves the non-hematopoietic tissues of the recipient intact. The feasibility and safety of this approach for cancer targeting in humans will essentially depend on whether this selectivity of p53-specific CTLs is conserved between mouse and man. The hematopoietic ablation observed upon transfer of p53-specific CTLs is reminiscent of the pathophysiology of aplastic anemia. Also this immune-mediated disorder involves the destruction of hematopoietic stem cells as a result of a type 1 T-cell response in the absence of overt auto-immune pathology to non-hematopoietic tissues (30). Studies with mouse models for aplastic anemia have shown that the destruction of hematopoietic progenitor and stem cells can occur through an IFN-γ-driven mechanism rather than through direct recognition (31-33). Our finding that transfer of stem cells from Bm1 or p53-/- origin can rescue mice infused with p53-specific CTLs suggests that in the current experiments direct recognition is responsible for the elimination of the host hematopoietic compartment. However, further research will be required to address this issue. For instance, it is conceivable that the high systemic IFN-γ levels associated with the expansion of p53-specific CTLs preferentially sensitize the host hematopoietic compartment for the cytolytic action of these CTLs. Furthermore, there is evidence suggesting that p53 is more highly expressed in the rapidly proliferating stem cells than in other normal tissues (34-37). This notion, and the higher levels of surface MHC class I on hematopoietic cells, may explain the increased sensitivity of the hematopoietic compartment to p53-specific CTLs. In view of the absence of toxicity to non-hematopoietic tissues, the potential for application of p53-specific TCRs overlaps with that of minor histocompatibility antigens (mHAgs) such as HA-1, the expression of which is restricted to the hematopoietic compartment and certain solid tumors (27). The use of p53-specific TCRs may have an advantage over those targeting mHAgs, in that the immunogenicity of mHAgs is restricted to the small polymorphic regions in these antigens, which are expected to result in immunogenic epitopes in only a limited repertoire of HLA class I epitopes. In contrast, the entire p53 sequence serves as substrate for antigen processing, making it conceivable that immunogenic p53-derived epitopes can be identified for a wide array of HLA class I molecules. In either case, TCR transfer into T cells of MHC-mismatched origin can result in off-target allo-reactivity directed by endogenous TCRs. This risk may be reduced by starting off with T-cell isolates that are highly enriched in T-cells specific for common infectious pathogens such as cytomegalovirus (CMV, 38).

In contrast to our failure to obtain potent p53-specific CTL responses through TCR gene transfer into murine p53+/+ lymphocytes (Figure 3), others have demonstrated successful transduction of a human p53-specific, HLA-A2.1-restricted TCR into human lymphocytes (8-12). Although differences in TCR, epitope and species could explain this discrepancy, it should be noted that the reports on gene transduction with the HLA-A2.1-restricted CTL do not specify the HLA-type of human lymphocytes used for the experiments, leaving the possibility that successful experiments particularly concerned HLA-A2.1-negative lymphocytes. Further pre-clinical evaluation of efficacy and safety of adoptive therapy with this TCR would require in vivo experiments in transgenic mice expressing both HLA-A2.1 and the human wild-type p53 antigen, involving transfer of TCR-tg or TCR-transduced primary T cells. Notably, on basis of our own previous studies, involving in vitro and in vivo studies with an established mouse p53-specific CTL clone (13, 14), we had not anticipated p53-specific TCR transfer to cause toxicity. We ascribe the discrepancy between our successive studies to the impressive capacity of the infused naive TCR-tg T cells to expand in vivo (39) (Figure 1) versus the negative impact of prolonged ex vivo culture on the in vivo activation,
p53 specific T-cell therapy of hematologic tumors

proliferation and survival of adoptively transferred CTLs (5, 40). Taken together, our findings stress the importance of testing the efficacy and safety of adoptive T-cell therapies involving auto-reactive TCRs in clinically relevant in vivo models.

Acknowledgements

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References


### Supplemental figure 2A

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Figure S1. Negative selection prevents development of p53 reactive CD8+ T cells.

Total lymphocytes from thymus and spleen of mice with indicated genetic backgrounds were analyzed for CD4+ and CD8+ positive cells by flow cytometry. Numbers below indicate percentage of cells in each quadrant. Representative examples of 6 mice analyzed per TCR-tg genotype are shown.

### Supplemental figure 2B

A) Thymic cell counts of non-transgenic mice and TCR-tg mice with indicated genetic backgrounds. Figures show mean values with SEM for groups of 8 mice. Results for p53-/- mice differ significantly from those for p53+/- and p53+/+ mice (p<0.0001) as determined by Students’ T-test. B) IFN-γ production by CD8+ and CD4+ splenocytes isolated from TCR-tg mice with the indicated genotype. Cells were stimulated overnight with synthetic p53<sup>158-166</sup> peptide or with tumor cells of B6 origin presenting the naturally processed peptide. Numbers indicate percentage of IFN-γ positive cells. Representative examples of 3 mice analyzed per TCR-tg genotype are shown.
Figure S3. Vaccination is required for expansion of p53-specific TCR-tg CD8+ T cells.

p53+/+ CD90.1+ non-transgenic mice were infused with CFSE-labeled TCR-tg splenocytes isolated from p53-/CD90.2+ donor mice. Where indicated, recipient mice received a single s.c. dose of p53-specific peptide vaccine at the day of infusion. Representative examples of 3 mice analyzed per group are shown. A) Proliferation and expansion was examined at days 4 and 7 after infusion by analyzing numbers and CFSE-intensity of donor-type CD90.2+ T cells in peripheral blood. Numbers in each plot indicate mean fluorescence intensity (MFI) of CFSE; vertical lines indicate CFSE MFI on day 4 to indicate progression of proliferation (or lack thereof) as detected on day 7. B) Activity of p53-specific CD8+ T cells in vaccinated mice was determined at day 4 after infusion by incubating peripheral blood cells in the presence (+) or absence (-) of p53¹⁵⁸⁻¹⁶⁶ peptide and Brefeldin A, and analyzing IFN-γ production by intracellular cytokine staining. Graphs depict staining of CD90.2+ subset for IFN-γ and for TCR Vβ6, the variable region expressed by the TCR-tg T cells. Numbers indicate percentages of IFN-γ positive cells. Similar analysis for non-vaccinated mice was negative, due to the lack of a significant CD90.2+ population in such mice (see panel A).
Figure S4. Fratricide causes functional failure of TCR-transduced p53+/+ T cells.

A) Efficiency of TCR gene transduction is comparable for p53-specific and chicken ovalbumin (OVA)-specific TCRs in splenocytes of p53+/+ and p53/- origin, as measured 18 hrs after retroviral infection. Numbers indicate percentage of TCR-β double-positive cells (upper right quadrant) compared to single endogenous TCR-β expressing cells (upper left quadrant). B) The capacity of p53-/- TCR-tg cells to eliminate lymphocytes from p53+/+ origin was determined by in vivo cytotoxicity assay performed as described previously (23). Non-transgenic CD45.1+ mice were infused with naive TCR-tg cells isolated from CD45.2+, p53-/- donor mice. Recipient mice (5/group) received a single s.c. dose of p53-specific peptide vaccine at the day of infusion. The control group was not vaccinated. Three days after vaccination, 1:1 mixture of CFSE labeled p53+/+ CD45.2 + (CFSE high) and p53-/- CD45.2 + (CFSE low) splenocytes was injected. Spleens were harvested on day 7 and the elimination of the CSFE-labeled target cells by the TCR-tg T cells was determined on the basis of the relative presence of CFSE high and CFSE low populations after gating on CD45.2+CD3+Vβ6- cells. Bars show mean (+ SD) in vivo killing of p53+/+ cells. C) Non-transgenic CD45.1+ mice were infused with splenocytes from p53-/- CD45.2+ or p53+/+ CD45.2+ origin that were retroviral transduced with genes encoding the ovalbumin-specific TCR (OVA TCR). Recipient mice (3/group) received a single s.c. dose of ovalbumin-specific peptide vaccine at the day of infusion. Bar graph shows the percentage of CD45.2+ donor cells (and SEM) 11 days after infusion. Where indicated results were statistically significant (p<0.05 *, p< 0.001***) as determined by Student’s T-test.
Figure S5. Presentation of p53 target peptide by bone marrow cells of wild-type and Bm1 origin.

Bone marrow cells from p53+/+ B6 mice or p53+/+ Bm1 mice were loaded for 4 hours with increasing amounts of p53\textsuperscript{158-166} peptide, after which free peptide was removed by thorough washing in medium. Peptide-loaded stimulator cells were co-cultured with p53\textsuperscript{-/-} TCR-tg cells at a 1:1 ratio for 16 hrs, after which culture supernatant was tested for IFN-γ content by ELISA as described previously (22). Cytokine production is expressed as percentage of maximal cytokine secretion. Lines indicate the peptide dose at which 50% of the maximal IFN-γ production was reached: p53+/+ B6 0.29 μg/ml (black line), p53+/+ Bm1 1.29 μg/ml (dotted line).
Chapter 4.3

Transduction of p53 \(^{158-166}\) TCR into naïve splenocytes

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*Supplement to chapter 4.2*
Introduction

Accumulating evidence shows however that the endogenous p53 specific cytotoxic T lymphocyte (CTL) response is largely blunted as a result of negative selection of self-reactive T cells (1). For several tumorantigens the transfer of T-cell receptors (TCR) into T cells can be a successful means of passive immunotherapy (2-4). For p53 directed immunotherapy, current efforts focus on the reconstitution of the p53 CTL repertoire by improving the function of infused p53 TCR transduced T cells (5-8). The original TCR that is used in these studies was originally obtained from a mouse that expresses the human class I HLA-A2 (1). Since major tolerance effects were circumvented this approach allowed the isolation of high avidity CD8 co-receptor independent CTL recognizing human p53. In chapter 4.1 we describe the generation of a transgenic mouse expressing a p53 TCR and in chapter 4.2 we study the in vivo effects of these cells after adoptive transfer. Clinical application of p53 TCR therapy in cancer patients will most likely involve adoptive transfer of engineered T cells such as by retroviral transduction. This chapter describes in detail the generation of cells expressing murine p53 158–166 TCR by retroviral transduction.

Materials and methods

Mice. p53 +/+ B6 (wild-type), p53-/- B6 (p53 knock out) and Bm1 (Kbm¹) mice (9) (all C57BL/6 Kh H-2b background) were bred in our own facilities (Leiden, Netherlands). Congenic CD45.1 mice (SJL) were purchased from Taconic. All experiments were performed in accordance with experimental guidelines and were approved in advance by an animal ethical committee.

Cell culture. All cells were cultured in complete medium consisting of Iscove’s Modified Dulbecco’s Medium (IMDM, Life Technologies, Rockville, MD) supplemented with 8% FCS (Greiner), 100 IU/ml penicillin, 2mM glutamine and 30 μM 2-Mercaptoethanol.

DNA vectors. Rearranged V(D)J α and V(D)J β regions of the TCRs from previously described p53158–166 specific CTL clone (10) and from the ovalbumine OT-1 specific CTL (11) were separately amplified by PCR and cloned into the pMX vector (12). Modified V(D)J segments of TCR genes were designed and produced by GeneArt (GeneArt GmbH) and cloned in a pMX vector. Unmodified p53 TCR is nominated p53 wt TCR. After gene optimization the construct is nominated p53 opt TCR. The ovalbumine TCR (OT-1) was only used in gene optimized conformation.

Production of retroviral supernatants and retroviral transduction. Retroviral supernatants were obtained by transfection of Phoenix-E packaging cells with the indicated retroviral vectors using the FuGENE 6 transfection reagent (Roche) as described previously (12). Total mouse splenocytes were isolated and cultured in 24-well plates (3*10⁶ cells/well) in complete medium in the presence of Concanavalin A (ConA, 2 μg/ml) and 2% supernatant of ConA stimulated rat splenocytes. After 48 hours splenocytes were resuspended in retroviral supernatant, transferred to RetroNectin-coated plates transduced with retroviral supernatant at 3*10⁶ cells/ml and centrifuged for 90 minutes at 2000 rpm. Plates were then incubated for 24 hours at 37°C. All procedures were performed as described previously (12).
Flow cytometric analysis. Phoenix packaging cells and transduced splenocytes were all routinely tested for presence of the introduced TCR as a measure for efficiency of transfection and transduction. Blood samples were depleted of erythrocytes before FACS analysis. For quantification of cell numbers standard amounts of unlabeled control beads (BD Calibrite, BD Pharmingen) were taken along. Direct extracellular labeling of cells was performed according to standard procedures. Labeling of intracellular products was performed according to manufacturer’s protocols by using BD Cytofix/Cytoperm to fix and permeabilize the cells. For Intracellular cytokine staining splenocytes were incubated with 2 μg/ml murine p53 158-166 peptide in the presence of 10 μg/ml Brefeldin A for 18 hours. Antibodies used; FITC-labeled Vβ3, Vβ8, Vβ11, Vβ13, Vβ14 (Vβ mix), CD45.2; PE-labeled Vβ6 (p53 TCR), Vα2 (OT-1 TCR); PerCP-conjugated CD8α; APC-conjugated IFN-γ, CD45.1 (all antibodies purchased from BD Pharmingen). Results were analyzed by using standard CellQuest software (BD).

Injection of TCR transduced cells and vaccination. Starting five days before adoptive transfer of transduced splenocytes, mice underwent lymphodepletion by treatment with 2 mg cyclophosphamide (Baxter) i.p. daily at days –5 and –4. Subsequently mice received 2 mg of fludarabine (Schering Plough) i.p. daily at days –3 to –1 (6). On day 0, transduced splenocytes were separated over a ficoll gradient to remove dead cells. Per recipient mouse 2*10⁶ total transduced splenocytes cells were injected in the tail vein. Subsequently mice were vaccinated with 50 μg CpG (kindly provided by Dimitri Filipov, Chemistry dept. Leiden University) and 50 μg α-CD40 antibody (FGK-45) in combination of 100 μg 30-mer p53¹⁴²⁻¹⁷¹ peptide (p53 opt TCR and mock) or 100 μg 30-mer ovalbumin²⁴¹⁻²⁷⁰ peptide (OT-1 TCR). All peptides were manufactured in our own facilities as described previously (13). A blood sample from the tail vein was taken every 3 days and mice were weighed every 1-4 days.

![Figure 1: TCR levels present in transfected Phoenix E cells](image).

Phoenix cells were transfected with p53 wt TCR, p53 opt TCR or OT-1 TCR. Untransfected cells were taken along as control (mock). Permeabilized cells were stained for expression of TCR chains by PE labelled TCR Vβ 6 (p53 TCR) or TCR Vα 2 (OT-1).
Results and discussion

Since the autologous p53 CTL repertoire seems to be blunted, immunotherapy will most likely have to involve adoptive cell transfer. One possible method to achieve this is by infusion of engineered T cells which express a tumor specific TCR (4, 5, 14), for instance by retroviral gene transfer. Safety and feasibility aspects of this approach have not been well documented for p53 TCR. In this study we therefore describe in detail the generation of murine p53\textsuperscript{158-166} TCR expressing cells by retroviral transduction. In combination with chapters 4.1 and 4.2 of this thesis, results herein provide important insights for the further development of p53 TCR cell therapy in cancer patients.

Our initial TCR transduction experiments showed lower expression levels of p53 wt TCR after transfection in virus producing cells compared to transfection of CEA specific TCR (15, and data not shown). This could be due to intrinsic properties of the TCR, for example unfavorable codon usage, which leads to impaired protein translation (16). Therefore we decided to codon optimize the gene construct of the p53 TCR (p53 opt TCR). Throughout this chapter we compare the \textit{in vitro} and \textit{in vivo} characteristics of the p53 TCR with that of the well-described ovalbumin\textsuperscript{257-264} specific TCR (OT-1 TCR). Transfection of the p53 wt TCR and p53 opt TCR constructs leads to comparable expression by virus packaging cells (Figure 1). However, expression of the OT-1 TCR leads to higher expression levels.

We next compared the expression levels of transduced TCR in T cells from p53+/+ B6 mice. We quantified the number of cells that express the introduced TCR next to an endogenous TCR by staining with a pool of TCR antibodies, since these are truly mature T cells (11, Figure 2A). Transduction of p53 wt TCR and p53 opt TCR constructs leads to efficient expression in p53 +/- splenocytes. The p53 opt TCR is expressed at similar levels as p53 wt TCR, despite improved codon usage. Expression of OT-1 TCR is markedly higher, again

![Figure 2. Expression of different TCR constructs in p53+/+ B6 splenocytes](image)

p53 wt TCR, p53 opt TCR or OT-1 opt TCR were introduced in p53 +/- B6 splenocytes. A) expression of introduced TCR in p53 +/- B6 splenocytes. Cells were gated on CD8 expression and staining for a mix of FITC labelled TCR V\textbeta\text{mix} specific antibodies and TCR V\textbeta\text{6} (p53TCR) or TCR Va2 (OT-1 TCR) are shown. B) transduced cells were restimulated with p53\textsuperscript{158-166} peptide (AIYSQHKM, p53 TCR) or ovalbumine\textsuperscript{257-264} (SIINFEKL, OT-1 TCR). Cells were gated on CD8 and staining of indicated TCR and IFN-\gamma are shown.
probably due to TCR or construct intrinsic properties. In addition, we tested the capacity of transduced cells to produce effector cytokines in vitro after specific stimulation (Figure 2B). Cells that express the OT-1 TCR produce high amounts of IFN-γ. Stimulation of cells expressing the p53 wt TCR and p53 opt TCR also leads to IFN-γ production of CD8+ cells bearing the introduced TCR. However, p53 TCR gene optimization does not significantly improve cytokine production after transduction in p53+/+ B6 splenocytes (Figure 2B).

In chapter 4.2 we have shown that p53 TCR transgenic cells efficiently recognize p53+/+ B6 hematopoietic cells. When immunotherapy by TCR transfer in autologous cells is considered, this could mean that p53 TCR transduced cells also recognize neighboring or ‘brother’ cells. Ultimately this could lead to cytolytic killing or fratricide (17), thereby abrogating the effective adoptively transferred population and subsequently the in vivo effect. To address this issue we introduced the p53 opt TCR in splenocytes from p53+/+ B6, p53 -/- B6 or p53+/+ Bm1 (Kbm1) mice. The latter expresses a mutated form of MHC class I Kb (9), which results in lower avidity T-cell recognition (see chapter 4.2). Since transduced murine splenocytes die rapidly in vitro we measured TCR expression levels shortly after introduction of the TCR (18 hours). Expression of the OT-1 TCR is equally high in all types of splenocytes tested. In addition, the expression of the p53 opt TCR is also not different in p53+/+ B6 or p53/- B6 or p53 +/- Bm1 splenocytes (Figure 3A). Therefore, presence of p53 does not seem to have an effect on the expression levels of p53 TCR at this time point. These results suggest that efficient presentation of p53 on splenocytes does not impair T-cell function in vitro shortly after transduction.

In chapter 4.2 we have shown that p53/- TCR-tg cells can cause a life threatening hematologic ablation in vaccinated p53 +/- B6 recipient mice. As a proof of principle we next
analyzed whether similar effects would also occur upon infusion of p53 opt TCR transduced splenocytes. Since we wished to study the feasibility and safety for clinical application of p53 TCR therapy we made use of a clinical gene therapy conditioning regimen (4). Subsequently we analyzed the effects of splenocytes from p53+/+ B6 or p53 -/- B6 mice expressing p53 opt TCR or OT-1 TCR upon infusion in p53 +/+ B6 mice. By using a congenic marker difference we were able to distinguish host hematopoietic cells (CD45.1+) from donor cells (CD45.2+). Cells from p53+/+ B6 or p53 -/- B6 mice expressing the OT-1 TCR expand robustly in vaccinated host mice (Figure 4). After several weeks the OT-1 T-cell response contracts and CD45.2+ cell numbers are hardly demonstrable. The contraction of the OT-1 TCR T-cell response occurs faster in p53 +/+ splenocytes than p53 -/- splenocytes, probably because the latter has a slightly better intrinsic expansion potential due to the lack of p53 cell cycle control. Markedly, there is a vast expansion of p53 opt TCR p53 -/- splenocytes in vivo (Figure 4). All expanded cell populations (p53 opt TCR in p53 -/- and OT-1 TCR) predominantly express CD8 and the introduced TCR. Strikingly, p53 +/+ splenocytes that express p53 opt TCR do not expand in vivo (Figure 4). A possible explanation could be that the cells commit fratricide, since they are also able to recognize brother cells, and consequently a very low number of cells survives.

As described in chapter 4.2 activated, p53 specific TCR-tg cells can cause acute hematopoietic ablation in p53 +/+ B6 mice. Likewise we observe that the robust expansion

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**Figure 4. Dynamics of p53 opt TCR and OT-1 TCR expressing cells in vivo**

Lymphodepleted mice (CD45.1+) received vaccination and 2*10⁶ transduced cells of p53+/+ or p53 -/- B6 splenocytes (CD45.2+) transduced with p53 opt TCR or OT-1 TCR. Mock transduced p53 -/- B6 cells were taken along as a control. Figure shows percentage of CD45.2+ donor cells in time after adoptive transduced cell transfer, measured in peripheral blood.
of p53 TCR expressing p53-/- B6 cells coincides with severe hematopoietic ablation and leading to the death of 2 out of 5 mice (Figure 5). These data confirm the results obtained by p53 opt TCR-tg cells described in chapter 4.2. Despite no measurable increase in p53 opt TCR in p53 +/- donor cells, these mice also have a reduced host blood cell count. Strikingly, mice that received OT-1 TCR transduced cells also have a reduced host cell population, however this group of mice retains normal bodyweight and all mice survive (Figure 5). Possibly this is the result of mild autoreactivity of TCR complexes that have emerged by heterodimerization of the engineered TCR with endogenous TCR chains, thereby introducing new self-specific TCR. Whether this is indeed the case, needs to be analyzed in more detail.

In conclusion, our data described in this chapter show that the murine p53 opt TCR can successfully be introduced in p53 +/- B6, p53-/- B6 and p53 +/- Bm1 splenocytes. However, in vivo survival of p53 opt TCR transduced p53 +/- B6 cells is impaired, probably due to fratricide. This implies that successful clinical application of p53 CTL therapy can only be accomplished by using allogeneic cells. Furthermore, our data show that infusion of p53 opt TCR expressing p53-/- splenocytes causes a similar hematopoietic ablation as p53-/- TCR-tg cells. As we show in chapter 4.2, adoptive cell transfer of p53 specific CTL can only be applied safely when the ablated host hematopoietic is reconstituted by non-sensitive bone marrow. This implies that patients receiving p53 TCR therapy will need to receive allogeneic engineered T cells in addition to allogeneic bone marrow transplantation, which is a challenging protocol but not impossible. Overall our data urge for meticulous safety and feasibility studies on the use of self-TCR gene therapy since as it appears even low expression levels of self-antigens such as p53 can potentially initiate recognition by engineered T cells.
References
