Tumor Eradication by Wild-type p53-specific Cytotoxic T Lymphocytes

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Summary

The tumor suppressor protein p53 is overexpressed in close to 50% of all human malignancies. The p53 protein is therefore an attractive target for immunotherapy. Cytotoxic T lymphocytes (CTLs) recognizing a murine wild-type p53 peptide, presented by the major histocompatibility complex class I molecule H-2Ka, were generated by immunizing p53 gene deficient (p53-/-) C57BL/6 mice with syngeneic p53-overexpressing tumor cells. Adoptive transfer of these CTLs into tumor-bearing p53+/- nude mice caused complete and permanent tumor eradication. Importantly, this occurred in the absence of any demonstrable damage to normal tissue. When transferred into p53+/- immunocompetent C57BL/6 mice, the CTLs persisted for weeks in the absence of immunopathology and were capable of preventing tumor outgrowth. Wild-type p53-specific CTLs can apparently discriminate between p53-overexpressing tumor cells and normal tissue, indicating that widely expressed autologous molecules such as p53 can serve as a target for CTL-mediated immunotherapy of tumors.

The efficacy of virus-specific CTLs to eradicate virus-induced tumors has been well documented (1–5). However, since the majority of tumors are not virus-induced, characterization of tumor-associated antigens encoded by cellular genes is important for the development of new immunotherapeutic strategies. Target antigens on non-virally-induced tumors recognized by CTLs were recently identified, notably in patients with melanoma (6–18). The fact that these antigens are lineage- or tumor-specific, limits the use of these targets in immunotherapy to a small group of cancers. On the other hand, the expression of some of these antigens on normal melanocytes demonstrates that self antigens can serve as targets for CTL-mediated destruction of tumors.

Mutations in the gene encoding the tumor suppressor protein p53 are found in ~50% of all human malignancies (19). Recently, a direct link has been established between mutational hot spots in the p53 gene leading to its overexpression, and carcinogenic metabolites derived from agents in cigarette smoke (20). In normal cells, p53 induces a cell cycle arrest, allowing DNA to be checked for irregularities, thereby guarding the integrity of the genome (21). Mutation of p53 abolishes its function as a suppressor of the cell cycle, promoting the escape of transformed cells from the normal restriction of controlled growth. Since these mutations, causing overexpression of p53, are present in a wide variety of cancers (22–25), a large group of patients would benefit from p53 directed immunotherapy. One could consider mutant p53 sequences as target antigens for tumor-specific CTLs. However, p53 mutations occur at many different sites in the p53 molecule, necessitating identification of the site of mutation in each patient before therapy. Furthermore, not all mutations are contained in MHC-binding CTL epitopes. If, in contrast, wild-type (wt) p53 sequences are used, the entire sequence of the p53 protein is available for properly processed immunogenic T cell epitopes. We hypothesized that the altered expression of p53, seen in many cancers, leads to modified processing and presentation of wt p53-derived peptides by MHC class I molecules. R ecently, wt p53 peptide-specific CTL were generated from human and murine responding lymphocytes, some of which recognized p53-overexpressing tumors in vitro (26–34).

Abbreviations used in this paper: aa, amino acids; FLVenv, Friend leukemia virus envelope; MEC, mouse embryo cell; wt, wild type.

M.P.M. Vierboom and H.W. Nijman contributed equally to this paper.
Here, we report the in vivo eradication of established p53-overexpressing tumors in C57BL/6 p53 (+/+) nude mice by a well-defined wt p53-specific CTL clone in the absence of any demonstrable immunopathology. These CTLs, generated in p53-deficient mice and recognizing the murine wt p53-derived epitope A1YK KSQ HM (amino acids [aa] 158–166) presented by the MHC class I molecule H-2Kb, were also capable of preventing the outgrowth of a more aggressive p53-overexpressing tumor in immuno-competent p53 (+/+ ) C57BL/6 mice.

Materials and Methods

Mice. C57BL/6 (B6, H-2b) mice were obtained either from the Netherlands Cancer Institute (Amsterdam, The Netherlands) or from IFFA Credo (Larbrese, France), C57BL/6 nu/nu (B6 nude, H-2b) mice were obtained from Bomholtgard (Ry, Denmark), and the p53 knockout (p53−/−; H-2b) mice were obtained from GenPharm (Mountain View, CA; 35) and held under specific pathogen-free conditions. Since offspring could only be obtained by crossing a p53 heterozygous female (p53+/−) with a p53 male (p53−/−), the mice had to be analyzed for their p53 status by PCR analysis. For the PCR analysis two primer sets were used. Primer set A consists of a forward primer binding to the neomycin resistance gene (5′ GCA TCG CCT TCT ATC GCC TCT TTC AGC 3′, neo fwd), with which the wt p53 gene was destroyed, and a reverse primer that binds to a sequence in exon 5 (5′ ATC ACC ATC GGA GCA GCC CTC ATG 3′, p53 exon 5 rev). Primer set A gives a band of 120 bp only when the p53 gene is present. Primer set B consists of a forward primer binding to a sequence in intron 4 of the wt p53 gene (5′ CAG TCC TCT TCT TCT TGC TGG CTC GCT C 3′, p53 intron 4 fwd), which is deleted by the insertion of the neomycin resistance gene in the wt p53 gene. This sequence is only present in an intact wt p53 gene. The same reverse primer used in primer set A was used for primer set B. Primer set B gives a band of 180 bp only when the wt p53 gene is present.

Cell Lines. Mouse embryo cells (MECs) of C57BL/6 origin were transfected by transfection with the following oncogenes: Hoxc3, Adenovirus type 5 E1 (2); C3, HPV16 E6 + E7; E[ras]36(36); 4, mut-p53 + H-ras 5A and 5D, mut-p53 + N-ras 6 and J3, mut-p53 + fos (van Hall, T., M.P.M. Vierboom, C.J.M. Melief, and R. Offringa, manuscript in preparation). The tumor cell lines 4J, 5A, 5D, and J3 express high levels of a murine p53 containing a mutation at position 135 (37). Cell line EL-4 originates from an H-2b thymoma (38). The cell line "Koko" was generated from a solid tumor that arose spontaneously in a p53 knockout mouse. The following CTL clones, all of C57BL/6 origin, were used: anti-MC1233 CTL clone BTM (provided by F. OSENDORF, University Hospital Leiden, Leiden, The Netherlands; reference 39), anti-Ad5SE1A CTL clone 5 (2), and anti-Ad5 E1B CTL clone 100B6 (5). Mouse cell lines were cultured in Iscove's modified Dulbecco medium (Gibco BOCULT, Glasgow, UK) supplemented with 10% FCS and 2% human serum. Dulbecco medium in the presence of 2% human serum to increase the proliferation of the T cells. Mouse cell lines were cultured in Iscove's modified Dulbecco medium (Gibco BOCULT, Glasgow, UK) supplemented with 10% FCS and 2% human serum. Dulbecco medium in the presence of 2% human serum to increase the proliferation of the T cells.

Peptides. Peptides were generated by solid phase strategies on an Applied Biosystems model 433 peptide synthesizer (Applied Biosystems, Foster City, CA) using, in the first step, a monoclonal antibody (Immunogenetics, Munich, Germany) by repetitive cycles in which addition of Fmoc protected amino acids was alternated with an Fmoc-deprotection procedure using Fmoc chemistry. The purity of the peptides was determined by reverse phase HPLC and was found to be routinely >90% pure. Peptides were dissolved in DMEM (final DMEM concentration 0.25%) and diluted in 0.9% NaCl to a peptide concentration of 2 mg/ml and stored at −80°C.

MHC Class I Peptide Binding Assay. The MHC class I peptide binding assay using the processing defective cell line RMA-S and the peptide competition assay were performed as described (41). In brief, RMA-S cells were cultured for 36 h at 37°C in culture medium in the presence of 2% human serum to increase the cell surface expression of "empty" class I molecules (42). In 96-well plates, RMA-S cells (2.5 × 103/well) were cultured in serum-free medium (40 μg/well) and peptide (10 μg/ml) for 4 h at 37°C. Subsequently, the cells were washed and stained for analysis on a FACscan flow cytometer (Becton Dickinson, Mountain View, CA) using, in the first step, a monoclonal antibody against H-2Kb (B8.24.3; reference 43) and, in the second step, FITC-labeled goat anti-mouse F(ab′)2 fragments.

Peptide Competition Cytotoxicity Assay. For the competition assay, competitor peptides were aliquoted in triplicate in 96-well U-bottomed plates at 10 times the final concentration, 10 μg/well. To each well, 10 μl of 50 μM of the reference peptide MCF1233 574–581 (KSPWFTTL; reference 39) was added. Triplicate wells were included that contained either no peptide or reference peptide only. EL-4 target cells (105 cells/well) were labeled with Europium (EuIII) and added to the peptides (40 μg/well). After 60 min of incubation, 5 × 103 CTL clone BTM cells (39) were added to the wells for another 4 h of incubation before supernatants were collected. By interpolation of the data obtained with each competitor peptide at different concentrations, we calculated the peptide concentration that inhibits 50% of the maximal CTL lysis in the presence of the reference peptide alone (IC50). Transporter A associated with Processing T cell lines was transfected by transfection with the following oncogenes: Hoxc3, Adenovirus type 5 E1 (2); C3, HPV16 E6 + E7; E[ras]36(36); 4, mut-p53 + H-ras 5A and 5D, mut-p53 + N-ras 6 and J3, mut-p53 + fos (van Hall, T., M.P.M. Vierboom, C.J.M. Melief, and R. Offringa, manuscript in preparation). The tumor cell lines 4J, 5A, 5D, and J3 express high levels of a murine p53 containing a mutation at position 135 (37). Cell line EL-4 originates from an H-2b thymoma (38). The cell line "Koko" was generated from a solid tumor that arose spontaneously in a p53 knockout mouse. The following CTL clones, all of C57BL/6 origin, were used: anti-MC1233 CTL clone BTM (provided by F. OSENDORF, University Hospital Leiden, Leiden, The Netherlands; reference 39), anti-Ad5SE1A CTL clone 5 (2), and anti-Ad5 E1B CTL clone 100B6 (5). Mouse cell lines were cultured in Iscove's modified Dulbecco medium (Gibco BOCULT, Glasgow, UK) supplemented with 10% FCS, penicillin (100 IU/ml), and β-mercaptoethanol (2 × 10−3 M) at 37°C in humidified air containing 5% CO2.

Generation of Tumor Cell Line-Specific CTL Cultures. Tumor cell line-specific CTL cultures were generated as described previously (2, 39). In brief, spleen cells from B6 and p53−/− mice were taken 3 wk after the second immunization with 106 irradiated 4J cells treated with IFN-γ (20 U/ml for 48 h) and brought into culture after enriching for T cells via passage over a nylon wool column. 5 × 104 responder cells were cocultured with 5 × 103 irradiated and IFN-γ-treated 4J cells in a total volume of 100 μl in 96-well U-bottomed plates. Bulk cultures were restimulated in vitro with irradiated and IFN-γ-treated 4J cells once a week, for 6 wk, which resulted in the CTL line 8. CTL clones, of which one was clone 1H11, were obtained by limiting dilution of the 4J-specific bulk culture at wk 3 as described (2). Long-term cultures were grown in medium with 10% FCS and 1.5% culture supernatant from PM A/Con A-stimulated rat spleen cells (40) and 25 μg/ml IL-2 (2). A1YK KSQ HM (amino acids [aa] 158–166) presented by the MHC class I molecule H-2Kb, were also capable of preventing the outgrowth of a more aggressive p53-overexpressing tumor in immuno-competent p53 (+/+ ) C57BL/6 mice.
release assay as described elsewhere (46). In brief, varying numbers of effector cells (harvested on Ficoll if necessary) were added to $10^3$ Eu$^{3+}$-labeled target cells in 100 μl of culture medium in 96-well U-bottomed plates. After 4 h incubation time, 20 μl culture supernatant was collected and mixed with 200 μl Enhancer Solution (W allac, Turku, Finland). Measurement of the samples took place in a fluorometer (1234 Delfia; W allac). The mean percentage specific lysis of triplicate wells was calculated as follows: percent specific lysis = ([cpm spontaneous release - cpm spontaneous release]) / [cpm maximum release - cpm spontaneous release] × 100. The spontaneous release of the Eu$^{3+}$-labeled target cells was <30% in all experiments. The figures shown are representative for experiments done in duplicate.

Transfection of COS-7 Cells. Transient transfection of COS-7 cells was performed as described elsewhere (5, 12). In short, 100 μg of plasmid pcDNA/Amp-p53 containing mut-p53 (37) together with 100 ng of plasmid pcDNA/Amp-D$^b$ or pcDNA/Amp-K$^b$ were transfected by the DEAE-dextran-chloroquine method into 106 COS-7 cells (47). Plasmid pcDNA/Amp-p53 harbors the p53 and plasmids pcDNA/Amp-D$^b$ and pcDNA/Amp-K$^b$ harbor the H-2D$^b$ and H-2K$^b$ genes, respectively. The COS-7 cells were incubated in 100 μl IMDM containing 10% FCS for 48–72 h at 37°C, after which 1,500 CTLs in 25 μl IMDM containing 50 μl rIL-2 were added. Upon specific stimulation of the CTL, TNF-α is released in the supernatant, as measured in a bioassay with the TNF-α-sensitive cells WEHI-164 clone 13 (5). Percent WEHI cell death was calculated by the following formula: percent specific lysis = 1 - ([OD 550–650 in sample wells/OD 550–650 in wells containing untransfected COS-7 cells and CTLs]) × 100.

Cold Target Inhibition Cytotoxicity Assay. Koko cells were loaded with peptide at a concentration of 50 μg/ml for 2 h. Cells were washed five times to remove unbound peptide. Effector cells were preincubated with 5 × 105 unlabeled “blocking” cells (50 times excess) for 60 min at 37°C, before addition of labeled cells. After a subsequent 4-h incubation period with labeled cells, the supernatant was collected.

Adoptive Transfer of Anti-p53-specific CTLs. wt p53-specific CTL clone 1H11 (2.0 × 107) was intravenously injected in either tumor-bearing or nonchallenged p53-deficient C57BL/6 nude mice and C57Bl/6 immunocompetent mice in combination with irradiated IFN-γ-treated 4J cells were restimulated in vitro with 4J, and the antigen specificity of the resulting bulk CTL cultures was analyzed. The CTL specifically lysed 4J, but not the tumor cell line Koko derived from a p53$^-/-$ mouse (Fig. 1 A). Specific recognition of p53 was tested by incubating these CTLs with COS-7 cells transiently transfected with pcDNAs encoding one of the two MHC class I molecules, H-2K$^b$ or H-2D$^b$, with or without mutant p53. Specific recognition was assayed by TNF-α production. Fig. 1 B shows that the CTL bulk cultures recognize COS-7 cells transfected with mutant p53 in an MHC class I H-2K$^b$-restricted manner. Several CTL clones, displaying shown). Spleen cells of mice immunized and boosted with irradiated IFN-γ-treated 4J cells were restimulated in vitro with 4J, and the antigen specificity of the resulting bulk CTL cultures was analyzed. The CTL specifically lysed 4J, but not the tumor cell line Koko derived from a p53$^-/-$ mouse (Fig. 1 A). Specific recognition of p53 was tested by incubating these CTLs with COS-7 cells transiently transfected with pcDNAs encoding one of the two MHC class I molecules, H-2K$^b$ or H-2D$^b$, with or without mutant p53. Specific recognition was assayed by TNF-α production. Fig. 1 B shows that the CTL bulk cultures recognize COS-7 cells transfected with mutant p53 in an MHC class I H-2K$^b$-restricted manner. Several CTL clones, displaying shown). Spleen cells of mice immunized and boosted with irradiated IFN-γ-treated 4J cells were restimulated in vitro with 4J, and the antigen specificity of the resulting bulk CTL cultures was analyzed. The CTL specifically lysed 4J, but not the tumor cell line Koko derived from a p53$^-/-$ mouse (Fig. 1 A). Specific recognition of p53 was tested by incubating these CTLs with COS-7 cells transiently transfected with pcDNAs encoding one of the two MHC class I molecules, H-2K$^b$ or H-2D$^b$, with or without mutant p53. Specific recognition was assayed by TNF-α production. Fig. 1 B shows that the CTL bulk cultures recognize COS-7 cells transfected with mutant p53 in an MHC class I H-2K$^b$-restricted manner. Several CTL clones, displaying shown). Spleen cells of mice immunized and boosted with irradiated IFN-γ-treated 4J cells were restimulated in vitro with 4J, and the antigen specificity of the resulting bulk CTL cultures was analyzed. The CTL specifically lysed 4J, but not the tumor cell line Koko derived from a p53$^-/-$ mouse (Fig. 1 A). Specific recognition of p53 was tested by incubating these CTLs with COS-7 cells transiently transfected with pcDNAs encoding one of the two MHC class I molecules, H-2K$^b$ or H-2D$^b$, with or without mutant p53. Specific recognition was assayed by TNF-α production. Fig. 1 B shows that the CTL bulk cultures recognize COS-7 cells transfected with mutant p53 in an MHC class I H-2K$^b$-restricted manner. Several CTL clones, displaying

Table 1. MHC Class I Kb-binding, Peptide Competition, and TAP-dependent Transport of Seven wt p53 Peptides

| K$^b$ binding peptides | UC$^{50}$ | CC$^{50}$ | IC$^{50}$ |
|------------------------|--------|--------|--------|
| aa 119–127: VM CYSPPL | 18 | 12 | 2.5 |
| aa 122–130: TY SPLNK L | 20 | 14 | 6 |
| aa 123–131: Y SPPLNK K LF | 4 | 0.5 | >100 |
| aa 127–134: LN KLFCQ L | 4 | 2 | 7 |
| aa 158–166: AI YK KSQ H M | 2.7 | 0.3 | 4.5 |
| aa 222–229: AGSE YTTI | 100 | 18 | 70 |
| aa 227–234: TT IH YK YM | 7 | 1.1 | 6 |
| SV9: FAPGN YPAL | 0.1 | 0.2 | ND |

Seven wt p53-derived were characterized in an MHC class I binding assay (column 1; reference 42), peptide competition cytotoxicity assay (column 2; reference 41), and TAP-dependent translocation assay (column 3; reference 44). (UC$^{50}$) Peptide concentration resulting in 50% of the maximal upregulation of H-2K$^b$ in the presence of the known H-2K$^b$-binding peptide Sendai virus N P 324–332 (61). (CC$^{50}$) Peptide concentration that inhibits 50% of the maximal lysis by CTL clone BTM recognizing the reference peptide KS PW FTTL derived from the MCF1233 virus (39). (IC$^{50}$) Concentration of competitor peptide that decreased the maximal amount of recovered glycosylated reporter peptide by 50% (44, 45). Indicated in bold are so-called anchor residues in the H-2K$^b$-binding motif described by Falk et al. (49).
The peptide specificity (A–C) and sensitivity (B) of p53-specific CTL clone 1H11 on peptide-pulsed target cells (A and B) and tumor cells (C). (A) A representative clone, 1H11, derived from line 8 by limiting dilution, was tested for lytic activity of Eu³⁺-labeled p53−/− Koko cells pulsed with one of seven H-2Kb binding wt p53 peptides (Table 1). Targets were unloaded Koko cells (open circles) or Koko cells loaded with peptides (given in aa sequence): VMCTYSPPL (open triangles), YSPPLNKL (filled triangles), YSPPLNLKF (open squares), LNKLFQOL (filled squares), AIYKKSQHM (filled circles), AGSEYTTI (open diamonds), TTHIYKYM (filled diamonds). As a control, Koko cells pulsed with K⁺-binding MCF1233-derived peptide KSPWFTTL (39) was taken along (asterisks). (B) Koko cells were incubated with titrated amounts of length variants of the wt mouse p53 epitope AIYKKSQHM (aa 158–166). CTL clone 1H11 was added after a preincubation of 30 min at an E/T ratio of 10:1. The following length variants were tested: AIYKKSQHM (dosed circles; aa 158–166), AIYKKSQHM T (open triangles; aa 158–167), AIYKKSQHM H (open squares; aa 158–165), IYKKSQHM (open circles; aa 159–166); AMAIYKKKS (dosed triangles; aa 156–163), (C) Cold target blocking of the p53-overexpressing tumor cell 4j was performed by preincubation of the CTL clone 1H11 with the following unlabeled cells: no cells (open squares), koko cells (open circles), koko cells (open circles), and Koko cells (open circles) pulsed with the wt p53 epitope AIYKKSQHM (dosed circles; aa 158–166) and 4j cells (dosed triangles). After 60 min, labeled 4j cells were added.

The specificity of the p53 epitope recognized by these CTLs is demonstrated by the fact that these peptides bind to the MHC class I molecules (Table 1, C.C59 column). These peptides were subsequently tested for their ability to sensitize Eu³⁺-labeled Koko cells for lysis by the p53-specific CTL clone 1H11. Only the peptide AIYKKSQHM, derived from the wt sequence of p53 (aa 158–166), was recognized by the CTLs (Fig. 2 A). Titrated amounts of length variants of this peptide were tested to establish the optimal length. The peptide AIYKKSQHM (aa 158–166), recognized at a concentration range of 0.1–1 pM (Fig. 2 B), was the optimal length peptide and one of the best binders (Table 1). Koko cells loaded with the peptide AIYKKSQHM were able to specifically block the recognition of the p53-overexpressing tumor cell lines 4j (Fig. 2 C) and 5D (data not shown) in a cold target inhibition cytotoxicity assay, demonstrating that this peptide is the naturally processed epitope presented by 4j and 5D.

Mutation-induced overexpression of p53 is not required for recognition by the p53-specific CTL clone 1H11. Mutant p53-transfected tumor cells and other tumor cells, with no known p53 overexpression, were tested for recognition. Lysis of HPV16 transformed tumor cell line C3 (36; Fig. 3 A) and the thymoma EL-4 (data not shown) by 1H11 was comparable to the lysis of other p53-overexpressing lines 4j, 5A, and 6J3 (Fig. 3 A). Strikingly, nontransformed B6M EC and C3H A-stimulated splenic cells were efficiently recognized by CTL clone 1H11 (Fig. 3 A), demonstrating the potential cross-reacting ability of these p53-specific CTLs to nonmalignant cells. On the other hand, freshly isolated thymocytes (Fig. 3 B) and freshly isolated splenic cells (data not shown) are not lysed by these CTLs.

A depletive transfer of wt p53-specific CTLs eradicates established tumors in p53−/+ B6 nude mice. Since these wt p53-specific CTLs cross-react on nontransformed cells.
we then assessed whether mice carrying a functional p53 gene would survive adoptive transfer of these potentially autoreactive CTLs and whether these CTLs could eradicate established 4J tumors in these mice without overt immunopathology. Adoptively transferred wt p53-specific CTLs were retrieved from the spleen of B6 p53+/+ nude mice up to 3 mo after intravenous administration (Fig. 4, A and B), but not from splenocytes of untreated B6 nude mice (Fig. 4 C). No signs of autoimmune-induced damage were observed in mice that had received wt p53-specific CTLs. We subsequently tested the possibility of eradicating established 4J tumors by adoptive transfer of wt p53-specific CTLs. Tumors grew progressively in untreated mice (Fig. 5 A) and in mice injected with an irrelevant CTL clone recognizing an Ad5 E1B-derived epitope (5; Fig. 5 B). Adoptive transfer by intravenous infusion of the wt p53-specific clone 1H11 resulted in complete and permanent (>5 mo) tumor eradication in mice with small- (average size = 41 mm²; Fig. 5 C) and medium- (average size = 140 mm²; Fig. 5 D) sized tumors. Intratumoral injection of similar numbers of wt p53-specific CTLs in combination with rIL-2 also led to the complete eradication of medium-sized tumors (data not shown). Even large established 4J tumors (average size = 427 mm³) were eradicated in three out of six mice (Fig. 5 E) after intravenous infusion of the wt p53-specific clone 1H11. Mice that had rejected p53-induced tumors after treatment with wt p53-specific CTLs retained long-term tumor-specific CTL immunity, since wt p53-specific CTLs could be retrieved from spleens of these animals one month after CTL treatment (Fig. 6, A and B).

Prevention of Outgrowth of a p53-Overexpressing Tumor in Immunocompetent C57BL/6 Mice. To investigate whether the wt p53-specific CTLs would also persist in immunocompetent p53+/- C57BL/6 mice or would be deleted in the presence of a T cell compartment absent in C57BL/6 nude mice, p53-specific CTLs were transferred into p53+/- C57BL/6 immunocompetent mice and the spleens of these mice were assayed for p53-specific activity after 14 d. wt p53-specific CTLs, recognizing the peptide and 4J tumor cells, could be retrieved from spleens of C57BL/6 mice injected with an irrelevant CTL clone recognizing an HPV16 E7-derived epitope, 12 out of 12 animals developed a progressively growing tumor and died within 3 wk. In the group of mice challenged intraperitoneally with 5D and simultaneously treated with control CTL clone 9.5, recognizing an HPV16 E7-derived epitope, 12 out of 12 animals developed a progressively growing tumor and died within 3 wk. In the group of mice challenged intraperitoneally with 5D and treated with the wt p53-specific CTL clone 1H11, only 1 out of 12 developed a progressively growing tumor, thus demonstrating in vivo activity against the tumor without demonstrable autoimmune pathology.

Evaluation of Autoimmune Pathology. 3 mo after adoptive transfer of the p53-specific CTL clone 1H11 into non-tumor-bearing nude animals and one month after successful eradication of small- and medium-sized tumors, tissues (liver, kidney, spleen, small intestine, large intestine, lung, stomach, heart, brain, skin, lymph nodes, and bone marrow) were collected and examined microscopically (hematoxylin and eosin stained). Examination of coded samples
by two independent investigators showed no evidence of immunopathology in normal tissues of these nude mice (Fig. 8). An increased infiltrate of mononuclear cells was observed in normal tissue of cured mice. This is probably caused by the administration of rIL-2, since mice treated with an irrelevant clone also show this infiltrate (data not shown). Similarly, tissues from C57BL/6 p53 +/+ immunocompetent mice did not show evidence of immunopathology at times when, after adoptive transfer, the wt p53-specific CTLs could be recovered (Fig. 7 A). Staining of spleen and skin for CD4 and CD8 showed no difference between treated animals and nontreated control animals (data not shown).

**Discussion**

The search for widely expressed tumor antigens as targets for MHC class I–restricted CTLs is of great importance for the development of T cell–mediated immunotherapy of cancer. The tumor suppressor protein p53 is potentially such an antigen, with altered expression in up to 50% of all human tumors (19). In the present study we demonstrate that CTLs recognizing a murine wt p53-derived epitope were able to eradicate a p53-overexpressing tumor in p53 +/+ B6 nude mice in the absence of demonstrable immunopathology.

wt p53-specific CTLs were generated by immunizing C57BL/6 p53 −/− mice with syngeneic p53-overexpressing 4J tumor cells. As expected, a wt p53-derived sequence was found to serve as an excellent epitope, the mutant p53 area being devoid of MHC class I–binding motif carrying sequences (49). The wt p53-specific CTLs recognized the H-2Kb-binding epitope A1YKKSQHM (aa 158–166; Fig. 2 A) with high affinity (Fig. 2 B), comparable to previously published CTL clones against viral epitopes that were able to eradicate established tumors (2, 4, 5).

C57BL/6 nude and immunocompetent mice carrying the wt p53 gene survived the adoptive transfer of wt p53-specific CTLs. These potentially autoreactive CTLs persist normally in p53 +/+ mice (Fig. 4, A and B, and 7 A) without damage to normal tissues (Fig. 8 C). This persistence of large amounts of self-reactive T cells was elegantly shown in a study of Ohashi et al. (51). They demonstrated that CTLs expressing a transgenic TCR recognizing a lympophytic chorionaditis virus glycoprotein-derived epitope remained functionally unresponsive towards β islet cells expressing the transgene lymphophtic chorionaditis virus glycoprotein. Similar observations were made by Governan et al. (52), who showed that animals with large amounts of functionally, autoreactive T cells expressing the transgenic TCR specific for the naturally expressed myelin basic protein can be present without causing experimental allergic encephalomyelitis. However, in both models the transgenic TCR–carrying T cells can be activated to cause autoimmunity (51, 52). In contrast, the wt p53-specific CTLs were active in tumor-bearing C57BL/6 nude mice and eradicated established tumors (Fig. 5, C–E) without autoimmunity. In this respect, our study corroborates observations in Friend leukemia virus envelope (FLV env) transgenic mice.

Table 2. W.t p53-specific CTL Incidence

| Tumor dose | Control clone 9.5 | wt p53-specific CTL clone 1H11 |
|------------|------------------|-------------------------------|
| 5 × 10⁴    | 4/4              | 1/4                           |
| 1 × 10⁴    | 4/4              | 0/4                           |
| 0.2 × 10⁴  | 4/4              | 0/4                           |

Immunocompetent B6 mice were simultaneously injected intraperitoneally with SD (p53/N-ras) tumor cells at various doses and a control clone 9.5 (2 × 10⁴) recognizing the HPV16 E7-derived epitope RAHYNIYTF or the wt p53-specific CTL clone 1H11 (2 × 10⁷). On the day of treatment and 7 d later, a subcutaneous injection of 6 × 10⁹ IU rIL-2 (Cetus Corp.) in 50% IFA was given.

Figure 6. Recovery of wt p53-specific CTLs from nude mice after successful tumor eradication. wt p53-specific CTL clone 1H11 was retrieved from the spleens of C57BL/6 nude mice 1 mo after tumor eradication as tested in an Eu³⁻ release assay. CTLs were administered intravenously (A) or intratumorally (B). A nontreated naive mouse (C) was taken along as a negative control. Spleen cells were restimulated in vitro with 4J cells and tested for their peptide specificity on p53 −/− Koko cells (open circles, filled circles), Koko cells pulsed with the p53 peptide A1YKKSQHM (filled circles), and recognition of 4J cells (filled squares) after 7 d of culture.

Figure 7. Recovery of wt p53-specific CTL activity after adoptive transfer of CTLs into immunocompetent mice. wt p53-specific CTL clone 1H11 was recovered from the spleens of C57BL/6 p53 +/+ immunocompetent mice 14 d after the CTLs were administered intravenously (A), as tested in an Eu³⁻ release assay. A nontreated C57BL/6 p53 +/+ immunocompetent mouse (B) was taken along as a negative control. Spleen cells were restimulated in vitro with 4J and tested for their peptide specificity on p53 −/− Koko cells (open circles, filled circles), Koko cells pulsed with the p53 peptide A1YKKSQHM (filled circles), and recognition of 4J cells (filled squares) after 7 d of culture.
in which adoptively transferred FLVenv-autoreactive T cells can eradicate FLVenv expressing tumor cells without damage to normal tissue expressing this artificial autoantigen (53).

A simple explanation for the observed tumor selectivity can be the increased expression of the p53 protein resulting from the p53 mutation. Alternatively, the lack of "danger" signals delivered by normal tissues (54) might protect against the destruction by the potentially autoreactive wt p53-specific CTL. In fact, in normal tissues, homeostatic mechanisms apparently control tissue damage by potentially autoreactive T cells. Only powerful inflammatory stimuli can provoke autoagression mediated by these otherwise dormant T cells, as illustrated by the examples of experimental allergic encephalomyelitis (55, 56) and adjuvant arthritis (57). The explanation why, in this particular instance, no autoimmune tissue damage occurs despite the infusion of a large number of activated cloned CTLs may lie in insufficient antigen display by the MHC class I molecules in combination with lack of proper costimulation (54) and downregulatory chemokine and cytokine conditions (55).

Recent reports show the induction of wt p53 peptide-specific responses with cross-reactivity on endogenously p53-expressing targets (28, 29, 31–34). However, the in vivo relevance of these responses remains to be demonstrated. In reports in which wt p53 was used as an immunogen (30, 58), therapeutic effects were found consisting of eradication of established tumors or protection against a subsequent tumor challenge. However, the mechanism of antitumor activity in these studies remains to be clarified. In

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**Figure 8.** Histology of various tissues after adoptive transfer of wt p53-specific CTLs. Hematoxylin and eosin-stained sections of liver, small intestine, skin, and lung of a nontreated control mouse (A; tested for CTL activity as shown in Fig. 4C); a mouse treated with an irrelevant clone directed against the Ad5E1B-derived epitope VNIRNYCI (aa 192–200; reference 5) (B; from the group depicted in Fig. 5B), and a mouse 1 mo after complete tumor eradication by the adoptive transfer of the wt p53-specific CTL clone 1H11 (C; tested for CTL activity as shown in Fig. 6A).
our model, a well-defined high affinity CD8+ CTL clone recognizing a wt p53 epitope eradicated established tumors in B6 nude mice and prevented the outgrowth of a more aggressive tumor injected simultaneously in the peritoneal cavity in B6 immunocompetent mice.

To generate these CTLs, responding lymphocytes from p53 knockout mice were used for the obvious reason that these mice are not tolerant of p53. Since p53 is a crucial protein serving as a checkpoint in the cell cycle of stimulated T cells (59) which are open to attack by wt p53-specific CTLs (Fig. 3A), one of the major challenges is to try to break tolerance of wt p53 and to generate high affinity wt p53-specific CTLs in normal p53 +/+ individuals.

Activation of CTLs to autoantigens seems feasible in cancer patients as evidenced by the recent analyses of responses against melanoma-associated antigens and against p53 (11–14, 16–18, 31). From the blood of healthy donors, CTLs reactive against the autoantigen tyrosinase and against wt p53 can be arosed from their unresponsive state by appropriate in vitro stimulation (29, 31, 60). Our data support the idea to use widely expressed tumor-associated antigens such as p53 for CTL-mediated immunotherapy.

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References

1. Melief, C.J.M. 1992. Tumor eradication by adoptive transfer of cytotoxic T lymphocytes. Adv. Cancer Res. 58:143–175.
2. Kast, W.M., R. Offringa, P.J. Peters, A.C. Voordouw, R.H. Melenen, A.J. van der Eb, and C.J.M. Melief. 1989. Eradication of adenovirus E1-induced tumors by E1A-specific cytotoxic T lymphocytes. Cell 59:603–614.
3. Greenberg, P.D. 1991. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. Adv. Immunol. 49:281–355.
4. Feltkamp, M.C.W., G.R. Vreugdenhil, M.P.M. Vierboom, E. Rais, S.H. van der Burg, J. Ter Schegget, C.J.M. Melief, and W.M. Kast. 1995. CTL raised against a subdominant epitope offered as a synthetic peptide eradicate human papillomavirus type 16-induced tumors. Eur. J. Immunol. 25:2638–2642.
5. Toes, R.E.M., R. Offringa, H.J.J. Blom, R.M.P. Brandt, A.J. van der Eb, C.J.M. Melief, and W.M. Kast. 1995. An adenovirus type 5 early region 1B-encoded CTL epitope-mediating tumor eradication by CTL clones is down-modulated by an activated ras oncogene. J. Immunol. 154:3396–3405.
6. van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. de Plaen, B. van den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytotolytic T lymphocytes on a human melanoma. Science (Wash. DC.). 254:1643–1647.
7. van den Eynde, B., B. Lethé, A. van Pel, E. de Plaen, and T. Boon. 1991. The gene coding for a major tumor rejection antigen of tumor P815 is identical to the normal gene of syngeneic DBA/2 mice. J. Exp. Med. 173:1373–1384.
8. Mandleboim, O., G. Berke, M. Fridkin, M. Feldman, M. Eisenstein, and L. Eisenbach. 1994. CTL induction by a tumor-associated antigen octa-peptide derived from a murine lung carcinoma. Nature (Lond.). 369:67–71.
9. Boon, T., J.-C. Cerottini, B. Van den Eynde, P. Van der Bruggen, and A. Van Pel. 1994. Tumor antigens recognized by T lymphocytes. Annu. Rev. Immunol. 12:337–365.
10. Van Pel, A., P. van der Bruggen, P.G. Coulié, V.G. Brichard, B. Lethé, B. Van den Eynde, C. Uyttenhove, J.-C. Renaud, and T. Boon. 1995. Genes coding for tumor antigens recognized by cytolytic T lymphocytes. Immunol. Rev. 145:229–250.
11. Traversari, C., P. van der Bruggen, I.F. Lüscher, C. Lurquin, P. Chomez, A. Van Pel, E. De Plaen, A. Amar-Costeseec, and T. Boon. 1992. A nonapeptide encoded by human gene MAGE-1 is recognized by cytolytic T lymphocytes directed against tumor antigen M2Z-E. J. Exp. Med. 176:1453–1457.
12. Brichard, V., A. Van Pel, T. Wolfel, C. Wolfel, E. De Plaen, B. Lethé, P. Coulié, and T. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytotoxic T lymphocytes on HLA-A2 melanomas. J. Exp. Med. 178:489–495.
13. Gaugler, B., B. van den Eynde, P. van der Bruggen, P. Romero, J.J. Gaforio, E. de Plaen, B. Lethé, F. Brasseur, and T. Boon. 1994. Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. J. Exp. Med. 179:921–930.
14. De Plaen, E., K. Arden, C. Traversari, J.J. Gaforio, J.-P. Szikora, C. De Smet, F. Brasseur, P. Van der Bruggen, B. Lethé, C. Lurquin, et al. 1994. Structure, chromosomal localization, and expression of 12 genes of the mag family. Immunogenetics. 40:360–369.
15. Kawakami, Y., S. Eliyahu, C.H. Delgado, P.F. Robbins, L. Rivoltini, S.L. Topalian, T. Miki, and S.A. Rosenberg. 1994. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc. Natl. Acad. Sci. USA. 91:3515–3519.
16. Kawakami, Y., S. Eliyahu, K. Sakaguchi, P.F. Robbins, L. Rivoltini, J.R. Yannelli, E. Appella, and S.A. Rosenberg. 1994. Identification of the immunodominant peptides of the
MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J. Exp. Med. 1991; 174:1357–1365.

27. Kawakami, Y., S. Eliyahu, C.H. Delgado, P.F. Roberts, K. Sakaguchi, E. Appella, J.R. Yanelli, G.J. Adema, T. Miki, and S.A. Rosenberg. 1994. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc. Natl. Acad. Sci. USA. 91:6458–6462.

28. Bakker, A.B.H., M.W.J. Schreurs, A.J. De Boer, Y. Kawakami, S.A. Rosenberg, G.J. Adema, and C.G. Figdor. 1994. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. J. Exp. Med. 179:1005–1009.

29. Nijman, H.W., S.H. Van den Burg, M.P.M. Vierboom, A.J. Levine, and L.A. Houbiers, J.G.A., H.W. Nijman, S.H. Van den Burg, J.W. Röpke, M., J. Hald, P. Guldenberg, J. Zeuthen, L. Norgaard, J.G.A. Houbiers, W.M. Kast, and C.J.M. Melief. 1994. Interactions of adenoviral proteins with Rb and p53. \textit{FASEB J.} 8:171–178.

30. Momand, M., G.P. Zambetti, D.C. Olson, D. George, and E. Moran. 1993. Interaction of adenoviral proteins with Rb and p53. Proc. Natl. Acad. Sci. USA. 90:7491–7495.

31. Kuerbitz, S.J., B.S. Plunkett, W.V. Walsh, and M.B. Kastan. 1994. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in p53. Science (Wash. DC). 274:430–432.

32. Zambetti, G.P., and A.J. Levine. 1993. A comparison of the biological activities of wild-type and mutant p53. \textit{FASEB J.} 7:855–865.

33. Noguchi, Y., Y.-T. Chen, and L.J. Old. 1994. A mouse mutant p53 product recognized by CD4+ and CD8+ T cells. Proc. Natl. Acad. Sci. USA. 91:3171–3175.

34. Nijman, H.W., S.H. Van den Burg, M.P.M. Vierboom, J.G.A. Houbiers, W.M. Kast, and C.J.M. Melief. 1994. p53, a potential target for tumor-directed T cells. Immunol. Lett. 40:171–178.

35. Noguchi, Y., Y.-T. Chen, and L.J. Old. 1994. A mouse mutant p53 protein recognized by CD4+ and CD8+ T cells. Proc. Natl. Acad. Sci. USA. 91:3171–3175.

36. Nijman, H.W., S.H. Van den Burg, M.P.M. Vierboom, J.G.A. Houbiers, W.M. Kast, and C.J.M. Melief. 1994. A potential target for tumor-directed T cells. Immunol. Lett. 40:171–178.

37. Theobald, M., J. Biggs, D. Dittmer, A.J. Levine, and L.A. Sherman. 1995. Targeting p53 as a general tumor antigen. Proc. Natl. Acad. Sci. USA. 92:11993–11997.

38. Houbiers, J.G.A., H.W. Nijman, S.H. Van den Burg, J.W. Drijfhout, P. Kemenans, C.J.H. Van de Velde, A. Brand, F. Momburg, W.M. Kast, and C.J.M. Melief. 1993. In vitro induction of human cytotoxic T lymphocyte responses against peptides of mutant and wild-type p53. Eur. J. Immunol. 23:2072–2077.

39. Ayorodeme, J.I., C. Leucos, H. Sakamoto, C.M. De Cesare, P.M. Appaamay, M.T. Lotze, W.J. Storkus, E. Appella, and A.B. DeLeo. 1996. Therapy of murine tumors with p53 wild-type and mutant sequence peptide-based vaccines. J. Exp. Med. 183:1357–1365.

40. Röpke, M., J. Hald, P. Guldenberg, J. Zeuthen, L. Norgaard, L. Fugger, A. Svegaard, S. Van der Burg, H.W. Nijman, C.J.M. Melief, and M.H. Claesson. 1996. Spontaneous human squamous cell carcinomas are killed by a human cytotoxic T lymphocyte clone recognizing a wild-type p53-derived peptide. Proc. Natl. Acad. Sci. USA. 93:14704–14707.

41. Bertholet, S., R. Iggo, and G. Corradin. 1997. Cytotoxic T lymphocytes responses to wild-type and mutant mouse p53 peptides. Eur. J. Immunol. 27:798–801.

42. Lacabanne, V., M. Viguier, J.-G. Guillet, and J. Choppin. 1996. A wild-type p53 cytotoxic T cell epitope is presented by mouse hepatocarcinoma cells. Eur. J. Immunol. 26:2635–2639.

43. Dahl, A.M., P.C.L. Beverley, and H.J. Stauss. 1996. A synthetic peptide derived from the tumor-associated protein mdm-2 can stimulate autoreactive, high avidity cytotoxic T lymphocytes that recognize naturally processed protein. J. Immunol. 157:239–246.

44. Donehower, L.A., M. Harvey, B.L. Slagle, M.J. McArthur, C.A. Montgomery, J.S. Butel, and A. Bradley. 1992. Mice deficient for p53 develop normally but are susceptible to spontaneous tumors. Nature (Lond.). 356:215–221.
46. Bouma, G.J., P.M. van der Meer-Prins, F.P.M.J. van Bree, J.J. van Rood, and F.H.J. Claas. 1992. Determination of cytotoxic T lymphocyte precursor frequencies using Europium labeling as a nonradioactive alternative to labeling with Chromium-51. Hum. Immunol. 35:85–92.

47. Seed, B., and A. Aruffo. 1987. Molecular cloning of the CD2 antigen, the T cell erythrocyte receptor, by a rapid immunoselection procedure. Proc. Natl. Acad. Sci. USA. 84:3365–3369.

48. Pennica, D., D.V. Goeddel, J.S. Hayflick, N. Reich, C.W. Anderson, and A.J. Levine. 1984. The amino acid sequence of murine p53 determined from a c-DNA clone. Virology. 134:477–482.

49. Falk, K., O. Rötschke, S. Stevanovic, G. Jung, and H.-G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature (Lond.). 351:290–296.

50. Kast, W.M., and C.J.M. Melief. 1991. Fine peptide specificity of cytotoxic T lymphocytes against adenovirus-induced tumours and peptide–MHC binding. Int. J. Cancer. 6(Suppl.): 90–94.

51. Ohashi, P.S., S. Oehen, K. Buerki, H. Pircher, C.T. Ohashi, B. Odermatt, B. Malissen, R.M. Zinkernagel, and H. Hengartner. 1991. Ablation of “tolerance” and induction of diabetes by virus infection in viral antigen transgenic mice. Cell. 65:305–317.

52. Goverman, J.A., L. Woods, L.P. Larson, H. Weiner, L. Hood, and D.M. Zaller. 1993. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. Cell. 72:551–560.

53. Hu, J., W. Kindsvogel, S. Busby, M.C. Bailey, Y. Shi, and P.D. Greenberg. 1993. An evaluation of the potential to use tumor-associated antigens as targets for antitumor T cell therapy using transgenic mice expressing a retroviral tumor antigen in normal lymphoid tissues. J. Exp. Med. 177:1681–1690.

54. Malzinger, P. 1994. Tolerance, danger, and the extended family. Annu. Rev. Immunol. 12:991–1045.

55. Olsson, T. 1995. Critical influences of the cytokine orchestration on the outcome of myelin antigen-specific T-cell autoimmunity in experimental autoimmune encephalomyelitis and multiple sclerosis. Immunol. Rev. 144:245–268.

56. Ridgway, W.M., H.L. Weiner, and C.G. Fathman. 1994. Regulation of autoimmune response. Curr. Opin. Immunol. 6:946–955.

57. van Eden, W., S.M. Anderton, R. van der Zee, B.J. Prakken, C.P.M. Broeren, and M.H.M. Wubben. 1996. (Altered) Self peptides and the regulation of self reactivity in the peripheral T cell pool. Immunol. Rev. 149:55–73.

58. Roth, J., D. Dittmer, D. Rea, J. Tartaglia, E. Paolelli, and A.J. Levine. 1996. p53 as a target for cancer vaccines recombinant canarypox virus vectors expressing p53 protect mice against a lethal tumor cell challenge. Proc. Natl. Acad. Sci. USA. 93:4781–4786.

59. Milner, J. 1984. Different forms of p53 detected by monoclonal antibodies in non-dividing and dividing lymphocytes. Nature (Lond.). 310:143–145.

60. Visseren, M.J.W., A. Van Elsas, E.I.H. Van der Voort, M.E. Resing, W.M. Kast, P.I. Schrier, and C.J.M. Melief. 1995. CTL specific for the tyrosinase autoantigen can be induced from healthy donor blood to lyse melanoma cells. J. Immunol. 154:3991–3998.

61. Kast, W.M., L. Roux, J. Curren, H.J.J. Blom, A.C. Voor- douw, R.H. Meulen, D. Kolakowski, and C.J.M. Melief. 1991. Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with an unbound peptide. Proc. Natl. Acad. Sci. USA. 88:2283–2287.

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