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Increased Generation of CD8+ T Cell Clones in p53 Mutant Mice

Xianzheng Zhou,* Stacey Wong,* Jurgen Walter,* Tyler Jacks,* and Herman N. Eisen2*

Very few cultured CD8+ T cell clones can normally be obtained from a single mouse and maintained in long-term culture. To improve the yield, we immunized p53 mutant mice with peptides of Sendai virus (FAPGNYPAL) and influenza virus (ASNNEMTM) origin. Substantially more clones could be derived from p53−/− mice than from similarly treated wild-type mice (p53+/+); an intermediate yield was obtained from heterozygous mice (p53+/−). CTL lines or clones from p53−/− mice exhibited greater proliferative activity and resistance to γ-irradiation than those from p53+/+ mice, and were cytolytically potent. The Journal of Immunology, 1999, 162: 3957–3960.

C ultured T cell clones have been studied extensively for over 15 years (for a review of early studies see Ref. 1); such clones have played a central role in advancing our understanding of Ag recognition by T cells and of the structure and function of Ag-specific TCRs. These contributions are all the more notable because of the difficulties normally encountered in deriving satisfactory clones. The number of clones that can be derived from a single mouse (or human) and successfully maintained in culture for prolonged periods is usually very small, typically only two or three per mouse. In part, this low yield seems to come about because the establishment and maintenance of these clones in culture requires stimulating the cells by ligating their TCRs at frequent intervals (e.g., once a week); most T cells that are subject to repeated antigenic stimulation undergo cell death (apoptosis) (2–4). In an effort to increase the yield of clones, we have explored in this study the possibility that there might be a higher cloning efficiency from p53 mutant mice (p53−/−) than from wild-type (wt) mice (p53+/+). Although p53 plays a role in the cell death of lymphoid cells, there is no evidence that TCR-mediated cell death requires p53 (5). Nevertheless, mice with p53 mutation(s) were chosen for this study because it is known that dysfunctional mutations or a loss of p53 may reduce the ability of cells to undergo apoptosis in response to a variety of stimuli (reviewed in Refs. 6 and 7).

Our results show that with mice immunized with two different peptides, and in different ways, the yield of peptide-specific CD8 CTLs was substantially higher from p53−/− mice than from wt mice. In comparison with p53+/+ lines, p53−/− lines proliferated more rapidly and were relatively resistant to γ-irradiation. Importantly, the cytolytic activity of p53−/− and p53+/+ clones was indistinguishable.

Materials and Methods

Mice and genotyping

p53 mutant mice (p53−/−, C57BL/6 × 129/SV, H-2b background) were generated by gene targeting; their phenotype has been described previously (8). For a genotypic analysis of offspring, tail DNA was isolated from offspring and examined by PCR (8). The wt p53 allele was amplified using PCR primers directed against exon 6.5 (5′-ACACCGCTGTTGTTACCT TAT-3′, p53X6.5) and exon 7 (5′-TATACGAGCCGGCCT-3′, p53X7), whereas the p53 mutant allele was amplified using a primer directed against exon 7 and neo (5′-CTCTCGTGCTTTACGGTATC-3′, p53Xneo18.5).

Synthetic peptides and cell lines

The synthetic peptides used here are: Sendai virus nucleoprotein (NP) peptide, FAPGNYPAL (termed SV9) (9); influenza virus A/PR/8/34 NP peptide, ASNNEMETM (termed NP 366) (10, 11); and adenovirus E1A peptide, SGPNTPPEI (termed EA234) (12, 13). These peptides were synthesized by solid phase tert-butyloxycarbonyl chemistry in the Massachusetts Institute of Technology Biopolymers Laboratory and then purified by reverse-phase HPLC. EL4 (thyoma, H-2b), RMA-S (TAP2-defective lymphoma, H-2b) (14), and RMA-S/B7-1 cells (RMA-S transfected with murine B7-1 gene cloned in a pBR322 plasmid (a gift of Drs. Klas Karre and Elisabeth Wolpert), Ref. 15) were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME (“K medium”), except for RMA-S/B7-1 transfectants, which were grown in K medium plus 320 μg/ml G418 (Life Technologies, Gaithersburg, MD).

CTL generation, cloning, and CTL assay

The generation of CTLs with synthetic peptides has been described previously (16). Briefly, p53−/− and p53+/+ mice were injected s.c. at the base of tail with 100 μg/mouse SV9 or NP 366 peptides emulsified in IFA. To generate peptide-specific CTLs with RMA-S/B7-1 cells, p53−/− and p53+/− mice were immunized twice i.p. with 1 × 106 peptide-loaded RMA-S/B7-1 cells that had been incubated with peptide at 100–200 μM overnight at 26°C (17). After 7–10 days, spleen cells were removed and restimulated with irradiated normal C57BL/6 splenocytes (2000 rad) in the presence of a low concentration of peptide (50 nM) or peptide-loaded RMA-S/B7-1 cells. After 5–6 days at 37°C, CTL assays were conducted. CTLs were stimulated weekly with peptide-loaded RMA-S or RMA-S/B7-1 cells (15,000 rad) in K medium supplemented with culture supernatants from Con A-stimulated rat spleen cells (termed complete medium). After 3–4 wk, FACS-sorted CD8+ CTLs from mice that had been injected with peptide were distributed into 96-well, flat-bottom or U-bottom microtiter plates at 100, 10, and 1 cell/well. Cells were distributed at 10 or 1 cell(s)/well with cells derived from mice injected with RMA-S/B7-1 cells loaded with either the SV9 or NP 366 peptide. Plates having 1 cell/well were always prepared and analyzed in duplicate. All cloning wells were stimulated weekly with RMA-S or RMA-S/B7-1 stimulator cells loaded

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3Abbreviations used in this paper: wt, wild type; NP, nucleoprotein.
with the appropriate peptides. Clones were expanded in 24-well plates with 5 × 10^5 T cells and 3 × 10^5 stimulator cells/well in 2 ml complete medium. CTL assays were performed using a standard 4-h ^51 Cr release test.

**Cell survival analysis**

The CTL lines obtained from p53^-/-, p53^+/+, and p53^-/- mice were stimulated for 6 days in culture and were subsequently plated in 24-well plates (5 × 10^5 cells/well) without adding stimulator cells. The lines were then exposed to 1000 rad γ-irradiation (^137 Cs) in complete medium. The irradiated and control nonirradiated cells were cultured for 1, 2, 3, 4, and 5 days at 37°C, and cell viability was determined by trypan blue staining. Percent survival was calculated as follows: (number of live cells from an irradiated sample/number of live cells from the corresponding nonirradiated sample) × 100. Each experiment was performed in duplicate.

**Cell proliferation assay**

Approximately 100,000, 50,000, 25,000, 12,500, 6,250, 3,125, 1,563, 781, 391, 195, or 0 cells/well were incubated with 4 × 10^5 SV9-loaded RMA-S/B7-1 cells/well (15,000 rad) for 48 h at 37°C; next, [3 H]thymidine was added to each well (1 μCi/well) for an additional 16 h of incubation at 37°C. Cells were harvested, and specific [3 H]thymidine incorporation was evaluated with a liquid scintillation counter. Each experiment was performed in triplicate.

**Results and Discussion**

The following description is representative of the general strategy we followed to evaluate CTL clonogenic efficiency. At 10 days after mice were injected s.c. with 100 μg of the Sendai virus peptide known as SV9 (FAPGNPYPAL) in IFA, spleen cells were removed and cultured with irradiated C57Bl/6 spleen cells in the presence of a low concentration of the peptide (50 nM). After 6 days in culture, CTL assays revealed that the T cells generated from p53^-/- and p53^-/- mice were indistinguishable in cytolytic activity using SV9-coated EL4 cells as target cells (data not shown). The cultured spleen cells were then restimulated weekly with SV9-loaded RMA-S cells. After 4 wk, virtually all the cells were CD8^+. Cells were then distributed by FACS (using anti-CD8 mAb to mark cells) into 96-well, flat-bottom microtiter plates with 100 cells per well (one plate), 10 cells per well (one plate), and 1 cell per well (two plates).

Initially (at wk 3) no difference between cells from p53^-/+ and p53^-/- mice could be seen by counting the numbers of cells in 10 wells randomly chosen from wells seeded at 100 cells/well (data not shown). Similarly, there was no significant difference between wells that had been seeded with 1 cell/well from mutant and wt mice (e.g., at wk 4, 19 of 120 wells from p53^-/+ mice were scored positive for growth, and 27 of 120 from p53^-/- mice were scored positive). Gradually, however, the cells from p53^-/- mice outgrew those from p53^-/+ mice. For example, as shown in Table I at wk 13 (expt. 1), cell growth was evident in 3% of the wells (4 of 120) from the p53^-/+ mice and in 29% of the wells (35 of 120) from the p53^-/- mice. Moreover, the cells in 15 of 28 wells from the mutant mice could be successfully expanded into 24-well plates (using 5 × 10^5 T cells and 3 × 10^5 stimulator cells in 2.0 ml/well), whereas none of 16 wells from the wt mice could be similarly expanded; this finding suggested that the CTLs from p53^-/- mice were more readily cloned, expanded, and maintained than those from p53^-/+ mice.

The difference between CTLs derived from mutant and wt mice was also reflected in split-well analyses (Fig. 1). At wk 10, cytolytically active wells were more frequent in wells seeded (10 cells/well) with cells from the mutant mice than from the wt mice. Thus, wells with >15% specific lysis (arbitrary cutoff value) were noted with 53 of 60 wells from mutant mice and with 15 of 60 wells from wt mice (Fig. 1). After 36 wk in cloning wells, there was an even greater difference in the number of clones that grew well enough for analysis (see below) and for storage in liquid nitrogen: 11 clones from the p53^-/- mice met these criteria, whereas only 1 such clone was obtained from the p53^-/+ mice (2F1).

In a second study, we compared the yield of clones from p53^-/- and p53^-/+ mice that had been injected once with the influenza virus NP peptide ASNNENMETM (A/PR/8/34 NP 366-374) plus IFA (Table I, expt. 2). After a total of 12 wk in culture (8 wk in cloning wells), we also found that there were four- to fivefold more clones from the p53^-/- mice than from wt mice. Furthermore, clones derived from p53^-/- mice could also be more readily expanded into 24-well plates and maintained in culture (e.g., 10 clones from p53^-/+ mice vs 40 clones from p53^-/- mice could be grown in 24-well plates (2.0 ml/well) from wk 15 to 19 (data not shown)). With further expansion into five such wells, 2 clones

### Table 1. Cloning frequency in p53 wt mutant, and heterozygous mice

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Immunogens</th>
<th>Time in Culture (wk)</th>
<th>Percent Positive&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FAPGNYPAL</td>
<td>4</td>
<td>15.8 22.5</td>
</tr>
<tr>
<td></td>
<td>(SV9)</td>
<td>6</td>
<td>14.1 23.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>6.6 25.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>3.3 25.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>3.0 29.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36</td>
<td>1 11</td>
</tr>
<tr>
<td>2</td>
<td>ASNNENMETM</td>
<td>3</td>
<td>7.2 34.3</td>
</tr>
<tr>
<td></td>
<td>(NP 366-374)</td>
<td>6</td>
<td>8.3 41.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>13.0 59.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>15.1 72.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td>2 12</td>
</tr>
<tr>
<td>3</td>
<td>SV9/RMA-S/B7-1</td>
<td>3</td>
<td>— 30.7 18.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>— 49.4 20.8</td>
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<tr>
<td></td>
<td></td>
<td>9</td>
<td>— 68.2 20.3</td>
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<tr>
<td></td>
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<td>12</td>
<td>— 63.0 19.8</td>
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<td>15</td>
<td>66 32</td>
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<sup>a</sup> Wells of 96-well plates were seeded by FACS at 1 cell/well (CD8<sup>+</sup>) and examined for growth at the times shown, scoring 120 wells (expt. 1) or 192 wells (expts. 2 and 3) at the indicated times.

<sup>b</sup> Average cloning efficiency was calculated on the assumption that wells were seeded with 1 cell/well on average. Hence, the percentage of wells that are positive divided by 63% (expected from the Poisson distribution to correspond to 100% cloning efficiency) is the observed cloning efficiency.

![Figure 1](http://www.jimmunol.org/)
from wt mice and 12 clones from mutant mice were ultimately obtained (Table I, wk 22).

In a third study, we compared the CD8 T cell cloning efficiency from p53\(^{+/+}\) and p53\(^{-/-}\) mice that had been injected with RMA-S/B7-1 cells loaded with the Sendai virus peptide FAPGNYPAL. As shown in Table I (expt. 3), there were threefold more clones from p53\(^{+/+}\) mice than from p53\(^{1/2}\) mice after 12 wk in culture; in addition, in one preliminary trial it appeared that more H-2b anti-H-2d alloreactive T cell clones were obtained from p53\(^{+/+}\) mice than from p53\(^{1/2}\) mice (J.W., unpublished observation). Therefore, from immunization with three or four different peptide-MHC combinations, we conclude that when CD8\(^{+}\) T cells were seeded at 1 cell/well, substantially more T cell clones were obtained from p53\(^{+/+}\) mice than from p53\(^{1/2}\) or p53\(^{1/2}\) mice.

In a preliminary test, we sought to determine whether anti-influenza CTL clones from mutant mice and wt mice had similar cytolytic activity and specificity. We compared six clones from mutant mice with the single clone (8H2) available from wt mice. As shown in Fig. 2, all of the clones lysed EL4 target cells that had been coated with the influenza virus peptide ASNENMETM (NP\(^{366-374}\)); however, clones did not lyse uncoated EL4 cells or those coated with a peptide from adenovirus E1A (SGPSNTPPEI).

Because it was important to examine more closely the cytolytic competence of the clones obtained from the mutant mice, we tested the effectiveness of eight anti-Sendai virus clones from these mice in standard 4-h CTL assays; EL4 cells were used as target cells (at an E:T ratio of 7.5:1), and the Sendai virus peptide was used at concentrations from \(5 \times 10^{-13}\) to \(1 \times 10^{-7}\) M. We found that maximum specific lysis varied from 40 to 60% at peptide concentrations of \(10^{-9}-10^{-11}\) M, and that for half-maximal lysis the peptide concentrations varied from \(~1\) to 50 pM. Similar half-maximal lysis values have been found for CTL clones from wt mice when TCRs have relatively high affinities for cognate peptide-MHC complexes (measured with the TCRs on live cells; Ref. 18). However, it was not possible to compare anti-Sendai virus clones from mutant and wt mice, because only a single clone of wt origin was obtained in this experiment (Table I, expt. 1).

To understand why there was a higher yield of T cell clones from p53\(^{-/-}\) mice than from wt mice, we compared the net growth of bulk CTLs from p53\(^{+/+}\), p53\(^{-/-}\), and p53\(^{-/-}\) mice (Fig. 3A). For this purpose, fresh cultures were initiated with the same number (\(5 \times 10^5\)) of bulk cultured CTLs from p53\(^{+/+}\), p53\(^{1/2}\), and p53\(^{1/2}\) mice. The cells were incubated with \(3 \times 10^6\) irradiated SV9-loaded APCs (RMA-S/B7-1) in 24-well plates. In the representative experiment shown, the number of cells from the p53\(^{+/+}\) cell line (\(1.34 \pm 0.3 \times 10^5\) ml) after 7 days in culture was fourfold lower than the number of cells from the p53\(^{-/-}\) cell line (\(4.52 \pm 1.15 \times 10^5\) ml). The number of cells from the heterozygous p53\(^{1/2}\) cell line was essentially indistinguishable from that obtained from homozygous mutants (Fig. 3A). Proliferation assays

![FIGURE 2. Specificity of CTL clones generated in p53\(^{+/+}\) and p53\(^{-/-}\) mice immunized with the influenza virus peptide (ASNENMETM). Similar results were obtained in three independent experiments.](http://www.jimmunol.org/)

![FIGURE 3. Net growth (A) and proliferation (B) of CTL lines generated from p53\(^{+/+}\), p53\(^{1/2}\), and p53\(^{1/2}\) mice immunized with Sendai virus peptide. A. The error bars refer to the SD. Results are representative of at least three independent experiments. B. Similar results were also obtained in bulk antilymphocytic choriomeningitis virus NP 118–126 peptide (RPQAS GVYM, L\(^{d}\)-binding) and antivesicular stomatitis virus NP 52–59 peptide (RGVYQGL, K\(^{b}\)-binding) CTLs generated in p53\(^{+/+}\), p53\(^{1/2}\), and p53\(^{1/2}\) mice (H-2\(^{d}\) or H-2\(^{b}\) background) (data not shown).](http://www.jimmunol.org/)
(Fig. 3B) conducted with anti-SV9 bulk CTL lines showed that cells from p53⁻/⁻ mice proliferated more actively than those from p53⁺/+ mice. Cells from p53⁺/+ mice proliferated to an intermediate extent (Fig. 3B).

In a preliminary experiment, we also compared mutant and wt CTLs with regard to their susceptibility to γ-irradiation. Anti-SV9 bulk CTLs from p53⁻/⁻ mice were more resistant to γ-irradiation (53% survival on day 5 after irradiation) than those from p53⁺/+ mice (11% survival); cells from p53⁻/- mice were intermediate (30% survival). These results differ from those of others (e.g., Strasser et al. (19)), who found that Con A-activated T cells from p53⁻/⁻ mice are as radiation-sensitive as those from wt mice (19, 20). That T cells stimulated by Con A (19, 20) and those stimulated with anti-CD3 (21) or Ag (the present study) might differ would not be surprising in view of an immense amount of recent data pointing to differences in T cell responses to different stimuli (e.g., to different anti-TCR Abs) (22, 23).

The p53 mutant effect could be due to several possibilities: 1) a lower rate of Ag-stimulated cell death (Table I, Fig. 3A), 2) a lower rate of stress-induced cell death (i.e., cells growing in culture are subject to a form of stress, perhaps due to deprivation of an optimal mix of growth factors), or 3) shortened doubling times leading to increased chances for subsequent mutations. Whether these possibilities explain the substantially greater cloning efficiency of T cells from p53⁻/⁻ mice compared with p53⁺/+ and p53⁻/- mice remains to be seen. Regardless of the explanation(s), our results indicate that efforts to obtain large numbers of T cell clones can benefit from the use of p53⁻/⁻ mice. In this regard, it should be noted that p53⁻/⁻ mice have been used previously to obtain murine NK cell clones, which are difficult to grow in culture when derived from wt mice (24). A long-term culture of lymphohemopoietic stem cells from p53⁻/⁻ mice has also been reported recently (25).

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References