Cancer Therapy: Preclinical

Spleen Cells from Young but Not Old Immunized Mice Eradicate Large Established Cancers

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Abstract

**Purpose:** Solid tumors that have grown two weeks or longer in mice and have diameters larger than 1 cm are histologically indistinguishable from autochthonous human cancers. When experimental tumors reach this clinically relevant size, they are usually refractory to most immunotherapies but may be destroyed by adoptive T-cell transfer. However, TCR-transgenic T cells and/or tumor cells overexpressing antigens are frequently used in these experiments. Here we studied the requirements for destroying clinical size, unmanipulated 8101 tumors by adoptive cell therapy.

**Experimental Design:** 8101 arose in an old mouse after chronic exposure to UV light. A cancer line was established, which was never serially transplanted. The immunodominant CD8+ T cell-recognized antigen of this tumor is caused by a somatic tumor-specific mutation in the RNA helicase p68. 8101 tumors were treated with spleen cells from young naive, or young and old immunized mice to ascertain the characteristics of immune cells that lead to rejection.

**Results:** Here we show that the mutant p68 peptide has an exceptionally high affinity to the presenting MHC class I molecule Kb and that spleen cells from immunized young syngeneic mice adoptively transferred to Rag2−/− or cancer-suppressed euthymic mice eradicate 8101 tumors larger than 1 cm in average diameter and established for several weeks. Spleen cells from naive young mice or from old and boosted (reimmunized) mice were ineffective.

**Conclusions:** Relapse-free destruction of large and long-established tumors expressing a genuine very high-affinity tumor-specific antigen can be achieved by using adoptive transfer of lymphocytes from immunized young individuals.

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Introduction

Most human tumors have reached at least 1 cm in diameter and contain at least 10^9 cancer cells at time of diagnosis. These tumors have been present in the patients for probably many months, if not years (1). However, most experimental tumors used for preclinical studies on the efficacy of immunotherapy do not reach that size, and most strategies fail at later stages of cancer progression (2). Adoptive T-cell therapy stands out as the most effective approach and thus offers the highest promise in a clinically realistic setting (2). In several experimental models, mice receiving syngeneic activated T cells rejected established tumors expressing potent, immunodominant artificial antigens (3–5). We have therefore focused on experimental adoptive cell therapy that could be effective against tumors at least 1 cm in diameter and containing approximately 10^9 cancer cells that express natural tumor-specific antigens arisen at an old age.

Most spontaneous human and animal tumors develop in older individuals (6–9). As people age, they are predisposed to developing cancer due to more mutations in their somatic cells as well as a decrease in their ability to mount effective immune responses against these malignant cells. Furthermore, several studies have shown that aged mice fail to reject similar tumor inocula that are eradicated by younger mice (10–12). These distinct age-related immune responses may be due to differences in costimulation, regulatory T cells (Treg), and/or the generation of effector T cells. As a consequence, tumors in elderly patients are likely to harbor tumor-specific antigens, which are not targeted because of deficits of the immune response.

In recent clinical trials, exogenously stimulated, autologous T cells caused regression of large tumor burdens in a
Translational Relevance

Preclinical models of immunotherapy are often successful at early stages of cancer, but fail when tumors become closer to the level of development at which they would be discovered in a clinical setting. Adoptive T-cell therapy stands out as one of the most successful immunotherapies for cancer. However, the characteristics that determine T-cell efficacy are insufficiently understood. In a genetically nonmanipulated preclinical model of long-established tumors, we found that T cells must be immune against the tumor antigens, and, importantly, come from young individuals. These findings show for the first time that the age of the donor is critical for the efficacy of adoptively transferred cells. Because most tumors develop in old age, this implies that for adoptive T-cell therapy of solid tumors, optimal efficacy might be achieved by T cells harvested from patient-related healthy young donors immunized against the tumor antigens, that is, by individualized immunization and therapy.

fraction of patients (13–17). However, experimental models of adoptive T-cell therapy often reject tumors much more effectively than when similar methods are applied to patients. One primary difference between these 2 observations lies in the age of the donor T cells. The experimental models use T cells often from adolescent or younger donor mice. By contrast, these clinical trials have isolated antitumor lymphocytes from the cancer patient. Because cancer patients are usually at a more advanced age, these donor T cells may not function as well as donor T cells from younger individuals. Furthermore, it is not clear to what extent the presence of a tumor could affect the quality of the T cells obtained from cancer patients. Here we show that adoptive transfer of spleen cells from young but not old immunized mice can eradicate large solid 8101 cancers that have grown for several weeks. These cancer cells express a natural immunodominant target peptide that binds to the presenting MHC class I molecule with nanomolar affinity. These findings suggest that clinically relevant size cancers can be eradicated by adoptive cell therapy also in a more realistic cancer model, and that the age of the immunized lymphocyte donor is critical.

Materials and Methods

Mice, cell lines, and reagents

C57BL/6 and C57BL/6 Rag1−/− mice were purchased from The Jackson Laboratory. B6C3F1 mice were obtained from Charles River Laboratories. C3H Rag2−/− mice were obtained from Douglas Hanahan (University of California, San Francisco, CA). All mice were maintained in a specific pathogen-free barrier facility at the University of Chicago according to the Institutional Animal Care and Use Committee guidelines.

PRO4L was originated in a C3H/HeN mouse and has been previously described (18). 8101 originated in UV-treated C57BL/6 and has been described (19, 20). P. Ohashi (University of Toronto, Toronto, Ontario, Canada), with permission of H. Hengartner (University Hospital Zurich, Zurich, Switzerland), provided the MC57G methylcholanthrene-induced, C57BL/6-derived fibrosarcoma (MC57). MC57-mp68-EGFP (M-mp68) was generated by retroviral transduction. Phoenix-ampho cells (21) were transduced with pMFG-(mp68-AAY)-EGFP using the CalPhos Mammalian Transfection Kit (Clontech). Repeated rounds of transduction of MC57 with viral supernatants and fluorescence-activated cell sorting (FACS) derived the highly peptide/fluorescent protein-expressing line.

pMFG-(mp68-AAY)-EGFP was constructed by inserting annealed oligonucleotides (IDT) encoding triple SNPFVAGI-AAY repeats into the Ncol-linearized (NEB) pMFG-EGFP vector kindly provided by R.C. Mulligan (Children’s Hospital Boston, Boston, MA; ref. 22).

Tumor challenge and treatment

For the experiments in Rag1−/− mice, 107 8101 cells were injected subcutaneously onto the shaved back of mice. Tumor volumes were measured along 3 orthogonal axes (a, b, and c) every 3 to 4 days and tumor volume was calculated as abc/2. Mice were treated intraperitoneally with naive or immune splenocytes (one spleen per recipient, around 1 × 106 cells). For the experiments in euthymic B6C3F1 mice, PRO4L tumors were grown in C3H Rag2−/− mice and were implanted subcutaneously as viable 1 mm3 fragments with a 12-gauge trocar (1 full trocar load) into the left flank of anesthetized B6C3F1 mice. Once PRO4L was established, 8101 tumors grown in C57BL/6 Rag1−/− mice were implanted in the right flank as fragments. Once 8101 was established (for details see Fig. 3A), PRO4L tumor was removed by tying off the tumor at its base ("stringing"). For the generation of memory T cells, 2 × 107 8101 cancer cells were injected subcutaneously into the flanks of B6C3F1 or C57BL/6 mice, and their spleens were used for adoptive transfer.

PCR analysis for mutant p68 expression

Genomic DNA and total RNA were isolated from cancer cell lines using QIAamp DNA mini and RNeasy mini kits. RNA was treated with DNase I (Roche) and reverse transcriptase (New England Biolabs) to synthesize the cDNA. PCR was done on the genomic DNA or cDNA using the following primers: Forward 5-GGGGATCCGCCATGAAGGACGATCGTCGTGACAG-3 and reverse primer 5-AGAATATCCTGTGGCAYTGG-3 amplify a 290 bp fragment only if the mutation is a point mutation at the nucleotide position 1812 of murine p68 RNA helicase. Forward primer 5-GGGGATCCGCCATGAAGGACGATCGTCGTGACAG-3 and reverse primer 5-AGAATATCCTGTGGCAYTGG-3 amplify a 425 bp fragment if the mutation is present. Vectors containing mutant and wild-type p68 minigenes on the pRES-EGFP vector backbone (Clontech) were used as controls.
T-cell analysis in peripheral blood

Percentages of T-cell subpopulations were measured in peripheral blood after lysis of red blood cells. For the determination of absolute numbers of cells, AccuCount Rainbow beads (Spherotech) were used according to the manufacturer’s instructions. For the analysis of the frequency of mp68-specific T cells, old or young immune or naive mice received 7 × 10^6 to 10 × 10^6 8101 or MC57-mp68-EGFP cancer cells and were subsequently bled at days 5, 9, and 19. Analysis before cancer cell injection served to determine the background staining (day 0).

Flow cytometry

Cells were stained using anti-CD3, CD4, CD8, CD44, and anti-CD62L mAb (all from BioLegend or eBioscience). Specific T cells were detected with a mp68-Kb tetramer (NIH Tetramer Core Facility). Treg were analyzed using the mouse Treg staining kit from eBioscience. Flow cytometry data were acquired on FACSAria (BD) or MoFlo-HTS (Beckman Coulter) at the Flow Cytometry Facility of The University of Chicago.

MHC peptide binding assays

MHC purification and quantitative assays to measure the binding affinity of peptides to purified H2-K^b, H2-D^b, and HLA-A*0201 molecules were carried out as previously described (23, 24).

Statistical analysis

Results of treatment of small groups of mice were analyzed using the 2-tailed P value calculated by Fisher exact test using Stata (P ≤ 0.05 is considered significant, P ≤ 0.01 highly significant). Differences between 2 sets of data were analyzed using the Student t test (paired for CD8^+ T-cell populations in the same mouse; unpaired for numbers and percentages of CD4^+ and CD8^+ T cells and Treg in different groups of mice).

Results

Young naive mice, but not an old naive mouse, reject a challenge with 8101 tumor fragments

Fragments of the cryopreserved autochthonous 8101 tumor were adapted to culture and then injected into an athymic nude C57BL/6 mouse (Fig. 1A). Fragments of this tumor were transplanted into a total of 20 naive 2- to 3-month-old euthymic C57BL/6 mice and 1 naive 2-year-old normal euthymic C57BL/6 mouse that we had available in our colony. Most (15 of 20) young naive mice rejected the inocula. We previously reported this group of 20 young naive mice challenged with 8101 tumor fragments with 13 rejecting and 5 growing the inoculum progressively. However, only 1 of these 5 progressing tumors had been analyzed previously to determine whether progressive growth was heritably acquired (19). The remaining 4 progressing tumors are first analyzed here. Also, none of these 5 variants had previously been analyzed by PCR for the mechanism of antigen loss (i.e., at DNA or RNA level). The same results were obtained injecting cryopreserved fragments from the original 8101 tumor directly in naive euthymic young animals (Supplementary Fig. S1). Fragments of the 5 tumors that progressed in young mice were (i) adapted to culture for later analysis and (ii) transplanted into 2-month-old normal C57BL/6 mice, 2 bilateral injection sites per mouse. Fragments of the 8101 tumor grown in the athymic mouse grew in the 2-year-old mouse, and fragments of this tumor were also transplanted into two 2-month-old normal C57BL/6 mice bilaterally (Fig. 1B). In contrast to the inocula of fragments from young mice, these inocula were rejected indicating the tumor that grew in the old host had not lost its antigenicity. We developed a mutation-specific PCR that identifies the single nucleotide substitution causing the immunodominant mutant p68 antigen (Supplementary Fig. S2A). All progressors that grew in young mice retained the mutant gene, except for one that lost the mutant gene but kept the nonmutated p68 (PRO1A). However, analysis at
Spleen Cells from Young Donors Eradicate Large Tumors

Table 1. The mutant p68 peptide binds MHC class I K\(^b\) with an affinity comparable with that of viral peptides that, when used for immunization, protect 100% of mice against a lethal challenge with vaccinia

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence</th>
<th>MHC</th>
<th>Affinity of peptide for MHC (IC(_{50}) nmol/L)(^a)</th>
<th>Geometric SD (times/divide)</th>
</tr>
</thead>
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<tr>
<td>mp68 (547-564, SF)</td>
<td>SNFVFAGI</td>
<td>K(^b)</td>
<td>0.48</td>
<td>2.63</td>
</tr>
<tr>
<td>p68 (547-554)</td>
<td>SNFVSAGI</td>
<td>K(^b)</td>
<td>22.00</td>
<td>2.06</td>
</tr>
<tr>
<td>A23R (597-360)</td>
<td>IGMMNLTFI</td>
<td>D(^b)</td>
<td>0.34(^b)</td>
<td>2.82</td>
</tr>
<tr>
<td>A6L (265-272)</td>
<td>YTLYRQL</td>
<td>K(^b)</td>
<td>6.00(^c)</td>
<td>4.43</td>
</tr>
</tbody>
</table>

\(^a\)IC\(_{50}\) values are the geometric mean of 5 or more experiments.

\(^b\)Vaccinia virus strain Copenhagen protein nomenclature.

\(^c\)Published in ref. 25.

mRNA level (Supplementary Fig. S2B) showed that all the lines derived from young mice were negative for the transcript of the mp68 antigen. By contrast, the tumor that developed in the old mouse and was rejected when retransplanted into young recipients (Fig. 1) had retained expression of the mRNA of the mp68 antigen (Supplementary Fig. S2B, right small panel). Tumors that lost expression of mp68 message were also resistant to lysis by 8101-specific T cells in a \(^{51}\)Cr-release assay (data not shown).

The mp68 peptide binds to K\(^b\) with an extremely high affinity

To further understand why the mutant peptide had to be lost before 8101 could form tumors in young naive mice, we analyzed the affinity of this peptide for the presenting MHC molecule K\(^b\). We found that the mutant peptide bound to K\(^b\) with an IC\(_{50}\) 0.48 nmol/L (Table 1) and is therefore considered to be a very good antigen, comparable in affinity to viral peptides that protect 100% of mice from lethality when used for immunization against vaccinia virus infection (25).

Lymphocytes from young immune but not from naive or old immune mice reject very large tumors

We next designed experiments to determine how effectively spleen cells from naive or immunized young or old mice could destroy clinical size 8101 tumors. The “uncloned” 8101 cell line (Fig. 1) was grown in Rag1\(^{-/-}\) C57BL/6 mice (Fig. 2A). Only 1 of the six 8101 tumor-bearing Rag1\(^{-/-}\) C57BL/6 mice rejected the established tumor when treated with naive young C57BL/6 spleen cells (Fig. 2B). Thus naive spleen cells from young mice are usually ineffective in eradicating established 8101 tumors. By contrast, all twelve 8101 tumor-bearing Rag1\(^{-/-}\) C57BL/6 mice rejected the established tumor when treated with spleen cells from young mice that had been immunized with live 8101 cancer cells. However, 4 mice that received spleen cells from older immunized donors (29-month-old), failed to reject the tumor. Remarkably, spleen cells from the older mice failed to eradicate the tumors even

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**Footnotes:**

4. The average tumor size and duration of growth of 8101 (mean ± SD) at time of treatment was 1,117 ± 336 mm\(^3\) and 41 ± 11 d for the “naive young” group; 1,219 ± 315 mm\(^3\) and 39 ± 7 d for the “young immune”; 1,205 ± 961 mm\(^3\) and 30 ± 6 d for the “old immune”.

**Figure 2.** Adoptive transfer of spleen cells from young immune but not naive or old immune mice leads to the eradication of large established 8101 tumors in Rag1\(^{-/-}\) mice. A, experimental design. B, tumor-bearing Rag1\(^{-/-}\) C57BL/6 mice were treated by adoptive transfer of spleen cells (one spleen per recipient, around 1 \(\times 10^7\) cells) from young naive donors (3- to 4-month-old), young donors (3- to 4-month-old) immunized once with 2 \(\times 10^7\) live 8101 cancer cells at the age of 2 months, or old immune mice (29-month-old) immunized when 4-month-old and boosted 2, 12, and 19 months later. Results are pooled from several experiments, one of which is sharing all 3 experimental groups and 2 sharing the young naive and young immune groups. The ⊕ symbol in the right panel designates a tumor that was reisolated and found to express the mutant p68 gene by reverse transcriptase PCR (RT-PCR).
Old and young immunized mice have similar numbers of mp68-specific T cells after immunization with 8101 cancer cells

We compared the frequency of mp68-specific CD8\(^+\) T cells in young versus old 8101-immune C57BL/6 mice (Fig. 4A). Mice primed with 8101 cancer cells required a secondary challenge (boosting) with the mp68 antigen before an expansion of mutant mp68-specific cells was detectable (day 9 and 19); a mp68-overexpressing MC57 cell line (M-Mp68) was used to make the specific response more prominent. No differences in the frequency of mp68-specific T cells were detected in young compared with old mice. Thus, failure of old T cells to reject 8101 tumors cannot be explained by a lower frequency of mp68-specific CD8\(^+\) T cells.

Young mice have more CD4\(^+\) T cells and respond to immunization with 8101 by increasing the percentage of effector memory cells

We then analyzed overall differences in T-cell subpopulations between young and old mice. Old mice had lower absolute numbers of circulating T cells than young mice, and CD4\(^+\) T cells were the most affected subset. Thus, the ratio CD8\(^+\) :CD4\(^+\) T cell was higher for old mice (Fig. 4B). Furthermore, although percentage of Treg among CD4\(^+\) T cells was increased in old mice, their absolute number was decreased (Supplementary Fig. S4A). Old and young mice, however, differed in the composition of their CD8\(^+\) T-cell pool. Consistent with what has been described before (26), young mice had more naive T cells, whereas the percentage of memory cells in the old mice was higher (Supplementary Fig. S4B). Interestingly, after boosting with the mp68 antigen-expressing cancer cells, the percentage of effector memory CD8\(^+\) T cells increased significantly in young mice (Fig. 4C). In contrast, the percentage of effector memory cells remained unaltered in old mice, and a tendency to increase was observed in the percentage of central memory cells (although not statistically significant).

Discussion

Probably all cancers have mutant genes and express epitopes that are not self. Such epitopes may bind to MHC molecules with high affinity, stimulate immunity effectively, and also serve as targets for effector cells. Some
As we show for the immunodominant tumor-specific antigen of the 8101 cancer, mutant epitopes such as the mutant p68 peptide may bind to MHC Class I molecules with very high affinity (below 1 nM/L). Unlike transfected and overexpressed model target antigens, this antigen originated during tumorigenesis in the autochthonous 8101 cancer. We further show in our study that this antigen was always lost before the cancer could grow in immunocompetent young mice. In contrast, 8101 cancers expressed the antigen in the old mouse in which it originated and in the old mouse receiving tumor fragments. Antigen-negative variants were probably present in tumors growing in both young and old hosts; however, only in young mice immunologic pressure selected for p68-negative variants, whereas in the old mice the variants remained a minority. Because the majority of common cancers are first diagnosed in older individuals, most human cancers may have retained strong antigens such as p68. Thus, our results are consistent with the possibility that old age should favor retention of strong rejection antigens.

Two conditions needed to be fulfilled by the donor of immune cells for successful therapy of 8101: to be young and to be immunized against the tumor being treated (truly individualized immunization and therapy). How could these conditions be fulfilled in patients?

Adoptively transferred cells should come from young donors. The age of the individual at the moment of immunization is known to be critical for effective vaccination (35). However, we did not find preclinical studies comparing the efficacy of lymphocytes from old and young donors in adoptive transfer. Our studies show that even when the first immunization took place when donors were young, immune lymphocytes from aged mice lost their efficacy for treatment of tumors upon adoptive transfer into young hosts. Interestingly, our cut-off point for age of donors was 9 months, which corresponds to a middle-age mouse. Most cancer patients eligible for adoptive T-cell therapy might also be between 40 and 60 (13–17). Importantly, T cells must be effective in the “old” environment of the patient (36). Our experiments tested the efficacy of transferred T cells only in young tumor-bearing hosts, and future experiments need to test whether adoptively transferred young immune spleen cells can be effective in old tumor-bearing mice. Previous studies (36) indicated that adoptively transferred young T cells proliferate poorly in the environment of old hosts. This problem could possibly be overcome by treating the old host with anti-type 1 IFN antibodies as suggested by Sprent and colleagues (36).

We immunized tumor-free syngeneic mice for adoptive transfer. Finding tumor-free human donors who are syngeneic will be impossible (unless an identical twin was available). Haploidentical patient-related tumor-free donors are more readily available and younger if they are children. It needs to be explored how lethal graft-versus-host effects by T cells from such donors can be circumvented. However, the success of strategies as allogeneic Epstein-Barr virus (EBV) nuclear antigen (EBNA)-specific T cells sharing major MHC allele with the patient, which
can treat successfully posttransplant lymphoproliferative disease (37) and even bulky EBV-positive lymphomas (38, 39), sets grounds for hope.

Why do old splenocytes fail? When we compared T-cell compartments in old and young mice, we found 2 main differences: old mice (i) had less T cells (especially CD4+ T cells) and (ii) did not increase the percentage of effector memory CD8+ T cells after boosting with the mp68 antigen, in contrast to young mice. This is consistent with the reduction in turnover observed in memory CD8+ T cells from aged mice (36). Proliferation and infiltration of effector cells must happen for tumor rejection. CD4+ T cells have been shown to be essential for expansion of memory cells (40), for tumor infiltration by CD8+ T cells (41), and optimal function of CD8+ T cells at the effector phase (41, 42). Aged CD4+ T cells form defective immunologic synapses (43). Thus, a defective response of the memory CD8+ T cells and ineffective help by CD4+ T cells could explain why old immune splenocytes failed to reject 8101 tumors.

Young naive euthymic splenocytes protect B6C3F1 mice against a 8101 tumor challenge. However, once the tumor is well established, transfer of naive spleen cells is no longer effective. Studies of the immune response to sporadic cancers expressing SV40 T antigen suggest that once the cancer is established, it is no longer immunizing but tolerizing (44). These cancers lack the proinflammatory type I cytokine environment caused by the initial injury of fragment or tumor fragments and a primary cell line. Also, the cancers we treated were truly long-established in the host. A systematic analysis of recent studies confirms the century old assertion (50) that many procedures are effective early after cancer cell inoculations but not later (2). Adoptive T-cell transfer was singled out as the most effective approach at later stages, consistent with findings of clinical studies. But even for adoptive T-cell therapy, we show here, stringent requirements must be fulfilled to eradicate clinically relevant tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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