Cancer Therapy: Preclinical

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

Karin Schreiber1, Ainhoa Arina1, Boris Engels1, Michael T. Spiotto2, John Sidney6, Alessandro Sette6, Theodore G. Karrison3, Ralph R. Weichselbaum2,4,5, Donald A. Rowley1, and Hans Schreiber1

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 Rag−/− 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. Clin Cancer Res; 18(9); 2526–33. ©2012 AACR.

Introduction

Most human tumors have reached at least 1 cm in diameter and contain at least 10⁹ 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 10⁹ 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...
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 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.

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.

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 × 108 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 QiAmp 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-GGGGATCCCGCCATGAAAGGACGATCGTCCGTGACAG-3 and reverse primer 5-AGAATTCTGTGCCATGG-3 amplify a 425 bp fragment of the murine p68 RNA helicase. Forward primer 5-GGGGATCCCGCCATGAAAGGACGATCGTCCGTGACAG-3 and reverse primer 5-AAGAATTCTGTGCCATGG-3 amplify a 123 bp fragment of the p68 RNA helicase. Forward primer 5-AAGAATTCTGTGCCATGG-3 and reverse primer 5-GGGGATCCCGCCATGAAAGGACGATCGTCCGTGACAG-3 amplify a 290 bp fragment only if the mutation is present. Vectors containing mutant and wild-type p68 minigens 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 $7 \times 10^6$ to $10 \times 10^6$ 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 FACS caliber or FACS Canto machines (BD) and data were analyzed using FlowJo (Tree Star) software. Cell sorting was done using FACS Aria (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-Kb, H2-Db, 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 ($P \leq 0.05$ is considered significant, $P \leq 0.01$ 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 naïve mice, but not an old naïve 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 naïve 2- to 3-month-old euthymic C57BL/6 mice and 1 naïve 2-year-old normal euthymic C57BL/6 mouse that we had available in our colony. Most (15 of 20) young naïve mice rejected the inoculum. We previously reported this group of 20 young naïve 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 naïve 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
from the older mice failed to eradicate the tumors even old), failed to reject the tumor. Remarkably, spleen cells from older immunized donors (29-month-old) with live 8101 cancer cells. However, 4 mice that received spleen cells from young immune mice rejected the established tumor when treated with naive young C57BL/6 spleen cells (one spleen per recipient, around 1 × 10^8 cells) from naive or old immune mice leads to the eradication of large established 8101 tumors in C57BL/6 mice when treated with spleen cells from naive 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 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".

**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 if they were resistant to lysis by 8101-specific T cells in a 51Cr-release assay (data not shown).

**Table 1.** The mutant p68 peptide binds MHC class I Kb 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 (IC50 nmol/L)(a)</th>
<th>Geometric SD (times/divide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mp68 (547-554, 5F)</td>
<td>SNFVFAGI</td>
<td>Kb</td>
<td>0.48</td>
<td>2.63</td>
</tr>
<tr>
<td>p68 (547-554)</td>
<td>SNFVSAGI</td>
<td>Kb</td>
<td>22.00</td>
<td>2.06</td>
</tr>
<tr>
<td>A23R (297-306)</td>
<td>IGFMNLTFI</td>
<td>Db</td>
<td>0.34c</td>
<td>2.82</td>
</tr>
<tr>
<td>A6L (265-272)</td>
<td>YTLIRYQL</td>
<td>Kb</td>
<td>6.00c</td>
<td>4.43</td>
</tr>
</tbody>
</table>

\(a\)IC50 values are the geometric mean of 5 or more experiments.

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

\(c\)Published in ref. 25.
Clinical Cancer Research
Clin Cancer Res; 18(9) May 1, 2012

虽然3/4的肿瘤用年轻免疫细胞治疗，肿瘤细胞从老免疫化小鼠中被成功抑制，但老免疫化的小鼠的肿瘤平均体积明显小于年轻的肿瘤。这种现象表明，老免疫化的小鼠的肿瘤细胞对年轻的肿瘤的抑制作用更强。

我们进一步研究了老免疫化的小鼠的肿瘤细胞对年轻的肿瘤的抑制作用。结果显示，老免疫化的小鼠的肿瘤细胞的杀伤作用比年轻的肿瘤细胞更强。

讨论

虽然所有的肿瘤都有异常基因和非已知的抗原，但这些抗原可能与已知的抗原不同。这些抗原可能与已知的抗原不同。这些抗原可能与已知的抗原不同。
tumor-specific somatic mutations affect genes expressed on the surface of cancer cells and are recognized by tumor-specific antibodies (27–29). The fact that most tumor-specific somatic mutations, however, seem to affect genes not expressed on the surface membrane of cancer cells (20, 30–32) should not matter, as such antigens could be effectively presented as mutant peptide/MHC molecule complexes on the surface of cancer cells or after cross-presentation on the surface of stromal cells in the tumor (3, 33, 34).

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 nmol/L). Unlike transfected and overexpressed model target antigens, this antigen originated during tumorigensis 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 mp68-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 mp68. 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 I 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 spleenocytes 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.

**References**


**Grants Support**

The work was supported by NIH grants R01-CA37516, P01-CA74182 and R01-CA22677, by the University of Chicago Cancer Center grant CA-14599, and in part by the Ludwig Center for Metastasis Research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 14, 2012; revised March 2, 2012; accepted March 8, 2012; published OnlineFirst March 13, 2012.

**Authors’ Contributions**

**Conception and design:** K. Schreiber, A. Arina, B. Engels, A. Sette, D.A. Rowley, H. Schreiber

**Development of methodology:** K. Schreiber, A. Arina, B. Engels, M.T. Spiotto

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** K. Schreiber, A. Arina, B. Engels, M.T. Spiotto, J. Sidney, A. Sette

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** K. Schreiber, A. Arina, B. Engels, J. Sidney, A. Sette, T. Karrison, R.R. Weichselbaum, H. Schreiber

**Writing, review, and/or revision of the manuscript:** K. Schreiber, A. Arina, B. Engels, J. Sidney, A. Sette, R.R. Weichselbaum, D.A. Rowley, H. Schreiber

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** K. Schreiber, J. Sidney, H. Schreiber

**Study supervision:** A. Sette, H. Schreiber

The authors thank Jonathan Schneck for his help with quantifying mp68-specific T-cell responses and the NIH Tetramer Core Facility for providing the mp68-K\(^d\) tetramer.

**Acknowledgments**

The authors thank Jonathan Schneck for his help with quantifying mp68-specific T-cell responses and the NIH Tetramer Core Facility for providing the mp68-K\(^d\) tetramer.

Downloaded from clincancerres.aacrjournals.org on April 14, 2017. © 2012 American Association for Cancer Research.
Spleen Cells from Young Donors Eradicate Large Tumors


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

Karin Schreiber, Ainhoa Arina, Boris Engels, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-0127

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/03/13/1078-0432.CCR-12-0127.DC1

Cited articles
This article cites 48 articles, 28 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/9/2526.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/18/9/2526.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.