Exuberated Numbers of Tumor-Specific T Cells Result in Tumor Escape

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Abstract

Cytotoxic T cells (CTL) play a major role in tumor rejection. Expansion of CTLs, either by immunization or adoptive transfer, is a prominent goal in current immunotherapy. The antigen-specific nature of these expansion processes inevitably initiates a clonotypic attack on the tumor. By injecting an Ovalbumin-expressing melanoma into OT-I mice, we show that >90% of CTLs recognize an Ovalbumin peptide, which shows that an increased number of tumor-specific CTLs causes emergence of escape variants. We show that these escape variants are a result of antigen silencing via a yet undetermined epigenetic mechanism, which occurs frequently and is spontaneously reversible. We further show that an increase in the time of tumor onset in OT-I compared with C57BL/6J is a result of immune selection. [Cancer Res 2008;68(9):3450–7]

Introduction

In the field of tumor immunology, two approaches are currently predominant, active specific and passive specific. The common denominator of these approaches is the endeavor to increase the number of tumor reactive T cells (1). Much effort has been directed at finding tumor-associated antigens (TAA) as targets for immunotherapy and indeed an extensive depository of those has been described in several studies (2). Because in both types of approaches, the heuristics are bottom-up, they both tend to evolve around a well-defined epitope and very often one extensively studied peptide.

The numbers of T cells that have been successfully induced in recent clinical trials of active specific approaches are significant and may reach 3% of CD8+ T cells, with an average increase of 45-fold (3). In passive-specific modalities, as many as 16 × 106 expanded tumor-infiltrating T cells have been transferred to lymphoablated patients, and these cells give rise to clones that constitute up to an impressive 68% of the total CD8 count (by Vβ staining; ref. 4). The underlying rationale is that more is better and current protocols focus on increasing the numbers of tumor-reactive CD8 T cells and their persistence in patients.

The flip side of the coin in cancer immunotherapy is the ability of the tumor to change in response to selective pressure induced by the immune system: a process called immune editing (5). According to this theory, the attack of the immune system on the tumor also drives immune escape by selectively killing tumor cells that express the recognized epitopes, whereas ignoring the ones that it does not. There are reports of variants emergence after therapy (4), and indeed, each metastasis (presumably originating from one detached cell) may differ from the primary tumor. Because the effect of the immune system is not only in killing the tumor but also in promoting escape variants, it stands to reason that the more pressure exerted on the tumor, the more likely is variant formation. Thus, in increasing the number of TAA-specific T cells in hope of therapy, the immunologist may very well be promoting tumor escape.

In this study, we show that established tumors expressing an immunodominant TAA cannot be treated by cell transfer of activated TAA-specific T cells. Moreover, we show that these tumors can grow in immunocompetent hosts in which >90% of the CD8 T cells are TAA-specific and that the mechanism by which they evade the immune system is epigenetic silencing of the TAA gene. We would therefore caution cancer immunologists to direct as much attention to diversifying the antitumor immune response as is directed at augmenting it against preselected TAs.

Materials and Methods

Mice and cell lines. B16.M05 (MO5) is B16 melanoma cell line transfected with the Ovalbumin gene (6). B16 and MO5 were cultured in DMEM with 10% FCS, 2 mmol/L glutamine, 25 mmol/L HEPES, 0.1% combined antibiotics, and 2 mg/mL. Neomycin for the MO5 only. B3Z, a T-cell hybridoma specific for the Ovalbumin-SIINFEKL peptide in H-2Kb context (7), were cultured in RPMI 1640 with 10% FCS, combined antibiotics, and 2 mmol/L L-glutamine. CTLL, an interleukin (IL)-2–dependent T-cell line, was maintained in RPMI 1640 with 10% FCS, 2 mmol/L glutamine, combined antibiotics, 1 mmol/L sodium pyruvate, 25 mmol/L HEPES, 5 × 10−5 mol/L 2-Mercaptoethanol, and 1% NEAA, added with 30% Concanavalin A supernatant.

C57BL/6j and B6SJL mice were obtained from the Jackson Laboratory. OT-I mice were a kind gift from Prof. Michael Bevan (University of Washington, Seattle, Washington). OT-I mice were bred by crossing with C57BL/6j and screening for T-cell receptor (TCR) Vα2. B6SJL-OT-I mice were produced by crossing OT-I and B6SJL and screening for Vα2 and CD45.1. A homozygotic colony was obtained by inbreeding of positive B6SJL-OT-I.

In vivo manipulations—tumor injection and adoptive transfer. Tumor injections in footpads or s.c. were performed in 50 μL PBS (>90% live cells by eosin staining). Tumor size was determined using calipers.

Fluorescence-activated cell sorting. MO5 grown in OT-I mice were excised and single-cell suspension was prepared by incubation with 2 mg/mL Collagenase D and 15 u/mL DNase I for 1 h at room temperature with shaking. Single-cell suspension was obtained by passing cells through a 40-μm cell strainer (BD Falcon). Cells were stained with anti-IL-2 antibodies (supernatants from clone 20-8-4) and FITC-goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.).

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Detection of Ovalbumin. MO5 tumors grown in OT-I mice were excised and minced. TRI reagent (Molecular Research Center, Inc.) was used to purify RNA and genomic DNA. cDNA was prepared from RNA by conventional reverse transcription-PCR. PCR for the Ovalbumin transcript was performed with the primers GGGGAAACACATCTGCCAAA and GGCCTCATCGGGCGCAAG. A PCR reaction was performed in 25 μL, and 5 μL were removed at the indicated cycles. As controls, cDNA from MO5 grown in C57BL/6J and in tissue culture were used as well as pcDNA3-Ovalbumin.

PCR for the ova gene was performed with the primers GCTGGGTG-GTGGTCATCT and TGCTTGTGTGTCTTCATCCTT to yield fragment containing a portion of the β-actin promoter used to express the gene in MO5 cells.

CTLL/B3Z assay was performed by coculturing 4 × 10⁴ C2 cell and 2.5 × 10⁵, 5 × 10⁵, or 10⁶ MO5 cells from tumors grown in OT-I (single-cell suspension prepared as described above). The following controls were used in the same amounts: MO5 from tissue culture and MO5 grown in C57BL/6J mice, also RMA-Ovalbumin and RMA both grown in tissue culture. After 24 h of incubation, supernatants were used to sustain a CTLL cell culture for 24 h. CTLL cells were added with ³H-Thymidine and incubated overnight. ²H-Thymidine uptake was detected using MATRIX96 β-counter (Packard Instrument Co., Inc.). Arbitary units were calculated as percent from maximum CTLL proliferation obtained by incubation with supernatants from B3Z incubated with 10 μg/mL SIINFEKL peptide (7).

Cytotoxicity assays. Cytotoxicity assays were performed as previously described (8, 9) with the following modifications. Splenocytes were sensitized for 48 h with 10 μmol/L peptides. Varying amounts of Lympholyte-M (Cedarlane Laboratories Ltd.) separated spleen cells were admixed with 5 × 10⁵ [³⁵S]-Methionine-labeled target cells in a 96-well microplate. Cells were incubated for 16 h and then 50 μL of the supernatants were added with MicroScint-40 (PerkinElmer). Luminescence resulting from radioactive material decay in the supernatants was measured by TopCount Luminescence counter (Packard Instrument Co., Inc.).

Results

Adoptive transfer of TAA-specific T cells does not alter tumor growth rate. The Ovalbumin-transfected B16.F1 melanoma cell line B16.MO5 (MO5) was shown to express significant levels of the Ovalbumin protein (6). An 8-mer peptide product of Ovalbumin degradation, SIINFEKL, was shown to bind MHC class I of type H-2Kb with very high affinity (1.56 × 10⁻⁹ mol/L; see ref. 10). In the OT-I TCR transgenic mice, >90% of the CD8 T cells express a transgenic TCR specific for the H-2Kb-SIINFEKL complex (11). These T cells efficiently lyse MO5 tumor cells in vitro and in various preclinical models (6, 12, 13).

To ascertain the validity of MO5 as a target for OT-I derived CTL, we performed PCR for the Ovalbumin transcript. The Ovalbumin transcript was found in MO5 cDNA but not in parental B16.F1 (Fig. 1A). The second required component, namely the expression of H-2Kb, was validated by fluorescence-activated cell sorting (FACS) analysis of MO5 cells grown in tissue culture (data not shown) and MO5 injected s.c to C57BL/6J (Fig. 1B, bottom).

To test the amount of T cells required to reject an established MO5 tumor, we injected C57BL/6J mice with 10⁶ MO5 cells intra-footpad at three different time points. When the average tumor diameter reached ~6 mm, the mice were lymphodepleted by total body irradiation and divided into two cohorts—small tumor and large tumor (3.9 ± 0.9 and 7.2 ± 1.4 mm footpads diameters, respectively). One day later, each cohort was randomly divided into
B6.SJL.OT-I TCR transgenic mice (5–6 mice per group). The experiment was terminated >150 d after tumor injection (>100 d after the appearance of the last tumor). Dark and light gray bars, two repeats. ND, not determined.

B. 5 × 10⁴ MO5 tumor cells were injected to B6.SJL.OT-I and C57BL/6j mice (6 mice per group), and tumor growth was monitored over time.

Figure 2. Tumor take of TAA-transfected tumor in anti-TAA TCR transgenic mice is dose dependent and occurs after a lag. A. MO5 tumor cells were injected s.c. at different doses to B6.SJL.OT-I mice (5–10⁰, 10⁴, 5–10⁵, and 5 × 10⁵). As a control, 5 × 10⁴ or 10⁶ MO5 were injected s.c. to C57BL/6j mice (5–6 mice per group). The experiment was terminated >150 d after tumor injection (>100 d after the appearance of the last tumor). Dark and light gray bars, two repeats. ND, not determined.

B. 5 × 10⁴ MO5 tumor cells were injected to B6.SJL.OT-I and C57BL/6j mice (6 mice per group), and tumor growth was monitored over time.

Mechanisms of tumor escape. Because the MO5 tumor cells express the Ovalbumin gene and because >90% of OT-I CD8 T cells are specific for the Ovalbumin peptide, it was evident that some escape mechanism was used by the tumor, otherwise it would have been destroyed by the immune system. Several tumor escape mechanisms were extensively reported, these include inhibition of protein degradation and presentation modulation (14), Fas expression (15), cytokine secretion, MHC down-regulation, and antigen loss (16). Initially, we tested whether the tumor expresses the MHC allele H-2Kb by FACS analysis. Both MHC class I H-2Kb and H-2Db were expressed at moderate to high levels in MO5 grown in C57BL/6j mice as well as in OT-I mice (Fig. 1B). We then tested expression of the Ovalbumin gene by semiquantitative PCR. Equal volume samples were removed at various cycles of a PCR reaction for the Ovalbumin transcript. Figure 3 shows that the Ovalbumin mRNA was found in MO5 grown in C57BL/6j (cycle 25), in MO5 grown in tissue culture (cycle 30), and that the transcript was completely absent in the MO5 grown in OT-I. This absence was persistent through the more stringent 35th cycle. A highly sensitive bioassay was then used in which the B3Z T-cell hybridoma, specific for the H-2Kb-SIINFEKL complex, was incubated with MO5 tumor cells grown in OT-I or C57BL/6j or in tissue culture. Upon activation of B3Z cells, they secrete IL-2 that can be assayed by the proliferation of the IL-2-dependent T-cell line CTLL. IL-2 secretion correlates with relative amounts of the H-2Kb-SIINFEKL complexes (7). This sensitive assay further confirmed that the Ovalbumin peptide was not expressed on OT-I–grown MO5 (Fig. 3B). Interestingly, C57BL/6j-grown MO5 showed markedly higher expression of the β-actin promoter–driven Ovalbumin gene compared with tissue culture–grown MO5. This may be attributed to the rapid rate of cell proliferation in vivo, a process that involves up-regulation of cytoskeletal components.

Because the cells used in the assays described grew in OT-I mice, it is reasonable that they escaped the OT-I clonogenic CD8 T cells and became impervious to the activity of OT-I CTLs. The alternate option is that the T cells in OT-I mice in which MO5 tumors developed are somehow deficient. It has been shown that the tumor has many potential adverse effects on the effector arm of the immune system. Such functional impairments of CD8 T cells have been shown to result from constant presence of antigen that
renders the CTLs exhausted (17). To test both the prowess of CTLs from MO5-bearing OT-I as effectors and the susceptibility of OT-I–grown MO5 as targets, we performed a cytotoxicity assay in which effectors were splenocytes from OT-I, which received a high dose or a low dose of MO5 cells, and in which tumor either developed or did not develop (tumor-bearing OT-I and tumor-rejecting OT-I, respectively). The targets in this assay were MO5 grown in C57BL/6J mice, in OT-I mice, and in tissue culture. Background cytotoxicity was measured against the Ovalbumin-negative B16.F1 melanoma. Two phenomena are evident from this assay: (a) CTLs from MO5-bearing OT-I are as effective as MO5-rejecting OT-I at lysing tissue culture MO5 targets and (b) MO5 grown in OT-I were not lysed by either type of effector (Fig. 3C).

In summary, although OT-I CTLs are capable of lysing MO5 melanoma, these tumors develop in OT-I mice, express H-2Kb normally, but have lost all expression of the Ovalbumin transcript.

Epigenetic silencing of a TAA gene as a result of selective pressure applied by T cells. The disappearance of the Ovalbumin TAA at the mRNA level in tumors grown in OT-I mice but not in C57BL/6J mice suggested that the expression of the gene was lost.

Figure 3. TAA gene expression is lost when tumor grows under T-cell selective pressure. MO5 tumor cells (5 × 10⁶) were injected into OT-I mice s.c. When tumor reached a volume of ~ 1 cm³, it was removed and single cells were separated. A, PCR for Ovalbumin transcript performed on cDNA from equal amounts (2 μg) of RNA from all sources. Samples were removed at the indicated PCR cycles. B, supernatants from coculture of B3Z hybridoma and targets from different sources were used to grow IL-2–dependent CTLL cells. IL-2 arbitrary units correlate with relative amounts of H-2Kb*SIINFEKL complexes (17). Shown are two repeats with two amounts of targets (B6, C57BL/6J; TC, tissue culture). C, MO5 from different sources were collected as in B and used as targets in a cytotoxicity assay in which effectors were T cells from either MO5 tumor-bearing mice or those that rejected small doses of this tumor (E:T ratio, 100:1, 50:1, and 25:1). PCR at 30 cycles repeated 8 times. B3Z/CTLL assay repeated more than five times. CTL assay repeated thrice.
due to a selection process mediated by the TAA-specific T cells in the OT-I mouse. As the only relevant difference between OT-I and C57BL/6J mice is the diversity of the TCR repertoire of their CTLs, it is likely to assume that CD8 T cells are the culprit. The time period required for formation of a palpable tumor in OT-I mice, which was at least 10 days longer than that in C57BL/6J mice combined with the fact that 100% of OT-I mice injected with \(5 \times 10^5\) MO5 cells developed the tumor, led us to believe that the process by which the escape variants emerged was a relatively straightforward one (rather than a rare event). We therefore first sought to determine whether the gene itself was partially or fully deleted from the genome. To test this, genomic DNA was prepared from MO5 grown in OT-I, and a genomic PCR was performed to detect the Ovalbumin gene. The full-length gene was detected using primers spanning the \(\beta\)-actin promoter and the end of the open reading frame (Fig. 4A). Cells from the same source as was used for genomic DNA preparation were cultured \textit{in vitro} (without selection antibiotics) for 2 weeks. After this time period, we prepared cDNA from these cells and performed a PCR for the Ovalbumin transcript. Surprisingly, the transcript reappeared after 2 weeks of tissue culture without any intervention (Fig. 4B). This re-expression of the TAA transcript adds to the belief that immune escape by variant formation is a relatively trivial feat by the tumor, in contrast to rare events such as gene deletion.

\textbf{Equilibrium phase is a dominant temporal factor in the processes involved in immune editing.} The process of immune editing is comprised of three stages: Elimination, Equilibrium, and Escape (5, 18). The lag in tumor appearance in OT-I compared with C57BL/6J suggests that at the initial stages of tumor development, Elimination was dominant. The OT-I (and C57BL/6J) mice to which the tumor was injected were naïve at the time of injection. The aforementioned growth lag duration is comprised of the Elimination phase (the time required for processes of antigen presentation and subsequent T-cell activation), Equilibrium, and Escape (emergence of antigen-silencing variants). To test which phases contribute to the duration of this lag, we immunized OT-I and C57BL/6J mice with RMA-S cells loaded with SIINFEKL peptide, MUT-D irrelevant peptide, or not immunized (six mice per group). Ten days after the last immunization, \(10^5\) or \(5 \times 10^5\) MO5 were injected s.c. to C57BL/6J and OT-I, respectively. The number of tumor-free mice at the end of the experiment (day 98) is presented.

\begin{table}[h]
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\begin{tabular}{|l|l|l|}
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\textbf{Strain} & \textbf{Immunization} & \textbf{Tumor-free mice} \\
\hline
C57BL/6J & None & 0/6 \\
& RMA-S/SIINFEKL & 6/6 \\
& RMA-S/MUT-D & 1/6 \\
OT-I & None & 0/6 \\
& RMA-S/SIINFEKL & 4/6 \\
& RMA-S/MUT-D & 1/6 \\
\hline
\end{tabular}
\caption{Table 1.}
\end{table}

\textbf{NOTE: Immune activation of TAA-specific CTLs protects from variant formation.} OT-I and C57BL/6J mice were immunized thrice at weekly intervals with RMA-S cells loaded with SIINFEKL peptide, MUT-D irrelevant peptide, or not immunized (six mice per group). Ten days after the last immunization, \(10^5\) or \(5 \times 10^5\) MO5 were injected s.c. to C57BL/6J and OT-I, respectively. The number of tumor-free mice at the end of the experiment (day 98; 40 days after the appearance of the last tumor).

All nontreated control mice had 100% tumor take. However, although all C57BL/6J mice were protected from the tumor, only 67% of preimmunized OT-I mice were protected. In preimmunized OT-I mice where tumors grew, they became palpable 30 days after the appearance of the first tumor in the control OT-I group and 37 days after the control C57BL/6J group. In this setup, the initial processes involved in Elimination—antigen presentation and T-cell activation—occurred before tumor challenge but the lag increased. When tumor grew in OT-I, its growth kinetics were similar to those in C57BL/6J mice, implying that the Escape phase was not the time-consuming phase.

The fact that tumor did grow in two of the six mice in this group shows that escape variants can arise even in the presence of a preactivated immune system. This negates the claim that escape variants can be overcome simply by improving the efficacy of TAA-specific T cells.

\textbf{TAA-silencing events occur in vivo.} To maintain the Ovalbumin plasmid, MO5 melanoma cells were constantly kept under antibiotics (Neomycin) selection in our laboratory. It is however possible that the effect of gene silencing occurs \textit{in vitro} and is restricted, for some reason, to the Ovalbumin gene. To rule out this

\begin{figure}[h]
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\includegraphics[width=0.8\textwidth]{figure4.png}
\caption{Figure 4. Tumor cells grown \textit{ex vivo} re-express the TAA Ovalbumin (Ova) mRNA. MO5 was injected s.c. to OT-I and C57BL/6J (\(5 \times 10^5\) and \(10^5\) cells, respectively) tumors were removed at \(\sim 1\) cm² and DNA and RNA were used for PCR. A, genomic PCR for the Ovalbumin full-length gene. B, PCR for the Ovalbumin transcript after 2 wk in tissue culture. B6, C57BL/6J. TC, tissue culture; 1 and 2, repeats. DDW, double distilled water. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Shown are two repeats.}
\end{figure}
possibility, we recloned the MO5 to yield subclones. Clone MO5.17 was selected for its expression of Ovalbumin mRNA, stable expression of H-2K\(\beta\), and uniform growth in C57BL/6J mice (data not shown). This clone was used shortly after its subcloning to ensure its monoclonal nature. Similarly to the parental MO5, low passage MO5.17 grown in OT-I mice with 5 \(\times\) 10\(^3\), 10\(^4\), 5 \(\times\) 10\(^4\), 5 \(\times\) 10\(^5\), and 10\(^6\) MO5 cells s.c. Figure 5B shows that the monoclonal MO5.17 tumor take was also dose dependent and reached 100% take at 5 \(\times\) 10\(^5\) as did the parental MO5. Moreover, the lower dose of 5 \(\times\) 10\(^3\) gives rise to escape variants in more mice than the parental MO5. This shows that the variant selection process occurs in vivo and is not a special case of tissue culture handling.

Discussion

Antitumor immune therapies rely on the fundamental immunologic principle of antigen recognition. Because this step is a prerequisite for any specific immune response, much effort has been directed at finding novel antigens and demonstrating their merit as immunogens (reviewed in ref. 19). The bulk of the effort in preclinical as well as clinical trials has been directed at immune monitoring, i.e., assessing the immune (T cell) aspect of the response, often neglecting the tumor aspect. Thus, simultaneous monitoring of the immune response and the tumor antigen profile is uncommon. When this is in fact done, insights into treatment nonresponsiveness prove most informative (4).

In this study, we examine the behavior of the tumor in a scenario where the variables by which the immune system is controlled are at their extremes. We believe that this setup shows the full potential of the immune system in fighting cancer and, therefore, can be used to pinpoint the inevitable pitfalls. The well-characterized TAA in this model system is the SIINFEKL peptide from the Ovalbumin protein, which is stably expressed in MO5 melanoma. This xeno-super antigen is not tolerized against in C57BL/6J and in OT-I TCR transgenic mice and is the target of most the CD8 T cells in the latter.

A major objective for tumor immunology is increasing the number of activated TAA-specific T cells, which serve as the effector arm of the immune system. The predominant zeitgeist is that more is better. Research that follows the expansion and migration of TAA-specific T cells in tumor-bearing mice triumphantly quote \(\sim\) 500-fold increase in the number of these cells upon in vivo stimulation (20) but concomitantly contrast this with the arguably low number of cells required for rejection of large tumors (4), an apparent self-contradiction.

We show here that by increasing the numbers of TAA-specific CD8 T cells, we could not mediate rejection of an established tumor. Indeed, increasing doses of these T cells up to 10\(^7\) cells did not result in tumor rejection regardless of tumor size (the smallest footpad diameter at time of T-cell transfer was 2.6 mm). This is despite the fact that the transferred cells persisted and proliferated extensively for at least 14 days (until the animal was euthanized) as measured by 5,6-carboxylfluorescein succinimidyl ester dilution (data not shown) and in contrast to the reported correlation of increased persistence with tumor regression (21–24).

In clinical trials, the proportion of TAA-specific T cells can be as high as 3%, as determined by Melan-A analogue peptide\(26–35\) multimer staining (3), or 8.89% by gp100\(239–243\) tetramer staining (25). However, one can claim that further increase in the amount of TAA-specific T cells would eventually cause tumor rejection—that is the premise of antitumor immunology. Therefore, in lieu of increasing the doses of transferred cells, we injected the tumor to OT-I mice where the frequency of TAA-specific T cells is approximately one, with the expectation that the tumor would fail to grow. It should be noted that the rejection of a newly injected tumor is a much simpler task than that of rejecting a well-established and vascularized one (26). Surprisingly, the MO5 tumor...
grew in OT-I mice s.c. This growth was dose dependent and reached 100% at $5 \times 10^5$ cells.

A prominent difference between the model system described and clinical situations is that in the latter case, tumors that do arise have already evaded the immune system. In our model, we have a priori knowledge that the tumor expresses a TAA that is recognized by all of the CD8 cells. Moreover, the xeno-super antigen TAA is acted upon by the immune system as is evident by the lag of tumor appearance between C57BL/6j and OT-I mice.

The fact that a tumor grows in an animal in which virtually all CTL are directed against it may present a paradox. However, this paradox can be explained by considering the effect the immune system has on the tumor environment. It has been suggested that the immune system "sculpts the tumor view" (5), i.e., that the immune-selective pressure results in emergence of escape variants. There are many works describing spontaneous tumor variant formation that lead to immune escape, usually attributed to immune selection pressure. However, a recent study by Chen et al. (27) showed immune escape variants formed in an immune privileged locus (the anterior chamber of the eye) in the absence of T cells. To elucidate the mechanism of immune escape in this case, we examined MHC class I down-regulation and tumor-induced T-cell dysfunction and found that these were not at play. Rather, the escape mechanism used by the tumor was found to be that of antigen silencing, as was determined by three different assays. The time frame for this event can be deduced from the lag of initial tumor appearance in OT-I compared with C57BL/6j mice. This period of $\geq 10$ days can be attributed to the sole difference between the two mouse strains, namely their CD8 T-cell populations. It appears that within this period, the Elimination phase occurs, which may, in low tumor doses, succeed. The appearance of the tumors in immunized OT-I was delayed by 30 days compared with the untreated OT-I. It is therefore likely that proper activation of the immune system is not the major time consuming factor in the race with antigen-silencing variants emergence. Rather, the processes involved in immune editing constitute the larger portion of the duration of the antitumor response.

Assuming one cell is sufficient to initiate tumor formation, because $5 \times 10^5$ cells are required for 100% tumor take in OT-I mice, it is deducible that the probability of antigen-silencing variant formation in this model system is $>1/5 \times 10^5$ and $\leq 1/5 \times 10^5$. This range reflects the situation in the extreme variables of this model system and may vary according to the proportion of TAA-specific T cells and the immunogenicity of the antigen. Thus, the emergence of antigen-silencing variants was not a rare event. This should be considered compared with the number of cells used in this model and more so compared with the number of cells that can be found in progressive human lesions. The frequent nature of these events gives some insight into the possible genetic mechanism underlying antigen silencing in this system. A PCR using cDNA from RNA of MO5 cells removed from OT-I and grown in tissue culture revealed that expression of the TAA was restored. This striking phenomenon further emphasizes the plasticity of the antigen profile expression of the tumor and consequently its importance.

To ascertain that the phenomena we described here were not a product of in vitro effects, we used a subclone of the MO5 (MO5.17), which was injected s.c. to OT-I mice and grew in a dose-dependent manner similar to that of the parental MO5. Moreover, the lowest doses tested ($5 \times 10^5$) resulted in higher tumor take than that of parental cells. If the variant formation process would have occurred in vitro, then a freshly cloned line would have produced less tumor take in smaller doses. Because the opposite effect was observed, this further supports the claim that the antigen-silencing event occurs in vivo.

The main issue this paper engages is the dogmatic belief that increasing the number of TAA-specific T cells will eventually mediate rejection of an established tumor. We show here that even by reaching the clinically implausible situation of $\sim 100\%$ TAA-specific T cells, the tumor progresses. The mechanism of tumor escape via antigen silencing described in this particular case serves mainly to show the relatively uncomplicated mechanisms involved in tumor escape.

Active specific cancer immunotherapy often uses a "single epitope at a time" approach. This approach, although beneficial in constructing preclinical models that involve small tumors, is doomed to failure in long-term therapy of large tumors. The more TAA-specific T cells the therapy induces, the greater the element of the immune system that exerts selective pressure on the tumor. Because this pressure is exerted over one specific antigen, antigen-silencing variants are expected to emerge. To overcome this, it could be suggested that more than one TAA should be used. This tactic may be problematic however because the immune system tends to select for T cells that are reactive to an immune-dominant epitope. These cells proliferate more and may overshadow T cells with other reactivities (28). However, in a recent pilot clinical study, intradermal immunization with dendritic cells loaded with multiple prostate TAA peptides showed increase in response to all peptides (albeit to various degrees; ref. 29). Thus, the true nature of the kinetics of an immune response to multiple epitopes is still vague.

A similar set of problems is induced in passive specific immunotherapy where large amounts of T cells are transferred to a lymphodepleted patient. Ongoing clinical trials with T-cell-adoptive transfer have led to the conjecture that the main hurdle to overcome is increasing the number of tumor-specific T cells and their persistence in the host (21, 22, 30). Recent advances in understanding the deficiencies of the transferred T cells in mice have led to the hypothesis that the limiting factor may be insufficient cytokines (21). Therefore, in current protocols, patients are irradiated before transfer to create "cytokine sinks" for the transferred T cells to bask in (24). The reconstitution of such a patient with "highly selected tumor-reactive T cells" creates a situation in which a majority of the T cells are of a very limited specificity (albeit to various degrees; ref. 31). Even when using a more varied population of tumor-infiltrating lymphocytes, the distribution of the specificities is far from homogenous as certain clones become predominant (24). This in turn leads to the same problem of the focused pressure of the immune system exerts on the tumor.

The question of how many TAAs are required to reject an established tumor is a complex theoretical issue. The variables are many and include the immunogenicity of the TAA, the presentation of the TAA on the tumor, the number of TAA-specific precursor CTLs in the patient, the site and mode of immunization or the timing of adoptive transfer, and the potential ease by which the specific TAA may be silenced. Quantification of CD8 immune responses upon immunization with more than one epitope reveals this complexity (28, 32, 33). It is possible that there is a threshold number of TAAs required for the rejection of a particular tumor, but this number and the exact nature of these TAAs are unclear. To further our understanding of the kinetics of immune pressure-induced tumor escape, we are currently conducting a similar line of experiments with the pmel-1 mouse in which most of the CD8 T cells...
cells express a TCR specific for gp100, which is a naturally expressed TAA and serves as a target in several clinical trials. Finally, there exist several means by which the limitations of immunizing with a single TAA may be solved. One possibility is selecting a TAA that is an essential protein. The problem is that such proteins are often expressed in normal cells, making them unsafe targets for immunotherapy. Another option is using the naturally occurring phenomenon of epitope spreading, reactivity after immunization toward antigens that were not in the vaccine, as a means to expand the CTL reaction against the tumor [intramolecular and intermolecular forms have been described in autoimmunity (34) and tumor immunology (8)]. For example, in analysis of a patient responsive to MAGE-3 A1 peptide vaccination, Coulie and Boon (35, 36) showed that the bulk of the antitumor response resulted from T-cell clones that were reactive to TAAs other than the peptide used for immunization (e.g., MAGE-C2 peptides, gp100209–217, and others). This phenomenon was hypothesized to be at the basis of the response. Thus, we would like to suggest that rather than selecting peptides just by virtue of their immunogenicity, it might be prudent to consider examining the potential of a peptide to induce an intermolecular epitope-spreading response (8). Furthermore, it is possible that an increase in clonal immune response may instigate antigen spreading as components of lysed targets become visible to the immune system; we are currently investigating this possibility in our laboratory.

Finally, to rearticulate Gilboa (37), the extent of the tumor escape problem in cancer patients will be revealed when potent vaccination strategies are in place (see also ref. 38). We may have indeed arrived at that era.

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