A single tumor contains a heterogeneous population of cancer cells. Some cancer cells express antigens and are susceptible to specific CTLs. However, other cancer cells are antigen-loss variants (ALVs) that escape these CTLs because they express little or no antigen. Here, we show that antigen-specific T cells can eliminate ALVs when the parental population expresses a model gp33 antigen (KAVYNFATM) at a level sufficient to be locally cross-presented by the nonmalignant stromal cells. That is, the ALVs are eliminated as bystanders because the stroma is destroyed. ALVs escape bystander killing when the bone marrow-derived and/or non-bone marrow-derived stroma does not express the appropriate MHC or when the amount of antigen is too low for effective cross-presentation. The rapid destruction of the stroma, including bone marrow-derived as well as sessile components, and of the parental cancer cells, may be essential for the complete rejection of established tumors by preventing variant escape.

Introduction

Immune responses and/or immunotherapy often fail to cause rejection of progressively growing or established tumors (1, 2, 3, 4, 5, 6, 7, 8, 9). Even when effective CTL responses are generated, established tumors may escape due to the outgrowth of ALVs (10, 11, 12, 13, 14, 15, 16, 17). These ALVs may fail to express the target antigen or may mutate the epitope, resulting in diminished MHC:peptide interactions or in diminished recognition of the MHC:peptide complex by the TCR (18). Despite the antigen specificity of immunotherapy, the decreased expression of targeted tumor antigens or of MHC-presenting molecules leads to tumor progression. Therefore, immunotherapies may fail unless ALVs can be eliminated.

Previously, we showed that antigen-specific T cells can indirectly eliminate ALVs by eliminating the tumor stroma that cross-presented an SIYYRYGL target antigen (19). Here, we extend our observations with a second antigenic model, the lymphocytic choriomeningitis virus (LCMV)-derived gp33 peptide KAVYNFATM, demonstrating that CTLs again eliminated ALVs by targeting the tumor microenvironment. The elimination of ALVs and of parental cancer cells coincided with the rapid infiltration of CTLs into the tumor, as well as with the rapid destruction of the tumor mass. Thus, it may be essential to target stromal cells rapidly to eliminate ALVs and reject established tumors.

Results

ALVs escape a systemic CTL response when the parental cancer cells express lower levels of the gp33 antigen

To study how ALVs can escape a CTL response, we used an MC57G fibrosarcoma cell line that was transfected with LCMV-derived gp33 antigen (KAVYNFATM). We generated an inducible gp33-Lo cell line that could be treated with 4-hydroxytamoxifen (4-OHTAM) to induce the gp33 antigen to 23-fold higher levels; these are referred to as gp33-Hi cells. Adoptive transfer of naive P14 T cells caused the rejection of 14-d established gp33-Hi tumors, but only a temporary regression followed by regrowth of the gp33-Lo tumors (Figure 1, panels A and B, and Table 1). The outgrowth of gp33-Lo tumors occurred in the same mouse that rejected gp33-Hi tumors, and despite the expansion of P14 T cells in the peripheral blood (Figure 1C). To determine why gp33-Lo tumors escaped rejection, we ascertained whether reisolated cancer cells expressed the gp33 antigen by treating the cells with 4-OHTAM. Since the gp33 epitope was fused to EGFP, the loss of EGFP fluorescence indicated that the cells no longer expressed the gp33 antigen. The gp33 antigen was induced by 4-OHTAM in only 4.4 ± 2.1% of cells that escaped P14 T cell immunity, but was induced in 67.0 ± 0.6% of tumor cells reisolated from mice that did not receive P14 T cells (Figure 1D and Table 1).

ALVs escape in gp33-Lo tumors despite the infiltration of cytotoxic P14 T cells

Shortly after T cell transfer (1, 2, and 4 d), tumors expressing higher or lower levels of antigen contained similar percentages of specific T cells (Figure 2A). Six days after T cell transfer, P14 T cells infiltrated both gp33-Lo and gp33-Hi tumors (20.1% versus 15.8% of the gp33-Lo and gp33-Hi tumors cells, respectively; Figure 2, panels A and B). Consistent with our previous observations (19), the number of infiltrating T cells on day 6 was lower in tumors expressing higher levels of the SIY (SIYRRYGL peptide recognized by 2C TCR) or gp33 antigen. We did not extend the kinetics past day 6, because the gp33-Hi tumors at this time-point were composed mostly of dead cells (Figure 3C) and were beginning to be resorbed. It is likely that this necrotic environment was hostile to the T cells that had infiltrated the
high-antigen tumors. T cells isolated from tumors expressing higher or lower levels of antigen produced IFN-gamma (48.4% and 62.3% of CD8+ T cells in gp33-Hi and gp33-Lo tumors respectively; Figure 2C) and possessed similar specific cytolytic activity (Figure 2D).

The elimination of ALVs correlates with increased stromal destruction and requires stromal cells cross-presenting the gp33 antigen. Within 6 d, it was observable that T cells had killed antigenic cancer cells that expressed higher levels of antigen (Figure 3A), because the number of fluorescent gp33-Hi cells was only slightly

**Figure 1**

**Tumors expressing lower levels of gp33 antigen escape rejection as ALVs.** 2C mice were injected with 10^6 gp33-Lo cells on the left flank and with 10^6 gp33-Hi cells on the right flank. Fourteen days after tumor challenge, 2C mice (mice 1, 2, and 3) received approximately 10^7 naive P14 T cells. Mouse 4 did not receive T cells. (A) gp33-Hi tumors were rejected, but gp33-Lo tumors escaped. The left and center pictures are of the same mouse, taken 2 wk apart. (B) P14 T cells delayed the growth of gp33-Lo tumors but rejected similarly sized gp33-Hi tumors. (C) gp33-Lo tumors escaped a CTL response despite a substantial expansion of P14 T cells (V-alpha2+ CD8+ cells) in the PRLs. (D) ALVs escaped a CTL response when the parental cancer cells expressed low levels of antigen.

**Table 1**

ALVs escape a systemic CTL response in gp33-Lo tumors

<table>
<thead>
<tr>
<th>Type of challenge</th>
<th>Cell line</th>
<th>T cells</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Tumor growth</th>
<th>Antigen retained</th>
</tr>
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<td>gp33-Lo</td>
<td>-</td>
<td>1/1</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>1/1</td>
<td>N.D.</td>
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<tr>
<td></td>
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<td>2/2</td>
<td>1/1</td>
<td>1/1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5/5</td>
<td>N.D.</td>
</tr>
<tr>
<td>Bilateral</td>
<td>gp33-Lo</td>
<td>-</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
<td>1/1</td>
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<td>2/2</td>
<td>6/6</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
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<td>+</td>
<td>1/1</td>
<td>1/1</td>
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<td>2/2</td>
<td>6/6</td>
<td>N.D.</td>
</tr>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>11/11</td>
<td>0/7</td>
</tr>
<tr>
<td></td>
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<td>+</td>
<td>0/4</td>
<td>-</td>
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<td>0/3</td>
<td>-</td>
<td>-</td>
<td>0/11</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

1. Mice bearing two week established tumors received approximately 10^7 adoptively transferred naive P14 T cells. Tumor outgrowth was assessed after 30 days.
2. In unilateral challenges, 2C mice received a single challenge of either 10^6 gp33-Hi cells or 10^6 gp33-Lo cells. In bilateral challenges, 2C mice received a challenge of 10^6 gp33-Lo cells on the left flank and a challenge of 10^6 gp33-Hi cells on the right flank.
3. The averages of each group are from two independent experiments.
4. The elimination of ALVs correlates with increased stromal destruction and requires stromal cells cross-presenting the gp33 antigen.
5. N.D. = Not done. N.A. = Not applicable.
6. "4OH-TAM" treatment induced 67.0 ± 0.6% of the cells above background. The average fold increase above the untreated control was 19.7 ± 10.2.
7. 4OH-TAM treatment induced 4.39 ± 2.1% of the cells above background. The average fold increase above the untreated control was 2.2 ± 0.8.

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greater than the number of neomycin-resistant cells expressing MerCreMer (Neo cells) (Figure 3A). Since T cells infiltrated the tumor between day 4 and day 6 (Figure 2B), the antigenic cancer cells were eliminated during this 48-h time-period. At that point in time, gp33-Hi tumors contained more nonviable cells than gp33-Lo tumors (Figure 3B). These nonviable tumor cells included stromal cells capable of cross-presenting antigens, as determined by CD11b expression (Figure 3C). P14 T cells lysed CD11b+ stromal cells purified from gp33-Hi tumors, but did not lyse CD11b+ stromal cells purified from gp33-Lo or Neo tumors. Therefore, the elimination of ALVs correlated with the cross-presentation of the gp33 antigen by stromal cells.

To determine if antigen presentation by the tumor stroma was necessary to eliminate ALVs, we injected C3H Rag2(-/-) mice or C57BL/6 Rag1(-/-) mice with gp33-Hi cells and, 15 d later, with transferred, activated P14 T cells. Whereas C57BL/6 Rag1(-/-) mice that received activated P14 T cells rejected gp33-Hi tumors, C3H Rag2(-/-) mice that received activated P14 T cells failed to reject gp33-Hi tumors (Figure 3D and Table 2). Cells recovered from tumors in C3H Rag2(-/-) mice that had received P14 T cells had lost the antigen because only 0.47 ± 0.1% of reisolated tumor cells fluoresced above background (Figure 3E).

To determine which stromal compartments are required to prevent tumor escape, we injected B6→C3H and C3H→B6 bone marrow chimeric mice with gp33-Hi cells and, 14 d later, transferred P14 T cells that had been activated in vitro (Table 2). Gp33-Hi tumors escaped rejection in both the B6→C3H and C3H→B6 bone marrow chimeric mice, indicating that both the bone marrow-derived and the non-bone marrow-derived cell compartments are required for tumor rejection.

Discussion

Cytotoxic T cells rejected established tumors by rapidly targeting highly expressed antigens that were presented by the parental cancer cells as well as by the tumor stroma. In coming to this conclusion, we observed that tumors escaped rejection when the cancer cells expressed antigen at levels insufficient for cross-presentation and when the tumor stroma expressed MHC molecules which are unable to present the antigen. In both cases, established tumors escaped rejection with the outgrowth of ALVs. This confirms our previous finding that tumor rejection occurs when T cells target the tumor stroma. Furthermore, our data suggests that T cells must eliminate the cancer cells within several days in order to prevent the outgrowth of ALVs. This rapid destruction likely prevented sufficient numbers of cancer cells from losing the antigen and thereby escaping tumor rejection. Thus, targeting antigenic tumor cells rapidly may prevent tumor escape.

Earlier studies have reported a so-called bystander effect on T cells during the induction phase of an immune response. In those cases, a T cell response was nonspecifically induced by CD40 ligation or through specific induction of a second, high-precursor frequency T cell response (20, 21). We studied the effector phase
Figure 3

Rejection of tumors expressing higher levels of the gp33 antigen required TILs targeting the tumor stroma. (A) As assessed by EGFP fluorescence, antigenic cancer cells in gp33-Hi tumors were eliminated between 4 and 6 d after T cell transfer. Shaded histograms are Neo tumor cells; unshaded histograms are gp33-Hi tumor cells. (B) gp33-Hi tumors contained more nonviable cells than gp33-Lo tumors after adoptive transfer of P14 T cells, as analyzed by PI uptake. (C) gp33-Hi tumors in which ALVs were eliminated contained more nonviable stromal cells than gp33-Lo tumors, as determined by CD11b staining. The numbers in the upper-right and lower-right quadrants indicate the percentages of CD11b+ PI+ and CD11b+ PI- cells, respectively. (D) gp33-Hi tumors escaped a CTL response when the stroma did not express the correct MHC-presenting molecule. (E) ALVs escaped a CTL response when the stroma did not express the correct MHC-presenting molecule. For these measurements, tumors from mice in (D) were reisolated and tested for gp33 antigen expression. Unshaded histograms represent the Neo cells. Shaded histograms represent the gp33-Hi cancer cells indicated.

of an immune response, and, in our case, bystander elimination of ALVs required effector T cells specifically recognizing stromal cells at the tumor site.

As had also been observed in the SIY antigen system (19), rejection ALVs required T cells targeting the bone marrow-derived and non-bone marrow-derived stromal cells. While bone marrow-derived stromal cells classically cross-present antigen, we and others have observed that non-bone marrow-derived stromal cells are also capable of cross-presenting antigens (19, 22). Still, it is unclear whether these non-bone marrow-derived stromal cells cross-presented the antigenic peptides or whether the antigenic peptides released from tumors were directly bound to MHC molecules on the cell’s surface.

Tumor rejection correlated with T cells killing the stromal cells isolated from tumors expressing high levels of antigen. Here, we observed that targeting these antigens was associated with the rapid destruction of the parental cancer cells, as well as with the destruction of the tumor stroma. This stromal targeting likely deprived the escaped variants of the nutrients essential for tumor growth. Targeting the tumor stroma appears to be important for tumor rejection because transplanted tumors containing antigenic stroma exhibited reduced tumorigenicity (23). Furthermore, cross-presentation of antigen to CTLs delayed the growth of antigenic tumor transplants and partially destroyed transplants of antigenic skin grafts (24, 25, 26). Targeting the stromal cells may negate the need to enhance T cell infiltration through local inflammatory mediators (27). In our case, cross-presentation by the tumor stroma was required to eliminate ALVs and cure the malignancy.

Our observations can explain previous studies that did not detect this bystander killing of nonantigenic cancer cells (28, 29). These studies used mixed inocula of antigenic and nonantigenic cells that had been recently injected and, therefore, had less time to load up the adjacent stromal cells with antigen. Furthermore, it was unclear whether the antigens that were targeted were even suitable for cross-presentation. According
to our observations, these nonantigenic cells may be killed only when these cells are surrounded by antigenic stroma in an established tumor. Similarly, nonantigenic metastatic cells may be more likely to escape than if the same cells were surrounded by antigenic stroma in an established tumor (30). Therefore, immunotherapies that induced significant tumor regression in cancer patients may have targeted antigens that can be presented by both the malignant cancer cells and by the tumor stroma. Since the rapid destruction of cancer cells correlated with the elimination of ALVs, our findings suggest that effective cancer therapies may require efficient targeting of the tumor stroma. Since nonmalignant stromal cells may also be genetically distinct from other somatic cells, unique biological, inflammatory, or angiostatic agents may efficiently target the tumor stroma. Stromal cell destruction may be mediated by other factors that disrupt the tumor/stromal cell interactions (31). Therefore, new agents that rapidly destroy the tumor stroma may prevent the outgrowth of ALVs and tumor escape.

Abbreviations
ALV, antigen-loss variant

Acknowledgements
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References


Materials and methods

Mice

C3H Rag2(-/-) mice were purchased from Taconic Biotechnology (Germantown, NY, USA). C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Dr. Avril Ma (The University of Chicago, IL, USA) and Dr. Matthew Mescher (The University of Minnesota, Minneapolis, USA) provided the OT-1 mice; Dr. Philip Ashton-Rickardt (The University of Chicago, IL, USA) provided the P14 mice. Dr. Thomas Gajewski provided the 2C x Rag2(-/-) mice that were subsequently bred to C57BL/6 mice to generate 2C x Rag2(+/-) mice. Mice were bred at The University of Chicago FMI Animal Research Facility. For B6→C3H bone marrow chimeras, C3H Rag2(-/-) mice were irradiated with 6 Gy, and 3 h later received 10^7 C57BL/6 Rag1(-/-) bone marrow cells. For C3H→B6 bone marrow chimeras, C57BL/6 Rag1(-/-) mice were irradiated with 6 Gy and 3 h later received 10^7 C3H Rag2(-/-) bone marrow cells. When analyzed, more than 95% of the peripheral blood leukocytes were found to have derived from the donor marrow.

Cell lines and reagents

Dr. Pamela Ohashi (University of Toronto, Canada), with the permission of Dr. Hans Hengartner (University Hospital, Zurich, Switzerland), provided the MC57G fibrosarcoma cell line. The gp33-Hi and gp33-Lo cells were described earlier. Briefly, these cells were engineered to express the lymphocytic choriomeningitis virus (LCMV)-derived gp33 epitope, KAVYNFATM (32). For our studies, the cysteine of the naturally occurring gp33 peptide (KAVYNFATC) was replaced with a methionine to prevent disulfide bond formation. The P14 TCR recognizes both the KAVYNFATM and KAVYNFATC peptides with similar efficiencies. These cell lines were maintained in cDMEM (Dulbecco’s Modification of Eagle’s Medium, Invitrogen, Carlsbad, CA, USA, supplemented with heat-inactivated 5-10% FCS). The PE anti-IFN-gamma (clone XMG 1.2), isotype control PE rat IgG1 (clone A110-1), FITC anti-CD8 (clone 53-6.7), PE antimouse IgG1 (clone A85-1), APC antimouse IgG1 (clone X56), PE anti-V-alpha2 (clone B20.1), FITC anti-CD11b (M1/70), isotype control FITC rat IgG2b (clone A95-1) and anti-Fc gamma receptor (clone 2.4G) antibodies were purchased from Pharmingen (San Diego, CA, USA). Helen Auer and Stephen Meredith synthesized the gp33 peptide KAVYNFATM and the mutant p68 peptide SFNVFAGI. Brefeldin A (BFA), ionomycin, and phorbol 12-myristate acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tamoxifen (TAM) and 4-hydroxytamoxifen (4-OHTAM) were also purchased from Sigma Chemical Co.

Adoptive P14 T cell transfer

Splenocytes were isolated from P14 mice, and CD8+ T cells were negatively selected using a CD8+ enrichment kit (StemCell Technologies, Vancouver, Canada). When analyzed, more than 90% of the enriched CD8+ cells expressed the respective P14 receptor. Between 5 x 10^6 and 1 x 10^7 naive T cells were injected intravenously into the retro-orbital plexus of the mice in a 0.1 ml volume. For transfer of activated cells, 2 x 10^6 NH4Cl-treated splenocytes from P14 mice were stimulated with 10 µM of the KAVYNFATM peptide in cRPMI medium supplemented with 10 U/ml IL-2. After 3-4 d, cells were collected, and approximately 10^7 cells were injected intravenously in the mice, as described above. Activation was confirmed by the upregulation of CD44 on the specific T cells.

Analysis of TILs

Tumors were harvested, diced into fragments, and digested with 400 U/ml Collagenase D in 10% FCS-DMEM for 3-4 h at 37°C. Lymphocytes were analyzed in bulk tumor cell suspensions or were purified by incubating cells with mouse CD90 (Thy1.2) MicroBeads (Miltenyi Biotec, Cologne, Germany) and sorting cells on an AutoMACS (Miltenyi Biotec) according to the manufacturer’s instructions. On average, approximately 70% of cells isolated from gp33-Hi or gp33-Lo tumors expressed V-alpha2. Bulk tumor cells were incubated with 2.4G2 and with a cocktail of anti-V-alpha2 and anti-CD8 antibodies in order to detect P14 T cells. Dead cells were excluded by positive propidium iodide staining. Thy1.2-purified cells were tested for IFN-gamma expression and cytolytic activity by plating cells with 10^6 Rag1(-/-) splenocytes and 1 µM of the gp33 peptide in the presence of 10 mg/ml BFA in cRPMI at 37°C. After 6 h, cells were stained with FITC anti-CD8 antibody, fixed with 4% paraformaldehyde, and permeabilized with 0.5% saponin in PBS with 1% BSA and 0.1% azide. In addition, Thy1.2-purified cells were tested for cytotoxic activity in a 51Cr release assay as described previously (33).

Contact

Address correspondence to:
Hans Schreiber
Department of Pathology
The University of Chicago
5841 South Maryland, MC 1089
Chicago, IL 60637
USA
Tel.: + 1 773 702-9214
Fax.: + 1 773 702-9224
E-mail: hszz@midway.uchicago.edu