Identification of a tumor-associated contact-dependent activity which reversibly downregulates cytolytic function of CD8+ T cells

Daniel L. Levey1, Heiichiro Udono2, Michael Heike3, and Pramod K. Srivastava

1Present address: Antigenics Inc., 630 Fifth Avenue, Suite 2100, New York City, NY 10111
2Present address: Department of Medical Zoology and Immunology, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
3Present address: I. Medizinische Klinik und Poliklinik, Johannes Gutenberg-Universität Mainz, Langenbeckstrasse 1, D-55101 Mainz, Germany
4Center for Immunotherapy of Cancer and Infectious Diseases, Mail Code 1601, University of Connecticut School of Medicine, Farmington, CT 06030

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Abstract

Tumors elicit an immune response in hosts and yet, paradoxically, often grow progressively with fatal consequences. This phenomenon has been attributed to the possible expression by tumor cells of immunomodulatory factors that overcome the anti-tumor effector functions of both specific and non-specific immune cells. This study reports on the ability of the methylcholanthrene-induced fibrosarcoma, Meth A, as well as other tumors of varied histological origins to downregulate the lytic activity of CD8+ T cells. The suppressive activity is contact-dependent and reversible. As tumor-bearing hosts are rarely immunosuppressed systemically, these findings may explain how local events within the tumor bed subvert the specific anti-tumor immune response.

Introduction

Tumor cells are capable of evading the immune system by numerous methods (1). These include: (i) downregulation of cell surface molecules (MHC class I proteins) which would otherwise alert the immune system to the presence of tumor antigens; (ii) secretion of immunomodulatory cytokines which impede anti-tumor effector cells; (iii) activation of host cells which suppress anti-tumor immunity, including T cells (2, 3), B cells (4) or macrophages (5, 6, 7, 8, 9); and (iv) expression of FasL which induces apoptosis in anti-tumor T cells (10).

Despite the mechanisms of immune evasion which allow tumors to grow progressively in naive hosts, a fundamental observation demonstrated in multiple tumor systems is the ability to specifically immunize against a viable tumor challenge. This phenomenon was defined classically using the methylcholanthrene-induced fibrosarcoma, Meth A, as well as other tumors of varied histological origins to downregulate the lytic activity of CD8+ T cells. The suppressive activity is contact-dependent and reversible. As tumor-bearing hosts are rarely immunosuppressed systemically, these findings may explain how local events within the tumor bed subvert the specific anti-tumor immune response.

It has been shown by depletion experiments in vivo that both CD4+ and CD8+ cells are required for the rejection of Meth A tumors (12). To further elucidate this pathway, attempts have been made to generate CTLs in vitro which are capable of lysing Meth A cells. This has led to the surprising observation that although immunization of syngeneic mice with Meth A cells elicits potent CD8+ anti-Meth A T cells in vivo, it has not been possible to generate anti-Meth A CTLs in vitro. Further studies demonstrated that Meth A is also resistant to lysis by allo-reactive C57BL/6 anti-BALB/c CTLs. These results have led to identification of a Meth A-associated immunosuppressive activity described in this report.
Results

CTLs against high MHC I-expressing Meth A cells could not be generated following Meth A tumor rejection in syngeneic hosts

Three groups of five BALB/cJ (H-2^d) mice were immunized subcutaneously with buffer or \(2 \times 10^7\) irradiated whole Meth A cells twice, at a one week interval. One week after the second immunization, all mice were challenged intradermally with \(1 \times 10^5\) live Meth A cells. Tumor diameter was measured at bi-weekly intervals (Fig. 1A). On day 10 post-tumor challenge, mixed lymphocyte tumor cultures (MLTCs) were subsequently performed with splenocytes of five mice which had rejected Meth A. Stimulator cells in MLTCs were irradiated Meth A cells. After six days culture, a \(^{51}\)Cr release assay was performed with Meth A targets (Fig. 1B). The tumor rejection data show that it is possible to induce tumor rejection in mice immunized with irradiated Meth A whole cells (Fig. 1A). However, after establishing MLTCs it has proven impossible to generate CTLs capable of lysing Meth A tumor cells \textit{in vitro} (Fig. 1B). This is not due to the protocol employed for the MLTC reaction, since tumor-specific CTLs have been generated in other systems by the same method (13 and unpublished observations).

A number of factors may explain this observation: (i) Meth A cells may not provide adequate stimulation in MLTCs, or may serve as a poor target during CTL assays because of loss of MHC I or adhesion molecules. This possibility has been ruled out by FACS analysis which reveals abundant MHC I expression on Meth A cells (data not shown); (ii) It is possible that an antigen processing defect could prevent peptides from being charged onto MHC molecules, thus blinding T cells to the presence of Meth A cells. However, as stable expression of MHC molecules is dependent on their being charged with peptides, it is unlikely that peptides are a limiting factor. Furthermore, an abundance of MHC I-extractable peptides were detected on Meth A cells in studies involving peptide sequencing (unpublished data); (iii) Meth A cells could actively suppress cell-mediated immune responses through an inhibitory molecule which is either secreted, or which is activated upon cell-cell contact.

Figure 1. Inability to generate CTLs against high MHC I-expressing Meth A cells after Meth A tumor rejection in syngeneic hosts. (A) Tumor diameter measured at bi-weekly intervals. (B) \(^{51}\)Cr release assays performed with Meth A targets.
While the first two possibilities have been discounted, the third one remains plausible: in order to test if such suppressive activity would also be seen in other T cells assays, an allogeneic system was used. Meth A cells are expected to be killed by allogeneic CTLs induced in C57BL/6 mice which had rejected a challenge of Meth A cells. To test this, mice were challenged intradermally with $2 \times 10^6$ live Meth A cells. After complete tumor regression, splenocytes of mice were cultured with mitomycin C-treated Meth A stimulator cells in MLTCs. It is observed that while CTLs are generated, they paradoxically killed all $^{51}$Cr-labeled H-2d-expressing cells in vitro except Meth A cells (Table 1).

**Table 1. Resistance of Meth A tumor cells to lysis by H-2d-specific CTLs**

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>% $^{51}$Cr release at E:T ratios:</th>
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<tr>
<td></td>
<td>Exp. 1</td>
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<tr>
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<td>C57BL/6 Con A blasts</td>
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<td>12</td>
</tr>
<tr>
<td>Meth A cultured</td>
<td>25</td>
</tr>
<tr>
<td>CMS4</td>
<td>60</td>
</tr>
<tr>
<td>CMS5</td>
<td>46</td>
</tr>
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</table>

1 C57BL/6 mice were challenged intradermally with $2 \times 10^6$ live Meth A cells. After tumor rejection, spleen cells were harvested and cultured with mitomycin C-treated Meth A cells (responder to stimulator ratio 20:1). On day 6 CTLs were tested against the indicated targets in $^{51}$Cr release assays. Shown are two representative experiments, of a total of five.

2 Con A blasts was prepared by culturing $2 \times 10^7$ whole spleen cells in 10 ml complete RPMI with Concanavalin A (final concentration 5 µg/ml). Blasts were harvested after 48 hours and $^{51}$Cr-labeled for use as target cells in CTL assays.

Additional experiments were carried out with H-2d-specific CTLs generated in mixed lymphocyte reactions (MLRs). C57BL/6 splenocytes were cultured with mitomycin C-treated BALB/cJ splenocytes, and on day five, CTLs were harvested and tested for cytolytic activity against a panel of $^{51}$Cr-labeled target cells. A similar pattern of cytolytic activity was observed, where all H-2d-expressing cells are killed except Meth A cells (not shown). Collectively, observations in this allogeneic system suggest that Meth A is an adequate stimulator of H-2d-specific CTL in MLTCs, but Meth A cells themselves are resistant to lysis by such CTLs.

To confirm that CD8+ T cells are involved in mediating allogeneic rejection of Meth A cells, in vivo depletion of T cell subsets was performed after tumor challenge. C57BL/6 mice were challenged intradermally with $2 \times 10^6$ live Meth A cells. To deplete cellular subsets, groups of five mice each received retro-orbital injection of serum-free RPMI, anti-CD8, anti-CD4, or both anti-CD8 and anti-CD4 monoclonal antibodies. Antibody injections were repeated at weekly intervals to assure depletion of T cell subsets. Perpendicular tumor diameters were measured at two to three day intervals until tumors were rejected, or reached approximately 20 mm in diameter.

It is observed that CD8+ T cells are the primary effectors in allogeneic tumor rejection, since tumors grow progressively when CD8+ cells are depleted (Fig. 2). Some T cell help is supplied by CD4+ cells, since tumor rejection takes three to four days longer in CD4-depleted mice compared to non-depleted mice. Furthermore,
depletion of both CD4+ and CD8+ cells enhances tumor growth compared to CD8-depletion alone. Collectively, these observations suggest that CD8+ T cells are required for rejection of Meth A tumor cells in both syngeneic and allogeneic hosts; further, although Meth A cells are adequate stimulators of H-2d-specific CTLs in MLTCs, Meth A cells themselves are resistant to lysis by such CTLs.

Figure 2. CD8+ T cells are required for allogeneic rejection of Meth A tumor cells. C57BL/6 mice (H-2b) were challenged intradermally with 2 x 10^6 live Meth A cells (H-2d). To deplete various T cell subsets, the indicated antibodies or RPMI were injected at the time of tumor challenge, and each week thereafter, in the retro-orbital sinus. Tumor diameter was measured every two to three days. Each line represents the average measurement of five mice.

Meth A cells can block killing of other targets non-specifically in bystander assays

To test the generality of Meth A cell-induced suppression observed in allogeneic T cell assays, a panel of bystander assays was established. In four separate experiments combinations of splenocytes from BALB/c, C57BL/6, and C3H-HeN mice were cultured in MLRs with mitomycin C-treated stimulator cells (C57BL/6 anti-BALB/c, C57BL/6 anti-C3H-HeN, BALB/c anti-C57BL/6, and BALB/c anti-C3H-HeN). On day five, CTL assays were performed with ^51^Cr-labeled targets consisting of splenocytes of the stimulating haplotype stimulated with Concanavalin A (Con A). "Cold" bystander targets consisted of Con A-stimulated splenocytes from H-2d, H-2b, and H-2k mice, and various tumor lines.

Figure 3. Meth A cells block CTL activity in an MHC-unrestricted manner in bystander assays. Shown is a representative experiment with H-2^d^ anti-H-2^b^ CTLs. The ^51^Cr-labeled target consists of Con A-stimulated H-2^b^ splenocytes, which in an unlabeled form serves as the control "cold" bystander target. Other cold targets are listed at the bottom of the figure. The diamond indicates CTL killing in the absence of cold target (effector to target ratio of 10).
Shown in Figure 3 is a representative experiment with BALB/c anti-C57BL/6 CTLs. The $^{51}$Cr-labeled target consists of Con A-stimulated H-2$^b$ splenocytes. Other cold targets are indicated in the figure. It is observed that the presence of cold Meth A cells inhibits cytolyis of labeled Con A targets, even more than the C57BL/6 Con A control cold target. In MLRs with H-2$d$-specific CTLs, Meth A cells are expected to be inhibitors since Meth A cells express H-2$d$ molecules. However, in MLRs with H-2$^b$ or H-2$k$-specific CTLs, Meth A cells are observed to inhibit CTLs in a non-MHC restricted manner (Fig. 3). Still, these experiments have not eliminated the possibility that Meth A cells suppress CTLs by secreting cytokines, with or without prior cell-cell contact.

**Meth A cell-induced T cell suppression is not mediated by transforming growth factor type beta (TGF-beta)**

Numerous tumor types secrete TGF-beta (14, 15, 16, 17, 18, 19, 20, 21). To test if the suppressive activity of Meth A cells is due to autocrine secretion of TGF-beta, a neutralization assay was employed using monoclonal anti-TGF-beta antibody (Fig. 4). Furthermore, to test if Meth A cells secrete a suppressor factor into the blood of tumor-bearing hosts which could suppress T cell function, serum from a Meth A tumor-bearing mouse (day 28) was analyzed. A cold target inhibition assay was performed as described in Figure 3 with H-2$d$ anti-H-2$^b$ CTLs.

It is observed that Meth A cells mixed with anti-TGF-beta inhibit cytotoxicity to the same degree as Meth A cells alone. To confirm that TGF-beta was not an active component of the suppressive activity, the supernatant of short-term cultured Meth A cells, as well as the supernatant of a Meth A-CTL mixture, were added to TGF-beta-sensitive mink lung epithelial cells for quantification of TGF-beta release in a bioassay. No TGF-beta was detected (data not shown). The serum from a tumor-bearing mouse has only a mildly suppressive effect (data not shown). These results show that TGF-beta is not responsible for the inhibitory activity mediated by Meth A tumor cells.

**Inhibition of cytolytic activity by Meth A cells requires cell-cell contact**

To determine whether or not Meth A cells inhibit CTLs through secretion of some soluble product other than TGF-beta, a cold target inhibition assay was performed using a 96-chamber, round-bottomed transwell with a 45 µM membrane. On a per plate basis, this prevents cell-cell contact, yet does not inhibit passage of secreted products between the two compartments (Fig. 5). H-2$d$ anti-H-2$^b$ CTLs were tested in this device using a modification of a cold target inhibition assay. The $^{51}$Cr-labeled target consisted of EL-4 (H-2$^b$) cells, while cold targets consisted of (i) Meth A cells, (ii) B/c-N cells (a normal, non-transformed immortalized clone), or (iii) EL-4 cells. Cells were arranged in the compartments as indicated in the figure.
Figure 5. Inhibition of cytolytic activity by Meth A cells requires cell-cell contact. H-2\textsuperscript{d} anti-H-2\textsuperscript{b} CTLs, generated as described in Materials and Methods, were tested in a transwell plate using a modification of a cold target inhibition assay. The \( ^{51} \text{Cr} \)-labeled target consisted of EL-4 (H-2\textsuperscript{b}) cells; cold targets are listed across the top of the figure. Cells were arranged in the compartments as indicated in the diagram at the bottom of the figure. In one experiment 50 µl of K44 (10 µg/ml), an MHC class I blocking antibody, was added to each well of the underlying compartment to demonstrate that soluble molecules pass through the transwell membrane. The diamond indicates CTL killing in the absence of cold target (effector to target ratio 10). It is observed that Meth A cells have no inhibitory effect on cytolysis when separated from CTLs by the membrane. However, Meth A cells clearly inhibit CTL activity when cocultured with the CTLs in the upper compartment. EL-4, a positive control for CTL inhibition, similarly inhibits CTLs when cocultured in the upper compartment, while B/c-N has no inhibitory effect. B/c-N was chosen as a negative control for suppression in this assay based on experiments described further on.

It is possible that Meth A cells secrete a suppressive factor only upon contacting CTLs. To test this, cold targets were cocultured with CTLs in the underlying compartment while additional CTLs and \( ^{51} \text{Cr} \)-labeled target were added to the upper compartment. No inhibition of CTL activity was observed. To confirm that the membrane allows passage of soluble molecules, K44 antibody specific for the alpha3 domain of MHC I was added in the underlying compartment, in the absence of cold target. It is observed that CTL activity is blocked, indicating that the antibody passed through the membrane and bound to MHC I molecules on the target cell in the upper compartment, inhibiting target cell-T cell interaction.

Other cold targets have been tested in this system, including an adherent Meth A line derived from a solid tumor, as well as Con A-stimulated BALB/c splenocytes (negative control for suppression). The adherent Meth A line shows the same contact-dependent inhibitory effect, while the Con A-stimulated splenocytes do not (data not shown). These experiments suggest a cell-cell contact-dependent mechanism of T cell suppression.
Meth A cell membranes inhibit cytotoxicity

The observations described above suggest that a non-secreted molecule contained within the cell surface of Meth A tumor cells mediates inhibition of CTL activity. It is possible that this molecule functions independently, or there may exist a cascade of intracellular signaling events which links the putative suppressive factor at the cell surface to downstream events within the tumor cell, ultimately leading to T cell suppression. To distinguish between these two scenarios, Meth A cells were metabolically fixed with paraformaldehyde prior to their inclusion in a cold target inhibition assay with alloreactive CTLs. It is observed that the paraformaldehyde-treated Meth A cells suppress CTL activity as effectively as non-treated Meth A cells (Fig. 6). These results favor the hypothesis that a suppressive factor residing at the surface of Meth A cells functions independently, without a requirement for intracellular signaling events.

Figure 6. Meth A cells fixed with paraformaldehyde suppress CTL activity. A cold target inhibition assay was performed as described in Figure 3 with H-2\(^d\) anti-H-2\(^b\) CTLs and EL-4 \(^{51}\text{Cr}\)-labeled target cells. Cold target cells are listed in the figure. The diamond indicates CTL killing in the absence of cold target (effector to target ratio 10).

Figure 7. Meth A cell whole membranes suppress CTL lytic ability while B/c-N membranes do not. Whole membranes were prepared as described in Materials and Methods. A cold target inhibition assay was prepared with H-2\(^d\) anti-H-2\(^b\) CTLs and EL-4 \(^{51}\text{Cr}\)-labeled target cells. The diamond indicates CTL killing in the absence of cold target (effector to target ratio 10).
To confirm that a cell surface-derived molecule mediates suppressive activity, whole membranes were prepared from Meth A tumor cells. In designing these experiments it was necessary to find a cell type which was negative for suppressive activity. While splenic B cells and peritoneal exudate cells (PEC) fit such criteria (see Table 2), the non-transformed, immortalized fibrosarcoma B/c-N was chosen due to its similarity to Meth A in terms of tissue origin. Thus, whole membranes were similarly isolated from B/c-N cells and tested in parallel with Meth A membranes. Cold target inhibition assays were performed with H-2d anti-H-2b CTLs and 51Cr-labeled EL-4 (H-2b) target cells. Included in the assay as cold targets were intact EL-4 cells, Meth A cells, B/c-N cells, Meth A membranes or B/c-N membranes. It is observed that Meth A whole membranes mediate a powerful suppressive activity which is titratable, while B/c-N membranes do not (Fig. 7).

**Kinetics of Meth A-induced T cell suppression**

In the experiments described above (Figs. 3-6), Meth A cells remain in continuous contact with CTLs for the duration of the cold target inhibition assay. It is possible, however, that the suppression of CTLs by Meth A cells requires only short term cell-cell contact. Further, the preceding assays did not address whether or not CTLs might recover cytolytic activity after tumor cells were removed from the assay. Two experiments were thus designed where Meth A cells were cultured with CTLs for variable amounts of time, followed by separation and testing of isolated CTLs in 51Cr release assays. In the first experiment, fresh Meth A cells were cocultured with H-2d anti-H-2b CTLs in round-bottomed tubes for 0.5, 1, 2 or 3 hours, followed by positive selection of CTLs using a high gradient magnetic cell sorter (MACS) and anti-Thyl.2 antibody. Isolated Thyl.2+ cells were then tested immediately for cytolytic activity against 51Cr-labeled EL-4 target cells in a 4 hour 51Cr release assay (Fig. 8). Separated T cells were checked for purity (>95% CD3+) by FACS analysis. T cells alone were similarly cultured, applied to the separating column, and tested for cytolytic activity. Suppressive activity is observed only when Meth A is cultured with CTLs for 3 hours; no suppressive activity was observed for coculture periods of 0.5, 1, or 2 hours. The reduction in CTL activity is most pronounced at effector to target ratios of 10:1 and 5:1, where it approaches 50%, as compared to T cells cultured alone.

![Figure 8. Kinetics of CTL suppression induced by coculture with Meth A tumor cells.](http://www.cancerimmunity.org/v1p5/010306.htm)

Coculture assays were performed with H-2d anti-H-2b CTLs as described in the text. T cells were cultured alone, or with Meth A cells for 0.5, 1, 2, or 3 hours, as indicated. CTLs were then positively selected by MACS and tested immediately in a 4 hour chromium release assay against EL-4 target cells.

In the second experiment the possibility that CTLs might recover lytic ability after separation from Meth A cells was addressed. CTLs were cultured alone, with Meth A cells, or with purified normal B cells, for 3 hours. After separation by MACS, CTLs were divided into two groups. The first group was tested immediately in a 4 hour 51Cr release assay. The second group was allowed to recover for 24 hours in the presence of 5% Con A supernatant, followed by a CTL assay. Con A supernatant contains IL-2 and was included to maintain T cell viability during the recovery period. It is observed that CTL activity is reduced by 50% after 3 hour coculture with Meth A cells (Fig. 9a). In contrast, T cells cocultured with B cells demonstrate heightened cytolytic activity. After overnight recovery,
T cells which were previously cultured with Meth A cells recover 90% of their activity; T cells cultured alone or with B cells retain cytolytic activity at least as strong as was observed immediately after the coculture period. The reversibility of Meth A cell-induced suppression is also observed with the CT26-specific CTLs, confirming the non-specific nature of this CTL downregulation (data not shown).

![Figure 9. CTL suppression induced by tumor cells in coculture assays is reversible.](image)

Figure 9. CTL suppression induced by tumor cells in coculture assays is reversible. CTLs were cultured alone, with Meth A cells, or with purified normal B cells for three hours (A). After isolation of T cells by MACS, half of the CTLs were tested immediately in a 4-hour chromium release assay against EL-4 target cells. The remaining cells were cultured overnight with 5% IL-2 and tested in a similar chromium release assay (recovery). A similar 3-hour coculture assay was established to test whether or not CTL suppression induced by other tumors is reversible (B).

To test the generality of the short-term suppression induced by tumor cells, H-2b-specific CTLs were cocultured with CMS4 or CMS5, two antigenically distinct methylcholanthrene-induced tumors. It is observed that after coculture with CMS4, CMS5, or Meth A cells, CTLs are suppressed by at least 50% compared to CTLs cultured alone (Fig. 9b). After separation with MACS and overnight recovery in the presence of 5% Con A supernatant, CTLs recover to within 85-90% of their maximal activity.

**A large panel of tumor cells, but not normal cells, is found to suppress CTLs in coculture assays**

The experiments described above (Figs. 8 and 9) provided additional basis for expanding the investigation of tumor-induced suppression to include a large panel of tumor cells. In total, two normal, tissue-derived cell types (splenic B cells and peritoneal exudate cells, consisting primarily of macrophage), one normal, non-transformed immortalized clone (B/c-N), and 8 transformed tumor cell lines were tested. Three-hour coculture assays were established utilizing anti-H-2b CTLs, as described in Figure 8.

Of the nine tumor and transformed lines tested in 3 hour coculture assays, eight are observed to suppress CTL
lytic ability by at least 25% at all effector to target ratios (Table 2). Of three normal cells (B/c-N, B cells, and peritoneal exudate cells) none exhibit suppressive activity. These results show that tumor-associated suppressive activity can be detected in nearly all transformed lines.

Table 2. A large panel of tumor cells suppresses CTL activity in short-term coculture assays

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<thead>
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<th>Suppression of CTL lytic ability²</th>
<th>Tumor lines</th>
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<tr>
<td>Meth A³</td>
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<td>CMS4³</td>
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<td>PEC⁴</td>
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</tr>
<tr>
<td>B/c-N</td>
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</table>

¹H-2d anti-H-2b CTLs were cocultured with the indicated tumor lines or normal cells for three hours, followed by selection of T cells with Thy1.2 antibody and the MACS cell sorting system. CTLs were immediately tested in a four hour ⁵¹Cr release assay against EL-4 target cells. ²Greater than 25% suppression of CTL lytic ability is indicated by a “+”; Less than 25% suppression is indicated by a “−”. ³In further analyses, the CTL suppression induced by these tumor lines was observed to be reversible. Thus, CTLs which are allowed to recover overnight in the presence of IL-2 regain 95% of their lytic ability (see Fig. 9b). ⁴Peritoneal exudate cells.

Discussion

Identification of structural mediators of immune suppression, of tumor or host origin, has remained particularly elusive. The studies presented here approach tumor-associated suppression from the perspective of the tumor cell, and began with the observation that although mice inoculated with whole irradiated Meth A tumor cells are able to reject a subsequent challenge with live Meth A cells, it is not possible to generate anti-Meth A CTLs in vitro. This observation raises the possibility that tumor-derived factors suppress the generation and activity of CTLs in vitro. Experiments were designed to characterize the activity of such factors on Meth A cells, and have subsequently demonstrated that many different tumor cells, of distinct histological origins, are capable of suppressing CTL activity.
The most detailed analyses of suppression were carried out with methylcholanthrene-induced tumors: live Meth A cells and two additional tumors, CMS4 and CMS5, were observed to downregulate T cell lytic ability in coculture assays. The nature of the suppressive activity can be summarized as follows: (i) suppression is observed only after CTLs are cocultured with tumor cells for at least three hours; (ii) CTLs recover 60 to 95% of their cytolytic activity after separation from tumor cells; (iii) Meth A-induced suppression requires continuous contact between tumor cells and T cells, and is not mediated by secretion of soluble factors; and (iv) suppressive activity is retained in the membrane component of Meth A tumor cells.

It is necessary to reconcile a key observation reported here, the inability to generate Meth A-specific CTLs, with the previously observed requirement for CD8+ T cells in the rejection of Meth A tumors in Meth A-immunized mice (12). It is possible that tumor-specific CD8+ T cells in vivo, although suppressed with regard to lytic activity, might still recruit non-specific effector cells to the site of tumor growth through secretion of cytokines. Evidence supporting such a role for non-specific effectors in tumor cell death derives from the observation that mice immunized with irradiated Meth A cells are capable of rejecting a subsequent challenge with a mixture of Meth A and CMS4 cells (unpublished data). The lack of an in vitro CTL-based correlate for CD8-dependent tumor rejection suggests that assays which measure other CD8+ T cell functions, such as cytokine secretion, may be a more appropriate indicator of lymphocyte activity in tumor response.

The phenomenon of immune suppression in the tumor-bearing state has been extensively investigated for at least forty years, and it is now clear that both the host immune system and the tumor itself can contribute to growth of tumor cells through the secretion of immunomodulatory cytokines. TGF-beta secretion by tumor cells can downregulate Th1 cytotoxic T cells, thus inhibiting important effector populations which mediate tumor eradication (15). IL-10 secretion by keratinocytes in mice exposed to UV light was observed to decrease APC function and suppress tumor rejection (22). Non-cytolytic CD8+ T cell clones secrete IL-10 which suppresses IFN-gamma secretion and anti-tumor activity of antigen-specific CTLs (23). While macrophage are capable of secreting nitric oxide which has tumoricidal activity against a mammary adenocarcinoma, both nitric oxide mRNA and protein are downregulated during tumor progression (24, 25). This downregulation appears to be mediated by a lipid factor secreted by the tumor (26). In at least one system, tumor-derived IL-10, TGF-beta, and prostaglandin E2 were responsible for decreased nitric oxide and TNF-alpha production by macrophages (14).

Downregulation of T cell function, as reported here, is not an all or none event since T cells recover 60 to 95% of their cytolytic activity after separation from tumor cells contained in coculture assays. This finding is consistent with the ability of lymphocytes infiltrating some cancers to recover activities such as cytokine secretion and proliferation when placed in culture (27, 28, 29). The suppressive activity described here differs from those described above in that downregulation of T cells by Meth A tumor cells requires cell-cell contact and is not mediated by a soluble factor. This report adds a new dimension to the phenomenon of suppression in the tumor-bearing state, and directs attention to tumor-associated factors which downregulate T cell lytic ability in vitro, and possibly in vivo, through contact-dependence. Such a mechanism could explain the specificity of immunosuppression in tumor-bearing hosts wherein antigen-specific tumor infiltrating lymphocytes are rendered dysfunctional through contact with the tumor while circulating lymphocytes with other specificities remain functional.

**Abbreviations**

Con A, Concanavalin A; MACS, high gradient magnetic cell sorter; Meth A, methylcholanthrene-induced fibrosarcoma; MLTC, mixed lymphocyte tumor culture; MLR, mixed lymphocyte reaction

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**Materials and methods**

**Animals and tumor cells**

BALB/cJ and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and C3H-HeN mice from Harlan Sprague Dawley, Inc. (Indianapolis, IN). CMS4, CMS5, 10ME, 10CR, UV-6139, UV-5117, UV-5125, macrophage line 63, RBL (gift of Richard Klausner), CT26, B/c-N, and B/c-NP were maintained *in vitro* in DMEM, and EL-4 thymoma and A20 lymphoma in RPMI, supplemented with 5% FCS, penicillin/streptomycin (50 U/ml), L-glutamine, sodium pyruvate (1 mM), nonessential amino acids (1 mM), 2-mercaptopethanol (5.5 x 10⁻⁵ M) and grown at 37°C, 5% CO₂. Meth A cells were serially passaged as ascites in the peritoneum of BALB/cJ mice.

**Tumor Cell Immunization and Challenges**

To induce tumor rejection, BALB/cJ mice were immunized two times at a one week interval with 2 x 10⁷ irradiated tumor cells. Mice were challenged intradermally on the dorsum one week after the last immunization with 1 x 10⁵ live Meth A cells suspended in serum-free RPMI. To induce alloreactive rejection of Meth A cells, 2 x 10⁶ live cells were injected intradermally on the shaved dorsum of C57BL/6 mice.

**Mixed Lymphocyte Reactions (MLR) and Mixed Lymphocyte Tumor Cultures (MLTC)**

To establish MLRs, BALB/cJ (H-2d), C57BL/6 (H-2b), or C3H-HeN (H-2k) spleens were removed from mice and red blood cells were expelled by perfusing with plain RPMI. After mechanical disruption of spleens, lymphocytes serving as stimulators were incubated with mitomycin C (50 µg/ml) for 45 min. at 37°C followed by three washes with plain RPMI. Cells were counted and plated in 24-well plates at 4 x 10⁶ stimulator cells/ well to which responder cells were added at 6 x 10⁶ cells/well. On day 5, CTLs were harvested and tested for cytolytic activity against ⁵¹Cr-labeled target cells as described below.

For establishing MLTCs, splenocytes (responders) were isolated from BALB/cJ mice which had been immunized two times at a one week interval with 2 x 10⁷ irradiated Meth A cells or from C57BL/6 which had rejected challenges of live Meth A cells. Stimulating tumor cells (Meth A) received 8000 rads and were cultured with responding spleen cells in upright T30 flasks at a responder to stimulator ratio of 40:1 or in round-bottomed tubes at a responder to stimulator ratio of 200:1. On day 6, CTLs were tested in ⁵¹Cr release assays as described below.

**Chromium release and cold target inhibition assays**

For cytotoxicity assays, CTLs were serially diluted and plated in 96-well microtiter plates. Target cells were incubated in 200 µl volume RPMI with 100 µCi ⁵¹Cr for 45 minutes, followed by two washes with RPMI. Cells were counted and added to the CTLs. After a 4-hour incubation, supernatants were harvested and counted in a gamma counter for determination of specific killing of target cells. For cold target inhibition assays, cold targets were plated by serial dilution in 96-well round-bottomed plates. Effector cells were added and the plate was incubated at room temperature for 30 min, followed by addition of ⁵¹Cr-labeled target cells and incubation at 37°C for 3 hours. 100 µl of supernatant was counted in a gamma counter.
Transwell assays

A 96-well filtration plate (transwell) was supplied by Millipore Corp. (Bedford, MA). For separation assays, 160 µl medium or medium with cold targets was plated in the underlying 96-well plate. After overlaying the 96-well membrane, CTLs and the 51Cr-labeled target, was added in 120 µl medium to the upper compartment. After 4 hours, 100 µl supernatant was collected from the underlying plate and counted in a gamma counter.

Antibodies

Anti-TGF-beta monoclonal antibody, specific for TGF-beta1, TGF-beta2, and TGF-beta3, was purchased from Genzyme (Cambridge, MA). Rat anti-mouse CD4 monoclonal antibody GK1.5 and anti-mouse CD8 Lyt2.2 were obtained as ascites fluid from E. Nakayama (Okayama University School of Medicine, Okayama, Japan). For testing the permeability of transwell membranes to soluble molecules, we used hybridoma supernatant of K44, which was concentrated 10-fold by 50% ammonium sulfate precipitation and dialyzed against PBS.

T cell depletion

C57BL/6 mice receiving retro-orbital antibody injections were anesthetized with ether. Antibodies (anti-CD4 and anti-CD8) were suspended in RPMI and injected in 200 µl volume.

Purification of T cells and FACS analysis

T cells were separated from tumor cells after coculture assays or were isolated from splenocytes by positive selection using magnetic bead-coupled anti-Thy1.2 antibody and the MACS cell sorting system (Miltenyi Biotec Inc., Auburn, CA). The purity of enriched T cells (>95%) was assessed by FACSscan (Becton Dickinson, San Jose, CA) using PE-conjugated anti-CD3 epsilon antibody (clone 145-2C11, Pharmingen, San Diego, CA).

Membrane purification

Cells were removed from tissue culture flasks by trypsinization or harvested from peritoneal cavities of mice and washed twice with PBS. Cells were lysed by Dounce homogenization (10-20 strokes) after incubation in 5 volumes of 30 mM sodium bicarbonate buffer, 1 mM PMSF. Residual intact cells and nuclei were removed by consecutive centrifugations at 1000 g for 5 min until pellets were free of nuclei and cells as determined microscopically. This procedure ensured that less than 100 cells or nuclei remained in 2 x 10^8 cell equivalent of membrane material. The postnuclear supernatant was pelleted by centrifugation at 100,000 g for 90 min. Membranes were resuspended in 8% sucrose, 5 mM Tris, pH 7.6 and frozen at -80°C until use.

Paraformaldehyde treatment of Meth A cells

5 x 10^6 Meth A tumor cells were suspended in 1 ml 1% paraformaldehyde in PBS (Sigma). Cells were incubated on ice for 15 minutes and subsequently washed three times with RPMI.

Contact

Address correspondence to:

Pramod K. Srivastava, Ph.D.
Center for Immunotherapy of Cancer and Infectious Diseases
Mail Code 1601
University of Connecticut School of Medicine
Farmington, CT 06030
Tel.: +1 860 679 44 44
Fax: +1 860 679 43 65
E-mail: srivastava@nso2.uchc.edu

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