Tumor Rejection and Immune Memory Elicited by Locally Released LEC Chemokine Are Associated with an Impressive Recruitment of APCs, Lymphocytes, and Granulocytes

Mirella Giovarelli, Paola Cappello, Guido Forni, Theodora Salcedo, Paul A. Moore, David W. LeFleur, Bernadetta Nardelli, Emma Di Carlo, Pier-Luigi Lollini, Steve Ruben, Stephen Ullrich, Gianni Garotta and Piero Musiani

*J Immunol* 2000; 164:3200-3206; doi: 10.4049/jimmunol.164.6.3200

http://www.jimmunol.org/content/164/6/3200

References

This article cites 29 articles, 10 of which you can access for free at:
http://www.jimmunol.org/content/164/6/3200.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Tumor Rejection and Immune Memory Elicited by Locally Released LEC Chemokine Are Associated with an Impressive Recruitment of APCs, Lymphocytes, and Granulocytes

Mirella Giovarelli, Paola Cappello, Guido Forni, Theodora Salcedo, Paul A. Moore, David W. LeFleur, Bernadetta Nardelli, Emma Di Carlo, Pier-Luigi Lollini, Steve Ruben, Stephen Ullrich, Gianni Garotta, and Piero Musiani

The human β chemokine known as LEC (also called NCC-4, HCC-4, or LMC) displays chemotactic activity for monocytes and dendritic cells. The possibility that its local presence increases tumor immunogenicity is addressed in this paper. TSA parental cells (TSA-pc) are poorly immunogenic adenocarcinoma cells that grow progressively, kill both nu/nu and syngeneic BALB/c mice, and give rise to lung metastases. TSA cells engineered to release LEC (TSA-LEC) are still able to grow in nu/nu mice, but are promptly rejected and display a marginal metastatic phenotype in BALB/c mice. Rejection is associated with a marked T lymphocyte and granulocyte infiltration, along with extensive macrophage and dendritic cell recruitment. NK cells and CD4+ T lymphocytes are unidirectional in TSA-LEC cell rejection, whereas both CD8+ lymphocytes and polymorphonuclear leukocytes play a major role. An antitumor immune memory is established very quickly after rejection, since 6 days later 75% of BALB/c mice were already resistant to a TSA-pc challenge. Spleen cells from rejecting mice display specific cytotoxic activity against TSA-pc and secrete IFN-γ and IL-2 when restimulated by TSA-pc. The ability of LEC to markedly improve recognition of poorly immunogenic cells by promoting APC-T cell cross-talk suggests that it could be an effective component of antitumor vaccines. The Journal of Immunology, 2000, 164: 3200–3206.

The chemokine superfamily contains more than 50 secreted 8–12-kDa proteins with 20–70% homology in amino acid sequences that regulate leukocyte trafficking, promote inflammation, and modulate angiogenesis and lympho- and hematopoiesis (1). This superfamily is currently divided into four families according to the position of cysteine residues in the cytokines. Two families (α and β chemokines) have been extensively characterized, β chemokines constitute the larger family. They display two contiguous cysteines (C-C motif) and most of them are coded by genes clustered on human chromosome 17q11-2 (2). The gene encoding the LEC β chemokine (also known as NCC-4, HCC-4, and LMC) has been located in this cluster (2), and its genomic organization (3) and amino acid sequence (4) have been determined. LEC is chemotactic for human monocytes and dendritic cells and not neutrophils (5–7). This suggests that LEC may be involved in inflammation. Paradoxically and by contrast with other chemokines, the expression of LEC mRNA is up-regulated by IL-10 (5).

Comparison of the effects of chemokines by transducing a variety of tumor cells with their genes has shown that their local release influences tumor growth, angiogenesis, and immunogenicity. The effect achieved is highly dependent on the type of chemokine (1). The present study was designed to determine whether local secretion and accumulation of LEC influences the growth and immunogenicity of TSA, an aggressive mouse adenocarcinoma line, whose cells have been extensively engineered to release many other cyto/chemokines and whose behavior and immunogenicity have been carefully compared (8–10).

We show that the release of LEC by engineered TSA cells quickly induces their rejection by syngeneic mice and inhibits their metastatic spread. Rejection is associated with an impressive infiltrate of macrophages, dendritic cells, T cells, and polymorphonuclear (PMN) leukocytes, and is attributable to the last two populations. It is followed by the establishment of an effective and specific immune memory against TSA wild-type parental cells (TSA-pc). The unique effectiveness of this rejection and induction of a specific immune memory suggest that LEC could be made an effective component of gene-engineered antitumor vaccines.

Materials and Methods

Tumor cells

TSA-pc are an aggressive and poorly immunogenic cell line established from a moderately differentiated mammary adenocarcinoma that arose spontaneously in a multiparous BALB/c mouse (10). They express MHC class I, but not class II molecules, secrete G-CSF and GM-CSF, TGF-β (11), basic fibroblast growth factor and vascular endothelial growth factor.

Abbreviations used in this paper: PMN, polymorphonuclear; iNOS, inducible NO synthase; Mit-C, mitomycin C; Spc, splenic cells; sbp, specific-binding potential; TSA-pc, TSA parental cells.

Copyright © 2000 by The American Association of Immunologists 0022-1767/00/$02.00.

Received for publication September 23, 1999. Accepted for publication December 30, 1999.

Address correspondence and requests for reprints to Dr. Mirella Giovarelli, Department of Clinical and Biological Sciences, University of Torino, Orbassano, Italy; telephone: +39-11-460320; fax: +39-11-460323; e-mail: giovarelli@mimetica.unito.it.
(12), but not LEC (data not shown) and do not stimulate a syngeneic antitumor response in vivo, nor in mixed lymphocyte-tumor cell cultures (11). FI-F is a newborn BALB/c mouse-derived skin fibroblast cell line that spontaneously transformed after the 15th in vitro passage. Its cells do not immunologically cross-react with TSA- pc and they were therefore used as controls (13). Confluent monolayers of TSA or FI-F cells treated with a 0.25% solution of trypsin in HBSS (Sigma, Milan, Italy) were used for in vitro and in vivo experiments. Their minimal 100% tumor-inducing doses in BALB/c mice are 5 × 10^6 and 1 × 10^6, respectively.

Transfection of TSA- pc cells

The LEC β chemokine gene used in this study was cloned by Human Genome Sciences (Rockville, MD) through an expressed sequence tags database search by a high degree of homology to the β chemokine genes. To generate the LEC expression vector, the LEC open reading frame was amplified as a HindIII/Xbalt fragment and subcloned into likewise digested pcDNA3. Insert sequences were confirmed by double-stranded sequencing. pcDNA3 expression vectors contain the neomycin resistance gene and were used for TSA- pc transfection. Cells were plated at a density of 6 × 10^5 cells/100-mm tissue culture plate and incubated overnight at 37°C in DMEM with 4.5 g/l glucose and 10% FBS (BioWhittaker, Walkersville, MD). They were then suspended in OptiMEM without FBS and transfected using LipofectAMINE reagent (Life Technologies, Rockville, MD). Forty-eight hours later, they were trypsinized (Sigma), split 1:10, and plated in DMEM with 10% FBS in triplicate. Cultures were isolated and subcloned by limiting dilution 15–20 days later. TSA-neo is a control clone transfected with the neomycin resistance gene only.

Clone selection

The amount of LEC protein secreted in 1 ml medium by 1 × 10^5 seeded transfected TSA cells after 48 h of culture was evaluated by ELISA using polyclonal rabbit Ab against LEC, and recombinant LEC proteins (all from PeproTech, Rocky Hill, NJ). One representative transfected clone denominated TSA-LEC was selected for use in the experiments, since it consistently releases 60–70 ng of LEC as determined by repeated determinations during the experimental period (data not shown).

Mice

Seven-week-old female nu/nu (CD1) and BALB/CAnCr (H-2b) mice (Charles River Breeding Laboratories, Calco, Italy) were treated in accordance with European Union guidelines. When required, starting 2 days during the experimental period (data not shown).

For histologic evaluation, tissue samples were fixed in 10% neutral buffered Formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa. For immunohistochemistry, acetone-

The Journal of Immunology 3201

For histologic evaluation, tissue samples were fixed in 10% neutral buffered Formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa. For immunohistochemistry, acetone-

The Journal of Immunology 3201

For histologic evaluation, tissue samples were fixed in 10% neutral buffered Formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa. For immunohistochemistry, acetone-

The Journal of Immunology 3201

For histologic evaluation, tissue samples were fixed in 10% neutral buffered Formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa. For immunohistochemistry, acetone-

The Journal of Immunology 3201

For histologic evaluation, tissue samples were fixed in 10% neutral buffered Formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa. For immunohistochemistry, acetone-

The Journal of Immunology 3201

For histologic evaluation, tissue samples were fixed in 10% neutral buffered Formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa. For immunohistochemistry, acetone-
data obtained with TSA-pc and TSA-neo never differed significantly. For the sake of simplicity, only those obtained with TSA-pc are shown.

To evaluate whether LEC secretion affects the ability of TSA cells to give rise to a tumor, syngeneic BALB/c mice were challenged s.c. with a TSA-pc dose two times higher than the minimal 100% tumor-inducing dose. All of these mice developed tumors that increased in size with a similar progression pattern, whereas only 20% of mice challenged with TSA-LEC cells developed tumors (Table I). These few tumors were characterized by delayed growth patterns and a latency time almost double that of TSA-pc.

The extent to which the selective lung homing of metastatic TSA cells is affected by chemokine release was evaluated by challenging BALB/c mice with TSA-LEC and TSA-pc i.v. All mice receiving TSA-pc displayed lung metastases compared with only 50% of those injected with TSA-LEC, whose median number of metastases was also markedly reduced (Table II).

Role of host reactivity in TSA-LEC rejection

Variously immunosuppressed mice were challenged with TSA-LEC cells to characterize the leukocyte subpopulations mainly involved in their rejection. T-deficient nu/nu mice challenged with TSA-LEC cells developed tumors that developed progressively but more slowly than those challenged with TSA-pc (Table I). TSA-LEC rejection by BALB/c mice was not impaired by CD4+ and asialo GM1+ depletions, whereas it was abolished by CD8+ depletion. Rejection does not simply rest on CD8+ cells, however, since it was also significantly impaired by PMN depletion (Table I).

Morphologic features associated with TSA-LEC cells growth and rejection

TSA-pc grow and kill BALB/c mice. Three days after challenge, TSA-pc cells have already formed solid tumors with numerous mitotic figures. At day 7, the tumor invaded subcutaneous fibro-fat, connective tissue and epidermis (Fig. 1a). This growth was sustained by a well-developed and evenly distributed vascular network. Macrophages and a few granulocytes and lymphocytes infiltrated the tumor mass peripherally, where endothelial microvessels faintly stained for the ICAM-1 adhesion molecule (Table III).

The growth of TSA-LEC cells is strongly impaired in BALB/c mice. Three days after challenge, aggregates of TSA-LEC cells were interspersed with a massive reactive cell infiltrate. On day 7, stromal blood vessels surrounding and penetrating TSA-LEC tumors were impressively clogged with numerous lymphocytes (Fig. 1b). A marked infiltration of dendritic cells was detected, particularly within the few surviving aggregates of tumor cells (Fig. 1d). CD8+ and CD4+ lymphocytes, macrophages, and granulocytes were widely recruited at the TSA-LEC tumor site (Table III and Fig. 1f) and penetrated to the center of the tumor. Lymphocytes were massively present and frequently tightly close to TSA-LEC cells. This impressive leukocyte recruitment was associated with an intense proinflammatory cytokine production and macrophagic inducible NO synthase (iNOS) activation (Table III). Inducible endothelial adhesion molecules, namely, endothelial leukocyte adhesion molecule-1, VCAM-1 and ICAM-1, were intensely expressed on most of the TSA-LEC tumor vessels (Table III and Fig. 1f) and penetrated to the center of the tumor. Microvessel density was not appreciably different from that observed for TSA-pc tumors.

In nu/nu mice, TSA-LEC cells grow progressively. Infiltrating macrophages and granulocytes were remarkably less numerous than in BALB/c mice. A weak production of proinflammatory cytokines was detected in the tumor microenvironment, where only iNOS was strongly expressed. No adhesion molecules were found on tumor microvessels (Table III).

Acquisition of an immune memory to TSA-pc

At progressive times after TSA-LEC challenge in the left flank, mice were rechallenged with TSA-pc in the right flank to evaluate the potential of the systemic reaction elicited by TSA-LEC. Tumor takes of TSA-pc were only evaluated in mice in which TSA-LEC cells did not grow in the left flank. No protection was found when TSA-pc were injected simultaneously with TSA-LEC cells or 1 day after, whereas 75% of mice rejected TSA-pc when challenged 6 days later. All mice were protected against a challenge performed after 10 or 30 days. A specificity check with the unrelated F1-F tumor cell challenge displayed the selectivity of this immune memory (Table IV).

Memory mechanisms elicited by TSA-LEC rejection

Mice that had rejected a primary TSA-LEC challenge in the left flank received a secondary TSA-pc challenge in the right flank 30

### Table I. Growth of TSA-LEC and TSA-pc in T-deficient nu/nu mice and in normal and selectively immunosuppressed BALB/c mice

<table>
<thead>
<tr>
<th>Recipient Mouse</th>
<th>Challenging Cells</th>
<th>Immunosuppressive Treatment</th>
<th>Tumor takes/Challenged mice</th>
<th>Latency Time (days)</th>
<th>Survival Time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nu/nu</td>
<td>TSA-pc</td>
<td></td>
<td>4/4 (0%)*</td>
<td>15 ± 1</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>nu/nu</td>
<td>TSA-LEC</td>
<td></td>
<td>4/4 (0%)</td>
<td>22 ± 3</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>BALB/c</td>
<td>TSA-pc</td>
<td></td>
<td>25/25 (0%)</td>
<td>12 ± 3</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>BALB/c</td>
<td>TSA-LEC</td>
<td></td>
<td>4/22 (82%)*</td>
<td>22 ± 3</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>BALB/c</td>
<td>TSA-LEC Anti-CD4</td>
<td></td>
<td>1/5 (80%)</td>
<td>32</td>
<td>50</td>
</tr>
<tr>
<td>BALB/c</td>
<td>TSA-LEC Anti-CD8</td>
<td></td>
<td>5/5 (0%)*</td>
<td>13 ± 3</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>BALB/c</td>
<td>TSA-LEC Anti-CD4 + anti-CD8</td>
<td></td>
<td>5/5 (0%)*</td>
<td>14 ± 3</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>BALB/c</td>
<td>TSA-LEC Anti-asialo GM1</td>
<td></td>
<td>1/5 (80%)*</td>
<td>36</td>
<td>57</td>
</tr>
<tr>
<td>BALB/c</td>
<td>TSA-LEC Anti-PMN</td>
<td></td>
<td>6/8 (25%)*</td>
<td>13 ± 3</td>
<td>41 ± 4</td>
</tr>
</tbody>
</table>

* Values significantly different (p < 0.001) from those observed in mice challenged with TSA-pc.

* Values significantly different (p < 0.001) from those observed in untreated BALB/c mice.

### Table II. Metastatic ability of TSA-pc and TSA-LEC 21 days after i.v. challenge

<table>
<thead>
<tr>
<th>Challenging Cells</th>
<th>Incidence</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA-pc</td>
<td>10/10</td>
<td>93</td>
<td>57–113</td>
</tr>
<tr>
<td>TSA-LEC</td>
<td>5/10</td>
<td>2</td>
<td>0–4</td>
</tr>
</tbody>
</table>

* Five × 10⁴ cells injected i.v.

* Values with lung nodules/total challenged mice.

* Values significantly different from that of mice challenged with TSA-pc (Wilcoxon test).
days later. At 72 h, the TSA-pc growth area was heavily infiltrated by reactive cells and a few aggregates of tumor cells with marked signs of injury were evident. The number of lymphocytes, and particularly that of CD8¹ lymphocytes, was significantly higher than that found after primary TSA-pc challenge in nonimmunized mice. Endothelial adhesion molecules along with proinflammatory cytokines, especially IFN-γ, were strongly expressed (Table III).

When mice that had rejected TSA-LEC cells 30 days before were selectively immunosuppressed during the secondary challenge, it was found that memory TSA-pc rejection was not affected by NK cell depletion, whereas it was impaired by CD4¹ and CD8¹ lymphocyte and PMN cell depletion (Table IV).

Both fresh and TSA-pc-restimulated Spc from mice that had rejected TSA-LEC cells displayed a marked and specific cytolytic activity against TSA-pc (Table V). Following restimulation with TSA-pc, these Spc released 5 times more IL-2 and 10 times more IFN-γ than restimulated Spc from normal mice (Fig. 2). Neither IL-4 nor IL-10 were released (data not shown).

Finally, as early as 7 days after a TSA-LEC challenge, anti-TSA-pc Abs were massively present. Thirty days after challenge, their level was still higher (Table VI).

**Discussion**

Our findings show that sustained secretion of LEC β chemokine in the tumor microenvironment markedly impairs the growth of TSA adenocarcinoma cells. This inhibition appears to depend on LEC’s ability to recruit and activate interacting leukocyte populations.

---

**Table III.** Reactive cell content, expression of endothelial adhesion molecules, and production of cytokines and mediators at the tumor area after TSA-pc and TSA-LEC cell challenge of nu/nu, untreated, and TSA-LEC-immunized BALB/c mice

<table>
<thead>
<tr>
<th>BALB/c Mice</th>
<th>mu/nu Mice</th>
<th>TSA-LEC-Immunized BALB/c Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSA-pc</td>
<td>TSA-LEC</td>
</tr>
<tr>
<td>TSA-pc</td>
<td>14 ± 4*</td>
<td>29 ± 6*</td>
</tr>
<tr>
<td>TSA-LEC</td>
<td>40 ± 7</td>
<td>88 ± 11*</td>
</tr>
<tr>
<td>Reactive cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>14 ± 2</td>
<td>62 ± 7*</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>6 ± 2</td>
<td>72 ± 8*</td>
</tr>
<tr>
<td>CD8¹ lymphocytes</td>
<td>2 ± 1</td>
<td>71 ± 9*</td>
</tr>
<tr>
<td>Endothelial adhesion molecules</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>ELAM-1¹</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Cytokines and mediators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>INF-γ</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>IL-6</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>iNOS</td>
<td>−</td>
<td>++</td>
</tr>
</tbody>
</table>

*Performed 7 days after challenge.

*Performed 3 days after challenge.

Cell counts performed at ×400 in a 0.180-mm² field on 10 randomly chosen fields/sample. Results are means ± SD of positive cells/field evaluated on cryostat sections by immunohistochemistry and/or Giemsa-stained sections (basophils and mast cells).

The expression of adhesion molecules, cytokines, and mediators was classed as absent (−), scarcely (+), moderately (++) or strongly (+++) present on cryostat sections decorated with the Abs.

ELAM-1, endothelial leukocyte adhesion molecule-1.

*Values significantly different (p < 0.005) from corresponding values in TSA-pc.
The in vitro proliferation of TSA-LEC cells is not influenced by the LEC release, whereas their growth in immunodeficient nu/nu mice is only slightly hindered as compared with TSA-pc.

In immunocompetent mice, TSA-LEC cells display an impressive ability to promote monocyte, dendritic cell, T cell, and granulocyte influx into the tumor site. These findings do not contradict the in vitro data on the chemotactic activity of LEC (5, 6), but certainly seem more remarkable than might have been expected. This consideration coupled with the marked differences in infiltrating cells observed in nu/nu and immunocompetent mice points to a major role for T cells in orchestrating the magnitude of the infiltrate and the efficacy of the reaction. In immunocompetent mice, TSA-LEC cells elicit a marked local production of IL-1β, TNF-α, and IFN-γ and induce the expression of adhesion molecules on tumor vessels. Leukocyte adhesion, transmigration, and recruitment appear to be guided by these two key events. In contrast, up-modulated expression of adhesion molecules on tumor vessels, recruitment of macrophages and granulocytes, and cytokine production are all almost negligible in T cell-deficient nu/nu mice.

Both in vitro (5, 7) and in vivo, LEC does not appear to be a typical chemotactic agent as it is endowed with a few peculiar features: 1) its in vitro chemotactic activity on macrophages and dendritic cells takes place at abnormally high concentrations when compared with other chemokines; 2) its mRNA is constitutively expressed by a wide range of tissues; and 3) in contrast to the down-regulatory effect of IL-10 on the expression of most chemokines, LEC expression is up-regulated by IL-10 (5, 7).

The sustained accumulation of LEC in the TSA-LEC tumor microenvironment may account for the recruitment of reactive cells leading to tumor rejection. Yet, how are T lymphocytes so impressively recruited despite the evidence that LEC is only slightly or occasionally chemotactic for resting T cells in vitro (5–7)? High local concentrations of LEC could induce downstream mediators, leading to T cell recruitment. Macrophages recruited and activated by LEC may produce proinflammatory factors, leading to dendritic cell maturation (15). By releasing DC-Ck1 and other chemokines, dendritic cells may subsequently attract and specifically activate T lymphocytes in the tumor microenvironment (16–19). This possibility is endorsed by immunohistochemical data showing that TSA-LEC cells also recruit macrophages and activate TNF-α, IL-1β, and iNOS production in T lymphocyte-deficient nu/nu mice. Alternatively, macrophages and dendritic cells attracted by LEC may migrate to lymphoid organs and present TSA Ag peptides to T cells (20). These activated T cells then home into the tumor area, release proinflammatory cytokines, and orchestrate tumor destruction.

The well-organized capillary network of a TSA-LEC tumor suggests that angiogenesis inhibition is not the means by which LEC induces TSA regression, unlike many other cytokines such as IFN-α, IFN-γ (21), and IL-12 (9). Data from selectively depleted syngeneic mice point to a major role of the CD8+ subset and underscore the significant role of PMN cells in TSA-LEC rejection. This finding offers further evidence of the importance of the PMN-T cell cross-talk in cytokine- and chemokine-elicited tumor rejection (8, 21).

TSA-LEC cells are poorly immunogenic in immunization-protection tests and unable to stimulate proliferation and lymphokine release by syngeneic lymphocytes (10). The early onset of a strong and systemic memory to TSA-pc following TSA-LEC challenge suggests that LEC accelerates events that underlie a cross-talk between T cells and APCs (22–23). Morphologic observations of TSA-pc memory rejection showed the absence of the macrophage and dendritic cell infiltrate that was the hallmark of the primary TSA-LEC rejection, whereas a conspicuous CD8+ and CD4+ lymphocyte infiltrate and a distinct expression of proinflammatory cytokines were evident. The importance of CD8+ T cells in secondary TSA-pc rejection, along with the strong lytic activity of Spc associated with their ability to release IFN-γ and IL-2 but not IL-4, suggests an immune memory deflected toward a Th1-type

### Table IV. Characteristics of immune memory to TSA-pc acquired following rejection of TSA-LEC cells

<table>
<thead>
<tr>
<th>Primary Challenge</th>
<th>Day of challenge</th>
<th>Immunosuppressive treatment during secondary challenge</th>
<th>Tumor takes/total mice challenged with (day 0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA-LEC</td>
<td>0</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>TSA-LEC</td>
<td>0</td>
<td>10/10</td>
<td>ND</td>
</tr>
<tr>
<td>TSA-LEC</td>
<td>1</td>
<td>10/10</td>
<td>ND</td>
</tr>
<tr>
<td>TSA-LEC</td>
<td>6</td>
<td>2/8</td>
<td>ND</td>
</tr>
<tr>
<td>TSA-LEC</td>
<td>10</td>
<td>0/8</td>
<td>ND</td>
</tr>
<tr>
<td>TSA-LEC</td>
<td>30</td>
<td>0/8</td>
<td>10/10</td>
</tr>
<tr>
<td>TSA-LEC</td>
<td>30</td>
<td>Anti-CD4</td>
<td>ND</td>
</tr>
<tr>
<td>TSA-LEC</td>
<td>30</td>
<td>Anti-CDS</td>
<td>7/8</td>
</tr>
<tr>
<td>TSA-LEC</td>
<td>30</td>
<td>Anti-asialoGM1</td>
<td>1/8</td>
</tr>
<tr>
<td>TSA-LEC</td>
<td>30</td>
<td>Anti-PMN</td>
<td>6/10</td>
</tr>
</tbody>
</table>

* One × 10⁵ cells injected s.c. in the left flank.

* Performs in the right flank with 1 × 10⁷ TSA-pc or 1 × 10⁴ F1-F cells.

* Performs as described in Materials and Methods before and after TSA-pc challenge.

### Table V. TSA-specificity CTL from spleen of mice immunized with TSA-LEC cellsa

<table>
<thead>
<tr>
<th>Spc from</th>
<th>In vitro restimulation</th>
<th>TSA-pc</th>
<th>F1-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mouse</td>
<td>9</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mice immunized with TSA-LEC</td>
<td>25b</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Normal mouse</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mice immunized with TSA-LEC</td>
<td>173c</td>
<td>29</td>
<td>29</td>
</tr>
</tbody>
</table>

* Spc from untreated mice or from mice challenged with 1 × 10⁵ TSA-LEC cells 30 days before were tested immediately or after 6 days of culture with Mit-C TSA-LEC stimulator cells (40:1 responder:stimulator ratio). Cytotoxicity was evaluated in a 48-h [³H]Tdr release assay and expressed as LU₃₀/₇ × 10⁷ from three replicates of the same experiment.

* The cytotoxicity percentage was uniformly higher at all four E:T ratios compared to corresponding values in naive mice. The LU₃₀ values were significantly different (p < 0.001).

* Spc from mice treated with 1 × 10⁷ TSA-LEC cells and 1 × 10⁴ F1-F cells.
reaction. However, depletion experiments also underscore a crucial role of CD4+ lymphocytes and PMN cells. This suggests that TSA-LEC triggers a more articulated memory mechanism. Indirect presentation of tumor Ags may trigger Th cells to release IFN-γ and other cytokines through which the antitumor activity helper T cells attract, activate, and guide PMN cells (13, 21, 24–26). Finally, the titer of anti-TSA Ab quickly elicited by TSA-LEC is markedly higher than that observed after challenge with TSA cells engineered to release cytokines eliciting a Th1 or Th2 response, such as IL-4 (13), IL-10 (27), and IL-12 (9). These Abs could provide further guidance for PMN-dependent tumor rejection (13).

The rejection of tumor cells engineered to release cytokines and chemokines is often followed by establishment of a systemic immune memory specific to a subsequent challenge by wild-type tumor cells. The features of this memory are dictated by the factor and other cytokines through which the antitumor activity helper T lymphocytes and PMN cells. This suggests that memory-inducing cytokines, such as IL-2, IFN-γ, and IL-12 (8–9, 27). This makes LEC a candidate for use as a very effective component of engineered tumor vaccines.

Acknowledgments

We thank Dr. R. L. Coffman, Dr. S. Landolfo, and Dr. A. Vecchi for providing reagents. We thank Dr. J. Iliffe for critical review of this manuscript.

### References


