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*J Immunol* 2000; 164:2619-2628; doi: 10.4049/jimmunol.164.5.2619

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Mice Bearing Late-Stage Tumors Have Normal Functional Systemic T Cell Responses In Vitro and In Vivo

Sasa Radoja,*† T. Dharma Rao,* Deborah Hillman,* and Alan B. Frey**

Immune suppression in tumor-bearing hosts is considered to be one factor causally associated with the growth of antigenic tumors. Support for this hypothesis has come from reports that spleen T cells in tumor-bearing mice are deficient in either priming or effector phase functions. We have reexamined this hypothesis in detail using multiple murine tumor models, including transplantable adenocarcinoma, melanoma, sarcoma, and thymoma, and also a transgenic model of spontaneous breast carcinoma. In both in vitro and in vivo assays of T cell function (proliferation, cytokine production, induction of CD8⁺ alloreactive CTL, and development of anti-keyhole limpet hemocyanin CD4⁺ T cells, rejection of allogeneic or syngeneic regressor tumors, respectively) we show that mice bearing sizable tumor burdens are not systemically suppressed and do not have diminished T cell functions. Therefore, if immune suppression is a causal function in the growth of antigenic tumor, the basis for escape from immune destruction is likely to be dependent upon tumor-induced T cell dysfunction at the site of tumor growth. The Journal of Immunology, 2000, 164: 2619–2628.

*Department of Cell Biology and Kaplan Cancer Center, New York University School of Medicine, New York, NY 10016; and † The Institute of Molecular Genetics and Genetic Engineering, Belgrade, Yugoslavia

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Received for publication July 30, 1999. Accepted for publication December 13, 1999.

T

cells of tumor-bearing mice have been described as hav-
ing defects in various molecular important in the TCR-
and IL-2R-mediated signal transduction pathways (1–3).
For example, diminished levels or activation status of TCR-prox-
imal protein tyrosine kinase molecules (p56lck, TCRz chain, or p59fyn) in spleen T cells have been described in murine tumor
models that were postulated to be responsible for an altered pattern of cellular protein tyrosine phosphorylation thought to reflect de-
f ective T cell function (4–6). Such defects in the T cell signal
transduction machinery are considered to be the biochemical basis
of suppression of immune response that is postulated to be causally
associated with tumor escape from immune destruction (1–3).

Although interest in the area of tumor-induced immune suppres-
sion has increased recently, the mechanism(s) by which tumor
growth induces the biochemical changes in T cells that are postu-
lated to underlie T cell dysfunction are largely unknown. Some
possibilities include production by tumor (or host cells that infil-
trate the tumor microenvironment) of soluble factors that inhibit
cell cycle progression, tumor-induced defects in proximal TCR-
mediated signal transduction molecules, tumor-induced defects in
intracellular signaling molecules, and activation of tumor-infiltrat-
ing macrophages that may express immunosuppressive factors
such as IL-10, PGE, hydrogen peroxide, or nitric oxide.

The physiological consequences of the tumor-induced T cell
defects are variable. In some murine tumor models TCR-mediated
proliferation is reduced in T cells obtained from spleens (7); in
other models cytokine expression from spleen T cells or induction
of CTL activity in MLR established in vitro is diminished (5, 8).

In several studies the reported diminished function of spleen
T cells in mice bearing late-stage tumors is postulated to correlate
with the development of a particular deficit in one of several mol-
ecules associated with proximal TCR-mediated cell signaling (5–7,
9). However, two reports persuasively argue that these biochemi-
cal changes in T cells of tumor-bearing mice represent normal
fluorochrome not attributable to the effects of tumor growth (8, 10).
In addition, several reports argue that, owing to its unusual sensi-
tivity to proteolysis, the apparent reduction of the TCRz levels in
spleen T cells from tumor-bearing mice may be an experimental
artifact resulting from contamination of T cell preparations with
neutrophils and/or macrophages (8, 10, 11). These observations,
considered together with an analysis of T cell function in cancer
patients in whom the loss of proteins involved in proximal TCR-
mediated signal transduction was not observed (12), prompted us
to carefully evaluate the function of T cells in spleens of tumor-
bearing mice. Our data demonstrate that tumor-bearing mice do
not have systemic T cell dysfunction.

Materials and Methods

Mice

C3H/HeN, C57BL/6, male, and FvBN female mice were obtained from
The Jackson Laboratory (Bar Harbor, ME). Mice were housed four per
cage in a barrier facility and maintained on a 12-h light, 12-h dark cycle
(0700–1900 h) with ad libitum access to food and water. A sentinel pro-
gram revealed that the mice were murine hepatitis virus negative. Exper-
iments involving animals were conducted with the approval of the New
York University Medical School committee on animal research.

Tumors

6-1 tumor was created by expression of plasmids encoding activated Ha-
Ras plus p53 genes in primary murine C3H/HeN embryonic fibroblasts.
The properties of this tumor have been described previously (13). Lp53
was created by transfection of L929 cells with a plasmid expressing acti-
vated murine p53 (13). MEFSV40TAg was created by transfection of pri-
mary C3H/HeN MEF with a plasmid expressing SV40 large T Ag (14).
MCA-38 cells and MC57G cells were gifts from S. Vukmanovic (New
York University Medical School, New York, NY). EL-4 cells were gifts
from A. Menoret (University of Connecticut, Farmington, CT). UV6138,
6130b, K1735, and K5222 were gifts from H. Schreiber (University of
Chicago, Chicago, IL). L929 (C3H/HeN) was purchased from American
Type Culture Collection (Manassas, VA). A-20 (BALB/c) was a gift from
Y. Liu (Ohio State University, Columbus, OH). Most adherent tumor cell
lines were removed from tissue culture plastic by incubation in HBSS containing 2 mM EDTA and were washed three times in HBSS. A-20 cells were grown in suspension culture, and 6139b were passaged in vivo in syngeneic nude mice by trocar injection of small tumor fragments (∼2 mm³). The viability of the cell lines was determined by trypan blue dye exclusion, and 2 × 10⁴ cells were injected i.p. in a volume of 0.2 ml of HBSS. Regressor tumor 6139b was injected as small tumor fragments using a trocar. Control mice received injections of HBSS only.

Transgenic FvBN mice expressing activated β-catenin under the mouse mammary tumor virus (MMTV) promoter (an amino-terminal deletion of 89 residues termed “ABCXM”) were created and provided by Alexandra Imbert and Pam Cowin (New York University Medical School). These mice develop multifocal breast carcinoma at 4–5 mo of age.

**Tissue culture**

RPMI 1640 medium (BioWhittaker, Walkersville, MD) was used for isolation and culture of T cells and was supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.002 mM l-glutamine, and 10% FBS (In- tergen, Purchase, NY). DMEM was used for culture of tumor cell lines. All tissue culture supplements were supplied by Life Technologies (Grand Island, NY).

**Isolation of T cells**

T cells were purified using immunomagnetic separation using type MS-5 or VS⁺ columns according to the manufacturer’s instructions (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany). In each experiment aliquots of isolated cells were analyzed for cell surface expression of various markers by flow cytometry and were routinely >95% CD3⁺. T cells purified thusly from splenocytes of control mice do not express activation Ags (CD25 and CD69), do not transcribe IL-2 mRNA, and do not incorporate thusly from splenocytes of control mice do not express activation Ags.

**Cytofluorometry**

For single-color analysis, splenocytes (10⁶) from control or tumor-bearing mice were once washed with FACS buffer (HBSS without phenol red (Bio-Whittaker)), 1% BSA (Sigma, St. Louis, MO), and 0.1% sodium azide (Sigma)) and analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) as previously described (16). 

**Proliferation assay**

Single-cell splenocyte suspensions were prepared and analyzed by measurement of incorporation of tritiated thymidine after stimulation with plate-bound purified anti-TCR mAb (H57-597, 0.01 mg/ml for 60 min at 37°C) as previously described (17).

**Generation of primary CTL in vitro**

Responder spleen cells (5 × 10⁶) from C3H/HeN or transgenic ΔBCM FvBN mice were cultured with the same number of irradiated stimulator splenocytes from C57BL/6 mice in 2 ml of complete RPMI 1640 medium. Responder cells from C57BL/6 mice were similarly stimulated with irradiated C3H/HeN cells. After 5 days cells were harvested, the viability was assessed by trypan blue exclusion, the percentage of CD8⁺ T cells was determined by flow cytometry of an aliquot (or in some cases, as indicated in the figure legend, CD8⁺ T cells purified by magnetic immunobeadning), and CTL activity was determined.

**Chromium release assay**

CTL activity of splenocytes was determined in standard ⁵¹Cr release assays. In brief, 10⁶ target cells (EL-4 or P815 for redirected assays) were incubated with 0.2 µCi of Na⁵¹CrO₄ in RPMI 1640 medium for 60 min at 37°C. Cells were washed twice with complete medium and transferred to round-bottom 96-well plates at 5 × 10⁴ cells/well. Effector cells were added at varying numbers as indicated in the figure legends in a final volume of 0.2 ml. After a 4-h incubation at 37°C, 0.1 ml of supernatants were harvested, and released radiolabel was determined by scintillation counting. Redirected cytolytic assays using anti-CD3e Ab 145-2C11 (at 0.001 mg/ml final concentration) were used to determine the CTL function of splenocytes from animals bearing MCA-38 tumors (because of lack of a suitable target cell) exactly as previously described (18). Purified hamster IgG was used as control Ab for redirected assay, and lysis of labeled targets in the presence of effector cells plus control IgG was equivalent to spontaneous release. Maximal release from target cells was determined by treatment of cells with 1% Triton X-100, spontaneous release was determined from cultures of labeled target cells incubated with medium only, and the formula used for determination of specific lysis was: [experimental release - spontaneous release]/(maximal release - spontaneous release) × 100.

**RNA isolation, RT, and PCR amplification**

Total cellular RNA was isolated from T cells without in vitro stimulation, used to prepare cDNA, and used to program PCR amplification as described previously (16). Actin was amplified for 30 cycles; IL-2, IL-10, TNF-α, and IFN-γ were amplified for 33 cycles; and IL-4 was amplified for 38 cycles. The sizes of the PCR fragments generated were 450 (actin), 247 (IL-2), 383 (IL-4), 354 (TNF-α), 186 (IL-10), and 237 (IFN-γ). The sequences of the PCR primers used are: actin, sense, 5'-GGT GGC CCC AGC CAC CA; and antisense, 5'-CTC ATT ATT GTC AGC CAC GAT TTC; IL-2, sense, 5'-GAG TCA AAT CCA GAA CAT GCC; and antisense, 5'-TCC ACT TCA AGC TCT ACA G; IL-4, sense, 5'-GAG TGT ACC AGG AGC CAT ATC; and antisense, 5'-CTC GTG ACT AGG AGT AAT CCA; IL-10, sense, 5'-CCT TTC TGA AAA TCG GAT TTC G; and antisense, 5'-CGG GAA GAC AAT AAC TG; TNF-α, sense, 5'-TTC TGT CTA CTA AAC TGG GGT ATG ATC GGT CC; and antisense, 5'-GTA TGA GAT AGG AAA TTC GCT GAC GGT GTG GG; and IFN-γ, sense, 5'-AAG GCT ACA CAC TGC ATC TTC G; and antisense, 5'-GAT CCC TAA GAG TCT GAG T. There were no cytokine PCR products in control reactions in which polymerase was omitted, as detected by Southern blot analyses.

**End labeling, agarose gel electrophoresis, and Southern blotting**

Oligonucleotide probes for all cytokine PCR products are internal to the PCR primers used for amplification (IL-2, 5'-CCC AGG ATG ATC ACC TTC TT; IL-4, 5'-AGG GCT TCT GGG CAC GGT AGG ATG T; TNF-α, 5'-AGC GCC ACC CAC TCT GTC GCC AAC GCC; and IFN-γ, 5'-GGA GAT GCC AAA AGG A; actin was visualized by ethidium bromide staining only). Probes were labeled with [γ-³²P]ATP (7000 mCi/mM; NEN/DuPont, Boston, MA) and used to analyze PCR-amplified DNA fragments by agarose gel electrophoresis followed by Southern blotting as previously described (16).

**Results**

**Tumor growth causes splenomegaly and accumulation of macrophages and neutrophils**

As a prelude to characterization of systemic T cell function in tumor-bearing mice we analyzed the effect of progressive tumor growth on spleen size and cellularity. Naive mice were seeded with tumor cells, and spleens were isolated and characterized by flow cytometry at increasing times of tumor growth. An inocula of ∼2 × 10⁶ 6-1 cells, a dose that caused progressive tumor growth and death after about 8 wk, resulted in tumors of ∼3 g after about 30–35 days. The total number of splenocytes increased progressively in tumor-bearing mice, such that by wk 2 of growth spleens had about 3-fold more cells than controls (Fig. 1). The increase was not due to metastasis of tumor, because spleens of mice bearing 6-1 tumor expressing neomycin resistance did not contain cells able to grow in the presence of G-418 in vitro (data not shown).

Spleens from a series of different murine transplantable tumors and a transgenic line that develop breast carcinoma spontaneously at about age 4–5 mo (termed ΔBCM) were analyzed for splenomegaly. In all 10 tumor models tested, encompassing four strains of mice and five tumor types (6-1, MCA-38, K1735, K5222, Lp53, UV6138, MEFSV40Tag, EL4, MC57G, and ΔBCM), splenomegaly was an inevitable consequence of tumor growth (data not shown).

The cellular composition of tumor-bearing mouse spleens as a function of tumor burden was analyzed by flow cytometry. For 6-1 sarcoma, at early times of tumor growth (1–2 wk) the percentages of Mac1⁺ and Gr-1⁺ were notably increased, whereas the abundance of other major cell types (CD4⁺ or CD8⁺ T cells and B cells) was not dramatically affected (data not shown). There was a
progressive accumulation of Mac1+ and Gr-1+ cells, such that in mice with large tumor burdens (> ~4 wk of growth) macrophages and neutrophils together accumulated to 50–60% of the total splenocytes. At early times of tumor growth there was only a modest effect on the percent abundance of CD4+ and CD8+ T cells, but by 3–4 wk CD3ε+ T cells were reduced from 25–30% to about 10% (Fig. 2). There was a similar reduction in abundance of B cells from ~50 to 10% by wk 4 of tumor growth. The mean fluorescence intensity of staining of T cell subsets did not diminish, but for B cells, as the abundance decreased, so did the mean fluorescence intensity (from ~750 to ~340).

Similarly we analyzed by flow cytometry splenocytes of animals bearing other tumor types. Although the relative abundance of different immune cells varied depending upon the individual tumor type (Fig. 2 and data not shown), in all models it can be generalized that with increasing time of tumor growth spleens accumulate macrophages and neutrophils simultaneously, with a decrease in T cells and in most cases also in B cells.

Spleen cells of tumor-bearing mice are deficient in proliferative response to TCR ligation in vitro

To determine whether the altered cellular composition of spleens reflected T cell function, the proliferative responsiveness of splenocytes from tumor-bearing mice was tested compared with that of cells from control mice (Fig. 3). Proliferation assay of 10⁵ splenocytes from mice bearing 6-1 tumor showed a progressive decrease in the incorporation of tritiated thymidine as a function of
time of tumor growth. At early times after tumor seeding the pro-
iferative deficit was modest (~25% that in control spleen cells at
2 wk), but in all mice analyzed (>40 individual 6-1 tumor-bearing mice) prolifera-
tion was reduced by about 40% by about 3 wk of tumor growth. At later times the deficit was even greater, ap-
proaching 90% at 5 wk. The proliferative deficit was not a function of the method of T cell activation in vitro (plate-bound anti-TCR Ab), because the deficit was also noted when soluble anti-TCR Ab (or anti-CD3e) plus anti-CD28 Ab, plate-bound anti-TCR plus anti-
CD28 Abs, or Con A were used to stimulate cells (data not shown). A similar proliferative deficit was noted in other tumor models.

Tumor-bearing mice prime anti-KLH T cells in vivo
The apparent proliferative deficit in tumor-bearing mice was ant-
icipated to reflect some phenotypic abnormality in T cell function, possibly related to escape of tumor from antitumor immune re-
sponse. To address this point we asked whether tumor-bearing mice can prime a T cell immune response in vivo. Tumor-bearing or control mice were injected with an antigenic protein, KLH, and the development of anti-KLH T cell immune response was mea-
sured by proliferation assay of splenocytes in vitro. We found that mice bearing tumors for 2 or 3 wk produced anti-KLH T cell immune response equivalent to that of non-tumor-bearing control mice (Fig. 4). This finding suggests that the dramatic change in spleen cellular composition and deficit in spleen T cell prolifera-
tion do not inhibit development of CD4+ T cells reactive with a soluble antigenic protein after injection.

Tumor-bearing mice reject allogeneic tumor challenge
We performed experiments designed to test the physiologically relevant function of T cell immune response in tumor-bearing mice by asking whether tumor-bearing mice could reject allogeneic tu-
mor challenge. Mice were seeded with 6-1 tumor cells and after 3 wk of tumor growth were injected with tumors originating in the
H-2B (Fig. 5a) or H-2D (data not shown) background. Mice syn-
geneic with the secondary tumors injected at the same time as mice bearing 6-1 tumor developed tumors with kinetics typical of the individual tumor. One hundred percent of both non-tumor-bearing control and tumor-bearing H-2K mice rejected allogeneic tumor challenges. This finding shows that tumor-bearing mice are com-
petent to reject allogeneic tumor challenge.
stimulus we also tested whether mice bearing 3-wk-old 6-1 tumor could reject challenge of syngeneic and non-cross-reactive tumors, which are rejected by mice with functional immune systems. The goal of this experiment was to test whether mice bearing tumors can mount a successful antitumor immune response to antigenically unrelated tumors. The choice of the specific second tumors, so-called regressor tumors (19), was made because they are known to require T cells for their rejection in intact (non-tumor-bearing) mice (20). If mice bearing tumors can reject secondary tumors, then it can be inferred that the T cells in the tumor-bearing mouse are not defective in the ability to recognize and reject antigenic tumors. The growth of regressor tumors was authenticated by seeding tumors in athymic syngeneic mice simultaneously with injection into tumor-bearing mice. As was found for the rejection of allogeneic tumors, mice bearing 6-1 tumors could reject challenge of three different syngeneic regressor tumors (Fig. 5b) and L929 and 1402RE tumors (data not shown). These results show that the immune function in tumor-bearing mice is sufficient to permit rejection of syngeneic, but not cross-reactive, tumors.

Splenocytes of tumor-bearing mice can develop CD8+ CTL after in vitro priming

The ability to develop spleen-derived CD8+ CTL response after in vitro priming has been found by others to be defective in tumor-bearing mice (4, 8). Because rejection of allogeneic and syngeneic tumor in vivo is dependent upon CD8+ T cells, this assay of systemic immune competency was tested in various tumor models. After 3 wk of 6-1 tumor growth (a point at which spleen cellularity is dramatically altered; Fig. 2) spleens were isolated from mice and primed in vitro with irradiated allogeneic H-2B splenocytes. After 5 days of coculture viable cells were isolated and analyzed by flow cytometry. Viable cells present after in vitro priming were 15% CD8+ using splenocytes from control mice and were 8% using splenocytes from tumor-bearing mice (day 21). Cells were used as effector cells in a standard cytolytic assay and were equal to or more active than control T cells in CTL activity (Fig. 6a). Target lysis was inhibited by inclusion of anti-CD8 Ab, but not by irrelevant Ig (data not shown).

In a similar manner in vitro priming of CD8+ CTL was tested for four other tumor models (MCA-38 and ΔBCM, Fig. 6, b and c; K1735 and EL4 tumors, data not shown). Splenocytes from mice bearing MCA-38 tumors were stimulated in vitro with C3H/HeN spleen cells; the others were primed with C57BL/6 spleen cells. Cytolytic activity induced in spleen cells obtained from MCA-38 tumor-bearing mice was analyzed by redirected CTL assay (using P815 tumor as target), and assay of the other tumor models used direct lysis of labeled EL4 targets (H-2B as were cells used for in vitro priming). In all tumor models tested efficient CTL activity was elicited from spleen cells of tumor-bearing mice. Alloreactivity induced in splenocytes from tumor-bearing mice was potent; when CD8+ T cells were purified after priming for use in CTL assay, significant killing was seen at E:T cell ratios as low as 2.5:1. These experiments demonstrate that although the cellular composition of spleens in tumor-bearing mice is changed compared with that in control mice (Fig. 2), the presence of abundant macrophages and neutrophils and the reduced levels of CD8+ T cells (even during priming in vitro) does not diminish functional priming of T cell-mediated cytolytic immune response.

Purified spleen CD3+ T cells from tumor-bearing mice are not defective in proliferative response in vitro

The results of the functional analyses of spleen T cell immune responses (shown above) motivated us to re-examine the proliferative deficit previously noted in Fig. 3. We reasoned that the presence of macrophages and neutrophils that accumulate in spleens may have artificially influenced T cell proliferation assays, perhaps due to elaboration of immunosuppressive chemokines and/or cytokines produced during in vitro culture. Alternatively, because of the progressive decrease in abundance of T cells as a function of tumor growth, the absolute number of T cells in spleen preparations of tumor-bearing mice may have been reduced below a minimum number required to reveal anti-TCR Ab-induced T cell proliferation in vitro. Therefore, we modified the proliferation assay to include analysis of the percentage of CD3ε+ T cells in splenocyte preparations, which permitted plating of known numbers of T cells from spleens of both control and tumor-bearing mice. When proliferation was assessed in this manner, spleen T cell responses (shown above) motivated us to re-examine the proliferative deficit previously noted in Fig. 3.
cells from tumor-bearing mice were not diminished in incorporation of tritiated thymidine compared with control T cells (Fig. 7, A and B).

Confirmatory experiments were also performed in which spleen T cells were purified by magnetic immunobead isolation using anti-Thy1 Ab before proliferation assay. T cells purified in this manner were >95% CD3ε+ and were not contaminated with Mac1+ cells (Fig. 7A) or Gr-1+ cells (15). In addition, incorporation of thymidine in vitro was dependent upon mitogen activation, demonstrating that isolation does not activate the T cells (15). Purified T cells obtained from tumor-bearing mice reproducibly proliferated better than T cells purified from control mice (Fig. 7B). In another confirmatory experiment CD3ε+ T cells purified from spleens of tumor-bearing mice by FACS also proliferated better than the equivalent cell population purified from control mice (data not shown).

To generalize this finding we performed proliferation assays using spleen cells from mice bearing 3-wk tumors of the following types: K1735, EL4, UV6138, L929/p53 (21), K5222, B16, SV40 large T Ag-transformed primary murine embryonic fibroblasts (21), MCA-38, and MC57G. In addition, we tested a transgenic murine line in which mutant β-catenin is expressed under the MMTV promoter and mice develop spontaneous multifocal breast carcinoma at 4–5 mo of age, ΔBCM. For all tumor models proliferation of spleen T cells from mice bearing tumors was equivalent to or greater than that of splenocytes from syngeneic control mice (Table I). Collectively, our analyses do not indicate a proliferative deficit in spleen T cells of mice bearing tumors up to 15–20% of body weight.

Cytokine analyses of Thy1+ spleen T cells from 6-1 tumor-bearing or control mice

The accumulation of cytokine RNA in spleen T cells of tumor-bearing mice RNA was isolated from purified Thy1+ cells and analyzed for the expression of mRNAs encoding various cytokines. Cells were purified by magnetic immunobeads from tumor-bearing or control spleens as described above, and an aliquot taken for flow cytometric confirmation of purity (cells were >95% CD3ε+; data not shown). The remainder of the cells were snap-frozen in liquid nitrogen before RNA isolation. We found that T cells obtained from spleens of tumor-bearing mice expressed a variety of cytokine RNAs, including IL-2, IL-4, IL-10, and IFN-γ (Fig. 8). Cytokine RNAs were strongly expressed without stimulation in vitro, but not in T cells of control mice, indicating that the T cells were transcribing these genes in situ. Importantly, cells from control mice did not express these RNAs, indicating that the isolation procedure does not induce transcription of these cytokine RNAs, which is indicative of activation.

Spleen T cells from tumor-bearing mice secrete bioactive IL-2

The cytokine RT-PCR analyses showed that several cytokine mRNAs were transcribed in T cells of tumor-bearing, but not control, mice without activation in vitro (Fig. 8). Because spleen T

### Table 1. Comparison of spleen T cell proliferation in different tumor models

<table>
<thead>
<tr>
<th>Tumor</th>
<th>cpm ± SD</th>
<th>% Proliferation Relative to Control</th>
</tr>
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<tbody>
<tr>
<td>K1735</td>
<td>87,362 ± 10,525</td>
<td>110</td>
</tr>
<tr>
<td>K5222</td>
<td>100,719 ± 4,276</td>
<td>127</td>
</tr>
<tr>
<td>Lp53</td>
<td>93,301 ± 10,007</td>
<td>117</td>
</tr>
<tr>
<td>UV6138</td>
<td>76,543 ± 3,588</td>
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</tr>
<tr>
<td>MEFs40TAg</td>
<td>122,801 ± 5,925</td>
<td>155</td>
</tr>
<tr>
<td>EL4</td>
<td>91,501 ± 2,364</td>
<td>116</td>
</tr>
<tr>
<td>MC57G</td>
<td>80,920 ± 4,109</td>
<td>103</td>
</tr>
<tr>
<td>MCA-38</td>
<td>82,982 ± 2,249</td>
<td>106</td>
</tr>
<tr>
<td>ΔBCM</td>
<td>85,500 ± 2,380</td>
<td>95</td>
</tr>
<tr>
<td>Control FvBN</td>
<td>89,200 ± 1,410</td>
<td></td>
</tr>
<tr>
<td>Control C57BL/6</td>
<td>78,285 ± 1,139</td>
<td></td>
</tr>
<tr>
<td>Control C3H/HeN</td>
<td>79,194 ± 4,970</td>
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Proliferation assays were performed using 2 × 10⁶ CD3ε+ splenocytes in triplicate wells prepared from animals bearing the indicated tumor types for 22–27 days (except for transgenic ΔBCM mice which were 4–5 mo old at the time of spleen isolation and tumors were ~1.5 cm²). Control mice for each strain were of the same litter and were injected with HBSS instead of tumor cells (except for FvBN which were nontransgenic littersmates). Aliquots of each splenocyte preparation was analyzed by flow cytometry and the percentage of CD3ε+ T cells was between 28 and 33% for each mouse. Cells derived from tumor-bearing spleens not stimulated by incubation with anti-TCR Ab did not incorporate thymidine at a greater level than unstimulated control splenocytes. This experiment, which has been performed at least twice for each tumor type with equivalent results, shows that when analyzed in comparison to the equivalent number of CD3ε+ cells, spleen T cells from tumor-bearing mice are not inhibited in proliferation relative to control splenocytes.
cells are apparently activated in situ, we considered the possibility that cytokine production upon further activation may be dampened, perhaps due to overstimulation in response to tumor growth. To make this determination T cells from MCA-38 tumor-bearing and control mice were purified before collection of culture supernatants either with or without activation in vitro by incubation on anti-TCR-coated plasticware and analysis of tissue culture supernatants for IL-2 content by CTLL-2 bioassay (22) (Fig. 9A). We found that highly purified CD4+ T cells of MCA-38 tumor-bearing mice secreted IL-2 without in vitro activation. The amount of IL-2 secreted ranged from 2 pg to 500 fg/ml/24 h. CD4+ spleen T cells from the other tumor models did not secrete IL-2 unless activated.

Because T cells are, for unknown reasons, activated in situ, we considered that the ability to respond to further activation stimuli may be altered relative to that of naive cells activated under similar conditions. To evaluate this we purified CD4+ T cells from spleens of mice bearing ΔBCM tumors (and 6-1, K1735, and EL-4; data not shown) and stimulated them with plate-bound anti-TCR plus anti-CD28 Abs before IL-2 analysis by bioassay (Fig. 9B). CD4+ spleen T cells in three of five tumor models tested produced IL-2 upon activation in vitro at levels close to those of control cells, for MCA-38 and 6-1 tumor models the production of IL-2 from CD4+ T cells was slightly less than that for controls. IL-2 secretion from T cells from mice bearing ΔBCM tumors required stimulation in vitro. However, although T cells from the ΔBCM model (and EL-4; data not shown) did not secrete IL-2 without in vitro activation, upon stimulation these T cells secreted greater levels of IL-2 than did syngeneic control cells. Collectively, these analyses demonstrate that IL-2 production from spleen T cells of tumor-bearing mice is not diminished compared with that in control animals. Furthermore, in several models systemic T cells of tumor-bearing mice secrete IL-2 without deliberate activation in vitro, implying a heightened activation status in vivo, an idea in concert with our other findings and which implies functional integrity in situ.

Discussion

Immune suppression in the tumor-bearing host is postulated to be the basis for growth of antigenic tumor despite antitumor immune response (2, 3). This hypothesis is based upon a variety of data showing that the antitumor immune response is demonstrable in the early phase of tumor growth but is down-regulated upon continuous growth (23–26). In recent years a number of reports have attempted to describe the phenomenon of tumor-induced immune suppression in biochemical terms. Mizoguchi et al. (5) reported that systemic peripheral T cells in tumor-bearing mice, isolated from spleens of mice bearing large burdens of the MCA-38 colon carcinoma, showed an altered pattern of protein tyrosine phosphorylation coincident with reduced expression of signal-transducing molecules p56lck and p59fyn. A key observation was that spleen T cells from tumor-bearing mice had also lost expression of TCRz, which, due to its fundamental position in the cascade of cytosolic signaling events, was postulated to be the basis for defective immune response, permitting antigenic tumor to grow. Subsequently, several independent reports described similar results (6, 9). These findings renewed enthusiasm into the search for a mechanistic understanding of tumor-induced escape from immune response.

However, it has been proposed that the apparent loss of TCR-proximal signal-transducing proteins may not accurately reflect expression in T cells of tumor-bearing mice or be truly tumor-induced functional immune suppression (8, 10, 26). The possibility of artifactual proteolysis of TCR-associated signaling proteins generated during T cell isolation and analysis was considered because of the observation that when splenic T cells are rigorously purified before detergent solubilization and immune precipitation analysis there is no loss of these proteins (8, 10). In support of this contention is the report that the inclusion of high levels of protease inhibitors during cell lysis resulted in enhanced recovery of TCRz (11). This issue is particularly acute if the contaminating cell types cause of the observation that when splenic T cells are rigorously purified before detergent solubilization and immune precipitation analysis there is no loss of these proteins (8, 10). In support of this contention is the report that the inclusion of high levels of protease inhibitors during cell lysis resulted in enhanced recovery of TCRz (11). This issue is particularly acute if the contaminating cell types...
tumors were extremely large, so the relevancy to putative tumor-induced down-regulation of antitumor immune response or an analogous situation in human patients is doubtful.

Previous analyses of putative systemic T cell dysfunction were limited to in vitro biochemical experiments and did not address the in vivo function of T cells of tumor-bearing mice as we have (4, 5, 7, 9, 11, 27). Assessment of T cell function in our experiments was made by in vivo assays, including T cell priming to exogenous protein Ag and rejection of allogeneic and syngeneic tumors. These physiological relevant characteristics, considered together with the in vitro analyses (including CD8⁺ CTL induction), clearly demonstrate that mice bearing sizable tumor burden have normal systemic T cell function in vivo. We have used at least one tumor model used by others (MCA-38) as well as nine other tumors of different histological origin to demonstrate that there is no systemic T cell dysfunction in tumor-bearing mice.

TIL from cancer patients have been reported to have decreased expression of TCRζ, which is suggested to correlate with decreased proliferation and effector phase functions of TIL (28–31). If decreased TCRζ renders TIL unable to effectively eliminate human tumors because of decreased CTL function, then understanding the biochemical basis for this phenomenon becomes important to develop effective immunotherapeutic strategies for the management of cancer that are dependent upon activation of antitumor T cell immune response (2). TCRζ has been recently shown to be a substrate for cleavage by caspase 3 resultant from activation of Fas, implying a mechanism by which TIL could be rendered dysfunctional by cell-to-cell contact in the tumor microenvironment (32). In this regard, there have been several reports that PBL T cells of patients with certain cancers also have decreased TCRζ levels, implying that systemic T cells are dysfunctional in cancer patients (33–35). If TCRζ is degraded in TIL by a Fas-dependent mechanism requiring contact with tumor, as suggested by the data presented by Rabinowich et al. (31), how TCRζ in PBL T cells could be degraded is hard to envisage, because this would mean that the whole body complement of T cells would have to have

FIGURE 9. IL-2 secretion from spleen T cells of tumor-bearing or control mice. A and B, IL-2 bioassay of splenocytes from mice bearing MCA-38 and ΔBCM tumors. CD4⁺ and CD8⁺ T cells were isolated by magnetic immunobeads from splenocyte preparations of individual tumor-bearing or control mice, and aliquots were plated in the presence of plate-bound anti-TCR (MCA-38) or anti-TCR plus anti-CD28 Abs for 48 h (2 × 10⁵ cells/ml) as described in Materials and Methods. Serial dilution of supernatants were analyzed in triplicate by CTLL-2 bioassay after 24 or 48 h of incubation as indicated using rIL-2 as standard (22). Anti-IL-2 Ab was added to some reactions as indicated in the form of S4B6 hybridoma-conditioned medium diluted 1/8 (which was titrated to inhibit 50–100 pg/ml of rIL-2).
contact with the Fas-activating environment of the tumor. In addition, loss of TCRζ presumably reflects diminished T-cell function, but cancer patients do not have loss of T-cell functions in vivo (except in very late stages), which would be reflected in enhanced opportunistic infection, which is not seen. Therefore, the issue of biochemical defects in PBL T cells of cancer patients and the relvancy to tumor escape from immune destruction are at present unresolved.

Despite the concerns that our data illustrate in consideration of the influence of tumor growth on systemic T-cell function, tumor-induced defective immune response is probably common in the early phase of immune response and is causally related to tumor escape from immune response because of tumor-induced defective T-cell function in tumor-specific T cells (2, 3). For example, in recent reports studying human TIL derived from late-stage renal cell carcinoma patients, the Finke laboratory has shown that diminished T-cell function in vitro is correlated with defective activation of NF-κB, which is postulated as the likely basis for diminished antitumor T-cell function (36). Those data illustrate a potentially important difference between cancer patients and tumor-bearing mice in terms of tumor-induced effects on systemic T-cell function.

In a variety of additional experiments that assessed the function of anti-tumor T cells in murine tumors in which hosts expressed transgenic TCR specific for tumor Ags, T cells were found to be ineffective in killing tumors, albeit for different reasons depending upon the specific model employed. For example, using adoptive transfer of T cells obtained from a transgenic TCR mouse model, Staveley-O’Carroll et al. (37) showed that systemic Ag-specific T-cell anergy is induced in CD4+ T cells in mice bearing early-stage A20 lymphoma modified to express influenza hemagglutinin as tumor Ag, although the biochemical basis underlying the anergic phenotype is still unknown. Ag-nonspecific T-cell responses were not defective in early-stage tumor-bearing mice. In a different transgenic TCR murine tumor model Prevost-Blondel and colleagues recently showed that B-16 melanoma (modified to express an MHC class I-restricted tumor Ag) grew in mice expressing a transgenic TCR reactive with the tumor Ag. TIL that accumulated in primary tumors were highly lytic for tumor in vitro in 18-h lysis assays, strongly implying that CTL function was inhibited in situ (38). Assessment of perforin-mediated cytolysis was not demonstrated, implying that cytolysis was Fas mediated. Finally,wick and colleagues showed, using a transgenic TCR mouse model, that antitumor T cells do not accumulate at the site of tumor growth, implying that the tumor microenvironment impedes T-cell recruitment, perhaps due to down-regulated inflammatory response at the tumor site (39). Collectively, these data highlight the important influence of the tumor microenvironment in both recruitment of T cells to the site of primary tumor and T-cell activation (of both priming and effector phase antitumor T-cell functions) and serve to focus our attention on the mechanism(s) by which Ag-specific T-cell anergy may be induced in situ.

Acknowledgments

We thank John Hirst for performing the flow cytometry analysis and FACS; Dan Levey for critical editorial comments; Alexandra Imbert and Pam Cowin for ABCM mice; Hans Schreiber, Antoine Menoret, Yang Liu, and Stanislav Vukanovic for providing several tumor cell lines; and S. Vukanovic for advice on the redirected CTL assay.

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