Tumor-Reactive CD8⁺ T Cells in Metastatic Gastrointestinal Cancer Refractory to Chemotherapy

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Abstract

Purpose: To evaluate whether patients with metastatic gastrointestinal adenocarcinomas refractory to chemotherapy harbor tumor-reactive cytotoxic T cells.

Experimental Design: Expansion of CD8⁺ tumor-infiltrating lymphocytes (TIL) and cancer cell lines was attempted from gastrointestinal cancer metastases in 16 consecutive patients for the study of antitumor immune recognition. Retroviral transduction of genes encoding T-cell receptors (TCR) was used to define HLA-restriction elements and specific reactivity.

Results: TIL were expanded from metastases in all patients, and new tumor cell lines were generated in 5 patients. Autologous tumor recognition without cross-reactivity against allogeneic HLA-matched gastrointestinal tumors was found in CD8⁺ TIL from 3 of these 5 patients. In a patient with gastric cancer liver metastases, the repertoire of CD8⁺ TIL was dominated by cytolytic sister clones reactive to 2 out of 4 autologous cancer cell lines restricted by HLA-C/C30701. From the same patient, a rare CD8⁺ TIL clone with a distinct TCR recognized all four cancer cell lines restricted by HLA-B/C34901. In a patient with bile duct cancer, two distinct antitumor cytolytic clones were isolated from a highly polyclonal CD8⁺ TIL repertoire. TCRs isolated from these clones recognized epitopes restricted by HLA-A/C30201. In a third patient, CD8⁺ TIL reactivity was progressively lost against an autologous colon cancer cell line that displayed loss of HLA haplotype.

Conclusions: This study provides a basis for the development of immunotherapy for patients with advanced gastrointestinal malignancies by first establishing the presence of naturally occurring tumor-reactive CD8⁺ TIL at the molecular level. Clin Cancer Res; 20(2); 331-43. ©2013 AACR.

Introduction

Gastrointestinal adenocarcinomas are among the 10 most common malignancies worldwide and the overall mortality associated with their high metastatic potential has not changed significantly over the last decades (1). Current multimodality treatments can slow disease progression but fail to cure patients with metastatic disease. Thus far, immune-based therapies have not shown clinical effectiveness in patients with gastrointestinal cancers (2–5). A positive association between the density of the tumor-infiltrating lymphocyte (TIL) infiltrate and better outcomes has, nonetheless, been reported in patients with adenocarcinomas arising in the esophagus, stomach, pancreas, liver, bile ducts, gallbladder, colon, and rectum (6). Multiple immune-escape mechanisms have however been proposed that may contribute to the absence, the depletion, and the dysfunction of tumor-reactive TIL in solid tumors (7–9). Thus, the specific recognition of human metastatic gastrointestinal cancers by naturally occurring, autologous, cytotoxic CD8⁺ T cells harvested from the tumor site has not been defined at the T-cell clonal level with defined HLA restriction elements.

Results from studies in patients with metastatic melanoma have stimulated us to investigate the antitumor reactivity of CD8⁺ TIL in common epithelial malignancies. Indeed, TIL isolated from melanoma metastases can exhibit direct in vitro tumor recognition of defined antigens presented by specific class I HLAAs (10–14), and tumor deposits seem to harbor antitumor T cells of sufficient avidity and in sufficient numbers to respond to nonspecific systemic modulation of immunity (15–18). In addition, as now reported by multiple institutions, the adoptive cell transfer of autologous TIL can mediate complete cancer regression in patients with metastatic disease considered incurable with standard therapy, with complete responders reported up to 10 years after treatment (19–23). The curative potential of TIL-based immunotherapy in advanced melanoma...
Detailed CD8+ in 16 patients with metastatic gastrointestinal cancer. How immunotherapy could be a relevant alternative to current therapeutics for these common malignancies is unclear. Conversely, adoptive transfer of tumor-infiltrating lymphocytes (TIL) can mediate durable complete regression of metastatic melanoma in patients with refractory disease. By overcoming the difficulty of establishing new cancer cell lines and by expanding TIL from the same patients, this study first provides evidence at the molecular level for the presence of cytolytic tumor-reactive CD8+ T cells in growing gastrointestinal cancer metastases. The difficulty in establishing tumor targets to test for T-cell reactivity, the heterogeneity and defects in antigen expression by cancer cell lines, the low frequency of tumor-reactive CD8+ TIL, and the lack of shared reactivity across HLA-matched allogeneic tumors seem as major challenges for the development of effective T-cell-based immunotherapy for patients with advanced gastrointestinal cancer.

This represents a paradigmatic shift on how solid cancer treatment is approached, and whether this strategy can be applied for common metastatic epithelial malignancies merits active investigations.

In the current report, an in vitro analysis of TIL was carried out in 16 patients with metastatic gastrointestinal cancer. Detailed CD8+ TIL reactivity to autologous gastrointestinal cancer metastases was carried out in 5 patients from whom, 13 new cancer cell lines were established. TIL from 3 of these patients exhibited specific immune reactivity against their autologous metastatic cancer. By defining immune features of metastatic gastrointestinal cancer cells and TIL, our findings have direct relevance to efforts to develop immunotherapies for patients with these malignancies.

**Materials and Methods**

**Patients and tumor processing**

Written informed consent was obtained from all patients enrolled under protocols approved by the Institutional Review Board of the National Cancer Institute (NCI) and U.S. Food and Drug Administration. Single-cell suspensions were obtained from freshly resected tumors by independent enzymatic digestion and mechanical dispersion as previously described for melanoma specimens (24).

**Primary human cancer cell cultures and culture of other cancer cell lines**

To develop cancer cell lines, 0.25e6 live nucleated cells were plated in multiple 25-cm² ultra-low attachment and standard treated canted neck flasks (Corning 3815 and 3056) in RPMI 1640–based medium supplemented with 20% FBS (Defined; HyClone Laboratories), 25 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all from Life Technologies, Invitrogen), 1.25 µg/mL amphotericin B (X-GEN Pharmaceuticals), and 10 µg/mL ciprofloxacin (Bedford Laboratories). After 6 to 12 weeks, cell aggregates/tumor spheres (~200 µm in diameter) were transferred into standard 25-cm² flasks for propagation under adherent conditions. For adherent conditions, fibroblast overgrowth was controlled by differential trypsinization (Trypsin–EDTA 1 ×, 0.05%; Gibco) and mechanical removal (17-mm blade cell scraper; Sarstedt), and cultures were fed weekly or according to need, and passaged into larger flasks when reaching confluence. The human cancer cell lines Kato III, NCI N87, NCI H508, Colo205, HCT15, SK-CO-1, KM12, HT29, SW480, SW620, HCC2998, SW1463, Capan1, and Panc 02.03 were purchased from the American Type Culture Collection and grown under the vendor's suggested conditions. Human melanoma cell lines, 3350 and 624, and human pancreatic cancer cell lines, 2596 and 2742-2, were established in our laboratory.

The authenticity of all cell lines was confirmed by HLA typing and testing expression of melanoma differentiation antigens (MART-1, Melan-A, and gp100) and gastrointestinal cancer antigens (CEA, pan-cytokeratins). Mycoplasma contamination was ruled out on all cell cultures by using MycoAlert (Lonza) according to the manufacturer's instructions.

**Generation of TIL**

TIL establishment and expansion followed techniques used for metastatic melanoma tumor deposits and are presented in Supplementary Methods (24, 25).

**Characteristics of fresh TIL and cultured lines**

Staining of paraffin-embedded tissue and cell pellets was done at the NCI Clinical Research Center (NCI/CRC) Pathology and Cytology Laboratory, following standard procedures with appropriate positive and isotype controls. Antibodies and staining conditions are presented in Supplementary Methods. For flow cytometry, the following monoclonal antibodies specific for human antigens and appropriate isotype controls were used: from BD Biosciences, APC-H7-conjugated anti-CD3 (SK7), APC anti-CD137/4-1BB (4B4-1); from Invitrogen: R-PE-Texas Red conjugated anti-CD8 (B48-1); Cell aggregates and dead cells were excluded by forward and side scatter, and with propidium iodide staining. Flow cytometry analysis was carried out with FlowJo F7.5.5 software (TreeStar). HLA typing of cell lines was done by the NCI/CRC HLA laboratory on genomic DNA following standard procedures (Supplementary Methods).

**Cancer-cell recognition by T cells coculture assays**

T-cell reactivity to cancer cell lines was assessed after 24-to 36-hour coculture assays in flat-bottom 96-well plates (1e5 T cells, 0.3e5 trypsinized cancer cells; final volume 200 µL). ELISA was carried out for measurement of IFN-γ release in supernatant, and flow cytometry was used to quantify CD137 (4-1BB) upregulation on T cells (Supplementary Methods). Chromium-51 release 4- to 6-hour cytolysis.
assays were used as described in previous studies (26, 27) and in Supplementary Methods. The percentage of specific lysis was calculated as: percent lysis = (sample release − spontaneous release)/(maximal − spontaneous release) × 100, average of triplicate samples.

**Limiting dilution cloning of 4-1BB−enriched TIL**

After stimulation assays with autologous cancer cell line, CD8+ TIL that exhibited reactivity by high 4-1BB expression were sorted by magnetic cell separation (anti-APC iMag; BD Biosciences) or fluorescence-activated cell sorting (FACSaria II; BD Biosciences). Briefly, between 1 and 20 TIL were plated in each well of a 96-well U-bottomed plate in 200 μL of conditioned medium containing 3,000 U/mL rhIL-2, 30 ng/mL of OKT3, with 5 × 10⁴ irradiated (40 Gy) allogeneic peripheral blood mononuclear cells (PBMC). On day 5 and every 3 to 4 days thereafter, half of the medium in each well was replaced with fresh medium containing interleukin (IL)-2. About 10 to 14 days after culture initiation, wells in which cell growth was visibly apparent were screened for reactivity to autologous and allogeneic cancer cell line. Clone reactive by IFN-γ secretion and/or 4-1BB expression were subsequently expanded by Rapid Expansion Protocol (REP; ref. 24).

**TCR sequencing and cloning, retroviral transduction of PBMC**

Clonotypic analysis of the TCR repertoire was done from the RNA purified from CD8+ TIL with an RNeasy mini kit (Qiagen) and reverse transcribed into cDNA for 5′ Rapid Amplification of cDNA Ends (RACE) by SMARTer RACE cDNA Amplification Kit as per manufacturer instructions (Clontech) and as described with subcloning into a pCRII vector by TA cloning (Life Technologies; ref. 28). Consensus regions were determined using the international ImmunoGeneTics information system (29) for sequence analysis of 96 colonies picked from each 5′ RACE product of both TCR α and β chains and their variable regions. Murine human TCRs were cloned into MSGV1 retroviral vectors as described in ref. (30) and detailed in Supplementary Methods. Transduction of PBMC was done as described in ref. (31), and efficiency was assessed after 1-week expansion by flow cytometry using an anti-mouse TCR β-chain antibody (H57-597; eBioscience). MSGV1 vector expressing the TCR recognizing the MART-1:27-35 epitope (31) and GFP were used as controls in all experiments, as well as untransduced PBMC.

**Statistical analysis**

Statistical analyses were performed on GraphPad Prism software version 5.04. Variances of mean values are presented as SEM. Two-tailed, nonparametric tests were used and P values of ≤0.05 were considered significant.

**Results**

**Patient and tumor clinicopathologic and immunologic characteristics**

Resected tumors from 16 patients with widespread, moderately to poorly differentiated metastatic adenocarcinomas originating from the stomach, the bile duct, or the colon were studied (Table 1). All patients had progressive disease after receiving at least one standard first-line chemotherapy regimen (median, 2). The expression of MHC class-I (MHC-I) molecules, required for CD8+ T-cell recognition, was heterogeneous across resected metastases and nearly undetectable in 35% of lesions (Table 1 and Fig. 1). All metastases were poorly infiltrated by TIL, which represented less than 5% of the tumor-cut surface on histologic assessment, with the exception of omental metastases from patients 5 and 10, which were more densely infiltrated by CD3+ T cells. Heterogeneity in antigenic expression by cancer cells within the same patient was exemplified in metastases resected in patient 10, as MHC-I was faintly detected on the liver metastasis but strongly on the omental metastasis (Fig. 1). Patient 11 had a mucinous adenocarcinoma characterized by fine reticulated bands of tumor cells separated by copious pools of mucin and surrounded by a fibrotic stroma containing rare T cells (Fig. 1).

**TIL expansion from gastrointestinal metastases**

Mined tumor fragments or cell suspensions obtained from gastrointestinal cancer metastases were cultured in IL-2-containing media. Overgrowth of tumor and other adherent cells by TIL was observed between 16 and 29 days from culture initiation (median, 21 days). An average of 25.0% ± 4.6% of the live cells in these cultures was CD3+ CD8+ (range, 3.0%–59.4%). TIL were further expanded to large numbers from all samples using soluble anti-CD3 and irradiated allogeneic PBMC feeder cells for 14 days. Separated CD8+ T cells that were positively selected with magnetic beads from the expanded bulk TIL cultures had a median cumulative fold expansion of 853 using clinical-grade reagents (range, 522–1,770; Table 1). Without CD8+ enrichment, TIL were similarly expanded from bulk cultures in patients 14, 15, and 16 with a median 1,454-fold expansion (range, 802–1,995). Overall, TIL from gastrointestinal cancer metastases in patients heavily pretreated with chemotherapy were found to have good in vitro proliferative potential.

**Establishment of new gastrointestinal cancer cell lines and MHC-I expression loss**

In parallel to setting up cultures for TIL expansion, as many cancer cell lines as possible were generated for each patient by initial culturing of tumor cell suspensions in ultra-low attachment flasks in addition to standard techniques in adherent flasks. When tumor spheroids were obtained, as in patients 3, 5, and 11, multiple cell lines were initiated from distinct spheroids, and thus 13 cancer cell lines were established in 5 patients (Table 1, Supplementary Fig. S1). Complete HLA genotyping of the cancer cell lines and PBMCs confirmed the parenthood of the lines for each patient (Supplementary Table S1). Loss of heterozygosity at the HLA loci (haplotype loss) was found in two out of the 13 newly established cancer cell lines (patients 9 and 10). Total loss of MHC-I protein expression was seen in addition to genomic HLA haplotype loss in the only one
Table 1. Patient demographics, tumor immune characteristics, and TIL expansion

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Adenocarcinoma characteristics</th>
<th>TIL first outgrowth from tumor</th>
<th>TIL clinical scale expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>Age, y/gender</td>
<td>Prior chemotherapy regimens</td>
<td>Primary cancer site</td>
</tr>
<tr>
<td>1</td>
<td>63/M</td>
<td>2</td>
<td>Colon</td>
</tr>
<tr>
<td>2</td>
<td>45/M</td>
<td>1</td>
<td>Colon</td>
</tr>
<tr>
<td>3</td>
<td>44/F</td>
<td>3</td>
<td>Stomach</td>
</tr>
<tr>
<td>4</td>
<td>45/F</td>
<td>3</td>
<td>Colon</td>
</tr>
<tr>
<td>5</td>
<td>57/M</td>
<td>3</td>
<td>Bile duct, intrahepatic</td>
</tr>
<tr>
<td>6</td>
<td>42/M</td>
<td>1</td>
<td>Colon</td>
</tr>
<tr>
<td>7</td>
<td>51/M</td>
<td>4</td>
<td>Rectum</td>
</tr>
<tr>
<td>8</td>
<td>51/M</td>
<td>2</td>
<td>Colon</td>
</tr>
<tr>
<td>9</td>
<td>57/F</td>
<td>3</td>
<td>Colon</td>
</tr>
<tr>
<td>10</td>
<td>51/F</td>
<td>1</td>
<td>Colon</td>
</tr>
<tr>
<td>11</td>
<td>53/F</td>
<td>2</td>
<td>Colon</td>
</tr>
<tr>
<td>12</td>
<td>52/F</td>
<td>6</td>
<td>Colon</td>
</tr>
<tr>
<td>13</td>
<td>41/M</td>
<td>5</td>
<td>Colon</td>
</tr>
<tr>
<td>14</td>
<td>37/F</td>
<td>2</td>
<td>Colon</td>
</tr>
<tr>
<td>15</td>
<td>63/M</td>
<td>2</td>
<td>Stomach</td>
</tr>
<tr>
<td>16</td>
<td>43/F</td>
<td>2</td>
<td>Bile duct, intrahepatic</td>
</tr>
</tbody>
</table>

Abbreviations: Fg, tumor fragments; n/a, not available; L, liver; O, omentum.

$^a$Underlined metastases designate the site of TIL harvest, and subsequent columns refer to these tumors.

$^b$Semi-quantitative measurements of MHC class I performed by immunohistochemistry, with intensity (1+ to 3+) reported in addition to the percentage of tumor cells expressing the surface marker (<5%, 5%–50%, or >50%).

$^c$Semi-quantitative measurement, percentage of surface of tumor occupied by tumor-infiltrating CD3$^+$ T cells (0%–1%, 1%–5%, or 5%–50%).

$^d$Fold expansion cannot be calculated for TIL expanded from tumor fragments.

$^e$Rapid expansion was obtained in all 16 patients, results shown for samples tested with clinical-grade reagents. Patient 1 to 13 had CD8$^+$ T-cell enrichment before clinical scale expansion.
colon cancer cell line generated from patient 10 (colon adenocarcinoma peritoneal metastasis, CAPM; Fig. 1), rendering this cell line "invisible" for CD8⁺ T cells.

CD8⁺ TIL recognition of newly established autologous cancer cell lines

After expansion, CD8⁺ TIL recognition of autologous gastrointestinal cancer was evaluated by examining their ability to secrete IFN-γ and to upregulate expression of the inducible activation marker 4-1BB (CD137; ref. 32) in response to tumor stimulation in the 5 patients for which new cancer cell lines had been established (Table 2). As expected by lack of MHC-I expression, CAPM failed to stimulate CD8⁺ TIL in patient 10 (Supplementary Table S2). The rare fraction of CD8⁺ TIL expanded from fragments of mucinous abdominal wall colon cancer metastasis in
patient 11 were not reactive to four autologous cancer cell lines (colon adenocarcinoma abdominal wall metastasis, CAAWM; Supplementary Table S2). TIL reactivity to autologous tumors was, however, detected at different levels in 3 patients with metastatic gastrointestinal adenocarcinoma originating from 3 different organs: stomach (patient 3), intrahepatic bile ducts (patient 5), and colon (patient 9; Table 2).

For patient 3, CD8+ T cells expanded from a gastric cancer liver metastasis secreted approximately 1,000 pg/mL of IFN-γ in response to stimulation by two of the four autologous tumor cell lines (GALMa and GALMd; Table 2; and Supplementary Table S2), and at least 65% upregulated 4-1BB, specifically. The other two autologous cancer cell lines GALMb and GALMc did not stimulate autologous 4-1BB, specifically. The other two autologous cancer cell lines expressed 4-1BB (0.3%), or the percentage of TIL upregulated at least twice as high as the unstimulated condition (media). Bold values for IFN-γ reflect secretion of at least 200 pg/mL, whereas bold 4-1BB+ reflects specific upregulation at least twice as high as the unstimulated condition (media). Abbreviation: pOKT3, plate-bound anti-CD3.

Table 2. Reactivity of bulk CD8+ tumor infiltrating lymphocytes to autologous cancer cell lines

<table>
<thead>
<tr>
<th>Patient 3 gastric cancer</th>
<th>Patient 5 biliary tract cancer</th>
<th>Patient 9 colon cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+ TIL</td>
<td>CD8+ TIL</td>
<td>CD8+ TIL</td>
</tr>
<tr>
<td>pg/mL IFN-γ (% 4-1BB+)</td>
<td>pg/mL IFN-γ (% 4-1BB+)</td>
<td>pg/mL IFN-γ (% 4-1BB+)</td>
</tr>
<tr>
<td>pOKT3</td>
<td>Medium</td>
<td>Autologous cancer cell lines</td>
</tr>
<tr>
<td>GALMa</td>
<td>1,236 (66.2)</td>
<td>BAOMa</td>
</tr>
<tr>
<td>GALMb</td>
<td>50 (1.7)</td>
<td>BAAM</td>
</tr>
<tr>
<td>GALMc</td>
<td>55 (1.0)</td>
<td></td>
</tr>
<tr>
<td>GALMd</td>
<td>2,046 (83.5)</td>
<td></td>
</tr>
<tr>
<td>Allogeneic cancer cell lines</td>
<td>Mel.624</td>
<td>37 (0.3)</td>
</tr>
<tr>
<td></td>
<td>CALM</td>
<td>200 (15.7)</td>
</tr>
<tr>
<td></td>
<td>BAOMa</td>
<td>66 —</td>
</tr>
</tbody>
</table>

NOTE: Mean IFN-γ secretion (pg/mL) in 24-hour coculture supernatants of CD8+ TIL with autologous and allogeneic cancer cell lines in representative independent experiments. After sampling each well for IFN-γ, TIL duplicates were pooled and harvested for 4-1BB staining for flow cytometry analysis (in parenthesis, percentage of CD8+ cells, gated on CD3+ cells). Bold values for IFN-γ reflect secretion of at least 200 pg/mL, whereas bold 4-1BB+ reflects specific upregulation at least twice as high as the unstimulated condition (media).

For patient 3, CD8+ T-cell clones reactive to autologous colon adenocarcinoma liver metastasis secreted approximately 1,000 pg/mL of IFN-γ in response to stimulation by two of four autologous tumor cell lines (GALMa and GALMd; Table 2; and Supplementary Table S2), and at least 65% upregulated 4-1BB, specifically. The other two autologous cancer cell lines GALMb and GALMc did not stimulate autologous CD8+ TIL to secrete significant amounts of IFN-γ, and only 1% to 2% expressed 4-1BB, compared with 0.3% or less in absence of tumor stimulation or against an allogeneic melanoma cell line (Table 2). Analysis of the diversity of TCR-BV (β chain gene, variable regions) sequences expressed by the CD8+ TIL from patient 3 revealed a relatively oligoclonal population, in which 65% of the TCRs corresponded to a single rearranged TRBV6-1 gene product (Fig. 2A).

In contrast with patient 3, studies carried out in patient 5 revealed that the repertoire of CD8+ TIL expanded from the malignant ascites and an omental metastasis was highly polyclonal (Fig. 2B, omental metastasis not shown). Between the ascites and the omental metastasis, only 2% of the TCR-BV sequences overlapped. Approximately 1% of CD8+ TIL expanded from the malignant ascites, nonetheless, upregulated 4-1BB after stimulation by the autologous cancer cell lines derived from the malignant ascites (biliary adenocarcinoma metastatic ascites, BAAM) and derived from the omental metastasis (BAOMa; Table 2). CD8+ TIL expanded from the omental nodule showed slightly lower expression of 4-1BB after stimulation with autologous tumor cell lines (data not shown). These percentages of 4-1BB expression upregulation, although low, were reproducible in sequential experiments, increased with prolonged autologous tumor stimulations, and were consistently higher than either the percentage of unstimulated TIL expressing 4-1BB (0.3%), or the percentage of TIL stimulated with allogeneic cancer cell lines, such as GALMa (0.1%; Table 2).

In patient 9, the CD8+ TIL expanded from a colon cancer liver metastasis were highly oligoclonal, as 93% (62 out of 67) sequences shared the same rearranged 7-9′03 TCR-BV sequence (Supplementary Fig. S2). Cocultures carried out with a low passage of the autologous colon cancer cell line colon adenocarcinoma liver metastasis (CALM) lead to the specific release of approximately 200 pg/mL of IFN-γ as well as upregulation of 4-1BB expression on 16% of the CD8+ TIL (Table 2). Although recognition of the autologous cancer cell line by CD8+ TIL in this patient was seen in three independent assays, it decreased and was lost by the sixth passage of the cancer line (Supplementary Fig. S2A). After documenting loss of HLA haplotype in this cancer cell line and that the initial recognition was likely restricted by the A allele (Supplementary Fig. S2B), we attempted to restore antigen recognition by treating the cell line with IFN-γ to induce gene expression, or by retrovirally transducing it with the HLA-A′0201 gene. Neither attempt restored recognition by TIL.

Isolation of CD8+ T-cell clones reactive to autologous cancers

We took advantage of the ability of CD8+ TIL to specifically upregulate 4-1BB, following tumor stimulation in
patients 3 and 5 to enrich and isolate reactive T cells. For patient 3, CD8⁺ TIL expressing 4-1BB after stimulation with GALMa were enriched by magnetic beads before limiting dilution cloning (Fig. 2C). Expansion occurred in 16% of the plated microcultures, allowing evaluation of the reactivity of 154 clonal T cells. Specific upregulation of 4-1BB expression was observed in 19 microcultures (12.3%). After reexpansion with soluble anti-CD3 and irradiated allogeneic PBMCs, the TCR repertoire of TIL found in malignant ascites from a bile duct cancer in pt. 5. C, for Pt. 3, FACS plot of 4-1BB expression upregulation in bulk CD8⁺ TIL after stimulation by autologous GALMa that allowed enrichment of the 4-1BB⁺ T cells for limiting dilution cloning (red rectangle). Inferior to the FACS plot, the bar graphs represent the reactivity of 19 CD8⁺ TIL clones to the autologous GALMb cancer cell line, a nonreactive (NR) clone (TRBV12-4), and the melanoma (F5) CD8⁺ TIL specificity control. FACS plot (middle) of the approximately 4% 4-1BB upregulation (red rectangle) seen in bulk CD8⁺ TIL from pt. 3 after stimulation by autologous GALMb. The bar graphs on the right side of the dotted line represent the low reactivity found in two expanded clones (1/C3, TRBV9; 2/C3, unsequenced) to GALMa and GALMb, the bulk CD8⁺ TIL, a NR clone, and the F5 specificity control by 4-1BB upregulation; none of these clones secreted significant amount of IFN-γ. D, for Pt. 5, FACS plot of the approximately 1% 4-1BB upregulation seen in the bulk CD8⁺ TIL. Limiting dilution cloning allowed isolation of two clones (TRBV28 and TRBV3-I) reactive to two autologous cancer cell lines established from an omental metastasis BAOMa and the malignant ascites BAAM. For bar graphs, left axis, IFN-γ ng/mL; right axis, %4-1BB⁺ of CD3⁺ CD8⁺. Unstim., unstimulated.

Figure 2. Reactivity of CD8⁺ TIL clones to autologous gastric and bile duct cancers. A, diversity of the TCR repertoire of CD8⁺ TIL expanded from patient (Pt.) 3 liver metastasis, in which distinct TCR variable β chains sequences are labeled by their TCR germline V consensus regions (TRBV). B, highly polyclonal TCRBV repertoire of TIL found in malignant ascites from a bile duct cancer in pt. 5. C, for Pt. 3, FACS plot of 4-1BB expression upregulation in bulk CD8⁺ TIL after stimulation by autologous GALMa that allowed enrichment of the 4-1BB⁺ T cells for limiting dilution cloning (red rectangle). Inferior to the FACS plot, the bar graphs represent the reactivity of 19 CD8⁺ TIL clones to the autologous GALMb cancer cell line, a nonreactive (NR) clone (TRBV12-4), and the melanoma (F5) CD8⁺ TIL specificity control. FACS plot (middle) of the approximately 4% 4-1BB upregulation (red rectangle) seen in bulk CD8⁺ TIL from pt. 3 after stimulation by autologous GALMb. The bar graphs on the right side of the dotted line represent the low reactivity found in two expanded clones (1/C3, TRBV9; 2/C3, unsequenced) to GALMa and GALMb, the bulk CD8⁺ TIL, a NR clone, and the F5 specificity control by 4-1BB upregulation; none of these clones secreted significant amount of IFN-γ. D, for Pt. 5, FACS plot of the approximately 1% 4-1BB upregulation seen in the bulk CD8⁺ TIL. Limiting dilution cloning allowed isolation of two clones (TRBV28 and TRBV3-I) reactive to two autologous cancer cell lines established from an omental metastasis BAOMa and the malignant ascites BAAM. For bar graphs, left axis, IFN-γ ng/mL; right axis, %4-1BB⁺ of CD3⁺ CD8⁺. Unstim., unstimulated.
feeder cells, each of the 19 clones still upregulated 4-1BB expression after stimulation by GALMa, but only six of the 19 clones secreted significant amounts of IFN-γ. The TCR of 18 reactive clones were sequenced and all shared the same dominant TRBV6-1*01 β chain rearrangement sequence (Supplementary Table S3 for complete TCR α and β chains). As expected, the TRBV6-1 clones were only stimulated by GALMa and GALMd, but not by GALMb and GALMc, reflecting the reactivity pattern initially found from the bulk CD8+ (Fig. 2C left bar graphs; TRBV6-1 clone reactivity against GALMd, nonreactivity against GALMa, and allogeneic specificity controls are not shown).

In an attempt to isolate rare CD8+ TIL clones reactive to GALMb, FACS sorting was carried out to enrich a population corresponding to approximately 4% of the bulk TIL that upregulated 4-1BB expression in response to stimulation by this autologous cancer cell line (Fig. 2C, right FACS plot). Clonal growth efficiency was 2.1% of the sorted TIL that were plated at 1 and 2 cells per well and led to expansion of 32 clones. Five clones could successfully be reexpanded, two of which demonstrating approximately 10% 4-1BB upregulation following coculture with all gastric adenocarcinoma liver metastasis (GALM) cell lines, without significant IFN-γ secretion (Fig. 2C, right bar graphs; clones 1* and 2* reactivity to GALMb and GALMd are not shown). The TCR of the CD8+ T-cell clones most reactive to GALM cell lines was sequenced and found to be derived from the TRBV9 germline gene (Supplementary Table S3). We estimated that the frequency of the TRBV9 TCR was of less than 1.3% of CD8+ TIL expanded from the metastasis, as this specific TCR sequence did not match any of the one initially analyzed (n = 76) from the bulk CD8+ TIL (Fig. 2A).

Thus, from the gastric cancer liver metastasis of patient 3, multiple TRBV6-1 sister clones constituting a dominant T-cell clonotype in CD8+ TIL recognized two out of four autologous cancer cell lines (GALMa and GALMd), whereas a genetically distinct TIL clone found at low frequency recognized all four autologous cell lines with lower reactivity. Of note, the TRBV6-1 sister clones, with genetically identical TCRs, upregulated 4-1BB to similar levels upon tumor recognition but differed in their capacity to secrete IFN-γ, implying distinct functional attributes regulated at the epigenetic level. Our findings also supported distinct antigen expression profiles found in four autologous cancer cell lines derived from a single liver metastasis constituted by heterogeneous cancer cells.

For patient 5 with bile duct cancer, approximately 4,000 4-1BB+ CD8+ TIL from the malignant ascites and the omental metastasis were FACs-sorted after stimulation with autologous cell lines (Fig. 2D). Overall, 604 clones grew at a clonal efficiency of 9%, 17%, and 32% when plated at 1, 2, and 5 cells per well. A preliminary assessment of the T-cell clone reactivity by stimulation with autologous tumor indicated that three clones (0.5%) were reactive by standard criteria for IFN-γ release and 10 (1.6%) clones possessed relatively low reactivity with IFN-γ secretion at least two times higher than the unstimulated condition, however, not reaching the 200 pg/mL cutoff. Among 32 microcultures assessed for 4-1BB upregulation, four clones (12.5%) upregulated the surface marker by at least 20%. After reexpansion, two out of the 17 clones showed signs of specific TCR engagement with autologous BAOma and BAAM cancer cell lines by 4-1BB upregulation, but only one clone secreted significant amounts of IFN-γ (Fig. 2D bar graphs; nonreactivity to allogeneic targets not shown). The two clones did not share the same TCR (TRBV28 and TRBV3-1; Supplementary Table S3) and were found at a frequency of less than 1.8% of the expanded CD8+ TIL. Again, the differential reactivity of these clones to autologous cancer cell lines here, established from distinct body compartments (omental metastases and malignant ascites), suggested a heterogeneous expression of immune epitopes by these cell lines.

Importantly, all of the CD8+ TIL clones isolated from the Patient 3 and 5 lysed autologous tumors with specificity, irrespective of their ability to specifically secrete IFN-γ (Fig. 3). For patient 3, the TRBV6-1 sister clones maintained the same pattern of reactivity noted by 4-1BB upregulation by not lysing the autologous GALMc line, whereas the TRBV9 clone could lyse both cell lines GALMa and GALMd (Fig. 3A). For patient 2, similarly, the 4-1BB upregulation seen by the TRBV28 and TRBV3-1 clones after stimulation by BAOma and BAAM was associated with cytolyis of tumor lines from patient 5 but not from patient 3. Thus, CD8+ TIL clones with specific lytic capacity toward autologous cancer cells were isolated from a metastatic gastric adenocarcinoma and a cholangiocarcinoma.

**TCR-transduced lymphocytes redirected to recognize gastric and bile duct cancers restricted to the autologous context by common HLA**

We next synthesized the genes encoding the TCRs derived from reactive TIL clones isolated from patients 3 and 5, as well as the dominant TCR found in patient 9 (Fig. 4A), and transduced these genes into PBMC. TCR-transduced PBMCs from patient 3 and 5 were cocultured with autologous cell lines in the presence of absence of HLA-blocking antibodies to define the HLA restriction elements mediating tumor recognition (Fig. 4B). For patient 3, the TRBV6-1 and TRBV9 TCRs seemed to recognize tumors in the context of HLA-A*0201, HLA-B*0701 and HLA-B*4901 class I alleles, respectively. For patient 5, TRBV28 and TRBV3-1 TCRs both seemed to recognize autologous tumor cells in the context of HLA-A*0201.

To define the specificity of the TCRs derived from the reactive clones and to investigate shared reactivities across cancer cell lines, large coculture assays were carried out to assess IFN-γ responses against a variety of targets, including autologous cancer cell lines, normal autologous or HLA-matched B cells, and a panel of 17 commercially available gastrointestinal cancer cell lines that were HLA genotyped (Supplementary Table S2). The reactivity observed from patient 3 bulk CD8+ TIL was recapitulated with the TRBV6-1 TCR-engineered T cells, specifically recognizing the autologous GALMa and GALMd, but not...
GALMb and GALMc, and T cells transduced with the TRBV9 TCR recognizing all four GALM tumor lines (Fig. 4C, top: orange bar, TRBV6-1; yellow bar, TRBV9; and red bar, bulk CD8\(^+\)). The TRBV6-1 lymphocyte reactivity was entirely specific to the autologous tumor, despite the fact that the restriction element HLA-C\(^*/C3\)\(^0701\) allele was potentially expressed by 13 other allogeneic tumor targets (Supplementary Table S1). For patient 5, the TRBV28 TCR-transduced PBMCs recognized the autologous cell lines established from this patient's omental nodule (BAOMa and BAOMb) and malignant ascites (BAAM; Fig. 4C, middle, lighter green bar). The reactivity of the TRBV3-1 was weaker but specific to the autologous tumor (Fig. 4C, dark green bar). Again, although 13 cancer cell lines potentially expressed HLA-A\(^*/C3\)\(^0201\), there was no reactivity suggestive of shared antigen recognition by T cells transduced with TRBV28 and TRBV3-1.

In addition, other CD8\(^+\) TIL derived from HLA-A\(^*/C3\)\(^0201\) patients or with known HLA-A\(^*/C3\)\(^0201\)–restricted epitopes (Patient 9, Patient 10, F5 mel with MART1 reactivity, ref. 31; CEA, ref. 33; and MAGE-A3, ref. 27 transduced T cells) failed to recognize biliary adenocarcinoma omental metastasis (BAOM) and BAAM T cell lines. PBMCs engineered to express the dominant TCR from patient 9 (TRBV7) failed to recognize CALM, even when the cancer cell line was transduced to express HLA-A\(^*/0201\), the expression that it had lost. Finally, native CD8\(^+\) TIL from patients 3, 5, 9, 10, and 11 failed to recognize allogeneic tumor cell lines.

Altogether, these results showed that it was possible to redirect PBMC specificity toward gastrointestinal cancer antigens presented by distinct HLA class I alleles, and that the antigens recognized in the autologous setting were likely unique to each patient.

**Discussion**

Whether common human epithelial malignancies such as gastrointestinal cancers harbor tumor-reactive T cells has been debated for decades, with arguments mainly relying on associative findings (6) rather than direct demonstration. Suggestive evidence has supported the idea that CD8\(^+\) TIL reactive to tumor cell suspensions could be expanded from metastatic gastrointestinal cancers (34, 35), but the lack of well-characterized cancer cell lines precluded assays necessary to define tumor reactivity and specificity at the TCR-HLA molecular level. Ten years ago, a colon cancer cell line established from a liver metastasis was used to identify CD4\(^+\) TIL clones reactive to a self-epitope restricted by HLA-DR\(^01\), but required engineering of the cancer cell line for expression of MHC Class II molecules (36). Here, we establish the presence of naturally occurring CD8\(^+\) TIL able to specifically recognize autologous metastatic gastric, bile duct, and colon adenocarcinomas by generating new cancer cell lines and TIL cultures. Three main observations can be made from our findings: first, tumor-reactive CD8\(^+\) TIL were found at low frequency; second, metastatic gastrointestinal cancer recognition by CD8\(^+\) TIL was seen only in the autologous setting, in absence of shared recognition across allogeneic cancer cell lines; and third, autologous cancer cell lines generated from given patients were not equally recognized by T cells, and in other patients were deficient in MHC-I expression.

Unlike melanoma, only anecdotal responses to systemic immunomodulation have been reported in patients with metastatic gastrointestinal cancers (2–5), implying that these tumors lacked tumor-reactive T cells in sufficient numbers or with the quality necessary to mediate tumor regression. One of the factors that may contribute to the low frequency of tumor-reactive TIL in gastrointestinal cancer is the relatively
low number of exomic mutations those tumors generally carry (14). Whole-exome sequencing studies have reported an average of approximately 200 mutations per melanoma compared with approximately 55 mutations in gastrointestinal cancers, representing less potential opportunities for antitumor CD8+ T-cell recognition (37). In silico-based epitope prediction analysis has suggested that 2 to 17 HLA-A’0201–mutated epitopes may be generated per colon cancer (38). In addition, even if a productive antitumor immune response occurred against mutated epitopes, it is...
possible that over time, a natural selection of the least immunogenic cancer cells occurs, as suggested in animal models (39, 40). Here, our ability to establish new cancer cell lines in a limited number of patients with advanced gastrointestinal cancers consistently supported a low frequency of tumor-reactive CD8\(^{+}\) TIL, but more samples should be tested to further examine this phenomenon. Higher frequencies of gastrointestinal tumor-reactive TIL may be found in earlier stages of disease, or in gastrointestinal cancer subsets, such as those with a high mutation rate due to mismatch repair gene deficiencies.

Our study thus demonstrates that tumor-reactive T cells with proliferative potential may be isolated from advanced gastrointestinal cancer, provided that suitable tumor targets are available for testing. Thus far, CD137 (4-1BB), a cell surface marker of recent TCR engagement, had been used for isolation of precursor T cells derived from PBMCs, using peptides from known tumor or viral antigens as stimulators (32, 41, 42). Without knowing the antigens recognized by polyclonal TIL, the use of 4-1BB expression here allowed to isolate polyclonal cytolytic CD8\(^{+}\) T cells, independent of their capacity to secrete IFN-\(\gamma\) (Figs. 2 and 3). Because the difficulty in generating cancer cell lines has limited the study of the immune recognition of epithelial cancers in vitro, it seems critical that new methods be developed to increase the yield of new cell line establishment, as shown recently by the use of a ROCK inhibitor with stromal cells (43, 44). As clinical-grade flow cytometry cell sorting becomes available for enriching cell products in tumor-reactive T cells (45), it may be possible to design adoptive cell transfer immunotherapy for patients with tumors that harbor a small fraction of tumor-reactive T cells that can be expanded in vitro.

By defining the reactivity of CD8\(^{+}\) T cells against gastrointestinal cancers at the TCR and the HLA molecular level and by testing allogeneic reactivity against a comprehensive panel of HLA-genotyped gastrointestinal cancer cell lines, our study highlighted an additional difference with melanoma, which is the absence of shared recognition across allogeneic tumors (Supplementary Table S2 and Fig. 4). TIL in melanoma not only recognize mutated epitopes, but many self-epitopes, such as cancer testis antigens (MAGE, NY-ESO1, etc.) and melanocyte-differentiation antigens (gp100, MART-1, etc.; refs. 10–13). Although ongoing studies are aimed at identifying the genes that encode the antigens recognized by gastrointestinal TIL, conceivably, these may consist of mutation-generated epitopes unique to each tumor, or self-antigens overexpressed by the tumor. Mining exomic mutation expression in this context could represent a powerful tool for defining new tumor antigens recognized by T cells (14). Thus, for advanced gastrointestinal cancers, T-cell based immunotherapy may have to rely on high throughput screening of unique reactive TIL, especially considering the infrequent expression of cancer-testis antigens (46) by gastrointestinal cancers and the potential toxicity seen in trial targeting shared self-differentiation antigens such as CEA (47). Further studies should also aim at clarifying whether tumor-reactive CD8\(^{+}\) TIL represent dominant clonotypes in freshly resected tumors, and if current in vitro TIL expansion protocols lead to overgrowth of nontumor reactive bystander rather than tumor-reactive T cells.

Although the genetic heterogeneity found in a given tumor and across distinct metastases in a given patient is now well established using second generation genomic sequencing (37, 48, 49), our data support that this heterogeneity can translate into the generation of various cell lines with the distinct potential of being recognized by autologous CD8\(^{+}\) TIL. For example, in the case of a gastric cancer metastatic to the liver (patient 3), two of four cancer cell lines seemed to express the gene encoding an epitope recognized by a TCR (TRBV6-1) that dominated the CD8\(^{+}\) TIL repertoire and was restricted by the HLA-C\(^\*\)0701 (Fig. 4B and C). A second epitope, restricted by HLA-B\(^\*\)4901, was present on the four autologous cancer cell lines and recognized by a distinct TCR (TRBV9) expressed by less than 1.3% of the CD8\(^{+}\) bulk TIL expanded from the tumor. Adding to the complexity of in vitro assessment of tumor recognition by T cells, the loss of HLA expression by cancer cells—a well-known mechanism of tumor immune-escape (9, 50)—was seen in 2 of the 5 patients in which new cancer cell lines were established, and in four of the 17 commercially available gastrointestinal cancer cell lines tested. HMC expression on paraffin-embedded gastrointestinal cancer metastases further suggested frequent defective expression in vivo (Fig. 1 and Table 1). Implications of these findings for advanced gastrointestinal cancer immunotherapy are 2-fold: first, a nonpolyclonal immunologic approach that only targets one tumor antigen is unlikely to mediate sustained tumor regression. Second, the antigen presentation capacity of cancer cells should be evaluated as a potential biomarker of response to immunotherapy, while strategies that aim at restoring antigen presentation have to be developed to broaden the use of T-cell based immunotherapy.

In conclusion, this study defines at the clonal and molecular level, the existence of naturally occurring cytolytic CD8\(^{+}\) TIL specifically reactive to autologous metastatic gastrointestinal cancers. These findings propose avenues for the development of T-cell based immunotherapies for gastrointestinal cancers by pointing to the main challenges to be addressed, such as the low frequency of tumor-reactive TIL, the absence of shared antigen recognition across allogeneic tumors, the difficulty in establishing suitable tumor targets for the selection of reactive TIL, the heterogeneity of antigen expression by tumors, and the loss of MHC-I expression by immune-escape tumor variants.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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