Phenotype and Function of T Cells Infiltrating Visceral Metastases from Gastrointestinal Cancers and Melanoma: Implications for Adoptive Cell Transfer Therapy

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Phenotype and Function of T Cells Infiltrating Visceral Metastases from Gastrointestinal Cancers and Melanoma: Implications for Adoptive Cell Transfer Therapy

Simon Turcotte, Alena Gros, Katherine Hogan, Eric Tran, Christian S. Hinrichs, John R. Wunderlich, Mark E. Dudley, and Steven A. Rosenberg

Adoptive cell transfer of tumor-infiltrating lymphocytes (TILs) can mediate cancer regression in patients with metastatic melanoma, but whether this approach can be applied to common epithelial malignancies remains unclear. In this study, we compared the phenotype and function of TILs derived from liver and lung metastases from patients with gastrointestinal (GI) cancers (n = 14) or melanoma (n = 42). Fewer CD3+ T cells were found to infiltrate GI compared with melanoma metastases, but the proportions of CD8+ cells, T cell differentiation stage, and expression of costimulatory molecules were similar for both tumor types. Clinical-scale expansion up to \(5 \times 10^9\) T cells on average was obtained for all patients with GI cancer and melanoma. From GI tumors, however, TIL outgrowth in high-dose IL-2 yielded \(22 \pm 1.4\%\) CD3+CD8+ cells compared with \(63 \pm 2.4\%\) from melanoma (\(p < 0.001\)). IFN-\(\gamma\) ELISA demonstrated MHC class I–mediated reactivity of TIL against autologous tumor in 5 of 7 GI cancer patients tested (9% of 188 distinct TIL cultures) and in 9 of 10 melanoma patients (43% of 246 distinct TIL cultures). In these assays, MHC class I–mediated up-regulation of CD137 (4-1BB) expression on CD8+ cells suggested that 0–3% of TILs expanded from GI cancer metastases were tumor-reactive. This study implies that the main challenge to the development of TIL adoptive cell transfer for metastatic GI cancers may not be the in vitro expansion of bulk TILs, but the ability to select and enrich for tumor-reactive T cells. The Journal of Immunology, 2013, 191: 2217–2225.

Cancers arising from the gastrointestinal (GI) tract mucosa rank in the 10 most frequent solid malignancies and causes of cancer-related death (1). Despite the combined use of surgery, chemotherapy, and targeted agents, the great majority of patients with GI adenocarcinomas will die of metastatic disease, and thus new therapies with curative potential are needed. In the past few decades, immunotherapy for solid tumors has emerged as a promising approach (2–4). Treatment goals for patients with metastatic melanoma are changing, given that the adoptive cell transfer (ACT) of autologous tumor-infiltrating lymphocytes (TILs) can mediate complete and durable cancer regression in patients with heavy disease burden, refractory to all other treatments (5). Cancer centers in and outside the United States have begun to offer this form of immunotherapy and report similar response rates, and multicenter trials are expected (6–9). Although the efficacy of TIL-based ACT may be linked to the more immunogenic nature of melanoma compared with other solid cancers, it remains to be determined whether this approach can be adapted for the treatment of common epithelial tumors, such as metastatic GI adenocarcinomas.

Indirect evidence suggests that T cell immunity may participate in controlling disease progression for GI adenocarcinomas arising in the esophagus, stomach, pancreas, liver, bile ducts, gallbladder, colon, and rectum (10–18). A redefinition of prognostic staging is being proposed based on the density of the TILs found in primary tumors, among which colorectal cancer has been studied most extensively (19). For example, patients with a primary colon cancer highly infiltrated by CD3+ TILs appear to have similar disease-free survival, irrespective of whether the tumor is confined to the colon (stage I and II) and are has spread to draining lymph nodes (stage III) (17). Genes involved in cytotoxicity and lysis, such as IFN-\(\gamma\) and granzyme B, appear to be transcribed at lower levels in tumors with aggressive features such as microvascular and perineural invasion compared with tumor with more favorable pathological features (16). Additionally, active mechanisms employed by colon cancer to evade immune recognition have been correlated with lower TIL infiltration and poorer clinical outcomes, such as down-regulation of MHC class I (MHC-I) expression by cancer cells (20), and high levels of immunosuppressive molecules such as IDO1 found in the tumor microenvironment (21). In colorectal cancer metastatic to the liver (stage IV), longer survival of patients after complete resection of metastases has been associated with a higher density of CD4+ and CD8+ TILs in metastases and their relative abundance compared with FOXP3+ T cells (putative regulatory T cells [Tregs]) (22, 23). However, in vitro evidence of MHC-restricted recognition of autologous GI cancers by TILs is limited. CD4+ TIL clones reactive to a self epitope presented by HLA-DRB1 expressed by an autologous colon cancer cell line derived from a liver metastasis and transduced to express MHC class II has been reported (24). More recently, a low frequency of cytotoxic CD8+ TIL clones were found to specifically recognize newly
established autologous cancer cell lines in three metastatic GI cancer patients, restricted by specific MHC-I molecules (S. Turcotte, A. Gros, E. Tran, C.-C.R. Lee, J.R. Wunderlich, P.F. Robbins, and S.A. Rosenberg, submitted for publication).

To test the feasibility of TIL-based ACT for patients with advanced GI cancers, we assessed the phenotype, the proliferative potential, and whether TILs reactive to autologous tumor could be identified. These features were evaluated with 14 GI adenocarcinomas metastatic to the liver and the lungs that were harvested in patients refractory to standard chemotherapy. To put these findings in perspective, we compared these GI TIL immune features with TILs derived from 42 contemporaneous melanoma metastases also harvested from the liver and the lungs.

**Materials and Methods**

**Patients, PBMCs, and tumor and normal samples**

Patients with metastatic GI cancer (n = 14) and melanoma (n = 42) underwent liver and lung metastases resections between December 2007 and March 2012 after giving their written informed consent under protocols approved by the Institutional Review Board of the National Cancer Institute (Tables I and II for patient clinicopathological characteristics). At least 4 wk had to have elapsed after the last dose of systemic chemotherapy prior to metastasectomy. PBMCs were obtained from patients with GI cancer on the morning of their surgery after venipuncture (sodium-heparin glass tubes, BD Vacutainer; BD Biosciences, San Jose, CA) and prepared over Ficoll-Hypaque (LSM gradient, ICN Biomedicals, Aurora, OH) gradient. Freshly resected tumors were sent from the surgery suite to the laboratory in sterile containers with saline and on ice. Under sterile conditions, tumors were dissected away from adjacent normal tissue and stroma. Tumor fragments were used for generating TILs. For large tumors, single-cell suspensions were obtained by enzymatic digestion and mechanical dispersion consisting of two 30-min incubations of 2.5 g minced tumor at 37°C, 5% CO2 in 5 ml RPMI 1640 (Lonza) supplemented with 1 mg/ml collagenase type IV (Sigma-Aldrich), 30 U/ml DNAse (Dornase alfa; Genentech, San Francisco, CA) and prepared over Ficoll-Hypaque (LSM gradient, ICN Biomedicals, Aurora, OH) gradient. Tumor cell suspensions were filtered through a 400-μm metal mesh (custom made) and cell strainers (100 or 50 μm). TILs were used: from BD Biosciences, allophycocyanin-H7–conjugated anti-CD3 (SK7), PE anti-CD25 (2A3), Fluor 700 anti-CD27 (M-T271), FITC anti-CD28 (CD28.2), PECy7 anti-CD45RO (UCHL1), allophycocyanin anti-CD69 (DREG-56), FITC anti-CD314-54 (ACT35), allophycocyanin anti-CD122 (BB (AB4-1), allophycocyanin anti-CD127 (BDH4); from Invitrogen: R-PE-Texas Red–conjugated anti-CD8 (35B); and from eBioscience, allophycocyanin anti-FOXP3 (236A/E7). Cells were resuspended in staining buffer (PBS containing 3% FBS) and blocked with mouse IgG mAbs (Caltag Laboratories, Burlingame, CA) for 15–30 min at room temperature. Cells were stained with mAb against surface Ags for 30 min at 4°C in the dark. For intracellular FOXP3 staining, cells were processed with the eBioscience staining buffer set. Acquisition of at least 20,000 events was done on a FACSCalibur II flow cytometer (BD Biosciences). Cell aggregates and dead cells were excluded by forward and side scatter, and with propidium iodide staining for unfixed cells. Flow cytometry analysis was carried out with FlowJo v.7.5.5 software (Tree Star, Ashland, OR), gating based on isotype control Ab staining and on unstained T cells for 4-IBB when used in simulation assays, and the number in each gate represents the percentage of cells.

**Coculture assays**

Cryopreserved cancer cell suspensions were thawed and plated immediately after cell count for 24-h coculture assays with TILs harvested at the end of their initial tumor outgrowth in round-bottom 96-well plates (∼1 × 10⁶ T cells, 1 × 10⁵ live tumor cells from cell suspension, final volume 200 μl culture media per well). A condition with pan–MHC-I blocking Ab (W6/32, 50 μg/ml) as well as an unstained TIL condition (without target) were included in all assays. The melanoma TIL cell line DM5 was used as a positive experimental control with the MART-1–expressing melanoma 624 cancer cell line and as a specificity control against the given fresh cryopreserved tumor in all assays.

For measurement of IFN-γ release in the coculture supernatant, ELISAs were carried out on 50 μl coculture supernatants in 96-well flat-bottom MaxiSorp plates (Nunc/Thermo Scientific) that were previously coated overnight at 4°C with 1 μg/ml anti–IFN-γ (2G7) and blocked 1 h with 5% BSA in PBS. Supernatants were incubated for 2 h and captured IFN-γ was detected with biotin-labeled IFN-γ detection Ab (B33.3; 1:2,000 dilution in 50 μl culture media), followed by a 30-min incubation with HRP-conjugated streptavidin (1:20,000 dilution), 1-Step Ultra TMB reaction, and the reaction was stopped with 0.18 M H₂SO₄ solution (all reagents from Thermo Fisher Scientific, Waltham, MA). IFN-γ levels were quantitated with a 5-point standard curve using recombinant human IFN-γ. For measurement of reactivity based on CD137/4-IBB T cell surface protein expression, cells were washed with PBS after the coculture supernatant was harvested for ELISA, kept on ice, and immediately stained for FACS acquisition in 96-well plates.

**Statistics**

Statistical analyses were performed on GraphPad Prism software version 5.04 (GraphPad Software, La Jolla, CA) and SPSS for IBM version 21. Variance of mean values are presented as SEMs. Statistical comparison of different T cell subsets in the same patients was calculated using a paired t test, whereas an unpaired t test was used to compare GI and melanoma samples. All tests were two-tailed, nonparametric, and p values ≤ 0.05 were considered statistically significant.

**Results**

Visceral metastases were resected from 14 patients with GI adenocarcinomas (Supplemental Table I). The median age of patients in perspective, we compared these GI TIL immune features with TILs derived from 42 contemporaneous melanoma metastases also harvested from the liver and the lungs.

**Flow cytometry**

Analyses of PBMCs and cell suspensions derived from metastases and adjacent normal tissues were performed after overnight incubation in complete media without IL-2 at 37°C in humidified atmosphere of 5% CO2. The following mAbs specific for human Ags and appropriate isotype controls were used: from BD Biosciences, allophycocyanin-H7–conjugated anti-CD3 (SK7), PE anti-CD25 (2A3), Alexa Fluor 700 anti-CD27 (M-T271), FITC anti-CD28 (CD28.2), PECy7 anti-CD45RO (UCHL1), allophycocyanin anti-CD69 (DREG-56), FITC anti-CD134-54 (ACT35), allophycocyanin anti-CD122 (BB (AB4-1), allophycocyanin anti-CD127 (BDH4); from Invitrogen: R-PE-Texas Red–conjugated anti-CD8 (35B); and from eBioscience, allophycocyanin anti-FOXP3 (236A/E7). Cells were resuspended in staining buffer (PBS containing 3% FBS) and blocked with mouse IgG mAbs (Caltag Laboratories, Burlingame, CA) for 15–30 min at room temperature. Cells were stained with mAb against surface Ags for 30 min at 4°C in the dark. For intracellular FOXP3 staining, cells were processed with the eBioscience staining buffer set. Acquisition of at least 20,000 events was done on a FACSCalibur II flow cytometer (BD Biosciences). Cell aggregates and dead cells were excluded by forward and side scatter, and with propidium iodide staining for unfixed cells. Flow cytometry analysis was carried out with FlowJo v.7.5.5 software (Tree Star, Ashland, OR), gating based on isotype control Ab staining and on unstained T cells for 4-IBB when used in simulation assays, and the number in each gate represents the percentage of cells.

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**Results**

Visceral metastases were resected from 14 patients with GI adenocarcinomas (Supplemental Table I). The median age of patients...
was 51 y, all patients had progressive disease resistant to at least one regimen of chemotherapy (range of one to six, median of two). Eleven liver and three lung metastases were harvested from 10 patients with primary cancer of the colon, 2 of the stomach, 1 of the rectum, and 1 of the intrahepatic bile ducts (cholangiocarcinoma). To establish an immunobiological comparison, we studied 19 liver metastases and 23 lung metastases contemporaneously resected in 42 patients with refractory melanoma (median age of 50 y; Supplemental Table II).

Similar MHC-I expression in GI and melanoma metastases, distinct HLA-DR expression

MHC-I expression by tumor cells, necessary for recognition of Ags by CD8+ T cells, was detected by immunohistochemistry (IHC) on >50% of cancer cells in 70 and 69% of GI and melanoma metastases, respectively, and lower MHC-I expression was also found in similar proportion for both tumor types (Fig. 1A). HLA-DR, an MHC class II molecule that is required for Ag recognition by CD4+ T cells and is known to be induced by IFN-γ and other cytokines, was expressed by <5% of cancer cells or none detectable in all of the GI metastases assessed. In contrast, 65% of melanoma metastases tested had at least 5% of their cancer cells expressing HLA-DR (p < 0.001 compared with GI), including nine samples (27%) in which HLA-DR was detected on >50% of the cancer cells (HLA-DRhigh) (Fig. 1A).

Fever TILs in GI metastases compare with melanoma

The density of the CD3+ TIL infiltrate was also assessed by IHC, with semiquantification based on the area of the tumor occupied by T cells. All GI cancer metastases assessed were poorly infiltrated by CD3+ T cells (>5% of the tumor area occupied by TILs), compared with 47% of melanoma metastases. In melanoma, moderate infiltration (5–50% of tumor area) and brisk infiltration (>50% of tumor area) were found in 22 and 31%, respectively, of tumors studied. Brisk TIL infiltration was similar in melanoma liver (4 of 17) and lung (7 of 20) metastases. Of note, brisk CD3+ T cell infiltration was found more frequently in HLA-DRhigh tumors than in all other melanoma metastases tested (67 versus 20%, respectively; p < 0.01). More viable cells were generally recovered from processing melanoma compared with GI cancer visceral metastases (Fig. 1B), and consistent with the IHC findings, the proportion of CD3+ cells was greater in melanoma when assessed by flow cytometry (Fig. 1C).

GI metastases are immunologically distinct from peritumoral normal tissue

To test whether the microenvironment of GI cancer metastases was an immunologically distinct milieu, we compared the phenotype of T cells infiltrating tumor, adjacent peritumoral normal tissue, and from the peripheral blood of 10 GI cancer patients with available samples (7 liver and 3 lung metastases) (Fig. 2A, Supplemental Table III). TILs in GI metastases shared some characteristics with T cells infiltrating peritumoral tissues, but they were significantly different from circulating blood lymphocytes for most markers assessed. Tumors nonetheless appeared to represent an immunologically distinct T cell niche compared with the peritumoral tissues, based on the expression of several markers. On average, the fraction of CD8+ in CD3+ T cells was lower in tumors compared with peritumoral tissues (37.7 ± 3.2 versus 62.0 ± 3.8%, p < 0.001) (Supplemental Table III). In both CD3+CD8+ and CD3+CD8– (hereafter CD8+ and CD4+ T cells), assessment of T cell differentiation based on the expression of CD45RO and L-selectin (CD62L) showed that tumors contained proportionally less terminally differentiated T cells (CD45RO–CD62L+) and more central memory T cells (CD45RO+CD62L+) than did the peritumoral tissues. The earlier state of T cell differentiation found in tumors compared with peritumoral tissues was consistent with the greater percentage of TILs coexpressing the costimulatory molecules CD27 and CD28 in tumors. In CD8+ T cells, the expression of the inhibitory molecule PD-1, the inducible activation markers 4-1BB (CD137) and OX40 (CD134), and the high-affinity chain of the IL-2 receptor (CD25) were not differently expressed in tumors compared with the peritumoral tissues. In CD4+ T cells, however, PD-1, 4-1BB, and OX40 were expressed at their highest level on TILs. The proportion of CD4+ T cells expressing high levels of CD25 and the transcription factor FOXP3, best representative of the Treg subset (CD4+CD25highFOXP3+), was not statistically different in GI compared with melanoma TILs.

GI cancer and melanoma metastasis harbor T cells with similar phenotype

TIL phenotype derived from GI cancer was then compared with 10 fresh melanoma visceral metastases that were processed and analyzed with the same methodology (Fig. 2B). The mean proportion of CD8+ in CD3+ TILs was similar in GI and melanoma visceral metastases (37.7 ± 3.2 versus 37.8 ± 5.8%, respectively) (Fig. 1C). The proportions of TILs falling into the four differentiation stages defined and expressing the costimulatory CD27 and CD28 molecules were similar. Additionally, there was no statistical difference in the expression of inhibitory and activation markers. The percentage of putative Tregs was not statistically different in GI compared with melanoma TILs. 

TIL first outgrowth from visceral metastases

We attempted to expand TILs with high-dose IL-2 from 12 liver and lung metastases in GI cancer patients using the same techniques
employed for metastatic melanoma. To initiate TIL cultures, 10 GI metastases were large enough to be processed into single-cell suspensions, 6 of which tumors were also minced into fragments, and 2 smaller tumors were only minced into fragments (Supplemental Table I). For melanoma metastases, 26 were processed into single-cell suspensions, 1 of which additionally was also processed into fragments, and 14 were processed into fragments to initiate TIL cultures (see Table II). At least one TIL-productive tumor fragment, defined by the growth of TIL from a single fragment up to confluence into 8 wells of a 24-well plate (∼16 × 10⁶ TILs), was obtained from all patients with GI cancer and melanoma when this technique was employed. The median number of TIL-productive fragments per GI metastasis was 29% (range, 12–62%) and 25% (range, 19–46%) per melanoma metastasis (Fig. 3A). In contrast, however, the mean days necessary for TIL to reach eight wells was significantly longer for GI compared with melanoma metastases (17.0 ± 0.9 versus 14.6 ± 0.6 d, p = 0.03).

Large tumors were also processed into cell suspensions to initiate TIL cultures. For these cultures, a longer time was necessary for TIL derived from GI compared with melanoma metastases to reach confluence with disappearance of adherent cells in cultures (21.6 ± 0.6 versus 17.3 ± 1.4 d, respectively, p = 0.004) (Fig. 3B, upper panel). The resulting mean cumulative fold expansion of GI cancer-derived TILs was greater than for melanoma (mean 27.5 ± 6.5 versus 9.4 ± 1.8, p = 0.01); however, this difference was not significant when the absolute cell expansion was normalized by the number of days of cultures (Fig. 3B, bottom panel). Noticeably, although flow cytometry analysis suggested that both GI and melanoma visceral metastases contained a similar fraction of CD8+ cells in CD3+ TILs (Fig. 1C), the mean percentage of CD3+CD8+ out of live cells that outgrew GI and melanoma metastases was 22 and 60%, respectively (p < 0.01) (Fig. 3C). This difference was not attributable to a higher proportion of CD3+CD56+ NK cells, but rather reflected a greater proportion of CD3+CD8− (CD4) TILs (data not shown). Initiating TIL expansion from fragments or tumor cell suspension did not impact significantly on the yield of CD8+ TILs.

Clinical-scale TIL expansion

After their initial tumor outgrowth, we tested whether GI cancer-derived TILs could be expanded to clinical scale (aiming for >10 × 10⁹ T cells) using a standard 14-d rapid expansion protocol (REP) with irradiated PBMC feeders and soluble anti-CD3 Ab. For the GI TIL samples tested with clinical-grade reagents, an overall 1144 ± 158-fold expansion was observed, providing on average 46.8 ± 6.3 × 10⁹ TILs for potential infusion into patients (range, 18.9–79.8 × 10⁹) (Fig. 3D). Positive selection of CD8+ TILs prior to REP (27) was performed with magnetic beads in seven of these samples, yielding 95% of CD3+CD8+ out of live cells (data not shown), and after REP they expanded on average by 1027 ± 170-fold. (Fig. 3D, CD8+). The 31 melanoma TIL cultures expanded for ACT with the same clinical-grade reagents expanded by a mean of 1407 ± 97-fold (p = 0.17 versus GI TILs), providing 51.8 ± 3.7 × 10⁹ absolute cells on average for infusion into patients (range, 11.1–84.6 × 10⁹). In the CD8+ TIL-enriched samples, the mean expansion fold was 1346 ± 114 (p = 0.2 versus GI CD8+-enriched TIL expansion). Overall, TILs derived from GI or melanoma metastases showed similar clinical-scale expansion.
MHC-I–mediated release of IFN-γ by TILs after stimulation with autologous tumors

We next studied the ability of TIL to secrete IFN-γ in the absence or presence of stimulation by autologous cryopreserved tumor cell suspensions, which were available in 7 GI cancer patients (188 TIL cultures after initial expansion) and in 10 contemporaneous metastatic melanoma patients (246 TIL cultures) (Table I). To determine whether the IFN-γ release was more likely mediated by CD8+ TIL TCR engagement with peptide/MHC-I, a third experimental condition was added in which the autologous tumor was preincubated with a pan–MHC-I blocking Ab. The average culture days at which the reactivities of GI TILs were tested was 17.4 ± 1.5 d compared with 13.8 ± 0.6 d for melanoma TILs (p = 0.08), reflecting growth kinetics of cultures. Significant release of IFN-γ by TIL cultures, defined as $\geq 200$ pg/ml and twice as much for the stimulated compared with the unstimulated conditions, was seen in at least one culture in all GI tumors, at frequencies ranging from 7–89% of cultures tested (median, 36.4%). Nine of 10 melanoma patient cultures yielded significant IFN-γ release, at frequencies ranging from 0 to 100% (median, 83%; p < 0.001 compared with GI).

Significant blockade of IFN-γ release, defined by ≥50% reduction in the presence of anti–MHC-I Ab, was found in five of the seven GI patients (17 out of 69 reactive cultures, 25%), compared with nine of the nine melanoma patients (105 out of 154 reactive cultures, 68%) (p < 0.001). Considering all cultures, there were 17 of 188 (9%) GI cancer–derived TIL cultures compared with 105 of 246 (43%) melanoma-derived TIL cultures that appear to harbor MHC-I–mediated CD8+ reactivity defined by this assay (p < 0.001). Of note, the mean percentage of CD8+ in CD3+ T cells assayed was 24.4 ± 6.8% for GI and 72.5 ± 6.7% for melanoma cocultures associated with significant IFN-γ release (p = 0.002). Additionally, the average amount of IFN-γ released in these assays tended to be higher in melanoma compared with GI cancer (Table I).

Development of a cell-based assay to identify CD8+ TILs reactive to autologous tumors

To assess at the single-cell level what proportion of CD8+ TILs derived from GI cancers could be reactive to autologous tumors irrespective of their functional capacity to secrete IFN-γ, TILs derived from tumor fragments were harvested after stimulation by autologous tumor in presence and absence of pan–MHC-I blockade and stained for the inducible activation marker 4-1BB+ (CD137). The expression of this TNFR family member has been shown in vitro on T cells to be highly restricted to recent activation by a signal through the TCR and has been successfully used to isolate Ag-specific CD8+ T cells from peptide-stimulated PBMCs (28–30).
Table I. TIL reactivity to autologous GI and melanoma visceral metastases

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>TIL Cultures Tested</th>
<th>Significant Release(c)</th>
<th>Mean Release(c)</th>
<th>CD8(a) (&gt;50%) Blockability</th>
<th>MHC-I–Mediated Reactivity</th>
<th>Mean percentage CD8+/CD3 in reactive TIL cultures</th>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>4</td>
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<td>18</td>
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<td>6</td>
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<td>69</td>
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</table>

| Melanoma Metastases |                     |                           |                   |                 |                     |                                              |
| 30         | 24                  | 22                        | 91.7              | 546 (4)         | 94.0                | 22                  | 100.0                             | 91.7                              |
| 31         | 36                  | 30                        | 83.3              | 468             | 57.0                | 5                  | 16.7                              | 13.9                              |
| 32         | 24                  | 3                         | 12.5              | 310             | 43.0                | 3                  | 100.0                             | 12.5                              |
| 34         | 18                  | 0                         | 0.0               | —               | —                   | —                  | —                                 | 0.0                               |
| 36         | 24                  | 24                        | 100.0             | 1069 (1)        | 76.4                | 22                  | 91.7                              | 91.7                              |
| 37         | 24                  | 24                        | 100.0             | 7560            | 94.8                | 24                  | 100.0                             | 100.0                             |
| 38         | 24                  | 5                         | 20.8              | 387             | 61.3                | 5                  | 100.0                             | 20.8                               |
| 39         | 24                  | 24                        | 100.0             | 1152 (17)       | 87.5                | 21                  | 87.5                              | 87.5                              |
| 40         | 24                  | 20                        | 83.3              | 390             | n/a                 | 2                  | 10.0                              | 8.3                               |
| 42         | 24                  | 2                         | 8.3               | 340             | 66.1                | 1                  | 50.0                              | 4.2                               |
| Total      | 246                 | 154                       |                   |                 |                     |                   | 105                                |

\(a\) IFN-\(\gamma\) (pg/ml) measured in the supernatant after 24 h coculture with autologous cryopreserved tumor cell suspension, considered significant when \(\geq 200\) pg/ml and at least twice as high in the stimulated condition compared with the unstimulated condition. 

\(b\) The number of off-scale values (>1000 pg/ml) are noted in parentheses.

\(c\) Mean percentage CD8+/CD3 in reactive TIL cultures.

The gating strategy and representative examples of 4-1BB upregulation seen in two CD8\(^+\) TIL cultures derived from distinct tumor fragments in four patients are shown in Fig. 4A. As shown, 4-1BB was expressed at low levels by TILs without stimulation in the great majority of cultures. However, upon autologous tumor stimulation, a distinct population of TILs upregulated 4-1BB expression, and this upregulation was decreased in the presence of the pan–MHC-I Ab in six of seven patients (range, 0–222). Upregulation of 4-1BB expression was also significant upregulation of 4-1BB, defined as expression in ≥50% of CD8\(^+\) TILs in at least twice as many cells in the stimulated compared with the unstimulated conditions, was detected in all the patients tested, ranging from 9.5 to 54.8% of cultures per patients tested. Upregulation of 4-1BB expression was also ≥50% blockable with the pan–MHC-I Ab in six of seven patients (range, 0–100% of reactive cultures). Thus, of 180 TIL cultures tested, 38 (21%) harbored CD8\(^+\) TILs that potentially engaged their TCR in an MHC-I–specific manner. However, the average frequency of CD8\(^+\)-4-1BB\(^+\) cells in these cultures was low, representing 10.5 and 2.1% of CD8\(^+\) and CD3\(^+\) expanded TILs, respectively.

Discussion

Whether ACT of TILs, an approach that can successfully mediate complete cancer regression in patients with metastatic melanoma (31, 32), can be developed for patients with metastatic GI malignancies has been little studied thus far (33). Using the same techniques as used for expansion of TILs from melanoma metastases, we demonstrate in this study that TILs derived from liver and lung metastases in consecutive patients with GI cancers, refractory to multiple chemotherapy regimens, are phenotypically distinct from T cells found in the peripheral blood or the adjacent normal tissue, and they have a good in vitro proliferative potential. Although the pre-expansion phenotype of TILs analyzed from fresh GI and melanoma visceral metastases was similar, fewer CD8\(^+\) T cells expanded from GI cancers, and MHC-I–mediated TIL reactivity to autologous tumors was found at low frequency. This study implies that the main challenge to develop effective ACT for metastatic GI cancers using TILs may not be the in vitro proliferation capacity of bulk TILs, but the selection and enrichment of tumor-reactive T cells.

TILs from GI visceral metastases were successfully expanded in vitro in all attempts from fresh liver and lung metastases cell suspensions, with a median fold expansion of 26 (range, 2–60) by 22 d of initial outgrowth. When tumor fragments were used to initiate cultures, approximately one of three produced enough TILs (~16 × 10\(^6\)) for further expansion. Clinical-scale expansion with irradiated PBMC feeders, anti-CD3, and IL-2 (REP protocol) of CD8\(^+\)-enriched or bulk TILs yielded ~50 × 10\(^9\) T cells on average (range, 18.9–79.8 × 10\(^9\) cells). These yields and rate of successful TIL outgrowth from tumor fragments are similar to those obtained from melanoma visceral metastases in this study and reported by us and other groups (5–8, 34). Compared to melanoma visceral metastases, however, 2 to 5 more days were necessary to complete the initial TIL expansion from GI metastases (Fig. 3). The lower density of TILs in GI cancers compared with melanoma metastases may at least partly account for this difference in growth kinetics (Fig. 1). Notably, however, although the proportion of CD8\(^+\) cells, the stage of differentiation, and the expression level of inhibition/activation markers found in TILs from GI and melanoma metastases were similar (Figs. 1B, 2B), the first outgrowth of TILs from tumors using identical culture conditions with IL-2 as a mitogenic factor yielded 3- to 4-fold more CD3\(^+\)CD8\(^+\) cells from melanoma compared with GI TILs (Fig. 3C). This observation supports the notion that despite general similarities in phenotype, intrinsic biological differences exist between TILs derived from the distinct microenvironment of melanoma and GI cancers.
Almost 20 y ago, TILs were successfully expanded in saturating doses of IL-2 from metastases in 19 colorectal cancer patients with an average of 606-fold expansion during 49 d in culture (range, 26–76 d), which yielded 40.1% of CD8+ T cells on average (35). Japanese investigators have also expanded lymphocytes harvested from 22 patients with gastric cancer lymph node metastases, FIGURE 4.

**A** Measurement of CD8+ TIL reactivity to autologous tumors by CD137 (4-1BB) upregulation. (A) Gating strategy: FACS plots (upper left) represent the effector TILs expanded with IL-2 from tumor fragments (average, 17 d) prior to tumor-recognition coculture assay. Effector TILs are typically bigger and more granular (red gate) than the uncultured T cells (green gate) present in autologous cryopreserved tumor cell suspension (Tumor) used as stimulators in this assay. At rest, the percentage of 4-1BB expression in effector CD8+ TILs is low (2%). After coculture with tumor, 6% of the effector CD8+ TILs (expanded 17 d) upregulate 4-1BB expression (red gates), compared with 2% in the unstimulated condition. The CD8+ TILs found in the fresh tumor do not upregulate 4-1BB expression (green gates). (B) Representative examples of 4-1BB upregulation by CD8+ TILs grown from metastatic GI cancers in four patients after 24 h stimulation with autologous tumor in the presence or absence of a pan–MHC-I Ab. For each patient in at least two TIL cultures derived from distinct fragments (F), 4-1BB expression (gated on CD8+ cells, percentage in CD3+ cells stated below fragment number) is upregulated by at least 2-fold and can appear as a distinct cell population, whereas MHC-I blockade prevents this upregulation by at least 50%. Melanoma TILs with known reactivity to the cancer cell line Mel624 were used as a positive control in all assays.

<table>
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<th>Patient No.</th>
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Table II. Upregulation of 4-1BB in CD8+ TILs derived from GI tumor fragments after autologous tumor stimulation

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- **4-1BB expression was considered significant when a 2-fold increased was measured on CD8+ TILs in the tumor-stimulated condition compared with the unstimulated condition and represented at least 2% of CD8+ TILs.**
- **Percentage decrease calculated by the difference in 4-1BB expression in stimulated versus blocked conditions over difference in 4-1BB expression in stimulated versus unstimulated (background) 4-1BB expression.**
- **Percentage 4-1BB+/CD8+ in stimulated condition divided by percentage 4-1BB+/CD8+ in unstimulated condition.**
malignant ascites, or pleural effusions with high-dose IL-2 in combination with mitomycin C–pretreated autologous tumor cells, and they generated on average 2.3 × 10⁶ cells during 42 d (range, 0.9–4.8 × 10⁶), yielding 62.7% CD8⁺ T cells on average (33). Successful TIL expansion from seven GI cancer patient liver metastases in high-dose IL-2 was also reported to yield 39-fold expansion in ~30 d with 20–40% of CD8⁺ cells (36). Although these pioneering studies have demonstrated the feasibility of expanding TILs from GI cancer metastases, in contrast with our study, patients had not previously been exposed to current multiple-agent chemotherapy regimens, which could potentially affect T cell recovery and/or growth. Moreover, cell suspensions obtained from fresh tumors in these prior studies were often processed with layered Ficoll gradients to enrich in lymphoid cells prior to culture initiation, and it had not been demonstrated that TILs could be expanded from tumor fragments as we have now described. Additionally, these prior studies did not test whether GI TILs could be expanded to numbers and with methods currently used in ACT protocols in melanoma patients (5–8). Interestingly however, the higher fractions of CD8⁺ TILs in prior studies compared with our study suggests that preferential expansion of T cell subsets may be achieved by changing culture techniques and mitogenic stimuli. In this respect, the use of different cytokines has not provided clear answers (36–38), but could be revisited in combination with agonistic and antagonistic Abs to PD-1, PD-1 ligands, 4-1BB,OX40, and CD25.

Our results support the notion that the ex vivo expansion of unselected TILs for therapy from GI cancer metastases may not represent a major hurdle, but they suggest that a greater challenge lies in selectively identifying and expanding a significant number of tumor-reactive T cells. The main factor limiting the assessment of TIL reactivity to autologous tumor, however, remains the availability of suitable autologous tumor targets. As known for many years (39) and shown in our study (Table I), cryopreserved tumor cell suspension has been used successfully in melanoma to demonstrate MHC-I–restricted TIL reactivity using lysis or various cytokine secretion readouts, owing to the unique feature that most melanomas harbor a substantial fraction of autologous tumor-specific CD8⁺ TILs that retain reactivity after in vitro expansion. For GI and other cancers, detecting low-frequency tumor-reactive TILs using cryopreserved cell suspensions has been less successful (35, 37, 40–42) for several reasons. First, cultures from primary GI tumors are more susceptible to bacterial contamination. Second, lymphocytes tested too early after activation with high-dose IL-2 often mediate non-MHC–restricted lysis. Third, innate immune cells present in the tumor cell suspension (used as a target in this assay) are not controlled for, and by secreting type I IFNs or other cytokines, they can trigger non-MHC–restricted IFN-γ release by T cells. Fourth, anergic CD8⁺ T cells may engage their TCRs without producing significant amounts of IFN-γ. Fifth, the proportion of CD8⁺ T cells in bulk TILs is often not controlled for, and measurement of IFN-γ in coculture supernatants may poorly reflect the function of a minor subset. Finally, the limited availability of cryopreserved tumor cell suspensions generally limits their use as allogeneic specificity controls and restricts the repeatability of the experiments. Despite all of these factors, MHC-I–restricted reactivity based on IFN-γ secretion was found in 17 of the 188 TIL cultures (9%) in five of seven patients tested. To better quantify the reactivity in CD8⁺ TILs, we used a well-validated marker of TCR engagement in CD8⁺ T cells, the inducible cell-surface marker 4-1BB (CD137) (28–30), and we adapted a cell-based flow cytometry assay to use cryopreserved tumor cell suspensions instead of peptides as T cell stimulators (Fig. 4).

Our work on GI cancer TILs focused on better defining the reactivity of CD8⁺ TILs to autologous tumors because recent trials in the melanoma setting have reported that the absolute number of CD8⁺ TILs infused to patients correlated with tumor regression (6, 7) and given that a randomized control trial comparing ACT of bulk TILs (CD8⁺ and CD4⁺) or CD8⁺ TILs alone for patients with metastatic melanoma suggested that CD4⁺ TILs were not necessary to mediate objective cancer regression (34). With an appropriate gating strategy to measure the upregulation and MHC-I blockability of 4-1BB expression by TILs (Fig. 4), CD8⁺ TILs appeared to upregulate 4-1BB in an MHC-I–restricted manner after stimulation by autologous tumor in a total of 36 of 180 TIL cultures derived from fragments (20%), representing 1–12 TIL cultures (median, 3) harboring reactive CD8⁺ TILs in six of seven patients tested (Table II). In those six patients, CD8⁺4-1BB⁺ autologous tumor–reactive T cells appeared to represent 1–3% of all TILs expanded from tumor fragments. The lack of established tumor cell lines from these patients, however, limited our ability to define tumor reactivity at the molecular level in this study. Containing with other cell surface markers, such as a surrogate of lytic capacity (CD107a), could increase the confidence by which assayed TILs are cytolytic and tumor-reactive. This assay could also potentially serve for investigating CD4⁺ TIL reactivity to GI cancer, which requires cell surface markers specific for CD4⁺ T cell activation such as CD154 (CD40L) (43) and MHC class II blocking Abs. Given the predominant expansion and apparent initial activation level of CD4⁺ TILs found in GI cancer metastases (Fig. 2A), functional characterization of this T cell subset should be carried out. Specifically, the role of inducible Tregs in these cultures needs to be better defined, especially given their ability to upregulate 4-1BB and other activation markers (44). Finally, as clinical flow cytometry cell sorting becomes available for cell therapy (45), its use could be most relevant for enriching cell products with the most appropriate subset of tumor-reactive T cells for use in adoptive cell transfer immunotherapy.

In conclusion, our results show that GI cancer metastases from heavily pretreated patients constitute an immunological milieu distinct from adjacent normal tissue, which harbor non–terminally differentiated TILs with a proliferative potential sufficient for clinical scale in vitro expansion for ACT. In most patients studied, a small fraction of autologous MHC-I–restricted tumor-reactive CD8⁺ TILs appeared detectable. The efficacy of ACT for solid cancers that are less immunogenic than melanoma may require enrichment of tumor-reactive T cells using dynamic tumor-recognition assays based on expression of activation markers such as 4-1BB.

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Disclosures
The authors have no financial conflicts of interest.

References