Detection and Characterization of a Novel Subset of CD8⁺CD57⁺ T-cells in Metastatic Melanoma with an Incompletely-Differentiated Phenotype

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Grant Support: This work was supported by National Cancer Institute (NCI) grant R01-CA111999 to PH and LR, by The University of Texas MD Anderson Cancer Center Support Grant (P30-CA16672) to the Flow Cytometry Core Facility, and Award No. TL1RR024147 from the National Center for Research Resources to RCW. We also acknowledge support from a Melanoma SPORE Developmental Grant (P50-CA093459-05-DRP21) and a Team Science Award from the Melanoma Research Alliance (MRA) to LR. Support from the Dr. Miriam and Sheldon Adelson Medical Research Foundation (AMRF) and the Mulva Foundation are also greatly appreciated.

Disclosure of Conflict of Interest: No potential interests relevant to this article were reported.

Running title: Novel non-senescent CD8⁺CD57⁺ subset in human cancer

Key words: Melanoma, tumor-infiltrating lymphocyte, CTL, CD8⁺ effector-memory, CD57

Abbreviations used in this paper:
ACT, adoptive T cell therapy; Perf, perforin; GB, Granzyme B; HNK-1, human natural killer-1; N-CAM, neural cell adhesion molecule; T₀, naïve T cells; T_EM, effector-memory T cells; T_TDE, terminally-differentiated effector cells; TIL, tumor-infiltrating lymphocyte; TIL-CM, TIL culture medium; MFI, mean fluorescence intensity; mAb: monoclonal antibodies.
ABSTRACT

PURPOSE: Tumor-specific T-cells are frequently induced naturally in melanoma patients and infiltrate tumors. It is enigmatic why these patients fail to experience tumor regression. Given that CD8+ T cells mediate antigen-specific killing of tumor cells, the focus of this study was to identify alterations in the differentiation of CD8+ residing at the tumor site, with emphasis on a population expressing CD57, a marker for terminal differentiation.

EXPERIMENTAL DESIGN: We performed flow cytometric analysis of CD8+ tumor-infiltrating lymphocytes (TIL) isolated from 44 resected melanoma metastases using known T-cell differentiation markers. For comparison, PBMC were isolated from matched melanoma patients. We sorted different CD8+ subsets found in TIL and determined their effector functions. In addition, we performed Vβ spectratyping of T-cell receptors to determine lineage relationship between the CD8+ TIL subsets.

RESULTS: The majority of CD8+ TIL were in the early effector-memory stage of differentiation. A significant population consisted of an oligoclonal subset of cells co-expressing early effector-memory markers and end-stage CTL marker, CD57, yet having low to absent perforin expression. These cells could be induced to proliferate, produce a high level of IFN-γ, and differentiate into CD27CD57+, perforin^{high} mature CTL in vitro. Addition of TGF-β1 prevented this further differentiation.

CONCLUSIONS: Our studies identified a novel subset of incompletely differentiated CD8+ CTL co-expressing early effector-memory and late CTL markers. This population resembles that found by in patients with uncontrolled chronic viral infections. TGF-β1, frequently produced by melanoma tumors, may be a key cytokine inhibiting the further maturation of this subset.
Translational Relevance

Uncovering how CD8$^+$ differentiation and effector function can be altered in the tumor microenvironment is critical to understand why most tumors continue to progress, despite being infiltrated with CD8$^+$ T cells. Although other investigators have examined CTL differentiation in lymph node (LN) metastases in melanoma, it is less clear whether the differentiation of the CD8$^+$ CTL is also defective in non-lymph node, visceral metastases. In this study, we elucidated the state of CD8$^+$ CTL differentiation in LN and non-LN melanoma metastases, and breast cancer pleural effusions. We discovered a novel population of incompletely differentiated CD8$^+$ tumor infiltrating lymphocytes. We found that TGF-β1, a key immunosuppressive cytokine in the tumor microenvironment, could inhibit the further differentiation of these cells. These results suggest that CD8$^+$ differentiation into potent CTL is altered in cancer. Thus, modifying this altered differentiation in the tumor microenvironment should also be the goal of current immunotherapeutic approaches.
CD8⁺ CTL are critical effector cells in anti-tumor responses known to mediate the antigen-specific cytolysis of tumor cells through the action of granzymes, such as Granzyme B (GB), and the pore-forming protein Perforin (Perf). Terminally-differentiated effector (T_{TDE}) CD8⁺CD57⁺ CTL, which highly express both GB and Perf, have been identified as the most potent cells for combating chronic viral infection as well as inducing effective cytotoxic killing of tumor cells (14,19,23-25,32). Most healthy adults contain a distinct population of long-lived CMV-specific CD8⁺CD57⁻CD27⁻CD28⁻GB^{high}Perf^{high} T_{TDE} lymphocytes detectable in the peripheral blood that exhibit spontaneous anti-viral CTL activity (3,20,25,32). Differentiation of CD57⁺ T_{TDE} CTL from CD27⁺ precursors has been shown to be critical for maintaining a pool of mature CTL in the blood, which controls CMV and EBV infections in humans (7,20,24,34-35). However, differentiation towards such T_{TDE} phenotype can be altered in cases of chronic antigen stimulation, as has been reported for individuals infected with HIV and EBV re-activation (3,21), where an accumulation of incompletely differentiated CD8⁺CD27⁺CD57⁺ CTL leads to a loss of control of viral replication (20).

In cancer patients, the role of the CD8⁺CD57⁺ T cells in the control of tumor growth has not been clearly defined (8). It was previously reported that in malignant melanoma, renal cell carcinoma, and gastric carcinoma, patients who harbored higher percentages of CD8⁺CD57⁺ lymphocytes in peripheral blood had shorter overall survival (2,9,10). However, in those studies, the exact sub-stage of differentiation of the CD8⁺CD57⁺ T lymphocytes was not further analyzed. CD8⁺CD57⁺ T cells in the peripheral blood of advanced gastric carcinoma patients have been shown to have lower Perf content and diminished IFN-γ production as compared to healthy individuals (11). On the other hand, CD8⁺CD57⁺ cells with a T_{TDE} phenotype (CD27⁻
CD28\textsuperscript{high}Perf\textsuperscript{high}) accumulate in the peripheral blood of normal aged individuals and are the main IFN-\(\gamma\)-producing CD8\textsuperscript{+} T lymphocyte subset (4). They have also been positively associated with potent anti-tumor effector function and improved relapse-free survival in leukemia patients after stem-cell transplant (14,45).

Little is known about the frequency and function of the CD8\textsuperscript{+}CD57\textsuperscript{+} T TDE CTL population in tumor-infiltrating lymphocytes (TIL) in solid tumors, such as melanoma or breast cancer. Recently, a distinct population of CD8\textsuperscript{+} CTL in human peripheral blood with fully mature T\textsubscript{TDE} characteristics (CD27\textsuperscript{-}CD28\textsuperscript{-}GB\textsuperscript{high}Perf\textsuperscript{high}) was found to express a NK cell marker, CD56 (25-26,32). CD8\textsuperscript{+}CD56\textsuperscript{+} T\textsubscript{TDE} CTL (CD3\textsuperscript{+}) that did not co-express CD57 exhibited an even higher spontaneous cytolytic activity than CD57\textsuperscript{+} CTL against tumor targets; they also showed higher Perf and GB expression and could re-express CD45RA (26). It is currently not known whether CD8\textsuperscript{+} TIL preferentially differentiate to become CD56 or CD57-expressing CTL.

We undertook a detailed analysis of the phenotypic differentiation of CD8\textsuperscript{+}CD57\textsuperscript{+} CTL in 43 melanoma and 5 breast cancer metastases using multicolor flow cytometry. Previous studies focused exclusively on T cells infiltrates in lymph node metastases (30). In our studies here, we included a large number of non-lymph node visceral metastases in order to exclude any possible contribution by non-tumor specific, resident lymph node immune cells. This is important because melanoma patients usually die from visceral, non-lymph node metastases. We did not find an appreciable population of CD8\textsuperscript{+}CD56\textsuperscript{+} CTL in freshly-isolated melanoma TIL, but frequently observed a significant population of CD57\textsuperscript{+} T cells. Moreover, we found that the majority of the CD57\textsuperscript{+} TIL in melanoma and breast cancer pleural effusions also co-expressed CD27 and CD28, markers of effector-memory T cells (T\textsubscript{EM}) (21,28,33), with moderate GB
expression and low-to-absent Perf expression, similar to the CD8⁺CD27⁺CD57⁺ T-cell population that was recently described in HIV-infected patients (20). These CD8⁺CD27⁺CD57⁺ T-cells could be induced to further differentiate into CD27⁻CD57⁺, Perf^high mature CTL after short-term TCR stimulation and exhibit enhanced cytotoxic capacity. In addition, we demonstrate that further differentiation of this lymphocyte subset could be inhibited by TGF-β₁, an immunosuppressive cytokine known to be produced by many types of tumor cells, including melanoma (1,5,13,48). These results suggest that CD8⁺ TIL show aberrant differentiation along the CD57⁺ CTL lineage, an effect that may result from suppressive factors found within the tumor microenvironment.
MATERIALS AND METHODS

Reagents. Flow cytometry antibodies were purchased from BD Biosciences (San Jose, CA), eBioscience (La Jolla, CA), or Beckman-Coulter (Brea, CA). Human recombinant IL-2 (ProLeukin™) was a generous gift from Novartis (East Hanover, NJ). KLRG1-Alexa 488 mAb was a gift from Dr. H. Pircher (Freiburg, Germany).

Patient TIL and PBMC samples. TIL and blood samples for laboratory studies were obtained from patients with Stage IIIc to Stage IV melanoma who were undergoing surgery at The University of Texas MD Anderson Cancer Center according to an Institutional Review Board–approved protocol and patient consent. The method for fresh isolation of TIL from melanoma metastases are described in detail on-line in Supplementary Methods. Typically 0.5 x 10^6 to 5 x10^6 lymphocytes were isolated initially from tumor fragments, depending on the size of tumors. The dividing TIL lines were cultured in high-dose (HD) IL-2 as described previously (41). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation with Ficoll-Isopaque from normal donors from buffy coats obtained from the Gulf Coast Regional Blood Center (Houston, TX) or peripheral blood from Stage IV melanoma patients collected in heparinized tubes (BD Biosciences). The PBMC were cryopreserved in 10% DMSO, 90% human AB serum until analysis.

Pleural effusions were also collected from metastatic breast cancer patients under an IRB-approved protocol. Lymphocytes in pleural effusions were isolated by centrifugation over 70% and 100% Ficoll-Isopaque double layers to separate tumor cells from lymphocytes. The lymphocytes were collected at the 100% Ficoll-Isopaque layer and washed in TIL-CM before flow cytometry staining.
Surface and intracellular staining by flow cytometry. Freshly-isolated TIL and TIL cultured and expanded with IL-2, as well as thawed normal donor or patient PBMC, were washed twice in D-PBS and stained using Live/Dead® Fixable Aqua Dead Cell Stain Kit (Life Technologies, CA) according to manufacturer’s instruction. Cells were then washed twice in FACS wash buffer (D-PBS + 1% BSA) and stained for 30 min at 4°C with antibodies conjugated with fluorochromes against different T-cell cell surface and intracellular markers. In the cases of HLA-A2.1+ patients, the TIL suspensions were additionally stained with MART-1 peptide (ELAGIGILTV) HLA-A2.1 tetramer, gp100 peptide (IMDQVPFSV) HLA-A2.1 tetramer, or control HLA-A2.1 tetramer containing HIV gag peptide (SLYNTVATL) (Beckman Coulter, Brea, CA). The cells were finally fixed in D-PBS, 1% p-formaldehyde and 0.25% ethanol. Intracellular staining for GB and Perf was done by according to manufacturer’s protocol (BD Biosciences). Flow cytometric analysis was performed using a FACSCanto II flow cytometer (Becton-Dickinson, San Jose, CA). The positive and negative regions of the staining of the indicated surface markers were determined by comparing against the unstained samples (Figure S1B, Supplementary Data online). Data was analyzed using FACSDiva (BD Biosciences) or FlowJo software (Tree Star Inc, Ashland, OR). Proliferation was assessed by intracellular staining for Ki67 using an anti-Ki67-APC antibody (BD Biosciences).

Sorting and ³H-thymidine incorporation assay. IL-2-cultured TIL were stained with anti-CD8-Pacific Blue, anti-CD27-APC-Cy7 and anti-CD57-FITC in PBS containing 1% BSA and 5% goat serum. The CD8⁺CD27⁺CD57⁺ and CD8⁺CD27⁺CD57⁺ subsets were sorted using an InFlux® cell sorter (BD Biosciences). 5 x 10⁴ viable cells per well were plated into 96-well
Costar 3361 High-bind plates (Sigma-Aldrich, St. Louis, MO) precoated overnight with anti-CD3 (OKT3; Ortho Biotech, Raritan, NJ) or anti-CD3 and anti-CD28 (eBioscience, La Jolla, CA) agonistic antibodies. After 3 days, the cells were pulsed with 1 µCi of [3H]-thymidine (methyl-T-thymidine, PerkinElmer Inc., Boston, MA) for 18 h. The incorporated [3H] thymidine was determined as counts per minute by using a beta liquid scintillation counter (Beckman Coulter, Brea, CA).

**Human Th1/Th2 multiplex cytokine analysis.** The tissue culture supernatants from a triplicate wells of sorted, TIL subsets from unstimulated and stimulated conditions were collected and plated on the multiplex ELISA plate configured to detect a panel of human Th1/Th2 cytokines (IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12 p70, and IL-13), according to the manufacturer’s instructions (Meso Scale Discovery, Gaithersburg, MD). The signals were captured and analyzed by the SECTOR Imager 2400 (Meso Scale Discovery, Gaithersburg, MD). The concentration of each cytokine was calculated from its each respective standard curves.

**Differentiation assay in vitro.** CD8+ TIL were sorted into subsets as described previously and stimulated with anti-CD3 or anti-CD3 and anti-CD28 antibodies precoated on Nunc® plates (Thermo Fisher Scientific, NY). IL-2 (200 IU/ml) was added to each culture to maintain cell viability. Human TGF-β1 (R&D Systems, Minneapolis, MN) was used at 1 ng/ml for the TGF-β1-treated group. After 7 days, the cells were stained for CD8, CD27, CD28, CD56, and CD57. Intracellular staining for GB and Perf was done as described above.
Redirected cytotoxic T-cell assay. Analysis of CTL activity on the sorted CD8\(^+\) subsets was done according to a novel flow cytometric method measuring the cleavage of caspase-3 in anti-CD3 coated target cells as described previously (40). Briefly, 5 x 10\(^6\) murine mastocytoma target cells (P815) were labeled with a fixable, far-red fluorescent tracer, CellTrace\textsuperscript{®} Far Red DDAO-Succinimidyl Ester (DDAO-SE; Invitrogen, Carlsbad, CA) according to manufacturer’s instructions, washed, resuspended at a density of 2.5 x 10\(^6\)/mL, and pulsed with 200 \(\mu\)g/mL of anti-CD3 mAb in a low-serum containing media (RPMI 1640 with 2% FBS) at RT for 30 min. Unpulsed target cells served as controls. Labeled, pulsed P815 target cells were added to the sorted TIL subsets at E:T ratios of 1:1 and 3:1, or 1:10 and 1:20 in a round-bottom 96-well plate and spun down for 5 min at low centrifuge speed (300 RPM) in order to maintain optimal contact between target cells and effector T cells. The cells were co-incubated for 3 h before harvesting. The 3 hour time point was chosen to prevent the apoptotic target cells from becoming necrotic and losing the cleaved caspase-3 signal. The cells were stained intracellularly with an anti-cleaved caspase-3-PE mAb (BD Biosciences). Target cells were distinguished from effector T cells by the far-red tracer DDAO-SE (which fluoresce in the APC channel), and the extent of the caspase-3 cleavage in the target cell population was analyzed by the FACSanto II flow cytometer (BD Biosciences).

Statistical analysis. GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA) was used for graphing and statistical analysis. The two-tailed, paired Wilcoxon rank-sum test was used to analyze the statistical significance in differences between two groups, and two-tailed nonparametric Kruskal-Wallis test followed by Dunn’s multiple comparison test were used for
more than two sample groups. A $p$-value of less than or equal to 0.05 was deemed to be statistically significant.
RESULTS

Patient tumor samples and experimental approach

In this study, tumors were surgically excised from Stage III/IIIc and Stage IV (M1a-M1c) melanoma patients as part of an ongoing adoptive T-cell therapy clinical trial at MD Anderson Cancer Center (Houston, TX). Table S1 (Supplementary Data online) shows the characteristics of the melanoma patients, including patient age, sex, tumor location, disease stage, and anatomical sites. The tumors were processed immediately after surgery for multicolor flow cytometry staining and expansion of TIL. The panels of fluorochromes-conjugated antibodies used in all experiments and their clone numbers are shown in Table S2 (Supplementary Data online).

Presence of a CD8⁺CD57⁺ subset highly positive for CD27 and CD28 and absence of CD8⁺CD56⁺ T cells in TIL freshly isolated from melanoma tumors

As CD8⁺ T cells transition from T_{EM} into fully-differentiated (T_{TDE}) CTL, they generally acquire GB and Perf expression, down-regulate CD27 and CD28, and express CD57, or in some cases, CD56. This T_{TDE} phenotype (CD27⁻CD28⁻CD56⁺ or CD27⁻CD28⁻CD57⁺) is associated with immediate cytolytic activity, with no requirement for antigenic re-stimulation to induce CTL function (23,26,32-33). To determine the extent of the T_{TDE} phenotype in tumors, we first gated on the live CD8⁺ lymphocytes, as determined by the Aqua Live/Dead® exclusion dye (Invitrogen), on TIL freshly isolated from tumors (Fig. S1; Supplementary data online). We then analyzed them for CD4, CD8, CD27, CD28, CCR7 and CD45RA expressions using multicolor flow cytometry. A majority of the bulk as well as tumor antigen-specific CD8⁺ TIL from
melanoma tumors displayed a T<sub>EM</sub> phenotype (CD27<sup>+</sup>, CD28<sup>+</sup>, CCR7<sup>-</sup>, CD45RA<sup>-</sup>) (Fig. S3 and S4; Supplementary Data online) (28,33). The specificity of the MART-1 and gp100 tetramer staining was verified against a negative control, HIV gag tetramer (data not shown) as well as comparing against HLA-A2<sup>-</sup> patients (Fig. S2, Supplementary data online). We then stained the TIL for CD8, CD27, CD28, CD56, and CD57 and analyzed the relationship between end-stage CTL markers (CD57 and CD56) vs. T<sub>EM</sub> markers (CD27 and CD28). We found that a significant population of CD57 single positive cells was seen without CD56 expression (see Fig. 1A for a representative example). Fig. 1B shows this predominance of CD8<sup>+</sup>CD57<sup>+</sup> cells (mean percentage ± SEM = 16.2 ± 3.5) over CD8<sup>+</sup>CD56<sup>+</sup> cells (mean percentage ± SEM: 1.0 ± 0.6) (paired Wilcoxon rank-sum test; \( p < 0.005 \)) in the CD8<sup>+</sup> subset in freshly-isolated TIL for ten separate patient samples. Upon further analysis of the staining profiles from the freshly-isolated TIL samples, we found that many CD8<sup>+</sup>CD57<sup>+</sup> T cells co-expressed CD27 and CD28, which could be considered as a hybrid of late-stage CTL and early T<sub>EM</sub> markers (Fig. 1C). We also found that PD-1, a marker for exhausted CD8<sup>+</sup> T cells or T<sub>EM</sub> in humans (6, 18, Supplementary ref. 1), was more enriched in the CD27<sup>+</sup>CD57<sup>+</sup> than the CD27<sup>+</sup>CD57<sup>-</sup> subset (Fig. S5A; Kruskal-Wallis test, \( p < 0.05 \)), in the tumors from ten additional metastatic melanoma patients (Fig. S5B), which suggests that the CD27<sup>+</sup>CD57<sup>+</sup> “hybrid” phenotype may represent a more end-stage effector phenotype. We also stained PBMC from patients and normal donors to determine the extent of CD27<sup>+</sup>CD28<sup>+</sup> CD8<sup>+</sup>CD57<sup>+</sup> T cells. Comparing TIL and PBMC from the same patient, we found that the CD8<sup>+</sup> T cells in the PBMC had only a few (<5%) of CD57<sup>+</sup> T cells co-expressing CD27 and CD28, while almost 60% of the CD8<sup>+</sup>CD57<sup>+</sup> TIL co-expressed CD27 and CD28 (Fig. 2A). Similarly, only a few (<5%) of the CD8<sup>+</sup>CD57<sup>+</sup> in PBMC of normal donors co-expressed CD27 and CD28 (Fig. 2A). In contrast, melanoma TIL contained, on average, >20%
of the CD8^+CD57^+ subset co-expressing CD27 and CD28 when the results of a larger number of
patient samples were analyzed (Fig. 2B). An examination of the melanoma antigen-specific
CD8^+ subsets (MART-1 and gp100) in fresh TIL isolates by using tetramer staining revealed
these tetramer^+ cells were either CD27^+CD57^- or CD27^+CD57^+, with a negligible fraction of
cells having a CD27^+CD57^- or CD57^-CD27^+ phenotype (Fig. 2C).

**CD8^+CD57^+CD27^+ melanoma TIL are GB^+, but Perf^low**

We next determined the intracellular GB and Perf expression, markers used to identify end-stage
(T_TDE) CTL, in CD8^+CD27^-CD57^- TIL in melanoma. We also obtained pleural effusions from
newly diagnosed metastatic breast cancer patients to determine whether CD8^+CD57^+ co-
expressing CD27 and CD28 were also found in other forms of cancer, and what their GB and
Perf expression might be. First, in melanoma TIL, we found that the majority of gated
CD8^+CD27^+CD57^+ and CD8^+CD27^-CD57^- cells expressed GB, but most of the cells in both
subsets had low or negative Perf expression (Fig. 3A). Using the same gating approach, we
analyzed fresh melanoma TIL samples from 7 Stage IV melanoma patients, PBMC samples from
17 Stage IV melanoma patients, and PBMC from 6 normal donors. Shown in Fig. 3B was an
example of the analysis that compared CD8^+CD57^+ T lymphocytes’ GB and Perf contents in a
patient’s TIL against the same patient’s PBMC and that of a normal donor. In Fig. 3C, 48.0 ±
8.6 % (mean ± SEM) of CD8^+CD27^-CD57^- TIL from 7 patients were found to express GB,
whereas only 20.5 ± 6.0 % expressed Perf. In contrast, averages of 94 ± 1.7 % and 80.9 ± 4.2 %,
of the CD8^+CD57^- lymphocytes in the peripheral blood of 17 melanoma patients were positive
for GB and Perf, respectively. The difference was statistically significant for both GB and Perf
(p<0.001, respectively; Kruskal-Wallis test) When we analyzed lymphocytes isolated from
breast cancer pleural effusions, we also found that many CD8^+CD57^+ cells co-expressed CD27 and CD28, and that these cells were predominantly GB^+ with very little or no Perf expression (Fig S6).

**CD8^+CD27^+CD57^+ TIL persist in culture with IL-2, can be induced to enter cell cycle, and are potent IFN-γproducers after TCR stimulation.**

Several studies have demonstrated that CD8^+CD57^+ cells do not divide appreciably, or at all, in response to TCR stimulation, indicating that they are senescent cells (7,42). We therefore asked whether the CD8^+CD27^+CD57^+ cells found in melanoma TIL were capable of further proliferation and cytokine production in the presence of IL-2. Culturing of isolated melanoma TIL in culture medium with high-dose (HD) IL-2 (3,000 IU/ml) is a standard method to expand TIL for adoptive cell therapy (39,41). Routinely, we find that a two-week culture period of isolated TIL with IL-2 expands the CD8^+ T cells between 200- to 300- fold (data not shown). We stained 2-week IL-2-expanded TIL for CD8, CD27, CD57, and Perf. The percentage of the CD27^+CD57^+ subset increased from 4% to 20% of the gated CD8^+ subset (see Fig. S7A). A small percentage of cells (~10%) in both CD27^+CD57^+ and CD27^+CD57^- subsets expressed another marker for senescence and terminal differentiation, KLRG-1 (Data not shown) (15,47). To further examine the proliferative capacity of these cells, we stained the cultured TIL with Ki67 at the end of the 2-week culture period and found that both CD8^+CD27^-CD57^+ and CD8^+CD27^-CD57^- subsets contained a significant frequency of Ki67^+ cells (Fig. S7B).

We then asked whether the CD8^+CD27^-CD57^+ TIL could enter cell cycle and produce cytokines after TCR stimulation. Two-week IL-2-cultured TIL were isolated, washed and then re-cultured with low-dose (LD) IL-2 (200 IU/ml) alone (to prevent apoptosis due to cytokine...
withdrawal), or re-stimulated with LD IL-2 plus plate-bound anti-CD3 or anti-CD3 plus anti-CD28 mAb for 7 days followed by Ki67 staining (all CD8+CD27+CD57+ T cells were CD28+; see also earlier results in Fig. 1 and 2). The gated CD8−CD27−CD57+ cells re-cultured with LD IL-2 alone were negative for Ki67 staining. However, a significant proportion of these cells were Ki67+ following stimulation with anti-CD3 or anti-CD3 and anti-CD28 (Fig. 4A).

The results above indicate that the CD8+CD27−CD57+ T-cells in isolated melanoma TIL have proliferative capacity, but it could also be that CD8+CD27+CD57− T cells proliferated and induced CD57 expression when cultured with IL-2. Thus, to more directly examine the proliferative capacity of CD8+CD27−CD57+ TIL, we sorted these cells and examined their response to TCR stimulation and IL-2. The CD8+CD27+CD57+ and CD8+CD27−CD57− TIL subsets from four different melanoma patients were each stimulated with anti-CD3 plus anti-CD28 mAbs, and labeled with [3H]-thymidine after 3 days. Both sorted subsets showed a marked induction of [3H]-thymidine incorporation after TCR stimulation, although the sorted CD8+CD27−CD57− subset demonstrated a higher proliferative capacity than the sorted CD8+CD27+CD57+ subset in response to CD3 and CD28 stimulation in a majority of patients (Fig. 4B). Analysis of the supernatants from a parallel set of cultures of activated cells found that both the sorted CD8+CD27−CD57+ and CD8+CD27+CD57− TIL produced Th1/Th2 cytokines (IFN-γ, IL-5 and IL-13) upon TCR stimulation, with the CD27+CD57+ subset producing significantly more IFN-γ compared to the CD27+CD57− subset in TIL in 3 out of the 4 patients studied (Fig. 4C; Fig. S8, Supplemental Data Online). Thus, the CD8+CD27+CD57+ T lymphocytes found in melanoma TIL were capable of continued cell division and were potent IFN-γ producers. In addition, we performed Vβ spectratyping and noted that the sorted CD8+CD27+CD57+ TIL from both the melanoma and breast cancer samples had a striking
oligoclonality in some Vβ families than the CD8^+CD27^+CD57^- TIL (data not shown), similar to observations on CD8^+CD57^+ T cells in other pathologic settings (45-46).

**TCR stimulation drives CD8^+CD27^+CD57^- TIL to differentiate into CD8^+CD27^-CD57^+Perf^{high} cells that are inhibited by TGF-β**

Here, we asked whether CD8^+CD27^-CD57^- TIL were capable of further differentiation towards CD8^+CD27^-CD57^- CTL expressing increased Perf. We first took bulk TIL in vitro and activated them in LD IL-2 medium with anti-CD3 plus anti-CD28 and tracked the fate of the cells after 7 days by staining and analyzing for changes in CD27, CD57 and Perf expression in the CD8^+ subset. This process induced further cell division (data not shown) associated with a decrease in the frequency of the CD27^-CD57^- and CD27^-CD57^- CD8^+ cells and an increase in the percentage of the CD27^-CD57^- and CD27^-CD57^- populations (Fig. S9A). Both the percentage and level (MFI) of Perf expression was also increased in each cell subset (Fig. S9B). To better delineate how TCR stimulation specifically affects the predominant CD8^+CD27^-CD57^- and CD8^-CD27^-CD57^- subsets in TIL, we sorted these subsets from melanoma TIL and re-cultured the sorted cells with LD IL-2 or IL-2 and anti-CD3 plus anti-CD28. The post-sort purity of the two populations was verified (Fig. S10, Supplementary Data Online). The sorted cells were also verified to have CD3 and CD28 expression, as previously shown (Fig. 1, and data not shown).

The 7-day TCR stimulation induced a large decrease in the percentage of the sorted CD8^-CD27^-CD57^- cells from 72% to 37% (Fig. 5A) or 66% to 6% (Fig. 5B), with a concomitant increase in the percentage of CD8^-CD27^-CD57^- cells from 6% to 49% (Fig. 5A, bottom panel), or CD8^-CD27^-CD57^- cells from 4% to 54% in other patient (Fig. 5B, bottom panel). On the other hand, the sorted CD8^-CD27^-CD57^- cells differentiated into a CD8^-CD27^-
CD57+ population with TCR stimulation (increase from 6% to 34% and 13% to 73%, respectively, in two different patients), although a minor subpopulation converted into CD8+CD27+CD57+ cells in LD IL-2 culture alone (Fig. 5A and 5B, top panel).

We also tracked changes in GB and Perf expression and found that both proteins increased in the sorted CD8+CD27+CD57+ and CD8+CD27+CD57- TIL populations following TCR stimulation for 7 days, although the extent of the increase in Perf expression was greater in the sorted CD8+CD27+CD57+ subset in three different patients (Fig. 6A). The increase in Perf expression in both sorted subsets led to an enhanced cytotoxic activity, in a redirected lysis assay using anti-CD3 antibody coated P815 target cells (Fig. 6B). We chose the redirected lysis assay to assess the cytotoxic activity between the TIL subsets to avoid erroneous interpretation of any differences due to differences in the percentages of tumor antigen-specific T lymphocytes in each subset. The sorted and TCR-activated CD8+CD27+CD57+ subset also exhibited higher or similar levels of cytotoxic activity against P815 target cells as compared to sorted CD8+CD27+CD57- subset in TIL from two different melanoma patients (Fig. 6B). Thus, TCR stimulation induces the phenotypic and functional maturation of the CD8+ CD27+CD57- and CD27+CD57+ subsets into more differentiated CD27-CD57- and CD27-CD57+ T cell populations with high Perf expression and cytotoxic activity.

TGF-β1 is an immunosuppressive factor produced by melanoma cells and is found in about 50% of metastatic melanomas (5,13,48). It has also been shown to inhibit CTL differentiation in human peripheral blood CD8+ T cells and inhibit the induction of Perf expression (1,22,31,37). We reasoned that TGF-β may also affect the further differentiation of the CD8+CD27+CD57+ cells found in melanoma TIL. We first tested the effect of adding TGF-β to bulk TIL stimulated with anti-CD3 and anti-CD28, and found that the differentiation of
CD8^+CD27^+ T cells subsets were arrested, with attenuated perforin expression (Fig. S11, Supplementary Data online). We then sorted CD8^+CD27^+CD57^+ and CD8^+CD27^+CD57^- melanoma TIL and treated them with TGF-β during TCR stimulation. In sorted TIL from two different melanoma patients, addition of TGF-β increased the frequency of the CD8^+CD27^+CD57^- memory-effector TIL (Fig 5A and B, top panels) as well as maintained the CD27^+CD57^- “hybrid” phenotype (Fig. 5A and B, bottom panels), which were both associated with low GB expression and more attenuated Perf expression (Fig. 6A). TGF-β prevented the loss of CD28 expression (data not shown). TGF-β1 also strongly diminished the cytotoxic activity of CD8^+CD27^+CD57^+ and CD27^+CD57^- TIL subsets after stimulation with anti-CD3 and anti-CD28 for 7 days, as seen in two different melanoma patients (Fig. 6B). Thus, the differentiation of the CD27^+CD57^- and CD27^+CD57^- CD8^+ TIL into mature CD27^- GB^highPerf^high CTL in vitro was blocked by TGF-β1, which resembled the phenotype of the Perf^low, CD8^+ TIL freshly isolated from melanoma tumors.
We found that the vast majority of CD8+ T cells infiltrating metastatic melanomas in a large number of patients had a CD27+CD28+GB+Perf-/low phenotype reminiscent of early effector memory cells (3,28,33). Importantly, a high percentage of the tumors analyzed were from non-LN visceral metastases. Very few highly differentiated CD8+ T cells that were CD27−CD28− with high Perf and GB co-expression were found. Unexpectedly, many of these high CD27-expressing CD8+ T cells also co-expressed CD57 (HNK-1), a marker usually attributed to highly differentiated end-stage CTL with high Perf and GB levels and potent cytolytic activity (25,32,33,42). A similar subset of CD8+ cells having this unusual early effector-memory phenotype with CD57 expression was found in pleural effusions of breast cancer patients. Some of these CD8+CD27+CD28+CD57+ TIL were also specific for melanoma tumor antigens, as found by co-staining with MART-1 and gp100 peptide tetramers, suggesting that they were not merely bystander CD8+ T cells that were not tumor-specific. However, many of the other CD8+CD27+CD28+CD57+ TIL may also have been tumor-specific, recognizing other undefined antigens; this will need to be addressed in future studies. The appearance of this subset in metastatic cancer patients has striking parallels to a population of CD8+CD27+CD28+CD57+ T cells found to accumulate in the peripheral blood of patients unable to control HIV, EBV, or CMV infection (20,24,34). The circulating gp100 tetramer+ CD8+ T cells containing a CD27+CD57+ subset has also been observed in the peripheral blood of early stage melanoma patients receiving gp100 peptide vaccination (Supplementary reference 2). Although it is important to examine how the state of CD8+ T-cell differentiation and the appearance of this CD8+CD27+CD28+CD57+ may change with tumor stage, our study was limited by the lack of access to early-stage melanoma tissue samples. We did not detect appreciable Foxp3 expression
in the CD8⁺CD27⁺CD57⁺ subset (data not shown), indicating that these are not “regulatory”
CD8⁺ T cells. In addition, a study by Anichini et al. found that a subpopulation of tumor-
infiltrating CD8⁺Foxp3⁺ T cells were CD57⁻ (30).

Overall, our results suggest that CD8⁺ CTL differentiation into potent Perfʰigh killer cells
in the melanoma tumor microenvironment may be arrested as effector-memory cells
(CD8⁺CD27⁺CD28⁺CD57⁻); some of these cells begin to express CD57 (an end-stage CTL
marker) but then fail to down-modulate CD27 and CD28 and induce Perf. Alternatively, it is
possible that CD8⁺57⁺ TIL with properties of T_TDE do arise, but these cells are too short-lived to
be detectable ex vivo. The reason why these cells begin to express CD57 is unclear at present.

CD57 (HNK-1) was originally found to be a marker of human NK cells. Recently, it has been
shown to be most highly expressed by the most mature CD8⁻CD56loCD16hi human NK cell sub-
population (29). The CD57 antigen is a terminally sulfated glycan carbohydrate epitope, and
little is known about its actual function in T cells, although studies on other cell types (e.g.,
motor neurons) have found that it appears to function as a cell adhesion molecule (36). One
study reported that CD57 might bind to IL-6 (12).

Previous investigators have shown that CD8⁺CD27⁻CD28⁻CD57⁺ differentiated CTL in
the peripheral blood accumulated with aging and is a senescent population incapable of further
cell division and prone to activation-induced cell death after TCR ligation (7,42). CD8⁺CD57⁺
cells have also been found to be an oligoclonal population resulting from multiple expansions of
specific clones, as a consequence of aging or chronic viral infection with EBV and CMV
(4,24,32,46). However, whether CD57⁺ T cells are truly senescent have come under debate with
newer studies showing that these cells could be induced to divide and produce cytokines after
TCR stimulation (43).
Based on these previous reports, we tested whether CD8⁺CD27⁺CD57⁺ melanoma TIL were capable of further cell division in comparison to CD8⁺CD27⁺CD57⁻ T cells, the other major subset found in melanoma TIL. Several pieces of evidence point to the fact that the CD57⁺ cells are indeed not senescent and are also capable of further cell division and differentiation in vitro. First, T cells with a CD8⁺CD27⁺CD57⁺ phenotype in the fresh TIL isolates persisted and expanded over a number of weeks in culture with IL-2. However, these IL-2-cultured CD8⁺ TIL did not show evidence of further differentiation into CD27⁻CD57⁺ or CD27⁻CD57⁻ cells, but did up-regulate Perf expression slightly, as expected after IL-2 signaling (49). Secondly, in bulk TIL cultures, or sorted pure populations of CD8⁺CD27⁺CD57⁻ T cells, we found that stimulation with anti-CD3 and CD28 antibodies also stimulated cell cycle entry and divisions of CD8⁺CD27⁺CD57⁻ T cells. Thirdly, the sorted CD8⁺CD27⁺CD57⁻ TIL produced IFN-γ, IL-5, and IL-13 after TCR stimulation, with significantly higher IFN-γ production than the CD27⁺CD57⁻ subset. Interestingly, TILs in breast and kidney carcinoma have also been observed to produce IL-13, which was hypothesized to negatively regulate IDO expression in tumors (27). Lastly, our sorting experiments revealed that TCR stimulation induced sorted CD8⁺CD27⁺CD57⁻ T cells to differentiate into CD8⁺CD27⁻CD57⁻GB⁺Perf⁺ high T cells, reminiscent of Tₜₜₑ CD57⁺ CTL in the peripheral blood; this observation was not due to CD8⁺CD27⁺CD57⁻ TIL contaminants, since sorted CD8⁺CD27⁺CD57⁻ T cells did not differentiate into CD27⁻CD57⁻ T cells, but rather, differentiated directly into CD27⁺CD57⁻ T cells, which further supports that CD8⁺CD57⁺ and CD8⁺CD57⁻ TIL belong to distinct lineages or subsets. We do not know whether these CD8⁺CD27⁺CD57⁺ TIL persist as short-lived effector cells at the tumor site or recirculate in the periphery. Addressing this question requires a murine model, but this CD8⁺CD57⁺ T cell subset does not exist in mice (35).
Based on our initial observation that incompletely differentiated Perf$^{\text{low}}$ CD8$^+$ TIL accumulate in the tumor microenvironment, we reasoned that some immunosuppressive mechanism may account for the lack of further differentiation. TGF-$\beta$1 is known to be secreted by many tumors, including melanoma cells and certain types of tumor-associated macrophages (1,5,13). Previous studies have also established that TGF-$\beta$1 inhibited the differentiation of naïve CD8$^+$ T cells into effector cells (22,37), impaired the production of inflammatory cytokines such as IFN-$\gamma$ and TNF-$\alpha$ by effector CD8$^+$ T cells (1), and attenuated Perf expression in lymphokine-activated killer (LAK) cells and murine CD8$^+$ T cells (31,44). The negative effect of TGF-$\beta$1 on CTL function is evident in studies showing enhanced tumor eradication in mice with T-cells lacking TGF-$\beta$ signaling (16,22). Indeed, we found that TGF-$\beta$1 prevented the differentiation of CD8$^+$CD27$^+$ subsets into more mature CD8$^+$CD27$^-$ CTL. This cytokine also potently inhibited the expression of Perf and GB to a lesser extent in CD8$^+$ TIL. In addition to TGF-$\beta$1, other factors in the tumor microenvironment, such as a lack of adequate cytokine signaling (e.g., IL-2, IL-15), or lack of proper costimulatory signals (e.g., through 4-1BB), or the presence of inhibitory costimulation through PD-1, BTLA, or TIM-3 (6,17) following TCR activation could also contribute to this arrest in CTL differentiation. Alternatively, the presence of other immunosuppressive cytokines, such as IDO, PGE$_2$, or IL-10 could also play a role (48,50).

In summary, we have identified a unique stage in which CD8$^+$ CTL differentiation seems to be arrested in the tumor microenvironment in metastatic melanoma and in breast cancer. This incompletely differentiated state of melanoma CD8$^+$ TIL was also associated with the appearance of an unusual CD8$^+$ effector-memory subset co-expressing a late terminally-differentiated CTL marker, CD57. These CD8$^+$CD27$^+$CD28$^+$CD57$^+$ TIL, although expressing...
GB, have little or no Perf expression and therefore seem to be a unique effector-memory subset in cancer. Immunosuppressive factors, such as TGF-β, may lead to incomplete CTL differentiation and the appearance of this unique CD57+ subset. It is possible that these unique CD57+ cells may also represent in part CD8+CD27+CD57+ TIL that have attempted to differentiate into CD57+ cells, but were blocked from further differentiation into CD27+Perf^high cells in the tumor microenvironment. On the other hand, the CD8+CD27+CD57+ effector-memory TIL, which are in fact the dominant CD8+ TIL subpopulation, may also represent a putative CTL precursor subset, which, like the hybrid CD27+CD57+ subset, is also functionally suppressed by the immunosuppressive factors in the tumor microenvironment. Finally, tumor antigen-specific CD8+ populations could be found in both CD27+CD57+ and CD27+CD57+ subsets. Thus, future studies will be needed to address whether the more differentiated CD8+CD27+ TIL exhibit better control of tumor growth in vivo than their CD27+ precursors. In addition, it will be of interest to further examine whether CD8+CD27+CD57+ TIL can differentiate into more long-lived CD8+CD27+CD57+ cells in vivo, which are reminiscent of the long-lived CD57+ T cells in the blood that effectively control CMV and EBV infection in humans (24,34).
ACKNOWLEDGEMENTS

The authors greatly appreciate the cooperation and efforts of the melanoma surgical staff at M.D. Anderson Cancer Center who have generously provided all the melanoma specimens for this study, including Drs. Jeffrey Gershenwald, Jeffrey Lee, Merrick Ross, Amy Heimberger, Paul Mansfield, Janice Cormier, and Anthony Lucci. We are also grateful to Renjith Ramachandran, Christopher Toth, OJ Fulbright, Rahmatu Mansaray, and Seth Wardell for processing tumor specimens. We also appreciate the breast cancer pleural effusion samples provided by Dr. James L. Murray from the Breast Medical Oncology Department. We also thank the M.D. Anderson’s Melanoma SPORE Tissue Bank personnel (Dr. Victor Prieto and Katie McNeil) for help in obtaining the melanoma samples used in this study. Lastly, we also thank Drs. Kevin Kim, Natalia Martin-Orozco, Chantale Bernatchez, and Willem Overwijk for critical reading of the manuscript. We also acknowledge help with flow cytometry and cytokine analysis by Dr. Luis Vence, Himabindu Pappu, Leslie Wiltz of the MD Anderson Cancer Center Immunomonitoring Core Lab (IMCL).

Authorship Contributions

R.C.W. and S.J.L. contributed equally to the phenotypic analysis of the TIL by flow cytometry. R.C.W. performed the functional studies of the TIL subsets. R.C.W. and S.J.L. co-wrote the manuscript. L.R. designed experiments, supervised the work, procured the melanoma tissue, and helped write and edit the paper. J.L.M provided the breast cancer pleural effusions patient samples. H.P. provided anti-human KLRG-1 mAb. J.L.M, G.A.L, J.J.M., and P.H. contributed to the editing of this manuscript. P.S. and J.J.M. conducted the Vβ spectratyping experiment.
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Figure legends

**Fig. 1.** Lack of CD56-expressing end-stage CTL and the appearance of a novel subset of CD8^+CD57^+ melanoma TIL co-expressing CD27 and CD28.  
(A) Fresh TIL isolates were stained with a panel of fluorochrome-conjugated antibodies for CD4, CD8, CD27, CD28, CD56, and CD57. Results were shown by gating on the live cells that were determined using Live/Dead® Fixable Aqua Dead Cell Stain Kit (Life Technologies, CA). A minimum of 100,000 gated light scatter events per sample were acquired on the flow cytometer. CD56 and CD57 expression in the CD8^+ subset in a representative patient TIL sample is shown.  
(B) A summary of the CD8^+CD56^+ and CD8^+CD57^+ populations in 10 freshly-isolated TIL from melanoma tumors. Significant difference was calculated by a two-tailed, paired Wilcoxon rank-sum test.  
(C) The gated CD8^+CD57^+ and CD8^+CD57^- sub-populations were further analyzed separately for CD27 versus CD28 expression. Examples of TIL from two melanoma patient tumor samples are shown.

**Fig. 2.** Relatively few fully differentiated CD8^+CD27^-CD28^-CD57^+ TIL were found in metastatic melanomas compared to peripheral blood of patients and normal donors. TIL isolated from melanoma metastases and PBMC from Stage III/IV melanoma patients were stained in a panel of fluorochrome-conjugated antibodies against CD4, CD8, CD27, CD28, CD56, and CD57 (same as in Fig. 1). A minimum of 100,000 gated light scatter events per sample were acquired on the flow cytometer. PBMC from 2 normal donors were similarly stained for comparison. Results were shown by gating on the CD8^+ live lymphocytes that were determined using Live/Dead® Fixable Aqua Dead Cell Stain Kit (Life Technologies, CA) and analyzed for CD27 and CD28 expressions.  
(A) The results from one representative TIL isolate from tumor #2163,
one representative patient PBMC sample, and two normal donors. (B) Analysis of the CD57+ subset co-expressing CD27 and CD28 in the CD8+ T-cell population in a larger group of melanoma TIL samples (n=18), melanoma patient PBMC (n=12), and normal donor PBMC (n=6) samples. The percentage of gated CD8+CD57+ subset co-expressing CD27, CD28, or both, was plotted for each sample type. Each dot represents a single sample with bars indicating the averages for each sample group. Results of a Student’s t-test between the TIL and patient PBMC are shown for each subset (p<0.05 indicates statistical significance). (C) Melanoma metastases from HLA-A2.1+ patients were stained with antibodies for the same markers as in panel A together with tetramer containing either a human HLA-A2.1 MART-1 epitope or the human gp100 epitope. A minimum of 100,000 gated light scatter per sample were acquired on the flow cytometer. The CD8+ T-cell subset was gated and analyzed for MART-1 or gp100 tetramer+ cells. The tetramer+ cells were then gated and analyzed for CD27 and CD57 expression. Shown are two examples of the analysis done using TIL from two different patient metastases (#2058 and #2276).

Fig. 3. CD8+CD57+ subset in the melanoma microenvironment has a unique GB+Perf/low phenotype compared to the same subset in patient and normal donor PBMC. (A) TIL isolates were stained for CD8, CD27, CD28, CD57, GB, and Perf expression, as described in Material and Methods. A minimum of 100,000 gated light scatter events per sample were acquired on the flow cytometer. Results were shown by gating on the live cells that were determined using Live/Dead® Fixable Aqua Dead Cell Stain Kit (Life Technologies, CA). The gated CD8+CD57+ and CD8+CD57- sub-populations were further analyzed separately for GB and Perf expression. One representative melanoma TIL sample is shown. (B) Results comparing GB and Perf
expression in the CD8⁺CD57⁺ TIL and CD8⁺CD57⁺ T cells in PBMC from the same patient (#2163) and in a representative normal donor. (C) Summary of GB and Perf expression in CD8⁺CD57⁺ TIL subsets from a number of melanoma metastases (n=7) compared to PBMC of patients (n=17) and PBMC from a group of normal donors (n=16). The percentages of CD8⁺CD57⁺ T-cells expressing GB or Perf are shown. Each dot represents a single sample with the bars indicating the averages and standard error on the means (SEM) for each TIL’s CD8⁺ subset. Significant differences between the groups were calculated by the two-tailed, nonparametric Kruskal-Wallis test followed by Dunn’s multiple comparison test. ns: not significant.

Fig. 4. The CD8⁺CD27⁺CD57⁺ subset in melanoma TIL was not anergic and could be induced to proliferate and produce a high level of IFN-γ and other Th2 cytokines after TCR stimulation with CD28 co-stimulation. (A) Bulk TIL were rested overnight with low-dose IL-2 and stimulated the next day with a combination of plate-bound anti-CD3 or anti-CD3 with anti-CD28 antibodies for 7 days. Proliferation was determined by the percentage of Ki67⁺ cells as compared to the isotype control out of the total gated CD8⁺CD27⁺CD57⁺ subset. A minimum of 50,000 gated light scatter events per sample were acquired on the flow cytometer (B) CD8⁺CD27⁺CD57⁺ and CD8⁺CD27⁺CD57⁻ TIL subsets from four different patients were purified by sorting and an equal number of both sorted subsets in triplicate were stimulated with anti-CD3 and anti-CD28 antibodies. [³H]-thymidine was added for 18 h on day 4 of the stimulation before harvesting and counting. The negative control was sorted TIL treated with low-dose (LD) of IL-2 (200 U/mL) only, which induced very low level of proliferation. Results shown were means ± standard error on the means. Note for patient #2184, no error bar was shown for the assay performed on sorted
CD8⁺CD27⁺CD57⁺ TIL due to insufficient number of cells for triplicate wells after sorting. Relevant patient information for TIL #2544 and #2545 can be found in Figure S5B. (C) The tissue culture supernatants from 5 x 10⁴ cells in each subset in triplicate wells after stimulation were collected on day 4 of the stimulation and cytokine levels were determined using MSD® human Th1/Th2 cytokine multiplex assay as described in Material and Methods. Results shown were means ± standard errors on the means on experiments done on two different patients (#2182 and #2206).

Fig. 5. Sorted CD8⁺CD27⁺CD57⁺ T cells in TIL could further differentiate into CD27⁻CD57⁺ CTL upon TCR stimulation, which was inhibited by the addition of TGF-β1. TIL were sorted into CD8⁺CD27⁺CD57⁻ and CD8⁺CD27⁺CD57⁺ subsets as before. Post-sort purity of the sorted populations is shown in Figure S10 (Supplementary Data online). (A) The sorted TIL subsets (from patient #2384) were stimulated with anti-CD3 and anti-CD28 antibodies for 7 days. The cells were then re-stained for CD8, CD27, and CD57 expression and analyzed by flow cytometry. To some cultures of sorted, re-stimulated cells, 1 ng/mL of TGF-β1 was added on day 0 with CD3 and CD28. Cells were harvested similarly on day 7 of the stimulation and stained for CD8, CD27, and CD57. A minimum of 50,000 gated light scatter events per sample were acquired on the flow cytometer. (B) Similar experiment performed on TIL from a different patient (#2541), whose relevant information was described in Figure S5B.

Fig. 6. CD3 and CD28 stimulation of melanoma TIL subsets induced Perf and GB expression and increased cytotoxic killing function of the sorted CD8⁺ TIL subsets, which was abrogated in the presence of TGF-β1. Intracellular flow cytometry staining for GB and Perf was performed as

33
described in Material and Methods. **(A)** Graph shows the percentage of GB$^+$ and Perf$^+$ cells in the sorted CD8$^+$CD27$^+$CD57$^+$ and CD8$^+$CD27$^+$CD57$^-$ subsets, from three different patients, before and after CD3 and CD28 stimulation for 7 days. TGF-β1 (1 ng/ml) was added to some cultures as indicated and the cells incubated and then stained in the same way. A minimum of 50,000 gated light scatter events per sample were acquired on the flow cytometer. *ND: Not determined due to insufficient cell number.** *(B)** A caspase-3 cleavage assay, described in Material and Methods, was used to assess the CTL killing activity. Sorted CD8$^+$ TIL subsets, cultured with LD IL-2 alone, or after TCR re-stimulation with anti-CD3 and anti-CD28, with or without added TGF-β1 (1 ng/ml) for 7 days, were co-incubated for 3 h at the indicated Effector: Target (E:T) ratios with P815 (murine mastocytoma) target cells, which were previously pulsed with 200 μg/mL of anti-CD3 antibody, in a redirected lysis assay. Target cells were distinguished from effector T cells by labeling with a fixable, far-red fluorescent tracer, CellTrace® Far Red DDAO-Succinimidyl Ester (DDAO-SE) at the beginning of the assay. Non-pulsed P815 cells were used as negative controls. Results were expressed as percent cleaved caspase-3 positive target cells out of the total target cell population. A minimum of 50,000 gated target cells per sample were acquired on the flow cytometer. Results shown were from experiments done on sorted TIL subsets from two different patients as indicated. Relevant patient information on TIL #2544 was described in Figure S5B.
Figure 1

A

TIL #2156

SSC

CD8

CD57

CD56

B

Gated on Live CD8+ TIL

% of CD8+ T-cell Subset

\[ p < 0.005 \]

C

TIL #2156

CD57

CD28

CD27

TIL #2276

CD57

CD28

CD27

CD8
Figure 3

A

CD57

GB

33

58

CD27

B

TIL #2163

GB

Perf

CD57

CD57

CD57

CD57

Pt. PBMC #2163

GB

Perf

CD57

CD57

CD57

CD57

ND PBMC

GB

Perf

CD57

CD57

CD57

CD57

C

% of CD8+ T-cell subset

TIL

Pt.

ND

PBMC

GB

Perf

TIL

Pt.

ND

PBMC

GB

Perf

p < 0.05

p < 0.001

p < 0.05

p < 0.001

ns

ns
**Figure 4**

A. Gated on CD8+CD27+CD57+ Subset

- LD IL-2
- LD IL-2 + CD3
- LD IL-2 + CD3 + CD28

![Flow cytometry plots](image)

B. Concentration (pg/mL) of cytokines

- **TIL #2206**
  - Control
  - CD3 + CD28

- **TIL #2544**
  - Control
  - CD3 + CD28

C. Concentration (pg/mL) of cytokines

- **TIL #2206**
  - IFN-γ
  - IL-5
  - IL-13

- **TIL #2545**
  - IFN-γ
  - IL-5
  - IL-13

- **TIL #2184**
  - IFN-γ
  - IL-5
  - IL-13

![Graphs showing cytokine concentrations](image)
Figure 5

Sorted CD8^+CD27^+CD57^- subset

TIL #2384

Sorted CD8^+CD27^+CD57^- subset

TIL #2541

CD27

Sorted CD8^+CD27^+CD57^+ subset

CD3 + CD28

TGF-β + CD3 + CD28

CD57

LD IL-2

66 26 6 2

42 13 34 11

77 3 20 1

56 25 18 2

377

Sorted CD8^+CD27^+CD57^+ subset

CD3 + CD28

TGF-β + CD3 + CD28

CD57

LD IL-2

20 72 6 2

7 37 8 4

35 42 12 11

5 66 10 6

1112

CD27
Figure 6

A

TIL #2384

TIL #2376

TIL #2541


B

TIL #2376

TIL #2544

% of CD8+ T-cell Subset

CD8+CD27+CD57- CD8+CD27+CD57+ CD8+CD27+CD57-

CD8+CD27+CD57+ CD8+CD27+CD57+ CD8+CD27+CD57+

% caspase 3 cleavage

CD8+CD27+CD57- CD8+CD27+CD57+ CD8+CD27+CD57-

CD8+CD27+CD57+ CD8+CD27+CD57+ CD8+CD27+CD57+

E:T ratio

IL-2

CD3 + CD28

TGF-β + CD3 + CD28

CD3 + CD27
Detection and Characterization of a Novel Subset of CD8+CD57+ T-cells in Metastatic Melanoma with an Incompletely-Differentiated Phenotype

Richard C Wu, Shujuan Liu, Jessica A Chacon, et al.

Clin Cancer Res Published OnlineFirst February 3, 2012.