

# Capture of Tumor Cell Membranes by Trogocytosis Facilitates Detection and Isolation of Tumor-Specific Functional CTLs

Arthur Machlenkin,<sup>1</sup> Ronny Uzana,<sup>1</sup> Shoshana Frankenburg,<sup>1</sup> Galit Eisenberg,<sup>1</sup> Lea Eisenbach,<sup>3</sup> Jacob Pitcovski,<sup>4</sup> Raphael Gorodetsky,<sup>1</sup> Aviram Nissan,<sup>2</sup> Tamar Peretz,<sup>1</sup> and Michal Lotem<sup>1</sup>

<sup>1</sup>Sharett Institute of Oncology and <sup>2</sup>Department of Surgery, Hadassah-Hebrew University Hospital, Jerusalem, Israel; <sup>3</sup>Department of Immunology, Weizmann Institute of Science, Rehovot, Israel; and <sup>4</sup>Migal, Kiryat Shmona, Israel

## Abstract

**The success of adoptive cell transfer in the treatment of metastatic cancer in humans is dependent on the selection of highly active tumor-specific cytotoxic T cells. We report here that CTLs capture membrane fragments from their targets while exerting cytotoxic activity and thus gain a detectable functional signature by which they can be identified. Fluorochrome labeling or biotinylation was used to tag tumor cells. CD8<sup>+</sup> T cells were coincubated with the tagged targets, sorted, and functionally evaluated. Our results show that membrane capture by CD8<sup>+</sup> lymphocytes is T-cell receptor dependent, epitope specific, and preferentially associated with highly cytotoxic clonal subsets. CTLs that captured membranes from unmodified melanoma exhibited enhanced cytotoxic activity against tumor cell lines and autologous melanoma. In a human melanoma *in vivo* model, adoptive transfer of membrane-capturing, peptide-specific T cells, but not noncapturing or bulk CD8<sup>+</sup> T cells, inhibits tumor progression. Membrane capture is therefore a signature of antigen-specific CTLs endowed with high functional avidity and may have direct relevance in the clinical application of adoptive immunotherapy. [Cancer Res 2008;68(6):2006–13]**

## Introduction

Tumor-reactive CTLs play a major role in antitumor responses, and the generation of ample amounts of these effectors is the desired outcome of immunotherapeutic strategies. The ability of CTLs to mediate cancer regression is best shown in adoptive cell therapy, a potent new anticancer immunotherapy yielding 50% objective responses in metastatic melanoma patients (1, 2).

Two approaches are widely applied for detection and isolation of antigen-specific CTLs. The first uses pMHC multimers to enumerate and separate antigen-specific CD8<sup>+</sup> T cells from the whole lymphocyte population (3, 4). The need for a priori knowledge of exact peptide-MHC specificity is a major limiting factor for evaluating complex T-cell responses against a broad repertoire of antigens. An alternative strategy, allowing both monitoring of immune response and isolation of antigen-specific CD8<sup>+</sup> T cells, is based on assessment of specific functions, such as cytokine (typically IFN- $\gamma$ ) production (5, 6), or activation-induced phenotypical alterations, such as cell surface expression of CD107a

(LAMP-1; ref. 7) and CD137 (4-1BB; ref. 8). IFN- $\gamma$  production is the most commonly used variable to detect T-cell reactivity against antigen-presenting targets. Cytokine-producing CD8<sup>+</sup> T cells are not necessarily cytotoxic (7, 9, 10), and consequently in the therapeutic context, it is important to distinguish T-cell reactivity from "functional cytotoxicity," specifically the capacity of CTLs to destroy target cells. Due to the diversity of effector functions used by T-cell subsets to kill their targets (11), a signature that integrates multiple function/molecules associated with the process of target destruction is required.

It has previously been shown that T cells acquire pMHC from antigen-presenting cells (APC; ref. 12). This process occurs in a MHC-restricted, antigen-specific manner and takes place at the immune synapse (13). Following the T cell-APC interaction, pMHC-T-cell receptor (TCR) complexes cluster at the cell-to-cell contact area and are internalized by lymphocytes. Other molecules, such as B7-1/2 and ICAM-1, are acquired in parallel with pMHC (14, 15). The mechanism underlying the acquisition of different molecules from APC into T cells was addressed by Hudrisier et al. (16). In their 2001 report, they showed that, following sustained TCR signaling, CTLs promptly capture target cell membranes from cells with which they interact. Transfer of cell membrane fragments from target to effector cell was termed "trogocytosis" and was shown to be TCR dependent (13, 16, 17). Trogocytosis forms the basis of an assay for T-cell recognition of APCs by protein transfer (18). Detection of antigen-specific T cells based on trogocytosis was shown in murine models and focused on APCs as target cells from which T cells acquire membranes (19). The membrane capture phenomenon has been described to occur between tumor cell lines and human bulk lymphocytes (20) and natural killer (NK) cells (21). Recently published reports used and supported the reliability of the trogocytosis phenomenon for detection of antigen-reactive CD8<sup>+</sup> T cells (18, 22).

In this report, we explore the membrane capture phenomenon in a human melanoma model. Our results show that CTLs capable of performing trogocytosis can be isolated and that they comprise a CD8<sup>+</sup> T-cell subset endowed with improved cytotoxic capacity. Thus, the phenomenon of trogocytosis can enable detection and isolation of functionally cytotoxic, tumor-specific CD8<sup>+</sup> T cells.

## Materials and Methods

**Mice and cell lines.** Male CD1<sup>nu/nu</sup> mice (8–12 wk old) were purchased from Harlan Laboratories. Mice were maintained under specific pathogen-free conditions. All experiments were conducted in accordance with Hadassah-Hebrew University Hospital Animal Facility and NIH guidelines. T2 is a TAP-2-deficient lymphoblastoid line of HLA-A2 genotype. The 624mel (HLA-A2<sup>+</sup>) melanoma cell line was provided by M. Parkhurst (Surgery Branch, NIH, Bethesda, MD). Melanoma cell lines M171, M181, and M579 (all HLA-A2<sup>-</sup>) were established in the Sharett Institute of Oncology/

**Requests for reprints:** Michal Lotem and Arthur Machlenkin, Sharett Institute of Oncology, Hadassah-Hebrew University Hospital, Jerusalem 91120, Israel. Phone: 972-2-6776781; Fax: 972-2-6422794; E-mail: mlotem@hadassah.org.il and arthurm@hadassah.org.il.

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Hadassah-Hebrew University Hospital laboratory as described (23). The M579-A2 clone is a stable HLA-A2 transfectant of M579 cells, established after transfection with pcDNA3-HLA-A2 plasmid (kind gift of P. Robbins, Surgery Branch, NIH). The expression of HLA-A2 was evaluated by fluorescence-activated cell sorting (FACS) using an HLA-A2-specific BB7.2 monoclonal antibody (mAb; AbD Serotec). The expression of MART-1 and gp100 was evaluated by immunostaining using A-103 and HMB-45 mAbs, respectively (Dako). All cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, and combined antibiotics. M579-A2 cells were maintained in the same medium supplemented with 1 mg/mL geneticin (Life Technologies). Human lymphocytes were cultured in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated human AB serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids, 25 mmol/L HEPES (pH 7.4), 50  $\mu$ mol/L  $\beta$ -mercaptoethanol, and combined antibiotics.

**Generation of peptide-reactive T-cell populations.** Generation of peptide-reactive T-cell populations from patients with metastatic melanoma was performed as previously described with minor modifications (24). gp100<sub>209-217(210M)</sub>, gp100<sub>154-162</sub>, gp100<sub>280-288(288V)</sub>, MART-1<sub>27-35(27L)</sub>, or MUC-1<sub>63-71</sub> peptides [all commercially synthesized and purified (>95%) by reverse-phase high-performance liquid chromatography by Biomer Technology] were used to stimulate HLA-A2<sup>+</sup> bulk melanoma patient-derived lymphocytes. Commercially available ELISA reagents (R&D Systems) were used to detect IFN- $\gamma$  secretion as an estimate for specific recognition of peptides by T cells following their coinoculation with peptide-pulsed T2 cells or HLA-A2<sup>+</sup> melanoma cells. The peptide-reactive cultures were expanded with 30 ng/mL ortho-anti-CD3 (eBioscience), recombinant interleukin-2 (IL-2; 300 IU/mL; Chiron B.V.), and  $5 \times 10^6$  allogeneic peripheral blood mononuclear cells (PBMC) as feeder cells.

**In vitro stimulation of melanoma-specific lymphocytes.** To enrich the tumor-reactive T-cell populations, cryopreserved PBMCs of melanoma patients that received autologous vaccine were thawed the day before stimulation and cultured in complete medium. Thereafter, the lymphocytes were plated in six-well plates ( $5 \times 10^6$  cells per well, 5 mL/well) in complete medium and stimulated by irradiated (17,000 rad) IFN- $\gamma$ -treated (50 units/mL; Prospec) autologous tumor cells ( $5 \times 10^5$  per well). Two days later, 300 IU/mL IL-2 was added and renewed every 2 d. In some experiments, CD8<sup>+</sup> lymphocytes were purified from thawed PBMC with magnetic beads (BD Biosciences) and then stimulated with autologous melanoma cells. On day 12, the enriched tumor-reactive T-cell cultures were harvested, washed in complete medium, and used in membrane capture assays.

**Membrane capture assays and flow cytometry.** T2 cells were pulsed with peptides (1  $\mu$ mol/L) in Opti-MEM (Life Technologies) for 1 h at 37°C and washed twice in PBS. Melanoma cells and peptide-pulsed T2 targets were labeled with a fluorescent lipophilic dye DiI (Molecular Probes) or surface biotinylated with biotin-7-NHS (Roche) according to the manufacturer's instructions with minor modifications. For DiI labeling,  $1 \times 10^6$ /mL target cells were suspended in 1  $\mu$ mol/L DiI in Opti-MEM and incubated for 5 min at 37°C and then for an additional 15 min at 4°C. For surface biotinylation, cells were suspended in buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 0.1 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub> in PBS) containing 1  $\mu$ g/mL biotin-7-NHS and incubated for 10 min at room temperature. In all cases, cold RPMI 1640 containing 10% AB serum was added to stop labeling (1:1 volume) for 10 min at 4°C and the cells were washed thrice with PBS containing 1% AB serum. Following resuspension in complete medium, labeled targets were cocultured with either peptide-reactive T-cell populations or melanoma-stimulated lymphocytes ( $5 \times 10^5$  per well in 200  $\mu$ L final volume) in U-shaped 96-well plates in E:T ratios ranging from 3:1 to 1:1. Following incubation for 1 to 5 h at 37°C, cells were washed twice in PBS containing 0.5 mmol/L EDTA, to ensure conjugate dissociation, resuspended in PBS supplemented with 0.5% bovine serum albumin and 0.1% sodium azide, and stained with FITC-conjugated or PE-Cy5-conjugated or APC-conjugated anti-human CD8 and/or CD4 (eBioscience), CD107a (BD PharMingen), or isotype control mAbs. For specific TCR staining, cells were incubated with FITC-conjugated HLA-A\*0201-MART-1<sub>26-35</sub> or HLA-A\*0201-gp100<sub>154-162</sub> dextramers (Dako) for 30 min at 4°C. The samples were washed

and analyzed by FACScan and "FCS Express" software (De Novo). Membrane capture by effector cells was measured by detection of DiI on the CD8<sup>+</sup>-gated population or by labeling with PE-Cy5-conjugated or PE-conjugated streptavidin (eBioscience) when biotinylated targets were used.

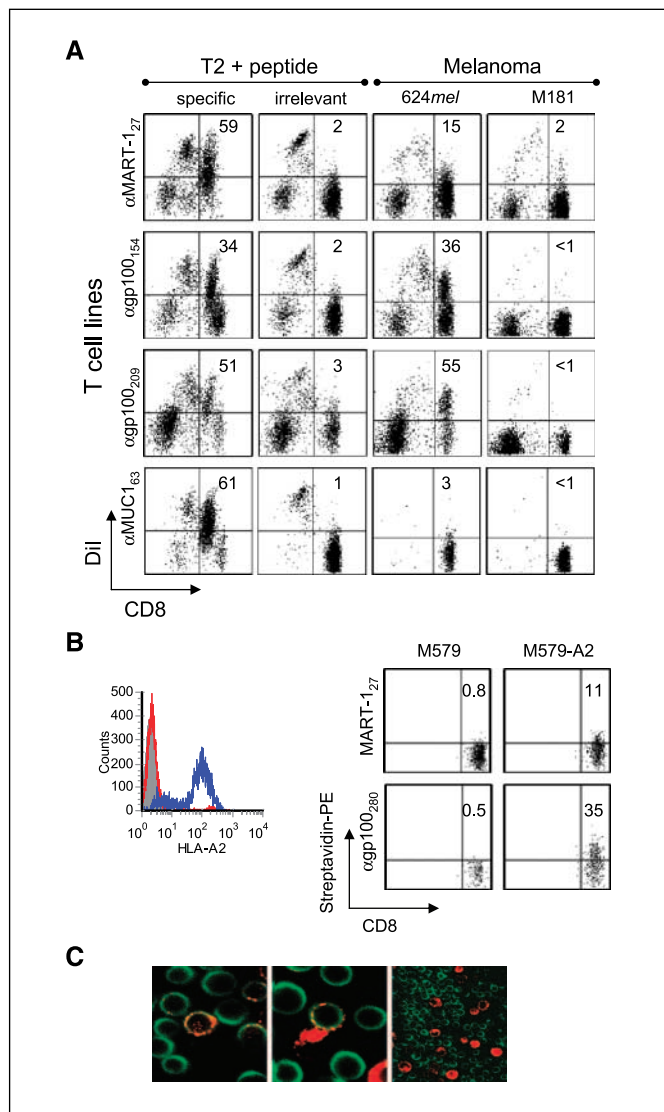
**Cell sorting and cytotoxicity assays.** Following coinoculation with irradiated DiI-labeled melanoma cells, DiI<sup>+</sup> (cap<sup>+</sup>) and DiI<sup>-</sup> (cap<sup>-</sup>) or bulk CD8<sup>+</sup> lymphocytes were sorted by FACS Aria (BD). When biotinylated targets were used, CD8<sup>+</sup> T cells were initially purified from autologous melanoma-stimulated bulk PBMC with EasySep CD8<sup>+</sup> negative selection kit (StemCell Technologies). Biotin<sup>+</sup> (cap<sup>+</sup>) and biotin<sup>-</sup> (cap<sup>-</sup>) CD8<sup>+</sup> lymphocytes were purified using positive selection with IMag streptavidin magnetic beads (BD Biosciences) according to the manufacturer's protocol. In both cases, purified cells were expanded *in vitro* with IL-2 and ortho-anti-CD3 and then examined for cytotoxic activity. *In vitro* cytotoxicity assays were performed as described (25). Briefly, lymphocytes were washed, resuspended in complete medium, and admixed at different ratios with 5,000 [<sup>35</sup>S]-methionine-labeled melanoma or peptide-pulsed T2 cells. CTL assays were done in U-shaped microtiter wells at 37°C, 5% CO<sub>2</sub> for 5 h. Percentage of specific lysis was calculated as follows: % lysis = (cpm in experimental well - cpm spontaneous release) / (cpm maximal release - cpm spontaneous release)  $\times$  100.

**Adoptive immunotherapy of CD1<sup>nu/nu</sup> mice bearing human melanoma.** Male CD1<sup>nu/nu</sup> mice were injected s.c. in the upper back with  $1 \times 10^6$  624mel cells admixed 1:1 with Matrigel (BD Biosciences). Five days later, mice were injected in the tumor area with  $1 \times 10^6$  cap<sup>+</sup>CD8<sup>+</sup> or cap<sup>-</sup>CD8<sup>+</sup> lymphocytes obtained after coinoculation of gp100<sub>154-162</sub>-reactive T cells and DiI-stained 624mel melanoma, sorting, and expansion as described above. Following T-cell transfer, mice were injected with 1,000 units IL-2 into the tumor site twice daily for 5 d. Survival of mice was monitored daily.

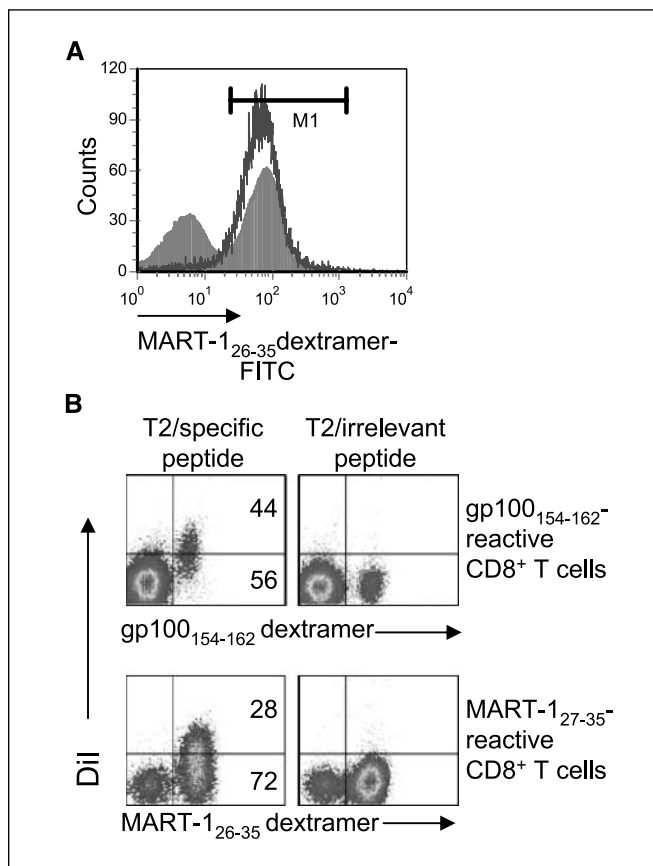
## Results

**Peptide-specific CD8<sup>+</sup> lymphocytes acquire membrane fragments from pulsed APCs and melanoma targets.** The antigen/TCR dependence of trogocytosis by CD8<sup>+</sup> lymphocytes has been shown in mouse models (18, 26). With the aim of studying the trogocytosis phenomenon in the context of human lymphocytes raised against melanoma-associated antigen/s, we made use of peptide-stimulated T cells. To this end, HLA-A\*0201 (A2<sup>+</sup>)-restricted T-cell populations recognizing gp100<sub>154-162</sub>, gp100<sub>209-217</sub>, gp100<sub>280-288</sub>, MART-1<sub>27-35</sub>, and melanoma-irrelevant MUC-1<sub>63-71</sub> peptides were generated from melanoma patient-derived PBMC using *in vitro* stimulations with antigen and IL-2, as described (24). Antigen-specific IFN- $\gamma$  secretion and cytotoxic activity of the populations were confirmed against cognate peptide-pulsed T2 cells and A2<sup>+</sup> melanoma cell lines (data not shown). To examine the ability of melanoma antigen-specific T cells to procure target cell membrane fragments, peptide-pulsed T2 and melanoma cells were labeled with lipophilic dye DiI and cocultured with the T cells. Trogocytosis was measured by detection of DiI on CD8<sup>+</sup> T cells using flow cytometry. CD8<sup>+</sup> T cells acquired DiI following coinoculation with the cognate but not with irrelevant peptide-pulsed T2 targets (Fig. 1A). The percentages of DiI<sup>+</sup>CD8<sup>+</sup> membrane-capturing (cap<sup>+</sup>) lymphocytes among the total CD8<sup>+</sup> population ranged from 34% to 61%, whereas nonspecific DiI staining was <3%. We next examined whether peptide-specific T cells can capture membrane fragments through binding with endogenously derived pMHC complexes on the surface of tumor cells. To this end, the lymphocytes were cocultured (1–18 h) with DiI-stained or biotinylated A2<sup>+</sup> and A2<sup>-</sup> melanoma targets followed by detection of the captured target membranes on the CD8<sup>+</sup> T cells. As shown in Fig. 1A, membrane capture by gp100-reactive and

MART-1-reactive lymphocytes was clearly detectable when 624mel melanoma cells (A2<sup>+</sup>, gp100<sup>+</sup>, and MART-1<sup>+</sup>) were used as targets. The A2<sup>-</sup> melanoma line M181 (A2<sup>-</sup>, gp100<sup>+</sup>, and MART-1<sup>+</sup>) and the melanoma-irrelevant anti-MUC-1 CTLs revealed only background levels of trogocytosis. The A2 transfectant of melanoma line M579 (MART-1<sup>+</sup>/gp100<sup>+</sup>) was used to emphasize the HLA restriction of trogocytosis (Fig. 1B). Eleven percent and 35% of MART-1<sub>27-35</sub>-



**Figure 1.** Membrane capture by antigen-specific T-cell populations from peptide-pulsed and melanoma targets. **A**, MART-1<sub>27-35</sub>-reactive, gp100<sub>154-162</sub>-reactive, gp100<sub>209-217</sub>-reactive, and MUC-1<sub>63-71</sub>-reactive T-cell populations were incubated with Dil-labeled, specific, or irrelevant peptide-pulsed T2 cells or with 624mel (A2<sup>+</sup>) and M181 (A2<sup>-</sup>) melanomas (see Materials and Methods). Numbers, percentage of Dil<sup>+</sup>CD8<sup>+</sup> T cells. **B**, left, HLA-A2 expression in human melanoma cell lines. M579 melanoma (red curve) and M579-A2 transfectant (blue curve) were stained with BB7.2 mAb followed by goat anti-mouse FITC and monitored by FACScan. Filled curve, background staining with the secondary antibody. Right, MART-1<sub>27-35</sub>-reactive and gp100<sub>280-288</sub>-reactive T-cell populations were incubated with biotinylated M579 (left) or M579-A2 (right) melanomas. Biotin capture by CD8<sup>+</sup> T cells was analyzed by flow cytometry following concomitant staining with streptavidin-PE and APC-conjugated anti-CD8 mAb. Numbers in top right quadrant, percentage of biotin<sup>+</sup>CD8<sup>+</sup> lymphocytes among total CD8<sup>+</sup> population. **C**, membrane capture was analyzed by confocal microscopy after staining of MART-1<sub>27-35</sub>-reactive (left and middle) or MUC-1<sub>63-71</sub>-reactive (right) CD8<sup>+</sup> T cells (green) that were incubated with Dil-labeled 624mel melanoma (red).



**Figure 2.** TCR staining of membrane-capturing and noncapturing CD8<sup>+</sup> T-cell populations. gp100<sub>154-162</sub>-reactive and MART-1<sub>27-35</sub>-reactive T-cell populations were concomitantly stained with PE-Cy5-conjugated anti-human CD8 and FITC-conjugated HLA-A\*0201-MART-1<sub>26-35</sub> or HLA-A\*0201-gp100<sub>154-162</sub> dextrans following coincubation with Dil-labeled cognate or irrelevant peptide-pulsed T2 cells. Lymphocytes were identified by forward and side scatter and gated for CD8<sup>+</sup>. **A**, expression of HLA-A\*0201-MART-1<sub>26-35</sub>-specific TCR on membrane-capturing (empty curve) and noncapturing (filled curve) CD8<sup>+</sup>-gated cells. **B**, dextramer staining of CD8<sup>+</sup> cells plotted against Dil staining. Numbers in top right and bottom right quadrant, percentage of CD8<sup>+</sup>dextramer<sup>+</sup> cells that are Dil<sup>+</sup> (membrane capturing) and Dil<sup>-</sup> (noncapturing), respectively.

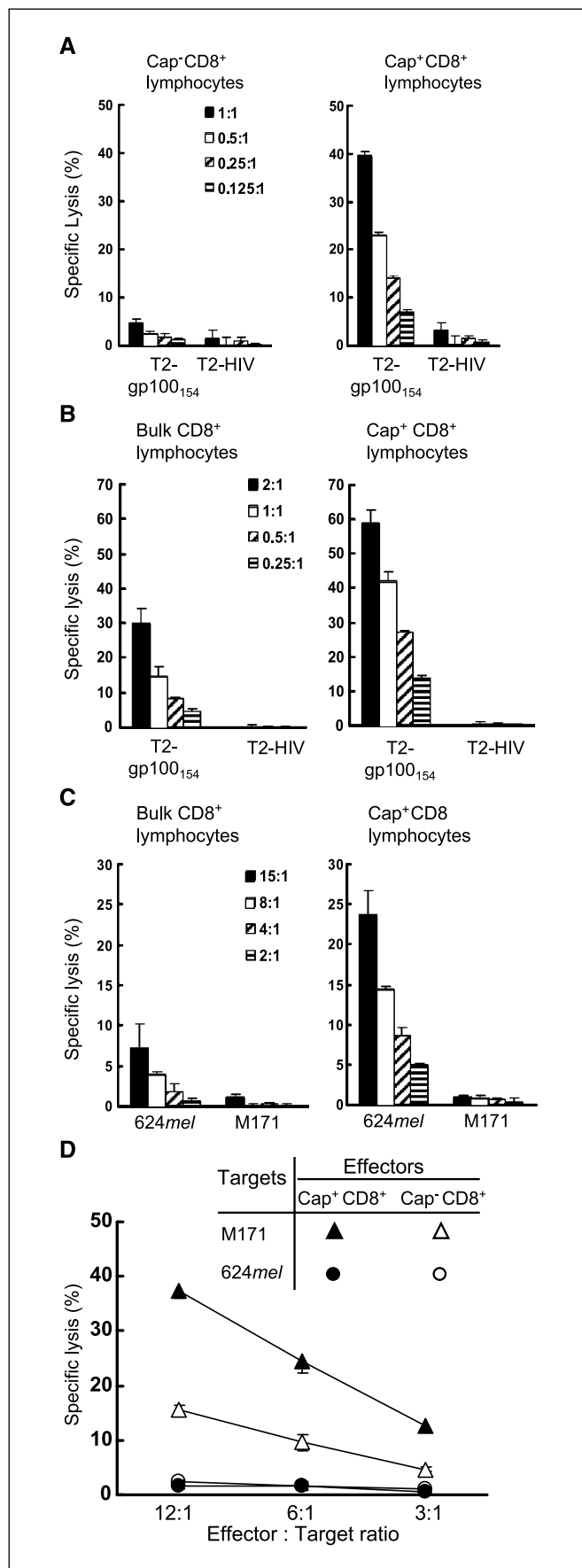
specific and gp100<sub>280-288</sub>-specific CD8<sup>+</sup> T cells procured membrane fragments from M579-A2<sup>+</sup> but not from the A2<sup>-</sup> parental M579 melanoma, excluding the effect of nonspecific target-related factors.

Antigen-dependent trogocytosis was evident after 15 min of incubation, peaked by 1 h, and remained unchanged during the following 1 to 5 h. Long-term incubation (>5 h) was associated with an increase of nonspecific dye acquisition (data not shown). By adding CD4<sup>+</sup> autologous T cells to the CD8<sup>+</sup> anti-MART-1<sub>27-35</sub> T-cell population, we have excluded “bystander” tagging of uninvolved T cells scavenging dye or membrane fragments (data not shown). Membrane capture from labeled 624mel by MART-1<sub>27-35</sub>-specific CD8<sup>+</sup> T cells was visualized by confocal microscopy (Fig. 1C, left and middle). Captured membrane fragments appear as single spots or tightly clustered regions on cap<sup>+</sup> lymphocytes. Consistent with flow cytometry results, there was no evidence of membrane capture by irrelevant T cells (Fig. 1C, right). Thus, these data show that human CD8<sup>+</sup> lymphocytes capture membrane fragments from melanoma cells expressing naturally processed antigens in peptide-specific and MHC-restricted manner.

**Trogocytosis is performed by a subset of CTLs derived from a population with identical TCR specificity.** The T-cell populations we used in these experiments were generated through repeated stimulations of bulk PBMCs with peptides, a process that increases the frequency of peptide-reactive T cells but does not yield a purified single clone. We assumed that the ability to perform trogocytosis would be conferred to all T cells with the same TCR and thus would separate peptide-specific from other T cells. pMHC dextramers were used for direct assessment of the TCR specificity of membrane-capturing T cells. The MART-1<sub>27-35</sub>-reactive and gp100<sub>154-162</sub>-reactive CD8<sup>+</sup> lymphocytes were analyzed according to their membrane capture capacity following coincubation with peptide-pulsed, DiI-stained T2 targets. As expected, all trogocytosis-performing CD8<sup>+</sup> T cells were dextramer<sup>+</sup>, whereas nearly all dextramer<sup>-</sup> lymphocytes were unable to trogocytose, attesting that TCR recognition is mandatory for this phenomenon. Surprisingly, however, 58% of the noncapturing (cap<sup>-</sup>) population consisted of dextramer<sup>+</sup> T cells (Fig. 2A). As shown in Fig. 2B, only 28% of the MART-1<sub>26-35</sub>-dextramer<sup>+</sup> and 44% of the gp100<sub>154-162</sub>-dextramer<sup>+</sup> CD8<sup>+</sup> T cells were membrane-capturing cells. This shows that TCR-specific recognition is an imperative factor but is not the sole determinant of membrane capture capacity of CD8<sup>+</sup> T cells.

**Trogocytosis is a feature of highly avid, functionally cytotoxic T cells.** The functional distinction of cap<sup>+</sup> and cap<sup>-</sup> T cells was addressed in the next set of experiments. cap<sup>+</sup> T cells were compared with cap<sup>-</sup> or bulk CD8<sup>+</sup> T cells in cytotoxicity assays against peptide-pulsed and melanoma targets. Peptide-reactive T-cell populations and PBMC of melanoma patients who previously participated in an adjuvant protocol of melanoma cell vaccination were used in these experiments (27). To increase tumor-specific T-cell frequencies, 12-day *in vitro* stimulation with autologous melanoma preceded T-cell evaluation. gp100<sub>154-162</sub>-reactive CD8<sup>+</sup> lymphocytes were coincubated with irradiated DiI-labeled 624mel cells and sorted by FACS into distinct fractions according to their membrane capture ability. The results of [<sup>35</sup>S]methionine release assay show that cytotoxicity was exclusively conferred to cap<sup>+</sup> lymphocytes with 40% specific lysis at 1:1 E:T ratio (Fig. 3A). cap<sup>-</sup>CD8<sup>+</sup> T cells failed to lyse specific peptide-pulsed T2 targets, yielding low reactivity similar to that of irrelevant effectors (Fig. 3A; data not shown). The improved reactivity of cap<sup>+</sup>CD8<sup>+</sup> cells was reproduced when compared with bulk CD8<sup>+</sup> T cells. Following sorting and *in vitro* expansion, as described above, the membrane-capturing and bulk lymphocytes were assayed against peptide-pulsed targets and 624mel (A2<sup>+</sup>) or M171 (A2<sup>-</sup>) melanomas. As shown in Fig. 3B, cap<sup>+</sup>CD8<sup>+</sup> T cells

**Figure 3.** Cytotoxic activity of membrane-capturing (cap<sup>+</sup>), noncapturing (cap<sup>-</sup>), or bulk CD8<sup>+</sup> T cells against peptide-pulsed targets and melanoma cell lines. gp100<sub>154-162</sub>-reactive CD8<sup>+</sup> T cells were coincubated with DiI-labeled 624mel melanoma, sorted into cap<sup>+</sup> and cap<sup>-</sup> fractions, and expanded *in vitro*. CTL assays were done with <sup>35</sup>S-labeled specific (gp100<sub>154-162</sub>) or irrelevant (HIV derived) peptide-pulsed T2 cells (A and B) or with A2<sup>+</sup> 624mel and A2<sup>-</sup> M171 melanoma cells (C) as targets, as described (25). Cytotoxic activity of cap<sup>+</sup> versus cap<sup>-</sup> cells (A) or of cap<sup>+</sup> versus bulk CD8<sup>+</sup> cells (B and C). D, melanoma patient-derived CD8<sup>+</sup> T cells were coincubated with biotinylated autologous melanoma cells followed by separation into cap<sup>+</sup> and cap<sup>-</sup> fractions by streptavidin-coated magnetic beads (see Materials and Methods). CTL assays were done with <sup>35</sup>S-labeled autologous (M171) and HLA-mismatched 624mel melanomas as specific and nonspecific targets. Points, mean percentage of target lysis at E:T ratios of 1:1 to 0.125:1 (A), 2:1 to 0.25:1 (B), 15:1 to 2:1 (C), and 12:1 to 3:1 (D; n = 3); bars, SD.



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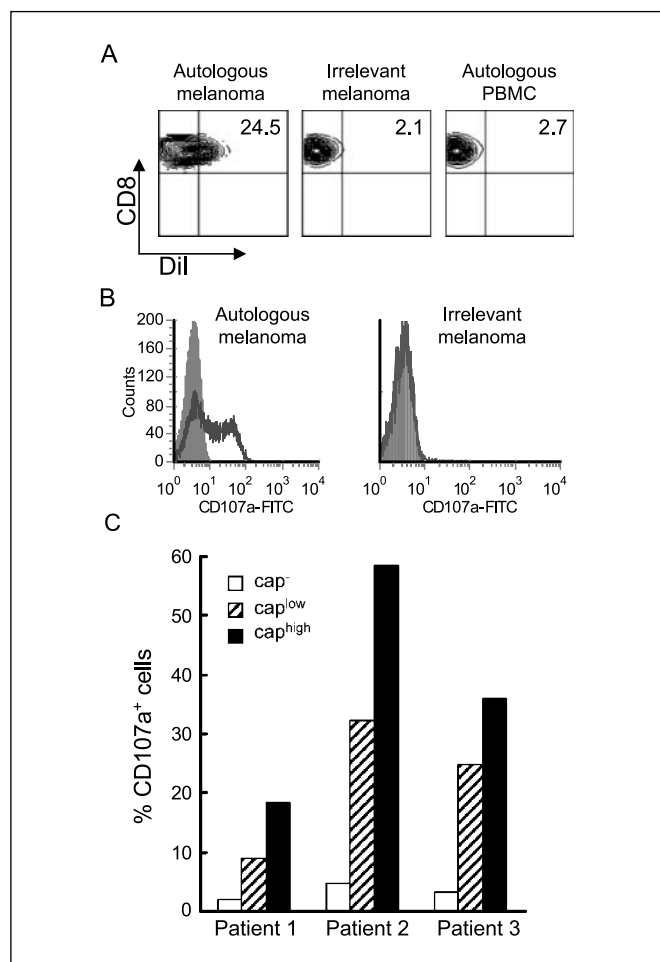
exhibited 2- to 3-fold higher lysis of cognate peptide-pulsed targets than unsorted lymphocytes. Moreover, the enriched lymphocytes were even more efficient (4- to 5-fold enhanced cytotoxic activity compared with bulk CD8<sup>+</sup> cells) when 624*mel* melanoma cells were used as targets (Fig. 3C).

At this point, it was evident that trogocytosis is a functional marker that can potentially be used for detection and isolation of high-avidity CTLs from patient-derived PBMC containing low frequencies of tumor-specific lymphocytes. To address this issue, bulk PBMCs were enriched for CD8<sup>+</sup> T cells using negative selection with magnetic beads followed by 12-day stimulation with autologous melanoma, as described in Materials and Methods. Due to the low frequency of tumor-specific T cells in stimulated bulk CD8<sup>+</sup> lymphocytes, we used the streptavidin-based detection method of trogocytosis. According to our results (data not shown) and to observations made by others (26), tagging of target membrane with biotin followed by its detection with streptavidin is more sensitive than DiI. With this system, melanoma patient-derived CD8<sup>+</sup> T cells were separated into cap<sup>+</sup> (biotin<sup>+</sup>) and cap<sup>-</sup> fractions after coincubation with biotinylated autologous M171 melanoma. The lymphocytes were then expanded *in vitro* and their cytotoxic activity against autologous melanoma was examined. As shown in Fig. 3D, cap<sup>+</sup> CTLs lysed autologous M171 but not HLA-mismatched 624*mel* melanoma cells in a dose-dependent manner. Compared with cap<sup>-</sup>CD8<sup>+</sup>, the cap<sup>+</sup>CD8<sup>+</sup> cells were 2- to 3-fold more effective at destroying the autologous melanoma (Fig. 3D). Taken together, these data show that CD8<sup>+</sup> T cells capable of trogocytosis contain high-avidity antitumor lymphocytes that exhibit enhanced cytotoxic activity against tumor cell lines and autologous melanoma.

**Membrane capture capacity of CTLs correlates with their activation-induced degranulation.** CD107a is a membrane glycoprotein of cytotoxic granules; its surface expression on CTLs correlates with degranulation and has been used as a surrogate marker for their cytolytic activity (7). We measured CD107a mobilization and membrane capture by melanoma patient-derived T cells following 5-h coincubation with DiI-labeled tumor cells. Membrane capture paralleled CD107a expression when both autologous M171 tumor (specific targets) and HLA-mismatched 624*mel* melanoma or autologous PBMCs (negative controls) were used (Fig. 4A and B). To correlate membrane capture with cytolytic activity of CTLs, CD8<sup>+</sup> T cells from three melanoma patients were incubated with labeled autologous tumor cells and concomitantly analyzed for DiI acquisition and CD107a mobilization. Based on DiI intensity, the lymphocytes were divided into three subsets (cap<sup>-</sup>, cap<sup>low</sup>, and cap<sup>high</sup>) and plotted against the corresponding CD107a expression. Supporting our data from CTL assays (Fig. 3), the vast majority of cytolytic T lymphocytes were detected in the membrane-capturing population. Furthermore, the percentage of CD107a<sup>+</sup> cells was 1.4- to 2-fold higher in cap<sup>high</sup> than in cap<sup>low</sup> CD8<sup>+</sup> T cells (Fig. 4C). More than 95% of noncapturing lymphocytes were CD107a<sup>-</sup>. Thus, the reliability of membrane capture as a measure of functional cytotoxicity is supported by CD107a mobilization results.

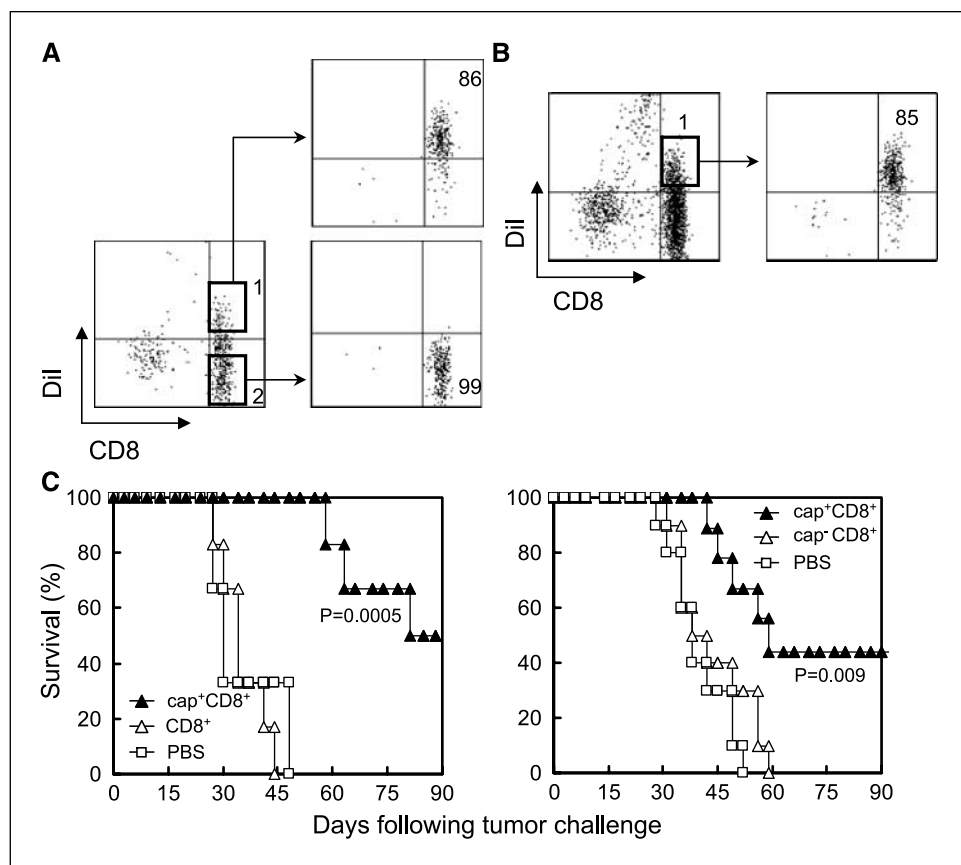
**Trogocytosis-performing CD8<sup>+</sup> T cells inhibit *in vivo* melanoma growth.** Finally, we tested the *in vivo* antitumor activity of membrane-capturing CD8<sup>+</sup> T cells in two experimental settings: compared with noncapturing cells (Fig. 5A) and with bulk CD8<sup>+</sup> lymphocytes (Fig. 5B). To this end, we established a model of adoptive transfer of human lymphocytes into nude mice bearing human melanoma. Testing different melanoma cell lines, we

found that 624*mel* cells at  $1 \times 10^6$  admixed with Matrigel formed homogenous s.c. tumors in 100% of nude mice. The adoptive transfer of CD8<sup>+</sup> T cells was carried out basically according to the previously proposed experimental scheme (25). Consistent with this scheme, nude mice were challenged s.c. with 624*mel* cells, and 5 days later, after formation of visible tumors, CD8<sup>+</sup> T cells were injected around the tumors followed by local s.c. administration of IL-2 twice daily for 5 more days. gp100<sub>154-162</sub>-reactive CD8<sup>+</sup> T-cell populations were separated based on membrane capture from labeled 624*mel* melanoma cells. Separated or bulk CD8<sup>+</sup> T cells were expanded *in vitro* as described and transferred to nude mice challenged with 624*mel* cells. Fifty percent (three of six) and



**Figure 4.** Analysis of membrane capture and CD107a mobilization in CD8<sup>+</sup> T cells of melanoma patients. A to C, melanoma patient-derived CD8<sup>+</sup> T cells were coincubated for 5 h with DiI-labeled autologous or irrelevant melanomas or autologous CD8<sup>-</sup> PBMC and then stained with PE-Cy5-conjugated anti-CD8 and FITC-conjugated anti-CD107a mAb and analyzed by flow cytometry. Lymphocytes were identified by forward and side scatter and gated on CD8<sup>+</sup> cells. A, numbers, percentage of DiI<sup>+</sup>CD8<sup>+</sup> lymphocytes among the total CD8<sup>+</sup> population. B, (empty curve), CD107a expression on CD8<sup>+</sup> lymphocytes following incubation with autologous M171 or irrelevant 624*mel* melanoma. Filled curve, background staining with the FITC-isotype mAb. C, concomitant analysis of membrane capture and CD107a mobilization by CD8<sup>+</sup> T cells from three melanoma patients after incubation with autologous melanoma. For each patient, CD8<sup>+</sup>-gated lymphocytes were subdivided into cap<sup>-</sup> (white columns), cap<sup>low</sup> (grid columns), and cap<sup>high</sup> (black columns) subsets and plotted against percentage of CD107a<sup>+</sup> cells. CD8<sup>+</sup> lymphocytes were defined as cap<sup>-</sup> based on DiI capture from labeled autologous PBMC. Based on DiI intensity, membrane-capturing CD8<sup>+</sup> lymphocytes were arbitrarily divided into cap<sup>low</sup> and cap<sup>high</sup> subsets.

**Figure 5.** Adoptive cell transfer of cap<sup>+</sup> T cells confers prolonged survival of 624mel melanoma-challenged mice. *A* and *B*, gp100<sub>154-162</sub>-reactive CD8<sup>+</sup> T cells were coincubated with Dil-labeled 624mel melanoma cells and sorted by FACS. Dil staining is plotted against CD8. Cells within enumerated boxes were sorted into cap<sup>+</sup> (*A* and *B*) and cap<sup>-</sup> (*A*) populations of CD8<sup>+</sup> T cells. *Numbers*, percentage of Dil<sup>+</sup>CD8<sup>+</sup> or Dil<sup>-</sup>CD8<sup>+</sup> cells following sorting. The sorted CD8<sup>+</sup> T cells were injected to nude mice challenged with 624mel melanoma (see Materials and Methods). *C*, survival curves of mice treated with cap<sup>+</sup> (filled triangles) or bulk CD8<sup>+</sup> T (empty triangles) cells (*n* = 6 per treatment group). *D*, survival data of mice treated with cap<sup>+</sup> (filled triangles) or cap<sup>-</sup> (empty triangles) CD8<sup>+</sup> T cells (*n* = 9–10). Control groups received PBS (empty squares), *n* = 6 (*C*) and *n* = 10 (*D*). Statistical significance was determined by the log-rank test analyzing survival of mice treated by cap<sup>+</sup>CD8<sup>+</sup> T cells in relation to other groups as follows: treated with bulk CD8<sup>+</sup> (*P* = 0.0005; *C*) and treated with cap<sup>-</sup>CD8<sup>+</sup> (*P* = 0.009; *D*).



44% (four of nine) of mice were alive at 90 days following transfer of cap<sup>+</sup> T cells compared with none in mice treated with bulk or cap<sup>-</sup>CD8<sup>+</sup> T cells, respectively (Fig. 5C and D). Therefore, only those CD8<sup>+</sup> T cells that capture membrane fragments from tumor targets inhibit melanoma growth *in vivo*.

## Discussion

This is the first study to elucidate the phenomenon of trogocytosis in the context of human melanoma-CD8<sup>+</sup> T-cell interactions. Our results show that membrane capture by CD8<sup>+</sup> lymphocytes is a signature of tumor antigen-specific T cells of the highest functional cytotoxicity. The immediate implication of the membrane capture phenomenon is that it opens a new option to identify and expand high-avidity antitumor CTLs. Various reports have described the membrane capture phenomenon in murine experimental systems *in vitro* (14, 15, 19, 28) and *in vivo* (29). In contrast, only a few cases have been reported for human T cells in the context of cell-to-cell interaction with human tumor cells. Acquisition of membrane patches from hematopoietic tumor cells has been reported for human NK and bulk CD4, CD8  $\alpha\beta$ , and  $\gamma\delta$  T cells (20, 30). To the best of our knowledge, the results presented here provide new evidence for antigen-specific transfer of labeled membrane patches from unmodified melanoma targets to cognate CD8<sup>+</sup> T cells (Figs. 1 and 4). To show this, we used T-cell populations of known specificity paired with HLA-matched tumor lines or patient's bulk PBMC paired with the autologous tumor. The HLA restriction of the capture by CD8<sup>+</sup> T cells was strongly confirmed using HLA-A2<sup>+</sup> transfectant of M579 cell line versus parental M579

melanoma (Fig. 1B). Our results are in line with murine studies showing TCR-MHC dependency of antigen-specific trogocytosis of peptide-pulsed targets (16, 26). Furthermore, the TCR molecule is directly involved in the process of trogocytosis (12). As was shown for other receptors of the tyrosine kinase receptor family, TCR is internalized following the linkage to its pMHC ligand (16, 31). Our results show decreased staining intensity of CD8 on membrane-capturing T cells, suggesting TCR internalization as a consequence of trogocytosis (Fig. 1A). Using multimer (dextramer) staining of melanoma antigen-specific T-cell populations, we further explored the relationship of TCR specificity to the trogocytosis phenomenon. Our data revealed that, although TCR specificity is vital for trogocytosis, it does not exclusively determine the ability of CTL to acquire membrane fragments (Fig. 2). T cells with the same TCR specificity are not necessarily identical in their overall functional avidity and, as a result, antigen reactivity (32). The relatively low TCR affinity and/or functional avidity could account for the lack of measurable trogocytosis by melanoma antigen-specific CD8<sup>+</sup> T-cell subsets.

TCR ligation is one component in the wider context of the immunologic synapse, occurring between an immune effector cell and its target. The "synapse" consists of an attachment zone between the two cellular parties where clusters of molecules gather to increase the likelihood of receptor-ligand interactions (33, 34). Assuming that the magnitude of trogocytosis will reflect the strength of the immunologic synapse, we looked for functional correlations of trogocytosis-performing and nonperforming CD8<sup>+</sup> T cells. Our results show a strong connection between the trogocytosis ability of CD8<sup>+</sup> T cells and their cytotoxic activity

against peptide-pulsed APC and, more importantly, unmodified tumor targets (Figs. 3 and 4). Recently, increased cytotoxic activity of trogocytosis-capable murine lymphocytes has been reported (19). In that study, ovalbumin-primed lymphocytes were sorted based on their trogocytosis activity against OVA<sub>257-264</sub>-pulsed APCs and then assayed against OVA<sub>257-264</sub>-pulsed EL4 targets. In contrast to this experimental system, we used human tumor cell lines and/or autologous melanoma for both lymphocyte sorting and as targets in cytotoxicity assays. The important hallmark of high-avidity functional CD8<sup>+</sup> T cells is their reactivity against tumor cells presenting endogenous antigens, additionally to peptide-pulsed artificial APCs (7). Our data illustrate increased cytotoxic activity of trogocytosis-capable CD8<sup>+</sup> T cells against tumor cell lines and autologous melanoma (Fig. 3). A use of melanoma cells for trogocytosis-based sorting of polyclonal tumor-specific lymphocytes represents a significant advantage over using peptide-pulsed APCs. The functional cytotoxicity of T cells capable of membrane capture was shown by direct cytotoxicity assays and was supported with the marker of degranulation, CD107a. This marker was extremely useful to detect lower-frequency, tumor-specific CD8<sup>+</sup> T cells within bulk PBMC of melanoma patients. The observed reduced killing of tumor compared with artificial APC by trogocytosis-capable effectors could be attributed to the apparently lower number of pMHC complexes presented on melanoma tumor cells versus peptide-pulsed APC (35). Alternatively, tumors can resist TCR-mediated lysis by impairment of antigen presentation or through the aberrant expression of PD-L1, a ligand of PD-1 receptor on CTLs, which normally inhibits TCR signaling (36, 37). Selection of cap<sup>+</sup> T cells by their ability to capture membranes from a tumor

target enables the demarcation of T cells that overcome tumor evasiveness.

Finally, to test the *in vivo* potential of CD8<sup>+</sup> T cells capable of trogocytosis, we used an adoptive transfer model (38) in which human melanoma-bearing mice were treated by lymphocytes transferred to the tumor vicinity. Our results clearly show that trogocytosing T cells confers the best inhibition of tumor growth compared with any other population tested (Fig. 5). This is the first evidence for the antitumor activity of T cells capable of membrane capture in the tumor-bearing host. This peritumoral adoptive cell transfer approach paves the road for the implementation of trogocytosis in systemic adoptive cell immunotherapy, currently under way in our laboratory.

In conclusion, we show that antigen-specific membrane capture by CD8<sup>+</sup> T cells is a feature of highly functional CTLs. Polyclonal tumor-reactive CTLs can be isolated from bulk lymphocytes using tumor targets expressing natural antigen levels. T cells with membrane capture ability that gain their functional signature from unmodified tumor cells *in vitro* may eventually mediate tumor regression *in vivo*. Understanding the mechanism of trogocytosis between CD8<sup>+</sup> T cells and tumor cells will benefit the future design of adoptive cell therapy of cancer.

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