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Trogocytosis Is a Gateway to Characterize Functional Diversity in Melanoma-Specific CD8+ T Cell Clones

Ronny Uzana,* Galit Eisenberg,* Yael Sagi,† Shoshana Frankenburg,* Sharon Merims,* Ninette Amariglio,§ Eitan Yefenof,§ Tamar Peretz,* Arthur Machlenkin,*,† and Michal Lotem*,†

Trogocytosis, the transfer of membrane patches from target to immune effector cells, is a signature of tumor–T cell interaction. In this study, we used the trogocytosis phenomenon to study functional diversity within tumor-specific T cell clones with identical TCR specificity. MART-126–35-specific CD8+ T cell clones, which differed in their trogocytosis capacity (low [2D11], intermediate [2G1], high [2E2]), were generated from melanoma patients. Functional evaluation of the clones showed that the percentage of trogocytosis-capable T cells closely paralleled each clone’s IFN-γ and TNF-α production, lysosome degranulation, and lysis of peptide-pulsed targets and unmodified melanoma. The highly cytotoxic 2E2 clone displayed the highest TCR peptide binding affinity, whereas the low-activity 2D11 clone showed TCR binding to peptide-MHC in a CD8-dependent manner. TCR analysis revealed VB16 for clones 2E2 and 2G1 and VB14 for 2D11. When peptide-affinity differences were bypassed by nonspecific TCR stimulation, clones 2E2 and 2D11 still manifested distinctive signaling patterns. The high-activity 2E2 clone displayed prolonged phosphorylation of ribosomal protein S6, an integrator of MAPK and AKT activation, whereas the low-activity 2D11 clone generated shorter and weaker phosphorylation. Screening the two clones with identical TCR VB by immunoreceptor array showed higher phosphorylation of NK, T, and B cell Ag (NTB-A), a SLAM family homophilic receptor, in clone 2E2 compared with 2G1. Specific blocking of NTB-A on APCs markedly reduced cytokine production by CD8 lymphocytes, pointing to a possible contribution of NTB-A costimulation to T cell functional diversity. This finding identifies NTB-A as a potential target for improving anti-cancer immunotherapy.

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Cytotoxic CD8+ T cells specific for a single antigenic epitope can originate from one or several progenitors, distinguishable by their TCR sequences (1). The structure of the TCR is the major determinant of T cell functional robustness, specifying its binding affinity to a cognate peptide presented by an appropriate MHC class I molecule (2). In vivo, many other factors affect the actual cytotoxic capacity of T cells, including their state of differentiation, shifting from naive cells to terminally differentiated effectors, length of activation (3), cytokine milieu (4), and costimulation (5). Hence, T cell competence reflects the overall outcome of all these variables.

Recently, it has been acknowledged that the initial encounter between a naive CD8+ T cell with its cognate peptide-MHC (pMHC) not only determines the activation capacity of the former, but also that of its clonal progeny (6). Short and loose interactions yield subclones of low potency whereas long and tight contact results in highly reactive T cells, thus leading to clonal diversity (2, 7). Although the mechanism underlying the functional imprint of T cells is not yet fully elucidated, it is clear that in addition to TCR affinity, other interactions take place at the initial immunological synapse, which shape T cell functional capacity. Immunological synapse refers to the focal contact between an immune effector cell and APCs. It is a contact-secretion process that leads to effector cell activation by reciprocal molecular clustering of ligands and receptors on the cytoplasmic membranes of the cellular partners involved (8, 9). The linkage between receptors and ligands leads to the unidirectional transfer of membrane fragments from APCs to effector cells, a process termed “trogocytosis” (10–12). It is readily visible by labeling Ag-loaded APCs with a membrane dye that can be detected on cognate T cells by immunofluorescence (13). Thus, trogocytosis provides a useful signature for T cells that have interacted with APCs.

We have previously shown that trogocytosis also occurs between tumor-reactive CD8+ T cells and melanoma target cells (14), and that it is preferentially associated with a highly cytotoxic T cell population. Notably, TCR specificity was not the only determinant of trogocytosis, as a proportion of Ag-specific T cells with the same TCR specificity did not capture target membranes. In the present study, we aim to elucidate the reasons for the functional diversity within CD8+ T cells with the same TCR specificity.

Materials and Methods

Tumor cell lines and lymphocyte cultures

Melanoma cell line M171 (HLA-A27/MART-1+) was established in the Sharet Institute of Oncology, Hadassah Medical Organization (Jerusalem, Israel; †Division of Oncology, School of Medicine, Stanford University, Stanford, CA 94305; ‡Cancer Research Center, Chaim Sheba Medical Center, Ramat Gan 52621, Israel; and †Lautenberg Center for General and Tumor Immunology, Hebrew University of Jerusalem, Jerusalem 91120, Israel)

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The online version of this article contains supplemental material.

Abbreviations used in this article: BD, Becton Dickinson; LCK, lymphocyte-specific protein tyrosine kinase; NTB-A, NK, T, and B cell Ag; PLC, phospholipase C; pMHC, peptide-MHC; rHLIL2, recombinant human IL-2; SLP-76, Src homology 2 domain-containing leukocyte protein of 76 kDa; TIL, tumor-infiltrating lymphocyte.

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Israel) (15). 62 µmol (HLA-A2+/MART-1+) was a gift of M. Parkhurst (Surgery Branch, National Institutes of Health, Bethesda, MD). T2 is a TAP-2–deficient lymphoblastoid line of HLA-A2 genotype. All cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mmol/l L-glutamine, and combined antibiotics. Tumor-infiltrating lymphocyte (TIL) microcultures were initiated and expanded from tumor specimens taken from resected metastases of melanoma patients, as described (16). Human lymphocytes were cultured in complete medium containing 10% FBS supplemented with 10% heat-inactivated human AB serum, 6000 IU/ml recombinant human IL-2 (rhIL-2; Chiron, Amsterdam, The Netherlands), 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 1% nonessential amino acids, 25 mmol/l HEPES (pH 7.4), 5.0 mmol/l 2-ME, and combined antibiotics (all from Invitrogen Life Technologies). The study was approved by the Institutional Review Board, and all patients gave their informed consent prior to initiation of lymphocyte and melanoma cell cultures.

Cloning of peptide-specific TIL

On day 14 of TIL initiation, the lymphocytes were washed with PBS, resuspended in PBS supplemented with 0.5% BSA, and stained with FITC-conjugated HLA-A*0201/MART-1b63 dextramer (Immudex, Copenhagen, Denmark) for 30 min at 4°C. The lymphocytes were then incubated with allophycocyanin-conjugated mouse anti-human CD8 (eBioscience) for an additional 30 min at 4°C and washed. CD8+ lymphocytes, positively stained in the dextramer “TIL” cells, were sorted by a Becton Dickinson (BD) FACS Aria and directly cloned at one or two cells per well in 96-well plates in the presence of 30 ng/ml Ortho anti-CD3 (eBiotech), 6000 IU/ml rhIL-2, and 4 Gy-irradiated 5 × 10⁴ allogeneic PBMCs as feeder cells. Five days later, 6000 IU/ml rhIL-2 was added and renewed every 2 days thereafter. On day 14, the clones were assayed for IFN-γ secretion in a peptide-specific manner following their coincubation with MART-1b63–pulsed T2 cells (commercially synthesized and purified [>95%] by reverse-phase HPLC by Biomer Technology) using commercially available ELISA reagents (R&D Systems, Minneapolis, MN). The MART-1b63–reactive clones were further expanded in a second-round exposure to 30 ng/ml Ortho anti-CD3 and 6000 IU/ml rhIL-2 in the presence of 50-fold excess irradiated feeder cells.

Trogocytosis assay and flow cytometry

T2 cells were pulsed with A2-restricted peptides MART-1b63-35, or HIV-1 Gag p24g11–23 for negative control, 1 µmol/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 either 1) labeled with PKH26 (Sigma-Aldrich) according to the manufacturer’s protocol or 2) surface bio- tinylated with N-hydroxysuccimide ester (biotin-7-NHS; Roche, Mannheim, Germany) according to the manufacturer’s instructions with minor modifications. Briefly, after being suspended in buffer (20 mM HEPES, 150 mM NaCl, 0.1 mM CaCl₂, and 1 mM MgCl₂ in PBS) containing 1 µg/ml biotin-7-NHS (Roche) and incubated for 10 min at room temperature, 200 µg/ml streptavidin (both from eBioscience) for 30 min at 4°C. The lymphocytes were then incubated with immune response. Cell lysates, conditioning of the membranes, and 10% human AB serum and kept on ice. Soluble mouse anti-human CD3 (clone OKT3; eBioscience) or T2 cells pulsed with 5 µmol MART-1b63-35 or HIV-1 Gag p24g11–23 were added to the clones while on ice. The cells plus mix was then transferred to a 37°C bath to perform yield incubation periods of 1, 4, 15, and 45 min. The reaction was terminated and signaling fixed by adding 1 ml fixation-permeabilization reagent (eBioscience). Signaling response was determined on the same day by flow cytometry using fluorochrome-labeled antibodies (BD Pharmingen). Anti-CD20 and anti-CD19 were used to gate on the T cell clones and exclude T2 cells from the signal analysis. The BD LSRII FACS cytometer and Cytobank Web site (http://www.cytobank.org) were used for data acquisition and analysis.

Profiling immunoreceptor phosphorylation in T cells

T cells were activated by plate-bound anti-CD3 mAb (eBioscience) for 30 min at 37°C. The cells were harvested and solubilized in lysis buffer, and sample protein concentrations were determined using Bradford’s reagent (Bio-Rad Laboratories). Then, 400 µl cell lysate of each clone was applied on a human phospho-immunoreceptor array (Proteome Profiler array; R&D Systems) that assesses a panel of phosphotyrosine molecules associated with immune response. Cell lysates, conditioning of the membranes, and
hybridization, and development were performed according to the manufacturer’s protocol. The array signals were visualized using luminescent image analyzer LAS-3000 (Fujiﬁlm) by exposure to ECL Western blotting substrate (Pierce), and then quantiﬁed with TINA 2.0 software using MiniBIS Pro (DNR Bio-Imaging Systems).

NK, T, and B cell Ag blocking on target cells
To mask NK, T, and B cell Ag (NT-B-A) on target cells, 5 μg/ml monoclonal anti–NTB-A Ab (clone NT-7) or mouse IgG1 x isotype control Abs (both from BioLegend, San Diego, CA) were added to 107/ml peptide-pulsed T2 or 624mel cells, suspended in complete medium. Following incubation for 30 min at 4°C, cells were washed twice in cold PBS and cocultured with effectors at 1:1 ratio for 6 h at 37°C.

Results
Generation of MART-126–35–specific TIL-derived clones with variable trogocytosis capacity
To elucidate key elements that contribute to functional diversity among CD8+ T cells of the same TCR speciﬁcity, we generated a panel of MART-126–35–speciﬁc T cell cultures by single cell sorting (Fig. IA). MART-126–35 speciﬁcity of the clones was conﬁrmed by dextramer staining, demonstrating that 5 of 10 growing clones were peptide speciﬁc (Fig. 1B). The dextramer-positive clones were examined for their ability to capture target membranes. The percentage of trogocytosis+ T cells following coculture with MART-126–35–pulsed T2 cells ranged from 11% for clone 2D11 to 44% for clone 2E2. Additional clones (2E6, 2G1, and 1C8) exhibited intermediate trogocytosis, ranging from 23 to 28% (Fig. 1C). To validate the observed interclonal difference in trogocytosis against T2 cells, we used as targets unmodiﬁed 624mel melanoma cells, which naturally present MART-126–35 on HLA-A2. The most powerful clone, 2E2, preserved its superiority (50% trogocytosis+), and the other clones were scaled in a similar order to that detected with peptide-pulsed T2 targets. Only 8% of trogocytosis+ cells were found in the 2D11 clone, whereas 13–30% were evident for 2E6, 2G1, and 1C8 cells (Fig. 1D). In all cases, coculture of the clones with control peptide-pulsed T2 cells or with irrelevant melanoma resulted in <1.2% nonspeciﬁc trogocytosis. Throughout the study, the clones underwent multiple rounds of expansion while consistently preserving their pattern of trogocytosis. Therefore, each pattern represents a coherent characteristic of a given T cell population rather than an inconsistent trait affected by in vitro conditions. Hence, we generated a panel of CD8+ T cell clones of the same TCR speciﬁcity but with distinct capacities to perform trogocytosis: from low (2D11), through intermediate (2G1), to high (2E2).

The extent of trogocytosis performed by T cell clones grades them along a functional scale
Recently, we showed that trogocytosis reﬂects the strength of dynamic interaction between a lymphocyte and a tumor cell and might be used as a functional signature of tumor-speciﬁc T cells (14). To gain a deeper insight into the link between trogocytosis intensity and tumor-speciﬁc responsiveness of distinct T cell clones, we compared 2D11, 2G1, and 2E2 clones in several functional assays: cytokine secretion, lysosomal degranulation, and cytotoxicity.

Ag-speciﬁc reactivity was initially evaluated by intracellular staining of IFN-γ and TNF-α following stimulation with 624mel cells. The percentage of IFN-γ-producing cells ranged from 20% for 2D11 to 54% for 2E2, with an intermediate value of 34% for 2G1 (Fig. 2A). The pattern for TNF-α production was similar, with a low cytokine production by 2D11 cells (13%) and a 2- and 3.5-fold increase in the percentage of cytokine-producing cells for 2G1 and 2E2 clones, respectively (Fig. 2B). To determine whether these results reﬂect a dampened machinery of cytokine production in 2D11 as compared with 2E2 cells, we measured IFN-γ and TNF-α production following stimulation with PMA/ionomycin. Our results revealed comparable potential for cytokine production by 2D11, suggesting intact ability to produce IFN-γ and TNF-α by this clone (Fig. 2A, 2B, histograms).

Next, we directly correlated trogocytosis capacity of the clones with their cytolytic activity as measured by cell surface expression of CD107A (LAMP-1), a surrogate marker of lysosomal degranulation (19). In this set of experiments, peptide-pulsed targets and melanoma cells were labeled with the lypophilic dye PKH26 and coincubated with T cells. Trogocytosis was measured by detection of PKH26 on CD8 lymphocytes and plotted against CD107A (Fig. 2C). In line with the results obtained with streptavidin (Fig. 1C, 1D), PKH26 acquisition was low in 2D11, intermediate in 2G1, and high in 2E2. Plotting trogocytosis (PKH26) against lysosomal degranulation (CD107A) exposed the highest percentage of
PKH26^CD107A^ cells in 2E2 and lowest in 2D11, further supporting a tight association between trogocytosis and cytolytic activity (Fig. 2C). To correlate trogocytosis with activation-induced phenotypic alterations, 2E2, 2G1, and 2D11 clones were compared with respect to CD137 expression following incubation with specific melanoma. CD137 has been described as a marker for activation of effector immune cells (20). In line with the data of Wolfl et al. (20), the vast majority of Ag-specific T cells displayed elevated levels of CD137 after incubation with 624mel cells, with a peak at 24 h (data not shown). Notably, 2D11 and 2G1, the clones with lower effector activity, exhibited a higher percentage of CD137^ cells, compared with the 2E2 clone (Fig. 2D, upper and lower right quadrants). However, the percentage of CD137^ cells was the highest for 2E2 (Fig. 2D). Thus, double staining for CD107A and CD137, which highlights Ag-reactive cells within the overall activated population, best correlates with trogocytosis levels of each clone.

Finally, we compared cytotoxic activity of 2E2, 2G1 and 2D11 against peptide-pulsed targets and MART-1^HLA-A2^ melanoma cells. Clone 2E2 displayed the highest lytic activity against peptide-pulsed T2 cells; 2G1 clone was 10–15% less effective than 2E2, and 2D11 cells exhibited only marginal CTL activity (Fig. 3A). Similarly, highest anti-melanoma cytotoxicity was displayed by clone 2E2, whereas 2D11 was entirely ineffective at killing melanoma cells (Fig. 3B). Reduced susceptibility of tumor cells to T cell attack may be due to low MHC expression or impaired Ag processing (21). To compare CTL clones in a scenario where tumor cells are less likely to escape T cell killing due to low expression of pMHC, 624mel cells were treated with IFN-γ prior to their usage as targets in cytotoxicity assays. Exposure to IFN-γ increased expression of MHC class I by 5-fold (data not shown). Whereas IFN-γ treatment notably increased the sensitivity of 624mel cells to 2E2 cytotoxicity, it barely affected killing by 2D11 (Fig. 3C). Therefore, low pMHC expression is unlikely to account for the impaired functionality of 2D11 cells, and IFN-γ pretreatment does not restore its poor functionality. Taken together, these data indicate that the level of trogocytosis reliably reflects anti-tumor reactivity and allows functional scaling of Ag-specific T cells.

**TCR analysis of T cell clones**

We next addressed the possible role of TCR binding affinity to pMHC in the functional diversity of T cell clones. TCR interaction with pMHC relies on intrinsic affinity and costabilization of the complex via the CD8 molecule and the α3 domain of the MHC class I H chain (22). The clones were evaluated for pMHC binding in a CD8-dependent and -independent manner. To this end, we used multimers consisting of either intact or α3-mutated HLA-A2,
Flow cytometry-based analysis.

Screening array of phosphorylated immunoreceptors.

To delineate the phosphorylation pattern of proteins associated with signal transduction in each T cell clone, we used two complementary approaches: 1) flow cytometry-based longitudinal analysis of phosphorylated proteins downstream of the TCR; and 2) screening array of phosphorylated immunoreceptors.

Flow cytometry-based analysis. To evaluate whether the functional differences between the clones are reflected in distinctive signaling patterns, phosphorylation of CD3ζ, SLP-76, ribosomal protein S6, LCK, PLCγ, and ERK1/2 proteins was recorded in the high- and low-reactive 2E2 and 2D11 clones, respectively. Differences in phosphorylation patterns were mainly observed for the ribosomal protein S6. Baseline phosphorylation of S6 (p-S6) was stronger in 2E2 than in 2D11, as evident in nontreated cells and in cells stimulated with control peptide-pulsed T2 cells. Additionally, a distinct timeline pattern of p-S6 in 2E2 and 2D11 was observed upon activation with peptide-pulsed T2 cells, with enhanced phosphorylation emerging between 15 and 45 min later (Fig. 5A). Peptide stimulation did not yield detectable levels of other TCR-related protein phosphorylation (data not shown). To exclude variability in phosphorylation patterns as a result of different TCR/pMHC affinities, anti-CD3 Ab, a potent activator of T cells, was used. p-S6 showed the most distinctive patterns differentiating 2E2 from 2D11. 2E2 showed an intense and protracted phosphorylation of S6 commencing after 1 min, peaking at 15 min, and continuing at 45 min. The functionally inferior 2D11 displayed stronger initial phosphorylation of S6, followed by a weaker and shorter course that ended after 45 min (Fig. 5B). Whereas minor differences in phosphorylation of SLP-76 and CD3ζ were observed between the clones, no differences in phosphorylation levels between the two clones were detected for LCK, PLCγ, and ERK1/2 (Fig. 5B and data not shown). These results indicate that even when T cell clones are triggered by anti-CD3 in a pMHC-independent manner, they show distinct signaling patterns, suggesting that factors other than TCR affinity affect the function level of each clone.

Screening array of phosphorylated immunoreceptors. T cell activation initiates cascades of tyrosine phosphorylation of ITAM and ITIM (reviewed in Ref. 24). To compare ITAM and ITIM patterns in 2D11, 2G1, and 2E2, cells were activated by anti-CD3 mAb and screened by a pheno-immunoreceptor array, TCR triggering resulted in strong CD3ζ phosphorylation in all tested clones (Fig. 6A). Phosphorylation of NTB-A was high in 2E2, lower in 2D11, and almost undetectable in 2G1 cells (Fig. 6A). Quantitative image analysis revealed a 2- and 4-fold increase of phosphorylated NTB-A in 2E2 compared with 2D11 and 2G1 cells, respectively (Fig. 6B). Total NTB-A expression in unstimulated and activated T cells was similar for all clones (Fig. 6C and data not shown). NTB-A is a surface receptor engaged in homophilic interactions (25). To ascribe a role for NTB-A–mediated signaling in CD8+ T cell activation, 2E2 cells were stimulated with MART-126–35–pulsed T2 cells whose NTB-A was masked by specific Ab (26). T2 cells

Table I. Characterization of MART-126–35–specific T cell clones of different avidity/affinity

<table>
<thead>
<tr>
<th>Clones</th>
<th>Vβ</th>
<th>Trogocytosis Intensity</th>
<th>Avidity (IFN-γ Curve)</th>
<th>CD8-Independent Tetramer Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>2E2</td>
<td>16</td>
<td>High</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td>2G1</td>
<td>16</td>
<td>Medium-high</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td>2D11</td>
<td>14</td>
<td>Low</td>
<td>Low</td>
<td>No</td>
</tr>
</tbody>
</table>
treated with isotype-matched Ab and NTB-A–negative 624mel melanoma were used as controls. As shown in Fig. 7, pretreatment of T2 cells with anti–NTB-A resulted in a decreased percentage of INF-γ– and TNF-α–producing cells, decreasing from 81 to 50% and from 34 to 13%, respectively. In an attempt to increase the inhibitory effect of the blocking Ab, in some experiments anti–NTB-A mAb was concomitantly added to target and effector cells. However, this did not lead to additional decrease in T cell activity (data not shown). Pretreatment of T2 cells with an isotype control mAb and 624mel treated with anti–NTB-A Ab did not affect cytokine production by T cells (Fig. 7). These results suggest that specific blocking of NTB-A homophilic interactions markedly reduces the responsiveness of CD8 T cells.

Discussion

In this study, we used the phenomenon of trogocytosis as a signature of function for clonal T cells. Trogocytosis was used to distinguish strong effector T cells from counterparts with reduced activity and to define molecules that partake in the functional diversification of cells with identical TCR specificity.

A potent tool developed to advance this study was a panel of MART-126–35–specific T cell clones endowed with distinct capacities to perform trogocytosis. The presence of membrane fragments on T cells that have recently interacted with target cells primarily reflects TCR specificity, avidity, and cytotoxic activity (14, 27, 28). In the present study, a correlation between the extent of trogocytosis and the functional status of T cells was established based on cytokine secretion, phenotypic activation-induced alterations, and cytotoxic activity. We conclude that the level of trogocytosis reliably reflects anti-tumor reactivity and allows functional scaling of Ag-specific T cells. This is exemplified in the present study by the 2E2, 2G1, and 2D11 clones of high, intermediate, and low activity, respectively. Importantly, the diverse functional traits of these clones were stable throughout repeated cycles of in vitro expansion. This observation reinforces the progeny functional consistency principle formulated by Beuneu et al. (6), which states that the activation level induced by peptide-loaded APCs is consistently displayed by the entire progeny.

The three T cell clones made two pairs: 2E2 and 2D11, which differed markedly in their function and bore different TCR Vβ; and 2E2 and 2G1, which expressed the same TCR Vβ but differed in function (Table I). In line with the fact that TCR affinity is the primary factor dictating T cell activation threshold (29), 2E2 and 2D11 express TCRs of strikingly different affinities, as demonstrated by CD8-dependent and -independent tetramer staining (Fig. 4) and dose-dependent IFN-γ secretion (not shown). In contrast, the second pair of clones, 2E2 and 2G1, varied in their degree of anti-tumor activity, despite having the same TCR Vβ region and similar TCR affinities. Several factors could contribute to the functional disparity between 2E2 and 2G1, including 1) TCR αβ expression levels, 2) ζ-chain downmodulation in the low-function clone (30), and 3) changes in the ratio between CD8 homodimers (αα) and heterodimers (αβ) (31, 32). The expression levels of TCR αβ, ζ, CD8α, and CD8β were indistinguishable.
between the two clones and therefore could not account for their functional diversity (data not shown). Experiments searching for other factors that could affect T cell function are currently underway.

Trogocytosis takes place at the immunological synapse, the focal point for signal initiation and integration that defines the level of T cell activation (33). The extent of trogocytosis could reflect changes in signaling pathways not necessarily related to pMHC/TCR affinity. In addressing this issue, we characterized the signaling pattern of clones 2E2 and 2D11 in a TCR/pMHC-dependent and –independent manner, using peptide-pulsed APCs and anti-CD3 mAbs as stimulators. Of the signals detected, phosphorylation of S6 ribosomal protein showed the most distinct difference between the clones upon peptide and anti-CD3 stimulation. 2E2, the most potent clone, endured longer phosphorylation, whereas 2D11 displayed only a brief signal. S6 is phosphorylated downstream of the AKT/mTOR pathway and integrates multiple signals, including those of the MAPK pathway (13, 14). Sustained activation of T cells, regardless of the initiating trigger, is associated with S6 kinase activity (34–36). The higher phosphorylation state of S6 in clone 2E2 is in accord with these data. Thus, S6 phosphorylation is a good indicator of the overall downstream signal propagation in activated T cells. The data suggest that different signaling properties of the low and high effector clones, regardless of TCR affinity, account for functional diversity.

TCR/CD3 signaling is augmented and sustained by a series of ligand–receptor interactions of costimulatory molecules on the surface of T cells and APCs (37). Screening by the phospho-immunoreceptor array technology revealed augmented phosphorylation patterns of NTB-A in clone 2E2 as compared with 2G1 and 2D11. Surprisingly, in the two clones with identical TCR affinities, 2E2 and 2G1, the differences in NTB-A phosphorylation were particularly prominent. NTB-A (SLAMF6) is a surface receptor of the SLAM family, which belongs to the CD2 superfamily, and is expressed on NK, T, and B lymphocytes (38–41). This receptor acts through homophilic interactions with other NTB-A molecules, providing a costimulatory signal for lymphocyte activation, with a focus on innate-like effectors (41, 42). Cross-linking of NTB-A results in its phosphorylation and association with the small adaptor molecules SAP and EAT2, as well as activation of Fyn and downstream transcription through NF-κB (43). NTB-A engagement in activated T cells leads to T cell proliferation, Tc1 cytokine secretion, and enhanced lytic function (25). Our data show that NTB-A blockade on APCs inhibits TNF-α and IFN-γ production, emphasizing the need for NTB-A costimulation (Fig. 7). Surprisingly, NTB-A blockade had a stronger impact on TNF-α than

FIGURE 6. NTB-A (SLAMF6) expression on MART-126–35-specific T cell clones. A, Phospho-immunoreceptor array of multiple tyrosine phosphorylated immunoreceptors in lysates prepared from designated T cell clones following activation with anti-CD3 mAb. B, Quantitative evaluation of phosphorylated NTB-A in the clones analyzing the array image file using image analysis software, as described in Materials and Methods. C, Expression of surface NTB-A on 2E2 (solid curve), 2G1 (broken curve), and 2D11 (dotted curve) cells following double staining with anti-CD8 and anti–NTB-A mAbs and flow cytometry on a BD LSRII. Filled histogram shows background staining with isotype control Ab. PC, positive control.

FIGURE 7. Interfering with the NTB-A/NTB-A homophilic interaction decreases cytokine production by CD8+ T cells. 2E2 T cells were stimulated with MART-126–35–pulsed T2 cells (NTB-A+ ) or 624mel melanoma (NTB-A−), pretreated with either anti–NTB-A or with matched isotype control mAb. Following 6 h stimulation in the presence of brefeldin A, the cells were double stained for CD8 and intracellular IFN-γ (A) or TNF-α (B) and subjected to flow cytometry on a BD LSRII. Columns show mean of three replicates for IFN-γ+CD8+ (A) or TNF-α+CD8+ (B) cells; bars indicate SE. Statistical significance of the effect of anti–NTB-A versus isotype control on T cell function was determined by an unpaired Student t test. *p < 0.001, **p < 0.0001. One representative experiment of three performed is shown.
on IFN-γ production. Although the reason for this finding is unclear, the impact of NTB-A on production of different cytokines was shown to be variable (44, 45).

To the best of our knowledge, this report is the first evidence for the importance of NTB-A as a costimulator in tumor Ag-specific CD8 T cells. Further studies are being conducted to explore the relevance of this molecule for anti-tumor response through direct engagement of NTB-A on tumor-specific T cells. Several costimulatory molecules, including CD28, ICOS, OX40, and 4-1BB, are recognized. Although CD28 is considered to be the most potent costimulatory pathway (46–48), its targeting with an agonistic Ab in a clinical trial tragically ended up with a fatal cytokine storm (49). The addition of NTB-A to the short list of costimulators contributes a new candidate to be evaluated for anti-cancer immunotherapy.

Understanding the different mechanisms that account for T cell diversification has a practical side: one of the main obstacles in cancer immunotherapy is the low affinity and impaired function of anti-tumor T cells. Even when a substantial number of T cells can be generated and transferred to the patient, cancer regression has not been guaranteed. Although the lack of specificity could be resolved with the advent of TCR engineering, the low reactivity and short survival of T cells still restricts the clinical benefit of cancer immunotherapy. Trogocytosis provides new opportunities to reveal and evaluate molecules that could improve this situation.

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Disclosures

The authors have no financial conflicts of interest.

References


Gate hierarchy in flow cytometry-based trogcytosis experiments. Doublets and cell debris are first eliminated based on scatter characteristics (forward scatter height versus forward scatter area and forward versus side scatters) followed by gating on clonal T cells based on expression of CD8.
Supplementary table I. Sequence of the primers used for multiplex PCR.

<table>
<thead>
<tr>
<th>V β region</th>
<th>Primer sequence</th>
<th>Primer in bp</th>
<th>Label</th>
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<tr>
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