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Defective Adhesion in Tumor Infiltrating CD8$^+$ T Cells[1]

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CD8$^+$ tumor-infiltrating lymphocytes (TIL) are defective in cytolysis due to tumor-induced inhibition of proximal TCR-mediated signaling, a defect that is relieved upon purification and brief culture. We show in this study that frequency of conjugation in vitro of nonlytic TIL with tumor cells is low in comparison with their lytic counterparts, and the strength of interaction and duration of conjugation are also reduced. Previous reports show that p56^{lck} activation is required for TCR-initiated LFA-1 avidity up-regulation, raising the question: is low LFA-1 avidity the basis of reduced TIL conjugation frequency? When stimulated with phorbol ester, nonlytic TIL bind purified ICAM-1 equivalently as lytic TIL, suggesting that LFA-1 can be activated if proximal TCR signaling is bypassed. However, when treated with phorbol ester, the conjugation frequency of nonlytic TIL does not increase. CD2 and CD8 also mediate T cell adhesion to cognate target cells and are both expressed at lower levels in nonlytic TIL in addition to being excluded from the immune synapse formed upon conjugation. Collectively, these results imply that adhesion defects in nonlytic TIL result from a combination of decreased cell surface levels of adhesion molecules, deficient LFA-1 activation, and the failure to recruit essential adhesion receptors to the membrane contact site formed with cognate target cells. *The Journal of Immunology,* **2006, 176: 6103–6111.**

Early TCR-dependent signaling events are essential in activation of the CD8$^+$ T cell effector phase, especially cytolysis and vectoral cytokine release, functions that are associated with formation of the immunological synapse (IS)[7] (1). Correlated with both cell activation and IS formation is clustering of F-actin coincident with coalescence of lipid rafts at the T cell: target cell contact site formed with nonhemopoietic target cells (1). Subsequent to target cell recognition, the microtubule organizing center (MTOC) mobilizes to the IS, which then directs release of lytic granules into tightly bound target cells (2). Analysis of lytic function in vitro has shown that the ability of T cells to form conjugates with cognate target cells is an essential requirement for TCR signaling and cytolysis (3).

Following recognition of cognate target cells, a role for adhesion-dependent events in the T cell effector phase is well known; however, the underlying mechanism(s) that links adhesion to lytic function is not understood. Two cell surface molecules, CD2 and LFA-1, are important in the initiation and maintenance of T cell adhesion (4). LFA-3 and CD48 are the counterligands for CD2 that when activated by ligand initiate a signaling program causing activation of LFA-1 (5, 6). Similarly, LFA-1 interaction with its ligands (ICAM-1, -2, and -3) causes rapid up-regulation of LFA-1 affinity (7). TCR-mediated inside-out signaling to LFA-1 has been shown to be required for adhesion (8) as well as for subsequent changes in T cell physiology, including regulation of cytoskeletal dynamics (8, 9); thus, CD2-LFA-3/CD48 interactions are considered to initiate T cell:target cell interactions. Subsequent MHC/peptide-induced TCR signaling is thought to perpetuate or potentiate T cell signaling via enhancement of LFA-1-mediated T cell: target cell interactions in addition to activation of tyrosine kinases and calcium flux (10).

The immune system has the ability to eliminate transformed cells, yet tumors still grow. Cancer patients frequently have CD8$^+$ antitumor T cells that infiltrate tumors (tumor-infiltrating lymphocytes (TIL)) and/or circulating antitumor Abs, proving that priming of antitumor immune response occurs (11, 12). However, tumors can use a variety of mechanisms to evade elimination by the immune response (13), including induction of lytic dysfunction in CD8$^+$ TIL (14, 15). Like most patients, tumor-bearing mice have normal systemic immune responses (16), but TIL are nonlytic, which indicates that inhibition of lytic function is induced within the tumor microenvironment. However, upon purification and brief culture in vitro, tumor-specific TIL cytolysis is re-established, showing that lytic dysfunction is both transient and most likely induced by factors in the tumor microenvironment (14, 15). We have shown previously that nonlytic TIL in conjugates with cognate tumor cells have defective F-actin localization and do not activate Wiskott-Aldrich syndrome protein or proline-rich tyrosine kinase-2, both essential for lytic function (14, 15). These deficiencies strongly suggest adhesion-dependent events are defective in nonlytic TIL, which may contribute to lytic dysfunction.

Although nonlytic TIL are deficient in degranulation, implying a defect in proximal TCR signaling, our earlier studies failed to identify TIL signaling defects (14). However, those analyses were performed using anti-CD3 stimulation to activate TIL in vitro, which ignores a potential contribution of inhibitory immune receptors (17). Therefore, to study TIL signaling under more physiologically relevant conditions, assays using stimulation of TIL in

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[7] Abbreviations used in this paper: IS, immunological synapse; BFA, brefeldin A; LAT, linker for activation of T cells; MFI, mean fluorescence index; MTOC, microtubule organizing center; PFA, paraformaldehyde; RT, room temperature; SMAC, supramolecular activation complex; TIL, tumor-infiltrating lymphocyte.

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vitro with cognate tumor cells were developed. In such experiments, we have determined recently that nonlytic TIL have a block in proximal TCR signaling due to abortive $p56^{lck}$ activation, resulting in the failure of TIL to flux calcium or activate protein tyrosine kinases (15). Therefore, because regulated vectoral cytokine secretion and release of lytic granules require intact proximal TCR signaling, lytic function is abrogated.

In this study, we have characterized the relationship between conjugation and the failure to degranulate. Because conjugate formation is essential for effector phase function, we analyzed the ability of nonlytic TIL and lytic TIL to form conjugates in vitro and find that nonlytic TIL form fewer conjugates than lytic TIL. We also assessed the stability of TIL:tumor conjugates and determined that nonlytic TIL interact with cognate tumor cells for a significantly shorter time than lytic TIL. In addition, we measured the width of the TIL membrane at the tumor target contact site and found that nonlytic TIL have a much weaker interaction with cognate targets compared with lytic TIL. In comparison with lytic TIL, nonlytic TIL have reduced cell surface expression of adhesion molecules CD2, CD8, and LFA-1, in addition to failing to localize CD2 and CD8 to the TIL:tumor contact site (15). The TIL proximal signaling defect prevents distal signaling events, including activation of LFA-1. However, PMA treatment fails to enhance conjugation frequency, supporting the notion that the decreased cell surface levels of adhesion molecules and the inability to localize CD2 and CD8 inhibit conjugation with cognate targets, possibly contributing to the lytic-defective phenotype.

Materials and Methods

Mice

C57BL/6 male mice were obtained from The Jackson Laboratory, were housed five per cage in a barrier facility, and were maintained on a 12-h light/dark cycle (7 a.m. to 7 p.m.) with ad libitum access to food and water. A sentinel program revealed that tumor-bearing mice were murine hepatitis virus negative. Experiments involving animals were conducted with the approval of the New York University School of Medicine Committee on Animal Research.

Tumors

MCA38 adenocarcinoma (a gift from N. Restifo, National Cancer Institute, Bethesda, MD) and MC57G (a gift from H. Schreiber, University of Chicago, Chicago, IL) were passaged from tissue culture plastic ware by incubation in HBSS containing 3 mM EDTA, followed by washing three times in HBSS. Viability was determined by trypan blue dye exclusion, and $2–3 \times 10^6$ cells were injected i.p. in a volume of 0.1 ml of HBSS for tumor induction. Cells were passaged in vitro for 3–5 wk, following which new frozen stocks were thawed for usage.

Tissue culture

RPMI 1640 medium (BioWhittaker) was used for growth of MCA-38 cells and for culture of T cells, as described previously (14).

Isolation of TIL

Tumors were dissected, mechanically disrupted by passage through a tissue press, and enzymatically digested into single-cell suspensions, and TIL were isolated by immunomagnetic separation using type LS^+ columns and anti-CD8α-conjugated magnetic beads (Miltenyi Biotec), as described previously (18). Aliquots of isolated T cells were analyzed by flow cytometry and were routinely ~95% $CD8^+$. TIL were used immediately after isolation for experiments (nonlytic), except in some experiments in which TIL were plated in complete RPMI 1640 medium ($\sim 2 \times 10^6$ cells/ml) for 6–18 h before usage (lytic). Isolation of TIL using magnetic immunobeading is unlikely to inhibit signaling or lytic function because TIL isolated using anti-Thy-1 magnetic beads are cytolytic, as determined by chromium release assay. Furthermore, as a control, CTL prepared by primary MLR in vitro, followed by treatment with digestive enzymes and isolation by anti-CD8 magnet immunobeading, are highly lytic (data not shown), indicating that the isolation protocol does not adversely affect cytolytic function. Lastly, lytic TIL have a small degree of beads that are not shed during the overnight in vitro recovery as determined by flow cytometry, and when additional beads are added, there is no effect on conjugate frequency (data not shown). Frequency of conjugation is 2- to 3-fold higher in lytic TIL than in nonlytic TIL even with the addition of immunobeads to lytic TIL before analysis of conjugate frequency.

Antibodies

Abs and reagents used for confocal microscopy and flow cytometry were CD2 (clone RM2-5; Caltag Laboratories), CD8 (clone 5H10; Caltag Laboratories), LFA-1 (clone I21/7; BD Pharmingen), phosphotyrosine (clone 4G10; Upstate Biotechnology), and phosphorylated linker for activation of T cells (LAT) (Y191, rabbit IgG; Upstate Biotechnology). Secondary reagents were obtained from Jackson ImmunoResearch Laboratories. Nonimmune control Ig were purchased from Caltag Laboratories.

Flow cytometric analysis

For flow analysis of extracellular molecules, cells were resuspended at 1×10^6/ml in complete RPMI 1640 medium, and primary Abs were added at empirically determined optimal concentrations. After incubation at 4°C for 30 min, cells were washed once with 1 ml of FACS wash (PBS, 2% FBS, 0.1% sodium azide) or complete medium, and, if primary Ab was unlabeled, resuspended in complete RPMI 1640 medium at 1×10^6/ml before addition of appropriate secondary Abs. Conditions for reaction with secondary reagents were identical as for primary Ab. Cells were given a final wash with complete medium before fixation with 4% paraformaldehyde (PFA) and analysis on a FACScan flow cytometer (BD Biosciences).

For flow cytometry analysis of intracellular molecules, cells were first fixed with 3% PFA (15 min on ice), washed with 1 ml of FACS wash or complete medium, permeabilized with 0.1% Triton X-100 for 5 min, washed with 1 ml of FACS wash or complete medium, and then reacted with primary Ab, as described above, without a further fixation step before analysis. Species-matched, control primary Ab was used to determine parameters and settings for flow cytometry. There is no staining of phospho-LAT Y191 if cells are not permeabilized, showing that PFA fixation does not permit Ab access to the interior of the cell.

FACS-based conjugate formation assay

Target cells were labeled with the PKH57 Green (or Red in some experiments) Fluorescence Cell Linker Kit (Sigma-Aldrich). T cells were labeled with anti-CD8-PE or TriColor. Cells were washed twice in medium without serum, were mixed at a 3:2 ratio, and were pulse spun at $16{,}000 \times g$ for 20 s. Cells were resuspended in 0.05 ml of medium without serum and incubated at 37°C for various times before fixation with 3–4% PFA and analysis. As a control, the frequency of conjugates with cognate tumor cells was compared with MC57G, a non-cross-reactive syngeneic tumor cell line.

TIL activation

TIL were isolated and incubated in cold RPMI 1640 medium containing 0.002 mg/ml anti-CD3ε (30 min on ice). Cells were washed twice with cold medium before incubation in 0.05 ml of medium containing 0.002 mg of affinity-purified goat anti-hamster (Jackson ImmunoResearch). To initiate signaling, cells were placed in a 37°C water bath for various times before fixation in PFA.

Extracellular and intracellular staining for immunofluorescence microscopy

$CD8^+$ TIL (3×10^5) were mixed with tumor cells (2×10^5), were centrifuged at $16{,}000 \times g$ for 20 s to promote conjugate formation, resuspended in 0.05 ml of RPMI 1640, transferred to poly(L-lysine) (Sigma-Aldrich)-coated cover slips, and incubated for different intervals of time at 37°C before fixation in 3–4% PFA (15 min at room temperature (RT)). Coverslips were washed thrice with PBS and were permeabilized with 0.1% Triton X-100 in PBS for 5 min at RT for analysis of intracellular molecules. Coverslips were then washed two times with PBS for 5 min, blocked in complete medium for 5–10 min before Abs were added, and incubated for 45–60 min at RT. If secondary Abs were required, samples were washed twice in PBS for 5 min and incubated with Ab for 45–60 min. Conjugates were also analyzed by flow cytometry (after labeling cells and permeabilization with Triton X-100), as described (14, 15).

Analysis of TIL:tumor cell contact size

Nonlytic and lytic TIL were prepared, and conjugates were formed for 15 min before fixation and analysis by confocal microscopy using a Carl Zeiss LSM 510 confocal microscope to obtain Z-stacks of conjugates with a ×63 Plan-Apochromat objective (1.4 oil). The width of the TIL cell membrane

at the contact site was measured as a function of the TIL length and expressed as a ratio.

Live imaging

TIL were added to MCA38 target cells grown on 35-mm glass-bottom microwell dishes (MatTek) for 24 h. Immediately before addition of TIL, target cells were washed with HBSS and placed into a Zeiss environmental chamber equilibrated with 5% humidified carbon dioxide maintained at 37°C (forced air and heated plate). A Zeiss Axiovert 220m microscope (equipped with a Chroma filter set and Zeiss ×40 oil immersion objective) was used to obtain images of conjugates for various times, which were subsequently analyzed using Zeiss Axioversion 4.0 software. Images were captured at 20-s intervals, and Axiovision LE was used for analysis of captured images.

Inhibitor treatment

TIL were purified and plated in vitro at a concentration of 10^6 cells/ml in the presence or absence of medium containing inhibitors for 6–18 h. Brefeldin A was added at 5 μg/ml, while lactacystin was added at 10 μM. After incubation, cells were washed a minimum of three times in complete medium before analysis by flow cytometry.

ICAM-1 adhesion assay

ICAM-1 binding was performed essentially as described by Dustin and Springer (7). Purified mouse transmembrane rICAM-1 in 1% octylglucoside was adsorbed to polystyrene 96-well plates (Sarstedt) in 0.025 M Tris (pH 8.0), 0.15 M NaCl, and 0.02% azide (Tris/saline/azide) to a final ICAM-1 density of 450 sites/μm^2. After overnight incubation at 4°C (or 2 h at RT), wells were washed three times with Tris/saline/azide, incubated for 1 h with heat-treated BSA (0.1% BSA in PBS with 2 mM $MgCl_2$ added subsequent to heating), and washed three times with PBS before addition of 0.05 ml of cold Leibovitz's L-15 medium without L-glutamine or phenol red (Invitrogen Life Technologies) either with or without 0.020 mg/ml PMA (10–20 min).

Nonlytic and lytic TIL (5×10^5 cells/ml) were labeled with CFSE (Molecular Probes). TIL were added to ICAM-1-coated wells in quadruplicate at a final density of 50,000–100,000 cells/well. The plates were centrifuged at 500 rpm for 3 min at 4°C, a baseline reading was obtained, and plates were incubated for 10–20 min at 37°C. Unbound cells were removed by washing with warm L-15, and the number of bound cells was visually assessed by light microscopy and quantified using a Cytofluor 2300/2350 Fluorescence Measurement System fluorometer (Millipore). The percentage of adherent cells was determined by dividing the final fluorometry reading of the sample minus the reading for medium alone by the initial (baseline) fluorometry reading for the sample minus the reading for medium alone ((final reading − medium reading)/(initial (baseline) reading − medium reading)).

Results

Differential activation of nonlytic and lytic TIL

Our prior experiments that studied TIL signaling used anti-CD3ε Ab for cell activation in vitro, and it was concluded that TCR signaling was intact in freshly isolated nonlytic TIL, including: calcium flux, tyrosine kinase activation, and protein kinase C translocation to the membrane (14). However, apparently normal TCR signaling could not be reconciled with lytic dysfunction; therefore, we compared signaling initiated via cross-linking anti-CD3ε with conjugation to cognate target cells. (After purification, signaling and lytic defects are restored if freshly isolated TIL are placed in culture for 3–6 h (14, 15)). Nonlytic TIL were stimulated with anti-CD3 before staining with Ab reactive with activated LAT (pY191), followed by analysis by flow cytometry (Fig. 1*A*).

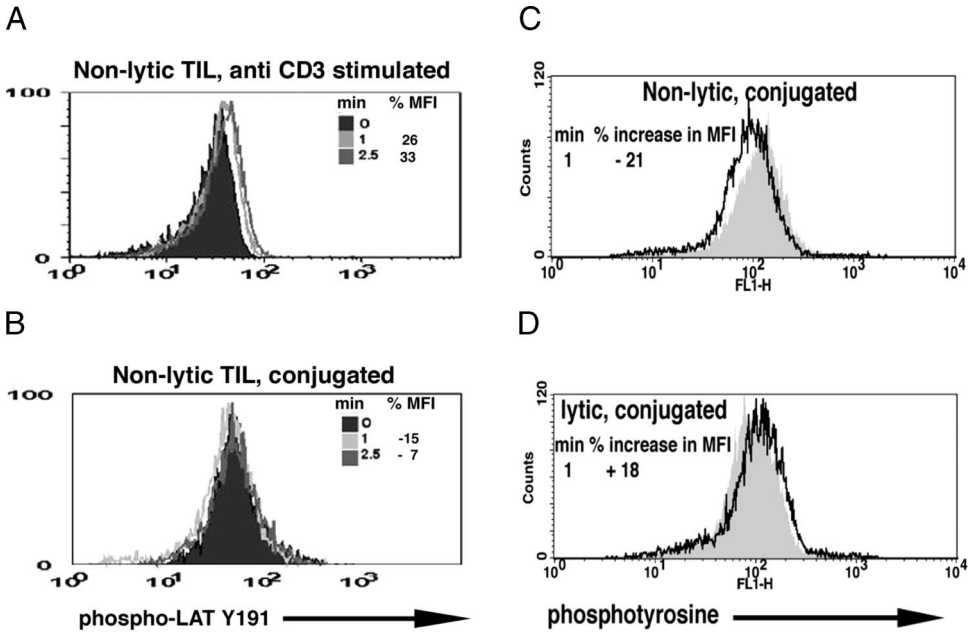

FIGURE 1. Differential activation of nonlytic TIL by anti-CD3ε and conjugation with cognate tumor cells. Nonlytic TIL were isolated and stimulated by anti-CD3ε cross-linking (*A*) or by conjugation with cognate MCA38 tumor cells (*B*), as described in *Materials and Methods*. Anti-CD3ε cross-linked TIL were incubated at 37°C for either 1 or 2.5 min before intracellular flow cytometric analysis for phosphorylated LAT (Y191). Shown in *B* is phospho-LAT staining of TIL after conjugation for either 1 or 2.5 min. The level of anti-phospho-LAT staining in nonactivated cells (i.e., after cross-linking Ab was added, but before warming to 37°C (*A*) or at time 0 of conjugation (*B*)) is shown in the filled histograms. The relative LAT phosphorylation was measured by comparison of MFI (which for the data shown in *A* was 30.5 at time 0, and which increased 26% at 1 min and 33% at 2.5 min relative to time 0, respectively). MFI for *B* data was 58 at time 0, which decreased 15% at 1 min and 7% at 2.5 min relative to time 0, respectively. (The higher baseline fluorescence of nonlytic TIL in conjugates at time 0 is due to the additive effect on the MFI from the tumor cells, which have a high autofluorescence.) *C* and *D*, Shows staining of conjugates of nonlytic (*C*) and lytic TIL (*D*) with anti-phosphotyrosine Ab at 1 min of conjugation. Similar to the data for LAT, upon conjugation the level of phosphotyrosine decreases in nonlytic TIL (−21%) and increases in lytic TIL (+18%). Filled histograms show staining with control Ig. This experiment has been repeated four times with identical results, and the average percentage change in MFI and SD for these experiments was: *A*, +20.5% (±4.5) at 1 min, +24.6% (±5.3) at 2.5 min; *B*, −10.9% (±5.1) at 1 min, −6.5% (± 3) at 2.5 min; *C*, −9.7% (±6) at 1 min; and *D*, +16.2% (±7.4) at 1 min.

Nonlytic TIL phosphorylate LAT when CD3ε is cross-linked; however, after conjugation with cognate tumor cells (a more physiological assessment of TIL activation), LAT is not phosphorylated (Fig. 1*B*). Analysis of TIL total phosphotyrosine levels also showed that nonlytic TIL are not activated by conjugation with cognate target cells (Fig. 1*C*), but in lytic TIL, conjugation caused rapid phosphotyrosinylation (Fig. 1*D*). Although the biochemical basis for differential signaling between these two methods of activation is not known, clearly nonlytic TIL fail to signal properly when stimulated with tumor cells, suggesting that the proximal signaling block is related to the inability to lyse cognate targets (14, 15).

Nonlytic TIL are deficient in conjugation with cognate target cells

To better understand the basis of defective TIL activation by cognate target cells, we characterized the frequency of conjugate formation by measuring the number of conjugates that form between TIL and cognate targets (MCA38) compared with a syngeneic antigenically distinct target (MC57G). The percentage of conjugates formed was determined by dividing the number of TIL in conjugates (*upper right quadrant*) by the total number of TIL (*upper right* plus *upper left quadrants*) (Fig. 2*A*). Typically, lytic TIL form 2- to 3-fold more conjugates than nonlytic TIL. In addition, the frequency of nonlytic TIL conjugation with cognate targets is approximately that of lytic TIL with syngeneic irrelevant targets (which are not killed by anti-MCA38 lytic TIL (14)) (Fig. 2*B*). When conjugate frequency of TIL is analyzed at various time points, lytic TIL form conjugates at 2- to 3-fold higher frequency at all time points compared with nonlytic TIL (Fig. 2*C*). Moreover, the lower frequency of both lytic and nonlytic TIL conjugates with control target cells indicates that conjugation is Ag specific, corroborating previous cytolysis specificity assays (14). A typical cytolysis assay comparing the two TIL populations is shown in Fig. 2*D*.

Live imaging analysis of conjugate stability

Decreased frequency of conjugates formed by nonlytic TIL in comparison with lytic TIL suggested that nonlytic TIL-adhesive properties may be deficient. Confocal microscopy or flow cytometry indicates only the number of conjugation-competent T cells in a population at a given point in time, but we wished to determine the amount of time that any given TIL interacts with its target. Live imaging permits examination of cell-to-cell interactions and was used to assess the stability of TIL conjugates with cognate tumor. Nonlytic and lytic TIL were allowed to contact tumor cells, and the time of interaction was recorded (a representative example is shown in Fig. 3*A*). When viewed in video format, nonlytic TIL bounce from one tumor cell to another with only a minor population making stable contacts (supplemental Fig. 1 data).[8] For both lytic and nonlytic TIL, the data are represented as a scatter plot of time in conjugation (Fig. 3*B*). Quantification of these data indicates that nonlytic TIL lack stability of interaction, having an average time of 4.9 min in conjugation with a given target, and the median time of interaction is 4 min. Even though the average time of conjugation for nonlytic TIL is 4.9 min, a few (~20%) continue to interact for an extended period (e.g., longer than 10 min), thereby increasing the population average. Conversely, lytic TIL not only form a greater percentage of conjugates with tumor cells (Fig. 2), but also interact with a given target cell for a longer period of time, on average 18.6 min (median = 17.3 min). Compared with nonlytic TIL, the scatter analysis reveals that lytic TIL have a

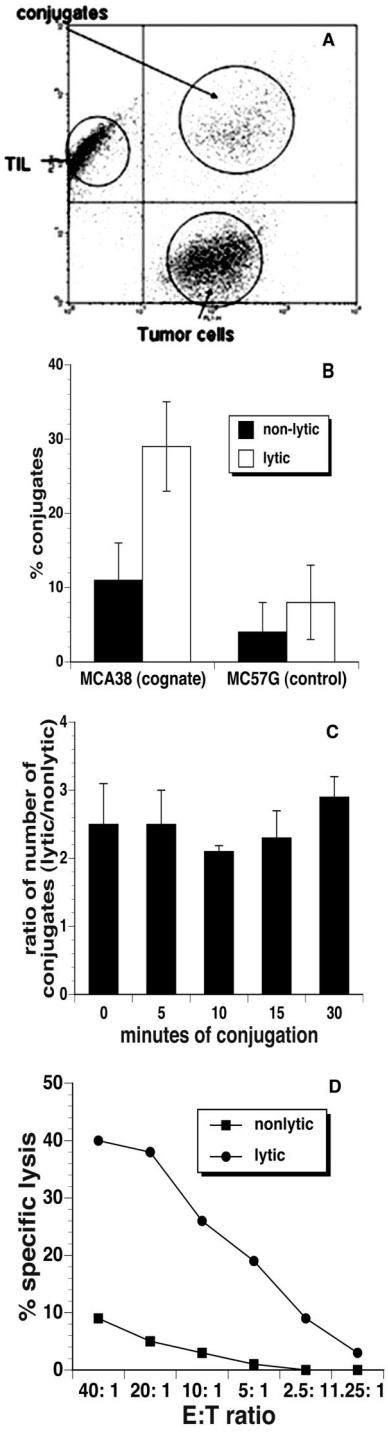

FIGURE 2. Nonlytic TIL form fewer conjugates than lytic TIL. Tumor cells and TIL were labeled and conjugates were formed for 15 min before analysis by flow cytometry, as described in *Materials and Methods* (*A*). Quantitation of TIL conjugation with either cognate MCA38 cells or syngeneic MC57G cells is shown in *B*. Lytic TIL form ~3-fold greater number of conjugates than nonlytic TIL. (This experiment has been repeated 12 times. The mean percentage of conjugation is shown, and the error bars show the SEM.) Comparison of the number of conjugates formed between nonlytic or lytic TIL and cognate MCA38 cells as a function of time in conjugation is shown in *C*. (The number of different experiments in which conjugates were formed at the indicated times were 0 min = 4; 5 min = 14; 10 min = 4; 15 min = 7; and 30 min = 5.) *D*, Typical ^{51}Cr release assay comparing nonlytic and lytic TIL for lysis of cognate MCA38 cells after overnight recovery (performed as described previously (14, 15)).

[8] The on-line version of this article contains supplemental material.

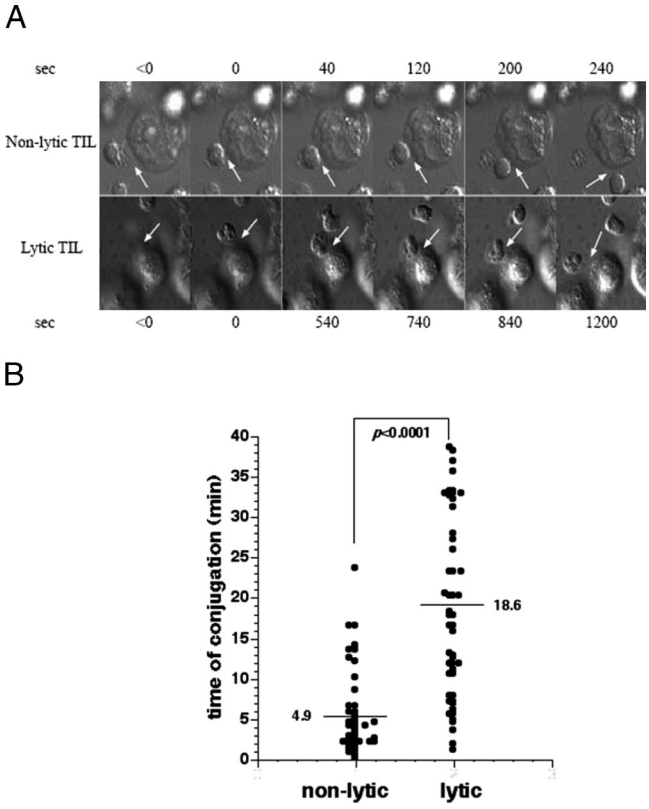

FIGURE 3. Live imaging analysis of conjugate stability. Nonlytic and lytic TIL were prepared, and the time of interaction with cognate MCA38 cells was analyzed by live imaging, as described in *Materials and Methods*. Single images of a given representative TIL are shown in *A* at the time of binding indicated (in seconds). The arrows show the TIL:tumor cell contact site. *B*, Data from individual TIL-tumor cell interactions; the mean time of interaction indicated for each T cell population. More than 40 individual nonlytic and lytic TIL conjugates were analyzed.

wider range of times of interaction with targets, indicating that lytic TIL show more variability. However, ~20% of lytic TIL interact for >30 min, and only ~10% interact for 4.9 min or less, the average time of contact for nonlytic TIL.

Confocal microscopic analysis of the TIL:target cell contact site

Normal T cells form very tight target cell interactions: Wulfing et al. (19) suggest that a tight membrane interface is defined as one whose width is greater than two-thirds of the T cell diameter. Tightness of the TIL:tumor cell interaction was assessed in conjugates by measurement of the TIL membrane contact area using confocal microscopic analysis of unlabeled cells. The contact size was expressed as a ratio of the width of the TIL membrane, which interacts with the target cell membrane divided by the perpendicular length of the T cell (Fig. 4) (19). The ratio of width to length for nonlytic TIL is 0.43, whereas for lytic TIL is 0.74, which indicates that lytic TIL form a significantly tighter interaction with tumor cells compared with nonlytic TIL. Considered with the previous analyses, this result suggests that while relatively few nonlytic TIL form conjugates with tumor cells (Fig. 2), the conjugates that do form are both less stable (Fig. 3) and less tight (Fig. 4) in comparison with lytic TIL.

Expression of TIL cell surface molecules

Looser and less stable interactions with target cells reflect weaker adhesive forces, which are mediated through binding of T cell adhesion molecules to their ligands. Therefore, we analyzed levels

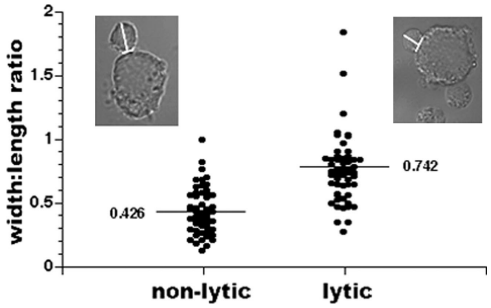

FIGURE 4. Analysis of TIL:tumor cell contact size. Nonlytic and lytic TIL were prepared and conjugates were formed for 15 min before fixation and analysis by confocal microscopy, as described in *Materials and Methods*. Nonlytic TIL showed a significantly smaller contact area compared with lytic TIL. $n = 54$ conjugates for both nonlytic and lytic TIL.

of various cell surface molecules, which are known to play a role in adhesion: TCRβ, CD2, CD8β, and LFA-1. Lytic and nonlytic TIL have very similar levels of TCR expression (Fig. 5; the mean fluorescence index (MFI) of cell surface TCR staining of nonlytic TIL ranges from 88 to 97% that of lytic TIL, $n = 5$, data not shown). Thus, increased conjugation frequency and recovery of TIL lytic function are not associated with up-regulation of TCR. However, upon recovery in vitro, the levels of CD2, CD8β, and

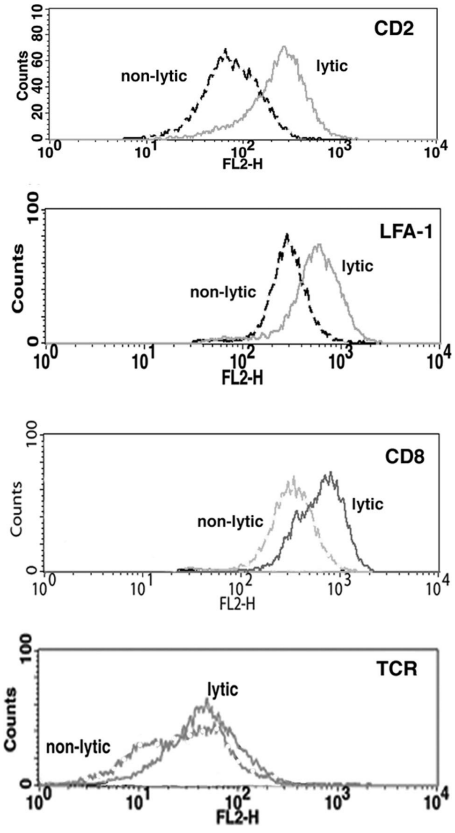

FIGURE 5. Expression of TIL cell surface molecules. Nonlytic and lytic TIL were isolated and analyzed by flow cytometry using the indicated fluorochrome-conjugated Abs. Tracings for nonlytic TIL are dashed lines; tracings for lytic TIL are solid lines. Lytic TIL have increased levels of adhesion molecules compared with nonlytic TIL. Isotype controls for both lytic and nonlytic TIL had an MFI <10. The cell surface levels of TCR are not significantly higher in lytic TIL compared with nonlytic TIL (*bottom histogram*). This experiment is representative of more than five experiments.

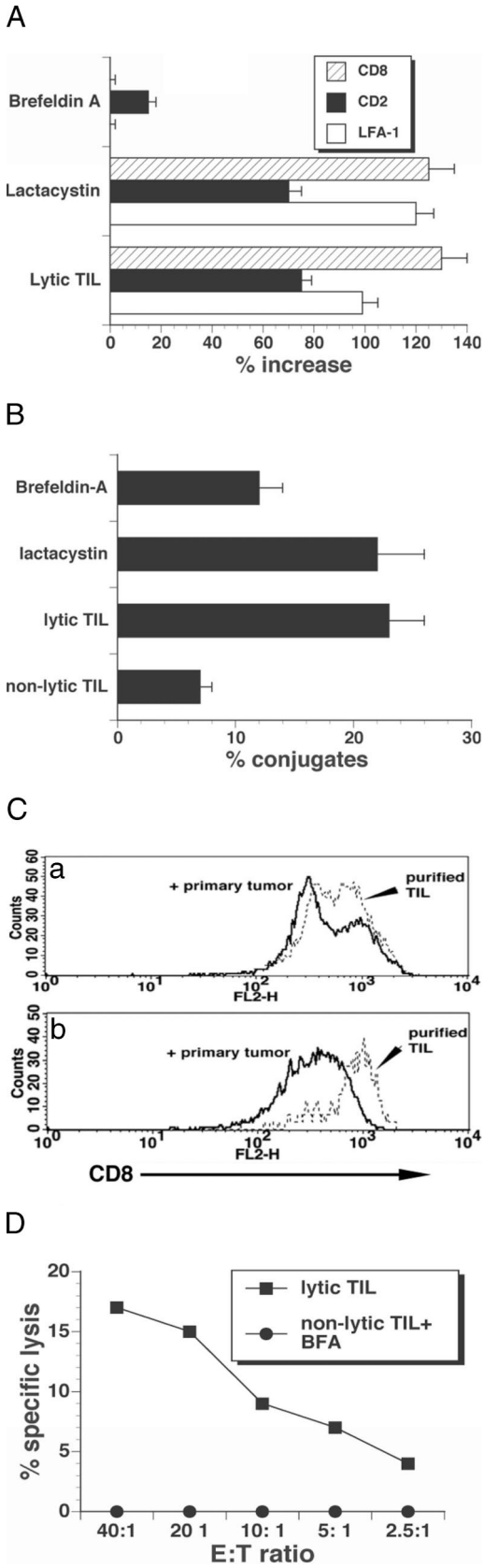

FIGURE 6. Expression of cell surface adhesion molecules and LFA-1 activation affects conjugation frequency. *A*, Effects of inhibitors on adhesion molecule expression. TIL were treated with the indicated inhibitors during 6 h or overnight recovery in vitro before analysis of the levels of cell surface molecules. Recovery of trypan blue-negative BFA-treated TIL ranged from 73 to 88%. The MFI of staining of lytic TIL (after culture) was compared with freshly isolated nonlytic TIL. *B*, Effects of inhibitors on conjugate formation. TIL were incubated with the indicated inhibitors before conjugate formation. TIL recovered in the presence of BFA form ~50% fewer conjugates, while lactacystin is without effect on conjugation frequency. *C*, Effects of primary tumor cells on TIL CD8 expression. *a*, *top histogram*, A single-cell suspension of primary tumor was prepared, washed, and cultured overnight (16 h). From the same primary tumor, $CD8^+$ TIL were isolated and cultured overnight. TIL were then recovered by magnetic immunobeading and analyzed for expression of CD8. *b*, *lower histogram*, Lytic TIL were incubated for 6 h in the absence (dashed tracing, MFI = 822) or presence (solid tracing, MFI = 391) of primary tumor single cell suspension. These experiments are representative of three repetitions. *D*, Effect of BFA treatment on TIL lytic function. TIL were isolated and plated in vitro for 6 h, as described previously, in the presence or absence of BFA before chromium release assay. This experiment has been repeated twice with identical results.

LFA-1 increase in lytic TIL compared with nonlytic TIL, an average of 2-fold. Like levels of TCR, cell surface levels of CD43 and CD45 do not change upon recovery of lytic function (data not shown).

To assess the basis for increased cell surface expression of CD2, CD8, and LFA-1 in lytic TIL, nonlytic TIL were cultured in the presence of brefeldin A (BFA), a vesicular transport inhibitor, before flow cytometric analysis (Fig. 6*A*). Treatment of nonlytic TIL with BFA prevented up-regulation of adhesion molecules during the in vitro culture (Fig. 6*A*). Treatment of nonlytic TIL with lactacystin, a proteasome inhibitor (or no treatment), has minimal or no effect on up-regulation of cell surface LFA-1, CD2, or CD8. These results show that inhibition of vesicular transport prevents up-regulation of adhesion molecules during TIL recovery in vitro, while proteosomal degradation is without effect.

To ask whether the increase in adhesion molecules during recovery influences TIL conjugation frequency, TIL were treated with BFA before conjugation assay (Fig. 6*B*). Control TIL (lactacystin treated, or TIL recovered in the absence of BFA) increased the frequency of conjugates formed compared with nonlytic TIL. However, preventing the increase of cell surface LFA-1, CD2, and CD8 reduced the ability to form conjugates in TIL. This observation suggests that the increased cell surface receptors in lytic TIL may be associated with enhanced conjugate formation.

Up-regulation of cell surface molecules occurs during in vitro culture of purified TIL, which implies that cell surface expression is down-regulated in situ. To determine the effects of the tumor microenvironment on cell surface expression of these receptors, single-cell suspensions of primary tumor were prepared and cultured in vitro before isolation of TIL and analysis of CD8 levels. After culture in the presence of tumor, TIL CD8 levels were demonstrably less than purified TIL cultured without tumor cells (MFI = 410 vs 578), suggesting the tumor milieu prevents up-regulation of CD8 expression (Fig. 6*Ca*). A similar experiment was performed in which lytic TIL were cocultured in the presence or absence of primary tumor single-cell suspension before analysis of CD8 levels (Fig. 6*Cb*). Lytic TIL plated in the absence of tumor maintain high levels of CD8 compared with TIL cultured in the presence of primary tumor (MFI = 822 compared with 391), suggesting that tumor cells may actively induce reduction in cell surface expression of CD8, and potentially other TIL adhesion molecules. Lytic function is inhibited in TIL plated in the presence of BFA (Fig. 6*D*), further supporting the notion that decreased cell surface levels of CD2, CD8, and LFA-1 impact on lytic function.

Nonlytic TIL and lytic TIL have equivalent PMA-induced binding to ICAM-1

Deficient adhesive properties of nonlytic TIL suggest that LFA-1 may not be activated. Because LFA-1 activity can be enhanced by

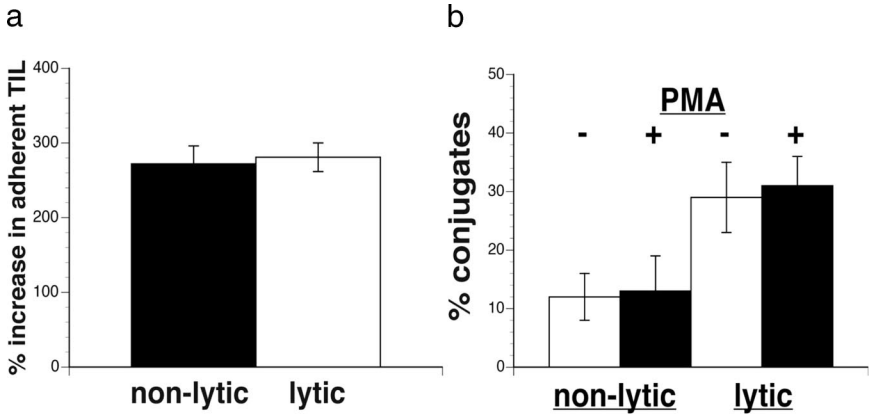

FIGURE 7. *a*, TIL binding to immobilized ICAM-1. TIL were isolated and either used immediately for binding experiments ($n = 3$), as described below, or a portion was plated in culture overnight. For ICAM-1-binding assays, TIL were treated with PMA or not, labeled with CFSE, and then incubated with plate-bound murine rICAM-1, as described in *Materials and Methods*. The number of TIL adhering to ICAM-1-coated plates was determined, and the data are shown as percentage of increase in TIL bound comparing PMA-treated with untreated cells. *b*, PMA treatment does not enhance conjugation frequency. TIL were isolated and used for 10-min conjugation assays immediately or after plating overnight. Some TIL samples were treated with PMA, as indicated (50 ng/ml for 5 min at 37°C), before addition of tumor cells.

treatment of cells with phorbol ester (20), nonlytic TIL were treated with phorbol ester before binding to purified ICAM-1 was measured (Fig. 7*A*). PMA treatment resulted in ~2.7-fold increased adhesion to ICAM-1 compared with untreated nonlytic TIL. Lytic TIL treated with PMA also had a similar increase in adhesion compared with cells not treated. This shows that TIL LFA-1 affinity and/or avidity can be up-regulated by PMA treatment in vitro. We then asked if PMA treatment could increase nonlytic TIL conjugation frequency. However, despite enhanced LFA-1 activation, the effect of PMA treatment was negligible: <3% more conjugates were formed after exposure to either PMA alone (Fig. 7*B*) or in combination with ionomycin (data not shown). These data show that PMA treatment of nonlytic TIL could activate LFA-1, which, combined with the failure of PMA-treated TIL to enhance conjugation frequency, suggests that although LFA-1 is not likely to be activated in nonlytic TIL upon conjugation owing to defective $p56^{lck}$ activation (15), the affinity status of LFA-1 is not the exclusive basis of reduced conjugation frequency of nonlytic TIL.

Discussion

The effector phase functions of T cells occur after recognition of cognate target cells, which rapidly induce recruitment of T cell signaling mediators, reorganization of the T cell cytoskeleton, and morphological changes in the cell. These changes initiate cellular programs, leading to both reorientation of the Golgi apparatus, to permit vectoral release of cytokine, and also recruitment of the MTOC to the IS, which then directs mobilization and vectoral release of lytic granules. The first contacts between a T cell and its cognate target are dependent upon interactions between adhesion molecules and counterligands that stabilize the T cell:target cell sufficiently to permit TCR engagement of Ag. The function of LFA-1 is crucial for mediating contact with target cells: signals resulting from both ligation of non-LFA-1 adhesion molecules and TCR signaling enhance LFA-1-dependent adhesion by causing a change in conformation required for high affinity binding to ICAM-1 (21).

The exact events that link TCR-mediated signaling to integrin activation have not been determined, although inside-out signaling is required for integrin-dependent adhesion. Recently, Morgan et al. (22) demonstrated that $p56^{lck}$ activity is necessary for LFA-1-mediated conjugate formation between Jurkat T cells and EBV-B cells presenting superantigen. Burkhardt and colleagues (22) suggest CD2 and LFA-1 are two important T cell adhesion molecules that act differentially in mediating interactions with target cells: while CD2 plays a crucial role in initiating cell-cell interactions before TCR signaling occurs, once TCR signaling is initiated LFA-1 strengthens the cell:cell contact via inside-out signaling. In addition to CD2, other molecules also participate in initial adhesion events through interaction with MHC class I or II proteins, such as the coreceptors CD4 and CD8.

Because the exact mechanism linking TCR stimulation to integrin activation is unknown, the differential pathways leading to enhanced integrin function vs other activation programs are difficult to dissect. However, various intermediate signaling molecules previously characterized as important for TCR-mediated gene transcription and cytoskeletal reorganization have been shown to impact upon integrin avidity, indicating that similarities also exist between these two processes in addition to poorly characterized differences. For instance, the actin cytoskeleton plays an important role in regulating integrin avidity, such that transient release of integrins from the cytoskeleton may allow for integrin clustering, while reattachment to the cytoskeleton potentially stabilizes adhesion (23).

Using flow cytometry, live imaging, and confocal analysis, we have determined that nonlytic TIL form fewer conjugates than lytic TIL. Additionally, conjugates of nonlytic TIL are less stable and weaker than those of lytic TIL, which most likely means that nonlytic TIL have adhesion defects. We have shown previously that initial TIL-target contacts are sufficient for triggering in that formation of the IS is initiated (15), but inside-out signaling needed for stronger interactions is deficient in nonlytic TIL, thereby preventing both the stabilization of conjugates and additional signaling events required for effector phase function. If $p56^{lck}$ function in nonlytic TIL is deficient, then LFA-1 cannot be activated, preventing both strengthening of the conjugate and downstream integrin-based signaling. Thus, other consequences of integrin-dependent signaling are abrogated, including reorganization of the actin cytoskeleton and mobilization of the MTOC, the phenotype of nonlytic TIL. We have shown recently that nonlytic TIL can initiate certain proximal TCR signaling events such as lipid raft coalescence and recruitment of CD3ζ, $p56^{lck}$, ZAP-70, and LAT to the IS, suggesting that initial contact is sufficient for triggering to occur (15). However, the transient and weak target

cell contacts may be directly related to the inability of nonlytic TIL to properly transduce an activating signal needed for lytic granule release (14, 15).

In this regard, the recent paper by Blank et al. (24) is intriguing in that they found that adoptive transfer of $CD8^+$ T cells that recognize a model tumor Ag into ICAM-$1^{-/-}$ mice was able to cause rejection of tumor. Functional targeting by $CD8^+$ T CTL in the absence of tumor cell ICAM-1 suggests that LFA-1-mediated T cell effects, while shown in other models to be important for T cell function, may be dispensable for the effector phase in vivo. Perhaps antitumor T cells can mediate tumor rejection in vivo indirectly by either recruitment of other effector cells or by release of cytostatic or cytotoxic factors other than granzymes. In addition, in the experiments of Blank et al. (24), the participation of LFA-1 ligands such as ICAM-2 or ICAM-3 in T cell activation (25), which results in tumor elimination, was not assessed, so the absolute absence of LFA-1 involvement remains still uncertain.

Furthermore, an unusual phenotype of nonlytic TIL is the failure to recruit two major adhesion molecules CD2 and CD8 into the IS upon conjugate formation (15). CD8, a component of the supramolecular activation complex (SMAC), interacts with both the TCR and $p56^{lck}$, thus providing for recruitment of the nonreceptor kinase to its substrate, CD3ζ. Because both $p56^{lck}$ and CD3ζ localize at the TIL IS (15), CD8 is not necessary to maintain association of the Ag receptor with its kinase. Similarly, CD2, which like CD8 localizes to the central SMAC (although close to the outer edge near the peripheral SMAC junction (26)), is also excluded from the TIL IS (15). Exclusion of CD2 and CD8 while LFA-1 properly localizes at the SMAC suggests that the molecular mechanism for recruitment of CD2 and CD8 differs from that of LFA-1. Coincident with exclusion of CD2 and CD8 from the SMAC is the failure to concentrate F-actin at the IS (15), an observation that permits the speculation that LFA-1 localization to the peripheral SMAC is independent of actin capping. By the same token, localization of CD2/CD8 to the SMAC may be dependent upon prior (or simultaneous) formation of the F-actin cap; thus, the inability to concentrate F-actin precludes recruitment of CD2 and CD8. Whatever the basis for differential recruitment of CD2/CD8 and LFA-1 to the IS, we consider that exclusion of CD2 and CD8, together with the lower cell surface expression of LFA-1, CD2, and CD8, and the likely failure to activate LFA-1 upon conjugation underlie the TIL adhesion defect, because even when activated by PMA treatment in vitro, LFA-1-mediated interactions are insufficient to increase conjugation frequency.

As we previously showed, upon purification and brief culture in vitro, TIL that infiltrate MCA38 tumor recover lytic function (14, 15). Killing of tumor by lytic TIL is Ag specific because if other syngeneic tumors are used as target cells in cytolysis assay, only cognate tumor is lysed (14). In experiments not shown, we have further tested the Ag specificity of MCA38 TIL cytolysis using additional syngeneic tumors (B16, EG.7, MCA205, MCA207, and MB49) and find killing of only cognate tumor (N. Monu, unpublished data). Those findings prove that TIL cytolytic function is Ag specific and eliminates a potential role for non-T cells in the lytic activity of TIL. We have not extended our findings of TIL adhesion defects to other tumor models, so our observations presented in this work may be relevant only to this specific tumor. However, we have tested lytic function of TIL isolated from the above-mentioned tumor models and find that freshly isolated TIL in those tumors also have transient defective cytolysis suggestive of similar effector phase defects as MCA38 TIL (E. Huang and N. Monu, unpublished data).

Although we cannot generalize our current findings to other tumor models, we are confident that they are authentic and not resultant from experimental artifact because our T cell isolation protocol (purification from enzymatically digested primary tumors by positive selection using magnetic immunobeads) does not impact upon TIL properties in that immunobead purification of primary CTL prepared by in vitro MLR does not diminish lytic activity (data not shown). Similarly, addition of additional magnetic immunobeads to lytic TIL immediately before cytolysis assay does not reduce lytic activity (data not shown).

An interesting finding that we made concerns the observation that proximal TCR signaling can be stimulated in nonlytic TIL by Ab cross-linking of TCR (or CD3ε), yet cytolytic function is not restored (14). In our original description of that phenomenon, we hypothesized that the TIL lytic defect was downstream of proximal signaling, perhaps reflecting the accumulation or activity of an inhibitor of MTOC function. Through the use of cognate tumor target cells as stimulator cells for biochemical analysis of signaling function, we know now that defective proximal signaling is in fact induced by tumor cells (15, 27). Thus, the observation that TCR Ab cross-linking induces proximal signaling implies that a negative signaling cascade is operative in nonlytic TIL when in contact with cognate tumor cells, but that can be overcome by very strong activating signals, as are provided by anti-TCR Ab. This putative mechanism of dysfunctional signaling in TIL is reminiscent of regulation of signaling in NK cells (28, 29) and is supported by our finding that Shp-1 localizes to the tumor cell:TIL contact site in nonlytic TIL, but is excluded in lytic TIL (15).

One factor that is crucial for in vitro recovery of lytic function in freshly isolated nonlytic TIL is purity of the TIL preparation. Contamination of TIL by infiltrating myeloid-derived cells, which can represent up to 40% of the single-cell suspension of enzymatically digested primary tumor, does not affect recovery (N. Monu, unpublished data). Addition of purified tumor-derived $CD11b^+F4/80^+$ cells to TIL during the brief in vitro culture does not prevent full recovery. However, if TIL are contaminated with primary tumor cells (or are incubated with the MCA38 tumor cell line), recovery of signaling and lytic function is abrogated (N. Monu, unpublished data). That observation implies that the inhibition on TIL function imposed by the tumor microenvironment is delivered to the TIL by the tumor cell, and identification of the tumor-derived factor responsible for induction of TIL signaling and lytic dysfunction is currently being pursued.

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Disclosures

The authors have no financial conflict of interest.

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