$\alpha_E\beta_7$ integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis

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Various T cell adhesion molecules and their cognate receptors on target cells promote T cell receptor (TCR)–mediated cell killing. In this report, we demonstrate that the interaction of epithelial cell marker E-cadherin with integrin α_E(CD103)β_7, often expressed by tumor-infiltrating lymphocytes (TILs), plays a major role in effective tumor cell lysis. Indeed, we found that although tumor-specific CD103$^+$ TIL-derived cytotoxic T lymphocyte (CTL) clones are able to kill E-cadherin$^+$/intercellular adhesion molecule 1$^-$ autologous tumor cells, CD103$^-$ peripheral blood lymphocyte (PBL)-derived counterparts are inefficient. This cell killing is abrogated after treatment of the TIL clones with a blocking anti-CD103 monoclonal antibody or after targeting E-cadherin in the tumor using ribonucleic acid interference. Confocal microscopy analysis also demonstrated that $\alpha_E\beta_7$ is recruited at the immunological synapse and that its interaction with E-cadherin is required for cytolytic granule polarization and subsequent exocytosis. Moreover, we report that the CD103$^-$ profile, frequently observed in PBL-derived CTL clones and associated with poor cytotoxicity against the cognate tumor, is up-regulated upon TCR engagement and transforming growth factor β1 treatment, resulting in strong potentiation of antitumor lytic function. Thus, CD8$^+$/CD103$^+$ tumor-reactive T lymphocytes infiltrating epithelial tumors most likely play a major role in antitumor cytotoxic response through $\alpha_E\beta_7$–E-cadherin interactions.

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Abbreviations used: ADC, adenocarcinoma; c-SMAC, central-supramolecular activation complex; ICAM, intercellular adhesion molecule; IS, immunological synapse; LCC, large cell carcinoma; NSCLC, non-small cell lung carcinoma; p-SMAC, peripheral-SMAC; SCC, squamous cell carcinoma; siRNA, small interfering RNA; TIL, tumor-infiltrating lymphocyte.

CD8$^+$ T cells play a critical role in antitumor immune response. Killing of tumor cells by CTLs is triggered after interaction of TCR with the specific tumor peptide–MHC-I complex. The TCR and several coreceptors thus become localized at the T cell surface, leading to the formation of signal complexes with intracellular molecules and the initiation of a transduction cascade, resulting in the execution of effector functions. For CTLs, the major effector function is mediated through directional exocytosis of cytotoxic granules, primarily containing perforin and granzymes, into the target leading to cell death (1). It has been widely documented that after initial TCR-dependent stimulation, adhesion/costimulatory proteins are repositioned at the T cell–APC contact site, referred to as the immunological synapse (IS). The TCR and associated signaling molecules, including protein kinase C θ and CD28, are clustered at the center of the T cell–target cell contact, an area referred to as the central-supramolecular activation complex (c-SMAC) (2), whereas LFA-1 (also known as CD11a/CD18 or α_L/β_2 integrin), CD2, CD8, and talin are localized at a ring-shaped structure surrounding the c-SMAC, referred to as the peripheral-SMAC (p-SMAC) (3). p-SMAC, which is formed upon ligation of LFA-1 on CTLs by high densities of intercellular adhesion molecule (ICAM)-1 on target cells, is essential for directing released cytolytic granules to the surface of tumor cells near c-SMAC and effective lysis of the latter cells by CTLs (4–6).

Most human lung cancers arise from the bronchial epithelium and belong to the categories

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of non-small cell lung carcinoma (NSCLCs), including adenocarcinomas (ADCs), large cell carcinomas (LCCs), and squamous cell carcinomas (SCCs). During cancer cell dissemination, NSCLCs frequently display reduced or absent MHC-I expression, which is often accompanied by loss of ICAM-1 (7). These tumors are often infiltrated by TCR-α/β^+, $CD8^+$, and $CD28^-$ T lymphocytes, and tumor-specific CTLs with high functional avidity were found to be selectively expanded at the tumor site, suggesting that they may contribute to control of the tumor (8). We previously isolated, from lymphocytes infiltrating an MHC-I^{low}/ICAM-I^- LCC and autologous PBL, two tumor-specific T cell clones expressing a unique TCR and displaying a $CD8^+/CD28^-/CD27^-/CD45RA^+/CD62L^-/CCR7^-$ terminally differentiated effector phenotype (9). Although both clones exhibited identical functional avidity and similar lytic potential, as measured by granzyme B and perforin intracellular expression and redirected cell killing, only the tumor-infiltrating lymphocyte (TIL)-derived clone mediated potent cytolytic activity toward autologous tumor cells (9). To gain further insight into molecular mechanisms underlying differential anti-tumor T cell reactivity, we conducted comprehensive microarray analysis using an Agilent oligonucleotide array. Functional studies indicated that the selective expression of integrin $\alpha_E(CD103)\beta_7$ by the TIL-derived clone was crucial for directional cytotoxic granule exocytosis in the ICAM-1^-/E-cadherin$^+$ tumor leading to cell death.

RESULTS

CD103 is differentially expressed in tumor-specific TIL- and PBL-derived T cell clones

Using mutated α-actinin-4 peptide–HLA-A2 tetramers, we isolated, from the PBLs and TILs of a lung cancer patient,

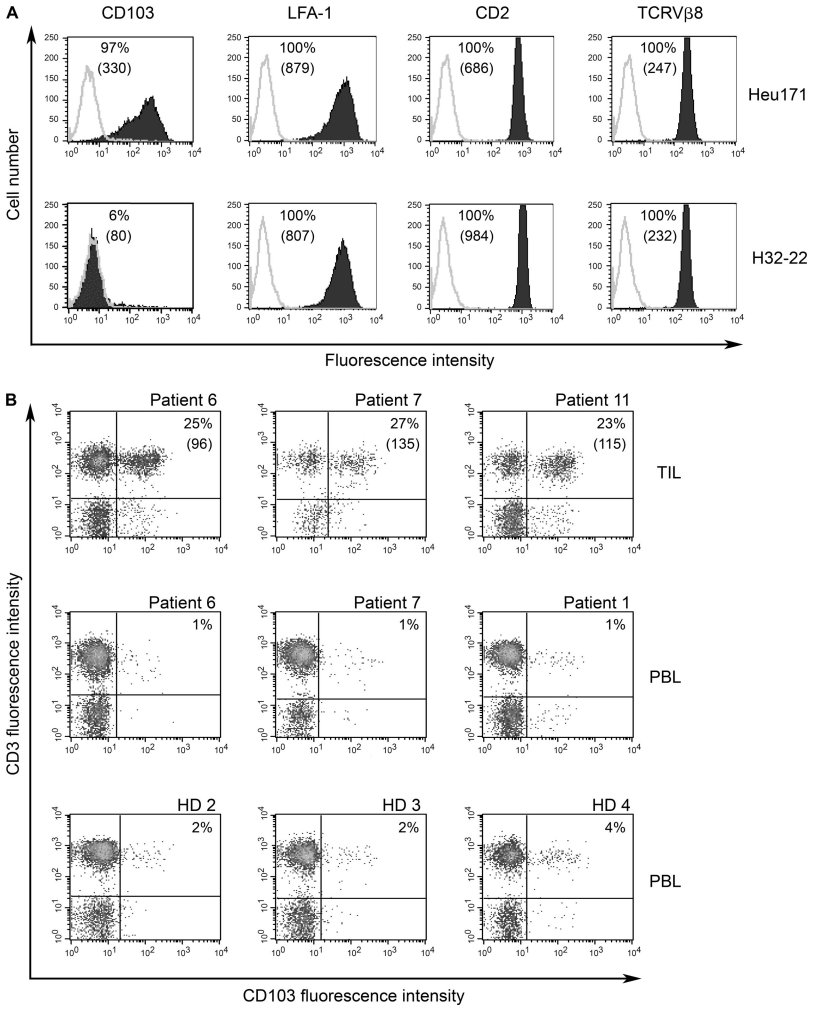

Figure 1. Surface expression of $\alpha_E\beta_7$ integrin on tumor-infiltrating and peripheral blood T cells. (A) Expression of $\alpha_E\beta_7$, LFA-1, CD2, and TCR-α/β on Heu171 and H32-22 T cell clones. Immunofluorescence analysis was performed using anti-CD103, anti-CD11a, anti-CD2, and anti-TCRVβ8 (filled) mAbs or isotypic control (open). (B) Detection of $CD103^+$ T cells in uncultured TILs and PBLs. Two-color flow cytometry analysis was performed using FITC-conjugated anti-CD103 and PE-conjugated anti-CD3 mAbs. Three uncultured NSCLC patient TIL and PBL samples and three healthy donor (HD) PBL samples are shown. Percentages of positive cells are indicated. Numbers in parentheses correspond to mean fluorescence intensities.

two tumor-specific T cell clones named H32-22 and Heu171, respectively. Although both clones expressed a unique TCR and displayed similar lytic potential, only the TIL clone, Heu171, elicited strong cytolytic activity toward the autologous IGR-Heu tumor cell line (9). To gain further insight into the molecular mechanisms underlying the differential functional activity of TIL and PBL clones, we compared their transcriptional profiles by microarray analysis using an Agilent 44000 human oligonucleotide array. Global gene expression studies performed with a p-value of $\leq 10^{-5}$ identified an expression profile of 491 genes, including a cluster of 241 genes that were less strongly expressed in TILs than PBLs, and a cluster of 250 genes that were more strongly expressed in the TIL than PBL clone (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20061524/DC1). Results acquired with a fold change of ≥ 2 identified an expression profile of 148 genes, including clusters of genes related to adhesion/recognition, cell death, locomotion/localization, metabolism, physiological processes, regulation, and response to stimulus (Fig. S1 B and Table S1, available at http://www.jem.org/cgi/content/full/jem.20061524/DC1). Among the adhesion/recognition genes, *ITGAE*, encoding the α_E (CD103) subunit of the $\alpha_E\beta_7$ integrin, was one of the most overexpressed genes in the TIL clone, with a sixfold stronger expression in the TIL than in the PBL clone, which was confirmed both by quantitative PCR analyses (unpublished data) and at the protein level (see below; Fig. 1 A). Moreover, such a stronger expression was consistently observed in several microarray analyses performed with additional TIL clones (Heu127 and Heu161; references 10 and 11, respectively) and a PBL cell line (H32; reference 9) derived against IGR-Heu (Table S2), making α_E (CD103) one of the best candidate molecules to be analyzed further.

We then assessed the expression of the $\alpha_E\beta_7$ protein on Heu171 and H32-22 by immunofluorescence analysis using an anti–human CD103 mAb (12). Results depicted in Fig. 1 A confirmed that $\alpha_E\beta_7$ was selectively expressed on the TIL clone. In contrast, both clones expressed similar levels of LFA-1 (CD11a/CD18), CD2, and TCR-α/β molecules (Fig. 1 A). NKG2D, CD8, CD44, and VCAM-1 were also expressed at similar levels by both clones (unpublished data), and CD5 was expressed at higher levels on PBLs than on TILs (9). CD31 (PECAM-1) was weakly expressed on only 29% of Heu171 cells, and CD40L was not expressed on both clones (unpublished data). It is worth noting that $\alpha_E\beta_7$ is widely expressed on most autologous and allogeneic $CD8^+$ tumor-specific TIL clones tested, and at a lower level on two autologous $tetramer^+/CD8^+$ PBL clones (H32-8 and H32-25; Table I and unpublished data). Importantly, 17–33% of uncultured $CD3^+$ TILs collected from eight NSCLC biopsies were found to express the integrin (Fig. 1 B and Table I). In contrast, only 1–2% and 2–6% of patient and healthy donor circulating T cells expressed CD103, respectively. These results indicate that $\alpha_E\beta_7$ is preferentially expressed by TILs and suggest that it may be induced upon T cell migration to the tumor site.

The $\alpha_E\beta_7$ integrin promotes TIL-mediated lysis of E-cadherin–expressing tumor cells

To determine the potential role of the $\alpha_E\beta_7$ integrin in TIL-mediated tumor cell lysis, we performed cytotoxic experiments in the absence or presence of anti-CD103–neutralizing mAb. Results indicate that Heu171 cells mediated strong cytotoxic activity toward IGR-Heu, which was abrogated in the presence of anti-CD103 ascite or purified mAb (Fig. 2 A and unpublished data). In contrast, a less dramatic inhibition was observed with anti–TCR-α/β and anti-CD8 mAb (unpublished data), and no inhibition was observed with anti-CD2 and anti-CD3 mAb (Fig. 2 A). It is noteworthy that anti-CD103 mAb also blocked the cytotoxicity of all specific TIL-derived clones against the autologous IGR-Heu target, and anti–LFA-1 mAb had no effect because IGR-Heu failed to express ICAM-1 (unpublished data). With regard to PBL clones, H32-22 mediated a weak cytotoxic activity toward

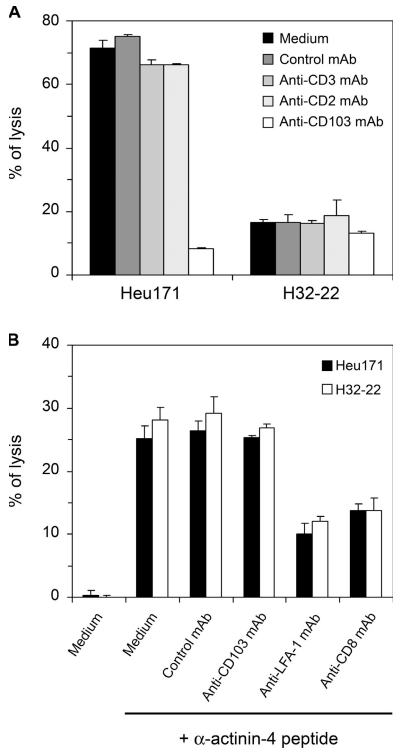

Figure 2. Role of adhesion/costimulatory molecules in T cell clone–mediated cytotoxicity. (A) Role of CD103 in Heu171 TIL clone–mediated lysis toward autologous IGR-Heu tumor cells. Cytotoxicity was determined by a conventional 4-h ^{51}Cr-release assay at a 20:1 E/T ratio. Experiments were performed either in medium or in the presence of indicated mAb. The Heu171 and H32-22 T cell clones were preincubated for 1 h with saturating concentrations of anti-CD3, anti-CD2, anti-CD103, or a control mAb. (B) Role of LFA-1 in Heu171 and H32-22 clone–mediated lysis against peptide-pulsed autologous EBV-transformed B cell line. Cytotoxicity was determined by a conventional 4-h ^{51}Cr-release assay at a 20:1 E/T ratio. The Heu-EBV B cell line was preincubated with mutated α-actinin-4 peptide. Experiments were performed either in medium or after preincubating effector cells with anti-CD103, anti–LFA-1, or anti-CD8 mAbs.

Table I. CD103 distribution among TIL and PBL T cell clones and uncultured TILs and PBLs

	T cell phenotype	% of $CD3^+/CD103^+$	MFI	References
TIL-derived T cell clones				
Patient 1 (Heu)				
Heu171	CD8	97	428	9
Heu127	CD8	100	785	10
Heu161	CD8	99	452	11, 59
Patient 2 (Bla)				
B90	CD8	100	677	19
B112	CD8	99	658	19
Patient 3 (Coco)				
CTL-C1	CD8	14	46	
Patient 4 (Pub)				
P62	CD4	1		19
PBL-derived T cells				
Patient 1 T cell clones				
H32-22	CD8	5		9
H23018	CD8	6		9
H32-8	CD8	96	126	9
H32-25	CD8	94	146	9
Patient 1 T cell line				
H32	CD8	2		9
Uncultured T cells				
TIL				
Patient 5		33	274	
Patient 6		25	96	
Patient 7		27	135	
Patient 8		17	299	
Patient 9		22	300	
Patient 10		22	374	
Patient 11		23	115	
Patient 12		22	93	
		Mean 23.7 ± 4.7	211	
Patient PBL				
Patient 1 (Heu)		1	104	
Patient 5		2	176	
Patient 6		1	74	
Patient 7		1	105	
Patient 13		2	146	
Patient 14		2	97	
Patient 15		1	149	
Patient 16		1	188	
		Mean 1.3 ± 0.5	130	
Healthy PBL				
Healthy donor 1		6	102	
Healthy donor 2		2	106	
Healthy donor 3		2	96	
Healthy donor 4		4	111	
Healthy donor 5		4	123	
Healthy donor 6		4	114	
Healthy donor 7		3	90	
Healthy donor 8		2	89	
		Mean 3.2 ± 1.6	104	

Mean percentage of $CD3^+/CD103^+$ cells was significantly higher in TILs than in patient PBLs ($P < 0.01$) and healthy donor PBLs ($P < 0.01$). Statistical analyses were performed using the Mann-Whitney test. MFI, CD103 mean fluorescence intensity.

the cognate tumor that was unaffected by all neutralizing mAbs used (Fig. 2 A). Importantly, H32-8 and H32-25 displayed a specific lysis that correlated with significant $\alpha_E\beta_7$ expression and was inhibited by anti-CD103 mAb (Table II). Furthermore, Heu171 and H32-22 clones killed the autologous EBV-transformed B cell line pulsed with the antigenic peptide, and this lysis was inhibited by anti–LFA-1 (anti-CD11a), but not by anti-CD103 mAb (Fig. 2 B), emphasizing

the role of LFA-1–ICAM-1 adhesion in this particular T cell–target cell interaction and ruling out a nonspecific effect of anti-CD103 ascite. These data strongly suggest that $\alpha_E\beta_7$ plays a major role in T cell–mediated tumor cell killing.

Thus far, the only reported ligand of the $\alpha_E\beta_7$ integrin is E-cadherin (CD324), which is expressed by normal epithelial cells but which is frequently down-regulated in metastatic cancer cells (13). To assess whether the cytotoxicity blocking effect of anti-CD103 mAb was due to inhibition of the $\alpha_E\beta_7$ interaction with E-cadherin, we tested E-cadherin expression on IGR-Heu and investigated its potential implication in TIL-mediated lysis. Fig. 3 A indicates that tumor cells displayed a high level of E-cadherin but a very low level of ICAM-1 (CD54) and moderate expression of LFA-3 (CD58) and HLA-A2 molecules. We then inhibited E-cadherin expression in IGR-Heu using specific small interfering RNA (siRNA; siRNA-E1 and siRNA-E2) and assessed target cell sensitivity to the Heu171 clone. Results depicted in Fig. 3 B indicate that siRNA-E1 and siRNA-E2 dramatically blocked E-cadherin expression in tumor cells, resulting in abrogation of TIL-mediated killing (Fig. 3 C). In contrast, luciferase siRNA (siRNA-Luc), used as a control, had no effect. It should be noted that E-cadherin knockdown did not affect tumor cell sensitivity to healthy donor NK cell–mediated lysis, excluding an effect on target cell susceptibility to apoptosis (unpublished data). In addition, transfection of the EBV-B cell line with siRNA-E1 and siRNA-E2 followed by loading with antigenic peptide did not alter T cell clone–mediated

Table II. CD103 levels reflect T cell clone antitumor cytolytic activity

	CD103 expression		% of lysis	
	%	MFI	Medium	Anti-CD103
PBL				
H32-22	3[a]		9[b]	7
H32-8	85	81	32	6
H32-25	72	119	49	21
TIL				
Heu171	95	293	60	8

Immunofluorescence and cytotoxic activity of T cell clones. Data shown are representative of three independent experiments.
[a]Percentages of $CD103^+$ T cells and mean fluorescence intensities (MFIs) are indicated.
[b]Cytotoxicity toward autologous IGR-Heu tumor cell line was determined by a conventional 4 h ^{51}Cr-release assay at a 20:1 E/T cell ratio.

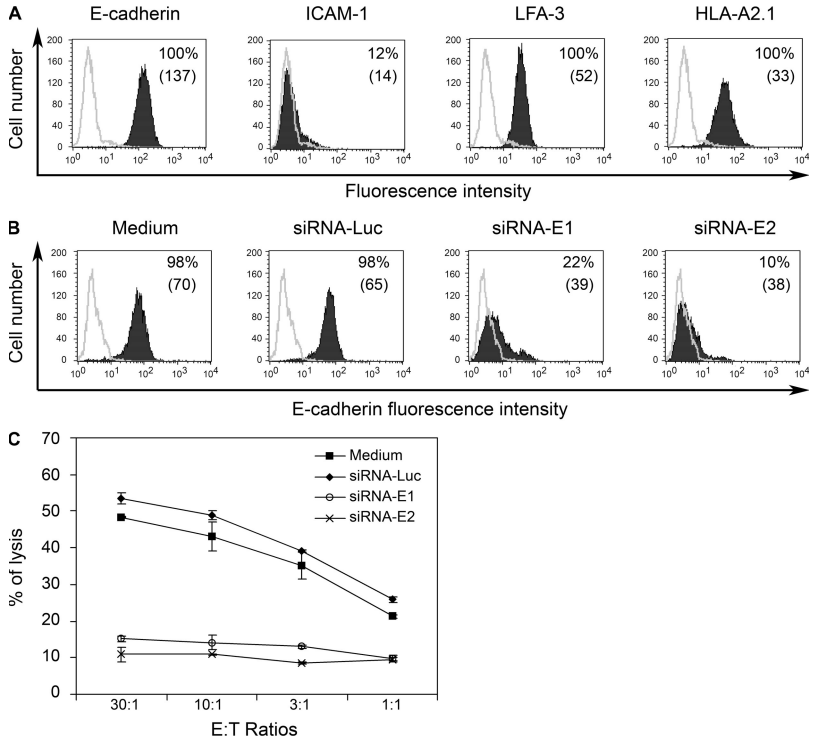

Figure 3. Down-regulation of E-cadherin expression on IGR-Heu results in inhibition of Heu171 cytotoxicity against its specific target. (A) Analysis of surface expression of E-cadherin (CD324), ICAM-1 (CD54), LFA-3 (CD58), and HLA-A2.1 on IGR-Heu tumor cells. Immunofluorescence analysis was performed using anti–E-cadherin, anti-CD54, anti-CD58, anti–HLA-A2.1 (filled) mAbs or an isotypic control (open). Percentages of positive cells are indicated. Numbers in parentheses correspond to mean fluorescence intensities. (B) Analysis of E-cadherin surface expression on IGR-Heu tumor cells electroporated or not with siRNA targeting E-cadherin, siRNA-E1, and siRNA-E2. Luciferase siRNA (siRNA-Luc) was used as a control. (C) Effect of E-cadherin extinction on tumor cell killing by the Heu171 TIL clone. Cytotoxicity was determined by a conventional 4-h ^{51}Cr-release assay at the indicated E/T ratios. IGR-Heu tumor cells electroporated or not with siRNA-E1, siRNA-E2, or siRNA-Luc were used as targets.

cytotoxicity (unpublished data). These data further support the notion that the $\alpha_E\beta_7$–E-cadherin interaction is crucial for effective cancer cell lysis; moreover, they suggest that it may substitute LFA-1–ICAM-1 adhesion in the promotion of TCR-mediated tumor cell killing.

TGF-β1–induced expression of CD103 potentiates PBL clone–mediated tumor cell killing

The above experiments clearly indicated that CD103 was widely expressed on $CD8^+$ T cells infiltrating NSCLC and suggested that it may be induced upon migration of antigen-specific T lymphocytes within the tumor. One factor abundantly secreted by neoplastic cells including IGR-Heu NSCLC (14 and unpublished data) is TGF-β1, known for its immunosuppressive effects but also for its capacity to induce CD103 expression upon T cell activation (15). We therefore assessed the effect of TGF-β1, associated or not with coated anti-CD3 mAb, on the expression of CD103 on the H32-22 clone. Immunofluorescence analyses revealed that TGF-β1 induced a sharp increase in CD103 expression on a subset of the H32-22 clone when combined with anti-CD3 mAb (Fig. 4 A). In contrast, TGF-β1 alone had only a slight effect on CD103 expression, and anti-CD3 alone had no effect.

Next, we performed cytotoxicity assays with H32-22, pretreated with coated anti-CD3, TGF-β1, or a combination of both, in the absence and presence of anti-CD103 mAb. The Heu171 TIL clone was included as a positive control. Data depicted in Fig. 4 B indicate that anti-CD3 mAb alone had no effect on H32-22 cytolytic activity toward autologous tumor cells, excluding its contribution to putative redirected target cell lysis. Interestingly, treatment of the PBL clone with a combination of anti-CD3 and TGF-β1 resulted in a strong increase in its cytotoxicity toward IGR-Heu. Such potentiation is dependent on $\alpha_E\beta_7$ induction because anti-CD103 mAb completely inhibited this killing (Fig. 4 B). TGF-β1 alone induced only a partial increase in H32-22–mediated lysis, which was significantly inhibited by anti-CD103 mAb. These data further support a role for TGF-β1 together with TCR engagement in the induction of CD103 on tumor-specific T lymphocytes upon their migration to the tumor microenvironment, and they point to the involvement of this integrin in TCR-mediated epithelial cancer cell lysis.

Interaction of E-cadherin with $\alpha_E\beta_7$ is essential for granule polarization and exocytosis

Failure of the H32-22 PBL-derived clone to kill autologous tumor cells might indicate that the interaction of E-cadherin with integrin $\alpha_E\beta_7$ is required for directional exocytosis of cytolytic granules. To test this hypothesis, we first assessed whether $\alpha_E\beta_7$ was engaged in TIL–tumor cell IS. For this purpose, Heu171 cells were incubated with IGR-Heu, and conjugates were stained with anti-CD103 mAb at different time intervals. Confocal microscopy analyses indicate that Heu171 cells formed stable conjugates with IGR-Heu and that the $\alpha_E\beta_7$ integrin progressively accumulated within the IS (Fig. 5 A). This was found in 91% of analyzed conjugates ($n = 200$). Next, to follow granule polarization and exocytosis, IGR-Heu cells were mixed with Heu171 or H32-22 cell clones, and

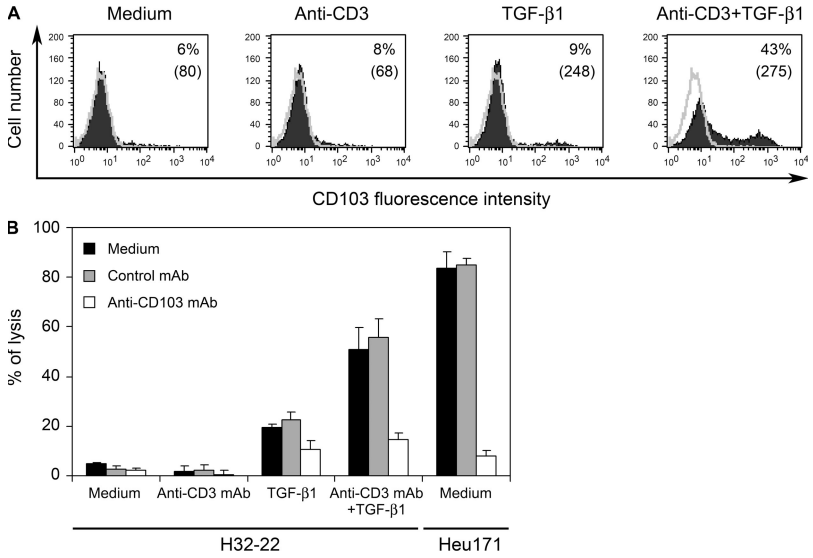

Figure 4. Role of TGF-β1 in CD103 induction and potentiation of T cell clone–mediated lysis. (A) H32-22 T cells were cultured in medium or in the presence of TGF-β1, coated anti-CD3 mAb, or a combination of both for 96 h. CD103 expression was then assessed by immunofluorescence analysis. Percentages of positive cells are indicated. Numbers in parentheses correspond to mean fluorescence intensities. (B) Role of TGF-β1–induced expression of CD103 in potentiation of H32-22–mediated killing. Cytotoxicity was determined by a conventional 4-h ^{51}Cr-release assay at a 20:1 E/T cell ratio. The H32-22 clone treated or not with TGF-β1 and/or coated anti-CD3 mAb and the Heu171 clone used as a control were preincubated for 1 h with saturating concentrations of anti-CD103 or a control mAb.

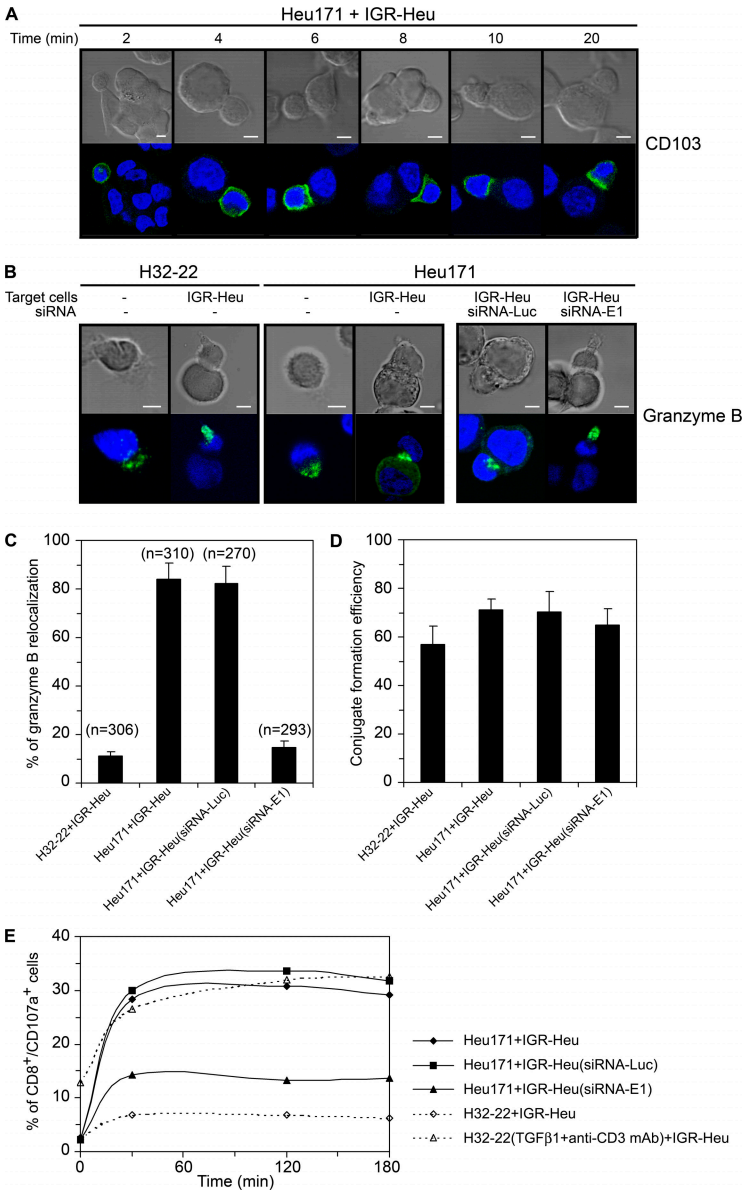

Figure 5. Engagement of $\alpha_E\beta_7$ in TIL–tumor cell IS is essential for granule polarization and exocytosis. (A) Recruitment of $\alpha_E\beta_7$ integrin in the IS formed between the Heu171 TIL clone and the IGR-Heu tumor cell line. Confocal microscopy analysis of CD103 localization (green fluorescence) in the contact area between the Heu171 and the IGR-Heu at the indicated time course. Nuclei were stained with TO-PRO-3 iodide (blue fluorescence). Bars, 5 μm. (B) On the left: TIL and PBL clones form stable conjugates with autologous tumor cells. Conjugates formed between the IGR-Heu and the H32-22 or Heu171 effector cells were analyzed by confocal microscopy after 15 min of co-culture. Granule polarization, as defined by the accumulation of granzyme B in the contact area between effector and tumor cells, was followed up using anti–granzyme B mAb (green fluorescence). On the right: E-cadherin siRNA does not affect conjugate formation between TIL and the cognate target but inhibits granule polarization. IGR-Heu tumor cells were pretreated with siRNA targeting E-cadherin (siRNA-E1) or luciferase (siRNA-Luc). Conjugates formed with the Heu171 TIL were then analyzed. Nuclei were stained with TO-PRO-3 iodide (blue fluorescence). Bars, 5 μm. (C) Percentages of CTLs displaying granzyme B relocalization during conjugate formation between the H32-22 and IGR-Heu or the Heu171 and IGR-Heu pretreated or not with siRNA-E1 or siRNA-Luc. Data shown represent mean ± SD of three independent experiments. Numbers of conjugates analyzed are indicated in parentheses. (D) Efficiency of conjugate formation between IGR-Heu and effector T cells was calculated by determining the E/T ratio × 100 as described in Materials and methods. Data represent the mean ± SD of quadruplicate fields including ~140 tumor cells each. (E) CD107a induction on Heu171 cells during co-culture with the IGR-Heu pretreated or not with siRNA targeting E-cadherin or luciferase, and on H32-22 stimulated or not with a combination of TGF-β1 and coated anti-CD3 mAb, during co-culture with IGR-Heu tumor cells. Immunofluorescence analysis was performed at the indicated time course. Staining with anti–human CD8 mAb was included to identify T lymphocytes.

polarization of cytotoxic granules, as defined by granzyme B accumulation in the contact area between effector and tumor cells, was analyzed by confocal microscopy. Data shown in Fig. 5 B indicate that polarization of cytotoxic granules occurred in the TIL-derived clone only, and delivery of Alexa-labeled granzyme B into target cells was even detected in most of conjugates analyzed. Granzyme B polarization was observed in 84 ± 7% of conjugates formed between Heu171 and IGR-Heu (n = 310), but only in 11 ± 2% of conjugates formed between H32-22 and tumor cells (n = 306) (Fig. 5 C). Importantly, silencing of E-cadherin with siRNA-E1 did not alter formation of conjugates between Heu171 and IGR-Heu (Fig. 5 D), but resulted in strong inhibition of cytotoxic granule polarization because only 15 ± 3% of conjugates (n = 293) displayed polarized granzyme B–containing granules (Fig. 5, B and C). In contrast, control siRNA (siRNA-Luc) had no effect on cytotoxic granule polarization because 82 ± 7% of the analyzed conjugates (n = 270) exhibited granzyme B relocalization.

Cytolytic granules are secretory lysosomes with a dense core containing perforin and granzymes surrounded by a lipid bilayer that contains lysosomal-associated membrane glycoproteins and FasL (16–18). Degranulation by CTLs results in CD107a (lysosomal-associated membrane glycoprotein 1) externalization at the cell surface and release of intracellular perforin and granzyme B. Therefore, we also assessed the role of the $\alpha_E\beta_7$ interaction with E-cadherin in granule exocytosis by monitoring CD107a levels in Heu171 and H32-22 cells. Immunofluorescence analysis indicated that CD107a was induced only at the surface of Heu171 TILs and that pretreatment of target cells with siRNA targeting E-cadherin partially inhibited this process (Fig. 5 E). Interestingly, pretreatment of H32-22 with a combination of anti-CD3 mAb and TGF-β1 followed by exposure to tumor cells strongly induced CD107a labeling to levels comparable to those observed on Heu171 (Fig. 5 E). These results emphasize the essential role of the $\alpha_E\beta_7$–E-cadherin interaction in granule polarization and exocytosis by tumor-specific TILs to kill their target.

Complementary role of $\alpha_E\beta_7$–E-cadherin and LFA-1–ICAM-1 interactions in TIL-mediated lysis

Our previous results clearly demonstrated a major role for $\alpha_E\beta_7$ in TCR-mediated killing of tumor-reactive CTLs infiltrating an ICAM-1$^-$/E-cadherin$^+$ carcinoma. To investigate the engagement of $\alpha_E\beta_7$–E-cadherin in a combination in which LFA-1–ICAM-1 adhesion is effective, we used NSCLC tumor cell line LCC-B2, expressing both ICAM-1 and E-cadherin (Table III), and a specific CD8$^+$/CD103$^+$ CTL clone, B90, isolated from autologous TILs (Table I; reference 19). B90 cells were mixed with LCC-B2 and conjugates stained with either anti–LFA-1 or anti–CD103 mAb. Confocal microscopy analyses of n = 100 conjugates indicated that both LFA-1 and $\alpha_E\beta_7$ integrins accumulated within the IS of 91 and 81% of TIL–tumor cell conjugates, respectively (Fig. 6 A).

Next, we assessed the effect of anti-CD103 and anti–LFA-1 mAb on CTL clone–mediated lysis toward the LCC-B2 autologous target. Results indicated that although each mAb had only a weak inhibitory effect on T cell clone–mediated killing, a combination of the two mAbs strongly blocked tumor cell lysis (Fig. 6 B). Of note, treatment of IGR-Heu with IFN-γ induced ICAM-1 expression and MHC-I molecule up-regulation resulting in an increase in H32-22–mediated lysis, which was partially inhibited by anti–LFA-1 but not by anti-CD103 mAb (unpublished data). In contrast, stable transfection of IGR-Heu with ICAM-1 had only a marginal effect on H32-22–mediated lysis (unpublished data), supporting our recent report emphasizing an inhibitory role of CD5 when the strength of the TCR/peptide–MHC interaction is weak (9). These results further emphasize a role for CD103 in TIL-mediated tumor cell killing and suggest that ICAM-1 and E-cadherin display complementary roles in T cell–tumor cell cytotoxic IS maturation.

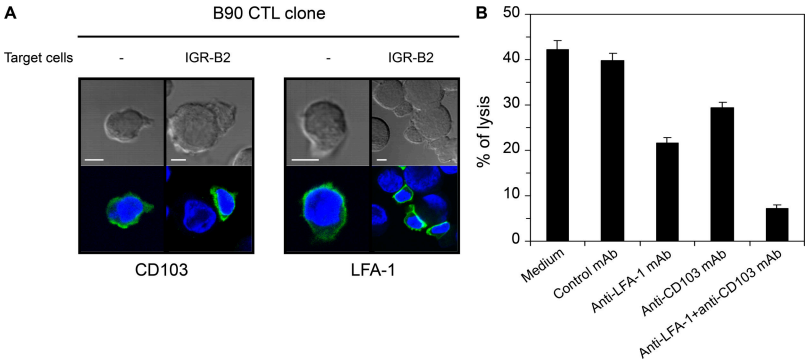

Figure 6. Both LFA-1 and $\alpha_E\beta_7$ integrins are engaged in TIL–tumor cell IS. (A) Confocal microscopy analysis of CD103 and LFA-1 localization (green fluorescence) in conjugates formed between the B90 TIL clone and the IGR-B2 autologous tumor cell line after 20 min of co-culture. Nuclei were stained with TO-PRO-3 iodide (blue fluorescence). Bars, 5 μm. (B) Role of LFA-1 and $\alpha_E\beta_7$ integrins in TIL-mediated lysis of the ICAM-1$^+$/E-cadherin$^+$ autologous tumor. Cytotoxicity was determined by a conventional 4-h ^{51}Cr-release assay at a 20:1 E/T ratio. The B90 TIL clone was preincubated or not for 1 h with saturating concentrations of anti–LFA-1, anti-CD103, a combination of anti–LFA-1 and anti-CD103, or a control mAb.

Table III. E-cadherin, ICAM-1, and MHC-I molecule expression on NSCLC and SCLC cell lines

	E-cadherin		ICAM-1		MHC-I	
NSCLC cell lines						
LCC						
IGR-Heu (patient 1)	100%[a]	(137)	12%	(14)	100%	(60)
IGR-B2 (patient 2)	45%	(146)	98%	(1619)	100%	(743)
LCC-M4	95%	(53)	ND		99%	(43)
H1155	1%		27%	(35)	100%	(855)
H460	1%		88%	(159)	100%	(412)
ADC						
ADC-Coco (patient 3)	1%		0%		1%	
IGR-Pub (patient 4)	20%	(31)	99%	(486)	100%	(393)
ADC-Tor	3%		43%	(46)	100%	(807)
ADC-Let	3%		ND		100%	(499)
A549	1%		ND		7%	(36)
H1355	34%	(89)	0%		100%	(294)
SCC						
SCC-Cher	93%	(93)	ND		82%	(27)
Ludlu	92%	(64)	34%	(19)	100%	(360)
SK-Mes	2%		53%	(47)	100%	(105)
SCLC cell lines						
DMS53	56%	(37)	46%	(43)	100%	(540)
DMS454	96%	(151)	58%	(25)	95%	(1198)

Numbers in parentheses correspond to mean fluorescence intensities.
ND, not done.
[a]Percentages of positive cells are indicated.

DISCUSSION

Adhesion between CTLs and specific target cells is thought to be a prerequisite for effective TCR recognition and subsequent cytotoxicity. This adhesion is often provided by the interaction of LFA-1 on T cells with ICAM-1 on APCs. The role of the most enigmatic integrin, $\alpha_E\beta_7$ (20), in CTL-mediated killing and antitumor immune response remains poorly understood. The $\alpha_E\beta_7$ integrin is expressed at high levels by mucosal T cells, in particular intestinal epithelium lymphocytes (21). It is also found on mucosal mast cells and DCs (22), $CD4^+/CD25^+$ T regulatory cells (23, 24), and on a large proportion of $CD8^+$ T cells infiltrating epithelial tumors, including bladder (25), colorectal (26), pancreatic (27), and lung carcinomas (this study). With regard to PBLs, we found that only a small subset of NSCLC patient and healthy donor $CD8^+$ T cells expressed the integrin. Our data also indicate that the expression level of $\alpha_E\beta_7$ on tumor-specific T cell clones correlated with their capacity to kill autologous ICAM-1^-/E-$cadherin^+$ tumor cells. This killing was abrogated by anti-CD103–neutralizing mAb and siRNA-targeting E-cadherin, pointing to a major role for the $\alpha_E\beta_7$–E-cadherin interaction in antitumor CTL response.

It has been reported that the α_E subunit can be induced by TGF-β1 upon T cell activation (28, 29). CD103 has been found on T cells residing in tissue microenvironments where this cytokine is abundant, such as in the vicinity of epithelia, in chronic inflammation (20), in renal allografts (15, 30, 31), and in epithelial tumors. Interestingly, incubation of the $\alpha_E\beta_7^-$ PBL clone with a combination of coated anti-CD3 mAb and TGF-β1 induced up-regulation of CD103 leading to potentiation of tumor-specific cytotoxicity. The paradoxical role of this cytokine, often described as an immunosuppressive factor used by neoplastic cells to escape from the immune system, but also as an important mediator of tissue remodeling and repair at sites of inflammation (32), in the control of the antitumor immune response warrants further investigation. $CD8^+/CD103^+$ T cells were also described as playing a critical role in selective destruction of host intestinal epithelium during graft-versus-host disease (33) and pancreatic islet allografts (34), in mediation of tubular injury after allogeneic renal transplantation (35), and in anti-EBV CTL response in the tonsil (36), and TGF-β1 most likely acts as a regulating factor in CD103 expression. In a previous study, we clearly showed a correlation between intratumoral down-regulation of the TCR inhibitory molecule CD5 and potentiation of antitumor T cell reactivity (9). Our overall results suggest that upon migration to TGF-β1–rich tumor microenvironment and TCR engagement, tumor-specific T cells undergo an adaptation process to tumor peptide/MHC-I levels by modulating CD5 expression together with concomitant induction of CD103, resulting in optimization of TCR-mediated cytotoxic activity.

Thus far, the only reported ligand of the $\alpha_E\beta_7$ integrin is E-cadherin (37–40). E-cadherin belongs to the type I family of cadherins, including E- (epithelial), N- (neuronal), P- (placental), M- (muscle), and R- (retinal) cadherins, and is expressed by epithelial cells forming homotypic bonds between adjacent cells. E-cadherin is known to possess a tumor suppressor function (41), and reduced expression during cancer development and metastatic invasion has been observed in many epithelial tumors (42–46). Among the 16 lung tumor cell lines tested, 5 displayed high levels of E-cadherin, 4 displayed moderate expression, and 7 were negative (Table III). These results indicate that lung carcinomas frequently express reduced levels of E-cadherin and suggest that its extinction may be associated with tumor escape from the intraepithelial CTL response. Indeed, the heterophilic adhesive interaction between the E-cadherin and the $\alpha_E\beta_7$ integrin plays a pivotal role in retention of $CD8^+$ T lymphocytes in epithelial tumors (20, 27), thus providing a local adaptive immune response (47). Furthermore, our results indicate that loss of E-cadherin expression, for example by specific siRNA, abolishes TCR-mediated tumor cell lysis by autologous $CD8^+/CD103^+$ CTLs. It is tempting to speculate that down-regulation of E-cadherin by epithelial neoplastic cells during the in vivo metastatic process could induce a decrease or even the failure of tumor-specific TILs to kill their target, suggesting a mechanism for immunological selection of cancer cells with reduced E-cadherin expression. It has been recently reported that E-cadherin is also a counterreceptor for one member of the C-type lectin-like receptor family, KLRG1 (48). Interestingly, it has been demonstrated that E-cadherin binding of

KLRG1 prevents lysis of E-cadherin–expressing targets by KLRG1$^+$ NK cells (49). These results suggest that tumors lacking E-cadherin expression may be less sensitive to CTL-mediated killing, but may become more susceptible to lysis by KLRG1$^+$ NK cells. Of note, the TIL clones used in the present study failed to express KLRG1 (unpublished data).

Although TCR engagement is necessary for inducing the cytotoxic activity of CD8$^+$ T lymphocytes, accessory molecules play a major role in mediating CTL degranulation and effective cytotoxicity (50–52). It has been reported that although the CD2–CD58 interaction is sufficient to trigger CTL degranulation, LFA-1–ICAM-1 ligation is necessary for effective target cell lysis by the released granules (53, 54). Indeed, blocking of the LFA-1–ICAM-1 interaction precludes pSMAC formation and leads to inhibition of specific lysis without a detectable decrease in granule release (54). Our results indicate that in the absence of ICAM-1, often lost during tumor cell dissemination, E-cadherin plays a pivotal role in target cell lysis by the $\alpha_E\beta_7^+$ tumor-specific TIL, most likely by promoting p-SMAC formation and subsequent directional cytolytic granule exocytosis. The interaction of E-cadherin on tumor cells with $\alpha_E\beta_7$ on specific TILs appears necessary for positioning of the cytotoxic granules near the interface and their release into the target. In the absence of both ICAM-1–LFA-1 and E-cadherin–$\alpha_E\beta_7$ adhesion, recruitment of cytotoxic granules and their release and delivery into the target cell are precluded. Indeed, although the LFA-1$^+$/$\alpha_E\beta_7^-$ PBL clone formed stable conjugates with ICAM-1$^-$/E-cadherin$^+$ autologous tumor cells, it was unable to lyse its specific target, most likely due to a defect in granule polarization and subsequent degranulation. This is further supported by experiments in which blocking of the $\alpha_E\beta_7$–E-cadherin interaction with siRNA targeting E-cadherin preserved TIL–tumor cell conjugate formation but precluded granule recruitment and release, as monitored by granzyme B relocalization and the expression of CD107a on the CTL surface. This result emphasizes the possibility that the interactions of TCR/peptide–MHC-I, likely in collaboration with CD8 and CD2-CD58, may not be sufficient to trigger CTL granule polarization. This may be associated with low expression levels of peptide–MHC-I and CD58 molecules by tumor cells used in this study. Distinct roles of ICAM-1 and E-cadherin in epithelial tumor cell killing by specific TILs are further supported by experiments performed with ICAM-1$^+$/E-cadherin$^+$ tumor cells. Indeed, both adhesion molecules were recruited at the CTL–tumor cell IS, and additional inhibitory effects of TIL-mediated lysis were observed using a combination of anti–LFA-1 and anti-CD103 mAb.

Collectively, our results demonstrate a major role for tumor-specific CD8$^+$/CD103$^+$ CTLs infiltrating epithelial cancers in antitumor immune response. Our data also emphasize a key role for the $\alpha_E\beta_7$–E-cadherin interaction in promoting TIL–tumor cell cytotoxic IS maturation correlated with granule polarization and directional exocytosis leading to an effective tumor cell lysis. Future studies investigating T cell–epithelial tumor cell conjugates, and in particular E-cadherin–$\alpha_E\beta_7$ and E-cadherin–KLRG1 interactions, will not only contribute to our understanding of the control of tumor progression, but may also provide novel targets for cancer immunotherapy.

MATERIALS AND METHODS

Derivation and culture of the tumor cell lines and T cell clones. The IGR-Heu tumor cell line was established from patient Heu suffering from LCC of the lung. Heu171, Heu127 (10), and Heu161 (11) T cell clones were isolated from autologous TILs. H32 T cell line and H32-22, H32-25, and H32-8 clones were isolated from autologous PBLs after stimulation with IGR-Heu and sorting with peptide–MHC tetramers (9). The IGR-B2 cell line was derived from patient Bla LCC, and the B90 CTL clone was isolated from autologous TILs (19). The NSCLC cell lines ADC-Coco (55), IGR-Pub, LCC-M4 (19), SCC-Cher, ADC-Tor, and ADC-Let were derived from tumor biopsies as described previously (9–11). A549 (ADC), SK-Mes, Ludlu (SCC), DMS53, and DMS454 (SCLC) were purchased from the European Collection of Cell Cultures. H460, H1155 (LCC), and H1355 (ADC) were provided by S. Rogers (Brigham and Women's Hospital, Boston, MA; reference 56). All tumor cell lines were maintained in culture as reported previously (10). The human experiments were approved by the Institutional Review Board of the Gustave Roussy Institute.

Antibodies and flow cytometry analysis. mAbs directed against CD103 and CD8 and coupled to fluorescein and purified anti-CD103 mAb were purchased from Immunotech. Anti-CD103 ascite was provided by N. Cerf-Bensussan (Hôpital Necker, Paris, France). Anti-CD107a coupled to CyChrome and anti-CD3 (UCHT1) mAb was provided by Becton Dickinson. Anti–E-cadherin mAb was purchased from R&D Systems, and anti–granzyme B mAb was from Caltag Laboratories. Anti–LFA-1, anti-CD2, anti–LFA-3, anti–ICAM-1, anti–HLA-A2.1 (MA2.1), anti-TCRVβ8, and anti-CD3 (OKT3) mAbs were reported previously (19, 57).

Phenotypic analyses of T cells and tumor cells were performed by direct or indirect immunofluorescence using a FACSCalibur flow cytometer. Data were processed using CELLQuest software (BD Biosciences). For CD103 induction, T cells were pre-stimulated with a combination of coated anti-CD3 mAb (UCHT1) and TGF-β1 (5 ng/ml; Abcys), and expression of CD103 was assessed at day 4. For granule exocytosis assay, IGR-Heu tumor cells were plated in flat-bottom 96-well plates. T cells were then added at different time points together with 3 μl anti-CD107a mAb at a 2:1 E/T ratio. T cells were then stained using a mouse anti–human CD8 mAb.

Cytotoxicity assay. The cytotoxic activity of the T cell clones was measured by a conventional 4-h ^{51}Cr-release assay using triplicate cultures. The autologous IGR-Heu tumor cell line and the autologous EBV-transformed B cell line (Heu-EBV), pulsed for 1 h at 37°C with 50 nM of the antigenic peptide, were used as targets in cytotoxicity experiments. E/T ratios were 30:1, 10:1, 3:1, and 1:1, or 20:1. Supernatants were then transferred to LumaPlateTM-96 wells (PerkinElmer), dried down, and counted on a Packard's TopCount NXT. Percent-specific cytotoxicity was calculated conventionally (58).

Oligo-microarray technology. Heu171 and H32-22 total RNA were directly compared using Agilent oligonucleotide dual color technology in running dye swap (duplicate experiments). Probe synthesis and labeling was performed by Agilent's low fluorescent low input linear amplification kit. Hybridization was performed on human whole genome 44 k oligonucleotide microarrays using reagents and protocols provided by the manufacturer. Feature extraction software provided by Agilent (version 7.5) was used to quantify the intensity of fluorescent images and to normalize results using the linear and lowest subtraction method. Primary analysis was performed using Resolver software (Rosetta Biosoftware). The microarray data related to this paper have been submitted to the Array Express data repository at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) and are available under the access codes E-TABM-207 and E-TABM-208.

RNA interference. Gene silencing of E-cadherin expression by the IGR-Heu cell line was performed using two sequence-specific siRNAs, siRNA-E1 (GCACGUACACAGCCCUAAUtt; no. 146381) and siRNA-E2 (GAGUGAAUUUUGAAGAUUGtt; no. 44988) purchased from Ambion. In brief, cells were transfected by electroporation with 0.8 nM siRNA in a gene Pulser Xcell electroporation system (Bio-Rad Laboratories) at 300 V, 500 μF, using electroporation cuvettes (Eurogentec). A second electroporation was performed after 24 h, and cells were then cultured for 48 h. Luciferase siRNA, siRNA-Luc (siRNA duplex, CGUACGCGGAAUACUUCGAdTdT, and UCGAAGUAUUCCGCGUACGdTdT), included as a negative control, was purchased from Sigma-Proligo.

Confocal microscopy. Tumor and effector cells were plated on poly-L-lysine–coated coverslips (Sigma-Aldrich) at a 2:1 E/T ratio. Cells were then fixed with 4% paraformaldehyde for 1 h and permeabilized with 0.1% SDS or Triton X-100 for 10 min, followed by blocking with 10% FBS for 20 min. The fixed cells were stained with anti-CD103 or anti–LFA-1 mAb and then with a secondary mAb coupled to Alexa Fluor 488 (Invitrogen). All antibodies were diluted in PBS containing 1 mg/ml BSA. Nuclei were stained with TO-PRO-3 iodide (Invitrogen). Coverslips were mounted with Vectashield (Vector Laboratories) and analyzed using a fluorescence microscope (LSM-510; Carl Zeiss MicroImaging, Inc.) the next day. Z-projection of slices was performed using LSM Image Examiner software (Carl Zeiss MicroImaging, Inc.). Polarization of cytotoxic granules was defined by the accumulation of granzyme B in the contact area between effector and tumor cells. Efficiency of conjugate formation between CTLs and IGR-Heu cells was calculated by determining the ratio of effector cells able to form conjugates with target cells to target cells × 100. In brief, 5×10^4 electroporated or not tumor cells were grown in Petri dishes for 48 h, and then 3×10^5 CTLs were added. After 20 min of co-culture, nonconjugated T cells were removed by gentle washing, and the remaining cells were fixed, permeabilized, and stained with Lysotracker Green (Invitrogen) for counting. Four confocal microscopy fields were analyzed for each condition, and SD were determined.

Online supplemental material. Fig. S1 illustrates global gene expression analysis in H32-22 and Heu171 T cell clones. Table S1 illustrates differential gene expression profiles of H32-22 PBL and Heu171 TIL clones. Table S2 shows the combined analysis of differential gene expression profiles of Heu171, Heu127, or Heu161 TIL clones compared with H32 PBL T cell line and Heu171 compared with H32-22 PBL clone. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20061524/DC1.

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