CD103 or LFA-1 engagement at the immune synapse between cytotoxic T cells and
tumor cells promotes maturation and regulates T-cell effector functions

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Running title: Role of CD103 and LFA-1 in maturation of the cIS.

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Abbreviations: APC: antigen-presenting cell; pMHC-I: peptide-major histocompatibility complex class I; IS: immune synapse; mcIS: mature cytotoxic IS; icIS: immature cIS; LFA-1: lymphocyte function-associated antigen-1; MFI: mean fluorescence intensity; TCR: T-cell receptor.

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Precis: This study shows how integrins that regulate the strength of the interaction between tumor cells and tumor-reactive T cells can alter their killing efficiency.
Abstract

T-cell adhesion/costimulatory molecules and their cognate receptors on target cells play a major role in T-cell receptor (TCR)-mediated activities. Here, we compared the involvement of CD103 and LFA-1, and their respective ligands, in maturation of the cytotoxic immune synapse (cIS) and in activation of CTL effector functions. Our results indicate that cytotoxicity towards cancer cells and to a lesser extent cytokine production by specific CTL require, along with TCR engagement, the interaction of either CD103 with E-cadherin or LFA-1 with ICAM-1. Flow-based adhesion assay demonstrated that engagement of CD103 or LFA-1, together with TCR, enhances the strength of the T-cell/target cell interaction. Moreover, electron microscopic analyses showed that integrin-dependent mature cIS (mcIS) displays a cohesive ultrastructure, with tight membrane contacts separated by extensive clefts. In contrast, immature cIS (icIS), which is unable to trigger target cell lysis, is loose, with multiple protrusions in the effector cell membrane. Confocal microscopy experiments revealed polarized cytokine release and degranulation at the mcIS associated with target cell killing, whereas icIS is characterized by failure of IFN-γ and granzyme B relocalization. Thus, interaction forces between CTL and epithelial tumor cells, mainly regulated by integrin engagement, correlate with maturity and the ultrastructure of the cIS and influence CTL effector functions. These results provide new insights into molecular mechanisms regulating antitumor CTL responses and may lead to the development of more efficient cancer immunotherapy strategies.
Introduction

CD8 T lymphocytes play a major role in defense against cancers through recognition by their TCR of specific antigenic peptides presented on the malignant cell surface by MHC class I (MHC-I) molecules and killing of the tumor target, mainly by releasing the content of secretory lysosomes. T-cell adhesion molecules, in particular lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18 or \( \alpha_\text{L}/\beta_2 \) integrin) and its cognate receptor, ICAM-1 (CD54), on target cells are involved in strengthening the interaction between CTL and antigen-presenting cells (APC). Subsequent to APC recognition, large-scale rearrangement of the cytoskeleton and reorganization of cell surface and cytoplasmic molecules result in formation of an “immune synapse” (IS) (1, 2). According to the bull-eye’s model of the IS, the TCR and associated signaling molecules are clustered at the central supramolecular activation complex (cSMAC) (3), while adhesion/costimulatory molecules, including LFA-1, are localized at the peripheral SMAC (pSMAC) (4). Upon CTL-APC adhesion, T cells rapidly polarize their microtubule organizing center (MTOC), Golgi complex and cytotoxic granules, containing perforin and granzymes, toward target cells (5, 6). Lysis of tumor cells then occurs through fusion of these granules with the T-cell plasma membrane at the contact site and release of their contents into the synaptic cleft formed between CTL and target cells (7). Accumulating evidence indicates that pSMAC, mainly through LFA-1 integrin, is essential for directing released cytolytic granules to the surface of target cells near cSMAC, and thus lysis of the latter cells by CTL (8-11).

We previously reported that CD8 T lymphocytes expressing \( \alpha_\text{E}(\text{CD103})\beta_7 \) integrin (also called CD103) selectively expand within the lung tumor microenvironment, and that the interaction of CD103 with its ligand, epithelial cell marker E-cadherin, on target cells plays an essential role in TCR-dependent cancer cell killing when ICAM-1 is lacking (12). Indeed,
αvβ7-integrin is recruited at the IS formed between CTL and epithelial tumor cells, and its interaction with E-cadherin is required for polarized exocytosis of lytic granules, leading to effective target cell killing. In the present study, we compared the role of CD103-E-cadherin and LFA-1-ICAM-1 interactions in the IS formed between human CTL clones and specific epithelial tumor cells, and in triggering activated CD8 T-cell functions. Our results demonstrated that CD103 or LFA-1 engagement is required for cytotoxic IS (cIS) maturation, resulting in synaptic release of both cytokines and lytic granules and thereby in target cell lysis. We also demonstrated that the CD103-E-cadherin or LFA-1-ICAM-1 interaction determines IS ultrastructure and thereby CTL effector functions. These data provide clear evidence of the role of integrins in antitumor immune response and may offer new opportunities for the design of more potent immunotherapeutic approaches in epithelial cancers.
Materials and methods

Tumor cell line and T-cell clones

The IGR-Heu cancer cell line was established in our laboratory in 1997 from a non-small-cell lung carcinoma sample of patient Heu as described (13). Heu171 and H32-22 T-cell clones, isolated respectively from autologous TIL and PBL in 1998 and 2002 (14), recognize a mutated α-actinin-4 tumor antigen (13). The T-cell clones were tested for their capacity to recognize IGR-Heu tumor cells and mutated α-actinin-4 peptide-loaded autologous EBV-transformed B-cell line in cytotoxicity and cytokine secretion assays during the present study and the last 6 months respectively.

Recombinant molecules, antibodies and flow cytometry

Human recombinant (r) E-cadherin-Fc and ICAM-1-Fc molecules were provided by R&D Systems.

Anti-CD107a and anti-CD3 (UCHT1) mAb were purchased from Becton-Dickinson. Anti-granzyme-B mAb was provided by Invitrogen. Anti-IFN-γ mAb was purchased from BD Pharmingen.

Phenotypic analyses of tumor cells were performed by direct or indirect immunofluorescence using a FACS Calibur™ flow cytometer. Data were processed using CellQuest software (BD Biosciences). For the granule exocytosis assay, T cells were stimulated with either autologous tumor cells or a combination of surface-bound UCHT1 mAb and rE-cadherin-Fc or rICAM-1-Fc in flat-bottom 96-well plates in the presence of anti-CD107a mAb and monensin A (Sigma-Aldrich) as described (15).
**RNA interference**

Gene silencing of E-cadherin expression by the IGR-Heu cell line was performed using specific siRNA-E1 (GCACGUACACAGCCCUAAUtt #146381) or siRNA-E2 (GAGUGAAUUUGAAGAUUGtt #44988) purchased from Ambion, as described (12). Briefly, cells were transfected by electroporation with 0.8 nM of siRNA in a gene Pulser Xcell electroporation system (Bio-Rad) 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, CGUACGCUGAUAUCUUCGAdTdT and UCGAAGUAUUCCGCGUCGCdTdT), included as a negative control, was purchased from Sigma-Proligo.

**Cytotoxicity experiments and cytokine release assays**

Cytotoxic activity of the T-cell clones was measured by conventional 4 h $^{51}$Cr-release assay as described (12). Autologous tumor cell line IGR-Heu, treated or not with specific siRNA, was used as a target. E:T ratios were 30:1, 10:1, 3:1 and 1:1. Cell supernatants were then transferred to LumaPlateTM-96 wells (PerkinElmer), dried down and counted on Packard’s TopCount® NXT™. Percent specific cytotoxicity was calculated conventionally (16).

For cytokine release, Heu171 and H32-22 T cells (3 x $10^3$/well) were co-cultured in the absence or presence of IGR-Heu (3 x $10^4$/well), transfected or not with specific siRNA or ICAM-1 encoding vector, for 6 h. For additional experiments, T-cell clones were stimulated with recombinant molecules, combined or not with anti-CD3 mAb, for 3 h. Culture supernatants were then tested for IFN-γ production by ELISA (eBioscience).

**T-cell adhesion assay under flow stress condition**
Shear stress adhesion experiments were carried out in a commercial flow chamber composed of six independent flow channels (µ-Slide VI 0.4 hydrophobic ibiTreat, IBIDI Biovalley). T-cell clones were stained by CellTracker green (Invitrogen) and incubated for 15 min on a monolayer of IGR-Heu cells pre-cultured for 48 h in IBIDI chambers. Adhesion was then recorded under a constant shear stress of 35 dyn/cm² for 1 min at 2 s intervals (corresponding to one time-lapse). Shear stress was initiated by a syringe pump filled with 37°C pre-warmed medium and calculated using the formula \[ \tau = 1,761 \sqrt{\Phi} \text{ dyn/cm}^2 \], where \( \tau \) is the shear stress and \( \Phi \) is the flow. Flow assay was visualized in real time using a fluorescence microscope (LSM-510; Carl Zeiss Microimaging Inc). Adherent cells were counted using the Image J program and values were normalized to the number of initial adherent cells, set at 100%.

**Electron microscopy**

CTL clones were mixed at a 2:1 E:T ratio with IGR-Heu tumor cells pretreated or not with siRNAs targeting E-cadherin, plated immediately into poly-L-lysine-treated slides (Sigma-Aldrich) and incubated at 37°C for 15 or 30 min to allow cells to form T cell/target cell conjugates. Conjugates were fixed by the addition of 2.5% gluteraldehyde in 0.1 M phosphate buffer, pH 7.4, directly into the incubation medium and left for 30 min at room temperature. Cells were post-fixed with 1% osmium tetroxide, dehydrated with 100% ethanol and embedded in epoxy resin. Finally, ultrathin slices (70 to 100 nm) were cut from the resin blocks with a Reichert Ultracut S ultramicrotome, stained with lead citrate and uranyl acetate and examined in a transmission electron microscope (JEOL 1011). Quantitative analyses of electron micrographs were performed as described previously (17). T cell-tumor cell intermembrane spaces were measured in nanometer (nm) at x 20,000 and x 25,000 enlargements.
Confocal microscopy

Tumor cells, treated or not with specific siRNA, and effector cells were plated on poly-(L-lysine)-coated coverslips (Sigma-Aldrich) at 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. Fixed cells were stained with anti-granzyme B or anti-IFN-γ mAb, to follow, respectively, polarization of cytotoxic granules or the cytokine in the contact area between effector and tumor cells, and then with a secondary mAb coupled to Alexa-Fluor-488 (Molecular Probes™, Invitrogen). For IFN-γ staining, T cells were first treated for 4 h with Brefeldin A to inhibit cytokine secretion before coculture with target cells for 4 h. All Abs were diluted in PBS containing 1 mg/mL of BSA. Nuclei were stained with TO-PRO®-3 iodide (Molecular Probes™, Invitrogen). Coverslips were mounted with Fluoromount-G (Southern Biotech) and analyzed the following day using a fluorescence microscope (Carl Zeiss LSM-510). Z-projection of slices was performed using LSM Image Examiner software (Zeiss). F-actin polymerization at the IS was monitored by phalloidin staining. The contact area formed between CTL and tumor cells was defined, and F-actin content (mean fluorescence intensity, MFI) was determined using Image J software.
Results

Role of accessory molecules in triggering the cytotoxic activity of antitumor CTL

We had previously reported that CD103 plays an essential role in TCR-mediated target cell lysis through interaction with its ligand, the epithelial cell marker E-cadherin, on autologous lung cancer cells (12). To further investigate the role of CD103 and LFA-1 and their respective ligands in the effective phase of TCR-mediated cytotoxicity, we used CD103+/LFA-1+ (Heu171 TIL) or CD103-/LFA-1+ (H32-22 PBL) T-cell clones and specific tumor cell line IGR-Heu (E-cadherin+/ICAM-1-) stably transduced or not with ICAM-1 or transiently transfected with siRNA targeting E-cadherin (siRNA-E1 or siRNA-E2) (Supplementary Fig. 1). Chromium release assay indicated that CD103+/LFA-1+ CTL (Heu171) lysed both parental and ICAM-1-transduced IGR-Heu cells and that siRNA-E1 and siRNA-E2 completely inhibited T-cell-clone-mediated cytotoxicity (Fig. 1A, left panel). Similar results were obtained with a CD103+/LFA-1+ PBL-derived CTL clone (data not shown). As expected, the CD103-/LFA-1+ clone (H32-22) was able to efficiently kill only ICAM-1-transduced IGR-Heu cells, but to a lesser extent than the Heu171 clone (Fig. 1A, right panel).

To further determine the role of CD103 and LFA-1 engagement in the release of secretory lysosomes, we incubated the two clones on plastic dishes coated with E-cadherin-Fc or ICAM-1-Fc (used at an optimal concentration of 5 μg/ml), in combination or not with a low dose of anti-CD3 mAb (0.5 μg/ml), and then evaluated lytic granule exocytosis by measuring lysosomal-associated membrane glycoprotein-1 (LAMP-1, CD107a) externalization on the T-cell surface (12). As a positive control, T cells were cultured with a high dose of UCHT1 mAb (10 μg/ml), which is able to trigger granule secretion. Immunofluorescence analyses did not reveal any CD107a induction at the surface of either clone (Heu171 and H32-22 T cells).
stimulated with the recombinant molecules alone or with the low concentration of UCHT1, even after 3 h of incubation (Fig. 1B). In contrast, a combination of immobilized ICAM-1-Fc and a suboptimal concentration of anti-CD3 mAb triggered CD107a externalization on both the CD103+/LFA-1+ (left panel) and CD103−/LFA-1+ (right panel) clone surface. In agreement with our previous results (18), immobilized E-cadherin-Fc combined with a low concentration of UCHT1 induced externalization of CD107a on the CD103+/LFA-1+ clone (Fig. 1B). As a control, fibronectin-Fc combined or not with anti-CD3 mAb had no effect on CD107a induction at the surface of either clone (data not shown). These results demonstrate that, together with TCR, engagement of integrins, namely CD103 or LFA-1, is required for lytic granule exocytosis and target cell lysis.

**Influence of accessory molecules on cytokine production by T-cell clones**

Next, we analyzed cytokine secretion by the two clones stimulated with parental IGR-Heu cells or with IGR-Heu stably transduced with ICAM-1 or transiently transfected with siRNA-E1 or siRNA-E2. Results indicated that both CD103+ and CD103− T-cell clones were able to secrete IFN-γ (Fig. 2A) and TNF-β (data not shown) following specific stimulation with autologous tumor cells transfected or not with ICAM-1. Figure 2A also shows that treatment of IGR-Heu with siRNA-E1 or siRNA-E2 partially inhibited IFN-γ release by CD103+ clone Heu171 suggesting that CD103 engagement potentiates cytokine production.

We then measured IFN-γ secretion by CD103+/LFA-1+ (Heu171 TIL) and CD103−/LFA-1+ (H32-22 PBL) clones stimulated with plastic-coated E-cadherin-Fc or ICAM-1-Fc, combined or not with anti-CD3 mAb. Results indicated that, similarly to cytotoxic granule exocytosis, both recombinant molecules failed to induce cytokine secretion by the two clones. In contrast, engagement of CD103 and/or LFA-1 with their respective ligands together with TCR, by low
concentrations of UCHT1 (0.5 μg/ml), triggered cytokine production by the CD103+/LFA-1+ (Fig. 2B, left panel) and CD103-/LFA-1+ clone (Fig. 2B, right panel), respectively. As expected, a high concentration of UCHT1 used as a positive control induced cytokine production by both clones. Together, these results support the hypothesis that cytokine production by activated CD8 T cells requires the interaction of integrins with their respective ligands. They also emphasize the crucial role of adhesion molecules in tuning the T-cell activation threshold when TCR displays low avidity for the specific peptide (p)MHC-I complex.

T-cell adhesion strength under flow conditions

As stability of the IS is critical for the outcome of T-cell activation, further experiments were performed to examine the adhesion strength of T cells to specific target cells using a shear force in flow chambers. For this purpose, T lymphocytes were pre-incubated for 15 min on a monolayer of autologous tumor cells to allow T-cell adhesion, and then submitted to a continuous shear force for 1 min. Figure 3 shows that CD103+/LFA-1+ (Heu171 TIL, left panel) adhered more firmly to tumor cells (82% ± 2% versus 28% ± 2% at time lapse 12, shear stress 35 dyn/cm²) than CD103-/LFA-1+ (H32-22 PBL, right panel). To evaluate the role of integrin engagement with its ligand in the adhesion strength of T cells to their specific targets, we used IGR-Heu, treated or not with siRNA-E1 or siRNA-E2, and IGR-Heu-ICAM-1. Results indicated that expression of ICAM-1 on tumor cells induced firm adhesion of the CD103-/LFA-1+ PBL clone, which reached levels similar to those of the CD103+/LFA-1+ TIL clone cultured on parental or ICAM-1-transfected IGR-Heu (90 ± 2% and 85% ± 2.5% respectively at time lapse 12; Fig. 3). In contrast, knockdown of E-cadherin with specific siRNA resulted in a strong decrease in the strength of adhesion of CD103+ Heu171 T cells to
siRNA-E1 or siRNA-E2-treated tumor cells (22% ± 2% or 20% ± 1.5% at time lapse 12 respectively).

We then examined the ability of the T-cell clones to adhere to E-cadherin-Fc or ICAM-1-Fc using coverslips coated with the recombinant molecules, combined or not with a low concentration of UCHT1 mAb and submitted to increasing shear flow. A high concentration of anti-CD3 mAb (5 μg/ml), which induced strong arrest of both TIL and PBL clones (Supplementary Fig. 2), was used as a positive control. Tight adhesion of the CD103+)/LFA-1+ TIL clone to ICAM-1-Fc or ICAM-1-Fc combined with a suboptimal concentration of UCHT1 was observed (Supplementary Fig. 2, left panel). Weaker but sustained T-cell adhesion was also observed with E-cadherin-Fc combined with anti-CD3 mAb. For the CD103−/LFA-1+ PBL clone, a high percentage of cells adhered to ICAM-1-Fc alone or combined with low concentrations of anti-CD3 mAb. As expected, marginal adhesion of the PBL clone to E-cadherin-Fc or E-cadherin-Fc conjugated with anti-CD3 was detected (Supplementary Fig. 2, right panel), and the two clones failed to adhere in the presence of low concentrations of anti-CD3 mAb (Supplementary Fig. 2). Overall, these data indicate that conjugates formed between IGR-Heu epithelial tumor cells and the CD103+ TIL clone are more stable than those formed with the CD103− PBL clone. They also demonstrate that the CD103-E-cadherin or LFA-1-ICAM-1 interaction, together with TCR engagement, plays a major role in tight adhesion of CTL to specific tumor cells.

**Ultrastructure of the IS formed between CTL and specific tumor cells**

To further investigate the role of CD103 integrin clustering in maturation of the cIS, we compared the ultrastructure of the contact zone in conjugates formed between the TIL or PBL clone and the specific tumor cell line by electron microscopy. Figure 4 indicates that the IS
formed between E-cadherin+ IGR-Heu and CD103+ Heu171 TIL, thereafter named mature cIS (mcIS), was characterized by a cohesive structure with tight membrane contacts separated by large clefts (upper panels). In contrast, the IS formed between IGR-Heu and CD103- H32-22 PBL, thereafter named immature cIS (icIS), was loose, with multiple protrusions in the effector cell membrane observed after 15 and 30 min of co-culture (Fig. 4, lower panels). We then examined the role of CD103 in the cohesive ultrastructure of the mcIS formed between TIL and tumor cells by silencing E-cadherin expression in IGR-Heu using specific siRNA. Results indicated that the IS formed between Heu171 TIL and siRNA-E1-treated IGR-Heu cells was similar to that formed between the H32-22 PBL clone and untreated IGR-Heu at both 15 and 30 min of coculture (Fig. 4). siRNA-Luc control had no effect on IS ultrastructure.

To examine more precisely the ultrastructure of the IS formed between the PBL or the TIL clone and tumor cells, treated or not with specific siRNA, we quantified the size of the synaptic cleft from electron micrographs. Results indicated that the averages contact lengths of the synapse formed between IGR-Heu and Heu171 TIL were 56 ± 36 nm and 52 ± 19 nm at 15 and 30 min of coculture respectively, and 784 ± 410 nm and 648 ± 352 nm for those formed with H32-22 PBL. Treatment of IGR-Heu with siRNA-E1 induced a looseness of the IS formed with the TIL clone with a contact length of 492 ± 313 nm and 743 ± 442 nm at 15 and 30 min of coculture respectively. In contrast, siRNA-Luc had only a marginal effect on the IS contact lengths (84 ± 40 nm and 82 ± 48 nm respectively, Fig. 4). These data further suggest that the αEβ7 integrin plays a major role in the cohesiveness of the mcIS formed between activated CTL and cognate epithelial tumor cells. They also demonstrate that mcIS and icIS display different morphologies, and that CD103 engagement at the IS determines its ultrastructure, and thereby T-cell effector functions.
The interaction of CD103 with E-cadherin is essential for cytokine and lytic granule polarization

We then compared the behavior of mcIS versus icIS by following cytotoxic granule and cytokine localization in conjugates formed between CD103+/LFA-1+ (Heu171 TIL) or CD103+/LFA-1+ (H32-22 PBL) T-cell clones and IGR-Heu parental cells, electroporated or not with siRNA-E1 or siRNA-E2, or IGR-Heu-ICAM-1 transfectants. Confocal microscopy analyses revealed polarization of lytic granules, as defined by granzyme B labeling (Fig. 5, left panels), and IFN-γ (Fig. 5, central panels) at the mcIS formed between Heu171 TIL and IGR-Heu cells. In contrast, no synaptic polarization of granzyme B (Fig. 5, left panels) or IFN-γ (Fig. 5, central panels) was observed in the H32-22 PBL clone conjugated with tumor cells. Granzyme B and IFN-γ polarization were observed, respectively, in 62% ± 1% and 57% ± 2% of conjugates (n=56) formed between Heu171 and IGR-Heu, but only in 26% ± 1% and 23% ± 1% of conjugates formed between H32-22 and tumor cells (Fig. 6A). Importantly, silencing of E-cadherin using siRNA-E1 or siRNA-E2 did not alter formation of conjugates between Heu171 and IGR-Heu (12), but resulted in strong inhibition of cytotoxic granule (Fig. 5, left panels) and cytokine (Fig. 5, central panels) polarization at the IS formed between CD103+ TIL and target cells. Indeed, only 24% ± 1% and 21% ± 1% (siRNA-E1) or 34% ± 1% and 30% ± 1% (siRNA-E2) of conjugates displayed polarized granzyme-B-containing granules and IFN-γ, respectively (Fig. 6A, left panel). In contrast, a marginal effect was obtained with the siRNA-Luc negative control, since 60% ± 1% and 54% ± 1% of analyzed conjugates exhibited lytic granules and cytokine polarization, respectively. Transfection of IGR-Heu with ICAM-1 induced relocalization of granzyme B (Fig. 5, left panels) and IFN-γ (Fig. 5, central panels) in, respectively, 58% ± 1% and 47% ± 1% (Fig. 6A, right panel) of conjugates formed with the CD103+/LFA-1+ PBL clone. CD103+/LFA-1+ TIL conjugated with
IGR-Heu-ICAM-1 cells displayed granzyme B and IFN-γ polarization at the IS (Fig. 5) in 62% ± 1% and 59% ± 1% of conjugates (Fig. 6A, left panel), respectively.

Protein synaptic polarization correlated with increased F-actin polymerization at the mcIS, as shown by phalloidin staining (Fig. 5, right panels). Indeed, we observed a high F-actin content (MFI= 3,195), which accumulated at the contact area between CD103⁺/LFA-1⁺ TIL and tumor cells (Fig. 6B, left panel). In contrast, weaker F-actin content (MFI= 2,020) was detected at the icIS formed between CD103⁻/LFA-1⁺ PBL and IGR-Heu (Fig. 6B, right panel). Moreover, treatment of tumor cells with siRNA-E1 or siRNA-E2 reduced F-actin content (MFI= 2,004 or MFI= 2,400) at the IS formed between Heu171 CTL and the autologous target (Fig. 6B, left panel). In contrast, siRNA-Luc had only a weak effect on F-actin polymerization at the contact zone formed between Heu171 TIL and IGR-Heu (MFI= 3,089). Notably, transfection of IGR-Heu with ICAM-1 increased F-actin content at the IS formed between H32-22 PBL and transduced cells (MFI= 3,078), but had no additional effect on F-actin polymerization (MFI= 3,450) at the IS formed with the TIL clone (Fig. 5, right panels and Fig. 6B). These results further emphasize the influence of CD103 and LFA-1 integrins in maturation of the cIS.
Discussion

Interaction of CTL with specific target cells involves several cellular events that take place in a sequential fashion. First, T cells migrate towards target cells and initial cell-to-cell contact is most likely antigen-independent, since the interaction between the TCR and the specific pMHC complex often displays very weak affinity (19-22). This contact probably relies on the interaction of CD2 with LFA-3, widely expressed in resting cells (23, 24), which may bring cell membranes to the close apposition necessary for TCR engagement (25, 26). LFA-1, which is usually in an inactive state on the T-cell surface (27), may be activated by antigen-specific and non-specific signals provided, respectively, by TCR occupancy and chemokines (25, 28, 29). Adhesion of activated LFA-1 to ICAM-1 on APC would then strengthen the T-cell/target cell contact following TCR recognition of specific pMHC (28, 30). This firm adhesion provided by the LFA-1-ICAM-1 interaction appears to be a prerequisite for effective target cell lysis by activated CTL (11, 31). Interaction of the CD103 integrin, induced in a large proportion of CD8⁺ TIL following TCR engagement within a TGF-β1-rich microenvironment, with E-cadherin on epithelial tumors, also provides T-cell/target cell tight adhesion and subsequent cytotoxicity (12, 32). In this report, we show that the interaction of LFA-1 or CD103 with its respective ligand is required for killing of autologous malignant cells (Fig. 1A) and, to a lesser extent, for cytokine production by CTL clones after stimulation with specific target cells (Fig. 2A). These results suggest that cytokine production is easier to trigger by activated T cells than lytic granule release. This may result in amplification of antitumor CTL responses, such as by enhancing MHC-I and ICAM-1 molecule expression on target cells induced by IFN-γ release. Previous reports showed that CTL activation to cytokine production is a slow process requiring prolonged TCR occupancy and sustained signaling, whereas secretory lysosome release occurs within a few minutes after initial contact between T cells and target cells (33, 34). It should, however, be noted that these studies were based on
antigenic peptide concentrations required to elicit different biological responses in human CTL clones, and did not take into account the involvement of integrins in triggering both T-cell activities. In addition, CD8 T lymphocytes must be activated so as to produce the lytic machinery necessary for target cell killing; such production may be a long process that is likely just as demanding as cytokine production.

Experiments based on stimulation of the CD103+/LFA-1+ TIL clone and the CD103−/LFA-1+ PBL counterpart with low concentrations of anti-CD3 mAb demonstrated that a combination with ICAM-1-Fc is required to trigger cytotoxic granule exocytosis (Fig. 1B) and IFN-γ production (Fig. 2B). As expected, activation of only the TIL clone with E-cadherin-Fc, combined with a low dose of UCHT1, induced CD107a externalization and cytokine secretion. These results further emphasize the role of integrins in triggering T-cell functions by reinforcing the strength of the rather weak TCR-pMHC interaction and by providing co-stimulatory signals that lower the activation threshold for both degranulation and cytokine production. Differences between the two integrins in granule exocytosis by the TIL clone may be due to the differential activation status of CD103 compared to LFA-1. Activation of LFA-1 involves diverse receptors and signaling pathways that result in transformation of the integrin from a bent, resting form to an extended conformation, displaying distinct states of ligand binding activity (31). This well-characterized, highly regulated activation process is essential for T-cell effector functions (35). In contrast, little is known about regulation of CD103 activity or the signaling events that drive integrin activation. We previously reported that CD103 ligation to E-cadherin triggers “outside-in” signals that promote phosphorylation of ERK1/2 kinases and phospholipase Cγ1, and that this integrin plays a unique co-stimulatory role in antitumor CTL activation (18).
Interactions between lymphocytes and the vascular endothelium are essential for recruitment of T cells to inflammatory tissues and ensuing achievement of an efficient immune response. A multistep process involving tethering and rolling of lymphocytes on endothelial cells and rapid activation of integrins, followed by firm arrest of the cells before extravasation into target tissues, has been widely documented (36-38). β2 integrins LFA-1 and Mac-1 (αMβ2) and α4 integrins such as VLA-4 (α4β1) and α4β7 play a major role in arrest of rolling leukocytes in blood vessels by binding to their respective available ligands (39-41). With regard to the αEβ7 integrin, it is assumed that it serves to locate T cells in epithelial tissues by engaging E-cadherin (42-44). A role of CD103 in shaping intra-epithelial morphogenesis of some leukocytes has also been reported (45). Here we show that CD103 mediates arrest of T cells in epithelial tumors under shear stress by interacting with E-cadherin (Fig. 3). Indeed, compared to the CD103− PBL clone, the CD103+ TIL clone adheres more firmly to autologous tumor cells in shear flow conditions, and silencing of E-cadherin with specific siRNA inhibits T-cell adhesion. Moreover, these cell adhesion forces appeared optimal, since transfection of tumor cells with ICAM-1 and its subsequent interaction with LFA-1 on the TIL clone surface did not further enhance T-cell arrest. Optimal firm adhesion under flow shear rates was observed with the two clones cultured on a combination of ICAM-1-Fc and low concentrations of anti-CD3 mAb. Sustained adhesion was also observed with the TIL clone to a combination of E-cadherin-Fc and UCHT1 (supplementary Fig. 2). These findings have relevance to our understanding of how CD103 mediates attachment of specific T cells in epithelial tissues under conditions of vascular shear flow and further emphasize the critical role of this integrin in T-cell retention in epithelial cancers and in optimization of the antitumor immune response.
The role of the IS formed between CTL and specific tumor cells remains elusive and might trigger several distinct biological functions. The LFA-1-ICAM-1 interaction plays a central role in maturation of the cIS, and thus in T-cell-mediated target cell killing (36-38, 46). CD103 also plays an important role in maturation of cIS formed between CD8+ TIL and epithelial tumor cells leading to target cell lysis. We show here that maturation of the cIS is also required for a synaptic secretion of IFN-γ by activated tumor-specific CTL and that formation of an icIS is associated with a multifocal cytokine release. Accordingly, it has been reported that formation of a cSMAC is not required for activation of naive CD8 T cells, supporting the notion that one of the major roles of a mature IS is directed delivery of effector functions (47). This is in agreement with the observation that mature (full) IS formation is not always required for activation of T cells (17, 22, 48) and that strong interaction forces would tend to favor optimal activation. Electron microscopy studies revealed morphological differences between mcIS and icIS, regulated by integrin engagement, which have direct consequences on T-cell effector functions (Fig. 4). Indeed, while the mcIS is characterized by a cohesive ultrastructure with tight membrane contacts separated by large clefts, the icIS is loose, with multiple protrusions in the effector cell membrane. Moreover, knockdown of E-cadherin in cancer cells by specific siRNA results in loss of the cohesiveness specific to a mcIS, which acquires morphology similar to that of the icIS. High-resolution electron microscopy showed that the mcIS formed between killer cells and specific target cells are characterized by exocytic and endocytic organelles polarized towards the centrosome at the plasma membrane, which forms a focal point for exocytosis and endocytosis within the synapse (8, 49). Release of cytolytic granules is confined to secretory clefts that provide a confined space in which cytotoxic proteins are kept concentrated for target cell attack (8, 50, 51). Directed delivery of other effector molecules, such as IFN-γ and other Th1 cytokines, through mcIS secretory clefts, has the advantage of increasing their local concentrations,
which may result in optimization of cancer cell recognition by CTL and ensuing potentiation of the adaptive antitumor immune response.

The differential morphological structures of mcIS and icIS have consequences for T-cell/target cell adhesive strength and molecular rearrangement, as revealed by confocal microscopy (Fig. 5). Indeed, the mcIS appeared more cohesive than the icIS and was characterized by increased F-actin polymerization and lytic granule and cytokine polarization at the contact area between CD103+/LFA-1+ T cells and tumor cells. Importantly, silencing of E-cadherin decreased the IS F-actin content and inhibited synaptic relocalization of IFN-γ and granzyme B, which correlated with abrogation of tumor cell killing. In contrast, icIS displayed weaker F-actin content, multifocal cytokine secretion and the inability to relocalize lytic granule machinery towards the opposing cells (Fig. 6). Protein polarization and tumor cell lysis by the CD103+/LFA-1+ PBL clone were achieved after transfection of target cells with ICAM-1, supporting the observation that the TCR-pMHC-I interaction is not sufficient to trigger granule polarization and that integrin engagement is a prerequisite for efficient target cell killing (12). Overall, these results provide biophysical values for the interaction forces between T cells and tumor cells, and correlate cell adhesion strength, mainly regulated by integrins, with maturity of the cIS and its ultrastructure, and with activation of CTL effector functions. Our results emphasize the crucial role of integrin-ligand pairs, in particular CD103-E-cadherin pair, in CTL functional activities and in arrest of tumor-specific CD8 T cells within epithelial tumors. Thus, by controlling retention of tumor-specific CTL, their cytokine production and their cytotoxicity towards cancer cells, CD103 contributes to the outcome of antitumor immune responses. A better knowledge of the molecular mechanisms that enhance the adhesion strength between CD8 T cells and epithelial tumor cells, and thus
stimulate CTL effector functions, may contribute to development of more effective adoptive T-cell therapies and vaccination strategies in the treatment of cancer.
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Figure Legends

Figure 1

A. Role of CD103 and LFA-1 in T-cell-clone-mediated cytotoxic activity towards autologous lung cancer cells. Cytotoxicity was determined by a conventional 4 h $^{51}$Cr-release assay at indicated E:T ratios. IGR-Heu tumor cells, stably transduced or not with ICAM-1 (IGR-Heu-ICAM-1) or transiently transfected with siRNA targeting E-cadherin (siRNA-E1 and siRNA-E2), were used as targets. siRNA-luciferase (siRNA-Luc) was used as a negative control. CD103$^+$/LFA-1$^+$ (Heu171, left panel) and CD103$^-$/LFA-1$^+$ (H32-22, right panel) T-cell clones were used as effector cells. B. CD107a induction on Heu171 (left panel) and H32-22 (right panel) clones during stimulation with plastic-coated E-cadherin-Fc (E-cadh-Fc) or ICAM-1-Fc (5 μg/ml), combined or not with a low concentration of anti-CD3 mAb (0.5 μg/ml). A high concentration of UCHT1 mAb (10 μg/ml) was used as a non-physiological positive control. Immunofluorescence analysis was performed at the indicated time course. Data correspond to means of 2 independent experiments.

Figure 2

Influence of CD103 and LFA-1 molecules in cytokine secretion by CD103$^+$/LFA-1$^+$ and CD103$^-$/LFA-1$^+$ T-cell clones. A. CD103$^+$/LFA-1$^+$ (Heu171, left panel) and CD103$^-$/LFA-1$^+$ (H32-22, right panel) CTL (3,000 cells) were stimulated for 24 h with autologous IGR-Heu, electroporated or not with siRNA-E1, siRNA-E2 or siRNA-Luc control, or with ICAM-1-transfected IGR-Heu (30,000 cells). IFN-$\gamma$ release in culture medium was measured by ELISA. * $p < 0.1$. B. Heu171 (left panel) and H32-22 (right panel) T cells (3,000 cells) were stimulated for 3 h with plastic-coated E-cadherin-Fc or ICAM-1-Fc (5 μg/ml), combined or not with 0.5 μg/ml of UCHT1 mAb, or with 10 μg/ml of anti-CD3 mAb used as a positive control. Data are representative of 3 independent experiments.
Figure 3
Adhesion of T-cell clones to autologous tumor cells under shear stress. CD103+/LFA-1+ (Heu171, panel left) and CD103+/LFA-1+ (H32-22, panel right) clones were stained by CellTracker green and incubated for 10 min on a monolayer of IGR-Heu cells, treated or not with indicated siRNA (siRNA-E1, siRNA-E2 and siRNA-luc) or transfected with ICAM-1, pre-cultured for 48 h in IBIDI chambers. Adhesion was then recorded under constant shear stress (35 dyn/cm² indicated by an arrow) for 1 min at 2 s intervals. Data correspond to 1 of 4 independent experiments.

Figure 4
CD103 shapes the morphology of the IS. Ultrastructure of the IS formed between T-cell clones and autologous target cells. Electron microscopic images of conjugates formed between CD103+ (Heu171, upper panels) or CD103- (H32-22, lower panels) T-cell clones (black arrow) and IGR-Heu tumor cells, treated or not with indicated siRNAs. CTL and target cells were incubated for 15 or 30 min, and 70 to 100 nm thin lead-stained sections were prepared as described in Materials and methods. The ultrastructure of the IS was analyzed using a transmission electron microscope. Scale represents 1µm. Values included under each panel correspond to mean size ± SD of synaptic clefts from indicated conjugate numbers of electron micrographs (n=). *** p < 0.001.

Figure 5
Interaction of CD103 or LFA-1 on T-cell clones with their respective ligands on target cells triggers cytotoxic granule and cytokine polarization at the IS and increased F-actin polymerization. Conjugates formed between CD103+/LFA-1+ (Heu171, upper panels) or
CD103+/LFA-1+ (H32-22, lower panels) effector T cells and IGR-Heu tumor cells, treated or not with indicated siRNA (siRNA-E1, siRNA-E2 and siRNA-luc) or transfected with human ICAM-1, were analyzed by confocal microscopy after 15 min of coculture. Lytic granule polarization, as defined by the accumulation of granzyme B in the contact area between effector and tumor cells (left panels), was followed up using anti-granzyme B mAb (green fluorescence). IFN-γ relocalization (central panels) was monitored using a specific mAb (green fluorescence). Nuclei were stained with TO-PRO®-3 iodide (blue fluorescence). F-actin polymerization at the IS (right panels) was detected by phalloidin staining (red fluorescence). Data correspond to 1 of 3 independent experiments. Bars correspond to 2.5 μm.

**Figure 6**

Engagement of integrins on CTL clones with their ligands on tumor cells triggers granzyme B and IFN-γ polarization and increased F-actin polymerization at the IS. **A.** Percentages of CTL displaying granzyme B or IFN-γ relocalization during conjugate formation between CD103+/LFA-1+ (Heu171, left panel) CTL and IGR-Heu cells, pretreated or not with indicated siRNA (siRNA-E1, siRNA-E2 and siRNA-luc) or transfected with ICAM-1, or between CD103+/LFA-1+ (H32-22, right panel) T cells and IGR-Heu or IGR-Heu-ICAM-1 targets. **B.** F-actin content at the IS formed T cells and tumor cells. F-actin polymerization was determined by phalloidin staining of conjugates formed between CD103+/LFA-1+ (Heu171, left panel) T cells and IGR-Heu cells, pretreated or not with the indicated siRNA or transfected with ICAM-1, or between CD103+/LFA-1+ (H32-22, right panel) T cells and IGR-Heu or IGR-Heu-ICAM-1 tumor cells. The contact area between T cells and target cells was defined and its F-actin content (MFI) determined using Image J software. Data shown represent mean ± SD of triplicate fields including 56 conjugates each (n= 56). **** p < 0.0001, *** p < 0.001.
Figure 1

(A) CD103^+LFA-1^+ (Heu171 TIL) and CD103^+LFA-1^+ (H32-22 PBL) cells were treated with various combinations of E-cadherin-Fc, ICAM-1-Fc, anti-CD3 (0.5 μg/ml), anti-CD3 (10 μg/ml), IGR-Heu siRNA-E1, IGR-Heu siRNA-E2, and IGR-Heu-ICAM-1. The percentage of lysis was measured at E:T ratios of 30:1, 10:1, 3:1, and 1:1.

(B) The percentage of CD107a^+ T cells was determined over time (30, 60, 120, 180 min) for cells treated with E-cadherin-Fc, ICAM-1-Fc, anti-CD3 (0.5 μg/ml), anti-CD3 (10 μg/ml), IGR-Heu siRNA-E1, IGR-Heu siRNA-E2, and IGR-Heu-ICAM-1.
Figure 3
Figure 6
CD103 or LFA-1 engagement at the immune synapse between cytotoxic T cells and tumor cells promotes maturation and regulates T-cell effector functions

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