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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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 the maturation of the cytotoxic immune synapse (cIS) and in the activation of CTL effector functions. Our results indicate that cytotoxicity toward cancer cells and, to a lesser extent, cytokine production by specific CTL require, together with TCR engagement, the interaction of either CD103 with E-cadherin or LFA-1 with ICAM-1. Flow-based adhesion assay showed 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. Experiments using confocal microscopy 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, interactive 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. Cancer Res; 73(2); 1–12. ©2012 AACR.

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 αβ 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 the formation of an "immune synapse" (IS; refs. 1, 2). According to the bull's eye model of the IS, the TCR and associated signaling molecules are clustered at the central supramolecular activation complex (cSMAC; ref. 3), while adhesion/costimulatory molecules, including LFA-1, are localized at the peripheral SMAC (pSMAC; ref. 4). Following 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 α7(CD103)β7 integrin (also called CD103) selectively expand within the lung tumor microenvironment, and that the interaction of CD103 with its ligand, the epithelial cell marker E-cadherin, on target cells plays an essential role in TCR-dependent cancer cell killing when ICAM-1 is deficient (12). Indeed,
αβ3-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 showed 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 showed 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 previously (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 cytoxicity 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 R&D Systems. Anti-CD8 mAb was purchased from BD Pharmingen.

Phenotypic analyses of tumor cells were carried out by direct or indirect immunofluorescence using a FACS Calibur (BD Biosciences) 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 previously (15).

RNA interference
Gene silencing of E-cadherin expression by the IGR-Heu cell line was carried out using specific siRNA-E1 (GACGCUAC-CAGCCCUAAUUtT; #146381) or siRNA-E2 (GAGUGAAUUU-GAAGAAUGtt; #44988) purchased from Ambion, as described previously (12). Briefly, cells were transfected by electroporation with 0.8 nmol/L of siRNA in a gene Pulser Xcell electroporation system (Bio-Rad) at 300 V, 500 μF using electroporation cuvettes (Eurogentec). A second electroporation was conducted after 24 hours, and cells were then cultured for 48 hours. Luciferase siRNA, siRNA-Luc (siRNA duplex, CGUAC-GCGGAUAUCUUCGAdTdT, and UCAGAAGUAUCCGCGUA-CGdTdT), included as a negative control, was purchased from Sigma-Prolo.

Cytotoxicity experiments and cytokine release assays
Cytotoxic activity of the T-cell clones was measured by a conventional 4-hour 51Cr-release assay as described previously (12). Autologous tumor cell line IGR-Heu, treated with specific siRNA or untreated, 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. Percentage of specific cytotoxicity was calculated conventionally (16).

For cytokine release, Heu171 and H32-22 T cells (3 x 10^3 /well) were cocultured in the absence or presence of IGR-Heu (3 x 10^5/well), transfected with specific siRNA or ICAM-1 encoding vector or nontransfected, for 6 hours. For additional experiments, T-cell clones were stimulated with recombinant molecules, alone or combined with anti-CD3 mAb, for 3 hours. 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 6 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 minutes on a monolayer of IGR-Heu cells precultured for 48 hours in IBIDI chambers. Adhesion was then recorded under a constant shear stress of 35 dyn/cm^2 for 1 minute at 2-second intervals (corresponding to 1 time-lapse). Shear stress was initiated by a syringe pump filled with 37°C prewarmed medium and calculated using the formula τ [dyn/cm^2] = 1,761 τ [mil/min], where τ is the shear stress and Φ 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 that were untreated or pretreated with siRNAs targeting E-cadherin, plated immediately into poly-L-lysine-treated slides (Sigma-Aldrich), and incubated at 37°C for 15 or 30 minutes to allow cells to form T cell/target cell conjugates. Conjugates were fixed by the addition of 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4, directly into the incubation medium and left for 30 minutes at room temperature. Cells were postfixed with 1% osmium tetroxide, dehydrated with 100% ethanol, and embedded in epoxy resin. Finally, ultrathin slices (70–100 nm thick) 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 carried out as described previously (17). T cell–tumor cell
intermembrane spaces were measured in nanometer (nm) at \( \times 20,000 \) and \( \times 25,000 \) magnification.

**Confocal microscopy**

Tumor cells, untreated or treated 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 hour and permeabilized with 0.1% SDS or Triton X100 for 10 minutes, followed by blocking with 10% FBS for 20 minutes. Fixed cells were stained with anti-granule mAb coupled to Alexa-Fluor-488 (Molecular Probes; Invitrogen). Coverslips were mounted with Fluoromount-G (Southern Biotech) and analyzed with a fluorescence microscope (Carl Zeiss LSM-510). Z-projection of slices was carried out using LSM Image Examiner software (Zeiss). F-actin polymerization was imaged with TCS-SP2 confocal microscopy (Carl Zeiss LSM-510). Z-projection of slices was carried out using LSM Image Examiner software (Zeiss). F-actin content (mean fluorescence intensity, MFI) was determined using Image J software.

**Results**

**Role of adhesion 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+ (Heu171 PBL) T-cell clones and specific tumor cell line IGR-Heu (E-cadherin-/+ICAM-1+) non-transduced or stably transduced with ICAM-1 or transiently transfected with siRNA targeting E-cadherin (siRNA-E1 or siRNA-E2; Supplementary Fig. S1). 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-cloned-mediated cytotoxicity (Fig. 1A, left). 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).

To further determine the role of CD103 and LFA-1 engagement in the release of secretory lysosomes, we incubated the 2 clones on plastic dishes coated with E-cadherin-Fc or ICAM-1-Fc (used at an optimal concentration of 5 \( \mu \)g/mL), alone or in combination with a low dose of anti-CD3 mAb (0.5 \( \mu \)g/mL), and then evaluated lytic granule exocytosis by measuring lysosome-associated membrane glycoprotein-1 (LAMP-1, the CD107a) externalization on the T-cell surface (12). As a positive control, T cells were cultured with a high dose of UCHT1 mAb (10 \( \mu \)g/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 hours 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 the surfaces of both CD103+/LFA-1+ (left) and CD103+//-LFA-1+ (right) clones. 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, either alone or combined with the low concentration of anti-CD3 mAb, had no effect on CD107a induction at the surface of either clone (data not shown). These results show that, together with TCR, engagement of integrins, namely CD103 or LFA-1, is required for lytic granule exocytosis and target cell lysis.

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

Next, we analyzed cytokine secretion by the 2 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-\( \gamma \) (Fig. 2A) and TNF-\( \beta \) (data not shown) following specific stimulation with autologous tumor cells transfected or not with ICAM-1. Fig. 2A also shows that treatment of IGR-Heu with siRNA-E1 or siRNA-E2 partially inhibited IFN-\( \gamma \) release by CD103− cell Heu171, suggesting that CD103 engagement potentiates cytokine production.

We then measured IFN-\( \gamma \) 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, alone or combined with anti-CD3 mAb. Results indicated that, similarly to cytotoxic granule exocytosis, both recombinant molecules failed to induce cytokine secretion by the 2 clones. In contrast, engagement of CD103 and/or LFA-1 with their respective ligands together with TCR, by low concentrations of UCHT1 (0.5 \( \mu \)g/mL), triggered cytokine production by the CD103+/+LFA-1+ (Fig. 2B, left) and CD103+//-LFA-1+ clones (Fig. 2B, right), 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 conducted 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...
preincubated for 15 minutes on a monolayer of autologous tumor cells to allow T-cell adhesion, and then submitted to a continuous shear force for 1 minute. Fig. 3 shows that CD103+/LFA-1+ (Heu171 TIL, left) adhered more firmly to tumor cells (82% ± 2% vs. 28% ± 2% at time lapse 12, with shear stress at 35 dyn/cm²) than CD103-/LFA-1+ (H32-22 PBL, right). 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, untreated or treated 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, alone or combined with a low concentration of anti-CD3 mAb. A high concentration of UCHT1 mAb (10 μg/mL) was used as a nonphysiologic positive control. Immunofluorescence analysis was carried out at the indicated time point. Data correspond to means of 2 independent experiments.

Figure 1. A, role of CD103 and LFA-1 in T-cell clone–mediated cytotoxic activity toward autologous lung cancer cells. Cytotoxicity was determined by a conventional 4 h 51Cr-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) and CD103+/LFA-1+ (H32-22, right) T-cell clones were used as effector cells. B, CD107a induction on Heu171 (left) and H32-22 (right) clones during stimulation with plastic-coated E-cadherin-Fc (E-cadh-Fc) or ICAM-1-Fc (5 μg/mL), without or combined 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 nonphysiologic positive control. Immunofluorescence analysis was carried out at the indicated time point. Data correspond to means of 2 independent experiments.
Fc conjugated with anti-CD3 was detected (Supplementary Fig. S2, right), and the 2 clones failed to adhere in the presence of low concentrations of anti-CD3 mAb (Supplementary Fig. S2). 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⁻/C0 PBL clone.

They also show 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. Fig. 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 (top). 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 minutes of coculture (Fig. 4, bottom). 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 minutes 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, untreated or treated 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 and 52 ± 19 nm at 15 and 30 minutes of coculture respectively, and 784 ± 410 and 648 ± 352 nm for those formed with H32-22 PBL. Treatment of IGR-Heu with siRNA-E1 induced a loosening of the IS formed with the TIL clone with a contact length of 492 ± 313 and 743 ± 442 nm at 15 and 30 minutes of coculture, respectively. In contrast, siRNA-Luc had only a marginal effect on the IS contact lengths (84 ± 40 and 82 ± 48 nm, respectively; Fig. 4). These data further suggest that the α7β7 integrin plays a major role in the cohesiveness of the mIS formed between activated CTL and cognate epithelial tumor cells. They also show that mIS and iIS 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 mIS with iIS 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, untreated or electroporated 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), and IFN-γ (Fig. 5, central panels) at the mIS formed between Heu171 TIL and IGR-Heu cells. In contrast, no synaptic polarization of granzyme B (Fig. 5, left) or IFN-γ (Fig. 5, center) 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. 5A). 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) and cytokine (Fig. 5, center) 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). 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) and IFN-γ (Fig. 5, center) in, respectively, 58% ± 1% and 47% ± 1% (Fig. 6A) 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), respectively.

Protein synaptic polarization correlated with increased F-actin polymerization at the mIS, as shown by phalloidin staining (Fig. 5, right). 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). In contrast, weaker F-actin content (MFI, 2,020) was detected at the iIS formed between CD103+/LFA-1+ PBL and IGR-Heu (Fig. 6B, right). Moreover, treatment of tumor cells with siRNA-E1 or siRNA-E2 reduced F-actin content (MFI, 2,040 or MFI, 2,400) at the IS formed between Heu171 CTL and the autologous target (Fig. 6B, left). 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 and Fig. 6B). These results further emphasize the influence of CD103 and LFA-1 integrins in the 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 toward target cells and initial cell-to-cell contact is most likely antigen-independent, because 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 nonspecific 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 lysis.
cell killing; such production may be a long process that is likely just as demanding as cytokine production.

Experiments conducted by stimulation of the CD103+/LFA-1+ TIL clone and the CD103-/LFA-1+ PBL counterpart with low concentrations of anti-CD3 mAb showed 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 costimulatory signals that lower the activation threshold for both degranulation and cytokine production. Differences between the 2 integrins in granule exocytosis by the TIL clone may be due to the differential activation status of CD103 compared with 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 costimulatory role in antitumor CTL activation (18).
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 intratumoral 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 with 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, because 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 2 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. S2). 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 biologic 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 1 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 morphologic differences between mclIS and icIS, regulated by integrin engagement, which have direct consequences on T-cell effector functions (Fig. 4). Indeed, while the mclIS 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 the mclIS, which acquires morphology similar to that of the icIS. High-resolution electron microscopy showed that the mclIS formed between killer cells and specific target cells are characterized by exo- and endocytic organelles polarized toward 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 mclIS 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 morphologic structures of mclIS and icIS have consequences for T-cell/target cell adhesive strength and molecular rearrangement, as revealed by confocal microscopy (Fig. 5). Indeed, the mclIS 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 toward 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 toward 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support

This work was financially supported by grants from the INSERM, the Association pour la Recherche sur le Cancer (ARC), the Institut National du Cancer (INCa), and the Ligue contre le Cancer and the Cancérologue Ile de France.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 2, 2012; revised October 15, 2012; accepted November 15, 2012; published OnlineFirst November 27, 2012.


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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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Cancer Res  Published OnlineFirst November 27, 2012.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-2569

Supplementary Material  Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/11/27/0008-5472.CAN-12-2569.DC1

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