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TGF-β signaling intersects with CD103 integrin signaling to promote T lymphocyte accumulation and antitumor activity in the lung tumor microenvironment

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Running title: Role of CD103 and TGF-β in CTL functions.

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Abbreviations: ICAM: intercellular adhesion molecule; ILK: integrin-linked kinase; IS: immune synapse; LFA: leukocyte function-associated antigen; mAb: monoclonal antibody; PBL: peripheral blood lymphocyte; Phospho: phosphorylated; PKB: protein kinase B; r:

recombinant; rE-cadh-Fc: rE-cadherin-Fc; TGFBR: TGR-beta receptor; TCR: T-cell receptor; TIL: tumor-infiltrating lymphocyte.

Abstract

Homing of $CD8^+$ T lymphocytes to the tumor microenvironment is an important step for mounting a robust antitumor immune response. TGF-β is responsible for CD103 ($\alpha_E\beta_7$) integrin induction in activated intraepithelial $CD8^+$ T lymphocytes. However, the interplay between TGF-β and CD103 and their contribution to T-cell infiltration and antitumor activity remain unknown. Here, we used viable human lung tumor slices and autologous tumor antigen-specific T-lymphocyte clones to provide evidence that CD103 is directly involved in T-lymphocyte recruitment within epithelial tumor islets and intratumoral early T-cell signaling. Moreover, TGF-β enhanced CD103-dependent T-cell adhesion and signaling, whereas it inhibited leukocyte function-associated antigen (LFA)-1 ($\alpha_L\beta_2$) integrin expression and LFA-1-mediated T-lymphocyte functions. Mechanistic investigations revealed that TGF-β bound to its receptors (TGFBR), which promoted the recruitment and phosphorylation of integrin-linked kinase (ILK) by TGFBR1. We further show that ILK interacted with the CD103 intracellular domain, resulting in protein kinase B (PKB)/AKT activation thereby initiating integrin inside-out signaling. Collectively, our findings suggest that the abundance of TGF-β in the tumor microenvironment may in fact engage with integrin signaling pathways to promote T-lymphocyte antitumor functions, with potential implications for T-cell-based immunotherapies for cancer.

Introduction

Adequate positioning of tumor antigen-specific T cells into tumor tissues plays a central role in an effective antitumor immune response. Hence, the number of $CD8^+$ T cells found in contact with tumor cells is usually associated with good outcome in cancer patients (1). Recruitment of T lymphocytes within the tumor involves multiple sequential steps regulated by molecular interactions with several stromal cell types and coordinated by chemotactic agents, cell surface adhesion molecules and extracellular matrix (ECM) proteins. Integrins are one of the major families of adhesion molecules that mediate cell-ECM and cell-cell interactions (2). Adhesion of T cells to ECM proteins or cellular integrin ligands is fundamental for lymphocyte development, migration, extravasation and activation (3, 4). Among the integrin family members, LFA-1 (α_L/β_2, CD11a/CD18) is essential for lymphocyte homing in lymphoid and non-lymphoid tissues, transmigration and antigen-specific T-cell responses (3). On naive T lymphocytes, LFA-1 has weak affinity for its ligands, the intercellular adhesion molecules (ICAM). However, T-cell stimulation through T-cell receptor (TCR) or chemokine receptors triggers an "inside-out" signal that results in LFA-1 activation by inducing integrin-extended conformation and clustering, thereby increasing its affinity for its ligands (5). Firm adhesion of LFA-1 to ICAM-1 (CD54) initiates an "outside-in" signal that has co-stimulatory functions in TCR signaling, thus contributing to T-cell stimulation, spreading and cytotoxicity (6, 7).

The α_E(CD103)β_7 integrin, hereafter referred to as CD103, plays a crucial role in TCR-mediated cytokine secretion and cytotoxic activity toward epithelial tumors by interacting with its ligand, epithelial cell marker E-cadherin, on target cells (8, 9). This integrin is essential in controlling $CD8^+$ tumor-infiltrating lymphocyte (TIL) activities, not only by promoting effector T-cell adhesion to tumor cells, but also by triggering intracellular

signaling events that co-stimulate TCR signals (10). A role for CD103 in T-cell homing into epithelia has been suggested (11, 12); however, accumulating evidence indicates a role for CD103 in retention of T-lymphocyte subpopulations in epithelial tissues (13). Consistently, CD103 mediates arrest of T lymphocytes by interacting with E-cadherin on epithelial tumors (9) and recruiting CCR5 at the immune synapse (IS) with cancer cells (14). Moreover, an enhanced $CD103^+$ TIL subset correlates with improved patient survival in ovarian (15) and lung (16) carcinomas and increased intraepithelial lymphocyte infiltration, suggesting that CD103 promotes recruitment of TIL within epithelial tumor islets. However, the true contribution of CD103 to promoting T-cell infiltration into tumor regions, and the influence of TGF-β, abundant within the tumor microenvironment and responsible for *ITGAE* gene expression in TCR-engaged $CD8^+$ T lymphocytes (10, 17, 18), on integrin functions remain poorly understood. Using viable human tumor slices (19) and autologous tumor antigen-specific CTL clones, we show here that CD103 promotes T-cell recruitment within epithelial tumor regions and enhances intratumoral T-cell early signaling. We also show that TGF-β upregulates CD103-dependent T-cell effector functions by triggering TGFBR1 (activin receptor-like kinase-5, ALK5)-mediated phosphorylation of ILK and its subsequent binding to CD103, thus initiating inside-out signaling leading to activation of the integrin and strengthening of CD103-E-cadherin adhesion.

Materials and methods

Tumor cell lines, T-cell clones and freshly isolated TIL

IGR-Heu and IGR-Heu-ICAM-1 tumor cell lines were derived in one of our laboratories in 1996 as previously described (8). Heu171 ($CD103^{+}$/$LFA\text{-}1^{+}$) and H32-22 ($CD103^{-}$/$LFA\text{-}1^{+}$) T-cell clones were generated from autologous TIL and peripheral blood lymphocytes (PBL), respectively. Cells were regularly tested and authenticated by immunofluorescence analysis and cytotoxic assay including in the present work. Fresh $CD8^{+}$ TIL were isolated using a FACS ARIA cell sorter.

Recombinant molecules, antibodies and chemical inhibitors

Recombinant (r) E-cadherin-Fc (rE-cadh-Fc), rICAM-1-Fc and rFibronectin-Fc molecules were provided by R&D Systems. rCXCL12 and rTGF-β was purchased from PeproTech and Abcys, respectively. Anti-CD103 and anti-β_2 mAb were purchased from Beckman Coulter, and PE-conjugated anti-CD103 from eBiosciences. Anti-CD3 (UCHT1) was provided by Ozyme and anti-CD8 by BioLegend. Anti-ILK and anti-phosphorylated (phospho)-ILK (Ser-246) mAb were purchased from Millipore. Anti-TGFBRI and secondary mAb were provided by Santa Cruz Biotechnology. Anti-AKT/PKB and anti-phospho-AKT were purchased from Cell-Signaling. Anti-KLRG1 was a gift from H Pircher (Freiburg, Germany). TGRBR1 kinase inhibitor SB-431542 (SB) and PI3K inhibitor Wortmanin were supplied by Sigma-Aldrich. Phospho-AKT inhibitor MK-2206 was purchased from Millipore and ILK inhibitor QLT-0267 (QLT) from Quadra Logic Technology.

Confocal microscopy analyses

For T-cell adhesion, poly-L-lysine slices were coated with rE-cadh-Fc, rICAM-1-Fc or rFibronectin-Fc (5 µg/ml) overnight at 4°C. T cells, non-stimulated or stimulated with

rCXCL12 (100 nM) or rTGF-β (2 ng/ml), were incubated on pre-coated slices as described (8, 9). Cells shape index was calculated as the ratio of the longer to the shorter axis measured via Image J sofware. For protein polarization, lymphocytes, untreated or pretreated with SB (1 μM), QLT (20 μM) or with rTGF-β, were preincubated with IGR-Heu cells, or rE-cadh-Fc-coupled protein G-Dynabeads, at a 2:1 effector to target cell (E:T) ratio and then plated on poly-(L-lysine)-coated coverslips. Cells were stained with mouse anti-CD103, rabbit anti-phospho-ILK or goat anti-TGFBRI mAb, followed by anti-mouse-Alexa-Fluor-546, anti-rabbit-Alexa-Fluor-647 or anti-goat-Alexa-Fluor-488 Ab, respectively. Coverslips were analyzed using a fluorescence microscope with x20 (adhesion) or x63 (spreading and protein relocalization) lenses as described (9).

Tumor slices and T-cell recruitment experiments

Xenograft tumor samples were embedded in low-gelling-temperature agarose as reported (19). Lymphocytes were stained with SNARF and added onto the cut surface of each slice. In some experiments, T cells were preincubated with neutralizing anti-CD103 (anti-α_E, 10 μg/ml), anti-β_2 (10 μg/ml) or anti-CD3 (1 μg/mL), or treated with rTGF-β. Slices were then stained with anti-E-cadherin mAb followed by a secondary Ab (Alexa-488). Images were acquired with a ×4 or x10 (S fluor; Nikon) objective and MetaVue imaging software (Universal Imaging).

Single cell calcium measurement

The calcium response was measured in the same tumor slice with T cells loaded with 1 μM Fura-2AM (Molecular Probes). One of the two clones was also labeled with CMFDA (5 nM). Fura-2AM-loaded lymphocytes and infiltrated into tumor slices were alternatively excited every 10 s at 350 and 380 nm on an inverted Eclipse TE300 microscope equipped with a x20

objective and a Metafluor imaging system. Emissions at 510 nm were used for the analysis of Ca^{2+} responses. Values were represented as a ratio: fluorescence intensity at 350 nm/fluorescence intensity at 380 nm.

Western blot and immunoprecipitation experiments

Lymphocytes, untreated or pretreated with rTGF-β, were unstimulated or stimulated with plastic-coated rE-cadh-Fc. Equivalent amounts of protein extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane as described (10). Blots were then probed with rabbit anti-phospho(Ser-246)-ILK, anti-phospho(Ser-473)-AKT/PKB, anti-AKT/PKB or anti-β-actin-peroxidase followed by secondary HRP-conjugated Ab.

For immunoprecipitation, T-cell extracts were incubated for 2 h at 4°C with cross-linked anti-TGFBRI or anti-CD103 beads, or protein G-Sepharose beads prebound with control goat or mouse IgG. Beads were then washed and eluted with reducing agent at 95°C.

Flow cytometric analysis and cytotoxicity assay

For granule exocytosis, T cells, untreated or pretreated with inhibitors or cultured with rTGF-β, were stimulated with a combination of rE-cadh-Fc or rICAM-1-Fc (2.5 µg/mL) and UCHT1 mAb (0.4 µg/mL) in the presence of anti-CD107 mAb and monensin A (10). A high concentration of anti-CD3 (10 µg/mL) was used as a control.

Cytotoxicity was evaluated with a conventional ^{51}Cr-release assay (8).

Statistical analysis

Statistical analyses were performed using the two-tailed Student's t-test. Two groups were considered as significantly different if $p<0.05$.

Results

Regulation of CD103-dependent T-cell adhesion and motility by TGF-β

Thus far, little is known about the involvement of CD103 in T-cell migration and regulation of integrin activities by TGF-β. Hence, experiments were performed to compare the effect of TGF-β on T-cell adhesion and the migratory behavior of the $CD103^{+}$/$LFA-1^{+}$ TIL clone Heu171 (8) on immobilized human rE-cadh-Fc or rICAM-1-Fc. The rFibronectin-Fc was included as a negative control. rCXCL12, rCCL5 and rCCL20 chemokines, the respective receptors (CXCR4, CCR5 and CCR6) of which are expressed on the T-cell clone surface, were used as positive controls. Indeed, chemokines can trigger integrin-dependent adhesion of lymphocytes by initiating an inside-out signaling leading to integrin activation (20, 21). Results indicated that T lymphocytes stimulated with rCXCL12 (Fig. 1A), rCCL5 or rCCL20 (Supplementary Fig. 1A), adhered more efficiently to both rE-cadh-Fc and rICAM-1-Fc than untreated cells. Of note, incubation of T cells for a short period of time (30 min) with the three chemokines had no effect on LFA-1 and CD103 integrin expression levels (data not shown). Remarkably, treatment of T lymphocytes with rTGF-β induced a sharp increase in T-cell adhesion to rE-cadh-Fc starting from 24 h of culture, while it inhibited lymphocyte adhesion to rICAM-1-Fc (Fig. 1A). Treatment with rTGF-β of $CD8^{+}$ TIL freshly isolated from three independent tumors also induced an increase in T-cell adhesion to E-cadherin-Fc, while it inhibited adhesion to rICAM-1-Fc (Fig. 1B). Opposing effects of TGF-β on the adhesion strength of $CD103^{+}$/$LFA-1^{+}$ TIL clone and $CD103^{-}$/$LFA-1^{+}$ PBL counterpart (clone H32-22) to autologous $E-cadherin^{+}$/$ICAM-1^{-}$ (IGR-Heu) and $E-cadherin^{+}$/$ICAM-1^{+}$ (IGR-Heu-ICAM-1) tumor cells, respectively, were also observed under flow conditions (Supplementary Fig. 1B). These results correlated with upregulation of CD103 expression and, in contrast, downregulation of LFA-1 following T-cell incubation with the cytokine

(Supplementary Fig. 2A). Notably, T-cell clones, untreated or pretreated with rTGF-β, failed to express detectable levels of the E-cadherin inhibitory receptor KLRG1 (data not shown).

Chemokines generate both pro- and anti-adhesive intracellular signaling events, whose equilibrium is relevant to cell movement (21). Therefore, we analyzed morphological changes in CD103$^+$/LFA-1$^+$ lymphocytes cultured on recombinant molecule-coated surfaces in the absence or presence of rCXCL12, rCCL5 or rCCL20. Compared to untreated T cells, rCXCL12-treated cells overlaid on rE-cadh-Fc or rICAM-1-Fc displayed more active motility behavior characterized by greater polarized cell morphology (Fig. 1C). Indeed, an increase in the percent of cells with larger protrusion sizes was obtained when T lymphocytes were stimulated with rCXCL12 and plated on either of the molecules. Similar results were observed with rCCL5 and rCCL20 (Supplementary Fig. 2B). In contrast, starting from 24 h of treatment, TGF-β induced a strong increase in lymphocyte locomotion behavior when T cells were exposed to rE-cadh-Fc, but not to rICAM-1-Fc (Fig. 1C). In addition, live-microscopy imaging revealed that motility was enhanced when T cells were pretreated with rTGF-β before culturing them on rE-cadh-Fc, whereas it was compromised when the same pretreated cells were added onto rICAM-1-Fc (Supplementary Fig. 2C and Movies). These results suggest that TGF-β promotes T-cell adhesion and cytoskeleton rearrangement by strengthening the interaction of CD103 with E-cadherin.

TGF-β enhances CD103-mediated T-cell recruitment in epithelial tumor islets

Next, experiments were undertaken to assess the influence of CD103 on T-cell recruitment in tumor regions via our previously described approach using tumor slices (19) obtained from E-cadherin$^+$ lung tumor cell line, untransfected or transfected with ICAM-1, engrafted into immunodeficient NOD-SCID mice. Fluorescent-labeled autologous CD103$^+$/LFA-1$^+$ and

CD103$^-$/LFA-1$^+$ CTL were overlaid onto fresh tumor slices generated from either E-cadherin$^+$/ICAM-1$^-$ or E-cadherin$^+$/ICAM-1$^+$ tumors. Slices were stained with anti-E-cadherin mAb to distinguish epithelial from stromal regions (Supplementary Fig. 3A), and then lymphocytes that infiltrated epithelial tumor regions and the stroma were counted. Results indicate that the CD103$^+$/LFA-1$^+$ TIL clone was much more efficiently recruited within E-cadherin$^+$/ICAM-1$^-$ tumor islets than the CD103$^-$/LFA-1$^+$ PBL clone, which was concentrated within stromal regions (Fig. 2A). Moreover, treatment of the CD103$^+$/LFA-1$^+$ clone with TGF-β enhanced T-cell recruitment in tumor regions starting from 24 h of pre-incubation. In contrast, treatment of the CD103$^-$/LFA-1$^+$ PBL clone with TGF-β was unable to induce CD103 expression (8) and thus it had no effect on T-cell recruitment within tumor islets (data not shown). Recruitment of the TIL clone in tumor regions was dependent on CD103 and TCR engagement, since neutralizing anti-CD103 and anti-CD3 mAb dramatically blocked lymphocyte migration by inhibiting T-cell adhesion to E-cadherin$^+$ target cells and peptide-MHC-I complex recognition by TCR, respectively. In contrast, neutralizing anti-β2 mAb, used as a negative control, had no effect on T-cell distribution (Fig. 2A).

We then analyzed the location of both T-cell clones plated on E-cadherin$^+$/ICAM-1$^+$ tumor slices and the effect of TGF-β on this process. Transfection of tumor cells with ICAM-1 only slightly increased recruitment of the CD103$^+$/LFA-1$^+$ clone in tumor islets, whereas it strongly enhanced CD103$^-$/LFA-1$^+$ clone migration (Fig. 2B). Moreover, anti-β_2 and anti-CD3 mAb strongly inhibited PBL clone recruitment, while anti-CD103, anti-β_2 or a combination of both mAb blocked TIL clone migration toward tumor regions. Notably, pertussis toxin, a bacterial toxin that blocks signaling from G protein-coupled receptors including chemokine receptors had no effect on T-cell distribution (data not shown). In contrast, treatment of the CD103$^-$/LFA-1$^+$ clone with TGF-β resulted in profound inhibition of

T-cell recruitment within E-cadherin^{+}/ICAM-1^{+} tumor islets, in particular after 96 h of pre-incubation (Fig. 2B). These results strongly suggest that CD103 is directly involved in T-lymphocyte recruitment in tumor islets and that TGF-β upregulates its functions, while it has opposing effects on LFA-1.

TGF-β potentiates intratumoral early signaling of CD103^{+} lymphocytes

Imaging of the intracellular calcium increase is a powerful means of monitoring the dynamics of T-lymphocyte activation. We therefore evaluated the role of CD103 and the influence of TGF-β on the Ca^{2+} response triggered in CTL CD103^{+}/LFA-1^{+} and CD103^{-}/LFA-1^{+}. T-cell clones, untreated or pretreated with rTGF-β, were labeled with Fura-2 (red) to monitor calcium response. The CD103^{-}/LFA-1^{+} clone was also labeled with CMFDA fluorescent dye (green; thus appearing in yellow) in order to identify each T-cell population on the same slice. The two clones were then added simultaneously to tumor slices, untransfected or transfected with ICAM-1. Results indicated that CTL CD103^{+}/LFA-1^{+}, added to the E-cadherin^{+}/ICAM-1^{-} slice and recruited within tumor islets, displayed a much stronger calcium response than CTL CD103^{-}/LFA-1^{+} (Fig. 3A). Moreover, neutralizing anti-CD103 induced a decrease in the calcium level of the TIL clone, whereas anti-β2 mAb, used as a negative control, had no effect. Remarkably, treatment of the TIL clone with rTGF-β induced a sharp increase in the T-cell calcium response. An increase in CD103^{+}/LFA-1^{+} Ca^{2+} response was also observed when the T-cell clone was pretreated with TGF-β and plated on a monolayer of E-cadherin^{+}/ICAM-1^{-} IGR-Heu tumor cells, which was inhibited when T lymphocytes were preincubated with neutralizing anti-CD103 mAb (Supplementary Fig. 3B). In contrast, treatment of the CD103^{-}/LFA-1^{+} PBL clone with TGF-β failed to induce CD103 expression and thus a marginal calcium response was observed when T cells were added to E-cadherin^{+}/ICAM-1^{-} tumor cells (data not shown).

We then monitored Ca^{2+} signaling of both clones plated on E-cadherin^{+}/ICAM-1^{+} tumor slices. As expected, a strong calcium response was obtained with the CD103^{+}/LFA-1^{+} clone, which was inhibited by both anti-CD103 and anti-β_2 blocking mAb. Transfection of tumor cells with ICAM-1 induced a strong Ca^{2+} increase in the CD103^{-}/LFA-1^{+} clone, which was blocked by neutralizing anti-β_2. Importantly, treatment of the PBL clone with TGF-β strongly inhibited early T-cell signaling (Fig. 3B). These results suggest a crucial role of CD103 in regulating TCR-mediated activities within a TGF-β-rich tumor ecosystem and that TGF-β is involved in the CD103 signaling pathway.

TGF-β regulates CD103 activation via ILK phosphorylation

The signaling pathway of CD103 and its contribution to T-cell migration and functions within epithelial tissues are unknown. ILK, a serine/threonine kinase, has been reported to mediate integrin signaling and to play a role in leukocyte recruitment (22) and T-cell chemotaxis (23). Moreover, a role for TGF-β in regulating ILK expression and activation has been reported (24). We therefore analyzed the involvement of ILK in CD103 signaling and the effect of TGF-β on integrin activation in CD103^{+}/LFA-1^{+} CTL. Initial experiments indicated that TGF-β upregulated *ILK* gene transcription starting from 6 h of T-cell clone treatment (Supplementary Fig. 3C). Moreover, stimulation of the clone with immobilized rE-cadh-Fc and/or rTGF-β triggered phosphorylation of ILK at Ser 246 (Fig. 4A), which was strongly inhibited by the ILK inhibitor QLT, the TGFBR1 inhibitor SB, the PI3K inhibitor Wortmanin (Fig. 4B) and anti-CD103 mAb (Supplementary Fig. 3D). Notably, inhibition of ILK phosphorylation resulted in a marked decrease in CTL adhesion on immobilized rE-cadh-Fc (Supplementary Fig. 3E). Stimulation of the TIL clone with rE-cadh-Fc also induced phosphorylation of the PKB/AKT at Ser-473, which was strongly inhibited by the AKT

inhibitor MK-2206 as well as QLT, SB and Wortmanin (Fig. 4C). Of note, incubation of lymphocytes for a short period of time (1 h) with all inhibitors had no effect on integrin expression level and T-cell viability, as checked by CD103 and annexin V labeling, respectively (data not shown).

Next, we evaluated the consequences of CD103 engagement with its ligand on ILK localization in $CD103^+/LFA\text{-}1^+$ TIL plated on a rE-cadh-Fc-coated slide. Results indicate that the CD103-E-cadherin interaction resulted not only in T-cell spreading, but also in recruitment of phospho-ILK, together with CD103, at the leading edge of migrating CTL presenting a high actin cytoskeleton rearrangement (Fig. 5A). Moreover, ILK and TGFBR1 inhibitors induced a marked decrease in phospho-ILK recruitment at the leading edge. Together, these results suggested that TGFBR1 serine kinase catalyzed, even in the absence of its ligand, phosphorylation of ILK through a PI3K-dependent pathway and that phospho-ILK and phospho-AKT were involved in CD103 signaling.

ILK recruitment and phosphorylation by TGFBR1 triggers binding to CD103

Because the interaction of ILK with β-integrins is important for intracellular signaling, we first monitored the localization of phospho-ILK and TGFBR1 in conjugates formed between $CD103^+/LFA\text{-}1^+$ T cells and $E\text{-}cadherin^+/ICAM\text{-}1^-$ tumor cells. Results revealed polarization of phospho-ILK, together with CD103 and TGFBR1, at the IS, which was inhibited by QLT and SB (Fig. 5B), as well as siRNA targeting E-cadherin (Supplementary Fig. 4A). Consistently, shRNA targeting ILK also inhibited CD103 polarization (Supplementary Fig. 4B and 4C) and AKT phosphorylation (data not shown). In contrast, treatment of the TIL clone with rTGF-β induced an increase in synaptic relocalization of CD103, phospho-ILK and TGFBR1 (Supplementary Fig. 4D).

To explore a potential link between TGFBR1, phospho-ILK and CD103, we searched for physical interactions between these proteins. Therefore, we performed immunoprecipitation experiments using anti-CD103 or anti-TGFBR1 mAb and immunobloting with anti-ILK. Results revealed that ILK bound to CD103 (Fig. 5C) and TGFBR1 (Fig. 5D), and that TGF-β, and to a lesser extent rE-cadh-Fc, enhanced ILK linking to CD103. Notably, no direct interaction between CD103 and TGFBR1 was observed, since CD103 was detected only in the supernatant of immunoprecipitation with anti-TGFBR1 (Fig. 5E). These results further support the hypothesis that the interaction of TGF-β with its receptors induces recruitment and phosphorylation of ILK by TGFBR1 and its subsequent binding to the CD103 intracellular domain, resulting in AKT phosphorylation and thereby initiating integrin inside-out signaling.

TGF-β regulates CD103-dependent CTL activities via PI3K-ILK-AKT pathway

Experiments were then conducted to evaluate the contribution of PI3K-ILK-AKT pathway in CD103-mediated T-cell functions. Using the tumor slice system, results indicated that Wortmanin, SB, QLT and MK-2206 inhibited T-cell recruitment in epithelial tumor islets (Fig. 6A). We then analyzed the effect of all the inhibitors and the influence of TGF-β on CD103-dependent T-cell clone degranulation. As shown in Fig. 6B, $CD103^{+}/LFA\text{-}1^{+}$ T cells cultured with rTGF-β and stimulated with a combination of anti-CD3 plus rE-cadh-Fc expressed higher surface levels of CD107a than T cells exposed to a combination of anti-CD3 and rICAM-1-Fc. Moreover, Wortmanin, SB, QLT and MK-2206 induced a decrease in T-cell degranulation triggered by a combination of anti-CD3 plus rE-cadh-Fc, whereas they had no effect on CD107a expression induced by a high dose of anti-CD3. Consistently, SB and QLT strongly inhibited cytotoxic granule polarization at the contact area between

$CD103^+/LFA\text{-}1^+$ CTL and rE-cadh-Fc-coupled beads (Supplementary Fig. 5A). In addition, while TGF-β inhibited granzyme B polarization at the contact area between the TIL clone and rICAM-Fc-coupled beads, it had no effect on serine protease relocalization at the contact zone with rE-cadh-Fc-coated beads.

We then evaluated the effect of TGF-β, PI3K, TGFBR1, phospho-ILK and phospho-AKT inhibitors on T-cell clone-mediated cytotoxicity toward target cells. Incubation of the $CD103^+/LFA\text{-}1^+$ clone with rTGF-β enhanced $E\text{-}cadherin^+/ICAM\text{-}1^-$ IGR-Heu tumor cell killing (Fig. 6C), which was inhibited in the presence of neutralizing anti-CD103 (Fig. 6D), whereas Wortmanin, SB, QLT and MK-2206 inhibited target cell lysis (Fig. 6C). Notably, all inhibitors had no effect on T-cell clone-mediated CD3-redirected cytotoxicity of the P815 target (Supplementary Fig. 5B). In contrast, culture of the $CD103^-/LFA\text{-}1^+$ CTL clone in the presence of TGF-β dramatically inhibited $E\text{-}cadherin^+/ICAM\text{-}1^+$ IGR-Heu-ICAM-1 target cell lysis (Fig. 6E). Notably, TGF-β had no effect on granzyme B expression levels in both T-cell clones (data not shown). These results further emphasize the role of PI3K, TGFBR1, phospho-ILK and phospho-AKT/PKB in CD103 signaling and the positive effect of TGF-β in antitumor CTL-mediated activities.

Discussion

In this report, we identified CD103 and its ligand E-cadherin as important adhesion molecules stimulating recruitment and effector functions of activated $CD8^+/CD103^+$ TIL inside human lung tumors. In agreement with our previous studies, we also show that $CD8^+/CD103^-$ T cells accumulate preferentially within the tumor stroma and display inefficient antitumor activities (19). Along the same line, it has been reported that mouse $CD103^{-/-}$ T cells failed to infiltrate allograft islets, suggesting that CD103 promotes intragraft migration of $CD8^+$ effectors into epithelial islets (25). Our data are consistent with a key role for CD103 in promoting entry of tumor antigen-specific $CD8^+$ T cells into epithelial tumor islets and in triggering antitumor T-cell functions. Recognition of tumor antigen by TCR also plays an important role, since anti-CD3 mAb inhibited T-cell recruitment into tumor regions. It is possible that CD103 contributes to intratumoral scanning of target cells for cognate antigen recognition.

CD103 and LFA-1 are the predominant integrins expressed by TIL and both are likely involved in T-cell adhesion and migration within and toward epithelial tumors, respectively. Indeed, we show here that LFA-1, through an interaction with ICAM-1, also induced T-cell infiltration within tumor nests. However, tumors often express low levels of ICAM (26) and produce high amounts of TGF-β (27) thus altering LFA-1 expression and functions ((28) and the present study). Indeed, this cytokine shifted the integrin expression profile of tumor-specific TIL from $LFA\text{-}1^{high}/CD103^{low}$ to $LFA\text{-}1^{low}/CD103^{high}$. Thus, CD103 most likely corresponds to an important integrin for adjusting T-cell adhesion and migratory potential in the tumor ecosystem, where TGF-β is abundant. Along this line, it has been reported that murine CD103 determines cell shape and motility, and confers the ability to move in an actin-polymerization-dependent manner (29). These results suggest that CD103-mediated cell movement probably involves signaling interactions with components of the cytoskeleton.

Likewise, we show here that human CD103 confers the capacity to $CD8^+$ T cells to form protrusions/filopodia in an E-cadherin-dependent fashion. Moreover, TGF-β strongly optimizes T-cell adhesion and motility by enhancing CD103 expression levels and initiating intracellular signals resulting in integrin activation.

TGF-β is known to inhibit cell growth in benign cells but promotes progression in cancer cells (30). Moreover, TGF-β signaling in peripheral T lymphocytes is essential for tolerance and homeostasis by restraining TCR activation-dependent Th1, cytotoxic and NKT cell differentiation program (31, 32). This cytokine, produced by stromal and tumor cells, is also described as an immunosuppressive factor used by malignant cells to escape from the immune response and to compromise CTL activities (33). Paradoxically, TGF-β is involved in promoting antitumor $CD8^+$ T-cell effector functions within epithelial tumors by inducing CD103 upon TCR engagement and by adjusting its expression levels. TGF-β also plays an essential role in the formation and long-term persistence of tissue resident memory T cells (T_{RM}), at least in part via induction of CD103 (12, 34). Importantly, our data indicate that TGF-β also contributes to T-cell migration toward epithelial tumor islets and subsequent cancer cell destruction. This paradoxical effect of TGF-β on T-cell functions is most likely associated with the use in the present study of $CD103^+$/LFA-1^+ lymphocytes while most of the previous studies were based on peripheral T cells, which generally express a $CD103^-$/LFA-1^+ phenotype. Consistently, we show here that TGF-β is responsible for arrest of LFA-1-dependent T-cell migration within stromal regions by preventing integrin expression and functions. It has been reported that TGF-β inhibits phosphorylation of Erk and, thereby, LFA-1-mediated T-cell adhesion and chemotaxis (35). In contrast, this cytokine contributes to CD103 signaling by upregulating ILK expression and inducing its phosphorylation. TGF-β initiates signaling by binding to TGFBR, leading to formation of a hetero-tetrameric complex

composed of TGFBR1 and TGFBR2 dimers, in which TGFBR2 catalyzes phosphorylation of the cytoplasmic domain of TGFBR1 (36). In addition to the Smad pathway, TGF-β utilizes multiple non-Smad pathways, including PI3K/AKT pathway, to regulate a wide range of cell functions (37). We show here that TGFBR1 recruits and phosphorylates ILK, which then binds to the α_E subunit of CD103, leading to AKT/PKB phosphorylation and thus initiating integrin inside-out signaling. It has been reported that ILK associates with TGFBR2 and that *ILK* gene inactivation results in a decrease in TGFBR2 signaling (38).

ILK is a serine-threonine kinase that interacts with intracytoplasmic domains of β1 and β3 integrins and cytoskeletal-associated proteins (39). This kinase is associated with integrin activation and intracellular signaling (40), and has been reported to mediate actin filament rearrangement and tumor cell migration and invasion through PI3K/AKT/Rac1 signaling (41). Indeed, ILK directly binds to AKT/PKB and contributes to its phosphorylation, thereby regulating several signaling pathways including spreading and migration (42). Consistently, our results indicated that ILK is directly involved in CD103 intracellular signaling by promoting phosphorylation of AKT/PKB and that the CD103-E-cadherin interaction induced phosphorylation of ILK and its subsequent polarization at the leading edge of migrating T cells. Implication of ILK in both inside-out and outside-in integrin signaling pathways (43, 44) and in β2 integrin-mediated polarization of lytic granules toward the target have also been reported (45). Our data suggest that ILK functions as a key component of a protein complex formed upon CD103 clustering that may connect extracellular ligands with the T-cell intracellular signaling complex. We also show here that TGF-β corresponds to an important factor that not only activates α_E (CD103) subunit expression, but also locally regulates integrin activation and signaling via the ILK and AKT/PKB pathway. This leads to strengthening of CD103-E-cadherin interaction, thereby initiating outside-in signaling that

promotes CD103-mediated T-cell functions such as T-cell spreading, migration and cytotoxicity.

Overall, our data expand our knowledge of $CD8^+$ T-cell locomotion within epithelial tissues and provide more insight into mechanisms that promote CD103 signaling within a TGF-β-rich tumor microenvironment. Thus, CD103-E-cadherin adhesive interactions regulate multiple local T-cell activities, including adhesion, Ca^{2+} response and recruitment within epithelial tumor regions. This integrin is also involved in T-lymphocyte proliferation, cytokine secretion, cytotoxic IS maturation and killing of epithelial target cells (8, 9, 14, 46). These findings are consistent with a general role of CD103 in promoting CD8 T-cell immune responses within epithelial tumors and may contribute to optimizing current T-cell-based cancer immunotherapy strategies.

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Figure legends

Figure 1

Adhesion and filopodia formation are increased following CD103^{+}/LFA-1^{+} T-cell stimulation with rCXCL12 or rTGF-β. **A.** Adhesion to rE-cadh-Fc or rICAM-1-Fc of the TIL clone, untreated or pretreated with rCXCL12 for 30 min, or with rTGF-β for indicated time points, was analyzed by confocal microscopy. rFibronectin-Fc was used as a negative control. *Lower panel*: mean numbers of adherent lymphocytes per field. Adhesion was evaluated by counting adherent lymphocytes stained with TO-PRO®-3 iodide. Statistical analyses are from 3 independent experiments (see Supplementary Table I). Bars, 100 µm, *,p<0.01 and **,p<0.001. **B.** T-cell adhesion is increased following treatment of freshly isolated TIL with rTGF-β. Adhesion of CD8^{+} TIL (including up to 60% of CD103^{+} T cells), untreated or pretreated with rTGF-β for 96 h, to rE-cadh-Fc or rICAM-1-Fc. Right panel: mean numbers of adherent cells per field. Results are from one experiment out of 3 performed with fresh TIL from 3 different patients. Bars, 500 µm. **C.** Migratory behavior of CD103^{+}/LFA-1^{+} lymphocytes, untreated or pretreated with rCXCL12 or rTGF-β, seeded on recombinant molecules. Polymerized F-actin was visualized with rhodamine phalloidin. Arrows indicate filopodia and/or cell protrusions. *Lower panel*: T-cell shape index. Statistical analyses are from 3 independent experiments. Bars, 5 µm, *,p<0.01, **,p<0.001 and ***,p<0.001.

Figure 2

Recruitment of CD103^{+}/LFA-1^{+} and CD103^{-}/LFA-1^{+} lymphocytes within epithelial tumor regions of human lung tumor slices. **A.** Fluorescent-labeled CD103^{+}/LFA-1^{+} T cells, untreated or pretreated with neutralizing anti-CD103, anti-β_2 or anti-CD3 mAb, or with rTGF-β, were plated on E-cadherin^{+}/ICAM-1^{-} tumor (IGR-Heu) slices. Stromal (S) and tumor (T) areas, identified by brightness contrast and confirmed by E-cadherin labeling (see

Supplementary Fig. 3A). Lymphocytes were stained with SNARF. *Right panel:* percentages of cells inside tumor areas are from 3 independent experiments. **B.** $CD103^+/LFA\text{-}1^+$ or $CD103^-/LFA\text{-}1^+$ lymphocytes, untreated or pretreated with neutralizing anti-CD103, anti-CD3 or anti-β_2 mAb, or with rTGF-β, were overlaid onto E-cadherin$^+$/ICAM-1$^+$ (IGR-Heu-ICAM-1) tumor slices. *Right panels*: percentages of T cells inside tumor areas are from 3 independent experiments out of 6. Bars, 100 μm; *,p<0.01, **,p<0.001 and ***,p<0.0001.

Figure 3

Calcium response of T cells within epithelial islets of lung tumor slices. **A.** $CD103^+/LFA\text{-}1^+$ and $CD103^-/LFA\text{-}1^+$ CTL were loaded with Fura-2 probe (red in upper panels). $CD103^-/LFA\text{-}1^+$ T cells were also labeled with CMFDA (green) to distinguish them from $CD103^+/LFA\text{-}1^+$ cells. Thus, $CD103^+/LFA\text{-}1^+$ lymphocytes appeared in red, whereas $CD103^-/LFA\text{-}1^+$ lymphocytes appeared in yellow (a merge of red and green dyes) in color merge panels. Loaded T cells were added to a tumor slice 1 h before the recording. Calcium response, represented in pseudo-colors (from green, average calcium level to red, strong calcium level), of $CD103^+/LFA\text{-}1^+$, untreated or pretreated with neutralizing anti-CD103 or anti-β_2, or with rTGF-β for 96 h, and $CD103^-/LFA\text{-}1^+$ lymphocytes within E-cadherin$^+$/ICAM-1$^-$ (IGR-Heu) tumor areas. White dotted circles correspond to $CD103^+/LFA\text{-}1^+$ responding cells. *Right panel*: quantification of intracellular Ca^{2+}. **B.** Calcium response of $CD103^+/LFA\text{-}1^+$ and $CD103^-/LFA\text{-}1^+$ lymphocytes, untreated or pretreated with neutralizing anti-CD103 or anti-β_2 mAb, or with rTGF-β, in tumor islets of E-cadherin$^+$/ICAM-1$^+$ (IGR-Heu-ICAM-1) slices. White dotted and full circles correspond to $CD103^+/LFA\text{-}1^+$ and $CD103^-/LFA\text{-}1^+$ responding lymphocytes, respectively. *Right panel:* quantification of intracellular calcium response. Statistical analyses are from 3 independent experiments. Bars, 100 μm; **,p<0.001, ***,p<0.0001.

Figure 4

Engagement of CD103 on CD103^{+}/LFA-1^{+} lymphocytes with rE-cadh-Fc induced phosphorylation of ILK and AKT. **A.** Engagement of CD103 with rE-cadh-Fc or treatment of the CD103^{+}/LFA-1^{+} clone with TGF-β induced phosphorylation of ILK. Lymphocytes, pretreated with rTGF-β for indicated time points, were stimulated with immobilized rE-cadh-Fc and then protein extracts were analyzed by western blot using anti-phospho-ILK or anti-ILK mAb. F-actin was used as a loading control. *Right panel:* normalization of phospho-ILK relative to total ILK. **B.** Wortmanin (Wortm), SB and QLT inhibit ILK phosphorylation induced by CD103 engagement with rE-cadh-Fc or rTGF-β stimulation. CD103^{+}/LFA-1^{+} lymphocytes were pretreated with PI3K, TGFBR1 or ILK inhibitors and then stimulated with immobilized rE-cadh-Fc or with rTGF-β. T-cell protein extracts were analyzed by western blot. *Lower panels:* normalization of phospho-ILK relative to ILK. **C.** Wortmanin, SB, QLT and MK-2206 inhibit AKT phosphorylation induced by CD103 engagement with rE-cadh-Fc. CD103^{+}/LFA-1^{+} lymphocytes were pretreated for 1 h with either of the inhibitors and then stimulated with immobilized rE-cadh-Fc. Protein extracts were analyzed by western blot using indicated mAb. *Right panel:* normalization of phospho-AKT relative to AKT. Statistical analyses are from 3 independent experiments.

Figure 5

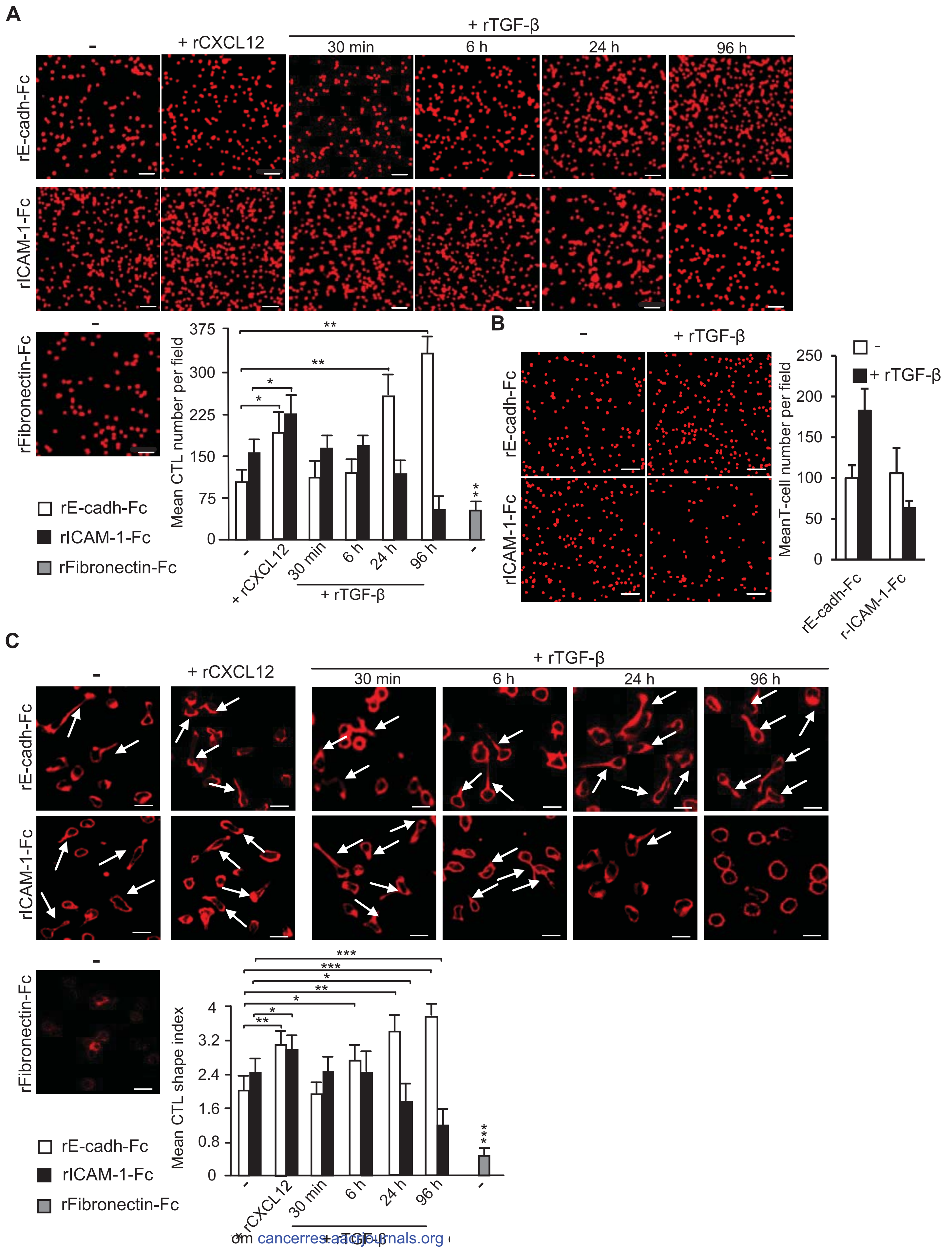
Engagement of CD103 on the T-cell surface with E-cadherin resulted in polarization of phospho-ILK and its subsequent binding to CD103. **A.** Engagement of CD103 with immobilized rE-cadh-Fc resulted in polarization of phospho-ILK at the leading edge. CD103^{+}/LFA-1^{+} T cells, untreated or pretreated with QLT or SB, were plated on plastic-coated rE-cadh-Fc, and then lymphocytes were analyzed for intracellular protein distribution

by confocal microscopy using anti-phospho-ILK, anti-CD103 or anti-F-actin. *Right panel*: percentages of T cells displaying polarized phospho-ILK at the leading edge (n=40). **B.** The CD103-E-cadherin interaction induced recruitment of phospho-ILK at the IS. Conjugates between $CD103^+/LFA-1^+$ CTL, untreated or treated with QLT or SB, and IGR-Heu tumor cells were analyzed for CD103, phospho-ILK and TGFBRI polarization. Right panel: percentages of cells displaying polarized proteins (n=35). Statistical analyses are from one experiment out of 3. Bars, 5 µm. **,p<0.001. **C.** ILK binds to CD103. Protein extracts from $CD103^+/LFA-1^+$ lymphocytes, stimulated with immobilized r-E-cadh-Fc for 30 min or rTGF-β for 96 h, were immunoprecipitated (IP) using anti-CD103 mAb and then immunoblotted (WB) with anti-ILK. An IgG negative control was included. **D.** TGFBR1 binds to ILK. $CD103^+/LFA-1^+$ cells were treated with rTGF-β and then protein extracts were immunoprecipitated with anti-TGFBR1, followed by immunoblotting with anti-ILK. Results are from one experiment out of 3. **E.** TGFBR1 does not bind to CD103. Protein extracts from $CD103^+/LFA-1^+$ lymphocytes were immunoprecipitated with anti-TGFBR1 mAb and then immunoblotted with anti-CD103. The $CD103^-/LFA-1^+$ clone and IgG were used as negative controls. *Right panel*: CD103 detected in the supernatant of $CD103^+/LFA-1^+$ CTL.

Figure 6

Phospho-ILK and phospho-AKT are involved in CTL activities. **A.** Recruitment of fluorescent-labeled $CD103^+/LFA-1^+$ lymphocytes, untreated or pretreated with Wortmanin, SB, QLT or MK-2206 inhibitors, within epithelial tumor regions of lung tumor (IGR-Heu) slices. Stromal (S) and tumor (T) areas were identified by brightness contrast. *Right panel:* percentages of T cells inside tumor areas were determined from 3 independent experiments. Bars, 100 µm. **B.** $CD103^+/LFA-1^+$ lymphocytes, untreated or pretreated with rTGF-β for 96 h or with indicated inhibitors for 1 h, were incubated with a combination of rE-cadh-Fc plus a

suboptimal concentration of anti-CD3 mAb. A combination of a suboptimal concentration of anti-CD3 plus rICAM-1-Fc or a high concentration of anti-CD3 was used as controls. Data correspond to percentages of CD107a^{+} lymphocytes from 2 independent experiments out of 3. **C**. Cytotoxic activity of CD103^{+}/LFA-1^{+} CTL, untreated or pretreated with rTGF-β and then with indicated inhibitors, towards autologous E-cadherin^{+}/ICAM-1^{-} (IGR-Heu) tumor cells. **D**. Cytotoxicity of CD103^{+}/LFA-1^{+} CTL, untreated or pretreated with rTGF-β and then either left in medium or preincubated with neutralizing anti-CD103 mAb, towards autologous E-cadherin^{+}/ICAM-1^{-} tumor cells. **E.** Cytotoxic activity of CD103^{-}/LFA-1^{+} CTL, untreated or pretreated with rTGF-β, towards autologous E-cadherin^{+}/ICAM-1^{+} (IGR-Heu-ICAM-1) tumor cells. *,$p<0.01$ and **,$p<0.001$.



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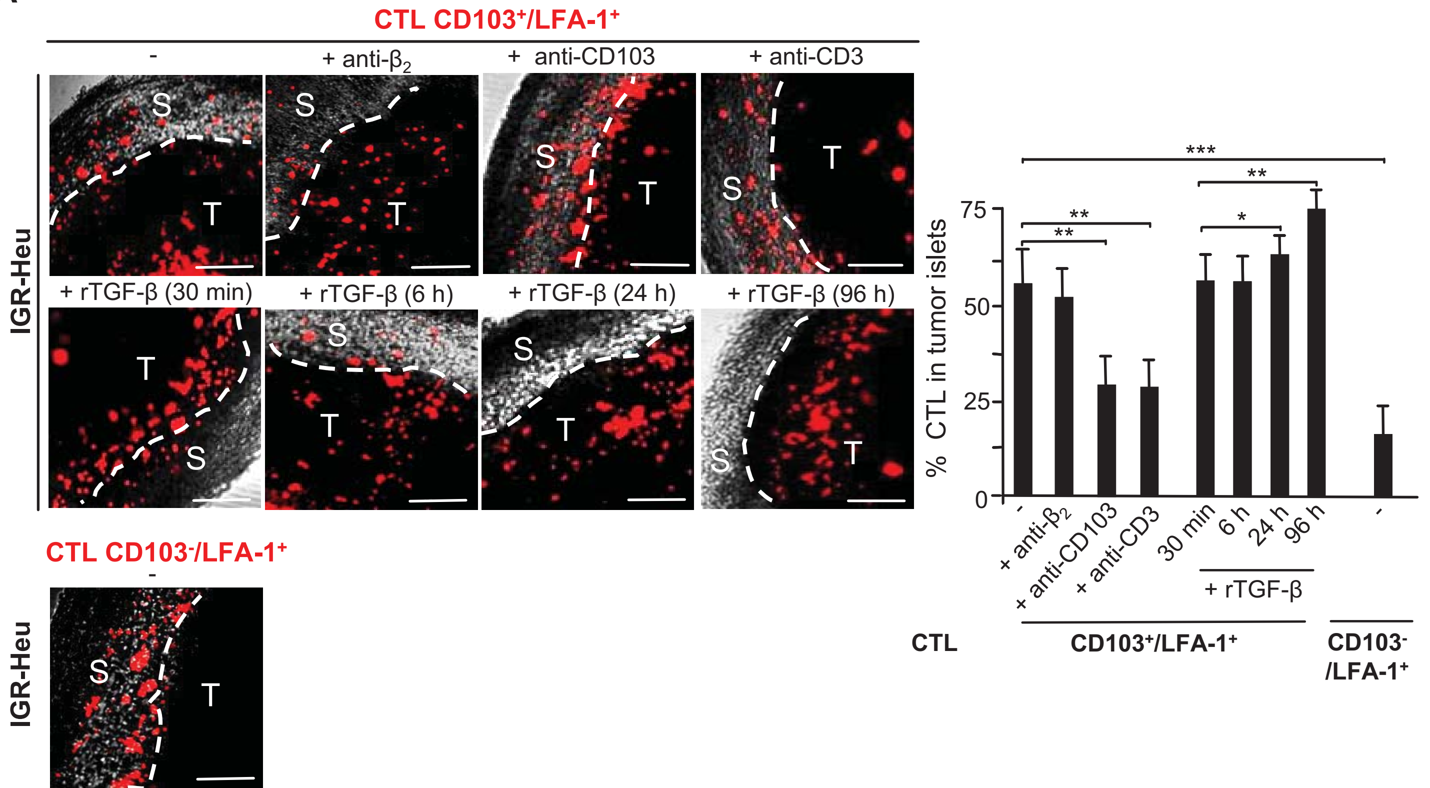


B

CTL CD103+/LFA-1+

IGR-Heu-ICAM-1

- | + anti-CD103 | + anti-β2 | + anti-CD103 + anti-β2

% CTL in tumor islets

75 50 25 0

** ** **

- + anti-CD103 + anti-β2 + anti-CD103 + anti-β2

CTL CD103-/LFA-1+

IGR-Heu-ICAM-1

- | + anti-CD103 | + anti-β2 | + anti-CD3

+ rTGF-β (30 min) | + rTGF-β (6 h) | + rTGF-β (24 h) | + rTGF-β (96 h)

% CTL in tumor islets

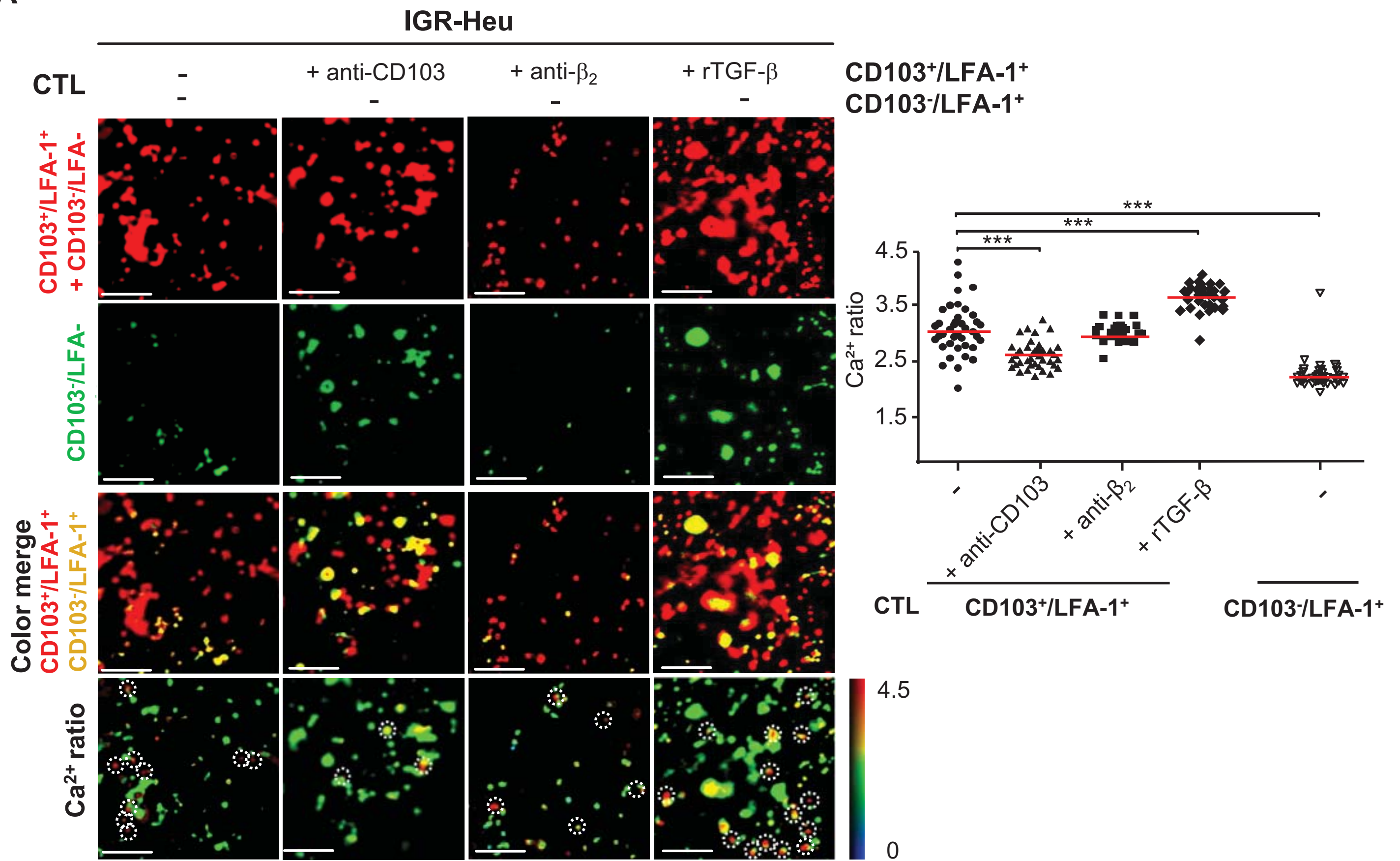
75 50 25 0

** ** ** *

- + anti-CD103 + anti-β2 + anti-CD3 30 min 6 h 24 h 96 h

+ rTGF-β

A



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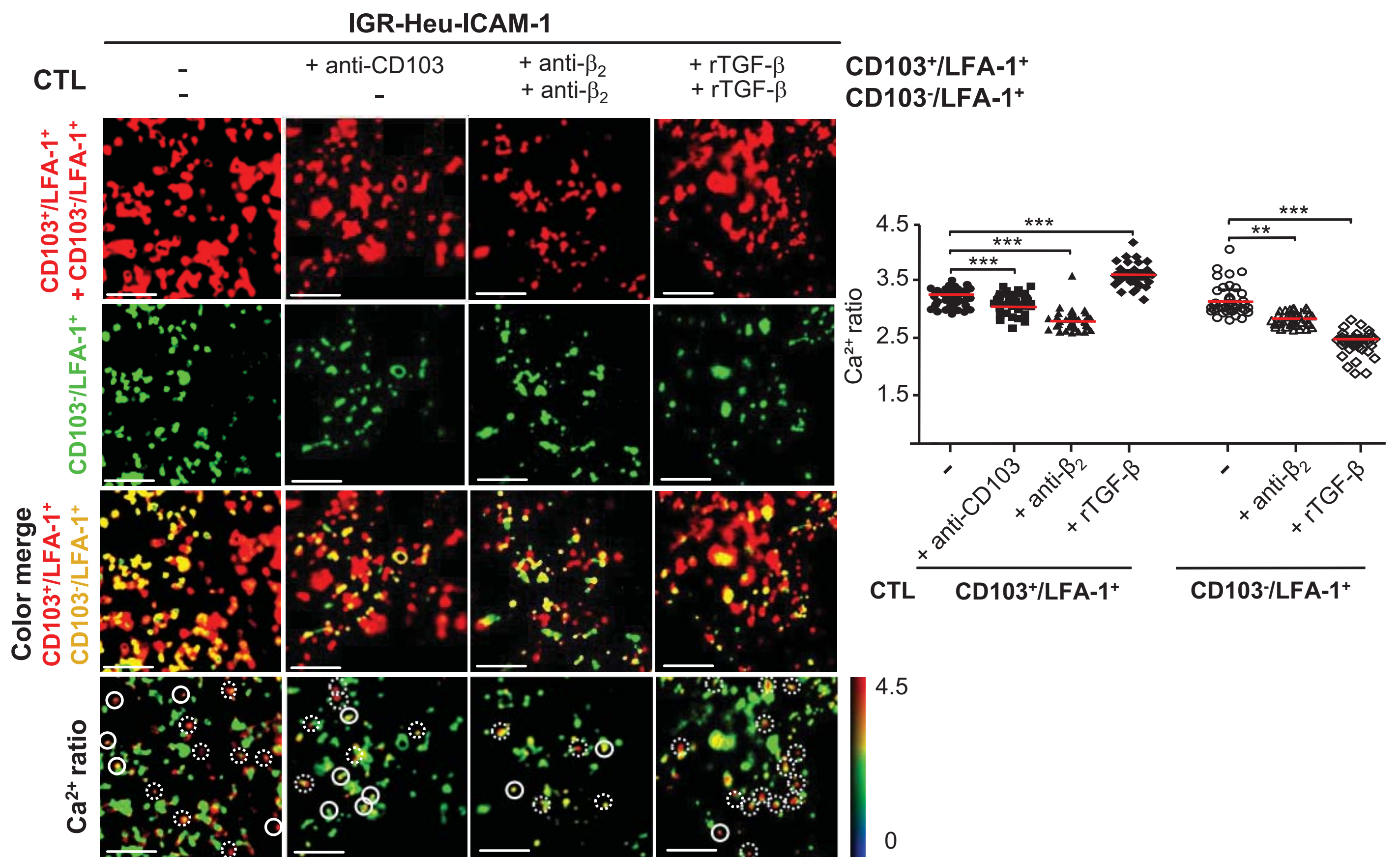

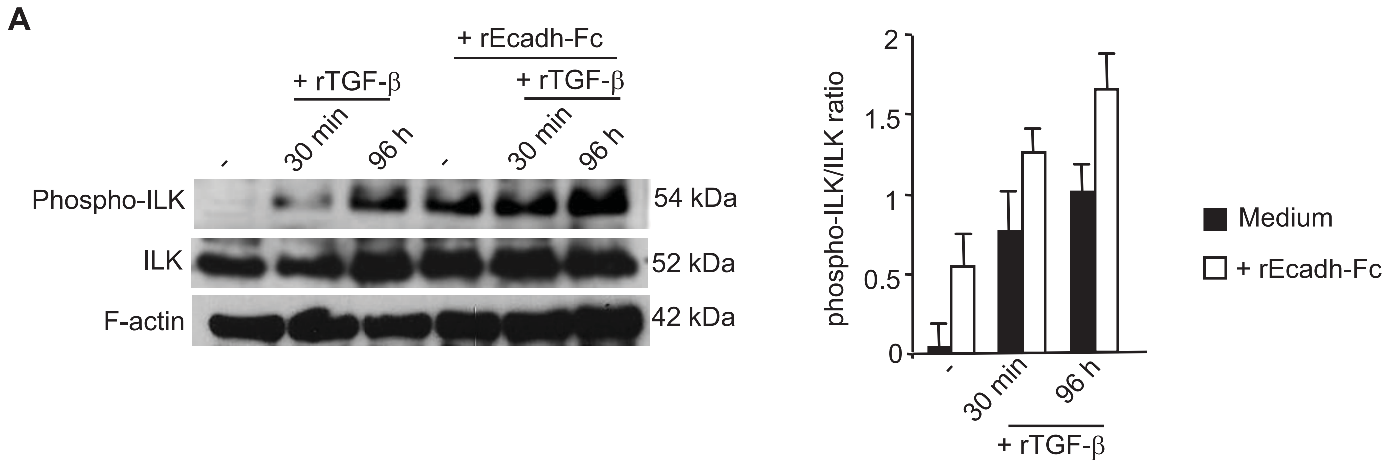
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96 h
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F-actin
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52 kDa
42 kDa
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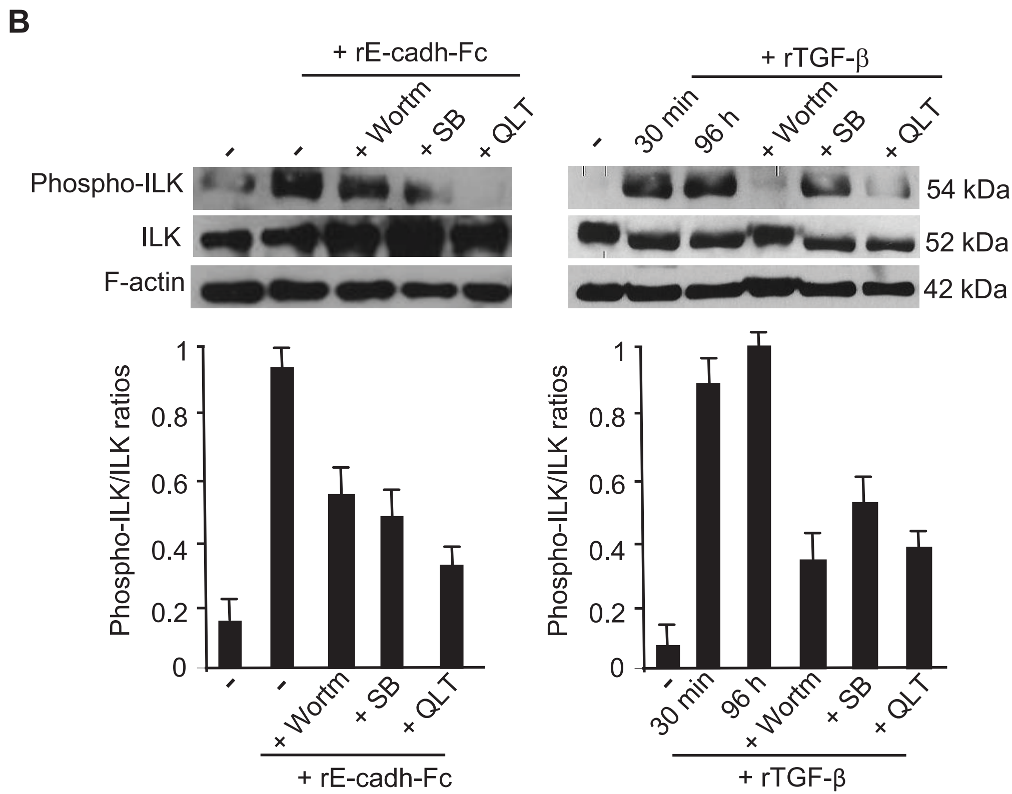
+ rTGF-β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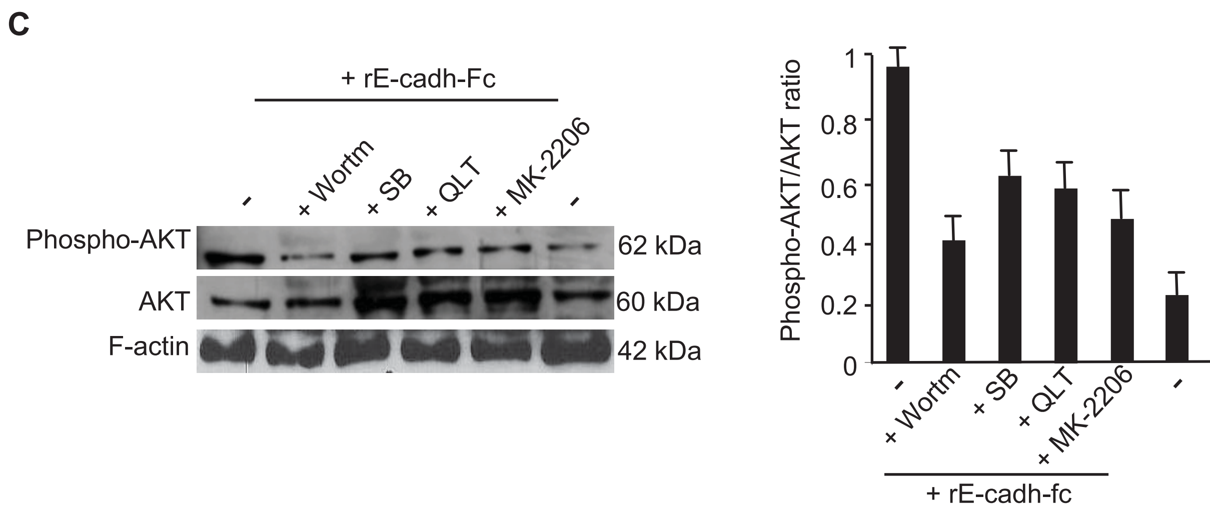
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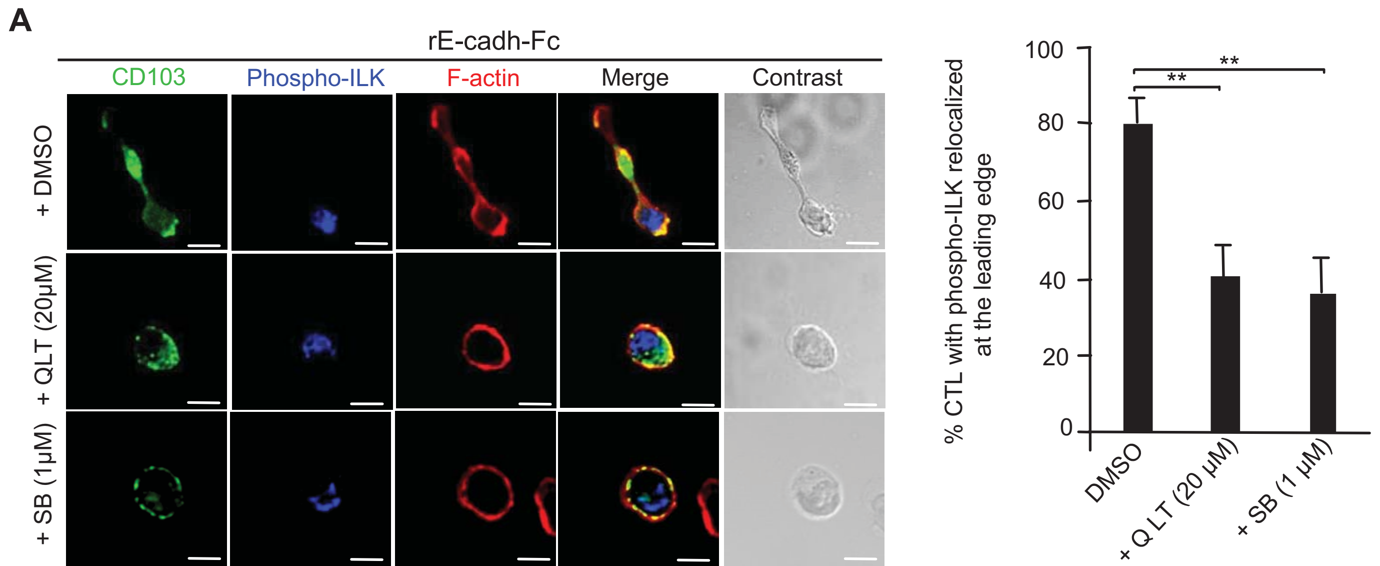
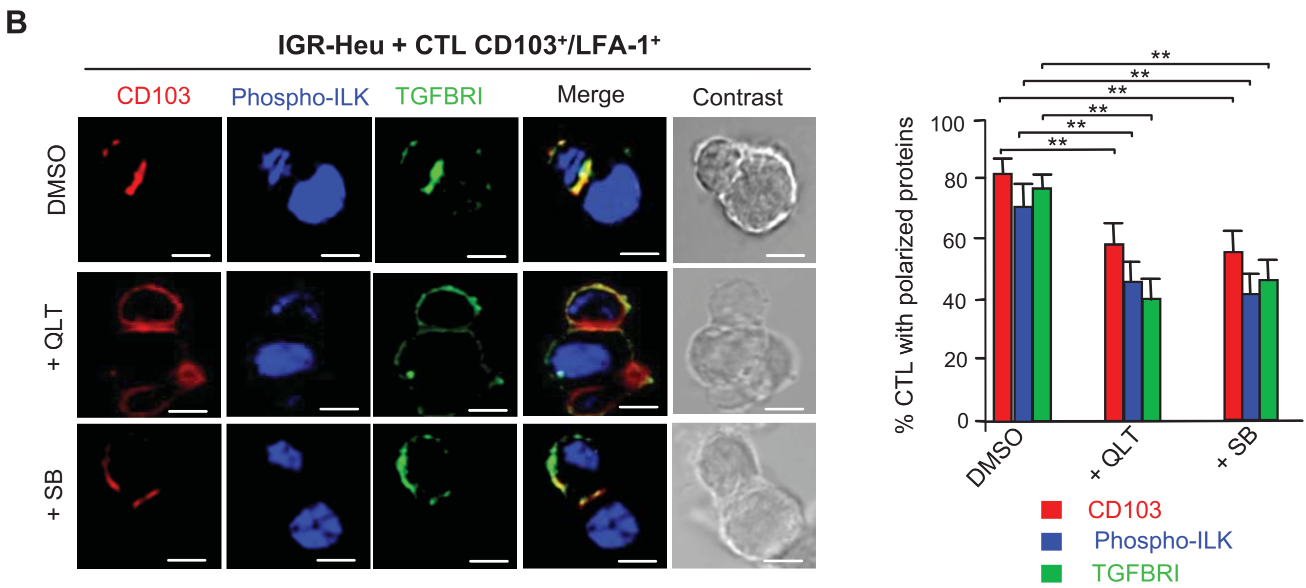
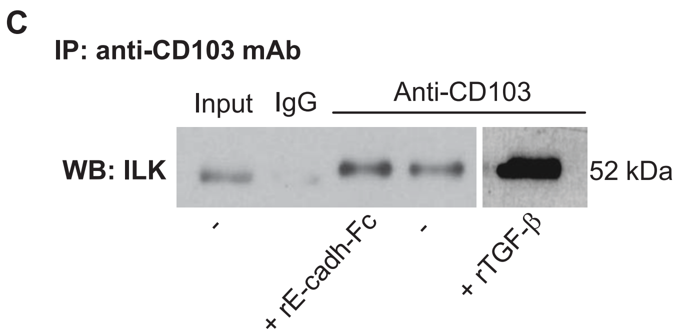
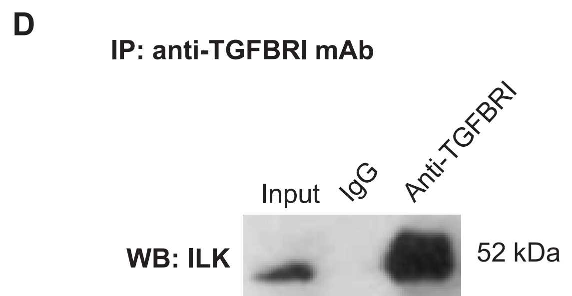
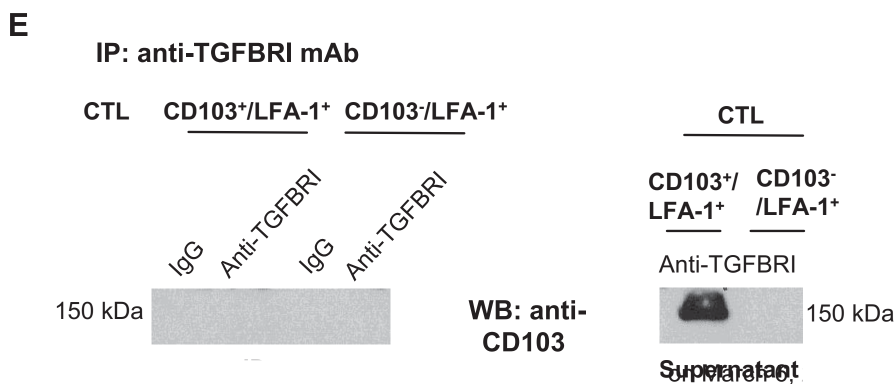
0
+ rE-cadh-fc


A
rE-cadh-Fc
CD103
Phospho-ILK
F-actin
Merge
Contrast
+ DMSO
+ QLT (20µM)
+ SB (1µM)
% CTL with phospho-ILK relocalized at the leading edge
100
80
60
40
20
0
**
**
DMSO
+ Q LT (20 µM)
+ SB (1 µM)

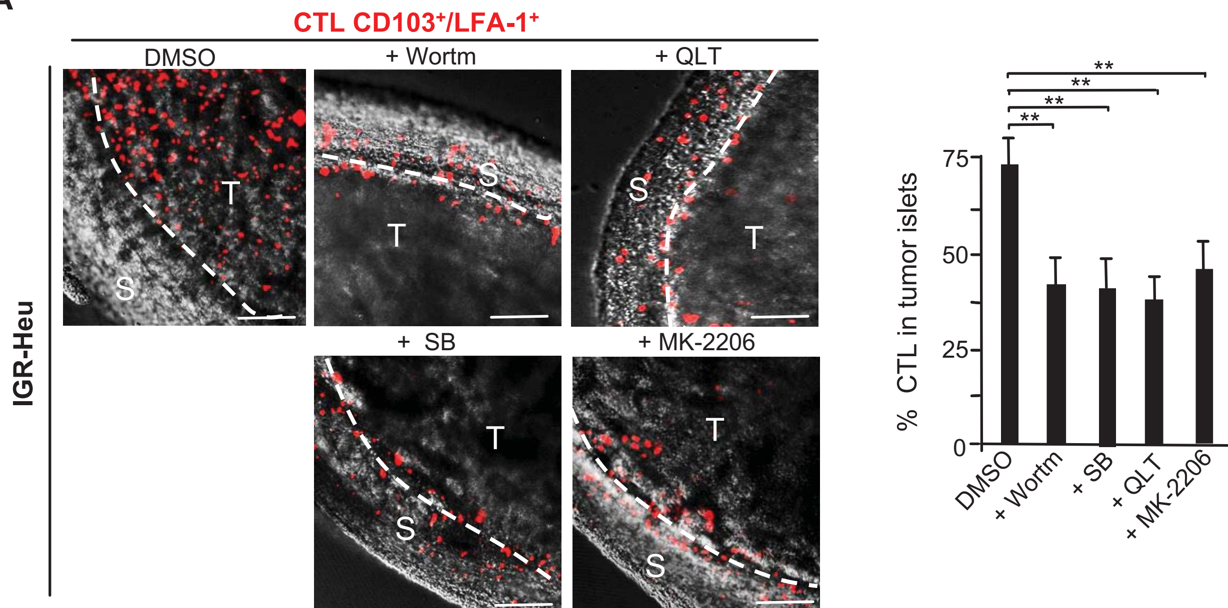

B
IGR-Heu + CTL CD103+/LFA-1+
CD103
Phospho-ILK
TGFBRI
Merge
Contrast
DMSO
+ QLT
+ SB
% CTL with polarized proteins
100
80
60
40
20
0
**
DMSO
+ QLT
+ SB
CD103
Phospho-ILK
TGFBRI


C
IP: anti-CD103 mAb
Input
IgG
Anti-CD103
WB: ILK
52 kDa
-
+ rE-cadh-Fc
-
+ rTGF-β

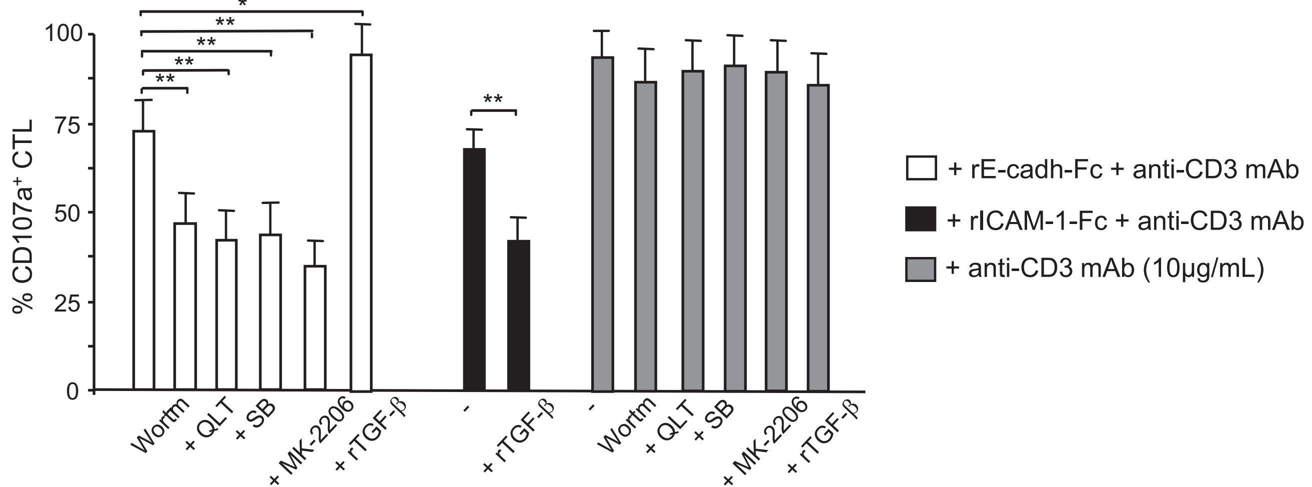

D
IP: anti-TGFBRI mAb
Input
IgG
Anti-TGFBRI
WB: ILK
52 kDa


E
IP: anti-TGFBRI mAb
CTL
CD103+/LFA-1+
CD103-/LFA-1+
IgG
Anti-TGFBRI
IgG
Anti-TGFBRI
150 kDa
WB: anti-CD103
CTL
CD103+/LFA-1+
CD103-/LFA-1+
Anti-TGFBRI
150 kDa
Supernatant

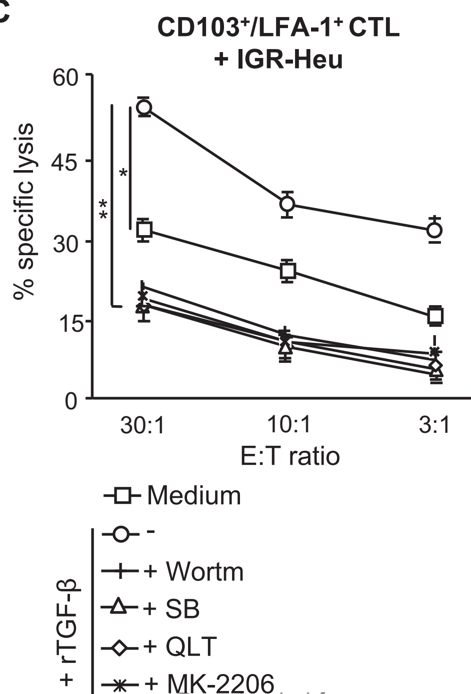
A



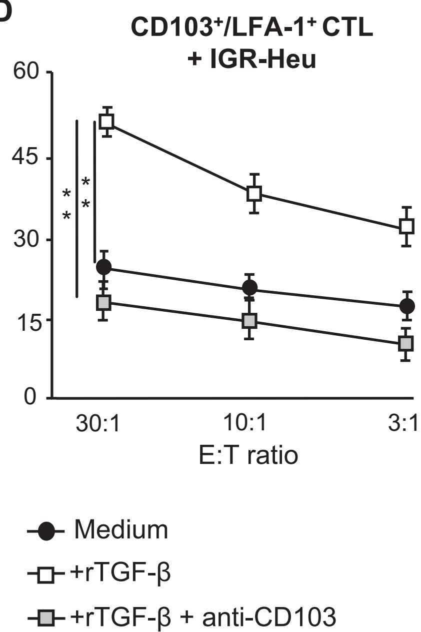
B



C



D



E