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TCR Complex-Activated CD8 Adhesion Function by Human T Cells

Jay C. Varghese and Kevin P. Kane

The CD8 receptor plays a central role in the recognition and elimination of virally infected and malignant cells by cytolytic CD8\(^+\) T cells. In conjunction with the TCR, the CD8 coreceptor binds Ag-specific class I MHC (MHC-I) molecules expressed by target cells, initiating signaling events that result in T cell activation. Whether CD8 can further function as an adhesion molecule for non-Ag MHC-I is currently unclear in humans. In this study, we show that in human CD8\(^+\) T cells, TCR complex signaling activates CD8 adhesion molecule function, resulting in a CD8 interaction with MHC-I that is sufficient to maintain firm T cell adhesion under shear conditions. Secondly, we found that while CD8 adhesive function was triggered by TCR complex activation in differentiated cells, including in vitro generated CTL and ex vivo effector/memory phenotype CD8\(^+\) T cells, naive CD8\(^+\) T cells were incapable of activated CD8 adhesion. Lastly, we examine the kinetics of, and signaling for, activated CD8 adhesion in humans and identify notable differences from the equivalent CD8 function in mouse. Activated CD8 adhesion induced by TCR signaling may contribute to the more rapid and robust elimination of pathogen-infected cells by differentiated CD8\(^+\) T cells. The Journal of Immunology, 2008, 181: 6002–6009.
Materials and Methods

Hybridomas, Abs, and flow cytometric analysis

The anti-CD8β monoclonal SF2 (16) (mouse IgG1) was purchased from Serotec and the anti-CD8α monoclonal 3B5 (mouse IgG2a) from Caltag Laboratories. Isotype control Abs A1 (17) (anti-mouse Ly49A, IgG2a) and Cwy3 (18) (mouse Ly49G2, IgG1) were generated from hybridomas in our laboratory. The anti-CD8α (OKT8) (19), mouse IgG2a, anti-HLA-A (ME1 (20), mouse IgG1), anti-HLA-B7 (BB7.1 (21), mouse IgG1), anti-HLA-B27 (ME2 (22), mouse IgG1), and anti-pan HLA (W6/32, mouse IgG2a) were prepared from American Type Culture Collection (ATCC) hybridomas. Abs prepared from hybridomas were purified using a protein G column. mAb UCHT1 (anti-CD3ε (23), mouse IgG1) was purchased from eBioscience, as were fluorochrome-conjugated mouse Abs Abs CD45RA-PE (HI100, IgG2b), CD62L-PE (HI100, IgG2b), CD28-PE-Cy7 (CD28.2, IgG1), CD27-allophycocyanin (UCHT1, IgG1), CD8ε-FITC (RPA-T8, IgG1), and CD3ε-allophycocyanin (UCHT1, IgG1). Anti-CCR7-FITC (Clone 150503, mouse IgG1) was purchased from R&D Systems. For flow cytometric analysis, cells were incubated with the indicated combinations of fluorochrome-conjugated Abs for 15 min at 4°C, followed by extensive washing and fixation (2% formaldehyde/PBS). Multicolor flow cytometry and cell sorting were performed using FACSCanto and FACSDiva. Materials and Methods

Protein purification and ELISA

HLA molecules were purified from RPMI 8866 cells (for HLA-A2) or HM2.21C1R HLA-B27 transfectants by immunoaffinity chromatography. Between 0.5 and 1 × 10^11 cells were lysed for 10 min on ice in 100-200 ml lysis buffer (150 mM NaCl, 0.5% Nonidet P-40, sodium polyethylenesulfonate fluoride (pH 7.3)). The lysate was cleared by centrifugation at 10,000 relative centrifugal force (RCF) for 20 min and again at 100,000 RCF for 45 min followed by 0.45 μm filtration. The filtered lysate was run at ~0.1 ml/min through a Sepharose 4B (Sigma-Aldrich) precolumn followed by a Sepharose 4B Ab-conjugated (MA2.1 for A2, or ME1 for B27) immunoaffinity column. The immunoaffinity column was washed extensively with 10 ml sodium polyethylenesulfonate fluoride, and 0.5% Triton X-100 (pH 7.3), and 10 mM Tris, 150 mM NaCl, and 0.5% Triton X-100 (pH 8.3). The HLA protein was then eluted in 25 ml diethylamine, 650 mM NaCl, and 0.5% octyl-β-D-glucoside (pH 11.5), and neutralized immediately with 2 M Tris (pH 8). HLA protein concentration was determined by MicroBCA (Pierce). Protein purity was monitored using Western blot and GelCode Blue protein gel stain (Pierce). Conformational integrity was assessed using W6/32 and MA2.1 or ME1 Abs by solid-phase ELISA as described previously (24). Peak fractions showing strong ELISA reactivity were pooled and used in adhesion assays.

Cells

Blood was collected from multiple HLA-A2 positive donors with informed consent in accordance with the University of Alberta Health Research Ethics Board approved protocol and the Helsinki Declaration as revised in 2005. Donor cell DNA was HLA typed at the University of Alberta Hospital Histocompatibility Laboratory. Human CD8+ T cells were isolated by negative selection of PBMC (obtained from venous blood) using the EasySep CD8+ T cell enrichment kit (StemCell Technologies). CD8+ T cell purity was greater than 98% by flow cytometry. Purified CD8+ T cells were either used immediately in assays or after weekly stimulation by coculture with irradiated human B lymphoblastoid RPMI 8866 cells (25) (gift from Dr. R. C. Bleackley, University of Alberta, Edmonton, Canada) and 80 U/ml IL-2, as described (26). Cell lines generated in this manner were named hCD8T-D0 through D7 (where “D” indicates anonymous donor number). The human B lymphoblastoid cell line HM2.21C1R (27) (ATCC), was stably transfected with genes encoding HLA-A2, HLA-B7, or HLA-B27 using Lipofectamine 2000 (Invitrogen).

Cytotoxicity assays

The HM2.21C1R and HM2.21C1R transfectants with HLA-A2 or HLA-B7 were labeled with 100 μCi Na3[^1]CrO4 ([^1]Cr; PerkinElmer Life and Analytical Sciences) for 1 h at 37°C, followed by extensive washing. Target cells were then incubated in 96-well V-bottom microtiter plates for 4 h at 37°C along with human CD8+ T cell lines. Following incubation, plates were centrifuged at 300 RCF for 5 min, after which 25 μl of culture supernatant was retained, mixed with a/well lysis mixture and then [[^1]Cr]release was determined by MicroBCA (Pierce), and counted in a Wallac MicroBeta counter (PerkinElmer). Percent specific lysis was calculated as follows: 100 × ([experimental release]−[spontaneous release])/[([maximal release]−[spontaneous release])]. Cytotoxicity assays were performed in triplicate wells and each experiment performed three separate times. Error bars indicate SE.

Adhesion assays

Adhesion assays were performed as previously described (10), with modifications. The MHC-I protein was diluted in PBS and immobilized on 96-well microplates (BD Biosciences) by incubation at 4°C for 4–16 h. Microplates were washed then blocked at room temperature with 1% BSA/ PBS (Sigma-Aldrich). Human CD8+ T cells labeled with Calcein AM fluorescent dye (Invitrogen) were treated with or without 0.5 μg/ml UCHT1 anti-CD3ε. Flow cytometry was performed using FACSCanto and FACSDiva flow cytometers, respectively, and data was analyzed using either FACSDiva (BD Biosciences) or FCS Express 3 (De Novo Software) software.

Results

Human CD8+ T cells undergo anti-CD3-triggered CD8+ adhesion to non-Ag HLA

Although CD8 is commonly regarded as a coreceptor as it binds to cognate MHC-I simultaneously with the TCR, it is not known whether on humans T cells, CD8 can also function as an adhesion molecule by binding to non-Ag MHC-I (i.e., MHC-I not bound with peptide Ag and not recognized by the TCR of the CD8+ T cell in question), and whether this function is constitutive or induced by TCR signals. To address this issue, we generated effector CD8+ T cell lines by coculturing purified human CD8+ T cells with RPMI 8866 lymphoblastoid cells (HLA-A2, HLA-A3, HLA-B7, HLA-Cw3, and HLA-Cw4), respectively, and was data analyzed using either FACSDiva (BD Biosciences) or FCS Express 3 (De Novo Software) software.

Although HLA-A2 is a self MHC-I for the hCD8T-D2 cell line, in the absence of peptide Ag it is not a cognate ligand for the TCR (Fig. 1). Consequently, we were able to use purified HLA-A2 as an exclusive ligand for CD8 adhesion. We used solid-phase adhesion assays to examine CD8 adhesion to HLA to circumvent confounding effects by other cell surface receptors such as LFA-1/ICAM-1 (1). Purified HLA-A2 protein was immobilized on microtiter wells at varying densities and native protein structure was confirmed by ELISA using the conformation-specific HLA Abs W6/32 (Fig. 2A) and MA2.1 (data not shown). The W6/32-specific ELISA reactivity as a function of HLA-A2 amount immobilized in microtiter wells was linear up to ~0.4 μg/well HLA-A2, at which point a plateau was reached where no further HLA-A2 could be immobilized (Fig. 2A). In the absence of a TCR stimulus, hCD8T-D2 cells showed little or no adhesion to HLA-A2 across the linear range of immobilized HLA-A2 (Fig. 2B). However, upon CD3/ TCR complex triggering, hCD8T-D2 cells were capable of adhering to non-Ag HLA-A2, suggesting that human CD8 can function as an adhesion molecule, but only subsequent to TCR activation.
Enhanced CD8 binding was not limited to hCD8T-D2 cells but also featured functionally distinct characteristics. For example, naive human CD8 T cells that have not encountered Ag are characterized by the expression of CD45RA, the lymph node homing receptors CCR7 and CD62L, and the costimulatory receptors CD28 and CD72 (29). Effector and memory CD8+ T cells, in contrast, express a complex and variable array of the above markers, most of which are typically reduced or absent from the cell surface. Functionally, differentiated cells have an enhanced ability to control pathogens upon secondary exposure owing to their increased frequency, rapid acquisition of effector function, and localization to peripheral sites of infection (11).

The preceding experiments demonstrated that effector CD8+ T cell lines have the ability to undergo activated CD8 adhesion. However, an important question is whether naive human CD8+ T cells are capable of TCR-triggered CD8 adhesion to non-Ag HLA, or whether this capacity is a unique property of CD8+ T cells that have differentiated. To address this issue, we began by comparing fresh ex vivo CD8+ T cells with in vitro generated effector CD8+ T cell lines obtained from the same donor. Purified resting CD8+ T cells from donor 2 were predominantly naive (>80%), appearing as a distinct population of CCR7highCD45RAhigh cells expressing the characteristic uniform high levels of CD27, CD28, and CD62L (L-selectin) (Fig. 3A). Conversely, 35-day hCD8T-D2 cytotoxic cells (generated by weekly stimulation with Ag), showed the characteristic effector cell staining profile that was negative/low for CCR7, CD45RA, CD62L, and CD28, and generally reduced in CD27 expression, compared with naive CD8+ T cells (Fig. 3A). Moreover, hCD8T-D2 behaved functionally as bona fide effector T cells since they demonstrated a robust Ag-specific cytolytic capacity (Fig. 1B). We found that fresh ex vivo (day 0) CD8+ T cells—the majority (83%) of which were naive—showed minimal CD3-triggered CD8 adhesion to HLA-A2 in comparison to the day 35 hCD8T cells (Fig. 3B). Naive and effector/memory

**FIGURE 1.** HLA-B7 but not HLA-A2 is a cognate allo-Ag for the human CD8+ T cell line hCD8T-D2. A. Flow cytometry measuring surface expression of HLA-A2 and HLA-B7 on HMy2.C1R or HMy2.C1R stably transfected to express HLA-A2 or HLA-B7. Cells were stained with MA2.1 (left panel) or BB7.1 (right panel), and their respective isotype control Abs. B, 51Cr release assay using hCD8T-D2 effector cells and target cells described in A.

**FIGURE 2.** Human CD8+ T cells undergo CD3-triggered CD8 adhesion to immobilized non-Ag HLA. A, ELISA using pan-HLA class I-specific Ab (W6/32) detects various densities of purified HLA-A2 immobilized on microtiter plates. B, Adhesion of hCD8T-D2 CTL to immobilized non-Ag HLA-A2 in the presence, but not absence, of soluble anti-CD3 (UCHT1). C, Assessment of soluble UCHT1 Ab-triggered CD8 adhesion by hCD8T-D2 to immobilized HLA-A2 (0.12 μg/well) with, or without, CD8 blocking. Cells were pretreated for 30 min at room temperature with 1–20 μg/ml anti-CD8a (OKT8) or isotype control (A1) Abs before UCHT1 Ab-triggering and plating on HLA-A2. D, Measurement of hCD8T-D0 CTL adhesion to immobilized purified non-Ag HLA-B27 with or without UCHT1 triggering, and with or without CD8 blocking. Cells were pretreated for 20 min at room temperature with 5 μg/ml anti-CD8a (3B5) or isotype control (A1) Ab before UCHT1 Ab-triggering and plating on HLA-B27.
CD8⁺ T cell subsets were then freshly isolated from donor blood to directly compare these subsets for CD3-triggered non-Ag HLA binding by CD8. Two distinct ex vivo subsets were identified and sorted based on surface marker staining: a naive subset expressing uniform and high levels of CCR7, CD45RA, CD27, CD28, and CD62L, and a subset primarily comprised of CD8⁺ T memory and/or effector cells (Fig. 4A and data not shown). Both sorted CD8⁺ T cell populations were between 94 and 98% pure. We found that the naive CD8⁺ T cell population was unable to adhere to non-Ag HLA-A2 in response to a soluble anti-CD3 stimulus, whereas the population containing memory/effector cells underwent adhesion to HLA-A2 (Fig. 4B). The CD3-triggered non-Ag HLA binding by sorted effector/memory cells (Fig. 4B) contrasts with minimal binding by bulk CD8⁺ T cells (Fig. 3B) and reflects the low frequency of memory/effector cells in the bulk CD8⁺ T cell population (Fig. 3A). The CD3-stimulated HLA binding by the ex vivo effector/memory subset are consistent with that found with the Ag-specific in vitro-stimulated CD8⁺ T cell lines (Fig. 3, A and B). We found that even with Ab cross-linked anti-CD3, sorted naive CD8⁺ T cells were unable to adhere to HLA-A2 (Fig. 4C). Conversely, upon cross-linked anti-CD3 treatment, the effector and memory CD8⁺ T cell population showed at least a 2-fold increase in adhesion to HLA-A2 (Fig. 4C), beyond their already significant adhesion when triggered with soluble anti-CD3 alone (Fig. 4B). Although the naive CD8⁺ T cell sorted subset was incapable of CD3-triggered CD8 adhesion, these cells acquired enhanced CD8 adhesion function after undergoing in vitro differentiation by weekly stimulation with APCs and IL-2 (data not shown), further indicating that the Ag-driven maturation state of a CD8⁺ T cell determines its capacity for CD3-triggered CD8 adhesion. Collectively, these findings demonstrate that ex vivo differentiated CD8⁺ T cells have the ability to undergo CD3-triggered adhesion to non-Ag HLA class I, unlike their naive CD8⁺ T cell counterparts. Furthermore, Ab-induced CD3 clustering augments CD3-triggered non-Ag HLA binding by the effector/memory CD8⁺ subset.

CD8 adhesion to non-Ag HLA following CD3-triggering of human CD8⁺ T cells is transient

We have previously shown that in mice, CD3-triggered CD8 adhesion to non-Ag MHC-I increases with time after stimulus, reaching a maximum at 60 min, which is sustained for over 5 h (4, 30). To determine whether these kinetics also held for human T cells, we performed the anti-CD3-triggered non-Ag HLA adhesion assay while varying the amount of time for which cells were allowed to bind before adhesion was measured. We used hCD8T-D2 effector CTL in these assays as they exhibited CD3-induced CD8 binding as observed with fresh ex vivo effector/memory CD8⁺ T cells; thus, they are likely functionally equivalent in this regard, and moreover, they could be produced in significant numbers. The hCD8T-D2 CTL showed increasing levels of adhesion to immobilized HLA-A2 in response to CD3 triggering, with maximal adhesion observed in multiple assays in a range spanning 30 to 60 min poststimulus (Fig. 5 and data not shown). However, in marked contrast to mouse CD3-triggered CD8 binding to non-Ag MHC-I (30), the equivalent in human was seen to decrease by 90 min and was nearly nonexistent by 4 h (Fig. 5). These observations indicate that CD3-activated CD8 binding by human T cells is transient, in comparison to mouse.

PI3K, PKC, and the actin cytoskeleton in the regulation of CD3-triggered CD8 binding to non-Ag HLA

The transient nature of CD3-triggered CD8 adhesion by human CD8⁺ T cells suggests that it may be regulated by a signaling mechanism similar to CD3-triggered LFA-1 adhesion to ICAM-1, which is also transient (31). To approach this issue, we began by focusing on phorbol ester stimulation, which is known to activate
and all remaining (effector/memory) CD8 and CD62L-allophycocyanin-Cy7 staining corresponding to gated (naive). Histograms (below plot) show CD27-allophycocyanin, CD28-PE-Cy7, nongated effector/memory cells showed 94.4% sorting efficiency (right dot), while the remaining middle dot plot showed 98.7% sorting efficiency (right dot plot). Postsort analysis on the gated naive cell population cells (left dot plot) sorted for the naive cell gated population, which comprised 66.9% of total /H11001jugated Abs recognizing CCR7 and CD45RA. The CD8 panels A undergo CD3-triggered CD8-mediated adhesion. CTL to HLA-A2 (Fig. 6A). Adhesion was maximal at 10 nM PMA in the low-dose range of 1–30 nM resulted in adhesion of hCD8T-D2 of CD3 engagement. We found that treatment with PMA at the concentration (9). We treated hCD8T-D2 CTL with the phorbol ester PMA in inhibition for mouse activated CD8 adhesion, promoting de-adhesion; however, phorbol ester treatment is inhibitory for mouse activated CD8 adhesion, promoting de-adhesion (9). We treated hCD8T-D2 CTL with the phorbol ester PMA as a potential triggering stimulus for CD8 adhesion in substitution of CD3 engagement. We found that treatment with PMA at the low-dose range of 1–30 nM resulted in adhesion of hCD8T-D2 CTL to HLA-A2 (Fig. 6A). Adhesion was maximal at 10 nM PMA and remained at this level as higher concentrations of phorbol ester were used. To determine whether PMA specifically activated CD8 to undergo adhesion to HLA-A2, we performed the same adhesion assay but with anti-CD8 blocking Abs (14, 32) (Fig. 6B). Blocking CD8 ablated PMA-induced hCD8T-D2 adhesion to HLA-A2 (Fig. 6B), indicating that phorbol ester is stimulatory for enhanced CD8 adhesion by human T cells.

PI3K is a signaling molecule downstream of the TCR/CD3 complex that is involved in TCR-activated adhesion by mouse CD8 T cells (9, 33). To examine the role of PI3K in human CD8-triggered CD8 adhesion, we used the irreversible PI3K-selective inhibitor Wortmannin. Treatment of hCD8T-D0 cells with 1–100 nM Wortmannin immediately before triggering with anti-CD3 resulted in a decrease of between 40 and 70% in CD8-mediated T cell adhesion to immobilized HLA-A2 (Fig. 7A). This result is consistent with previous studies implicating a role for PI3K in TCR/CD3-triggered cell adhesion pathways (9, 33, 34).

The actin cytoskeleton has an essential function in the formation of stable T cell conjugates and cytoskeletal rearrangements are important for T cell activation (35). We found that CD3-activated CD8 adhesion by hCD8T-D0 CTL was exquisitely sensitive to treatment with Cytochalasin D, an actin filament disrupter/polymerization inhibitor, as a low dose of 0.5 μM Cytochalasin D was sufficient to nearly ablate adhesion to HLA-A2, while higher doses completely prevented hCD8T-D0 from adhering to non-Ag HLA (Fig. 7B). These results indicate a critical role for the actin cytoskeleton in CD3-activated CD8 adhesion to non-Ag HLA by human CTL.

As a whole, these findings demonstrate that TCR/CD3 activation of CD8 adhesion to non-Ag HLA is regulated in a manner similar to that of other adhesion molecules such as LFA-1 by the involvement of the actin cytoskeleton and downstream signaling molecules from TCR engagement including PI3K and possibly PKC. Furthermore, the stimulatory role of PMA in human TCR-triggered CD8 adhesion is in direct contrast to mouse, where PMA induces the de-adhesion of CD8 from non-Ag MHC-I (9).

TCR/CD3 downstream targets including PKC. Phorbol ester stimulation has been shown to activate T cell adhesion by adhesion molecules such as LFA-1 (31); however, phorbol ester treatment is inhibitory for mouse activated CD8 adhesion, promoting de-adhesion (9). We treated hCD8T-D2 CTL with the phorbol ester PMA as a potential triggering stimulus for CD8 adhesion in substitution of CD3 engagement. We found that treatment with PMA at the low-dose range of 1–30 nM resulted in adhesion of hCD8T-D2 CTL to HLA-A2 (Fig. 6A). Adhesion was maximal at 10 nM PMA and remained at this level as higher concentrations of phorbol ester were used. To determine whether PMA specifically activated CD8 to undergo adhesion to HLA-A2, we performed the same adhesion assay but with anti-CD8 blocking Abs (14, 32) (Fig. 6B). Blocking CD8 ablated PMA-induced hCD8T-D2 adhesion to HLA-A2 (Fig. 6B), indicating that phorbol ester is stimulatory for enhanced CD8 adhesion by human T cells.

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Discussion

It was not known whether the TCR can activate CD8 adhesion molecule function in human T cells and, moreover, which CD8⁺ T cell populations may have this capacity. In this report, we show that human CD8⁺ T cells undergo TCR/CD3-activated CD8 adhesion to non-Ag HLA molecules. Additionally, we found that while CD8 adhesion was enhanced by CD3 triggering in differentiated cells, including in vitro-generated CTL and ex vivo effector/memory phenotype CD8⁺ T cells, naive CD8⁺ T cells were incapable of CD3-enhanced CD8 adhesion. Lastly, we report that CD8 adhesion function in humans is inducible by PMA stimulus and is kinetically distinct, both of which are in contrast to mouse CD8 adhesion function (9, 30).

Freshly isolated effector/memory phenotype CD8⁺ T cells were capable of CD8 adhesion to non-Ag MHC-I, as were naive CD8⁺ T cells that had undergone Ag-mediated T cell differentiation. The acquisition of enhanced CD8 adhesion functions became apparent as early as after two rounds of stimulation of naive CD8⁺ T cells by APCs (e.g., day 10 in vitro) and correlated with the emergence of an effector cell population exhibiting the loss of CD45RA, CCR7, CD62L, and CD28 cell surface markers (data not shown). These results reinforce our finding that naive cells are incapable of TCR-enhanced CD8 adhesion but must undergo differentiation into an effector or memory cell before acquiring this function. The mechanism(s) underlying this difference in CD8 function between subsets is unclear but may involve physical properties of the CD8 molecule itself and/or differences in signaling between CD8⁺ T cell subsets. Human memory phenotype CD8⁺ T cells do not express greater levels of cell surface CD8α and β-chains than naive CD8⁺ T cells (data not shown), thereby excluding CD8 expression levels as an explanation for differential CD8 adhesion by T cell subsets. Differential glycosylation by the sialyltransferase ST3Gal-I has been proposed to regulate the affinity of CD8 for noncognate MHC-I during thymocyte development (36, 37); however, the postulated role of ST3Gal-I in regulating CD8 affinity for MHC-I is controversial and likely more complex than predicted (38). Nevertheless, differential glycosylation of CD8 on naive and effector/memory peripheral T cell subsets could be a possible explanation for the different functional capacities of CD8 we observed between naive and differentiated T cells (39, 40). Aside from changes in CD8 itself, inherent signaling differences between naive and effector/memory cells in response to TCR induction may account for CD8 adhesion function in the latter T cell subset. For example, PKC activity has been shown to be greater in CD45RO⁺ differentiated human T cells compared with CD45RA⁺ naive cells, in response to an anti-CD3 stimulus (41). Enhanced PKC activity in differentiated T cells may provide sufficient signaling to mobilize CD8 adhesion function, and such a mechanism would be in keeping with our finding that a PMA stimulus can activate CD8 adhesion function. Irrespective of mechanism, TCR-regulated CD8 adhesion in differentiated T cell subsets may contribute to the rapid and robust memory recall and effector cytolytic response to Ag.

We found that TCR-induced regulation of CD8 function in human T cells differs from that of mouse with respect to the transient nature of adhesion and the stimulatory effect of PMA. Nevertheless, human CD8⁺ T cells also share similarities with their mouse counterparts (9, 10) as both use the PI3K pathway and require the actin cytoskeleton to mediate TCR-activated enhanced CD8 adhesion. We found Wortmannin inhibition of enhanced CD8 adhesion to be significant, although incomplete, suggesting that CD8 adhesion is likely regulated by PI3K and additional pathways. In marked contrast to our findings in human, mouse TCR-activated CD8 adhesion is inhibited by PMA and is not transient but rather stable for over 3 h (9, 30). It is not readily apparent why the kinetics of TCR-activated CD8 adhesion would differ between mouse and human. The discrepancy may simply be a reflection of differential regulation by signaling intermediates. Additionally, the murine CD8 interaction with MHC-I is higher affinity than that of human due to a substantially lower dissociation constant (42); this may result in a more stable, and consequently longer-lived, adhesion response as seen in the mouse. Whether the kinetic difference observed between species serves functionally different roles or is merely incidental is not clear. It may be that transient TCR-activated CD8 adhesion in humans preferentially contributes to conjugate formation during CTL lytic function, whereas TCR-activated CD8 adhesion in mouse may be involved not only in CTL lytic function but also more sustained interactions observed between CTL and dendritic cells (43). However, it is also likely that de-adhesion signals (9), from extracellular stimuli present in vivo may alter the kinetics of activated CD8 adhesion to suit the desired function. Additional studies correlating the duration of cellular contact by memory and effector CD8⁺ T cells with short- and long-term functional outcomes will be required to determine whether TCR-activated CD8 adhesion fulfills different functional roles in humans vs mice.

The mechanism by which TCR stimulation enhances CD8 binding to MHC-I is not known. Although modification of CD8 glycosylation can alter CD8 affinity for MHC-I, these changes are unlikely to account for the rapid kinetics of TCR-activated CD8 adhesion indicated by our data. It is possible that enhanced CD8 adhesion involves multiple mechanisms, perhaps including CD8 conformational changes resulting in higher binding affinity (44, 45), and localization in membrane microdomains such as lipid rafts (46, 47).

The coupling of CD8 with the TCR has been shown to occur via CD3ζ and the TCR α-chain connecting peptide motif, with the latter required for optimal MHC-I binding and functional responses including thymocyte positive selection (48, 49). The interaction between CD8 and non-Ag MHC-I may also enhance association between the CD8 cytoplasmic domain and the CD3ζ complex when local TCR are also engaged, thereby allowing the CD8-associated kinase Lck to phosphorylate ITAM motifs on the CD3ζ-chain for efficient T cell signaling and activation (42, 50). Recent studies have shown that CD8 interaction with non-Ag MHC-I augments T cell-APC conjugate formation, as well as the kinetics and magnitude of intracellular Ca²⁺ mobilization during T cell activation (50, 51). Although the CD8 receptor was shown to bind non-Ag MHC-I in the absence of TCR engagement, cell conjugate formation and activation required the presence of cognate...
MHC-I Ag (50). Our data show that TCR engagement is essential for CD8-mediated adhesion by human T cells, since we observed little or no adhesion to non-Ag MHC-I in the absence of triggering of the TCR complex. Collectively, these results suggest that while CD8 can interact weakly with MHC-I in a constitutive TCR-independent manner at the synapses of cell-cell contact (50, 52), TCR-induced signals are required for optimal CD8 orientation and/or cell morphological changes necessary for tight adhesion between cells. In further support of this model is our finding that cytoskeleton disruption abrogates CD8-mediated human T cell adhesion to non-Ag MHC-I.

The density of cognate MHC-I is typically low in comparison to non-Ag MHC-I on virally infected and malignant cells (53, 54); however, the CD8+ T cell response exhibits remarkable sensitivity and specificity in that as few as 1–3 cognate MHC-I are sufficient to elicit activation (55, 56). Indeed, studies have shown that when cognate MHC-I Ag is limiting, CD8 interactions with non-Ag MHC-I are essential for T cell conjugate formation and activation, thereby supporting a model in which CD8 binding to non-Ag MHC-I contributes to the sensitivity of the T cell response (7, 50, 51). Our data with human T cells also support this model as we found that a low dose of soluble anti-CD3, known to be substimulatory (7), was sufficient to trigger CD8-mediated T cell adhesion to non-Ag MHC-I. In addition to likely increasing the sensitivity of the T cell response, we show that CD8 binding to non-Ag MHC-I contributes to the adhesive aspect of T cell function. The adhesion of human CD8 induced by TCR engagement was seen not only to non-Ag self MHC-I but also to non-Ag allogeneic MHC-I (Fig. 2D). The latter CD8 interaction may contribute to allogeneic CD8+ T cell responses in transplant rejection by CTL.

In summary we have found that differentiated human CD8+ T cells mediate CD8-dependent firm adhesion to non-Ag HLA when stimulated through the TCR/CD3 complex. Enhanced CD8 adhesion likely contributes to the sensitivity of Ag recognition and adhesive forces for T cell conjugate formation and activation.

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Disclosures
The authors have no financial conflict of interest.

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