

Very Late Antigen 4-dependent Adhesion and Costimulation of Resting Human T Cells by the Bacterial β 1 Integrin Ligand Invasin

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Summary

Bacteria and viruses often use the normal biological properties of host adhesion molecules to infect relevant host cells. The outer membrane bacterial protein invasins mediates the attachment of *Yersinia pseudotuberculosis* to human cells. In vitro studies have shown that four members of the very late antigen (VLA) integrin family of adhesion molecules, VLA-3, VLA-4, VLA-5, and VLA-6, can bind to invasins. Since CD4⁺ T cells express and use these integrins, we have investigated the interaction of CD4⁺ T cells with purified invasins. Although VLA integrin-mediated adhesion of T cells to other ligands such as fibronectin does not occur at high levels unless the T cells are activated, resting T cells bind strongly to purified invasins. The binding of resting T cells to invasins requires metabolic activity and an intact cytoskeleton. Although CD4⁺ T cells express VLA-3, VLA-4, VLA-5, and VLA-6, monoclonal antibody (mAb) blocking studies implicate only VLA-4 as a T cell invasins receptor. Like other integrin ligands, invasins can facilitate T cell proliferative responses induced by a CD3-specific mAb. These results suggest that the nature of the integrin ligand is a critical additional factor that regulates T cell integrin activity, and that direct interactions of T cells with bacterial pathogens such as *Yersinia* may be relevant to host immune responses to bacterial infection.

The immune response is dependent on the physical interaction of its various cellular members with each other and with the surrounding microenvironment (1). Integrins are a large family of α/β heterodimeric proteins that mediate the adhesion of many different cell types to other cells and to components of the extracellular matrix (ECM) such as fibronectin (FN), laminin, and collagen (1-3). Since T cells must be able to rapidly alternate between adhesive and nonadhesive states, the functional activity of integrins expressed on T cells is tightly regulated. T cell activation rapidly modulates the functional activity of four integrins expressed on peripheral T cells: the β 2 integrin LFA-1 and the β 1 integrins very late antigen (VLA)-4 (also designated α 4 β 1), VLA-5 (α 5 β 1), and VLA-6 (α 6 β 1). Whereas resting T cells demonstrate minimal integrin-mediated adhesion, activation results within minutes in strong T cell adhesion to integrin ligands without a change in the level of cell surface expression of the integrin receptors themselves (4-6). Multiple activation signals can induce this rapid change in integrin activity, including treatment with the phorbol ester PMA, the Ca²⁺ ionophore A23187, mitogenic pairs of CD2-specific mAbs, mAb cross-linking of the CD3-TCR complex, or mAb cross-linking of the T cell surface molecules CD7, CD28, or CD31

(4-8). To date, this dependence on activation for strong T cell integrin function has been demonstrated for all of the integrin ligands tested, including the LFA-1 ligand intercellular adhesion molecule 1 (ICAM-1), the VLA-4 cell surface ligand vascular cell adhesion molecule (VCAM)-1, the VLA-4 and VLA-5 ligand FN, and the VLA-6 ligand laminin (4-7, 9).

Whereas adhesion molecules play a fundamentally critical role in immune system function, their adhesive properties are often used by bacteria and viruses as a means of entry and survival in the host organism (10, 11). Recent studies of the intracellular bacterial pathogen *Yersinia pseudotuberculosis* have shown that the bacterial outer membrane protein invasins mediates the attachment and internalization of *Yersinia* by normally nonphagocytic cells by binding to four different VLA integrins: VLA-3, VLA-4, VLA-5, and VLA-6 (12, 13). Since CD4⁺ T cells express all of these VLA integrins (6), and VLA-4 in particular is expressed predominantly by lymphoid cells (2), we hypothesized that the interaction of T cells with invasins may be critical to our understanding of integrin function and immune responses to this bacterial pathogen. We show in this study that: (a) strong T cell binding to invasins occurs without activation, unlike binding to all

other T cell integrin ligands tested to date; (b) the predominant invasin receptor on CD4⁺ T cells is VLA-4; and (c) invasin can facilitate CD3-mediated T cell proliferative responses.

Materials and Methods

Human T Cells. Peripheral CD4⁺ T cells were isolated by negative magnetic immunoselection from either leukapheresis packs or buffy coats as previously described (6, 7, 14). The T cell populations were typically >96% CD3⁺CD4⁺ as assessed by flow cytometric analysis. Complete depletion of monocytes in the T cell preparation was determined by the lack of proliferative response to optimal concentrations (1:200 dilution) of PHA (M form) (Gibco-BRL, Grand Island, NY).

Antibodies and Other Reagents. The following mAbs were used as purified IgG: anti-CD3 mAb OKT3, anti-CD7 mAb 3A1, and anti-LFA-1 α chain mAb TS1/22 (American Type Culture Collection, Rockville, MD); anti-VLA-5 α chain mAb MAB16 (15) and anti-VLA β chain mAb MAB13 (15) (provided by Dr. K. Yamada, National Institutes of Health [NIH], Bethesda, MD); and anti-VLA-4 α chain mAb NIH49d-1 (8) (Drs. S. Shaw and Y. Tanaka, NIH). The following mAbs were used as dilutions of ascites fluid: anti-CD28 mAb 9.3 (J. Ledbetter, Bristol Myers Squibb, Seattle, WA); anti-CD2 mAbs 95-5-49 and 9-1 (Dr. B. Dupont, Memorial Sloan Kettering Cancer Center, New York, NY); anti-VLA-3 α chain mAb P1B5, anti-VLA-5 α chain mAb P1D6, and anti-VLA-4 α chain mAb P4G9 (Telios Pharmaceuticals, Inc., San Diego, CA); and anti-VLA-4 α chain mAb L25 (16) (Dr. P. Estess, Becton Dickinson & Co., Mountain View, CA). The following mAbs were used as dilutions of culture supernatant: anti-VLA-6 α chain mAb GoH-3 (Dr. A. Sonnenberg, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands); anti-VLA-5 α chain mAbs BIIG2 and B1E5, and anti-VLA β chain mAb AIIB2 (Dr. C. Damsky, University of California, San Francisco, CA); the anti-VLA-4 α chain mAbs HP2/1, HP1/2, HP2/4, HP1/1, HP1/7 (17), and the anti-VLA β chain mAbs LIA1/2, ALEX1/4, LIA1/5, and TS2/16 (18) (kindly provided by Dr. F. Sanchez-Madrid, Universidad Autonoma de Madrid, Madrid, Spain).

The invasin utilized in this study is a fusion protein consisting of the COOH-terminal end of invasin (479 amino acids in length) and maltose binding protein (MBP) (13). MBP and collagen (Sigma Chemical Co., St. Louis, MO) were used as negative control proteins. FN was purchased from the New York Blood Center (New York, NY). Cytochalasin B was purchased from Sigma Chemical Co., and staurosporin was purchased from Boehringer Mannheim Diagnostics (Indianapolis, IN). Both were dissolved in DMSO before use. Dibutyryl cAMP (Calbiochem-Behring Corp., San Diego, CA) was dissolved in PBS before use.

Adhesion Assays. Adhesion assays were performed as described (6, 7). Each well contained 50,000 ⁵¹Cr-labeled T cells in a final volume of 0.1 ml PBS/0.5% HSA. For PMA activation, cells were added to wells containing 10 ng/ml PMA (Sigma Chemical Co.). All data are expressed as the mean percent of cells binding from three replicate wells plus SEM. In all mAb blocking experiments, mAbs were added to wells at 10 μ g/ml (for purified IgG), 1:500 dilution (for ascites fluid) or 1:5 dilution (for culture supernatants). Inhibitors were present throughout the assay at the concentrations indicated.

T Cell Proliferation Assays. Proliferation assays were performed essentially as previously described (19). During the last 18 h of the assay, the culture was pulsed with 1 μ Ci/well of [³H]thymidine

(New England Nuclear/Dupont, sp act of 6.7 Ci/mmol). Results are expressed as the arithmetic mean cpm of triplicate cultures. Microtiter plates (Costar Corp., Cambridge, MA) were prepared by first incubating indicated concentrations of the CD3 mAb OKT3 overnight at 4°C in PBS, washing away the unbound mAb, and adding the indicated concentrations of invasin or FN (diluted in PBS with Ca²⁺ and Mg²⁺) for an additional 2–3 h incubation at room temperature. Plates were washed three times with PBS before the start of culture. mAbs were added at the same concentrations used in the adhesion assays, except for culture supernatants, which were used at a 1:8 final dilution.

Results

Binding of Resting As Well As Activated T Cells To Purified Invasin. Previous studies have shown that strong adhesion of human CD4⁺ T cells to relevant VLA ligands such as FN (via VLA-4 and VLA-5), laminin (via VLA-6), and VCAM-1 (via VLA-4) requires activation of the T cell by a stimulus such as PMA (6, 7, 9). In contrast to the results obtained with these previously described VLA integrin ligands, resting CD4⁺ T cells bound strongly and in a dose-dependent fashion to purified invasin (Fig. 1). Treatment of T cells for 10 min with 10 ng/ml PMA dramatically increased adhesion to FN, but only slightly increased adhesion to invasin (Fig. 1). An increase in binding to invasin after PMA activation of T cells was most evident at low doses of immobilized invasin. Similar results were obtained after activation of T cells with other stimuli that increase VLA integrin-mediated binding to FN (6, 7, and data not shown).

VLA-4 Is the Predominant Integrin Invasin Receptor on Human T Cells. mAb blocking studies were performed using a large panel of VLA β chain- and VLA α chain-specific mAbs. The data in Fig. 2 show that the VLA β chain-specific mAb AIIB2, which has previously been shown to block the binding

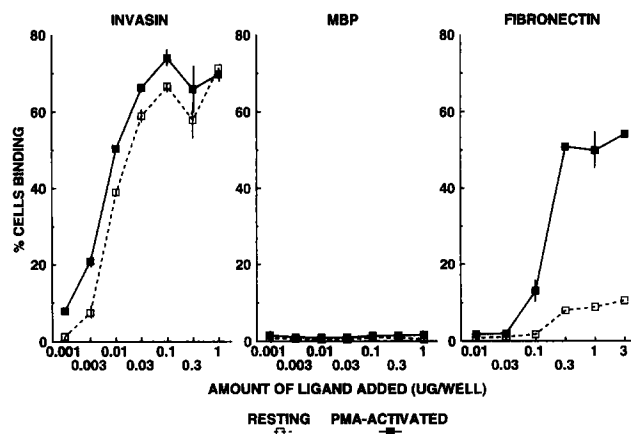


Figure 1. Binding of resting and PMA-activated CD4⁺ T cells to purified invasin. Binding of ⁵¹Cr-labeled CD4⁺ T cells to purified invasin, MBP, or FN (applied at the indicated concentrations) after no activation (open squares) or after activation for 10 min at 37°C with 10 ng/ml PMA (solid squares). Binding of resting and PMA-activated T cells to collagen was <8%. Data are representative of a minimum of four experiments with different donors.

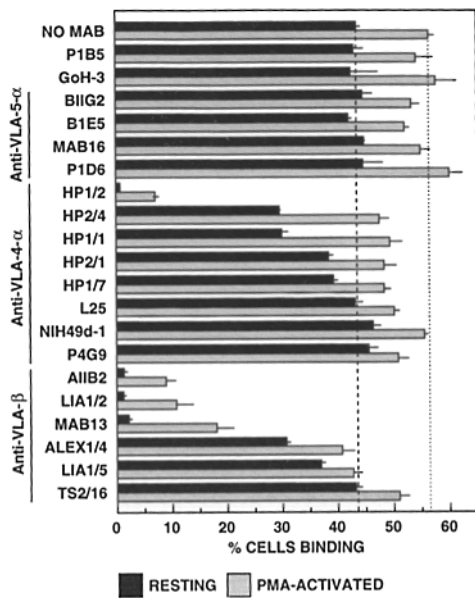


Figure 2. mAb blocking of T cell adhesion to invasin by VLA α chain and VLA β chain mAbs. Binding of resting (solid bars) and PMA-activated (shaded bars) CD4⁺ T cells to invasin (added at 30 ng/well) was assessed in the continuous presence of the indicated mAbs. Binding of resting and PMA-activated T cells to either collagen or MBP was <5%. Data are representative of a minimum of three experiments with different donors.

of the erythroleukemia cell line K562 to invasin (12), can also completely inhibit T cell adhesion to invasin. In addition, two other VLA β chain-specific mAbs, LIA1/2 and MAB13, can inhibit T cell adhesion to invasin. Three other VLA β chain-specific mAbs only partially inhibit T cell adhesion to invasin, suggesting that these mAbs recognize epitopes on the VLA β chain that are minimally involved in binding to invasin.

Studies with various VLA α chain-specific mAbs demonstrate that one VLA-4 α chain-specific mAb, HP1/2, can inhibit T cell adhesion to invasin as effectively as the inhibitory VLA β chain-specific mAbs (Fig. 2). A panel of VLA-5 α chain-specific mAbs, all of which inhibit cell adhesion to FN, did not inhibit T cell adhesion to invasin. This panel includes one VLA-5-specific mAb (BIIG2) that was previously shown to inhibit the binding of K562 cells to invasin (12). In addition, the VLA-3 α chain-specific mAb P1B5 and the VLA-6 α chain-specific mAb GoH-3, both of which are functionally inhibitory mAbs (6, 20, and Y. Shimizu, unpublished results), did not inhibit T cell adhesion to invasin. Thus, these data strongly suggest that VLA-4 is the predominant invasin receptor on human T cells.

Binding of Resting T Cells to Invasin Requires Metabolic Activity and an Intact Cytoskeleton. We assessed the ability of various metabolic inhibitors to inhibit the binding of resting T cells to invasin as a first test of whether engagement by invasin generates intracellular signals that might rapidly up-regulate VLA integrin activity. The data in Fig. 3 demonstrate that the increased adhesion to invasin observed with

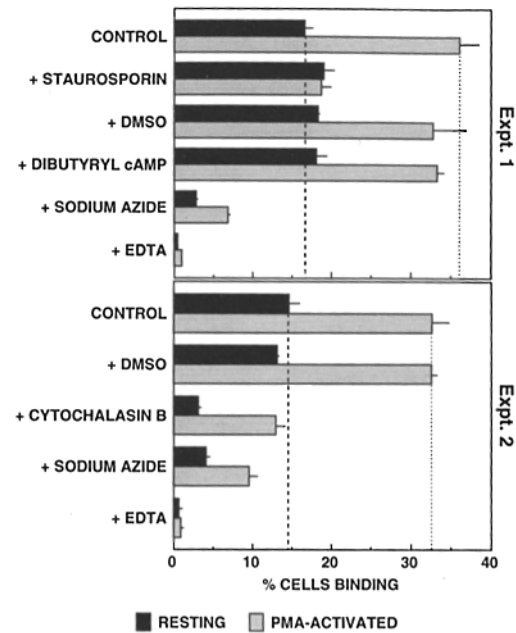


Figure 3. Binding of resting T cells to invasin requires metabolic activity and an intact cytoskeleton. Binding of CD4⁺ T cells was assessed as described in Materials and Methods. Invasin was immobilized at 30 ng/well in both experiments shown. This suboptimal amount of invasin was chosen to maximize the differences in binding between resting and PMA-activated T cells. For dibutyryl cAMP, T cells were preincubated for 30 min at 37°C. Each of the indicated inhibitors was present throughout the adhesion assay. Staurosporin was used at a final concentration of 1 μ M, dibutyryl cAMP at 3 mM, sodium azide at 0.1%, cytochalasin B at 5 μ g/ml, and EDTA at 5 mM. Data are representative of a minimum of three experiments testing each inhibitor.

PMA-activated T cells can be inhibited to the level of resting T cell binding by the protein kinase C (PKC) inhibitor staurosporin. However, staurosporin did not inhibit resting T cell adhesion to invasin, suggesting that adhesion of resting T cells to invasin is not dependent on PKC activation. Treatment of T cells with the cAMP analogue dibutyryl cAMP, which has been shown to inhibit activation-dependent T cell adhesion to FN and ICAM-1 (4, 7), or the tyrosine kinase inhibitor herbimycin A also did not inhibit the binding of resting T cells to invasin (Fig. 3 and data not shown). However, treatment of T cells with sodium azide significantly inhibited invasin binding by resting T cells, suggesting that T cell adhesion to invasin requires a metabolically active T cell. Furthermore, the ability of the cytoskeletal disrupting agent cytochalasin B to inhibit the binding of both resting and PMA-activated T cells to invasin is consistent with earlier data implicating the cytoskeleton in integrin-mediated adhesive events (21). Treatment of T cells with EDTA also abolishes T cell binding to invasin, consistent with the divalent cation requirements of integrin receptors.

Invasin Facilitates CD3-mediated T Cell Proliferation. The interaction of VLA integrins with a relevant ligand can also facilitate T cell proliferative responses. This has been most effectively demonstrated in an in vitro system where purified

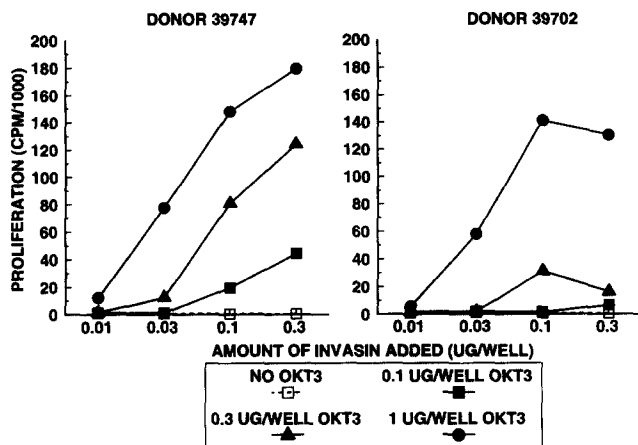


Figure 4. Purified invasin facilitates CD3-mediated T cell proliferation. Proliferative responses of CD4⁺ T cells were assessed as described in Materials and Methods. Microtiter wells were coated with either no OKT3 mAb (open squares), 0.1 μ g/well OKT3 (solid squares), 0.3 μ g/well OKT3 (solid triangles), or 1 μ g/well OKT3 (solid circles) and then the indicated amount of invasin before wells were washed and cells added. [³H]Thymidine incorporation by T cells in the absence of any additional stimulus was 2361 cpm (donor 39747) and 579 cpm (donor 39702) and has not been subtracted from the data shown. Thymidine incorporation by T cells in the presence of PHA (1:200 dilution) was 3,053 cpm (donor 39747) and 880 cpm (donor 39702). Thymidine incorporation by T cells to any concentration of OKT3 alone tested (or a combination of OKT3 and MBP) was <2,060 cpm (donor 39747) and 1,767 cpm (donor 39702). Data are representative of a minimum of four different experiments with different donors.

T cells are activated through the CD3-TCR by the CD3-specific mAb OKT3 immobilized on plastic. In the absence of accessory cells, human T cells fail to proliferate in response to immobilized CD3 mAb alone (9, 19). However, vigorous T cell proliferation occurs when a VLA integrin ligand, such as FN, laminin, or VCAM-1, is coimmobilized with the CD3 mAb (9, 15, 19, 22). Fig. 4 shows that as with other VLA integrin ligands, coimmobilization of invasin with the CD3 mAb OKT3 can also result in T cell proliferation. The same VLA4 α chain- and VLA β chain-specific mAbs that inhibit T cell adhesion to invasin (Fig. 2) also inhibit T cell proliferation induced by coimmobilized invasin and CD3 mAb (data not shown).

Discussion

We have shown in this study that human CD4⁺ T cells bind via VLA integrins to the bacterial protein invasin. However, unlike other VLA integrin ligands, there is strong adhesion of resting T cells to invasin with a relatively small increase in the overall strength of adhesion after modes of activation that dramatically increase T cell adhesion to other integrin ligands such as FN, VCAM-1, and laminin. Thus, these findings illustrate an important additional regulatory component to integrin receptors. Other properties common to integrin ligands, such as the ability to facilitate CD3-mediated T cell proliferative responses (Fig. 4), are also exhibited by invasin.

The exact mechanism by which invasin promotes such vig-

orous adhesion of resting T cells to invasin currently remains undefined. One possibility is that the high affinity of invasin for its VLA integrin receptors can overcome the low functional activity of VLA integrins on resting T cells. This mechanism would suggest that any integrin ligand with sufficiently high affinity would be able to mediate the adhesion of resting T cells. A second possibility is that binding of resting T cells to invasin results in the generation of a signal that upregulates VLA integrin activity. This hypothesis is supported indirectly by: (a) the requirement for a metabolically active T cell to observe binding to invasin (Fig. 3); and (b) numerous reports of mAbs directed against the VLA-4 α chain or the VLA β chain that can upregulate adhesion to relevant ligands (17, 18, 23–25). We are continuing to investigate the potential of invasin as an integrin regulatory molecule. Nevertheless, our results clearly illustrate that in addition to the already well established role of activation in regulating T cell integrin function, the nature of the ligand itself also plays an important role in regulating adhesion. Thus, integrin-mediated adhesion to distinct ligands can be regulated in very different ways.

mAb blocking studies indicate that the VLA-4 integrin is the predominant invasin receptor on CD4⁺ T cells (Fig. 2). Even though CD4⁺ T cells express VLA-3, VLA-5, and VLA-6, and these VLA integrins have previously been shown to bind to invasin (12), we were unable to identify any mAbs specific for VLA-3, VLA-5, or VLA-6 that were able to inhibit T cell binding to invasin. This may be due to the higher levels of VLA-4 expressed on CD4⁺ T cells compared with VLA-3, VLA-5, and VLA-6 (6). However, the contribution of specific VLA integrins to adhesion to a specific ligand does not always correlate with the level of integrin expression. For example, T cell adhesion to FN is mediated predominantly by VLA-5, even though it is expressed at lower levels on T cells than VLA-4, which also binds FN (6). There may also be cell type-specific differences in the ligand specificity of individual VLA integrins since VLA-2 has been shown to differentially recognize laminin depending upon the specific type of cell in which VLA-2 is expressed (26). Thus, it is conceivable that VLA-3, VLA-5, and VLA-6 on human T cells do not function as invasin receptors as they do on other cell types that express these receptors. The inability of the VLA-5-specific mAb BIIG2 to inhibit T cell binding to invasin is consistent with this hypothesis, since this mAb was previously shown to inhibit the binding of K562 cells to invasin (12).

Although enteropathogenic bacteria such as *Yersinia* are thought to mainly interact with epithelial and other connective tissue cells, T cell interactions with bacteria may also be relevant to our understanding of bacterial pathogenicity. Our results suggest that invasin allows *Yersinia* to potentially interact with any human cells that express the appropriate VLA integrins, even cells that normally express these receptors in a low activity functional state. This strategy allows invasin-bearing bacteria to overcome the normal mechanisms that regulate integrin functional activity. The ability of invasin to facilitate CD3-mediated T cell proliferation (Fig. 4) suggests that *Yersinia* might also be able to modulate the im-

immune response in vivo by VLA integrin-mediated interactions with invasins. Our data, coupled with other indirect lines of evidence such as localization of *Yersinia* to anatomic sites rich in T cells such as Peyer's patches (27) and the isola-

tion of *Yersinia*-specific T cell clones (28), suggest that further investigation of the significance of lymphocyte interactions with bacterial pathogens is warranted.

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