EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 32, No. 1, 18-22, March 2000

Cross-linking of CD4 induces cytoskeletal association of CD4 and p56^{lck}

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Accepted 31 January 2000

Abbreviation: TCR, T cell receptor; MHC, major histocompatibility complex; GAM, goat anti-immunoglubulin antibody; PTK, protein tyrosine kinase; PI3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C

Abstract

A membrane glycoprotein CD4 functions as a co-receptor of a T lymphocyte. The co-receptor function has been attributed to a protein tyrosine kinase, p56^{lck}, which is activated upon CD4 binding to MHC molecule. In this study, we present evidences that one of the pathways through which CD4 transmits its signal is cytoskeleton association of p56^{lck} tyrosine kinase as well as CD4 itself. Cytoskeletal association of both proteins is inhibited by a tyrosine kinase inhibitor, genistein, indicating that tyrosine protein kinase activation is important for cytoskeletal association of CD4 and p56^{lck}. Cytoskeletal association of these proteins by CD4 cross-linking is not affected by inhibitors of protein kinase C nor PI3-kinase. Taken together, these results suggest that CD4 cross-linking activates a tyrosine kinase which then induces the simultaneous association of CD4 and p56^{lck} with cytoskeleton.

Keywords: T cells, cytoskeletal association, tyrosine kinase inhibition, CD4, p56^{lck}

Introduction

The CD4 molecule is a 55 kD membrane glycoprotein expressed on a subset of T cells, macrophages, and astrocytes. In T cells, CD4 exerts its signal by association with a TCR, when it recognizes the same MHC molecule as TCR. When a CD4 and a TCR are engaged in the same antigen-MHC complex, the TCR mediated signals are enhanced by 30-300 folds compared to the ones initiated by TCR alone. This greatly reduces the density of ligands required for the T-cell activation (Janeway *et al*., 1989). This co-receptor function of CD4 has been attributed to a PTK, p56^{lck}, which is associated with the cytoplasmic domain of CD4 and is activated upon CD4 aggregation (Veillette *et al*., 1988).

CD4 can also induce independent signals. Many studies have revealed that CD4 cross-linking with anti-CD4 antibodies prior to TCR engagement generates negative signals which inhibit proper T cell activation induced by TCR as well as by mitogens (Bank and Chess, 1985; Haughn *et al*., 1992; Racioppi *et al*., 1996). It has been shown that HIV binding to CD4 generates similar negative signals which might cause T cell depletion during the pathogenesis of AIDS (Kornfeld *et al*., 1988; Banda *et al*., 1992). The negative signal is likely due to the sequestration of p56^{lck} by CD4 cross-linking (Goldman *et al*., 1997). Such sequestration may interfere with the TCR signaling by uncoupling CD4-p56^{lck} signals from some critical substrates involved in the full activating process. CD4 cross-linking is shown to generate different patterns of tyrosine phosphorylation from CD3 or CD3-CD4 co-crosslinking (Thompson *et al*., 1992; Hubert *et al*., 1995; Goldman *et al*., 1997).

Many T cell surface molecules such as CD2, CD3, CD4, CD8, CD11a/CD18, CD44, and class I MHC molecules can be induced to associate with detergent-insoluble cytoskeletal fraction (Marano *et al*., 1989; Geppert and Lipsky, 1991; Offringa and Bierer, 1993). Recently, it has been shown that the engagement of CD28 and LFA-1 with their ligands triggers an active accumulation of these molecules at the interface of the T cell and antigen-presenting cell, during T cell activation. This movement appears to depend on myosin motor proteins which then increase the overall amplitude and duration of T cell signaling (Wulfing and Davis, 1998). However, the signals, which mediate cytoskeletal associations of these molecules, have not been elucidated.

The surface CD4 is not associated with cytoskeleton in normal unstimulated condition (Geppert and Lipsky, 1991). Upon cross-linking, its surface expression is down modulated and it becomes associated with cytoskeleton. Since CD4 exerts its function through p56^{lck}, we have examined whether p56^{lck} is associated with cytoskeleton and what mediates such association. We found that upon CD4 cross-linking, both p56^{lck} and CD4 were associated

with cytoskeleton. Cytoskeletal association of both proteins is inhibited by a tyrosine kinase inhibitor, genistein, indicating that tyrosine protein kinase activation is important for cytoskeletal association of CD4 and p56lck. Cytoskeletal association of CD4 and p56lck induced by CD4 cross-linking is not affected by inhibitors of protein kinase C nor PI3-kinase. Taken together, these results suggest that CD4 cross-linking activates a tyrosine kinase which then induces the simultaneous association of CD4 and p56lck with cytoskeleton.

Materials and Methods

Cells, reagents and antibodies

The T lymphoblast leukemia cell line, CEM-T4 (NIH AIDS research program), has been maintained in RPMI containing 10% fetal calf serum. Antibodies and reagents used are OKT4 (ATCC), 4G10 (UBI), rabbit anti-p56lck antibody (a generous gift from Dr. Young-Dae Yoon), FITC conjugated monoclonal anti-CD4 antibody (Pharmingen), goat anti-mouse immunoglobulin Fc antibody (GAM; Sigma), staurosporin (UBI), phorbol 12-monomuristyl 13-acetate (PMA; Sigma), geneistein (UBI), wortmanin (Sigma)

Analysis of cell surface expression and cytoskeletal association of CD4 by FACS

For an analysis of the cell surface expression of CD4, CEM-T4 cells were incubated with a saturating concentration (10 μg/ml) of FITC conjugated anti-CD4 antibody at 4°C for 30 min. Cells were washed 3 times with ice-cold RPMI 1640 media and CD4 molecules were cross-linked by incubation with 10 μg/ml goat anti-mouse antibody for 15 min at 4°C. Basal levels of the surface CD4 expression were determined by flow cytometry without cross-linking. A total of 10,000 events were acquired for an each sample on FACScan (Becton Dickinson). For the analysis of cytoskeleton associated CD4, cells were incubated with FITC conjugated anti-CD4 antibody**,** with or without cross-linking by GAM and were treated with a lysis buffer containing 0.5% Triton X-100 for 30 min. The CD4 molecules that were not associated with cytoskeleton were solublized in Triton lysis buffer and removed. CD4 molecules associated with detergent-insoluble fraction**s** were analyzed with flow cytometry.

Cell lysis and separation of detergent-soluble and -insoluble fractions

CEM-T4 cells were incubated in serum-free RPMI media at 37°C for 4 h. All of kinase inhibitors; genistein (300 M), staurosporin (100 nM) and wortmannin (100 μM) were added 30 min prior to CD4 cross-linking or PMA (100 ng/ml) treatment. Cells were harvested and resuspended in ice-cold RPMI at 1×10^7 cells/ml. An anti-CD4 antibody (OKT4) were added at the concentration of 10 μg/ml and incubated for 1 h at 4°C. CD4 molecules were cross-linked by incubation with 20 μg/ml goat anti-mouse antibody (GAM) for 15 min at 4°C. Various times after incubation at 37°C, cells were quickly chilled and lysed by the Triton-lysis buffer (0.5% Triton X-100, 1 mM Na_3VO_4, 50 mM NaF, 2 mM aprotinin, 2 mM leupeptin, 1 mM PMSF, 20 mM Tris, pH 7.4, 120 mM NaCl). The lysates were centrifuged for 15 min at 10,000 × g at 4°C. The detergent-soluble fractions were analyzed by SDS-PAGE. The detergent-insoluble fractions were resuspended in SDS-PAGE sample buffer, boiled for 5 min and centrifuged at 15,000 × g for 30 min. The supernatants were analyzed prior to an analysis by SDS-PAGE.

Western analysis

Proteins were separated by 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. CD4, p56lck and tyrosine-phosphorylated proteins were detected by immunoblotting with anti-CD4 antibody, p56lck antibody or anti-phosphotyrosine mAb respectively followed by incubation with HRP-conjugated secondary antibodies and ECL reaction (Amersham kit).

Results

CD4 cross-linking induces cytoskeletal association of p56lck

It has been previously shown that CD4 cross-linking induces down-modulation of the surface CD4 expression as well as the cytoskeletal association of CD4 molecules. Since CD4 is associated with p56lck by its cytoplasmic domain, we have examined a possible interaction of p56lck with the cytoskeletal fraction upon CD4 cross-linking.

For this examination, surface CD4 molecules were cross-linked with monoclonal anti-CD4 antibody, OKT4, and goat anti-immunoglubulin antibody (GAM). At different times following the cross-linking, the Triton X-100-soluble cytosol or membrane fractions and Triton-insoluble cytoskeleton fractions were separated and analyzed by immunoblotting.

In a control where cells were treated with GAM alone (Figure 1A and B, lanes 1-4), CD4 and p56lck were present mainly in the cytosol fraction. Upon CD4 cross-linking (Figure 1: lanes 5-8), both CD4 and p56lck were partitioned into the cytoskeletal fraction. Such partitioning occurred even on ice during cross-linking. We were able to detect an immediate loss of p56lck and CD4 from soluble fraction (Figure 1: lanes 5). Cytoskeletal association of CD4 and p56lck did not occur when cells were treated with OKT4 alone without cross-linking (Figure 1: lanes 9-12).

When the same membranes were analyzed with anti-phosphotyrosine antibody (α-p-tyr), the tyrosine phosphorylated proteins at 56 kD was detected only when

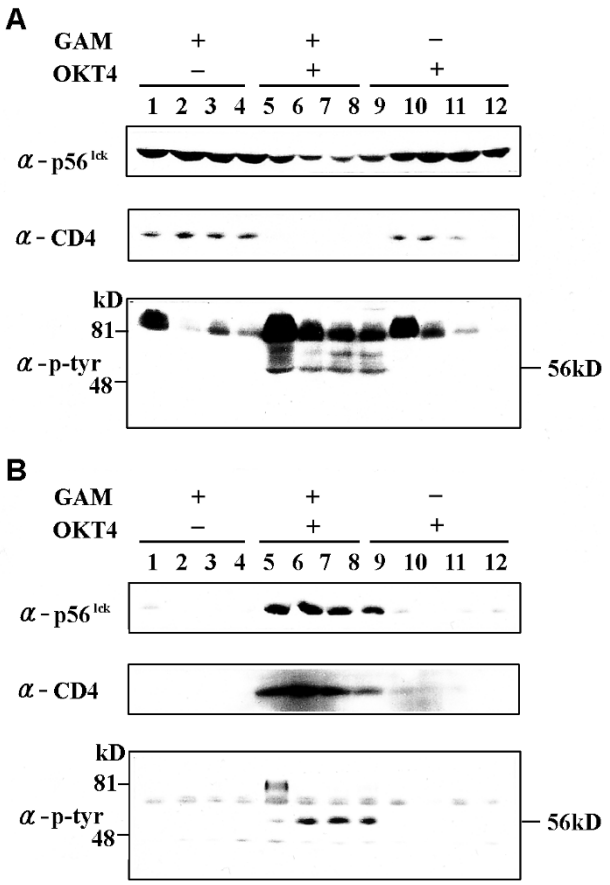

Figure 1. Associations of CD4 and p56^{lck} with detergent-insoluble cytoskeletal fraction. CEM-T4 cells were treated with GAM alone (lanes 1-4), anti-CD4 mAb (OKT4) alone (lanes 9-12), or cross-linked by incubation with OKT4 followed by GAM (lanes 5-8). Various times following incubation at 37°C, the detergent-soluble (A) and -insoluble fractions (B) were prepared. p56^{lck} (α-p56^{lck}), CD4 (α-CD4) or phosphotyrosine (α-p-tyr) was detected by western analysis. (A) 30 μg of total protein was loaded in each lane. (B) proteins from 6×10^6 cells/lane. lanes 1, 5, 9: 0 time. lanes 2, 6, 10: 3 min. lanes 3, 7, 11: 8 min. lanes 4, 8, 12: 15 min. 0 time indicates the cells on ice with all the antibodies added respectively but without incubation at 37°C.

CD4 was cross-linked (Figure 1A and 1B, lanes 5-8). The tyrosine phosphorylated p56 protein was detected in cytoskeletal fraction (Figure 1B), as well as in the cytosol fraction (Figure 1A) and its band was also detected with anti-p56^{lck}, indicating that it was most likely p56^{lck}. The above results showed that CD4 cross-linking activated tyrosine kinase and induced tyrosine-phosphorylation and cytoskeletal association of both p56^{lck} and CD4.

PMA stimulation down-modulates CD4 but does not induce cytoskeletal association of CD4 nor p56^{lck}

Since CD4 cross-linking induced down-modulation of CD4, it was possible that the cytoskeletal association is related to CD4 down-modulation. To test this, a PKC activator, PMA that is known to down-modulate surface CD4 expression, was employed. PMA induces down-modulation

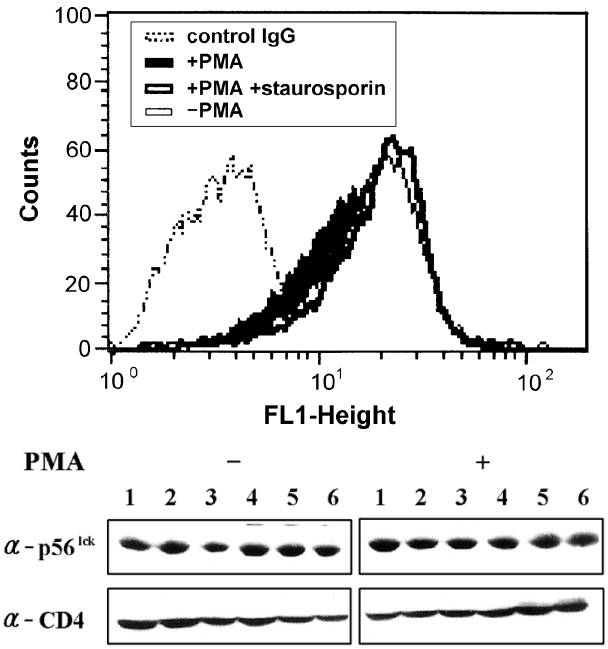

Figure 2. Effect of PMA on CD4 down-modulation and cytoskeleton association. (A) CEM-T4 cells were cultured with (thick line) or without (filled line) staurosporin 30 min prior to PMA treatment. 30 min after PMA treatment, cell surface CD4 molecules were labeled with FITC anti-CD4 antibodies and analyzed by FACS. No PMA treated control (thin line), control antibody (dotted line). (B) CD4 and p56^{lck} in detergent-soluble fraction at different times after PMA treatment. (-): no PMA control. (+): PMA treated. Lanes 1, 0 min; 2, 1 min; 3, 2 min; 4, 6 min; 5, 15 min; 6, 2 h.

of CD4 by dissociation of p56^{lck} hence exposing endocytic recognition sequence of CD4 (Aiken *et al.*, 1994).

As shown in Figure 2A, the PMA down-modulated CD4 within 15 min and the CD4 down-modulation could be blocked by a PKC inhibitor, staurosporin. This result indicates that CD4 down-modulation is due to PKC activation by PMA. The western analysis of the cytosol fraction (Figure 2B), however, did not reveal reduction of either CD4 or p56^{lck} following PMA treatment. The amounts of both proteins in cytosol remained unchanged regardless to the PMA treatment, showing that down-modulation of CD4 is not necessarily required for cytoskeletal association of CD4 and p56^{lck}.

CD4 down-modulation can also be induced by OKT4 treatment without cross-linking, however we have not observed cytoskeletal association of CD4 either by western analysis (Figure 1; lanes 9-12) or by FACS analysis (data not shown).

The cytoskeletal association of CD4 p56^{lck} following CD4 cross-linking is dependent on the PTK activity

In order to further define the mechanism of the cytoskeletal association of CD4 and p56^{lck}, inhibitors for different kinases were tested for the ability to inhibit cytoskeletal association. For this, cells were pretreated

with various kinase inhibitors before CD4 cross-linking, and then the detergent-soluble fractions were analyzed.

When CD4 cross-linking was carried out in the presence of a PTK inhibitor, genistein, more p56lck proteins were left in Triton-soluble fraction (Figure 3A, top). However, there was no increase in the amount of p56lck in the detergent-soluble fraction when a PKC inhibitor, staurosporin (middle), or a PI3-kinase, wortmannin (bottom), were tested. These results suggest that cytoskeletal association induced by CD4 cross-linking requires PTK activation. The cytoskeletal association did not appear to be mediated by PKC or PI3-kinase activation. In the presence of genistein, tyrosine phosphorylation of the 56 kD protein was reduced comparable to the level of cytoskeleton associated p56lck (data not shown), again supporting the idea that the substrate of PTK activated by CD4 cross-linking is p56lck.

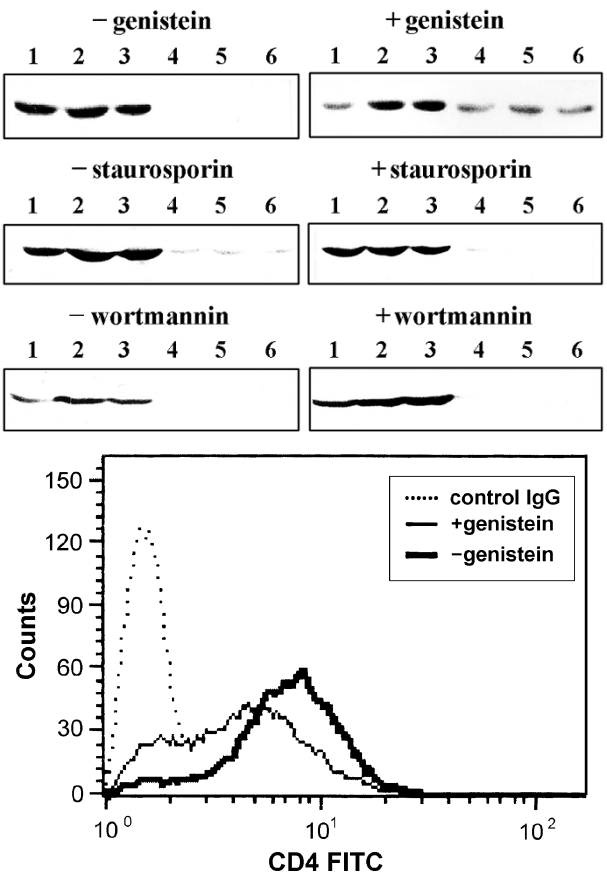

Figure 3. Effect of various inhibitors for cytoskeletal association of p56lck. (A) CEM-T4 cells were treated with genistein, staurosporin, or wortmannin 30 min prior to CD4 cross-linking. At various times after cross-linking, the presence of p56lck in detergent-soluble fraction (lanes 1, 2 and 3) and in -insoluble fraction (lanes 4, 5 and 6) were analyzed by western analysis. Lanes 1 and 4, 1 min; lanes 2 and 5, 3 min; lanes 3 and 6, 15 min after cross-linking. (B) The cytoskeleton associated CD4 was analyzed by FACS as described in Materials and Methods. Cells were pretreated with genistein (thick line) or without genistein (thin line) 30 min prior to cross-linking that was carried out with FITC conjugated anti-CD4 mAb and GAM. Antibody control is shown with dotted line.

To confirm the inhibitory effect of genistein for the cytoskeletal association of CD4, the cytoskeletal association of CD4 was also analyzed by flowcytometry. CD4 molecules were labeled with FITC-conjugated anti-CD4 antibody and cross-linked by GAM for 15 min. CD4 molecules that were not associated with cytoskeleton were washed away by the Triton X-100 treatment and cytoskeleton associated CD4 was analyzed by FACS. As shown in Figure 3B, when CD4 molecules were cross-linked in the presence of genistein, the cytoskeleton associated CD4 was greatly reduced compared to CD4 cross-linking without genistein.

Discussion

Several studies revealed that association of cytoskeleton with surface molecules plays an important role to transmit the signals generated at the membrane to the cytoskeletal structure and therefore modulate cellular responses.

In this study, we demonstrated that CD4 cross-linking induced a simultaneous cytoskeletal association of both CD4 and CD4 associated tyrosine kinase p56lck. Our result further showed that the association of both CD4 and p56lck with cytoskeleton required tyrosine kinase activation, probably that of p56lck.

TCR ligation induces association of TCR and tyrosine phosphorylated TCR ζ with the detergent-insoluble cell fraction (Rozdzial *et al*., 1995; Marano *et al*., 1997; Rozdzial *et al*., 1998). P56lck has been implicated in CD3 ζ-chain phosphorylation and ζ-cytoskeletal binding (Rozdzial *et al*., 1998).

CD4 is mainly known to function as a co-receptor of TCR. However CD4 can also transmit an independent signal. The interaction of CD4 and MHC class II molecules without TCR engagement appears to negatively regulate antigen-independent adhesion of T cells. The negative regulation of adherence can be blocked by wortmannin, a PI3-kinase inhibitor, indicating that PI3-kinase participates in the regulation of cytoskeleton structure. The antigen-independent adhesion of T cells also requires the tyrosine kinase activity of the p56lck (Doyle and Strominger, 1987; Mazerolles *et al*., 1994; Kinch *et al*., 1994). Based on these results, it was postulated that activation of PI3-kinase associated with p56lck leads to a cytoskeleton organization unfavorable of maintaining adherence. Unlike the negative regulation of adherence by antigen independent interaction of CD4 and MHC class II molecules, the cytoskeletal association of p56lck induced by CD4 cross-linking was not affected by a PI3-kinase inhibitor but affected by a PTK inhibitor. One likely explanation is that an intial CD4 and MHC class II interaction requires p56lck activation which subsequently induces cytoskeletal association. p56lck activation then regulates cytoskeletal organization unfavorable for further

adhesion.

It is not clear yet whether CD4 and $p56^{lck}$ bind to the cytoskeleton independently or in a CD4-$p56^{lck}$ complex form. The co-segregation of CD4 and $p56^{lck}$ with cytoskeletal fraction and the slow dissociation of CD4 from $p56^{lck}$ (Juszcaak *et al.*, 1991) suggest that CD4 and $p56^{lck}$ are still in complex form.

Clearly there are many different pathways by which CD4 mediates different signals generated by different stimulations. CD4 cross-linking induces down modulation of CD4, but cytoskeletal association is clearly not related to CD4 down modulation process. PMA induces CD4 down-modulation without the cytoskeletal association of $p56^{lck}$ or CD4. Our results shown here strongly suggest that one of the pathways through which CD4 transmits its signals is cytoskeletal association of CD4 and $p56^{lck}$.

Acknowledgements

This work was partly supported by Good Health R&D Project HMP-96-M-2-0030, Ministry of Health & Welfare, R.O.K.

References

Aiken, C., Konner, J., Landau, N. R., Lenbury, M. E. and Trono, D. (1994) Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell* 76: 853-864

Banda, N. K., Bernier, J., Kurahara, D. K., Kurrle, R., Haigwood, N., Sekaly, R.-P. and Finkel, T. H. (1992) Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. *J. Exp. Med.* 176: 1099-1106

Bank, I. and Chess, L. (1985) Perturbation of the T4 molecule transmits a negative signal to T cells. *J. Exp. Med.* 162: 1294-1303

Doyle, C. and Strominger, J. L. (1987) Interaction between CD4 and class II MHC molecules mediates cell adhesion. *J. Immunol.* 330: 256

Geppert, T. D. and Lipsky, P. E. (1991) Association of various T cell-surface molecules with the cytoskeleton. *J. Immunol.* 146: 3298-3305

Goldman, F., Crabtree, J., Hollenback, C. and Koretzky, G. (1997) Sequestration of p56lck by gp120, a model for TCR desensitization. *J. Immunol.* 158: 2017-2024

Haughn, L., Gratton, S., Caron, L., Sekaly, R.-P., Veillete, A. and Julius, M. (1992) Association of tyrosine kinase p56lck with CD4 inhibits the induction of growth through the ab T-cell receptor. *Nature* 358: 328-331

Hubert, P., Bismuth, G., Korner, M. and Debre, P. (1995) HIV-1 glycoprotein gp120 disrupts CD4-p56lck/CD3-T cell receptor interactions and inhibits CD3 signaling. *Eur. J. Immunol.* 25: 1417-1425

Janeway, C. A. J., Rojo, J., Saizawa, K. and al., e. (1989) The co-receptor function of murine CD4. *Immunol. Rev.* 109: 77

Juszcaak, R. J., Turchin, H., Truneh, A., Culp, J. and kassis, S. (1991) Effect of human immunodeficiency virus gp120 glycoprotein on the association of the protein tyrosine kinase $p56^{lck}$ with CD4 in human T lymphocytes. *J. Biol. Chem.* 266: 11176-11183

Kinch, M. S., Sanfridson, A. and Doyle, C. (1994) The protein tyrosine kinase p56lck regulates cell adhesion mediated by CD4 and major histocompatibility complex class II proteins. *J. Exp. Med.* 180: 1729-1739

Kornfeld, H., Cruikshank, W. W., Pyle, S. W., Berman, J. S. and Center, D. M. (1988) Lymphocyte activation by HIV-1 envelope glycoprotein. *Nature* 335: 445-448

Marano, N., Crawford, M. and Govindan, B. (1997) Characterization of the detergent insolubility of the T cell receptor for antigen. *Mol Immunol* 34: 967-976

Marano, N., Holwka, D. and Baird, B. (1989) Bivalent binding of an anti-CD3 antibody to Jurkat cells induces association of the T cell receptor complex with the cytoskeleton. *J. Immunol.* 143: 931-938

Mazerolles, F., Barbat, C., Meloche, S. and Sekaly, and al, e. (1994) LFA-1-mediated antigen-independent T cell adhesion is regulated by CD4 and p56lck tyrosine kinase. *J. Immunol.* 152: 5670-5679

Offringa, R. and Bierer, B. (1993) Association of CD2 with tubulin. Evidence for a role of the cytoskeleton in T cell activation. *Biol Chem* 268: 4979-4988

Racioppi, L., Matarese, G., D'Oro, U., Ke-Pascale, M., Masci, A. M., Fontana, S. and Zappacosta, S. (1996) The role of CD4-lck in T-cell receptor antagonism: evidence for negative signaling. *Proc. Natl. Acad. Sci. USA* 93: 10360-10365

Rozdzial, M., Pleiman, C., Cambier, J. and Finkel, T. (1998) Pp56lck mediates TCR zeta-chain binding to the microfilament cytoskeleton. *J. Immunol.* 161: 5491-5499

Rozdzial, M. M., Malissen, B. and Finkel, T. H. (1995) Tyrosine-phosphorylated T cell receptor zeta chain associates with the actin cytoskeleton upon activation of mature T lymphocytes. *Immunity* 3: 623-633

Thompson, P. A., Gutkind, J. S., Robbins, K. C., Ledbetter, J. A. and Bolen, J. B. (1992) Identification of distinct populations of PI-3 kinase activity following T-cell activation. *Oncogene* 7: 719-725

Veillette, A., Bookman, M. A., Horak, E. M. and Bolen, J. B. (1988) The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. *Cell* 55: 301-308

Wulfing and Davis (1998) A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science* 282: 2266-2269