CD46/CD3 Costimulation Induces Morphological Changes of Human T Cells and Activation of Vav, Rac, and Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase

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CD46/CD3 Costimulation Induces Morphological Changes of Human T Cells and Activation of Vav, Rac, and Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase

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Efficient T cell activation requires at least two signals, one mediated by the engagement of the TCR-CD3 complex and another one mediated by a costimulatory molecule. We recently showed that CD46, a complement regulatory receptor for C3b as well as a receptor for several pathogens, could act as a potent costimulatory molecule for human T cells, highly promoting T cell proliferation. Indeed, we show in this study that CD46/CD3 costimulation induces a synergistic activation of extracellular signal-regulated kinase mitogen-activated protein kinase. Furthermore, whereas T lymphocytes primarily circulate within the bloodstream, activation may induce their migration toward secondary lymphoid organs or other tissues to encounter APCs or target cells. In this study, we show that CD46/CD3 costimulation also induces drastic morphological changes of primary human T cells, as well as actin relocation. Moreover, we show that the GTP/GDP exchange factor Vav is phosphorylated upon CD46 stimulation alone, and that CD46/CD3 costimulation induces a synergistic increase of Vav phosphorylation. These results prompted us to investigate whether CD46/CD3 costimulation induced the activation of GTPases from the Rho family. Indeed, we report that the small GTPase Rac is also activated upon CD46/CD3 costimulation, whereas no change of Rho and Cdc42 activity could be detected. Therefore, CD46 costimulation profoundly affects T cell behavior, and these results provide important data concerning the biology of primary human T cells. The Journal of Immunology, 2001, 167: 6780–6785.

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Abbreviations used in this paper: GEF, GDP/GTP exchange factor; anti-P-Tyr, anti-phosphotyrosine; CRIB, Cdc42/Rac interactive binding domain; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase; ERK, protein kinase C; RaM, rabbit anti-mouse Ig; RBID, Rho-binding domain.
on its phosphorylation (27, 28), which occurs after TCR stimulation (29), as well as following CD28 costimulation (30, 31). Indeed, CD28 costimulation increases the ZAP-70-mediated Tyr phosphorylation of Vav, and then synergistically induces Rac GDP/GTP exchange that further activates p38 mitogen-activated protein kinase (MAPK) (32).

In this study, we first report that CD46 costimulation leads to a synergistic activation of extracellular signal-regulated kinase (Erk) MAPK, and that indeed the inhibition of this pathway abrogates CD46/CD3 costimulation effect. Furthermore, CD46/CD3 costimulation induces profound changes in cell morphology of human primary T cells, as well as actin relocalization. Importantly, we used fresh primary T cells, and not cell lines, and therefore these results provide new data concerning human T cell biology. We show that CD46 stimulation induces Vav Tyr phosphorylation, and that CD46/CD3 costimulation leads to a synergistic increase of Vav phosphorylation. These results prompted us to investigate whether the GTPases from the Rho family were involved in the signaling cascade induced by CD46/CD3 ligation. We show that Rac is activated upon CD46/CD3 costimulation. These results suggest that the Vav/Rac pathway is a potential candidate for the costimulatory cross-talk between CD46 and TCR. Therefore, CD46 costimulation profoundly affects T cell behavior, and this might be taken in account for its costimulatory effects.

Materials and Methods

Cell lines

PBL were purified from blood of human healthy donors (ETS, Lyon, France) by Ficoll/Hypaque and then Percoll centrifugation. Purified T cells were obtained by immunomagnetic bead depletion of B cells, monocytes, and NK cells, as previously described (33).

Antibodies

The mouse mAbs used in this study were 20.6, IgG1 directed against CD46 (7), OKT3, IgG1 directed against CD3 (obtained from the American Type Culture Collection, Manassas, VA), and anti-CD28 Ab (clone CD28.2) that was kindly given by Dr. D. Olive (Marseille, France). Irrelevant IgG1 Abs were purchased at Immunotech, Beckman Coulter (Marseille, France). Affinity-purified rabbit anti-mouse Ig (RaM) and anti-CD28 Ab (26C4) were a generous gift from Dr. J. Bertoglio (Paris, France). Anti-Cdc42 mAbs were obtained from Transduction Laboratories (Lexington, KY). Anti-CDC42 mAbs were purchased at Immunotech, Beckman Coulter (Marseille, France). Anti-cocave Abs in TBS-T (20 mM Tris (pH 7.6), 130 mM NaCl, and 0.1% Tween 20) and incubated for 1 h with specific Abs. Immunoreactive bands were visualized by using secondary HRP-conjugated Abs (Promega, Madison, WI) and chemiluminescence (ECL; Amersham, Little Chalfont, U.K.). The membranes were then stripped and reblotted with another Ab.

GTPase activity assays by affinity precipitation

The Rac and Rho activity assays were performed as previously described (34, 35). Briefly, to evaluate Rac, Rho, and Cdc42 activity, cell lysates were incubated for 1 h at 4°C with 20 μg of GST-Cdc42/Rac interactive binding domain (CRIB), corresponding to the Rac and Cdc42-binding domain of human PAK1B (kindly obtained from Dr. J. Collard, The Netherlands Cancer Institute, Amsterdam, The Netherlands) (34), GST-RBD (Rho-binding domain of mouse Rhotekin; kindly given by Dr. M. Schwartz, The Scripps Research Institute, La Jolla, CA), and GST-ACK42-Lys34 (minimum Cdc42-binding domain of the ACK1 kinase (36) (kindly provided by Dr. H. Maruta, Ludwig Institute for Cancer Research, Melbourne, Australia), respectively, bound to glutathione-coupled Sepharose beads. After several washes with lysis buffer and elution with sample buffer, bound proteins were then loaded on 8% SDS-PAGE and analyzed by Western blot using anti-Rac, anti-Rho, or anti-Cdc42 Abs.

F-actin localization

Cells (1 × 10⁶) were incubated in 12-well plates with coverslips previously coated with anti-CD3 (10 μg/ml) and/or anti-CD28 or anti-CD28 (10 μg/ml) and cultured for 24 h. Cells were then stained with FITC-phalloidin for 20 min at room temperature and extensively washed with PBS. Coverslips were then mounted with a Prolong antifade kit (Molecular Probes, Eugene, OR). Cells were then analyzed by confocal microscopy (LSM510 microscope; Zeiss, Oberkochen, Germany), using a ×63 (numerical aperture 1.4) Zeiss Plan Neofluor objective.

Proliferation assay

Cells (2 × 10⁶) were incubated in 96-well plates coated with anti-CD3 (OKT3) and/or anti-CD46 or anti-CD28 (10 μg/ml), or an irrelevant IgG1 and cultured for 3 days, in the presence or absence of U0126 (mitogen-activated protein/Erk kinase 1 (MEK1) inhibitor; Promega). Cells were then incubated with 1 μCi of [³H]Thymidine for 16 h and harvested on 96-hole papers using a Tomtec Instruments (Orange, CT) cell harvester. [³H]Thymidine incorporation was measured using a 1450 Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD).

Results

CD46/CD3 costimulation leads to a synergistic activation of Erk1/2 MAPK

We previously reported that CD46/CD3 costimulation strongly promotes T cell proliferation, as compared with CD3 stimulation alone. We had also shown that LAT was phosphorylated after CD46 stimulation. In this study, we further investigated the signaling cascade induced by CD46 and CD46/CD3 stimulation. We analyzed whether Erk1/2 MAPK could be activated by CD46 stimulation, since Erk MAPK are involved in the control of proliferation (37, 38). Human PBL were stimulated with anti-CD46, anti-CD3, anti-CD3 and anti-CD46, or anti-CD3 and anti-CD28 as a control for 1, 10, and 30 min before lysis. An aliquot of each cell lysate was then analyzed by Western blot using anti-phospho-Erk1/2 Abs (Fig. 1A), which reflect Erk1/2 activation. The membranes were then analyzed with anti-Erk1/2 and anti-actin Abs to estimate the levels of proteins in each lane (Fig. 1, B and C). A very faint activation of Erk1/2 could be observed when the cells were stimulated by anti-CD46 alone or by anti-CD3 stimulation alone. However, CD46/CD3 costimulation induced a more dramatic activation of Erk1/2 (1 and 10 min, arrow). As a control, we also show that CD3/CD28 costimulation induced a strong Erk activation that was more important than the one induced by CD3/ CD46 costimulation. Therefore, CD46 costimulation induced a synergistic activation of Erk1/2 MAPK.
We therefore analyzed whether the inhibition of Erk1/2 would affect the costimulatory role of CD46. Human T cells were cultured in 96-well plates coated with anti-CD3, anti-CD3 and anti-CD46, or anti-CD3 and anti-CD28, in the presence or absence of several concentrations of U0126 that inhibits Erk1/2 activation via the inhibition of MEK1. Costimulated T cells did not grow and did not acquire morphological changes when cultured in presence of U0126 (data not shown). However, the drug was not toxic since the cells were still alive, as observed after trypan blue exclusion counting (data not shown). Proliferation was estimated by [3H]thymidine incorporation. As shown on Fig. 2, similarly to CD28, addition of U0126 inhibits CD46 costimulation in a dose-response manner, indicating that stimulation of Erk1/2 is indeed required for CD46 costimulation.

**CD46/CD3 costimulation induces morphological changes in human T cells**

Furthermore, besides promoting proliferation, CD46/CD3 costimulation also induced morphological changes of human T cells, as depicted on Fig. 3, in which pictures have been taken from representative cell cultures. Indeed, whereas CD3-stimulated or CD28/CD3-costimulated cells grew in clonal suspension with apparent homotypic aggregates, CD46/CD3-costimulated cells were not aggregated and adhered to the substratum. In presence of anti-CD46 alone, the cells were similar to the unstimulated cells (data not shown). Therefore, CD46 costimulation induces signals that modulate adhesion of human T cells. Furthermore, when observed at high magnification (Fig. 4A), CD46/CD3-costimulated cells appeared spread on the bottom of the well and exhibited membrane protrusions such as lamellipodia and filipodia (arrows). This effect was not observed when the cells were costimulated by CD28/CD3, although they were clearly stimulated, as observed by the enlargement of the cells compared with CD3 stimulation alone (Fig. 4A). These cells remained mainly spherical, although few cells presented a flatter aspect than CD3-stimulated cells, and some of them were attached to the substratum. To better characterize the morphological changes induced after CD46/CD3 costimulation, we analyzed the actin organization after FITC-phalloidin staining. Cells cultured with either anti-CD3, anti-CD3 and anti-CD46, or anti-CD3 and anti-CD28 for 24 h were analyzed by FITC-phalloidin staining.
staining and confocal microscopy. Whereas actin was detected at the periphery of CD3-stimulated cells, it was found mostly polarized at one edge in CD28/CD3-stimulated cells. In CD46/CD3-stimulated cells, actin was found in lamellipodia-like structures, as well as in thin protrusions resembling long retraction fibers (arrow) usually seen at the rear edge of highly motile cells. Furthermore, CD46/CD3-costimulated T cells were spread on the substratum and presented a completely different morphology than CD3- or CD28/CD3-costimulated T cells that remained mostly spherical (Fig. 4B).

Therefore, CD46/CD3 costimulation induces a dramatic synergistic effect on human T cell morphology, compared with CD3 alone.

**CD46 stimulation leads to Vav phosphorylation**

Vav has been shown to play a crucial role in T cell activation, as well as being a GEF for the small GTPases of the Rho family that are also involved in cytoskeletal rearrangements. As we have previously shown that CD46 plays an important role in T cell activation, and that morphological changes occur after costimulation, we investigated whether Vav was phosphorylated either after CD46 stimulation alone or after CD46/CD3 costimulation, and after CD28/CD3 costimulation as a control. Human PBL were stimulated with anti-CD46 mAb, anti-CD3, both Abs, as well as with anti-CD28 and anti-CD3 for 3 min at 37°C, and then lysed. Vav was immunoprecipitated and analyzed by anti-P-Tyr immunoblotting. Although CD46 as well as CD3 stimulation induced Vav tyrosine phosphorylation (Fig. 5A, arrow), CD46/CD3 costimulation led to a more dramatic increase of Vav phosphorylation. The same result was obtained after CD28/CD3 costimulation, as already described (32). The membrane was then stripped and reprobed with anti-Vav Abs (Fig. 5A) to confirm that equivalent amounts of Vav protein had been loaded in each lane. Therefore, Vav phosphorylation is induced by CD46 and CD3 aggregation in human PBL, but CD46/CD3 costimulation has a synergistic effect on Vav phosphorylation.

We then compared the kinetic of Vav phosphorylation induced by CD46/CD3 or CD28/CD3 costimulation. Human PBL were stimulated with anti-CD46 and anti-CD3 or anti-CD28 and anti-CD3 for 1, 10, and 30 min before lysis. Vav was immunoprecipitated, and analyzed by anti-P-Tyr and anti-Vav immunoblotting (Fig. 5B). Whereas the same level of phosphorylation could be observed after 3 min of stimulation under both stimuli, CD28/CD3 costimulation induced a stronger effect on Vav phosphorylation at 10 min.

**CD46 costimulation induces Rac activation**

Once Tyr-phosphorylated, Vav is a GEF for the Rho family proteins Rac, Rho, and Cdc42 that are involved in TCR activation. We therefore investigated whether Rac, Rho, and Cdc42 could also be stimulated after CD46/CD3 costimulation. To analyze Rac, Rho, and Cdc42 activities, cells were stimulated for 3 min with CD3 and/or CD46 or CD28/CD3 as a control, and their cell lysates were then pulled down with glutathione-Sepharose beads coupled with GST-fusion proteins containing the binding domain of each GTPase: GST-CRIB, GST-RBD, and GST-ACK42 fusion proteins, to precipitate the respective GTP-bound GTPases. Proteins were then eluted and analyzed by Western blot using anti-Rac (Fig. 6A), anti-Rho A (Fig. 6B), or anti-Cdc42 Abs (Fig. 6C). As a loading control, the membranes were stripped and reblotted with anti-GST Abs, as indicated. Furthermore, an aliquot of each cell lysate was also loaded on the gel to quantify the amount of each GTPase. The ratio of GTP/GDP-bound proteins was then determined after densitometry analysis. Although a basal level of Rac-GTP could be detected, a 2.5-fold increase was observed when cells were costimulated by CD46/CD3 or after CD28/CD3 costimulation (densitometric values, representative of three independent experiments). However, no activation of Rho or Cdc42 was detected after CD46/CD3 costimulation, whereas both GTPases were activated after CD28/CD3 costimulation, as already described (32, 39). CD46 stimulation alone had a very mild effect on Rac activation compared with CD46/CD3 costimulation (Fig. 6A, right panel). Therefore, CD46 costimulation differs from CD28 costimulation that induces activation of each GTPase tested, whereas only Rac is activated by CD46 costimulation.

**Discussion**

We had previously reported that CD46 ligation in human PBL induces Tyr phosphorylation of intracellular proteins and identified...
two of them, p120CBL and LAT, two adapter molecules involved in T cell activation. We had also shown that proliferation of T cells is dramatically enhanced after CD46/CD3 costimulation (4). We now identified other signaling compounds of the cascade linked to CD46, such as Vav, Rac, and Erk MAPK. We report that CD46/CD3 costimulation leads to a synergistic activation of Vav and Erk MAPK, and that inhibition of Erk1/2 abrogates CD46 costimulation. A recent paper showed that stimulation by the complement of the K562 cell line leads to Erk activation (40). Therefore, this pathway might at least in part explain the costimulatory effect of CD46/CD3 stimulation. Rac also controls the activity of the MAPKs and cell cycle progression through G1 (41, 42). In addition, it has been recently shown that Vav was required for the activation of Erk in T cells (43). The authors showed that in Vav-deficient T cells, TCR-induced calcium fluxes and Erk activation were inhibited. Another potential mechanism of the costimulation induced by CD46 could also be the activation of protein kinase C0 (PKC0) that has been recently shown to play an important role in the CD28/CD3-induced stimulation of MAPK (44). After CD28/CD3 stimulation, Vav promotes PKC0 translocation from the cytosol to the membrane and induces its activation in a pathway that is dependent on Rac and on actin cytoskeleton reorganization. The authors described too very recently that translocation of PKC0 in the rafts was also required for T cell activation (45). Since we report that Vav and Rac are activated and that actin reorganization is observed after CD46/CD3 costimulation, one can imagine that a similar recruitment of PKC0 occurs after CD46 engagement. Finally, it has been recently reported that TCR and Rac activation also led to protein kinase B/AKT activation (46). The authors report that Rac is implicated in this pathway, connecting the TCR with the lipid kinase phosphatidylinositol 3-kinase. Further investigations will tell whether PKC0 and AKT are also activated under CD46 costimulation.

Activation of T cells requires actin cytoskeleton for changing their cellular shape and migration that will allow the formation of the immune synapse (for review, see Refs. 20 and 47). In this study, we show that CD46 stimulation induces Vav phosphorylation, and Vav is implicated in the cytoskeletal reorganization mediated by the TCR (48, 49). Vav might possibly be activated by LAT since it has been reported that phosphorylated LAT plays a role in the recruitment of Vav to the membrane of T cells, enabling Vav to activate Rac (50). Indeed, we show that Rac, a member of the Rho family, is activated by CD46/CD3 costimulation. More importantly, CD46/CD3 costimulation has a synergistic effect on Vav phosphorylation, suggesting a synergistic effect on its activation (27, 28). CD46 activation might act like CD28 by increasing the ZAP-70-mediated Tyr phosphorylation of Vav and then synergistically induces Rac GDP/GTP exchange (32). Although an activation of p38 by CD28/CD3 costimulation has been reported (32), we could not observe any increase of p38 activity after CD46/CD3 ligation compared with CD3 stimulation alone (data not shown).

CD46/CD3 costimulation induced drastic morphological changes of human T cells. Costimulated cells do not grow in homotypic aggregates, but stick to the bottom of the wells. This effect appears to be specific to CD46 since CD28/CD3-costimulated T cells also mainly grow within aggregates, although some cells tend to stick after longer time of culture (data not shown). Regulation of adhesion of human T cells is essential for the immune response. Indeed, circulating lymphocytes have to adhere to components of the extracellular matrix at sites of inflammation and in lymphoid tissues. This requires a regulation of the integrins’ affinity for their ligands, resulting in an alteration of cell spreading. It has been shown that spreading of T cells is specifically dependent on Rac (51). The authors showed that expression of an activated mutant of Rac triggered a dramatic spreading of Jurkat T cells. The Rac-induced spreading is accompanied by cytoskeletal rearrangements, and no effect was observed with Rho- or Cdc42-activated mutants. Similarly, we found that CD46 did not induce any activation of these two last GTPases, only Rac is activated, and we indeed observed a spreading of the cells. Actin localization in purified human T cells after CD46/CD3 costimulation also showed a reorganization of actin within the cells. Importantly, our results were obtained with primary T cells and not Jurkat cells, and therefore importantly reinforce a role for T cell spreading in the immune system. Moreover, it has been reported that in Jurkat T cells, Vav participates in the regulation of cytoskeletal organization, and that its phosphorylation is strongly dependent on adhesion to fibronectin (52). Furthermore, Vav overexpression enhances the formation of lamellipodia and increases the cellular growth rate in an adhesion-dependent manner. When both the TCR and CD46 are engaged, a transduction pathway leads to Vav and Rac activation. One can imagine that CD46 costimulation modulates T cell spreading at sites of inflammation and lymphoid tissues. Indeed, integrin ligation induces the activation of the Erk1/2 MAPK in different cells (53–55) as well as in T cells (56, 57). Furthermore, recent studies report a role of Erk activation in the modulation of affinity of integrins (58, 59). It will be interesting to analyze now the effects of CD46 on the avidity and affinity of different integrins, as well as the potential migration of T cells costimulated by CD46 and CD3.

Our results demonstrate that CD46 costimulation induces a drastic Vav Tyr phosphorylation and activates the small GTPase Rac. Furthermore, we show drastic morphological changes and actin reorganization in human primary T cells following costimulation. Therefore, CD46/CD3 costimulation profoundly affects T cell behavior. These biochemical events suggest that the Vav/Rac pathway is involved in the cross-talk between TCR and CD46, leading to cytoskeletal and morphological changes, and might help to better understand the way CD46/CD3 costimulation occurs in T cells. Finally, these data could help to define more precisely T cell adhesion function.

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References


