AILIM/ICOS-mediated elongation of activated T cells is regulated by both the PI3-kinase/Akt and Rho family cascade

Yuko Nukada1,2, Naokazu Okamoto1,2, Shu Konakahara1,2, Katsunari Tezuka3, Kazumasa Ohashi4, Kensaku Mizuno4 and Takashi Tsuji1,2

1Department of Biological Science and Technology, Faculty of Industrial Science and Technology and 2Tissue Engineering Research Center, Research Institute of Biological Science, Tokyo University of Science, Yamazaki 2641, Noda, Chiba 278-8510, Japan
3Pharmaceutical Research Laboratory, JT Inc., Takatsuki, Osaka 569-1125, Japan
4Department of Biomolecular Sciences, Graduate School of Life Sciences, Tohoku University, Miyagi 980-8578, Japan

Keywords: cell elongation, cell polarization, co-stimulatory molecule

Abstract

T-cell migration and movement is a critical component of a fully functional immune system. Activation-inducible lymphocyte immunomediatory molecule/inducible co-stimulator (AILIM/ICOS), which is a member of CD28 co-stimulatory receptor family, induces both activated T-cell migration underneath tumor necrosis factor α-treated human umbilical vein endothelial cell layers and also the morphological polarization of activated T cells. In our current study, we have investigated the signaling mechanisms underlying the morphological polarization of activated T cells, initiated by AILIM/ICOS signaling. AILIM/ICOS signaling induces the activation of phosphoinositide-3 (PI3)-kinase, the product of which, phosphatidylinositol 3,4,5-trisphosphate (PIP3), was found to be localized in the lamellipodia at the front part of the cells. Phosphorylated Akt is also co-localized with PIP3 and filamentous actin in lamellipodia and the PI3-kinase/Akt signaling cascade has critical roles in T-cell polarization and lamellipodia formation via the re-organization of the actin cytoskeleton. Rho family members and their downstream effectors, Rho-associated kinase and p21-activated kinase (PAK), are also involved in AILIM/ICOS-mediated elongation. The PAK family members are serine/threonine kinase downstream effectors of both Rac and Cdc42. PAK3 is induced by the activation of T cells, whereas PAK1 is constitutively expressed in both naive and activated T cells. During the elongation, not only PAK1 but also PAK3 play an essential role through the phosphorylation of their conservative autophosphorylation sites and catalytic domain. Ser-244 phosphorylation, which is a putative Akt phosphorylation site, on PAK3 but not on PAK1 also regulates the morphological polarization of activated T cells by AILIM/ICOS signaling. Both the PI3-kinase/Akt and Rho family cascades operate coordinately to induce the forward migration of activated T cells by AILIM/ICOS signaling.

Introduction

During an immunological response, circulating T cells in the vascular system adhere to and subsequently migrate into the endothelium, and then move either into the secondary lymphoid organs or to sites of inflammation (1, 2). The acquisition of a polarized phenotype by T cells is an essential signal for cell migration and for the movement of migrating T cells. Previous studies demonstrated that the integrin family members, the transmembrane glycoproteins, such as CD44, and also a number of chemokines contribute to the regulation of the rapid cytoskeletal re-organization and polarity formation along the front-rear axis in motile T cells (3, 4).

Activation-inducible lymphocyte immunomediatory molecule/inducible co-stimulator (AILIM/ICOS) is the third member of the CD28 family (5–8). Its expression on T cells is induced by T-cell activation upon antigen recognition via both the TCR–CD3 complex and a second co-stimulatory signaling mechanism through CD28 (9, 10). AILIM/ICOS-mediated signaling is thought to contribute mainly to the regulation of activated T cells and to effector T-cell functions (8). Consistent with the predicted roles for AILIM/ICOS in the immune response, the AILIM/ICOS ligand, B7h, is expressed not only in lymphoid tissues and antigen-presenting cells but also in
non-lymphoid cells, such as fibroblasts and endothelial cells (8). Additionally, B7h is induced in endothelial cells by inflammatory mediators such as tumor necrosis factor-α (TNF-α), IL-1β and lipopolysaccharide (8, 11).

The interaction of AILIM/ICOS and B7h on endothelial cells has an important physiological role in the reactivation of memory/effector T cells in the endothelium and also in the regulation of both effector T-cell responses and of the entry of memory/effector T cells into inflamed tissue sites in peripheral areas (8, 12). Recently, we have demonstrated that AILIM/ICOS signaling regulates both activated T-cell migration underneath TNF-α-treated human umbilical vein endothelial cell layers and also the morphological polarization of activated T cells (13). AILIM/ICOS stimulation also preferentially induces polarization in CD4+CD45RO+ memory T cells and Tn1, but not CD4+CD45RA+ naive T cells. These findings suggest that AILIM/ICOS has a novel and distinct functional role during T-cell migration and polarization, and that it has important physiological roles in the regulation of Tn1 in the endothelium. Moreover, the evidence also suggests that AILIM/ICOS controls the selective entry of Tn1 into areas of inflamed peripheral tissue.

Phosphoinositide-3 (PI3)-kinase is now known to play an essential role in T-cell activation and cell motility in cells of the immune system, and also in other cell types, through cytoskeletal rearrangements (14, 15). In the intracellular region of the AILIM/ICOS protein, PI3-kinase binds to the YFMF sequence between residues 180 and 183 (5, 6). The activation of PI3-kinase is in fact essential for T-cell activation of AILIM/ICOS-mediated signaling (16, 17). The activation of PI3-kinase also induces the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate (PIP2), which then binds to the pleckstrin homology (PH) domains of intracellular signaling molecules and thus has essential roles in the rearrangement of the cytoskeleton (14, 15, 18, 19). PIP3 binds to the PH domain of the PDK-1 kinase which then activates Akt1 by phosphorylation on the plasma membrane. The PI3-kinase/Akt pathway is thought to be involved in the regulation of neutrophil chemotaxis (20). However, the roles of Akt in both cell polarization and motility are still poorly understood in mammalian cells.

The Rho family of GTPases, including Rho, Rac and Cdc42, are known to play a central role in adhesion, cell shape formation and motility in a variety of mammalian cell types, such as fibroblasts, neural cells and hematopoietic cells (21–23). They regulate actin cytoskeletal re-organization through Rho-associated kinase (ROCK) and via the serine/threonine kinase, p21-activated kinase (PAK), which are downstream effectors of Rho and Rac/Cdc42, respectively (24). The ROCK cascade is known to be involved in a broad spectrum of cell contraction phenomena (18, 25). The PAK family members are serine/threonine kinases that function as downstream effectors of both Rac and Cdc42. PAK1, PAK2 and PAK3, based on their conserved amino acid residues, are classified into the group 1 PAKs (26, 27).

In our current study, we show that AILIM/ICOS signaling induces the morphological polarization of activated T cells, which is defined by the asymmetry of cellular components induced by both the PI3-kinase/Akt and Rho family cascades. We demonstrate through pharmacological and dominant-negative mutant analyses that PIP3 and phosphorylated Akt co-localize with filamentous actin in the lamellipodia at the leading edge, and that the PI3-kinase/Akt cascade is involved in AILIM/ICOS-mediated elongation. We further show that the Rho family members and their downstream effectors, ROCK and PAK, are also essential for AILIM/ICOS-mediated elongation. Moreover, among the PAK family members, PAK3, which is induced by T-cell activation, plays a role in AILIM/ICOS-mediated elongation through the activation of not only conserved autophosphorylation sites and catalytic domains but also via the phosphorylation of Ser-244 in the putative Akt phosphorylation site of the PAK3 protein.

**Methods**

**T-cell preparation**

Highly purified T cells (>97%) were isolated from healthy human peripheral blood and activated T cells were prepared as described previously (7). Briefly, T cells were cultured in 96-well flat-bottomed plates (Asahi Techno Glass, Tokyo, Japan) precoated with 50 ng per well of anti-CD3 mAb (OKT3) plus 250 ng per well of anti-CD28 mAb (clone 28.2, BD PharMingen, San Diego, CA, USA) for 48 h (7).

**Plasmid construction and transfection**

The SRα-Dp85 plasmid, which lacks amino acids 479–513 of PI3-kinase p85α and acts as a dominant negative, was a gift from W. Ogawa at Kobe University (28), pRK5-FlagAkt1/PKBα and pRK5-FlagAkt1/PKBα AA, containing Ala substitutions at Thr308 and Ser473, were kindly provided by U. Kikkawa at Kobe University (29). The RhoN19, RacN17 and Cdc42N17 were kindly provided by S. Kuroda and K. Kaibuchi at Nagoya University and sub-cloned into pEYFP-C1 plasmid. The ROCK KD-IA was a gift from S. Narumiya at Kyoto University (30). The PAK-Al was kindly provided by H. Sumimoto at Kyushu University. Several of our PAK3 mutants, shown in Fig. 5c, were generated from pEYFP-PAK3-WT using a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The expression vector, pEYFP-C1 (BD Clontech, Palo Alto, CA, USA) was used as a mock control. For transient expression in activated T cells, the cells were suspended with 2 µg of plasmid in 100 µl of Human T-cell Nucleofector Kit (Amaxa, Koeln, Germany) and then applied to Program T-23 equipped with Nucleofector (Amaxa). Cells were cultured for 8 h in 10% FBS–RPMI-1640 prior to subsequent stimulation.

**Short-hairpin RNA constructs**

PAK1 or PAK3-specific knockdowns were performed by expression of short-hairpin RNAs (shRNAs) using a pSuper gfp+neo in pSuper RNAi system (OligoEngine, Seattle, WA, USA). Unique sequences within the PAK genes, derived from PAK3 mRNA, were synthesized and cloned into pSuper gfp+neo vector according to the manufacturer’s instructions.

**T-cell elongation assay**

Activated T cells as described above were stimulated by anti-AILIM/ICOS (clone SA12) (7), anti-CD11a (clone G43-25B,
BD PharMingen) or anti-CD44 (clone 7, Immunotech, Marseilles, France) mAbs pre-coated onto 96-well flat-bottomed plates (Asahi Techno Glass) at concentrations of 62.5 and 250 ng per well, respectively. Cells in which the length of the protrusion was 2-fold longer than the width of the cell body were counted as elongated cells. The maximum frequency of AILIM/ICOS- or CD11a- and CD44-mediated elongation of activated T cells was reached at 120 or 60 min following stimulation (13), so the measurements were made at these time points.

To analyze the effects of the inhibitors LY294002 (Promega Biotech, Madison, WI, USA) for PI3-kinase (31) and 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (Akt inhibitor, Calbiochem, San Diego, CA, USA) for Akt (32) on activated T-cell elongation, treatments were performed on pre-cultures for 30 min at 37°C before stimulation of activated T cells as described above. To analyze the effects of several mutants (Ap85 for PI3-kinase, Akt1/PIKbα AA, RhoN19, RacN17, Cdc42N17, ROCK KD-IA or PAK-AI for small GTPase family and several PAK3 mutants for PAK3) or knockdown experiments using shRNA on the elongation of activated T cells, live cells were purified using a Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA, USA) and then stimulated under various conditions. The ratios of elongated cells were calculated from the number of elongated cells and the total number of adherent cells by microscopic observation.

Fluorescence microscopy
Activated T cells were stimulated with anti-AILIM/ICOS mAb that were pre-coated at a concentration of 10 μg ml⁻¹ onto a cover slip (Matsunami, Tokyo, Japan) for 2 h. Cells were then fixed, permeabilized and incubated with FITC-conjugated anti-α-tubulin mAb (1:200 dilution; Sigma, Tokyo, Japan), rhodamine-conjugated phalloidin (1:100 dilution; Molecular Probes, Eugene, OR, USA) and Hoechst33258 (1:2000 dilution; Sigma) for 30 min. Cells were also stained with anti-CD44 mAb or anti-phospho-Akt polyclonal antibody (pAb) (Cell Signaling Technology, Danvers, MA, USA). After washing, cells were stained with rhodamine- or FITC-conjugated goat anti-IgG pAb (1:200 dilution; ICN/Cappel, Aurora, OH, USA). Cells were analyzed by fluorescent microscopy using an Axiosvert 200M (Carl Zeiss, Jena, Germany). Image acquisition from the Zeiss inscribe was made with a cooled CCD camera using AxioCAM MRm (Carl Zeiss), and the images were processed with AxioVision software (Carl Zeiss). To observe the localization of ezrin or PIP3, expression plasmids harboring a fusion protein of ezrin and EGFP (pEzrin-EGFP) or the PH domain of Akt and EYFP (pEYFP-PHAkt) were used, and the cells were then stimulated and protein localization was analyzed by fluorescent microscopy as described above (33, 34). The pEYFP-PHAkt was constructed as described previously (35).

Immunoprecipitation and immunoblotting
After the stimulation as described above, naive T cells or activated T cells were lysed as described previously (16). For the immunoprecipitation of PI3-kinase, PAK1 or PAK3, the cell lysates were incubated with an anti-PI3-kinase p85 sub-unit mAb (BD Transduction), anti-PAK1 pAb (Cell Signaling) or anti-PAK3 pAb (Cell Signaling) and then incubated with protein-G Sepharose beads (Amersham Biosciences, UK). The beads were washed with lysis buffer and the bound proteins were solubilized by boiling for 5 min in SDS sample buffer. Immunoblotting was performed according to methods described previously (16). The membranes were then incubated with the primary antibodies anti-p85 subunit of PI3-kinase, anti-phospho-tyrosine pAbs (Upstate Biotechnology, Lake Placid, NY, USA), anti-Akt pAb (Cell Signaling Technology), anti-phospho-Akt pAb (Cell Signaling Technology), anti-PAK1 pAb or anti-PAK3 pAb according to the manufacturer’s instructions. After washing, the bound primary antibodies were detected with a secondary HRP-conjugated F(ab')2 fragment of IgG (ICN/Cappel) and visualized using enhanced chemiluminescence (Amersham Biosciences).

RNA isolation and real-time reverse transcription–PCR
The expression of PAK1/PAK3, suppressed by shRNA in HB-ALL cells, was assessed by real-time reverse transcription–PCR. Total RNA was prepared from cells under various conditions using an SV Total RNA Isolation System (Promega). First-strand cDNA, primed with pd(N)₆ random primers, was synthesized using a First-Strand cDNA Synthesis Kit (Amersham Biosciences). PCR mixtures were prepared using SYBR Premix Ex Taq (Takara, Tokyo, Japan) containing 0.2 μM of each primer and amplification reactions were performed at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. The sequences of both primers were as follows—PAK1: forward, 5'-GGATGATGATGATGATGAT-3' and reverse, 5'-GGAATGGGAGATGTAGCCAC-3' and PAK3: forward, 5'-GTAACAACCGGGATCTTCTAG-3' and reverse, 5'-CTGGGAAGATAGAGCGAAGC-3'. The gene expression levels of PAK1 and PAK3 were measured using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). PCR product levels were estimated by measurement of the intensity of SYBR green fluorescence, and the expression levels of PAK1 and PAK3 were normalized to β-actin mRNA.

Results
AILIM/ICOS signaling induces the polarization of activated T cells
We investigated the defined asymmetry of filamentous actin in lamellipodia, and also the localization of microtubules, ezrin and CD44 along the front-rear axis, during morphological polarization induced by AILIM/ICOS signaling. AILIM/ICOS signaling, which is stimulated by engagement with plate-coated anti-AILIM/ICOS mAb in the absence of CD3 stimulation, was found to dramatically induce the morphological polarization of highly elongated membrane protrusions in activated T cells (Fig. 1a), whereas cell adhesion and morphological change were not observed in unstimulated cells (data not shown). We next analyzed the actin and microtubule cytoskeletal reorganization during the AILIM/ICOS-mediated polarization of activated T cells (Fig. 1b). T cells stimulated by AILIM/ICOS signaling show a defined asymmetric morphology with distinct membrane protrusions, long rod-like stem regions and leading edges from the cell bodies which contain both nuclei.
and lamellipodia with filamentous actin (Fig. 1b, blue and red). The microtubule-organizing center was found to be localized behind the nuclei, with the microtubule extended at the front and rear edges, of these polarized T cells (Fig. 1b, green). Furthermore, ezrin and CD44, which are known to be selectively compartmentalized at the trailing edge (36–39), were also observed to be localized in the trailing edges of polarized T cells induced by AILIM/ICOS signaling (Fig. 1c, green and red, respectively). These results indicate that AILIM/ICOS signaling drastically induces T-cell polarization via the well-defined asymmetry of cellular components along the front-rear axis.

PI3-kinase is involved in cell elongation induced by AILIM/ICOS signaling in activated T cells

We have previously demonstrated that the PI3-kinase cascade is involved in the activation of naive T cells by AILIM/ICOS signaling, in the presence of CD3 stimulation, and in ATLL-type multi-lobulated nuclei formation by AILIM/ICOS stimulation (16, 17). We next investigated in our present study whether PI3-kinase is also involved in cell elongation induced by AILIM/ICOS in activated T cells. We therefore assessed the tyrosine phosphorylation status of PI3-kinase following stimulation with either AILIM/ICOS or CD28 for 5 min, in the absence of CD3 stimulation in activated T cells. These tyrosine phosphorylation levels were markedly up-regulated by AILIM/ICOS stimulation, but were not induced by CD28 (Fig. 2a).

PI3-kinase inhibitors, such as LY294002, have been shown to block the transendothelial migration of T cells and also inhibit T-cell elongation induced by CD44 cross-linking (40). In this study, treatment with LY294002 also significantly inhibited the elongation of activated T cells induced by CD11a, CD44 and AILIM/ICOS (Fig. 2b). Moreover, the transduction of dominant-negative p85 \((\Delta p85)\), lacking amino acids 479–513 of the p85\(a\) subunit of PI3-kinase (28), also significantly inhibited T-cell elongation, induced by AILIM/ICOS (Fig. 2c). These data indicate that PI3-kinase activation is essential for AILIM/ICOS-induced T-cell elongation.

PIP3 molecules, which are converted from PIP2 by activated PI3-kinase, anchor to the inner face of the membrane where they bind and recruit various proteins with PH domains (41). Significantly, in elongated T cells induced by AILIM/ICOS ligation, PIP3, detected by transiently expressed PHAkt-EYFP (29), was found to co-localize with filamentous actin in lamellipodia at the leading edge (Fig. 2d).

The Akt pathway is involved in AILIM/ICOS-induced elongation

Previous studies have shown that Akt is markedly activated by AILIM/ICOS-mediated co-stimulation, in the presence of CD3 stimulation, in peripheral T cells and that it is involved in IL-10 production, but not T-cell proliferation, and in the escape from activation-induced T-cell apoptosis (16, 42). We therefore investigated the possible involvement of the Akt pathway in T-cell elongation induced by AILIM/ICOS.
AILIM/ICOS stimulation induced the phosphorylation of Akt within 15 min and the phospho-Akt levels were subsequently maintained for 120 min, whereas CD11a- and CD44-mediated stimulation could not induce Akt phosphorylation in activated T cells (Fig. 3a). Although CD28 could not induce either T-cell polarization or polarity formation, Akt phosphorylation was slightly increased following stimulation with this cytokine. In addition, neither Erk1/2 nor p38, which are signaling molecules activated in human peripheral T cells by the engagement of both CD3 and AILIM/ICOS (16), could activate T-cell elongation induced by AILIM/ICOS (data not shown).

We next analyzed the effects of the regulation of Akt activity upon T-cell elongation induced by CD11a, CD44 and AILIM/ICOS, in the absence of CD3 stimulation in activated T cells (Fig. 3b). Treatment with an Akt inhibitor significantly inhibited AILIM/ICOS-mediated T-cell elongation, but not T-cell elongation induced by either CD11a or CD44 ligation. Furthermore, we examined the effects of the transduction of both dominant-negative and active forms of Akt upon AILIM/ICOS-mediated T-cell elongation (Fig. 3c). The transduction of Akt AA, a dominant-negative form of Akt1 in which both Thr308 and Ser473 are substituted by alanine, significantly reduced T-cell elongation induced by AILIM/ICOS.

We then investigated the localization of phospho-Akt in AILIM/ICOS-induced elongated T cells and found that these molecules localized at lamellipodia in the leading edges and co-localized with filamentous actin in the lamellipodia (Fig. 3d). These observations strongly suggest that the regulation of Akt is directly involved in T-cell elongation induced by AILIM/ICOS stimulation.

Rho GTPases and their downstream effectors mediate AILIM/ICOS-induced T-cell elongation

To investigate whether members of the Rho family of GTPases, such as Rho, Rac and Cdc42, contribute to AILIM/ICOS-induced T-cell elongation, we expressed dominant-negative forms of these factors (RhoN19, RacN17 and Cdc42N17) in activated T cells and examined their inhibitory effects. Activated T cells expressing YFP-fused dominant-negative forms of Rho GTPases and their downstream effectors were isolated using a cell sorter, following a deprivation procedure for dead cells, at >95% purity (data not shown). Each of the dominant-negative species was found to

![Fig. 2. PI3-kinase mediates activated T-cell elongation induced by AILIM/ICOS signaling.](image-url)
significantly inhibit AILIM/ICOS-mediated elongation (Fig. 4a). In particular, RacN17 and Cdc42N17 also significantly reduced these elongation ratios (Fig. 4a).

We next investigated the involvement in AILIM/ICOS-induced elongation of ROCK and PAK, which are downstream effectors of Rho and Rac/Cdc42, respectively. The expression of the dominant-negative form of ROCK (ROCK KD-IA) or an autoinhibitory domain of the PAKs (PAK-AI) was observed to significantly inhibit T-cell elongation (Fig. 4b). ROCK KD-IA markedly reduced the ratio of T-cell elongation to 32% of the mock control value. These results indicate that the Rho family GTPases play important roles during AILIM/ICOS-mediated activated T-cell elongation.

The involvement of both PAK1 and PAK3 in the AILIM/ICOS-mediated elongation of activated T cells

Previous reports using microarray analysis have shown that the expression of PAK3, but not other PAK family members, is induced in CD4+ T cells that have been activated by co-stimulation (43). We thus analyzed the expression levels of PAK1 and PAK3 in both naive and activated T cells by western blotting. The expression of PAK1 was detectable in both naive and activated T cells, but PAK3 was evident only in activated T cells (Fig. 5a). To investigate the involvement of PAK3 during the AILIM/ICOS-mediated elongation of activated T cells, PAK1 and PAK3-specific knockdowns in activated T cells were performed by the expression of shRNA molecules. We confirmed the specific down-regulation of each transcript following the expression of the PAK1-105

---

**Fig. 3.** Akt is involved in the AILIM/ICOS-mediated elongation of activated T cells. (a) Activated T cells by CD3/CD28 ligation for 2 days were stimulated by AILIM/ICOS, CD28, CD11a or CD44 ligation. Cell lysates were prepared at the indicated times. Phosphorylation of Akt was detected by anti-phospho-Akt pAb. The data shown are representative of at least three experiments using independent donors with similar results. (b) Activated T cells were pre-treated with various concentrations of Akt inhibitor for 30 min and then stimulated by AILIM/ICOS, CD11a or CD44 ligation. After incubation for 2 h following AILIM/ICOS stimulation (filled lozenge), or 1 h following CD11a (open square) and CD44 (filled triangle) induction, the elongation ratios were analyzed as described. Mean ± SEM. **P<0.001 compared with inhibitor-untreated cells (AILIM/ICOS, 44.1 ± 2.5%; CD11a, 27.1 ± 4.0%; CD44, 34.8 ± 1.5%). (c) Activated T cells were transfected with pYFP-C1 (mock control), pRK5-Akt1/PKBα or pRK5-Akt1/PKBα AA, and then stimulated by AILIM/ICOS for 2 h. Cells were then stained with anti-Akt pAbs. Highly fluorescent cells, compared with mock transfected cells, were subjected to elongation ratio analysis. Means ± SEMs. *P<0.005 and **P<0.001 compared with mock transfected cells. (d) Activated T cells were stimulated by AILIM/ICOS for 2 h and the localization of phospho-Akt was detected with anti-phospho-Akt antibodies. AILIM/ICOS-stimulated T-cells were then stained with rhodamine–phalloidin and Hoechst33258. Cells were analyzed by fluorescent microscopy using an Axiovert 200M and the images were processed with AxioVision software. Bar, 10 μm.
AILIM/ICOS-induced T-cell elongation. (a) Expression plasmids for pEYFP-RhoN19, RacN17 and Cdc42N17 were transfected into T cells activated by CD3/CD28 ligation for 2 days, and cultured for a further 8 h. The expression plasmids were transfected as a mock control. The cells were then plated onto anti-AILIM/ICOS mAb-coated cover slips for 2 h. EYFP-positive cells were used to determine the elongation ratios. Mean ± SEM. *P < 0.001 compared with the mock control. (b) Expression plasmids for pEYFP-ROCK KD-IA and PAK-AI were transfected into activated T cells and the cells were stimulated as described above. Fluorescence-positive cells were again used to determine the elongation ratios. Mean ± SEM. *P < 0.001 compared with mock control.

Fig. 4. The Rho small GTPase family members are involved in the AILIM/ICOS-induced elongation of activated T cells. (a) Expression plasmids for pEYFP-RhoN19, RacN17 and Cdc42N17 were transfected into T cells activated by CD3/CD28 ligation for 2 days, and cultured for a further 8 h. The expression plasmids were transfected as a mock control. The cells were then plated onto anti-AILIM/ICOS mAb-coated cover slips for 2 h. EYFP-positive cells were used to determine the elongation ratios. Mean ± SEM. *P < 0.001 compared with the mock control. (b) Expression plasmids for pEYFP-ROCK KD-IA and PAK-AI were transfected into activated T cells and the cells were stimulated as described above. Fluorescence-positive cells were again used to determine the elongation ratios. Mean ± SEM. *P < 0.001 compared with mock control.

and PAK3-479 siRNA constructs, respectively, in the T-cell lymphoma cell line, HPB-ALL, which expresses both molecules (Fig. 5b). Treatment with siRNA constructs for PAK1 and PAK3 was found to significantly reduce the ratio of the AILIM/ICOS-mediated elongation of activated T cells to 62 and 59% of the mock control levels, respectively (Fig. 5b). These results indicate the involvement of not only PAK1 but also PAK3 in AILIM/ICOS-induced elongation of activated T cells.

PAK1 and PAK3 contain high levels of conservation in their amino acid sequence, including their domain structures and the autophosphorylation sites (26, 27). Figure 5c shows a schematic representation of these structural features of PAK1 and PAK3, including each of the non-kinase autophosphorylation serine residues (26, 27) and the threonine residues (Thr-422 in PAK1 and Thr-421 in PAK3), the phosphorylation of which is required for trans-activation of the catalytic domain. The expression of the K297L mutant, which acts as dominant-negative forms of both PAK1 and PAK3, in activated T cells led to a measurable reduction in the ratio of AILIM/ICOS-induced T-cell elongation (Fig. 5d). The expression of the dominant-negative forms of the autophosphorylation sites at Ser-50, Ser-139 and Thr-421 in PAK3, which are conserved between both molecules, also drastically reduced AILIM/ICOS-induced elongation and the combined mutation of these sites had an additional inhibitory effect on elongation (Fig. 5d). These results indicate that these conserved autophosphorylation sites and catalytic domain in PAK1 and PAK3 are essential for AILIM/ICOS-induced activated T-cell elongation.

Previous studies have reported that PAK3, but not PAK1, contains a consensus Akt phosphorylation motif, Arg-X-Arg-X-Ser/Thr, at amino acid positions 239–244 which includes the putative Akt phosphorylation site at Ser-244 (27; Fig. 5c). Significantly, the expression of a dominant-negative S244A mutant markedly reduced the frequency of T-cell elongation to 70% of the mock transfectant levels (Fig. 5d).

Discussion

During the immune response, the recruitment of activated T cells to the inflammatory sites in the periphery is thought to be an important biological phenomenon (1, 2). We have previously shown that the interaction between AILIM/ICOS, which is induced in activated T cells, and its ligand B7h, expressed in TNF-α-activated endothelial cells, induces the migration of activated T cells into endothelial cells (13). Recently, it was also shown that AILIM/ICOS-mediated T-cell migration can contribute to the onset of graft versus host disease (44). In our current study, we have revealed that AILIM/ICOS-induced migration of activated T cells is mediated by both the PI3-kinase/Akt cascade and the Rho family members of small GTPases through cytoskeletal rearrangement. AILIM/ICOS stimulation has also been reported to induce a similar morphological polarization event in activated T cells as previously identified polarization-inducible molecules, such as CD44 and LFA-1 (36–39). However, AILIM/ICOS-induced elongation is mediated by the PI3-kinase/Akt cascade, whereas both CD44 and LFA-1-induced elongation signals are regulated by the activation of PI3-kinase and PI-specific phospholipase C cascades in T₃,1 (40). These results suggest the possibility that elongation by the AILIM/ICOS-B7h system is differently regulated and has separate roles from other polarization mechanisms in the immune system.

PI3-kinase is well known to play an essential role in cell motility in not only immune cells but also other cell types (15). The activation of PI3-kinase induces the production of PIP3 from PIP2, which then binds to PH domains in intracellular signaling molecules and localizes at lamellipodia in leading edges (14, 18). In our present study, PHAkt-EYFP immediately translocated and co-localized with filamentous actin at lamellipodia in the leading edges of activated T cells upon AILIM/ICOS treatment. Phospho-Akt was also found to translocate and co-localize with filamentous actin and PHAkt-YFP at lamellipodia in polarized T cells, induced by AILIM/ICOS stimulation (Fig. 3d). Previous studies have demonstrated that Akt is essential for cell polarization and movement along chemoattractant gradients in Dictyostelium (19). Our present results further suggest that activated Akt regulates factors that control actin cytoskeletal re-organization and the resulting cell polarization and motility.
The Rho family members of small GTPases, Rho, Rac and Cdc42, are known to be essential for cell motility through the regulation of cell polarization and the cytoskeletal rearrangement of actin (21, 45). In this study, Rho family members and their effectors were found to be essential for AILIM/ICOS-induced cell elongation in activated T cells. In the polarized T cells induced by AILIM/ICOS signaling, ezrin and CD44, which have an ezrin/radixin/moesin-binding motif in their intracellular domains (36–39), were selectively compartmentalized at the trailing edge (Fig. 1). The expression of dominant-negative forms of Rho and ROCK was then shown to drastically down-regulate AILIM/ICOS-induced cell elongation (Fig. 4). It has been shown that the ‘backness’ signal is generated by Rho activation in neutrophils (18, 46), and that Rho-ROCK signaling is required for the establishment of membrane ‘posteriority’ in cooperation with ezrin compartmentalization in the trailing edge (38). Our findings presented herein also suggest that Rho-ROCK signaling induced by AILIM/ICOS plays an important role in the establishment of the rear portion of the cell during AILIM/ICOS-induced cell elongation.

PAKs are a highly conserved family and are activated by Rac and Cdc42 (26, 27, 47). The conventional PAKs (PAK1–PAK3) are known to be involved in the formation of the leading edge during cell motility and generate a protrusive force by Rac signaling and induce filopodia by Cdc42 signaling through cytoskeletal rearrangements of actin (26, 27, 45, 47). Previous studies have reported that PAK3 is expressed only in brain and that mutations in the PAK3 gene are associated with X-linked, non-syndromic mental retardation syndrome in humans (26, 27). In this study, the expression of PAK3 was induced by the activation of T cells, whereas PAK1 is constitutively expressed in both naive and activated T cells. Previous reports have also shown that PAK3 expression is induced by co-stimulation in the presence of a CD3 engagement of CD4+ T cells (43). However, the roles of PAK3 in cell motility remain poorly understood. The specific depletion of PAK3, which is induced by the activation of T cells using shRNA, reduces the AILIM/ICOS-induced elongation of activated T cells as efficiently as PAK1, indicating that both PAK1 and PAK3 are independently involved in the AILIM/ICOS-induced elongation of activated T cells (Fig. 5b).

![Fig. 5. Both PAK1 and PAK3 are involved in AILIM/ICOS-induced T-cell elongation.](image-url)
The regulatory amino acid residues, such as the catalytic domain and autophosphorylation sites, are highly conserved between PAK1 and PAK3 and dominant-negative mutants of those sites markedly reduce AILIM/ICOS-induced elongation through the down-regulation of both PAK1 and PAK3 activities (Fig. 5c and d). We have demonstrated in this study that the mutation of Ser-244 in PAK3, but not in PAK1 (27, Fig. 5c), significantly reduces the frequency of T-cell elongation (Fig. 5c). This finding suggests that Ser-244 is also involved in AILIM/ICOS-induced elongation in addition to other regulatory amino acid residues, which are conserved in PAK3 and PAK1. Recently, it has been shown that Akt1 can phosphorylate Ser-21 on PAK1, and that this phosphorylation event modulates Nck binding and cell migration (48). Furthermore, mutations in Akt2 and/or PAK4 are involved in the generation of colorectal cancer (49). We speculate, therefore, that active Akt will also modulate Ser-244 in PAK3, which is putative Akt phosphorylation motif, and thus has a role in AILIM/ICOS-mediated elongation of activated T cells.

Further studies that explore the downstream effectors of the PI3-kinase/Akt cascade and the regulation of PAK3 through Ser-244 will provide valuable insights into cell motility and into the signaling pathways underlying AILIM/ICOS-induced elongation of activated T cells.

Acknowledgements

We thank U. Kikkawa (Kobe University), W. Ogawa (Kobe University), S. Kuroda (Nagoya University), K. Kaibuchi (Nagoya University), S. Narumiy (Kyoto University) and H. Sumimoto (Kyushu University) for donating plasmids. We also thank Y. Nishi of JT Inc. for useful discussions and encouragement.

Abbreviations

AILIM activation-inducible lymphocyte immunomediatory molecule
ICOS inducible co-stimulator
PAb polyclonal antibody
PAK p21-activated kinase
PH pleckstrin homology
PI3-kinase phosphoinositide-3-kinase
PIP2 phosphatidylinositol 4,5-bisphosphate
PIP3 phosphatidylinositol 3,4,5-trisphosphate
PI3-kinase phosphoinositide-3-kinase
ROCK Rho-associated kinase
shRNA short-hairpin RNA
TNF-α tumor necrosis factor-α

References


