AILIM/ICOS signaling induces T-cell migration/polarization of memory/effector T-cells

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Abstract
AILIM/ICOS has critical roles in the regulation of T-cell differentiation and effector T-cell function in various immune responses. The counter-ligand for AILIM/ICOS, B7h, is widely expressed in not only lymphoid tissue and antigen-presenting cells, but also in fibroblast and endothelial cells in various organs. Here, we demonstrate that activated human T-cells migrate beneath TNF-α-treated HUVEC and display morphological polarization via AILIM/ICOS signaling. AILIM/ICOS stimulation, in the absence of antigen stimulation, also induced T-cell polarization. Importantly, AILIM/ICOS-mediated polarization was evident in CD4+CD45RO+ memory T-cells and generated Th1 cells, but not in CD4+CD45RA+ naive T-cells and generated Th2 cells. Furthermore, AILIM/ICOS signaling is involved in transendothelial migration of Th1 cells, but not Th2 cells. Our data suggest that AILIM/ICOS–B7h interactions play an important role in the endothelium in controlling the entry of memory/effector T-cells into inflamed tissues in the periphery.

Introduction
During the immune response, T-cells are optimally activated in secondary lymphoid tissues in order to properly migrate into areas of inflamed tissue (1). Upon antigen recognition via the T-cell receptor/CD3 complex, a second costimulatory signal from antigen-presenting cells (APCs) is necessary for activation of naive T-cells (2,3). Priming of naive T-cells in lymphoid organs depends on the interaction between CD28, which is constitutively expressed in T-cells, and both CD80 and CD86, which induces subsequent IL-2 production and clonal expansion (2,3). T-cell activation also induces other costimulatory molecules, including the activation-inducible lymphocyte immunomediatory molecule (AILIM)/inducible costimulator (ICOS), which is the third member of the CD28 family and is only expressed at very low levels on naive T-cells (4–7). AILIM/ICOS-mediated signal is thought to contribute mainly to the regulation of activated T-cells and to effector T-cell functions (5).

Previous studies have demonstrated that AILIM/ICOS-mediated signaling also functions in the generation of T helper 2 (Th2) responses (8,9). Effector Th2 responses have now been shown to be primarily regulated via AILIM/ICOS-mediated signaling from studies using Th2-mediated disease models such as chronic graft-versus-host disease and lung mucosal inflammation (10,11). AILIM/ICOS-mediated signals also play critical roles in the regulation of effector Th1 cell functions, as shown by experiments with the Th1-mediated autoimmune diseases, EAE and acute GVHD (10,12,13). These studies demonstrate that AILIM/ICOS has important roles in regulating Th1 and Th2 effector responses in vivo and in tissue-specific immune responses in the periphery.

Consistent with the predicted roles for AILIM/ICOS in immune responses, the AILIM/ICOS ligand, B7h, has a noteworthy expression profile in comparison to CD80 and CD86. B7h is expressed on not only lymphoid tissues and APCs but also on non-lymphoid cells, such as fibroblasts and endothelial cells, in heart, lung, kidney and testis, whereas the expression of CD80 and CD86 is restricted to lymphoid cells and APCs (5). Additionally, B7h is induced in endothelial cells...
and in non-lymphoid tissues by inflammatory mediators such as TNF-α, IL-1β and lipopolysaccharide (LPS) (5, 11). Recently, it was reported that B7h expressed on human umbilical vein endothelial cells (HUVEC) costimulated Th1 and Th2 cytokine production by memory CD4+ T-cells in the presence of superantigen (14). 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 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 (5, 14).

In this study, we have demonstrated that AILIM/ICOS signaling regulates both activated T-cell migration underneath TNF-α-treated HUVEC layers and also the morphological polarization of activated T-cells. Furthermore, AILIM/ICOS stimulation preferentially induced T-cell polarization of CD4+CD45RO+ memory T-cells and Th1 cells, but not CD4+CD45RA+ naive T-cells. The findings reported here indicate that AILIM/ICOS has a novel and distinct functional role in T-cell migration and polarization when compared with other costimulatory molecules. Our study also suggests the possibility that AILIM/ICOS has important physiological roles in the regulation of Th1 cells in endothelium and in the control of the selective entry of Th1 cells into inflamed peripheral tissue.

**Methods**

**T-cell preparation**

Highly purified T-cells (>97%) were isolated from healthy human peripheral blood as described previously (15). CD4+ T-cells, CD4+CD45RA+ T-cells and CD4+CD45RO+ T-cells were purified by negative selection using anti-CD8 microbeads (Miltenyi Biotech, Germany), and negative and positive selection using anti-CD45RA microbeads (Miltenyi Biotech), respectively. Activated T-cells were prepared as described previously (6).

**Migration assay**

HUVEC was purchased from Cambrex Bioscience (Walkersville, MD), cultured according to the manufacturer’s instructions and then stimulated with 10 ng/ml of TNF-α (Peprotech, Rocky Hill, NJ) for 48 h. The cells were co-cultured with activated T-cells or generated Th1/Th2 cells (5 × 10^5 cells/well) in 10% FBS–RPMI-1640 for 4 h. For analysis of the effects of neutralizing antibodies against integrins and AILIM/ICOS, those T-cells were pre-incubated for 30 min at a concentration of 10 and 20 μg/ml, and then seeded onto a HUVEC-layer. Using phase-contrast microscopy, activated T-cells adhering on the HUVEC layer were observed as bright cells and the migrating T-cells were observed as flat, dark and large cells, in which nuclei and granules can be easily observed, as described previously (16). The migration ratios were calculated from the numbers of migrating and adherent activated T-cells.

**Plasmid transfections**

The expression vector, pEYFP-C1 (Clontech, Palo Alto, CA), was used as a MOCK control. For transient expression in activated T-cells, the cells were suspended with 1 μg of plasmid in 100 μl of human T-cell Nucleofector Kit (Amaza, Koeln, Germany) and then applied to Program T-23 equipped with Nucleofector (Amaza). Cells were cultured for 8 h in 10% FBS–RPMI-1640 prior to subsequent stimulation.

**T-cell elongation assay**

Activated T-cells were stimulated by anti-AILIM/ICOS (clone SA12) (10), anti-CD11a (clone G43-25B, BD Pharmingen, SanDiago, CA), anti-CD44 (clone 7, Immunotech, Marseilles, France) or anti-CD28 (clone 28.2, BD Pharmingen) mAbs precoated onto 96-well type plates (Asahi Techno Glass, Tokyo, Japan) at concentrations of 250, 62.5, 250 and 250 ng/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 after 1 h of CD44 and CD11a stimulation, and after 2 h of AILIM/ICOS and CD28 stimulation. The elongated cell ratios were calculated from the number of elongated cells and the total number of adherent cells by microscopic observation.

**Cell staining**

Activated T-cells were stained with 10 μg/ml of either anti-AILIM/ICOS mAb (clone SA12) or a human AILIM/ICOS-IgFc chimera for analysis of either AILIM/ICOS or B7h expression and then stained with FITC-conjugated anti-mouse IgG goat or anti-human IgFc goat (Fab’)2 fragments, respectively. After staining, the cells were analyzed on a FACS Calibur flow cytometer (BD). For microscopic observations, the cells were fixed after the stimulations and incubated with rhodamine-conjugated phalloidin (1:100 dilution; Molecular Probes) and Hoechst 33258 (1:2000 dilution; Sigma) for 30 min. Cells were also stained with anti-α-tubulin mAb (1:200 dilution; Sigma). Co-cultured cells were observed by confocal microscopy using a LSM 510 META (Carl Zeiss, Jena, Germany). The images acquired by confocal microscopy were processed with Imaris 4 software (Zeiss). Cells were also analyzed by fluorescent microscopy using an Axiovert 200M (Carl Zeiss). Image acquisition from the Zeiss inscribe was made with a cooled CCD camera using AxioCAM MRm (Zeiss) and the images were processed with AxioVision software (Zeiss).

**Th1/Th2 differentiation**

CD4+CD45RA+ T-cells were activated with anti-CD3, anti-CD28 and anti-AILIM/ICOS mAb coated plates at concentrations of 50, 125 and 250 ng/well, respectively, incubated in 10% FCS–RPMI-1640 supplemented with 50 U/ml IL-2 (Peprotech) for 2 days. To generate Th1 or Th2 effector cells, activated CD4+CD45RA+ T-cells were subjected to subsequent stimulation with 5 ng/ml IL-12 (Peprotech) and 2.5 μg/ml anti-IL-4 mAb (BD Pharmingen), or to 12.5 ng/ml IL-4 (R&D systems, Minneapolis, MN) and 5 μg/ml anti-IFN-γ mAb (BD Pharmingen), respectively, for 4 days. Isolated Th1 or Th2 cells were then stimulated with anti-CD3, anti-CD28 and anti-AILIM/ICOS mAbs, as described above, in the presence of 50 U/ml IL-2 (Peprotech) for 2 days. After 3 days of culture, Th1 or Th2 cells were subjected to elongation assays.

**Semi-quantitative RT-PCR**

Expression of t-bet, c-maf and GATA3 was determined by semi-quantitative RT-PCR as described previously (15). Total
RNA was collected from Th1 and Th2 cells and amplification of β-actin, t-bet, c-maf and GATA3 cDNA was performed by PCR using specific primers. Primer sequences are as follows: β-actin: 5’-CAAGGCCAACCGCGAGAAGA-3’ and 5’-GCACTGTGTTGGCGTACAGGT-3’; t-bet: 5’-CACTACAGGAT-GTTGTGGACGTG-3’ and 5’-CCCCCTTGTTTGTGAGCTTT- TAG-3’; c-maf: 5’-GGAGAAATACGAGAAGTTGGTGAGC-3’ and 5’-ACAGAAGTCAGGGGTAGGTGGTTC-3’; GATA3: 5’-AACTGTCAGACCACCACAACCACAC-3’ and 5’-GGATG-CCTTCTTTCATAGTCAGG-3’ (17).

Fig. 1. Activated T-cells migrate beneath TNF-α-treated HUVEC. (A) Purified human peripheral T-cells were activated by both anti-CD3 and anti-CD28 mAbs, or by PMA and calcium ionophore for 2 days (upper panel). The profiles obtained after treatments by CD3/CD28 (thick line) and PMA/Ca-ionophore stimulation (thin line). HUVEC was stimulated with 10 ng/ml of TNF-α for 2 days and stained with a biotinylated AILIM/ICOS-IgFc chimera (thick line). Control-staining with isotype-matched IgG is shown by a dotted line. (B) A phase-contrast image of a coculture of activated T-cells and TNF-α-treated HUVEC. Representative images of adherent T-cells on HUVEC layers, partially migrated cells and fully migratory T-cells beneath HUVEC are indicated by black arrowheads, hatched arrows and white arrows, respectively. Bar, 20 µm. (C) EYFP-expressing activated T-cells (green) were seeded onto a confluent layer of TNF-α-treated HUVEC. After 4 h of coculture the cells were stained with rhodamine-conjugated phalloidin and Hoechst 33258 for filamentous actin (red signal) and nuclei (blue signal), respectively. The images in the upper and row represent 3-dimensional views at 45° from x-z projections. The images in the lower row represent x-z sliced sections by viewing from the arrows in upper row images. Bar, 10 µm.
Results

AILIM/ICOS signaling is involved in activated T-cell migration

Cell surface AILIM/ICOS expression is strongly induced in activated T-cells which are stimulated by either CD3 and CD28 interactions or treatment with phorbol 12-myristate 13-acetate (PMA) and calcium ionophores (Fig. 1A). In contrast, HUVEC were found to constitutively express B7h on their cell surfaces at relatively low levels (5,14) which were induced by TNF-α and reached a maximal level at 24–48 h following stimulation (Fig. 1A).

To further elucidate the roles of AILIM/ICOS–B7h interactions in transendothelial migration, we developed a co-culture of T-cells, activated by both CD3 and CD28 engagement, and HUVEC stimulated with or without TNF-α. Using phase-contrast microscopy, activated T-cells that had adhered to the TNF-α-treated HUVEC cell layer were observed as bright cells (Fig. 1B, closed arrowheads) and migrating T-cells were observed as flat, dark and large cells, in which nuclei and granules can be easily observed (Fig. 1B, open arrows).

We confirmed these analyses using confocal laser microscopy to determine three-dimensional morphologies (Fig. 1C). To enable fluorescent microscopy analysis, the expression plasmid for yellow fluorescent protein (YFP; green signal) was transduced in activated T-cells for 8 h, which were then seeded onto a confluent layer of TNF-α-treated in activated T-cells for 8 h, which were then seeded onto a confluent layer of TNF-α-treated HUVEC. The cells were stained for filamentous actin using rhodamine-conjugated phalloidin to detect stress fibers in the HUVEC layer and also to visualize the actin cytoskeleton reorganization of both cell types (red signal). Representative images of YFP-activated T-cells co-cultured with TNF-α-treated HUVEC were viewed using x-z projections of the entire complement of optical sections from the co-culture with confocal laser microscopy (Fig. 1C). Fully migratory YFP-activated T-cells beneath the TNF-α-treated HUVEC layers appear under a stress fiber (red signal, left in Fig. 1C) of the HUVEC. Interestingly, YFP-activated T-cells were found to be flattened beneath the HUVEC layers and showed a clear cell polarity, with the leading edges of the cell bodies containing lamellipodia. We also generated images of partially migrated T-cells (middle and right panels in Fig. 1C) which showed clear cell polarities with distinct cell protrusion and a long rod-like stem region. Activated T-cells migrated from the cell edges of HUVEC, with protrusions under the stress fibers, whereas the cell bodies of partially migrated T-cells, containing nuclei, remained on the outside of the HUVEC edges.

The migration ratio of activated T-cells in these experiments was dramatically increased to 23% of the input cells, whereas transendothelial migration beneath non-stimulated HUVEC was only 3.5% of the input cells (Fig. 2). Additionally, activated T-cell migration in these co-cultures was significantly inhibited by pretreatment with anti-CD11a or anti-AILIM/ICOS (clone SA12) antibodies (Fig. 2). This inhibitory effect was not enhanced, however, by co-treatment with both antibodies (Fig. 2).

AILIM/ICOS signaling induces polarization of activated T-cells

We investigated whether AILIM/ICOS signaling induces polarity formation by reorganizing the actin cytoskeleton in T-cells activated by both CD3 and CD28 engagement. T-cells were seeded onto culture slides pre-coated with anti-AILIM/ICOS mAb, and both CD44 and CD11a ligations induced cell polarization with a highly elongated morphology (Fig. 3), which was consistent with a previous study (18). The frequency of elongation following either CD44 or CD11a ligation in activated T-cells was 37% and 27%, respectively. AILIM/ICOS ligation, in the absence of CD3 ligation, also dramatically induced cell polarization with highly elongated membrane protrusions at a frequency of 42% in activated T-cells, whereas CD28 did not induce polarization of these cells (Fig. 3A and B). T-cell elongation and polarization following AILIM/ICOS stimulation could also be induced by a B7h-IgFc chimera (data not shown). However, the maximal frequency of AILIM/ICOS-mediated polarization was reached at 120 min following stimulation, whereas polarization induced by CD44 or CD11a stimulation reached peak levels after 60 min (data not shown).

We next observed filamentous actin and tubulin in the elongated T-cells stimulated with either CD44, CD11a or AILIM/ICOS ligation (Fig. 3C). In each case, the resulting morphological features were identical with the elongated T-cells showing clear cell polarity with distinct membrane protrusions, long rod-like stem regions and leading edges from the cell bodies containing nuclei and lamellipodia with filamentous actin (Fig. 3C). Following tubulin staining, the microtubule organizing centre (MTOC) was found to be localized behind the nuclei of the polarized cells with filamentous tubulin extended at the front and rear edges of elongated T-cells. However, the protrusion formations of partially migrated T-cells, which have distinct long protrusions at the front of the cells (Fig. 1C), would occur via a different mechanism to that of migrating T-cells and T-cell movement with lamellipodia.

AILIM/ICOS-mediated T-cell polarization preferentially occurs in CD4+CD45RO+ memory/effector T-cells

We next examined the elongation ability of diverse T-cell populations induced by AILIM/ICOS ligation. Pan-T-purified T-cells, CD4+CD45RA+ and CD4+CD45RO+ T-cells were activated by CD28 in the presence of CD3 for 2 days. Activated T-cell populations were then stimulated by AILIM/ICOS ligation in the absence of CD3 for 2 h and the frequency of elongated
T-cells was determined in each population of cells (Fig. 4). AILIM/ICOS stimulation preferentially induced elongation and polarization in CD4+CD45RO+ T-cells when compared with CD4+CD45RA+ T-cells.

AILIM/ICOS-mediated T-cell transendothelial migration and polarization preferentially occurs in Th1 cells

To independently identify the levels of polarization in response to AILIM/ICOS stimulation in both Th1 and Th2 cells, we purified these cell types from CD4+CD45RA+ T-cells using in vitro cultures. Briefly, CD4+CD45RA+ T-cells were activated via both CD28 and AILIM/ICOS costimulation in the presence of CD3 and then cultured in the presence of either recombinant human IL-12 and anti-IL-4 or IL-4 and anti-IFN-γ antibodies to differentiate between Th1 and Th2 cells, respectively. Th1 and Th2 cells were then analyzed for the expression of specific transcription factors by RT–PCR. It has been previously shown that t-bet is selectively expressed in Th1 cells and that c-maf
and GATA3 are selectively expressed in Th2 cells (17,19). The results of the RT-PCR of these factors demonstrated that we successfully generated both Th1 and Th2 cells (Fig. 5A). Furthermore, the cell-surface expression of AILIM/ICOS for both Th1 and Th2 cells was detected in both Th1 and Th2 cells (Fig. 5B) and was at relatively higher levels than CD4⁺CD45RA⁺ T-cells (data not shown).

We investigated transendothelial migration of Th1 and Th2 cells and whether AILIM/ICOS-B7h signaling is involved in endothelial migration of Th1 cells. The generated Th1 and Th2 cells successfully migrated underneath TNF-α-treated HUVEC at a ratio of 18.5% and 15.5%, respectively (Fig. 6A). Additionally, transendothelial migration of Th1 cells, but not Th2 cells, was significantly inhibited by pre-treatment with neutralizing antibody for AILIM/ICOS. Finally, we also investigated the polarizing ability of Th1 and Th2 cells induced by AILIM/ICOS. More than 50% of Th1 cells were morphologically altered, whereas the proportion of elongated Th2 cells was found to be <10% (Fig. 6B). These findings strongly suggest that AILIM/ICOS signaling is involved in polarization and transendothelial migration of Th1 cells.

**Discussion**

T-cell recruitment in blood is a central event in the regulation of immune responses (1). Endothelial cells play an important role in the recruitment of activated T-cells at the sites of inflammation in the periphery, and transendothelial migration is well known to occur via sequential interactions between cell-surface adhesion molecules on T-cells and endothelial cells (1).

In the present study, we have revealed that AILIM/ICOS expressed on T-cells activated by CD3, CD28 and PMA interacts with B7h on TNF-α-stimulated HUVEC and results in cell polarization and transendothelial activity. Surprisingly, these functions of AILIM/ICOS-B7h interactions in activated T-cells are induced only by AILIM/ICOS signaling in the absence of CD3 stimulation. The AILIM/ICOS-B7h system also has an essential role in controlling the migration of activated T-cells and therefore has a novel and distinct role in the regulation of T-cell activation, differentiation and immune function compared with other costimulatory molecules.

Morphological polarization is necessary for both chemotaxis and motility in immune cells (20,21). T-cells, neutrophils and other motile cells respond to a chemoattractant gradient by rapidly adopting a polarized morphology with leading and trailing edges oriented with respect to this gradient, and subsequent migration into inflamed tissues (20). Adhesion of not only LFA-1, but also the hyaluronate receptor, CD44, induces chemokine-independent polarization of Th1 cells in an outside-in manner (19). In these cells, remarkable asymmetric shapes are generated with filamentous actin, which is polymerized preferentially at the leading edge as lamellipodia (22). Although AILIM/ICOS stimulation also induced similar morphological polarization in activated T-cells, its signaling...
cascade differed from both chemokine-dependent (Gαi-dependent) and adhesion-dependent cell polarization (19,20).

Recently, it was reported that B7h expressed on endothelial cells costimulates Th1 and Th2 cytokine production by resting memory T-cells in the presence of superantigen (14). This report suggested that an important physiological role of B7h is the reactivation of effector/memory T-cells in the endothelium and the control of T-cell entry into inflamed tissue (5,14). Interestingly, AILIM/ICOS stimulation preferentially induced morphological polarization of CD4+CD45RO+ memory T-cells and Th1 cells in comparison with CD4+CD45RA+ naive T-cells, whereas the expression levels of AILIM/ICOS between Th1 and Th2 cells were not so different (Figs 4–6). In polarization experiments of T-cell subsets, CD44 or LFA-1-mediated T-cell polarization preferentially occurs in Th1 cells, but not Th2 cells (18). Transmigration and polarization of T-cells induced by not only AILIM/ICOS signaling but also by some adhesion molecules preferentially occurs in Th1 cells, but not Th2 cells. From these observations, we speculate that the signaling molecules regulating for these functions might have different expression profiles between Th1 and Th2 cells, and these conditions will lead to preferentially responsiveness for AILIM/ICOS and some adhesion molecules in Th1 cells. Furthermore, Th1-tropic chemokines, such as RANTES, MIP-1β and IP-10, selectively attract Th1 cells and mainly contribute to the selective accumulation of Th1 cells in chronic inflamed tissue (23–25). Our findings also suggest that AILIM/ICOS has an important role in selective homing of T-cells to non-lymphoid inflamed tissues and also the generation of effector T-cell responses at inflamed sites in peripheral tissue.

In previous studies, AILIM/ICOS–B7h interactions have been shown to have essential roles in both Th1 and Th2 dependent immune and autoimmune diseases (5,26). Th1-mediated inflammation was evaluated using EAE (13,27,28) and collagen-induced arthritis (29,30). Disruption of AILIM/ICOS signaling during the effector phase abrogated progression of the disease as a result of the suppression of Th1 effector functions (13). Experiments with AILIM/ICOS knockouts demonstrated that the mice were quite resistant to CIA and had no inflammation in tissues at the joints (30). T-cells, in rheumatoid synovitis with rheumatoid arthritis, were mainly polarized into CD4+CD45RO+ T-cells and Th1 cell types (31–34). Furthermore, peripheral blood T-cells from patients with rheumatoid arthritis markedly induced the expression of AILIM/ICOS to higher levels than healthy donors and AILIM/ICOS-expressing T-cells were drastically increased as a proportion of the migratory CD4+ cells in the synovial fluid (34). In addition, triggering of AILIM/ICOS by interaction with B7h on endothelial cells near inflamed tissues had a profound effect on cell polarization and transendothelial migration, a finding that supports the hypothesis that AILIM/ICOS-B7h interactions not only have a role in the regulation of Th1/Th2 differentiation, but also have a critical function in the control of Th1 polarization and migration and in the generation of Th1-effector responses in inflamed tissue.

Further studies that explore the contribution of AILIM/ICOS-mediated migration and polarization during inflammation will provide valuable information for understanding the role of AILIM/ICOS in the regulation of T-cell effector functions and immune responses.

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Abbreviations
AILIM/ICOS activation-inducible lymphocyte immunomediatory molecule/inducible costimulator
HUVEC human umbilical vein endothelial cells
Th1/2 T helper-1/2

References
AILIM/ICOS induces Th1 cell migration/polarization