integrins undergo inside-out conformational activation and ligand-triggered outside-in stabilization (Alon and Feigelson, 2002; Carman and Springer, 2003). Separation of the LFA-1 cytoplasmic domains by talin could serve to stabilize the high affinity conformation (Kim *et al*, 2003). This study indicate that Mst1$^{-/-}$ lymphocytes have a normal initial arrest step but are defective in establishing a subsequent stable attachment. A similar result was also obtained with RAPL$^{-/-}$ lymphocytes (in preparation). These results suggest that Rap1-RAPL-Mst1 signalling is critical for the conversion from transient arrest to stable arrest.

The reduced numbers of lymphocytes in the spleen of Mst1-deficient mice was in contrast to those exhibiting increased splenic lymphocytes in LFA-1-deficient mice (Schmits *et al*, 1996). As lymphocyte homing/retention is mediated by adhesion through both LFA-1 and α4 integrins to ICAM-1 and VCAM-1 (Lo *et al*, 2003), defective adhesion through these integrins and low peripheral blood T cells in Mst1-deficient mice likely result in the hypocellular spleen. Alternatively, integrin-independent mechanism might play a role in homing of lymphocytes to spleen, which is also dependent on Mst1 through the regulation of interstitial migration.

We showed earlier that Mst1 was associated with and activated by Rap1 and RAPL and colocalized with LFA-1 at the leading edge and in the immune synapse (Katagiri *et al*, 2003, 2006). An Mst1 deficiency in lymphocytes resulted in defective integrin clustering by chemokines, which likely impairs the adhesion strength by modulating the avidity of integrins. It should be noted that Rap1, RAPL and Mst1 are mostly present in vesicle compartments containing β2 integrin in primary lymphocytes (Katagiri *et al*, 2006). Intracellular transport of integrin-containing vesicles towards the nascent contact site might be involved in surface clustering and thereby facilitate the transition from a labile to stable attachment. Alternatively, recruiting Mst1 in proximity to LFA-1 through RAPL could facilitate binding of integrins to talin and the actin-cytoskeleton through phosphorylation of integrin cytoplasmic tails or its associated molecules by Mst1. Identification of Mst1 kinase substrates will be useful to further dissect this process.

We showed that stable adhesion through α4β1 and α4β7 was also reduced in Mst1$^{-/-}$ lymphocytes, but the severity was less compared with LFA-1-dependent adhesion (Figures 3D, 4A and B; Supplementary Figure 5), suggesting involvement of the other signalling pathways triggered by chemokines in controlling α4 integrins. We reported earlier that constitutively active PI3 kinase activates LFA-1, but its effect was rather weak, compared with VLA-4 (Katagiri *et al*, 2000), suggesting that LFA-1 might be more tightly regulated by Rap1 signalling than VLA-4. Indeed, in human T cells, inactivation of Rap1 blocked chemokine-stimulated LFA-1-dependent adhesion, but not adhesion through VLA-4 (Ghandour *et al*, 2007). Thus, chemokines may use signalling pathways to VLA-4 distinct from LFA-1.

Coordination of front-back cell polarity and regulation of integrin-dependent attachment at the front and detachment and pulling at the back is the prototype of amoeboid movement in directed cell migration (Lauffenburger and Horwitz, 1996; Sanchez-Madrid and del Pozo, 1999). Using *in vitro* models with the LN-derived FRC cell line, we showed that LFA-1 and VLA-4 were partly involved in stromal-dependent migration of lymphoblasts (this study) as well as active migration of naive B cells (Katakai *et al*, 2008). Adoptive transfer experiments using β2$^{-/-}$ T cells and ICAM-1$^{-/-}$ mice showed a modest reduction in median velocity of T-cell migration in LN (approximately 20 and 34%) (Woolf *et al*, 2007). These results suggest that both integrin-dependent and -independent components are involved in stromal cell-dependent migration. Integrin-independent attachment could be mediated by other adhesion molecules and/or chemokine receptors (Woolf *et al*, 2007). As an Mst1 deficiency affected lymphocyte motility *in vitro* and *in vivo* to levels more than expected from integrin contribution, Mst1 likely contributes to both integrin-dependent and -independent migration in the LN.

The requirement for integrins in lymphocyte interstitial migration within the LN has been recently challenged by a study using DCs lacking integrins. (Lammermann *et al*, 2008). These DCs displayed integrin-independent chemotactic migration in a three-dimensional collagen gel model (Lammermann *et al*, 2008). DCs are relatively sessile in the paracortex with much slower velocities than T cells (average velocity in LN is 4 μm/min versus 11–15 μm/min). The mechanisms regulating DC migration in the LN are likely distinct from T cells. Nonetheless, amoeboid movement of DCs, supported by coordinated regulation of cell protrusion at the front and contraction at the rear, is similar to those observed in lymphocytes, suggesting a common mechanism that governs the development of cell polarity in lymphocytes and DCs (Lammermann *et al*, 2008). If this is the case, impaired front-back polarity could be more detrimental than defective integrin regulation in tissues. This notion is supported by studies showing that a deficiency in the actin-regulator DOCK2 inhibited T-cell migration without affecting integrin function (Nombela-Arrieta *et al*, 2004, 2007). Activated Rap1 is capable of inducing cell polarization with the development of a leading edge and uropod (Shimonaka *et al*, 2003). This Rap1 function, which is independent of cell attachment, requires RAPL and Mst1 (Katagiri *et al*, 2004, 2006). Immobilized LN chemokines potently stimulated lymphocyte polarization and migration of human resting T cells without integrin ligands. (Woolf *et al*, 2007), suggesting the cellular mechanism of integrin-independent migration in lymphoid tissues. We showed that Mst1 deficiency led to decreased motility of T cells on the immobilized CCL21 (Supplementary Figure 9). Defective polarization might explain why an Mst1 deficiency severely reduced lymphocyte motility to greater levels than is expected by blocking integrin function as well as skin DC migration from skin to draining LNs through lymphatics, which was reported to be integrin-independent process (Lammermann *et al*, 2008).

Cdc42 and Rac induce lamellipodia and a leading edge. Rho is important for uropod formation and detachment, the pulling force and integrin activation (Laudanna *et al*, 2002; Smith *et al*, 2003; Morin *et al*, 2008). The Rap1-RAPL-Mst1 pathway might link with actin-regulatory proteins through the regulatory or effector proteins of these small GTPases. Indeed, the effect of Rap1 on cell polarization was inhibited by dominant negative forms of Cdc42 or Rac (Gerard *et al*, 2007). It is still unclear how Rap1 signalling is related to other small GTPases in immune cells.

Although it is still possible that Mst1 is involved in the proliferation and apoptosis of T cells, the functions of murine Mst1, which include the regulation of integrins, cell polarity

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and the motility of lymphocytes, appear to be distinct from the functions of the fly Hippo pathway, which regulates cell contact inhibition and organ size through negative regulation of cell proliferation and apoptosis (Zeng and Hong, 2008). It will be helpful to examine the roles of Mst2 in mice to clarify whether the functions of Hippo are conserved in the immune system. These studies will shed light on the coordinated regulation of lymphocyte trafficking and proliferation/apoptosis through Mst1/2, and further elucidate how dynamic homeostasis of the immune system is maintained through coordination of cell–cell interactions and proliferative responses during antigen responses and tolerance.

Materials and methods

Mice

C57BL/6 mice were obtained from Shimizu Laboratory Supplies and used as wild-type mice. CAG-Cre mice were provided by Dr S Yamada (Akita University, Akita, Japan). All mouse protocols were approved by the Committee on Animal Research of Kansai Medical University (Osaka, Japan). Floxed Mst1 mice and CAG-Cre mice were maintained and bred under specific pathogen-free conditions at Kansai Medical University. Homozygous mice were obtained by interbreeding the heterozygous mice. For all experiments, 7- to 8-week-old littermates were used.

Antibodies and immunofluorescence staining

Monoclonal antibodies to B220 (RA3-6B2), CD3 (2C11), CD28, IgM (eB121-15F9), IgD (11-26.c), L-selectin, αL (M17/4), α4, β7, CD4 (GK1.5), CD8 (53-6.7), CD24 (30-F1), CD23 (2G8), MHC class II (M5/114.15.2) (eBioscience), CD21 (7G6) and CD11c (HL3) (BD Pharmingen) were used for flow cytometry and tissue staining. Anti-laminin (LSL, Rabbit polyclonal), PNAd (MECA-79) (Pharmingen), LYVE1 (Goat polyclonal, R&D systems), VCAM-1 (BAF643, Goat polyclonal) (R&D systems), ICAM-1 (YN1/1) (ATCC) and MAdCAM-1 (MECA-367) (Serotec) were used for tissue staining. Mst1 (Upstate) and Mst2 (Cell Signalling) antibodies were used for immunoblotting. Staining of CCR7 and CXCR4 was described earlier (Katagiri *et al*, 2003; Shimonaka *et al*, 2003). Secondary antibodies conjugated with Alexa 488 and Alexa 546 were obtained from Invitrogen. For flow cytometry, single cell suspensions from spleens, LNs, thymus and bone marrow were incubated with the antibodies indicated in the figures and analysed on a FACSCalibur (Becton Dickinson).

Cryostat sections of frozen tissues (10 μm) were fixed with acetone, air-dried and stained with the indicated antibodies. Chemokine-stimulated lymphocytes were stained with phycoerythrin-labelled anti-LFA-1, FITC-labelled anti-CD44 (Pharmingen) and anti-Talin (Sigma) as described (Katagiri *et al*, 2003). Stained samples were observed with a confocal laser microscope (LSM510 META, Zeiss). Cells with segregated LFA-1 and CD44 accompanied with elongated cell shapes were considered polarized cells.

Gene targeting

Mouse Mst1/Stk4 was isolated from a BAC clone derived from C57/BL6 mice (Invitrogen) using a full-length cDNA probe (Katagiri *et al*, 2006) and used to generate the targeting vector containing exon 1 flanked with a loxP1 site and the floxed neomycin-resistant gene. The loxP1 site and floxed neomycin-resistant gene were inserted into the Ssp1 and EcoRV sites upstream and downstream of exon 1, respectively. The targeting vector was electroporated into C57BL/6 ES cells (Bruce 4) obtained from Dr F Koentgen (Ozgene Pty Ltd, Australia), and targeted ES-cell clones were identified by Southern blot analysis. Isolated ES-cell clones were transiently infected with Cre-expressing adenovirus and subsequently selected for a conditional floxed allele by Southern blotting and PCR. Appropriate ES clones were then injected into blastocysts to generate chimeric mice. The chimeric mice were then bred with C57BL/6 mice to achieve germline transmission. These mice were subsequently crossed with CAG-Cre mice to delete exon 1. Mice were screened for the respective genotype by PCR and Southern blotting and for Mst1 protein expression by immunoblotting.

Immunoblotting

Mouse organs were homogenized with 1% Triton-X100 buffer (1% Triton X-100, 50 mM Tris pH8.0, 100 mM NaCl, 1 μg/ml aprotinin, 1 mM PMSF, 1 μg/ml leupeptin). T and B cells were purified from splenocytes by MACS (Miltenyi Biotec) according to the manufacturer's protocols, and lysed with 1% Triton-X100 buffer. Tissue and cell lysates were subjected to immunoblotting as described earlier (Katagiri *et al*, 2000).

Homing and cell adhesion assays

Lymphocytes were adoptively transferred as described earlier (Katagiri *et al*, 2004). Purified T or B cells from spleens and LNs of control $Mst1^{f/f}$ and $Mst1^{-/-}$ mice were differentially labelled with 1 μM 5,6-carboxyfluorescein diacetate (CFSE, Invitrogen) and 10 μM (5-(and-6)(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) (CMTMR, Invitrogen). An equal number of labelled control and Mst1-deficient cells (5×10^6 each) was injected intravenously into wild-type mice. After 1 h, peripheral LN (inguinal and auxiliary) cells, splenocytes and peripheral blood mononuclear cells were analysed by flow cytometry. Reversal of the fluorescent dyes gave the same results. In some experiments, intravital epifluorescent microscopy of mesenteric LNs was performed as described earlier (Kanemitsu *et al*, 2005) to observe attachment of transferred lymphocytes using an epifluorescence microscope (IX70; Olympus, Tokyo, Japan) equipped with a CCD camera (EM-CCD E9100; Hamamatsu Photonics). Image acquisition was performed using Aquacosmos software (Hamamatsu Photonics).

Chemokine-stimulated lymphocyte adhesion assays were performed as described earlier using a temperature-controlled parallel late flow chamber (FCS2, Bioptecs Inc.) with immobilized recombinant ICAM-1Fc and VCAM-1-Fc (0.5 μg/ml) (Katagiri *et al*, 2004). Purified T and B cells were incubated with 100 nM CCL21 and CXCL12, respectively, for 10 min and then shear stress was applied for 1 min at 2 dyne/cm^2. Splenic DCs enriched by centrifugation over BSA density gradients were subjected to static adhesion assays as described (Pribila *et al*, 2004).

In flow adhesion assays, a monolayer of LS12 cells, an endothelial cell line expressing PNAd (Kimura *et al*, 1999) and murine ICAM-1 by gene transfer was prepared in the parallel plate flow chamber with pretreatment with chemokines (100 nM CCL21) for 10 min before perfusion of purified T (1×10^6 cell/ml) in prewarmed RPMI1640 medium containing 10% FCS at 2 dyne/cm^2 with an automated syringe pump (Harvard Apparatus). Phase-contrast images in a 0.32-mm^2 microscopic field were recorded with an Olympus Plan Fluor DL 10 × /0.3NA objective, CCD camera (C2741, Hamamatsu Photonics) and VHS recorder. The analog videos were digitized with 30-ms intervals, and frame-by-frame displacements and velocities of lymphocyte movements were calculated by automatically tracking individual cell for 2 min using a MetaMorph software (Molecular Devices). In some experiments, lymphocytes were pretreated with 5 μg/ml of anti-L-selectin antibody (MEL14) (Caltag) and anti-LFA-1 antibody (FD441.8) (ATCC) for 30 min for determination of L-selection or LFA-1-dependent interaction, or with 200 ng/ml of pertussis toxin (Calbiochem) to inhibit Gi signalling. A flow adhesion assay using immobilized recombinant MAdCAM-1-Fc (1 μg/ml) was also performed essentially as with LS12 cells.

Thymocyte emigration was measured using thymic lobes as described earlier (Fukui *et al*, 2001). Thymic lobes isolated from $Mst1^{f/f}$ and $Mst1^{-/-}$ mice without disrupting the capsule were incubated in the upper chamber of a transwell (0.5 μm pore size) with CCL19 (100 nM) in the lower chamber. After 3 h, cells in the lower chamber were recovered and counted, and then immunostained with FITC-labelled CD4 and phycoerythrin-labelled CD8. The cell numbers was calculated as the frequencies of the respective population.

Lymphocyte migration on a stromal cell monolayer

The LN-derived stromal cell line BLS12 was seeded on fibronectin-coated (20 μg/ml) ΔT dishes (Bioptechs) and cultured for at least 5 days to construct a monolayer. Total splenocytes were stimulated with 1 μg/ml anti-CD3 (2C11) or 2 μg/ml LPS for 2 or 3 days, respectively. After dead cells were removed with an M-SMF solution, 5×10^5 lymphoblasts were loaded onto the activated BLS12 monolayer. Phase-contrast images were obtained every 30 s for 30 min at 37 °C using a LSM510 confocal laser microscope (Carl Zeiss) equipped with a heated stage for ΔT dishes (Bioptechs).

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Image data were analysed by ImagePro Plus software (Media Cybernatics). In each field, 50 randomly selected cells were manually tracked to measure the median velocity and displacement from the starting point.

Interstitial migration by two-photon microscopy

Purified T or B cells from $Mst1^{f/f}$ and $Mst1^{-/-}$ mice were labelled with 10 μM CMTMR (Molecular Probes) or 1 μM CFSE (Molecular Probes), respectively, for 15 min at 37 °C. Because of defective homing of $Mst1^{-/-}$ cells to LNs, $Mst1^{f/f}$ and $Mst1^{-/-}$ lymphocytes were mixed at the ratio of 1.5 to 1, and cells (5×10^6 cells/ml) were injected i.v. into mice (200 μl/mouse). Twenty-four hours after transfer, the LN was removed without disrupting the capsule and perfused with 95% O_2/5% CO_2 equilibrated RPMI1640 and imaged through the capsule by two-photon microscopy. For two-photon excitation, a Ti:sapphire laser with a 10-W MilleniaXs pump laser (Maitai, XF-1, Spectra-Physics) was tuned to 810 nm. For four-dimensional analysis of interstitial migration, stacks of 27–34 *x*–*y* sections (300 × 300 μm, 256 × 256 pixels) with 3 μm *z*-spacing were acquired every 20 or 30 s for 30 min by Olympus FV1000, using emission wavelengths of 500–540 nm (for CFSE-labelled cells) and 570–640 nm (for CMTMR-labelled cells). Image stack sequences were transformed into volume-rendered four-dimensional movies using Volocity (Improvision), which was also used for semi-automated tracking of cell motility in three dimensions. From the *x*, *y* and *z* coordinates of cell centroids, cellular motility parameters were calculated as described earlier (Mempel *et al*, 2004).

Statistical analysis

A student's two-tailed *t*-test was used to compare experimental groups, and *P*-values <0.05 were considered to be statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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