Mst1 controls lymphocyte trafficking and interstitial motility within lymph nodes

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The regulation of lymphocyte adhesion and migration plays crucial roles in lymphocyte trafficking during immunosurveillance. However, our understanding of the intracellular signalling that regulates these processes is still limited. Here, we show that the Ste20-like kinase Mst1 plays crucial roles in lymphocyte trafficking *in vivo*. Mst1^{-/-} lymphocytes exhibited an impairment of firm adhesion to high endothelial venules, resulting in an inefficient homing capacity. *In vitro* lymphocyte adhesion cascade assays under physiological shear flow revealed that the stopping time of Mst1^{-/-} lymphocytes on endothelium was markedly reduced, whereas their L-selectin-dependent rolling/tethering and transition to LFA-1-mediated arrest were not affected. Mst1^{-/-} lymphocytes were also defective in the stabilization of adhesion through $\alpha 4$ integrins. Consequently, Mst1^{-/-} mice had hypotrophic peripheral lymphoid tissues and reduced marginal zone B cells and dendritic cells in the spleen, and defective emigration of single positive thymocytes. Furthermore, Mst1^{-/-} lymphocytes had impaired motility over lymph node-derived stromal cells and within lymph nodes. Thus, our data indicate that Mst1 is a key enzyme involved in lymphocyte entry and interstitial migration.

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Introduction

Naive lymphocytes continuously circulate between secondary lymphoid tissues and vasculatures in search of foreign antigens (Butcher *et al*, 1999). Lymphocyte trafficking in the peripheral lymph nodes (LNs) is generally divided into four steps: entry through the high endothelial venules (HEV), interstitial migration, antigen scanning and exit through the

efferent lymphatics (von Andrian and Mempel, 2003). Our understanding of the lymphocyte trafficking has been greatly advanced by the identification of adhesion molecules, chemokines, phospholipids and their receptors. During lymphocyte homing to the peripheral LN, naive lymphocytes are first captured by weak binding between L-selectin and a sulphated sialyl Le^x-related carbohydrate, resulting in rolling on the HEV. When rolling lymphocytes are exposed to chemokines on the luminal side of the HEV, chemokine signalling coupled with Gi hetero-trimeric G proteins activates LFA-1, resulting in a complete stop. In gut-associated lymphoid tissues, $\alpha 4 \beta 7$ and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) also support lymphocyte rolling and arrest. Within seconds to minutes, lymphocyte adhesion is stabilized and these cells transmigrate through the HEV into the tissues. Recent observations using multiphoton microscopy have revealed a robust random walk-like motility of naive lymphocytes within the LNs (Sumen *et al*, 2004). Lymphocytes appear to move in close proximity to intricate stromal networks composed of fibroblastic reticular cells in the paracortex and follicular dendritic cells (DCs) in the follicles (Bajenoff *et al*, 2006), suggesting that cell–cell interactions and/or tissue-derived factors enhance cell motility.

Chemokines, in either gradients or nongradients, can activate integrins and induce lymphocyte polarized morphology, generating a leading edge and uropod and stimulating cell motility (Sanchez-Madrid and del Pozo, 1999; Stachowiak *et al*, 2006). Chemokine signalling is coupled with pertussis toxin-sensitive Gi/o hetero-trimeric G proteins, as illustrated by the ability of pertussis toxin treatment to inhibit chemokine-triggered integrin activation and attachment to the HEV (Butcher *et al*, 1999) as well as lymphocyte motility within the LN (Okada and Cyster, 2007). Gene targeting of *Gxi2* impairs B-cell lymphocyte homing and interstitial motility (Han *et al*, 2005). Chemokines activate multiple signalling pathways, including the Ras/Rho family of small GTPases. For example, DOCK2 is a Rac guanine exchange factor that is critical for actin cytoskeletal rearrangements in lymphocytes (Fukui *et al*, 2001), integrin activation and trafficking in B cells (Nombela-Arrieta *et al*, 2004), and directional high-velocity lymphocyte movement within the LN (Nombela-Arrieta *et al*, 2007). Rho GTPase signalling also plays important roles in lymphocyte adhesion and migration (Laudanna *et al*, 2002). Indeed, the actin-nucleating and polymerization protein, mDia1, acts as a downstream Rho GTPase effector and is required for efficient chemokine-stimulated actin polymerization and T-cell trafficking *in vivo* (Sakata *et al*, 2007). Coronin1, an Arp2/3 inhibitory protein, is required for efficient T-cell trafficking *in vivo*, uropod formation and cell survival (Foger *et al*, 2006).

In addition to actin regulators, the Rap1 small GTPase activates integrins and stimulates lymphocyte polarization and motility (Bos *et al*, 2001; Kinashi, 2005). A deficiency in the Rap1-specific CalDAG-GEFI through gene targeting in

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mice (Crittenden *et al*, 2004; Bergmeier *et al*, 2007) or abnormal splicing in human LAD-III patients (Pasvolosky *et al*, 2007), severely impairs the functions of leukocyte and platelet integrins. We reported earlier that RAPL (also known as RASSF5b), a Rap1-GTP binding protein expressed predominantly in lymphoid tissues, was required for lymphocyte adhesion through LFA-1 and $\alpha 4$ integrins and cell polarization triggered by chemokines. RAPL^{-/-} lymphocytes showed defective lymphocyte homing to peripheral LN (Katagiri *et al*, 2003, 2004). We identified mammalian Ste20-like kinase (Mst1, also known as Stk4) as a critical RAPL effector. RAPL associates with Mst1 and regulates the localization and kinase activity of Mst1. Knockdown of Mst1 showed that it is required for polarized morphology and integrin-dependent lymphocyte adhesion (Katagiri *et al*, 2006).

Mst1 was originally identified as a protein kinase homologous to yeast sterile 20 that acts downstream of the pheromone-linked G protein in the mating pathway (Creasy and Chernoff, 1995). The mammalian Ste20 group of kinases regulates diverse biological functions including proliferation, differentiation, apoptosis, morphogenesis and cytoskeletal rearrangement. Mst1 was reported earlier to be involved in apoptosis through caspase-mediated proteolytic activation and histone H2B phosphorylation (Cheung *et al*, 2003), or in a proapoptotic pathway of Ki-Ras through Nore1 (Khokhlatchev *et al*, 2002). Hippo, the *Drosophila* ortholog of mammalian Mst1 and Mst2, has been shown to be involved in cell contact inhibition and the determination of organ size through negative regulation of cell proliferation and apoptosis (Zeng and Hong, 2008). To clarify the physiological roles of Mst1 in primary lymphocytes and trafficking *in vivo*, we generated Mst1-deficient mice. Mst1-deficient mice grew normally with no gross abnormalities. However, peripheral lymphoid organs were hypoplastic. Mst1^{-/-} lymphocyte trafficking to the peripheral LN was defective due to impairment at a transition from transient arrest to stable attachment of lymphocytes on HEV. Furthermore, Mst1^{-/-} lymphocytes exhibited defective motility on LN-derived stromal cells and interstitial migration within LNs. Our study also reveals that Mst1 is required for proper localization of marginal zone B (MZB) cells and splenic CD11c⁺ DC. Thus, these results show the crucial roles of Mst1 in regulating lymphocyte trafficking.

Results

Hypoplastic secondary lymphoid organs in Mst1^{-/-} mice

The Mst1 protein was expressed predominantly in lymphoid tissues, and both T and B cells expressed Mst1. Mst1 was also detected at lower levels in the lung and brain but was below detectable levels in the kidney, liver, heart and skeletal muscle (Figure 1A). To generate Mst1^{-/-} mice, mice carrying floxed Mst1 alleles (Mst1^{f/f}) were produced by gene targeting, in which exon 1 containing the initiation codon was flanked with loxP sites, and then mated with CAG-Cre transgenic mice to delete exon 1 ubiquitously (Supplementary Figure 1). Southern blot analysis confirmed that exon 1 was completely deleted in Cre⁺ Mst1^{f/f} mice (Supplementary Figure 1). Although Mst1 was comparably expressed in wild-type and Mst1^{f/f} mice, the Mst1 protein was not detected in the tissues of Cre⁺ Mst1^{f/f} mice with no apparent generation of a

truncated Mst1 protein (Figure 1A). We hereafter refer to Cre⁺ Mst1^{f/f} mice as Mst1^{-/-} mice. Mst2, which is homologous to Mst1 (78% amino acid identity), was expressed in all tissues examined, and there were no concomitant changes in expression with the Mst1 deficiency (Figure 1A). Mst1^{-/-} mice were born with expected Mendelian frequencies and grew normally without gross abnormalities. Analysis of Mst1^{-/-} tissues revealed hypoplastic lymphoid tissues, whereas there were no apparent abnormalities in other tissues including the lung and brain (data not shown). The number of both T and B cells was decreased in the peripheral LN, Peyer's patches and spleen (Figure 1B, D and F). Immunohistology showed decreases in the sizes and cellular densities of B-cell follicles and T-cell areas of these secondary lymphoid tissues (Figure 1C, E and G). The architecture of lymphoid tissues, including the segregation of T and B cells, distribution of the HEV, and stromal networks appeared normal (Figure 1; Supplementary Figure 2). The proliferative response of Mst1^{-/-} B cells stimulated with BCR ligation was normal, whereas T-cell growth responses were rather augmented compared with control cells when stimulated with TCR ligation (Supplementary Figure 3). There were no significant differences in spontaneous apoptosis of mature T and B cells, although double-positive thymocytes from Mst1^{-/-} mice displayed slightly enhanced cell survival (see below). Therefore, proliferation and apoptosis could not account for the reduced lymphocyte numbers in lymphoid tissues.

Defective lymphocyte trafficking to the peripheral LN

As the cellularity in peripheral lymphoid tissues was reduced, we examined whether an Mst1 deficiency could impair lymphocyte homing to secondary lymphoid organs. T and B lymphocytes were isolated from the LNs and spleens of Mst1^{f/f} and Mst1^{-/-} mice, both of which exhibited naive phenotypes for T cells (CD62L^{hi}CD44^{lo}CD69⁻) and B cells (CD62L⁺IgM⁺IgD^{hi}). Control Mst1^{f/f} and Mst1^{-/-} lymphocytes were differentially labelled and adoptively transferred into normal mice. Trafficking of Mst1^{-/-} T cells to the peripheral LNs and spleen was reduced to one fourth and one third, respectively, of control T cells (Figure 2A). Mst1^{-/-} B-cell trafficking to the LN was also reduced to one fourth compared with that of control B cells (Figure 2A). These data suggest that hypoplastic lymphoid tissues are due to impaired homing capacity of Mst1-deficient lymphocytes.

We examined attachment to the HEV for control and Mst1^{-/-} lymphocytes simultaneously by intravital microscopy. Although accumulation of attached control T cells (green) on the HEV in the mesenteric LN was obvious 20 min after transfer, Mst1^{-/-} T cells (red) poorly attached to the HEV (Figure 2B). We quantified the number of attached cells using images of several microscopic fields taken 30 min after cell transfer. The number of attached Mst1^{-/-} T cells was decreased by approximately 65% compared with control cells (Figure 2B). Although B cells tended to be less efficient than T cells in attaching to the HEV, attachment of Mst1^{-/-} B cells to the HEV was reduced by approximately 75% compared with control B cells (Figure 2B).

Impaired integrin-dependent firm adhesion of Mst1^{-/-} T and B cells

Naive lymphocyte interaction to the HEV in peripheral LN is regulated by adhesive cascades initiated by L-selectin-

Figure 1 Hypoplastic lymphoid organs in Mst1-deficient mice. (A) Expression of Mst1 and Mst2 in organs of wild-type (+/+), Mst1^{flox/flox} (f/f) and Cre⁺ Mst1^{flox/flox} (-/-) mice. Tubulin served as a loading control. (B) Total and CD3⁺ and B220⁺ subset cell numbers in the inguinal lymph nodes. Total and subset numbers of axillary, popliteal, cervical and mesenteric lymph nodes in Mst1-deficient (-/-) mice were decreased to similar extents. *n* = 5 for each, *P < 0.001, **P < 0.005, compared with the corresponding Mst1^{flox/flox} (f/f) fractions. (C) Immunofluorescence staining of frozen tissue sections of axillary lymph nodes for B cells (B220; green), T cells (CD3; red) and laminin (blue). (D) Total and CD3⁺ and B220⁺ subset cell numbers in Peyer's patches. *n* = 5 for each, *P < 0.001, compared with the corresponding Mst1^{flox/flox} (f/f) fractions. (E) Immunofluorescence staining of frozen tissue sections of Peyer's patches for B cells (B220; green), T cells (CD3; red) and laminin (blue). (F) Total and CD3⁺ and B220⁺ subset cell numbers in spleens. *n* = 5 for each, *P < 0.01, **P < 0.005, compared with the corresponding Mst1^{flox/flox} (f/f) fractions. (G) Immunofluorescence staining of frozen tissue sections of the spleen for B cells (B220; green), T cells (CD3; red) and laminin (blue).

mediated tethering and rolling, followed by chemokine-triggered integrin activation and integrin-dependent arrest. Expression of L-selectin, LFA-1, $\alpha 4$ integrin and chemokine receptors CCR7 and CXCR4 was not affected in Mst1^{-/-} lymphocytes (Supplementary Figure 4). To clarify specifically which step in the interaction with the HEV is impaired in Mst1^{-/-} lymphocytes, we established an *in vitro* assay that reconstitute the lymphocyte adhesion cascade using endothelial cells (Kimura *et al*, 1999; Shamri *et al*, 2005) that express peripheral node addressin (PNAd) and ICAM-1, as intravital microscopic experiment was not found to be suitable for dissection of each step of adhesion cascades quantitatively.

T cells were perfused into a parallel plate flow chamber coated with the endothelial monolayer with immobilized CCL21. The interactive processes were video-recorded and digitized with 30-ms intervals for a frame-by-frame cell tracking analysis. Representative profiles of the interactive processes were shown in Figure 3A. A fraction of T cell transiently attached, rolled and stopped under physiological shear stress (2–6 dyne/cm²), whereas low shear stress (<0.5 dyne/cm²) did not support rolling efficiently (data not shown), as reported before (Finger *et al*, 1996). As transition from the rolling to the arrest was best observed at 2 dyne/cm² in this system, the experiments were per-

Figure 2 Defective homing of Mst1-deficient lymphocytes. (A) Adoptive transfer of T cells. T cells from Mst1^{flox/flox} (f/f) and Mst1-deficient (-/-) mice were labelled with CFSE and CMTMR, respectively. They were mixed in equal numbers and injected into the tail veins of wild-type (Wt) mice. After 1 h, lymphocytes from the peripheral lymph nodes, spleen and blood were analysed by flow cytometry. Representative flow cytometry profiles of blood, lymph nodes, and spleen are shown. Numbers beside the boxed areas indicate the ratio of Mst1-deficient cells to Mst1^{flox/flox} (f/f) cells (upper panel). Adoptive transfer of B cells. B cells from Mst1^{flox/flox} (f/f) and Mst1-deficient (-/-) mice were similarly analysed as the T cells (lower panel). (B) Appearance of lymphocyte attachment to the HEV of the mesenteric lymph node. Intravital images of lymphocyte attachment to the HEV were taken 20 min after intravenous transfer of lymphocytes from Mst1^{flox/flox} (f/f) (green) and Mst1-deficient (-/-) (red) mice (top). Representative images of three independent experiments are shown. The number of attached Mst1^{flox/flox} (f/f) or Mst1^{-/-} (-/-) T and B cells to the HEV. The number of attached cells were counted using images of more than five microscopic fields taken 30 min after cell transfer (bottom). Representative data of three independent experiments are shown. *P < 0.01, **P < 0.005, compared with the corresponding Mst1^{flox/flox} (f/f) fractions. A full-colour version of this figure is available at *The EMBO Journal* Online.

formed under this condition. The addition of anti-L-selectin antibody completely abolished adhesive events, as cells travelled at velocities approximately equivalent to theoretical velocities of noninteracting cells (> 500 $\mu\text{m/s}$) (Figure 3A, B and D; Supplementary video 1). L-selectin-dependent interactions in the presence of anti-LFA-1 antibody resulted in a brief stop, which was < 0.5 s (tether), or rolling with variable velocities (average 69.7 $\mu\text{m/s}$) (Figure 3A, B and D; Supplementary video 2). In the presence of immobilized CCL21, 60% of control T cells were attached to the endothelial cells for > 1 s. A few cells detached within 10 s, but 94% of the attached cells stopped for > 10 s, mostly over 2-min

Figure 3 Defective integrin-dependent stable adhesion of Mst1-deficient lymphocytes. (A) Time-displacement profiles of individual T-cell movement over LS12 endothelial monolayers under shear flow. Primary T cells from control mice perfused at 2 dyne/cm² on LS12 monolayers immobilized with CCL21. Representative profiles of the cellular displacements over time were shown in four categories (no interaction, rolling, tether, transient and stable arrest), as described in the text. (B) The noninteracting and rolling velocities of control T cells movements on LS12 in the presence of anti-L-selectin and anti-LFA-1 antibody. (C) Stopping time of Mst1^{flox/flox} (f/f) or Mst1-deficient (-/-) T cells arrested on LS12 endothelial cells were shown. More than 100 cells were measured in three independent experiments, and representative distribution of stopping time is shown. (D) Effects of anti-L-selectin, anti-LFA-1, PTX and Mst1-deficiency on the interactions of T cells with LS12 endothelial cells. Control Mst1^{flox/flox} (f/f) T cells were pretreated with anti-L-selectin, LFA-1 and pertussis toxin (PTX), as described in Materials and methods. Mst1^{flox/flox} (f/f) T cells and Mst1-deficient (-/-) T cells perfused at 2 dyne/cm² on LS12 monolayers, which was immobilized with CCL21. The adhesive events of > 100 cells were measured and categorized as described in (A). Data represent the means and s.e.m. of three independent experiments. *P < 0.001, compared with Mst1^{flox/flox} (f/f) lymphocytes.

observation time (Figure 3C, D; Supplementary video 3). Therefore, we categorized the LFA-1-dependent adhesion into the transient (0.5–10 s) and stable arrest (> 10 s), depending on dwell time on endothelial cells. As expected, PTX

treatment inhibited the arrest, both transient and stable, without affecting tether/rolling (Figure 3D), indicating that LFA-1 is activated by the intracellular signalling mediated through the Gi family. Mst1^{-/-} T cells tethered and rolled normally, indicating that L-selectin-dependent adhesive interaction is not affected. However, the LFA-1-dependent adhesion was found to be unstable with >80% of Mst1^{-/-} T cells were detached within 5 s (Figure 3C, D; Supplementary video 4), indicating that Mst1 plays a critical role in stabilization of the transient arrest.

We also examined under flow adhesion to the $\alpha 4\beta 7$ ligand MAdCAM-1, the major ligand for homing to Peyer's patches. Control T and B cells displayed tethering/rolling, which efficiently resulted in stable arrest in the presence of chemokines (Supplementary Figure 5). Although the frequencies of the transient arrest were rather increased, both Mst1^{-/-} T and B cells had defective stable arrest that was reduced to approximately one third of control cells (Supplementary Figure 5). Taken together, these data indicate that the reduced homing capacity of Mst1^{-/-} lymphocytes is due to the impairment in stabilization of integrin-dependent arrest on HEV.

Mst1^{-/-} lymphocytes are defective in LFA-1 clustering and talin accumulation at the contact sites

Under flow conditions, lymphocytes have to develop integrin-dependent stable adhesion within seconds. We also examined roles of Mst1 in the stabilization of lymphocyte adhesion under static conditions, in which cells might develop stable adhesion by other mechanisms. The lymphocytes were allowed to adhere to immobilized integrin ligands for several minutes before subjected to shear flow. Compared with control lymphocytes, there were few Mst1^{-/-} T and B cells that exhibited shear resistant, firm attachment to ICAM-1 after a 10-min incubation in the presence of CCL21 for T cells and CXCR4 ligand CXCL12 for B cells (Figure 4A). The stable adhesion of both Mst1^{-/-} T and B cells to the VLA-4 ligand VCAM-1 were also severely decreased (Figure 4B), compared with those of control T and B cells. Thus, Mst1 plays a nonredundant role in adhesion stabilization under static as well as flow conditions.

We reported earlier that Mst1 is required for cell polarization and LFA-1 clustering triggered by chemokines but not involved in the regulation of LFA-1 affinity changes measured by binding to soluble ICAM-1 (Katagiri *et al*, 2006). We examined whether an Mst1 deficiency in primary lymphocytes affected LFA-1 clustering and lymphocyte polarization in response to chemokines. T and B cells were treated with CCL21 and CXCL12 for 5 min in suspension and were then fixed and stained for LFA-1 and CD44. Approximately 20–25% of chemokine-stimulated T and B cells from control mice showed polarized morphologies with a leading edge and uropod, to which LFA-1 and CD44 were clustered, respectively (Figure 4C). Although talin tended to be accumulated at the leading edge, it was not precisely colocalized with clustered LFA-1 (data not shown). The majority of Mst1^{-/-} T and B cells remained unpolarized, and the redistribution of LFA-1 was not clearly observed (Figure 4C). The defects in cell polarization and LFA-1 clustering were also observed when incubated on ICAM-1 in the presence of chemokines (Figure 4D). In control cells, LFA-1 clustering was observed at the contact sites on ICAM-1, where talin was colocalized

(Figure 4D), in agreement with the important role of talin in the final common step of integrin activation (Tadokoro *et al*, 2003). In contrast, colocalization of LFA-1 with talin was not observed clearly on the contact site of Mst1^{-/-} cells upon attachment to ICAM-1 (Figure 4D). These data suggest that the chemokine-triggered lymphocytes attach to ICAM-1 through LFA-1 clustering, and the impaired LFA-1 clustering in Mst1^{-/-} cells result in defective talin recruitment to the contact sites, leading to unstable adhesion.

Reduced B-cell subsets and DC in the spleen

Segregation of T cells and follicular B cells in the peripheral LN requires chemokine signalling (von Andrian and Mempel, 2003), but the contribution of integrins to this process is unclear. In contrast, MZB cells were reported to localize in the marginal sinus of the spleen in a manner dependent on ICAM-1 and VCAM-1 (Lu and Cyster, 2002). MZB cells are characterized by high IgM and CD21 expression and low IgD and CD23 expression (Martin and Kearney, 2002). FACS analysis revealed that the B220⁺ CD21^{hi} CD23^{lo} population corresponding to MZB cells was scarcely present in Mst1^{-/-} mice (Figure 5A). In control mice, IgM^{hi} IgD^{lo} MZB cells were clearly detected in the marginal sinus at the border between the white and red pulp of the spleen (Figure 5B). However, there were few cells at the corresponding sites in the spleens of Mst1^{-/-} mice (Figure 5B). There were no irregular structures of the marginal sinus, which normally express ICAM-1, MAdCAM-1 and VCAM-1 (Supplementary Figure 6). These results support the notion that defective adhesion to ICAM-1 and VCAM-1 results in a MZB cell deficiency in Mst1^{-/-} mice.

As Mst1 was expressed abundantly in BM-derived DCs (Supplementary Figure 7), we examined whether an Mst1 deficiency affected the adhesion of DCs. CD11⁺ splenic DCs were enriched from Mst1^{-/-} and control mice and subjected to static adhesion assays using recombinant ICAM-1Fc or fibronectin. DCs from control mice adhered to ICAM-1 and fibronectin, but not substantially to BSA (Supplementary Figure 7). In contrast, Mst1^{-/-} DCs poorly adhered to ICAM-1 and fibronectin. As LFA-1, VLA-4 and VLA-5 expression was comparable between control and Mst1^{-/-} DCs (data not shown), these results indicate that splenic Mst1^{-/-} DCs have defective stable adhesion. In the Mst1^{-/-} spleen, the total number of CD11c⁺ B220⁻ conventional DCs (cDCs) was decreased to approximately 65% of the control spleen (Figure 5C). Among the three cDC subsets in the spleen, which are defined by the surface expression of CD4 and CD8 in addition to CD11c, CD8⁻CD4⁺ and CD8⁻CD4⁻, DCs were substantially decreased (Figure 5C). CD11c⁺B220⁺ plasmacytoid DCs were not affected in Mst1^{-/-} mice (data not shown). CD11c⁺CD8⁻ DCs are usually located at the bridging channel of the marginal zone and red pulp (Metlay *et al*, 1990), as was evident in the spleens of control mice (Figure 5D). In contrast, CD11c⁺ DCs were scarcely found in the bridging channel of the Mst1^{-/-} spleen (Figure 5D). Marginal zone DCs are mobile with a high turnover rate (De Smedt *et al*, 1996); therefore, the deficiency of this DC subset could be due to impaired retention and/or homing caused by defective integrin function in Mst1^{-/-} DCs.

Skin DC migration into draining LN was also found to be defective in Mst1^{-/-} mice. In Mst1-deficient mice, the numbers of Langerhans DCs were equivalent to those of control

mice (Figure 5E). Skin DCs migrate into draining LNs at the peak 24 h after inflammatory stimulation such as painting of the skin with fluorescein isothiocyanate (FITC) (Macatonia *et al*, 1987; Katagiri *et al*, 2004). FITC⁺ major histocompatibility complex (MHC) class II-positive DCs appeared in the draining LNs in control mice at 24 h after painting (Figure 5E). However, in Mst1-deficient mice, this population was reduced to 20% of that of control mice (Figure 5E), indicating that Mst1 is required for efficient trafficking of skin DCs to LNs.

Defective emigration of thymocytes

In the peripheral blood, the number of B cells was comparable between Mst1^{-/-} mice and control mice, but T cells were

reduced by approximately 60% (Figure 6A). On the other hand, thymic cellularity was significantly increased in Mst1^{-/-} mice (Figure 6B). T-cell development was assessed by measuring surface expression of CD4 and CD8. Although CD4⁺CD8⁺ double-positive cells were modestly increased in number, single-positive CD4 and CD8 cells in Mst1^{-/-} mice were increased in number and proportion by approximately two-fold, suggesting that T-cell maturation was not affected in Mst1^{-/-} mice (Figure 6B and C). As impaired thymocyte egress could lead to an increase in mature, single-positive cells, we examined thymocyte egress in transwell assays using thymic lobes (Fukui *et al*, 2001). Both CD4 and CD8 single-positive cells from Mst1^{-/-} mice displayed severe defects in emigration from the thymus in response to the

Figure 4 Defective stable adhesion and LFA-1 clustering in Mst1-deficient cells. (A) CCL21-stimulated T-cell adhesion (left) or CXCL12-stimulated B-cell adhesion (right) to ICAM-1. After incubation with 100 nM CCL21 or CXCL12 for 10 min, shear stress-resistant adhesion was measured as described in Materials and Methods. Data represent the means and s.e.m. of triplicate experiments. None, no stimulation. * $P < 0.001$, compared with Mst1^{flox/flox} (f/f) T cells stimulated with CCL21; ** $P < 0.001$, compared with Mst1^{flox/flox} (f/f) B cells stimulated with CXCL12. (B) CCL21-stimulated T-cell adhesion (left) or CXCL12-stimulated B-cell adhesion (right) to VCAM-1. Shear stress-resistant adhesion was measured as described above. Data represent the mean and s.e.m. of triplicate experiments. None, no stimulation. * $P < 0.002$, compared with Mst1^{flox/flox} (f/f) T cells stimulated with CCL21; ** $P < 0.002$, compared with Mst1^{flox/flox} (f/f) B cells stimulated with CXCL12. (C) Redistribution of LFA-1 (red) and CD44 (green). Mst1^{flox/flox} (f/f) and Mst1-deficient (-/-) T and B cells were stimulated with CCL21 or CXCL12 for 5 min, then fixed and analysed by confocal microscopy quantitatively for cells showing a polarized distribution of LFA-1 and CD44 (top). Representative cell morphology and distribution of LFA-1 and CD44 (bottom). Data represent the means and s.e.m. of triplicate experiments. * $P < 0.001$, compared with Mst1^{flox/flox} (f/f) lymphocytes. (D) Confocal microscopic analysis of LFA-1 and talin distribution of T cells from Mst1^{flox/flox} (f/f) (left panel, upper) and Mst1-deficient (-/-) (left panel, bottom) mice. T cells were incubated on cover glass coated with ICAM-1 in the presence of CCL21 for 5 min, and then fixed and stained for LFA-1 and talin. DAPI was used for nuclear staining. A series of Z-stack images at 1-μm intervals from the glass surface are shown above (left panels). Right panels showed the LFA-1 and talin distribution on contact sites of Mst1^{flox/flox} (f/f) and Mst1-deficient (-/-) T cells on ICAM-1.

Figure 5 Deficient numbers of MZB cells and dendritic cells in the spleen of Mst1^{–/–} mice. (A) Flow cytometry profiles of B220⁺ splenic B cells from Mst1^{flx/flx} (f/f) and Mst1-deficient (–/–) mice stained with anti-CD21 and anti-CD23. The numbers beside the boxed areas indicate the percentages of CD21^{hi}CD23^{low} MZB cells, CD21^{hi}CD23^{hi} mature B cells and CD21[–]CD23[–] immature B cells of the total number of B220⁺ cells. (B) Spleen sections stained with IgM (green), IgD (red) and laminin (blue). IgM^{hi} and IgD[–] marginal zone B cells were not observed in Mst1-deficient mice (bottom). (C) Total splenic DCs and numbers of splenic DCs in the CD8⁺, CD8[–]CD4[–], CD8[–]CD4⁺ subsets. *P < 0.002, compared with corresponding Mst1^{flx/flx} (f/f) fractions. (D) Immunofluorescence staining of frozen tissue sections of Mst1^{flx/flx} and Mst1^{–/–} spleens for B cells (B220; green), DCs (CD11c; red) and laminin (blue). (E) Impaired DC trafficking from skin to draining lymph node. Epidermal sheets from Mst1^{flx/flx} and Mst1-deficient mice stained with anti-MHC class II (upper, left panel). The number of skin-derived DCs migrated to lymph nodes after painting of shaved abdomens of Mst1^{flx/flx} and Mst1-deficient mice with 1% FITC (lower, left panel). Data represent the absolute number of FITC⁺ MHC class II^{high} cells that appeared in draining lymph nodes (axillary and inguinal). *N* = 3; *P < 0.005. Representative flow cytometry profiles are presented in right panel.

CCR7 ligand CCL19 (Figure 6D). We also examined the apoptosis and proliferation of thymocytes. The proliferative response of Mst1^{–/–} thymocytes to anti-CD3 was comparable to that of control thymocytes (data not shown). Although apoptosis of single-positive thymocytes was not affected, Mst1^{–/–} double-positive thymocytes showed reduced apoptosis compared with control cells (45 ± 1.5 versus 34 ± 1.5%, *P* < 0.02), as judged by annexin V staining (Supplementary Figure 8). This reduction might contribute to a mild increase in double-positive thymocytes in Mst1^{–/–} mice (Figure 6B). Thus, the accumulation of single-positive thymocytes is likely due to impaired thymic egress, which could lead to peripheral T-cell lymphopenia.

Defective lymphocyte interstitial migration

As combined defects in integrin clustering and cell polarization could lead to inefficient migration, we investigated whether the lack of Mst1 influenced lymphocyte interstitial migration. To this end, we first examined lymphocyte motility *in vitro*. As LN tissues are composed of an intricate network of stromal cells, which might support lymphocyte migration (Bajenoff *et al*, 2006), we used BLS12, a stromal cell line established from LNs (Katakai *et al*, 2004) (Katakai *et al*, 2008). As primary naive lymphocytes are generally immotile *in vitro*, we used cultured lymphoblasts in migration assays. T-cell blasts from control mice actively migrated on the BLS12

Figure 6 Decreased thymocyte emigration in Mst1-deficient mice. (A) Total and CD3⁺ and B220⁺ subset cell numbers in the peripheral blood of Mst1^{flx/flx} (f/f) and Mst1-deficient (-/-) mice. *P < 0.03, compared with Mst1^{flx/flx} (f/f) T lymphocytes. (B) Total, CD4⁺CD8⁺ double-positive (DP), CD4⁺ or CD8⁺ single-positive cells in thymi from Mst1^{flx/flx} (f/f) and Mst1-deficient (-/-) mice. *P < 0.05, compared with the corresponding fractions. (C) CD4 and CD8 flow cytometry profiles of thymi from Mst1^{flx/flx} (f/f) and Mst1-deficient (-/-) mice. The numbers beside the boxed areas indicate the percentages. (D) Emigration of thymocytes towards CCL19 from thymic lobes. Thymic lobes from Mst1^{flx/flx} (f/f) or Mst1-deficient (-/-) mice were put in the upper chamber of transwell chemotactic chambers. CD4 and CD8 profiles of cells recovered from the lower chamber containing CCL19 were measured after 3 h (left), and the total numbers of emigrated cells (right) are shown. *P < 0.001, compared with Mst1^{flx/flx} (f/f) single-positive cells.

monolayer with an average velocity of $8.1 \pm 3.4 \mu\text{m}/\text{min}$ (Figure 7A; Supplementary video 5). This motility was reduced by approximately 39% after treating with anti-LFA-1 and anti- $\alpha 4$ integrin antibodies (data not shown). Compared with control cells, Mst1^{-/-} T-cell blasts moved inefficiently with an average velocity of $5.2 \pm 2.3 \mu\text{m}/\text{min}$ and a significantly reduced distance compared with control cells (Figure 7A; Supplementary video 6). Similarly, Mst1^{-/-} B-cell blasts had impaired cell migration over the stromal layer with significant reductions in both velocity and displacement (Figure 7A; Supplementary videos 7 and 8). Control T- and B-cell blasts migrating over stromal layers displayed polarized phenotypes with a leading edge and uropod (Supplementary videos 5 and 7). However, most Mst1^{-/-} T- and B-cell blasts failed to develop polarized cell shapes and displayed rather oscillated movements (Supplementary videos 6 and 8).

As chemokine-stimulated migration of lymphocytes includes integrin-independent processes (Lammermann *et al*, 2008), we also examined whether Mst1 could be involved in chemokine-dependent motility without integrin ligands (Woolf *et al*, 2007). Substantial numbers of control naive T cells adhered and actively migrated on the immobilized CCL21 (Supplementary Figure 9). Mst1-deficient T cells attached similarly on the immobilized CCL21, but the motility was poor, compared with those of control cells (Supplementary Figure 9), suggesting that Mst1 is required for both integrin-dependent and -independent lymphocyte motility triggered by chemokines.

We then examined whether the Mst1 deficiency could influence primary naive lymphocyte motility within the LNs. A multiphoton microscopic analysis was performed

using LN explants that had been adoptively transferred with differentially labelled lymphocytes from control and Mst1^{-/-} mice (Figure 7B and C). As reported earlier (Miller *et al*, 2002; Stoll *et al*, 2002; Bousso and Robey, 2003; Mempel *et al*, 2004; Okada and Cyster, 2007), control T cells showed robust random walk-like movements within LN explants with an average velocity of $15.7 \pm 4.2 \mu\text{m}/\text{min}$ (Figure 7B; Supplementary video 9). Under the same conditions, Mst1^{-/-} T cells displayed inefficient migration; the average velocity was reduced by 40% ($9.5 \pm 4.8 \mu\text{m}/\text{min}$), and the distance displaced from the initial tracking point was reduced by 68% compared with those of control T cells (Figure 7B; Supplementary video 9). B-cell migration was measured similarly by adoptive transfer of differentially labelled B cells from control and Mst1^{-/-} mice. Most B cells were found in follicular areas. Compared with T cells, B cells were less motile with an average velocity of approximately $7.3 \pm 2.6 \mu\text{m}/\text{min}$ (Figure 7C; Supplementary video 10), which was consistent with earlier reports (Miller *et al*, 2002; Han *et al*, 2005). Mst1^{-/-} B cells had a reduced mean velocity of $4.5 \pm 2.3 \mu\text{m}/\text{min}$ and decreased displacement (Figure 7C; Supplementary video 10). Taken together, these results indicate that Mst1 is required for efficient interstitial migration of both T and B cells.

Discussion

This study shows that the major role of Mst1 *in vivo* is to control lymphocyte adhesion and migration. T and B cells required Mst1 to attach firmly to the HEV when entering the LN. Mst1 deficiency in lymphocytes impaired their motility over stromal cells as well as within the intact LN. In addition

Figure 7 Defective interstitial migration of Mst1-deficient T and B cells. (A) Cell motility over monolayers of the BLS12 FRC cell line. Representative tracks of Mst1^{flox/flox} (f/f) and Mst1^{-/-} (-/-) T-cell blasts (left) and B-cells blast (right) over BLS12 cells as indicated (top). Each line represents a single cell track. Displacements and velocities of Mst1^{flox/flox} (f/f) and Mst1-deficient (-/-) T and B cells (bottom). Sixty cells of each type were tracked for 10 min for each data set. The velocity data were obtained from movements every 30 s. *P < 0.001, **P < 0.05, compared with Mst1^{flox/flox} (f/f) lymphocytes. (B) Multi-photon microscopic analysis of Mst1^{flox/flox} (f/f) and Mst1-deficient (-/-) lymphocyte migration within LN explants. Representative tracks of Mst1^{flox/flox} (f/f) T cells (red) and Mst1-deficient (-/-) T cells (green) are shown. Each line represents a single T-cell track (left). Velocities and displacements of Mst1^{flox/flox} (f/f) and Mst1-deficient T cells (-/-) (right). Sixty-five cells of each type were tracked for each data set. *P < 0.001, compared with Mst1^{flox/flox} (f/f) T cells. (C) Multi-photon microscopic analysis of Mst1^{flox/flox} (f/f) and Mst1-deficient (-/-) B-cell migration as in (B). Representative tracks of Mst1^{flox/flox} (f/f) B cells (red) and Mst1-deficient (-/-) B cells (green). Each line represents a single B-cell track (left). Velocities and displacements of Mst1^{flox/flox} (f/f) and Mst1-deficient B cells (-/-) (right). Fifty-two cells of each type were tracked for each data set. *P < 0.001, compared with Mst1^{flox/flox} (f/f) B cells. A full-colour version of this figure is available at *The EMBO Journal* Online.

to lymphocyte homing, Mst1 was required for localization of MZB cells and DCs in the marginal zone as well as thymocyte emigration. Thus, Mst1 is a key enzyme that controls proper immune cell localization and motility.

We previously identified Mst1 as a RAPL effector molecule that mediates integrin-dependent adhesion using lymphoid cell lines and lymphocytes (Katagiri *et al*, 2006). Our findings that the phenotype of Mst1-deficient mice was similar to that of RAPL-deficient mice (Katagiri *et al*, 2004) provides genetic evidence that indicates a critical link between Mst1 and RAPL in the regulation of lymphocyte trafficking. Although the phenotypes of these two mutant mice are similar, the phenotype of lymphocyte homing and lymphocytopenia in the

peripheral LN appears to be more pronounced in Mst1^{-/-} mice than RAPL^{-/-} mice. As a RAPL deficiency reduced, but did not abrogate Mst1 phosphorylation (Katagiri *et al*, 2006), this may explain why the phenotype of Mst1-mutant mice is more severe than that of RAPL^{-/-} mice.

Our study indicates that lymphocyte arrest is a transient process that must be sustained by intracellular signalling through Mst1. It is well established that the transition from leukocyte rolling to arrest is controlled by integrin activation through Gi signalling triggered by chemokines (Butcher *et al*, 1999). However, the development of a stable attachment from a nascent, labile attachment has not been recognized as an important process, which occurs within seconds as

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 $\alpha 4$ 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 $\beta 2$ 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 $\alpha 4\beta 1$ and $\alpha 4\beta 7$ 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 $\alpha 4$ 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 $\beta 2^{-/-}$ 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 μ m/min versus 11–15 μ m/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

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, α L (M17/4), α 4, β 7, 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), PNA^d (MECA-79) (Pharmin-gen), 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 μ m) 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 (Pharmin-gen) 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 μ g/ml aprotinin, 1 mM PMSF, 1 μ g/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 μ M 5,6-carboxyfluorescein diacetate (CFSE, Invitrogen) and 10 μ M (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 plate flow chamber (FCS2, Biopetec Inc.) with immobilized recombinant ICAM-1-Fc and VCAM-1-Fc (0.5 μ g/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/cm². 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 PNA^d (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 pre-warmed RPMI1640 medium containing 10% FCS at 2 dyne/cm² with an automated syringe pump (Harvard Apparatus). Phase-contrast images in a 0.32-mm² microscopic field were recorded with an Olympus Plan Fluor DL 10 $\times$ /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 μ g/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 μ g/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 μ m 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 μ g/ml) Δ T dishes (Biopetec) and cultured for at least 5 days to construct a monolayer. Total splenocytes were stimulated with 1 μ g/ml anti-CD3 (2C11) or 2 μ g/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 $^{\circ}$ C using a LSM510 confocal laser microscope (Carl Zeiss) equipped with a heated stage for Δ T dishes (Biopetec).

Image data were analysed by ImagePro Plus software (Media Cybernetics). 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} and Mst1^{-/-} mice were labelled with 10 μ M CMTMR (Molecular Probes) or 1 μ M CFSE (Molecular Probes), respectively, for 15 min at 37 °C. Because of defective homing of Mst1^{-/-} cells to LNs, Mst1^{+/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 μ l/mouse). Twenty-four hours after transfer, the LN was removed without disrupting the capsule and perfused with 95% O₂/5% CO₂ equilibrated RPMI1640 and imaged through the capsule by two-photon microscopy. For two-photon excitation, a Ti:sapphire laser with a 10-W MillenniaXs 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 $\times$ 300 μ m, 256 $\times$ 256 pixels) with 3 μ m *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 Velocity (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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