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WIP and WASP play complementary roles in T cell homing and chemotaxis to SDF-1α

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Abstract

Homing of lymphocytes to tissues is a biologically important multistep process that involves selectin-dependent rolling, integrin-dependent adhesion and chemokine-directed chemotaxis. The actin cytoskeleton plays a central role in lymphocyte adhesion and motility. Wiskott–Aldrich syndrome protein (WASP), the product of the gene mutated in Wiskott–Aldrich syndrome, and its partner, the Wiskott–Aldrich syndrome protein-interacting protein (WIP), play important roles in actin re-organization in T lymphocytes. We used mice with disruption of the WASP and WIP genes to examine the role of WASP and WIP in T cell homing. T cell homing to spleen and lymph nodes *in vivo* was deficient in WASP$^{-/-}$ and WIP$^{-/-}$ mice and severely impaired in WASP$^{-/-}$WIP$^{-/-}$ double knockout (DKO) mice. Deficiency of WASP, WIP or both did not interfere with selectin-dependent rolling or integrin-dependent adhesion of T cells *in vitro*. Chemotaxis to stromal cell-derived factor-1α (SDF-1α) *in vitro* was mildly reduced in T cells from WASP$^{-/-}$ mice. In contrast, it was significantly impaired in T cells from WIP$^{-/-}$ mice and severely reduced in T cells from DKO mice. Cellular F-actin increase following SDF-1α stimulation was normal in WASP$^{-/-}$ and WIP$^{-/-}$ T cells, but severely reduced in T cells from DKO mice. Actin re-organization and polarization in response to SDF-1α was abnormal in T cells from all knockout mice. Early biochemical events following SDF-1α stimulation that are important for chemotaxis and that included phosphorylation of Lck, cofilin, PAK1 and extracellular regulated kinase (Erk) and GTP loading of Rac-1 were examined in T cells from DKO mice and found to be normal. These results suggest that WASP and WIP are not essential for T lymphocyte rolling and adhesion, but play important and partially redundant roles in T cell chemotaxis *in vitro* and homing *in vivo* and function downstream of small GTPases.

Introduction

Cell migration is a fundamental biological multistep process involving membrane polarization and changes in the cytoskeleton (1, 2). Lymphocyte recirculation into lymphoid and non-lymphoid tissues begins with blood lymphocytes interacting transiently and reversibly with vascular endothelium selectins in a process called rolling (3). Rolling brings lymphocytes into contact with the endothelium where they are activated by tissue-derived chemokines displayed on the surface of endothelial cells. Chemokine activation up-regulates the affinity of lymphocyte integrins to their ligands on endothelial cells, resulting in firm adhesion of the lymphocyte to the vessel wall and subsequent migration across the endothelium where tissue-associated chemokine gradients may also direct localization (4, 5).

Chemokines are a class of molecules that binds to receptors coupled to heterotrimeric G proteins to induce the movement of immune cells toward a concentration gradient of the cognate ligand (6). Stromal cell-derived factor-1α (SDF-1α) is thought to be the primordial chemokine (7). It is highly conserved in mammals (with a homology of >95% between

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human and mouse), and is the only chemokine that is requisite for survival. Disruption of either SDF-1α or its receptor CXCR4 is lethal in mice and is accompanied by several defects (8, 9). CXCR4 is expressed on all hematopoietic cells (10) and has also been identified as a principal co-receptor, in addition to CD4, for the entry of the T cells-tropic HIV into its target cells (11, 12).

The actin cytoskeleton plays a central role in cell motility, morphology, phagocytosis and cytokinesis (13). It is spatially and dynamically organized, providing force for the shape change and surface movement in most eukaryotic cells (14, 15). Rearrangement of actin is evoked rapidly by extracellular stimuli, including chemokines (16). Wiskott–Aldrich syndrome protein (WASP), the product of the gene mutated in Wiskott–Aldrich syndrome (WAS), plays an important role in actin polymerization in hematopoietic cells (17, 18). Lymphocytes from patients with WAS have abnormal cytoskeletal architecture (19). T cells from these patients and from WASP-deficient mice have defective actin polymerization and impaired proliferation in response to engagement of their TCR (20–22).

WASP has an N-terminal Ena/VASP homology domain 1 (EVH1) domain, a Cdc42/Rac GTPase-binding domain (GBD), a proline-rich domain, a G-actin-binding verprolin homology (V) domain, a cofilin homology (C) domain and a C-terminal acidic (A) segment. WASP interacts with the Wiskott–Aldrich syndrome protein-interacting protein (WIP) via its EVH1 domain (23), with Cdc42–GTP via its GBD domain, with multiple SH3 domain containing proteins via its proline-rich region (24) and with actin and the Arp2/3 complex via its VCA domain. WASP exists in cells in a closed inactive conformation due to intramolecular interactions that prevent the C-terminal acidic domain from interacting with the Arp2/3 complex. Binding of Cdc42–GTP or of SH3 domain of proteins such as Nck, Grb2 and cortactin is thought to cause a conformational change in WASP, which allows the VCA domain to interact with and activate the Arp2/3 complex (25–27) to regulate actin polymerization.

WIP is expressed at high levels in lymphoid tissues (23). WIP binds actin via its VH domain located at the N-terminal end of the molecule (a.a. 1–151) and binds WASP via a sequence in its C-terminal end (a.a. 460–485). In lymphocytes, >95% of WASP is complexed with WIP (28). WIP plays an important role in the recruitment of the WIP–WASP complex to ZAP-70 following TCR ligation. TCR ligation causes protein kinase Cθ-dependent WIP phosphorylation and disengagement of WASP from the WIP–WASP complex, allowing WASP activation by the small GTPase Cdc42 (28). A role for WIP in T cell activation was demonstrated by the study of WIP knockout (KO) mice. T cells from these mice fail to polymerize F-actin, proliferate, polarize and extend protrusions following TCR ligation. Furthermore, the actin cytoskeleton is disrupted in $WIP^{-/-}$ T cells (29).

Monocytes from WAS patients have a reduced chemotactic response to MCP-1, MIP-1α and FMLP (30, 31) and their T lymphocytes display an abnormal chemotaxis in response to SDF-1α (32). These data suggest that WASP is important for chemotaxis. Given the role of WASP and WIP in actin reorganization in T cells, we took advantage of the availability of mice deficient in WASP, WIP or both to examine the role of WASP and WIP in the homing of T cells to peripheral lymphoid organs and in T lymphocyte rolling, adhesion and chemotaxis.

Methods

Mice

The generation of WASP- and WIP-deficient mice has been described (21, 29). $WASP^{-/0}WIP^{+/-}$ males were bred with $WASP^{-/-}WIP^{+/-}$ females to generate $WASP^{-/-}WIP^{-/-}$ double knockout (DKO) mice. All mice were from mixed 129S × C57Bl6 background. Wild-type (WT) littermates were used as controls.

Antibodies and FACS analysis

Fluorochrome-labeled mAbs [anti-CXCR4–PE, anti-VLA-4–PE (CD49d), anti-VLA-5–PE (CD49e) and anti-CD62L–PE] were obtained from BD Biosciences (San Diego, CA, USA) and used to stain cells which were analyzed by FACS. Expression of E-selectin ligands was determined by flow cytometry using recombinant mouse E-selectin Fc chimera (R&D Systems, Inc., Minneapolis, MN, USA). Cells were incubated sequentially (30 min at 4°C) with Fc-block (CD16/CD32, BD Biosciences), E-selectin chimera (5 μg ml^{-1}), goat $F(ab')_2$ anti-human IgG–biotin (3 μg ml^{-1}; Caltag, Burlingame, CA, USA), streptavidin–PE (2.5 μg ml^{-1}, BD Biosciences) and conjugated antibodies, anti-CD3–FITC or hamster IgG–FITC (BD Biosciences). Analysis was performed on a Becton Dickinson FACScan IV using CellQuest software. Aliquots of cells were processed in HBSS containing 10 mM HEPES and 5% FCS plus either 2 mM $CaCl_2$ or 5 mM EDTA, to confirm calcium-dependent binding. As an additional negative control, aliquots of cells were stained without E-selectin chimera (secondary antibody, streptavidin–PE and conjugated antibodies only) in 2 mM $CaCl_2$.

In vivo homing of T cells

T cells were purified (usually >90% $CD3^+$ cells) from spleen by negative selection using the T Cell Enrichment Colums kit (R&D Systems). Purified T cells from WT mice were labeled for 15 min with 10 μg ml^{-1} of Alexa-Fluor 488 (Molecular Probes) or for 10 min with 0.8 μg ml^{-1} of tetramethylrhodamine-5(and 6)-isothiocyanate (TRITC, Molecuar Probes), at 37°C. T cells from $WASP^{-/-}$, $WIP^{-/-}$ and DKO were labeled with TRITC. Cells were then centrifuged, washed and re-suspended in RPMI. A total of 8×10^6 TRITC-labeled WT, $WASP^{-/-}$, $WIP^{-/-}$ and DKO T cells were mixed with an equal number of Alexa-fluor-labeled WT T cells and injected intravenously into recipient mice. After 1 h, recipient mice were killed and spleen, inguinal lymph nodes (LNs) and mesenteric LNs were harvested. Single-cell suspensions were analyzed by flow cytometry, gating on 3×10^5 live lymphocytes and the percentages of $TRITC^+$ and $Alexa\text{-}fluor^+$ cells were determined. T cell homing index was calculated as the ratio of TRITC-labeled to Alexa-labeled cells.

Laminar flow assay for T cell attachment to selectin

Selectin ligand function was determined in a parallel-plate flow chamber using substrates prepared with E-selectin or P-selectin IgG chimeras as described (33). T cells were washed in HBSS containing 10 mM HEPES (H/H) and aliquots were suspended at $1–2 \times 10^6$ ml^{-1} in H/H containing 2 mM $CaCl_2$ for individual assay runs. Cells were loaded into the flow

chamber and allowed to settle for 2 min. Flow was then initiated at 0.28 dyne cm^{-2} for 1 min followed by stepwise increases in wall shear stress every 15 s up to 1.40 dyne cm^{-2}. Cells expressing functional selectin ligand remain attached to the substrate and roll in response to the application of shear. Calcium-dependent binding was confirmed at the end of each run by perfusing the chamber with EDTA (5 mM in H/H) at 1.40 dyne cm^{-2}. Experiments were observed in real time and videotaped for subsequent analysis. Percentage of cells bound was defined as (cells remaining bound in shear flow minus cells remaining in EDTA divided by the total number of cells settled onto the plate surface at the onset of shear) × 100. New cells attaching or rolling into the field of view during the application of shear were not included in the analysis.

Adhesion to fibronectin

A total of 5×10^5 purified spleen T cells from WT or $WASP^{-/-}$, $WIP^{-/-}$ and DKO mice and suspended in 2.5% BSA in PBS were added to 96-well Nunc Maxisorp Immuno Module flat-bottom plates (Costar, Cambridge, MA, USA) coated with 10 μg ml^{-1} fibronectin (FN) (R&D systems) and allowed to settle with treatment [medium alone or SDF-1α (100 ng ml^{-1})] for 1 h at 4°C. Samples were incubated at 37°C for 15 min followed by washing with 2.5% BSA in PBS. Adherent cells were released with 10 mM EDTA in calcium- and magnesium-free PBS and were counted by Trypan blue exclusion.

Chemotaxis assay

In vitro chemotaxis was assayed using transwell chambers (diameter, 6.5 mm; pore 5 μm) obtained from Costar. A total of 5×10^5 purified splenic T cells in 125 μl RPMI 1640 with 1% FCS were added to the upper chamber and 500 μl RPMI 1640 with 1% FCS medium with or without SDF-1α (PrepoTech, London, UK) was added to the bottom chamber. After 3 h at 37°C, cells that migrated to the lower chamber were collected and counted. The experiments were performed in duplicate and were repeated a minimum of three times.

Actin polymerization

Purified splenic T cells were stimulated with SDF-1α (500 ng ml^{-1}) for the indicated times and the reaction was stopped by adding 10% formalin (Sigma). The cells were fixed for 15 min on ice, washed with PBS and stained with 5 μg ml^{-1} TRITC–phalloidin in 0.1% TritonX-100/PBS. Cells were then washed and examined by FACS. Values were expressed as the ratio of mean fluorescence intensity in stimulated versus unstimulated cells (fold increase over baseline).

Intracellular immunofluorescence

T cells purified from spleen were suspended in RPMI 1640 medium containing 1% FCS and incubated for 30 min at 37°C. The cells were plated on cover slips and allowed to attach for 30 min at 37°C. Attached cells were stimulated with 10 nM SDF-1α, fixed with 10% formalin and then permeabilized with 0.2% TritonX-100 in PBS for 3 min. After washing with PBS, the cells were blocked with 2.5% BSA and then incubated with 1 μg ml^{-1} TRITC–phalloidin for 20 min at room temperature and examined using a fluorescence microscope. A minimum of 200 cells were counted in each sample.

Protein tyrosine phosphorylation after SDF-1α stimulation of T cells

Purified T cells were left unstimulated or were stimulated with 10 nM SDF-1α. Lysates were prepared in 1% NP-40 lysis buffer supplemented with protease and phosphatase inhibitors and resolved by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and probed with the indicated antibodies. Phosphorylated PAK1, cofilin and Erk were detected using rabbit phospho-specific antibodies to PAK1 (Biosource), cofilin and Erk (both from Cell Signaling), followed by reprobing the membranes with antibody to PAK1 (Cell Signaling), cofilin (Cell Signaling) or Erk (Santa Cruz Biotechnology, Inc.) as loading controls. Lck immunoprecitates using mAb to Lck (BD Transduction Laboratories) were probed with anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology) and reprobed with anti-Lck antibody as loading control. Rac-1 GTP was pulled down from lysates using a fusion protein of glutathione-S-transferase and the PVB domain of PAK1 (Pierce Laboratories), following the manufacturers' instructions. An equivalent aliquot of lysates was probed with anti-Rac-1 antibody (BD Transduction Laboratories) as loading control.

Statistical analysis

Student's *t*-test was used to compare the differences between groups. A *P*-value <0.05 was considered statistically significant. Time curves of F-actin content in KO mice and WT controls were analyzed by analysis of variance (ANOVA) using the GraphPad PRISM software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Defective homing of $WASP^{-/-}$, $WIP^{-/-}$ and DKO T cells to lymphoid organs

To examine the role of WASP and WIP in T cell homing to peripheral lymphoid organs, equivalent numbers of TRITC-labeled T cells from KO mice and Alexa-488-labeled T cells from WT control mice were injected intravenously into WT recipients. As a control, equivalent numbers of TRITC-labeled and Alexa-labeled WT T cells were injected into WT recipient mice. One hour later, spleens, mesenteric LNs and inguinal LNs were removed and analyzed by FACS. T cell homing index was calculated as the ratio of TRITC-labeled to Alexa-labeled cells. As expected, the homing index for WT T cells was close to 1 in all three lymphoid organs tested (Fig. 1). In contrast, the homing index of $WASP^{-/-}$, $WIP^{-/-}$ and DKO T cells was significantly decreased in all three organs. These results suggest that WIP and WASP play an important role in homing of T cells to lymphoid organs.

Tethering to selectin-coated surfaces is normal in $WASP^{-/-}$, $WIP^{-/-}$ and DKO T cells

Interaction between selectin ligands on circulating cells and endothelial selectins mediates T cell tethering and rolling over endothelial surfaces (34–36). Figure 2(A and B) shows that expression of L-selectin (CD62L) and E-selectin ligand is

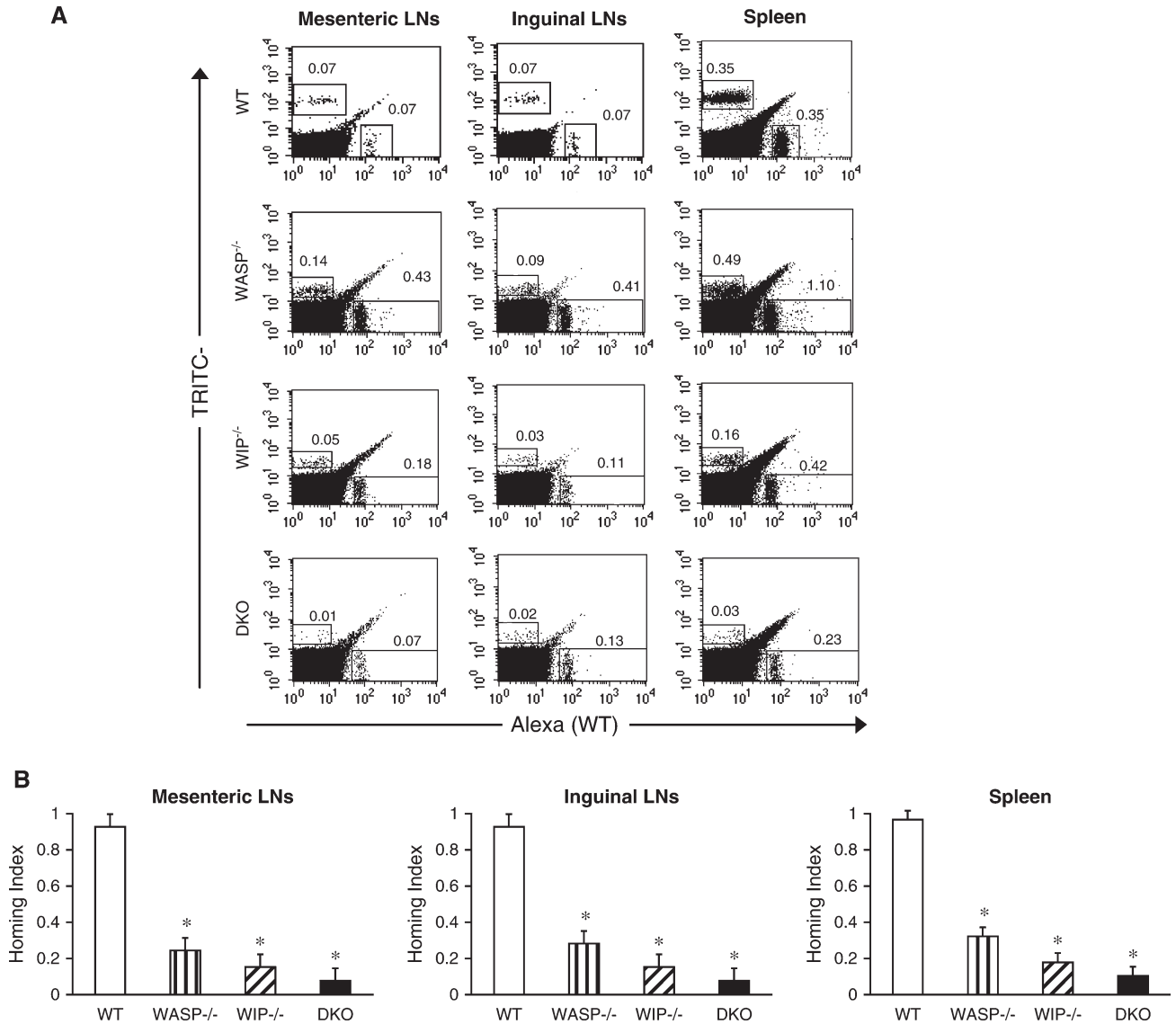

Fig. 1. Homing of T cells to peripheral lymphoid tissues. A mixture of equal numbers of Alexa-488-labeled WT cells and TRITC-labeled WT or KO (WASP$^{-/-}$, WIP$^{-/-}$ and DKO) purified splenic T lymphocytes was injected into genetically matched WT recipients. (A) FACS analysis of recipient spleen, mesenteric LNs and inguinal LNs. (B) Homing index derived from data of three independent experiments. The index represents the ratio of TRITC-labeled T cells (from WT and KO mice) to Alexa-488-labeled WT cells. Columns and error bars represent mean ± SD. *$P < 0.05$ compared with WT cells.

normal on WASP$^{-/-}$, WIP$^{-/-}$ and DKO T cells. In the case of E-selectin ligand, only a fraction of the T cells stained with E-selectin Fc and staining was slightly lower in all three KO strains compared with WT. Figure 2(C) shows that WASP$^{-/-}$, WIP$^{-/-}$ and DKO T cells tethered normally over E-selectin-coated and P-selectin-coated surfaces. These results suggest that WIP and WASP are not essential for selectin-mediated tethering of T cells on endothelial cells.

Spontaneous and SDF-1-α-induced adhesion to FN are normal in WASP$^{-/-}$, WIP$^{-/-}$ and DKO T cells

T cells express the β1 integrins VLA-4 (α4 β1) and VLA-5 (α5 β1) which mediate adhesion to FN (37, 38). VLA-4 and VLA-5 expressions were normal in WASP$^{-/-}$, WIP$^{-/-}$ and DKO T cells (Fig. 3A). Figure 3(B) shows that the adhesion of unstimulated T cells to FN is normal in T cells from all three KO mice strains. The chemokine SDF-1α up-regulates the affinity of β1 integrins to their ligand (39–41). SDF-1α up-regulated normally the adherence of T cells to FN in all three KOs mice strains (Fig. 3B). These results suggest that WIP and WASP are not essential for baseline or chemokine-stimulated T cell adhesion mediated by β1 integrins.

In vitro chemotaxis to SDF-1α in vitro is defective in WASP$^{-/-}$, WIP$^{-/-}$ and DKO T cells

The chemokine SDF-1α has been implicated in the migration of T lymphocytes across LN high endothelial cells (42) and to lymphoid organs *in vivo* (43). To determine whether WIP and WASP play a role in this response, we examined the chemotactic response of T cells from WASP$^{-/-}$, WIP$^{-/-}$ and

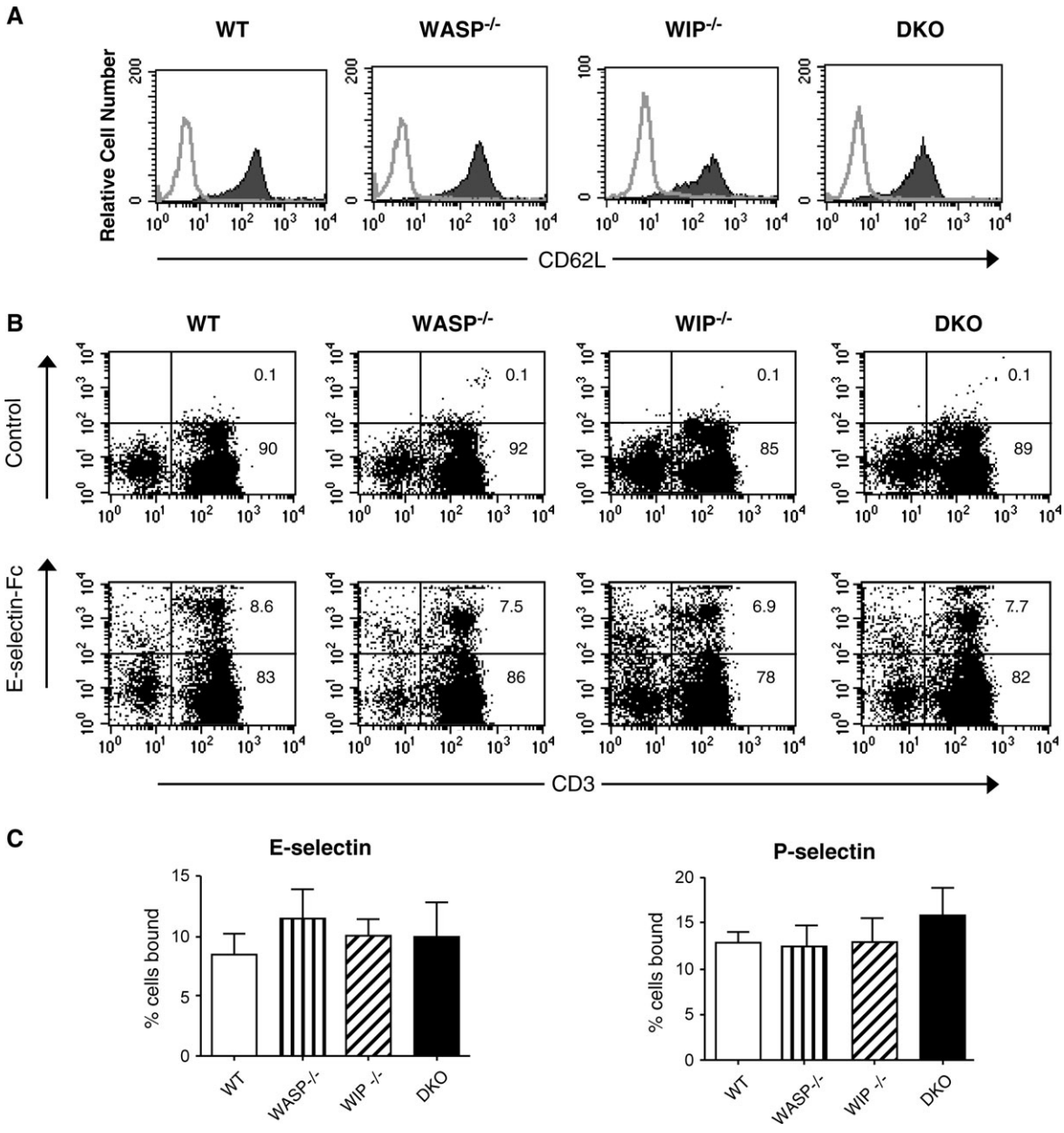

Fig. 2. Selectin ligand expression and function in T cells from WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice (A and B). FACS analysis of L-selectin (CD62L) and E-selectin ligand expression on purified splenic T cells. Splenocytes were double stained with anti-CD3–FITC and anti-CD62L–PE (A) or with biotinylated E-selectin : IgG chimeric protein (E-selectin : Fc) followed by streptavidin–PE. As control, incubation with the chimeric protein was omitted (B). Results are representative of two independent experiments. (C) Attachment of T cells to E-selectin- and P-selectin-coated flow chambers. Results represent the mean ± SD of three experiments.

DKO mice to SDF-1α. FACS analysis using mAb to the SDF-1α receptor CXCR4 revealed that T cells from all three (KO) strains expressed CXCR4 at a level similar to that of T cells from WT controls (Fig. 4A). Figure 4(B) shows that the chemotactic response of WASP$^{-/-}$ T cells to SDF-1α was slightly, but not significantly, decreased. The chemotactic response of WIP$^{-/-}$-deficient T cells was significantly reduced to ~50% of control. The chemotactic response of DKO T cells was dramatically reduced to ~10% of the response of control. These results suggest that WIP and WASP play partially redundant roles in *in vitro* T cell chemotaxis to SDF-1α.

Actin polymerization in response to SDF-1α is defective in T cells double deficient in WASP and WIP

Actin polymerization is required for chemotaxis (13, 18). One of the first events involved in the migration of T cells in response to chemokines is an increase in cellular F-actin content. Purified splenic T cells from WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice were stimulated with SDF-1α, then stained with TRITC–phalloidin and analyzed by FACS for F-actin content. As expected (16), SDF-1α stimulation of WT T cells resulted in an increase in F-actin content within 15–30 min of stimulation. The level of F-actin then progressively decreased and returned to baseline

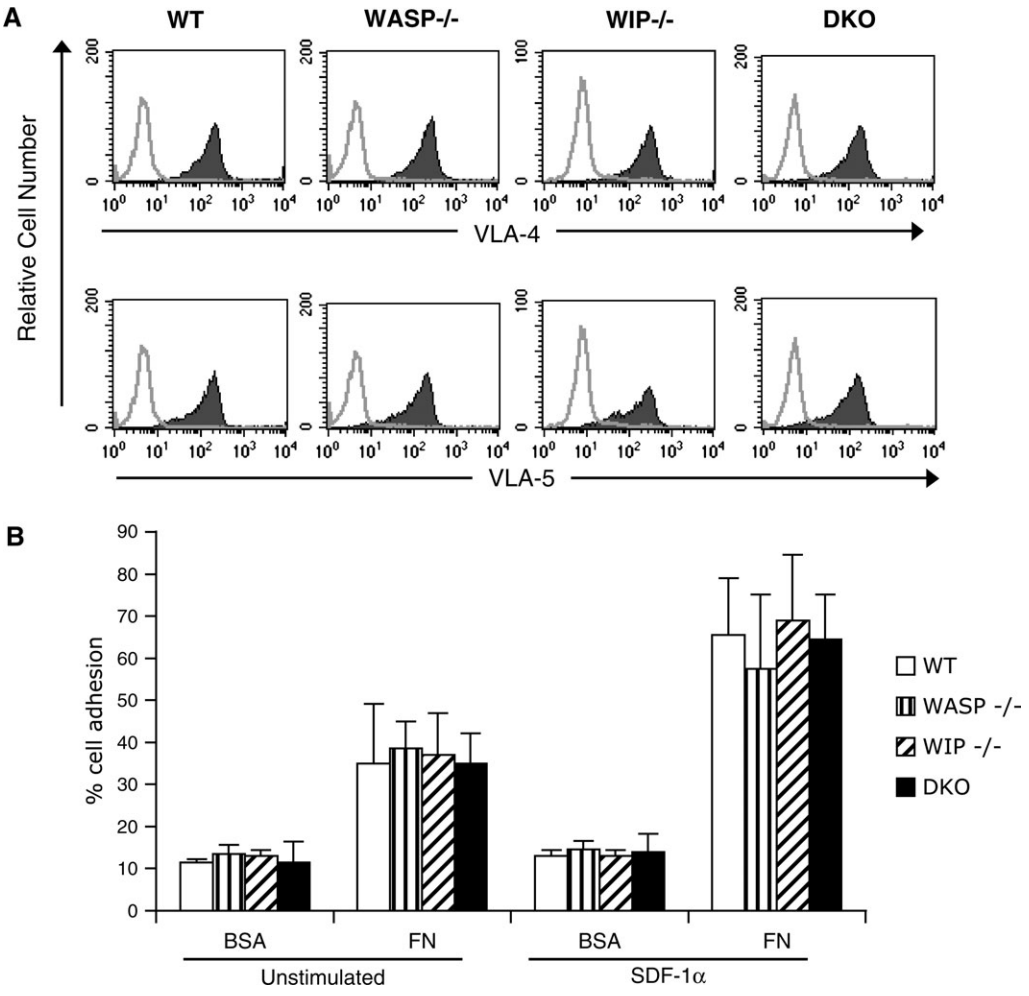

Fig. 3. Expression of β1 integrins and adhesion to FN of T cells from WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice. (A) FACS analysis of VLA-4 and VLA-5 expression. Purified splenic T cells were stained with anti-CD3–FITC and anti-CD49d–PE or anti-CD49e–PE. (B) Adhesion of unstimulated and SDF-1α-stimulated purified splenic T cells to BSA- versus FN-coated plates. Results represent the mean ± SD of two experiments.

60 min after stimulation (Fig. 5). SDF-1α induced a normal F-actin increase in WASP$^{-/-}$ and WIP$^{-/-}$ T cells. In contrast, T cells from DKO mice were severely impaired in their ability to increase their content of F-actin following SDF-1α stimulation. ANOVA revealed a significant difference between the time curves of F-actin content of DKO and controls. These results suggest that WIP and WASP play redundant roles in actin polymerization triggered by SDF-1α.

Re-organization of the actin cytoskeleton in T cells in response to SDF-1α

SDF-1α induces actin re-organization and polarization in cells (44). To investigate actin cytoskeleton rearrangement, purified T cells were stimulated with SDF-1α for 0, 1 and 5 min, then were fixed, stained with TRITC–phalloidin and examined by fluorescence microscopy. Unstimulated cells from WT, WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice exhibited a ring of peripheral actin. Small and comparable fractions of these cells (~10%) exhibited polarized actin (Fig. 6A and B). After 1 min of stimulation with SDF-1α, T cells from WT mice started to lose their round shape, and ~50% of them polarized actin to one pole. At 5 min, there was a further increase in the fraction of polarized cells and a distinct uropod was formed in the majority of WT cells (Fig. 6A and B). Significantly more cells from WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice retained their rounded shape and significantly less of them accumulated F-actin at one pole and formed uropods 1 min after SDF-1α stimulation (Fig. 6A and B). This trend persisted 5 min after stimulation. A fraction (>15%) of T cells from all three KO mice strains, exhibited abnormal polarization with multiple patches (two or more) of F-actin after SDF-1α stimulation. Only a negligible fraction of WT T cells (<3%) exhibited more than one pole of F-actin at 5 min post-stimulation. These results suggest that WIP and WASP are important for normal actin cytoskeleton re-organization after SDF-1α stimulation.

WASP and WIP are not essential for early signaling events following CXCR4 ligation in T cells

SDF-1α ligation of its G-protein-coupled receptor CXCR4 results in the activation of multiple signaling pathways. They include the phosphorylation and activation of PAK1. PAK1 is upstream of two pathways that are involved in actin

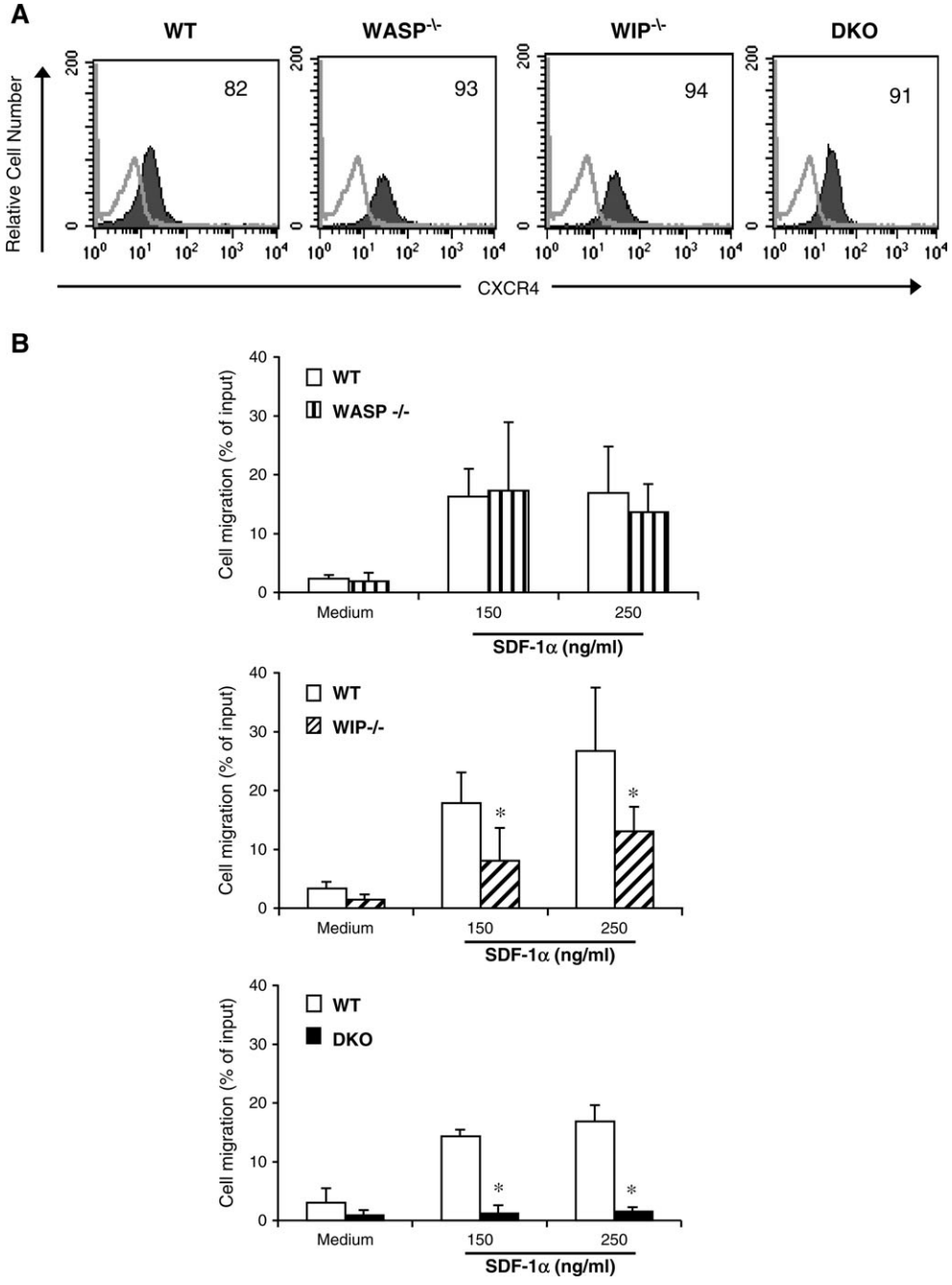

Fig. 4. CXCR4 expression and chemotaxis to SDF-1α of T cells from WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice. (A) Purified splenic T cells were double stained with anti-CD3–FITC and anti-CXCR4–PE. CXCR4 expression on gated CD3^{+} cell is shown. Results shown are representative of two independent experiments. (B) Percent of splenic T lymphocytes from WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice and WT controls that migrated through the filter to the SDF-1α-containing lower compartment of a transwell chamber. Results represent the mean ± SD of four experiments. Columns and error bars represent mean ± SD. *$P < 0.05$.

re-organization and chemotaxis following SDF-1α stimulation: activation of the small GTPases Rac-1 and Cdc42 with subsequent WASP-mediated, Arp2/3 complex-dependent actin polymerization (32, 45) and activation of LIM kinase-mediated cofilin phosphorylation and inactivation (46, 47). CXCR4 ligation also causes phosphorylation and activation of phospholipase Cβ, which initiates a biochemical cascade that results in the phosphorylation and activation of the mitogen-activated protein kinase Erk, which may play a role in chemotaxis to SDF-1α (48, 49). Finally, SDF-1α causes ZAP-70 phosphorylation and activation of src kinases, including Lck, which also have been implicated in chemotaxis (50, 51). Figure 7 shows that phosphorylation of PAK1, cofilin, Erk and Lck after SDF-1α ligation was normal in T cells from DKO mice. Figure 7 also shows that GTP loading of Rac-1 after SDF-1α stimulation was normal in DKO T cells. These data suggest

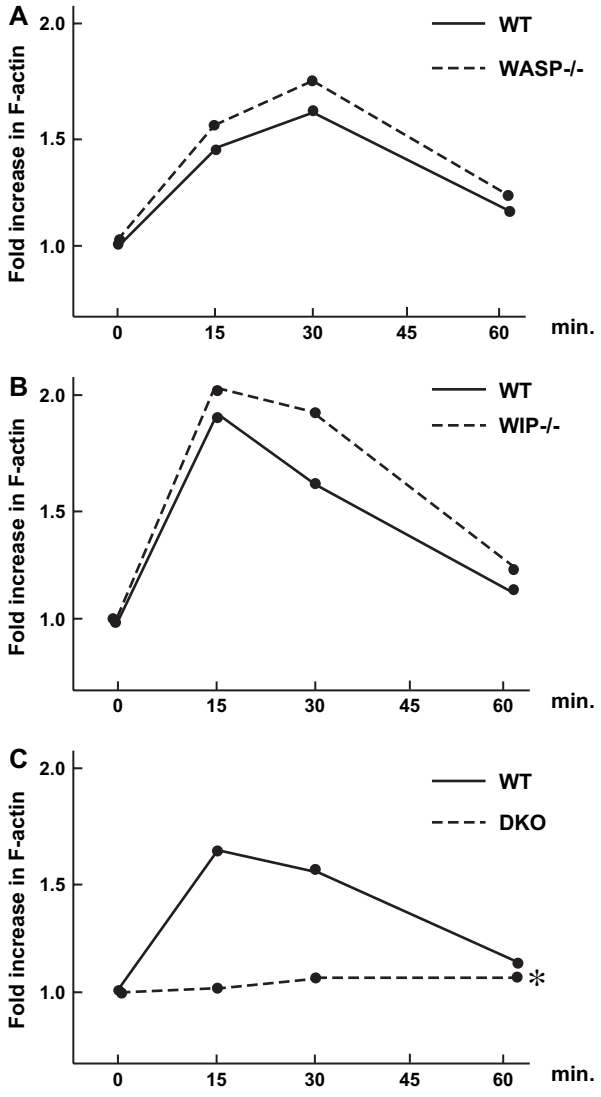

Fig. 5. Changes in F-actin content in T cells stimulated with SDF-1α. Purified splenic T lymphocytes from (A) WASP$^{-/-}$, (B) WIP$^{-/-}$ and (C) DKO mice and littermate WT controls were stimulated with SDF-1α for indicated times. Cells were then fixed, permeabilized, stained with phalloidin–TRITC and analyzed by FACS. Results are expressed as fold increase in mean fluorescence intensity over baseline (0 time). The results shown are representative of four independent experiments. *$P < 0.05$ (by ANOVA).

that WASP and WIP function downstream of small GTPases in SDF-1α chemotaxis.

Discussion

The results of this study show that both WASP and its partner WIP play important and partially redundant roles in T cell homing and in chemotaxis of T cells to SDF-1α.

T cells from WASP$^{-/-}$ and WIP$^{-/-}$ mice exhibited defective homing *in vivo* to spleen and LNs of comparable extent. T cell homing was reduced to 44% of normal in WASP$^{-/-}$ T cells and to 38% of normal in WIP$^{-/-}$ T cells. The homing defect was more severe in T cells from WASP$^{-/-}$/WIP$^{-/-}$ DKO mice, being reduced to 13% of normal. The more severe reduction in homing of DKO T cells compared with single KO cells suggests that WIP and WASP play complementary roles in the homing of T cells to peripheral lymphoid organs. The T cell homing defect in WASP$^{-/-}$ mice observed is consistent with the observation that T cell areas are reduced in spleens and LNs from WAS patients (52). The observation that the total number of CD3^{+} T cells in spleen and peripheral LNs is normal in WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice (21, 29; data not shown) suggests the presence of compensatory mechanisms *in vivo*. In fact, the size and weight of the spleen are significantly increased in WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice (M.-D.G., unpublished results).

WASP is expressed predominantly in hematopoietic cells while WIP is expressed in all tissues (23). It is therefore possible that hematopoietic non-T cells, e.g. dendritic cells which are known to secrete T cell chemoattractants (53) may contribute to the T cell homing defect in WASP$^{-/-}$ and that both hematopoietic non-T cells and non-hematopoietic cells, e.g. endothelial cells and stromal cells, contribute to the T cell homing defect in WIP$^{-/-}$ and DKO mice. However, we have observed that WT T cells home normally to the peripheral lymphoid organs of WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice (our unpublished results). This suggests that non-T cells do not substantially contribute to defective T cell homing in these mice.

In vitro studies revealed that two of the processes involved in T cell homing to peripheral tissues, namely selectin ligand-mediated attachment and β1 integrin-mediated adhesion, were normal in T cells from WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice. The percentages of T cells from all three strains that expressed L-selectin and E-selectin ligands on their surface were comparable to those observed in WT T cells (Fig. 2A and B). In the case of E-selectin, there was a slight reduction in the intensity of expression in all three KO strains. T cells from all three KO strains tethered normally to E-selectin- and P-selectin-coated surfaces (Fig. 2C), although tethering was tested at only a single concentration of ligands coating the plate. The apparently normal tethering to P-selectin suggests that, although it could not be measured directly due to lack of suitable reagent, P-selectin ligand expression was intact in all three strains of KO mice. Recently, tethering of WASP$^{-/-}$ lymphocytes to peripheral node addressin was reported to be reduced by ~20%, suggesting that lack of WASP may differentially affect tethering to various ligands (54).

T cells from all three strains expressed normal amounts of the β1 integrins VLA-4 and VLA-5 (Fig. 3A). More importantly, they adhered normally to FN and up-regulated normally their adhesion to FN after SDF-1α stimulation (Fig. 3B). Previous work has shown that WASP$^{-/-}$ T cells adhered normally to ICAM-1 and FN after stimulation with anti-CD3 (55). Thus, WASP is not important for either the up-regulation of β1 integrin adhesive activity by chemokine or antigen receptors. Taken together, these results suggest that neither WIP nor WASP is essential for T cell tethering and rolling or for integrin-mediated adhesion to FN in response to SDF-1α.

In vitro chemotaxis to SDF-1α was defective in WIP$^{-/-}$ T cells, being reduced to ~50% of normal, and severely defective in DKO T cells, being reduced to ~10% of normal. Despite their *in vivo* homing defect, WASP$^{-/-}$ T cells did not

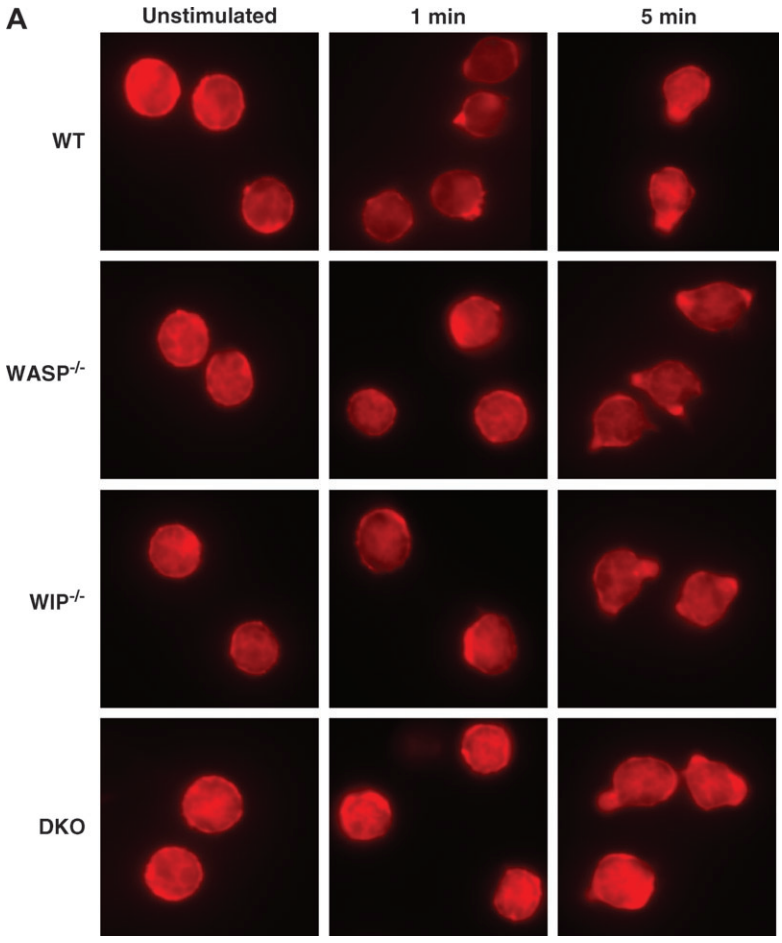

Fig. 6. Actin cytoskeletal re-organization after stimulation with SDF-1α. Purified splenic T lymphocytes from WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice and WT controls were stimulated for 1 and 5 min with SDF-1α, fixed, permeabilized and stained with phalloidin–TRITC and then examined by fluorescent microscopy at ×60 magnification. (A) Representative images. (B) Quantitation of results from three experiments. *$P < 0.05$ compared with WT cells.

exhibit a significant decrease in chemotaxis to SDF-1α *in vitro*. This is not entirely surprising because homing *in vivo* involves a multitude of signals and complex requirements compared with *in vitro* chemotaxis to a single chemokine. In this regard, it was recently shown that lymphocytes from WASP$^{-/-}$ mice have defective chemotaxis to CCL19. (21). The virtually normal chemotaxis of murine WASP-deficient T cells to SDF-1α is in contrast to the defective chemotaxis exhibited by T cells from WAS patients in response to this chemokine (32). It is possible that differences in the expression of N-WASP may explain the difference between the response of WASP-deficient human and mouse T cells to SDF-1α. Higher expression of N-WASP in mouse platelets than in human platelets is thought to account for the preservation of circulating platelet numbers in WASP-deficient mice (21).

Actin polymerization after SDF-1α stimulation was normal in T cells from WASP$^{-/-}$ and WIP$^{-/-}$ mice. This finding in WASP$^{-/-}$ T cells is consistent with the observation that over-expression of the CRIB domain of WASP in a T cell line does not inhibit SDF-1α actin polymerization, although it inhibits SDF-1α chemotaxis (45). The normal actin polymerization in WIP$^{-/-}$ T cells in response to SDF-1α suggests that actin polymerization is not sufficient for chemotaxis. In contrast to the results in WASP$^{-/-}$ and WIP$^{-/-}$ T cells, actin polymerization in response to SDF-1α was severely impaired in T cells from DKO mice. This suggests that WIP and WASP play redundant roles in actin polymerization in response to CXCR4 ligation in T cells. In contrast, WASP and WIP play non-redundant roles in the actin polymerization response of T cells to TCR ligation, as evidenced by the observation that this response is impaired in both WASP$^{-/-}$ and WIP$^{-/-}$ T cells (21, 22, 29).

Examination of the actin cytoskeleton revealed defective actin cytoskeleton re-organization in T cells of WASP$^{-/-}$, WIP$^{-/-}$ and DKO mice in response to SDF-1α. These results suggest that WASP and WIP play complementary roles in actin cytoskeletal changes induced by SDF-1α. The importance of both WASP and WIP in actin re-organization after TCR ligation is well documented (21, 22, 29). The fact that T cells from WASP$^{-/-}$ mice exhibited a significant defect in their ability to re-organize their actin cytoskeleton, despite their near-normal chemotactic response to SDF-1α *in vitro*, is reminiscent of observations on megakaryocytes from WAS patients, which exhibit abnormal filopodia formation, and actin re-distribution in response to SDF-1α, but migrate normally in response to this

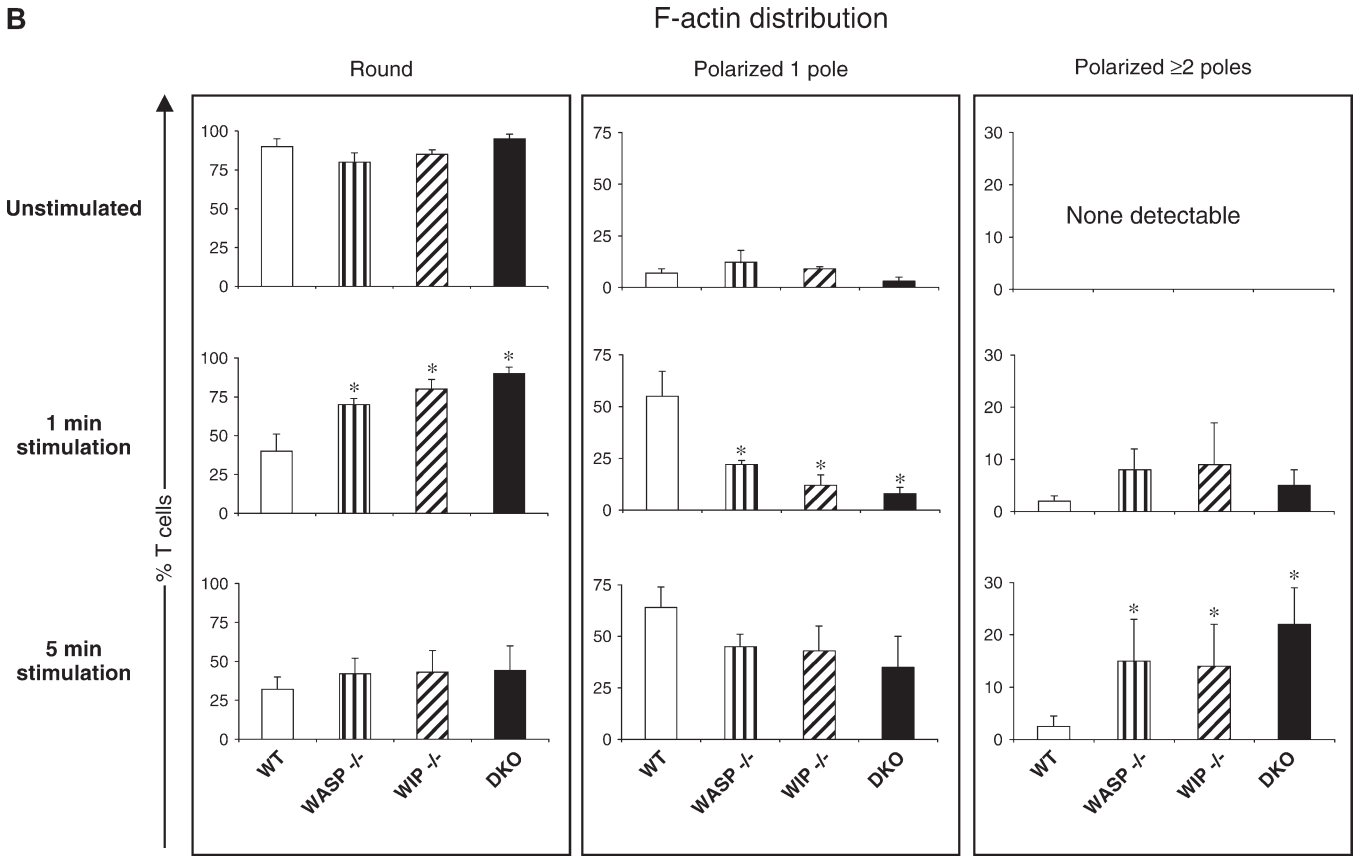

Fig. 6. *Continued.*

chemokine (56). The fact that the defect in actin cytoskeleton re-organization of WASP$^{-/-}$ T cells may not be sufficient to result in abnormal chemotaxis in an *in vitro* assay, but is associated with abnormal *in vivo* homing, suggests that the requirement for an intact actin cytoskeleton is more stringent for homing *in vivo*. Alternatively, the response to other chemokines that may be also important in homing to lymphoid organs may be more affected than the response to SDF-1α in WASP$^{-/-}$ T cells.

SDF-1α causes the activation of Erk and Lck, which may play a role in chemotaxis to SDF-1α (51, 57). Both Erk and Lck activations were normal in SDF-1α-stimulated T cells from DKO mice (Fig. 7). SDF-1α also activates Pyk2, which is essential for up-regulation of β1 integrin-mediated adhesion to FN (58, 59). The observation that the latter was normal in WASP$^{-/-}$, WIP$^{-/-}$ and DKO T cells (Fig. 3B) suggests that Pyk2 activation is normal in these cells. An important early event after CXCR4 ligation is the induced association of the β and γ subunits of the trimeric G-protein with the serine threonine kinase PAK1 and its cofactor PIX (60). This results in the phosphorylation and activation of PAK1, which is important for directional sensing and migration in neutrophils and macrophages. PAK1 phosphorylation in response to SDF-1α was normal in T cells from DKO mice, suggesting that WASP and WIP act downstream of PAK1. PAK 1 is involved in the activation of two pathways that are involved in actin re-organization following SDF-1α stimulation: the LIM kinase–cofilin phosphorylation and inactivation pathway (46, 47) and the Cdc42/Rac-1–WASP–Arp2/3

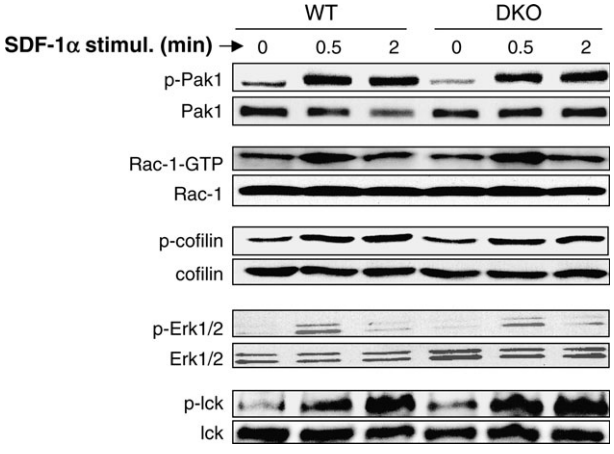

Fig. 7. SDF-1α signaling in T cells. Phosphorylation of PAK1, cofilin, Erk and Lck and GTP loading of Rac-1 in response to SDF-1α stimulation of T cells from DKO mice and WT controls. Splenic T cells were stimulated for the indicated times and lysed in SDS-PAGE sample buffer. Cell lysates were immunoblotted with phospho-specific antibodies to PAK1, cofilin and Erk, and then reprobed with antibodies to PAK1, cofilin and Erk. Lck immunoprecipitates from cell lysates were probed with mAb 4G10, and then reprobed with antibody to Lck to control for loading. Rac-1 GTP was pulled down from lysates using a fusion protein of glutathione-S-transferase and the PVB domain of PAK1 and probed with anti-Rac-1 antibody. An equivalent aliquot of lysates was probed in parallel with anti-Rac-1 as loading control.

complex pathway (32, 45). Cofilin phosphorylation in response to SDF-1α was normal in T cells from DKO mice, suggesting that WASP and WIP are not involved in this pathway. GTP loading of Rac-1 in response to SDF-1α was also normal in DKO T cells, suggesting that both WASP and WIP are downstream of the Cdc42/Rac-1 small GTPases in SDF-1α signaling. In the case of WASP, this is consistent with the observation that over-expression of the Cdc42 CRIB domain which disrupts the interaction of WASP with small GTPases inhibits SDF-1α chemotaxis (32, 45). The role of WIP in actin re-organization is complex. WIP inhibits WASP activation by Cdc42 (61). We have previously reported that virtually all WASPs are complexed with WIP in resting T cells and that following TCR ligation the complex dissociates allowing WASP activation by Cdc42 (28). However, we have preliminary evidence that WIP freed from WASP binds and activates the Arp2/3 complex (N. Ramesh, unpublished results) and thus may contribute to actin polymerization. This is consistent with the observation that over-expression of WIP in lymphocytes results in increased cellular F-actin content (23).

We have recently observed that WASP levels are reduced to ~10% in WIP$^{-/-}$ T cells. In contrast, WIP levels are normal in WASP$^{-/-}$ T cells (our unpublished results). The observation that WIP$^{-/-}$, but not WASP$^{-/-}$, murine T cells exhibits defective chemotaxis to SDF-1α strongly argues for a distinct role for WIP in this response. Similarly, the fact that the *in vivo* homing defect was more severe in T cells from DKO mice than in cells from single KO mice strongly suggests a distinct role for WIP in T cell homing. The observation that SDF-1α-induced actin polymerization was normal in WIP$^{-/-}$ T cells, but severely impaired in DKO T cells, suggests that the residual amount of WASP in WIP$^{-/-}$ T cells was sufficient for normal actin polymerization in response to SDF-1α. Comparable levels of residual WASP in T cells in patients with X-linked thrombocytopenia are compatible with normal T cell function (62). It is difficult at present to assess the role of the secondary WASP deficiency of WIP$^{-/-}$ T cells in their defective chemotaxis and homing. A genetic approach to restoring WASP levels in WIP$^{-/-}$ T cells is needed to determine the contribution of WASP deficiency to the migration defects we have observed in these cells.

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Abbreviations

ANOVA	analysis of variance
DKO	double knockout
EVH1	Ena/VASP homology domain 1
FN	fibronectin
GBD	GTPase-binding domain
H/H	HBSS containing 10 mM HEPES
KO	knockout
LN	lymph node
SDF-1α	stromal cell-derived factor-1α
TRITC	tetramethylrhodamine-5(and 6)-isothiocyanate
WAS	Wiskott–Aldrich syndrome
WASP	Wiskott–Aldrich syndrome protein
WIP	Wiskott–Aldrich syndrome protein-interacting protein
WT	wild type

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