

WASP deficiency leads to global defects of directed leukocyte migration in vitro and in vivo

Scott B. Snapper,^{*,1} Parool Meelu,^{*,†} Deanna Nguyen,^{*} Britt M. Stockton,[‡] Patricia Bozza,[§] Frederick W. Alt,[‡] Fred S. Rosen,[‡] Ulrich H. von Andrian,[‡] and Christoph Klein[†]

^{*}Gastrointestinal Unit and the Center for Inflammatory Bowel Diseases, Massachusetts General Hospital, and
[†]Center for Blood Research, Department of Pathology, Harvard Medical School, Boston, Massachusetts; [‡]Department of Pediatric Hematology/Oncology, Hannover Medical School, Germany; and [§]Departamento de Fisiologia e Farmacodinamica, IOC, Fundacao Oswaldo Cruz, Rio de Janeiro, Brazil

Abstract: Intact cellular migration is critically important for the induction and regulation of the immune response. The Wiskott-Aldrich syndrome protein (WASP) regulates surface receptor signaling to the actin cytoskeleton in hematopoietic cells and thus plays a pivotal role in cellular locomotion. WASP deficiency causes WAS, characterized by immunodeficiency, thrombocytopenia, and eczema. Cell migration defects may contribute to the pathophysiology of WAS. In this study, we used a variety of in vitro and in vivo assays to comprehensively analyze migration properties of lymphocytes, dendritic cells (DC), and neutrophils from WASP-deficient mice. We provide evidence that WASP-deficient lymphocytes show a marked reduction in tethering in an in vitro flow chamber assay as well as decreased migration of T cells in response to the CC chemokine ligand 19 (CCL19). In vivo, compared with wild-type lymphocytes, WASP-deficient lymphocytes showed significantly impaired homing to Peyer's patches upon adoptive transfer into recipient mice. In addition, bone marrow-derived DC migrated less efficiently in response to CCL19. In vivo studies showed decreased migration of DC from skin to draining lymph nodes in WASP-deficient animals. Finally, we also document decreased neutrophil migration in vitro and in vivo. In summary, our studies suggest that WASP plays an important role in the locomotion of lymphocytes, DC, and granulocytes in vitro and in vivo and thus, reveal a crucial role of WASP in physiological trafficking of various hematopoietic cell lineages. These results further delineate immunological abnormalities in WASP-deficient mice, which will be useful to assess preclinical gene therapy studies. *J. Leukoc. Biol.* 77: 000–000; 2005.

Key Words: primary immunodeficiency · Wiskott-Aldrich syndrome · gene therapy · chemokines

INTRODUCTION

Intact cell migration plays a key role for the function of the immune system: Maturation of cells of the innate and adaptive

immune system takes place in distinct, anatomical compartments, and immunological effector functions have to be operative wherever the integrity of the organism is challenged. Thus, immune cells are constantly “en route” to complete their maturation and to exert their effector functions. Migration of cells is orchestrated by a complex interplay of interactions of cell-surface receptors with their respective cognate ligands [1]. Disturbed migration of immune cells can lead to immunodeficiency diseases, as exemplified by leukocyte adhesion deficiency type I (LADI) [2] and LADII [3].

The Wiskott-Aldrich syndrome (WAS) is an X-linked disorder characterized by immunodeficiency, thrombocytopenia, eczema, and lympho-reticular malignancies. Hematopoietic cells from WAS patients show characteristic abnormalities in signaling and cytoskeletal rearrangement [4, 5]. The protein defective in patients with this syndrome, WASP, is expressed exclusively in cells of hematopoietic origin and constitutes an important link between cell-surface signaling events and the rearrangement of the actin-cytoskeleton.

A defect in neutrophil and monocyte chemotaxis was already noted in early studies using cells from WAS patients [6, 7], suggesting that the clinical phenotype of WAS reflects a general defect of signaling pathways involving the regulation of cell motility and the cytoskeleton. More recently, these studies were extended to macrophages and dendritic cells (DC) derived from WAS patients. Monocytes fail to migrate and polarize their cytoskeleton in response to a variety of chemotactic agents such as formyl methionyl-leucyl-phenylalanine (fMLP), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), and colony-stimulating factor-1 (CSF-1) [8, 9]. A reduced translocational motility was also seen in DC in vitro [10]. A defect in podosome formation reflects the aberrant cytoskeletal rearrangement in macrophages and DC from WAS patients [11, 12]. Furthermore, T cells from WAS patients exhibit decreased chemotaxis in response to chemoattractants [13].

These findings suggest, at least in part, that the complex immunodeficiency underlying WAS might result from aberrant

¹ Correspondence: Massachusetts General Hospital, Jackson 706, 55 Fruit Street, Boston, MA 02114. E-mail: ssnapper@partners.org

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migration of immune cells [4]. In this study, we use WASP-deficient mice to comprehensively analyze the role of WASP in the directed cellular locomotion of lymphocytes, DC, and neutrophils in vitro and in vivo to further delineate the phenotype of murine WASP deficiency. These studies will also help to set the stage for preclinical gene therapy studies aiming at the correction of WAS using transplantation of genetically modified, hematopoietic stem cells.

MATERIALS AND METHODS

Animals

WASP-deficient (WKO) mice (129SvEv background) were described previously and bred in specific pathogen-free facilities at the Center for Blood Research (Harvard Medical School, Boston, MA), Massachusetts General Hospital (Boston, MA), or Hannover Medical School (Germany) [14]. No variation in the clinical or immune phenotype has been identified in WKO mice bred in different facilities. WKO animals used for these studies had no macroscopic evidence of colitis. The size of spleens and lymph nodes was comparable with wild-type (WT) control mice. 129 SvEv control (WT) mice were purchased from Taconic Farms (Germantown, NY). All experiments were carried out using age- and sex-matched animals (4–8 weeks of age).

Cells and monoclonal antibodies (mAb)

Where indicated, T cells were purified from mouse peripheral lymph nodes (PLN) using negative depletion of B cells by immunomagnetic bead-labeling with anti-immunoglobulin antibodies and negative depletion (DynaL Biotech, Oslo, Norway). The purity of T cells exceeded 90% in all experiments and was confirmed using cell-surface markers against Thy-1 and B220. DC were obtained from murine bone marrow culture using previously published protocols [15]. In brief, mononuclear bone marrow cells were incubated in the presence of murine recombinant granulocyte macrophage (GM)-CSF and interleukin-4 (IL-4) for 7–8 days. In some experiments, conditioned medium from a genetically engineered cell line expression murine GM-CSF and murine IL-4 was used. Fluorescein-activated cell sorter (FACS) staining for CD11c, major histocompatibility complex (MHC) class II, CD40, CD80, and CD86 confirmed the purity of DC. In some experiments, further purification of DC was performed using a CD11c magnetic bead isolation system following the manufacturer's instructions (Miltenyi, Bergisch-Gladbach, Germany). All antibodies were purchased from BD/PharMingen (Franklin Lakes, NJ).

Flow chamber assay

Tethering assays were performed essentially as reported previously [16]. Peripheral node addressin (PNA_d) was immunopurified from human tonsils and coated on a plastic slide to study tethering and rolling of murine lymphocytes in a flow chamber apparatus as described [17]. WT and WKO PLN lymphocytes were perfused over immobilized PNA_d at 1×10^6 cells/ml at a constant shear stress (1.6 dyne/cm²) and recorded on videotape. The number of tethered cells was counted during a 3-min period after the frequency of rolling had reached equilibrium. Infusions of assay media containing 5 mM EDTA were used to clear the field of view of all adherent cells between recordings. WT and WKO cells were compared while rolling through the identical field of view. Lymphocytes that displayed erratic rolling behavior (skipping or a rolling distance of less than three cell diameters) were not included.

In vivo migration assays

In vivo homing assays were performed as previously reported [16, 18]. Briefly, WT and WKO mice were killed to prepare single-cell suspensions of PLN lymphocytes. First, 5×10^7 cells of each population were labeled for 30 min with calcein-AM (20 μ M) or tetramethylrhodamine isothiocyanate (TRITC; 30 μ g/ml, Molecular Probes, Eugene, OR), washed three times with cold RPMI 1640 containing 10% fetal calf serum (Life Technologies, Gibco, Grand Island, NY), mixed, and injected through a catheter in the right jugular vein of anesthetized WT-recipient mice. Cells remaining in the catheter were saved to

control for potential differences in cell input. After 2 h, recipients were anesthetized and exsanguinated again by puncture of the retro-orbital plexus. Peripheral blood lymphocytes were isolated by density gradient separation on Lympholyte-M (Cedar Lane, Ontario, Canada). Spleen, PLN, mesenteric lymph nodes (MLN), Peyer's patches (PP), and bone marrow were dissected and pressed through fine wire mesh to yield single-cell suspensions, which were analyzed on a flow cytometer (Becton Dickinson) after gating for viable lymphocytes by forward- and light-scatter characteristics. The relative frequency of the two donor cell populations was determined for each individual organ and corrected for differences in cell input in the same animal. Identical results were obtained when WT and WKO cells were stained with the alternative-labeling agent. Adhesion receptor expression on isolated PLN lymphocytes was analyzed using biotinylated mAb CD62L, lymphocyte function-associated antigen-1 (LFA-1), α 4, β 7, and CD44, followed by appropriate second-stage reagents.

Neutrophil migration into the pleural cavity was investigated as recently described [19]. Briefly, intrathoracic injections were performed in 8- to 12-week-old WT (n=8) and WKO (n=8), anesthetized mice with lipopolysaccharide (LPS; 250 ng/cavity, *Escherichia coli* 0127:BB, Sigma Chemical Co., St. Louis, MO) or control vehicle (sterile saline). Animals were killed after 4 h, blood, bone marrow, and pleural cells were recovered and counted, and differential analyses were performed.

DC migration was studied using previously published protocols [20]. In brief, the ventral abdomen and thorax were stained with 450 μ l fluorescein isothiocyanate (FITC; 5 mg/ml dissolved in equal volumes of dibutylphthalate and acetone). After 24 h, the mice were killed, and the percentage of CD11c-positive, FITC-positive cells was determined in cell suspensions of draining lymph nodes.

Transwell migration assays

In vitro chemotaxis assays were performed using Costar Transwell inserts (see ref. [21]) in 24-well plates. Cells were washed three times and resuspended in serum-free medium [Dulbecco's modified Eagle's medium (DMEM) 1% albumin, Pen+Strep]. For T cell migration, 5×10^5 T cells were put in the upper well in a volume of 100 μ l, using 5 μ m pore-size Transwell inserts (Costar, Cambridge, MA). The lower well contained 1 ml serum-free DMEM supplemented with various concentrations of chemokines [CC chemokine ligand 9 (CCL19), R&D Systems, Minneapolis, MN]. The plates were incubated at 37°C for 4 h before harvesting the migrated cells in the lower chamber. Quantification of input and output cells was performed using FACS analysis in a time-triggered acquisition setting. The absence of adherent cells to the under-surface of the Transwell plate was ascertained by light microscopy.

For DC migration assays, 1×10^6 bone marrow-derived DC were added in serum-free medium containing 1% low endotoxin bovine serum albumin (Sigma Chemical Co.) to 5 μ m pore-size Transwell inserts (Costar) in a 24-well plate. After 120 min incubation time, the Transwell inserts were removed, and 10^4 15 μ m microsphere beads (Dynospheres, Bangs Laboratories, Fishers, IN) were added to each well. Cells were stained with anti-CD11c and anti-MHC II mAb (BD/PharMingen) and analyzed by FACS. Numbers of cells in the input and transmigrated populations were calculated as: (no. of cells acquired/no. of Dynospheres acquired) $\times 10^4$ Dynospheres/sample.

Bone marrow neutrophils were isolated from the hindlimbs using a previously described Percoll gradient technique [22]. The purity of cells was confirmed using FACS analysis (Gr1 and CD11b expression) and Giemsa stains. Migration experiments were performed in serum-free medium in 48 Transwell plates with 3.0 μ m pore-size tissue culture-treated polycarbonate membrane (Corning). Migrated cells were quantified by FACS, by addition of inert beads as described above (Dynospheres).

RESULTS

Defective homing of WKO lymphocytes to PP

We first analyzed the expression pattern of cell-surface receptors known to play a role in lymphocyte homing by flow cytometry. WKO lymphocytes did not differ from WT lymphocytes with respect to the surface expression of L-selectin (CD62L), LFA-1, α 4 and β 7 integrins, CD43, and CD44 (data

not shown). We then investigated whether adoptively transferred WKO lymphocytes had migration defects to PLN nodes using an *in vivo* migration assay [16]. Equal numbers of WT and WKO lymphocytes were labeled with TRITC (red fluorescence) or calcein-AM (green fluorescence). Both populations were mixed at a ratio of 1:1 immediately prior to intravenous injection into a WT recipient mouse. To monitor the homing characteristics, the recipient mouse was killed 2 h after the adoptive lymphocyte transfer. Lymphoid organs were harvested, and single-cell suspensions were analyzed using flow cytometry (**Fig. 1A**). Figure 1B represents a synopsis of seven independent experiments. Whereas the ratio of WT:WKO cells was not statistically different in peripheral lymphoid organs, a significant decrease of WKO cells was seen in PP. As expected, concomitant with this decrease in migration, there was a statistically significant increase in the percentage of WKO cells in peripheral blood. Defective homing to PP but not other peripheral lymphoid nodes may result from the increase in flow rates (i.e., shear stress) unique to this lymphoid compartment.

Defective tethering of WKO lymphocytes and decreased chemotaxis of WKO T cells

To assess whether tethering of lymphocytes is impaired by WASP deficiency, we used a dynamic flow chamber system that allows the monitoring of lymphocytes on a PNAd-coated surface under physiological flow forces. **Figure 2A** shows the

result of seven independent experiments. Compared with WT lymphocytes, WKO cells were defective in tethering on a PNAd-coated surface.

As directed T cell migration of naïve T cells is critically dependent on the interaction of CC chemokine receptor 7 with its cognate ligands CCL19 and CCL21, we next tested the ability of T cells to migrate through a chemokine gradient using a Transwell migration assay. As shown in **Figure 2B**, WT T cells migrated effectively in response to a CCL19 gradient. In contrast, WKO T cells migrated at a significantly reduced rate through a wide range of chemokine concentrations. These experiments suggest that WASP plays an integral role in regulating cell-cell adhesion and cellular migration in response to chemokines. Similar defects in B cell migration have been identified recently (L. Westerberg and E. Severinson, personal communication).

Aberrant migration of WKO DC

T cells and DC migrate to the paracortex of lymph nodes, where they meet to initiate an antigen-dependent immune response. Mature DC are known to migrate in response to CCL19 [21]. We therefore used a CCL19-dependent Transwell migration assay to assess the chemotactic response of bone marrow-derived DC. The percentage of migrated class II⁺ CD86⁺-expressing DC is shown in **Figure 3A**. Similar to the results seen with WKO T cells, WKO DC were defective in their migratory response to CCL19.

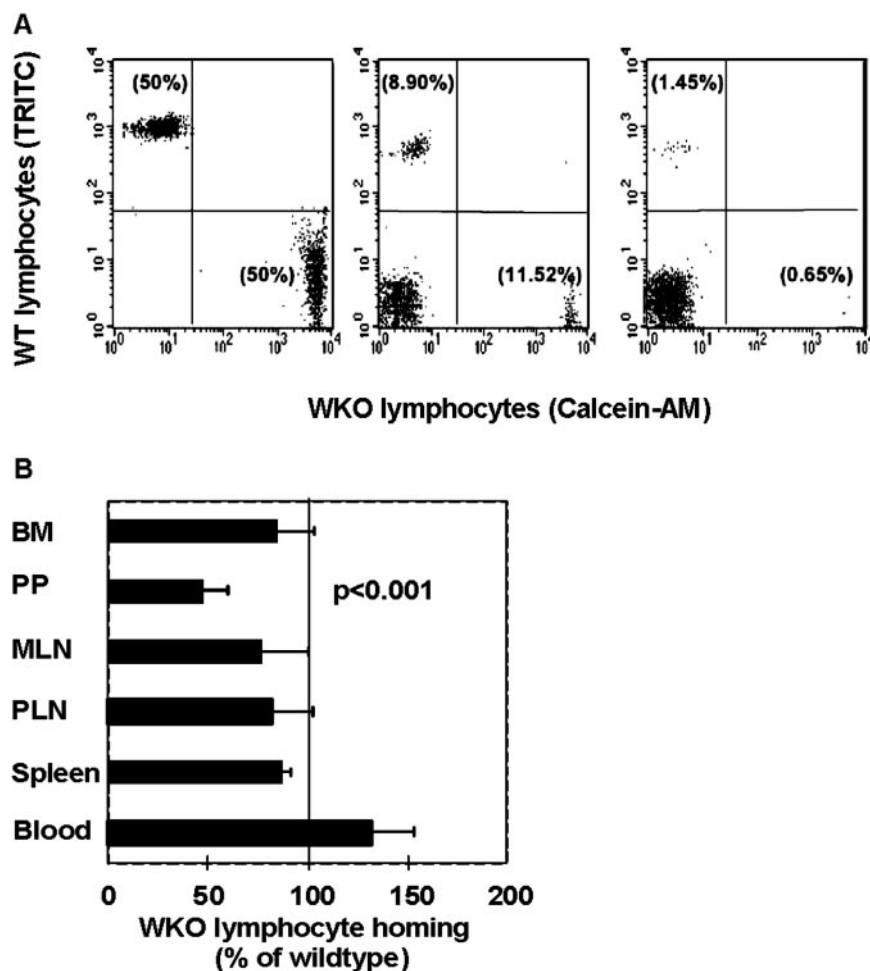
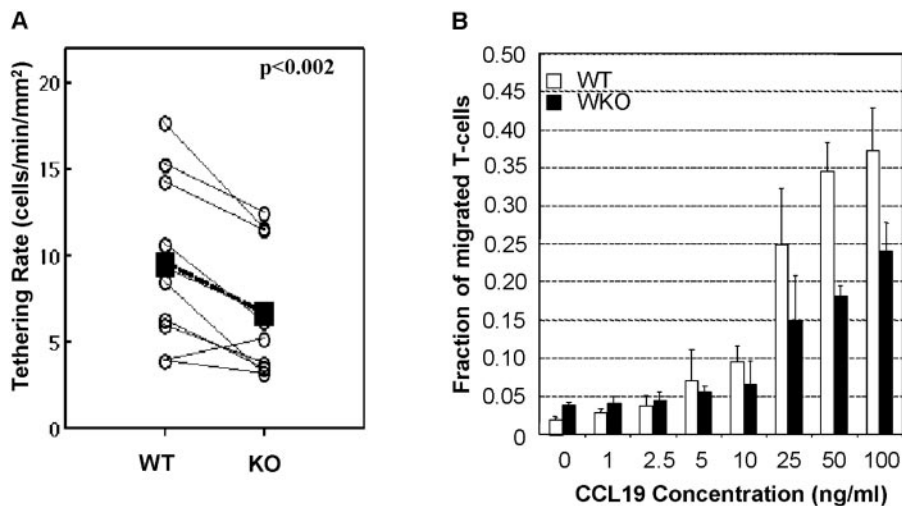


Fig. 1. Defective lymphocyte migration *in vivo*. (A) Representative dot-blot showing the relative amounts of WT and WKO cells in the catheter used for the injection (left panel), as well as in blood (middle panel) and in PP (right panel) 2 h after injection. In this representative experiment, WT and WKO cells were stained prior to injection with TRITC and calcein-AM, respectively. Note the relative decrease in homing of WASP-KO cells to PP (right panel) with a concomitant increase in WKO cells in the blood (middle panel). (B) WKO lymphocytes are defective in homing to PP. Cumulative data of seven independent experiments are shown. The relative contribution of lymphocytes for each data point was normalized by the precise amount of WT and WKO cells in the catheter before injection (A, left panel). BM, Bone marrow.

Fig. 2. Defective lymphocyte migration in vitro. (A) WKO lymphocytes tether less efficiently to PNAd under shear flow in vitro. Lymphocytes from WT and WKO lymph nodes were isolated and allowed to flow over purified PNAd in an in vitro flow chamber apparatus. Data pairs connected by solid lines indicate tethering fractions of WT and WKO lymphocyte samples in the same experiment. (■—■) Average tethering fractions. Displayed is the paired tethering rate in seven individual experiments. (B) Defective chemotaxis of WKO T cells in response to CCL19 in a Transwell migration assay. Displayed are the fraction WT and WKO T cells migrating in response to increasing concentrations of CCL19 as quantified by flow cytometry.



Next, we analyzed the in vivo trafficking of DC and checked the capacity of Langerhans cells to migrate from skin to draining lymph nodes. We determined that WT and WKO mice show similar numbers of epidermal skin Langerhans cells by immunohistochemical analysis (data not shown). The abdominal walls of WT and WKO mice were painted with FITC-labeled isomers. Draining lymph nodes were removed after 24 h and analyzed by flow cytometry for FITC-positive DC. As shown in Figure 3B, WKO mice showed a markedly reduced number of FITC-loaded DC compared with WT control animals, suggesting that WASP-deficient Langerhans cells were impaired in their trafficking capacity.

Defective granulocyte migration in WKO mice

To assess the trafficking properties of neutrophils, we analyzed their migration using an in vitro Transwell migration assay. WKO neutrophils showed a reduced capacity to migrate toward the chemoattractant fMLP (Fig. 4). Next, we injected *E. coli* LPS into the pleural cavity of WT and WKO mice and determined the number of migrated neutrophils (Fig. 5). Whereas WKO mice and WT mice respond equally to LPS with respect to the release of neutrophils from bone marrow to peripheral blood, we found a

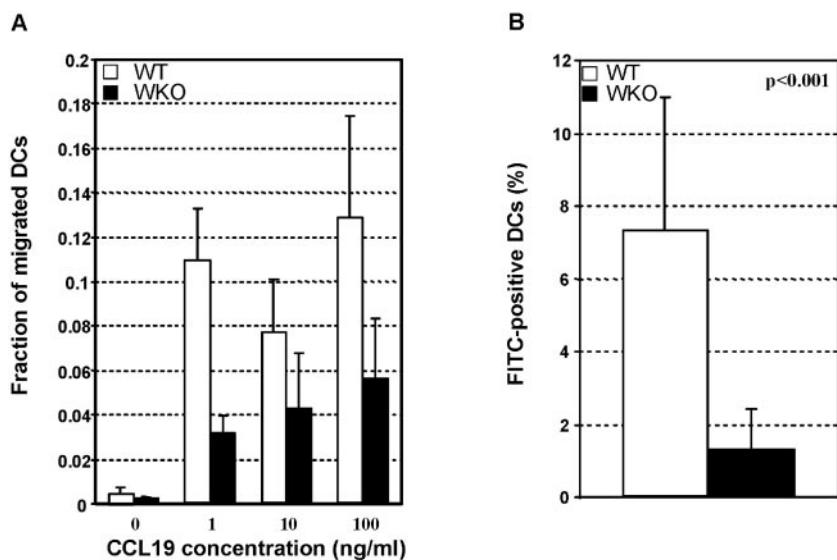
significant difference in the capacity of neutrophils to accumulate in the pleural cavity. These results suggest that the release of neutrophils from bone marrow is intact, whereas the accumulation of neutrophils in inflammatory target organs is impaired in WKO mice. It is interesting that there was an increase in WKO neutrophils in the pleural cavity of mock-treated mice. This may reflect a mild degree of pleural inflammation associated with WASP deficiency reminiscent of inflammatory changes in the colon of young WKO mice.

DISCUSSION

Our results document that murine WKO cells of various hematopoietic lineages show aberrant migration in vitro and in vivo. These findings bear implications not only for the clarification of the immunopathology of WAS but also for the development and assessment of therapeutic strategies using transplantation of genetically transduced hematopoietic stem cells.

In particular, WKO lymphocytes show altered tethering and chemotaxis in vitro and decreased migration to PP in vivo. In contrast, although reduced, migration of WKO lymphocytes to

Fig. 3. Defective DC migration in vitro and in vivo. (A) Defective chemotaxis of WKO DC in response to CCL19. Bone marrow-derived WT and WKO cells were assayed for their ability to migrate across Transwell plates in response to various concentrations of CCL19. The data indicate the fraction of migrated MHC II+ CD86+ cells. (B) Defective in vivo migration of WKO epidermal DC to draining lymph nodes. The abdominal and thoracic skin of WKO WT animals was shaved and painted with a solution of FITC isomers. The animals (n=4) were killed, and the proportion of FITC-positive cells in lymph node suspensions was determined by flow cytometry.



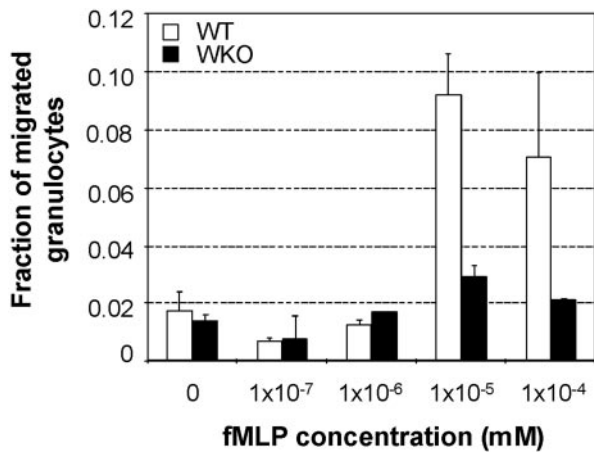


Fig. 4. Detective granulocyte migration in vitro. Peripheral blood granulocytes were purified from WT and WKO mice and analyzed for their migratory response to fMLP in a Transwell migration assay. Displayed is the fraction of cells migrating in response to fMLP. Data are representative of three independent experiments.

PLN did not achieve statistical significance. Homing of circulating lymphocytes to PP and PLN via specialized, high endothelial venules (HEV) is orchestrated by distinct receptor-ligand interactions. Whereas the predominant PP HEV ligand for $\alpha 4\beta 7$ and L-selectin is mucosal addressin cell adhesion molecule-1 [23, 24], homing to PLN is primarily dependent on the PNA^d [25, 26]. Whether our findings showing site-specific homing differences reflect WASP-dependent intracellular signaling pathways or cellular reactions toward physical conditions, such as different intravascular shear forces, is unknown. Our observation is intriguing, given the fact that WKO mice develop chronic colitis associated with a massive infiltration of all hematopoietic lineages. Although WKO lymphocytes are required for colitis induction and T cells sufficient to mediate disease, the contribution of aberrant lymphoid trafficking to the pathophysiology of colitis remains unknown (D. Nguyen and S. B. Snapper, submitted).

In the absence of WASP, human T lymphocytes display abnormal chemotaxis in response to the T cell chemoattractant stroma-derived factor 1 (SDF-1) [13], and SDF-1-mediated chemotaxis is dependent on WASP-Cdc42 interactions and is associated with WASP phosphorylation [13, 27]. Our studies suggest that the T cell migration defect is not restricted to SDF-1 but rather reflects a general underlying inability for directed migration.

Several investigators have analyzed human WASP-deficient cells of the monocyte/macrophage lineage. Zicha et al. [9] have shown that directional motility of WASP-deficient macrophages on a glass surface was abolished, although translocational locomotor capacity appeared preserved. Similarly, monocytes from WAS patients show impaired migration in response to fMLP, MCP-1, and MIP1- α [8]. Furthermore, macrophages and DC from WAS patients show absent podosomes and are unable to polarize in response to fMLP or regulated on activation, normal T expressed and secreted [10–12]. Despite these migratory defects, it has been difficult by some to document clear, functional alterations of antigen-presenting cells (APC) [28]. Nonetheless, defects in phagocytosis have been reported in WASP-deficient APC [29]. In this study, we show that in vivo migration of DC and granulocytes is severely affected in WKO mice. Furthermore, the in vitro and in vivo defects in directed DC migration by WKO DC described here are consistent with and extend the abnormalities in cell adhesion and podosome formation recently reported [30].

Mechanistically, it remains challenging to prove whether a defect in cell migration is directly responsible for the immunodysregulation and associated clinical phenotypes associated with WAS deficiency in mice and men [4]. Our comprehensive analysis along with previous in vitro experiments [6–10] suggest that WASP deficiency is responsible for a severe defect in cell migration in vitro and in vivo in multiple cell types. However, this defect is not absolute, and it will be interesting to see whether the presence of WASP homologues, such as WASP family Verprolin-homologous protein (WAVE) and neural WASP, accounts for the residual migration capacity. Clearly, proper cell migration requires a highly complex interplay of multiple signaling cascades

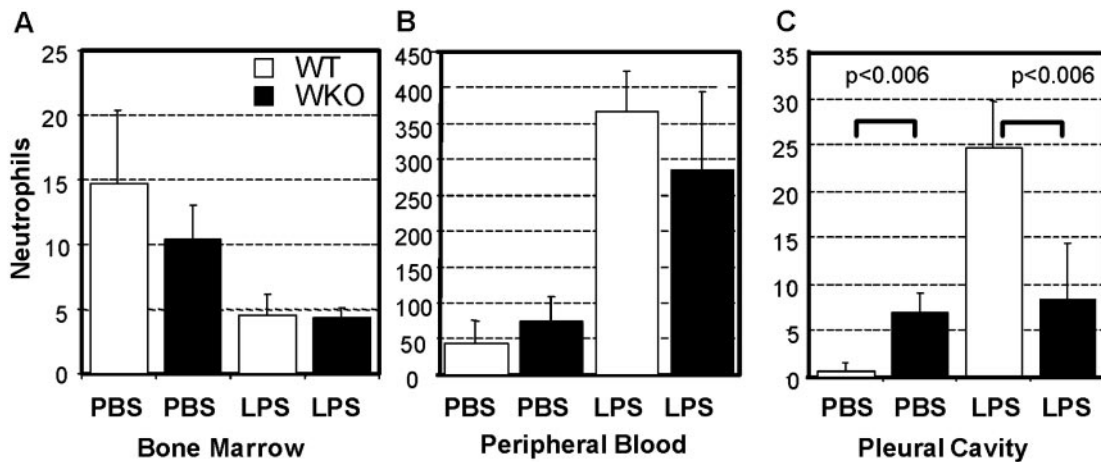


Fig. 5. Detective granulocyte migration in vivo. WT and WKO mice (groups of eight) were injected with LPS or mock-injected into the pleural cavity. Four hours later, the mice were killed, and the number of neutrophils was determined in the bone marrow (A, counts/ 0.5×10^6 /femur), blood (B, counts/ 10×10^3 /ml), and pleural cavity (C, counts/ 10×10^6 /cavity). WT and WKO mice respond equally to LPS with release of neutrophils from the bone marrow (A) and a concomitant increase of neutrophils in the blood (B). However, WKO neutrophils are defective in migrating into the pleural cavity (C). PBS, Phosphate-buffered saline.

and is not merely determined by WASP family proteins. Regulated by Rac1, WAVE proteins have been implicated in the formation of lamellipodial protrusions and thus, may play a crucial role in leukocyte migration [31, 32].

In summary, our results underline the importance of WASP for directed cellular locomotion in hematopoietic cells and shed new light on the pathophysiology of WASP deficiency. In addition, they have implications for the preclinical and clinical assessment of gene therapy studies aimed at correction of the WAS phenotype using hematopoietic stem cells. This is especially critical, given recent *in vivo* data by Lacout et al. [33], demonstrating that WAS deficiency affects the *in vivo* migration of hematopoietic stem cells and may be responsible for the nonrandom X inactivation characteristic of female carriers of the WAS mutation.

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