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Signaling protein SWAP-70 is required for efficient B cell homing to lymphoid organs

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The migration of B cells into secondary lymphoid organs is required for the generation of an effective immune response. Here we analyzed the involvement of SWAP-70, a Rac-interacting protein involved in actin rearrangement, in B cell entry into lymph nodes. We noted reduced migration of *Swap70*$^{-/-}$ B cells into lymph nodes *in vivo*. *Swap70*$^{-/-}$ B cells rolled and adhered, yet accumulated in lymph node high endothelial venules. This defect was not due to impaired integrin expression or chemotaxis. Instead, *Swap70*$^{-/-}$ B cells aberrantly regulated integrin-mediated adhesion. During attachment, *Swap70*$^{-/-}$ B cells showed defective polarization and did not form uropods or stabilize lamellipodia at a defined region. Thus, SWAP-70 selectively regulates processes essential for B cell entry into lymph nodes.

Migration of B cells into secondary lymphoid organs is required for the induction of an antigen-specific humoral immune response. In secondary lymphoid organs, B cells encounter antigen-presenting cells and cognate T cells. B cells migrate directly from the blood stream to the lymph nodes by extravasation through high endothelial venules (HEVs). Lymphocyte transendothelial cell migration is a multistep process. Before diapedesis, B cells roll in an L-selectin-dependent way, receive signals through CCR7 and CXCR4, and then adhere to HEVs via integrin $\alpha_L\beta_2$ (LFA-1)[1–5]. The presence of B cells in secondary lymphoid organs is critical for the homeostatic and inflammatory regulation of these tissues[6–8]. B cell accumulation is required for the normal hypertrophic response of an inflamed lymph node[8]. Although it is apparent that B cell migration to and within secondary lymphoid organs is important during the generation of an immune response, relatively little is known about the mechanisms underlying such movements.

Many molecules involved in the transduction of signals from cell surface receptors to the actin cytoskeleton regulate cell adhesion and transmigration[9–11]. Notably, small G proteins of the Rho family (such as Rac1 and Rac2), together with their regulators, are important in hematopoietic cell migration. Although highly homologous, Rac1 and Rac2 have slightly different functions, with Rac1 being required for directed migration and Rac2 being required for cell motility[12–14].

'Upstream' activators of Rho proteins, such as Dbl homology domain proteins of the Vav family, are required for β-integrin-mediated activation of the Rac, Cdc42 and Rho small G proteins and for the stable adhesion of cells in flow conditions[15]. Other indirect regulators of Rac proteins, including phosphatidylinositol-3-OH kinase (PI(3)K) and the Tec and Pyk-2 tyrosine kinases, are required for both chemokine- and integrin-mediated cell migration *in vitro* and *in vivo*[9,11,13,14,16,17]. Although integrin- and chemokine-activated signaling pathways for activation of Rac and its effector proteins are common among hematopoietic cells, functions of individual members of those pathways vary among cell types.

Migration is differentially regulated even in closely related cells such as B cells and T cells. For example, the Rac activator Dock2 has different functions in B cell and T cell transendothelial migration[18]. *Dock2*$^{-/-}$ B cells show reduced adhesion *in vitro* and *in vivo* whereas *Dock2*$^{-/-}$ T cells are able to adhere, yet are unable to transmigrate across HEVs *in vivo*. Requirements for different isoforms of PI(3)K also vary between B cells and T cells. For example, unlike B cells, T cells specifically require PI(3)K-γ for efficient migration[18].

As B cell transendothelial migration is uniquely regulated, it is important to identify the signaling molecules involved. SWAP-70 is a Rac-interacting protein expressed in B cells but not in T cells[19,20]. SWAP-70, along with its only close homolog SLAT ('SWAP-70-like adaptor of T cells'; also called IBP)[21,22], are unique because they contain a pleckstrin homology domain N-terminal of their Dbl homology–like domain. The pleckstrin homology domain specifically binds phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3). In addition, SWAP-70 binds nonmuscle F-actin[23,24]. Dbl homology domain–containing proteins are involved in the activation of small GTPases of the Rho family. Accordingly, SWAP-70 specifically interacts with and regulates Rac and is required during remodeling of the F-actin cytoskeleton[20,23–25]. Hence, we have analyzed and characterized the effects of SWAP-70 deficiency on B cell adhesion and migration into secondary lymphoid organs.

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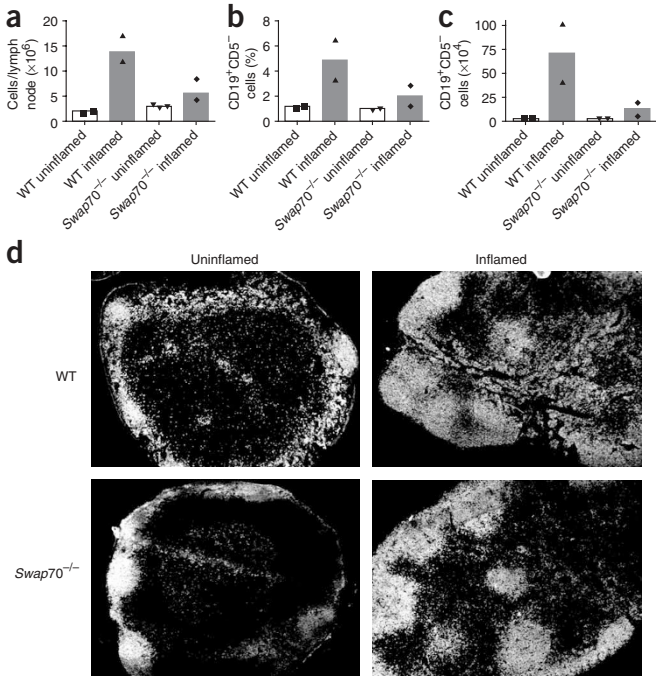

Figure 1 Inflammation-induced accumulation of *Swap70*$^{-/-}$ B cells in lymph nodes. (**a**–**c**) Flow cytometry to determine total numbers of cells present (**a**) and percentages (**b**) and absolute numbers (**c**) of CD19^{+} B cells. Data are representative of two individual experiments. Symbols indicate individual experiments; bars indicate the average. (**d**) Cryosections of inflamed and uninflamed lymph nodes stained with anti-B220. Original magnification, ×10. Sections were obtained from the inflamed and uninflamed lymph nodes of three wild-type and *Swap70*$^{-/-}$ mice, and a minimum of five images were recorded per lymph node.

RESULTS

Impaired *Swap70*$^{-/-}$ B cell migration into lymph node

To assess the migration of *Swap70*$^{-/-}$B cells *in vivo*, we injected keyhole limpet hemocyanin emulsified in complete Freund's adjuvant into the footpads of mice. In wild-type mice this stimulus induces a robust inflammatory reaction, resulting in the accumulation of B cells in the draining lymph node. Uninflamed lymph nodes from *Swap70*$^{-/-}$ and wild-type mice were similar in size and contained the same amount of B cells (**Fig. 1a–c**). This suggests that in adult *Swap70*$^{-/-}$ mice, constitutive homing of B cells to lymph nodes is unimpaired. Inflammation of wild-type lymph nodes increased the percentage of B cells present and increased cell numbers 16-fold relative to that of uninflamed, wild-type lymph nodes (**Fig. 1b,c**). In contrast, inflammation induced an increase of only threefold in the number of B cells in *Swap70*$^{-/-}$ lymph nodes. Wild-type and *Swap70*$^{-/-}$ uninflamed lymph nodes have similar structures, with both containing distinct B cell follicles. As inflamed wild-type lymph nodes filled with B220^{+} cells, distinct follicles were lost (**Fig. 1d**). In contrast, although *Swap70*$^{-/-}$ lymph nodes began to fill with B220^{+} cells, obvious B cell follicles remained. Thus, during inflammation *Swap70*$^{-/-}$ lymph node hypertrophy is not as great as that of wild-type lymph nodes, partly because of notably reduced B cell accumulation.

To compare migration into lymphoid tissue with migration into a peripheral site we analyzed the presence of B1 and B2 B cells in peritoneal lavage fluid from wild-type or *Swap70*$^{-/-}$ mice before and after intraperitoneal injection of thioglycollate. Untreated wild-type and *Swap70*$^{-/-}$ mice had no differences in peritoneal B cell subsets (**Supplementary Fig. 1** online). At 9 h and 18 h after injection, wild-type lavage fluid contained more B2 B cells than did lavage fluid from *Swap70*$^{-/-}$ mice. Only at 9 h were more B1 cells present in peritoneum. Thus, *Swap70*$^{-/-}$ B cells, particularly the B2 subset, show impaired migration into inflamed peritoneum.

To study *Swap70*$^{-/-}$ B cell migration into lymph nodes, we did competitive migration assays. We labeled purified wild-type and *Swap70*$^{-/-}$ splenic B cells with the membrane dye PKH-26 or carboxyfluorescein diacetate succinimidyl diester and injected the cells intravenously into wild-type mice at a ratio of 1:1. At 2 h after injection, more wild-type than *Swap70*$^{-/-}$ B cells entered the lymph nodes and spleen even though equal numbers of wild-type and *Swap70*$^{-/-}$ B cells were present in the circulation (**Fig. 2**). At 6 h after injection, similar numbers of wild-type and *Swap70*$^{-/-}$ B cells were present in the spleen and blood, but lymph nodes had a wild-type/*Swap70*$^{-/-}$ B cell ratio of 2:1. At 24 h after cell transfer, there were no differences in the numbers of wild-type and *Swap70*$^{-/-}$ B cells in the blood or spleen and only slightly more wild-type cells in the lymph nodes. These results demonstrated a kinetic delay in *Swap70*$^{-/-}$ B cell entry into secondary lymphoid organs, most obviously into lymph node.

When we analyzed expression of the chemokine receptors and adhesion molecules necessary for B cell migration into lymph nodes we found no substantial differences for wild-type versus *Swap70*$^{-/-}$ (**Supplementary Fig. 2** online). Also, *Swap70*$^{-/-}$ B cells showed no detectable difference relative to wild-type B cells in chemotaxis toward CXCL12, CCL19 and CXCL13 *in vitro*. Thus, the reduced migration of *Swap70*$^{-/-}$ B cells cannot be explained by failure to respond to chemokines or of adhesion molecule expression (**Supplementary Note** online).

Defective *Swap70*$^{-/-}$ B cell polarization

Stimuli including chemokines, mitogens (such as LPS), interleukin 4 (IL-4) and CD40L activate integrins[26–28]. After activation *in vitro* by CD40L and IL-4, splenic B cells form tight homotypic aggregates that

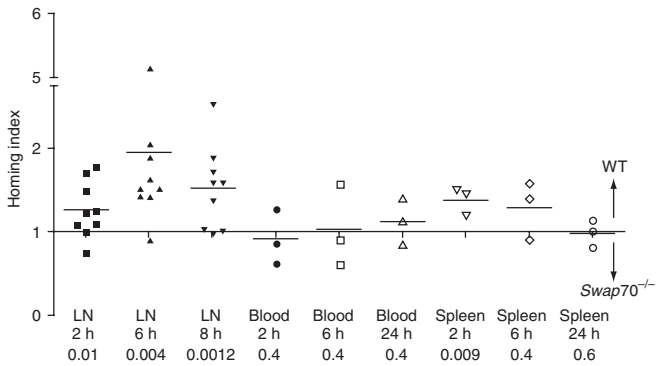

Figure 2 Migration of *Swap70*$^{-/-}$ B cells to lymph nodes. Variously labeled purified wild-type and *Swap70*$^{-/-}$splenic B cells were injected together through the tail vein into wild-type mice at a ratio of 1:1. At various time points (horizontal axis), superficial cervical, brachial and inguinal lymph nodes (LN), blood and spleens were collected and wild-type and *Swap70*$^{-/-}$ B cells were counted by flow cytometry. Data are representative of three adoptive transfer experiments. Symbols indicate individual tissue samples collected. The homing index of wild-type/*Swap70*$^{-/-}$ cells was calculated and plotted, with '1' designating the input ratio. Raw data are presented for each tissue, and arrows indicate the direction of a relative increase in wild-type or *Swap70*$^{-/-}$ cells. Bottom row, significance of the variation from the input ratio of 1.

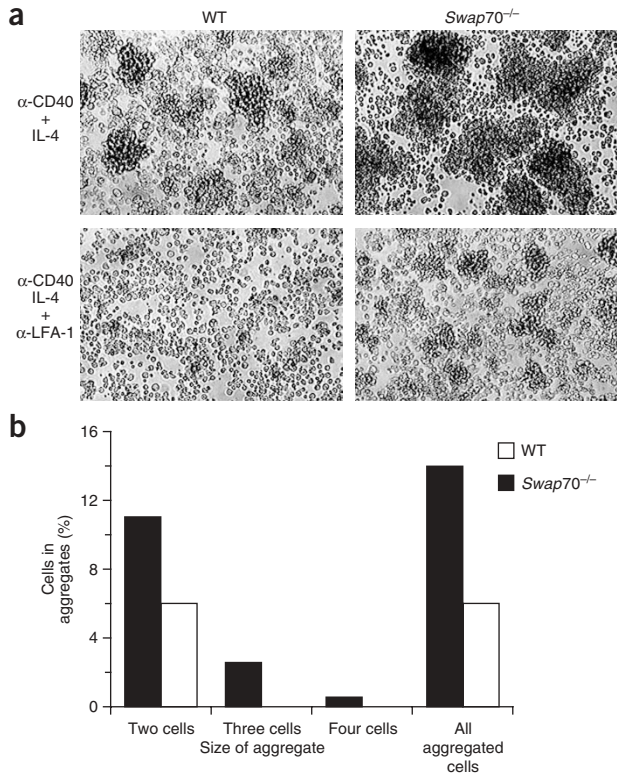

Figure 3 Integrin-mediated adhesion of *Swap70*$^{-/-}$ B cells. (**a**) Aggregates of wild-type and *Swap70*$^{-/-}$ B cells after 48 h incubation with various stimuli (left margin). α-, antibody to. Original magnification, ×20. Images represent two experiments done in triplicate. (**b**) Percentage of cells in aggregates 30 min after disruption of existing B cell aggregates. Three experiments were done in triplicate.

become large visible clusters by 48–72 h; such aggregation depends mainly on the integrin $\alpha_L\beta_2$ (refs. 29,30). In contrast to wild-type B cells, *Swap70*$^{-/-}$ B cells aggregated much more *in vitro*, yet like wild-type cells depended on $\alpha_L\beta_2$, as aggregation was mostly blocked by inhibitory antibodies to $\alpha_L\beta_2$ (**Fig. 3a**). As *Swap70*$^{-/-}$ B cells proliferated marginally faster after CD40 stimulation[31], we did reaggregation assays, which showed that *Swap70*$^{-/-}$ B cells formed more aggregates that were also larger in size (up to four cells) than those of wild-type B cells (**Fig. 3b**). Thus, whereas expression of $\alpha_L\beta_2$ is normal on *Swap70*$^{-/-}$ B cells its regulation is altered.

As a consequence of integrin engagement, lymphocytes polarize through coordinated rearrangements of their cytoskeleton[26,32]. Such processes are likely to be very important during B cell transendothelial migration. As integrin attachment was 'misregulated' in *Swap70*$^{-/-}$ B cells, we examined polarization of the cells. After *in vitro* stimulation with antibody to CD40 (anti-CD40) and IL-4, B cells polarize on integrin ligands, antibodies to integrins, or substrates such as immunoglobulin E[33]. After 72 h with anti-CD40 and IL-4, wild-type B cells attached to anti-CD44 and spread, forming many polarized bodies (**Fig. 4a**). In contrast, whereas *Swap70*$^{-/-}$ B cells spread more than wild-type cells, they failed to polarize as much as wild-type cells. We counted polarized and irregularly spread B cells, defining a 'polarized cell' as a cell with one dendrite at least equal to the width of the cell body and an 'irregular cell' as a cell substantially deformed from a round cell body. We found 20% more *Swap70*$^{-/-}$ B cells spread on the surface coated with anti-CD44 yet only 30% polarized (**Fig. 4b**). In comparison, fewer wild-type B cells spread on the surface coated with anti-CD44, but over 50% of spread B cells became polarized. We obtained similar patterns of irregular attachment using immunoglobulin E–coated surfaces and the same conditions (data not shown).

The irregular attachment of *Swap70*$^{-/-}$ B cells to anti-CD44 was emphasized by staining of the F-actin of spread B cells with TRITC (tetramethylrhodamine isothiocyanate)–phalloidin. Polarized wild-type

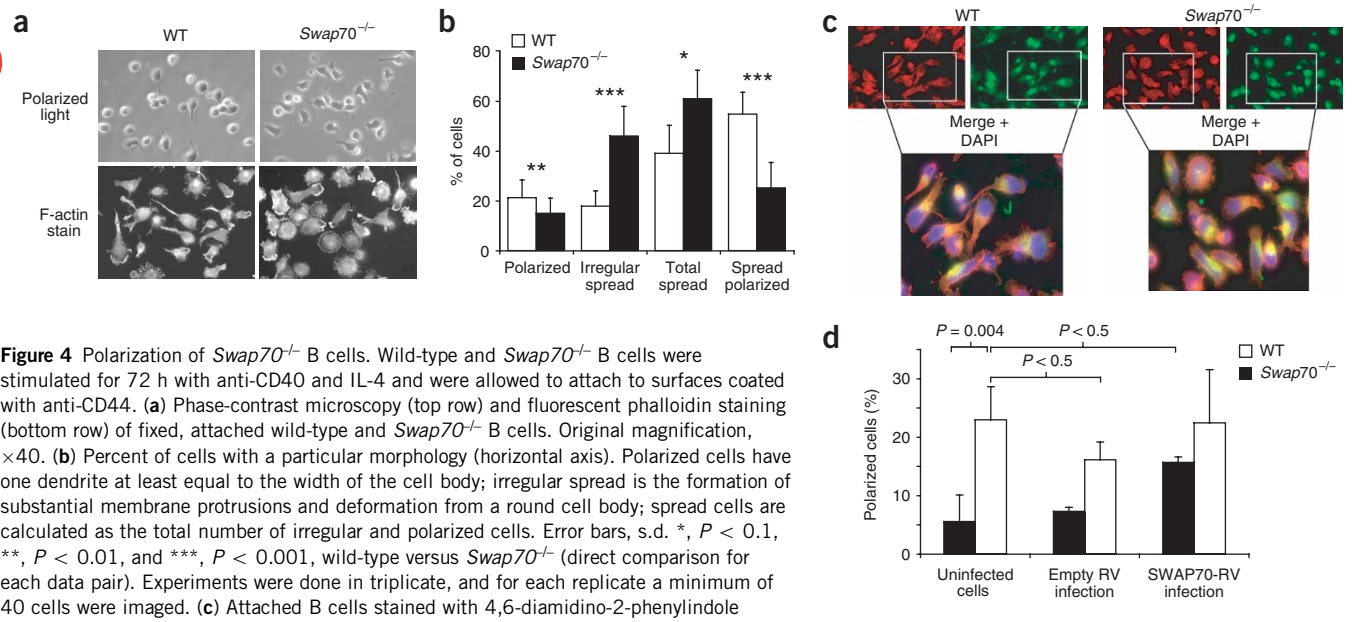

Figure 4 Polarization of *Swap70*$^{-/-}$ B cells. Wild-type and *Swap70*$^{-/-}$ B cells were stimulated for 72 h with anti-CD40 and IL-4 and were allowed to attach to surfaces coated with anti-CD44. (**a**) Phase-contrast microscopy (top row) and fluorescent phalloidin staining (bottom row) of fixed, attached wild-type and *Swap70*$^{-/-}$ B cells. Original magnification, ×40. (**b**) Percent of cells with a particular morphology (horizontal axis). Polarized cells have one dendrite at least equal to the width of the cell body; irregular spread is the formation of substantial membrane protrusions and deformation from a round cell body; spread cells are calculated as the total number of irregular and polarized cells. Error bars, s.d. *, $P < 0.1$, **, $P < 0.01$, and ***, $P < 0.001$, wild-type versus *Swap70*$^{-/-}$ (direct comparison for each data pair). Experiments were done in triplicate, and for each replicate a minimum of 40 cells were imaged. (**c**) Attached B cells stained with 4,6-diamidino-2-phenylindole (DAPI; blue), TRITC-phallodin (red) and anti-LFA-1 (green). Bottom row, merged image and enlargement of boxed area in top row. Original magnification, ×40. Data are typical images from three independent experiments. (**d**) Polarization of B cells infected with retroviruses (RV; horizontal axis) on coverslips coated with anti-CD44. Error bars, s.d. Experiments were done in triplicate, and for each replicate a minimum of 30 cells were counted.

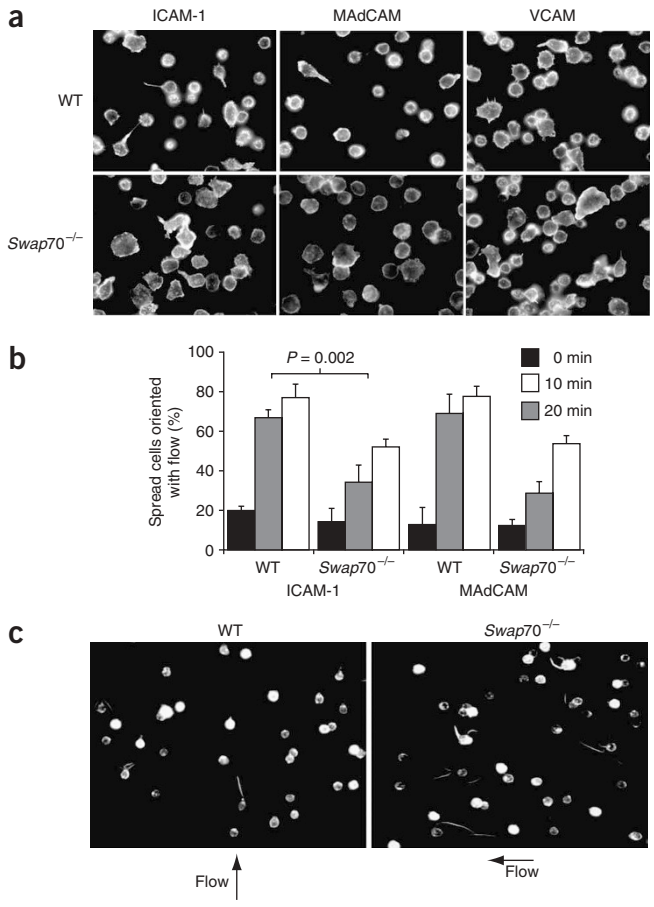

Figure 5 Adhesion of *Swap70*$^{-/-}$ B cells. (**a**) Reorganization of the F-actin cytoskeleton assessed by phalloidin staining of CCL19-stimulated B cells that were allowed to attach and spread on ICAM-1, MAdCAM or VCAM. Images represent three experiments done in triplicate. (**b**) Percent of attached cells orientated in the direction of flow, for CCL19-stimulated B cells adhered to ICAM subjected to a constant shear stress force of 2 dyn/cm^2. Data from two experiments are plotted; error bars, s.d. Similar results were obtained on MAdCAM. (**c**) Cytoskeletal rearrangements assessed by phalloidin staining of B cells fixed and stained after 20 min of exposure to shear stress. Images represent five independent experiments. Original magnification, ×40 (**a**) and ×20 (**c**).

cells had lamellipodia localized to one side of the cell body and a uropod to the rear of the cell (**Fig. 4a**). In contrast, *Swap70*$^{-/-}$ B cells formed lamellipodia that were often simultaneously on several sides of the B cell. Most *Swap70*$^{-/-}$ cells had no obvious uropod structure.

During the attachment and polarization of cells, various molecules and organelles redistribute as the cell polarizes[10,34]. In polarized wild-type B cells, nuclei moved toward the front of the cell, whereas $\alpha_L\beta_2$ localized to the rear of the nucleus near the beginning of the uropod (**Fig. 4c**). In *Swap70*$^{-/-}$ B cells, nuclei were often not present near the front of the cell, and $\alpha_L\beta_2$ did not localize toward a defined structure, perhaps because of the absence of a uropod. Staining of wild-type and *Swap70*$^{-/-}$ B cells with anti-CD44 showed patterns similar to that of $\alpha_L\beta_2$ staining (data not shown). These observations suggest that *Swap70*$^{-/-}$ B cells do not form a stable uropod during cell attachment.

To confirm that *Swap70*$^{-/-}$ B cells fail to polarize normally because of the absence of SWAP-70, we reintroduced SWAP-70 using retroviral vectors. During the initial 48 h of stimulation with anti-CD40 and IL-4, we infected wild-type and *Swap70*$^{-/-}$ splenic B cells with retroviruses bearing expression cassettes of either green fluorescent protein or SWAP-70–internal ribosomal entry site–green fluorescent protein. After 70 h, we isolated green fluorescent protein–positive infected B cells and allowed the cells to 'rest' for 2 h before assessing spreading on surfaces coated with anti-CD44. Infection of wild-type cells with either retrovirus had no effect on cell polarization (**Fig. 4d**). However, infection of *Swap70*$^{-/-}$ B cells with the retrovirus encoding SWAP-70 resulted in restored polarization to nearly wild-type amounts.

The aberrant regulation of integrin activity, coupled with the abnormal polarization on surfaces coated with anti-CD44, suggest that *Swap70*$^{-/-}$ B cells have defective adhesion molecule–mediated interactions. To investigate that possibility, we examined chemokine-stimulated interactions of wild-type and *Swap70*$^{-/-}$ B cells with various adhesion molecules (MAdCAM, VCAM and ICAM-1). Chemokine-stimulated lymphocyte adhesion to ICAM-1 is transient, peaking between 2 and 3 min after initial contact[35]. Wild-type and *Swap70*$^{-/-}$ B cells stimulated with CCL19 or CCL21 had similar kinetics of attachment, which peaked at around 1 min and then decreased (data not shown).

Although we noted no difference in the kinetics of B cell attachment, the morphology of the attached B cells seemed different. Unstimulated wild-type and *Swap70*$^{-/-}$ B cells were regular in shape and of similar size. After CCL19 stimulation on ICAM-1, 10.9% of wild-type B cells had polarized (**Fig. 5a**), and the remaining wild-type B cells had a regular shape. In contrast, only 5.2% of *Swap70*$^{-/-}$ B cells had polarized, although they covered a larger area (average diameter of wild-type cells was 10.4 μm, versus 12.2 μm for *Swap70*$^{-/-}$ B cells; $P < 0.0001$) and had lamellipodia on several sides of the cell. Wild-type and *Swap70*$^{-/-}$ B cells attached to MAdCAM had characteristics similar to those on ICAM-1 (3.6% of wild-type polarized and 1.1% of *Swap70*$^{-/-}$ B cells). On VCAM, both wild-type and *Swap70*$^{-/-}$ B cells infrequently polarized; we noted some irregularity and spreading of wild-type B cells (average diameter, 9.21 μm), although the spread area was significantly larger for *Swap70*$^{-/-}$ B cells (average diameter, 11.6 μm; $P < 0.0001$).

To determine how those alterations in *Swap70*$^{-/-}$ B cells might affect migration under flow, we examined wild-type and *Swap70*$^{-/-}$ B cells after attachment under shear stress similar to that found in HEVs (2.0 dyn/cm^2). Cells were pretreated with CCL19 before shear was applied, and wild-type and *Swap70*$^{-/-}$ B cells oriented randomly. After application of shear, cells began to align parallel to the flow. After 10 min, 67% of wild-type B cells and 34% of *Swap70*$^{-/-}$ B cells on ICAM-1 were oriented parallel to the flow ($P = 0.0022$; **Fig. 5b**). This increased to 77% for wild-type and 52% for *Swap70*$^{-/-}$ B cells after 20 min ($P = 0.002$). We obtained similar results for cells on MAdCAM (**Fig. 5b**).

To identify cytoskeletal changes occurring under shear stress, we stained B cells with phalloidine. Almost all wild-type cells aligned with the flow and some had filopodia originating from behind the cell (**Fig. 5c**). In contrast, *Swap70*$^{-/-}$ B cells often formed more filopodia that originated from different regions of the cell, and although some cells oriented with the flow, many did not. The filopodia of *Swap70*$^{-/-}$ B cells were also often longer than those of wild-type cells. These data show that *Swap70*$^{-/-}$ B cells have irregular chemokine-stimulated attachment to adhesion molecules known to be involved in cell migration, both in static conditions and when exposed to shear stress.

Unstable *Swap70*$^{-/-}$ B cell lamellipodia

To understand the dynamics of B cell polarization, we collected time-lapse images of cells after 25 min of attachment on surfaces coated with anti-CD44. Lamellipodia could be identified at one end of wild-type

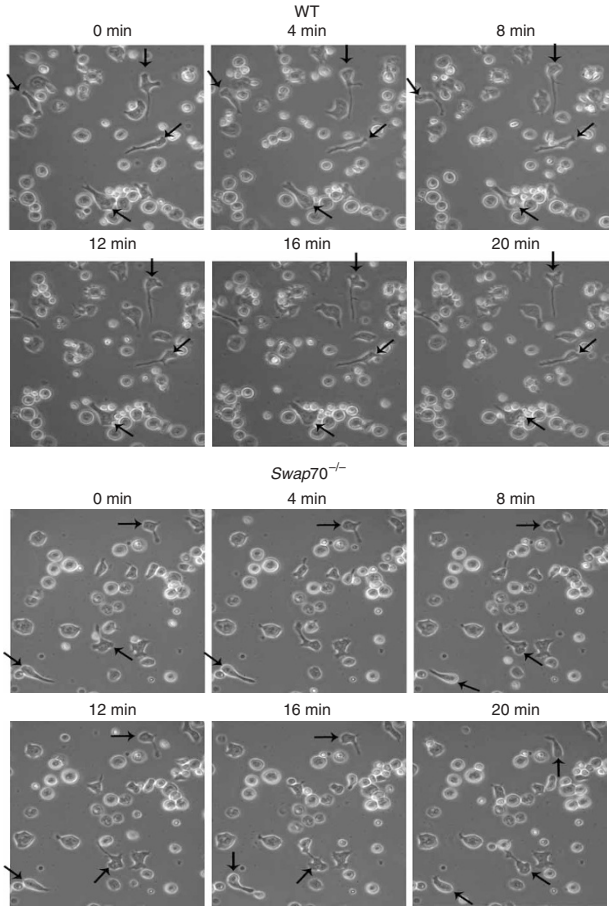

Figure 6 Lamellipodia formation. Images of B cells attached to anti-CD44-coated surfaces were collected every 10 s for 20 min after a 25-minute attachment period (**Supplementary Videos 1,2**). Arrows indicate the leading edge of the cell. Original magnification, ×40. Data are representative of three independent time-lapse experiments.

B cells (where the main protrusive activity localized), and a uropod formed at the opposite end (**Fig. 6** and **Supplementary Video 1** online). The uropod in wild-type cells showed little protrusive activity and seemed to be retractile. As a consequence of localization of the leading edge to one side of the B cell, it moved in that direction. *Swap70*$^{-/-}$ B cells also formed lamellipodia, but they were unstable and, during a 20-minute interval, moved around the cell (**Fig. 6** and **Supplementary Video 2** online). Some *Swap70*$^{-/-}$ B cells contained uropods, but unlike wild-type B cell uropods, *Swap70*$^{-/-}$ uropods formed many protrusions and had little retractile activity. Possibly as a consequence of the observations described above, little coordinated movement of *Swap70*$^{-/-}$ B cells occurred, which suggests movement of *Swap70*$^{-/-}$ B cells on endothelial cells is impaired *in vivo*.

The leading edge of migrating neutrophils is established by a feedback loop involving Rac and F-actin. As the leading edge of *Swap70*$^{-/-}$ B cells was unstable, and SWAP-70 binds Rac and F-actin, it is possible SWAP-70 might stabilize this feedback loop. Overall cellular actin polymerization and Rac1 activation occurred to the same degree in wild-type and *Swap70*$^{-/-}$ B cells (**Supplementary Fig. 3** online and **Supplementary Note**). However, SWAP-70 was essential for the localization of Rac1 in B cells stimulated with anti-CD40 and IL-4 and polarized on surfaces coated with anti-CD44 (**Supplementary Fig. 3**). Rac1 localized mainly to the leading edge of wild-type B cells but remained diffuse in *Swap70*$^{-/-}$ B cells. This observation suggests that whereas in these conditions SWAP-70 is dispensable for Rac1 activity, SWAP-70 is essential for the localization of activated Rac1 to the leading edge of B cells.

Swap70$^{-/-}$ B cell accumulation in HEVs

Swap70$^{-/-}$ B cells showed aberrant patterns of adhesion *in vitro*, suggesting that adhesion is also impaired *in vivo*. To examine adhesion *in vivo*, we used intravital microscopy. *Swap70*$^{-/-}$ and wild-type mice had similar numbers of rolling and adhering B cells (**Fig. 7a,b**), which shows that *Swap70*$^{-/-}$ B cells roll and adhere normally *in vivo* and suggests subsequent processes, such as polarization, are abnormal. The fate of adhered *Swap70*$^{-/-}$ B cells *in vivo* remained unknown.

We speculated that the inability of cells to polarize efficiently after attachment would cause a failure of transmigration across HEVs into lymph nodes. In this case, an accumulation of B cells in the lumen of HEVs might be expected. To test that, we labeled wild-type and *Swap70*$^{-/-}$ B cells with carboxyfluorescein diacetate succinimidyl diester and transferred the cells into wild-type mice. We collected lymph nodes after 30 min and then stained lymph node cryosections for the HEV marker PNAd. Few wild-type B cells were near HEVs, whereas *Swap70*$^{-/-}$ cells were often associated with endothelial cells and were often in the HEV lumen (**Fig. 7c**). We counted transferred B cells as being either closely associated with HEVs (**Fig. 7c**) or unassociated with HEVs. The proportion of *Swap70*$^{-/-}$ B cells closely associated with HEV was nearly twice that of wild-type B cells (**Fig. 7d**). This accumulation of B cells in HEV shows that after attachment *Swap70*$^{-/-}$ B cells failed to move efficiently through the endothelial cell layer.

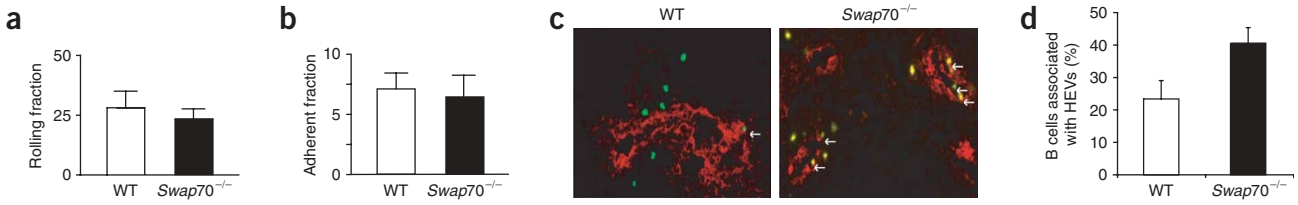

Figure 7 B cell accumulation in HEVs. (**a**,**b**) Intravital microscopy to determine the fraction of rolling (**a**) and adherent (**b**) wild-type and *Swap70*$^{-/-}$ B cells adoptively transferred into wild-type mice. Ten venular branches of greater than the third order were analyzed in four independent experiments (wild-type and *Swap70*$^{-/-}$). (**c**) Typical images of cryosections of lymph nodes from wild-type mice into which carboxyfluorescein diacetate succinimidyl diester–labeled (green) wild-type or *Swap70*$^{-/-}$ cells were transferred. HEVs were stained with anti-PNAd (red). Arrows indicate B cells closely associated with HEVs. Original magnification, ×20. (**d**) Percent wild-type or *Swap70*$^{-/-}$ B cells associated with HEVs. Error bars, s.d. $P = 0.0001$, wild-type versus *Swap70*$^{-/-}$. Data in **c**,**d** were obtained from a minimum of three tissue sections per lymph node from two lymph nodes in three independent experiments.

DISCUSSION

Here we have described a unique function of a signaling protein expressed in B cells. We hypothesized that SWAP-70, a protein capable of interacting with Rac, PtdIns(3,4,5)P_3 and F-actin[20,23,24], regulates B cell migration, adhesion and homing into secondary lymphoid tissues. Indeed, SWAP-70 is required for proper B cell polarization, B cell interactions with other molecules, B cell integrin function and B cell transmigration into lymph nodes. Our study has provided insights into the mechanism(s) by which SWAP-70 regulates lymphoid tissue inflammation *in vivo*.

A first indication for involvement of SWAP-70 in integrin-mediated homotypic aggregation and in adhesion to an extracellular matrix was obtained in studies of cultured mast cells, which also express SWAP-70. However, no data on *Swap70*$^{-/-}$ mast cell interaction with integrin ligands, rolling, spreading, polarization, static or flow adhesion, or homing to lymphoid tissues in inflamed or uninflamed conditions, were reported[25]. After injection into the tail veins of mice, *Swap70*$^{-/-}$ bone marrow–derived mast cells show decreased migration into the peritoneum, which is mainly through reduced chemotaxis[25]. Thus, the nature of the *Swap70*$^{-/-}$ mast cell defect seems to be distinct from that found in *Swap70*$^{-/-}$ B cells.

Here, *Swap70*$^{-/-}$ B cells showed not a complete failure but instead a modest impairment in polarization and attachment *in vitro*. However, that partial defect had a substantial effect on B cell migration *in vivo*. In many transgenic mice, seemingly minor perturbations in cellular activity, obtained by shifting of the balance between cell populations, can have considerable effects on tissue homeostasis and may have profound consequences outside pathogen-free environments. For example, a similar reduction in lymph node homing of adoptively transferred *Dock2*$^{-/-}$ T cells causes a substantial reduction in lymphocyte numbers in lymph nodes[18] and the T cells show impaired entry into lymph nodes and prolonged adhesion to HEVs.

We found that *Swap70*$^{-/-}$ lymph nodes did not substantially increase in size after immune stimulation, demonstrating a requirement for SWAP-70 during inflammation. The involvement of SWAP-70 is at least in part through the regulation of B cell migration into the lymph node, which is required for normal lymph node swelling in response to inflammation[8]. However, mast cells, which also express SWAP-70 and contribute to inflammation-induced lymph node hypertrophy, may also influence the hypertrophic response of *Swap70*$^{-/-}$ mice[36].

Polarization fails when cells do not stabilize their leading edge to a defined region and fail to efficiently form a uropod. Transwell migration assays showed no impairment in *Swap70*$^{-/-}$ B cell migration, which suggests that *Swap70*$^{-/-}$ B cells can polarize and migrate toward a source of chemokine; failure to polarize occurs only after contact with another surface. However, because the assays described here do not discriminate between chemotaxis and chemokinesis, it is possible that *Swap70*$^{-/-}$ B cells fail to move by chemotaxis but still undergo chemokinesis. Notably, a similar phenomenon has been described for *Rac1*$^{-/-}$ neutrophils, which can undergo chemokinesis but fail to show directed migration. In addition, *Rac1*$^{-/-}$ neutrophils form unstable lamellipodia and often have two leading edges. Conversely, *Rac2*$^{-/-}$ B cells show little chemokinesis, but move in a directed way[12].

Stabilization of the leading edge of migrating cells is associated with the accumulation of PtdIns(3,4,5)P_3 in leading-edge membranes and involves a positive feedback loop requiring activation of PI(3)K and Rac1 and polymerization of F-actin[37,38]. As SWAP-70 has been shown to bind Rac1, F-actin and PtdIns(3,4,5)P_3, SWAP-70 might regulate such a positive feedback loop, thereby contributing to leading-edge stabilization. Indeed, although wild-type and *Swap70*$^{-/-}$ activated B cells have similar amounts of Rac1-GTP, the absence of SWAP-70 alters the intracellular localization of Rac1-GTP. Rac localization has been shown to be important for Rac functionality[14].

Attached *Swap70*$^{-/-}$ B cells had defects in forming a distinct urpod, perhaps because of their defects in leading-edge stabilization. It is unclear, however, if SWAP-70 is required directly or indirectly during uropod formation. The structures at the rear of a cell are important for resolution of adhesions and contraction[10]. Myosin II, which is regulated by Rho and Rho kinase, is important for both the contraction of the rear of a cell and for adhesion breakage, which is coupled to protrusive activity at the front of a cell[38,39]. Failure to resolve adhesions might be in part responsible for the accumulation of *Swap70*$^{-/-}$ B cells in HEVs.

Adhesion molecules involved in firm integrin-mediated attachment to endothelial cells are also required for locomotion over and through endothelial cells. Blockade of integrin $\alpha_L\beta_2$ renders monocytes unable to migrate over endothelial cells *in vitro*[40]. Locomotion of B cells is seen on ICAM-1 (ref. 41), and this is likely to be important during their transendothelial migration. Here we have shown that *Swap70*$^{-/-}$ B cells have defective polarization and leading-edge stabilization, as well as increased spreading and lamellipodia formation after adhesion to ICAM-1, VCAM and MAdCAM. Failed leading-edge localization might prevent efficient movement over and through the endothelial cell barrier, thereby resulting in arrest and accumulation in HEVs.

Efficient migration of chemokine stimulated cells on endothelial cells *in vivo* also requires a cellular response to shear stress[42]. Our data have indicated that chemokine-stimulated *Swap70*$^{-/-}$ B cells attached to ICAM-1 have defects in rearranging F-actin and orienting themselves in the direction of flow. This inappropriate response to shear stress is also likely to impair *Swap70*$^{-/-}$ B cell transmigration in HEVs.

It is not apparent at present precisely how B cells move from the apical to basal surface of HEVs. Although most cell types move by means of cell junctions, transcellular migration has also been described and varies depending not only on the migrating cell but also on the type of endothelial cell[43]. There is no primary mouse endothelial cell system that properly represents the endothelial cell layer present in an HEV. Available mouse endothelial cell lines derived from the skin or brain form junctions different from those in the HEV. Thus, exploration of HEV transendothelial migration *in vitro* must await the development of appropriate HEV-derived endothelial systems.

Although B cell entry into lymph node was affected by SWAP-70 deficiency, the total accumulation of B cells during inflammation or after adoptive transfer is also regulated by cell retention in the organ[44]. The fate of *Swap70*$^{-/-}$ B cells after migration into the lymph node remains to be determined. As cell-cell interactions in the lymph node involve many of the integrins involved in transendothelial migration[27], future studies should examine the efficiency of *Swap70*$^{-/-}$ B cell migration throughout the lymph node and analyze *Swap70*$^{-/-}$ B cell immune synapse formation.

METHODS

Mice. *Swap70*$^{-/-}$ mice backcrossed onto a 129/Sv background have been described[31]. Mice were bred in the animal facilities of Mount Sinai School of Medicine (New York, New York) and of the Medical Faculty, Dresden University of Technology (Dresden, Germany), and all experiments were done with the approval of and according to institutional guidelines.

Lymph node hypertrophy. Equal volumes of complete Freund's adjuvant and sterile PBS containing keyhole limpet hemocyanin (2.5 mg/ml) were emulsified and 20 μl was injected into one forward footpad. Then, 3 d after injection, draining and contralateral lymph nodes were collected and then

either cryosectioned, stained and mounted in fluoromont (Southern Biotech) or homogenized, quantified and analyzed on an LSRII flow cytometer (Becton Dickinson).

B cell isolation, culture and chemotaxis. Small resting B cells were isolated from splenic cell suspensions by negative selection with MACS beads (Miltenyi Biotec) or DynaBeads (Dynal Biotech) with equal results. Purity was checked by staining with anti-B220 followed by flow cytometry; only isolates of more than 90% purity were used. Isolated B cells were resuspended at a density of 5×10^5 cells/ml in hybridoma-SFM medium (Invitrogen) supplemented with 5% FCS and stimulated with 5 μg/ml of anti-CD40 and IL-4. Chemotaxis of B cells toward CXCL12, CCL19 and CXCL13 was analyzed in Transwell plates with a pore size of 5 μm (Corning) as described[25].

***In vivo* competitive migration.** Purified wild-type and *Swap70*$^{-/-}$ B cells were labeled with either 5,6-carboxyfluorescein diacetate (Molecular Probes) or PKH-26 red fluorescent cell linker (Sigma). Equal numbers of cells (1×10^7) were injected into the tail veins of 129/Sv mice. After 2, 6 and 24 h, fluorescent cells in lymph node, spleen and blood were quantified by flow cytometry. Fluorescent dyes were reversed with similar results.

Cell aggregation. Images of clusters of B cells were collected 72 h after stimulation in the presence or absence of anti-LFA1 (clone M17/5.2; American Type Culture Collection). Reaggregation assays were done after 72 as described[29].

Cell morphology. After 72 h of stimulation, cells were resuspended at a density of 1×10^5 cells/ml in hybridoma-SFM medium and were added to glass coverslips coated with either anti-CD44 (clone KM201) or anti–immunoglobulin E (clone 84.1.C; both from American Type Culture Collection). Coverslips were coated by incubation overnight at 4 °C in 50 μg/ml of antibody in PBS. For analysis of morphology, cells were allowed to spread for 30 min before fixation by addition of 5% paraformaldehyde. After being imaged on an inverted microscope, cells were made permeable and stained. For analysis of the cytoskeleton, cells were stained with TRITC-phallodin. For analysis of integrin redistribution during spreading, cells were stained with anti-LFA1 (M17/5.2) or anti-CD44 (KM201; both from American Type Culture Collection), followed by the appropriate fluorescein isothiocyanate–labeled secondary antibody, TRITC-phallodin and 4,6-diamidino-2-phenylindole. Cover slips were mounted in fluoromount (Invitrogen) before image acquisition. Time-lapse images were collected beginning at 25 min after attachment; images were collected every 10 s for 20 min with an inverted microscope. During imaging, the temperature was maintained at 37 °C in a heated δT dish (Bioptechs).

Cell attachment. After 96-well plates were coated for 4 h at 4 °C with 20 μg/ml of protein L (Sigma) in PBS, pH 8.5, they were incubated overnight with 10 μg/ml of recombinant Fc–adhesion molecule fusion proteins (Fc-MAdCAM, Fc-ICAM-1 or Fc-VCAM; R&D Systems) in PBS, pH 8.5. Purified B cells were labeled with 5,6-carboxyfluorescein diacetate, added to coated wells and stimulated with 1 μM CCL19 or CCL21 as described[35]. The number of B cells in each well was determined by fluorimetry (Fluostar Optima; BMG Labtech). For analysis of B cell morphology after CCL19-stimulated attachment, for 1 h cells were left in contact with glass coverslips coated as described above, after which they were fixed with 5% paraformaldehyde, made permeable, stained with TRITC-phallodin and mounted in fluoromount. For cell size determination, the diameter of the five largest cells in each field was measured with Axiovision LE software (Carl Zeiss) for a minimum of 75 cells. Further description of cell analysis under flow is in the **Supplementary Methods** online.

Intravital microscopy. Intravital microscopy was done as described[45,46] (detailed description, **Supplementary Methods**).

B cell–HEV interaction in lymph nodes. Purified wild-type or *Swap70*$^{-/-}$ B cells (1×10^7) labeled with 5,6-carboxyfluorescein diacetate were injected into the tail veins of wild-type 129/Sv mice. After 30 min, inguinal and axillary lymph nodes were collected and frozen in optimum cutting temperature compound (Sakura Finetek). Sections 20 μm in thickness were fixed with 5% paraformaldehyde and were stained with anti-PNAd (clone MECA-79; American Type Culture Collection), which was visualized with a TRITC-labeled secondary antibody. Sections were mounted in fluoromount before analysis by confocal microscopy.

Retroviral infection. Internal ribosomal entry site–green fluorescent protein and SWAP-70–internal ribosomal entry site–green fluorescent protein retroviruses were prepared in Phoenix cells and were used to infect B cells as described[47] (details, **Supplementary Methods**).

Statistics. For direct comparison of the activity of wild-type and *Swap70*$^{-/-}$ cells, statistical significance was determined with the Mann-Whitney test for two-tailed data. *P* values less than 0.1 are considered significant, *P* values less than 0.01 are considered very significant and *P* values less than 0.001 are considered highly significant.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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