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Abl Family Kinases Modulate T Cell–Mediated Inflammation and Chemokine-Induced Migration Through a HEF1-Rap1 Signaling Module

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Abstract

Chemokine signaling is critical for T cell function during homeostasis and inflammation, and directs T cell polarity and migration through the activation of specific intracellular pathways. Here, we uncovered a previously uncharacterized role for the Abl family tyrosine kinases Abl and Arg in the regulation of T cell-dependent inflammatory responses, and we showed that the Abl kinases were required for chemokine-induced T cell polarization and migration. Our data demonstrated that Abl and Arg were activated downstream of chemokine receptors and controlled the chemokine-induced tyrosine phosphoylation of human enhancer of filamentation 1(HEF1), an adaptor protein that modulates the activity of the guanosine triphosphatase (GTPase) Rap1. Furthermore, Abl-mediated tyrosine phosphorylation of HEF1 and activation of Rap1 were required for chemokine-induced T cell migration. T cells isolated from conditional knockout mice lacking Abl and Arg exhibited defective homing to lymph nodes and impaired migration to sites of inflammation. These findings suggest that Abl family kinases are potential therapeutic targets for

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the treatment of T cell–dependent immune disorders that are characterized by chemokine-mediated inflammation.

INTRODUCTION

Continuous circulation of T lymphocytes in the blood and secondary lymphoid organs, as well as their migration to sites of inflammation are crucial processes for immune surveillance and protective immunity (1). Chemokines bind to their cognate G protein-coupled receptors (GPCRs) and promote the activation of signaling networks important for actin polymerization and cytoskeletal remodeling that are required for T cell polarity and migration (2). Among the principal intracellular regulators of chemokine-induced polarization and migration of T cells are the small guanosine triphosphatases (GTPases) of the Rap and Rho families (3). Rap1 is activated by distinct guanine nucleotide exchange factors (GEFs) in response to diverse stimuli, which lead to changes in cell adhesion, polarization, and migration (4). Chemokine-induced Rap1 activation in T cells requires the Chat-H adaptor protein, and the interaction of Chat-H with the scaffold protein HEF1 (also known as CasL or NEDD9) is important for chemokine-induced T cell migration (5). Activation of Rap1 by the stimulation of T cells by chemokines functions upstream of the GTPases Cdc42 and Rac1, which are required for T cell polarization (6). Additionally, Rac1 and Rac2 control T cell migration to and within lymph nodes, and Rac-deficient T cells exhibit defective migration in the presence (chemotaxis) and absence (chemokinesis) of a chemokine gradient (7). Although roles for Rap1, Cdc42, and Rac in T cell motility are well-established, the signaling molecules that link the activation of chemokine receptors to the activation of GTPase-dependent pathways critical for actin polymerization and cellular polarization during T cell migration have not been fully elucidated.

The Abl family of tyrosine kinases were first identified as oncogenes in mouse and human leukemias, and subsequent work has shown that the endogenous kinases Abl (Abl1) and Arg (Abl2) act to link activation of diverse cell-surface receptors to the cytoskeletal rearrangements that are critical for cell morphogenesis and motility (8, 9). Abl family kinases are also required for actin-dependent processes that are promoted by pathogens such as *Shigella flexneri* in host cells (10). We reported that Abl family kinases are activated downstream of the T cell receptor (TCR) and are required for maximal TCR signaling (11). T cells lacking Abl and Arg exhibit impaired TCR-induced cell proliferation and cytokine production in vitro and in vivo (12, 13). Abl family kinases modulate T cell adhesion and immunosynapse formation in response to engagement of the TCR (14, 15). Here, we showed that Abl and Arg linked chemokine signals to T cell polarization and migration in vitro and in mice. We identified an Abl-HEF1-Rap1 signaling module that was required for chemokine-induced T cell migration. Thus, we suggest that inhibition of the Abl family kinases may be useful in the treatment of autoimmune diseases that are characterized by T cell-mediated inflammation.

RESULTS

Abl family kinases are required for T cell chemotaxis in vitro and are activated by chemokines

Given the role that Abl family kinases play in the regulation of actin dynamics and cytoskeletal remodeling processes, we questioned whether they were required for T cell motility. We first examined whether Abl family kinases regulated directional T cell migration in response to chemokines. T cells lacking both Abl and Arg exhibited impaired directional migration towards both the CXC chemokine stromal cell-derived factor1 (SDF-1α) and the CC chemokine ligand 21 (**CC**L21) compared to that of wild-type T cells

in an transwell migration assay (Fig. 1A). The impaired chemotaxis of T cells from mice deficient in both Abl and Arg (Abl/Arg null mice) was more substantial than that of T cells from mice singly-deficient in either Abl or Arg (Fig. 1B). These data suggest that Abl and Arg are both required for maximal chemokine-induced T cell migration.

To determine whether the Abl family kinases signaled downstream of chemokine receptors, we examined whether Abl kinase activity was altered after stimulation of cells with chemokine. We found that Abl kinase activity was increased in primary T cells in response to either SDF-1α or CCL21 as was demonstrated by the enhanced phosphorylation of endogenous CrkL at its Abl-specific target site (Fig. 1C). The chemokine-induced increase in CrkL phosphorylation was absent in primary T cells isolated from the T cell-conditional Abl/Arg null mice (Fig. 1D). CCL21-induced CrkL phosphorylation was primarily dependent on Abl, because it was markedly decreased in Abl-deficient, but not Arg-deficient, T cells (fig. S1A).

To determine whether Abl kinase activity was important for regulating T cell migration, we treated human H9 T cells with the Abl family kinase inhibitor STI571 (also known as imatinib) and observed markedly reduced chemotaxis to SDF-1α (Fig. 1E). Chemokine-induced activation of the Abl kinases was dose-dependent and was completely inhibited by STI571 (Fig. 1F). Consistent with previous reports (16), chemokines elicited marked activation of the extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) pathways, but chemokine-induced activation of these pathways was not affected by loss of Abl family kinases in T cells (fig. S1A). It was previously reported that the Src family kinase Lck is required for chemokine-induced chemotaxis in T cells (17). We previously showed that TCR-induced activation of Abl family kinases is Lck-dependent (13). Thus, we evaluated whether chemokine-dependent activation of the Abl kinases required Lck in experiments with Lck-deficient Jurkat J.CaM1.6 cells. In contrast to the parental Jurkat cells, chemokine stimulation failed to activate Abl family kinases in the Lck-deficient cells (fig. S1B).

Abl family kinases are required for T cell chemokinesis

To directly measure chemokine-induced T cell motility (chemokinesis), wild-type and Abl/Arg null T cells were differentially labeled with fluorescent dyes and their motilities were analyzed after they were plated onto surfaces that were coated with bovine serum albumin (BSA) as a control or with intercellular adhesion molecule 1 (ICAM-1), a ligand for the αLβ2 integrin and CCL21, a ligand for the CCR7 chemokine receptor. Whereas wild-type and Abl/Arg null T cells displayed little movement on BSA-coated control wells (movie S1), most (70–80%) wild-type T cells traveled longer distances than did Abl/Arg null T cells on ICAM- and CCL21-coated surfaces (Fig. 2A and movie S2; cell tracking was overlaid in movies S3 and S4). Furthermore, Abl/Arg null T cells exhibited a significant ($p<0.001$)reduction in migration velocity on ICAM-1- and CCL21-coated surfaces compared to that of wild-type cells (Fig. 2B) and they displayed impaired chemokinesis in a transwell migration assay (Fig. 2C). Defective migration of Abl/Arg null T cells was not a result of developmental defects, because conditional deletion of Abl in vitro (fig. S2A) by transduction of T cell blasts derived from $Abl^{flox/flox}/Arg^{-/-}$ mice with retroviruses encoding Cre recombinase resulted in decreased chemokine-induced migration compared to that of vector-transduced, wild-type T cell blasts derived from $Abl^{flox/flox}/Arg^{+/+}$ mice (fig. S2B). Together, these findings showed that Abl family kinases acted downstream of chemokine receptors and were required for T cell chemotaxis and chemokinesis.

Impaired chemokine-induced migration in the absence of Abl family kinases might have been a result of the reduced cell-surface abundance of chemokine and adhesion receptors. To examine this possibility, we measured the cell-surface amounts of CXCR4, the receptor

for SDF-1α, and CCR7, the CCL21 receptor. Chemokine receptor abundances were similar in wild type and Abl/Arg null T cells (fig. S3A). Furthermore, inhibition of Abl family kinases with STI571 did not alter the abundances of CXCR4 or the β1 integrin on the surface of human T cells (figs. S3B and C). Thus, Abl family kinases regulate chemokine-dependent migration without affecting the cell-surface amounts of the chemokine and adhesion receptors that we tested.

Abl family kinases are required for chemokine-induced T cell polarization and F-actin polymerization

Chemokines elicit a polarized T cell morphology that is characterized by a leading edge, where chemokine receptors and activated integrins are clustered, and a trailing uropod that contains accumulated ICAM-3 and CD44 (18). Abl family kinases have been implicated in the regulation of cell shape in neuronal cells and fibroblasts (19). Thus, we stained human H9 T cells for the uropod marker ICAM-3 to examine whether loss of functional Abl family kinases might affect chemokine-induced polarization (Fig. 3A). H9 T cells exhibited relatively high basal polarization, which was further increased by SDF-1α (Fig. 3B). Notably, both resting and SDF-1α-induced polarization were markedly reduced in cells pretreated with STI571 (Fig. 3A and B). Similarly, mouse primary T cells treated with STI571 as well as T cells isolated from Abl/Arg null mice had markedly decreased chemokine-induced polarization compared to that of wild-type T cells (Fig. 3C and movie S2).

Defective chemokine-induced T cell polarization in the absence of the Abl kinases may be due in part to abnormal actin polymerization. We found that human T cells pretreated with STI571 exhibited substantially reduced actin polymerization in response to SDF-1α than did control cells (Fig. 3D). Similarly, Abl/Arg null primary T cells were impaired in their ability to promote actin polymerization in response to CCL21 compared to wild-type T cells (Fig. 3E). T lymphocytes deficient in Rac1 and Rac2 exhibit defective migration and reduced amounts of F-actin in response to chemokine (7), and we previously showed that Abl family kinases mediate Rac activation in response to adhesion signals (20) and bacterial infection (10). Therefore, we analyzed whether chemokine-induced Rac1 activation in primary T cells was modulated by Abl family kinases. We found that T cells treated with STI571 or that were devoid of Abl and Arg proteins exhibited a marked decrease in chemokine-induced Rac1 activity compared to that of wild-type cells (Fig. 3F). Thus, Abl kinases are required for chemokine-induced T cell polarization, which may be mediated in part by Abl- and Arg-dependent Rac1 activation and actin polymerization.

Abl family kinases are required for maximal chemokine-induced Rap1 activation

The GTPase Rap1 is a key component in the regulation of chemokine-induced T cell polarization and migration (4). Futhermore, Rap1 acts upstream of Rac activation in the regulation of cell polarity in T lymphoma cells (6). Thus, we examined whether Abl family kinases mediated Rap1 activation in response to chemokine. To this end, we first examined whether chemokine-induced Rap1 activation was dependent on Abl kinase activity. We found that SDF-1α-induced Rap1 activation was markedly inhibited in human H9 T cells treated with STI571 compared to that in untreated cells (Fig. 4A). Similarly, mouse primary T cells treated with STI571 or isolated from T cell-conditional Abl/Arg null mice were profoundly impaired in chemokine-induced Rap1 activation (Fig. 4B).

To evaluate whether reduced activation of Rap1 in the absence of Abl family kinases was linked to the abnormal chemokine-dependent T cell polarization and migration observed in Abl/Arg null T cells, we analyzed whether these defective phenotypes could be rescued by active Rap1. We transduced H9 T cells with lentiviruses encoding a constitutively active

form of Rap1 (Rap 1a-63E) (21), and cells showing a modest increase in the amount of Rap1 bound to guanosine triphosphate (GTP), the active form of Rap1 (GTP-Rap1) (Fig. 4C) were used in assays of chemokine-induced polarization and migration. Expression of Rap1a-63E substantially restored SDF-1α-induced polarization and migration in the presence of STI571 (Fig. 4D and 4E). In addition, expression of active Rap1a-63E also rescued SDF-1α-induced migration in Abl/Arg null T cells (fig. S4). These results showed that Abl family kinases modulated chemokine-induced Rap1 activation, and that this was sufficient to induce T cell polarization and migration.

Rap1 activation downstream of antigen and chemokine receptors mediates integrin-induced adhesion (22, 23). However, treatment of human T cells with STI571 did not affect their binding to soluble VCAM or their adhesion to VCAM-coated surfaces regardless of whether they were exposed to SDF-1α (figs. S5A, B). The extent of adhesion of Abl/Arg null primary T cells to VCAM and ICAM was similar to that of wild-type cells (figs. S5C, D). Abl/Arg null T cells exhibited slightly reduced adhesion to ICAM in response to CCL21 compared to that of wild-type T cells (fig. S5D). Together, these findings suggest that the markedly reduced migration and polarization of T cells lacking functional Abl kinases is likely to be mediated by pathways independent of integrin-mediated adhesion.

Abl family kinases mediate the tyrosine phosphorylation of HEF1 induced by chemokine signaling

The scaffold protein HEF1 is required for chemokine-induced T cell migration (5). To assess whether HEF1 was also required for chemokine-induced Rap1 activation, we analyzed chemokine-induced Rap1 activation in HEF1-depleted human Molt-4 T cells. We found that HEF1-depleted T cells exhibited a marked decrease in Rap1 activation in response to SDF-1α compared to that of control cells (Fig. 5A). The protein kinase(s) that link chemokine signaling to the tyrosine phosphorylation of HEF1 remain to be identified. The requirement for Abl family kinases in chemokine-induced T cell migration, polarization, and Rap1 activation prompted us to examine whether Abl kinases linked chemokine receptor stimulation to HEF1 phosphorylation. We found that the extent of tyrosine phosphorylation of HEF1 increased in H9 T cells in response to SDF-1α in a dose-dependent manner, and was completely inhibited by STI571 (Fig. 5B). Depletion of Abl family kinases by RNA interference (RNAi) resulted in decreased SDF-1α-induced tyrosine phosphorylation of HEF1 and impaired activation of Rap1 (Fig. 5C). Similarly, SDF-1α-induced tyrosine phosphorylation of HEF1 was markedly decreased in primary mouse T cells lacking Abl and Arg and in wild-type primary T cells treated with STI571 (Fig. 5D). Thus, Abl family kinases mediate chemokine-induced tyrosine phosphorylation of HEF1 and activation of Rap1.

To evaluate whether activated Abl family kinases promoted HEF1 tyrosine phosphorylation in cells, we used a constitutively active form of Abl, Abl-PP, which contains point mutations in the interlinker region (24). Abl-PP, but not the kinase-inactive form of Abl (Abl-KM), induced the tyrosine phosphorylation of HEF1 in transfected human embryonic kidney (HEK) 293T cells (Fig. 6A). Wild-type Abl induced low-level tyrosine phosphorylation of HEF1, which correlated with its reduced kinase activity compared to that of Abl-PP. Furthermore, expression of Abl-PP in H9 T cells enhanced both HEF1 tyrosine phosphorylation and Rap1 activation (fig. S6A). To evaluate whether enhanced tyrosine phosphorylation of HEF1 might promote its interaction with Abl, we performed coimmunoprecipitation experiments. Consistent with a report that the Src homology 2 (SH2) domain of Abl interacts with phosphorylated HEF1 in vitro (25), we found that Abl-PP associated with hemagglutinin (HA)-tagged HEF1 in cotransfected HEK 293T cells (fig. S6B). To assess whether the endogenous Abl and HEF1 proteins interacted with each other, we treated H9 T cells with or without SDF-1α and subjected cell lysates to reciprocal

coimmunoprecipitations. We found that Abl associated with HEF1 only in the SDF-1α treated samples (fig. S6C), thus suggesting that a stable interaction between both proteins may require tyrosine phosphorylation induced by chemokine.

To define the region in HEF1 that was phosphorylated in an Abl-dependent manner, we performed experiments with various recombinant HEF1 fragments (fig. S6D). Active Abl induced the tyrosine phosphorylation of the HEF1 N-terminal region (E and B fragments) encompassing part of the SH3 domain and the entire substrate domain, but did not phosphorylate the serine-rich and C-terminal domains (I and J fragments) (fig. S6E). The N-terminal substrate domain of HEF1 (amino acids 90–350) contains multiple potential sites of tyrosine phosphorylation (26). Mass spectrometric analysis of the HEF1 B fragment co-expressed with Abl-PP revealed that active Abl promoted the phosphorylation of 15 tyrosines within the substrate domain of HEF1 (fig. S7). Abl may induce HEF1 tyrosine phosphorylation in cells indirectly by downstream kinases or it could phosphorylate HEF1 directly. Thus, we used purified Arg to phosphorylate the isolated HEF1-B fragment in vitro and then performed mass spectrometric analysis. Phosphorylation of the HEF1 substrate domain by purified Arg resulted in a marked shift in protein mobility when analyzed by SDS-PAGE (fig. S7A). Mass spectrometric analysis revealed that purified Arg phosphorylated 9 tyrosine residues within the substrate domain of HEF1 (figs. S7B, C). These data suggest that a subset of the tyrosines within the HEF1 substrate domain are directly phosphorylated by Abl family kinases.

Abl-mediated tyrosine phosphorylation of HEF1 is required for chemokine-induced T cell migration

To evaluate whether Abl-mediated phosphorylation of HEF1 was involved in the regulation of chemokine-induced T cell migration, we subjected seven tyrosine (Y) residues on HEF1 that were phosphorylatable by active Abl family kinases to site-directed mutagenesis to phenyalanines (F) (fig. S7C). Phosphorylation of GFP-tagged wild-type HEF1 (GFP-HEF1) by Abl-PP or Arg-PP induced a mobility shift in SDS-PAGE gels, which is characteristic of highly phosphorylated proteins (Fig. 6B). In contrast, GFP-HEF1 was not phosphorylated by kinase-inactive mutants of Abl (Abl-KM) or Arg (Arg-KR) (Fig. 6B). We found that whereas the 3YF-HEF1 mutant (3YF: mutated at tyrosines 164, 166, and 177) exhibited retarded mobility in the presence of active Abl and Arg, mutation of additional tyrosines in the 5YF-HEF1 mutant protein (which contained the same mutations as the 3YF mutant with the addition of mutations in tyrosines 189 and 317) and the 7YF-HEF1 mutant protein (tyrosines 92 and 279 in addition to those of 5YF) abolished the mobility shift of HEF1 in cells coexpressing Abl-PP or Arg-PP (Fig. 6B), which suggested decreased HEF1 phosphorylation.

Next, we analyzed whether loss of specific HEF1 phosphotyrosines could affect T cell migration. To this end, we first depleted human Molt-4 T cells of endogenous HEF1 with lentiviral-mediated, HEF1-specific short hairpin RNA (shRNA). We found that although the amount of endogenous HEF1 protein was reduced only by ~50% in T cells expressing HEF1-specific shRNA (Fig. 6C), T cell migration to SDF-1α was inhibited by ~60% compared to that of control cells expressing scrambled shRNA (Fig. 6D). To evaluate whether Abl- or Arg-mediated phosphorylation of HEF1 played a role in the regulation of chemokine-induced migration, we transfected control and HEF1-depleted T cells with plasmids encoding GFP-tagged wild-type HEF1 or GFP-tagged 7YF-HEF1 (Fig. 6C). Reconstitution of HEF1-depleted cells with wild-type HEF1, but not the 7YF-HEF1 mutant, rescued defective chemokine-induced migration (Fig. 6E). Thus, Abl-mediated tyrosine phosphorylation of HEF1 is required for maximal chemokine-induced T cell migration.

Abl/Arg null T cells exhibit defective homing to lymph nodes and impaired migration to sites of inflammation in vivo

To determine whether Abl family kinases mediated T cell migration in vivo, we first examined whether their loss might affect the distribution of circulating T cells (homing) to lymphoid organs during homeostasis. Wild-type and Abl/Arg null T cells were differentially labeled with fluorescent dyes, and equal numbers of wild-type and null cells were adoptively transferred into C57BL/6 recipient mice. We found that Abl/Arg null T cells consistently exhibited decreased migration to peripheral lymph nodes compared to that of wild-type cells (Fig. 7A). Mice treated with STI571 (imatinib) exhibit a reduced delayed-type hypersensitivity response compared to that of control mice (27). To determine whether Abl family kinases mediated T cell migration in response to inflammation, we used a mouse model of contact hypersensitivity (CHS), which is an inflammatory, T cell-mediated skin reaction to hapten (28). This assay measures the recruitment of hapten-specific T cells to the skin after challenge in sensitized mice, and was modified to enable the direct comparison of the migration of adoptively transferred, fluorescently labeled wild-type and Abl/Arg null T cells. In this model, wild-type cells migrated into the hapten-challenged right ears and exhibited little migration into the vehicle-treated left ears (Fig. 7B, upper panels). Migration of adoptively transferred Abl/Arg null T cells to the right ear was markedly impaired compared to that of wild-type cells (Fig. 7B, lower panels). Quantification of the data showed that migration of Abl/Arg null T cells was ~50% decreased compared to that of wild-type cells (Fig. 7C). Similar results were obtained when equal numbers of differentially labeled wild-type and Abl/Arg null T cells were mixed in the adoptive transfer experiment (fig. S8A). Furthermore, flow cytometric analysis of cells from the draining lymph nodes of the challenged recipient mice revealed decreased numbers of both CD4+ and CD8+ adoptively transferred Abl/Arg null T cell populations compared to those of wild-type T cells (Fig. 7D and fig. S8B). We also performed the CHS assays directly on wild-type and T cell-conditional Abl/Arg null mice and found that, consistent with the adoptive transfer CHS model, the Abl/Arg mutant mice displayed a statistically significant ($p<0.05$) reduction in swelling in the challenged right ear compared to that of wild type mice (fig. S8C).

To determine whether lack of Abl kinases affected the generation of effector T cells in the CHS model, we measured the secretion of interferon-γ (IFN-γ) as a marker of effector T cell function. We isolated the draining lymph nodes of wild-type or Abl/Arg null mice after hapten challenge, and we restimulated total lymph node cells in vitro with plate-bound antibody against CD3 to stimulate the TCR complex. The production of IFN-γ by Abl/Arg null T cells was markedly impaired compared to that of wild-type T cells (Fig. 7E). To evaluate whether the extent of activation of Abl/Arg-deficient and wild-type T cells were equivalent after initial exposure to antigen, we isolated lymph nodes and spleens from mice after initial hapten immunization and analyzed cells for T cell activation markers, integrins, and homing receptors. We did not detect substantial differences in the relative cell-surface abundances of T cell activation markers (CD44, CD62 and CD25), L-selectin, β1 and β2 (LFA) integrins, or chemokine receptors between CD3+ cells isolated from wild type and Abl/Arg null mice (fig. S9). Thus, lack of Abl family kinases did not affect the cell-surface expression of T cell activation markers, integrins, or chemokine receptors after initial immunization and before adoptive transfer. Together, these results demonstrate that Abl family kinases play a role in the regulation of optimal T cell responses to inflammation.

DISCUSSION

Chemokine-induced polarization and motility are essential for T cell trafficking during homeostasis and in response to inflammation (1). Here, we identified a previously unappreciated role for the Abl family of tyrosine kinases as mediators of chemokine signaling required for optimal T cell migration in vitro and in mice. Although it has been

demonstrated that Rap1 is activated in response to chemokines and promotes T cell migration (29), the mechanism linking chemokine receptor engagement to Rap1 activation has not been fully defined. Here, we showed that Abl famiy kinases in T cells were activated by chemokines and were required for chemokine-dependent Rap1 activation. Abl family kinases might regulate Rap1 activation by modulating its GEF activity. In this regard, Abl family kinases phosphorylate the Rap1-GEF, C3G, downstream of TCR engagement (15), and Lyn, another non-receptor tyrosine kinase, controls activation of Rap1 in neutrophils by recruiting the CrkL-C3G complex (30). However, we did not detect increased C3G tyrosine phosphorylation in T cells in response to chemokine. Thus, distinct Rap1 GEFs might be involved in Rap1 activation downstream of chemokine receptors.

Chemokine-induced T cell migration requires the scaffold protein, HEF1. Here, we demonstrated that HEF1 was required for chemokine-induced Rap1 activation and we identified the Abl family kinases as upstream regulators of HEF1 in response to chemokine receptor engagement. The HEF1/CasL protein has been implicated in the regulation of cell polarity, adhesion, motility, and invasion in multiple cell types (31). In lymphocytes, engagement of integrins or the TCR induces HEF1 phosphorylation, which is mediated by Fyn and Lck and is essential for T cell migration (32). Here, we showed that the Abl family kinases phosphorylated HEF1 on multiple tyrosines within its substrate domain in vitro and in cells, and we showed that mutation of seven of these tyrosine residues impaired chemokine-induced T cell migration. HEF1 may function downstream of chemokine receptors to recruit protein complexes required for Rap1 activation at the plasma membrane (5). It is possible that the direct or indirect interaction of Abl family kinases with HEF1 might promote Rap1 activation in response to chemokines through the recruitment of Rap1-specific GEFs to the tyrosine-phosphorylated HEF1 (Fig. 7F). Although Abl kinase activity promotes Rap1 activation in response to TCR or chemokine receptor stimulation in T cells, over-expression of constitutively active Arg inhibits Rap1 activity in epithelial cells (24). Thus, Abl family kinases may promote or dampen Rap1 activity depending on the cellular context and the type of stimuli.

Accumulating reports have demonstrated that pharmacological inhibitors of the Abl family kinases, such as imatinib and nilotinib, which are approved for the treatment of human chronic myeloid leukemia (33), also exhibit immunomodulatory activities (34). Imatinib induces anti-inflammatory effects in diverse autoimmune conditions, including arthritis (35). To date, the therapeutic effects of imatinib and related kinase inhibitors in the treatment of chronic inflammation have been primarily attributed to inhibition of the receptor tyrosine kinases platelet-derived growth factor receptor (PDGFR) and c-Kit (36). Our findings suggest that the Abl family kinases are likely candidates to mediate the immunosuppressive effects of imatinib and related compounds on T cells. Thus, inhibition of Abl family kinases with selective pharmacological inhibitors may attenuate inflammatory responses in immune pathologies driven by chemokine-mediated T cell stimulation.

A role for the Abl-HEF1-Rap1 signaling axis in the transmission of chemokine-induced signals required for T cell polarity, motility, and invasion may extend to cancer cells. In this regard, Abl family kinases, HEF1 (NEDD9/CasL), and Rap1 have been independently implicated in the regulation of cancer cell invasion (26, 37, 38). Furthermore, the chemokine receptors CXCR4 and CCR7 have emerged as key components in tumor metastasis, and inhibition of CXCR4 signaling is being tested as an antimetastatic therapy (39). Thus, disruption of the chemokine-activated Abl-HEF1-Rap1 signaling module with Abl kinase inhibitors may be exploited to effectively impair migration and invasion by T cells and cancer cells.

In summary, our findings have identified the Abl family of tyrosine kinases as mediators of chemokine signaling and the regulation of chemokine-induced T cell migration. Aberrant T cell activation is a common feature of autoimmune disorders and other conditions associated with increased T cell motility and invasion into lymphoid and non-lymphoid tissues. We propose that inhibition of the Abl family kinases with available pharmacological inhibitors may represent a useful strategy in the treatment of autoimmunity and other pathologies characterized by deregulated chemokine signaling.

MATERIALS AND METHODS

Mice

T cell-conditional Abl/Arg null mice were generated as previously described (12). Briefly, $abl^{flox/flox}$ $arg^{-/-}$ mice were crossed with the Lck-Cre transgenic mice to generate conditional loss of Abl kinases in the T cell lineage. Mice were housed under specific pathogen-free conditions in the Duke University Cancer Center Isolation Facility. All studies with mice followed the protocols reviewed and approved by the Duke Institutional Animal Care and Use Committee.

In vitro T cell migration assays

Transwell migration assays were performed with 5-μm polycarbonate membranes and 6.5-mm inserts (Costar, Corning Inc., Corning NY). T cells were added to the upper chamber and allowed to migrate at 37°C for 3 hours in the presence or absence of chemokines in the bottom chamber alone (to measure chemotaxis) or in both the upper and bottom chambers (to measure chemokinesis). T cells that migrated to the bottom chamber were quantified by flow cytometry. Where indicated, upper chamber inserts were pre-coated with fibronectin (FN) (20 μg/ml) at 37°C for 3 hours. Both SDF-1α and CCL21 were used at concentrations of 30 to100 ng/ml. Where indicated, cells were pre-treated with 10 μM STI571 at 37°C for 1 to 2 hours.

Time-lapse microscopy analysis of live cell migration

Glass-bottom microwell dishes (MatTek Corp. Ashland, MA) were coated with recombinant mouse ICAM-1-Fc (5μg/ml) and CCL21 (0.2 to 2 μg/ml) at 4°C overnight, and blocked with 2% BSA in PBS. Wild-type and Abl/Arg null T cells were differentially labeled with PKH-67 (green) and PKH-26 (red), respectively, and mixed in equal numbers before being plated onto the wells. To exclude the possibility that the dyes could differentially affect cell motility, the dyes were switched for the labeling of wild-type and Abl/Arg null T cells in separate experiments; similar results were obtained regardless of the dyes used for the labeling. Cells were allowed to settle for at least 20 min before recording with a Zeiss Axio Observer Live Cell Station in a chamber at 37°C and with a 5% CO_2 atmosphere. Time-lapse images were collected at 15-s intervals for 30 min. Individual cell movements were tracked, and their velocity was calculated with MetaMorph software (Downingtown, PA).

T cell polarization and F-actin polymerization assays

Chemokine-induced T cell polarization and actin polymerization were assayed as previously described (5, 6), with the modifications detailed in the Supplementary Materials.

GTPase pull-down assays

T cells were stimulated with SDF-1α or CCL21 (100 ng/ml for 30 s) and lysed with 1% NP-40 buffer. Fresh lysates were mixed with Sepharose 4B–coupled GST-Ral-GDS (a gift from Daniel Billadeau, Mayo Clinic, Rochester, MN) for GTP-Rap1 pull-down assays or with Sepharose 4B–coupled GST-p21-activated protein kinase 1-p21 binding domain (GST-

Pak1-PBD) for GTP-Rac1 pull-down assays. After incubation at 4°C for 1 hour, samples were washed and analyzed by SDS-PAGE and Western blotting with antibodies against Rap 1 and Rac1.

T cell homing and CHS assays

T cell homing and CHS assays were performed as described previously (5, 28), with the modifications detailed in the Supplementary Materials.

Statistical analysis

The Student's *t* test was used to determine statistical significance. Significance was defined as $*P < 0.05$, $** P < 0.01$, and $*** P < 0.001$. Protein gels and Western blots were quantified with NIH ImageJ software.

Supplementary Material

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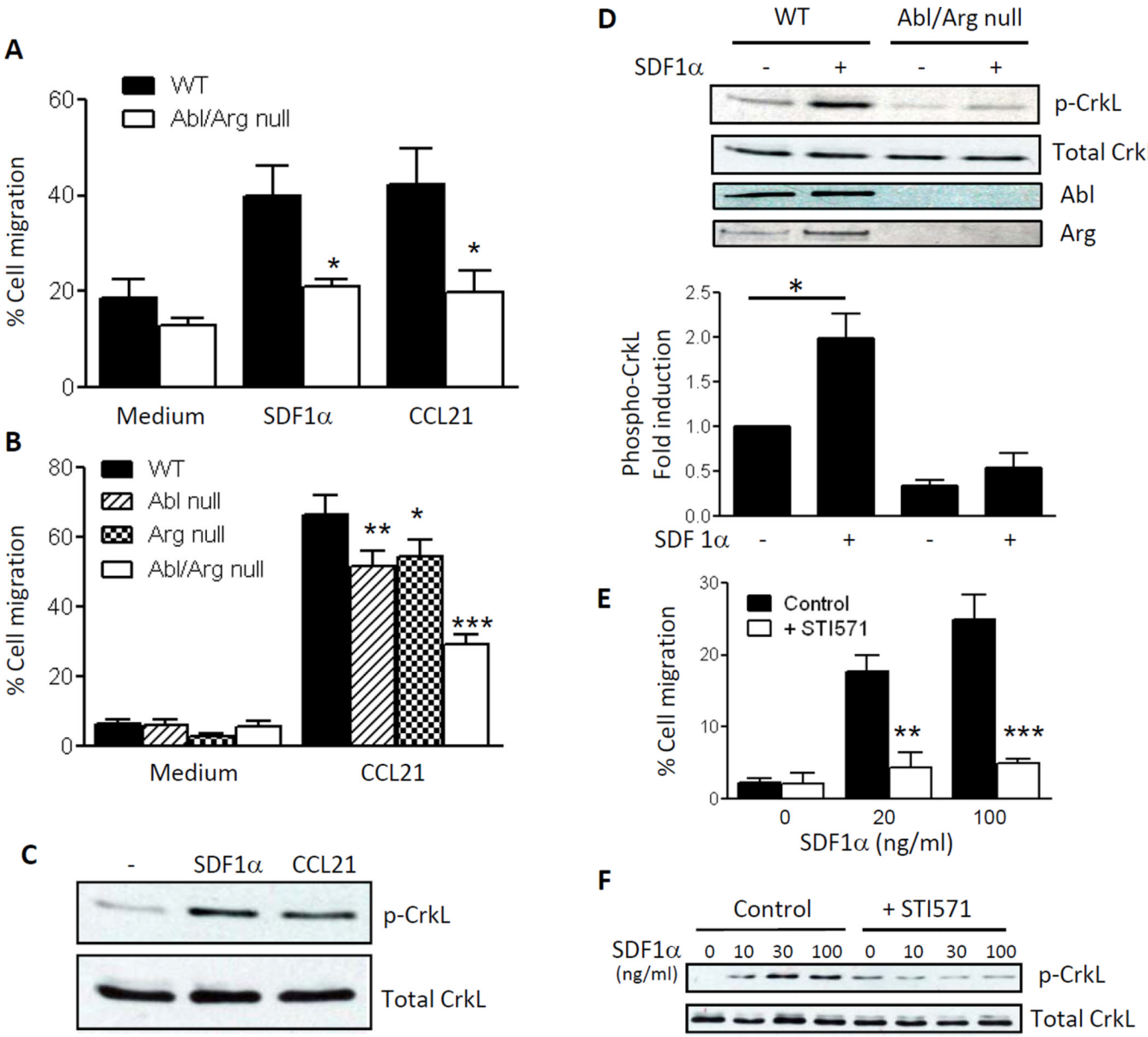

Fig. 1.

Abl family kinases are required for chemokine-induced T cell migration and are activated by chemokines. (**A**) Primary wild-type (WT) and Abl/Arg null T cells were tested for their ability to migrate to either SDF-1α or CCL21 in transwell chambers. Data are means ± SEM (n = 5 experiments). (**B**) WT, Abl-deficient, Arg-deficient, and Abl and Arg doubly deficient (Abl/Arg null) T cells were tested for migration to CCL21. Data are means ± SEM (WT, n=4; $Abl^{-/-}$, n=3; $Arg^{-/-}$, n=4; $Abl/Arg^{-/-}$, n=3 experiments). (**C**) Mouse primary T cells were stimulated with SDF-1α or CCL21 for 30 s, and activation of Abl family kinases was detected by Western blotting analysis of the phosphorylation of CrkL (pCrkL, Y207). Blots were stripped and then analyzed for total CrkL protein. (**D**) WT or Abl/Arg null T cells were stimulated with SDF-1α and analyzed by Western blotting for the presence of pCrkL as described for (C). Total cell lysates were analyzed by Western blotting for Abl and Arg. Quantification of the amount of pCrkL normalized to that of total CrkL is shown in the bar graph below (n = 4 experiments). (**E**) Human H9 T cells that were untreated or were

pretreated with STI571 were plated onto transwell inserts precoated with fibronectin. Migration of the cells to increasing amounts of SDF-1α is shown. Data are means ± SD of triplicate values. (**F**) H9 T cells that were untreated or were pretreated with STI571 were stimulated with SDF-1α at the indicated concentrations for 30 s, and lysates were analyzed by Western blotting for pCrkL as described for (C). Blots were then analyzed for total CrkL protein. Data are representative of three (C and E) or two (F) independent experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

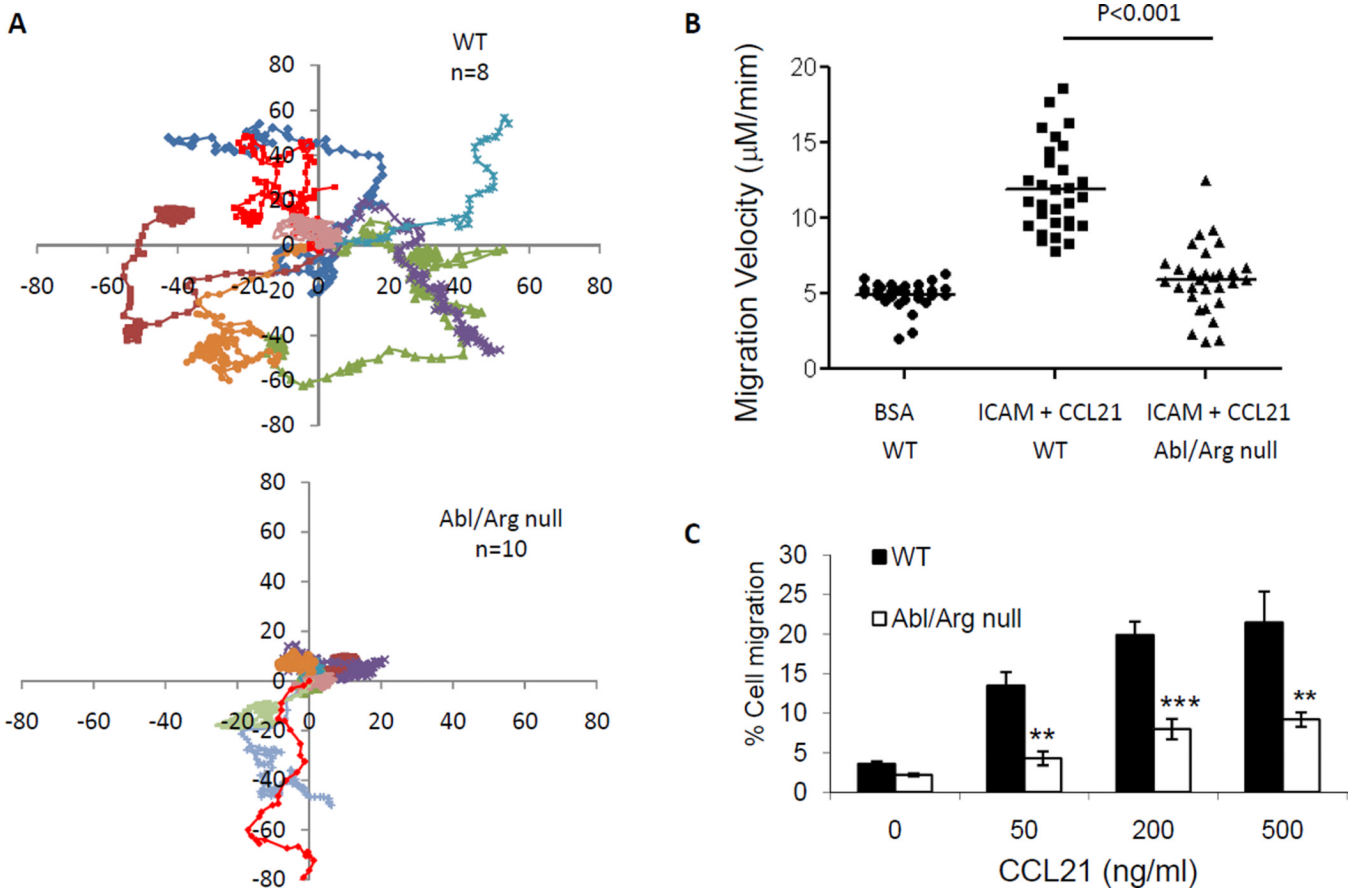

Fig. 2.

Abl/Arg null T cells exhibit decreased migration velocity and impaired chemokinesis. Primary T cells isolated from either WT or Abl/Arg null mice were differentially fluorescently labeled, mixed, and plated onto microwell dishes that were precoated with BSA as a control or with ICAM-1 and CCL21. Cell movement was tracked by time-lapse video microscopy. (**A**) Cell tracking of WT (top) and Ab/Arg null (bottom) T cells on ICAM-1- and CCL21-coated surfaces. (**B**) Migration velocities of WT T cells plated on BSA, or of WT and Abl/Arg null T cells plated on ICAM-1- and CCL21-coated wells were calculated with Metamorph software (n = 30 cells). Data are representative of three independent experiments. (**C**) T cells isolated from either WT or Abl/Arg null mice were tested in a transwell migration assay with the indicated amounts of CCL21 present in both the upper and bottom chambers. Data are representative of four independent experiments and are presented as the mean ± SD of triplicate values.

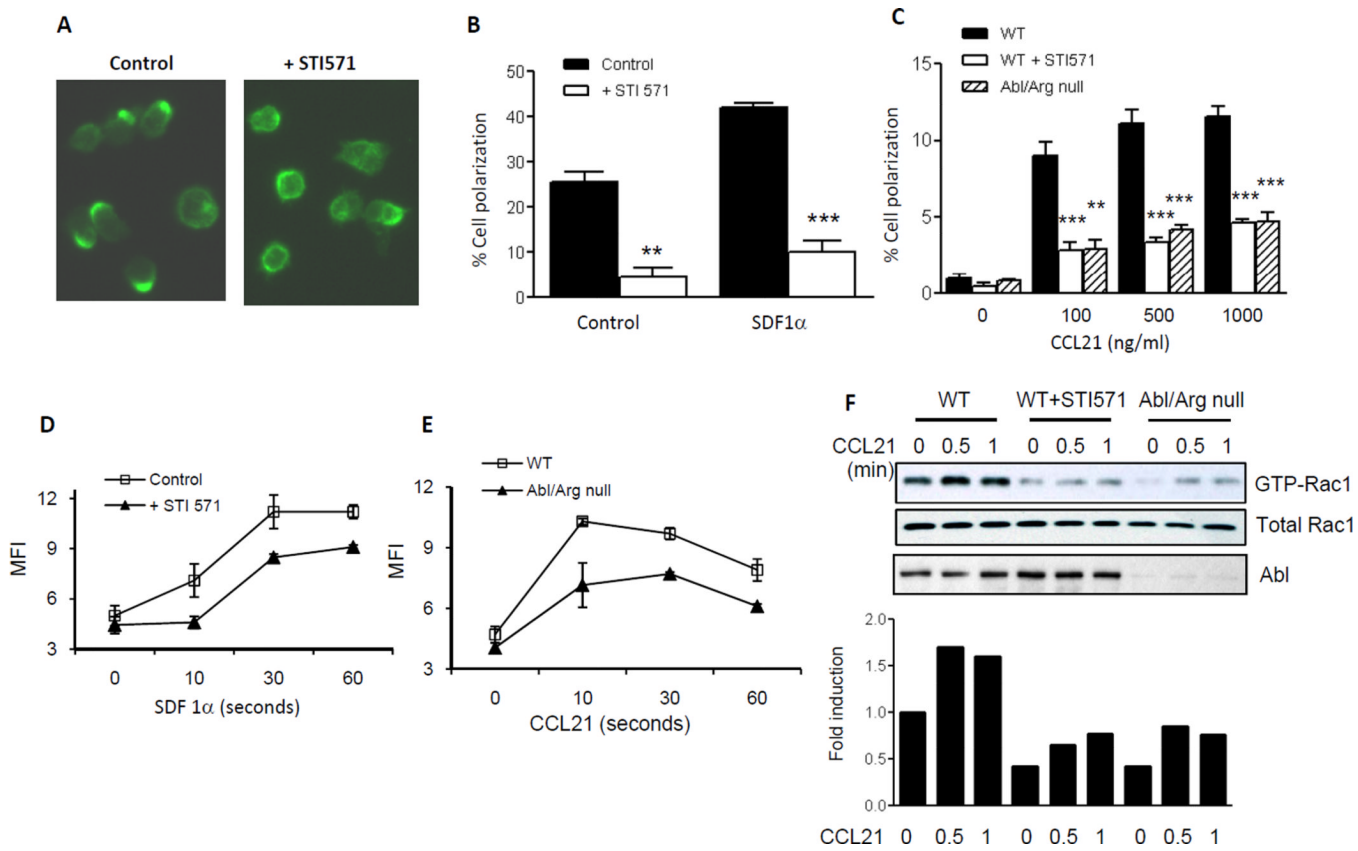

Fig. 3.

Abl family kinases are required for chemokine-induced T cell polarization and F-actin polymerization. (**A**) H9 T cells that were untreated or were pretreated with STI571 were stimulated with SDF-1α. Polarization was analyzed by staining the uropod with antibody against ICAM-3 for human T cells. Representative immunofluorescence images are shown. (**B**) Quantification of polarization for control or STI571-treated H9 T cells in the presence or absence of SDF-1α. Data are presented as mean ± SEM (n=3 experiments). (**C**) WT mouse primary T cells that were untreated or were pretreated with STI571 and Abl/Arg null T cells were stimulated with CCL21 at the indicated concentrations. Polarization was analyzed by staining the uropod with antibody against CD44 for mouse T cells. Data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$. (**D**) Molt-4 T cells that were untreated or were pretreated with STI571 were stimulated with SDF-1α for the indicated times. Cells stained with phalloidin (to detect F-actin) were analyzed by flow cytometry. The y-axis indicates mean fluorescence intensity (MFI). (**E**) WT or Abl/Arg null T cells were stimulated with CCL21, and actin polymerization was assayed as described for (D). Data are means ± SD of triplicate values. (**F**) WT primary T cells that were untreated or were pretreated with STI571 and Abl/Arg null T cells were stimulated with CCL21, and GTP-bound Rac1 was detected by pull-down assay with GST-PBD and analyzed by Western blotting with antibody against Rac1. Total cellular lysates were analyzed by Western blotting for total Rac1 and Abl proteins. Quantification of the amount of GTP-Rac1 normalized to that of total Rac1 l is shown in the bar graph below. Data are representative of three (C and E) or two (D and F) independent experiments.

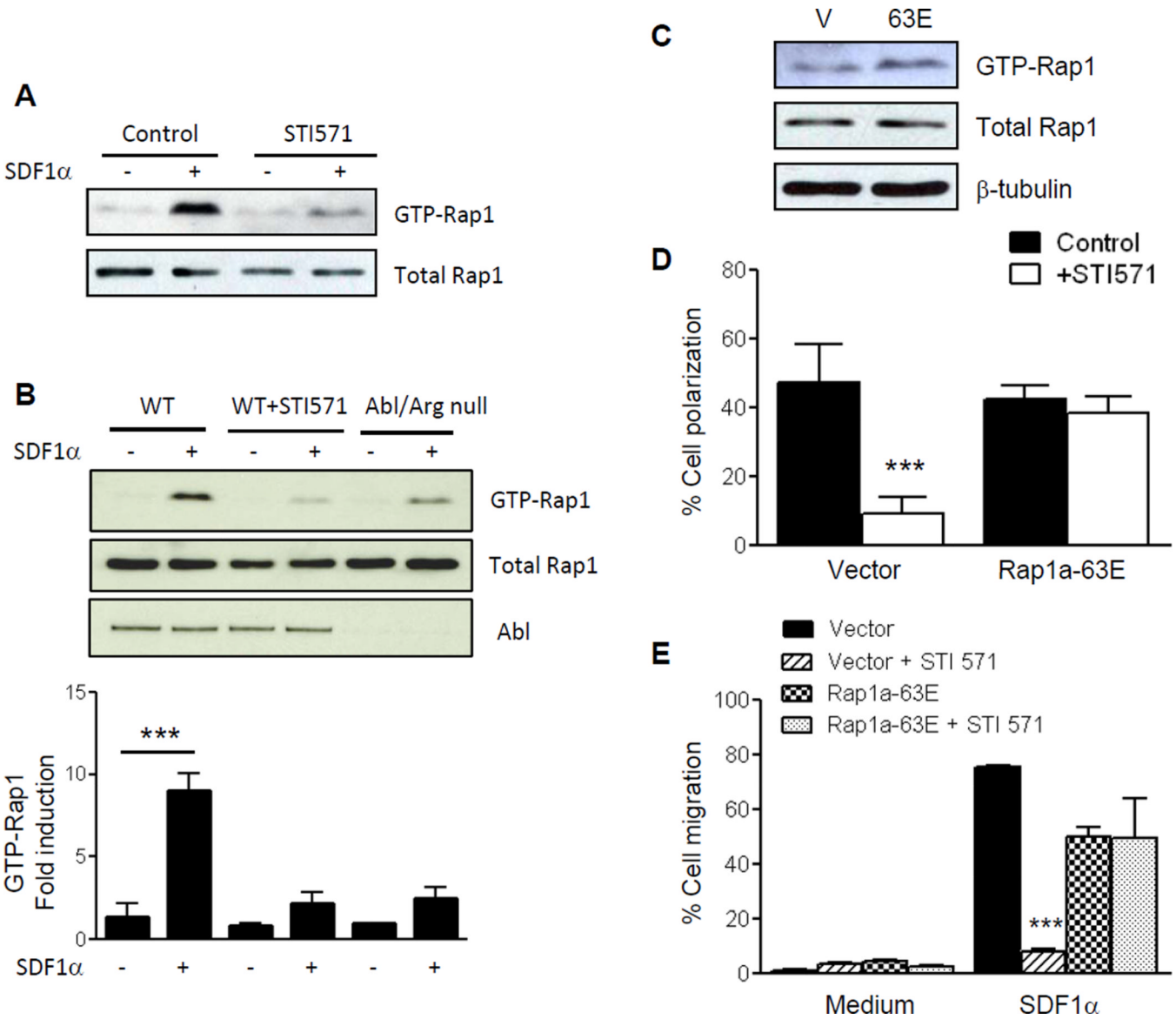

Fig. 4.

Abl-mediated activation of Rap1 in response to chemokine is required for T cell polarization and migration. (**A**) H9 T cells that were untreated or were pretreated with STI571 were stimulated with SDF-1α. GTP-bound Rap1 was pulled down with GST-Ral-GDS and detected by Western blotting analysis with an antibody against Rap1. Data are representative of three independent experiments. (**B**) WT primary T cells that were untreated or were pretreated with STI571 and Abl/Arg null T cells were stimulated with SDF-1α, and GTP-Rap1 amounts were measured as described for (A). Quantification of GTP-Rap1 abundance normalized to that of total Rap1 is shown in the bar graph below (n = 3 experiments). (**C**) H9 T cells were transduced with lentiviruses encoding the pCSCGW-GFP vector (V) or constitutively active Rap1a-63E (63E). Active GTP-bound Rap1 was detected as described for (A), and total cell lysates were analyzed by Western blotting for the presence of Rap1 and β-tubulin. (**D** and **E**) H9 T cells transduced with empty plasmid or with plasmid encoding Rap1a-63E were left untreated or were pretreated with STI571 and then were analyzed for (D) polarization in the presence of SDF-1α and (E) migration in the presence

or absence of SDF-1α. Data are means ± SD of triplicate values and are representative of two independent experiments. ***$P < 0.001$.

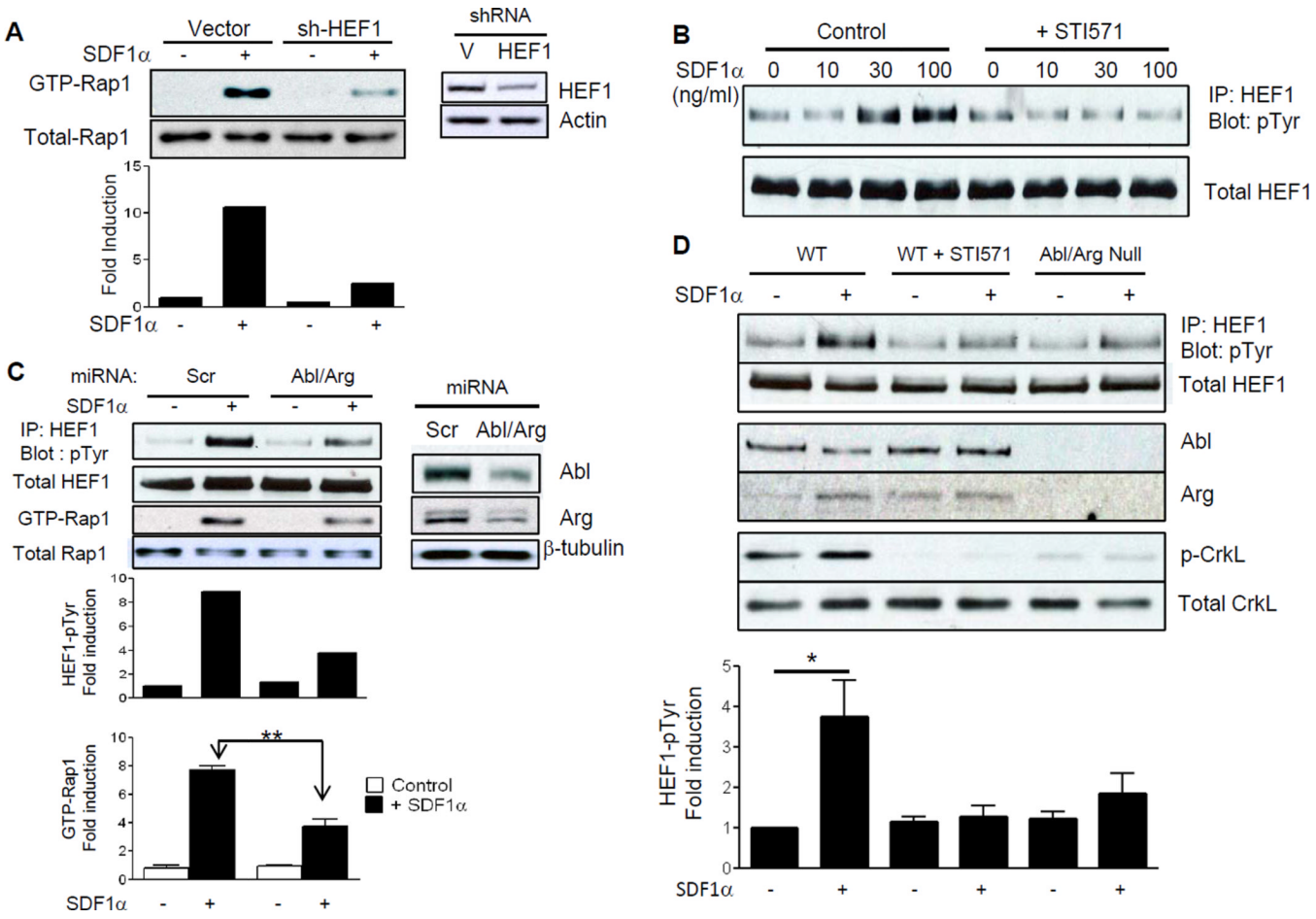

Fig. 5.

Abl kinases are required for chemokine-induced tyrosine phosphorylation of HEF1. (**A**) Human Molt-4 T cells were transduced with lentiviruses encoding either the pLKO vector (V) or HEF1-specific shRNA (sh-HEF). Total cells lysates were analyzed by Western blotting for HEF1 and actin (right panel). Transduced cells were untreated or were stimulated with SDF-1α, and active GTP-Rap1 was detected as described for Fig. 4A. Total cells lysates were analyzed by Western blotting for Rap1 (left panel). Quantification of GTP-Rap1 abundance normalized to that of total Rap1 is shown in the bar graph below. (**B**) Human H9 T cells that were untreated or were pretreated with STI571 were stimulated with SDF-1α at the indicated concentrations for 30 s. HEF1 protein was immunoprecipitated (IP) and samples were analyzed by Western blotting with phospho-tyrosine-specific antibody (pTyr). Total cellular lysates were analyzed by Western blotting for HEF1. (**C**) H9 T cells were transduced with lentiviruses encoding either scrambled miRNA (Scr) or miRNAs specific for Abl and Arg. GFP-sorted cells were left untreated or were stimulated with SDF-1α, and tyrosine phosphorylation of HEF1 was detected as described for (B). GTP-bound Rap1 was analyzed as described for (A). Total cell lysates were analyzed by Western blotting for HEF1 and Rap1 (left panel). Depletion of Abl and Arg in the knockdown cells was verified by Western blotting analysis (right panel). Quantification of the abundance of tyrosine-phosphorylated HEF1 normalized to that of total HEF1 protein and of the amount of GTP-Rap1 normalized to that of total Rap1 is shown in bar graphs below (n = 3 experiments). (**D**) WT primary T cells that were untreated or were pretreated with STI 571 and Abl/Arg null T cells were stimulated with SDF-1α. Tyrosine phosphorylation of HEF1

was detected as described for (B). Total cellular lysates were analyzed by Western blotting for the indicated proteins. Quantification of the abundance of HEF1-pTyr normalized to that of total HEF1 protein is shown in the bar graph below (n = 4 experiments). Data in (A) to (C) are representative of at least two independent experiments.

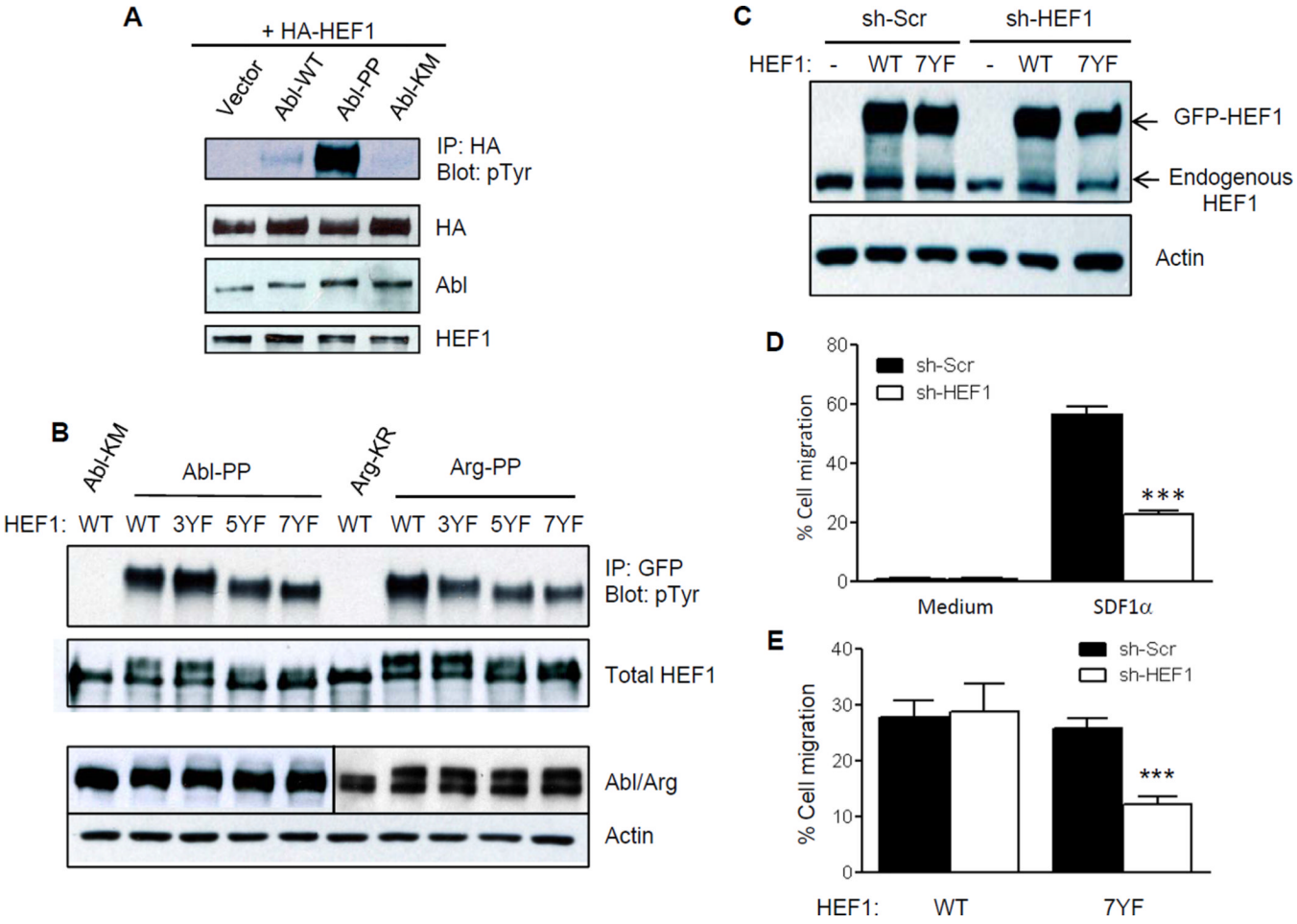

Fig. 6.

Abl-dependent tyrosine phosphorylation of HEF1 is required for chemokine-induced T cell migration. (**A**) **HEK** 293T cells were cotransfected with plasmids encoding HA-tagged HEF1 and the indicated Abl constructs. Phosphorylated HEF1 was detected by immunoprecipitation with an antibody against HA and Western blotting with antibody against pTyr. HEF1 protein was detected by incubating stripped blots with antibody against HA. Total cell lysates were analyzed by Western blotting for Abl and HEF1. (**B**) HEK 293T cells were transfected with plasmids encoding GFP-tagged WT HEF1 or the indicated (Y-F) HEF1 mutants, and cotransfected with plasmids encoding the inactive kinase mutants Abl-KM and Arg-KR or the constitutively active kinases Abl-PP and Arg-PP. Immunoprecipitation of GFP-HEF1 with antibody against GFP was followed by Western blotting analysis with antibody against pTyr. Total cellular lysates were analyzed by Western blotting for the indicated proteins. (**C**) Human Molt-4 T cells were transduced with lentiviruses encoding either the pGZ vector with scrambled shRNA (sh-Scr) or HEF1-specific shRNA (sh-HEF1). These cells were then transfected with plasmids encoding HEF1-WT or HEF1-7YF constructs. Total cellular lysates were analyzed by Western blotting for HEF1 and actin. (**D**) Migration of control and HEF1-depleted T cells in the presence or absence of SDF-1α. (**E**) T cells transduced with lentiviruses expressing the indicated shRNAs and then transfected with plasmids encoding HEF1-WT or HEF1-7YF proteins were analyzed for their ability to migrate in the presence of SDF-1α. Data are means ± SD of triplicate values. Data are representative of three (A and B) or two (C to E) independent experiments. ***$P < 0.001$.

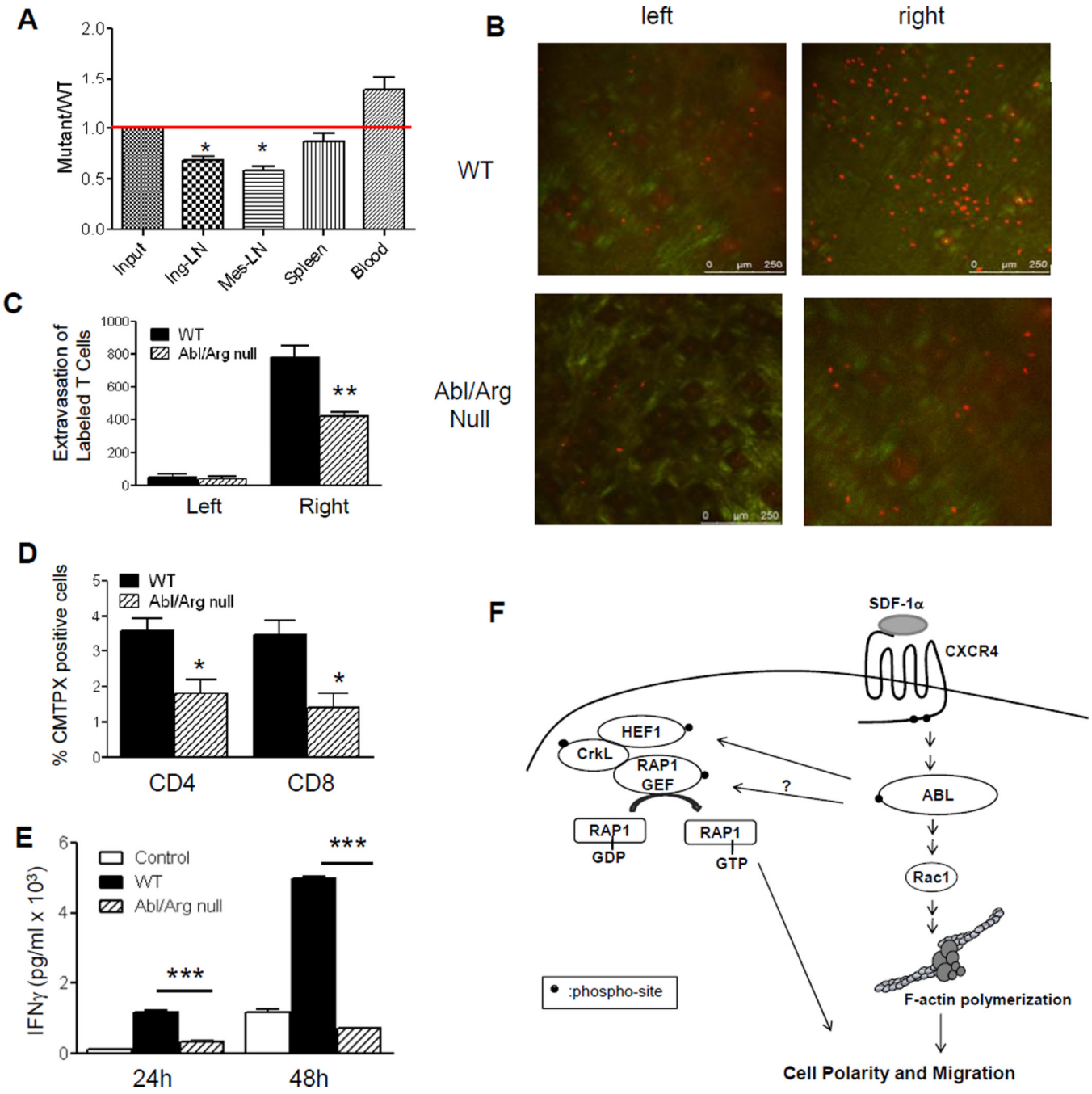

Fig. 7.

Abl/Arg null T cells exhibit defective homing to lymph nodes and impaired migration to sites of inflammation in mice. (**A**) WT or Abl/Arg null T cells were differentially fluorescently labeled and injected into recipient mice. Inguinal lymph nodes (Ing-LN), mesenteric lymph nodes (Mes-LN), spleen, and blood were collected after 2 hours and analyzed by flow cytometry. Input indicates the initial 1:1 ratio of mutant to WT T cells injected. Data are means ± SEM (n = 3 experiments). (**B**) In the adoptive transfer CHS model, extravasation of adoptively transferred CMTPX-labeled T cells (red) in each ear was visualized by confocal microscopy. Representative images are shown. (**C**) Infiltrated and labeled T cells were quantified with Metamorph software. Data are means ± SEM (WT: n=4

mice; Abl/Arg null: n=5 mice). The numbers of infiltrating cells were normalized to the total numbers of cells injected. (**D**) Draining lymph node cells were isolated from recipient mice 20 hours after challenge with hapten. Cells were stained with FITC-conjugated antibodies against CD4 or CD8. Double positive T cells for adoptively transferred CD4-CMTPX or CD8-CMTPX were plotted as percentages of the total numbers of CD4+ or CD8+ T cells. Data are means ± SEM (WT: n=4 mice; Abl/Arg null: n=5 mice). (**E**) WT and Abl/Arg null mice were immunized with hapten, and draining lymph node cells were isolated and stimulated in vitro with antibody against CD3. Culture media were assayed for the presence of IFN-γ by ELISA. Control WT mice were not exposed to hapten. Data are representative of two independent experiments and are presented as means ± SD of triplicates. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (**F**) Proposed model for Abl-mediated signaling during chemokine-induced polarization and migration.