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Impaired function of Fanconi anemia type C-deficient macrophages

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ABSTRACT

FA is a genetic disorder characterized by BM failure, developmental defects, and cancer predisposition. Previous studies suggest that FA patients exhibit alterations in immunologic function. However, it is unclear whether the defects are immune cell-autonomous or secondary to leukopenia from evolving BM failure. Given the central role that macrophages have in the innate immune response, inflammation resolution, and antigen presentation for acquired immunity, we examined whether macrophages from *Fancc*−/− mice exhibit impaired function. Peritoneal inflammation induced by LPS or sodium periodate resulted in reduced monocyte/macrophage recruitment in *Fancc*−/− mice compared with WT controls. *Fancc*−/− mice also had decreased inflammatory monocytes mobilized into the peripheral blood after LPS treatment compared with controls. Furthermore, *Fancc*−/− peritoneal macrophages displayed cell-autonomous defects in function, including impaired adhesion to FN or endothelial cells, reduced chemoattractant-mediated migration, and decreased phagocytosis. Moreover, dysregulated F-actin rearrangement was detected in *Fancc*−/− macrophages after adhesion to FN, which was consistent with an observed reduction in RhoA-GTP levels. Importantly, these data suggest that impaired cytoskeletal rearrangements in *Fancc*−/− macrophages may be the common mechanism responsible for cell-autonomous defects detected in vitro, as well as altered monocyte/macrophage trafficking in vivo. *J. Leukoc. Biol.* 91: 333–340; 2012.

Introduction

FA is a genetic disorder, characterized clinically by progressive BM failure, cancer predisposition, and congenital anomalies. The majority of FA patients has homozygous mutations in one of 15 FA genes, and rare males have a mutation on the X-chromosome [1–4]. FA complementation type C was the first FA gene cloned with subsequent murine model development [5]. Given that the majority of the life-threatening features of FA is hematologic, it is not surprising that *Fancc*−/− mice exhibit several defects in steady-state and stress hematopoiesis. *Fancc*−/− progenitors undergo enhanced apoptosis in response to physiologic concentrations of TNF-α, IFN-γ, ROS, and other inflammatory mediators in vitro and in vivo [6–8]. Moreover, *Fancc*−/− hematopoietic stem cells exhibit an impaired short- and long-term multilineage repopulating ability, reduced self-renewal capacity, increased cycling, and reduced survival [9–11]. Collectively, these findings suggest that primary defects in FA hematopoietic stem and progenitor cell functions contribute to the progressive BM failure observed in patients.

Astute clinical observations have long suggested that FA patients may have immune defects [12], which require intact function of differentiated hematopoietic cells [12–15]. A number of clinical studies indicate that FA patients have altered levels of circulating cytokines [16–18]. In addition, it has been suggested that FA patients may have an increased susceptibility to a variety of pathogens [12], although it is unclear whether this observation is a result of a subtle immunodeficiency or secondary to leukopenia from evolving BM failure. Studies in *Fancc*−/− mice provide support for a primary defect in the immune response in FA. *Fancc*−/− mice challenged in vivo with LPS at doses that induce septic shock have increased peripheral blood levels of inflammatory mediators [14], although it remains unknown what cell type(s) is responsible. Limited data suggest that *Fancc*−/− macrophage function may be altered. *Fancc*−/− macrophages stimulated in vitro with IFN-γ and LPS have increased iNOS expression and nitrite release [13]. In addition, *Fancc*−/− splenic macrophages overexpress TNF-α in response to TLR8 agonists [15]. Although these studies suggest dysfunction of *Fancc*−/− macrophages in vitro,

Abbreviations: BM=bone marrow, FA=Fanconi anemia, *Fancc*−/−=Fanconi anemia-type C-deficient, FN=fibronectin, IUSM=Indiana University School of Medicine, Mac1=Macrophage 1 antigen, MPE=maximum permissible exposure, SOZ=serum-opsonized zymosan

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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other important macrophage functions required for an intact immune response have not been examined.

Macrophages are a primary line of defense in the innate immune system [19]. The biologic functions of macrophages are complex, including elimination of pathogens via phagocytosis and cytokine/chemokine production and repairing damaged tissues during inflammation [19, 20]. Most of these functions require macrophages to migrate to an inflammatory site [20, 21]. Therefore, the ability to adhere and migrate properly is of utmost importance through the macrophage life cycle. At steady-state, monocytes originate from the BM and mobilize into the blood, where cells circulate for several days before lodging in peripheral sites to replenish resident macrophage populations [19]. After an inflammatory stimulus, subsets of monocytes or inflammatory monocytes are actively mobilized from the BM and are recruited to an inflammatory site [19, 20]. Given previous human and murine data suggesting alterations in FA immunity, together with the integral role that macrophages have in orchestrating an intact immune response, we hypothesized that *Fancc*−/− macrophages would exhibit cell-autonomous, functional deficits that contribute to an altered inflammatory response in vivo.

MATERIALS AND METHODS

Animals and peritoneal cell isolation

Fancc−/− mice were described previously [5, 6]. All studies were approved by the IUSM Animal Care and Use Committee (Indianapolis, IN, USA). Age-matched WT and *Fancc*−/− mice were injected i.p. with a single dose of LPS (1 mg/kg) or a single dose of sodium periodate (5 mM, 1 mL) to induce inflammation [14, 22]. Fresh peritoneal cells were isolated for adhesion, migration, and phagocytosis assays, as described previously [22].

RNA isolation and real-time PCR

Total RNA from macrophage was isolated using the RNeasy micro kit (Qiagen, Valencia, CA, USA). Inflammatory gene expression was evaluated by real-time PCR (see Supplemental Material).

Antibodies and reagents

The following antibodies were used for flow cytometry analyses: CD11b (BD PharMingen, San Diego, CA, USA), CD115 (eBioscience, San Diego, CA, USA), F4/80 (Invitrogen, Carlsbad, CA, USA), integrin α4 chain (BD PharMingen), integrin α5 chain (BD PharMingen), integrin β1 chain (BD PharMingen), and PE streptavidin (BD PharMingen). The following antibodies were used for GST pull-down assays: RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Rac1 (BD PharMingen), and Cdc42 (Santa Cruz Biotechnology).

Adhesion assays

Adhesion assays were performed on FN (recombinant human fragment CH296, Takara Bio, Madison, WI, USA) or on endothelial cells as described [23, 24] (see Supplemental Material). Human endothelial colony-forming cells [25] were used as the source of endothelial cells, similar to previous studies [24].

Transwell motility assays

Assays for migration were performed in transwell cell culture chambers with polycarbonate filters (24-well, 8-μm pore, Corning Costar, Cambridge, MA, USA), as described before [23] (see Supplemental Material).

Phagocytosis assay and superoxide detection

Phagocytosis was conducted with *Escherichia coli* bioparticles BODIPY FL conjugate (Sigma-Aldrich, St. Louis, MO, USA). *E. coli* bioparticles (20 mg/mL) were incubated with peritoneal macrophages for 1 h. Phagocytosis was stopped by placing cells on ice, and nonphagocytosed bioparticles were washed off with cold PBS. Cells were fixed with 4% paraformaldehyde and stained with rhodamine phalloidin (Invitrogen) and DAPI. Phagocytosis of *E. coli* was imaged using a fluorescent microscope with a 20× objective lens. Images were quantified by counting at least 100 cells in each condition for each experiment. The production of superoxide was monitored by a lucigenin chemiluminescence assay [22] (see Supplemental Material).

F-actin immunocytochemistry

Macrophage were adhered to glass coverslips previously coated with FN (2 μg/mL) for indicated times. Adherent cells were fixed with 4% paraformaldehyde and stained with rhodamine phalloidin (100 ng/mL). The slides were mounted in mounting solution (Dako, Cambridgeshire, UK) and imaged with confocal microscopy using an Olympus FV1000-MPE confocal/multiphoton microscope.

GST pull-down assay for activated RhoA, Rac1, and Cdc42

For a single, small GTPase activation assay, peritoneal cells from 10 mice/genotype were pooled. Activation of RhoA, Rac1, and Cdc42 was determined using kits from Millipore (Billerica, MA, USA), as described previously [23]. For Western blotting studies, time-lapsed exposure was conducted, and optimal blots were selected for densitometric analysis based on band intensity. Densitometric analysis was conducted using NIH ImageJ software to quantitate arbitrary density units.

Statistical analyses

Parametric data are presented as mean ± SEM, unless otherwise stated. For all data shown, an unpaired Student's *t* test was conducted to evaluate for differences between treatment groups. A P value <0.05 was considered significant.

RESULTS

Fancc−/− mice exhibit attenuated monocyte/macrophage accumulation in vivo

To evaluate whether *Fancc*−/− macrophages exhibit altered trafficking in vivo, we examined macrophage distribution in *Fancc*−/− mice at steady-state and after peritoneal inflammation induced by LPS. At baseline, differential peritoneal cell counts after Giemsa staining indicated that nearly 80% of resident cells were macrophages, and no differences were detected between WT and *Fancc*−/− mice (Supplemental Fig. 1A and B). Flow cytometric analyses showed a similar expression pattern and absolute number of $Mac1^+$, $CD115^+$, and $F4/80^+$ between WT and *Fancc*−/− mice (Supplemental Fig. 1C and D). After peritoneal inflammation induced by LPS, a time-dependent accumulation of monocytes/macrophages was observed (**Fig. 1A**). In WT mice, LPS caused extravasation of monocytes/macrophages into the peritoneal cavity, peaking at 96 h and declining to basal levels by 120 h. *Fancc*−/− mice displayed similar kinetics for monocyte/macrophage accumulation; however, monocyte/macrophage numbers were lower in the later phases of inflammation (72–120 h) compared with WT con-

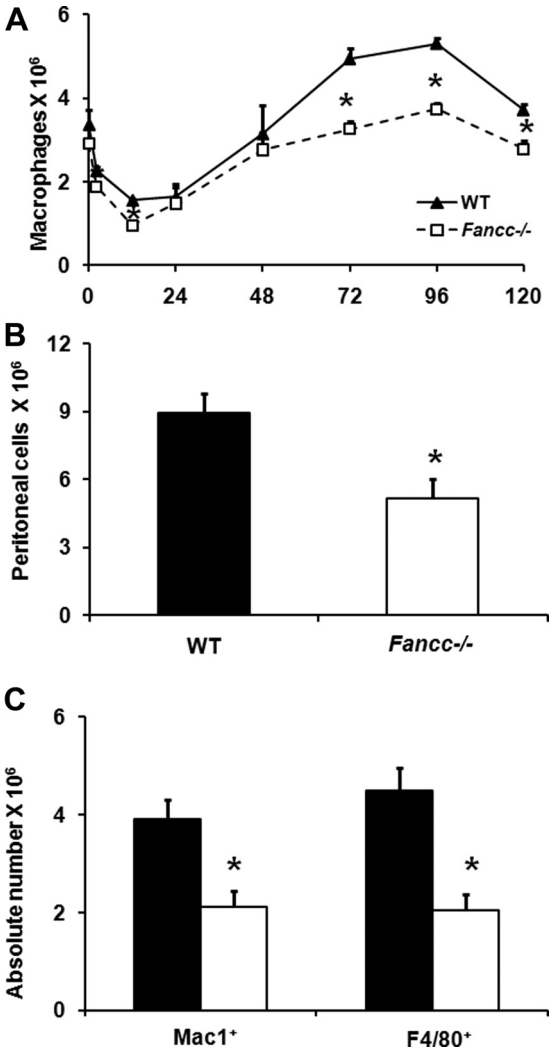

Figure 1. *Fancc*−/− mice exhibit impaired monocyte/macrophage recruitment. (A) LPS induced macrophage recruitment. Total numbers of macrophages in the peritoneum after LPS injection of WT and *Fancc*−/− mice are shown. Macrophage number was calculated by multiplying total peritoneal cells by the percentage of macrophage determined by Giemsa staining. Each time-point includes at least six mice/genotype; $*P < 0.05$. (B) Total number of peritoneal cells and (C) phenotypically defined macrophages in the peritoneum, 96 h after sodium periodate injection of WT (solid bars) and *Fancc*−/− (open bars) mice is shown. At least eight mice/genotype were evaluated; $*P < 0.05$.

trols (Fig. 1A). As a second inflammatory stimulus, WT and *Fancc*−/− mice were injected with sodium periodate [22]. Under these conditions, *Fancc*−/− mice also had fewer peritoneal macrophages, 96 h after injection compared with WT mice (Fig. 1B and C). Together, these data support the hypothesis that macrophage recruitment is altered in *Fancc*−/− mice during inflammation.

Inflammatory monocytes, defined as Mac-1$^+$F4/80$^+$Ly-6C^{high} cells, are mobilized from the BM into the peripheral blood and recruited to inflamed tissues [26]. Therefore, we examined whether *Fancc*−/− mice had altered circulating inflammatory monocytes after LPS treatment. **Figure 2A** illustrates the gating strategy for flow cytometry studies. By 24 h, WT and *Fancc*−/− mice exhibited an increase in the percentage and total number of Mac-1$^+$F4/80$^+$Ly-6C^{high} cells in the peripheral blood compared with untreated controls (Fig. 2B and C). However, *Fancc*−/− mice had fewer circulating Mac-1$^+$F4/80$^+$Ly-6C^{high} cells compared with WT mice (Fig. 2B and C). Collectively, these data demonstrate that monocyte/macrophage recruitment to the inflamed peritoneum is reduced substantially in *Fancc*−/− mice during LPS-induced inflammation, which may be attributed, in part, to reduced monocyte mobilization from the BM.

Fancc−/− peritoneal macrophages exhibit impaired adhesion, migration, and phagocytosis

Our data demonstrate reduced *Fancc*−/− monocyte/macrophage recruitment after LPS-induced peritonitis; however, it is unclear whether this observation is a result of cell-autonomous defects in *Fancc*−/− macrophages. To examine whether *Fancc*−/− macrophages exhibit cell-autonomous defects that contribute to the impaired accumulation in vivo, we tested whether *Fancc*−/− macrophages had altered inflammatory cytokine/chemokine production, adhesion, migration, and/or phagocytosis. LPS-induced inflammatory

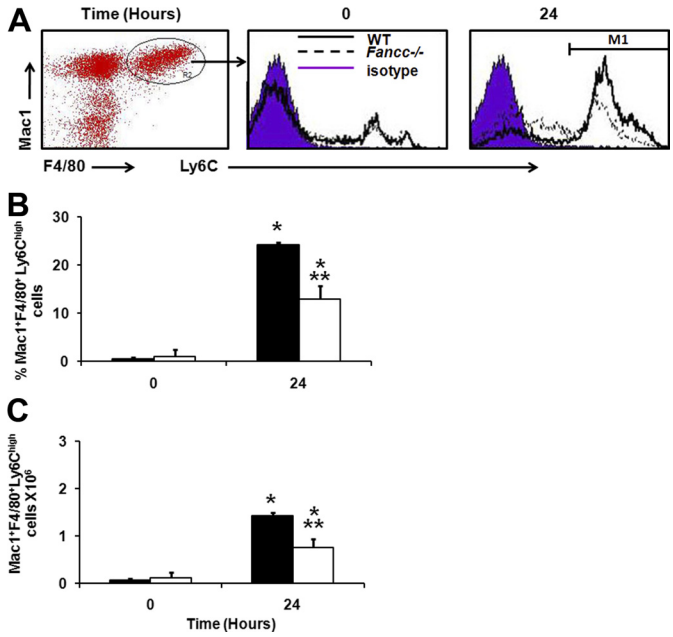

Figure 2. *Fancc*−/− mice have fewer LPS-induced inflammatory monocytes in the peripheral blood. Inflammatory monocytes (Mac1$^+$F4/80$^+$Ly-6C^{high}) were evaluated in the peripheral blood of WT (solid bars) and *Fancc*−/− (open bars) mice at baseline and after LPS treatment. (A) Representative gating strategy for flow cytometry studies is shown. The percentage (B) and absolute number (C) of Mac1$^+$F4/80$^+$Ly-6C^{high} cells in the peripheral blood are shown. Absolute number of Mac1$^+$F4/80$^+$Ly-6C^{high} cells was calculated by multiplying the percentage of Mac1$^+$F4/80$^+$Ly-6C^{high} cells by total white blood cell count; $n = 3$ mice/genotype; $*P < 0.05$ compared with time 0; $**P < 0.05$ compared with WT.

cytokine/chemokine expression was no different between WT and *Fancc*−/− peritoneal macrophages (Supplemental Fig. 2), consistent with previous studies [15]. To evaluate whether adhesion of *Fancc*−/− macrophages is compromised, resident macrophages were adhered to uncoated tissue-culture dishes, FN (an important component of the ECM), or endothelial cells. No differences were detected in the adhesion of *Fancc*−/− and WT macrophage to uncoated tissue-culture plates (data not shown). In contrast, fewer *Fancc*−/− macrophages adhered to FN compared with WT cells (**Fig. 3A** and **B**). Impaired adhesion of *Fancc*−/− macrophage was not a result of altered expression of integrins known to bind FN, including $\alpha4\beta1$ and $\alpha5\beta1$ (Supplemental Fig. 3), suggesting that alterations downstream of ligand-receptor binding may account for decreased adhesion of *Fancc*−/− cells.

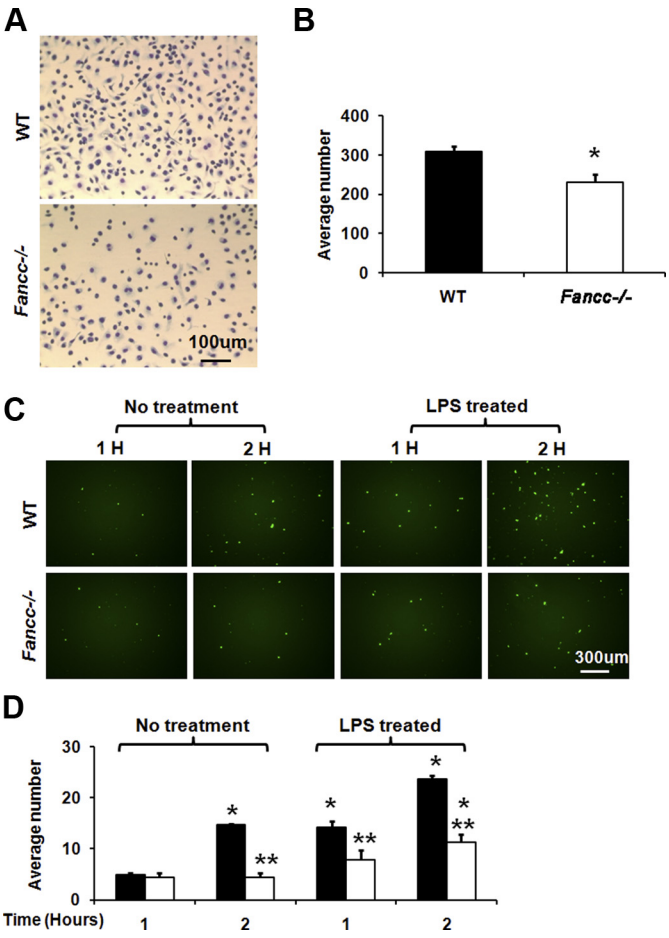

Figure 3. *Fancc* −/− macrophages exhibit decreased adhesion to FN and endothelial cells. Resident macrophages were collected from WT and *Fancc*−/− mice and adhered to FN or endothelial cells. For FN studies, adherent cells were stained with Giemsa to quantify cell number. (A) Representative photomicrographs from FN studies are shown (20× original magnification). (B) Quantified data for FN studies are illustrated; three independent experiments were conducted; $*P < 0.05$. For endothelial cell studies, WT (solid bar) and *Fancc*−/− (open bar) macrophages were labeled with the fluorescent membrane dye, CFSE, prior to coculture with endothelial cells. (C) Representative photographs are illustrated (10× objective). (D) Quantified data for endothelial cell studies are shown; three independent experiments were conducted; $*P < 0.05$ compared with 1-h adherence to untreated endothelial cells; $**P < 0.05$ compared with WT in same endothelial cell condition.

During inflammatory stress, circulating leukocytes are recruited to activated endothelium, where they adhere and traverse between endothelial cells to enter an inflammatory site [27]. Therefore, we next questioned whether cell–cell adhesion was also altered in *Fancc*−/− macrophages by conducting macrophage–endothelial cell coculture assays. In addition to conducting assays under baseline conditions, endothelial cells were stimulated with LPS prior to macrophage coculture to model the up-regulation of adhesion molecules that occurs on endothelial cells during inflammation [28]. In conditions with untreated endothelial cells, WT macrophages had increased adherence after 2 h of coculture compared with the 1-h time-point (Fig. 3C and D). In contrast, *Fancc*−/− macrophages had no observable increase under the same conditions and were decreased significantly compared with WT cells. The impairment in *Fancc*−/− macrophage adhesion was even more apparent when evaluated on LPS-activated endothelial cells. As alterations in adhesion are highly associated with impaired motility, we next examined whether *Fancc*−/− macrophages displayed altered migration. WT and *Fancc*−/− macrophages were subjected to a transwell motility assay with soluble chemoattractants (MCP-1 and M-CSF). Within 24 h of migration, fewer *Fancc*−/− migrated cells were observed in the presence of MCP-1 and M-CSF compared with similar conditions with WT cells (**Fig. 4A** and **B**), suggesting that loss of *Fancc* impairs M-CSF- and MCP-1-induced macrophage migration.

Phagocytosis is a critical biological function that macrophages perform for pathogen clearance and for inflammation resolution. Therefore, we evaluated whether *Fancc*−/− macrophages exhibit impaired phagocytosis by assesseing uptake of fluorescent *E. coli* bioparticles. Compared with WT macrophages, *Fancc*−/− macrophages had reduced cells that ingested fluorescent *E. coli* bioparticles (**Fig. 5A** and **B**), implying dysfunctional phagocytosis mediated by TLR4. As superoxide production occurs during and after phagocytosis, we next examined whether *Fancc*−/− macrophages had decreased superoxide production during phagocytosis. SOZ- and zymosan-induced superoxide production is illustrated in Fig. 5C and D. *Fancc*−/− macrophages had attenuated superoxide production in response to SOZ with a trend toward reduced superoxide production during the uptake of zymosan. Together, these in vitro studies suggest that *Fancc*−/− macrophages exhibit impaired adhesion, migration, and phagocytosis in response to multiple ligands that signal via distinct receptors. Moreover, the identification of *Fancc*−/− macrophage deficits in vitro supports macrophage-autonomous defects in *Fancc*−/− mice, which likely participate in the alterations observed in vivo. Collectively, these data provide strong evidence for a primary defect in the function of the *Fancc*−/− macrophage.

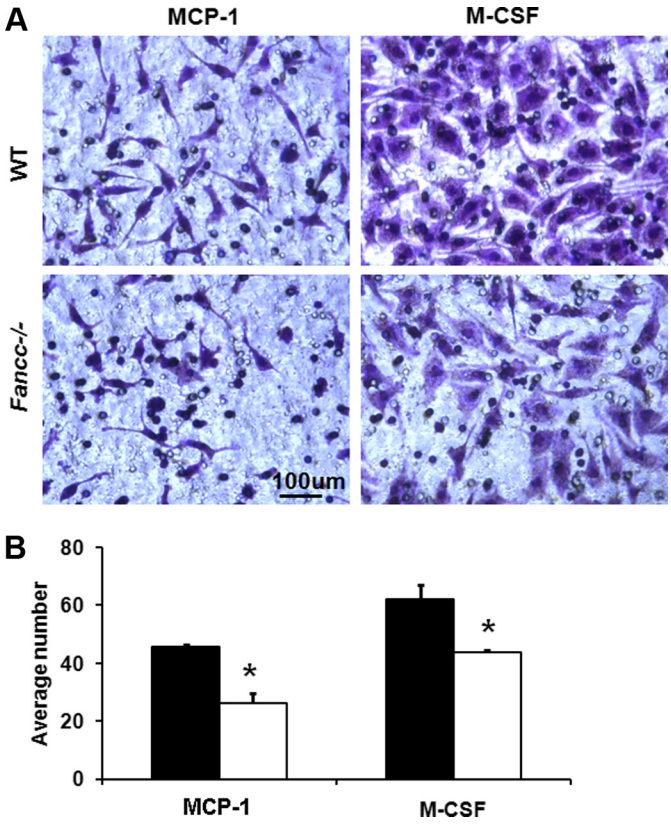

Figure 4. *Fancc*−/− macrophages display impaired migration. Macrophage from WT (solid bars) and *Fancc*−/− (open bars) mice were plated in transwell assays, with the lower chamber containing MCP-1 (20 ng/ml) or M-CSF (50 ng/ml). Migrated cells were stained with crystal violet. (A) Representative photomicrographs of migrated cells are shown (20× original magnification). (B) Quantitation of migrated cells is shown; three independent experiments were conducted; $*P < 0.05$.

The implications of these findings are significant, given the central role that macrophages have in innate and acquired immunity. Furthermore, macrophages orchestrate the initiation and resolution phases of an acute inflammatory response [19]. The severity of inflammation is dependent on pathogen type, efficiency of pathogen clearance, and competent inflammation resolution [29]. Crucial macrophage functions are to kill pathogens, phagocytose apoptotic neutrophils, and repair tissues. Therefore, modest defects in *Fancc*−/− macrophage function may not result in overt immunodeficiency but rather, subtle inefficiencies in pathogen clearance and inflammation resolution, resulting in a predisposition toward chronic inflammation or a lower threshold for progression to systemic inflammation. Studies reporting increased inflammatory cytokines in FA patients are consistent with a chronic inflammatory state [16, 17, 30]. In addition, inflammatory cytokine overproduction as well as hypersensitivity to LPS-induced shock/death are consistent with a lower threshold for progression to a severe systemic inflammatory response in FA [12, 14, 18, 31].

Fancc−/− macrophages exhibit altered actin rearrangements and impaired RhoA activation

Complex cytoskeletal rearrangements are essential for intact adhesion, migration, and phagocytosis [32]. Given that the defects in *Fancc*−/− macrophage were induced by numerous extracellular stimuli (i.e., FN, cytokines, *E. coli*), we speculated that dysregulation of cytoskeletal organization was the unifying mechanism responsible for the functional deficits of *Fancc*−/− macrophage, as well as altered trafficking in vivo. Initially, to test this hypothesis, F-actin content was assessed by rhodamine-labeled phalloidin staining and flow cytometry. *Fancc*−/− resident peritoneal cells and adherent macrophage had normal F-actin content (data not

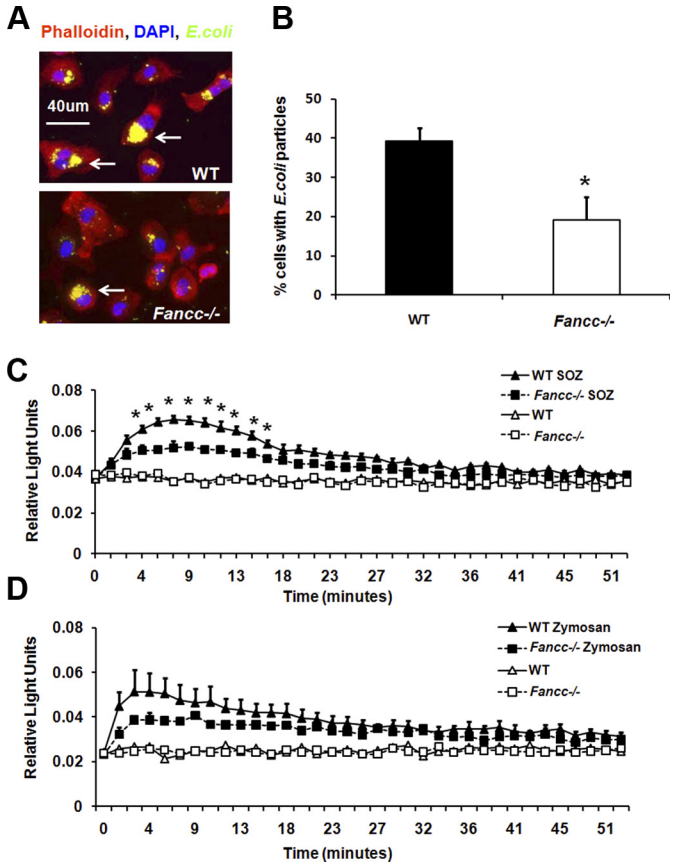

Figure 5. *Fancc*−/− macrophages have attenuated phagocytosis. Resident peritoneal macrophages were collected from WT and *Fancc*−/− mice and adhered to a plastic tissue-culture dish. (A and B) Phagocytosis of *E. coli* bioparticles. Macrophages were cocultured with fluorescent *E. coli* bioparticles and then stained with rhodamine-labeled phalloidin and DAPI. Phagocytosis was evaluated by fluorescent microscopy (20× original magnification). Representative photomicrographs are shown in A. Arrows point to ingested *E. coli* bioparticles. Percent of macrophages that phagocytosed *E.coli* is shown in B; three independent experiments were conducted; $*P < 0.05$ (C and D) Superoxide production after SOZ and zymosan is shown. Macrophages were cultured with SOZ or zymosan at 37°C for 60 min. The production and relative amount of superoxide were monitored and determined by the Lmax microplate luminometer. Three independent experiments were conducted; $*P < 0.05$.

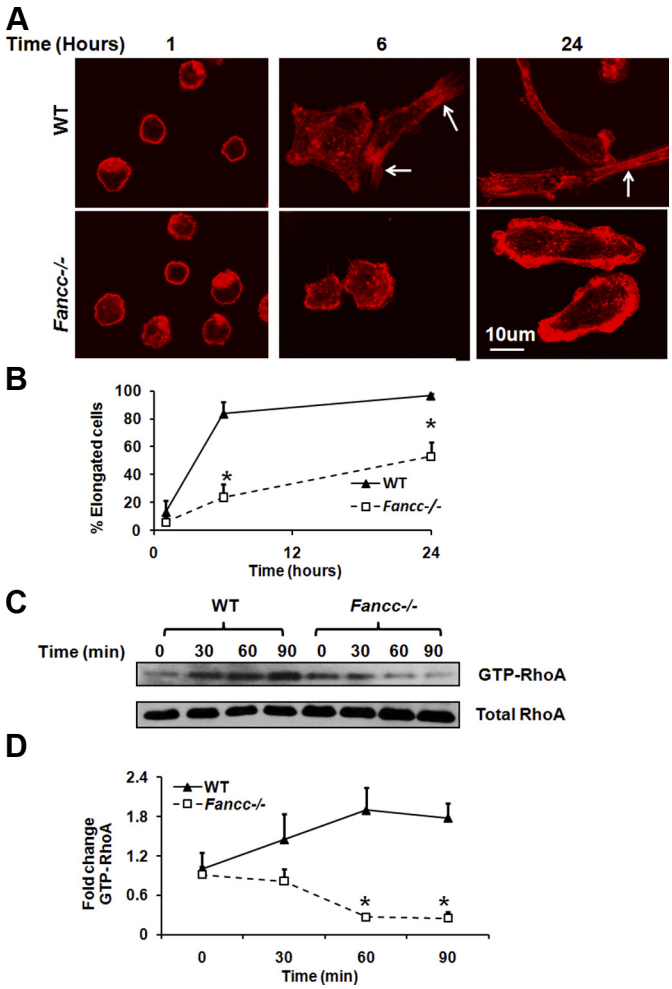

Figure 6. *Fancc*−/− macrophages exhibit impaired F-actin reorganization, cell elongation, and RhoA activation. (A) F-actin localization in macrophages, which were adhered to FN and stained with rhodamine-labeled phalloidin. Representative images using confocal microscopy (Olympus FV1000-MPE confocal/multiphoton microscope, 60× original magnification) are shown. Arrows point to the elongated cells with concentrated staining of phalloidin. (B) The percentage of elongated cells on FN is shown. Three independent experiments were performed; $*P < 0.05$. (C) Representative RhoA activation assay is shown. GTP-RhoA was immunoprecipitated with GST-Rho-binding domain of Rhotekin and detected by RhoA antibody. For a single, small GTPase activation assay, peritoneal cells from 10 mice/genotype were pooled. Three independent experiments were performed. (D) Quantification of densitometry data is shown. GTP-RhoA was normalized to total RhoA, and the relative change was normalized to WT time 0; $n = 3$; $*P < 0.005$.

shown). Subsequently, we examined whether F-actin reorganization was altered in *Fancc*−/− macrophages. F-actin localization was evaluated by rhodamine-labeled phalloidin staining and confocal microscopy. By 1 h of adhesion, WT and *Fancc*−/− cells were round with pericellular F-actin distribution (**Fig. 6A**). WT macrophages began to polarize and elongate from 6 to 24 h and developed multiple protrusions, which were rich in phalloidin staining by 6 h. In contrast, *Fancc*−/− macrophages remained predominantly circular in shape, had fewer protrusions within the entire 24 h, and continued to exhibit pericellular phalloidin staining (Fig. 6A). To quantitate these differences, the percentage of elongated cells was examined. WT cells had a dramatic increase in the percentage of elongated cells within 6 h, compared with the 1-h time-point. Over 90% of WT cells finished cell spreading by 24 h. Interestingly, *Fancc*−/− cells displayed fewer elongated cells by 6 h, with only one-half of them completing cell spreading by 24 h (Fig. 6B), supporting disrupted F-actin cytoskeletal rearrangements in *Fancc*−/− macrophages.

Rho GTPases have a central role in controlling actin cytoskeleton reorganization, cell shape, contractility, and polarity [33–36]. Thus, we examined whether *Fancc*−/− macrophages had impaired activation of small GTPases involved in F-actin regulation. Specifically, Rac1, Cdc42, and RhoA activities were measured in WT and *Fancc*−/− peritoneal macrophages. GTP-Rac1 and GTP-Cdc42 increased 10 min after serum stimulation and declined to basal levels by 30 min, with no significant differences between WT and *Fancc*−/− macrophages (Supplemental Fig. 4). On the other hand, RhoA activation was observed 30 min after stimulation in WT macrophage and was sustained until 90 min. However, in *Fancc*−/− macrophage, RhoA activation was reduced significantly compared with WT cells (Fig. 6C). In Fig. 6C, baseline GTP-RhoA appeared increased in *Fancc*−/− macrophage compared with WT cells. However, this observation was not detected in all experiments, as demonstrated by mean densitometry data (Fig. 6D). The consistent finding in all experiments was that stimulation of *Fancc*−/− macrophages does not appropriately activate RhoA. These data suggest that the reduced adhesion, migration, and phagocytosis may be a result of, at least in part, the impaired RhoA activation. Consistent with these data, macrophages expressing a dominant-negative RhoA mutant exhibit reduced migration and phagocytosis [33, 37]. Collectively, these data suggest that diminished RhoA activation and altered cytoskeletal rearrangements may be the underlying mechanism responsible for altered macrophage functions observed in vitro and in vivo.

DISCUSSION

Although no previous studies have demonstrated alterations in the activation of Rho GTPases in *Fancc*−/− macrophages, a previous study reported decreased cdc42 activity and normal Rac1 activity in human lymphoblastoid cells from FA type A patients, which was associated with decreased adhesion in vitro and homing of hematopoietic cells to the BM in vivo [37]. Our data, together with this previous observation, suggest that dysregulated cytoskeletal organization may have a more global, mechanistic role in the FA hematopoietic phenotype. However, future studies that examine whether expression of a constitutively active RhoA cDNA in *Fancc*−/− macrophage rescues cytoskeletal defects would begin to clarify whether altered RhoA activity was responsible for disrupted *Fancc*−/− macrophage function.

The molecular mechanism by which the Fancc protein regulates macrophage function is not clear. Previous studies show that FA proteins are multifunctional and are involved in the regulation of multiple intracellular signaling pathways, including DNA damage/repair, redox signaling, and cytokine signaling [3, 7, 38]. We found that loss of *Fancc* alters multiple critical macrophage functions, impairs cytoskeletal rearrangements, and reduces RhoA activation. How Fancc regulates RhoA activation remains unknown, although it is possible that Fancc may serve as a chaperone to control molecular events involved in regulating the cytoskeleton, as has been suggested previously for other cytokine signaling pathways [4].

In summary, this study provides compelling evidence for a cell-autonomous defect in *Fancc−/−* macrophages. Specifically, functions requiring dynamic cytoskeletal changes are impaired, including adhesion, migration, and phagocytosis, as well as in vivo inflammatory monocyte mobilization and recruitment. Future studies investigating whether dysregulation of cytoskeletal-based functions exists in other *Fancc−/−* hematopoietic cells are warranted. In addition, these data provide novel insights into FA immunologic dysfunction, which may lead to an improved understanding of FA hematopoietic disease pathogenesis, as well as identify future immunologic, therapeutic targets.

AUTHORSHIP

Y.L. performed research, designed experiments, analyzed data, and prepared the manuscript. K.B., S.K., and E.D-Y. conducted experiments and analyzed data. D.L. provided technical assistance with fluorescent microscopy and confocal microscopy. W.S. provided critical guidance on fluorescent imaging studies and data analysis of cell elongation. L.S.H. designed experiments, analyzed data, and prepared the manuscript.

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DISCLOSURES

The authors have no conflict of interest in this article to declare.

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