IMPAIRED FUNCTION OF FANCONI ANEMIA TYPE C DEFICIENT MACROPHAGES

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ABSTRACT

Ying Liu

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Fanconi anemia (FA) is a genetic disorder characterized by bone marrow (BM) failure. Previous studies suggest that FA patients exhibit alterations in immunologic function. However, it is unclear whether the immune defects are immune cell autonomous or secondary to leucopenia from evolving BM failure. The aim of the current study was to determine whether FA type C deficient (*Fancc-/-*) macrophages exhibit impaired function and contribute to an altered inflammatory response. In this study, primary peritoneal macrophage function and the inflammatory response of *Fancc-/-* immune cells after *in vivo* intraperitoneal (IP) administration of lipopolysaccharide (LPS) were assessed. *Fancc-/-* peritoneum exhibit normal macrophage distribution at baseline. However, *Fancc-/-* macrophages exhibit reduced adhesion both on fibronectin and endothelial cells, impaired migration toward monocyte chemotactic protein-1 (MCP-1) and macrophages-colony stimulating factor (M-CSF), and altered phagocytosis of *E.coli* and ImmunoglobulinG (IgG)-labeled latex beads compared to WT. An altered F-actin reorganization and impaired activation of RhoA were observed in *Fancc-/-* macrophages. After single LPS injection IP, *Fancc-/-* mice exhibited decreased macrophage recruitment, reduced peripheral inflammatory monocytes and impaired myeloid colony formation in presence of M-CSF. Upon M-CSF stimulation, *Fancc-/-* BM derived macrophages (BMDM) showed a decreased phosphorylation of AKT and ERK compared to WT, leading to reduced proliferation. Collectively, these data suggest that *Fancc-/-*

macrophages and subsequent defects in adhesion, migration, phagocytosis, and recruitment *in vivo*. These data also support a *Fancc-/-* macrophage cells autonomous defect predisposing to an altered inflammatory response.

Laura S. Haneline M.D., Chair

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LIST OF ABBREVIATIONS

BMDM	Bone marrow derived macrophage
CFSE	Carboxy-fluorescein diacetate, succinimidyl ester
CFU-G	Colony forming unit–granulocyte
CFU-M	Colony forming unit–macrophage
CFU-GM	Colony forming unit–granulocyte macrophage
CMP	Common myeloid progenitor
DAPI	4',6-Diamidino-2-phenylindole
DEB	Diepoxybutane
FA	Fanconi anemia
FANCC	Fanconi anemia type C
Fancc-/-	FA type C deficient
GEF	Guanosine exchange factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GST	Glutathione S Transferase
HSC	Hematopoietic stem cell
ID	FANCI/FANCD2
IMDM	Iscove modified Dulbecco medium
IP	Intraperitoneally
LPS	Lipopolysaccharide
LSK	$Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage-colony stimulating factor

MMC	Mitomycin C
MMP	Multipotent progenitor
MNC	Mononuclear cells
NO	nitric oxide
PAMPs	Pathogen associated molecular patterns
PBD	P21 binding domain
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRRs	Pathogen recognition receptors
RBD	Rho binding domain
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
SOZ	Serum opsonized zymosan
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 8
TLR8	Toll-like receptor 4

INTRODUCTION

I. Overview of Fanconi anemia

A. Clinical features of FA

Fanconi anemia (FA) was first reported by Dr. Guido Fanconi (Fanconi *et al*., 1927), who described 3 siblings with short stature, skin pigmentation, and pancytopenia. Subsequently, FA is defined as a rare genetic disorder characterized by progressive bone marrow failure, developmental anomalies, and increased predisposition to malignancy (Alter, 2002). Although multiple systems are affected in FA, the life-threatening features of this disease are hematologic. Clinically, 98% of FA patients will develop myelodysplastic syndromes, acute myeloid leukemia or bone marrow (BM) failure by the age of 40 (Young and Alter, 1994). Though blood counts are usually normal at birth, the size of red blood cells is often large (macrocytosis) within the first decade of life. By 20 years of age, over 50% of patients will develop pancytopenia (Young and Alter, 1994). The symptoms appear progressively and often lead to BM failure. The incidence of FA is estimated to be 1 per 350,000 live births (Verlander *et al.*, 1995). Though FA is found in all ethnic groups, it is highly prevalent in two populations: Ashkenazi Jews and the Afrikaans of South Africa (Moustacchi *et al.*, 1990) (Moustacchi *et al.,*2002).

A key cellular feature of FA is chromosomal instability. Chromosomal breakage induced by DNA cross-linking agents, including mitomycin C (MMC) and diepoxybutane (DEB), has been exploited to study mechanisms underlying the disease manifestations and to confirm the clinical suspicion for FA. Indeed, DEB testing of peripheral blood cells is the gold-standard clinical test used to make a definitive diagnosis

of FA. FA cells treated with DEB exhibit marked chromosome breakage on cytogenetic analyses.

The long-term treatment for FA is hematopoietic stem cell transplantation. Over the last two decades, the modifications to standard allogeneic stem cell transplantation have reduced regimen-related toxicity and increased survival . However, finding a suitable donor (HLA compatibility) only occurs in 30% of patients (Gluckman *et al.*, 1995), which obviously remains a major hurdle for treatment. Alternatively, umbilical cord blood offers a potential source of hematopoietic stem cells for FA patients without an HLA match (Broxmeyer *et al.*, 1989). Though hematopoietic stem cell transplantation improves hematologic outcomes, patients are still at increased risk of developing malignancies including head and neck, gynecologic, and gastrointestinal cancers. Therefore, further pathogenetic investigations of FA are needed to improve clinical outcomes for patients.

B. Molecular mechanisms of FA

For the past two decades, extensive investigation has focused on elucidating the function of FA proteins. Current data demonstrate that FA is caused by autosomal recessive mutations in any of 15 FA genes (FANCA, C, D1, D2, E, F, G, I, J, L, M,N and O) (de Winter and Joenje, 2009; Green and Kupfer, 2009; Mace *et al.*, 2007; Smogorzewska *et al.*, 2007; Meindl *et al.*, 2010; Vaz *et al.*, 2010; Stoepker *et al.*, 2011) or a rare X-linked recessive mutation in *FANCB*. FA proteins are involved in a common pathway which plays multiple distinct roles (Li and Stollar, 2007). Eight FA proteins (A, B, C, E, F, G, L, and M) form a nuclear multi-protein core complex after activation by

DNA damage. The FA core complex functions as an E3 ubiquitin ligase and mediates the monoubiquitination of FANCD2 and FANCI (Levitus *et al.*, 2006; Dorsman *et al.*, 2007), the so-called ID complex (FANCI/FANCD2) (Sims *et al.*, 2007). The activated ID complex subsequently co-localizes with downstream FA proteins (FANCD1/BRCA2, FANCN, and FANCJ) and other DNA-repair proteins such as RAD51. Defects in one of the FA core complex proteins results in lack of FA core complex formation and ID complex monoubiquitination.

Understanding FA pathway function in DNA damage response has received significant attention in recent years. However, numerous studies demonstrate that several FA proteins have alternate functions that are independent of DNA damage response. For instance, Fanconi anemia type C (FANCC) has an important role in cellular response to oxidative stress, which is mediated in part through altered redox regulation and ASK1 activation (Saadatzadeh *et al.*, 2004). Other novel investigations have shown that FANCC facilitates phosphorylation of STAT proteins, which has been suggested to indicate that FA proteins participate in cell growth arrest, cell cycle progression, and survival (Pang *et al.*, 2000; Fagerlie *et al.*, 2001; Fagerlie *et al.*, 2004). In addition, FANCC forms a cytoplasmic subcomplex with FANCA, which stabilizes nucleophosmin, a protein that regulates centrosome duplication and genomic stability (Du *et al.*, 2010; Grisendi *et al.*, 2005; Grisendi and Pandolfi, 2005). In addition, FANCC is involved in the regulation of telomere length (Rhee *et al.*, 2010). Collectively, these findings indicate that FANCC possesses functions independent of DNA damage response.

C. Fanconi anemia type C (FANCC)

FANCC is a commonly mutated FA gene, which is conserved among vertebrates (de Winter and Joenje, 2009; Green and Kupfer, 2009; Mace *et al.*, 2007; Smogorzewska *et al.*, 2007; Meindl *et al.*, 2010). FANCC encodes a 63 kDa protein with no recognizable motifs or domains (Patel and Joenje, 2007; Wang, 2007). It is localized in both cytoplasmic and nuclear cellular compartments (Yamashita *et al.*, 1994; Youssoufian, 1994; Hoatlin *et al.*, 1998). The biologic consequences of lacking FANCC have been investigated in a murine model containing homozygous disruption of the *Fancc* gene (Chen *et al.*, 1996). This model recapitulates many aspects of the human disease including hypersensitivity to DNA cross-linking agents (Carreau *et al.*, 1998; Marathi *et al.*, 1996; Haneline *et al.*, 1998; Haneline *et al.*, 2003; Bijangi-Vishehsaraei *et al.*, 2005; Rathbun *et al.*, 1997), defects in hematopoiesis (Haneline *et al.*, 1999; Haneline *et al.*, 2003), and a predisposition to malignancies (Freie *et al.*, 2003; Rogers *et al.*, 2004; van der Heijden *et al.*, 2004; Wreesmann *et al.*, 2007). This model is frequently used as a preclinical platform to examine FA hematologic disease pathogenesis *in vivo*. Specifically, several studies demonstrate that *Fancc-/-* BM cells display the characteristic hypersensitivity to MMC and DEB (Chen *et al.*, 1996; Carreau *et al.*, 1998; Marathi *et al.*, 1996; Haneline *et al.*, 1998; Haneline *et al.*, 2003; Bijangi-Vishehsaraei *et al.*, 2005). *Fancc-/-* hematopoietic stem cells (HSCs) exhibit decreased self-renewal capacity, impaired short- and long-term multilineage repopulating ability, and reduced survival (Li *et al.*, 2007; Haneline *et al.*, 1999; Haneline *et al.*, 1998; Haneline *et al.*, 2003). In addition, *Fancc-/-* progenitors are exquisitely sensitive to low doses of TNF-α, IFN-γ, reactive oxygen species (ROS), and other inflammatory mediators *in vitro* and *in vivo*

(Haneline *et al.*, 1998; Li *et al.*, 2004; Haneline *et al.*, 2003; Haneline *et al.*, 1999). Furthermore, long-term exposure of *Fancc-/-* HSCs to TNF-α causes the outgrowth of cytogenetically abnormal clones and predisposition to leukemic transformation after transplantation (Li *et al.*, 2007). In summary, studies using the *Fancc-/-* murine model have yielded novel insights into hematopoietic disease pathogenesis in FA. Importantly, emerging data from FA patients and *Fancc-/-* mice suggest that FA patients may have defects in immunity as well.

D. Immune defects in FA

A growing body of literature suggests that mutations in FA genes may also impair the immune system, which requires intact function of differentiated hematopoietic cells (Hadjur and Jirik, 2003; Sejas *et al.*, 2007; Zhang *et al.*, 2007). A number of clinical studies indicate that FA patients have altered levels of circulating cytokines (Stark *et al.*, 1993; Bagnara *et al.*, 1993; Rosselli *et al.*, 1994). The most recent study from Dufour and colleagues demonstrated that BM mononuclear cells from FA patients overproduce TNF-α and IFN-γ after *in vitro* activation (Dufour *et al.*, 2003). In addition, it has been suggested that FA patients may have an increased susceptibility to a variety of pathogens (Fagerlie and Bagby, 2006; MacMillan *et al.*, 2000; Dufour *et al.*, 2003; Schultz and Shahidi, 1993; Rosselli *et al.*, 1994; Sejas *et al.*, 2007; Froom *et al.*, 1987; Hersey *et al.*, 1982; Lebbe *et al.*, 1993; Castello *et al.*, 1998; Perussia *et al.*, 1987; Pedersen *et al.*, 1977), though it is unclear whether this observation is due to a subtle immunodeficiency or secondary to leukopenia from evolving BM failure.

Studies in *Fancc-/-* mice provide compelling support for a primary defect in the innate immune response in FA. *Fancc-/-* mice challenged *in vivo* with lipopolysaccharide (LPS) exhibit increased systemic inflammation and death compared to controls (Sejas *et al.*, 2007), though it remains unknown what cells are responsible. Limited data suggest that *Fancc-/-* macrophage function may be altered. *Fancc-/-* macrophages stimulated *in vitro* with IFN-γ and LPS have increased inducible nitric oxide synthase expression and nitrite release (Hadjur and Jirik, 2003). In addition, Vanderwerf *et al*. demonstrated that splenic macrophages from *Fancc-/-* mice overexpress TNF-α in response to Toll-like receptor 8 (TLR8) agonists, but not TLR4 agonists (Vanderwerf *et al.*, 2009). While these studies show dysfunction of *Fancc-/-* macrophages *in vitro*, other important macrophage functions required for an intact immune response were not examined. Furthermore, no studies have been done to examine whether the *in vivo* physiologic function of *Fancc-/-* macrophages is altered. Therefore, the overall hypothesis being tested in this proposal is that *Fancc-/-* macrophages exhibit defective function predisposing to an altered inflammatory response.

II. Monocytes/Macrophages

A. Function and generation of macrophages

The immunologic system is divided into two major branches: the innate immune system and the adaptive immune system. Innate immunity is not specific to the type of organism it fights and is ready to be activated upon initial infection. Macrophages are a major component of innate immunity. They are equipped with a broad range of pathogen recognition receptors (PRR) and are capable of increasing phagocytic efficiency and

inflammatory cytokine production after pathogen exposure (Greaves and Gordon, 2002; Gordon and Read, 2002; Platt *et al.*, 2002). Macrophages identify a "danger" by detecting an organism's pathogen-associated molecular patterns and endogenous signals released from damaged tissues via PRR. Additionally, macrophages also play essential roles in tissue homeostasis at steady state via clearance of apoptotic cells and cellular debris as well as through production of growth factors (Swirski *et al.*, 2009; Gordon and Taylor, 2005). Last but not least, activated macrophages initiate an acute inflammatory response to recruit neutrophils and natural killer cells and to facilitate the maturation, differentiation and migration of dendritic cells, which proceed to activate the adaptive immune response (Ni and O'Neill, 2001; Matzinger, 2002; Whelan *et al.*, 2003). Therefore, macrophages are the unique cell type that serves as a direct link between the innate and adaptive immune systems.

As professional mononuclear phagocytes, macrophages are primarily described as a population of long-lived myeloid cells that are derived from the BM. Like other hematopoietic cells, macrophages originate from HSCs, which sequentially differentiate into multipotent progenitors incapable of self-renewal. Successive lineage commitment steps during monocyte/macrophage development include common myeloid progenitor (CMP), granulocyte macrophage progenitor (GMP), and BM monocytes, which are illustrated in Figure 1(Varol *et al.*, 2007; Iwasaki and Akashi, 2007; Geissmann *et al.*, 2003). CMPs are multipotent cells that generate megakaryocytes, erythrocytes, granulocytes and monoycte/macrophages (Chang, 2009). GMPs are more committed and give rise to granulocytes and macrophages only (Varol *et al.*, 2007; Iwasaki and Akashi, 2007). The process of lineage designation and restriction are regulated by selective and

gradual modulation of lineage-specific transcription factors and cytokine signaling pathways.

Colony stimulating factor-1 [(CSF-1) also known as macrophage colony stimulating factor (M-CSF)] regulates differentiation, proliferation, and survival of the monocyte/macrophage lineage (Bonifer and Hume, 2008). The critical role for M-CSF in macrophage generation is documented in CSF-1 and CSF-1 receptor deficient mice, which have a reduction in several macrophage populations (Umeda *et al.*, 1996) (Yu *et al.*, 2008). As an essential growth factor for macrophage differentiation, M-CSF stimulates BM cells to differentiate into macrophages *in vitro*, bone marrow derived macrophages (BMDM), which serve as a powerful model system to study macrophage differentiation and function (Zhu *et al.*, 2008; Ikeda *et al.*, 2008; Uemura *et al.*, 1993).

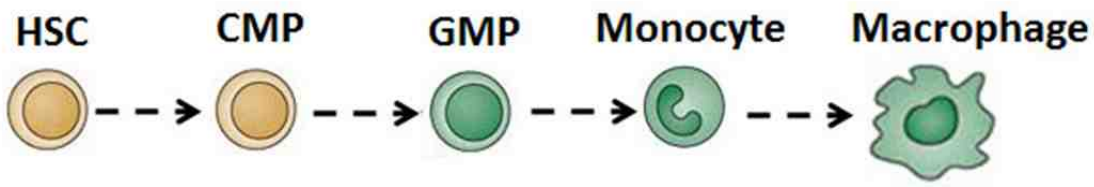
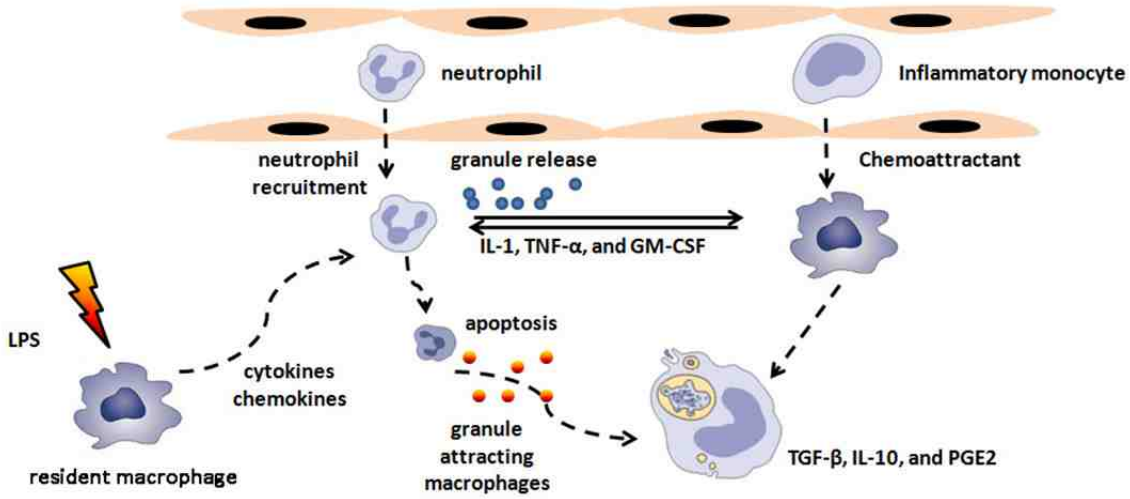


Figure 1. Simplified schematic of monocytes/macrophages generation in the BM. The BM consists of HSCs, which are pluripotent cells capable of self-renewal, and committed progenitor cells. HSCs give rise to CMP that differentiate into GMP which eventually mature into monocytes and macrophages. HSC: hematopoietic stem cell; CMP: common myeloid progenitor; GMP: granulocyte macrophage progenitor.

B. Innate immune response during inflammation

Inflammation is a tightly controlled process that is initiated following tissue injury or infection. The synchronized action of professional phagocytes, e.g., neutrophils, macrophages, and monocytes, is crucial for effective elimination of intruders and cell debris. To initiate an inflammatory response, resident macrophages recognize pathogens and produce inflammatory cytokines/chemokines. Subsequently, neutrophils are recruited during inflammation and release a large array of granules and then become apoptotic, which in turn promotes monocyte influx. After removal of cellular debris, the inflammatory response is terminated by macrophages via release of anti-inflammatory mediators following ingestion of apoptotic neutrophils (Gordon and Taylor, 2005). Multi-functional macrophages also manipulate the adaptive immune response and repair damaged tissues during inflammation (Gordon, 2007; Gordon and Martinez, 2010; Schneberger *et al.*, 2011; Taylor *et al.*, 2005). An illustration of a local inflammatory response, including leukocyte recruitment after LPS challenge, is shown in Figure 2.



Adapted from Soehnlein and Lindbom review, Nature Immunology, 2010

Figure 2. Leukocyte recruitment after LPS treatment. LPS activated resident macrophages release inflammatory cytokines/chemokines which promote the recruitment of neutrophils to the inflamed site. Neutrophils release granules, which recruits monocytes from the peripheral blood and promotes the activation of monocytes to macrophages. Activated macrophages control the life-span of neutrophils by producing GM-CSF and are also responsible for removing apoptotic cells by phagocytosis. Macrophages produce anti-inflammatory cytokines and growth factors during phagocytosis to promote tissue-repair and leave the inflamed site from draining lymph nodes.

C. The generation of monocytes/macrophages during inflammation

During inflammation, the origination of monocytes/macrophages from the BM is influenced by the immunologic storm and stress (Chang, 2009). For instance, *in vivo* studies show that lipopolysaccharide (LPS) is sufficient to activate tissue resident macrophages to produce a large amount of inflammatory cytokines/chemokines. Mobilization of white cells from the BM to the peripheral blood is increased immediately as a result. Inflammatory monocytes are an important inflammatory cell type that is mobilized with subsequent migration towards local inflammatory loci. Based on Ly6C expression, murine monocytes can be differentiated into two discrete subpopulations: $Ly6C^{hi}$ and $Ly6C^{lo}$ (Yona *et al.*, 2010; Ziegler-Heitbrock, 2007; Auffray *et al.*, 2007; Nahrendorf *et al.*, 2007). The ratio of $Ly6C^{lo}$ to $Ly6C^{hi}$ is fairly stable at steady state. However, following inflammation, $Ly6C^{hi}$ monocytes (also known as inflammatory monocytes) egress from the BM into the peripheral blood in a CCR2-dependent fashion (Tsou *et al.*, 2007; Serbina *et al.*, 2003; Serbina and Pamer, 2006; Geissmann *et al.*, 2003), which is likely determined by the inflammatory milieu and PRRs (Auffray *et al.*, 2009).

In vivo studies also demonstrate that local inflammatory response is sufficient to induce an expansion of HSCs and progenitors in the BM as defined by the LSK phenotype (cells negative for lineage markers and positive for Sca1 and c-kit) (Rodriguez *et al.*, 2009). HSC and progenitor expansion is primarily due to enhanced cytokine/chemokine production during inflammation. In addition, Nagai *et al*., showed that CMP and GMP differentiate into $F4/80^{hi}$ macrophages *in vitro* in the presence of LPS or pam3csk4 (a TLR2 agonist) (Abramson *et al.*, 1977), suggesting that TLR ligands

directly regulate hematopoietic cell proliferation and fate determination. In summary, TLR activation of hematopoietic progenitors constitutes an important innate immune response to expand the monocyte/macrophage lineage during an infection. The differentiation and proliferation of monocytes/macrophages are regulated by a network of cytokines, chemokines, and immunological stresses (Figure 3A-B).

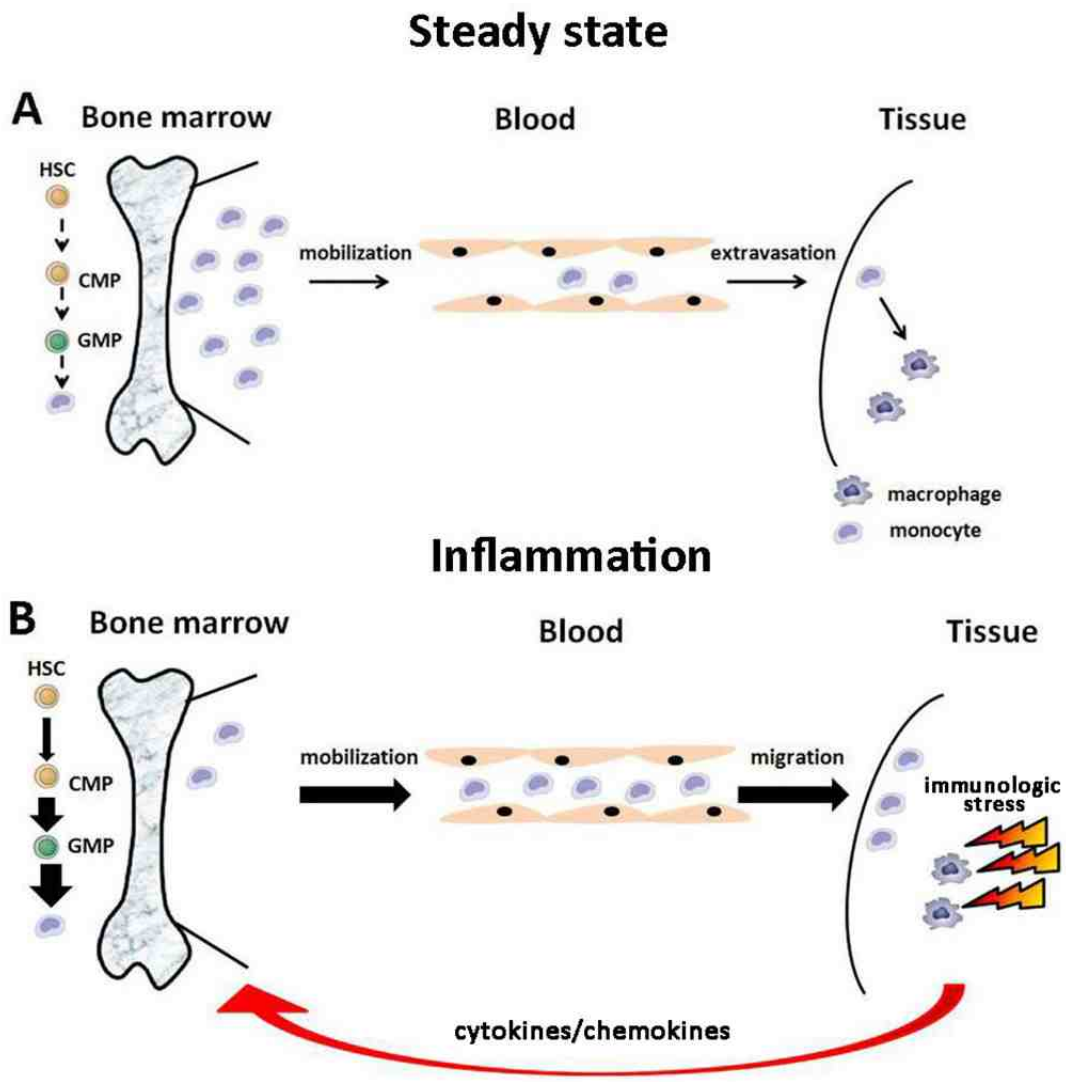


Figure 3. Life-cycle of macrophages. A) Steady state monocyte life-cycle. Monocyte generation from HSCs, mobilization into the peripheral blood, and extravasation into tissues occurs at a relatively low frequency in steady state conditions. B) Life-cycle of monocytes during inflammation. Monocytes in the BM are mobilized quickly after an inflammatory stress and migrate to locally inflamed tissues where they become activated macrophage. Concurrently, expansion of HSCs, CMP, and GMP occurs in the BM after an inflammatory stimulus. Abbreviations: HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte macrophage progenitor.

III. Cytoskeletal reorganization regulates macrophage function

A. Cytoskeletal rearrangement in macrophages

Actin cytoskeletal rearrangement is fundamental for many aspects of immune response to infection. For instance, adhesion, spreading of immune cells on the vascular endothelium, migration to the inflammatory site, and formation of phagosomes are dependent on proper organization of the actin cytoskeleton (Burkhardt *et al.*, 2008; Fooksman *et al.*, 2010; Batista *et al.*, 2010; Hidalgo and Frenette, 2007; Bokoch, 2005). The clinical significance of active reorganization of the actin cytoskeleton in immune cells is accentuated by several human diseases where this process is disrupted. For instance, individuals with mutations in the Wiskott–Aldrich syndrome gene (*WASP*) suffer recurrent infections due to altered cytoskeletal rearrangements resulting in dysfunctional migration, proliferation, and survival of immune cells (Notarangelo *et al.*, 2008; Thrasher and Burns, 2010). Likewise, a dominant-negative mutation in the *RAC2* gene results in recurrent infections due to a cytoskeletal alteration of phagocytes (Williams *et al.*, 2000).

Several cell structures composed of F-actin including lamellipodia and filopodia are present in macrophages. Lamellipodia are thin, broad projections at the edge of a mobile cell and are dynamic structures that constantly change shape. They contain an extensively branched array of actin filaments, oriented with their plus ends toward the plasma membrane. Forward extension of a lamellipodium occurs by growth of actin filaments adjacent to the plasma membrane. Filopodia (microspikes) are long, thin and transient processes that extend out from the cell surface. They are enriched with bundles

of parallel actin filaments that are closely spaced and provide stiffness (DeFife *et al.*, 1999) .

In fact, a successive migration of macrophages requires the synergistic coordination of all cytoskeletal structures described above. Specifically, the forward movement of a cell can be divided into several sequential steps. First is the protrusion of filopodia and lamellipodia, which are full of actin filaments located at the leading edge of a cell. Secondly, the protrusions adhere on the substratum via focal complexes. Lastly, the cell contracts cytoplasmic actomyosin and releases contact sites at the tail of the cell (Allen *et al.*, 1998; Sheetz *et al.*, 1999). Dynamic and integrated coordination of the actin cytoskeleton in macrophages has an essential role in cell movement.

In addition to adhesion and migration having an integral role in regulating the *in vivo* trafficking of monocytes/macrophages, a major function of macrophages once in tissue beds is phagocytosis of cell debris, pathogens, and apoptotic cells (Savill *et al.*, 1989; Dockrell *et al.*, 2001). Phagocytosis is also tightly regulated by the actin cytoskeleton (Yamada *et al.*, 1989; Haberzettl *et al.*, 2007). The phagocytic process is comprised of several sequential and complex events initiated by specific receptors on the surface of phagocytic cells. The signal generated by the receptors clustering in turn leads to the local polymerization of actin filaments and to particle internalization (Niedergang and Chavrier, 2005). Formation of phagosomes requires the assistance of local reorganization of the actin cytoskeleton. Although the mechanisms associated with ingestion are diverse according to different particles and receptors, studies have highlighted the significance of small GTP-binding proteins of the Rho family in phagocytosis (Niedergang and Chavrier, 2005).

B. Regulators of cytoskeletal rearrangement in macrophages

1. Overview of Rho GTPases

Rho GTPases belong to the Ras-like families that link signal transduction pathways through multiple cell-surface receptors to a variety of intracellular signaling proteins. Rho GTPases function as molecular switches between an inactive form (GDP-bound) and an active form (GTP-bound) (Bar-Sagi and Hall, 2000). They are regulated by guanosine exchange factors (GEFs) and GTPase-activating proteins which promote GTP loading and accelerate GTP hydrolysis, respectively (Cerione and Zheng, 1996). Along with a variety of effectors, the GTP-bound Rho GTPases regulate actin cytoskeletal reorganization, cell shape, elongation, polarization, and motility (del Pozo *et al.*, 1999; Yang *et al.*, 2001a). To date, the best studied Rho GTPases are RhoA, Rac, and Cdc42. All of these small GTPases are of importance for cell shape, adhesion, motility and cell-cycle progression. Specifically, RhoA is essential for cell shape and adhesion by assembling contractile actin microfilaments into stress fibers (Hall, 1998). Formation of lamellipodial and filopodial protrusions are mainly dependent on Rac and Cdc42 GTPases (Hall, 1998). In summary, the balance of Rho GTPase activities determines cellular morphology and migratory behavior (Evers *et al.*, 2000).

2. Role of RhoA, Rac, and Cdc42 in macrophages

Three Rho isoforms have been identified in mammals, RhoA, RhoB and RhoC (Wheeler and Ridley, 2004). Macrophages only express RhoA and RhoB (Wheeler and Ridley, 2007), which have distinct cellular localization. RhoA is mainly in the cytosol, complexed to Rho GDP-dissociation inhibitors which maintain Rho GTPases as soluble

inactive cytosolic proteins. RhoB is mainly restricted in membranous compartments (Adamson *et al.*, 1992). Macrophages expressing a RhoA dominant-negative mutant display impaired CR3-mediated phagocytosis and migration. Mice with conditionally deleted RhoA in macrophages have not been described to date. RhoB is unique among this subclass of small GTPases because expression is induced in response to genotoxic agents as well as mitogens (Fritz *et al.*, 1999). Indeed, RhoB is required for DNA-damage-induced apoptosis of mouse embryonic fibroblasts (Liu *et al.*, 2001).

The Rac subfamily of GTPase includes Rac1, Rac2, and Rac3 that share high homology. Macrophages lacking Rac1 are elongated but have normal migration (Wells *et al.*, 2004). Macrophages expressing a Rac1 dominant-negative mutant exhibit impaired migration and Fc-γ-dependent phagocytosis (Jones *et al.*, 1998; Caron and Hall, 1998). Similarly, Rac1/Rac2-null macrophages appear to be elongated and star-like cells with multiple long protrusions, but with normal migration (Wheeler *et al.*, 2006). Macrophages lacking only Rac2 have a relatively normal shape, but are deprived of podosomes, implicating a migration defect upon contact with the extracellular matrix (Linder, 2007). Rac2 null mice display hematopoietic defects in formyl-methionyl-leucyl-phenylalanine-induced migration and superoxide production, chemokine-induced chemotaxis, and cell survival (Yang *et al.*, 2001a; Roberts *et al.*, 1999; Yang *et al.*, 2000; Yang *et al.*, 2001b; Kim and Dinauer, 2001). However, in murine macrophages, studies have shown that Rac1 is the predominant isoform (Yamauchi *et al.*, 2004; Pradip *et al.*, 2003).

In dendritic cells and macrophages, Cdc42 is required for podosome assembly through WASP complex-induced actin polymerization. Indeed, the interaction with

WASP separates Cdc42 from Rac (Weed *et al*., 1998). Macrophages with Cdc42 dominant-negative mutants exhibit disrupted polarity and reduced chemotaxis (Allen *et al.*, 1998). In summary, Rho, Rac, and Cdc42 small GTPases are essential for cell shape, adhesion, and motility of macrophages.

IV. RESEARCH RATIONALE

Although FA hematologic dysfunction is primarily considered to be a disease affecting the function of HSCs and progenitors, studies in both FA patients and FA gene murine models suggest that immune defects exist (Hadjur and Jirik, 2003; Sejas *et al.*, 2007; Zhang *et al.*, 2007; Stark *et al.*, 1993; Bagnara *et al.*, 1993; Rosselli *et al.*, 1994; Dufour *et al.*, 2003; Fagerlie and Bagby, 2006; MacMillan *et al.*, 2000; Schultz and Shahidi, 1993; Froom *et al.*, 1987; Hersey *et al.*, 1982; Lebbe *et al.*, 1993; Castello *et al.*, 1998; Perussia *et al.*, 1987; Pedersen *et al.*, 1977). However, it is not clear from previous studies whether innate immune defects observed in FA are immune cell autonomous or secondary to leukopenia from evolving BM failure. Limited studies in *Fancc-/-* mice provide support for a primary defect in macrophages. *Fancc-/-* mice challenged *in vivo* with LPS have increased serum inflammatory mediators (Sejas *et al.*, 2007; Vanderwerf *et al.*, 2009), though it remains unknown what cells are responsible. *Fancc-/-* macrophages stimulated *in vitro* with IFN-γ and LPS have increased inducible nitric oxide synthase expression and nitrite release (Hadjur and Jirik, 2003). In addition, splenic macrophages from *Fancc-/-* mice overexpress TNF-α in response to toll-like receptor 8 (TLR8) agonists, but not TLR4 agonists (Vanderwerf *et al.*, 2009). While these studies show dysfunction of *Fancc-/-* macrophages *in vitro*, other important macrophage functions required for an intact immune response have not been examined. Furthermore, no studies have examined whether the *in vivo* physiologic function of *Fancc-/-* macrophages is altered. Therefore, the overall goal of these studies was to assess whether *Fancc-/-* macrophages exhibit altered function, thereby leading to an impaired inflammatory response *in vivo*.

V. HYPOTHESIS AND AIMS

Hypothesis: *Fancc-/-* macrophages exhibit defective function predisposing to an altered inflammatory response.

Aim 1: To examine whether *Fancc-/-* mice exhibit an altered inflammatory response *in vivo*.

Aim 2: To determine whether *Fancc-/-* macrophages exhibit cell autonomous defects on adhesion, migration, and phagocytosis.

MATERIALS AND METHODS

1. Mice

Fancc-/- mice were previously described (Chen *et al.*, 1996). *Fancc+/-* mice were intercrossed with C57BI/6J mice for more than 10 generations to develop an inbred strain. Since *Fancc-/-* mice are infertile, *Fancc+/-* mice were bred to generate *Fancc-/-* and WT mice. The genotype of mice was determined by PCR as described (Haneline *et al.*, 1998). The following primers were used: 5'CCTGCCATCTTCAGAATTGT3' (exon 8 primer), 5'GAGCAACACAAATGGTAAGG3' (intron 8 primer), and 5'TTGAATGGAAGGATTGGAGC3' (neomycin resistance gene primer).
These mice were maintained under specific pathogen-free conditions in the Indiana University Laboratory Animal Research Center, Indianapolis, IN. All studies were approved by the Indiana University School of Medicine Animal Care and Use Committee.

2. Treatment of mice and isolation of resident peritoneal cells

Age-matched (6 to 12 weeks) WT and *Fancc-/-* mice were used for all experiments. Mice were injected intraperitoneally (IP) with either a single dose of LPS (1 mg/kg suspended in 500 μL PBS) or a single dose of sodium periodate (5 mM, 1 mL) to induce inflammation (Sejas *et al.*, 2007; Suh *et al.*, 2006). An equal volume of the vehicle control (phosphate buffered saline, PBS) was used for both models. Peritoneal lavage was conducted with 10 mL of ice cold PBS to collect cellular exudates for *in vitro* analyses at times indicated. Peritoneal cells were washed with PBS twice and resuspended in Roswell Park Memorial Institute medium (RPMI) 1640 (Invitrogen, Carlsbad, CA)

containing 10% fetal calf serum (FCS, Lonza Walkersville, Walkersville, MD). Recovered peritoneal cells were diluted and counted on an electronic cell counter (Beckman Coulter). Each sample was counted in duplicate. Cells were then prepared for adhesion, migration, and flow cytometry assays. An aliquot of cells was plated with macrophage media (RPMI containing 10% FCS). After a 2-hour incubation at 37°C, 5% CO_2, non-adherent cells were removed by washing with PBS, and the remaining adherent cells were cultured for 24 hours before conducting functional assays. For phagocytosis assays, fresh peritoneal cells were plated in either chamber slides (Thermo Scientific, Rochester, NY) for phagocytosing *E.coli* bioparticles or coverslips (Schott, Louisville, KY) for uptake of IgG latex beads.

3. Differential counts of peritoneal cells

An aliquot of recovered peritoneal cells was used to make cytospin preparations and for flow cytometry analyses. The cytospin slides were stained with Giemsa for differential counts. A representative photomicrograph is shown in Figure 4. Two hundred cells per slide were counted.

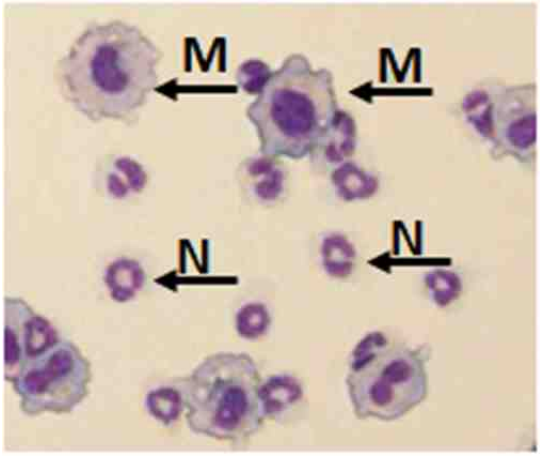


Figure 4. Differential counts of peritoneal cells. Giemsa stain of peritoneal cells after LPS injection. Macrophages indicated by M are large, round and heavily vacuolated cells. Neutrophils indicated by N are smaller cells with a fragmented nucleus.

4. Flow cytometry

Resident peritoneal cells were incubated with antibodies that recognize macrophage antigens including CD11b or Mac1, CD115, and F4/80. Specific antibodies used for these studies were FITC-conjugated mouse CD11b (BD, San Diego, CA), PE-conjugated mouse CD115 (eBioscience, San Diego, CA), and APC-conjugated mouse F4/80 (Invitrogen). To quantitate inflammatory monocytes, peripheral blood was collected from WT and *Fancc-/-* mice. A small aliquot was used for total white blood cell count determination as described (Haneline *et al.*, 1999). The majority of sample was treated with ACK lysing buffer (Lonza Walkersville, Walkersville, MD) to remove red blood cells. The remaining cells were incubated with antibodies that distinguish inflammatory monocytes (Hokeness *et al.*, 2005). Specific antibodies used for these studies were FITC-conjugated mouse Ly-6C (BD), PE-conjugated CD11b (BD), and APC-conjugated F4/80 (Invitrogen). Isotype control antibodies were used to correct background fluorescence and set analysis gates. Cells were acquired using a FACSCalibur (BD) and analyzed with CellQuest software (BD).

5. Myeloid colony formation assay

WT and *Fancc*-/- BM low-density mononuclear cells (MNCs) were prepared and plated in hematopoietic progenitor assays as described with minor modifications in the cytokines used (Haneline *et al.*, 1998; Haneline *et al.*, 2003). Briefly, MNCs from the BM of WT and *Fancc*-/- mice were resuspended in Iscove modified Dulbecco medium (IMDM; GIBCO) supplemented with 20% FCS. Cells were plated (5×10^4 cells/mL) in methylcellulose (STEMCELL Technologies Inc, Vancouver, British Columbia, Canada) in the presence of M-CSF (50 ng/mL) and/or GM-CSF (10 ng/mL). Total colonies, including colony forming unit–granulocyte (CFU-G), colony forming unit–macrophage (CFU-M), and colony forming unit–granulocyte macrophage (CFU-GM), were scored 7 days after plating under a light microscope. Colony morphology was used to determine individual hematopoietic colony type as illustrated in Figure 5.

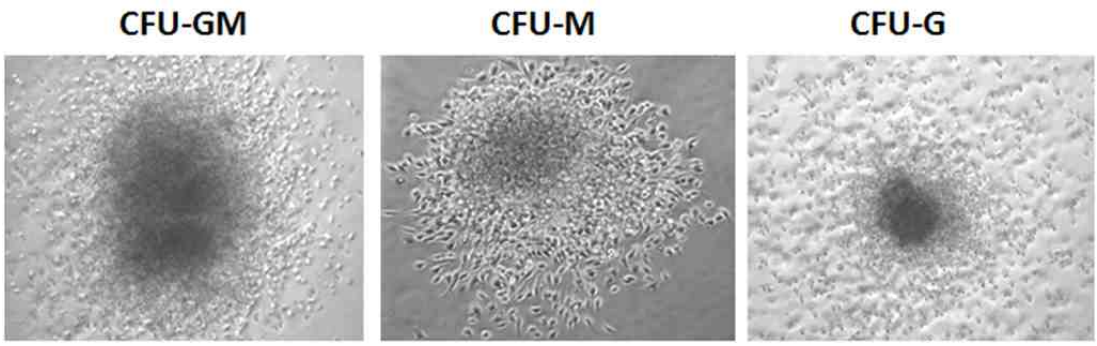


Figure 5. Representative photographs of individual hematopoietic progenitor colony. M-CSF and GM-CSF induced colonies are mainly divided into three types – CFU-GM, CFU-M, and CFU-G. CFU-GMs are relatively large colonies which consist of more cells with various sizes. CFU-Ms are recognized as colonies with an aggregation of large dispersed macrophages. The smallest colonies are CFU-Gs which are comprised of dispersed granulocytes.

6. Differentiation of bone marrow derived macrophages (BMDM)

BMDM were obtained using the conditions adapted from Racoosin and Swanson (Racoosin and Swanson, 1989). Total bone marrow cells flushed from mouse femurs were washed twice in IMDM. Total bone marrow cells from each mouse were suspended in 20 mL IMDM with 20% FCS, 50 ng/mL M-CSF, 1% penicillin, and 100 μg/mL streptomycin and plated on a single 100 mm petri dish (non-tissue culture plastic) (Day 0). Following 12 hours at 37°C, non-adherent cells were collected and transferred to a new 100 mm tissue culture dish, whereas adherent cells were discarded. After 3 days of differentiation, non-adherent cells were collected, centrifuged, re-suspended in fresh differentiation media, and added back to the original tissue culture dish. On day 6 of differentiation, adherent cells were either collected using cold PBS for experiments or new media added. Day 6-8 differentiated BMDMs were used for all experiments.

7. Apoptosis assay

Apoptosis of BMDM was examined by terminal deoxynucleotidyl transferase (tDt)-mediated dUTP nick end-labeling (TUNEL) assay (Roche Diagnostics, Indianapolis, IN) (Saadatzadeh *et al.*, 2009; Li *et al.*, 2004). Cells were treated with mitomycin C (50 nM and 500 nM) as positive controls. In detail, BMDM were fixed with 4% formaldehyde for 30 minutes at room temperature and permeabilized with 0.1% sodium citrate (Fisher Scientific, Fair Lawn, NJ), 0.1% Triton-X100 (Boehringer Mannheim, Mannheim, Germany) for 2 minutes at 4°C. Cells were incubated with Tdt and dUTP-FITC in a humidified environment at 37°C, 5% CO_2 for 1 hour. After incubation, cytospins were evaluated by fluorescence microscopy. Photographs were

obtained of each condition and scored for apoptotic cells. A total of at least 100 cells from at least three independent experiments were analyzed for apoptotic cells.

Apoptosis assay of peritoneal cells were tested by expression of annexin V (BD) and 7AAD (BD) in cells. Cells stain positive for both antibodies by flow cytometry are defined as early apoptotic cells.

8. Proliferation assay

Starved BMDM were stimulated with M-CSF (50 ng/mL) for 24 hours and pulsed with 0.037 MBq (1.0 μCi) of [^{3}H]-thymidine (Amersham Biosciences, Piscataway, NJ) for 6 hours at 37°C prior to harvesting. The cells were washed 4 to 5 times with PBS. Thymidine incorporation was measured using a Beckman Coulter LS 6500 Multipurpose Scintillation counter (Beckman Coulter, Fullerton, CA) (Munugalavadla *et al.*, 2005).

9. Immunoblot analysis

BMDMs and peritoneal cells were lysed and quantitated using a bicinchoninic acid (BCA) protein assay (Pierce Chemical, San Diego, CA). Lysis buffer includes: 0.5 M Hepes, 5 M NaCl, 50% Glycerol, 25% Triton X, 1 M $MgCl_2$, 250 mM EDTA, and Protease inhibitors and PMSF. Equal amounts of proteins were separated on a 4-12% sodium dodecyl sulfate (SDS)–polyacrylamide gradient gel (Invitrogen) by electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with Tris-buffered saline Tween containing 5% nonfat milk for 1 hour. For immunodetection of ERK and AKT, anti-ERK (phospho and total), and anti-AKT (phospho and total) were used at a 1:1000 dilution for 12 hours. All primary antibodies

were from Cell Signaling (Danvers, MA). The secondary antibody, anti-rabbit-horesradish peroxidase (HRP) (Amersham Biosciences), was used at a 1:5000 dilution for 1 hour before visualizing by ECL chemiluminescence.

10. Adhesion assays

Adhesion assays were performed on fibronectin (FN, recombinant human fragment CH296, Takara Bio Inc, Madison, WI) or on endothelial cells as described (Munugalavadla *et al.*, 2005; Hollingsworth *et al.*, 2007). For assay on FN, flat-bottom 96-well polystyrene plates were coated with FN at a concentration of 2 μg/mL at 37°C for 1 hour and then washed twice. Peritoneal cells (1×10^5) were added to each well and allowed to adhere at 37°C for 1 hour. Unbound cells were removed by aspiration, and wells were washed twice with cold PBS. Adherent cells were fixed with 4% paraformaldehyde (Sigma, St. Louis, MO) and stained with 0.1% crystal violet (Sigma). Adherent cells were photographed in 5-6 random fields/well with a Leica microscope using a 10X objective lens. For assay on activated endothelial cells, human endothelial colony-forming cells (Ingram *et al.*, 2008) were cultured in a 96-well plate to 100% confluence followed by treatment with LPS (1 μg/mL) or vehicle control for 24 hours. Carboxy-fluorescein diacetate, succinimidyl ester (CFSE, Sigma) was used to label peritoneal cells at a concentration of 1 μM for 8 minutes. CSFE-stained peritoneal cells (5×10^4 cells) were added to endothelial monolayers and co-cultured for 1 and 2 hours. Non-adherent cells were washed off, and adherent cells were photographed in 5-6 random fields/well with a 10X objective lens.

11. Transwell motility assay

Migration assays were performed in transwell cell culture chambers with polycarbonate filters (24-well, 8-μm pore, corning Costar, Cambridge, MA). The filters were coated with FN (20 μg/mL) for 2 hours and washed with PBS. Peritoneal cells (5×10^5 cells) in 100 μL RPMI were added to the upper chamber. RPMI (500 μL) with or without chemotaxis reagents (100 ng/mL M-CSF, 20 ng/mL MCP1) was placed in the lower chamber. After 24 hours of culture, cells remaining on the membrane in the upper chamber were scraped off, and cells that had migrated to the membrane in the lower chamber were stained with crystal violet and counted under a Leica microscope. Ten random fields/well were counted.

12. Phagocytosis assay

Phagocytosis was conducted with either *Escherichia coli* (*E.coli,* K-12 strain) bioparticles BODIPY FL conjugate (Sigma) or human serum coated latex beads (3.30 μm, Bangs Lab, Fishers, IN). *E.coli* bioparticles were suspended at a concentration of 20 mg/mL in PBS containing 2 mM sodium azide and incubated with macrophages for 1 hour. Latex beads were incubated with human serum at a concentration of 4 mg/mL in PBS at 37°C for 1 hour (Suh *et al.*, 2006). Serum coated latex beads were then re-suspended in RPMI 1640 medium and incubated with macrophages. Phagocytosis was stopped by placing cells on ice and non-phagocytosed bioparticles and beads were washed off with cold PBS. Cells were fixed with 4% paraformaldehyde and stained with rhodamine phalloidin (Invitrogen) and diamidino-2-phenylindole (DAPI) (Sigma). Phagocytosis of *E.coli* was imaged using a fluorescent microscope with a 20X objective

lens. Ingestion of latex beads was analyzed by confocal microscopy (Olympus FV1000-MPE confocal/multiphoton Microscope) after phalloidin staining. Images were quantified by counting at least 100 cells in each condition for each experiment.

13. Superoxide detection

The production of superoxide was monitored by a lucigenin chemiluminescence assay (Suh *et al.*, 2006; Vazquez-Torres *et al.*, 2000). WT and *Fancc-/-* peritoneal macrophages were plated in a 96-well flat bottom tissue culture-treated plate at a density of 2×10^5 cells per well (Corning Inc.) for 24 hours. After the addition of Zymosan particles (Invitrogen) and serum opsonized Zymosan (SOZ), cells were then incubated at 37°C for 60 minutes in an Lma microplate luminometer. The relative amount of superoxide produced over 60 minutes was determined by integrating the chemiluminescence unit signals using a SoftMax PRO software (Molecular Devices). Human serum (Sigma) was used to coat Zymosan particles (Sigma) at 37°C for one hour to achieve SOZ.

14. F-actin reorganization immunocytochemistry

Peritoneal macrophage were adhered to glass coverslips previously coated with FN (2 μg/mL**)** for indicated times. Adherent cells were fixed with 4% paraformaldehyde for 10 minutes, washed with PBS, and stained with rhodamine phalloidin (100 ng/mL, Sigma) at 37°C for 10 minutes. The slides were washed with PBS, air dried, and mounted in mounting solution (DAKO, Cambridgeshire, United Kingdom). Confocal microscopic imaging with Olympus FV1000-MPE confocal/multiphoton equipped with an Argon

laser and three diode lasers were used to determine phalloidin staining. Image J was used to process the micrographs. Images acquired from confocal systems were opened in Image J and single plane images were saved as individual TIFF files only after cropping without any alterations. A total of at least 100 cells from at least three independent experiments were analyzed unless otherwise stated in the figure legend.

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

Activation of RhoA, Rac1 and Cdc42 was determined using kits from Millipore following the instructions provided by the manufacturer (Roberts *et al.*, 1999). Briefly, WT and *Fancc-/-* peritoneal macrophages were harvested from 10 mice per genotype per experiment. Macrophages were quiesced by serum deprivation for 12 hours. Cells were activated by the addition of serum and then harvested at multiple time points by lysing with ice-cold buffer (50 mM Tris-HCl, pH 7.2, 1% TritonX-100, 0.1% SDS, 0.5% sodium deoxycholate, 500 mM NaCl, 10 mM $MgCl_2$, 5 μg/mL each of leupeptin and aprotinin, and 1 mM PMSF). Rac1 and Cdc42 activation were analyzed by affinity precipitation of a GST fusion-protein corresponding to p21-binding domain (PBD 67-150) of human PAK-1 bound to glutathione agarose (GST-PBD). RhoA-GTP was immunoprecipitated with a GST-tagged fusion protein, corresponding to residues 7-89 of mouse Rhotekin Rho Binding Domain (GST-RBD). Cell lysates were immunoprecipitated at 4°C for 1 hour. Immunoprecipitated complexes were subjected to 4-12% SDS-PAGE electrophoresis. Activated Rho GTPases were detected by immunoblotting using antibodies against RhoA (Santa Cruz, CA), Rac1 (BD

Pharmingen, San Diego, CA), and Cdc42 (Santa Cruz). Blots were developed by enhanced chemiluminescence and bands were quantified with ImageJ software.

16. RNA isolation and reverse transcription-PCR (RT-PCR)

Total RNA from cells was isolated using RNeasy Micro Kit (QIAGEN, Valencia, CA). Isolated RNA was quickly evaluated by agarose gel, and RNA concentration was measured using a spectrophotometer. cDNA was generated by reverse transcription with oligo dT as a primer using the SuperScript First-Strand synthesis system (Invitrogen, Carlsbad, CA). The cDNA were then used as templates for PCR to detect mRNA expression. The RT-PCR primer sets are listed in Table 1. PCR amplification was carried out in a total volume of 15 μL, containing 2 μL of cDNA, 10 μL of 2X FastStart Universal SYBR Green Master Mix (Roche), and 0.2 μM of each primer. The PCR cycling conditions were 95°C for 5 minutes followed by two-step cycling 40 cycles of 95°C for 10 seconds, and 60°C for 30 seconds. All PCR assays were performed in duplicate or triplicate. Fold change of gene expression was analyzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). $\Delta\Delta C$T = (CT.Target - CT.Actin) Time χ - (CT.Target - CT.Actin) Time 0. Time χ is any time point and Time 0 represents the expression of the target gene normalized to β-actin.

Table 1. Primer sequences used for RT-PCR studies.

MIP-2	**Forward**	**5' - GAA CAA AGG CAA GGC TAA CTG - 3'**
	Reverse	**5' - AAC ATA ACA ACA TCT GGG CAA T - 3'**
IL-1β	**Forward**	**5' - TCT TCT TTG GGT ATT GCT TGG - 3'**
	Reverse	**5' - TGT AAT GAA AGA CGG CAC ACC - 3'**
IL-6	**Forward**	**5' - CCA GGT AGC TAT GGT ACT CCA GAA - 3'**
	Reverse	**5' - GCT ACC AAA CTG GAT ATA ATC AGG A - 3'**
MIP-1α	**Forward**	**5' - GTG GAA TCT TCC GGC TGT AG - 3'**
	Reverse	**5' - TGC CCT TGC TGT TCT TCT CT - 3'**
GAPDH	**Forward**	**5' - TTT GAT GTT AGT GGG GTC TCG - 3'**
	Reverse	**5' - ATC TTG TCA TCA ACG GGA AG - 3'**
KC	**Forward**	**5' - CTT GAA GGT GTT GCC CTC AG - 3'**
	Reverse	**5' - TGG GGA CTA CTT TTA GCA TC - 3'**
MCP-1	**Forward**	**5' - ACT GAA GCC AGC TCT CTC TTC CTC - 3'**
	Reverse	**5' - TTC CTT CTT GGG GTC AGC ACA GAC - 3'**
TNF-α	**Forward**	**5' - AGC CCC CAG TCT GTA TCC TT - 3'**
	Reverse	**5' - CTC CCT TTG CAG AAC TCA GG - 3'**

17. Cytokine multiplex analysis of mouse serum

Blood was collected at indicated times after LPS treatment from WT and *Fancc-/-* mice. Serum was separated by centrifugation (3,000 RPM, 10 minutes). Levels of TNF-α, IL-6, IL-1β, MCP-1, IFN-γ, and KC were measured using Multiplex Bead Immunoassays as per the manufactuer's protocol (Millipore, Billerica, MA). A luminex 200 instrument (Luminex Corp, Austin, TX) was used for data acquisition and analysis. Cytokine concentrations were calculated using a Bio-Plex Manager 2.3 software (Bio-Rad, Hercules, CA) with a 5-parameter curve-fitting algorithm applied for standard curve calculations.

18. Statistical analyses

Parametric data are presented as mean plus or minus standard error of the mean (SEM), unless otherwise stated. For all data, an unpaired Student *t* test was conducted to evaluate for differences between treatment groups. A *P* value less than 0.05 was considered significant.

RESULTS

AIM 1: To examine whether *Fancc-/-* mice exhibit an altered inflammatory response *in vivo*. To create a model of peritoneal inflammation, a low dose of LPS (1 mg/kg suspended in 500 μL PBS) was injected into WT and *Fancc-/-* mice intraperitoneally (IP) similar to previous studies (Sejas *et al.*, 2007; Hollingsworth *et al.*, 2007; Hadjur and Jirik, 2003). Figure 6 illustrates the strategy of experiments for this aim. In brief, after one LPS injection, serum and peritoneal cells were collected at indicated time points to assess the systemic and local inflammatory response *in vivo*.

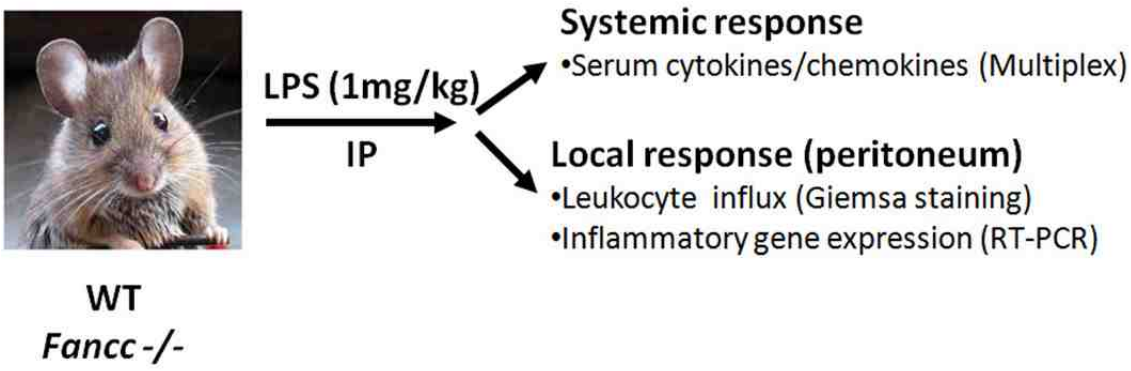


Figure 6. Experimental design for Aim 1. WT and *Fancc-/-* mice were injected with LPS (1mg/mL, in 500 μL PBS) IP. To evaluate for evidence of a systemic inflammatory response, serum samples were collected (0-120 hours) and tested for content of inflammatory cytokines/chemokines by Multiplex analysis. To evaluate the local inflammatory response, peritoneal cells were counted, phenotyped by Giemsa staining, and evaluated for inflammatory gene expression by RT-PCR.

I. *Fancc-/-* mice exhibited an altered inflammatory response *in vivo*.

A. Normal systemic levels of inflammatory cytokines/chemokines after LPS injection

LPS administered IP induced a systemic and local response in WT and *Fancc-/-* mice. The levels of inflammatory cytokines and chemokines in the serum were measured by cytokine Multiplex analysis. In both genotypes, the levels of TNF-α, IL-6, and Il-1β showed a dramatic increase at 2 hours with a quick decline to basal levels at 12 hours (Figure 7). The immediate response of these inflammatory cytokines parallels the early recruitment of inflammatory cells to the inflamed tissue as shown by others (Lukacs *et al.*, 1995). In contrast, MCP-1 levels increased at 12 hours and recovered to baseline by 48 hours, which is consistent with the role of this chemokine in macrophage recruitment (Figure 7). In summary, these serum cytokines/chemokines recovered to basal levels by 24 hours, suggesting that the LPS induced inflammatory response was controlled locally in the peritoneum without causing significant systemic inflammation.

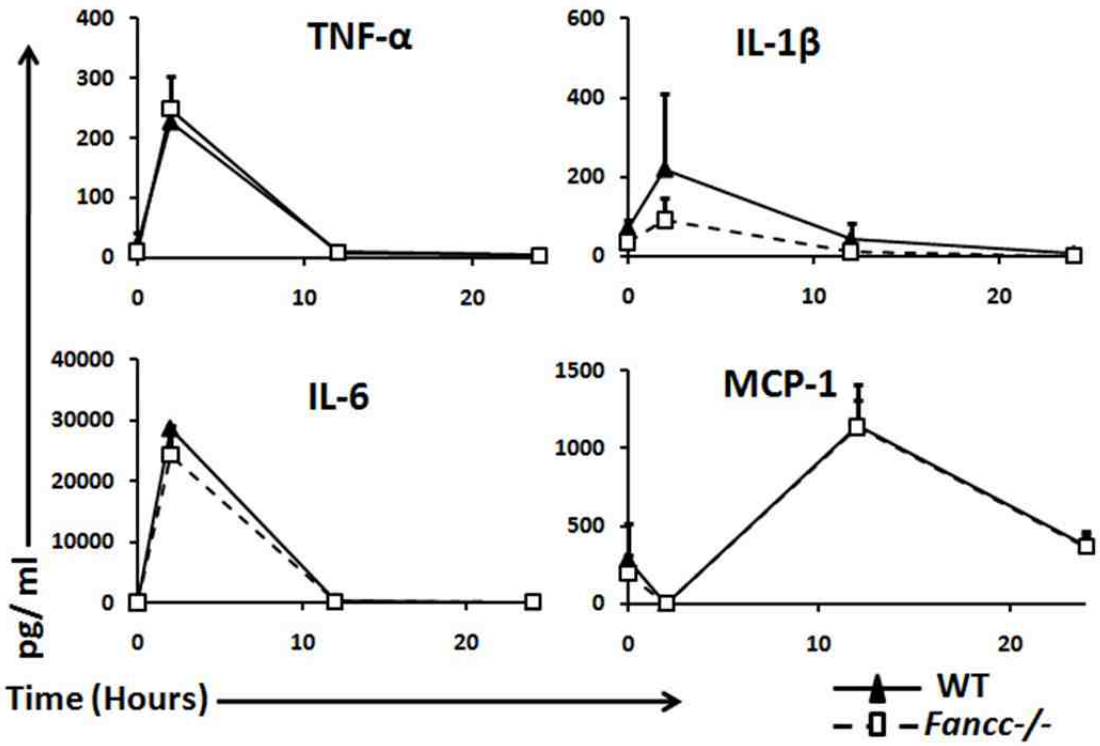


Figure 7. *Fancc-/-* and WT mice had similar serum levels of inflammatory mediators after LPS treatment. WT and *Fancc-/-* mice were injected with LPS (1 mg/kg) IP. Levels of TNF-α, IL-6, IL-1β, and MCP-1 were measured over time by Multiplex analysis. Six to nine mice per genotype were tested at each time point.

B. Local accumulation of monocytes/macrophages in LPS induced peritonitis

Using a low dose of LPS, serum inflammatory cytokines and chemokines were found to be no different between WT and *Fancc-/-* mice, suggesting that *Fancc-/-* mice had a similar systemic inflammatory response to LPS as WT mice. We subsequently questioned whether *Fancc-/-* mice exhibit an altered local inflammatory response to LPS in the inflamed peritoneum. LPS injection induced a time-dependent accumulation of leukocytes in the peritoneum. Giesma staining of peritoneal cells from WT mice demonstrated the normal kinetics of leukocyte influx after LPS induced peritonitis (Figure 8A). After LPS induced peritoneal inflammation, neutrophil recruitment increased quickly. The total number of peritoneal neutrophils peaked at 24 hours and dropped to low levels by 72 hours (Figure 8B). Interestingly, *Fancc-/-* mice exhibited a slightly higher number of neutrophils compared to WT mice (Figure 8B). As described in the background, the number of neutrophils in the inflamed site is largely influenced by the rate of recruitment from the peripheral blood and the rate of elimination via apoptosis and subsequent phagocytosis by macrophage. Therefore, we hypothesized that increased neutrophils in the inflamed peritoneum of *Fancc-/-* mice may be due to increased neutrophil recruitment or reduced neutrophil apoptosis. Since *Fancc-/-* neutrophils exhibit impaired granulocyte colony-stimulating factor induced mobilization from the BM (Pulliam *et al.*, 2008), the possibility that neutrophil recruitment was enhanced seemed less likely. Thus, we subsequently examined the apoptosis of peritoneal cells at indicated time points after LPS injection. The data showed that *Fancc-/-* cells had a lower percentage of apoptotic cells compared to WT at 24 hours (Figure 8C), a time point when >75% of peritoneal cells are neutrophils. These data support the hypothesis that reduced

neutrophil apoptosis may contribute to the increased number of neutrophils observed in the peritoneum of *Fancc-/-* mice after LPS treatment. These data also suggest that *Fancc-/-* neutrophils may exhibit autonomous defects that alter the inflammatory response *in vivo*.

As expected, LPS induced peritonitis caused an influx of monocytes/macrophages into the peritoneum at later time points. In WT mice, LPS caused a significant extravasation of monocytes/macrophages into the peritoneal cavity, peaking at 96 hours and declining sharply to near basal levels by 120 hours, similar to previous studies (Figure 8D) (Ajuebor *et al.*, 1999; Takahashi *et al.*, 2009; Parsons *et al.*, 2007; Mortier *et al.*, 2005). *Fancc-/-* mice displayed similar kinetics for monocyte/macrophage recruitment after LPS injection (Figure 8D). However, the number of monoyctes/macrophages was significantly lower in *Fancc-/-* mice compared to WT in the later phase of inflammation (i.e. 72 to 120 hours). These data are intriguing given that *Fancc-/-* mice had more neutrophils in the peritoneum after LPS treatment, which should correlate with increased macrophage recruitment. We speculate that intrinsic defects in monocytes/macrophages may account for this observation.

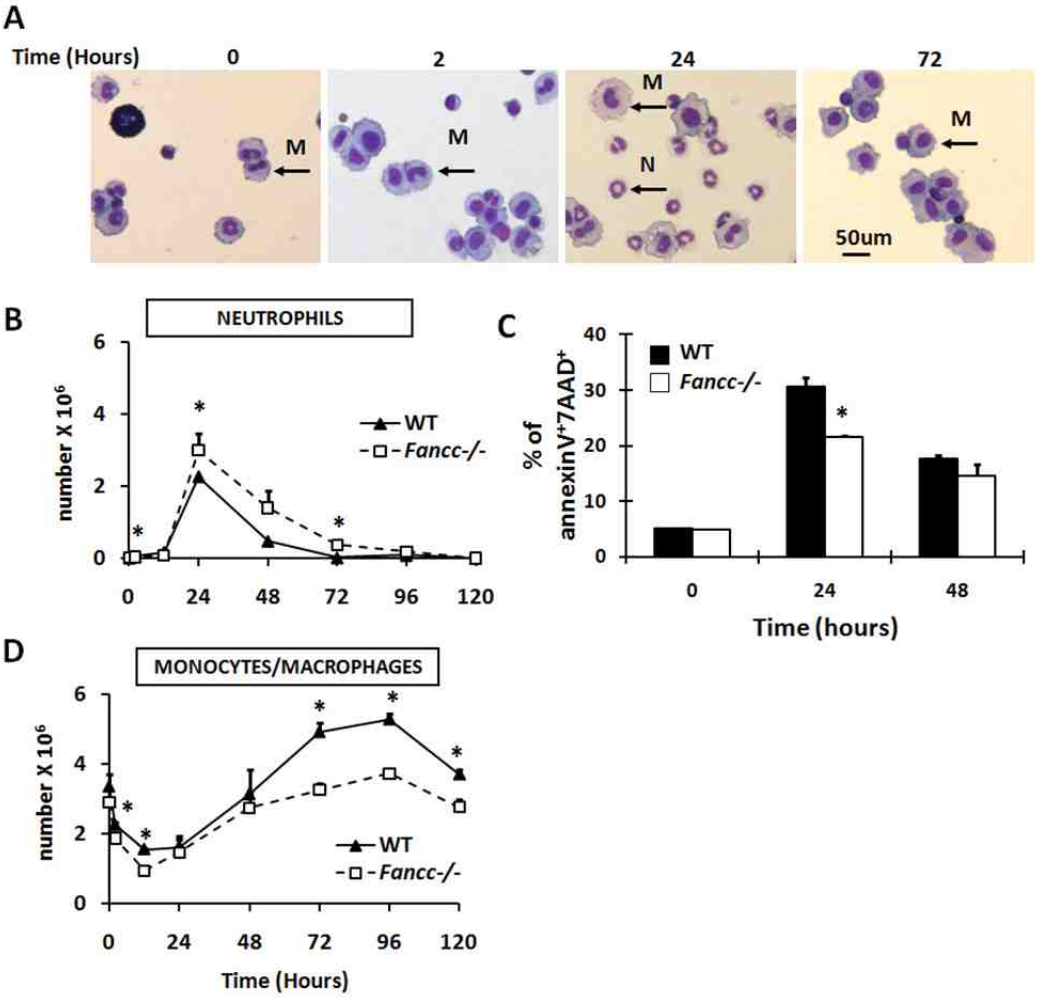


Figure 8. *Fancc-/-* mice had altered number of leukocytes in LPS inflamed peritoneal cavity. (A) Leukocyte distribution in LPS inflamed peritoneum. At baseline and after LPS injection, peritoneal cells were harvested (0-72 hours) and stained with Giemsa for morphologic evaluation of peritoneal leukocyte distribution. Representative photomicrographs of peritoneal cells from WT mice are shown (M: macrophage, N: neutrophil). (B) Total number of peritoneal neutrophils. Neutrophil number was obtained by multiplying total peritoneal cells by the neutrophil percentage determined by Giemsa staining. Six to twelve mice per genotype were used in each of the three independent experiments, *P<0.05. (C) Total number of monocytes/macrophages.

Monocyte/macrophage number was calculated by multiplying total peritoneal cells by the monocyte/macrophage percentage determined by Giemsa staining. Six to twelve mice per genotype were used in each of the three independent experiments, *P<0.05. (D) Apoptosis of peritoneal cells after LPS treatment. Apoptosis was evaluated by flow cytometry. Cells staining positively for both annexinV and 7AAD were scored as apoptotic cells. Three mice per genotype were used in each of the three independent experiments, *P<0.05.

C. Accumulation of monocytes/macrophages in sodium periodate induced inflammatory response

To further evaluate whether loss of *Fancc* alters monocyte/macrophage accumulation to sites of inflammation, WT and *Fancc-/-* mice were injected IP with sodium periodate as a second inflammatory stimulus, similar to previous studies (Yamauchi *et al.*, 2004; Suh *et al.*, 2006). Previous reports demonstrate that the number of monocytes/macrophages in the peritoneum after sodium periodate injection peaks at 96 hours (Suh *et al.*, 2006). Thus, we used this time point to examine whether *Fancc-/-* mice had impaired monocyte/macrophage recruitment in response to sodium periodate. *Fancc-/-* mice had fewer peritoneal macrophages demonstrated by decreased peritoneal exudate cellularity and decreased absolute number of $Mac1^+$ and $F4/80^+$ cells compared to WT mice (Figure 9A-B). Together these data support the hypothesis that macrophage accumulation is impaired in *Fancc-/-* mice during inflammation.

Given the numerous roles that macrophages have in responding to and resolving inflammation together with the complex life cycle of monocytes/macrophages, several potential mechanisms could account for the observed reduction of *Fancc-/-* peritoneal macrophages after LPS induced inflammation. In the early phase of inflammation, *Fancc-/-* resident macrophages and neutrophils may release altered levels of inflammatory cytokines/chemokines, which would result in decreased recruitment of monocytes to the inflamed site. In addition, the number of inflammatory monocytes, which are the precursors of peritoneum macrophages, may be reduced in *Fancc-/-* mice. In the later phases of inflammation, BM expansion of monocytes/macrophages may be reduced in *Fancc-/-* mice due to impaired HSC and progenitor responsiveness to

inflammatory stimuli. The next series of experiments were designed to test each of these possibilities.

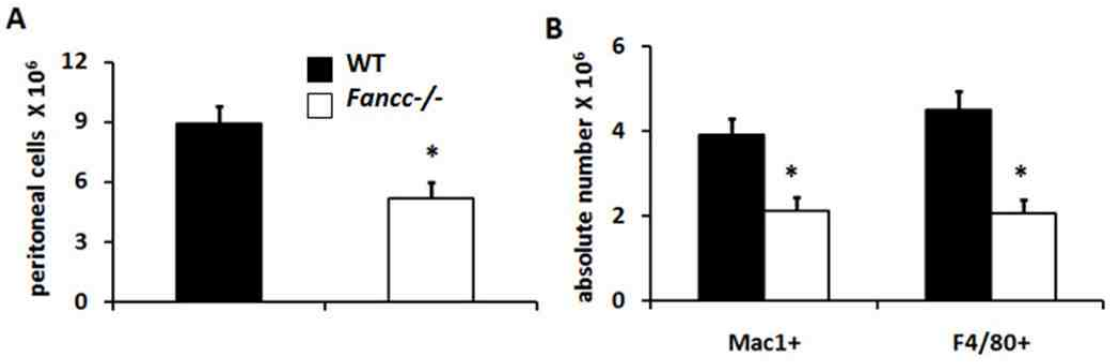


Figure 9. *Fancc-/-* mice exhibit impaired monocyte/macrophage accumulation in sodium periodate induced peritonitis. Peritoneal cells were harvested 96 hours after IP injection of sodium periodate. (A) Total peritoneal cell number is shown. Eight mice per genotype were used in each of the three independent experiments, *P<0.05. (B) The absolute number of peritoneal cells expressing macrophage antigens, Mac1 or F4/80, is shown. The absolute number of $Mac1^+$ or $F4/80^+$ is calculated by multiplying the total cell number by percentage of Mac1+ or F4/80+ cells determined by flow cytometry. Six mice per genotype were used in each of the three independent experiments, *P<0.05.

1. Reduced levels of inflammatory cytokines/chemokines in the early phase of inflammation

Because accumulation of leukocytes in the inflamed site is largely regulated by cytokine/chemokine production, gene expression of multiple inflammatory mediators was assessed in local peritoneal cells. Initially, gene expression was examined in peritoneal cells at baseline and 2 hours after LPS treatment because resident macrophage account for the majority of cells in the peritoneum at these time points (see Figure 8). No differences were detected between WT and *Fancc-/-* peritoneal cells in the baseline mRNA expression of cytokines/chemokines tested. In both WT and *Fancc-/-*peritoneal cells, most transcripts of inflammatory cytokines/chemokines had a significant increase after LPS treatment compared to PBS controls (Figure 10A). Among them, KC had a dramatic increase, consistent with the role that KC serves as a neutrophil chemoattractant. Surprisingly, compared to WT, *Fancc-/-* peritoneal cells displayed a significant decrease in mRNA expression of TNF-α, KC, MIP-1α, and MIP-2, with a similar trend for IL-6, IL-1β, and MCP-1. To determine whether the reduced expression of inflammatory cytokines/chemokines was either impaired or delayed, we assessed expression in peritoneal cells at later time points (0-120 hours) (Figure 10B).These studies showed that *Fancc-/-* peritoneal cells exhibited reduced inflammatory gene expression at multiple time points, implying an altered inflammatory response *in vivo*. Additionally, we noticed that KC, IL-6, TNF-α, and MIP-1α exhibit different kinetics of expression patterns after LPS injection in both WT and *Fancc-/-* mice. KC expression peaked at 2 hours when resident macrophages were the majority cell type, indicating resident macrophages are important for neutrophil recruitment during inflammation. TNF-α and MIP-1α peaked at

24 hours when half of the peritoneal cells were neutrophils, suggesting that neutrophils contribute to monocyte recruitment in LPS induced peritonitis.

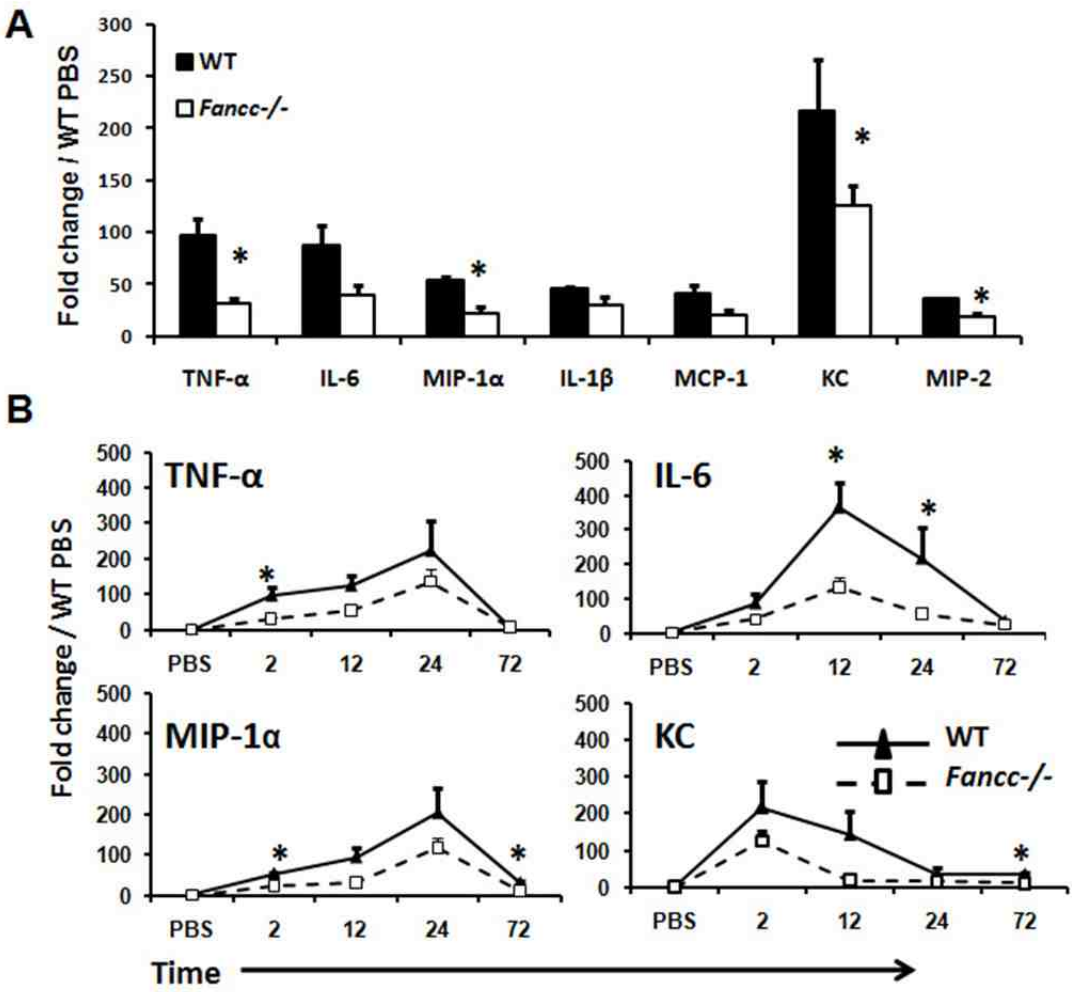


Figure 10. *Fancc-/-* peritoneal cells exhibited a significant decrease in gene expression of multiple inflammatory cytokines/chemokines after LPS treatment. Transcripts of inflammatory cytokines/chemokines were examined in peritoneal cells by RT-PCR over multiple time points. (A) Inflammatory gene expression of peritoneal cells 2 hours after LPS treatment. Fold change in transcripts relative to WT PBS control is shown. Six mice per genotype were used per genotype, *P<0.05. (B) Inflammatory gene expression over time in peritoneal cells after LPS treatment. Fold change in transcripts relative to WT PBS control is shown. Six mice per genotype were used per genotype, *P<0.05.

2. Decreased inflammatory monocytes in *Fancc-/-* mice

a. Inflammatory monocytes in the peripheral blood

Given the observation that *Fancc-/-* peritoneal cells had reduced expression of chemokines required for monocyte recruitment, we speculated that *Fancc-/-* mice may have fewer circulating monocytes in the peripheral blood after LPS treatment. Inflammatory monocytes, defined as Mac-1^{+}F4/80^{+}Ly-$6C^{high}$ cells, are mobilized from the BM into the peripheral blood and specifically recruited to inflamed tissue sites (Crane *et al.*, 2009; Geissmann *et al.*, 2003; Gordon and Taylor, 2005; Sunderkotter *et al.*, 2004; Tacke and Randolph, 2006). Therefore, we examined whether *Fancc-/-* mice had reduced circulating inflammatory monocytes after LPS treatment. Figure 11A illustrates the gating strategy for flow cytometry studies. By 24 hours, 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 to controls (Figure 11B-C). However, *Fancc-/-* mice had fewer circulating Mac-1^{+}F4/80^{+}Ly-$6C^{high}$ cells compared to WT mice. By 48 hours, circulating Mac-1^{+}F4/80^{+}Ly-$6C^{high}$ cells recovered to near baseline levels.

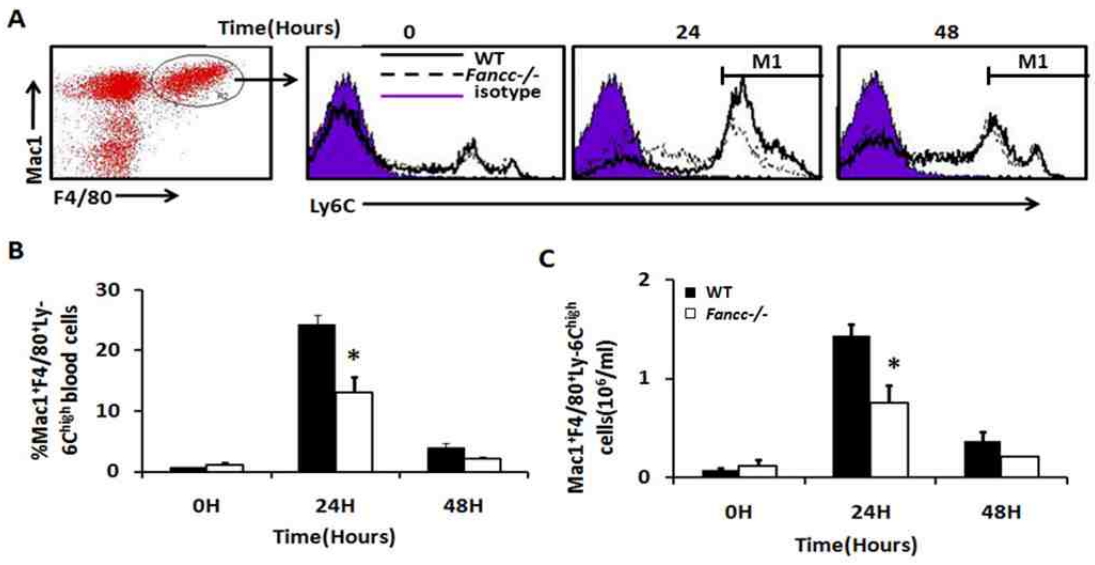


Figure 11. *Fancc-/-* mice exhibited decreased inflammatory monocytes in the peripheral blood. (A) Multiparameter flow cytometry to identify inflammatory monocytes. Gating strategy for flow cytometry studies is shown. Inflammatory monocytes were defined as $Mac1^{+}F4/80^{+}Ly\text{-}6C^{high}$ cells. M1 gate represents high expression of Ly-6C. (B) Percentage of inflammatory monocytes in the peripheral blood. The percentage of $Mac1^{+}F4/80^{+}Ly\text{-}6C^{high}$ cells determined by flow cytometry is shown. Three mice per genotype were used for each independent experiment, *P<0.05. (C) Frequency of inflammatory monocytes in the peripheral blood. Total $Mac1^{+}F4/80^{+}Ly\text{-}6C^{high}$ cells/mL of blood is shown. Total white blood cells were counted using a Hemavet. $Mac1^{+}F4/80^{+}Ly\text{-}6C^{high}$ cells were calculated by multiplying the frequency determined by flow cytometry with the total white blood cell count/mL of blood. Three mice per genotype were used for each independent experiment, *P<0.05.

b. Inflammatory monocytes in the BM

Because inflammatory monocytes are mobilized from the BM during inflammation, we subsequently evaluated the BM for Mac-1^{+}F4/80^{+}Ly-$6C^{high}$ cells after LPS injection. These studies demonstrated that no dramatic changes were observed within the BM of mice from either genotype (Figure 12). By 48 hours, *Fancc-/-* BM cells had a slightly higher percentage of inflammatory monocytes compared to WT, though the absolute number of inflammatory monocytes in a femur was not different between genotypes. Collectively, these data suggest that defective monocyte mobilization from the BM into the peripheral blood or impaired monocyte activation to an inflammatory phenotype may occur in *Fancc-/-* mice during inflammatory stress. However, further studies need to be done to understand the underlying mechanisms.

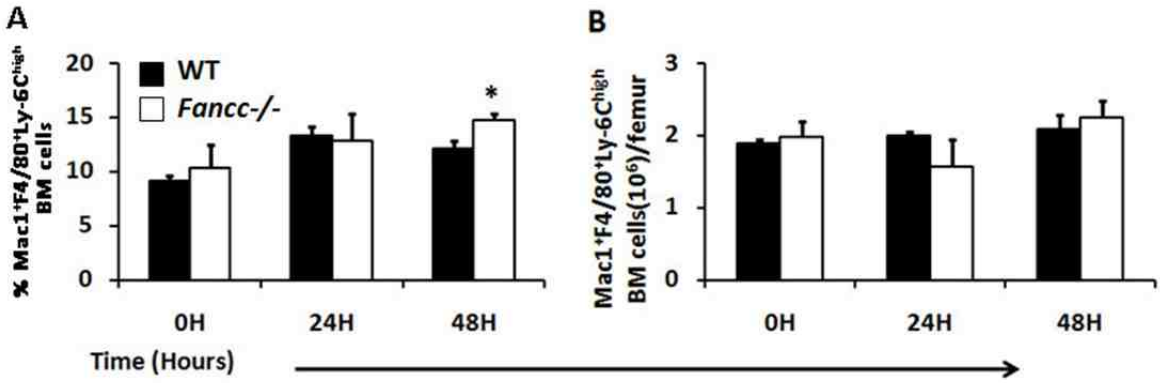


Figure 12. *Fancc-/-* mice exhibited no significant alterations in the number of BM inflammatory monocytes. BM cells were collected at indicated time points after LPS injection. Inflammatory monocytes were defined as $Mac1^{+}F4/80^{+}Ly\text{-}6C^{high}$ cells. (A) Percentage of inflammatory monocytes in the BM. The percentage of $Mac1^{+}F4/80^{+}Ly\text{-}6C^{high}$ cells determined by flow cytometry is shown. Three mice per genotype were used for each independent experiment, *P<0.05. (B) Absolute number of inflammatory monocytes per femur. Total $Mac1^{+}F4/80^{+}Ly\text{-}6C^{high}$ cells/femur is shown. Three mice per genotype were used for each independent experiment, *P<0.05.

3. Reduced monocyte progenitors in the BM

a. BM cellularity after LPS treatment

During inflammation, leukocytes are quickly mobilized from the BM into the peripheral blood. Concurrently, hematopoietic stem and progenitor cells are stimulated to promote enhanced production of mature myeloid cells to protect from ongoing inflammation and to replenish the BM cell pool of leukocytes. We hypothesized that *Fancc-/-* BM cells may exhibit altered inflammation induced expansion of HSCs and progenitors. Therefore, we examined whether total BM cellularity was altered in *Fancc-/-* mice after LPS treatment. Femur cellularity was used as a surrogate measure of total BM cellularity because it is easily accessible and should be representative of other BM environments. Total BM cells from one femur were collected and counted from mice treated with LPS and compared to baseline controls. A decrease in femur cellularity was observed in mice from both genotypes at 24 and 48 hours compared to baseline controls (Figure 13). Interestingly, femur cellularity recovered to near baseline levels by 72 hours. However, no significant differences were observed between WT and *Fancc-/-* mice. The rapid decline in BM cellularity is consistent with mobilization of mature cells from the BM into the peripheral blood. By 72 hours, the recovery of BM cell number to basal levels suggests that hematopoietic stem and progenitor cells responded to the inflammatory stress via differentiation and proliferation to replenish the BM of differentiated leukocyte populations.

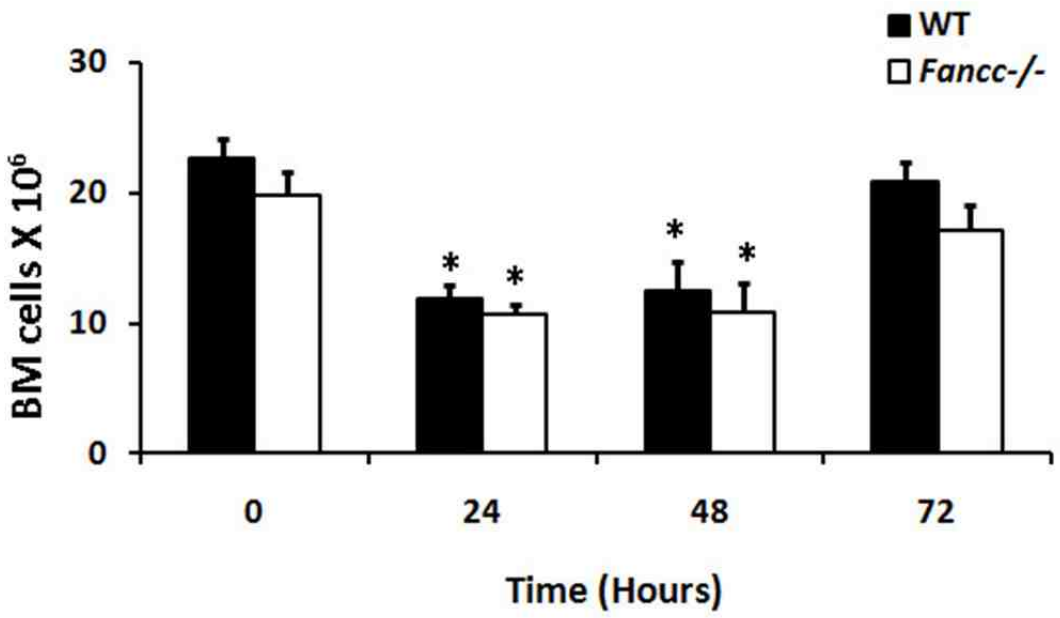


Figure 13. *Fancc-/-* mice had normal BM cellularity after LPS injection. Total BM cells from one femur were collected and counted from WT and *Fancc-/-* mice treated with LPS and untreated controls. Mice were sacrificed 24, 48, and 72 hours following LPS treatment. Six mice per genotype were used for three independent experiments, *P<0.05 compared to time 0.

b. Phenotypic characterization of monocyte progenitors in the BM after LPS treatment

Previous data from our laboratory demonstrate that *Fancc-/-* HSC and myeloid progenitors exhibit defective function (Haneline *et al.*, 1999; Haneline *et al.*, 1998; Saadatzadeh *et al.*, 2004; Saadatzadeh *et al.*, 2009; Haneline *et al.*, 2006; Haneline *et al.*, 2003). Therefore, we hypothesized that *Fancc-/-* myeloid progenitors may inadequately expand in response to inflammatory stimuli. As described in the background (Figure 1), the BM HSCs give rise to CMP that differentiate into GMP which eventually mature into monocytes and macrophages. Thus, we next questioned whether *Fancc-/-* BM cells exhibit altered myeloid population during LPS induced inflammatory response. Phenotypically-defined populations including LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) which is enriched with stem cells, CMP (LKS^- Fc-$\gamma R^{lo}CD34^+IL\text{-}7R^-$) and GMP ($LKS^-$ Fc-$\gamma R^+CD34^+IL\text{-}7R^-$) were examined by multiparameter flow cytometry. Both WT and *Fancc-/-* BM had an increase in the absolute number of LSK cells by 24 hours, and recovered to steady state levels by 72 hours (Figure 14). The number of CMP and GMP population, on the other hand, showed a dramatic increase at 72 hours, suggesting that the expansion of CMP and GMP takes about three days after LPS treatment. However, no significant differences were observed between WT and *Fancc-/-* mice.

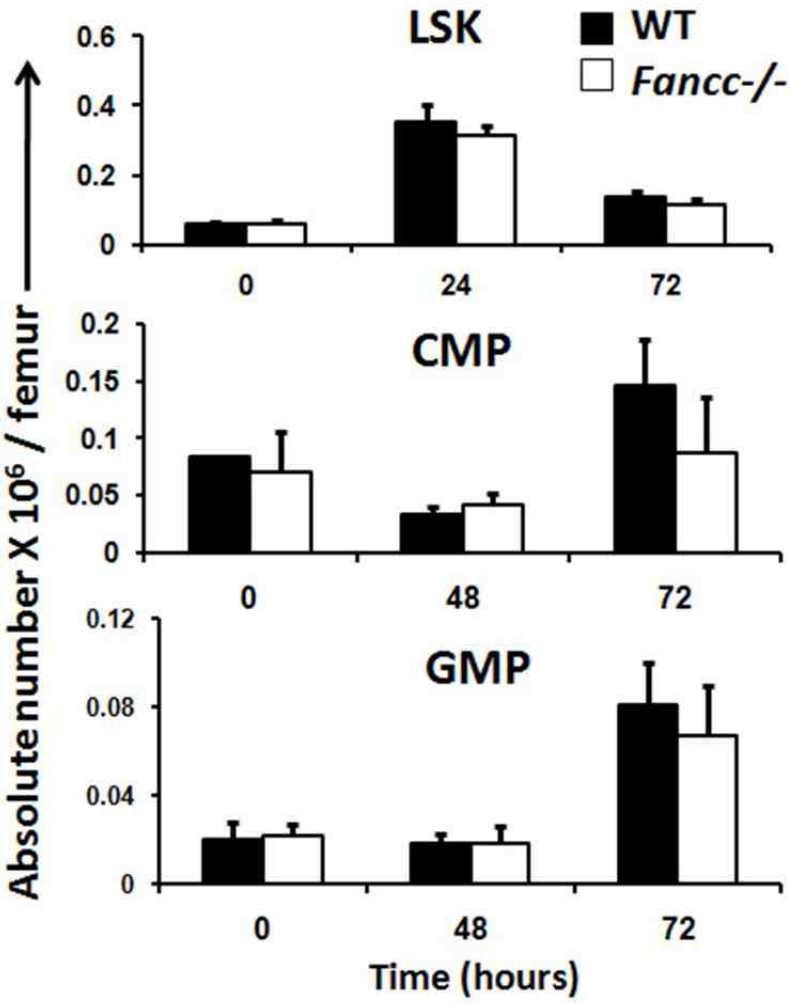


Figure 14. Phenotypically-defined progenitors from *Fancc-/-* mice exhibited normal LPS-induced expansion. Total BM cells from one femur were counted from control and LPS treated WT and *Fancc-/-* mice. BM cells were labeled with antibodies for the measurement of LSK, CMP, and GMP by multi parameter flow cytometry. Absolute numbers were calculated by multiplying percentage of each cell population determined by flow cytometry by femur cellularity. LSK was defined as $lin^{-}sca1^{+}c\text{-}kit^{+}$. CMP was defined as LKS^{-} Fc-$\gamma R^{lo}CD34^{+}IL\text{-}7R^{-}$. GMP was defined as LKS^{-} Fc-$\gamma R^{+}CD34^{+}IL\text{-}7R^{-}$. Six mice per genotype were used in three independent experiments.

c. Functional analysis of BM progenitors after LPS treatment

Since quantitative analyses of hematopoietic stem/progenitor cell populations by flow cytometry were not altered in *Fancc-/-* mice treated with LPS, we next questioned whether functional impairments existed in *Fancc-/-* myeloid progenitors by examining colony forming ability in response to monocyte/macrophage growth factors BM mononuclear cells from LPS treated mice were cultured in methylcellulose with M-CSF and /or GM-CSF and assessed for the growth of myeloid progenitors after 7 days of culture. Because the expansion of myeloid progenitors needs time to occur after an inflammatory stress, we collected BM cells 48 hours after LPS injection. At this time point, BM cells from WT mice displayed an increase in the total number of colonies formed in the presence of M-CSF, GM-CSF, and M-CSF+GM-CSF compared to baseline levels (Figure 15). In contrast, no expansion of myeloid progenitors was observed in *Fancc-/-* BM. By 72 hours, the differences in colony formation between WT and *Fancc-/-* BM cells persisted only in conditions that included M-CSF. These data suggest the reduced number of colonies from *Fancc-/-* mice was influenced most by M-CSF. To determine whether specific myeloid progenitor populations were affected in *Fancc-/-* mice, colonies were scored based on morphology as CFU-GM, CFU-M, and CFU-G (Figure 16). As expected, the majority of colonies grown in the presence of M-CSF were CFU-M and CFU-GM. In M-CSF cultures, *Fancc-/-* BM cells had a significant reduction in CFU-M colonies at 48 and 72 hours after LPS treatment compared to WT. CFU-GM colonies were rarely observed in M-CSF cultures. In the presence of GM-CSF, with or without M-CSF, CFU-GM colonies were the major colony type observed from both WT and *Fancc-/-* BM cells. However, fewer CFU-GM were noted from *Fancc-/-* BM cells

collected 48 hours after LPS treatment compared to WT. Together these data indicate that *Fancc-/-* mice are capable of preserving normal phenotypic distributions of hematopoietic stem/progenitor cells after *in vivo* LPS exposure. However, *in vivo* LPS treatment significantly impairs the responsiveness of *Fancc-/-* myeloid progenitors to growth promoting cytokines, especially M-CSF.

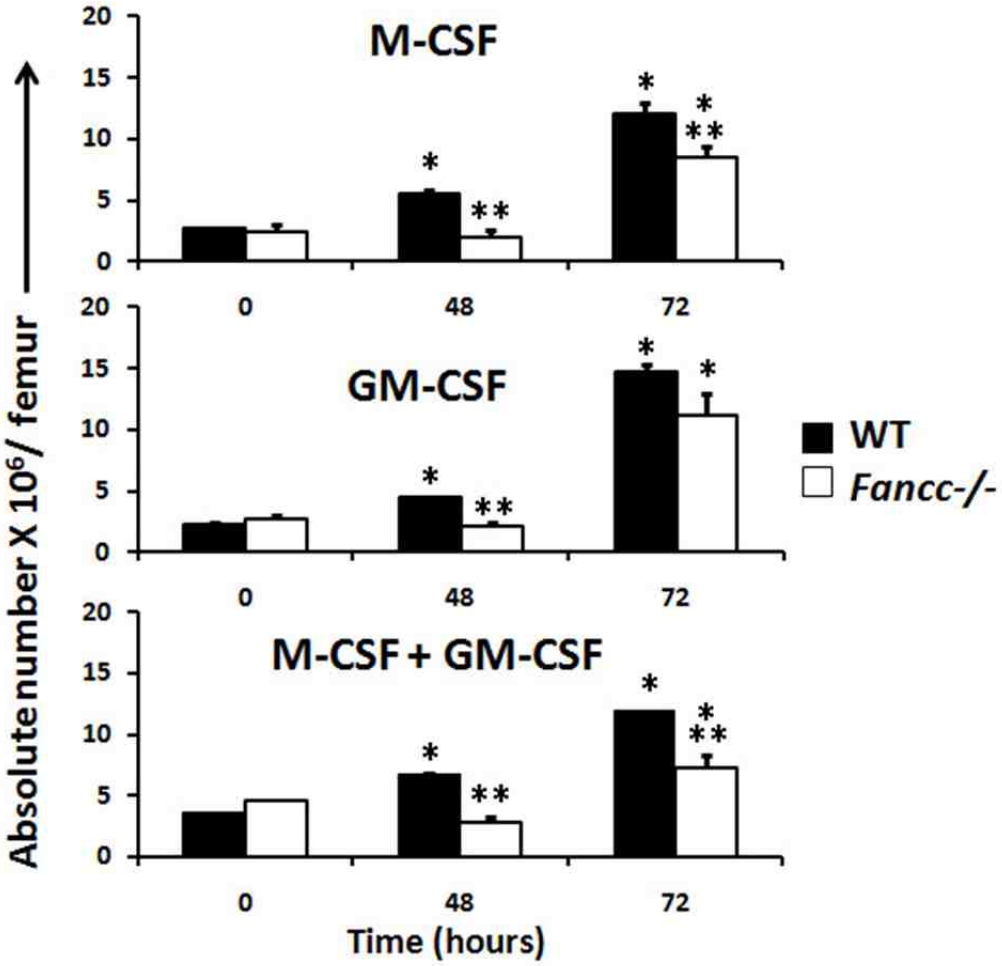


Figure 15. *Fancc-/-* progenitors exhibited impaired colony forming ability after *in vivo* LPS exposure. BM cells were collected from untreated and LPS treated mice and were cultured in methylcellulose (50,000/dish) in the presence of M-CSF (50 ng/mL), GM-CSF (10 ng/mL), or M-CSF and GM-CSF for 7 days. Colonies were scored under an Olympus microscope with a 4X objective lens. Total colonies per femur were calculated by multiplying femur cellularity with the number of colonies scored per 50,000 BM cells plated. Three mice were used for each of the three independent experiments, *P<0.05 compared to time 0, **P<0.05 compared to WT.

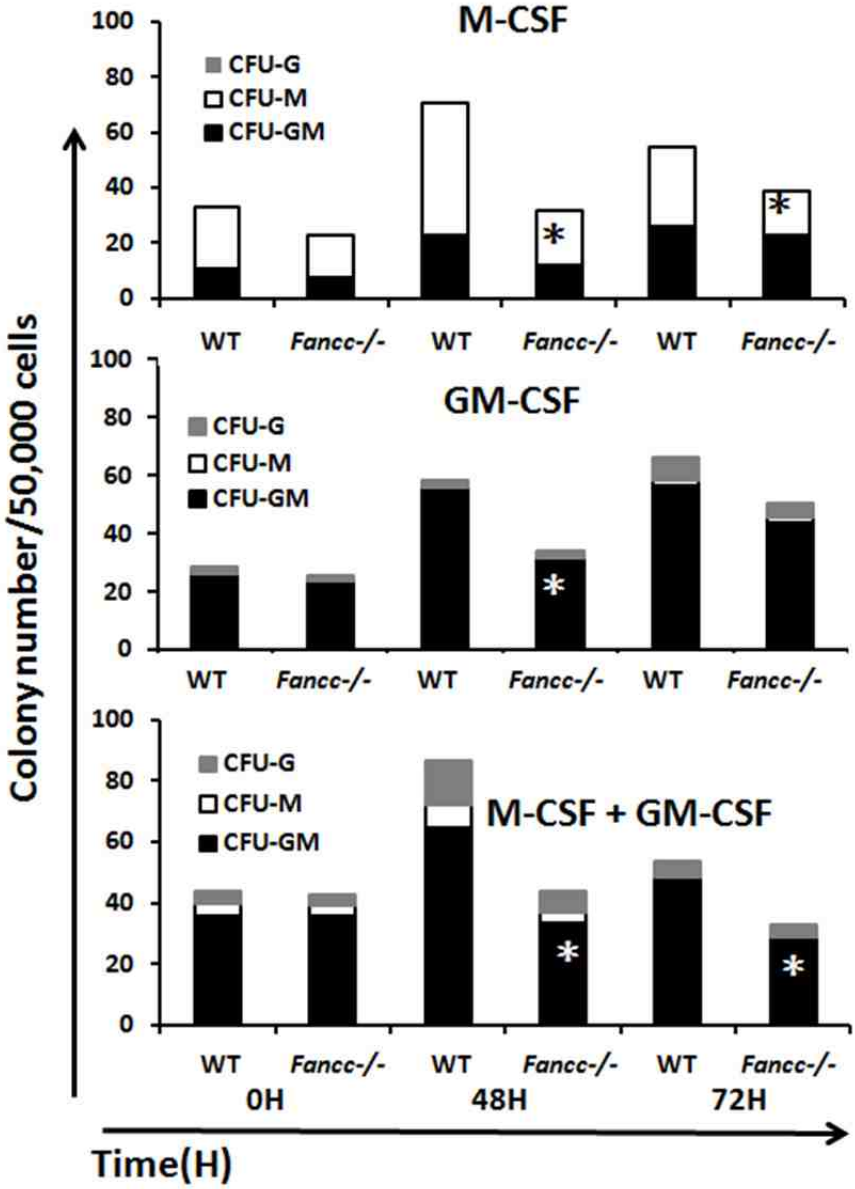


Figure 16. *Fancc-/-* progenitors exhibited altered distribution after *in vivo* LPS treatment. BM cells were collected from untreated and LPS treated mice and were cultured in methylcellulose in the presence of M-CSF (50 ng/mL), GM-CSF (10 ng/mL), or M-CSF and GM-CSF for 7 days. Colonies were differentially scored by morphology as CFU-M, CFU-G, and CFU-GM. Colony type frequencies are shown. Three mice were used for each of the three independent experiments, *P<0.05.

II. *Fancc-/-* BM cells exhibited an impaired response to M-CSF *in vitro*

A. Decreased number of BMDM in the presence of M-CSF

Given our data demonstrating impaired colony formation in the presence of M-CSF together with the central role that M-CSF has in monocyte/macrophage differentiation, we tested whether *Fancc-/-* BM cells exhibited impaired M-CSF induced differentiation using a well-established model of macrophage differentiation *in vitro*, bone marrow derived macrophages or BMDM. For these studies, BM cells were cultured with M-CSF for 6 days to establish BMDM. BMDM were counted and tested for macrophage purity by evaluating co-expression of Mac1 and F4/80. Nearly 90% of WT and *Fancc-/-* BMDM were $Mac1^+$ $F4/80^+$ (Figure 17A), suggesting that *Fancc-/-* BM cells exhibit normal M-CSF induced macrophage differentiation *in vitro*. However, *Fancc-/-* BMDM had a significant reduction in cell number compared to WT (Figure 17B).

B. Reduced M-CSF stimulated proliferation

The decreased number of BMDM from *Fancc-/-* BM cells was speculated to either be caused by reduced proliferation or decreased survival induced by M-CSF. To examine whether *Fancc-/-* BM cells exhibited reduced proliferation, H^3 thymidine incorporation assays were conducted on BMDM stimulated with or without M-CSF. Upon M-CSF stimulation, WT and *Fancc-/-* cells had increased proliferation (Figure 17C). However, a significant decrease was observed in *Fancc-/-* BMDM compared to WT, indicating that altered proliferation contributes to the observed reduction in the number of *Fancc-/-* BMDM. To evaluate whether *Fancc-/-* BM cells had decreased

survival in response to M-CSF, TUNEL assays were conducted to measure apoptosis. WT and *Fancc-/-* BMDM exhibited very low rates of apoptosis (<2%). Together, these data suggest that the major factor contributing to decreased BMDM cell number is impaired M-CSF induced proliferation and not a survival defect.

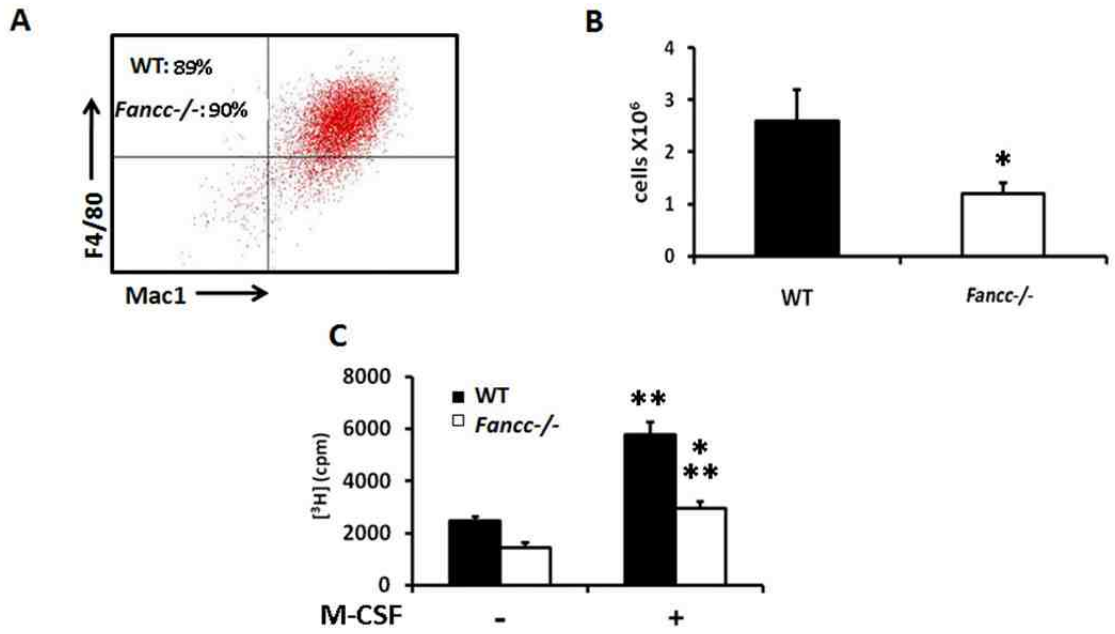


Figure 17. *Fancc-/-* BM cells exhibited reduced proliferation in response to M-CSF. BM mononuclear cells were cultured in the presence of M-CSF (50 ng/mL) for 6 days to establish BMDM. (A) Phenotypic analysis. A representative dot plot illustrating Mac1 and F4/80 expression is shown. (B) BMDM cellularity. The number of viable cells is shown. Six independent experiments were performed, *P<0.05. (C) Proliferation assays. Thymidine incorporation assays were used to evaluate proliferation of BMDM at baseline and after M-CSF stimulation (50 ng/mL for 24 hours). Six independent experiments were performed, *P<0.05, compared to WT, **P<0.05, compared to no M-CSF.

C. Impaired phosphorylation of ERK and AKT upon M-CSF stimulation

ERK and AKT activation are the major downstream targets of M-CSF signaling that regulate the proliferation of BMDM (Pixley and Stanley, 2004). Therefore, we next examined whether *Fancc-/-* BMDM exhibited altered phosphorylation of ERK and AKT. BMDM established from WT mice exhibited robust phosphorylation of ERK and AKT after M-CSF stimulation (Figure 18A-B). In contrast, *Fancc-/-* BMDM had reduced phosphorylation of ERK and AKT (Figure 18A-C). To ensure that differences detected in ERK and AKT phosphorylation were not due to impaired expression of the M-CSF receptor or C-Fms (CD115), flow cytometric analysis of CD115 on BMDM was conducted. *Fancc-/-* BMDM had similar expression levels of the M-CSF receptor compared to WT (Figure 18D). Collectively, these data indicate that *Fancc-/-* BMDM have altered M-CSF induced ERK and AKT phosphorylation which likely contributes to the proliferation defect observed.

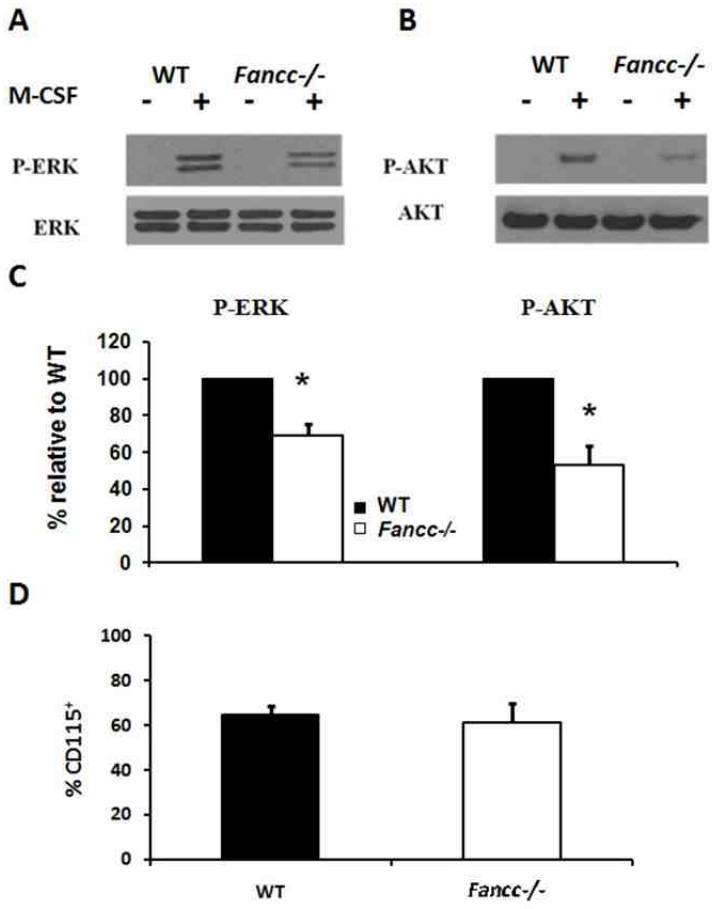


Figure 18. *Fancc-/-* BMDM exhibited impaired phosphorylation of ERK and AKT after M-CSF stimulation. WT and *Fancc-/-* BMDM were either untreated or stimulated with M-CSF (50 ng/mL) for 15 minutes. Representative western blots of (A) phosphorylated ERK/total ERK and (B) phosphorylated AKT/total AKT are shown. (C) Densitometry analysis. Quantified data show the level of phosphorylation of ERK and AKT in *Fancc-/-* BMDM compared to WT. Three independent experiments were conducted,*P<0.05. (D) M-CSF receptor expression. WT and *Fancc-/-* BMDM were stained with anti-CD115-PE conjugated and evaluated by flow cytometry. The mean percentage of cells expressing CD115 is shown. Three independent experiments were performed, *P<0.05.

SUMMARY OF AIM 1:

In the peritoneum after LPS induced peritonitis, *Fancc-/-* mice had increased neutrophils in early inflammatory stages and fewer monocytes/macrophages in later phases of inflammation. The inflammatory gene expression of *Fancc-/-* peritoneal cells was reduced, which was associated with fewer circulating inflammatory monocytes in the peripheral blood of *Fancc-/-* mice. In addition, *Fancc-/-* mice were capable of preserving normal phenotypic distributions of hematopoietic stem/progenitor cells after *in vivo* LPS exposure, however *Fancc-/-* myeloid progenitor responsiveness to M-CSF was significantly impaired. Studies using an *in vitro* BMDM model showed that *Fancc-/-* BMDM had reduced M-CSF induced proliferation and decreased phosphorylation of ERK and AKT. Overall, these observations indicate that *Fancc-/-* mice have an altered LPS induced inflammatory response and impaired M-CSF signaling. However, the question of whether *Fancc-/-* macrophages exhibit autonomous defects in function that contributes to the *in vivo* phenotype remains.

AIM 2: To determine whether *Fancc-/-* macrophages exhibit cell autonomous defects in adhesion, migration and phagocytosis.

Macrophage motility is essential for normal development and immune function. We questioned whether *Fancc-/-* peritoneal macrophages have impaired cell motility including adhesion and migration as well as phagocytosis since this is a critical cellular function for macrophages. The experimental design is illustrated in Figure 19. In brief, peritoneal macrophages were collected and assessed for adhesion on FN and endothelial cells, migration toward MCP-1 and M-CSF, and phagocytosis of *E.coli* particles and IgG latex beads. These analyses were instrumental to determine whether *Fancc-/-* macrophages exhibit intrinsic alterations in function. These studies also help to clarify whether reduced monocyte/macrophage in the local inflamed site is due to impaired motility of *Fancc-/-* cells.

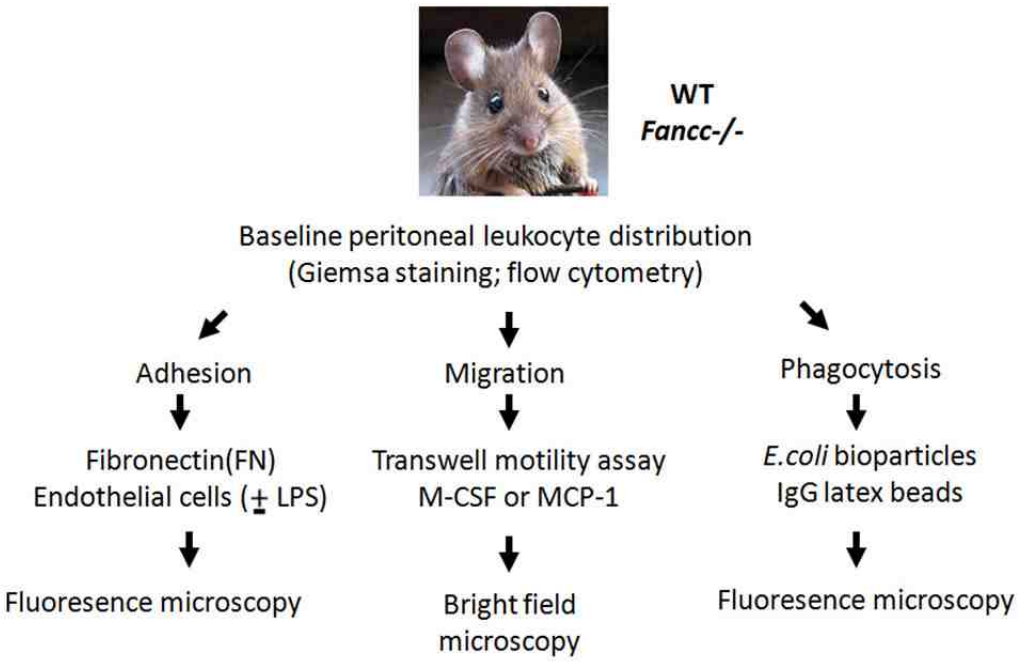


Figure 19. Experimental strategy for Aim 2. Peritoneal cells from age-matched WT and *Fancc-/-* mice were assessed for macrophage distribution at baseline. Three types of functional assays were evaluated on these peritoneal macrophages. For adhesion assays, peritoneal cells were allowed to adhere on either FN or endothelial cells treated with or without LPS (1 μg/mL). For migration assays, MCP-1 and M-CSF were chosen for the chemoattractant of macrophages. For phagoyctosis assays, peritoneal cells were adhered overnight. Adherent peritoneal macrophages were cultured with either *E.coli* bioparticles or IgG latex beads. All of these assays were measured by microscopy (bright field or fluorescent as indicated).

III. Impaired motility and function of *Fancc-/-* monocytes/macrophages

A. Characterization of *Fancc-/-* peritoneal cells at the steady state

Before examining whether *Fancc-/-* peritoneal macrophages exhibit altered function, the steady-state distribution of macrophages in the peritoneal cavity was examined. At baseline, Giemsa staining of peritoneal cells indicated that the majority of cells in the peritoneum were macrophages, accounting for nearly 80% of cells, and no differences were detected between WT and *Fancc-/-* mice (Figure 20A-B). Flow cytometric analyses of peritoneal cells from WT and *Fancc-/-* mice showed a similar phenotypic expression of macrophage antigens (Figure 20C) In addition, the absolute number of these phenotypically-defined cells were also similar between both genotypes (Figure 21D). These data demonstrate the distribution of monocyte/macrophage at steady state is normal in *Fancc-/-* mice compared to WT.

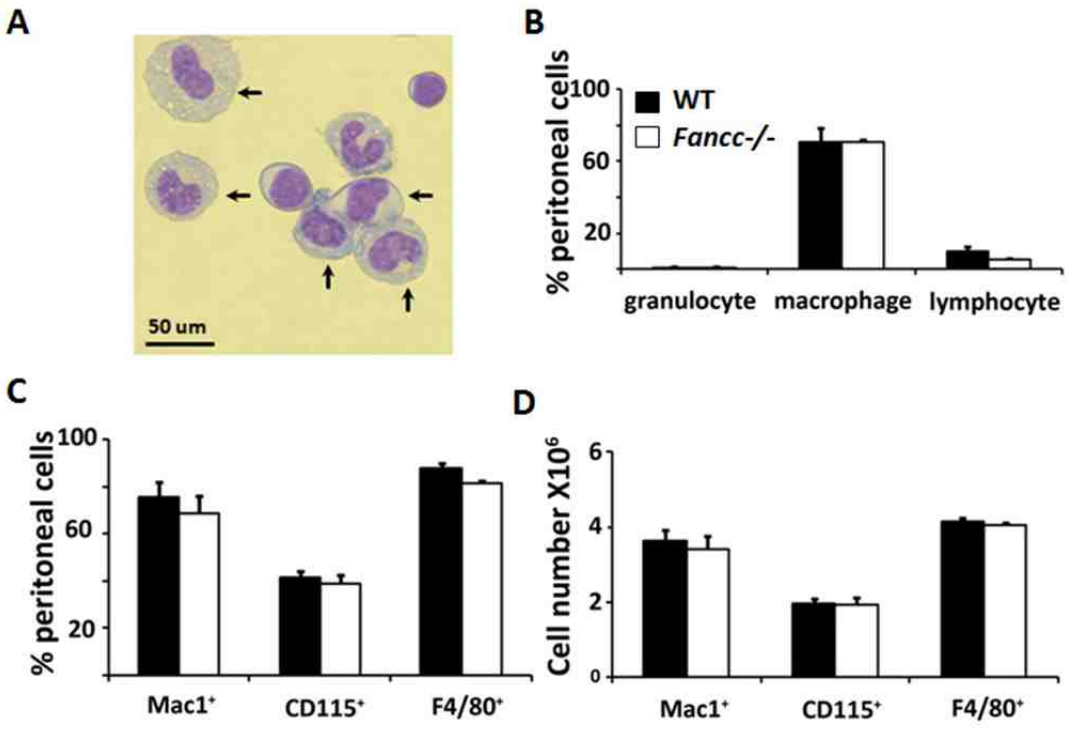


Figure 20. *Fancc-/-* peritoneum exhibited a normal leukocyte distribution. (A) Giemsa staining of peritoneal cells. Representative photomicrograph of peritoneal cells from a WT mouse is shown. Arrows indicate peritoneal monocytes/macrophages. (B) Differential counts determined by morphology after Giemsa staining. Six mice per genotype were tested in each experiment. (C) Expression of macrophage antigens in peritoneal cells. Peritoneal cells were evaluated for the expression of macrophage antigens including $Mac1^+$, $CD115^+$ and $F4/80^+$. Three mice per genotype were tested in each experiment. (D) Absolute number of $Mac1^+$, $CD115^+$ and $F4/80^+$ cells was calculated by multiplying total peritoneal cells by the percentage of each cell population determined by flow cytometry. Three mice per genotype were tested in each experiment.

B. Impaired adhesion of *Fancc-/-* peritoneal macrophages

1. Impaired adhesion on FN

As professional phagocytes, monocytes/macrophages are recruited to the inflamed site to effectively eliminate intruders and cell debris. Our data demonstrate that *Fancc-/-* mice exhibit significantly reduced monocytes/macrophages in LPS induced peritonitis. However, it is not clear whether this observation is due to cell autonomous defects in *Fancc-/-* macrophages. To examine whether *Fancc-/-* macrophages exhibit cell autonomous defects that contribute to the impaired accumulation *in vivo*, we next examined whether *Fancc-/-* macrophages have altered adhesion and migration. To evaluate whether adhesion of *Fancc-/-* macrophages is compromised, resident macrophages were adhered to either FN, which is an important component of the extracellular matrix, or to endothelial cells, which represents a physiologically-relevant cell type. For adhesion to FN, fewer *Fancc-/-* macrophages adhered to FN after 1 hour compared to WT (Figure 21A-B).

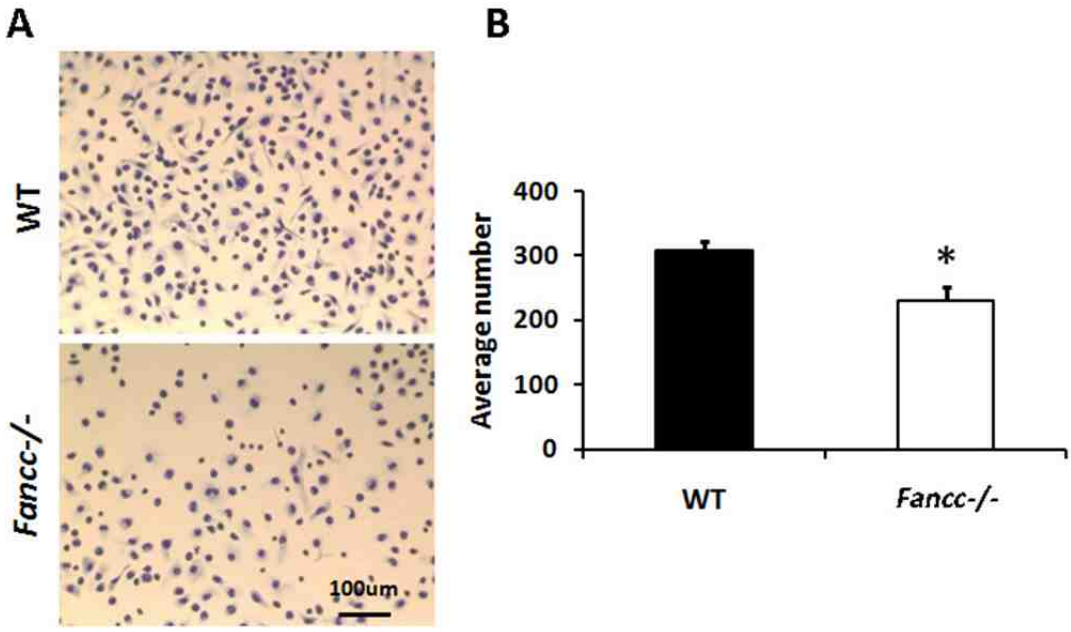


Figure 21. *Fancc-/-* macrophages exhibited reduced adhesion on FN. Fresh peritoneal cells were collected from WT and *Fancc-/-* mice in the steady state. Cells were allowed to adhere on FN (CH296) coated and uncoated wells for one hour. Nonadherent cells were removed by washing with PBS. Adherent cells were estimated after Giemsa staining. Photomicrographs were taken on a Leica microscope (20X). (A) Representative photomicrographs are shown. (B) Quantitative data of levels of adhesion for *Fancc-/-* cells compared to WT are shown. Three independent experiments were performed,*$P<0.05$.

2. Impaired adhesion on endothelial cells

Given that circulating leukocytes are recruited to the inflamed endothelium, where they adhere and traverse between endothelial cells to enter the inflammatory site (Kubes, 2002), we next questioned whether cell-cell adhesion was also altered in *Fancc-/-* macrophages by conducting macrophage-endothelial cell co-culture assays. In addition to conducting assays under baseline conditions, endothelial cells were stimulated with LPS (1 μg/mL) prior to co-culture with macrophages to model the upregulation of adhesion molecules that occurs on endothelial cells during inflammation (Lush *et al.*, 2000). In conditions with untreated endothelial cells, WT macrophages had increased adherence after 2 hours of co-culture compared to the 1 hour time point (Figure 22A-B). In contrast, *Fancc-/-* macrophages had no observable increase under the same conditions and were significantly decreased compared to WT cells. The impairment in *Fancc-/-* macrophage adhesion was even more apparent when evaluated on LPS-activated endothelial cells. WT macrophages exhibited enhanced adhesion to LPS-activated endothelial cells at 1 and 2 hour time points compared to untreated endothelial cell controls. However, *Fancc-/-* macrophages had a dramatic reduction in the number of adherent cells at both time points compared to WT cells (Figure 22A-B). Taken together these data indicate that *Fancc-/-* macrophages exhibit impaired adhesion to FN and endothelial cells.

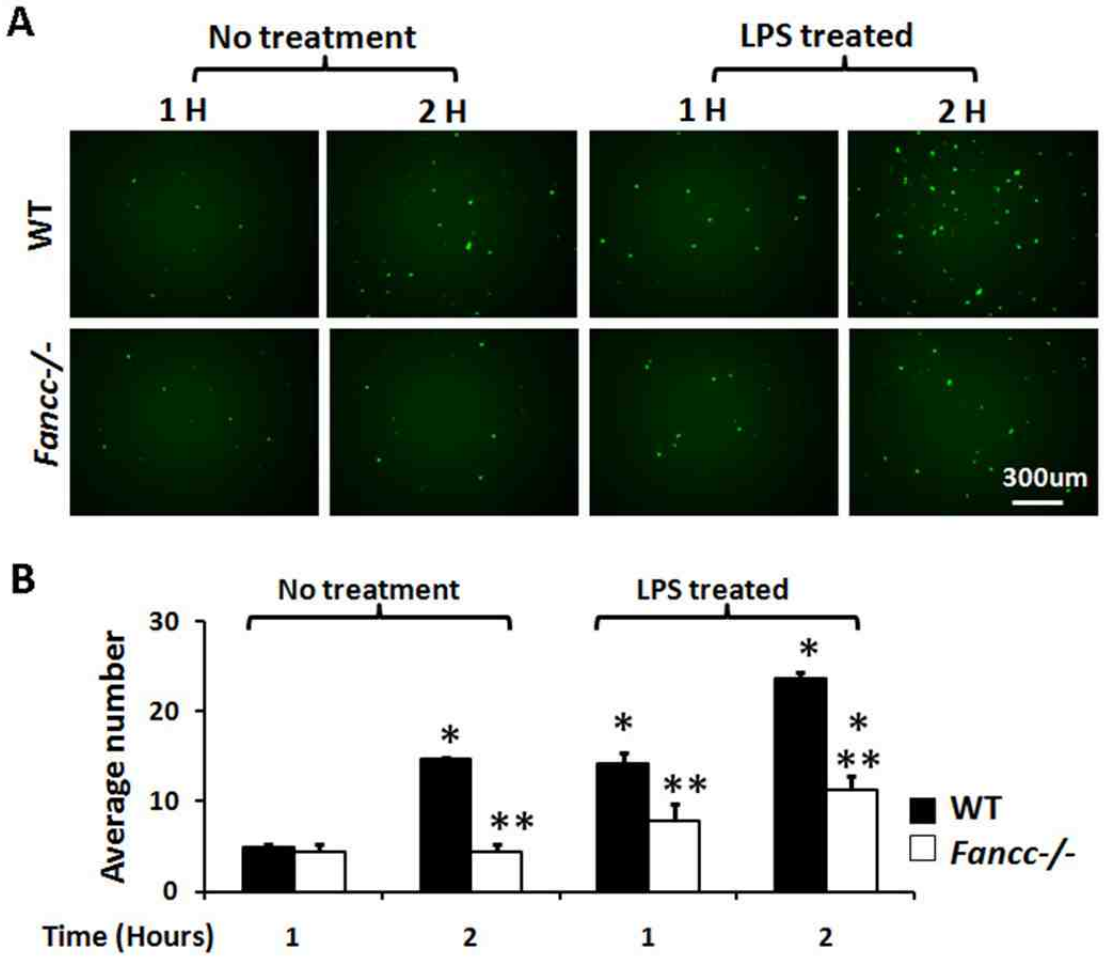


Figure 22. *Fancc-/-* macrophages exhibited reduced adhesion on endothelial cells. CFSE-labeled peritoneal cells were co-cultured with endothelial cells that had been treated with or without LPS. (A) Adhesion on endothelial cells. Representative photographs demonstrate the accumulation of peritoneal cells on untreated and LPS treated endothelial cells. Photomicrographs were taken on a Leica fluorescent microscope (10X). (B) Quantified levels of adhesion. Three independent experiments were performed; *P<0.05 compared to WT cells adhered to untreated endothelial cells for 1 hour, **P<0.05 compared to WT cells in same conditions.

C. Impaired migration of *Fancc-/-* peritoneal macrophages

Because alterations in adhesion are highly associated with impaired motility, we 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 hours of migration, *Fancc-/-* and WT cells had an increase in migrated cells in response to both chemoattractants compared to the media control (Figure 23A-B). However, fewer *Fancc-/-* migrated cells were observed in the presence of MCP-1 and M-CSF compared to similar conditions with WT cells (Figure 23A-B). These data suggest that loss of *Fancc* impairs M-CSF and MCP-1-induced macrophage migration, which is consistent with *Fancc-/-* myeloid progenitor defects detected in the presence of M-CSF (Figure 18). Our previous data showed M-CSF induced proliferation was impaired in *Fancc-/-* BMDM, suggesting potential defects in the signaling pathway downstream of M-CSF receptor. These data showing that M-CSF induced migration was impaired in *Fancc-/-* macrophage provides additional evidence that signaling pathways downstream of the M-CSF receptor is altered in the absence of *Fancc*.

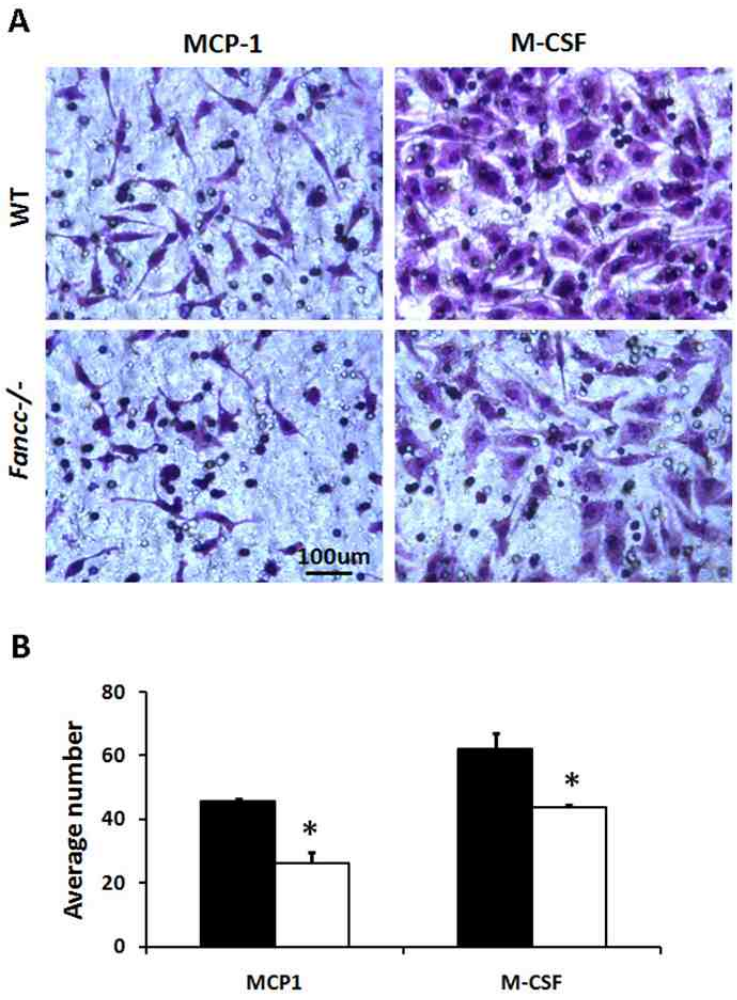


Figure 23. *Fancc-/-* macrophages exhibited reduced migration. Peritoneal cells from mice in the steady state were collected and plated in transwell inserts with a pore size of 8 μM. Cells were allowed to migrate toward MCP1 (20 ng/mL) or M-CSF (50 ng/mL) in the bottom chamber of the transwell for 24 hours. Migrated cells were stained with crystal violet for visibility and scored by microcopy. (A) Representative photomicrographs of migrated cells are shown. Photomicrographs were taken on a Leica microscope (20X). (B) MCP1 and M-CSF induced migration of WT and *Fancc-/-* cells is shown. Three independent experiments were performed; *$P<0.05$.

D. Impaired phagocytosis of *Fancc-/-* peritoneal macrophages

1. Phagocytosis of *E.coli* bioparticles

As a professional phagocyte, phagocytosis is a critical biologic function that macrophages perform for pathogen clearance and for inflammation resolution. Therefore, we evaluated whether *Fancc-/-* macrophages also exhibit impaired phagocytosis assessed by the uptake of fluorescent *E.coli* bioparticles and IgG-coated latex beads. Compared to WT macrophages, *Fancc-/-* macrophages had reduced cells that ingested fluorescent *E.coli* particles after 1 hour of co-culture (Figure 24A-B), implying dysfunctional phagocytosis mediated by TLR4.

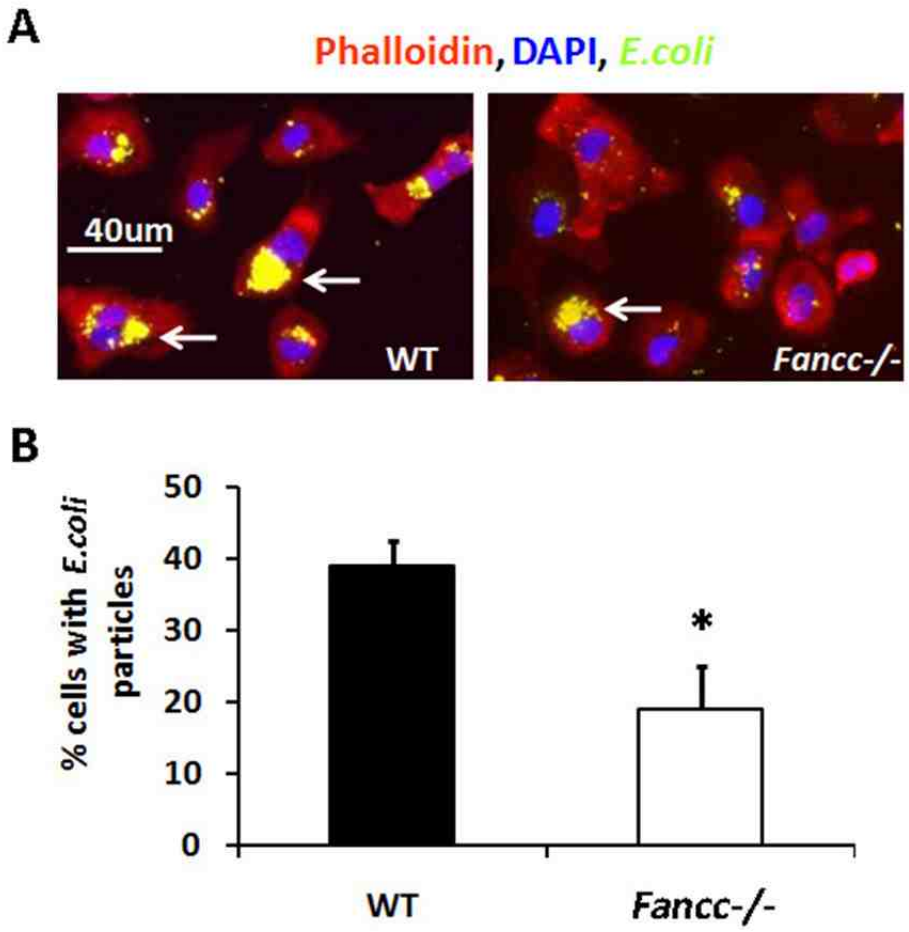


Figure 24. *Fancc-/-* macrophages exhibited decreased phagocytosis of *E.coli* bioparticles. Peritoneal macrophages were cultured for 24 hours and allowed to uptake *E.coli* particles for1 hour. Phagocytosis was estimated by fluorescent microscopy. Photomicrographs were taken on a Leica fluorescent microscope using a uniform exposure time for all slides under comparison (40X). Rhodamine phalloidin stains red. DAPI stains blue. (A) Up-take of *E.coli* particles. Representative photomicrographs of WT and *Fancc-/-* macrophages phagocytosing *E.coli* particles are shown. Arrows indicate engulfed fluorescent particles in macrophages. (B) Quantified data for *E.coli* particle uptake are shown. Three independent experiments were performed, *P<0.05.

2. Phagocytosis of IgG latex beads

The impaired phagocytosis of *E.coli* suggests TLR4 mediated phagocytosis is altered in *Fancc-/-* macrophages. Macrophages express a variety of pathogen receptors which initiate phagocytosis. To examine whether the phagocytosis defect of *Fancc-/-* macrophages was specific to TLR4 ligands or a more global defect, we examined whether Fc-γ receptor mediated phagocytosis was altered in *Fancc-/-* macrophages. To address this issue, phagocytosis of IgG-coated latex beads, which mainly signals through Fc-γ receptor, was assessed. Macrophages were stained with rhodamine labeled phalloidin to visualize intracellular F-actin which encircles ingested beads (Figure 25A). Macrophages that ingested one or multiple beads were quantified in WT and *Fancc-/-* macrophages. Although phagocytosis of one bead was normal in *Fancc-/-* macrophages, fewer *Fancc-/-* macrophages ingested multiple beads compared to WT cells (Figure 25B), Together these data indicate that both TLR4 and Fc-γ receptor mediated phagocytosis are altered in *Fancc-/-* macrophages, supporting the possibility that loss of *Fancc* results in a more global defect in phagocytosis.

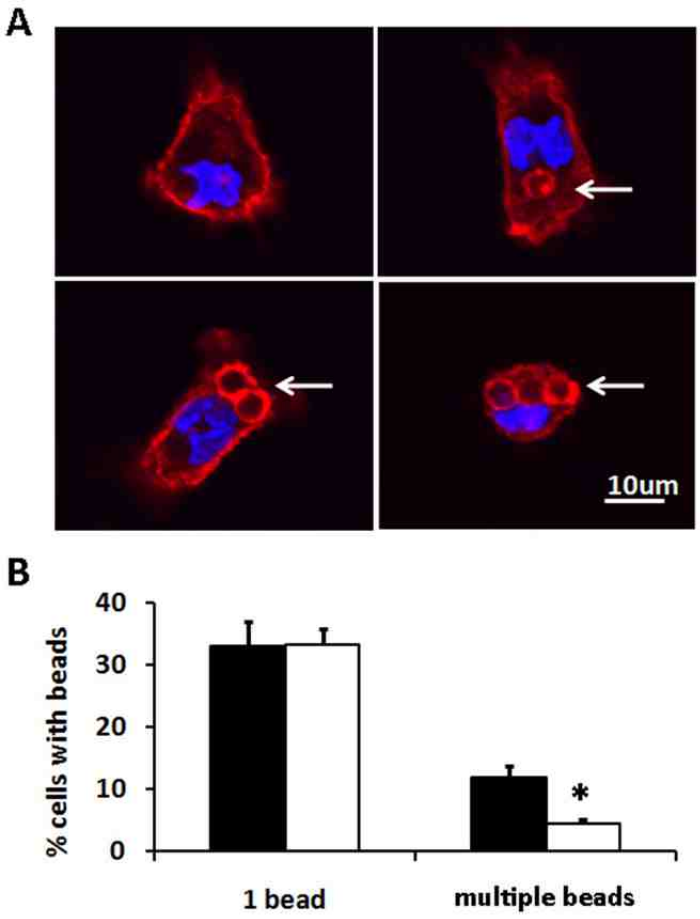


Figure 25. *Fancc-/-* macrophages exhibited decreased phagocytosis of IgG beads. Peritoneal macrophages were cultured for 24 hours and allowed to uptake IgG labeled beads for 1 hour. Macrophages were fixed and stained with phalloidin and DAPI. Phagocytosis was evaluated by confocal microscopy. Photomicrographs were taken on an Olympus FV-1000 MPE fluorescent microscope using a uniform exposure time for all slides under comparison (60X). (A) Representative photomicrographs of ingestion of IgG labeled beads in WT macrophages are shown. Arrows indicate the beads phagocytosed by macrophages. Cells were scored by the number of beads ingested. (B) Quantified data for number of IgG beads phagocytosed are shown. Three independent experiments were performed, *$P<0.05$.

3. Reduced phagocytosis mediated superoxide production

Superoxide production is a key event for pathogen clearance that occurs during and after phagocytosis. If *Fancc-/-* macrophages have decreased phagocytosis, we speculated that superoxide production would also be reduced. To test this hypothesis, *Fancc-/-* and WT macrophages were evaluated for superoxide production during phagocytosis of serum opsonized Zymosan (SOZ) and Zymosan. Macrophages from both genotypes showed an immediate increase of superoxide production after treatment with SOZ and Zymosan compared with no treatment (Figure 26A-B). However, *Fancc-/-* macrophages had attenuated superoxide production in response to SOZ, with a trend towards a decreased response to Zymosan (Figure 26A-B) compared to WT cells. These data are consistent with the impaired phagocytic activity of *Fancc-/-* macrophages in response to multiple ligands.

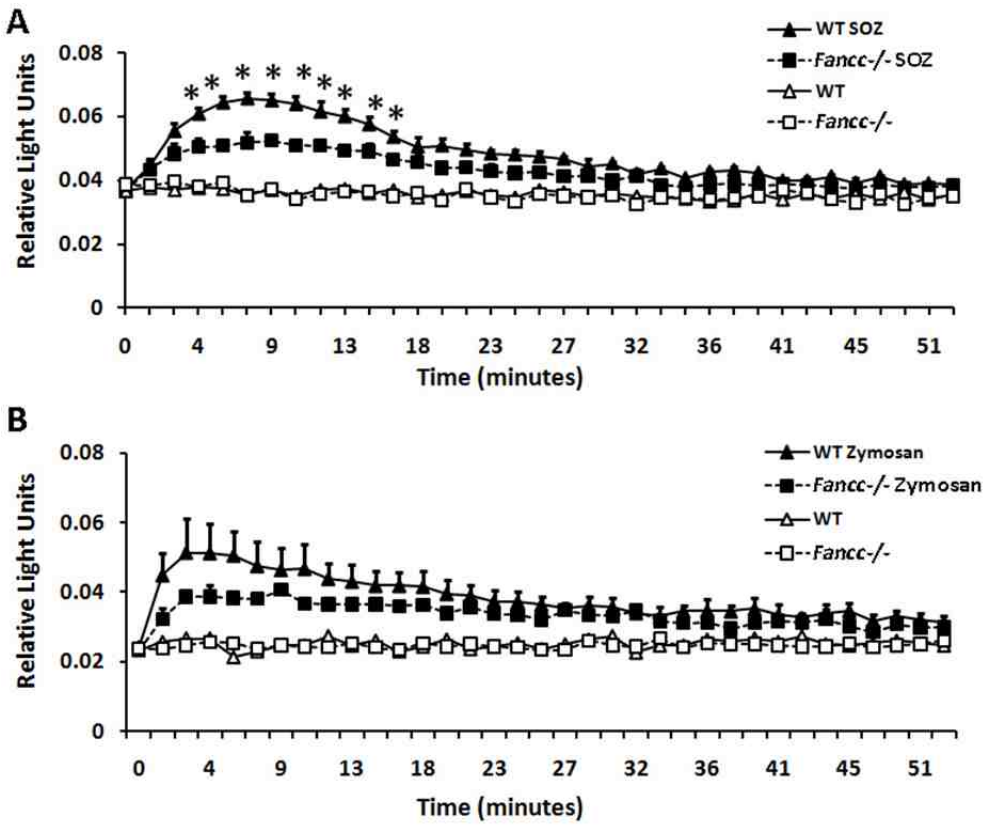


Figure 26. *Fancc-/-* macrophages exhibited reduced superoxide production. Peritoneal macrophages were cultured for 24 hours and allowed to uptake SOZ, Zymosan, or no treatment for one hour. The production and relative amount of superoxide was monitored and determined by a lucigenin chemiluminescence assay. (A) Superoxide production after phagocytosis of SOZ is shown. Three independent experiments were performed, *P<0.05. (B) Superoxide production after phagocytosis of Zymosan is shown. Three independent experiments were performed.

E. Dysfunctional cytoskeleton reorganization in *Fancc-/-* macrophages

The cellular processes of adhesion, migration, and phagocytosis require complex actin cytoskeletal rearrangement to occur (Worthylake and Burridge, 2001; Jones, 2000; Worthylake *et al.*, 2001). Thus, we speculated that disrupted actin cytoskeletal rearrangement may be the unifying mechanism responsible for the functional abnormalities detected in *Fancc-/-* macrophages. 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 no significant changes in F-actin content compared to WT cells (Figure 27A-B). Therefore, we questioned whether F-actin reorganization was altered in *Fancc-/-* macrophages. The localization of F-actin was evaluated by rhodamine labeled phalloidin staining followed by fluorescence microscopy. By one hour of adhesion, WT and *Fancc-/-* cells were round with F-actin in a pericellular distribution (Figure 28A). WT macrophages began to polarize from 6 to 24 hours and developed multiple protrusions, which were enriched with phalloidin staining at 6 hours. However, *Fancc-/-* macrophages remained predominantly circular in shape. By 24 hours, WT cells demonstrated cell spreading with a dramatic elongation. F-actin staining displayed a fiber-like staining in WT cells and was primarily located in the longitudinal axis. In contrast, *Fancc-/-* macrophages had fewer protrusions at 24 hours, and the phalloidin staining remained pericellular in distribution. Moreover, the fiber-like staining of phalloidin was rarely observed in *Fancc-/-* macrophages. In order to quantitate qualitative defects observed in *Fancc-/-* macrophage elongation, we measured the spreading and elongation of peritoneal macrophages on FN as described by others (Yu *et al.*, 2005) (Figure 28B). Adherent cells were measured for long axis and short axis by

Adobe photoshop. The cells with the ratio of Long/Short axes greater than 1 were defined as elongating or polarizing cells. Round cells were defined as having a ratio of 1. Figure 28C illustrates the percentage of elongated cells within 24 hours. WT cells had a dramatic increase in the percentage of elongated cells within 6 hours compared to 1 hour. Over 90% of WT cells finished cell spreading by 24 hours. In contrast, *Fancc-/-* cells displayed a dramatic reduction in percentage of elongated cells by 6 hours and only half of them completed elongation by 24 hours. These data indicate that *Fancc-/-* macrophages have insufficient F-actin cytoskeletal rearrangements induced by FN, which may be the underlying mechanism responsible for dysfunctional adhesion, migration, and phagocytosis.

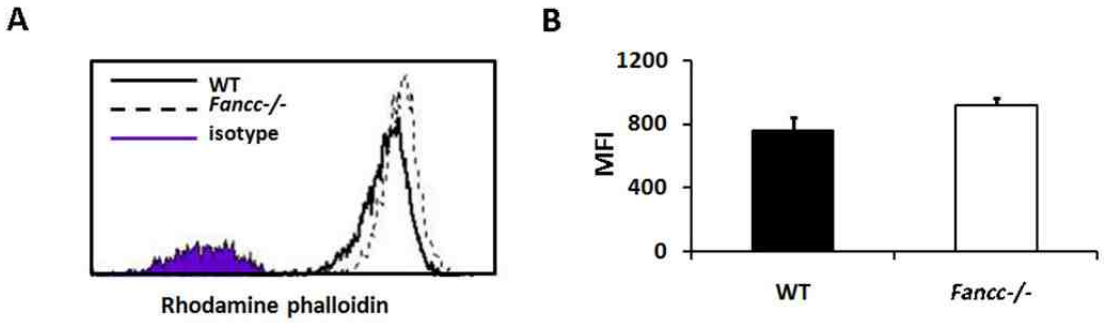


Figure 27. *Fancc-/-* peritoneal macrophages exhibited normal F-actin content. Fresh peritoneal cells from WT and *Fancc-/-* mice were stained with rhodamine phalloidin and examined by flow cytometry to quantitate F-actin content. (A) Representative histogram of rhodamin-phalloidin staining. (B) Mean fluorescence intensity (MFI) of rhodamine-phalloidin staining. MFI of phalloidin staining is shown. Three independent experiments were performed.

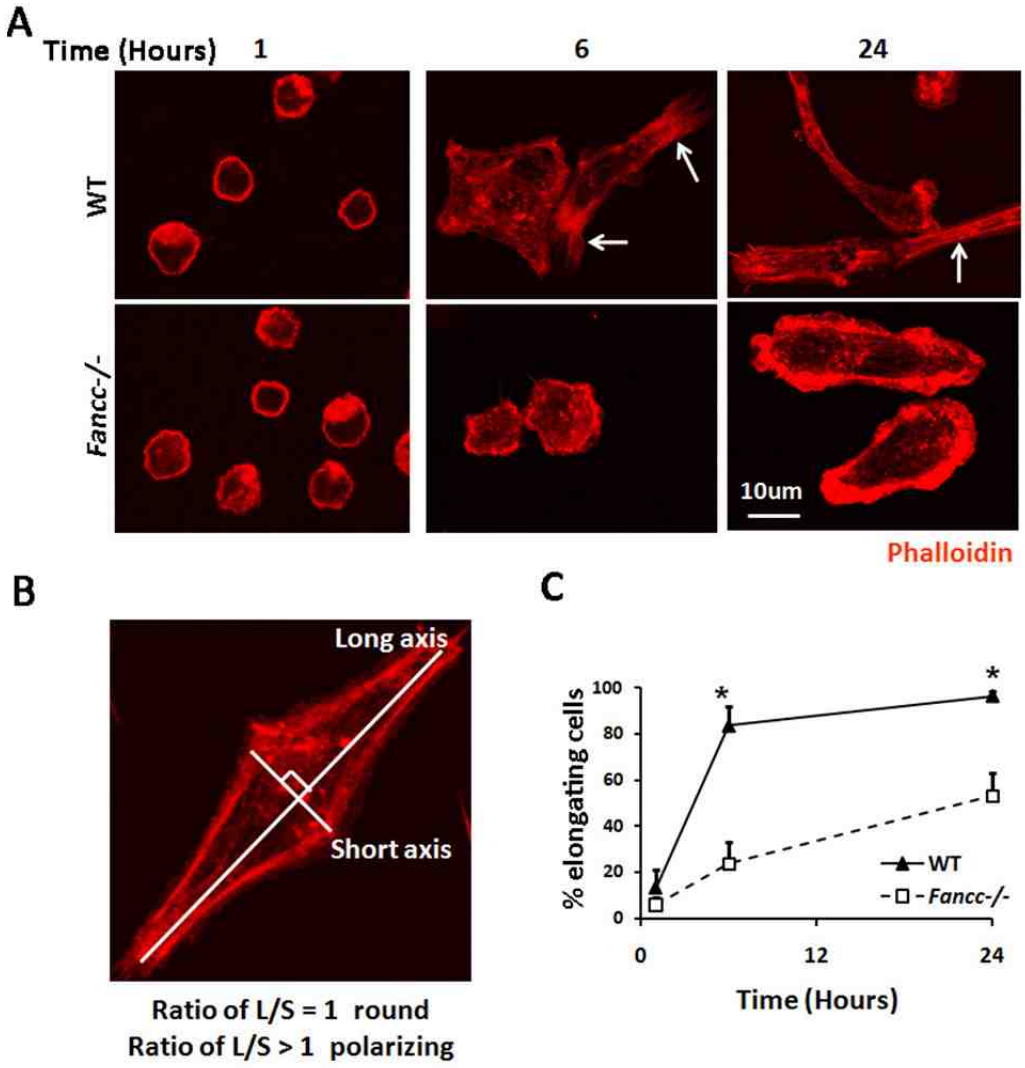


Figure 28. *Fancc-/-* peritoneal macrophages exhibited impaired elongation. (A) F-actin localization in macrophage. Macrophages were adhered to FN and stained with rhodamine phalloidin. Representative confocal images are shown. Arrows point to the elongated cells with concentrated staining of phalloidin. (B) Long axis (L) and short axis (S) were measured by Adobe photoshop. Ratio of L/S =1 defined a round cell. Ratio of L/S >1 defined an elongating cell. (C) The percentage of elongating cells on FN is shown. Three independent experiments were performed, *P<0.05.

F. Impaired activation of RhoA in *Fancc-/-* macrophages

A variety of proteins are involved in the regulation of actin cytoskeleton reorganization and dynamics. Among them, Rho GTPases, including Rac1, Cdc42 and RhoA interact with a spectrum of effectors to control actin cytoskeleton reorganization, cell shape, and cell polarity (Wheeler and Ridley, 2007; Kao *et al.*, 1999; Liao *et al.*, 2003; Munugalavadla *et al.*, 2005; Okada *et al.*, 2010; Sulahian *et al.*, 2008; Suzuki and Umezawa, 2006). 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 after serum stimulation by 10 minutes and declined to basal levels by 30 minutes with no significant differences between WT and *Fancc-/-* macrophages (Figure 29A). On the other hand, RhoA activation initiated at 30 minutes in WT and remained sustained until 90 minutes (Figure 29C-D). However, in *Fancc-/-* macrophage the activation of RhoA was not increased at 30 minutes and significant reduction was observed at later time points compared to WT cells.

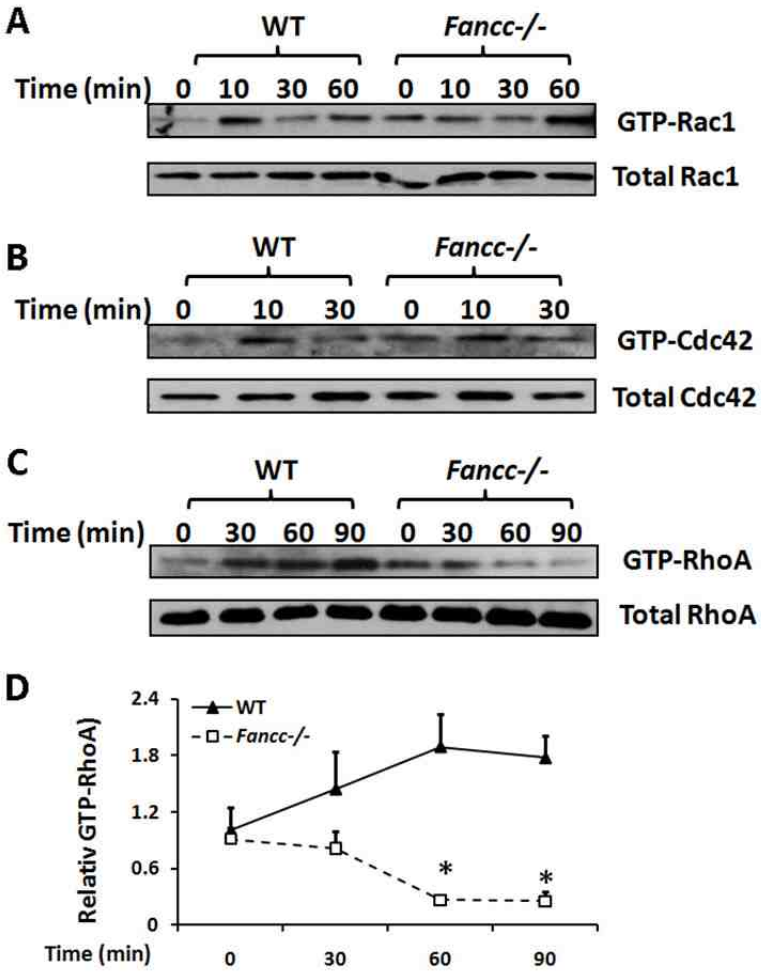


Figure 29. Impaired activation of RhoA was observed in *Fancc-/-* macrophages. For a single small GTPase activation assay, peritoneal cells from 10 mice per genotype were pooled. (A) Representative Rac1 and (B) Cdc42 activation assays are shown. GTP-Rac1 and GTP-Cdc42 were immunoprecipitated with GST-PBD and detected by Rac1 and Cdc42 specific antibodies. (C) Representative RhoA activation assay is shown. GTP-RhoA was immunoprecipitated with GST-RBD and detected by RhoA antibody. (D) Quantification of densitometry data is shown. GTP-RhoA was normalized to total RhoA, and the relative change was normalized to time 0. Three independent experiments were performed,*P<0.005.

SUMMARY OF AIM2

Baseline leukocyte distribution in the peritoneum of *Fancc-/-* mice was normal, however numerous functional deficits were elucidated in resident *Fancc-/-* macrophages, including impaired adhesion, migration, phagocytosis, and superoxide production. The unifying mechanism responsible for these phenotypes is speculated to be altered F-actin reorganization, which is supported by our data showing abnormal elongation of *Fancc-/-* peritoneal macrophages after adhesion to FN and impaired RhoA activation . Collectively, these data support the contention that loss of *Fancc* not only affects hematopoietic stem/progenitor cell functions, as previously thought, but also severely impairs immune cell function resulting in an altered inflammatory response *in vivo*.

DISCUSSION

Astute clinical observations have long suggested that FA patients may have immune defects (reviewed in Fagerlie SR, 2006). However, FA genetic heterogeneity together with leukopenia from evolving BM failure has made formal proof of this concept challenging. Limited previous studies in murine models of FA support altered immunologic function (Sejas *et al.*, 2007; Zhang *et al.*, 2007; Hadjur and Jirik, 2003; Vanderwerf *et al.*, 2009). However, none of these reports included a systematic functional evaluation of macrophages, which are an integral cellular component of the innate immune system as well as an antigen presenting cell that participates in the coordination of acquired immunity.

1. Altered inflammatory response in *Fancc-/-* mice

In the current studies, we show that LPS induced recruitment of *Fancc-/-* monocytes/macrophages to an inflamed peritoneum *in vivo* is significantly reduced. Previous studies demonstrated alterations in the response of *Fancc-/-* cells to toll-like receptor ligands such as LPS and R848 (Vanderwerf *et al.*, 2009; Sejas *et al.*, 2007; Hadjur and Jirik, 2003). Decreased *Fancc-/-* monocyte/macrophage recruitment was not specific to toll receptor signaling since a similar response was detected after injection of sodium periodate, which induces a non-specific, chemical inflammation. The decrease in circulating *Fancc-/-* inflammatory monocytes after LPS injection suggests that impaired monocytes mobilization from the BM may be a contributing factor for fewer macrophages observed in the local inflammatory site. However, there are other possible explanations for the reduced local monocytes/macrophages which have not been tested.

First of all, the protein level of cytokines/chemokines in the inflamed peritoneum cavity has not been examined. We have tested the gene expression of cytokines/chemokines in the peritoneal cells. Our data showed reduced mRNA production in *Fancc-/-* cells. However, the total protein levels in the peritoneal fluid could be different from mRNA expression in peritoneal cells, because other cell types in the peritoneum may contribute to the protein levels of cytokines/chemokines (i.e. endothelial, epithelial and T cells). To determine the levels of cytokines/chemokines, Multiplex technique could be used with LPS injected peritoneal fluid samples. Secondly, the survival of peritoneal monocytes/macrophages were not evaluated in our studies. We showed *Fancc-/-* mice exhibited a decreased number of monocytes/macrophages in the later phase of inflammation, which could be due to either decreased recruitment from peripheral blood or increased apoptosis of monocytes/macrophages. Although we showed that *Fancc-/-* peritoneal neutrophils had decreased apoptosis in the early phase, the survival of macrophages was not assessed. A similar method of testing apoptosis of neutrophils could be used for determining the monocytes/macrophages survival. Moreover, the identification of *Fancc-/-* macrophage deficits *in vitro*, including impaired adhesion, migration, and phagocytosis, support macrophage autonomous defects in *Fancc-/-* mice that likely participate in the alterations observed *in vivo*. Collectively, these data provide strong evidence for a primary defect in the function of *Fancc-/-* macrophage.

Previously, Sejas and colleagues examined *Fancc-/-* mice for evidence of enhanced systemic inflammation after LPS treatment (Sejas *et al.*, 2007). In these studies, investigators reported high serum levels of inflammatory cytokines, especially TNF-α, in *Fancc-/-* mice compared to WT after LPS injection. Therefore, our data demonstrating no

differences in serum inflammatory cytokines and reduced inflammatory cytokine/chemokine expression in peritoneal cells from *Fancc-/-* mice after LPS was initially surprising. However, the overall goal of our studies was to evaluate for alterations in the local inflammatory response, not systemic inflammation. Therefore, potential explanations for the apparent discrepant results between our studies and previous data include type of inflammatory response initiated, type of LPS used, and environment that *Fancc-/-* mice are housed. IP injections are highly reproducible for LPS induced inflammatory response studies in mice (Holmgren *et al.*, 2008; Marquez-Velasco *et al.*, 2007). However, handling mice for a proper IP injection can be challenging. To keep the results consistent, we treated mice with isoflurane which anaesthesizes the mice allowing for the proper treatment of LPS injection and avoiding subcutaneous or abdominal organs injection which will cause complete different inflammatory responses compared to peritoneal cavity injection. Additionally, we also adjusted the LPS dose according to mouse body weight in order to have consistent results. Although we use the LPS from the pharmaceutical company, the lot number and the potential quality may be different from previous studies. Overall, these are the contributors for the difference we observed in *Fancc-/-* mice from previous studies. However, our findings are consistent with a more recent study that reported normal LPS induced TNF-α production from *Fancc-/-* splenic macrophages (Vanderwerf *et al.,* 2009). Interestingly, these studies did show that *Fancc-/-* splenic macrophages treated with a TLR8 agonist overproduced TNF-α, suggesting that TLR8 signaling may be altered. Taken together, these studies emphasize that *Fancc-/-* immune cells do exhibit altered functions including higher levels of TNF-α. Yet, the mechanisms involved needs to be further investigated.

2. Impaired adhesion, migration and phagocytosis of *Fancc-/-* macrophages

The implications of our findings are significant given the central role that macrophages have in both innate and acquired immunity. Furthermore, macrophages orchestrate the initiation and resolution phases of an acute inflammatory response (Cailhier *et al.*, 2005). The severity of local inflammation is dependent on the type of pathogen exposure, efficiency of pathogen clearance, and competent resolution of inflammation (Cox *et al.*, 1995; Tabas, 2010). Crucial functions of macrophages in these processes are to kill pathogens, phagocytose apoptotic neutrophils, and repair tissue injury as a result of the inflammation. Therefore, the 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 towards chronic inflammation or a lower threshold for progression to a severe systemic inflammatory response. Studies demonstrating increased serum inflammatory cytokines in FA patients are consistent with a chronic inflammatory state in FA (Stark *et al.*, 1993; Bagnara *et al.*, 1993; Rosselli *et al.*, 1994). In addition, studies showing 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 (Fagerlie and Bagby, 2006; Sejas *et al.*, 2007; Zhang *et al.*, 2007; Dufour *et al.*, 2003). In our studies, LPS is the only stimulus that we tested for an altered inflammatory response in *Fancc-/-* mice. Since LPS is only part of gram-negative bacteria, the inflammation induced by real pathogen and endotoxin may be very different. Therefore, it is necessary to examine whether *Fancc-/-* mice exhibit altered inflammation in response to a bacterial (eg. *E.coli*) infection.

3. Classical and alternative macrophage activation

In the acute-phase of inflammation, pro-inflammatory mediators are released primarily by PMNs and inflammatory macrophages. Over time, the inflammatory response is dampened by resolving macrophages. Consequently, macrophage activation can be pro-inflammatory or anti-inflammatory, which is dependent on intrinsic macrophage characteristics and extrinsic environmental cues. Dysregulated activation of macrophages contributes to local tissue destruction, systemic inflammation, and chronic inflammation. Thus, macrophages have an essential role in initiating, instructing, and terminating the adaptive immune response.

Classically activated or M1 macrophages develop in response to concomitant stimulation with IFN-γ and microbial products such as LPS (Adams, 1989; Yamazaki *et al.*, 2008; Jeannin *et al.*, 2011). Functionally, M1 macrophages secrete significant amounts of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-12 (Benoit *et al.*, 2008). In addition, M1 macrophages are characterized by enhanced endocytic functions and ability to kill intracellular pathogens, which is mediated by a variety of mechanisms including phagocytosis, phagosome acidification, and nitric oxide (NO) synthesis (Groesdonk *et al.*, 2006; Raisanen *et al.*, 2005). NO synthesis in macrophages is regulated by the balance between inducible NO synthetase (iNOS) and arginase, which sequesters NO by competing the same substrate L-Arginine (Colton *et al.*, 2006). Similar to M1 cells, M2 or alternatively activated macrophages change cellular morphology and secretory pattern after being stimulated by multiple anti-inflammatory regulators such as IL-4, IL-13, TGF-β, and IL-10 (Pixley and Stanley, 2004; Martinez *et al.*, 2008). Importantly, M-CSF stimulation of macrophages is critical for intact activation towards

an M2 phenotype (Kinashi *et al.*, 1991; Yamazaki *et al.*, 2008). M2 macrophages release chemokines including CCL17, and CCL18 (Imai *et al.*, 1999). In addition, M2 macrophages counteract ongoing inflammation by releasing factors such as Ym1, Ym2, IL-10, and TGF-β, which also indirectly promotes tissue repair (Raes *et al.*, 2002a; Raes *et al.*, 2002b; Stein *et al.*, 1992).

Interestingly, previous studies in *Fancc-/-* peritoneal macrophages demonstrated increased iNOS expression and NO production in response to LPS and IFN-γ stimulation. Though not discussed in the manuscript, these findings suggest that *Fancc-/-* macrophages exhibit increased M1 activation. Our data showing reduced M-CSF induced proliferation, ERK and AKT phosphorylation in *Fancc-/-* macrophages suggest reduced M2 activation. Therefore, we hypothesize that *Fancc-/-* macrophages have increased classical activation and reduced alternative activation leading to an altered inflammatory response. Future studies to test this hypothesis will be important.

4. Association of altered cytoskeleton rearrangement and decreased RhoA activation in *Fancc-/-* macrophages

Dramatic morphologic differences were identified between *Fancc-/-* macrophage and controls, which are attributed to disrupted actin rearrangement in *Fancc-/-* macrophage. Complex cytoskeletal rearrangements are essential for numerous cellular processes to be intact including adhesion, migration, and phagocytosis. Given that the functional defects in *Fancc-/-* macrophage were induced by numerous extracellular stimuli (i.e. FN, M-CSF, MCP-1, SOZ, *E.coli*), we speculate that dysregulation of cytoskeletal organization is the underlying mechanism responsible for the functional defects in *Fancc-/-* macrophage *in vitro* as well as altered trafficking *in vivo.* The recruitment of monocytes/macrophages to local inflammatory sites is dependent on dynamic cell shape changes, which promote cell movement from the blood through the endothelium into local tissue environments. Several important steps are involved in this process with each individual step requiring dynamic changes in the actin cytoskeleton (Vicente-Manzanares and Sanchez-Madrid, 2004; Worthylake *et al.*, 2001; Worthylake and Burridge, 2001). Reduced adhesion of *Fancc-/-* monocytes/macrophages to endothelial cells as well as decreased motility of macrophages through the endothelium will significantly impact mobilization and recruitment.

A variety of proteins are involved in the complex regulation of the cytoskeleton. Among them, Rho GTPases have a central role in controlling actin cytoskeleton reorganization, cell shape, contractility, and polarity (Wheeler and Ridley, 2007; Kao *et al.*, 1999; Liao *et al.*, 2003; Munugalavadla *et al.*, 2005; Okada *et al.*, 2010; Sulahian *et al.*, 2008; Suzuki and Umezawa, 2006). Interestingly, *Fancc-/-* macrophage had reduced

RhoA-GTP, while Rac1-GTP and Cdc42-GTP levels were normal. Consistent with these data, macrophages expressing a dominant negative RhoA mutant exhibit reduced migration and phagocytosis (Caron and Hall, 1998; Jones *et al.*, 1998). While no previous studies have demonstrated alterations in the activation of Rho GTPases in *Fancc-/-* macrophages, a previous study reported decreased cdc42 activity in FA-A Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines. Reduced Cdc42 activity was associated with impaired adhesion of *Fanca-/-* hematopoietic stem/progenitor cells (Zhang *et al.*, 2008). In addition, previous data showed granulocyte-colony stimulating factor induced mobilization of neutrophils and HSC/progenitors was decreased in FA patients and murine models (Croop *et al.*, 2001; Kang *et al.*, 2001; Pulliam *et al.*, 2008; Muller and Williams, 2009). Our data together with these previous observations support the hypothesis that deregulated cytoskeletal organization may have a more global mechanistic role in the FA hematopoietic phenotype.

5. Attenuated M-CSF responsiveness of *Fancc-/-* myeloid progenitors and macrophages

Interestingly, our studies demonstrate that *Fancc-/-* mice challenged with LPS exhibit reduced expansion of M-CSF responsive progenitors in the BM. However, quantification of hematopoietic stem and progenitor cells by multi-parameter flow cytometry showed that absolute numbers of LSK, CMP, and GMP in the BM of WT and *Fancc-/-* mice treated with LPS are not different. We speculated that these findings were due to altered M-CSF intracellular signaling in myeloid progenitors. While our studies do not directly address whether M-CSF signaling is abnormal in *Fancc-/-* myeloid

progenitors, data from BMDM are supportive of this hypothesis. Fewer numbers of *Fancc-/-* BMDM were obtained after 6 days in culture with M-CSF compared to WT. Although M-CSF regulates differentiation, survival, and proliferation of BMDM, only proliferation was impaired in *Fancc-/-* cells. Reduced M-CSF induced ERK and AKT phosphorylation were observed in *Fancc-/-* BMDM, which is consistent with the central role that these two proteins have in regulating proliferation of BMDM. Taken together these data suggest that monocyte/macrophage generation *in vivo* is altered under conditions of stress. To determine whether M-CSF signaling is also impaired in *Fancc-/-* myeloid progenitors, the next set of experiments would require isolation of LSK, CMP, and GMP by flow cytometry followed by stimulation with M-CSF and analyzing for activation of downstream signaling molecules such as ERK and AKT.

Additional clinical evidence suggests that FA patients may exhibit impaired macrophage function. Skeletal studies of FA patients demonstrate a high prevalence of osteopenia (Giri *et al.*, 2007; Brim *et al.*, 2007). Osteoclasts are reduced, and the balance of osteoclast to osteoblast function is altered (Brim *et al.*, 2007). However, the potential mechanisms have not been elucidated. As specific BM resident macrophages, osteoclasts have an important role in bone resorption. M-CSF is required for intact differentiation, proliferation and survival of osteoclasts via activation of ERK and AKT (Bradley *et al.*, 2008; Umeda *et al.*, 1996). Importantly, adhesion of osteoclasts to the bone surface and migration along the bone are integral for intact bone remodeling to occur. Reduced adhesion, migration, and phagocytosis of *Fancc-/-* peritoneal macrophages suggests that osteoclasts may also have similar defects that disrupt the balance of bone remodeling *in*

vivo. Taken together, our data may provide a potential mechanism for skeletal defects observed in FA, which warrants further investigation.

6. A limitation of our study: the molecular function of FANCC was not evaluated

A number of groups have shown that FANCC have non-FA binding partners indicating that FANCC is involved in signaling pathways independent of DNA repair. For instance, *Fancc* mutant hematopoietic and embryonic fibroblast cells exhibit suppressed STAT1 activation in response to IFN-γ stimulation (Fagerlie *et al.*, 2001; Pang *et al.*, 2000). These studies also showed that FANCC and STAT1 physically interact in cells stimulated with cytokines. In our studies, we observed suppressed ERK and AKT activation in response to M-CSF. To determine the actual link of FANCC and M-CSF signaling, it is necessary to investigate where these alterations are initially observed downstream of M-CSF stimulation. We speculate FANCC serves as a chaperone protein to regulate multiple survival, growth, and proliferation pathways, as has been previously suggested (Bagby, 2003).

FUTURE STUDIES

Given the data discussed above several future aims become evident. These aims are outlined below with a brief rationale and approach comment.

1. To evaluate whether *Fancc-/-* mice have altered inflammation and immunity to bacterial pathogen challenge *in vivo*

LPS is an integral component of the outer cell wall membrane of gram-negative bacteria. The toxicity of LPS is associated with Lipid A, and LPS immunogenicity is associated with the polysaccharide component (Freudenberg *et al.*, 2008). Humans injected with purified LPS develop a cytokine cascade in the serum reminiscent of bacterial sepsis (Wilson *et al.*, 1997). The early cytokine response (TNF-α, IL-6, and IL-8) to LPS in humans coincides with the onset of fever and the activation of blood neutrophils, monocytes and lymphocytes. Therefore, IP injection of LPS in mice is a popular model to investigate the local inflammatory response followed by the recruitment of white blood cells. However, an LPS induced inflammatory response is different from gram-negative bacteria induced inflammation. For example, the components for antigen presentation are not evaluable in the host by pure LPS injection. In addition, bacterial pathogen infection significantly challenges the integrity of the immune system, which needs to work as a synergistic network to clear pathogens and resolve ongoing inflammation. Therefore, challenging *Fancc-/-* mice with a local gram negative bacterial infection would provide a clinically-relevant test to determine whether immunity and inflammation resolution were compromised in FA.

2. To determine whether the altered function of macrophages is due to the intrinsic role of FANCC

We identified defects in *Fancc-/-* macrophages *in vivo* and *in vitro*. However, these findings do not necessarily mean that the dysfunctional phenotypes observed are caused by deficient *Fancc* expression. Rather, altered *Fancc-/-* macrophage function could be secondary to other mutations given the well-established genomic instability of *Fancc-/-* cells. To address this issue, it is essential to restore *Fancc* expression in *Fancc-/-* macrophages and assess whether functional deficits of *Fancc-/-* macrophages are rescued. The initial approach for these experiments would be to transduce *Fancc-/-* peritoneal macrophage with a bicistronic lentiviral vector encoding recombinant *Fancc* and enhanced green fluorescent protein. Transduced *Fancc-/-* cells would be green when evaluated by fluorescent microscopy and would be examined for correction of adhesion and migration defects, including an evaluation of cytoskeletal rearrangement by confocal microscopy after adhesion to FN.

3. To assess whether the dysfunctional cell adhesion of *Fancc-/-* macrophage is secondary to deficient RhoA activation

Our data demonstrated that *Fancc-/-* macrophages had impaired adhesion on FN, altered F-actin distribution, and impaired RhoA activation, suggesting that defective RhoA activation is responsible for altered cytoskeletal rearrangement and subsequent dysfunctional adhesion. However, these studies did not assess whether the altered adhesion of *Fancc-/-* macrophages is directly mediated through RhoA. To address this important point, it will be necessary to restore RhoA function in *Fancc-/-* macrophage.

To accomplish this goal, we propose to transducer *Fancc-/-* macrophages with a bicistronic lentiviral vector encoding a constitutively active RhoA cDNA and enhanced green fluorescent protein (Ghiaur *et al.*, 2006; Melendez *et al.*, 2011) followed by an assessment of adhesion and cytoskeletal reorganization. If macrophage elongation and adhesion to FN of *Fancc-/-* macrophage are restored to WT levels, we will conclude that altered adhesion and cytoskeletal defects are caused by reduced activation of RhoA.

4. To measure whether M-CSF signaling is altered in myeloid progenitors

We found that M-CSF signaling was altered in *Fancc-/-* BMDM characterized by reduced phosphorylation of ERK and AKT. These data suggest that activation of ERK and AKT may be partially responsible for the decreased number of *Fancc-/-* BMDM in response to M-CSF. In addition, our data show that *Fancc-/-* myeloid progenitors exhibit reduced M-CSF responsiveness after immunologic stress *in vivo*. However, a direct evaluation of M-CSF signaling was not conducted in *Fancc-/-* myeloid progenitors. Therefore, it is necessary to assess M-CSF signaling in monocyte/macrophage progenitors. These experiments would require sorting CMPs and GMPs by flow cytometry from the BM of WT and *Fancc-/-* mice injected with either LPS or PBS as controls. Sorted cells would be stimulated with M-CSF. Then, the phosphorylation status of ERK and AKT would be determined by flow cytometry as previously described (Arroyo *et al.*, 2010; Stritesky *et al.*, 2011). Additional studies to evaluate whether M-CSF induced survival and proliferation on CMP and GMP would also be conducted. Again, sorted CMP and GMP from the BM of WT and *Fancc-/-* mice injected with either LPS or PBS would be cultured with M-CSF for six days. During this time, proliferation

will be examined by thymidine incorporation assays, and survival will be measured using a TUNEL assay.

5. To examine whether *Fancc-/-* macrophages exhibit altered M1 or M2 activation

As described in the discussion, M-CSF is important for M2 activation (Kinashi *et al.*, 1991; Yamazaki *et al.*, 2008; Pixley and Stanley, 2004). Our data showing that *Fancc-/-* BMDM and myeloid progenitors exhibit altered responsiveness to M-CSF suggest that M1/M2 activation may be altered in *Fancc-/-* mice. Therefore, to determine whether *Fancc-/-* macrophages exhibit altered M1 or M2 activation, we would evaluate for mRNA markers of M1/M2 activation, ROS/NO production, and arginase activity in WT and *Fancc-/-* macrophages. Additional details are outlined below.

To assess cytokine profile. Circulating monocytes will be collected by magnetic activated cell sorting selection of $CD14^+$ cells from the peripheral blood of WT and *Fancc-/-* mice. These cells will be stimulated with either LPS and IFN-γ or IL-4 and TGF-β to induce an M1 and M2 phenotype respectively, as previously described (Benoit *et al.*, 2008; Yamazaki *et al.*, 2008). Cytokine profile will be performed by RT-PCR. M1 cytokines include TNF-α, IL-1β, IL-12, and IL-23. M2 cytokines include IL-10, IL-4, and IL-13.

To evaluate expression of cluster genes for M1 and M2 activation. Emerging data demonstrate that M1 and M2 macrophage exhibit distinct gene expression profiles (Pixley and Stanley, 2004; Martinez *et al.*, 2008). For example, M1 macrophages highly express Cox2 and iNOS as well as inflammatory cytokines while M2 macrophages highly express FIZZ1, Ym, MMR, and arginase as well as anti-inflammatory cytokines (Raes *et*

al., 2002a; Raes *et al.*, 2002b; Stein *et al.*, 1992), Using the same cells and RNA from a), the gene expression of M1 and M2 markers would be performed by RT-PCR.

ROS and NO production.ROS and NO production are sensitive measures of M1 activation (Groesdonk *et al.*, 2006; Raisanen *et al.*, 2005). Production of ROS and NO will be assessed using CM-H2DCFDA and DAF-FM fluorescent probes respectively. The percentage of cells positive for ROS/NO probes and mean fluorescence intensity would be evaluated by flow cytometry. In addition, ROS and NO production within macrophages would be determined by confocal microscopy.

Arginase activity. Increased arginase activity is one of the most important characteristics of alternative or M2 activation (Colton *et al.*, 2006; Chang *et al.*, 2001; Abdallahi *et al.*, 2001). Arginase activity assay is a functional analysis of arginase, which catalyzes the conversion of arginine to orithine and urea.

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3. The Montreal Children's Hospital Research Institute (McGill University Health Center, Canada): Research assistant in Dr. Rima Rozen's lab (Dept. of Human Genetcs).

4. Shanghai Tissue Engineering Research & development Center: Research assistant, Faculty of the information section.

Conferences Attended

1. **Ying Liu**, Kimberly Ballman, Ethel Derr-Yellin, Shehnaz Khan and Laura Haneline. Impaired Function of *Fancc-/-* Macrophage. The Pediatric Academic Societies and Asian Society For Pediatric Research. Annual Joint Meeting, Denver, CO, April, 2011 [**poster**]
2. **Ying Liu**, Kimberly Ballman, Ethel Derr-Yellin, Shehnaz Khan and Laura Haneline. Impaired Function of *Fancc-/-* Immune Cells. The American Society of Hematology, 50th Annual Meeting, Orlando, FL, December, 2010 [**poster**]
3. Twentieth Annual Fanconi Anemia Scientific Symposium, Eugene, OR, 2008
4. Nineteenth Annual Fanconi Anemia Scientific Symposium, Chicago, IL, 2007

Publications

1. ***Liu Y***, Ballman K, Li D, Khan S, Derr-Yellin E, Shou W and Haneline LS. Impaired Function of Fanconi Anemia Type C Deficient Macrophages. J Leukoc Biol. (under review)
2. Li D, ***Liu Y***, Maruyama M, Zhu W, Chen H, Zhang W, Reuter S, Lin SF, Haneline LS, Field LJ, Chen PS and Shou W. Restrictive loss of Plakoglobin in

cardiomyocytes leads to Arrhythmognic Right Ventricular Cardiomyopathy. Hum Mol Genet. (accepted)

3. Li D, Hallett MA, Zhu W, Rubart M, ***Liu Y***, Yang Z, Chen H, Haneline LS, Chan RJ, Schwartz RJ, Field LJ, Atkinson SJ and Shou W. Dishevelled-associated activator of morphogenesis 1 (Daam1) is required for heart morphogenesis. Development. 2011, 138(2):303-315.
4. Li D, Pickell L, ***Liu Y***, Wu Q, Cohn JS and Rozen R. Maternal methylenetetrahydrofolate reductase deficiency and low dietary folate lead to adverse reproductive outcomes and congenital heart defects in mice. Am J Clin Nutr. 2005, 82(1):188-95.
5. Li D, Pickell L, ***Liu Y*** and Rozen R. Impact of methylenetetrahydrofolate reductase deficiency and low dietary folate on the development of neural tube defects in splotch mice.Birth Defects Res A Clin Mol Teratol. 2006, 76(1):55-9.