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Inflammatory Reactive Oxygen Species-Mediated Hemopoietic Suppression in Fance-Deficient Mice

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Patients with the genomic instability syndrome Fanconi anemia (FA) commonly develop progressive bone marrow (BM) failure and have a high risk of cancer. Certain manifestations of the disease suggest that the FA immune system is dysfunctional and may contribute to the pathogenesis of both BM failure and malignancies. In this study, we have investigated inflammation and innate immunity in FA hemopoietic cells using mice deficient in Fanconi complementation group C gene (Fancc). We demonstrate that Fance-deficient mice exhibit enhanced inflammatory response and are hypersensitive to LPS-induced septic shock as a result of hemopoietic suppression. This exacerbated inflammatory phenotype is intrinsic to the hemopoietic system and can be corrected by the re-expression of a wild-type FANCC gene, suggesting a potential role of the FANCC protein in innate immunity. LPS-mediated hemopoietic suppression requires two major inflammatory agents, TNF-α and reactive oxygen species. In addition, LPS-induced excessive accumulation of reactive oxygen species in Fance−/− BM cells overactivates the stress kinase p38 and requires prolonged activation of the JNK. Our data implicate a role of inflammation in pathogenesis of FA and BM failure diseases in general.


F
ancconi anemia (FA) is a genetic disorder characterized by progressive bone marrow (BM) failure and cancer predisposition (1, 2). Somatic cell fusion studies show that FA is genetically heterogeneous, with at least 13 complementation groups identified thus far (3). The genes encoding the groups A (FANCA), B (FANCB), C (FANCC), D1 (FANCD1/BRCA2), D2 (FANCD2), E (FANCE), F (FANCF), G (FANCG/XRCC9), J (BACH1/BRIP1), L (FANCL/PHF9), M (FANCN/Hef), and N (PALB2) have been cloned (4–17). Studies on the function of these FA proteins have shown that they function to protect against genotoxic stress by forming complexes with each other (18–20) and that they protect hemopoietic cells from apoptotic cues by both suppressing apoptotic signaling pathways and enhancing survival signaling pathways (21–26). Functional inactivation of any of these proteins leads to clinical phenotypes of FA and cellular phenotype of genomic instability (1, 2).

The most important clinical features of FA are hematological because the progressive BM failure represents the hallmark of the disease and leading cause of patient death (1). It has been proposed that BM failure in aplastic anemia, including FA, results from hemopoietic stem cell (HSC) depletion (27). Because FA hemopoietic progenitor and stem cells have high rates of stress-induced apoptosis and reduced repopulating ability (21, 28, 29), the FA proteins are believed to play important roles in the maintenance of hemopoiesis. Indeed, consistent with the observations that the cells derived from FA patients are intolerant of oxidative stress, it has been reported that FA proteins, particularly the complementation group C (FANCC) protein, play a crucial role in oxidative stress signaling in a variety of cell types, including hemopoietic cells (30–36). More recently, cytokine hypersensitivity of FA hemopoietic cells to apoptotic cues has been proposed as a major factor in the pathogenesis of BM failure in three FA mouse models (Fanca−/−, Fance−/−, and Fancg−/−) (37, 38).

The cytokine TNF-α is a vital member of the multifunctional TNF superfamily and has important roles in immunity and cellular remodeling as well as influencing apoptosis and cell survival (reviewed in Ref. 37). The biological activities of TNF-α are mediated by two structurally related but functionally distinct receptors, designated the p55 and p75 TNF-α receptors. The activation TNF-α receptor triggers a complex array of signaling events, giving rise to the pleiotropic effects of TNF-α on cells (39). TNF-α is a major mediator of inflammation and plays a key role in the pathogenesis of such inflammatory diseases as rheumatoid arthritis, Crohn’s disease, and psoriasis, as demonstrated by the successful treatment of such conditions with Abs to TNF-α or with a soluble TNF-α receptor fusion protein (40). With respect to abnormal hemopoiiesis, it is well recognized that TNF-α is involved in many disease situations, including anemia, myelodysplasia (MDS), and leukemia (41). TNF-α exerts many of its biological effects through the activation of the MAPK stress signaling cascade, including JNK, p38MAPK, and ERK (42), as well as the NF-κB transcription factor (43). Signal transduction triggered by TNF-α also induces an increase in intracellular reactive oxygen species (ROS). It is established that TNF-α-induced ROS production involves the JNK and NF-κB pathways (44, 45).
Patients with FA have abnormally high levels of proinflammatory TNF-α, low levels of NK cell activity, and reduced lymphocyte counts and are highly susceptible to bacterial infection (46–57). Although these clinical manifestations suggest that the FA immune system may be dysfunctional, the mechanism(s) underlying these abnormalities and the signaling pathways involved in FA innate immunity have not been elucidated. In this study, we used mice deficient in FA complementation group C gene (Fancc) to investigate inflammation and innate immunity in Fancc−/− hemopoietic cells. We demonstrate that Fancc−/− mice exhibit enhanced inflammatory response and are extremely sensitive to LPS-induced septic shock as a result of hemopoietic suppression. Our data implicate a role of inflammation in pathogenesis of FA and BM failure diseases in general.

Materials and Methods

Mice and treatments

Wild-type (WT) and Fancc−/− mice were generated by interbreeding the heterozygous Fancc+/− mice (provided by Dr. M. Buchwald, University of Toronto, Toronto, Ontario, Canada) (58). The genetic background of the mice is C57BL/6 (CD45.2+). Fancc−/− Tnfa−/− mice were generated by mating Fancc−/− with Tnfa−/− (The Jackson Laboratory), followed by mating of F1 heterozygous siblings. All of the mice were used at 10–14 wk of age. For septic shock studies, mice were injected i.p. with LPS in PBS at a single dose of 1 mg/kg. The number of RBCs and concentrations of hemoglobin and hematocrit in peripheral blood were determined on day 3 after LPS injection. Kaplan-Meier survival curves are shown for a single dose (25 mg/kg) of i.p.-injected LPS. Experiments were repeated three times, each with 6 animals (total 18 mice) for WT or Fancc−/− mice (10–14 wk old). The log-rank test indicated a statistically significant difference (p < 0.01) in survival between the two genotype groups. WT, LPS reduces blood counts in Fancc−/− mice. WT or Fancc−/− mice were injected i.p. with LPS in PBS at a single dose of 1 mg/kg. The number of RBCs and concentrations of hemoglobin and hematocrit in peripheral blood were determined on day 3 after LPS injection. Our data implicate a role of inflammation in pathogenesis of FA LPS-induced septic shock as a result of hemopoietic suppression. We demonstrate that Fancc−/− mice exhibit enhanced inflammatory response and are extremely sensitive to LPS-induced septic shock as a result of hemopoietic suppression. Our data implicate a role of inflammation in pathogenesis of FA and BM failure diseases in general.

Flow cytometric analysis of HSC and lineage differentiation

Low-density BM mononuclear cells were suspended in FACS buffer (0.1% FCS in 0.02% sodium azide) and incubated with the indicated Abs on ice for 30 min, followed by two washes. Data were collected on a FACSCalibur (BD Biosciences). Abs used were (Miltenyi Biotec), Sca-1-PE, c-κt-allophycocyanin, B220/CD3ε, Gr-1/CD11b, and Ter119 (all from BD Pharmingen).

Retroviral vectors and infection

The full-length human FANCC cDNA (GenBank sequence accession no. NM000136) was amplified by PCR, using Pfu DNA polymerase (Stratagene) and subcloned into the Ncol site of retroviral vector SF591 (a gift from Dr. C. Baum, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH) to create SF591-FANCC. The retroviral vectors HA-MKK6-KM and Flag-MKK7-KM have been reported elsewhere (42). Retroviral supernatant was collected at 36, 48, and 60 h, respectively, after transfection. BM mononuclear cells were plated onto Retronectin (Takara Shuzo)-coated nontissue culture 6- or 12-well plates and prestimulated for 2 days in IMDM containing 20% FCS, 100 ng/ml SCF, 20 ng/ml IL-6, and 50 ng/ml Flt-3L (PeproTech). Cells were then exposed to the retroviral supernatant for 3 h at 37°C in the presence of 4 μg/ml polybrene (Sigma-Aldrich). Cells were centrifuged at 600 x g for 45 min. Infection was repeated twice, and infection efficiency was assessed by the detection of GFP-positive cells by flow cytometry.

Clonogenic progenitor assays

BM progenitor cells were cultured in a 35-mm tissue culture dish in 4 ml of semisolid medium containing 3 ml of MethoCult M 3134 (Stem Cell Technologies) and the following growth factors: 100 ng/ml SCF, 10 ng/ml IL-3, 100 ng/ml G-CSF, and 4 U/ml erythropoietin (PeproTech). On day 10 after plating, the colony number was counted and photographed. Colony growth results were expressed as mean (of triplicate plates) ± SD of three independent experiments.

BM transplantation

Age-matched congenic B6.J-PrtcaPep3b/BoyJ (B6.BoyJ; CD45.1+) mice (The Jackson Laboratory) were used as transplant recipients. These
mice were lethally irradiated (9.5 Gy, 110 cGy/min, 137Cs gamma rays) and injected i.v. with $2 \times 10^6$ test BM mononuclear cells (CD45.2+), mixed with $1 \times 10^6$ competitor cells (BoyJ; CD45.1+). Donor-derived repopulation in recipients was assessed by the proportion of leukocytes in peripheral blood that expressed the CD45.2 marker by flow cytometry. Short- and long-term engraftment and multilineage repopulation analysis of donor cells were performed at 4- and 16-wk posttransplantation, respectively.

**Apoptosis assay**

Cells were stained with annexin V and 7-aminoactinomycin D (7-AAD) using BD ApoAlert Annexin V kit (BD Pharmingen) in accordance with the manufacturer’s instructions. Apoptosis was analyzed by quantification of annexin V-positive cell population by flow cytometry.

**Determination of ROS production**

BM cells were incubated with CM-H2DCFDA (Molecular Probes) in the dark for 15 min at 37°C. After washing, the cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Data were analyzed by using the CellQuest program (BD Biosciences).

**Histology and immunohistochemistry**

During necropsy, organs were removed, preserved in formalin, and then embedded in paraffin blocks. Sections were stained with H&E. For immunohistochemistry, paraffin sections were deparaffinized, rehydrated, incubated in 0.1 mM sodium citrate (pH 6.0), washed, and incubated with peroxidase blocking reagent (VectaStain Elite ABC kit; Vector Laboratories). After washing in PBS, the slides were incubated with the primary Ab...
HNE (11-S; Alpha Diagnostic International) or myeloperoxidase (MPO; catalog no. RB-373-A0; LAB Vision). Following three PBS washes, slides were incubated with secondary Ab and then detected with the VectaStain Elite ABC reagents.

**NF-κB activation**

Nuclear protein extracts were prepared from BM cells using a Transfactor Extraction kit (BD Biosciences). Nuclear extracts were incubated with DNA specific for the NF-κB consensus sequence, and the DNA-binding activity of NF-κB was measured using a Transfactor kit (BD Biosciences).

**Immunoblotting**

Cells were solubilized in radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Nonidet P-40) containing a mixture of protease inhibitors (Calbiochem). Equal amounts of protein were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and blotted with Abs against p65 or IκBα (Santa Cruz Biotechnology), phosphorylated p38, phosphorylated JNK, the pan kinases p38 and JNK (all from Cell Signaling Technology), and β-actin (Sigma-Aldrich).

**Real-time PCR analysis**

Total RNA was prepared with RNeasy kit (Qiagen) following the manufacturer’s procedure. Following treatment with RNase-free DNase, RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen Life Technologies). Real-time PCR was performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems) with SYBR green PCR master mix (Applied Biosystems), according to the manufacturer’s instructions. Samples were normalized to the level of GAPDH mRNA, and the relative expression levels were determined by the standard curve method. Primer sequences are available upon request.

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**FIGURE 3.** Exacerbated inflammatory responses in hemopoietic cells of LPS-treated Fanc−/− mice. A, Fanc-deficient mice release significantly more proinflammatory cytokines than WT animals following LPS challenge. WT or Fanc−/− mice were injected i.p. with PBS or LPS in PBS at a dose of 1 mg/kg. The mice were then sacrificed 2 h later, and serum was assessed for the indicated cytokines by ELISA. Data are expressed as mean ± SD of two independent experiments with n = 3 animals per group for each experiment. *p < 0.05. B, Kinetics of circulating TNF-α release induced by LPS. WT or Fanc−/− mice were injected i.p. with LPS (1 mg/kg), and serum samples were collected at the indicated time points. TNF-α concentration was determined by ELISA. C, Expression of genes encoding inflammatory proteins was examined in BM cells of LPS-treated mice. WT or Fanc−/− mice were injected i.p. with PBS or LPS in PBS at a dose of 1 mg/kg. The mice were then sacrificed 2 h later, and low-density BM mononuclear cells were isolated, total RNA was prepared, and gene expression was analyzed by real-time PCR. Data are presented as the fold increase in mRNA expression in Fanc−/− BM cells relative to WT cells, which was given an arbitrary level of 1.0 for each gene. Results are means ± SD of three independent experiments. D, Paraffin-embedded liver sections of PBS- or LPS-treated WT or Fanc−/− mice were stained with Ab against the neutrophil marker MPO (×40). Right panel, MPO-positive cell counts in liver and lung of LPS-treated mice. The numbers are represented as mean ± SD of three experiments. E, Kaplan-Meier survival curves for radiation chimera mice. WT mice were lethally irradiated and reconstituted with WT (WT-WT), Fanc−/− (WT-Fanc−/−), or FANCC-transduced Fanc−/− (WT-Fanc−/−+FANCC) BM cells. Ten weeks post-transplantation, recipient mice were injected with a single dose (25 mg/kg) of LPS. Experiments were repeated two times, each with 6 recipients (total 12 mice/group).
immune response in mice deficient for the Fance gene. Fance<sup>−/−</sup> mice were extremely sensitive to septic shock by LPS, an immunological endotoxin from Gram-negative bacteria (Fig. 1A). LPS-treated Fance<sup>−/−</sup> mice exhibited cytoplasia, as evidenced by decrease in red cell counts, hemoglobin, and hematocrit values (Fig. 1B). Consistent with this, analysis of BM of LPS-treated Fance<sup>−/−</sup> mice revealed a decrease in BM cellularity accompanied by extensive areas of necrosis (data not shown). In another set of experiments, we analyzed hemopoietic recovery in mice injected with LPS. We found that Fance<sup>−/−</sup> mice recovered from hemosuppression slowly, taking as much twice the time as WT mice to reach the pretreatment level (Fig. 1C).

We next asked whether LPS suppressed hemopoietic progenitor activity. As shown in Fig. 2A, total number of colonies formed by BM cells from LPS-treated Fance<sup>−/−</sup> mice was >3-fold lower than that of WT mice. We then performed BM transplantation to evaluate the in vivo effect of LPS on hemopoietic reconstitution. Analysis of short-term (4-wk) engraftment demonstrated that LPS significantly compromised the BM repopulating ability of BM cells isolated from Fance<sup>−/−</sup> mice injected with a single dose of the endotoxin (Fig. 2B). More dramatically, LPS almost completely disabled long-term hemopoietic reconstitution of Fance<sup>−/−</sup> BM cells with few donor-derived cells detected in the peripheral blood cells of the recipients at 16 wk after transplantation (Fig. 2C). However, analysis of the composition of lymphoid (B220/CD3e), myeloid (Gr-1/CD11b), and erythroid (Ter<sup>119</sup>) showed that LPS did not compromise multiple lineage reconstitution (data not shown). We next asked whether LPS reduced stem cell pool or induced excessive cell

**Table I. Inflammatory cytokines in recipient mice**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Treatment</th>
<th>Serum Cytokines</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>TNF-α</td>
<td>IL-6</td>
<td>MIP-2</td>
</tr>
<tr>
<td>WT</td>
<td>PBS</td>
<td>41.2 ± 4.1</td>
<td>9.3 ± 2.5</td>
<td>31.2 ± 5.7</td>
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<tr>
<td></td>
<td>LPS</td>
<td>303 ± 52.6</td>
<td>49.3 ± 7.4</td>
<td>34 ± 36.8</td>
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<tr>
<td>Fance&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>PBS</td>
<td>28.8 ± 4.4</td>
<td>11.3 ± 3.2</td>
<td>34.8 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>LPS</td>
<td>485 ± 62.8</td>
<td>76.4 ± 23.6</td>
<td>225 ± 38.3</td>
</tr>
<tr>
<td>Fance&lt;sup&gt;−/−&lt;/sup&gt; +</td>
<td>PBS</td>
<td>29.6 ± 6.4</td>
<td>14.3 ± 4.1</td>
<td>39.2 ± 7.3</td>
</tr>
<tr>
<td>FANCC</td>
<td>LPS</td>
<td>342 ± 47.6</td>
<td>55.4 ± 18.6</td>
<td>168 ± 29.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>TNF-α and MIP-2, pg/ml; IL-6, ng/ml.

**Results**

**LPS suppresses hemopoietic functions**

Because mutations in the FA complementation group C gene FANCC repress clonal growth of hemopoietic progenitor cells and disruption of the Fance gene, in mice, renders hemopoietic progenitor cells hypersensitive to the proapoptotic effect of IFN-γ and TNF-α (21–23, 26, 37, 38, 46, 49), we studied innate

**FIGURE 4.** TNF-α is the mediator of LPS-induced hemopoietic suppression. A, WT or Fance<sup>−/−</sup> mice or their littermates deficient for Tnfα were injected with PBS or LPS (1 mg/kg). The mice were then sacrificed 2 h later, and serum was assessed for the indicated cytokines by ELISA. Data are expressed as mean ± SD of at least two independent experiments with n = 3 animals per experiment. B, Neutralization of circulating TNF-α. WT or Fance<sup>−/−</sup> mice were injected with PBS or LPS (1 mg/kg), and 30 min later, the mice were injected with 20 μg of an anti-mouse TNF-α-neutralizing Ab (LPS + anti-TNF) or control IgG (LPS + IgG). The mice were then sacrificed 2 h later, and serum was assessed for TNF-α by ELISA. Data are expressed as mean ± SD of 3 and 2 independent experiments with 3 animals per experiment, respectively. Neutralization or ablation of LPS-induced TNF-α rescues progenitor growth. WT or Fance<sup>−/−</sup> mice or their littermates deficient for Tnfα were injected with PBS or a single dose of LPS (1 mg/kg). Mice were injected with 20 μg of a TNF-α-neutralizing Ab 30 min after LPS injection. Twenty-four hours after LPS injection, BM cells were isolated and subjected to clonogenic assay. Data show represent the number (mean ± SD) of total number of colonies from three independent experiments. D, Neutralization or ablation of LPS-induced TNF-α restores HSC self-renewal ability. A total of 2 × 10<sup>6</sup> BM mononuclear cells isolated from the mice described in C was transplanted, along with 1 × 10<sup>6</sup> competitor cells from B6.BoyJ mice (CD45.1<sup>−</sup>), into lethally irradiated recipient mice, and long-term engraftment was evaluated at 16 wk posttransplantation. Data represent mean ± SD of three independent experiments with n = 3 recipients per group for each experiment.
death in F ance−/− hemopoietic progenitor cells. Although LPS did not cause notable change in stem cell pool in WT mice, ~2-fold reduction of BM stem/progenitor (lineage-negative, Sca-1-positive, c-kit-positive; LSK) cell frequencies was observed in LPS-treated F ance−/− mice compared with untreated littermate controls (Fig. 2D). In addition, BM stem/progenitor cells of LPS-treated F ance−/− mice showed increase in apoptosis (7-AADlowAnnexin V+), and necrosis (7-AADhighAnnexin V+) (Fig. 2E). Collectively, these results indicate that LPS suppresses hemopoietic reconstitution in F ance−/− mice, at least in part, through induction of stem/progenitor cell death.

LPS-induced inflammation is exacerbated in F ance−/− mice

The hypersensitivity of F ance−/− mice to LPS-induced septic shock prompted us to investigate whether these mice display an enhanced inflammatory response. LPS activates hemopoietic innate immune cells such as macrophages and dendritic cells to produce large amounts of TNF-α, IL-1β, and IL-6, which then mediate a cascade of inflammatory responses leading to endotoxic shock (59). Thus, we determined the levels of these inflammatory cytokines in serum from mice treated with LPS. F ance−/− mice showed significant increase in the levels of TNF-α, IL-6, and MIP-2 compared with WT littermate controls (Fig. 3A). Albeit statistically insignificant, increased serum levels of IL-1β were also observed in treated F ance−/− mice. Importantly, TNF-α levels in serum of LPS-treated F ance−/− mice peaked at 2 h and were maintained at high levels for 10 h post-LPS injection (Fig. 3B). This was in contrast to WT littermate controls, where TNF-α levels dropped off markedly after peaking at 2 h. In addition, real-time quantitative PCR analysis of the F ance−/− BM cells cultured in the presence of LPS showed prolonged high expression of other known proinflammatory genes, including MIP-1α, cyclooxygenase-2, and inducible NO synthase (Fig. 3C). Consistent with this, liver and lung tissues of LPS-treated F ance−/− mice showed increased immunoreactivity for MPO, a major marker of inflammation (Fig. 3D). These results suggest that long-lasting high levels of proinflammatory cytokines may mediate prolonged and exacerbated inflammatory response in F ance−/− mice, which might account for the hypersensitivity of these animals to LPS-induced septic shock.

To determine whether the exacerbated inflammatory phenotype observed in LPS-treated F ance−/− mice was intrinsic to the hemopoietic system, we reconstituted lethally irradiated congenic
C57BL/6 mice with BM from Fancc−/− mice or WT littermate controls. BM transplantation was also performed with Fancc−/− BM cells that had been functionally corrected with a DNA repair-proficient FANCC gene, as tested by mitomycin C sensitivity assay (1, 2). Mice receiving Fancc−/− BM were significantly more susceptible to LPS-induced septic shock than those transplanted with WT marrow (Fig. 3E). These Fancc−/− BM-transplanted recipients also displayed abnormal high levels of serum TNF-α, IL-6, and MIP-2 (Table I). Remarkably, complementation of Fancc−/− BM cells with the DNA repair-proficient FANCC gene significantly mitigated these deregulated innate immune responses. Thus, the exacerbated inflammatory phenotype seen in Fancc−/− mice is due to an intrinsic defect in the hematopoietic system.

Hemopoietic suppression by LPS is mostly mediated by TNF-α

TNF-α is a major proinflammatory cytokine whose production is strongly induced by LPS (39, 59). We proposed that LPS-induced TNF-α was responsible in part for the observed hemopoietic suppression. LPS administration to WT and Fancc−/− mice resulted in robust TNF-α induction, whereas this induction was ablated in mice deficient for the Tnfa gene (Fig. 4A). Administration of an anti-TNF-α Ab 30 min after LPS injection effectively neutralized most of the circulating TNF-α (Fig. 4B). To determine whether LPS-mediated hemopoietic suppression required TNF-α, we examined the proliferative potential of hemopoietic progenitors using two established assays: clonogenic progenitor assay and competitive hemopoietic repopulation. Indeed, LPS-mediated progenitor growth inhibition through TNF-α, as ablation of TNF-α production in WT (Tnfa−/−) or Fancc−/− (Fanco−/− Tnfa−/−) mice rescued progenitor growth (Fig. 4C). Neutralization of circulating TNF-α with the anti-TNF-α Ab also reduced, albeit less profoundly, the inhibitory effect of LPS in progenitor growth (Fig. 4C). Similar results were obtained with BM reconstitution assay in which cells from LPS-injected WT Tnfa−/− mice were able to reconstitute irradiated BM as efficiently as those from untreated counterparts (Fig. 4D). Again, Tnfa deficiency in Fancc−/− mice also abrogated the negative effect of LPS on BM repopulation (Fig. 4D).

We conclude that TNF-α is an important mediator of LPS-induced hemopoietic suppression.

The role of TNF-α-induced ROS in hemopoietic suppression by LPS

One mechanism by which LPS mediates inflammatory effect is to increase the cellular oxidative stress (60), which has been known to be very harmful to hemopoietic cells particularly to those from Fanconi patients (1). We suspected that TNF-α-induced ROS was the source of LPS-generated cellular oxidative stress responsible in part for the observed hemopoietic suppression. To test this notion, we pretreated the LPS-injected mice with the ROS scavenger NAC. NAC rescued both progenitor growth (Fig. 5A) and repopulating ability (Fig. 5B) of the BM cells from LPS-injected WT and Fancc−/− mice. Notably, NAC did not have any affect on
progenitor growth or hematopoietic reconstitution of BM cells from those mice that could not produce TNF-α (Fig. 5, A and B), indicating that TNF-α is the source of LPS-generated oxidative stress.

To directly ask whether LPS-generated ROS required TNF-α, we stained BM cells freshly isolated from LPS-injected mice with CM-H2DCFDA, a cell-permeable fluorescence dye that reacts to a broad spectrum of ROS. LPS induced substantially more ROS in Fancc mice than in WT mice (Fig. 5C). TNF-α was required for this ROS production, as administration of the neutralizing anti-TNF-α Ab or deletion of the Tnfa gene in these mice significantly reduced ROS accumulation (Fig. 5C).

**Excessive ROS accumulation in Fancc−/− BM cells overactivates p38 and requires prolonged JNK activation**

We further investigated the molecular mechanism that leads to excessive ROS production in Fancc−/− BM cells. It has been reported that activation of the transcription factor NF-κB inhibits TNF-α-induced ROS production (43, 44). We wondered whether NF-κB activity might be defective in Fancc−/− BM cells. LPS-induced NF-κB activation was indistinguishable in BM cells freshly isolated from LPS-injected WT and Fancc−/− mice, as assessed by the degradation of NF-κB inhibitor IκBα and the DNA-binding activity of NF-κB (data not shown). However, we observed overactivation of the p38 kinase in BM cells isolated from Fancc−/− mice sacrificed 1 h after LPS injection (Fig. 6A). Strikingly, LPS-induced JNK activation was persistent in BM cells isolated from LPS-injected Fancc−/− mice at this time point but disappeared in their WT counterparts (Fig. 6A). Kinetics study with in vitro culture of isolated BM cells in the presence of LPS further demonstrated enhanced p38 and prolonged JNK activation in Fancc−/− cells (Fig. 6B). Thus, LPS-induced p38 overactivation or/and prolonged JNK activation may be responsible for the difference in oxidative stress conditions in WT and Fancc−/− mice.

The excessive levels of oxidative stress observed in BM cells from LPS-injected Fancc−/− mice may be resulted from p38 overactivation or prolonged JNK activation. To distinguish between these possibilities, we treated LPS-injected mice with antioxidant NAC, p38 inhibitor SB203580, or JNK inhibitor SP600125. Administration of SB203580 effectively abrogated LPS-induced p38 activation in both WT and Fancc−/− mice (Fig. 6C). Notably, NAC significantly reduced p38 activity in LPS-injected Fancc−/− mice. LPS-induced activation of p38 was also reduced by NAC treatment in WT mice, albeit less dramatic. However, p38 inhibitor did not affect LPS-induced ROS production in these mice (Fig. 6D). In contrast, the JNK inhibitor, which suppressed LPS-induced JNK activation in both WT and Fancc−/− mice (Fig. 6C), dramatically reduced LPS-induced ROS production in Fancc−/− mice and, to a less degree, in WT mice (Fig. 6D). Interestingly, NAC administration to LPS-injected mice had no effect on JNK activation (Fig. 6C).

To unambiguously define the contribution of p38 and JNK to LPS-induced ROS production and hematopoietic suppression, we inhibited specifically the kinases using dominant-negative mutants of their upstream activators, MKK6-KM (for p38) and MKK7-KM (for JNK). We expressed these mutants in BM mononuclear cells by retroviral gene transfer. Expression of the mutant proteins was verified by Western blotting (Fig. 7A). The functionality of the

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**FIGURE 7.** p38 and JNK play distinct roles in LPS-mediated hemopoietic suppression in Fancc-deficient BM cells. A. Low-density BM cells from WT or Fancc−/− mice were transduced with retroviruses carrying vector alone, MKK6-KM, or MKK7-KM, and protein extracts were prepared and analyzed by immunoblotting with anti-HA (for MKK6-KM), anti-Flag (for MKK7-KM), and anti-actin Abs. B. Inhibition of LPS-induced p38 and JNK activation by overexpression of dominant-negative MKK6 and MKK7 in BM cells. Cells described in A were cultured in the presence of LPS (100 μg/ml) for 10 min, and protein extracts were prepared and analyzed by immunoblotting with anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, and anti-actin Abs. NAC (100 μM) was added to the culture before LPS treatment. C. Inhibition of JNK but not p38 reduced LPS-induced ROS production. Cells described in A were cultured in the presence of LPS (100 μg/ml) for 10 min and labeled with CM-H2DCFDA, followed by flow cytometry. NAC (100 μM) was added to the culture before LPS treatment. Data represent mean ± SD of three independent experiments. D. Inhibition of LPS-induced JNK or p38 activation partially rescues progenitor growth. Cells described in A were cultured in the presence of LPS (100 μg/ml) for 10 min and subjected to clonogenic assay described in Materials and Methods. NAC (100 μM) was added to the culture before LPS treatment. Data represent mean ± SD of three independent experiments.
dominant-negative mutants was demonstrated by their ability to inhibit the activity of the endogenous kinases (Fig. 7B). Consistent with the in vivo results (Fig. 6), inhibition of JNK by MKK7-KM but not p38 by MKK6-KM reduced LPS-induced ROS production (Fig. 7C). Clonogenic progenitor assay demonstrated that inhibition of either p38 or JNK was sufficient to rescue progenitor growth (Fig. 7D). Collectively, these results suggest that the MAPK kinases p38 and JNK play distinct roles in LPS-mediated hemopoietic suppression in Fancc−/− cells: prolonged JNK activation increases ROS accumulation leading to p38 overactivation.

Discussion

The present study demonstrates that mice deficient in the Fanconi gene Fancc exhibited enhanced inflammatory response and were extremely sensitive to LPS-induced septic shock. Inflammation as a consequence of the activation of innate immune system is essential for host survival yet has the potential for devastating consequences if not precisely controlled or resolved. The fact that patients with FA frequently show overproduced TNF-α in their serum and plasma (46–49) suggest that these patients may consistently be subjected to inflammatory cues. LPS-treated Fancc-deficient mice not only exhibit elevated levels of TNF-α secretting into serum, but the production of ROS is also enhanced. Overproduction of these two major inflammatory agents results in deregulation of the stress kinases p38 and JNK, leading to hemopoietic suppression, heightened septic shock, and animal mortality. Our study thus implicates a functional deficiency in FA innate immunity.

The observed prolonged induction of TNF-α in Fancc-deficient mice suggested that the inflammatory cytokine is responsible in part for LPS-induced hemopoietic suppression and subsequent septic shock. Indeed, the in vivo findings presented here clearly demonstrate that deletion of Tnfa gene or neutralization of TNF-α in LPS-treated Fancc−/− mice effectively rescued progenitor growth and hemopoietic reconstitution. This finding underscores pathogenic roles of TNF-α in clinical manifestations in BM failure-related diseases, including FA (39–41). Bone marrow cells from FA patients show overproduction of TNF-α (46–49). Overproduction of TNF-α has also been implicated in other pathological conditions related to chronic inflammation, cancer, and aging (39–41, 61). Our finding also highlights a regulatory role of TNF-α in hemopoiesis. TNF-α has been shown to decrease cytokine-driven HSC expansion, interferes with HSC self-renewal, and compromises the ability of HSC to reconstitute hemopoiesis (62–66). Therefore, overproduction of TNF-α may play a pivotal role in pathogenesis of certain BM failure diseases through inhibiting hemopoiesis.

We demonstrated that ROS, another primary inflammatory agent, was overproduced in LPS-treated Fancc−/− mice, which was dependent on TNF-α and largely responsible for LPS-induced hemopoietic suppression. Direct, compelling evidence has suggested that TNF-α-mediated cytotoxicity is due to the induced ROS production in a variety of cell types (44, 45). Because FA cells, including BM hemopoietic cells, are hypersensitive to oxidative stress (1, 30–36), it is conceivable that ROS-mediated inflammation contributes to the observed hemopoietic suppression and toxicity in LPS-treated Fancc−/− mice. Alternatively, ROS can cause oxidative DNA damage. Recent studies have shown that the production of ROS by TNF-α at inflammatory sites causes DNA damage (39, 67–69). We believe that in FA cells, in which the repair of oxidative DNA damage may be deficient or whose DNA may be susceptible to oxidative attack, the ability of TNF-α-induced ROS to damage DNA is a potential mechanism through which ROS mediate their effects on the inflammatory process.

An extensive body of evidence has suggested that FA cells are in an in vivo pro-oxidant state (36) and that the FA proteins play important roles in cellular responses to oxidative stress. For example, the FANCC protein has been found to interact with NADPH cytochrome P450 reductase and GST P1-1 (30, 31), two enzymes involved in either triggering or detoxifying reactive intermediates, including ROS. In addition, mice with combined deficiencies of the antioxidative enzyme Cu/Zn superoxide dismutase and Fancc genes demonstrated a defective hemopoiesis (32). Another FA protein, FANCG, interacts with cytochrome P450 2E1 (33) and mitochondrial peroxiredoxin-3 (70), suggesting a possible role of FANCG in protection against oxidative DNA damage. Significantly, Saadatzadeh et al. (34) recently showed that oxidant hypersensitivity of Fancc−/− cells was due to an altered redox regulation and ASK1 hyperactivation. Moreover, oxidative stress induces complex formation by two major FA proteins, Fanca and Fancc (36). Our present finding that LPS/TNF-α-generated ROS induces hemopoietic suppression in Fancc−/− mice corroborates a critical role for oxidative stress in FA phenotype and disease progression.

Perhaps more importantly, inflammatory ROS may contribute to the progression of cancer-related BM failure diseases such as FA. ROS have been associated with the initiation or aggravation of diverse pathological states, including cancers (71–76). In FA, the disease typically progresses from anemia to MDS then to acute myeloid leukemia (1, 2). High levels of ROS production in patients with anemia would be consistent with the observation implicating ROS production as a mechanism of TNF-α-induced cell death (1, 30–36). ROS are known to cause carcinogenic mutations, which may promote clonal evolution in MDS and leukemic transformation.

Intriguingly, our study indicates that LPS induced sustained activation of JNK and p38 overactivation in Fancc−/− mice. This deregulation of the stress kinases required LPS-induced ROS production and sustained JNK activation correlated with high ROS production in BM cells from LPS-treated Fancc−/− mice. It has been reported recently that TNF-α induced a high level of p38 activation in FA cells (26, 77). Consistent with previous observations that abnormal p38 and JNK activation is predominantly pro-apoptotic (78–80), we showed that LPS-induced activation of these two stress kinases in Fancc−/− mice inhibited progenitor proliferation. Notably, we found that LPS-induced ROS could activate p38 but required prolonged JNK activation. Simultaneous and quantitatively balanced induction of JNK/p38 and NF-κB pathways by TNF-α has been demonstrated in different cell types (81, 82). Disruption of this balance (e.g., by inhibition of the NF-κB or JNK pathway) may tip the cell toward apoptosis or proliferation. It is established that the production of inflammatory ROS involves the JNK and NF-κB pathways (43–45). For instance, TNF-α-induced ROS activate JNK, which in turn leads to more ROS production and exacerbated inflammation (45). In certain disease situations where inflammation plays a pathogenic role, ROS production stimulates NF-κB activation (83, 84). Although we did not see a diminished NF-κB activation in the BM cells from LPS-treated Fancc−/− mice, it would be intriguing to know whether NF-κB activation is necessary for the emergence of abnormal cell clones and the subsequent progression of FA to MDS and acute myeloid leukemia. Nevertheless, our results provide new insight into the role of p38 and JNK in FA innate immunity.

The results presented here suggest that antagonizing proinflammatory TNF-α and/or ROS may have therapeutic benefit in patients with FA. The elimination of TNF-α by either neutralizing Ab or deleting the Tnfa gene not only abrogated the negative effect of LPS on progenitor proliferation but also restored the ability
the progenitor cells to reconstitute irradiated BM. Likewise, inhibition of ROS production rescued hematopoietic function otherwise suppressed by LPS. Therefore, a pharmacological ablation of TNF-α and/or ROS will potentially limit the severity of inflammatory phenotype by transiently controlling these primary proinflammatory signals. These findings may be extended to other BM failure disease such as aplastic anemia and MDS.

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