Inflammatory ROS promote and cooperate with the Fanconi anemia mutation for hematopoietic senescence

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Summary
The proinflammatory cytokine tumor necrosis factor α (TNFα) inhibits hematopoietic stem cell (HSC) expansion, interferes with HSC self-renewal and compromises the ability of HSC to reconstitute hematopoiesis. We have investigated mechanisms by which TNFα suppresses hematopoiesis using the genomic instability syndrome Fanconi anemia mouse model deficient for the complementation-group-C gene (Fanc). Examination of senescence makers, such as senescence-associated β-galactosidase, HP1-γ, p53 and p16INK4A shows that TNFα induces premature senescence in bone marrow HSCs and progenitor cells as well as other tissues of Fanc−/− mice. TNFα-induced senescence correlates with the accumulation of reactive oxygen species (ROS) and oxidative DNA damage. Neutralization of TNFα or deletion of the TNF receptor in Fanc−/− mice (Fanc−/−;Tnfr1−/−) prevents excessive ROS production and hematopoietic senescence. Pretreatment of TNFα-injected Fanc−/− mice with a ROS scavenger significantly reduces oxidative base damage, DNA strand breaks and senescence. Furthermore, HSCs and progenitor cells from TNFα-treated Fanc−/− mice show increased chromosomal aberrations and have an impaired oxidative DNA-damage repair. These results indicate an intimate link between inflammatory reactive oxygen species and DNA-damage-induced premature senescence in HSCs and progenitor cells, which may play an important role in aging and anemia.

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Key words: DNA damage and repair, Fanconi anemia, Genomic instability, Hematopoietic stem cells, Inflammation, Reactive oxygen species

Introduction
The genomic instability syndrome Fanconi anemia (FA) shares the common feature of having defective DNA damage response/repair processes with other premature aging syndromes such as Bloom (Bischof et al., 2001) and Werner (Chen et al., 2003), ataxia telangiectasia (Ito et al., 2004) and BRCA1-deficient breast cancer (Cao et al., 2003). The FA pathway has been shown to functionally interact with the proteins responsible for Bloom syndrome (BLM) (Meetei et al., 2003), ataxia telangiectasia (ATM) (Taniguchi et al., 2002) and breast cancer (BRCA1) (Seal et al., 2003). In fact, one of the FA genes, FANCD1, turned out to be the breast cancer gene BRCA2 (Howlett et al., 2002). This suggests a biological link between the FA disease and organisal aging. The FA disease model is unique in that defects are especially profound in the rapidly proliferating hematopoietic system. Thus far, aging studies have not yet focused on hematopoietic stem cells (HSCs) in FA or these other premature aging syndromes. Nevertheless, HSCs are capable of self-renewing, have a high risk of accumulating mutations and, thus, may require fewer events to become senescent or transformed (Pellici, 2004). In addition, HSC aging can be assessed in a quantitative fashion with well-established assays. Since senescence can cause stem cell depletion, which can conceivably lead to organisal aging, such studies may yield important insights to the mechanisms of aging.

Functional failure of HSCs can result in the decrease in number and function of HSCs and progenitor cells leading to the development of anemia, neutropenia and thrombocytopenia, and has been implicated in hematologic diseases such as bone marrow (BM)-failure diseases such as FA, myelodysplastic syndromes (MDS) and aplastic anemia (Young, 2002; Bagby, Jr, 2003; Chen, 2005). Indeed, it has been shown that FA HSCs and progenitor cells have high rates of stress-induced apoptosis and reduced repopulating ability (Haneline et al., 1998; Haneline et al., 1999; Haneline et al., 2003). The FA proteins are thus believed to play important roles in the maintenance of hematopoiesis. Consistent with the observations that 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 hematopoietic cells (Kruyt et al., 1998; Cuming et al., 2001; Hadjur et al., 2001; Futaki et al., 2002; Park et al., 2004; Saadatzadeh et al., 2004; Pagano et al., 2005).

More recently, cytokine hypersensitivity of FA hematopoietic cells to apoptotic cues has also been proposed as a major factor in the pathogenesis of BM failure in three FA mouse models.
specific (Fanca<sup>−/−</sup>, Fancc<sup>−/−</sup> and Faneg<sup>−/−</sup>) (Li et al., 2004; Si et al., 2006).

It is believed that cellular and organismal senescence occurs as a result of chronic exposure to extrinsic environmental factors, primarily of oxidative stress (Sohal and Weindruch, 1996). The degree of oxidative damage has been found to increase exponentially with senescence and aging in a variety of cells, and tissues of different species (Chen et al., 1995). Endogenously formed reactive oxygen species (ROS) continuously damage cellular constituents including DNA. These challenges, coupled with exogenous exposure to agents that generate ROS, are both associated with normal aging processes and linked to cardiovascular disease, age-related anemia and cancer (Maccio et al., 2005; Sablina et al., 2005; Wallace, 2005). In the aged and certain disease states, ROS produced by proinflammatory cytokines – such as tumor necrosis factor-α (TNFα) – at inflammatory sites often cause DNA damage (Goossens et al., 1999; Suematsu et al., 2003; Wheelhouse et al., 2003). However, the mechanism through which ROS mediate their effects on the aging process is unclear.

Overproduction of the proinflammatory cytokine TNFα has been implicated in pathological conditions related to anemia and aging that is characteristic of HSC function failure (Oster et al., 1989; Kitagawa et al., 1997; Young, 2000; Dufour et al., 2003). Here, we have employed the disease model of genomic instability syndrome FA to investigate the cellular mechanism by which TNFα-induced inflammatory ROS influence hematopoiesis. We demonstrate that ROS induce premature senescence in BM cells enriched for HSCs and progenitor cells through prolonged oxidative DNA-damage response and increased genomic instability.

**Results**

**TNFα suppresses HSC/progenitor cell activity**

To examine the effect of TNFα on HSC/progenitor cell activity, we injected intraperitoneally (i.p.) wild-type (WT) or Fancc<sup>−/−</sup> mice with mouse recombinant TNFα in PBS at a dose of 0.1 mg/kg per day for two consecutive days. The mice were then sacrificed 24 hours later and BM mononuclear cells were prepared and stained with antibodies against lineage markers (Lin) and Sca-1-PE and c-Kit-PE-Cy7. Cells were then analyzed by flow cytometry to obtain fractions representing HSCs. The frequencies of LSK cells as a percentage of total BM mononuclear cells are indicated. (A) TNFα suppresses HSC/progenitor cell activity. (A) Effect of TNFα on HSC frequency. Wild-type (WT) or Fancc<sup>−/−</sup> mice were injected intraperitoneally (i.p.) with mouse recombinant TNFα in PBS at a dose of 0.1 mg/kg per day for two consecutive days. The mice were then sacrificed 24 hours later and BM mononuclear cells were prepared and stained with antibodies against lineage markers (Lin) and Sca-1-PE and c-Kit-PE-Cy7. Cells were then analyzed by flow cytometry to obtain fractions representing HSCs. The frequencies of LSK cells as a percentage of total BM mononuclear cells are indicated. (B) TNFα inhibited BM-progenitor proliferation. LSK cells isolated from TNFα-injected mice were analyzed for series-plating efficiency of hematopoietic progenitor cells. Data represents the mean ± s.d. of three experiments. (D) TNFα inhibited HSC renewal. 2×10<sup>5</sup> BM cells isolated from control (PBS-injected) or TNFα-injected mice were analyzed for series-plating efficiency of hematopoietic progenitor cells. Data represents the mean ± s.d. of three experiments. Numbers in the corners indicate percent of events in that quadrant.

TNFα-treated Fancc<sup>−/−</sup> mice exhibited mild cytopenia, as evidenced by a decrease in red cell counts, hemoglobin and hematocrit values (see supplementary material Fig. S1). Consistent with this, BM analysis of TNFα-treated Fancc<sup>−/−</sup> mice revealed a decrease in the number of cells compared with PBS-injected Fancc<sup>−/−</sup> mice (see supplementary material Table S1). In another set of experiments we analyzed hematopoietic recovery in mice injected with TNFα. We found that Fancc<sup>−/−</sup> mice recovered slowly from TNFα-induced hemo-suppression compared with WT mice (see supplementary material Fig. S2). Furthermore, Fancc<sup>−/−</sup> mice showed reduced rates of multilineage recovery (see supplementary material Table S2).

The frequencies of BM HSC/progenitor [lineage-negative, Sca-1-positive, c-Kit-positive (Lin<sup>−</sup>Sca-1<sup>−</sup>-c-Kit<sup>+</sup>)] cells (LSK cells) were reduced by more than twofold in TNFα-treated Fancc<sup>−/−</sup> mice compared with PBS controls (Fig. 1A). We thus asked whether TNFα suppressed clonogenic progenitor activity. As shown in Fig. 1B, the total number of colonies formed by LSK cells from TNFα-treated Fancc<sup>−/−</sup> mice was
more than threefold lower than that of WT mice. We also observed significantly decreased series-plating efficiency with BM LSK cells of TNFα-treated Fancc−/− mice compared with WT cells (Fig. 1C). To evaluate the in vivo effect of TNFα on HSC/progenitor cell activity, we performed BM-stem-cell transplantation. Long-term engraftment analysis shows that Fancc−/− BM HSCs were decreased in their ability to repopulate compared with WT cells (Fig. 1D), which is consistent with previous reports (Haneline et al., 1998; Haneline et al., 1999). Significantly, BM HSCs from TNFα-treated Fancc−/− mice constituted less than 10% of the peripheral blood cells at 16 weeks after transplantation compared with more than 40% reconstitution by those from TNFα-treated WT mice (Fig. 1D), indicating that TNFα impaired long-term hematopoietic reconstitution of Fancc−/− HSCs. Collectively, these results suggest that TNFα suppresses hematopoietic functions by inhibiting the self-renewal or proliferative potential of HSCs and progenitor cells.

TNFα induces premature senescence in HSC/progenitor cells

Given that TNFα suppressed the proliferative potential of HSCs and progenitor cells, we wondered whether TNFα affected differentiation or apoptosis of these primitive cells. Fig. 2A shows that BM HSCs from TNFα-injected WT or Fancc−/− donors had the ability of multilineage reconstitution, suggesting that TNFα does not affect the differentiation of long-term repopulating HSCs. Whereas TNFα induced apoptosis in BM LSK cells of treated mice, no statistically significant difference was observed between WT and Fancc−/− mice (Fig. 2B). Thus, apoptosis was not the major consequence of TNFα-mediated inhibitory effect on the self-renewal or proliferative potential of Fancc−/− HSCs and progenitor cells. However, BM LSK cells from TNFα-injected Fancc−/− mice clearly exhibited a prolonged G2-M accumulation with decreased cells in S phase (Fig. 2C), suggesting that the G2 checkpoint is compromised in these BM HSC/progenitor cells. This result reminisces of DNA-damage-induced G2-M arrest, a hallmark of FA (Bagby, Jr, 2003; Kennedy and D’Andrea, 2005).

It has been suggested that HSC senescence plays an important role in the pathophysiology of hematologic diseases, such as aplastic anemia and MDS (Maciejewski and Risitano, 2003; Zhang et al., 2005a; Zhang et al., 2005b). To determine whether HSC senescence is a defining feature of TNFα-mediated hematopoietic suppression, we examined two established senescence markers: senescence-associated β-galactosidase (SA-β-gal) (Dimri et al., 1995) and senescence-associated heterochromatin foci (Narita et al., 2003). We found that approximately 10% of LSK cells from TNFα-injected WT mice and more than 20% of those from TNFα-injected Fancc−/−
animals stained positive for SA-β-gal activity (Fig. 3A). LSK cells from TNFα-treated Fancc−/− mice also stained strongly positive for HP1-γ, indicating the formation of senescence-associated heterochromatin foci (Fig. 3B) (Narita et al., 2003). In addition, analysis of these two senescence markers in BM sections from TNFα-injected Fancc−/− mice revealed abundant positive staining in those cells (Fig. 3C) accompanied with a weak proliferation index, as identified by the proliferation marker Ki-67 (Fig. 3D). We also examined two established effectors of stress-induced senescence, p53 and p16\(^{INK4A}\) (Randle et al., 2001; Serrano and Blasco, 2001). We observed that the BM LSK cells of TNFα-injected Fancc−/− mice stained...
positive for p53 and p16INK4A, whereas those from untreated mice stained essentially negative (Fig. 3E). These results suggest that senescence is a defining feature of TNFα/H9251-mediated hematopoietic suppression in Fancc–/– mice.

Premature senescence of HSC/progenitor cells is linked to TNFα-induced ROS production

Telomere shorting has been proposed to be a cause of HSC senescence (Lansdorp, 2005). The telomere length of BM cells from TNFα-injected mice was comparable to that of untreated mice, regardless of genotype (see supplementary material Fig. S3A). Overexpression of the mouse telomere reverse transcriptase (mTERT) in TNFα-treated Fancc–/– BM cells enriched for HSCs and progenitor (Lin−) cells failed to abrogate senescence (see supplementary material Fig. S3B) or to restore long-term repopulating ability of these HSCs and progenitor cells (see supplementary material Fig. S3C). The production of reactive oxygen species (ROS) has been

Fig. 4. TNFα-induced senescence is mediated by oxidative stress in Fancc-deficient HSCs and progenitor cells. (A) The ROS scavenger NAC mitigated TNFα-induced senescence in HSCs and progenitor cells. WT and Fancc−/− mice were injected i.p. with TNFα (100 μg/kg per day) for 2 consecutive days. NAC (1 mg/mouse per day) was administered 30 minutes before and after each TNFα injection. The isolated BM LSK cells (top and middle groups of panels) or paraffin-embedded BM sections (bottom group of panels; magnification, 40×) were stained with the antibody against the senescence marker HP1-γ. The LSK cell slides were also counterstained with DAPI. (B) BM LSK cells from WT and Fancc−/− mice were cultured in the absence or presence of H2O2 (100 μM) for 45 minutes, washed and further cultured in fresh medium for 24 hours. Cells were then stained with the antibody against the senescence marker HP1-γ. Shown is quantification of HP1-γ-positive cells by scoring >100 cells in random fields on a slide for each of three independent experiments; *P<0.05. (C) BM LSK cells from WT and Fancc−/− mice were cultured in the absence or presence of H2O2 (100 μM) for 45 minutes, washed and further cultured in fresh medium for 24 hours. 1000 cells were mixed with 1×10⁶ competitor cells were transplanted into lethally irradiated recipient mice and long-term engraftment was evaluated 16 weeks after transplantation. Data are expressed as mean ± s.d. of two independent experiments, each with six recipients (12 mice per group). P<0.05 between untreated and treated Fancc−/− samples. (D) ROS production. BM cells from PBS- or TNFα-treated WT and Fancc−/− mice were labeled with CM-H2DCFDA followed by flow cytometry. (E) ROS production was mediated by TNFα. WT, Fancc−/− mice or their littermates lacking the Tnfr1 gene were injected with PBS or TNFα at 0.1 mg/kg per day for 2 consecutive days. 24 hours after TNFα injection, BM cells from individual animals were isolated and labeled with CM-H2DCFDA followed by flow cytometry. Data represent the mean ± s.d. of two independent experiments, each with six mice (total 12 mice per group). (F) WT, Fancc−/− mice or their littermates lacking the Tnfr1 gene were injected with TNFα (0.1 mg/kg per day) for 2 consecutive days. TNFα-treated mice were injected with 20 μg of a TNFα-neutralizing antibody 30 minutes after each TNFα injection. At 24 hours after TNFα injection, frozen spleen sections were prepared and subjected to SA-β-gal staining.
implicated as a mechanism of TNFα-induced cell death (Sakon et al., 2003; Ventura et al., 2004). To address whether ROS might be relevant for TNFα-induced hematopoietic senescence, we pretreated the TNFα-injected mice with the ROS scavenger N-acetyl-L-cysteine (NAC). Remarkably, NAC almost completely abrogated the induction of senescence-associated heterochromatin foci (HP1-γ) by TNFα in both BM LSK cells and BM sections of Fancc−/− mice (Fig. 4A), indicating that TNFα-induced senescence was mediated by oxidative stress. Examination of the effect of exogenous peroxide (H₂O₂; a potent ROS producer) on BM LSK cells demonstrated that, indeed, Fancc−/− HSC/progenitor cells were vulnerable to oxidative-stress-induced senescence (Fig. 4B) and inhibition of hematopoietic reconstitution (Fig. 4C). Analysis of ROS production by flow cytometry revealed that TNFα did not cause substantial ROS production in the BM of WT mice (Fig. 4D). By contrast, there was a significant ROS accumulation in the Fancc−/− BM. In vitro culture of isolated BM LSK cells in the presence of TNFα further demonstrated increased ROS production in Fancc−/− HSC/progenitor cells (see supplementary material Fig. S4).

To ascertain that it was TNFα that induced ROS production in hematopoietic cells, we took two approaches: pretreatment of mice with neutralizing anti-TNFα antibody and inactivation of TNFα signaling in Fancc−/− mice. For the latter, Fancc−/− mice were crossed with mice carrying a null mutation in the type 1 TNF receptor (Tnfr1−/− mice). Nearly complete prevention of TNFα-induced ROS accumulation (Fig. 4E) and senescence induction (Fig. 4F) was obtained with TNFα-injected mice deficient for Tnfr1. Whereas treatment of mice with anti-TNFα antibody 30 minutes after each TNFα injection did not completely neutralize serum TNFα [mean ELISA values for anti-TNFα and vehicle-injected animals were 78.6±12.4 pg/ml and 245±21.5 pg/ml, respectively, in TNFα-treated WT mice (n=6) compared with 63.9±17.2 pg/ml and 227±23.8 pg/ml, respectively, in TNFα-treated Fancc−/− mice (n=6); the basal level of serum TNFα in either WT or Fancc−/− mice was approximately 20 pg/ml], administration of the anti-TNF monoclonal antibody resulted in significant reduction of senescence in the spleen (Fig. 4F). In vitro assays further demonstrated that Fancc−/−;Tnfr1−/− BM HSC/progenitor cells were resistant to TNFα-induced ROS production and senescence (see supplementary material Fig. S5). Thus, oxidative stress is responsible for TNFα-induced hematopoietic senescence in Fancc-deficient mice.

To specifically assess TNFα-induced oxidative damage in hematopoietic organs, we immunostained for 4-hydroxy-2-nonenal (HNE), an established marker of ROS-induced tissue damage and an aldehyde product of polyunsaturated fatty acid oxidation (Cauwels et al., 2003). HNE staining was evident in the spleen of WT mice treated with TNFα (see supplementary material Fig. S6), in which we did not observe significant induction of senescence (Figs 3, 4). We found further increase in HNE immunoreactivity in the treated Fancc−/− mice (see supplementary material Fig. S6). This increase in HNE immunoreactivity was largely prevented by anti-TNFα antibody or NAC treatment (see supplementary material Fig. S6). Thus, oxidative organ damage induced by TNFα, as detected by fatty acid oxidation, appears to be proportional to the level of ROS production but not the extent of induction of senescence.

To determine the effect of TNFα-induced ROS on Fancc−/− hematopoietic function, we performed two established assays: clonogenic progenitor assay and competitive hematopoietic repopulation assay using BM cells from double-knockout (Fancc−/−;Tnfr1−/−) mice. Inactivation of TNFα signaling in Fancc−/−; (Fancc−/−;Tnfr1−/−) mice rescued progenitor growth (Fig. 5A) and stem cell repopulating ability (Fig. 5B). Pretreatment of TNFα-injected mice with the ROS scavenger NAC also significantly reduced the inhibitory effect of TNFα in progenitor growth and hematopoietic reconstitution. Interestingly, NAC did not further improve progenitor proliferation or hematopoietic reconstitution of BM cells from mice deficient for the Tnfr1 gene (Fig. 5A,B).

TNFα induced senescence of HSC/progenitor cells as a result of accumulation of oxidative DNA damage and increased genomic instability

Oxidative stress can induce DNA damage that causes growth arrest and cellular senescence (Ames et al., 1993; Chen, 2000). To determine whether TNFα-generated ROS induced DNA damage in HSC/progenitor cells that appeared to have
Fig. 6. See next page for legend.
Fig. 6. TNFα-induced senescence of HSCs and progenitor cells is associated with oxidative DNA damage and genomic instability. (A) WT or Fancc–/– mice were injected i.p. with two doses of TNFα (100 μg/kg per day) for 2 consecutive days. NAC (1 mg/mouse per day) was administered 30 minutes before and after each TNFα injection. The mice were then sacrificed 24 hours later and BM cells from individual mice were analyzed for DNA strand breaks in a comet assay. Larger tails represent higher levels of DNA damage. For each treatment, at least 100 cells were scored from random sampling. Data are expressed as the mean ± s.d. of two independent experiments, each with three mice (six mice per group). (B) WT or Fancc–/– mice were injected i.p. with two doses of TNFα (100 μg/kg per day) for 2 consecutive days. NAC (1 mg/mouse/day) was administered 30 minutes before and after each TNFα injection. The mice were then sacrificed 24 hours later and BM LSK cells were isolated and stained for the oxidative DNA damage marker 8-oxo-deoxyguanosin (8-oxoG). The bar graph shows the percentages of the cells stained positive for 8-oxoG and quantified by counting >100 cells in random fields on a slide for each of two experiments with total 6 mice. (C) Examples of metaphase chromosomes prepared from TNFα-treated WT and Fancc–/– BM cells. Arrows indicate aberrant chromosomes. (D) WT or Fancc–/– mice were injected i.p. with two doses of TNFα (100 μg/kg per day) for 2 consecutive days. NAC (1 mg/mouse/day) was administered 30 minutes before and after each TNFα injection. Mice were sacrificed 24 hours later and BM LSK cells were then isolated and stained for p53Ser20 and γH2AX. Bar graphs show the percentages of the cells stained positive for p53Ser20 and γH2AX. Cells were quantified by counting >100 cells in random fields on a slide for each of two experiments with total six mice. *P<0.05. (E) Kinetics of DNA repair of oxidative DNA damage and DNA strand breaks. BM cells from WT and Fancc–/– mice were treated with or without H2O2 (100 μM) for the indicated time periods, and protein extracts were prepared and analyzed by immunoblotting with antibody against phosphorylated p53Ser20 (p53Ser20) and anti-γH2AX and anti-antibodies. Extracts were also prepared from cells 2 hours (2-) after H2O2 withdrawal after the cells had been treated with H2O2 for 4 hours.

Table 1. Chromosome aberrations in Lin– BM cells from TNF-treated mice

| Aberrations          | Frequency (%) |
|----------------------|---------------|
|                      | WT            | Fancc–/–       |
| ≤1 aberration        | 3             | 8             | 3              | 56             |
| ≥2 aberrations       | 0             | 1             | 1              | 35             |
| ≥3 aberrations       | 0             | 0             | 0              | 23             |
| ≥9 chromosomes       | 1             | 2             | 0              | 5              |
| ≥15 chromosomes      | 1             | 4             | 1              | 9              |

TNFα-induced oxidative DNA damage may increase genomic instability leading to premature senescence of HSC/progenitor cells. To test this possibility, we conducted karyotype analysis of BM Lin– (enriched for HSC/progenitor) cells from TNFα-injected WT and Fancc–/– mice. Chromosomal aberrations were rare in cells from treated WT mice, with fewer than 10% of the cells showing one aberration and almost no cells containing two or more aberrations (Table 1). By contrast, BM HSC/progenitor cells from TNFα-injected Fancc–/– mice showed significant increase in chromosomal aberrations (56%) of the cells contained at least one aberration, 35% had two or more aberrations, and 23% had three or more aberrations; Table 1). FISH analysis of the chromosomal damages indicated that the TNFα-induced aberrations in Fancc–/– cells consisted mostly of chromatid breaks, gaps, chromosomal breaks, dicentric chromosomes, double minutes, chromosome fragments and fusions (Fig. 6C; Table 2), suggesting that the efficiency and accuracy of DNA strand break/repair may have been compromised in these cells.

A biochemical consequence of genomic damage is the activation of DNA-damage response markers, which include phosphorylated kinases ATM and Chk2, and phosphorylated histone H2AX (γH2AX) and p53 (Shiloh, 2003; Bartkova et al., 2005). Phosphorylation of p53 at Ser20 (p53Ser20) is considered a specific indicator of oxidative DNA damage (Shieh et al., 1999; d’Adda di Fagagna et al., 2003); whereas the formation of γH2AX foci constitutes a robust marker of DNA strand breaks (Banin et al., 1998; Celeste et al., 2003). In HSC/progenitor cells from TNFα-injected WT mice, about 15% of cells stained positive for p53Ser20 and less than 10% stained positive for γH2AX (Fig. 6D). However, a majority of the cells from TNFα-injected Fancc–/– mice were intensively stained for p53Ser20 (62%) or γH2AX (80%). Treatment of the mice with NAC reduced these damage responses to near basal levels (Fig. 6D).

The persistent high levels of oxidative DNA damage observed in HSC/progenitor cells from TNFα-injected Fancc–/– mice suggest that a deficiency in the FA pathway renders chromosomal DNA susceptible to ROS attack, thereby increasing oxidative DNA damage. Alternatively, a defect in the FA function might compromise the damage response/repair process. To distinguish between these possibilities, we treated BM cells from WT and Fancc–/– mice with H2O2, and conducted a time-course study to assess DNA-repair kinetics by examining the levels of p53Ser20 and γH2AX. Compared with WT cells, Fancc–/– cells consistently showed a significant delay in the kinetics of DNA-damage response/repair as evidenced by the much slower clearance of the markers of oxidative DNA damage (p53Ser20) and DNA strand break
cells from TNF production and senescence. Moreover, HSCs and progenitor cells from TNF-induced senescence of HSCs and progenitor cells correlated with the accumulation of inflammatory ROS, and oxidative DNA damage. ROS are involved in the induction of a senescent phenotype characterized by irreversible growth arrest (Chen et al., 1999; Sohal and Weindruch, 1996). The question naturally arises as to whether the inhibition of ROS can rescue HSCs and progenitor cells from TNF-mediated senescence. Our findings revealed that pretreatment of TNF-induced Fanc-deficient mice with antioxidant abrogated the exacerbated inflammatory phenotype, and prevented oxidative DNA damage and hematopoietic senescence. It is clear that the persistent inflammatory response, as reflected by prolonged production of inflammatory molecules, is primarily responsible for the generation of ROS and senescence, because mice deficient for both FA function and TNF signaling (Fanc−/−;Tnfr1−/−) were resistant to TNF-induced ROS production and senescence. Moreover, HSCs and progenitor cells from TNF-treated Fanc-deficient mice showed increased genomic instability and an impaired repair of oxidative DNA damage. Our study thus provides a link between inflammatory ROS and HSC senescence.

Compelling evidence indicates that ROS are widely implicated in the inflammatory process (Lavrovsky et al., 2000). However, the mechanism by which ROS contribute to the proinflammatory states of the aging process is not well defined. Our results demonstrated that TNF-induced ROS contributed to the increase in oxidative DNA damage in Fanc-deficient HSCs and progenitor cells. Inflammatory ROS have been associated with the initiation or aggravation of diverse pathological states including aging and cancers (Cutler, 2005; Wallace, 2005; Yoshida et al., 2005). We believe that in cells with compromised DNA repair capacity, the ability of inflammatory ROS to damage DNA is the mechanism through which ROS mediate their effects on the inflammatory and, thus, aging process. Indeed, studies have shown that the production of ROS by TNF at inflammatory sites causes DNA damage (Goossens et al., 1999; Suematsu et al., 2003; Wheelhouse et al., 2003). The role of ROS in TNF-induced inflammation and HSC senescence was validated by the use of the ROS scavenger NAC, which showed that inhibition of ROS accumulation reduced oxidative DNA damage and cellular senescence. In addition, mice treated with TNF accumulated high levels of ROS, and administration of NAC significantly reduced the amounts of secreted pro-inflammatory cytokines (Y.Q. and Q.P., unpublished results), indicating that the inflammatory response is, at least in part, mediated by ROS. Thus, these results suggest that ROS serves as a link between inflammation and senescence.

### Discussion

We used the disease model of the genomic instability syndrome Fancc−/− to investigate the role of TNFα-mediated inflammation in HSC senescence. We demonstrated that TNFα induced premature senescence in BM HSCs and progenitor cells as well as other tissues of mice deleted for the Fancc gene, which is required for the maintenance of genetic integrity (Bagby, Jr, 2003; Kennedy and D’Andrea, 2005). Importantly, TNFα-induced senescence of HSCs and progenitor cells correlated with the accumulation of inflammatory ROS, and oxidative DNA damage. ROS are involved in the induction of a senescent phenotype characterized by irreversible growth arrest (Chen et al., 1999; Sohal and Weindruch, 1996). The question naturally arises as to whether the inhibition of ROS can rescue HSCs and progenitor cells from TNFα-mediated senescence. Our findings revealed that pretreatment of TNFα-injected Fanc-deficient mice with antioxidant abrogated the exacerbated inflammatory phenotype, and prevented oxidative DNA damage and hematopoietic senescence. It is clear that the persistent inflammatory response, as reflected by prolonged production of inflammatory molecules, is primarily responsible for the generation of ROS and senescence, because mice deficient for both FA function and TNFα signaling (Fanc−/−;Tnfr1−/−) were resistant to TNFα-induced ROS production and senescence. Moreover, HSCs and progenitor cells from TNFα-treated Fanc-deficient mice showed increased genomic instability and an impaired repair of oxidative DNA damage. Our study thus provides a link between inflammatory ROS and HSC senescence.

Our study raises an important question: can inflammatory response be a link between aging and HSC function? It is long known that general inflammatory stress tends to increase with age (Chung et al., 2001). Under inflammatory conditions, HSCs must be able to produce a large number of leukocytes, which are then activated to fight against invaders or stressing agents. This, ultimately, may lead to premature exhaustion of the HSC pool. In the meantime, the HSCs become targets of the toxicity of inflammatory ROS. The ensuing consequence will depend upon the capacity of HSCs to repair ROS-induced damage, particularly DNA damage. In aged and certain disease states, inefficient repair of the oxidative damage may lead to the decrease of the HSC quality (self-renewal capacity). Hence, the toll of inflammatory stress consists in premature senescence of HSCs.

### Table 2. TNF-induced chromosome instability in Fanc−/− HSC/progenitors

| Aberration                    | WT (%) | Fanc−/− (%) |
|------------------------------|--------|-------------|
| Chromosome breaks            | 1      | 15          |
| Chromosome fragment          | 2      | 8           |
| Chromatid break              | 2      | 13          |
| Chromatid gap                | 2      | 10          |
| Complex rearrangement        | 0      | 3           |
| Deletions                    | 1      | 8           |
| dicentric chromosome        | 0      | 5           |
| Double minute                | 0      | 1           |
| Translocation                | 0      | 22          |

By investigating four well-established senescence makers (SA-β-gal, HP1-γ, p53 and p16INK4A), we provided evidence that TNFα induced premature senescence in BM HSCs and progenitor cells and also other tissues of Fanc−/− mice. Recently, another senescence marker protein, SMP30, has been shown to play an important role in senescence and aging, and SMP-deficiency renders mice highly susceptible to oxidative stress (Sato et al., 2006). It would be interesting to examine the effect of TNFα on the expression pattern of this new senescence marker in HSCs and progenitor cells of Fanc−/− mice.

The persistent DNA damage response and increased genomic instability in senescent HSCs, and progenitor cells are two important features of our study. BM progenitor cells from TNFα-treated Fanc−/− mice contained high levels of DNA strand breakage and oxidative DNA damage. Moreover, studies of repair kinetics revealed much slower clearance of the oxidative DNA damage and DNA strand break markers in Fanc−/− HSCs and progenitor cells than in WT cells. Consistent with this, DNA damage response was persisted in these Fanc−/− cells. This suggests that premature senescence observed in Fanc−/− HSCs and progenitor cells may result from the prolonged activation of the oxidative DNA damage and DNA double-strand-break checkpoints. However, this prolonged checkpoint activation did not facilitate damage repair. Instead, we found dramatically increased genomic instability in Fanc−/− cells. Thus, we conclude that it is unrepaird DNA damage that results in the persistent DNA damage response in these cells.

Our study raises an important question: can inflammatory response be a link between aging and HSC function? It is long known that general inflammatory stress tends to increase with age (Chung et al., 2001). Under inflammatory conditions, HSCs must be able to produce a large number of leukocytes, which are then activated to fight against invaders or stressing agents. This, ultimately, may lead to premature exhaustion of the HSC pool. In the meantime, the HSCs become targets of the toxicity of inflammatory ROS. The ensuing consequence will depend upon the capacity of HSCs to repair ROS-induced damage, particularly DNA damage. In aged and certain disease states, inefficient repair of the oxidative damage may lead to the decrease of the HSC quality (self-renewal capacity). Hence, the toll of inflammatory stress consists in premature senescence of HSCs.

### Chronic inflammation and oxidative stress are important features in the pathogenesis of BM diseases such as FA

(Lavrovsky et al., 2000; Bagby, Jr, 2003; Chen, 2005). The
increased oxidative stress in FA patients may be the result of an increased burden of endogenously produced oxidants as well as increased amounts of ROS generated by various inflammatory cytokines – as suggested in our study. Therefore, understanding the relationship between ROS and inflammation in the context of HSC senescence and aging in these disease states provides a unique opportunity to mechanistically comprehend, and potentially intervene in these physiologically important processes. In addition, our results suggest that, antioxidant compounds may be of therapeutic value in monitoring disease progression, and antioxidant therapy could be used to stop the initiation and propagation of inflammatory response in these diseases.

Materials and Methods

Mice and treatments

Generation of FancE knockout mice has been described by Chen et al. (Chen et al., 1996). FancE+/− mice were intercrossed with C57Bl/6 mice for more than ten generations to develop an inbred strain. FancE−/− mice and their WT littermates were used at approximately 8 weeks of age. Mice were maintained on a C57Bl/6 (CD45.2 +) background. All mice were used at approximately 10-14 weeks of age. Mice were injected i.p. at 1 mg per mouse 30 minutes before and after each TNF injection.

Apoptosis assay and cell cycle analysis

Cells were stained with annexin V and 7-AAD using BD ApoAlert Annexin V kit (BD Pharmingen) in accordance with the manufacturer’s instructions. Apoptosis was determined as quantification of annexin-V-positive cell population by flow cytometry. For cell cycle analysis, cells were permeabilized with 0.3% Nonidet P-40 (NP-40), and then stained with propidium iodide (PI) containing 1 mg/ml RNase A, followed by FACS analysis of the G0-G1, S and G2-M populations.

Analysis of HSC senescence

Cells were cytospun onto slides and fixed in ice-cold methanol for 5 minutes at −20°C. After air drying, cells were blocked for 1 hour with 5% normal serum. Then cells were incubated with antibodies against H2A-H2AX (Upstate Biotechnologies). Cells were incubated with CM-H2DCFDA (Molecular Probe) in the dark for 15 minutes at 37°C. After washing, cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Data were analyzed by using the CellQuest program (BD Biosciences).

Immunohistochemistry

During necropy, organs were removed, preserved in formalin and then embedded in paraffin blocks. Paraffin sections were deparaffinized, rehydrated, incubated in 0.1 mM sodium citrate (pH 6.0), washed and incubated with peroxidase blocking solution (Vector Laboratories, Vectastain Elite ABC kit). After washing in PBS, slides were incubated with primary antibodies against HNE (11-S; Alpha Diagnostic International, San Antonio, TX) or H1P-γ (07-332; Upstate Cell Signaling Solutions, Lake Placid, NY). Following three PBS washes, slides were incubated with secondary antibody and then detected with theVectaStain Elite ABC reagents.

Retroviral vectors and infection

The retroviral vector expressing the mTERT was kindly provided by Fuyuki Ishikawa (Kyoto University, Japan). Retroviruses were prepared by the Vector Core at Cincinnati Children’s Research Foundation. Retroviral supernatant was collected 36 hours, 48 hours and 60 hours after transfection. Cells were plated onto non-tissue culture 24-well plates coated with Retronectin (Takara-Shuzo, Shiga, Japan) and pre-stimulated for 2 days in Iscove’s modified Dulbecco’s medium (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 hours at 37°C in the presence of 4 μM Polybrene (Sigma). Cells were centrifuged at 600 g for 45 minutes. Infection was repeated twice and infection efficiency was assessed by the detection of green fluorescent protein (GFP)-positive cells by FACS.

Immunoblots

Cells were solubilized in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet NP-40) containing a cocktail of protease inhibitors (Calbiochem, San Diego, CA). Equal amounts of protein were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane and blotted with antibodies against p53/p21 (Santa Cruz Biotech), γ-H2AX (Upstate Biotechnologies), and β-actin (Sigma).

Serum levels of cytokines

Serum levels of inflammatory cytokines were measured using enzyme-linked immunosorbent assays (ELISA) kits (R&D Systems).

Comet assay

Generation of DNA strand breaks was assessed by the single-cell gel electrophoresis (comet) assay (Fairbairn et al., 1995), using a Fpg-FLARE (fragment-length analysis using repair enzymes) comet assay kit in accordance with the manufacturer’s instructions (Trevigen, Gaithersburg, MO). For each experimental point at least three different cultures were analyzed, and 100 cells were evaluated for comet-tail length from each culture.

Cytogenetic analysis

Cells were incubated in 0.1 mg/ml colcemid for 30-60 minutes and then incubated for 75 min at 37°C. Chromosomes were subsequently fixed in methanol/acetic acid (3:1) and dropped onto glass slides. Metaphase chromosomes were Giemsa-stained and examined for abnormalities. Chromosome aberrations were defined using the nomenclature rules from the Committee on Standard Genetic Nomenclature for Mice.

Statistics

Data were analyzed statistically using a two-tailed Student’s t-test. The level of statistical significance stated in the text was based on the P values. P<0.05 was considered statistically significant.
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