Thrombopoietin-Increased DNA-PK-Dependent DNA Repair Limits Hematopoietic Stem and Progenitor Cell Mutagenesis in Response to DNA Damage

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SUMMARY

DNA double-strand breaks (DSBs) represent a serious threat for hematopoietic stem cells (HSCs). How cytokines and environmental signals integrate the DNA damage response and contribute to HSC-intrinsic DNA repair processes remains unknown. Thrombopoietin (TPO) and its receptor, Mpl, are critical factors supporting HSC self-renewal and expansion. Here, we uncover an unknown function for TPO-Mpl in the regulation of DNA damage response. We show that DNA repair following γ-irradiation (γ-IR) or the action of topoisomerase-II inhibitors is defective in Mpl−/− and in wild-type mouse or human hematopoietic stem and progenitor cells treated in the absence of TPO. TPO stimulates DNA repair in vitro and in vivo by increasing DNA-PK-dependent nonhomologous end-joining efficiency. This ensures HSC chromosomal integrity and limits their long-term injury in response to IR. This shows that niche factors can modulate DNA repair processes to improve the safety of anticancer DNA agents and reduce the risk of cancer. Moreover, DNA-damaging agent-induced bone marrow (BM) injury is one of the major limiting factors for cancer therapies that use DNA-damaging agents, such as IR and chemotherapeutic drugs. DNA-PK-dependent repair pathways that use nonhomologous end-joining (NHEJ) and homologous recombination (HR) are critical to maintain HSC integrity during IR. This work reveals the importance of DNA-PK signaling in the modulation of DNA damage response and the coupling of DNA repair with Ito et al., 2004; Nijnik et al., 2007; Rossi et al., 2007. Therefore, approaches for improving the safety of anticancer DNA agents require careful analysis of HSC response to DNA damage. Limited data are available on the mechanisms involved in DNA repair in HSCs. Recent data demonstrated that DSB repair through NHEJ is necessary for HSC maintenance, given that mice deficient for DNA Ligase IV or Ku80/70 heterodimer, and the DNA Ligase IV/XRCC4/XLF complex. NHEJ is considered intrinsically error prone. However, although it frequently causes small alterations in DNA sequence around the break site, the classical NHEJ usually does not join unlinked DNA ends. In the absence of key canonical NHEJ components, a backup DNA-PK-independent version of NHEJ accounts for residual end-joining of DSBs and results in major genomic rearrangements, including chromosomal deletions and translocations like those at the origin of leukemia (Ferguson et al., 2000; Guirouilh-Barbat et al., 2004; Iliakis et al., 2004; Weinstock et al., 2007).

Bone marrow (BM) injury is one of the major limiting factors for cancer therapies that use DNA-damaging agents, such as IR and chemotherapeutic drugs. Besides acute myelosuppression, resulting from apoptosis of proliferating hematopoietic progenitor cells (HPCs) and reversible for nonlethal doses, IR induces residual BM injury due to loss of hematopoietic stem cell (HSC) reserves or functions (Shao et al., 2010; Simonnet et al., 2009; Wang et al., 2006). Moreover, DNA-damaging agent-induced risk of cancer is very high for the hematopoietic tissue. Indeed, secondary acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) are concerning complications of these therapies. Because they maintain hematopoiesis throughout their life span, HSCs have an increased risk of accumulating genomic abnormalities. Maintenance of genomic integrity is also crucial for preservation of HSC self-renewal (Ito et al., 2004; Nijnik et al., 2007; Rossi et al., 2007). Therefore, approaches for improving the safety of anticancer DNA agents require careful analysis of HSC response to DNA damage. Limited data are available on the mechanisms involved in DNA repair in HSCs. Recent data demonstrated that DSB repair through NHEJ is necessary for HSC maintenance, given that mice deficient for DNA Ligase IV or Ku80 display age-progressive loss of HSC functions (Nijnik et al., 2007; Rossi et al., 2007). However, the obligatory use of NHEJ to repair IR-induced DNA damage in quiescent HSCs has been shown to promote high levels of mutagenesis (Mohrin...
HSCs are also regulated through interactions with the niche environment and growth factors controlling the balance between quiescence, proliferation, and differentiation. How these signals contribute to the HSC-intrinsic DNA repair process is currently unknown.

Thrombopoietin (TPO) and its receptor Mpl are master regulators of both megakaryopoiesis and HSCs. TPO has been shown to increase HSC interaction with the osteoblastic niche and to support HSC quiescence and expansion posttransplantation (Fox et al., 2002; Qian et al., 2007; Yoshihara et al., 2007). Mice deficient for TPO or Mpl display increased HSC cycling and age-progressive loss (Qian et al., 2007; Yoshihara et al., 2007). Inactivating mutations of the Mpl receptor in humans cause thrombocytopenia and BM failure in early childhood, a syndrome called congenital amegakaryocytic thrombocytopenia (CAMT) (Maserati et al., 2008), whereas they induced rapid death of all Mpl−/− mice (Figure 1A). Clonogenic assays showed that Mpl−/− Lin−Sca1+Kit+ cells (hereafter referred to as HSPCs or LSK cells) were less resistant than WT LSK cells to low doses of IR, indicating a radiosensitive phenotype (Figure 1B). The dose of 2 Gy was chosen for further examination of this phenotype.

We then asked whether the lower radioresistance of Mpl−/− HSPCs could be due to DNA repair defects. Thus, we first examined the presence of γH2AX nuclear foci, a commonly used surrogate marker of DSB formation. A slightly higher proportion of Mpl−/− LSK cells expressed spontaneous γH2AX foci compared to WT cells, although this remained low. Twenty-four hours after IR, a significantly greater number of WT LSK cells harbored γH2AX foci, and this number was further increased in Mpl−/− mice (Figure 1C). The number of γH2AX foci detected by flow cytometry was also increased in Mpl−/− HSC-enriched LSK-CD34+ cells (Figure 1D).

Second, we assessed DNA repair by analyzing γH2AX foci disappearance kinetics. Thus, LSK cells were irradiated in vitro and analyzed at various times of culture in a medium containing a cocktail of cytokines including TPO (complete medium). Maximum γH2AX foci formation was observed immediately after IR, and it was not significantly different between WT and Mpl−/− cells. WT LSK cells had already started resolving their foci at 5 hr post-IR, and 62 ± 3% of cells harboring foci had disappeared at 24 hr. By contrast, at that time, the majority of...
Mpl−/− cells still stained positively for γH2AX, and the majority of them contained four or more foci (Figures 1E and 1F), indicative of prolonged DNA damage in Mpl−/− HSPCs. Interestingly, a similar defect was observed in Mpl+/− cells (Figure 1E), indicating that the level of Mpl expression is critical for correct DNA repair in response to IR.

To analyze whether this persistent DNA damage resulted from defective DSB repair, we performed single-cell comet assays in neutral conditions that measure DSBs specifically. Just after IR, all the cells displayed comparable amounts of DNA breaks. However, whereas WT cells rejoined 2/3 of the breaks in 2 hr, the great majority of them remained unrepaired in Mpl−/− cells (Figure S1A available online). In addition, when the extent of damage was quantified on a 0 (undamaged) to 3 (very damaged) scale according to the tail length, it appeared that the severity of IR-induced DNA damage was greatly enhanced in Mpl−/− (Figure S1B).

**TPO-Mpl Signaling Specifically Regulates DSB Repair in HSCs**

To exclude the possibility that the constitutive lack of Mpl leads to increased DNA damage due to chronic changes, we analyzed the effect of acute TPO signaling upon IR. Thus, we switched the cells from the complete medium to the same medium containing all cytokines but TPO. The presence of TPO did not affect the maximum levels of γH2AX foci or break formation, showing Mpl−/− cells. Removal of TPO to Mpl−/− LSK cell cultures had no effect (Figure S2B), showing that TPO acts through Mpl only. In agreement with the higher Mpl expression in HSCs than in progenitors (Qian et al., 2007), TPO had an even more striking effect in improving DNA damage resolution in LSK-CD34− cells (Figure 2C). This indicates that the DNA repair defect of Mpl−/− cells results from a specific loss of TPO signaling. TPO also increased γH2AX foci disappearance in CD34−CD38− human HSPCs (Figures 2E and S2C) and in a human cell line expressing Mpl, UT7-Mpl (Figure S2D), which can be grown in erythropoietin (EPO) or TPO (Hamelin et al., 2006). Kinetics analysis indicated that the protective effect of TPO required the presence of TPO shortly before IR and was abolished when TPO was added to the medium after IR (data not shown).

TPO could also favor DNA repair in vivo. Indeed, TPO injection to WT but not to Mpl−/− mice just before TBI or DXR treatment (Figure 3A) significantly reduced the number of LSK cells displaying γH2AX foci at 5 or 16 hr compared to mice treated with PBS (Figures 3B and 3C). The TPO agonist Romiplostim (Léon et al., 2012) induced a similar effect (Figure 3B). As observed in vitro, an even more striking effect of TPO was observed in HSC-enriched LSK-CD34− cells (Figure 3D).

In contrast with TPO, removal of stem cell factor (SCF; Figures 2A, 2D, and S2B) or Flt3 ligand (Flt3-L; data not shown) did not impair the kinetics of IR-induced γH2AX foci or DSB resolution (Figure 2B). As a consequence, the majority of LSK-CD34− cells still stained positively for γH2AX to increased DNA damage due to chronic changes, we analyzed the extent of acute TPO signaling upon IR. Thus, we switched the cells from the complete medium to the same medium containing all cytokines but TPO. The presence of TPO did not affect the maximum levels of γH2AX foci or break formation, showing that TPO did not prevent DNA damage. However, LSK cells irradiated and cultured in TPO-free medium were greatly impaired in their capacity to resolve γH2AX foci (Figure 2A). Similar results were found by using etoposide and doxorubicin (DXR) for inducing DSBs (Figures 2B and S2A). DNA comet tail repair was also greatly altered in the absence of TPO (Figure 2D). The defects were similar in their extent to those observed with...
disappearance in WT or Mpl⁻/⁻ LSK cells. Likewise, injection of SCF or Flt3-L (Hérodin et al., 2003) to mice before TBI did not reduce γH2AX foci accumulation in LSK-CD34⁻ cells (Figure 3D). Injection of TPO-neutralizing antibodies significantly increased TBI-induced γH2AX foci accumulation, whereas the c-Kit neutralizing antibody ACK2 (Xue et al., 2010) had no effect (Figure 3E). This indicates that TPO has a unique effect on HSC DNA damage responses in vivo and in vitro. Confirming these results, HSPCs from Kit⁺W⁻/W⁻ mutant mice did not display increased DNA damage upon TBI (Figure S2E).

In addition to defects in DNA repair, decreased DNA damage and radiosensitivity can result from altered apoptotic or cell-cycle responses. Thus, we next tested whether TPO signaling could also modulate these cellular outcomes after IR. WT and Mpl⁻/⁻ LSK cells showed similar degrees of caspase 3 and caspase 7 activation at 24 hr post-IR (2 Gy) in vitro (Figure S3A). Likewise, a low and similar percentage of WT and Mpl⁻/⁻ LSK cells isolated 5 hr (data not shown) or 24 hr (Figure S3B) after 2 Gy TBI stained positive for annexin V. Thus, the Mpl⁻/⁻ HSPC radiosensitivity is not due to increased IR-induced early apoptosis. Confirming these results, TPO had no effect on apoptosis of WT LSK cells 24 hr after IR in vitro (Figure S3C). Accordingly, induction of p53-dependent apoptotic genes involved in IR-induced HSPC death (Shao et al., 2010) was similar in the presence or the absence of TPO (Figure S3D).

At 5 hr after TBI, the proportions of LSK and LSK-CD34⁻ cells recovered from the BM of PBS- or TPO-treated mice were not significantly different (data not shown). Cell-cycle analysis using Hoechst and Pyronin Y staining confirmed previous data showing that the LSK-CD34⁻ HSC population contains a majority of G0 quiescent cells and fewer proliferative cells than the LSK cell population (Figures 3F and 3G). Although TBI increased the number of LSK cells in S-G2/M, it has no significant effect on the total number of quiescent LSK and LSK-CD34⁻ cells. Importantly, TPO treatment pre-TBI did not alter the cell-cycle status of either LSK or LSK-CD34⁻ cells compared to PBS treatment, at a time when it already reduced γH2AX foci. Similar results were observed at 16 hr post-TBI (Figure S3E). Moreover, expression of the cell-cycle regulators p57Kip2 and p27kip1, involved in HSC quiescence (Qian et al., 2007; Yoshihara et al., 2007), remained unchanged after TPO injection and TBI (Figure S3F). Likewise, although the proportion of quiescent LSK cells decreased significantly after 24 hr culture in vitro, the presence of TPO in the medium had no effect on this phenomenon (Figure S3G). For determining whether TPO could give more time to the cells to repair damaged DNA by slightly slowing down the cell cycle, cell-division history of LSK cells irradiated in vitro was also assessed using CFSE dilution assays. No difference was observed in cultures containing TPO or not, even at early time points (Figure S3H). Finally, we could not detect senescence by SA-β-galactosidase staining in WT or Mpl⁻/⁻ LSK cells (data not shown).
Thus, TPO-mediated decrease in DNA damage in HSPCs and HSCs is unrelated to their cell-cycle status and is not due to changes in quiescence, proliferation, or apoptosis in vivo and in vitro. Rather, this effect seems to result specifically from an alteration in the DNA repair process.

**TPO Regulates a DNA-PK-Dependent NHEJ Pathway**

We next examined the mechanism involved in TPO-mediated DSB repair. HR and NHEJ are the two major DSB repair pathways. The above data show that TPO regulates DNA damage in HSC-enriched, mostly quiescent LSK-CD34^- cells (65%–80% G0), HSPCs (LSK cells, 30%–40% G0), proliferating Lin^- progenitors and UT7-Mpl cells. This suggests that the mechanism involved is different from HR which acts only during the G2 and S phases. Supporting this hypothesis, TPO did not increase IR-induced Rad51 foci formation, an in vivo functional marker of HR ([Figure S4](#)), and it could not improve repair of DSBs induced by replicative stresses such as camptotecin and hydroxyurea (data not shown).

NHEJ is the predominant repair mechanism for DSBs resulting from IR ([Iliakis et al., 2004](#)). Thus, we then tested this pathway by analyzing the involvement of its main enzyme, DNA-PK. Addition of the specific DNA-PK inhibitor NU7441 to TPO-containing cultures of both LSK and LSK-CD34^- cells abolished TPO-improved γH2AX foci resolution to the level observed in the absence of TPO ([Figures 4A and 4B]). Similarly, NU7441 inhibited the fast rejoining of DSBs induced by TPO, as measured by neutral comet assays (Figure 4C). Interestingly, in the absence of TPO a slow repair process occurred on which NU7441 had no effect, showing that this repair process takes place in a DNA-PK-independent manner. Confirming the importance of DNA-PK activation in TPO-mediated DNA repair, TPO injection to severe combined immunodeficiency (SCID) mice, which are NHEJ repair deficient as a result of a DNA-PKcs mutation, did not decrease TBI-induced LSK γH2AX foci
IR has been shown to induce phosphorylation of DNA-PK at Ser2056 and Thr2609, and pDNA-PK foci formation can be used as an in vivo functional marker of NHEJ activity (Chan et al., 2002; Chen et al., 2005). To test whether TPO could affect DNA-PK activation, we used human cells, because the available pDNA-PK antibodies could not detect DNA-PK in mouse cells. According to previous studies (Chen et al., 2005), pDNA-PK is rapidly induced upon IR and accumulates in nuclear foci (Figure S4B). In UT7-Mpl as well as in CD34+ and CD34+C38 human primary HSPCs, the presence of TPO led to a significant increase in IR-induced pSer2056- and pThr2609-DNA-PK foci formation (Figures 4E–4H and S4C). By contrast, total DNA-PK gave a diffuse signal that did not change upon IR or TPO treatment (Figure S4B).

To confirm the activation of NHEJ repair by TPO, we examined NHEJ activity. First, we transfected WT and Mpl-deficient Lin−Kit+ cells with a plasmid-based episomal DSB end-rejoining assay, in which the religation leads to green fluorescent protein (GFP) expression (Seluanov et al., 2004). This assay showed that NHEJ activity in response to IR was significantly decreased in Mpl−/− and Mpl+/− progenitors (Figure 5A). Second, to assess NHEJ activity more thoroughly, we used an intrachromosomal substrate (Figure 5B) stably introduced in UT7-Mpl cells and introduced DSBs using the meganuclease HA-I-SceI (Guirouilh-Barbat et al., 2004). NHEJ events scored by CD4 or CD8 expression were greatly enhanced in cells treated with TPO (Figures 5C and 5D), whereas I-SceI expression was similar in both conditions (Figure 5E). As a control, NU7441 completely abrogated NHEJ activity. Taken together, these results show that TPO stimulates DNA-PK activity and NHEJ-mediated DNA repair.

TPO Confers Genomic Stability in Response to DNA Damage

Defects in DNA-PK-dependent NHEJ repair lead to a dramatic increase in large deletions and translocations (Ferguson et al., 2000; Weinstock et al., 2007). This suggests that the loss of TPO signal may lead to enhanced generation of misrepaired HSPCs. Indeed, cells derived from IR-exposed Mpl−/− LSK cells showed significantly higher chromosomal aberrations than WT controls (Figure 6A). Spectral karyotyping analysis using fluorescence in situ hybridization (FISH) probes specific for chromosomes 2, 6, and 12 showed that BM cells isolated from Mpl−/− mice 24 hr post-TBI displayed three times more chromosomal translocations than those from WT mice (Figure 6B). No significant difference was found between WT and Mpl−/− mice in the absence of IR (Figures 6B and 6C). Lin− progenitors isolated from WT mice 5 months post-TBI showed increased genomic instability compared to their nonirradiated age-matched counterparts, and this was further accentuated in cells from Mpl−/− mice (Figure 6C). This confirms the increased genomic instability in Mpl−/− cells and shows that a greater number of Mpl−/− cells expressing more aberrations could persist for several months in vivo.

As Mpl−/−, WT HSPCs irradiated in vitro and cultured in the absence of TPO harbored high numbers of chromosomal rearrangements (Figure 6A), showing that TPO-increased NHEJ...
could prevent IR-mediated genomic instability in HSPC descendants. We next examined whether TPO-induced DSB repair at early times after IR could lead to long-term protection in vivo. Thus, WT CD45.1 LSK cells cultured in the presence or absence of TPO for short times before and after exposition to IR in vitro (Figure S5A) were injected into lethally irradiated congenic CD45.2 mice. Metaphase analysis of donor progenitor progeny 4 months posttransplantation showed that exposure to TPO led to decreased genomic aberrations (Figure S5B).

To determine whether TPO could improve long-term adverse effects of IR in vivo, mice were treated with TPO or PBS prior to TBI and analyzed 3 months later (Figure 7, stage 1). It has been reported that despite seeming recovery of phenotypically defined HSC number and cell cycle, DNA damage may persist and HSC function remains altered several months after IR (Marusyk et al., 2009; Simonnet et al., 2009). Accordingly, both LSK and LSK-CD34− cells isolated from TBI mice harbored more γH2AX foci than cells from nontreated mice (Figures 7B and 7C), whereas their frequencies and proliferation and quiescence statuses at that time were not significantly different (Figures S5C and S5C). Injection of TPO or Romiplostim almost completely abolished persistent DNA damage in HSCs and HSPCs but had no effect on their number and cycle. In addition, the in vitro LSK clonogenic potential was similar among the three groups of mice (data not shown). Thus, TPO did not reduce the numbers of γH2AX-positive LSK cells by inhibiting senescence (Wang et al., 2006) or inducing proliferation. Three months after TBI, metaphases from progenitor cells isolated from TPO-injected mice also displayed greatly reduced chromosomal aberrations (Figure 7D). This shows that TPO injection before TBI limits IR-induced long-lasting DNA damage and genomic instability in HSC/HSPCs.

Previous studies have shown that sublethal IR induces intense HSC cycling to reconstitute hematopoiesis following acute ablation of differentiated leukocytes (Ban and Kai, 2009; Marusyk et al., 2009; Michalak et al., 2010). Because designation of HSCs requires contribution to hematopoiesis for at least 3 months, the results above suggest that the progenitors isolated 5 months (Figure 6C) or 12 weeks (Figure 7D) after IR may represent HSC progeny and that TPO might restrain genomic instability in these cells. However, to distinguish this hypothesis from a bystander effect (Lorimore et al., 2005) and determine whether the reduced DNA damage in cells from TPO-treated mice represents HSCs capable of long-term hematopoietic reconstitution, LSK cells from stage 1 (Figure 7A) were used to reconstitute lethally irradiated CD45.2 recipients. Four months after reconstitution (Figure 7A, stage 2), the progenitor progeny of LSK cells from the group that had received TBI and PBS displayed significantly increased levels of genomic rearrangements compared to their nonirradiated counterparts or those treated with TPO.

Figure 6. Increased Genomic Instability in Mpl−/− HSPCs in Response to DNA Damage

(A) Chromosomal aberrations in metaphases prepared from WT and Mpl−/− LSK cells exposed to IR (2 Gy) in vitro and cultured in complete medium or without TPO for 48 hr. Top: Means ± SEM of aberrations per cell from three independent experiments with three WT or Mpl−/− mice. At least 50 complete metaphases were scored. Bottom: representative images with aberrations indicated by arrows.

(B) Summary table and representative pictures of chromosomal translocations measured by three-color FISH in WT and Mpl−/− BM cell metaphases 24 hr after TBI.

(C) Chromosomal aberrations analyzed as in (A) in WT and Mpl−/− Lin− progenitors 5 months after TBI or in NIF age-matched controls. Each point represents an individual mouse. Means ± SEM.
with TPO before TBI (Figure 7E). These results support previous data showing that IR induces genomic instability in HSCs that can be transmitted to their progeny (Mohrin et al., 2010). They also indicate that TPO injection before TBI significantly reduced this effect.

Increased DNA damage is linked to HSC dysfunction (Nijnik et al., 2007; Rossi et al., 2007). We therefore assessed whether TPO could also improve the reconstitution capacity of irradiated cells. Supporting this possibility, the long-term engraftment of LSK cells exposed to TPO before and after IR in vitro was enhanced by almost 2-fold as compared to cells not treated with TPO (Figure S5C). Competitive transplant experiments (Figure 7A, stage 2) showed that LSK cells isolated from mice that had received PBS and TBI 3 months before were far less efficient in outcompeting recipient cells, with a mean 9-fold decrease in the level of donor contribution to peripheral blood leukocytes 4 months after transplantation (Figure 7F). TPO injection partially restored this defect with a 3- to 5-fold increase in donor CD45.1 chimerism in the peripheral blood and in BM LSK cells 4 months posttransplantation (G), although this level remained decreased compared to results in mice reconstituted with LSK cells from nonirradiated donors. No difference was found between the TPO and PBS groups at 6 weeks posttransplantation (Figure 7E), suggesting that TPO

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**Figure 7. TPO Confers Long-Term Protection and Genomic Stability to HSCs in Response to IR**

(A) Experimental design to test TPO and TBI effects on genomic instability and long-term reconstitution in vivo. (B and C) Frequencies of γH2AX-positive LSK (B) and LSK-CD34− (C) cells at stage 1 from mice that received PBS, TPO, or Romiplostim (Romi) before TBI. Each dot represents an individual mouse. Means ± SEM are shown with the values above the dots indicating mean numbers. (D) Frequencies of chromosomal aberrations in stage 1-Lin− progenitors, detected by DAPI staining (n = 3 mice for each group). (E–G) Analysis at stage 2. (E) Frequencies of chromosomal aberrations in CD45.1+Lin− progenitor metaphases measured as in (D). Means ± SEM of three independent pools containing three mice per group are shown. (F and G) CD45.1 chimerism in peripheral blood (F) and in BM LSK cells 4 months posttransplantation (G). Means ± SEM are shown (n = 7–9 mice per group). See also Figure S5.
treatment improved HSC function of irradiated cells rather than their short-term proliferation.

Altogether, these results show that the presence of TPO in vitro or its injection in vivo just before IR reduced IR-induced HSPC mutagenesis and loss function.

DISCUSSION

Management of acute myelosuppression following radiotherapy has been significantly improved in recent years by the use of growth factors. TPO injection shortly after IR has been shown to accelerate progenitor recovery and prevent short-term pancytopenia (Hérodin et al., 2003; Neelis et al., 1998; Shibuya et al., 1998) by inhibiting acute p53-dependent apoptosis (Pestina et al., 2001). However, how TPO affects the HSC response to DNA-damaging agents has never been studied. In addition, protection from immediate IR-induced ablation does not always correlate with maintenance of long-term proliferative capacities (Marusyk et al., 2009). Much remains to be elucidated regarding how cytokines and environmental signals integrate the DNA damage responses in HSCs and regulate their long-term defects following radiotherapy. In this study we uncover a specific function for TPO-Mpl in the regulation of DNA-PK-dependent NHEJ activity that ensures efficient DNA repair and chromosomal integrity of HSPCs and limits their residual injury in response to IR or topoisomerase-II inhibitors. This is the first demonstration that a cytokine involved in HSCs’ maintenance may also regulate their response to external DNA-damaging insults by controlling the DSB repair machinery. A single injection of TPO prior to TBI was sufficient to significantly improve DNA damage repair, suggesting that endogenous HSCs in the BM niche respond more similarly to DSB inducers and TPO signaling than isolated HSCs ex vivo.

Recent data have shown that NHEJ activity is higher in quiescent HSCs than in proliferating HPCs (Mohrin et al., 2010). Although our results do not call this into question, increased HR does not seem to be the mechanism by which TPO favors DSB repair. Indeed, TPO increases DSB repair similarly in myeloid progenitors (Lin ‘Kit’), mouse and human HSPC- and HSC-enriched cell populations, and UT7-Mpl cells containing various proportions of quiescent cells, without modifying their cell cycle upon IR in vivo and in vitro. Moreover, we could not observe a difference in Rad51 foci formation in LSK cells treated with TPO or not, or in Mpl−/− cells (data not shown). Rather, our results indicate that TPO increases DNA repair in these different cell types by modulating the efficiency of the NHEJ pathway. This is shown by (1) the complete inhibition of TPO-promoted γH2AX resolution and DSB rejoining in both LSK and LSK-CD34+ cells by a specific inhibitor of DNA-PK; (2) the loss of the TPO effect in vivo in DNA-PKcs-deficient SCID mice; (3) the increased NHEJ efficiency in irradiated myeloid progenitors and in UT7-Mpl upon TPO treatment; and (4) the increased IR-induced DNA-PK phosphorylation at Ser2056 and Thr2609 in the presence of TPO. A Ser to Ala mutation at both sites has been shown to compromise both radiation resistance and NHEJ (Chan et al., 2002; Chen et al., 2005), suggesting that TPO may be required for full DNA-PK activity. Additional studies are required for determining how TPO signaling increases DNA-PK phosphorylation. However, whatever the mechanism, the TPO effect on DNA-PK translates into a major effect on NHEJ activity, as shown by the extent of decrease in NHEJ efficiency in the absence of TPO. This fits with reports showing that a partial defect in DNA-PK expression or phosphorylation may be sufficient to underlie DSB repair defects and radiosensitive phenotypes (Burma et al., 2006; Chan et al., 2002; Chen et al., 2005).

In contrast to HR, NHEJ is intrinsically error prone. However, if chromosomal integrity is the endpoint, it can be considered as a conservative error-free process accommodating non-fully complementary ends at the cost of limited mutagenesis. Indeed, in the absence of major classic NHEJ actors, cells use a slower backup DNA-PK-independent NHEJ pathway that has been shown to be the primary mediator of IR-induced genome rearrangements (Iliakis et al., 2004; Martin et al., 2005). The genomic instability and the significant increase in chromosomal translocations observed in the in vivo Mpl−/− HSPC progeny or in cells irradiated in the absence of TPO suggest that TPO-Mpl signaling may favor DSB shunting in rapid classic DNA-PK-dependent NHEJ repair. In agreement with this possibility, neutral comet assays show that the DSB repair occurring in the absence of TPO is slow, with 20%–30% of breaks still unrepaired 8–16 hr after IR. Furthermore, this slow DNA repair is DNA-PK independent given that it could not be inhibited by NU7441. HSCs, which cannot use HR because of their quiescent status, are more vulnerable than progenitors to the acquisition of IR-induced genome rearrangements (Mohrin et al., 2010). However, our data suggest that environmental BM signals can act to limit this phenomenon by increasing the global efficiency of NHEJ-mediated repair through its fast DNA-PK-dependent component. Interestingly, expression of the main DNA-PK-dependent classic NHEJ proteins Ku80 and DNA-Ligase IV has been shown to be downregulated by Flt3-ITD and Bcr-Abl oncopgenes (Fan et al., 2010). Recent data have shown that leukemic stem cells can arise not only from HSCs but also from committed primitive progenitors (Passegué and Weisman, 2005). TPO was found to regulate DNA-PK-dependent DSB repair in both myeloid progenitors and HSC-enriched cells, and this pathway is downregulated in Mpl−/− and Mpl−/+ cells. This suggests that the loss or reduced expression of Mpl could lead to hematopoietic malignancies and a tumor-suppressor role for this receptor in response to IR. This possibility is in agreement with the increased propensity of CAMT patients to develop AML (Maserati et al., 2008). Interestingly, increased sensitivity to preconditioning treatments and the presence of chromosomal aberrations resembling that found in therapy-induced MDS and AML have been described for those patients (Maserati et al., 2008; Steele et al., 2005). We could not observe malignancies in Mpl−/− mice sacrificed 5 months after TBI. This might be due to the long latency for IR-induced leukemia development in mice and the resistance to C57B/6 strain to AML and MDS. Thus, additional studies are required for validation of this hypothesis.

Full Mpl expression is required for TPO-mediated DNA repair. This requirement for high TPO signaling may explain why an acute injection of TPO to TPO-proficient mice can increase DNA repair. However, the loss of DSB repair function of Mpl−/− cells is intriguing given that these mice, in contrast with Mpl−/− mice (Kimura et al., 1998; Qian et al., 2007), have normal...
numbers of progenitors (Alexander et al., 1996) and HSCs and also have reconstitution ability (B.d.L., L.P.-C., M.S., and F. Porteu, unpublished data). Thus, Mpl is haplosufficient for HSC maintenance and expansion posttransplantation but not for DNA damage response to IR, suggesting that these functions are separately controlled. In agreement with this possibility, the critical role of TPO in adult HSC maintenance has been shown to primarily reflect its ability to regulate HSC quiescence (Qian et al., 2007; Yoshihara et al., 2007), whereas the TPO-induced DNA repair reported here and HSC radiosensitivity (Mohrin et al., 2010) are independent of their quiescence.

Mice or humans exposed to moderate or high doses of IR exhibit residual defects in HSC function even after hematopoiesis has recovered months after exposure (Marusyk et al., 2009; Simonnet et al., 2008; Wang et al., 2006). In addition, IR increases the probability of the occurrence of oncogenic mutations. A recent report has demonstrated that HSPCs forced to proliferate before IR exposure through the application of an in vivo mobilization treatment to mice with granulocyte colony-stimulating factor (G-CSF) and cyclosphosphamide have significantly decreased mutation rates because of enhanced DNA-repair fidelity in conjunction with the preservation of their relative radiosensitivity in vitro (Mohrin et al., 2010). However, G-CSF injection has also been shown to promote HSPC proliferation and differentiation at the expense of HSC self-renewal (van Os et al., 2000), inducing a differentiation checkpoint in the context of DNA damage (Wang et al., 2012). Abrogation of this checkpoint prolongs HSC self-renewal but results in DNA-damage accumulation. Thus, the routine use of G-CSF to hasten recovery from radiotherapy or chemotherapy-induced neutropenia may decrease HSCs’ mutagenesis but impair their function. Our results show that short-term specific exposure to TPO in vitro or in vivo protects HSCs from IR-induced genomic instability and rescues, at least partially, their loss of activity. TPO also increases the repair of DSBs induced by etoposide and DXR. Romiplostim was found to recapitulate TPO effects on both short-term and long-term TBI-induced accumulation of DNA damage in HSCs. Thus, our results may have important clinical implications in opening new avenues for the use of TPO agonists prior to therapy with DNA-damaging agents to minimize the risk of secondary AML and long-term residual HSPC injury. Importantly, although we have not tried to optimize the dose of TPO used, a single injection just before exposure to IR proved sufficient to induce some long-term protection against DNA damage. This may avoid the described adverse complications induced by prolonged, but not by acute, treatments with TPO or its agonists (Ikeda and Miyakawa, 2009; Léon et al., 2012).

**EXPERIMENTAL PROCEDURES**

**Mice, Cell Culture, and Reconstitution Assays**

Mpl**−/−** backcrossed on C57BL/6 (CD45.2) background (kindly provided by Dr F. de Sauvage) and 129S2/Sv Kit**−/−**/Kit**+/−** mice were described previously (Petit-Cocault et al., 2007; Alix et al., 2008). C57BL/6J (CD45.2 and CD45.1) and CB17-Prkdc-scid mice were purchased from Charles River and Harlan laboratories, respectively. Unless otherwise specified, mice of 8–10 weeks of age were used. All procedures were approved by the Animal Care Committee (registered no. P2.MG.137.10, 2010) and the department director of veterinary services of Paris (agreement no. 75-1064). Lin−, LSK, and LSK-CD34− cells were isolated from BM and stained as described (Saulnier et al., 2012). The cells were cultured in StemSpan serum-free expansion medium (SFEM, STEMCELL Technologies) supplemented with recombinant Flt3 ligand (100 ng/ml), interleukin-3 (IL-3, 10 ng/ml), IL-6 (10 ng/ml), SCF (100 ng/ml), and TPO (50 ng/ml), all from PeproTech (Rocky Hill, NJ, USA), (complete medium) or in the same medium without TPO, CD34− and CD34+/CD38− human HSPCs were isolated from cord blood or cytapheresis, as described (Hamelin et al., 2006). U77-Mpl cells were cultured in α-MEM supplemented with 10% fetal calf serum (FCS) and 2 U/ml EPO (Boehringer Ingelheim). Cell IR in vitro was carried out in a BIOBEAM 8000 irradiator (Gamma-Service Medical GmbH, Leipzig, Germany). Mouse TBI was performed in an IBL 637 cesium irradiator (Curie Institute, Paris). For competitive reconstitution experiments, 3,000 LSK cells sorted from C57BL/6 CD45.1 mice subjected to TBI (2 Gy) or not as well as TPO injection (8 μg/kg body weight) were injected in lethally irradiated (10 Gy) C57BL/6 CD45.2 congenic mice, together with 3 × 10^5 BM CD45.2 competitor cells, and analyzed 4 months later.

**Immunofluorescence**

Immunofluorescence was performed as described (Pawlowskia et al., 2010), with cells cytospun on glass slides. Slides were visualized with a Leica DMI 6000 microscope (Wetzlar, Germany) equipped with a 63 × 1.6 oil-immersion objective and a Micromax charge-coupled device camera (Princeton Instruments, Trenton, NJ, USA). Pictures were analyzed using ImageJ software.

**Comet and Cytogenetic Assays**

The neutral comet assay and chromosomal-aberration analysis in metaphase spreads were performed as described (Pawlowskia et al., 2010). Three-color FISH was performed using whole-chromosome probes for mouse chromosomes 2 (fluorescein isothiocyanate [FITC]), 6 (Texas red), and 12 (FITC:Texas red) (MetaSystems, Altusheim, Germany), and counterstaining was performed with DAPI-Antifade solution (Oligogene, Illkirch, France), as reported (Pouzoulet et al., 2007). Fluorescence was analyzed with a Zeiss Axioplan epifluorescent microscope and Isis-multicolor FISH (mFISH) imaging system (MetaSystems).

**Cell-Cycle Analysis**

Cell cycle was analyzed by staining Lin− cells with Hoechst and Pyronin Y and with anti-CD34, Sca1, and c-Kit (Saulnier et al., 2012). Analysis was performed using an LSRII flow cytometer (BD Biosciences) and FlowJo software (TreeStar).

**NHEJ Assays**

U77-Mpl cells were electroporated with the NHEJ pCOH substrate (Guirouilh-Barbat et al., 2004), and clonal populations were selected in the presence of blasticidin (40 μg/ml). Expression of the meganuclease I-SceI was achieved by electroporation of pcDNA-HA-I-SceI, using Amaxa Kit V (Lonza, Amaxa, Basel). The cells were then cultured in medium containing 2 U/ml EPO or 3 nM TPO peptide (Hamelin et al., 2006). End-joining was analyzed 48 hr to 1 week later. For assessing I-SceI expression, the cells were permeabilized by treatment with 0.1% Triton X-100 (10 min, room temperature [RT]) and methanol (50%, 10 min, 4°C) and stained with anti-hemagglutinin (HA) followed by anti-mouse-FITC antibodies. For testing NHEJ activity on primary cells, WT and Mpl−/− Lin− Kit− cells were cultured in StemSpan SFEM containing 25 ng/ml SCF, 25 ng/ml IL-11, 10 ng/ml IL-3, 50 ng/ml TPO, and 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). After 24 hr, cells were irradiated and electroporated with 1.5 μg of HindIII-digested pEGFP-Perm1-A2d plasmid (Seluanov et al., 2004) and 0.5 μg of pDsRed-Express (Clontech Laboratories, Mountain View, CA, USA) using Amaxa Kit V. Cells were analyzed by flow cytometry 24 hr later.

**Statistical Analysis**

Results were evaluated using either one-way ANOVA and Tukey comparison test or unpaired t test by GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA). For FISH analysis, a specific chi-square test was adapted to Poisson statistics was used (Pouzoulet et al., 2007). Results are shown as means and SEM and the value of "p < 0.05" was determined as significant, and "**p < 0.01 or ***p < 0.001 as highly significant."
SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.10.012.

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