Low-dose irradiation prior to bone marrow transplantation results in ATM activation and increased lethality in Atm-deficient mice

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Ataxia telangiectasia is a genetic instability syndrome characterized by neurodegeneration, immunodeficiency, severe bronchial complications, hypersensitivity to radiotherapy and an elevated risk of malignancies. Repopulation with ATM-competent bone marrow-derived cells (BMDCs) significantly prolonged the lifespan and improved the phenotype of Atm-deficient mice. The aim of the present study was to promote BMDC engraftment after bone marrow transplantation using low-dose irradiation (IR) as a co-conditioning strategy. Atm-deficient mice were transplanted with green fluorescent protein-expressing, ATM-positive BMDCs using a clinically relevant non-myeloablative host-conditioning regimen together with TBI (0.2–2.0 Gy). IR significantly improved the engraftment of BMDCs into the bone marrow, blood, spleen and lung in a dose-dependent manner, but not into the cerebellum. However, with increasing doses, IR lethality increased even after low-dose IR. Analysis of the bronchoalveolar lavage fluid and lung histology revealed a significant enhancement in the number of inflammatory cells and oxidative damage. A delay in the resolution of γ-H2AX-expression points to an insufficient double-strand break repair capacity following IR with 0.5 Gy in Atm-deficient splenocytes. Our results demonstrate that even low-dose IR results in ATM activation. In the absence of ATM, low-dose IR leads to increased inflammation, oxidative stress and lethality in the Atm-deficient mouse model.

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INTRODUCTION

Ataxia telangiectasia (A-T) is a genetic instability syndrome that exhibits progressive neurodegeneration as well as immunodeficiency, connective tissue abnormalities, recurrent respiratory tract infections, oxidative stress, hypersensitivity to radiotherapy and elevated risk of malignancies.1–4 Leukemia and lymphoma, as well as lung failure, are the main causes for morbidity and mortality.5 At present, only symptomatic therapies are available, but no cure for A.T exists.6

A promising approach that might offer an avenue leading to a possible therapy for A-T could be bone marrow transplantation (BMT). In addition to the securely established effects on cancer and immunodeficiency, engraftment of bone-marrow-derived cells (BMDC) into lung tissue and cerebellum has been proposed.7 At the face of Purkinje cell loss and progressive lung destruction several studies have shown that BMDC are able to contribute to neogenesis of cerebellar Purkinje neurons or lung tissue regeneration and protection which is of special interest for a therapeutic approach for A-T.8,9 In Atm-deficient mice, BMT significantly inhibited tumorigenesis and improved the immune, weight gain and fitness.10–11 Our results further showed the migration of CD31+CD45+ endothelial cells and EpCAM+ epithelial cells into the lung tissue of transplanted Atm-deficient mice.12 However, BMT using a non-myeloablative host-conditioning regimen with cyclophosphamide (CP) and anti-thymocyte globulin did not result in full donor chimerism. In addition, the engraftment of donor-derived cells into the lung tissue was low and no cells migrated into the cerebellum.13 Experience in the transplantation of A-T patients so far is limited and no transplantation strategy exists.14

In other genomic instability syndromes, such as Fanconi anemia (FA), Nijmegen breakage syndrome (NBS) or DNA ligase IV deficiency, BMT has been successfully used.15–18 In FA, continuous improvements to the approach to BMT over the last 20 years have resulted in reduced regimen-related toxicity, superior engraftment and less GVHD, leading to improved survival.19–21 However, in contrast to BMT with HLA-identical siblings, using alternate donors has been markedly less successful, due to the high rates of graft failure, regimen-related toxicity, GVHD and opportunistic infection.19 For this purpose a more intensive conditioning with additional low-dose irradiation (IR) has been demonstrated to exert the immunosuppressive effect that enables durable engraftment.22 Conditioning is a critical factor in the clinical setting of BMT getting the balance right between toxicity and ablative effects. To date, there is a lack of data as to what conditioning regimen is

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immunohistochemistry, the lung sections were incubated in PBS with 10% normal goat serum and 0.5% Triton X-100 and were stained with anti-CD45 (purified rat anti-mouse, Ab, BD Pharmingen, San Diego, CA, USA) for 60 min at room temperature (RT) and Alexa647-conjugated secondary Ab (donkey anti-mouse IgG Ab, Invitrogen, Carlsbad, CA, USA). Migration and inflammation were ranked by 10 blinded observers from 1 to 6 (1 = lowest, 6 = highest) on subjective amounts of GFP⁺ cells and CD45⁺ ceils. The omission or primary antibodies served as a negative control and resulted in no detectable staining. Digital micrographs of GFP and Alexa647 fluorescence were obtained on a conventional fluorescence microscope (OXX41, Olympus, Hamburg, Germany) equipped with a camera (Fetigo 2000 R Fast 1394, Q-Imaging, Surrey, BC, Canada).

Bronchoalveolar lavage fluid (BALF)
Twelve weeks after transplantation (Atm⁻/⁻ n = 6, per group, WT n = 3), the animals were anesthetized with an IP injection of Ketamine and Rompun dissolved in a 0.9% sodium chloride solution (dose: 100 mg/10 kg body weight). They were fixed in a supine position and anesthetized locally with Xylocain in the ventral abdominal area. The trachea was dissected and a venous catheter (21G) was inserted. Then, 700 μl of cold PBS with 0.1% NaN₃ EDTA was administered slowly into the lungs. The procedure was repeated twice, with three aliquots per mouse kept on ice. The cells were cytacentrifuged on to slides at 400 g using a cytopsin and dried for at least 12 h and stained according to Pappenheim. Then, the slides were stained in filtered May-Grünwald solution for 8 min and rinsed three times with tap water. Afterwards, the slides were incubated with Giemsa solution for 35 min and again rinsed three times with tap water. After 1 h or dying at RT, the sides were briefly immersed in xylene and coverslipped directly with Eukitt (Sigma-Aldrich, St Louis, MO, USA). Percentage of leukocyte differential in bronchoalveolar lavage fluids (BALFs) was determined using a hemocytometer, and differential cell counts were determined after cytopsin with Wright-Giemsa staining.

Histone H2AX phosphorylation
Wild-type and Atm-deficient splenocytes were mock-irradiated or irradiated with 0.2 to 2.0 Gy (n = 3, per group). Cells were cultured in RPMI1640 at 37 °C and 5% CO₂ for 3 and 24 h after IR. A total of 1 x 10⁶ cells were fixed for 10 min at RT (BD Cytofix, BD Pharmingen), washed with PBS and permeabilized with −20°C cold BD Phosflow Perm III buffer (BD Pharmingen, San Diego, CA, USA) for 5 min at RT. The cells were washed with PBS and blocked with 3% fetal calf serum in PBS for 30 min at RT. The anti-γH2AX (5139) Ab (Alexa Flour 647 Mouse anti-γH2AX (pSer139), BD Pharmingen) was added and the samples were incubated for 60 min at RT in the dark. After a further washing step, the probes were analyzed using the FACSCount II flow cytometer.

MTT assay. Splenocytes (n = 3, per group) were treated with different doses of IR and cultured for 24 h. PBS with 0.5% dimethylsulfoxide (DMSO) (D5877, Sigma-Aldrich) was added and the cells were incubated further for 4 h at 37 °C. The blue-colored water-insoluble product that is converted from the yellow MTT by the cells was dissolved in 0.04 M HCl in isopropanol for 5 min and colorimetrically quantified (absorbance 570 nm, reference 630 nm).

8-hydroxy-2'-deoxyguanosine
Lung tissues (n = 6 per group) were harvested 12 weeks post transplantation and oxidative DNA damage was quantitatively determined using an 8-hydroxy-2'-deoxyguanosine (8-OH-dG) ELISA (OxSelect Oxidative Damage ELISA Kit, B-8OH-G Quantification, Cell Biolabs, San Diego, CA, USA) according to the manufacturer’s instructions.

Statistics
Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Values are presented as the means (±s.e.m) and were analyzed using paired or unpaired tests or the corresponding Mann-Whitney U-test or a Wilcoxon-Mann-Whitney test. For multiple comparisons, we used Kruskal-Wallis testing. Survival curves comparing the effect of IR on survival of Atm-deficient mice were calculated by the Kaplan-Meier method. The following symbols indicate significant differences: “P < 0.05,” “P < 0.01” and “P < 0.001.”
RESULTS

Engraftment into lymphoid tissues

Engraftment of GFP-positive cells was higher in Atm-deficient mice compared with wild-type mice (Figures 1a–d). IR significantly improved the engraftment of BMDCs in a dose-dependent manner in the bone marrow (P < 0.05), in the blood (P < 0.05) and in the spleen (P < 0.05) of Atm-deficient mice starting at 1.0 Gy. IR with 2.0 Gy consistently caused a higher percentage of migrated donor cells in Atm-deficient mice compared with wild-type mice in the bone marrow (P < 0.01), in the blood (P < 0.05) and in the spleen (P < 0.01). The comparison of the radiation dose with the percentage of migrated cells into the bone marrow of Atm-deficient mice showed a highly significant correlation (r = 0.92, P < 0.001) (Figure 1d).

Migration into the lung and neuronal tissue

Cryosections of the lung tissues showed increasing migration of GFP-positive BMDCs in a dose-dependent manner after IR starting at 0.5 Gy (P < 0.05) (Figures 2a and b). At 2.0 Gy, migration was significantly higher in the Atm-deficient mice compared with wild-type mice (P < 0.01) (Figure 2b). Quantification of GFP-positive cells by flow cytometry revealed a significant increase at 2 Gy compared with unirradiated Atm-deficient mice (P < 0.05) (Figure 2c). As observed for the cryosections of the lung tissues, 2 Gy IR led to a higher percentage of migrated BMDCs into the lung of Atm-deficient mice compared with wild-type mice (P < 0.05) (Figure 2c). In contrast, neither GFP-positive cells (data not shown) nor ATM-expression after IR could be detected in the cerebellum of Atm-deficient mice (Figure 2d).
Lethality
The lethality of Atm-deficient mice after co-conditioning with increasing IR doses was 0% for mice treated with 0 to 0.5 Gy, 12.5% for mice treated with 1.0 Gy and 37.5% (P < 0.05) for mice treated with 2.0 Gy and monitored until 6 weeks after transplantation (Figure 3a). Monitored until 12 weeks after transplantation, the lethality of Atm-deficient mice treated with 0.5 Gy was 37.5% (P < 0.05), whereas none of the animals died in the group without IR in the observation period (Figure 3b).

γH2AX-expression and viability
Low-dose IR (0.05 Gy) led to γH2AX-generation in Atm-deficient splenocytes as well as in wild-type splenocytes, as seen after treatment (Figures 4a and b). After 24 h, the resolution of γH2AX-expression was significantly delayed (P < 0.05) and cell viability (P < 0.001) was not affected following IR with 0.5 Gy in Atm-deficient cells in contrast to wild-type cells (Figure 4c).

Inflammation and oxidative damage
CD45-staining of lung tissue cross-sections showed increasing infiltration of inflammatory cells after IR of 2.0 Gy (Figure 5a). The quantification of the lung cross-sections determined a significantly higher number of CD45+ cells in the irradiated recipients, starting at 0.5 Gy (P < 0.05) (Figure 5b). At 2.0 Gy, the number of CD45+ cells was significantly higher in the Atm-deficient mice and in the wild-type mice (P < 0.01). The number of inflammatory cells in the BALF was higher in the lungs of mice co-conditioned with 0.5 Gy compared with unirradiated, Atm-deficient mice 6 weeks after transplantation (Figures 5c–e). IR with 0.5 Gy resulted in a shift in the cellular distribution in the lungs of Atm-deficient mice. The percentage of neutrophils (P < 0.001) and lymphocytes (P < 0.05) increased significantly 6 weeks after transplantation, and this increase was associated with a significant decrease in the number of macrophages (P < 0.001) in the BALF of these mice. No differences between
Figure 3. Lethality of Atm<sup>-/-</sup> mice after co-conditioning with increasing IR doses. (a) Survival of Atm<sup>-/-</sup> mice treated with IR kinetic (n = 8, per group) was monitored until 6 weeks post transplant. (b) Survival of transplanted Atm<sup>-/-</sup> mice (n = 14) treated with a non-myeloablative regimen compared with Atm<sup>-/-</sup> mice (n = 16) treated with an additional low-dose IR of 0.5 Gy monitored until 12 weeks post transplant. The mice were killed at termination of the experiments. Results are shown as Kaplan-Meier plots.

Figure 4. γH2AX-expression and viability. Analysis of ATM-dependent DNA double-strand break repair capacity of Atm<sup>-/-</sup> (n = 3, for each treatment group) and Atm<sup>+/+</sup> (n = 3, for each treatment group) splenocytes after treatment with 0.5 Gy of IR using the γ-H2AX assay. (a) Histograms of γH2AX-expression of untreated and IR-treated Atm<sup>-/-</sup> and Atm<sup>+/+</sup> splenocytes after 3 h and 24 h, respectively, as measured by flow cytometry. (b) γ-H2AX-positive Atm<sup>-/-</sup> and Atm<sup>+/+</sup> splenocytes 24 h after treatment with 0, 0.2, 0.5, 1.0 and 2.0 Gy doses of IR. (c) Cell viability of Atm<sup>-/-</sup> and Atm<sup>+/+</sup> splenocytes 72 h after treatment with 0, 0.2, 0.5, 1.0 and 2.0 Gy doses of IR, as measured by MTT assay. *P < 0.05, ***P < 0.001.

The genotypes could be detected without IR. In addition, no differences were found between unirradiated Atm-deficient treated with the non-myeloablative host-conditioning regimen mice and Atm-deficient mice without any treatment (data not shown).

The determination of oxidative DNA damage by analysis of 8-OH-dG revealed a significant higher amount of 8-OH-dG in the BALF of untreated Atm-deficient mice compared with wild-type animals (P < 0.05) (Figure 5). IR with 0.5 Gy led to a significant increase in oxidative DNA damage in the Atm-deficient mice (P < 0.05).
DISCUSSION

BMT has been successfully used for years in the treatment of genetic instability syndromes, such as FA or NBS, to correct hematological manifestations, whereas experience in A-T is scarce and no transplantation strategy for A-T patients exists. In the few published approaches, it becomes clear that the conditioning regime prior to the clinical application of bone marrow cells is the critical factor that determines patient outcome and that the
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balance between the toxicity and the ablative effect is essential.\textsuperscript{12-14}

Recently, we and others have demonstrated that BMT significantly improves the immunological phenotype, weight gain and fitness, and inhibits tumorigenesis in Atm-deficient mice.\textsuperscript{10,11} However, BMT using a non-myeloablative host-conditioning regimen failed to reach a full donor chimerism. In addition, engraftment of donor-derived cells into the lung tissue was low, and no migrated cells could be found in the cerebellum. In this regard, the aim of the present study was to evaluate whether a co-conditioning strategy with low-dose IR improves donor cell migration of BMT, especially in the bone marrow, lung tissue and cerebellum, in relation to its toxic side effects in Atm-deficient mice. Although the effect of IR on Atm-deficient mice or Atm-deficient cells is well-described in the literature, very few studies about low-dose IR in Atm-deficient animals have been performed so far.\textsuperscript{24,27} TBI has become a well-established approach for conditioning patients prior to BMT, and a large variety of techniques, dose rates and dose-fractionation schemes are employed.\textsuperscript{26} Although TBI is uniquely challenging for patients with genetic instability disorders, low-dose radiation conditioning regimens have been established, especially when patients are transplanted from unrelated donors.\textsuperscript{15,20} In FA, the use of a FLU-based conditioning regimen containing low-dose radiation (200--400 cGy) is one method of choice prior to BMT.\textsuperscript{20} Even in NBS, conditioning with FLU in combination with a low-dose thoracoabdominal IR followed by successful BMT has been reported.\textsuperscript{18} On the other hand, radiation therapy for patients with genomic instability syndromes is akin to replacing one evil with another. In this regard, Pollard and Gatti\textsuperscript{30} summarized 32 cases of radiotherapy-induced mortality in DNA-repair disorders, such as A-T, NBS, FA and DNA Ligase IV deficiency, of which 13 were diagnosed with A-T.\textsuperscript{20,32} Our present data underline the skepticism towards IR in A-T. Although radiation improved donor cell migration into bone marrow, blood, spleen and lung tissue in a dose-dependent manner, it increased lethality of Atm-deficient mice, even at low doses of 0.5 Gy. That was not necessary to be expected because of the findings of Hada et al,\textsuperscript{31} who showed that A-T cells, in contrast to NBS cells, are not radiosensitive for simple chromosomal exchanges at doses < 0.5 Gy. Moreover, Kikur et al\textsuperscript{42} found that chromosomal aberration analysis did not demonstrate low-dose radiosensitivity in ATM mutation carrier cells or A-T patient cells. In contrast to these reports, our yh2AX data showed activation of the DNA damage response pathway and involvement of ATM, even at low doses of radiation.\textsuperscript{33} Nakamura et al\textsuperscript{33} also demonstrated significant and dose-dependent numbers of yh2AX foci in A-T cells, suggesting that DNA damage was not completely repaired during low-dose radiation. These finds go in line with Short et al,\textsuperscript{20} who found that ATM-dependent signaling to downstream targets, such as TP53, CHK1 and CHK2, occurs even at low doses of 0.2 Gy IR.\textsuperscript{35} In this regard, Suzuki et al\textsuperscript{36} showed that the activated and phosphorylated ATM protein in the nucleus between doses of 10 mGy and 1 Gy is connected to multiple signal transduction pathways.

Although our data demonstrate that migration of BMDCs into the lung tissue was significantly enhanced by IR doses as low as 0.5 Gy, the data further show that low-dose IR was accompanied by oxidative stress and damage, as well as the infiltration of inflammatory neutrophils and lymphocytes.

In particular, the alveolar/capillary complex of the lung is highly radiosensitive.\textsuperscript{37} IR-induced reactive oxygen species (ROS) leading to lipid peroxidation, oxidation of DNA and proteins, as well as activation of pro-inflammatory factors are directly toxic to parenchymal cells and initiate a cascade of molecular events altering the cytokine milieu of the microenvironment, creating a self-sustaining cycle of inflammation and oxidative stress.\textsuperscript{37} Unfortunately, in our experimental setting we cannot provide data about wild-type mice irradiated with 0.5 Gy. However, as proposed by Kataska et al\textsuperscript{38} and others low-dose radiation rather activates the anti-oxidative defense systems in the body, thus counteracts oxidative stress and therefore contributes to preventing or reducing ROS-related injuries. In contrast, enhanced oxidative stress and reduced oxidative capacity is one hallmark in A-T patients and the deficiency in mitochondrial DNA damage also contributes to the phenotype in Atm-deficient mice.\textsuperscript{39,40} Thus, even low-dose IR led to an increase in ROS and inflammation, as shown in the bronchial tissue, and is responsible for increased lethality of the transplanted Atm-deficient mice. This is in line with our recent finding that ATM plays a key role in the inflammatory response after airway mucosal injury.\textsuperscript{41,42}

Another major issue of A-T is neurodegeneration. Due to microscopical atrophy of the cerebellar cortex resulting from a reduction in Purkinje cells, granular cells and basket cells, most patients are wheelchair bound before they reach the second decade of life.\textsuperscript{43} In this context, several studies have reported that BMDCs migrate into the brain, raising the possibility of using them as a new tool for the repair of damaged brain tissue.\textsuperscript{43,44} In one of the first studies, Priller et al\textsuperscript{45} demonstrated the neogenesis of cerebellar Purkinje neurons derived from BDMCs in vivo. They found fully developed Purkinje neurons after a long-term follow-up of 15 months post-BMT in chimeric C57BL/6 mice. Their data suggest that BM cells can differentiate into Purkinje cells but that neuronal transdifferentiation occurs late, after BMT. In their study, Alvarez-Dolado et al\textsuperscript{46} showed that BMDCs may fuse in vivo with Purkinje neurons in the brain, resulting in the formation of multinucleated cells. In contrast to these findings and the enhanced migration of BMDCs into bone marrow, blood, spleen and lung, we did not observe any ATM-competent or GFP+ cells in the cerebellum of the transplanted Atm-deficient mice.\textsuperscript{46} Several reasons could have accounted for the failure of donor cell migration in our experimental setting. First, the dose of IR that we used was too low. In their review of BMT strategies, Dias et al\textsuperscript{46} favor a minimal dose of 7.5 Gy or even higher for detecting integration of BMDCs into the brain. However, as shown by the present study and others, conventional doses of IR are neither practicable in the Atm-deficient mice nor advisable for the treatment of A-T patients.\textsuperscript{47} Second, the route of BMT is crucial, and the direct engraftment of BMDCs by intrathal injection is the method of choice. However, when intracerebellar and intrathal injection was performed in a young A-T patient, he developed a donor-derived brain tumor following neural stem cell transplantation.\textsuperscript{48} A third reason could be the low numbers of mesenchymal stem cells in the bone marrow. As described by Alvarez-Dolado et al.\textsuperscript{49} and others in addition to neuronal stem cells, mesenchymal stem cells are the primary cell population directly involved in nerve cell regeneration. Furthermore, mesenchymal stem cells rescue Purkinje cells and improve motor functions in a mouse model of cerebellar ataxia.\textsuperscript{49,50} Thus, it is tempting to speculate whether prior enrichment of mesenchymal stem cells might achieve an improvement in homing and engraftment in the targeting tissues, including migration into the cerebellum.

In summary, we have shown that low-dose IR used as the co-conditioning strategy significantly improved engraftment of BMDCs in a dose-dependent manner into the bone marrow, blood, spleen and lung, but not into the cerebellum, of Atm-deficient mice. However, our study provides evidence that even low-dose IR causes ATM activation and BMT after low-dose IR was accompanied by elevated inflammation, oxidative stress and increased mortality. Therefore, the use of low-dose IR is a double-edged sword and is not recommended for use in A-T patients.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.
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