

Pharmacological targeting of the thrombomodulin-activated protein C pathway mitigates radiation toxicity

Hartmut Geiger^{1,2,10}, Snehalata A Pawar^{3,10}, Edward J Kerschen^{4,10}, Kalpana J Nattamai^{1,10}, Irene Hernandez⁴, Hai Po H Liang⁴, Jose Á Fernández⁵, Jose A Cancelas¹, Marnie A Ryan¹, Olga Kustikova⁶, Axel Schambach⁶, Qiang Fu³, Junru Wang³, Louis M Fink⁷, Karl-Uwe Petersen⁸, Daohong Zhou³, John H Griffin⁵, Christopher Baum⁶, Hartmut Weiler^{4,10} & Martin Hauer-Jensen^{3,9,10}

Tissue damage induced by ionizing radiation in the hematopoietic and gastrointestinal systems is the major cause of lethality in radiological emergency scenarios and underlies some deleterious side effects in patients undergoing radiation therapy^{1,2}. The identification of target-specific interventions that confer radiomitigating activity is an unmet challenge. Here we identify the thrombomodulin (Thbd)-activated protein C (aPC) pathway as a new mechanism for the mitigation of total body irradiation (TBI)-induced mortality. Although the effects of the endogenous Thbd-aPC pathway were largely confined to the local microenvironment of Thbd-expressing cells, systemic administration of soluble Thbd or aPC could reproduce and augment the radioprotective effect of the endogenous Thbd-aPC pathway. Therapeutic administration of recombinant, soluble Thbd or aPC to lethally irradiated wild-type mice resulted in an accelerated recovery of hematopoietic progenitor activity in bone marrow and a mitigation of lethal TBI. Starting infusion of aPC as late as 24 h after exposure to radiation was sufficient to mitigate radiation-induced mortality in these mice. These findings suggest that pharmacologic augmentation of the activity of the Thbd-aPC pathway by recombinant Thbd or aPC might offer a rational approach to the mitigation of tissue injury and lethality caused by ionizing radiation.

TBI is associated with the dysfunction of radiosensitive organs²⁻⁵. To identify new genes and pathways protecting hematopoietic stem and progenitor cells (HSPCs) against radiation injury, we performed retroviral insertional mutagenesis screens in mice using a replication-deficient virus bearing a strong internal promoter expressing enhanced GFP (eGFP)⁶ (Supplementary Fig. 1a). At weeks 4, 7 and 10 after the transfer of donor bone marrow cells transduced with the

retrovirus, we exposed the recipient mice to a single dose of 3-Gy TBI, which resulted in three consecutive cycles of radiation-induced contraction and re-expansion of the hematopoietic system. In mice with a substantially augmented relative abundance of eGFP⁺ cells in the peripheral blood or bone marrow⁶ (Supplementary Fig. 1b-e), we used ligation-mediated PCR (LM-PCR)⁶ to identify viral integration sites in the genomic DNA of their bone marrow cells. Among the loci targeted by integration, we identified genes known to have a role in the radioprotection of either hematopoietic or neuronal cells^{2,7,8}, such as *Puma* (also known as *Bbc3*; Supplementary Fig. 2b-d) and *c-Jun* (also known as *Jun*; data not shown). In the bone marrow cells of one mouse, designated animal 9 (Fig. 1a and Supplementary Fig. 1b), LM-PCR revealed integration of the retrovirus 31.6 kb upstream of the thrombomodulin (*Thbd*) gene (Fig. 1b). This integration event was associated with increased abundance of endogenous Thbd transcript and protein in radioselected eGFP⁺ cells, whereas it had little effect on the expression of neighboring genes, such as the somatostatin receptor 4 gene (*Sstr4*) and the C-type lectin transmembrane receptor gene, *CD93* (Fig. 1c,d).

To ascertain whether augmented Thbd expression in HSPCs was sufficient to confer a competitive selection advantage to hematopoietic cells in response to TBI, we transduced HSPCs with lentiviral Thbd expression constructs and transplanted cells overexpressing Thbd into preconditioned C57BL/6-CD45.1 recipient mice (Fig. 1e and Supplementary Fig. 3). We administered one dose of 3-Gy TBI 4 weeks after this transplantation and analyzed the peripheral blood for eGFP⁺ cell chimerism 3 weeks after TBI. Cells overexpressing Thbd were 1.5-fold enriched in the peripheral blood as compared to vector-only-transduced cells (Fig. 1f,g), indicating that elevated expression of Thbd in hematopoietic cells was sufficient to confer a selective advantage after radiation injury. However, HSPCs overexpressing Thbd were not protected from the effects of ionizing radiation *in vitro*, as determined by the survival, apoptosis and

¹Division of Experimental Hematology and Cancer Biology, Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, Ohio, USA. ²Department of Dermatology and Allergic Diseases, University of Ulm, Ulm, Germany. ³Division of Radiation Health, College of Pharmacy, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA. ⁴Blood Research Institute, Blood Center of Wisconsin and the Medical College of Wisconsin, Milwaukee, Wisconsin, USA. ⁵Scripps Research Institute, La Jolla, California, USA. ⁶Department of Experimental Hematology, Hannover Medical School, Hannover, Germany. ⁷Desert Research Institute, Las Vegas, Nevada, USA. ⁸PAION Deutschland GmbH, Aachen, Germany. ⁹Surgical Service, Central Arkansas Veterans Healthcare System, Little Rock, Arkansas, USA. ¹⁰These authors contributed equally to this work. Correspondence should be addressed to H.G. (hartmut.geiger@cchmc.org).

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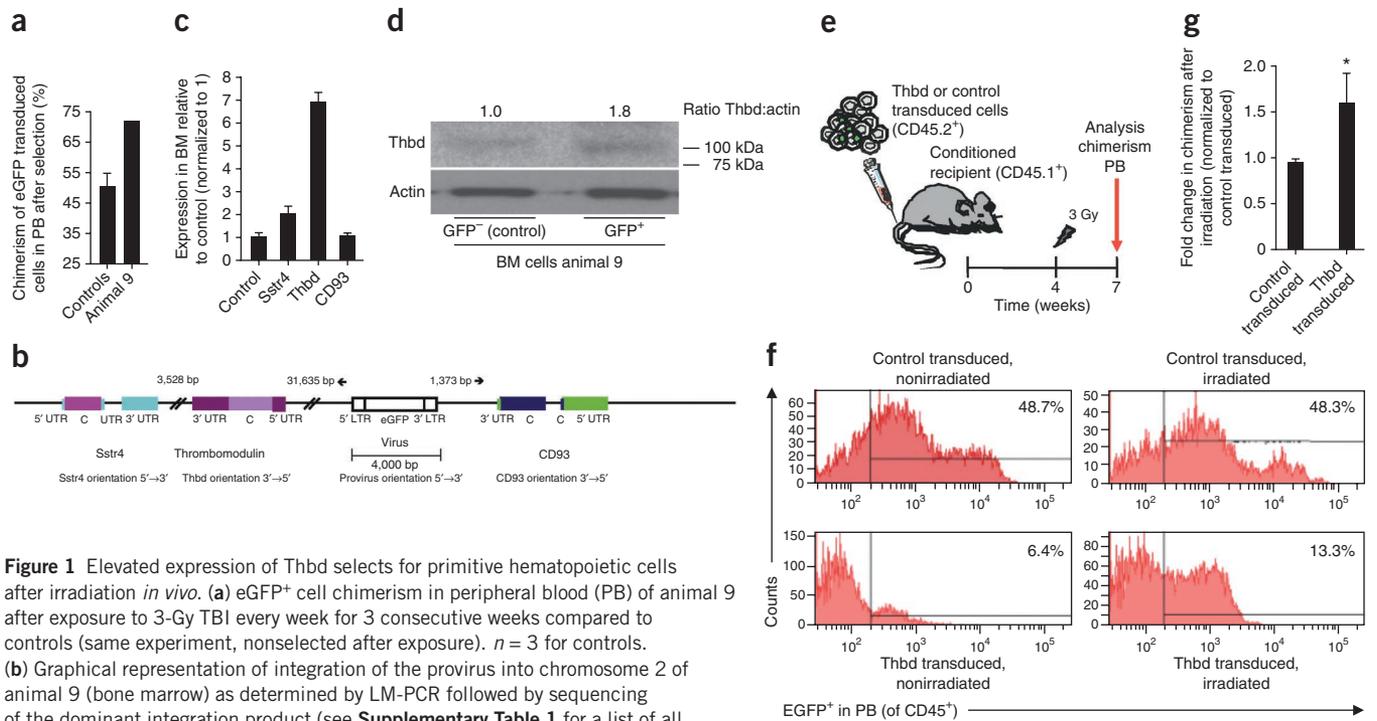


Figure 1 Elevated expression of Thbd selects for primitive hematopoietic cells after irradiation *in vivo*. **(a)** eGFP⁺ cell chimerism in peripheral blood (PB) of animal 9 after exposure to 3-Gy TBI every week for 3 consecutive weeks compared to controls (same experiment, nonselected after exposure). $n = 3$ for controls. **(b)** Graphical representation of integration of the provirus into chromosome 2 of animal 9 (bone marrow) as determined by LM-PCR followed by sequencing of the dominant integration product (see **Supplementary Table 1** for a list of all integration sites in animal 9). The numbers listed above the genes indicate the distances between the genes. C, coding region; UTR, untranslated region. **(c)** Transcriptional level of expression of Thbd and the two neighboring genes in bone marrow (BM) cells from animal 9, as determined by quantitative real-time RT-PCR. Expression was normalized to the level of expression of each of the gene measured (set to one) in a pool of control samples (bone marrow cells from C57BL/6 CD45.1 mice and mice 11 and 18, which were transplanted with transduced HSPCs but were not irradiated). **(d)** Expression of Thbd protein in the bone marrow of animal 9 compared to control mice (C57BL/6) as determined by western blot analysis. Actin served as a loading control. **(e)** Diagram of the experimental setup. Thbd was overexpressed in HSPCs by retroviral transduction, followed by transplantation and subsequent selection by irradiation. Recipient mice were preconditioned by irradiation with 11.75 Gy. Transplanted mice had a graft chimerism (CD45.2⁺ cells in peripheral blood) of higher than 90% 3 weeks after transplantation (data not shown). **(f)** Representative data showing the level of eGFP expression and selection among CD45.2⁺ cells in the peripheral blood of individual mice with and without irradiation in Thbd-transduced compared to control-transduced transplanted cells. **(g)** Quantification of the radioselection of Thbd-transduced hematopoietic cells in peripheral blood *in vivo* after irradiation relative to control (GFP)-transduced hematopoietic cells. $n = 3$ independent experiments with at least three recipients per single experiment. * $P \leq 0.05$ (t test).

proliferation of these cells in response to irradiation (**Supplementary Fig. 4**). Therefore, the beneficial effects of Thbd on HSPCs *in vivo* must require additional cells or molecules.

Endogenous Thbd is a multifunctional cell-surface-associated receptor that regulates the activities of several physiological protease systems, including complement, fibrinolysis and blood coagulation⁹. Biochemically, Thbd functions as a high-affinity receptor for thrombin. The Thbd-thrombin complex activates thrombin activatable fibrinolysis inhibitor (TAFI) and also converts the plasma zymogen protein C into the natural anticoagulant aPC^{10–12}. aPC inhibits blood coagulation through proteolysis of blood coagulation factors V and VIII, indirectly promotes the activity of the fibrinolytic system and exerts potent anti-inflammatory and cytoprotective effects on endothelial cells, neurons and various innate immune cell populations¹³. These latter effects are mediated by the interaction of aPC with signaling-competent receptors, such as the protease activated receptors Par1, Par2, and Par3, integrins and the endothelial protein C receptor (Epcr)^{13,14}.

In view of the beneficial effects of Thbd overexpression in HSPCs *in vivo*, we tested whether systemically-administered Thbd would have beneficial effects after radiation injury. Administration of recombinant soluble forms of THBD to baboons and humans is safe and has anticoagulant and antithrombotic activities^{15–17}. Using an oxidation-resistant form of soluble, recombinant human THBD (Solulin, International Nonproprietary Name (INN) sothrombomodulin

alpha; **Supplementary Fig. 5**), we found that administration up to 30 min after an 8.5-Gy or 9.5-Gy TBI resulted in significant ($P < 0.05$) radioprotection of wild-type mice compared to vehicle-treated controls, with a 40–80% survival benefit over vehicle-treated controls (**Fig. 2a,b**). Solulin has been shown to serve as a cofactor for the conversion of the plasma zymogen protein C into the natural anticoagulant aPC^{10,16,18}. To determine whether the protective effects of soluble THBD could be related to activation of protein C, we investigated whether infusion of recombinant aPC could reproduce the radioprotective effect of soluble THBD. In independent experiments conducted in three different laboratories, administration of recombinant mouse aPC to C57BL/6 mice (at 5 μ g per mouse intravenously (i.v.), equivalent to 0.4 mg per kg body weight) conferred a substantial survival benefit compared to vehicle-treated controls (**Fig. 2c,d**). We obtained similar data with genetically distinct CD2F1 mice (at 0.35 mg per kg body weight i.v. 30 min after TBI; data not shown), indicating that the effect of aPC was not dependent on a specific genetic background. In addition, after exposure to 10-Gy TBI, 40% of irradiated C57BL/6 mice survived after multiple injections of aPC (at 30 min, 1 h and 2 h after TBI) (**Fig. 2d**). Notably, even when the first injection of aPC was delayed until 24 h after TBI and a second injection of aPC was given at 48 h after TBI, we still observed significant radiomitigation (**Fig. 2e,f**).

The radiation doses used in our experiments resulted in death occurring 12–20 d after radiation exposure as a result of severe

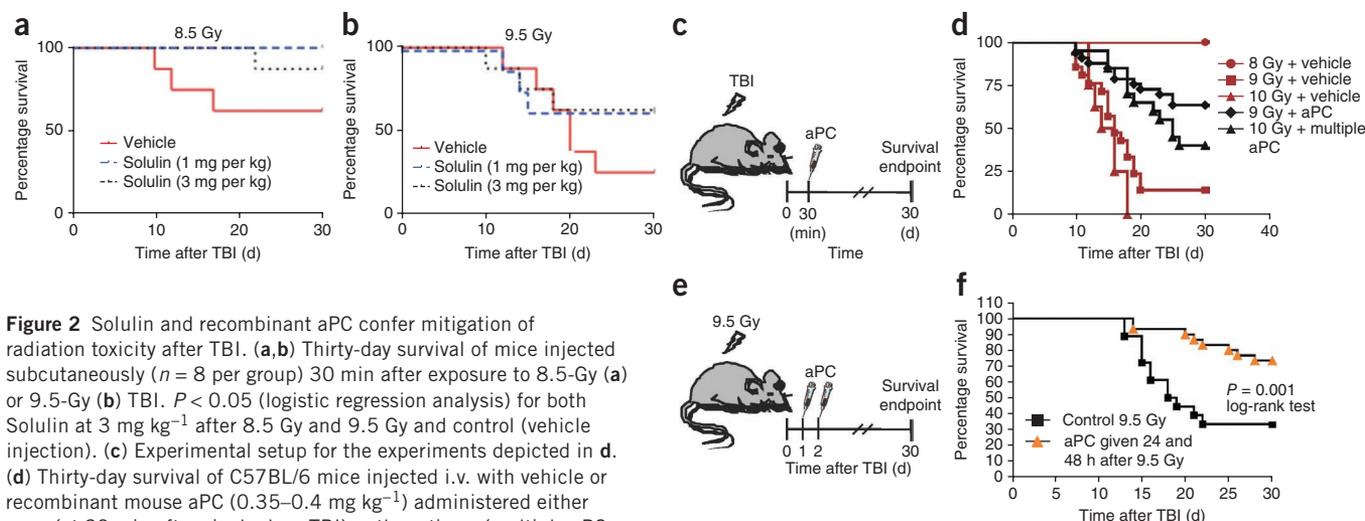


Figure 2 Solulin and recombinant aPC confer mitigation of radiation toxicity after TBI. **(a,b)** Thirty-day survival of mice injected subcutaneously ($n = 8$ per group) 30 min after exposure to 8.5-Gy **(a)** or 9.5-Gy **(b)** TBI. $P < 0.05$ (logistic regression analysis) for both Solulin at 3 mg kg⁻¹ after 8.5 Gy and 9.5 Gy and control (vehicle injection). **(c)** Experimental setup for the experiments depicted in **d**. **(d)** Thirty-day survival of C57BL/6 mice injected i.v. with vehicle or recombinant mouse aPC (0.35–0.4 mg kg⁻¹) administered either once (at 30 min after single dose TBI) or three times (multiple aPC, administered at 30 min, 1 h and 2 h after TBI) or vehicle (PBS). $n = 8$ for 8 Gy + vehicle; $n = 33$ for 9 Gy + aPC; $n = 23$ for 9 Gy + vehicle; $n = 8$ for 10 Gy + vehicle; $n = 20$ for 10 Gy + multiple aPC. $P < 0.0001$ for 9 Gy + vehicle compared to 9 Gy + aPC; $P < 0.0001$ for 10 Gy + vehicle compared to 10 Gy + multiple aPC (log-rank (Mantel-Cox) test). **(e)** Depiction of experimental setup to test whether aPC given no earlier than 24 h after TBI can mitigate TBI toxicity. **(f)** Thirty-day survival of C57BL/6 mice injected with vehicle or mouse aPC at a dose of 5 μ g per mouse through a single i.v. injection at 24 h and at 48 h after 9.5-Gy TBI (log-rank (Mantel-Cox) test); $n = 18$ vehicle-treated mice, $n = 30$ aPC-treated mice.

damage to bone marrow, suggesting that probable candidates for the relevant cellular targets of systemically administered Solulin and aPC in radiation mitigation include bone marrow hematopoietic cells and epithelial and/or endothelial structures of the bone marrow or gut. Administration of Solulin or aPC to lethally irradiated mice had no detectable effect on basic blood cell parameters at either day 3 or day 10 after radiation exposure (**Supplementary Fig. 6a** and data not shown), except for marginally elevated numbers of white blood cells in the bone marrow of mice treated with aPC at day 10 after radiation exposure (**Fig. 3a,b**). Hematopoietic progenitor cells (HPCs) in the bone marrow, determined either by flow cytometry (Lin⁻Sca1⁺c-Kit⁻ cells; **Fig. 3c**) or functionally by colony-forming unit cell (CFU-C) assays (**Fig. 3d**), were almost undetectable at 3 d after irradiation (data not shown); however, at 10 d after irradiation, the number of these cells was substantially higher in aPC-treated mice than in vehicle-treated controls. The frequency of mice with more than ten CFU-C colonies per 5×10^4 bone marrow cells at day 10 after TBI was also substantially higher in the aPC-treated group than in controls (data not shown), and this frequency of CFU-C colonies correlated with the frequency of aPC-treated mice surviving exposure to lethal radiation doses (**Fig. 2d,f**). We obtained similar findings in lethally irradiated mice receiving Solulin (**Fig. 3e,f**), consistent with the notion that activation of protein C constitutes a relevant downstream effector of soluble THBD. Infusion of aPC did not alter biomarkers of radiation-induced gut injury, such as plasma citrulline concentrations and the integrity of the gut epithelial surface (**Supplementary Fig. 6c–e**), indicating that mitigation of radiation damage to the intestine probably did not substantially contribute to the efficacy of aPC.

To gain more insight into the molecular mechanisms of aPC action, we compared recombinant variants of aPC with respect to their radiomitigating activity. The mouse 5A aPC variant, which has full EPCR- and Par1-mediated cytoprotective function but only residual (~8%) anticoagulant activity^{19,20}, did not prevent radiation-induced mortality (**Fig. 3g**). In contrast, infusion of the Glu149Ala aPC variant (E149A aPC), which has augmented anticoagulant activity but deficient signaling activities (for example, only ~5% of normal anti-apoptotic

activity)²¹, conferred a significant ($P < 0.001$) survival benefit that was comparable to that of wild-type aPC (**Fig. 3g**). The biological activity of aPC that mediates radiomitigation is thus preserved in the E149A aPC variant but is compromised in the 5A aPC variant.

Both Par1 and Epcr are expressed on primitive bone marrow cells^{22–25}. HSPCs lacking Par1 (ref. 26) or with greatly diminished expression of Epcr²⁷ were not impaired but, rather, were slightly favored compared to wild-type control HSPCs in competitive transplantation and radiation-injury experiments (**Supplementary Fig. 7a–c**). This result provides additional support for the notion that the radiomitigation activity of aPC does not involve Epcr- and Par1-dependent signaling on HSPCs, consistent with the failure of the signaling-selective 5A aPC variant to confer radioprotection.

Compared to the 5A aPC variant, the E149A aPC variant has increased anticoagulant effects and may also have increased coagulation-independent effects, such as in contributing to the degradation of cytotoxic histone-DNA complexes released from damaged cells²⁸. However, inhibition of the cytotoxic histones 3 and 4 with the function-blocking BWA3 antibody under conditions shown previously to reduce mortality in sepsis^{28,29} did not result in radioprotection (**Fig. 3h**). Similarly, inhibition of the intrinsic coagulation pathway with an antibody to factor XIa (14E11; ref. 30; **Fig. 3i**) or with low-molecular-weight heparin (data not shown) were both ineffective in conferring radioprotection. Biomarkers indicative of the activation state of the blood coagulation system in the peripheral blood (plasma thrombin-antithrombin complexes and the fibrin D dimer) were unaltered over a 24-h time window after exposure to lethal doses of radiation (**Supplementary Fig. 8**), suggesting that the radioprotective effect of the E149A aPC variant is not due to its antithrombotic actions. Although it is possible that optimized dosing with antibodies that block thrombosis or histone-induced inflammation might reveal some beneficial effect on radiation injury, it seems unlikely that these pathological mechanisms are the crucial targets of aPC in mediating the accelerated recovery of HPC activity and survival. The relevant targets of aPC therefore remain unknown and probably involve new functions associated with wild-type aPC and the E149A aPC variant^{20,31}.

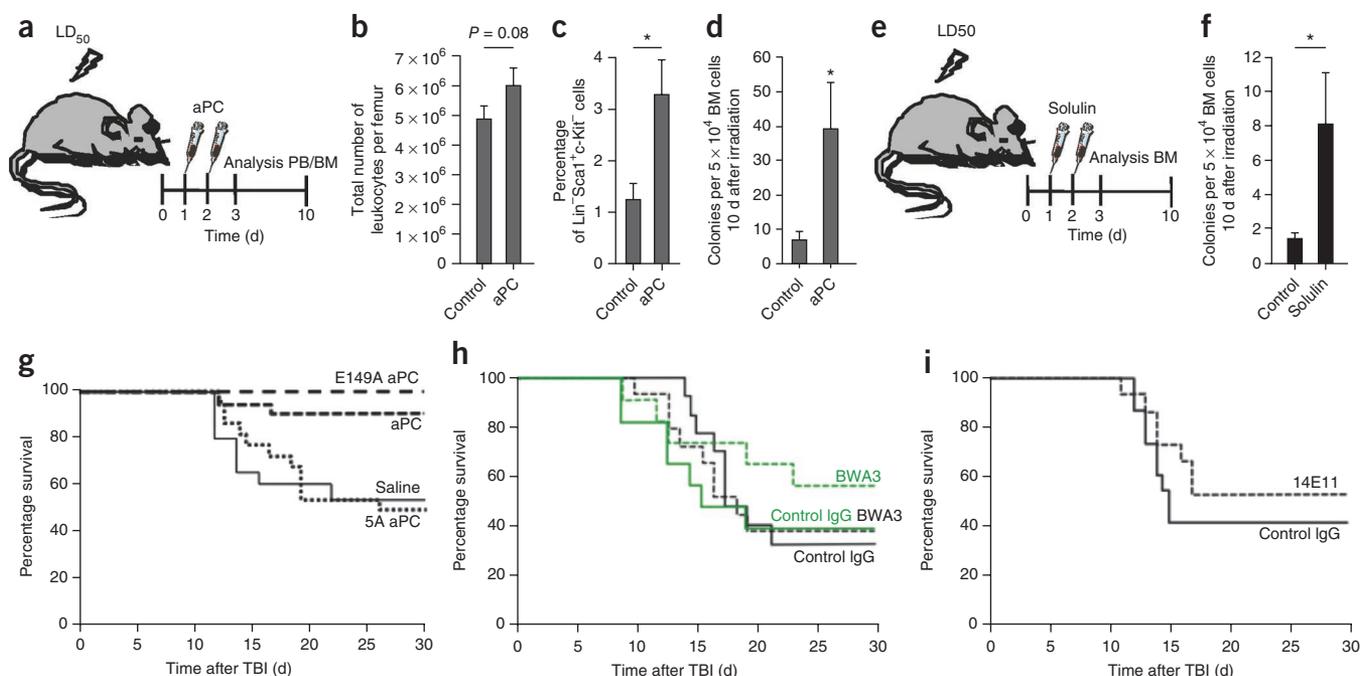
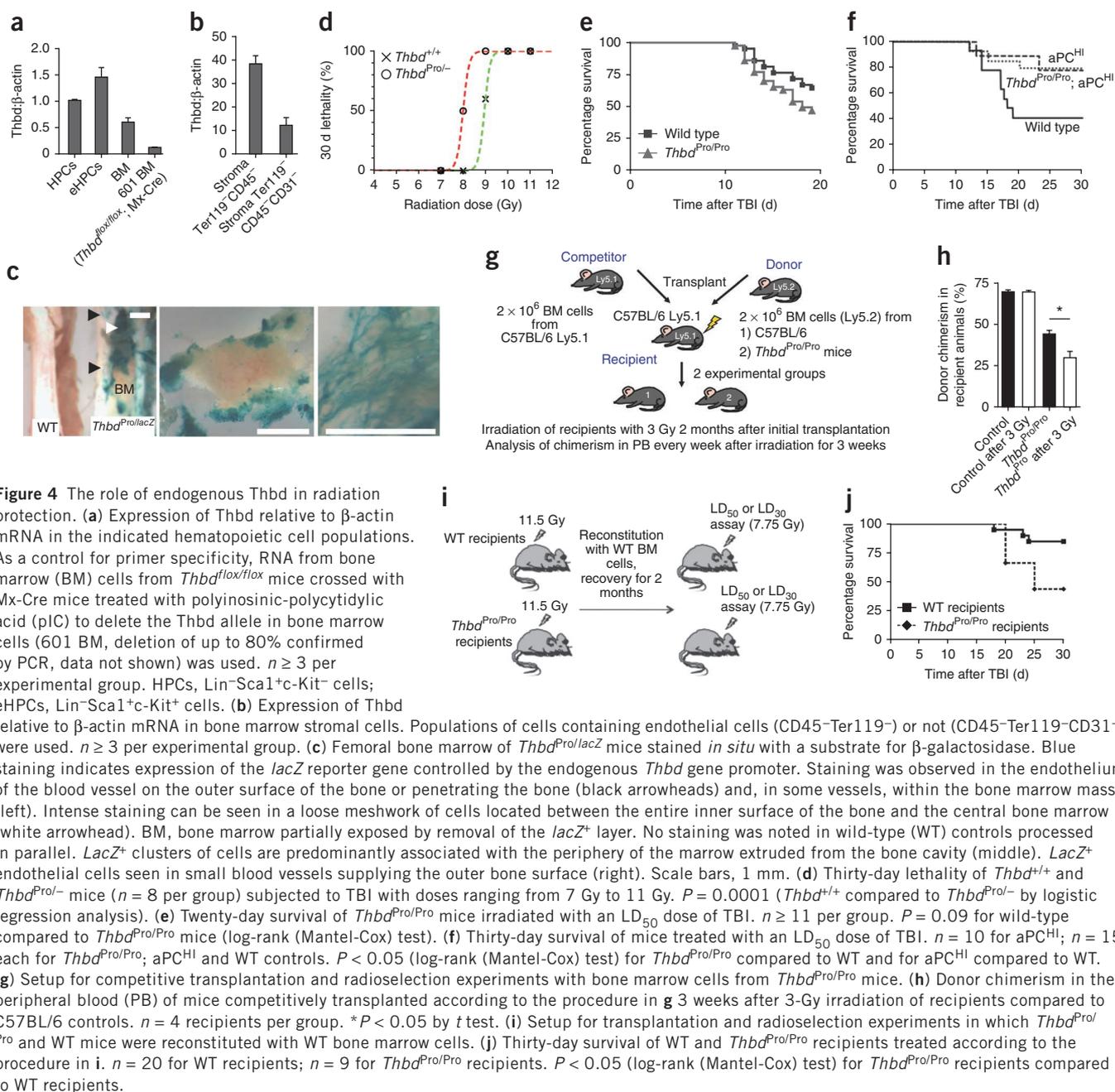


Figure 3 Mechanisms of the action of toxicity mitigation by soluble Thbd and aPC. **(a)** Experimental design for the data presented in **b–d**. **(b)** Total number of leukocytes per femur at 10 d after irradiation in irradiated and vehicle-treated control mice and in aPC-treated mice (*t* test). *n* = 18 for both vehicle-treated and aPC-treated mice. **(c)** Frequency of Lin⁻Kit⁺ cells in the bone marrow of the same mice 10 d after irradiation **P* ≤ 0.05 by *t* test. *n* = 18 for both vehicle-treated and aPC-treated mice. **(d)** Frequency of colony-forming cells (CFCs) in bone marrow (BM) cells 10 d after irradiation. **P* ≤ 0.05 by *t* test. *n* = 18 for vehicle-treated and aPC-treated mice. **(e)** Experimental design for the data presented in **f**. **(f)** Frequency of CFCs in bone marrow cells 10 d after irradiation and treatment with Solulin or vehicle control. **P* ≤ 0.05 by *t* test. **(g)** Thirty-day survival of mice (*n* = 15 per group) irradiated with an LD₅₀ dose receiving either 5 μg per mouse of the E149A aPC or 5A aPC variants or vehicle control through intravenous injection 30 min after TBI. *P* < 0.001 (Wilcoxon test) for E149A compared to saline, E149A compared to 5A, aPC compared to saline and aPC compared to 5A. **(h)** Thirty-day survival of mice given an LD₅₀ dose of TBI and treated with the histone-blocking antibody BWA3 (30 mg kg⁻¹ intraperitoneally) 0 h (black lines) or 16 h (green lines) after irradiation compared to injection of control isotype-matched, non-immune IgG. *n* ≥ 12 per experimental group. **(i)** Thirty-day survival of mice given an LD₅₀ dose of TBI and treated with an antibody to factor XIa (14E11) at 0 h after irradiation (30 mg kg⁻¹ intraperitoneally) compared to injection of control antibody. *n* ≥ 16 per experimental group.

Given the beneficial effects of THBD and aPC administration, the endogenous Thbd-aPC pathway might have a previously unrecognized role in mitigating the lethal consequences of radiation-induced bone marrow failure. In adult mice and humans, Thbd is expressed ubiquitously in endothelial cells of small blood vessels, except in certain brain microvascular beds, in which its expression is low³². Within the human hematopoietic system, THBD is expressed in a subpopulation of human dendritic cells, in monocytes and in a small subset of neutrophils^{33,34}. Western blot analysis of bone marrow from radiation-exposed mice indicated the presence of Thbd protein in their bone marrow cells (Fig. 1d), and we detected Thbd transcripts in differentiated bone marrow cells and bone marrow hematopoietic progenitor and early hematopoietic progenitor cells (Fig. 4a), as well as in bone-marrow-derived CD45⁻Ter119⁻CD31⁺ endothelial cells and in the CD45⁻Ter119⁻CD31⁻ stromal cell compartment of bone marrow (Fig. 4a,b). An *in situ* survey of β-galactosidase expression in the femurs of *Thbd^{lacZ}* knock-in mice indicated abundant Thbd expression within the endosteal region, as well as in femoral blood vessel endothelial cells supplying the bone marrow (Fig. 4c). Flow cytometry confirmed Thbd expression in Ly-6G⁻Gr1⁺CD115⁺ bone marrow macrophages, as well as in B220- and CD19-positive B cell precursors (Supplementary Fig. 9a and data not shown). Thbd-expressing macrophages are distinct from two previously described macrophage populations with the surface phenotype CD169⁺CD115^{intermediate}F4/80⁺Gr1⁻ or

CD11b⁺Ly-6G⁺F4/80⁺ that are involved in the maintenance of the hematopoietic niche (Supplementary Fig. 9b). Within the CD45⁻CD31⁺ endothelial cell population in bone marrow, we detected Thbd expression in Sca1⁻ sinusoidal endothelium but not in Sca1⁺ arterial endothelium (Supplementary Fig. 9c). This analysis of Thbd mRNA, Thbd antigen and *lacZ* reporter gene expression is consistent with the presence of Thbd on both hematopoietic and nonhematopoietic cells within the bone marrow.

We next investigated whether selective genetic disruption of protein C activation by endogenous Thbd^{35–37} would modify survival after TBI. Mice with the genotype *Thbd^{Pro/lacZ}* (ref. 36) carry only one functional Thbd allele, which encodes a Thbd variant (Thbd Pro) with severely reduced ability to activate protein C. These mice had increased sensitivity to TBI, such that the dose of radiation needed to elicit a 50% lethality shifted from ~8.75 Gy in wild-type mice to ~7.5 Gy in *Thbd^{Pro/lacZ}* mice (Fig. 4d). *Thbd^{Pro/Pro}* mice, which have a less severe Thbd deficiency than do *Thbd^{Pro/lacZ}* mice, showed elevated sensitivity to TBI compared to wild-type mice (Fig. 4e). In contrast, aPC^{HI} transgenic mice, which have constitutively high plasma aPC concentrations caused by the expression of a human protein C variant that is efficiently activated by thrombin even in the absence of THBD³⁸, were protected from radiation-induced bone marrow failure to a similar extent as wild-type mice treated with recombinant aPC (Fig. 4f). Expression of the aPC^{HI} transgene also rescued the



increased radiation sensitivity of *Thbd*^{Pro/Pro} mice (Fig. 4f), providing direct genetic evidence that the increased radiation sensitivity of *Thbd*-deficient mice is caused by inadequate activation of endogenous protein C.

We next performed competitive hematopoietic reconstitution experiments in which lethally irradiated wild-type recipients were transplanted with bone marrow from *Thbd*^{Pro/Pro} and wild-type mice, followed by exposure to 3-Gy TBI given 8 weeks after transplantation (Fig. 4g). *Thbd*^{Pro/Pro} cells showed less recovery than did wild-type cells (Fig. 4h). The initially lower contribution of *Thbd*^{Pro/Pro} bone marrow cells to chimerism before irradiation might be caused by additional functions of *Thbd* in HSC biology that have not yet been investigated. In noncompetitive reconstitution experiments, we transplanted irradiated wild-type or *Thbd*^{Pro/Pro} recipients

with wild-type bone marrow, followed by a second exposure to a lethal dose (LD₅₀) of TBI (Fig. 4i). Transplantation into *Thbd*^{Pro/Pro} recipients resulted in a significantly ($P < 0.05$) increased 30-day mortality rate compared to transplantation into wild-type recipients (Fig. 4j). Hence, endogenous *Thbd* expression on hematopoietic cells as well as on nonhematopoietic stroma cells confers protection against radiation, mirroring the protection conferred by forced *Thbd* overexpression in HSPCs or by therapeutic administration of soluble *Thbd* or aPC.

In summary, we have identified the *Thbd*-aPC pathway as a physiologically relevant mechanism that accelerates HSPC recovery in response to lethal TBI, resulting in substantial radiomitigation. Our data are consistent with a mechanism by which endogenous *Thbd* expressed on stromal endothelial or hematopoietic cells promotes protein C

activation and the release of aPC into the bone marrow microenvironment, leading to aPC-stimulated recovery from radiation-induced hematopoietic suppression. Based on the effect of Thbd deficiency on 30-d mortality (Fig. 4j), Thbd expression on stromal endothelial cells seems to make a more crucial contribution to overall survival than does Thbd expression in hematopoietic cells. Nevertheless, our findings also indicate that Thbd expression on HSPCs supports hematopoietic recovery after TBI in a cell-autonomous manner, with unknown effects on animal survival. Although the cellular and molecular mechanisms underlying the effect of soluble Thbd or aPC on the recovery of HSPC activity need to be studied in more detail, they are probably distinct from previously explored pathways involved in radioprotection, including those mediated by agonists of toll-like receptor 5 (ref. 39), inhibitors of cyclin-dependent kinase 4 and 6 (ref. 40) or various antioxidant compounds. Although the effects of endogenous aPC may remain largely confined to its site of formation, that is, the local microenvironment of Thbd-expressing cells, systemic administration of soluble Thbd or aPC can reproduce and augment the radioprotective effect of the endogenous protein C pathway.

Recombinant human aPC has undergone extensive clinical testing in patients, and recombinant soluble human THBD is currently being investigated for efficacy in antithrombotic therapy in humans. Our data encourage the further evaluation of these proteins for their radiomitigating activities. Moreover, these agents, possibly in combinations with other compounds targeting other pathways, may provide new medical countermeasures against radiation-induced pathologies.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.G. and H.W. designed and performed experiments, and wrote the paper. S.A.P. collected, analyzed and summarized data. E.J.K., I.H., H.P.H.L., J.A.C. and M.A.R. performed experiments. K.J.N. performed experiments and wrote parts of the paper. J.Á.F., A.S. and J.H.G. provided reagents and advised on experimental design. O.K., D.Z. and C.B. advised on experimental design and provided experimental expertise. Q.F. and J.W. performed *in vivo* studies. L.M.F. advised on experimental design and participated in writing the manuscript. K.-U.P. performed pharmacokinetics studies with Solulin and advised on experimental details. M.H.-J. designed experiments and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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- Mauch, P. *et al.* Hematopoietic stem cell compartment: acute and late effects of radiation therapy and chemotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* **31**, 1319–1339 (1995).
- Kirsch, D.G. *et al.* p53 controls radiation-induced gastrointestinal syndrome in mice independent of apoptosis. *Science* **327**, 593–596 (2010).
- Schuller, B.W. *et al.* Selective irradiation of the vascular endothelium has no effect on the survival of murine intestinal crypt stem cells. *Proc. Natl. Acad. Sci. USA* **103**, 3787–3792 (2006).
- Rotolo, J.A. *et al.* Regulation of ceramide synthase-mediated crypt epithelium apoptosis by DNA damage repair enzymes. *Cancer Res.* **70**, 957–967 (2010).
- Paris, F. *et al.* Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. *Science* **293**, 293–297 (2001).
- Kustikova, O.S. *et al.* Retroviral vector insertion sites associated with dominant hematopoietic clones mark “stemness” pathways. *Blood* **109**, 1897–1907 (2007).
- Yu, H. *et al.* Deletion of Puma protects hematopoietic stem cells and confers long-term survival in response to high-dose gamma-irradiation. *Blood* **115**, 3472–3480 (2010).
- Kanzawa, T. *et al.* Ionizing radiation induces apoptosis and inhibits neuronal differentiation in rat neural stem cells via the c-Jun NH₂-terminal kinase (JNK) pathway. *Oncogene* **25**, 3638–3648 (2006).
- Weiler, H. & Isermann, B.H. Thrombomodulin. *J. Thromb. Haemost.* **1**, 1515–1524 (2003).
- Wang, W., Nagashima, M., Schneider, M., Morser, J. & Nesheim, M. Elements of the primary structure of thrombomodulin required for efficient thrombin-activable fibrinolysis inhibitor activation. *J. Biol. Chem.* **275**, 22942–22947 (2000).
- Esmon, N.L., Owen, W.G. & Esmon, C.T. Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. *J. Biol. Chem.* **257**, 859–864 (1982).
- Esmon, C.T., Esmon, N.L. & Harris, K.W. Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalyzed fibrin formation and factor V activation. *J. Biol. Chem.* **257**, 7944–7947 (1982).
- Mosnier, L.O., Zlokovic, B.V. & Griffin, J.H. The cytoprotective protein C pathway. *Blood* **109**, 3161–3172 (2007).
- Weiler, H. Regulation of inflammation by the protein C system. *Crit. Care Med.* **38**, S18–S25 (2010).
- Tanaka, K.A. *et al.* Soluble thrombomodulin is antithrombotic in the presence of neutralising antibodies to protein C and reduces circulating activated protein C levels in primates. *Br. J. Haematol.* **132**, 197–203 (2006).
- van Iersel, T., Stroissnig, H., Giesen, P., Wemer, J. & Wilhelm-Ogunbiyi, K. Phase I study of Solulin, a novel recombinant soluble human thrombomodulin analogue. *Thromb. Haemost.* **105**, 302–312 (2011).
- Su, E.J. *et al.* The thrombomodulin analog Solulin promotes reperfusion and reduces infarct volume in a thrombotic stroke model. *J. Thromb. Haemost.* **9**, 1174–1182 (2011).
- Foley, J.H., Rea, C.J., Petersen, K.U., Nesheim, M.E. & Sorensen, B. Solulin increases clot stability in whole blood from humans and dogs with hemophilia. *Blood* **119**, 3622–3628 (2012).
- Mosnier, L.O., Yang, X.V. & Griffin, J.H. Activated protein C mutant with minimal anticoagulant activity, normal cytoprotective activity, and preservation of thrombin activable fibrinolysis inhibitor-dependent cytoprotective functions. *J. Biol. Chem.* **282**, 33022–33033 (2007).
- Kerschen, E. *et al.* Activated protein C targets CD8⁺ dendritic cells to reduce the mortality of endotoxemia in mice. *J. Clin. Invest.* **120**, 3167–3178 (2010).
- Mosnier, L.O. *et al.* Hyperantithrombotic, noncytoprotective Glu149Ala-activated protein C mutant. *Blood* **113**, 5970–5978 (2009).
- Ramallo-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R.C. & Melton, D.A. “Stemness”: transcriptional profiling of embryonic and adult stem cells. *Science* **298**, 597–600 (2002).
- Kent, D.G. *et al.* Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. *Blood* **113**, 6342–6350 (2009).
- Balazs, A.B., Fabian, A.J., Esmon, C.T. & Mulligan, R.C. Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow. *Blood* **107**, 2317–2321 (2006).
- Iwasaki, H., Arai, F., Kubota, Y., Dahl, M. & Suda, T. Endothelial protein C receptor-expressing hematopoietic stem cells reside in the perisinusoidal niche in fetal liver. *Blood* **116**, 544–553 (2010).
- Darrow, A.L. *et al.* Biological consequences of thrombin receptor deficiency in mice. *Thromb. Haemost.* **76**, 860–866 (1996).
- Iwaki, T., Cruz, D.T., Martin, J.A. & Castellino, F.J. A cardioprotective role for the endothelial protein C receptor in lipopolysaccharide-induced endotoxemia in the mouse. *Blood* **105**, 2364–2371 (2005).
- Xu, J. *et al.* Extracellular histones are major mediators of death in sepsis. *Nat. Med.* **15**, 1318–1321 (2009).
- Xu, J., Zhang, X., Monestier, M., Esmon, N.L. & Esmon, C.T. Extracellular histones are mediators of death through TLR2 and TLR4 in mouse fatal liver injury. *J. Immunol.* **187**, 2626–2631 (2011).
- Luo, D. *et al.* Factor XI-deficient mice display reduced inflammation, coagulopathy, and bacterial growth during listeriosis. *Infect. Immun.* **80**, 91–99 (2012).
- Kerschen, E.J. *et al.* Endotoxemia and sepsis mortality reduction by non-anticoagulant activated protein C. *J. Exp. Med.* **204**, 2439–2448 (2007).
- Ismail, J.A. *et al.* Immunohistologic labeling of murine endothelium. *Cardiovasc. Pathol.* **12**, 82–90 (2003).

33. Conway, E.M., Nowakowski, B. & Steiner-Mosonyi, M. Human neutrophils synthesize thrombomodulin that does not promote thrombin-dependent protein C activation. *Blood* **80**, 1254–1263 (1992).
34. Yerkovich, S.T. *et al.* Allergen-enhanced thrombomodulin (blood dendritic cell antigen 3, CD141) expression on dendritic cells is associated with a T_H2-skewed immune response. *J. Allergy Clin. Immunol.* **123**, 209–216 (2009).
35. Weiler-Guettler, H. *et al.* A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. *J. Clin. Invest.* **101**, 1983–1991 (1998).
36. Weiler, H. *et al.* Characterization of a mouse model for thrombomodulin deficiency. *Arterioscler. Thromb. Vasc. Biol.* **21**, 1531–1537 (2001).
37. Weiler-Guettler, H., Aird, W.C., Husain, M., Rayburn, H. & Rosenberg, R.D. Targeting of transgene expression to the vascular endothelium of mice by homologous recombination at the thrombomodulin locus. *Circ. Res.* **78**, 180–187 (1996).
38. Isermann, B. *et al.* Activated protein C protects against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis. *Nat. Med.* **13**, 1349–1358 (2007).
39. Burdelya, L.G. *et al.* An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. *Science* **320**, 226–230 (2008).
40. Johnson, S.M. *et al.* Mitigation of hematologic radiation toxicity in mice through pharmacological quiescence induced by CDK4/6 inhibition. *J. Clin. Invest.* **120**, 2528–2536 (2010).

ONLINE METHODS

Mice. Mice were housed under standardized conditions with controlled temperature and humidity and a 12-h, 12-h day, night light cycle. C57BL/6, C57BL/6CD45.1 and CD2F1 mice were obtained from commercial vendors (Charles River, The Jackson Laboratories and Harlan Sprague Dawley, respectively). *Thbd*^{Pro/Pro}, *Thbd*^{lacZ}, ECPR-low and *Par1*^{-/-} mice have been described previously^{26,27,35,36,41,42} and were on a C57BL/6 background (>14 backcrosses). Because of the early embryonic lethality of *Thbd*-null mice, we generated mice with minimal residual *Thbd* function, *Thbd*^{Pro/lacZ} mice. These mice carry a *Thbd*^{Pro} allele that encodes a *Thbd* variant with a greatly diminished ability to support the activation of protein C^{35,36} and a *Thbd*-null allele in which the *Thbd* gene is disrupted by the insertion of a *lacZ* reporter³⁷. The *Thbd*^{Pro} allele is a point mutation resulting in the substitution of proline for glutamic acid in the EGF3-4 interdomain loop of *Thbd* and a substantial suppression of *Thbd*-dependent aPC generation, although additional functions associated with *Thbd* (such as effects of the lectin domain on complement regulation and the leukocyte-endothelial interaction) are retained. *Thbd* antigen concentrations in the lung are twofold to threefold lower in *Thbd*^{Pro/Pro} mice than in wild-type mice³⁵. For all mouse experiments, male and female mice were used in an unspecified ratio. Mouse experiments were approved by the Institutional Animal Care and Use Committees at CCHMC, the University of Arkansas for Medical Sciences Central Arkansas Veterans Healthcare System or the Medical College of Wisconsin.

Reagents. Recombinant human *Thbd* and protein C for *in vitro* protein C activation assays were purchased from American Diagnostica, and thrombin was purchased from Sigma. Production and characterization of recombinant mouse wild-type aPC, the signaling-selective aPC variant 5A aPC and the hyperantithrombotic E149A aPC variant have been described previously^{19,21}. Solulin (soluble human recombinant thrombomodulin, ZK 158 266, INN: sothrombomodulin alpha; PAION GmbH, Germany) is a modified recombinant human *Thbd* molecule composed of the extracellular portion of *Thbd* (the N-terminallelectin-binding domain, the six EGF-like repeats and the serine/threonine-rich domain) but lacks the transmembrane and intracellular domains, as well as the chondroitin sulfate moiety. It derives from the molecule originally described by Glaser *et al.* and referred to as TMLEO⁴³ and is distinguished from TMLEO by the following changes: deletion of the first three amino acids of the N terminus, Met388Leu, Arg456Gly, His457Gln, Ser474Ala and deletion of the last seven amino acids of the C terminus⁴⁴. Hybridoma cells secreting the rat monoclonal antibodies to mouse *Thbd* (273-34A and 411-201B)⁴⁵ were generously provided by S.J. Kennel (University of Tennessee Graduate School of Medicine, Knoxville, TN, USA). *Thbd* detection by flow cytometry was accomplished with anti-mouse *Thbd* antibodies 273-34A, 411-201B and allophycocyanin-labeled anti-rat IgG secondary antibody; or with phycoerythrin-conjugated, monoclonal rat anti-mouse *Thbd* antibodies (clone 461714, R&D Systems). Detection of *Thbd* in western blots used affinity-purified polyclonal goat anti-mouse *Thbd* antibody (clone M-17, Santa Cruz Biotechnology). Inhibitory mouse anti-histone H4 antibody BWA3 was kindly provided by M. Monestier (Temple University School of Medicine, Philadelphia, PA). Purified mouse anti-FXI antibody 14E11, was provided by D. Gailani and A. Gruber (Vanderbilt University, Nashville, TN). All commercial antibodies were used at the manufacturer-recommended dilution (usually between 1:100 and 1:200), protein G-purified monoclonal rat anti-mouse *Thbd* antibodies 34A and 201B were used at 100 ng/ml for flow cytometry.

TBI of mice. Irradiation was performed at three different locations using either a Shepherd Mark I, model 25, cesium-137 irradiator (J.L. Shepherd & Associates), as described⁴⁴, with an average dose rate was 1.37 Gy per minute (at the Central Arkansas Veterans Healthcare System); using a Shepherd Mark I-68 cesium-137 irradiator with an average dose rate of 0.52 Gy per minute (at CCHMC); or using a Gammacell 40 Extractor cesium-137 irradiator (Best Theratronics) with an average dose rate 0.97 Gy min⁻¹ (at the Blood Research Institute, Milwaukee, WI). All irradiators are calibrated at least once a year. For TBI mitigation experiments, mice were monitored for up to 30 d after TBI. The number of dead or moribund

mice was recorded twice daily. Kaplan-Meier survival curves, median survival times and lethality at 30 d after TBI were recorded.

Retroviral and lentiviral transduction of hematopoietic cells. HSPCs derived from bone marrow were transduced with SFbeta⁶ virus containing an internal ribosome entry site (IRES)-eGFP control construct or a *Thbd*-IRES-eGFP construct at a multiplicity of infection of about 2–4. This results, on average, in one or two integration sites per single genome. These cells were subsequently transplanted into recipient mice that had been preconditioned with 11.75 Gy (the first irradiation was 7 Gy and, 4 h later, the second was 4.75 Gy) as previously described^{6,46,47}. LM-PCR and sequencing of the integration sites was performed as previously described⁶.

Blood parameters, flow cytometry, isolation of stromal cells and quantitative real-time RT-PCR. Blood parameters were determined by a Hemavet Instrument (Drew Scientific Inc). Immunostaining and flow cytometry analyses were performed according to standard procedures and analyzed on a FACSCanto flow cytometer (BD Biosciences). Monoclonal antibodies to Ly5.2 (104, BD Biosciences, FITC conjugated) and Ly5.1 (A20, BD Biosciences, phycoerythrin (PE) conjugated) were used to distinguish donor from recipient and competitor cells. For lineage analysis in hematopoietic tissues, antibodies to CD3-ε (145-2C11, PE-Cy7 conjugated), B220 (RA3-6B2, APC conjugated), CD11b (M1/70, APC-Cy7 conjugated) and Gr1 (RB6-8C5, APC-Cy7 conjugated) as well as CD19 (clone 1D3) and CD31 (Mec13.3), all from BD Biosciences, were used. All antibodies were used at a 1:100 dilution. RNA expression was determined by real-time RT-PCR using TaqMan Universal PCR and reverse transcriptase reagents (Life Technologies Corporation). RNA was purified with the QIAGEN RNeasy Micro Kit (QIAGEN Inc). The TaqMan probes listed in **Supplementary Table 2** were used to determine the expression of selected genes in the neighborhood of the viral integration site near the *Thbd* gene.

Effects of TBI on *Thbd*^{+/+}, *Thbd*^{Pro/lacZ}, ECPR-low and *Par1*^{-/-} mice or bone marrow cells. Mice were subjected to TBI at 7 Gy or 11 Gy. For experiments involving competitive transplantation followed by TBI for radiosélection, bone marrow cells harvested from the tibias and femurs of 6- to 8-week-old *Thbd*^{Pro/Pro} (donor) or B6.SJL(BoyJ) (competitor) mice (2 × 10⁶ cells from each group) were transplanted into BoyJ recipient mice that had been lethally irradiated with a total dosage of 11.75 Gy (7 Gy and 4.75 Gy, 4 h apart). Cells were injected into the retro-orbital sinus or injected into the tail vein in a volume of 200 μl in Iscove's Modified Dulbecco's Medium (IMDM) and 2% FCS. Three to four weeks after transplantation, chimerism of the peripheral blood was analyzed by flow cytometry using an antibody panel detecting CD45.2 and B220 for B cells, CD3-ε for T cells and Mac1 and Gr1 combined for the myeloid lineage. Mice were subsequently irradiated with 3 Gy TBI for radiosélection and peripheral blood was analyzed for chimerism at the time points after TBI indicated.

Effects of recombinant aPC, aPC variants and Solulin on survival in response to TBI. Mice subjected to TBI at the doses indicated were subsequently randomly assigned to receive wild-type aPC, signaling-selective aPC (5A aPC), signaling-defective aPC (E149A aPC) or vehicle. aPC or its variants were dissolved in vehicle buffer (50 mM Tris and 100 mM NaCl, pH 7.4). Mice received a single dose or, where indicated, multiple doses of either recombinant aPC, an aPC variant at 0.35–0.4 mg per kg of body weight or vehicle buffer in a volume of 200 μl or less by tail vein (i.v.) injection at the time points indicated after TBI. Male mice were injected subcutaneously with Solulin (1 mg/kg or 3 mg/kg) or vehicle 30 min after TBI. The mice were returned to their cages with free access to food and water and monitored twice daily for 30 d for weight loss, apparent behavioral deficits and survival.

Apoptosis and cell survival assays. Transduced cells sorted for eGFP were subjected to 1.5 Gy *in vitro* and cultured overnight in IMDM supplemented with 10% FBS, 2% penicillin-streptomycin, 100 ng/ml mouse stem cell factor (mSCF), 100 ng/ml thrombopoietin (TPO) and 100 ng/ml granulocyte colony stimulating factor (G-CSF). 18 h after irradiation, cells were harvested and

washed twice with ice cold PBS and 1×10^5 cells were resuspended in PBS. Cells were stained with APC-conjugated antibody to c-Kit (CD117, 2B8, BD Pharmingen) for 20 min on ice. Then cells were stained with PE-conjugated annexin V (BD Pharmingen) and 7-amino-actinomycin D (BD Pharmingen) and analyzed using a BD FACSCanto flow cytometer. All antibodies were used at a 1:100 dilution.

Cell proliferation assay. Triplicates of 2×10^5 to 5×10^5 transduced cells were resuspended in 2 ml IMDM supplemented with 10% FBS, 2% penicillin-streptomycin, 100 ng/ml mSCF, 100 ng/ml TPO and 100 ng/ml G-CSF. Cell counts were determined on days 3, 5 and 7.

CFC assays. CFC assays were performed using MethoCult (M3234, Stem Cell Technologies Inc) containing a final concentration of 50 ng/ml mSCF, 10 ng/ml mouse interleukin-3 (mIL-3) and 10 ng/ml mIL-6 (PeproTech). The cells were plated in triplicate in six-well plates and irradiated with 1.5 Gy and 3 Gy using a cesium137 source. Plates were incubated at 37 °C in 5% CO₂ and colonies were counted between 7 and 10 d after plating.

In vitro irradiation. *In vitro* irradiation experiments were performed in a cell-free system (a system not confounded by transcriptional regulation or ectodomain shedding of Thbd) as described previously⁴⁸. Briefly, all Thbd and Solulin samples were dissolved in buffer (described above) and irradiated in 1 ml polypropylene microcentrifuge tubes in a total volume of 500 µl. At least three independent samples each for Thbd and Solulin were irradiated at each dose for all experiments, not including optimization and validation studies.

Thbd activity assay. Recombinant Thbd and Solulin were dissolved to a final concentration of 50 nM in a buffer containing 10 mM Tris-HCl, 0.2 M NaCl, 5 mM CaCl₂ and 0.1% polyethylene glycol, pH 8.0. The effect of single-dose irradiation on Thbd functional activity was assessed using a protein C activation assay after exposure to 0 Gy, 1.77 Gy (0.3 min radiation exposure), 10 Gy (1.7 min), 20 Gy (3.4 min) or 40 Gy (6.8 min). The protein C activation assay was performed as follows: irradiated and sham-irradiated samples were diluted to a final concentration of 2.5 nM Thbd and incubated with 200 nM protein C and 1.4 nM thrombin for 60 min at 37 °C in a 96-well plate to generate aPC. The amount of aPC generated was measured by monitoring hydrolysis of the chromogenic substrate S-2366 (Diapharma) at 405 nm in a microplate reader (Bio-Tek Instruments) at 5-min intervals for 60 min. The results were expressed as the mean optical density at 60 min. All assays were performed in triplicate, and the average was considered to be a single value for statistical purposes.

Solulin pharmacokinetics. As the intravenous pharmacokinetics of Solulin in mice and rats are highly comparable (PAION Deutschland GmbH; K.-U.P., unpublished data), the dose selection for subcutaneous administration of Solulin in mice was based on available rat data (see below). Solulin, injected subcutaneously at 3 mg per kg body weight in male rats, reached plasma concentrations of about 40 nM at 6 h after injection and of about 100 nM at 26 h after injection (maximal observed value)⁴⁹. As these concentrations are associated with pronounced aPC-mediated effects of intravenous Solulin in rat models of thrombotic vessel occlusion⁴⁹, doses of 1 mg/kg and 3 mg/kg were selected for the mouse radiation studies.

Determination of plasma citrulline concentrations. Citrulline plasma concentrations were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described⁵⁰.

Intestinal mucosal surface area measurements. Mucosal surface area was measured in vertical sections of the jejunum stained with H&E using a

projection and cycloid method, as described⁵¹. The method has previously been validated by us specifically for surface area determination of the intestinal mucosa after irradiation⁵².

HSPC staining after irradiation. Two million total bone marrow cells were blocked with 2% mouse serum (M5905, 5 ml, Sigma) for 10 min, followed by staining for 20 min with biotin-conjugated antibodies to the following lineage markers: CD5 (eBiosciences, 53-7.3), CD45R (eBiosciences, B220 RA3-6B2), Mac1 (eBiosciences, CD11b M1/70), Gr1 (eBiosciences, RB6-8C5), Ly-6G and Ly-6c (eBiosciences, RB6-8C5), CD8a (eBiosciences, 53-6.7) and Ter119 (BD Pharmingen, TER-119). The cells were then washed once and stained for 30 min with antibodies to c-Kit (BD Pharmingen, CD117 2B8, FITC conjugated), Sca1 (eBiosciences, Ly-6A/E, PE-Cy7 conjugated) and streptavidin (BD Pharmingen, 554063, APC-Cy7 conjugated). Analysis was on a BD FACSCanto flow cytometer. All antibodies were used at a 1:100 dilution.

Statistical analyses. The sample size for the TBI experiments was estimated according to a recent publication on radiation countermeasures studies⁵³. Statistical analyses were performed using NCSS 2004 for Windows (NCSS). Data are presented as the mean \pm the s.e.m. Two-sided tests were used throughout, and differences were considered statistically significant at $P < 0.05$. Pairwise (univariate) comparisons were performed with the Student's *t* test or the Mann-Whitney *U* test, as appropriate. Mouse survival curves were constructed using the Kaplan-Meier method, and 30-d survival rates were compared with the logistic regression analyses in GraphPad Prism (Graphpad Software). The LD₅₀ values for the comparisons of mouse survival after radiation exposure were calculated with logit-transformed data, and standard error estimates were obtained by the Δ method.

- Weiler, H. Mouse models of thrombosis: thrombomodulin. *Thromb. Haemost.* **92**, 467–477 (2004).
- Connolly, A.J., Ishihara, H., Kahn, M.L., Farese, R.V. Jr. & Coughlin, S.R. Role of the thrombin receptor in development and evidence for a second receptor. *Nature* **381**, 516–519 (1996).
- Glaser, C.B. *et al.* Oxidation of a specific methionine in thrombomodulin by activated neutrophil products blocks cofactor activity. A potential rapid mechanism for modulation of coagulation. *J. Clin. Invest.* **90**, 2565–2573 (1992).
- Weisel, J.W., Nagaswami, C., Young, T.A. & Light, D.R. The shape of thrombomodulin and interactions with thrombin as determined by electron microscopy. *J. Biol. Chem.* **271**, 31485–31490 (1996).
- Kennel, S.J., Lankford, T., Hughes, B. & Hotchkiss, J.A. Quantitation of a murine lung endothelial cell protein, P112, with a double monoclonal antibody assay. *Lab. Invest.* **59**, 692–701 (1988).
- Modlich, U. *et al.* Cell-culture assays reveal the importance of retroviral vector design for insertional genotoxicity. *Blood* **108**, 2545–2553 (2006).
- Kustikova, O.S. *et al.* Cell-intrinsic and vector-related properties cooperate to determine the incidence and consequences of insertional mutagenesis. *Mol. Ther.* **17**, 1537–1547 (2009).
- Ross, C.C. *et al.* Inactivation of thrombomodulin by ionizing radiation in a cell-free system: possible implications for radiation responses in vascular endothelium. *Radiat. Res.* **169**, 408–416 (2008).
- Solis, M.M. *et al.* Recombinant soluble human thrombomodulin: a randomized, blinded assessment of prevention of venous thrombosis and effects on hemostatic parameters in a rat model. *Thromb. Res.* **73**, 385–394 (1994).
- Gupta, P.K. *et al.* Development of high-throughput HILIC-MS/MS methodology for plasma citrulline determination in multiple species. *Anal. Meth.* **3**, 1759–1768 (2011).
- Baddeley, A.J., Gundersen, H.J. & Cruz-Orive, L.M. Estimation of surface area from vertical sections. *J. Microsc.* **142**, 259–276 (1986).
- Langberg, C.W. & Hauer-Jensen, M. Optimal interfraction interval to minimize small bowel radiation injury in treatment regimens with two fractions per day: an experimental study in a rat model. *Radiother. Oncol.* **41**, 249–255 (1996).
- Kodell, R.L., Lensing, S.Y., Landes, R.D., Kumar, K.S. & Hauer-Jensen, M. Determination of sample sizes for demonstrating efficacy of radiation countermeasures. *Biometrics* **66**, 239–248 (2010).