

## RESEARCH ARTICLE

# Revisiting emergency anti-apoptotic cytokinotherapy: erythropoietin synergizes with stem cell factor, FLT-3 ligand, thrombopoietin and interleukin-3 to rescue lethally-irradiated mice

Michel Drouet, Nancy Grenier, Francis Hérodin

Institut de recherche biomédicale des armées, CRSSA  
Department of Radiobiology, Cell Therapy Unit

**Correspondence:** M. Drouet, Institut de Recherche Biomédicale des Armées, CRSSA. 24, avenue des Maquis du Grésivaudan, 38702, La Tronche Cedex, France  
<micheldrouet@crssa.net>

Accepted for publication April 27, 2012

To cite this article: Drouet M, Grenier N, Hérodin F. Revisiting emergency anti-apoptotic cytokinotherapy: erythropoietin synergizes with stem cell factor, FLT-3 ligand, thrombopoietin and interleukin-3 to rescue lethally-irradiated mice. *Eur. Cytokine Netw.* 2012; 23(2): 56-63 doi:10.1684/ecn.2012.0306

**ABSTRACT.** We have re-evaluated the benefit of using erythropoietin (Epo) as a pleiotropic cytokine to counteract hematological and extra-hematological toxicity following lethal irradiation. B6D2F1 mice were exposed to a dose of 9 Gy gamma radiation resulting in 90% mortality at 30 days, and then injected with stem cell factor, FLT-3 ligand, thrombopoietin and interleukin-3 [*i.e.* SFT3] at two and 24 hours with or without Epo (1,000 IU/kg) at 2 hours and day 8. As controls, two groups of irradiated mice were given only Epo or Phosphate-buffered saline. Epo synergized with SFT3 to rescue lethally-irradiated mice from radiation-induced death (survival: 60%, 95% and 5% respectively for SFT3, SFT3+Epo and controls at 30 days,  $p < 0.05$ ), whereas Epo alone exhibited no protective effect. Hematopoietic parameters did not differ significantly between SFT3 and SFT3+Epo groups during the animal death period. Some beneficial effects on gastro-intestinal toxicity were noticed following administration of Epo, although lung, liver and kidney were not protected. Further studies are necessary to understand fully the mechanisms involved in these effects of Epo in order to optimize treatment with cytokines following high-dose irradiation.

**Keywords:** cytokine, irradiation, mice, erythropoietin

The pathophysiology of accidental irradiation has been recently revisited. Indeed, irradiation has to be considered as more complex than simply combinations of hematological, gastro-intestinal, neurovascular or cutaneous syndromes [1-3]. This was particularly illustrated in the Tokaimura case in which two, highly irradiated victims died respectively at day 82 and day 211 from a complex, multi-organ distress, then failure syndrome (MODS/MOFS), in spite of transient, hematopoietic chimerism following hematopoietic stem cell (HSC) transplantation [4]. The term MODS was first used in the mid-seventies to describe a syndrome that combined the dysfunction of multiple organs with sepsis, shock or inflammation [5, 6]. Radiation-induced (RI) MODS is characterized by a progressive and sequential loss of function of one or more vital organs. Thus, in a recent review of 110 cases, the 65 irradiated victims who exhibited the higher MODS score were reported to be suffering from the consequences of hematological, neurovascular, gastrointestinal, and skin damage [7]. MODS also includes metabolic disorders such as mitochondrial dysfunction (*i.e.* diminished capacity to produce adenosine triphosphate and reactive oxygen species (ROS)/nitric oxide production). Importantly, RI MODS

may result from direct or indirect damage (abscopal effect).

In fact, the pathophysiology of MODS strongly overlaps the systemic inflammatory response syndrome (SIRS), which is mainly the consequence of extensive endothelial cell damage [8]. Following a major stress, including high dose irradiation, proinflammatory cytokines and chemokines are released into the systemic circulation and disorders such as microvascular occlusion, inflammatory infiltrates, fibrosis and necrosis seem to be a constant feature [9-11]. MODS is thought to result from the strong activation of leukocytes and endothelial cells, as well as complement coagulation and fibrinolytic pathways. In addition to skin, the gastro-intestinal tract and lung are highly radiosensitive, and are key organs involved in death following high-dose irradiation [12-15]. Even a single, 10 Gy exposure of the thorax has been demonstrated to result in decreased oxygen uptake and early pulmonary vascular pathology in rat [16, 17]. More recently, it has been shown in adult mice that small, intestinal crypts displayed two waves of apoptosis following a single 2 Gy whole-body irradiation [18]. This confirms the severe damage observed following whole body or abdominal exposure at higher doses [19].

Clearly, there is a need to develop new therapeutic strategies to counteract such extrahematological toxicity that is associated with RI, in addition to treating the hematopoietic disorders [20-22]. It is within this context that we propose to re-evaluate Epo as a pleiotropic cytokine. Initially described as an erythropoietic factor, Epo is thought to exhibit pleiotropic, tissue-protective effects, although this point remains controversial [23-26]. For example, Epo has been reported to prevent RI-apoptosis at the endothelial cell level (gastro-intestinal tract), and it may recruit endothelial progenitor cells from the bone marrow that would ultimately home to damaged areas [27-29]. Epo has also been described as an efficient neuroprotective factor and may exhibit anti-inflammatory properties [30, 31]. In this study, Epo was assessed in a mouse model of whole-body, high-dose irradiation alone or in combination with a reference cytokine cocktail. Lung and gastro-intestinal tract were particularly screened. No clear reduction – marginal jejunum effect – in radiation damage at these levels was observed.

## METHODS

### Animals

Animals were taken care of at the accredited animal facility at the Institut de Recherche Biomédicale des Armées-CRSSA (La Tronche, France) Six-week-old, female B6D2F1 mice (Janvier, LeGenest, France) were housed in groups of 10 per cage. Experiments were approved by the French Army Ethics Committee (protocol n°1999/11.1 and 11.2) in accordance with European rules and regulations.

### Irradiation

Mice placed in ventilated Plexiglas boxes were whole-body irradiated using a  $^{60}\text{Co}$  gamma source (9-11 Gy total body irradiation/TBI; dose rate 33 cGy/min): 9 Gy resulted in  $95\% \pm 5\%$  mortality at 30 days.

### Cytokines

Recombinant murine stem cell factor (SCF), FLT-3 ligand (FL), interleukin 3 (IL-3) and recombinant human thrombopoietin (Tpo) were purchased from R&D systems (Abingdon, UK), and epoietin beta from Roche (Neuilly, France).

### Study groups and treatment

For survival analysis, study groups of 40 mice were randomly assigned to different cytokine treatments: SFT3, SFT3+Epo, Epo. The SFT3 combination was given at 2 hours and 24 hours post-TBI (each cytokine at 50  $\mu\text{g}/\text{kg}$ ); Epo (1,000 IU/kg) was given at 2 hours and day 8. Early administration was based on putative anti-apoptotic effects. The administration at day 8 takes into account Epo pharmacokinetics and was aimed to achieve prolonged protection. The Epo dose was chosen from recommendations used in the clinic to correct anemia and adapted for the mouse model.

Control groups received PBS. Mice were monitored daily for survival for 100 days. Surviving mice were killed by cervical dislocation.

### Hematopoietic parameter evaluation

Complete blood cell counts (CBC) were performed on a Pentra 120 analyzer, (ABX, Montpellier, France).

### Histology

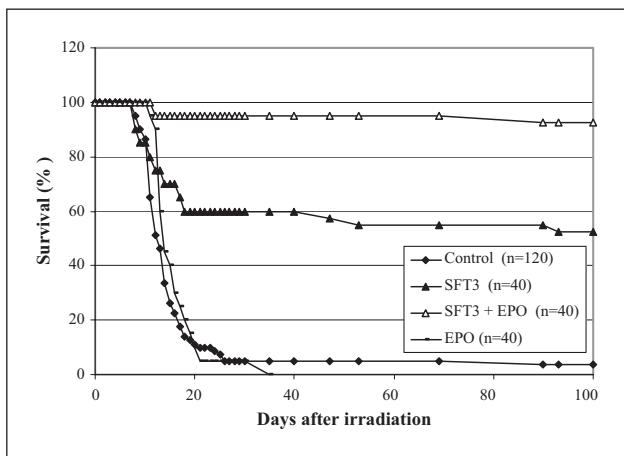
On day 15 and day 30 post-irradiation five mice per group were euthanized and samples were collected. Lung tissue samples were fixed, embedded in paraffin, and sectioned at 5  $\mu\text{m}$ . Sections were stained with hematoxylin and eosin (H&E) for analysis. Each sample was assigned a histological score from 0 to 3 based on the degree of neutrophilic infiltration, hemorrhage, and edema in the interstitial and alveolar spaces [32]: normal appearing lung- 0; mild congestion, interstitial edema, neutrophilic infiltrate with rare red blood cells and/or neutrophils only in the alveolar spaces- 1; moderate congestion and interstitial edema, with neutrophils partially filling the alveolar spaces- 2; marked congestion and interstitial edema, with neutrophilic infiltrate nearly or completely filling the alveolar spaces- 3.

Jejunum samples were fixed and then embedded in paraffin. Sections (5  $\mu\text{m}$ ) were stained with H&E for analysis. Scoring parameters were as followed: villus/crypt height ratio, which represents the main parameter; other parameters: chorion (edema, inflammation, macrophages), villus (height, thickness, necrosis, inflammation), crypts (distension, necrosis, inflammation, mitosis).

## RESULTS

### *Epo synergizes with SFT3 to promote survival in lethally-irradiated mice*

Hundred-day survival rates are presented in figure 1. As previously reported [33], the anti-apoptotic SFT3 combination provided a significant protective effect (60 %  $p < 0.05$  versus control). Epo as a single treatment did not improve the survival of irradiated mice (survival rate 30 days after irradiation was 5% for both Epo- and PBS-injected mice). In contrast, addition of Epo to SFT3



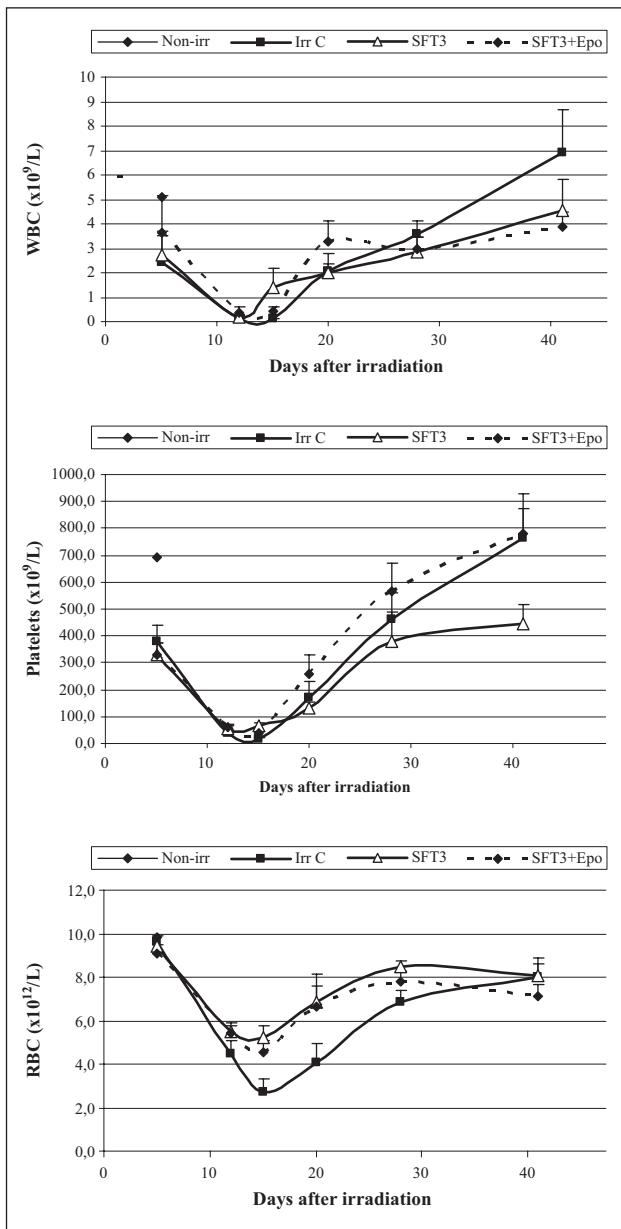
**Figure 1**

Effect of treatments with SFT3  $\pm$  Epo on the survival of B6D2F1 mice after 9 Gy  $^{60}\text{Co}$  whole body gamma irradiation. Stem cell factor, FLT-3 ligand, thrombopoietin and interleukin-3 (SFT3) at 50  $\mu\text{g}/\text{kg}$  each, Epo at 1,000 IU/kg. SFT3 was given at 2 and 24 hours post-irradiation, and Epo at 2 hours and 8 days post-irradiation. Groups were compared two-by-two using the Chi-square ( $\chi^2$ ) test with Yates' correction.

(2 hours and day 8) significantly enhanced the survival rate (95%,  $p<0.05$ ) when compared with SFT3-treated animals.

### Hematopoietic parameters

Irradiated mice experienced prolonged neutropenia and thrombocytopenia from day 5 to day 15 (*figure 2*). Also on day 15, severe neutropenia ( $0.2 \pm 0.1 \times 10^9/L$ ), thrombocytopenia ( $16.3 \pm 5.1 \times 10^9/L$ ) and anemia ( $2.7 \pm 0.6 \times 10^{12}/L$ ) were observed in untreated animals, whereas SFT3 and SFT3+Epo mice showed a multilineage recovery from day 12. Hematopoietic parameters did not differ between the SFT3 and the SFT3+Epo groups during this phase, which corresponded to the animal death period in these groups. On day 20, CBCs were as followed: white blood cells [WBC]:  $2 \pm 0.4 \times 10^9/L$  and  $3.3 \pm 0.8 \times 10^9/L$ ; platelets [PLT]:  $132 \pm 24 \times 10^9/L$



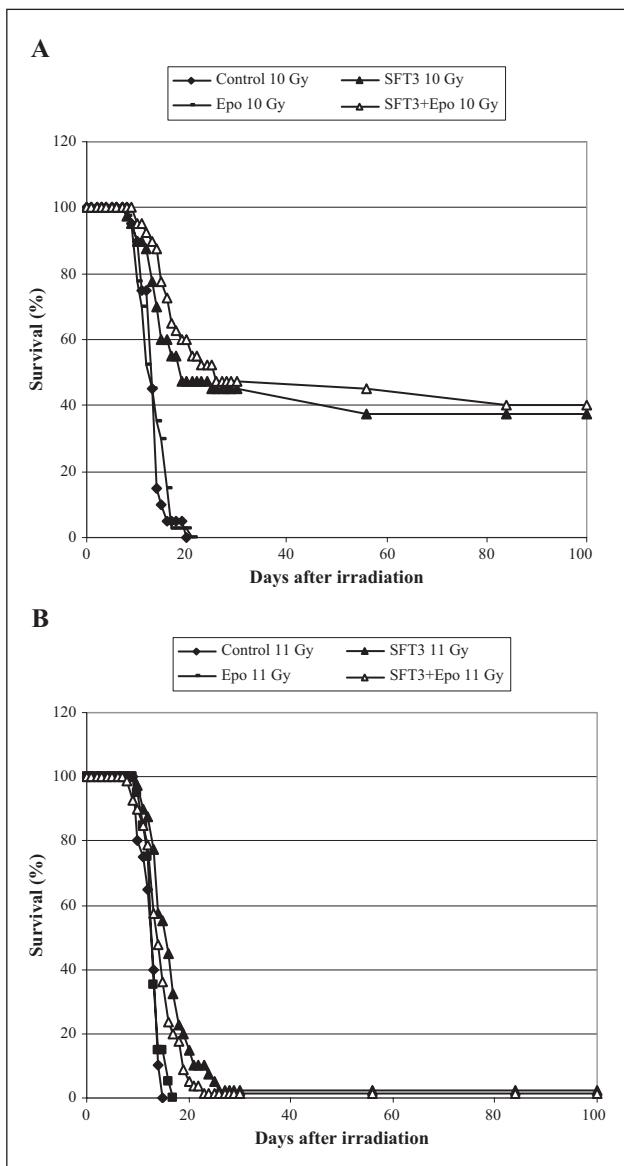
**Figure 2**

Hematopoietic parameters of irradiated mice. Blood cell counts for white blood cells [WBC], platelets [PLT] and red blood cells [RBC] are shown. No statistical difference was observed between the SFT3 and SFT3+Epo groups during the first two weeks. Values presented as mean  $\pm$  SD (irradiated controls  $n>8$ , SFT3  $n>5$ , SFT3+Epo  $n>4$ ).

and  $258.5 \pm 74 \times 10^9/L$ ,  $p<0.05$ ; red blood cells [RBC]:  $6.8 \pm 1.3$  and  $6.6 \pm 0.9 \times 10^{12}/L$ , hemoglobin [Hb]:  $11.1 \pm 2$  and  $10.1 \pm 1.5 \text{ g/dL}$  for the SFT3 and SFT3+Epo groups respectively; WBC:  $2.1 \pm 0.7 \times 10^9/L$ ; PLT:  $169 \pm 59 \times 10^9/L$ , RBC:  $4.1 \pm 0.9 \times 10^{12}/L$ , Hb:  $6.5 \pm 1.4 \text{ g/dL}$  for irradiated controls at the same time. PLTs were significantly higher ( $p<0.05$ ) in the SFT3+Epo group than in the SFT3 group from day 20, but RBCs and Hb values were higher in the SFT3 group. By day 28 all groups had recovered.

### SFT3+Epo combination following supralethal irradiation

SFT3+Epo and SFT3 preserved about 40% of mice from death 30 days after a 10 Gy irradiation. At the higher dose of 11 Gy, no beneficial effect in terms of survival was observed whatever the cytokine treatment (*figure 3*).



**Figure 3**

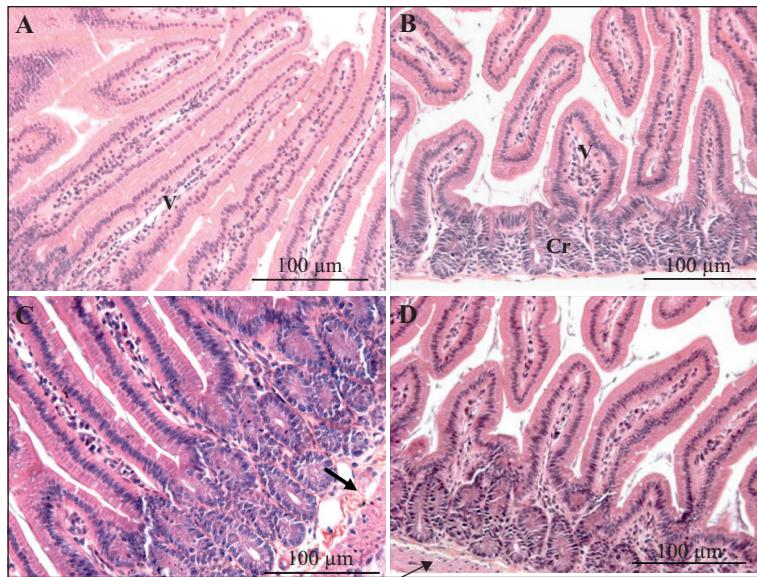
Effect of treatments with SFT3  $\pm$  Epo on the survival of B6D2F1 mice after 10 Gy (A) and 11 Gy ( $^{60}\text{Co}$ , whole body gamma irradiation. Stem cell factor, FLT-3 ligand, thrombopoietin and interleukin-3 (SFT3) at 50  $\mu\text{g}/\text{kg}$  each, Epo at 1,000 IU/kg. SFT3 was given at 2 and 24 hours post-irradiation and Epo at 2 hours and 8 days post-irradiation. Groups were compared two-by-two using the Chi-square ( $\chi^2$ ) test with Yates' correction. Each group:  $n = 40$ .

### Extrahematological toxicity

Damage at the jejunum level was noticeable on day 15 and day 30 post-irradiation in the SFT3 and SFT3+Epo groups (figure 4). On day 30, only cytokine-treated animals survived. No significant differences in term of total histological scores were observed between the two groups in spite of slightly decreased scores in the SFT3+Epo group at both times ( $12.8 \pm 2.4$  versus  $15.5 \pm 0.9$  on day 15 and  $14.6 \pm 3.3$  versus  $17.6 \pm 2.2$  on day 30 for SFT3+Epo and SFT3 respectively). In the SFT3+Epo group, damage was

marginally mitigated at the chorion (day 15) and crypt level (day 30), and animals exhibited a greatly reduced collagen deposition in muscular mucosa at both times ( $P < .05$  at day 15; table 1).

As regards lung tissue, untreated, irradiated animals exhibited mild damage (score 1- to 1+) on day 15. By day 30 only cytokine-treated animals survived and all mice exhibited a grade 2 damage with marked congestion, interstitial edema and bronchiolar alveolar/alterations regardless of the cytokine treatment given (table 2, figure 5).



**Figure 4**

Intestinal damage in 9 Gy  $^{60}\text{Co}$ , whole body-irradiated mice given the cytokine combination. Mice were euthanized on days 15 or 30 after irradiation and jejunum sections were stained with hematoxylin and eosin. Non-irradiated control (A); representative animals given SFT3 (B) showing reduced villus height (V) and crypt (Cr) number 15 days after irradiation. At this time, SFT3-treated animals (C), but not SFT3+Epo (D) exhibited strong collagen deposition in muscular mucosa [arrow] (SFT3: 2 and 24 hours; Epo: 2 hours and day 8).

**Table 1**

Whole body-irradiated mice receiving cytokines survived, but exhibited severe intestinal damage 15 and 30 days following irradiation; jejunum damage can be observed whatever the cytokine treatment as shown by the reduction in the villus/crypt height ratio. Addition of Epo marginally improved the histological score.

(0 to 4 score)	Baseline	Day 15 post-TBI			Day 30 post-TBI	
		Non-irradiated	9 Gy	9 Gy SFT3	9 Gy SFT3+Epo	9 Gy SFT3
V/C	4	3 $\pm$ 0	2.25 $\pm$ 0.5	2.8 $\pm$ 0.4	2 $\pm$ 0.4	2.2 $\pm$ 0.2
<b>Chorion</b> [edema, inflammatory infiltration macrophages]	0	1.6 $\pm$ 0.3	3 $\pm$ 1	2.2 $\pm$ 0.2	3.75 $\pm$ 0.2	3.4 $\pm$ 0.7
<b>Villi</b> [height thickness necrosis inflammation]	0	2.6 $\pm$ 0.3**	4.75 $\pm$ 0.6	4.2 $\pm$ 1	4.75 $\pm$ 0.9	4.8 $\pm$ 1.3
<b>Crypts</b> [size, epithelial necrosis inflammatory infiltration mitosis]	1	3.2 $\pm$ 0.8	3.5 $\pm$ 0.9	4 $\pm$ 0.9	5 $\pm$ 1.1	3.4 $\pm$ 0.7
<b>Collagen deposition</b> [mucosa, muscular]	0	0.8 $\pm$ 0.2	1.75 $\pm$ 0.2*	0.6 $\pm$ 0.3	1.5 $\pm$ 0.3*	0.6 $\pm$ 0.3
<b>Cellular debris</b> in lumen + infiltration	0	1.4 $\pm$ 0.3	1.25 $\pm$ 0.2	1 $\pm$ 0.3	1.25 $\pm$ 0.2	1.4 $\pm$ 0.7
Architecture	0	1 $\pm$ 0	1.25 $\pm$ 0.2	0.8 $\pm$ 0.2	1 $\pm$ 0	1 $\pm$ 0.3
Total score		10 $\pm$ 1.2	15.5 $\pm$ 0.9	12.8 $\pm$ 2.4	17.6 $\pm$ 2.2	14.6 $\pm$ 3.3

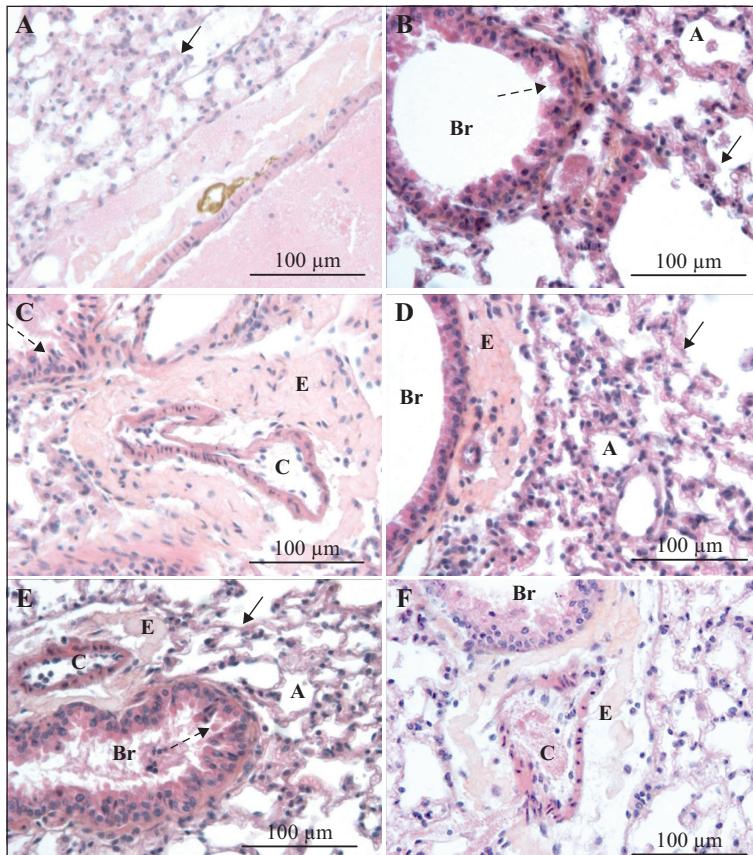
N=5 each group; hematoxylin and eosin staining; V/C: villus/crypt height ratio; for other parameters, scoring increases from 0 (non-irradiated) to 4 (major lesions), when damage increases. SFT3: stem cell factor + FLT-3 ligand + thrombopoietin + interleukin-3 (50  $\mu\text{g}/\text{kg}$ ); Epo: erythropoietin (1,000 IU/kg). \* = Statistical significance  $P < 0.05$  between SFT3 and SFT3+Epo groups [\*] and between untreated irradiated and cytokine-treated animals [\*\*]. Results are expressed as mean value  $\pm$  SD.

**Table 2**

Irradiated mice receiving cytokines that survived and exhibited mild pulmonitis: 30 days following irradiation, mild pneumonitis was observed in both cytokine groups.

Baseline	Day 15 post-TBI			Day 30 post-TBI		
Non-irradiated	9 Gy	9 Gy SFT3	9 Gy SFT3 + Epo	9 Gy SFT3	9 Gy SFT3 + Epo	
0	1	0 (0 to 0+)		1 (1 to 1+)	1.4 ± 0.27 (1 to 2)	2

N=5 each group; hematoxylin and eosin staining; scoring was performed according to Broccard *et al.* [32] from 0 (non-irradiated) to 3 (severe damage). TBI: total body irradiation. SFT3: stem cell factor + Flt3 ligand + thrombopoietin + interleukin-3 (50 µg/kg); Epo: erythropoietin (1,000 IU/kg). Results are expressed as mean value ± SD.

**Figure 5**

Irradiated mice given cytokine combination exhibited mild pulmonitis. Mice were euthanized on day 30 after irradiation, and lung sections were stained with hematoxylin and eosin: (A) non-irradiated animals with normal alveoli; (B-E) representative animal given SFT3+Epo or SFT3 (F) after a whole body, 9 Gy gamma radiation exposure (SFT3: 2 and 24 hours; Epo: 2 hours and day 8) and showing disorganization of the bronchial epithelial cell lining and interstitial/perivascular edema. A : alveoli ; Br : bronchiole ; E : edema ; C : capillary ; A : alveoli ; Br : bronchiole ; E : edema ; C : capillary

Liver damage was severe, and included necrotic hepatocytes, sinusoidal dilation, and collagen deposition at both times whatever the treatment and with no improvement when Epo was added (*figure 6*). Kidney lesions, including necrosis, fibrosis/sclerosis, ischemia, were evident in the two groups on day 30 (data not shown).

## DISCUSSION

The aim of this study was to assess in a mouse model the benefit of using Epo to prevent the development of RI extrahematologic toxicity, based upon the hypothesis of the pleiotropic action/character of Epo. Epo is a cytokine produced primarily in the fetal liver and in the adult kidney in a hypoxia-dependent manner, via the induction of hypoxia-inducible factor 1 [34]. Two types of Epo receptors (EpoR) have been demonstrated so far, which mediate

the multiple effects of Epo: EpoR2, located on hematopoietic cells, and an alternative EpoR complex that includes the β common receptor (βc or cluster of differentiation 131), are expressed in numerous tissues, and especially in brain, heart and kidney [35].

In our model of high-dose, whole body irradiation (9 Gy TBI) hematopoietic syndrome is the major cause of death within 30 days. We therefore tested Epo added to the previously described anti-apoptotic combination, SFT3, and compared the effect with injection of Epo as a single treatment. Interestingly, day 30 survival was significantly increased in SFT3+Epo (2 hour + day 8) -treated mice when compared with the SFT3 group, whereas Epo alone did not protect mice from radiation-induced death. Animals were initially checked for hematopoietic recovery as Epo has been described as an erythroid factor required particularly for early burst-forming unit erythroid survival; dose-dependent Epo effects on thrombopoiesis

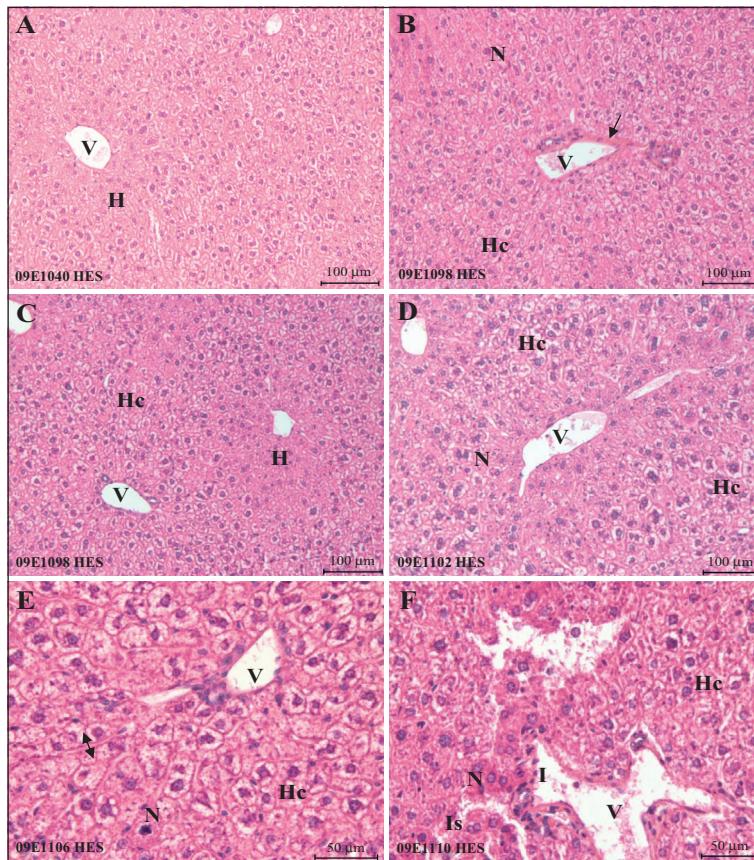


Figure 6

Irradiated mice given cytokine combinations exhibited markedly delayed liver damage. Mice were euthanized after a whole body, 9 Gy gamma irradiation. Liver sections were stained with hematoxylin and eosin. On day 30, extensive areas of cellular lysis could be observed in addition to sinusoidal dilation, ischemia/hemorrhagic areas, thrombi and mild inflammation (monocytes), whatever the treatment. (A) non-irradiated animals; SFT3 (B-D) or SFT3+Epo (E,F) treated animals. Undamaged (H), ballooned (Hc) or necrotic (N) hepatocytes; I: inflammation; Is: ischemia; V: centrilobular vein (with fibrosis = single arrow); sinusoidal dilatation (double arrow).

have also been reported in mice [36]. In addition, Epo has been reported to mobilize hematopoietic stem cells (HSC) and accelerate hematopoietic recovery [37]. SFT3-treated mice showed a limited reduction in thrombocytopenia and anemia over the first three weeks following TBI when compared with controls, mainly as a consequence of Tpo activity. In fact, the control group values specifically reflected the hematopoietic recovery in the cohort of surviving controls not taking into account the numerous dead animals. Importantly, no significant improvement in RBC and PLT recovery was seen over the first two weeks corresponding to the phase when animals died. Thus, addition of Epo to SFT3 did not appear to increase survival via the enhancement of hematopoietic recovery. As suspected, the protection from hematological death diminished then vanished at doses higher than 9 Gy, which suggests damage at the niche level, above the repair threshold. At 10 and 11 Gy, the SFT3 combination, with or without Epo, failed to counteract the death of hematopoietic stem and progenitor cells. We then looked for pulmonary and gastro-intestinal damage, as this may have been responsible for the increase in early RI death, and because the pleiotropic activity of Epo, including reduction in apoptosis following different stresses in different tissues, has been recently challenged [30]. Here we report a mild RI pneumonitis following whole body-irradiation at a dose of 9 Gy TBI, in accordance with the Moulder group [16, 17] who used a 5-10 Gy thorax irradiation model and a longer follow-up period. At the 9 Gy TBI dose, pneumonitis is not lethal *per se*, but

combination with bone marrow aplasia may be detrimental. However, we did not notice any differences between the two cytokine groups as regards the hematopoietic recovery time, at day 30 post-irradiation. Regarding the jejunum, severe damage was observed in all groups; again on day 15 the control group values (villi height for example) in surviving controls did not take into account the numerous dead animals. The global histological score did not significantly differ between the two cytokine groups. However, addition of Epo had some effect at the jejunum level as (a) collagen deposition at the muscular mucosa level was significantly reduced in the SFT3+Epo group in which (b) two out of five animals exhibited greatly reduced histological scores on day 15 (7/8). Interestingly, a relative gastro-intestinal protective effect of Epo has been reported in irradiated swine, which was postulated to be mainly indirect via stem cell mobilization [38]. At the higher whole body dose of 10 Gy, injection of SFT3 with or without Epo, only moderately mitigate RI death of HSCs and progenitor cells. At the supralethal dose of 11 Gy, overwhelming HSC apoptosis and medullar cell eradication resulted in a prompt death, which could only be averted by stem cell transplantation.

As regards other vital organs, we then checked liver and kidney (data not shown). Damage was severe at both times, whatever the treatment and with no mitigation when Epo was added.

Overall, our observations have demonstrated the benefit of adding Epo to anti-apoptotic cytokines, using the

SFT3 combination as a reference, to increase the survival rate of highly-irradiated mice. However, the mechanisms involved are still only partially understood. Other mechanisms, such as ROS and iron metabolism mitigation [39], endothelial progenitor mobilization, modulatory effects on macrophages and their functions [40], fibrinolysis/coagulation and SIRS reduction, as well as proangiogenic morphogenesis induction [41], are likely to have also played a role and deserve further investigation. Lymphocyte subpopulations and immune functions should also be explored in detail. In preliminary studies involving neuropathology at day 1/day3, Epo alone failed to mitigate the severe cell death seen in the replicating neural progenitors located in the subgranular zone of the dentate gyrus and the subventricular zone.

**Acknowledgements.** We are grateful to Sylvie Guerret and Aurelie Nallet (Novotec, Lyon, France) who performed the histology, and to Mylene Vivier for technical assistance.

**Disclosure.** Financial support: supported by a grant from DGA (Délégation Générale pour l'Armement). Conflict of interest: none.

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