This project has received funding from the Euratom research and training programme 2014-2018 under grant agreement No 662287.

EJP-CONCERT

European Joint Programme for the Integration of Radiation Protection Research

H2020 – 662287

D9.97 - Evaluating major cellular and molecular mechanisms mediated by irradiated EVs

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Work package / Task	**WP9**	**T9.5**	**ST 9.5.1**
Deliverable nature:	**Report**		
Dissemination level: (Confidentiality)	**Public**		
Contractual delivery date:	**M54**		
Actual delivery date:	**M54**		
Version:	**1**		
Total number of pages:	**21**		
Keywords:	**cellular and molecular mechanisms, bone marrow-derived EVs**		
Approved by the coordinator:	**M54**		
Submitted to EC by the coordinator:	**M54**		

Abstract

The main objective of the deliverable was to study cellular and molecular mechanisms how extracellular vesicles (EVs) originating from irradiated mice can transmit bystander signals upon injection into naïve mice. Bone marrow-derived EVs were isolated from mice total-body irradiated with low doses (0.1 and 0.25 Gy) and with high dose (2 or 3 Gy) and injected intravenously into naïve mice 24 hours after irradiation. Both direct radiation and EV-mediated effects were investigated 24 hours or 3 months after treatment. Phenotypical changes after EV treatment in the different cellular subpopulations in the bone marrow were very similar to direct radiation effects both acutely (24 hours after treatment) and chronically (3 months after treatment). EVs induced an increased apoptotic frequency in the haematopoietic stem cells and lymphoid progenitors. EV effects were manifest in the spleen as well, where, similarly to direct radiation effects, EVs originating from mice irradiated with high dose induced a reduction in the gene expression of major proteins involved in the antioxidant system. We showed that irradiation of either EVs or EV-acceptor bone marrow cells significantly reduced EV uptake rate, however, within the cells taking up EVs myeloid progenitors were over-represented.

In conclusion, we showed that bone marrow-derived EVs could stably transmit direct irradiation-mediated signals. These effects were cell type-specific, where haematopoietic stem cells and myeloid progenitors were preferentially affected.

CONTENT

1 Introduction

Bystander signaling is particularly important in the manifestation of low dose radiation effects in the bone marrow and thus it is reasonable to assume that has its role in low dose-induced leukaemogenesis as well. Extracellular vesicles (EVs) due to their complex protein and RNA cargo are major mediators of bystander signals. The main objective of LEU-TRACK is to study the role of bone marrow (BM)-derived EVs in radiation leukaemogenesis in a murine model genetically prone to develop leukaemia after irradiation.

The objective of the present deliverable is to study the types of signals and the mechanisms how EVs transmit radiation-related signal within the haematopotoietic system. The deliverable will present data on the following investigations:

- phenotypical changes within the bone marrow of mice treated with BM-derived EVs, analyzing both acute (24 hours after EV treatment) and chronic (3 months after EV treatment) effects;
- changes in the apoptotic rate, proliferation index and DNA damage level of haematopoietic cells treated with EVs;
- changes in the oxidative status and antioxidant system in the spleen of mice directly irradiated or treated with EVs;
- the rate of EV uptake in the bone marrow and major EV acceptor cells.

2 Phenotypical changes within the bone marrow of mice treated with BM-derived EVs, analyzing both acute (24 hours after EV treatment) and chronic (3 months after EV treatment) effects

3 Methods

10-12 week old male mice were irradiated with low dose (0.1 Gy, 0.25 Gy) and high dose (2 or 3 Gy) X-rays. Part of the irradiated animals was used to evaluate direct radiation effects. The other part was used for isolation of EVs with Exoquick precipitation kit. Naïve mice were injected with the isolated EVs iv. 24 hours after irradiation of mice. EV-mediated effects in the EV-injected animals were investigated either 24 hours or 3 months after EV injection. Additionally, a further group of mice were treated with EVs isolated 3 months after irradiation of mice and EV effects were investigated 24 hours later.

Bone marrow (BM) was isolated from irradiated animals 24 hours after irradiation. Immune phenotyping of bone marrow single cell suspension was performed and the following subpopulations analyzed by flow cytometry: lymphoid progenitors (CD45+CD90.2+), granulocyte/monocyte progenitors (Gr1+CD11b+), erythroid precursors (Ter119+CD71+), megakariocyte progenitors (CD41+CD61+), haematopoietic stem cells (HSCs) (Lin-Sca1+cKit+), short-term HSCs (Lin-Sca1+cKit+CD35-CD34+), long-term HSCs (Lin-Sca1+cKit+CD35-CD34-), multilineage progenitors (Lin-Sca1+cKit+CD35+CD34+).

3.1 Results

Formerly, we have reported that BM EV injection induced acute effects in the different BM subpopulations, which were similar to direct radiation effects [1]. In short, we could show a dose-dependent decrease in the HSCs and lymphoid progenitors, while myeloid progenitors, erythroid and megakaryocyte precursors were not affected. This time we extended our investigations of acute EV effects to other cell populations in the BM (mesenchymal stem cells and different HSC subpopulations) and investigated chronic bystander signaling within the BM mediated by EVs. This was done by two

scenarios: either EVs isolated from irradiated animals were injected in naïve mice 24 hours after irradiation and effects were investigated 3 months later (24hours/3months) or EVs isolated from irradiated animals were injected in naïve mice 3 months after irradiation and effects were investigated 24 hours later (3months/24 hours). Mice irradiated only and investigated 3 months after irradiation were used as controls to study direct radiation effects in these latter two groups.

a. *Changes in the different HSC subpopulations*

Acutely after irradiation total HSCs decreased both in the directly irradiated and EV-treated mice dose-dependently (Figure 1A). 3 months after irradiation a significant decrease in the total HSC subpopulation could only be seen in mice irradiated with high dose (Figure 1B). In the 24hours/3months EV-treated mice HSCs decreased dose-dependently, while in 3months/24H EV-treated mice no changes were seen compared to controls (Figure 1C).

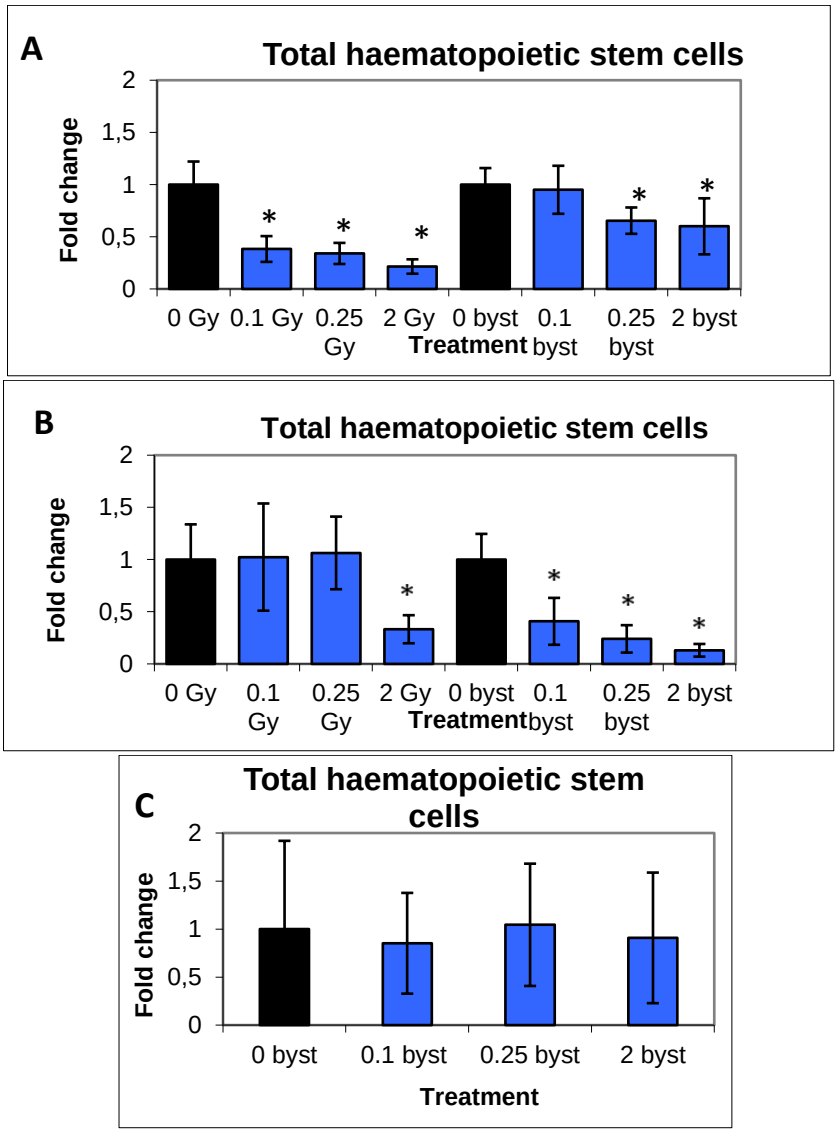


Figure 1: Changes in the fraction of haematopoietic stem cells in directly irradiated and EV-treated mice. Mice were total-body irradiated with the indicated doses. EVs were isolated from directly irradiated animals and injected into naïve mice either 24 hours after irradiation (Figure 1A and 1B) or 3 months after irradiation (Figure 1C). EV effects were investigated either 3 months after EV injection (Figure 1B) or 24 hours after EV injection (Figure 1A and 1C). Bone marrow cells were isolated, immune phenotyped and analyzed as indicated in the Methods section. Individual treatment groups comprised of a minimum of 6 animals. Error bars represent standard deviations. Statistical significance was evaluated by Student T-test. Statistically significant data points ($p<0.05$) are indicated by *.

Acutely after irradiation the proportion of long-term HSCs increased with the dose in the directly irradiated mice, while the fraction of multilineage progenitors decreased with the dose. Short-term HSCs were not affected. In the EV-treated mice the same tendency was seen than in directly irradiated mice, indicating that EVs transmitted radiation effects in the HSC subpopulations (Figure 2A). Chronic changes were similar to 24 hour effects but milder both in the directly irradiated mice and in the 24 hour/3months EV-treated group (Figure 2B). No changes compared to unirradiated controls could be detected in the 3months/24 hour EV-treated group (Figure 2C).

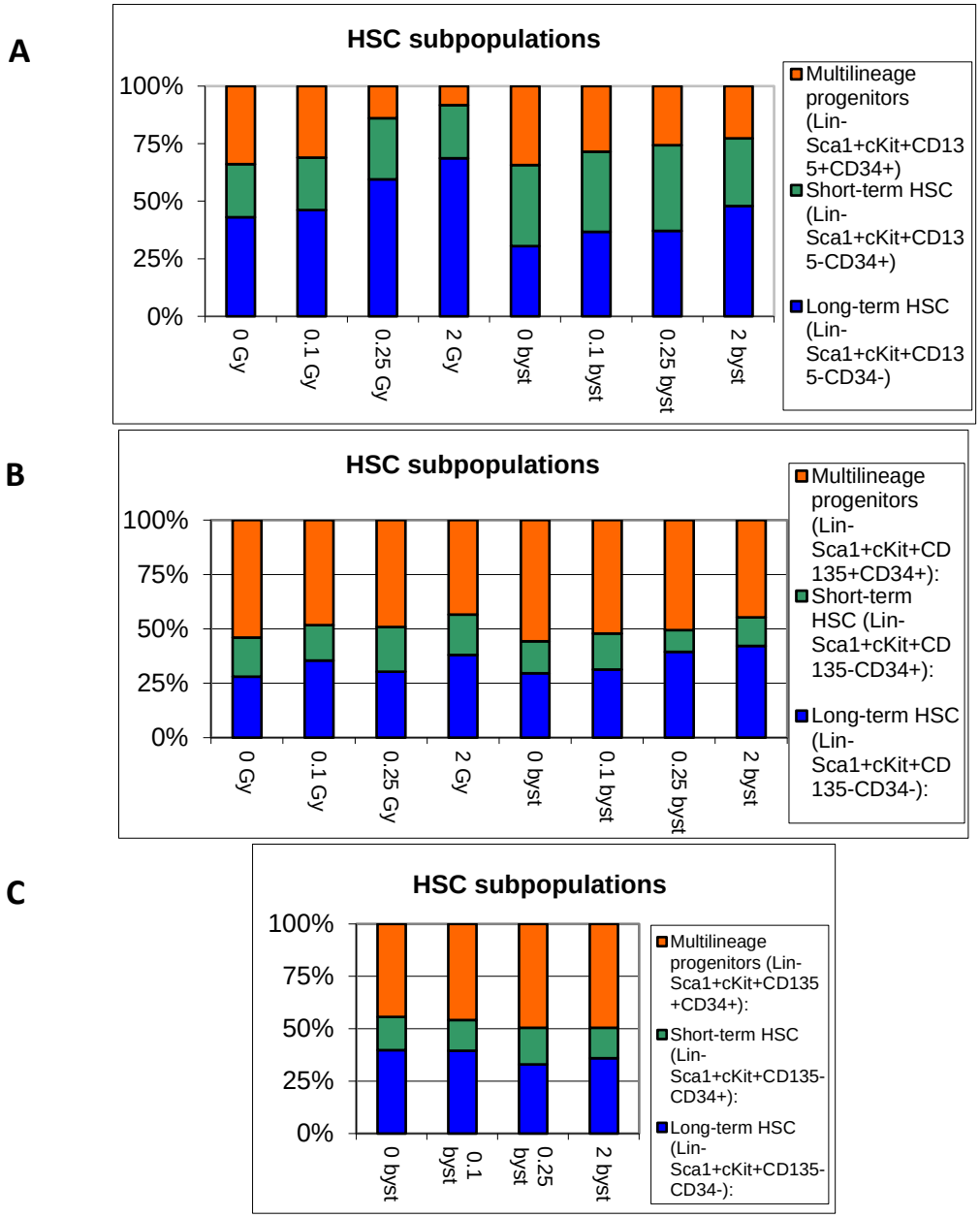


Figure 2: Changes in the fraction of haematopoietic stem cell subpopulations in directly irradiated and EV-treated mice. Mice were total-body irradiated with the indicated doses. EVs were isolated from directly irradiated animals and injected into naïve mice either 24 hours after irradiation (Figure 2A and 2B) or 3 months after irradiation (Figure 2C). EV effects were investigated either 3 months after EV injection (Figure 2B) or 24 hours after EV injection (Figure 2A and 2C). Bone marrow cells were isolated, immune phenotyped and analyzed as indicated in the Methods section. Individual treatment groups comprised of a minimum of 6 animals.

b. *Changes in the fraction of lymphoid progenitors*

Acutely after irradiation lymphoid progenitors decreased both in the directly irradiated and EV-treated mice only after high dose irradiation (Figure 3A). The same changes persisted 3 months after irradiation and in mice injected with EVs and analyzed 3 months after EV injection (24 hours/3months group) (Figure 3B). No effects were seen if EVs were injected 3 months after irradiation and investigated 24 hours later (3months/24 hour group) (Figure 3C).

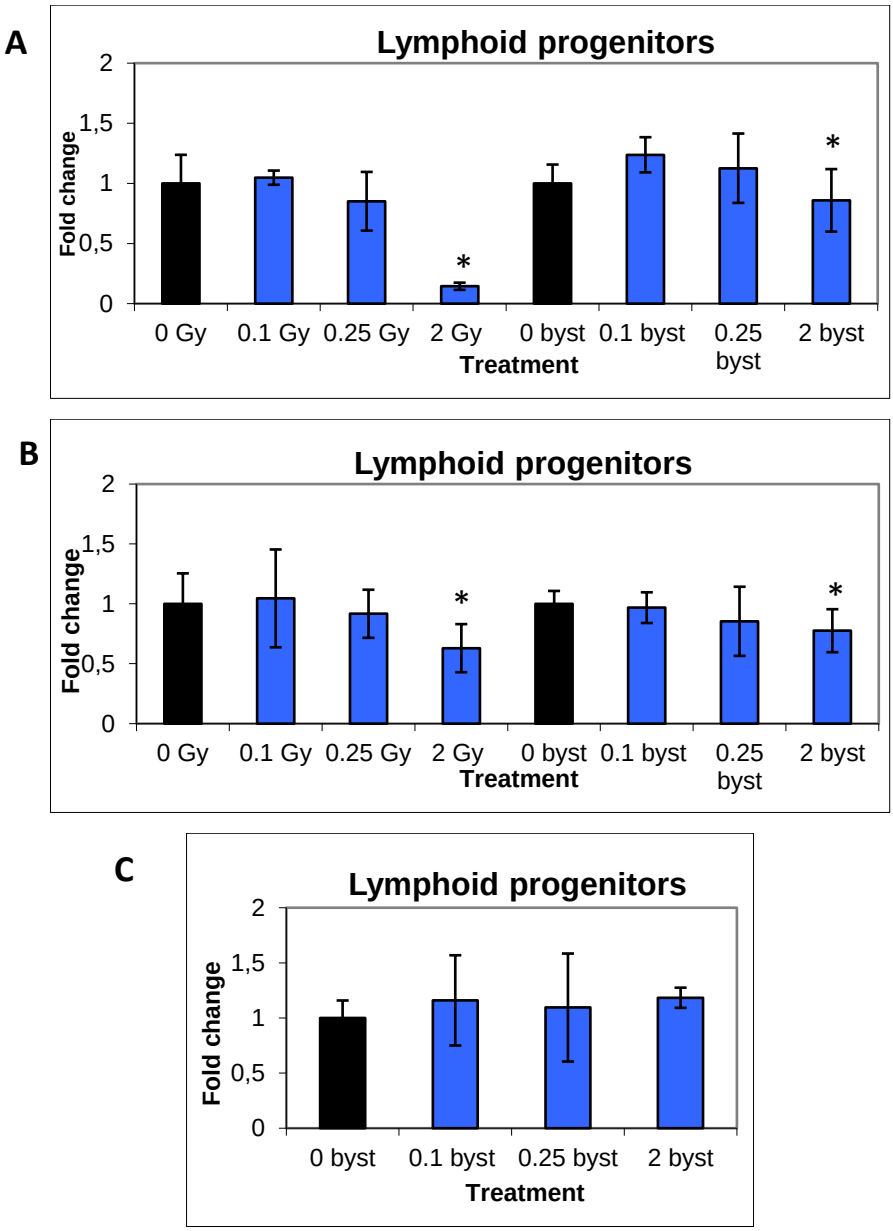
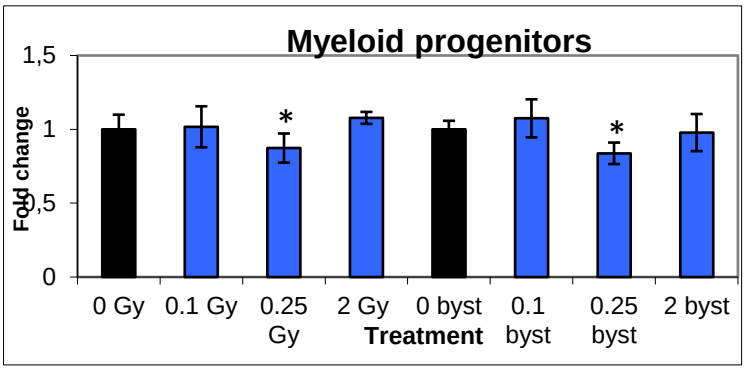


Figure 3: Changes in the fraction of lymphoid progenitors in directly irradiated and EV-treated mice. Mice were total-body irradiated with the indicated doses. EVs were isolated from directly irradiated animals and injected into naïve mice either 24 hours after irradiation (Figure 3A and 3B) or 3 months after irradiation (Figure 3C). EV effects were investigated either 3 months after EV injection (Figure 3B) or 24 hours after EV injection (Figure 3A and 3C). Bone marrow cells were isolated, immune phenotyped and analyzed as indicated in the Methods section. Individual treatment groups comprised of a minimum of 6 animals. Error bars represent standard deviations. Statistical significance was evaluated by Student T-test. Statistically significant data points ($p<0.05$) are indicated by *.

c. *Changes in the fraction of myeloid progenitors*

Acutely after irradiation changes were mild, both in the directly irradiated and EV-treated mice and interestingly 0.25 Gy had the strongest effect (Figure 4A). Three months after irradiation myeloid cells decreased strongly after high dose irradiation and the same effects were seen if mice were injected with EVs from 3 Gy-irradiated animals (24 hours/3months group) (Figure 4B). No effects were observed if EVs were injected 3 months after irradiation and investigated 24 hours later (3months/24H group) (Figure 4C).

A



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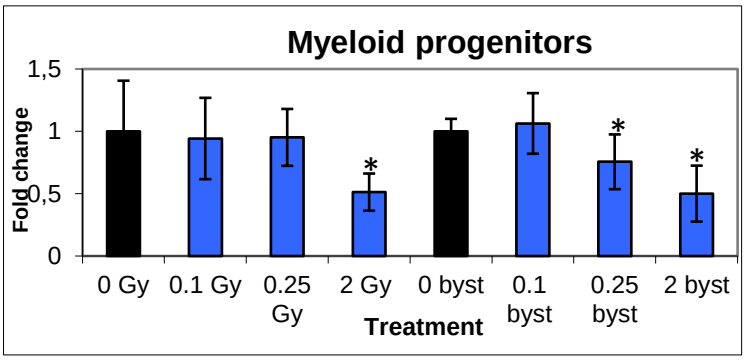

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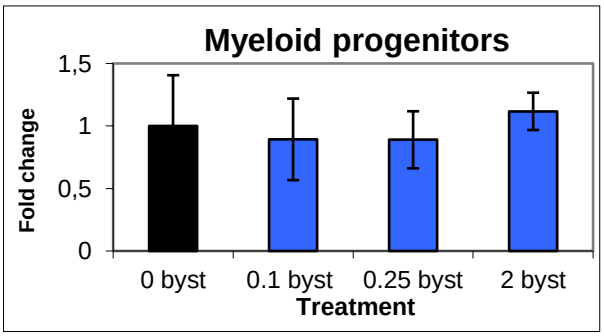


Figure 4: Changes in the fraction of myeloid progenitors in directly irradiated and EV-treated mice. Mice were total-body irradiated with the indicated doses. EVs were isolated from directly irradiated animals and injected into naïve mice either 24 hours after irradiation (Figure 4A and 4B) or 3 months after irradiation (Figure 4C). EV effects were investigated either 3 months after EV injection (Figure 4B) or 24 hours after EV injection (Figure 4A and 4C). Bone marrow cells were isolated, immune phenotyped and analyzed as indicated in the Methods section. Individual treatment groups comprised of a minimum of 6 animals. Error bars represent standard deviations. Statistical significance was evaluated by Student T-test. Statistically significant data points ($p<0.05$) are indicated by *.

d. Changes in the fraction of erythroid and megakaryocyte precursors

Acutely after irradiation a moderate but statistically highly significant increase was seen in the fraction of erythroid precursors after high dose irradiation. In mice, which received EVs from the directly irradiated animals, the fraction of erythroid precursors decreased significantly after low dose irradiation (0.1 and 0.25 Gy) (Figure 5). Chronic effects were absent.
Irradiation and EV treatment did not induce statistically significant changes in the pool of megakaryocyte precursors either acutely or chronically after irradiation and EV treatment.

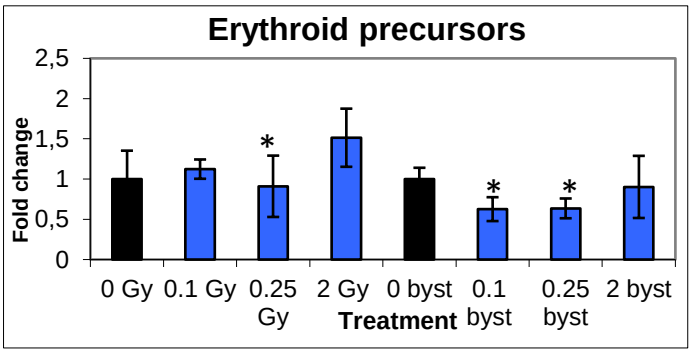
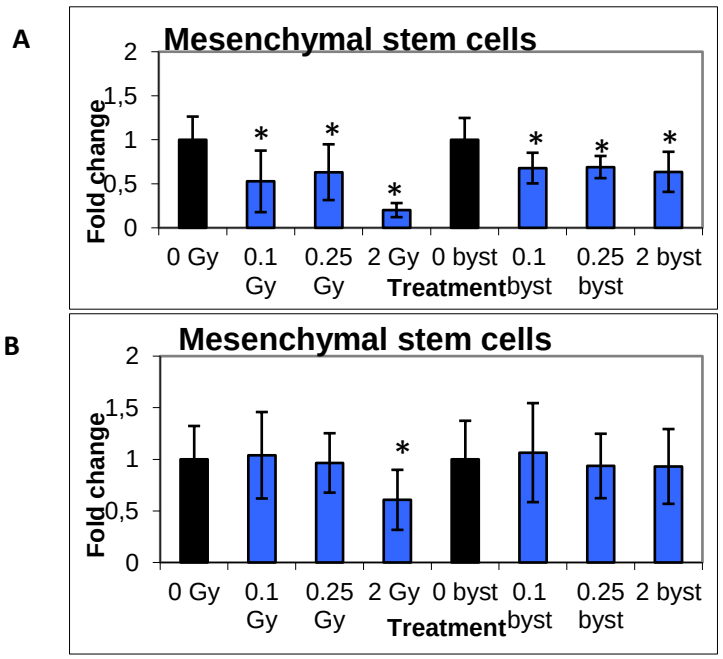


Figure 5: Changes in the fraction of erythroid precursor cells in directly irradiated and EV-treated mice. Mice were total-body irradiated with the indicated doses. EVs were isolated from directly irradiated animals and injected into naïve mice 24 hours after irradiation. EV effects were investigated 24 hours after EV injection. Bone marrow cells were isolated, immune phenotyped and analyzed as indicated in the Methods section. Individual treatment groups comprised of a minimum of 6 animals. Error bars represent standard deviations. Statistical significance was evaluated by Student T-test. Statistically significant data points ($p<0.05$) are indicated by *.

e. Changes in the fraction of mesenchymal stem cells (MSC)

Both direct irradiation and EV-mediated bystander signals induced a significant reduction of the MSC pool in the bone marrow after all doses acutely after treatment (Figure 6A). Changes were not dose dependent. Three months after irradiation only high dose irradiation induced a moderate but significant reduction in the MSC pool (Figure 6B). Chronic EV-mediated bystander effects were absent (Figure 6B and 6C).



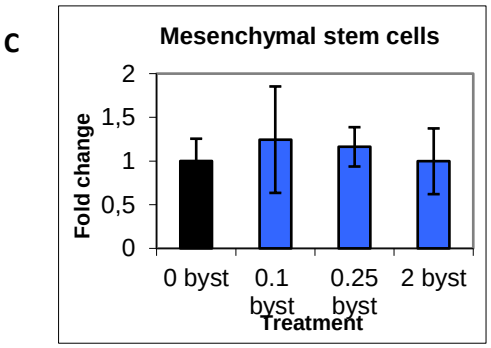


Figure 6: Changes in the fraction of mesenchymal stem cells in directly irradiated and EV-treated mice. Mice were total-body irradiated with the indicated doses. EVs were isolated from directly irradiated animals and injected into naïve mice either 24 hours after irradiation (Figure 5A and 5B) or 3 months after irradiation (Figure 5C). EV effects were investigated either 3 months after EV injection (Figure 5B) or 24 hours after EV injection (Figure 5A and 5C). Bone marrow cells were isolated, immune phenotyped and analyzed as indicated in the Methods section. Individual treatment groups comprised of a minimum of 6 animals. Error bars represent standard deviations. Statistical significance was evaluated by Student T-test. Statistically significant data points ($p<0.05$) are indicated by *.

3.2 Conclusion

Our data show that EVs can transmit radiation-related signals in the bone marrow and significantly alter the proportion of the different subpopulations in the stem cell compartment. It is very important to highlight that radiation-related signals transmitted by EVs were persistent and could be detected even 3 months after irradiation. However, if EVs were isolated from the animals 3 months after irradiation, EVs could not transmit any bystander signal.

4 Changes in the apoptotic rate, proliferation index and DNA damage level of haematopoietic cells treated with EVs

4.1 A: In the bone marrow

4.1.1 Methods

Apoptosis in the BM single cells prepared as described above was evaluated using the Tunnel assay. The presence of γ-H2AX foci, indicating DNA damage was investigated by flow cytometry using fluorescently labelled antibodies against the S139 phosphorylated H2AX protein.

4.1.2 Results

Apoptosis was an early event both after direct irradiation and EV treatment. While direct irradiation led to a dose-dependent increase in the rate of apoptosis in most of the studied cell populations, EV effects were detectable only in certain cell subpopulations. The rate of apoptosis in HSCs increased strongly both in directly irradiated and EV-treated mice but only after high dose irradiation (Figure 7A). Apoptosis in the lymphoid progenitors increased dose-dependently after direct irradiation. EVs induced increase in apoptotic frequency only after high dose irradiation (Figure 7B).

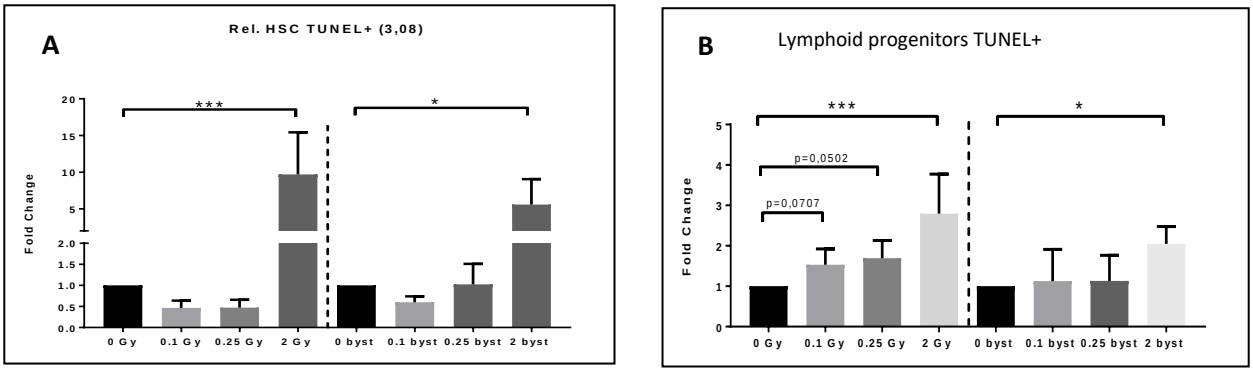


Figure 7: Evaluation of apoptosis in the different bone marrow subpopulations in the directly irradiated and EVC-treated mice. Mice were total-body irradiated with the indicated doses. EVs were isolated from the directly irradiated animals as described in the Methods and were injected in naïve mice. Both direct radiation effects and EV-mediated effects were investigated 24 hours after treatment. Bone marrow single cell suspensions were immune phenotypes as described above and apoptosis in the individual cell subpopulation were measured by flow cytometry using the TUNNEL assay. A: Apoptosis in the haematopoietic stem cells; B: Apoptosis in the lymphoid progenitors. Individual treatment groups comprised of a minimum of 6 animals. Error bars represent standard deviations. Statistical significance was evaluated by Student T-test. Statistically significant data points ($p<0.05$) are indicated by *.

Previously we have shown that EVs can transmit DNA damage in the spleen [1]. Here we investigated whether EVs can transmit DNA damage in the bone marrow. While direct irradiation yielded a dose-dependent increase in the frequency of γ-H2AX foci in all studied BM subpopulations, EV-mediated effects were either absent or statistically not significant.

4.2 B: In the spleen of leukaemic mice

An immunohistochemistry analysis of mouse tissues derived from animals treated with total body irradiation was performed.
First spleen tissue from leukaemic mice was compared to non-leukaemic mice.
Due to the fact, that one mouse (non-leukaemic) showed enlarged and morphological altered kidneys, we further investigated the normal kidney compared to the tumor kidney of the same mouse. Analysis covered quantification of double-stranded DNA breaks by γ-H2AX staining, proliferating cells detected by Ki-67 and apoptosis detected by cleaved Caspase 3. For morphological evaluation of the tissues, hematoxylin-eosin (HE) staining was performed in addition.

4.2.1 Methods

Tissue extraction

The spleen and kidney tissue were removed and were fixed in 1% paraformaldehyde for 24 h and then stored in 70% Ethanol until they were embedded in paraffin to generate formalin-fixed, paraffin-embedded (FFPE) tissue.

Immunohistochemistry

(FFPE) tissues were subjected to a standardized staining procedure, using an automatic staining procedure with Leica Autostainer ST5010 for hematoxylin-eosin staining and BOND-MAX (Leica) for immunohistochemistry.
Primary antibodies were used for either Ki-67 (Mib1, Cell Signaling), phospho-Histone H2AX (γ-H2AX, Cell Signaling) and cleaved caspase 3 (Asp175, Cell Signaling). Next, dextran polymer conjugated

horseradish peroxidase and 3,3-diamino-benzidine (DAB) chromogen was used for visualization and hematoxylin-eosin (HE) for counterstaining.
Marker expression was evaluated semi-quantitatively by digitalization of the slides with an Axio ScanZ.1 slide scanner utilizing the Axio Zen2.3 software (Carl Zeiss Microscopy). Next, the proportion of cleaved Caspase 3, phospho-Histone H2AX and Ki-67 positive cells were calculated by the software QuPath-0.2.0. Data are given as percentages of positive cells per total tissue scan.

4.2.2 Results

The spleen morphology of the leukaemic mice was different to that observed in the non-leukaemic mice (Fig. 8 A a-d) with a more compact structure in leukaemic mice. The proportion of apoptotic (cleaved Caspase 3-positive) cells in the leukaemic mice was increased compared to that observed in the non-leukaemic mice (Fig. 8 A e-h, B and C). In contrast, the proportion of proliferating (Ki-67-positive) cells was not altered while DNA damage (γ-H2AX) was slightly reduced in spleens derived from leukaemic mice.

HE staining revealed a different morphology of the tumor-bearing kidney when compared to the normal kidney of a non-leukaemic mouse (Fig. 9 A a-d) based on the density of the tissues. Immunohistochemical staining showed a significantly increased proportion of apoptotic (cleaved Caspase 3-positive) cells in the tumor-bearing kidneys was compared to that observed in the normal kidney (Fig. 9 A e-h and B). The number of double-strand breaks detected by γ-H2AX was also increased in the tumor kidney compared to the normal kidney (Fig. 9 A m-p and B). Notably, we observed a reduced number of Ki-67-positive cells in malignant tissue (Fig. 9 A i-l).

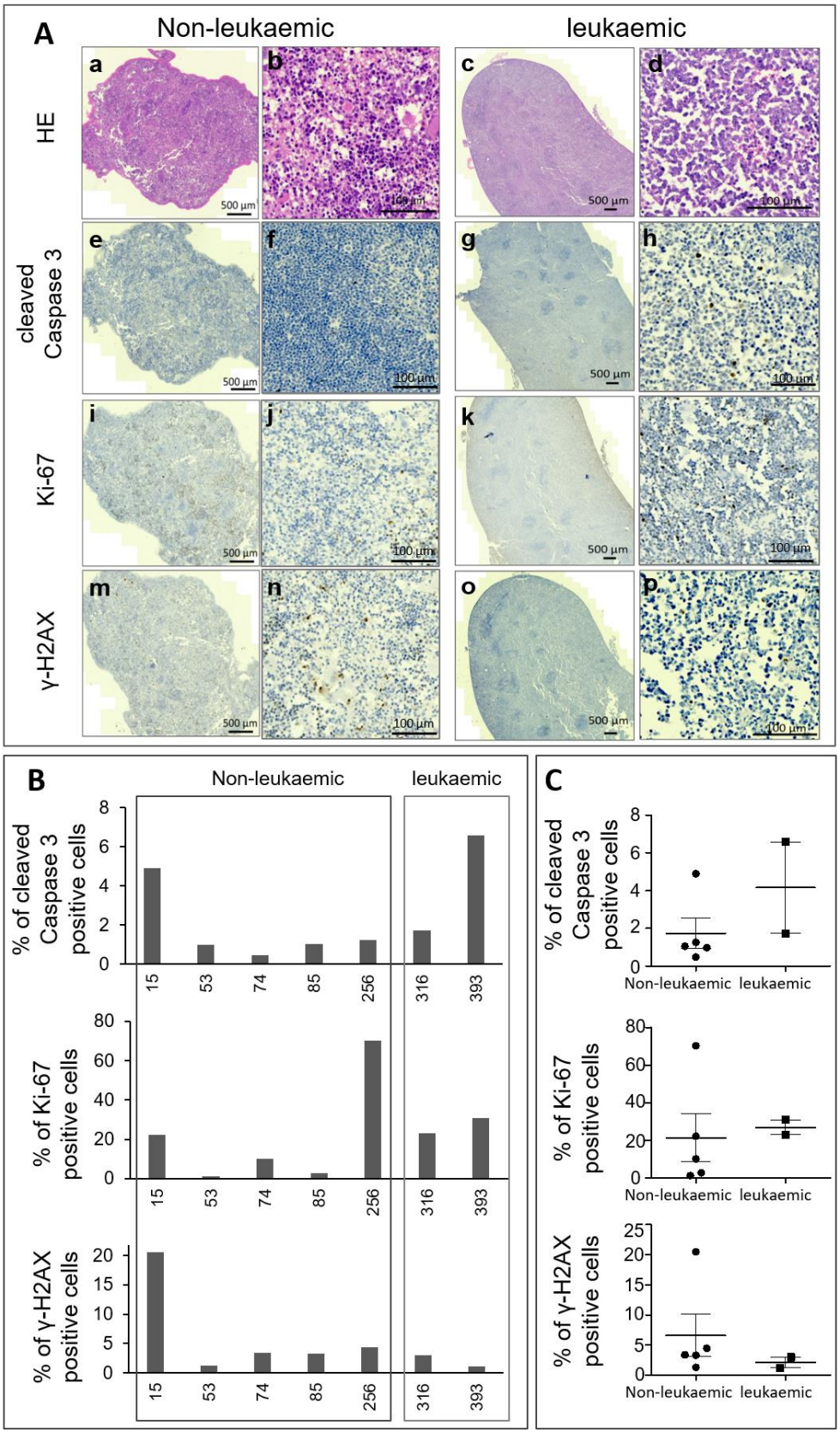


Figure 8: A Examples of HE staining and immunohistochemistry of leukaemic and non-leukaemic mice spleens. Tissue stained with Hematoxylin-eosin (HE) (a-d) and immunohistochemical staining with cleaved Caspase 3 (e-h), Ki-67 (i-l) and γ-H2AX (m-p). **B** Quantitative analysis of non-leukaemic and leukaemic mice of individual markers (Ki-67, cleaved Caspase 3 and γ-H2AX) was performed by the software QuPath V0.2. **C** Scatter plots showing distribution of cleaved Caspase 3, Ki-67 and γ-H2AX of non-leukaemic and leukaemic mice. Data are derived from 5 normal mice and 2 leukaemic mice.

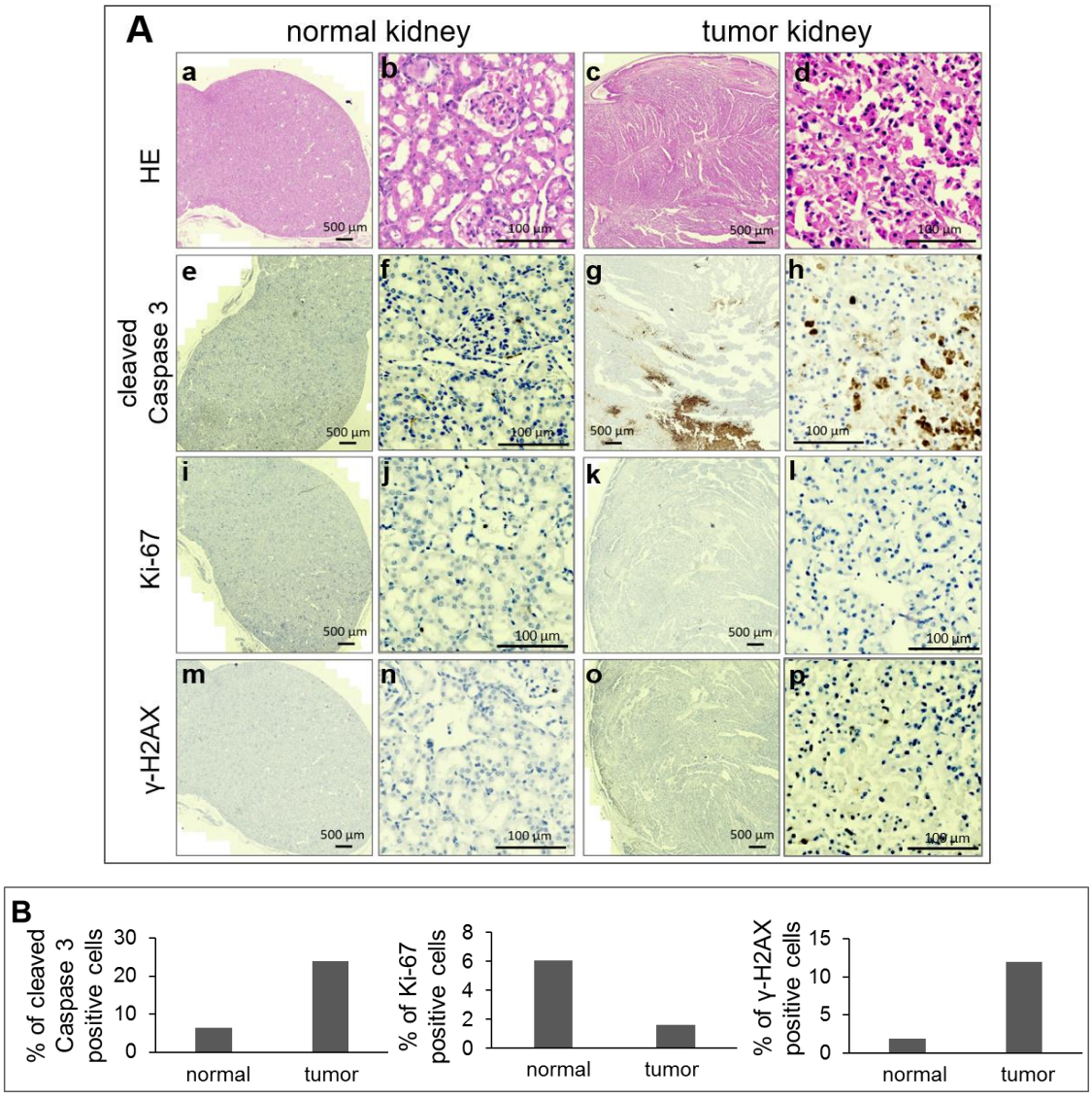


Figure 9: (A) Examples of HE staining and immunohistochemistry of normal and tumor mouse kidney. Tissue stained with Hematoxylin-eosin (HE) (A-D) and immunohistochemical staining with cleaved Caspase 3 (e-h), Ki-67 (i-l) and γ-H2AX (m-p). B Quantitative analyses of normal kidney compared to tumor kidney of individual markers (Ki-67; cleaved Caspase 3; γ-H2AX) were performed by the software QuPath V0.2.0 (Belfast, Northern Ireland). Data are derived from one mouse.

5 Changes in the oxidative status and antioxidant system in the spleen of mice directly irradiated or treated with EVs

Since previously we showed that EVs can transmit DNA damage signals in a bystander manner in the spleen, we investigated whether these effects are due to the transmission of oxidative stress. Although these investigations were carried out in the spleen so far, similar experiments are in progress in the bone marrow as well.

5.1 Methods

Male mice were exposed to X-ray irradiation of 0 Gy, 0.1 Gy and 2 Gy at the age of 11-13 weeks. Spleen tissue was collected 24 hours after irradiationand EV was isolated from bone marrow of mice 24 hours after irradation with Exoquick TC kit. 10 µg bone marrow-derived EV of 0 Gy, 0.1 Gy or 2 Gy irradiated mice was next injected into the tail vein of naïve animals, and these animals were later called 0 Gy

bystander, 0.1 Gy bystander, and 2 Gy bystander mice, respectively. At 24 hrs after irradiation and 24 hrs after injection of bystander mice, spleen tissue was collected and immediately snap-frozen in liquid nitrogen and stored at -80 °C until analyses. RNA was isolated from spleen with RNeasy Mini kit (Qiagen) and cDNA was transcribed with Revert Aid First Strand Kit (Thermo), according g to the manufacturer's instructions. Real-time PCR analyses were conducted with Maxima SYBR Green master mix (Thermo) in a Rotor-Gene Q (Qiagen) real-time PCR thermocycler. Oligonucleotide primers were obtained from Integrated DNA Technologies. Polg2 (DNA polymerase subunit gamma-2) was used as an endogenous control gene for normalization. SOD enzyme activity was analysed with SOD Assay kit (Sigma) according to the manufacturer's instructions. Protein carbonyl concentration was analysed with an Oxiselect Protein Carbonyl ELISA kit (Cell Biolabs) according to the manufacturer's instructions.

5.2 Results

5.2.1 A. Gene expression

a. Antioxidant enzymes (SOD2, CAT, GSTs)

We found that exposure to 0.1 Gy did not affect the expression of any of the studied antioxidant enzymes in the spleen 24 hours after exposure (Figure 10-12)24 hours after exposure to 2 Gy irradiation the expressions of SOD2 (superoxide dismutase 2, mitochondrial) (Figure 10) and CAT (catalase) genes (Figure 11) were reduced in the spleen, and expression of GSTs (glutathione S-transferase) also showed a decreasing tendency, although it did not reach a level of significance (Figure 12)while the expression of SOD2, CAT and GSTs did not change in the spleen (Figure 10-12) 24 hrs after exposure to bone marrow-derived EVs of mice exposed to 0.1 Gy.

24 hrs after exposure to bone marrow-derived EVs of mice exposed to 2 Gy, the expression of all studied antioxidant enzymes (SOD2, CAT and GSTs) was significantly reduced (Figure 10-12).

These results suggest that EVs from irradiated cells may transfer signals that may reduce the expression of antioxidant enzymes, similarly as found in the directly irradiated tissue.

The reduced expression of antioxidant enzymes may elevate cellular level of oxidative stress, which may induce DNA damage that was observed in bystander cells via gamma-H2AX analysis in our earlier studies.

Figure 10. Median (± SD) relative gene expression of superoxide dismutase 2 (mitochondrial) enzyme in the spleen of mice 24 hrs after exposure to irradiation (0.1 Gy, 2 Gy) or exposure to bone marrow-derived EVs of irradiated mice (0.1 Gy Byst; 2 Gy Byst). 0 Gy and 0 Gy Byst were considered to be the baseline expression in the directly irradiated and bystander groups, respectively. N = 6. Statistical significance was evaluated by Student T-test. Statistically significant data points ($p<0.05$) are indicated by *.

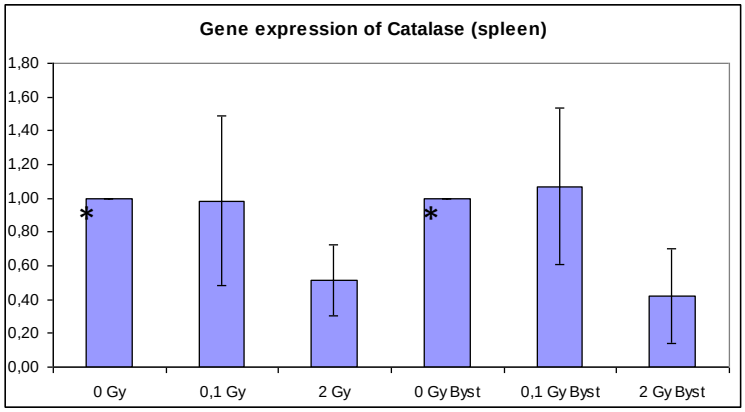


Figure 11. Average (± SD) relative gene expression of catalase enzyme in the spleen of mice 24 hrs after exposure to irradiation (0.1 Gy, 2 Gy) or exposure to bone marrow-derived EVs of irradiated mice (0.1 Gy Byst; 2 Gy Byst). 0 Gy and 0 Gy Byst were considered to be the baseline expression in the directly irradiated and bystander groups, respectively. N = 6. . Statistical significance was evaluated by Student T-test. Statistically significant data points ($p<0.05$) are indicated by *.

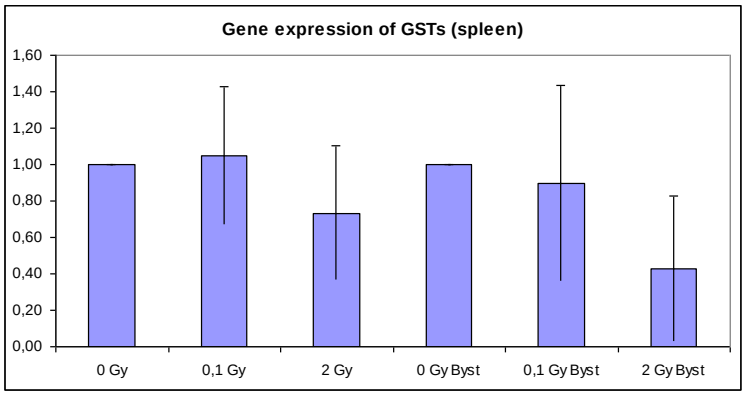


Figure 12. Average (± SD) relative gene expression of glutathione S-transferase enzyme in the spleen of mice 24 hrs after exposure to irradiation (0.1 Gy, 2 Gy) or exposure to bone marrow-derived EVs of irradiated mice (0.1 Gy Byst; 2 Gy Byst). 0 Gy and 0 Gy Byst were considered to be the baseline expression in the directly irradiated and bystander groups, respectively. N = 6.

b. DNA methyltransferase 1 (DNMT1)

We found that the expression of DNMT1 in the spleen did not change 24 hours after exposure to 0.1 Gy dose, but it significantly decreased after exposure to 2 Gy dose (Figure 12). 24 hours after exposure to bone marrow-derived EVs of mice exposed to 2 Gy dose, the expression of DNMT1 in the spleen tended to decrease, although it was not significant (Figure 13). The decreased expression of DNMT1 may lead to global hypomethylation, which can induce genetic instability.

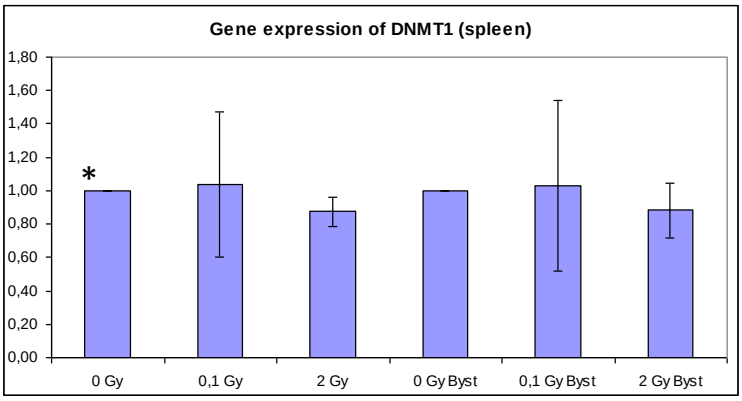


Figure 13. Average (± SD) relative gene expression of DNA methyltransferase 1 enzyme in the spleen of mice 24 hours after exposure to irradiation (0.1 Gy, 2 Gy) or exposure to bone marrow-derived EVs of irradiated mice (0.1 Gy Byst; 2 Gy Byst). 0 Gy and 0 Gy Byst were considered to be the baseline expression in the directly irradiated and bystander groups, respectively. N = 6. Statistical significance was evaluated by Student T-test. Statistically significant data points ($p<0.05$) are indicated by *.

c. *Senescence marker gene (P16)*

We found that the expression of P16 (cyclin dependent kinase inhibitor 2A; Cdkn2a) in the spleen did not change significantly 24 hours after exposure to 0.1 Gy dose or after exposure to bone marrow-derived EVs of 0.1 Gy irradiated mice, although a tendency for an elevated expression was observed. 24 hours after exposure to 2 Gy we detected a highly significant elevation in the expression of P16 gene in the spleen. Similarly, expression of P16 gene showed a significant increase 24 hours after exposure to bone marrow-derived EVs of 2 Gy irradiated mice (Figure 14).

These results indicate that exposure to irradiation elevate the number of cells that had slowed down the cell cycle in order to repair DNA damage, and this phenomenon was similarly observed in the spleen of mice exposed to EVs of 2 Gy-irradiated mice suggesting that EVs from irradiated cells may transfer signals that may slow down the cell cycle.

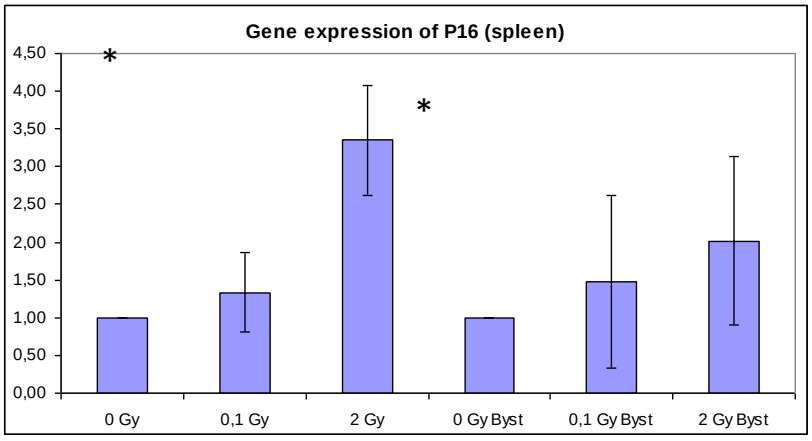


Figure 14. Average (± SD) relative gene expression of P16 in the spleen of mice 24 hrs after exposure to irradiation (0.1 Gy, 2 Gy) or exposure to bone marrow-derived EVs of irradiated mice (0.1 Gy Byst; 2 Gy Byst). 0 Gy and 0 Gy Byst were considered to be the baseline expression in the directly irradiated and bystander groups, respectively. N = 6. Statistical significance was evaluated by Student T-test. Statistically significant data points ($p<0.05$) are indicated by *.

5.2.2 B: Antioxidant enzyme activity

We found that the activity of superoxide dismutase (SOD) enzyme in the spleen did not change in the directly irradiated group. In bystander animals, we observed a slight decrease in 2 Gy bystander group, however, it was not significant (Figure 15).

We intend to increase the samples size of this test and we also aim to study the activity of catalase enzyme.

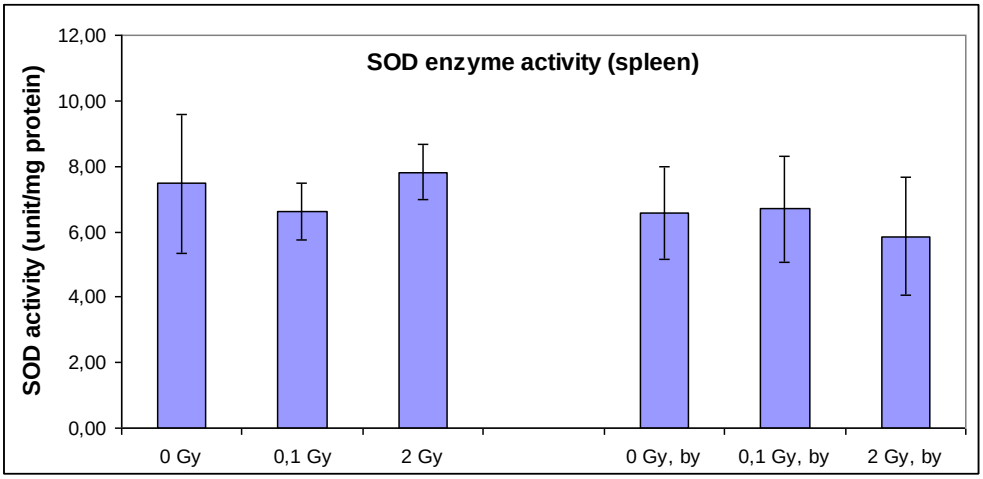


Figure 15. Average (± SD) activity of SOD enzyme (unit/mg protein) in the spleen of mice 24 hrs after exposure to irradiation (0 Gy, 0.1 Gy, 2 Gy) or exposure to bone marrow-derived EVs of irradiated mice (0 Gy Byst; 0.1 Gy Byst; 2 Gy Byst). N = 4.

5.2.3 C: Protein carbonylation

We observed that protein carbonyl concentration in the spleen tended to be higher in 2 Gy Byst animals, which may be related to the higher level of oxidative stress in this group. However, due to the high intragroup variation, the effect was not significant (Figure 16).

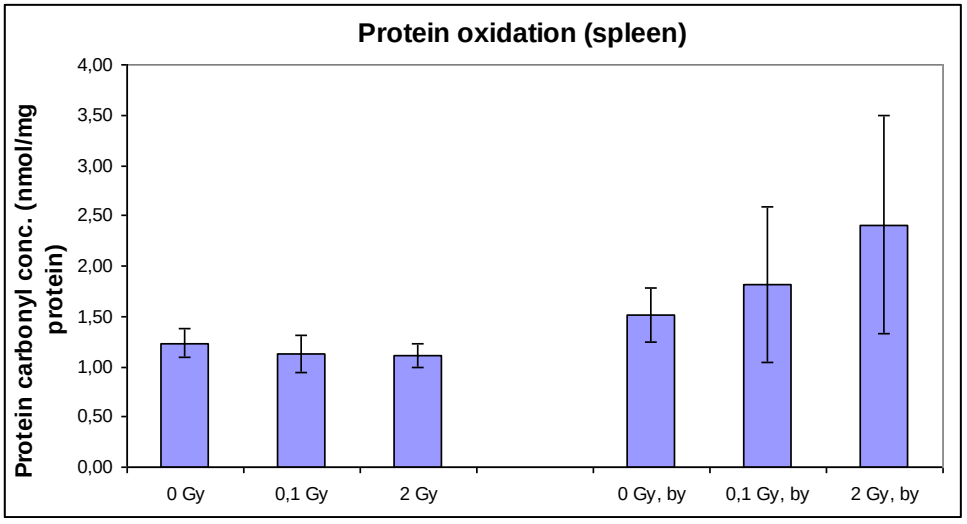


Figure 16. Average (± SD) protein carbonyl concentration (nmol/mg protein) in the spleen of mice 24 hrs after exposure to irradiation (0 Gy; 0.1 Gy; 2 Gy) or exposure to bone marrow-derived EVs of irradiated mice (0 Gy Byst; 0.1 Gy Byst; 2 Gy Byst). N = 6.

5.3 Conclusions

In summary ,we found indications that that level of oxidative stress in the spleen 24 hours after exposure to high dose (2 Gy) radiation was elevated, but this effect could not be observed in the low-dose (0.1 Gy) group.Similarly, mice exposed to EVs derived from the bone marrow of 2 Gy irradiated animals showed reduced expression of antioxidant enzymes, elevated expression of a senescence marker, and a tendency for lower SOD activity and higher concentration of oxidatively-damaged proteins (protein carbonyls).

In conclusion, our results suggest that EVs may transfer signals to recipient cells that may elevate level of oxidative stress.

Further studies are needed to elucidate the EV cargo that may evoke these effects.

6 The rate of EV uptake in the bone marrow and major EV acceptor cells

Our previous results indicated a differential effect of EVs in the different cellular subpopulations in the bone marrow. This suggests a differential uptake of EVs by the various cell types. Thus, we aimed to study EV uptake in the bone marrow and to identify EV uptake rate by individual cell types.

6.1 Methods

BM-derived EVs were fluorescently labeled and co-cultured with BM single cell suspensions. After the end of the incubation period BM cells were labelled with cell surface specific antibody markers and marker expression and EV uptake were evaluated by flow cytometry.

6.2 Results

BM single cell suspension derived from mice irradiated with low or high doses were incubated with EVs derived from mice irradiated with low or high doses. Regarding EV uptake by the unfractionated BM cell population we could show that:

- if non-irradiated BM cells were co-cultured with irradiated EVs, a minor decrease in EV uptake was seen after incubation with EVs from 2 Gy-irradiated mice,
- if irradiated BM cells were co-cultured with EVs isolated from non-irradiated mice, a major decrease in EV uptake was seen in the cells irradiated with 2 Gy;
- if the two scenarios were combined, a further decrease was seen in the cells which were irradiated with 2 Gy and were co-incubated with EVs from mice irradiated with 2 Gy. The effect was synergistic (Figure 17).

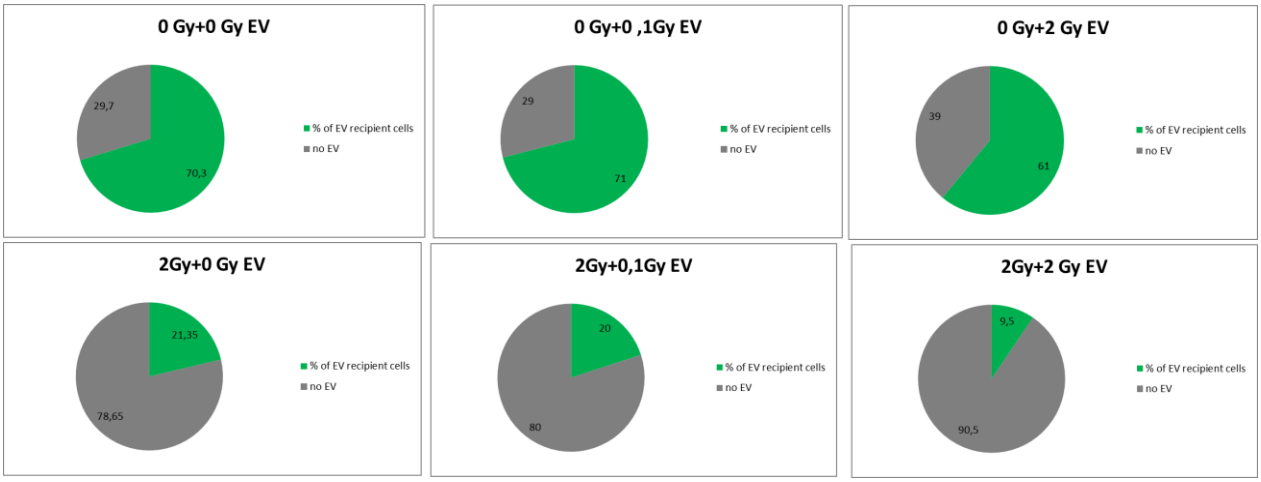


Figure 17. EV uptake by bone marrow cells. EVs originating from control or irradiated mice were fluorescently labelled and co-incubated in vitro with BM single cell suspension from non-irradiated or irradiated mice. The fractions of cells which have taken up the EVs were quantified by flow cytometry. Data represent the average of three independent experiments.

Next we investigated the distribution of the different BM subpopulation within the fraction of cells which picked up the EVs. Our data unravels the following:

- irradiation of either the cells directly or the EVs had the greatest impact on the EV uptake of two cell populations: the erythroid precursors and the myeloid progenitors. The rate of EV-acceptor erythroid progenitors decreased either after direct irradiation or after incubation with EVs from irradiated animals. In contrast, the rate of EV-acceptor myeloid progenitors strongly increased if either were originating from mice irradiated with 2 Gy or if were incubated with EVs originating from 2 Gy-irradiated mice.

This indicates that both irradiation of acceptor cells and irradiation of EVs influence EV uptake but irradiation of acceptor cells has a stronger effect.

7 Final conclusions

Within this deliverable we investigated mechanisms how EVs can transmit radiation-related signals in the haematopoietic system. Our most important conclusions can be summarized as follows:

- bystander signals transmitted by EVs are persistent and can be detected even 3 months after EV transfer;
- effects are cell-type specific and do not manifest uniformly in the different BM subpopulations;
- effects transmitted by the BM-derived EVs can be detected in the peripheral haematopoietic organs (spleen), where they induce gene expression changes relevant in oxidative stress and senescence induction,
- the rate of EV uptake differs in the various BM subpopulations and irradiation has a significant impact on the rate of EV uptake.

8 References

Szatmari T, Kis D, Bogdandi EN, Benedek A, Bright S, Bowler D, Persa E, Kis E, Balogh A, Naszalyi LN *et al*: **Extracellular Vesicles Mediate Radiation-Induced Systemic Bystander Signals in the Bone Marrow and Spleen**. *Front Immunol* 2017, **8**:347.