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ADVANCES IN RADIATION BIOLOGY – HIGHLIGHTS FROM 16TH ICRR SPECIAL FEATURE: FULL PAPER

The effect of ionising radiation on the phenotype of bone marrow-derived extracellular vesicles

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Objectives: Ionising radiation-induced alterations affecting intercellular communication in the bone marrow (BM) contribute to the development of haematological pathologies. Extracellular vesicles (EVs), which are membrane-coated particles released by cells, have important roles in intercellular signalling in the BM. Our objective was to investigate the effects of ionising radiation on the phenotype of BM-derived EVs of total-body irradiated mice.

Methods: CBA mice were irradiated with 0.1 Gy or 3 Gy X-rays. BM was isolated from the femur and tibia 24 h after irradiation. EVs were isolated from the BM supernatant. The phenotype of BM cells and EVs was analysed by flow cytometry.

Results: The mean size of BM-derived EVs was below 300 nm and was not altered by ionising radiation. Their

phenotype was very heterogeneous with EVs carrying either CD29 or CD44 integrins representing the major fraction. High-dose ionising radiation induced a strong rearrangement in the pool of BM-derived EVs which were markedly different from BM cell pool changes. The proportion of CD29 and CD44 integrin-harboring EVs significantly decreased and the relative proportion of EVs with haematopoietic stem cell or lymphoid progenitor markers increased. Low-dose irradiation had limited effect on EV secretion.

Conclusions: Ionising radiation induced selective changes in the secretion of EVs by the different BM cell subpopulations.

Advances in knowledge: The novelty of the paper consists of performing a detailed phenotyping of BM-derived EVs after *in vivo* irradiation of mice.

INTRODUCTION

Extracellular vesicles (EVs) are small (50–1000 nm) membrane-coated bodies released by most cell types.¹ EVs are traditionally divided based on their size and their biogenesis in three categories: exosomes (50–100 nm), microvesicles (100–1000 nm) and apoptotic bodies (above 1000 nm).² Although, there is a large overlap in the dimensions of these particles, for this reason, the most recent position paper of the International Society of EVs suggests two categories: vesicles below and above 200 nm.³ The former exosomes are mostly below 200 nm, while apoptotic bodies are mostly above 200 nm. EV cargo consists of nucleic acids (RNA, microRNA, DNA), proteins and lipids originating from the EV-releasing cells.^{4,5} By travelling in the extracellular space and blood, EVs transfer

their cargo to other distant cells, thus facilitating intercellular communication.^{6,7} Information transfer by EVs is realised through different mechanisms, such as receptor-mediated intracellular signal transduction by surface-expressed ligands, or delivering functional proteins, RNAs, and lipids into the target cells by fusion with plasma membrane or internalization into the endocytic compartment.⁸

EVs are secreted by cells both under physiological conditions and in various disease states. It has been shown that in cancerous microenvironment or under systemic stress conditions including ionising radiation, the release of EVs is increased.^{2,9} Irradiation also alters the protein and RNA content of EVs.^{10–12}

Radiation-induced bystander effects (RIBE) are the manifestation of ionising radiation effects on cells not directly hit by radiation.^{13,14} The mechanism of RIBE is attributed to signals transmitted by damaged cells directly via gap junctions or factors released into the extracellular space, such as reactive oxygen species, chemokines, cytokines, circulating-free microRNAs (miRNAs) and other danger signals.^{15,16} Several groups studied the role of EVs in RIBE mostly under *in vitro* conditions.^{17–20} Recently, we established and validated an *in vivo* bystander model suitable for investigating the role of bone marrow-derived EVs in mediating systemic RIBE both in the bone marrow (BM) and plasma.^{11,12}

In this paper, we investigated the effect of ionising radiation on the phenotype of BM-derived EVs in order to identify ionising radiation-induced changes in the EV secretion pattern of different BM subpopulations. The originality of the paper consists in the detailed phenotypical analysis of the different EV subpopulations released by the different BM cell subpopulations of *in vivo* irradiated mice.

METHODS

Handling and irradiation of mice

10- to 12-week-old CBA/Ca male mice were used in the experiments. Mice were kept and investigated in accordance with the guidelines and all applicable sections of the Hungarian and European regulations and directives. All animal studies were approved and permission was issued by Budapest and Pest County Administration Office Food Chain Safety and Animal Health Board (number of the ethical permission: PE/EA/392-7/2017).

Five mice were irradiated simultaneously by inserting them into a pie cage and subjected to total-body irradiation with 0.1 or 3 Gy using a X-RAD 225/Xli X-ray source (Precision X-ray, North Branford, United States). Control mice were handled in a similar way to the irradiated ones but not subjected to irradiation. Irradiated and control animals were kept in their standard conditions with food and water *ad libitum* for 24 h. 24 h later mice were sacrificed by intraperitoneal injection of 20 µL Euthanal 40% solution (Alfasan, JaWoerden, The Netherlands).

Extraction of BM cells and isolation and characterisation of BM-derived EVs

BM was isolated from the femur and tibia of directly irradiated and control mice as described previously.¹¹ Briefly, BM was flushed from the diaphyses, cells were pelleted by centrifugation at 400g, 4°C for 10 min and the BM supernatant was used for EV isolation. BM supernatants from five mice/dose were pooled to obtain sufficient amount of EVs. Cell debris and apoptotic bodies were eliminated by centrifugation at 3000g, 4°C for 15 min. EVs were isolated from the obtained supernatant using ExoQuick-TC™ kit (System Biosciences, Palo Alto CA, USA) according to manufacturer's instruction. Briefly, 1 volume ExoQuick-TC™ solution was added to five volumes of supernatant and incubated overnight at 4°C. EVs were pelleted by centrifugation at 1500 g, 4°C for 30 min and resuspended in 150–180 µL of 0.1 µm filtered PBS. GE Healthcare PD SpinTrap G-25 desalting columns (GE Healthcare, Life Science, WI, USA)

were used to remove ExoQuick polymers from the EV solution, based on the manufacturer's protocol. A negative control, containing solely PBS, was processed similarly to the EV-containing samples.

The amount of isolated EVs was determined based on the protein content measured by Bradford protein assay kit (Thermo Scientific, MA, USA) using a Synergy HT (Biotek, Winooski, USA) plate reader. The size of EVs was determined by dynamic light scattering (DLS) using an Avid Nano W130i DLS instrument (Avid Nano, High Wycombe, UK) and transmission electron microscopy (TEM). For TEM, EV samples were applied to nickel grids and stained with a 2% uranyl acetate (v/v) solution for 5 min. Grids were air-dried and viewed using a JEOL TEM 1011 TEM (JEOL, Peabody, MA, USA) operated at 80 kV. The camera used for image acquisition was a Morada TEM 11 MPixel from Olympus (Olympus, Tokyo, Japan) using iTEM5.1 software for metadata analysis.

Flow cytometry measurement

5 × 10⁵ BM cells and 40 µg of EVs were used for the individual staining combinations. EVs were first labelled with the EV-specific cell surface markers CD9, CD63 and CD81 (BioLegendSan Diego, CA, USA). Since these are all validated EV markers but their expression rate on the different EV subpopulations might be different, in order to easily identify all EVs in one step, we used antibodies conjugated to the same fluorescent label. Next, EVs and BM cells were labelled with antibodies specific for the different BM cell subpopulation surface markers. The following directly labelled anti-mouse monoclonal antibodies were used: CD90.2-APC and CD45-PE/Cy7 for lymphoid progenitors, CD61-APC and CD41-FITC for megakaryocytic population, Ter119-FITC and CD71-PE for erythroid precursors, CD11b-PE and Gr1-FITC for granulocytes/monocytes progenitors, Lineage Cocktail (Lin)-PB (CD3, Ly-6G/Ly-6C, CD11b, CD45R, Ter119), Sca1-PE and cKit (CD117)-APC for haematopoietic stem cells (HSCs), as well as Lin-PB, CD29-PE and CD44-APC for mesenchymal stromal cells (MSCs). MSCs were identified as CD44 + CD29 + double positive cells within the lineage negative cell fraction.^{21–23} All antibodies were purchased from BioLegend (San Diego, CA, USA) and Sony Biotechnology (San Jose, CA, USA). A custom cell surface staining protocol was applied for both cell and EV phenotyping, consisting of 30–60 min incubation at 4°C in the dark. In order to remove unbound antibodies, EV samples were pelleted by ultracentrifugation at 100,000g, 4°C for 70 min (Beckman Coulter, Brea, CA, USA) and resuspended in 0.1 µm filtered PBS. Both cell and EV preparations were measured immediately after staining using a CytoFlex flow cytometer (Beckman Coulter, Brea, CA, USA). Analysis of samples was performed using the CytExpert software (Beckman Coulter, Brea, CA, USA).

EVs were identified based on their size and the presence of EV-specific markers (CD9/CD63/CD81). For size gating of EVs Megamix Plus-SSC and Megamix Plus-FSC commonly called Gigamix beads (BioCytex, Marseilles, France) and hollow organosilica beads (HOBs)²⁴ were used.

Table 1. The effect of ionising radiation on the number of cells in the different bone marrow subpopulations.

	MSC	HSC	LP	GMP	EP	Eryt	MP
	Lin- CD29 +CD44+ (x10 ³)	Lin- Sca1 +cKit+ (x10 ³)	CD45 +CD90.2+ (x10 ⁶)	Gr1 +CD11b+ (x10 ⁶)	Ter119 +CD71+ (x10 ⁶)	Ter119+ (x10 ⁶)	CD41 +CD61+ (x10 ⁶)
0 Gy	35.368 (±6.473)	36.801 (±6.347)	0.392 (±0.048)	5.202 (±1.072)	3.793 (±1.173)	10.320 (±1.229)	0.340 (±0.040)
0.1 Gy	30.303 (±5.244)	25.636 (±9.061) *	0.371 (±0.085)	5.331 (±1.546)	3.606 (±0.864)	9.826 (±0.601)	0.307 (±0.079)
3 Gy	0.861 (±0.412) ***	0.524 (±0.258) ***	0.031 (±0.005) ***	0.408 (±0.128) ***	0.353 (±0.159) **	4.430 (±0.752) ***	0.050 (±0.009) **

Mice were irradiated with the indicated doses and 24h after irradiation BM cells were isolated from BM and labelled with antibodies against cell-type specific antibodies. The fraction of mesenchymal stromal cells (MSC), haematopoietic stem cells (HSC), lymphoid progenitors (LP), granulocyte – monocyte progenitors (GMP), erythroid progenitors (EP), erythroid lineage cells (Eryt) and megakaryocyte progenitors (MP) was measured by flow cytometry and the numbers of the individual cell populations were calculated based on total BM cell numbers. Data represent cell numbers within the lineage negative cell population (MSC and HSC), and within the total BM cells (all the other cells).

The mean (±SD) of data from five independent experiments is shown. Statistical significance was calculated with Student's t-test, changes are significant if *p < 0.05, **p < 0.01, ***p < 0.001 compared to 0 Gy controls

Statistical analysis

Data are presented as mean ± standard deviation (SD). Unpaired, one-sample Student's t-test and Mann-Whitney test were applied to determine statistical significance, using GraphPad Prism v.6.00 for Windows (GraphPad Software, La Jolla, CA, USA), if not stated otherwise. Data were considered statistically significant if *p*-value was lower than 0.05.

RESULTS

Ionising radiation-induced changes in the pool of BM cells

Alterations in the BM cells were studied 24h after radiation exposure of mice focusing on HSCs, MSCs, lymphoid, erythroid, granulocyte/monocyte progenitors and megakaryocytes. Low-dose (0.1 Gy) irradiation did not influence the pool of the studied cell populations with the exception of the HSCs, which decreased 1.4-fold (Table 1). High-dose (3 Gy) irradiation induced a drastic decrease in BM cell numbers but changes in the individual cell subpopulations greatly varied. By far the most sensitive cells were the HSCs, which decreased 70-fold compared to non-irradiated mice, followed by the MSCs (41-fold decrease). Progenitor cells were much less sensitive to high-dose irradiation with 11- and 12-fold reduction in the erythroid as well as lymphoid and granulocyte/monocyte progenitors, respectively, while megakaryocyte progenitors were even more resistant (approximately 7-fold reduction in the cell pool) (Table 1).

Absolute cell number changes of the studied cell subpopulations were very well reflected in their distribution among the total BM cells. The fraction of HSCs and MSCs in the Lin- BM cells decreased 18-fold and 10-fold, respectively, after 3 Gy irradiation compared to controls, while progenitor cell changes ranged between 1.6-fold reduction (megakaryocytes) and approximately 3-fold reduction (lymphoid, erythroid and granulocyte/monocyte progenitors) (Table 2).

In conclusion, these data indicate that HSCs are the most sensitive cell population in the BM, with statistically significant damage in the cell pool after low-dose irradiation already. Progenitor cells, on the other hand, are more resistant and their pool is not affected by low-dose irradiation, although, response of the different progenitor cell types to irradiation is heterogeneous.

Standardisation of EV analysis by flow cytometry
EVs were isolated from the BM supernatant and characterised as described previously.¹¹ DLS measurements indicated that the average diameter of the EVs was 228 nm (±52 nm). TEM images confirmed DLS measurements and showed that although EVs were diverse in size, the majority of the particles had diameters below 300 nm (Figure 1). EV phenotype was investigated by flow cytometry using a dedicated flow cytometer suitable for visualisation of vesicles above 100 nm.²⁵ In order to define a suitable gating for EV identification, two types of validation beads were used: the Gigamix polystyrene beads, which consisted of a commercially available mixture of beads with different diameters (between 100 nm and 900 nm) able to identify EVs with diameters within these limits; and the hollow-organosilica beads (HOBs), which consisted of beads with nominal diameter of 200 nm and 400 nm. Gigamix beads and HOBs had different refractive indices, which explain their different light scattering behaviour on the Violet SSC channel. Based on these two types of validation beads, we identified two populations: populations with a bigger diameter (identified by the Gigamix beads) and populations with a smaller diameter (identified by the HOBs) (Figure 2). Histogram plots showed that size range of HOBs (200 and 400 nm) was equivalent with polystyrene beads of around 100 nm or below (Figure 2). Despite the fact that these two gates had overlapping regions, we used both to delineate the EVs.

The number of events recorded in the two different types of gates allowed us to distinguish between negative controls (consisting of samples containing PBS only), background noise (consisting of samples containing PBS and residual polymers used for EV

Table 2. Percentage of subpopulations of bone marrow cells 24h after irradiation.

	MSC	HSC	LP	GMP	EP	Eryt	MP
	Lin- CD29 +CD44+ %	Lin- Sca1 +cKit+ %	CD45 +CD90.2+ %	Gr1 +CD11b+ %	Ter119 +CD71+ %	Ter119+ %	CD41 +CD61+ %
0 Gy	1.508 (±0.210)	1.582 (±0.308)	1.680 (±0.213)	22,200 (±3.997)	16.202 (±4.720)	44.144 (±4.248)	1.456 (±0.183)
0.1 Gy	1.356 (±0.270)	1.124 (±0.307) *	1.622 (±0.189)	23.292 (±4.331)	16.268 (±4.861)	43.942 (±5.283)	1.338 (±0.213)
3 Gy	0.152 (±0.067) ***	0.088 (±0.031) ***	0.570 (±0.168) ***	7.036 (±1.054) ***	6.222 (±2.329) **	77.878 (±2.705) ***	0.902 (±0.259) **

Mice were irradiated with the indicated doses and 24h after irradiation BM cells were isolated from BM and labelled with antibodies against cell-type specific antibodies. The fraction of mesenchymal stromal cells (MSC), hematopoietic stem cells (HSC), lymphoid progenitors (LP), granulocyte – monocyte progenitors (GMP), erythroid progenitors (EP), erythroid lineage cells (Eryt) and megakaryocytic population (MP) was measured by flow cytometry. Data represent percentages within the lineage negative cell population (MSC and HSC), and within the total BM cells (all the other cells).

The mean (±SD) of data from five individual experiments is shown. Statistical significance was calculated with Students's t-test, changes are significant if *p < 0.05, **p < 0.01, ***p < 0.001 compared to 0 Gy controls

isolation) and EV isolates (Figure 2). Figure 2 shows that negative controls had very few events in both gates, samples detecting background noise including residual polymers had events mainly in the upper regions of the Gigamix beads, while EV isolates had more events in the lower region of Gigamix gate and in the HOB gate, which is consistent with our DLS and TEM data indicating that the average size of our EV isolates were below 300 nm.

Within the EV gates defined on size and light scattering properties, EVs were further identified using the CD9, CD63 and CD81 EV-specific surface markers, which also indicated that the majority of the marker-positive events fell in the HOB gate (Figure 2). While distribution of these markers on the different EV subpopulations might vary, presence of any of them is an obligate criterion for EV identification³ and our preliminary

Figure 1. Characterisation of EVs by transmission electron microscope (A and B) and by dynamic light scattering (C and D). EVs were isolated from bone marrow of 0 Gy (A and C) and 3 Gy (B and D) irradiated mice using ExoQuick-TC solution by precipitation. Transmission electron microscopy was performed by applying the EVs on 300 mesh nickel grids, which were stained with 2% aqueous uranyl-acetate solution, and analysed by JEOL1011 transmission electron microscope. Scale bar shows size of the vesicles. Size distribution of EVs was determined measuring the hydrodynamic size by dynamic light scattering using an Avid Nano W130i DLS instrument. Images represent one typical capture of the samples.

Figure 2. Gating strategy of extracellular vesicles (EVs). (A) Size distribution of the Gigamix and hollow-organosilica beads (HOBs) using the side scatter channel of violet laser. (B) Distribution of events within the gates defined by the the Gigamix beads and HOBs. Percentages show the gated fraction within all detected events. All measurements were performed for 30 sc with constant flow rate. For PBS background, PBS with EQ polymer and EVs the total events were approximately 3000, 200000 and 360000, respectively. (C) Distribution of CD9/CD63/CD81 positive events in the defined EV HOB gate. EV-containing samples were labelled with the EV-specific CD9/CD63/CD81 antibodies as described in Methods.

investigations indicated that all three markers can be detected individually on the isolated BM-derived EVs (Figure 3). Thus, our approach was to use antibodies conjugated to the same fluorochrome for the detection of the above-mentioned three markers, which allowed us to determine the fraction of particles positive for any of these EV markers but we could not determine the ratio of EVs positive only for one marker within the population. This approach helped us to evaluate radiation-induced phenotypical changes in all EV subpopulations (Figure 3).

Ionising radiation induced changes in the phenotype of BM-derived EVs

Phenotypical evaluation was performed on smaller particles (identified based on the HOB gate) since these events are supposed to represent BM-derived EVs also in conformity with our DLS data (Table 3).

Most BM-derived EVs from control mice expressed integrins (30.5% and 42.1% of EVs were CD29+ and CD44+, respectively) typical for MSCs but present on other BM subpopulations as well (Table 3 and Figure 4). Since the fraction of MSCs represents less than 1.5% of the Lin- BM cells, this indicates that most probably EVs harbouring integrin markers are released by different cell types, not only MSCs and represent an abundant EV fraction in the BM. EVs carrying CD11b or Gr1 markers can be attributed to granulocyte-myeloid progenitors (GMP), although these markers are also present on other, more differentiated cells of myeloid origin. While the fraction of GMPs in the BM of control mice was 22% (Table 2), the fraction of EVs harbouring GMP markers were between 3.1% and 4% (Table 3). Ter119+ cells are of erythroid origin consisting of erythroid progenitors and more differentiated erythroid cells. Their fraction in the

BM was altogether 60.3% (Table 2). Although EVs carrying the Ter119 marker represented only 0.227% of total BM-derived EVs (Table 3). The presence of CD90.2 is rather specific to lymphoid progenitors (LPs), so EVs carrying CD90.2 can be attributed to LPs. Similarly, CD41 is also specific for megakaryocytes, thus EVs carrying this marker are considered of megakaryocyte origin. The fraction of LP- and megakaryocyte-derived EVs was 4.2% and 5.5%, respectively (Table 3), while the fraction of LP cells and megakaryocytes in the BM was 1.6% and 1.4%, respectively (Table 2). These data indicate that EV production by LPs and megakaryocytes were more abundant than EV production by myeloid or erythroid cells.

Ionising radiation did not have a major influence on EV size as it can be seen on TEM images and also indicated by DLS measurements (Figure 1). Although a precise comparison of the size of EV particles would require further analysis by other methods, such as nanoparticle tracking analysis. Low-dose ionising radiation did not change significantly the fraction of the different types of EVs in the BM with the exception of EVs harbouring lymphoid progenitor (LP) markers, which increased significantly (Table 3). High-dose (3 Gy) irradiation induced a significant decrease in EVs carrying the CD29 or CD44 integrin markers. Other EVs either did not change or increased after irradiation with 3 Gy. The most pronounced increase in EV production after 3 Gy was noted in HSC-related EVs carrying the c-kit marker. While the HSC pool decreased 18-fold after irradiation with 3 Gy, EV secretion increased with 4.2 fold. Strong increase was seen also in EV secretion by myeloid cells (carrying the CD11b or Gr1 marker) and LPs (carrying the CD90.2 marker) after 3 Gy, albeit changes were milder than in HSC-derived EV secretion (Figure 4).

Figure 3. Percentage of EV-marker positive events in EV samples. EVs were isolated from bone marrow tissue (of control mice) and labelled with EV-specific antibodies (CD9, CD63 and CD81). EVs were measured in HOB gate. Histogram gates were compared to PBS control sample. Pictures show data from one typical experiment.

In conclusion, high-dose irradiation-induced changes in the fraction of EVs carrying markers characteristic for the different BM subpopulations were markedly different from changes in the pools of the corresponding cellular subpopulations. This indicates that ionising radiation effects on the EV secretion by the different BM cell subpopulations were cell-type-specific and did not correlate with the radiosensitivity of the particular cell subpopulation.

DISCUSSION

Ionising radiation damages the BM not only by inducing cell death but also by altering intercellular signalling which in the long term can lead to various ionising radiation-related BM pathologies. EVs may play an important role in this process. EVs produced in the BM are very heterogeneous both in size and origin, originating from different cellular fractions. In order to study EV-mediated signalling networks in the BM, it is important to identify EVs of interest with the highest relevance for ionising radiation-related information transfer. Due to the fact that ionising radiation induces apoptosis in certain BM subpopulations, apoptotic bodies might be present at higher concentration in the irradiated samples. As stated in the Introduction section, apoptotic bodies are mainly larger vesicles with diameter above 200 nm. Currently, there is no well-defined method to separate apoptotic bodies from other vesicle types in a way to maintain EV

Table 3. The effect of ionising radiation on the percentage of EVs with typical bone marrow cell markers.

	CD29	CD44	cKit	CD90.2	Ter119	CD11b	Gr-1	CD41	CD61
0 Gy	30.50 (± 5.64)	42.07 (± 18.20)	0.83 (± 0.41)	4.29 (± 2.71)	0.23 (± 0.17)	3.16 (± 1.05)	4.06 (± 1.26)	5.54 (± 2.74)	0.09 (± 0.03)
0.1 Gy	34.46 (± 9.40)	47.06 (± 14.20)	0.65 (± 0.34)	11.25 (± 1.14)*	0.33 (± 0.17)	3.19 (± 1.39)	6.35 (± 1.36)	11.44 (± 5.25)	0.10 (± 0.01)
3 Gy	13.86 (± 4.05 ***)	24.71 (± 9.75 *)	3.48 (± 1.52)	9.15 (± 4.44)	1.35 (± 0.44 *)	3.75 (± 0.49)	8.18 (± 4.31)	9.00 (± 3.89)	0.37 (± 0.11)

Mice were irradiated with the indicated doses and 24 h after irradiation EVs were isolated from BM supernatant and labelled with EV-specific markers followed by antibodies against typical BM cell markers and analysed by flow cytometry as described in the Methods. Data represent mean ($\pm$ SD) percentages of EVs within the HOB gate. Four mice were pooled for one sample and minimum four parallels were used for statistical analysis.

Statistical significance was calculated with Student's t-test, changes are significant if * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to 0 Gy controls

Figure 4. Relative changes in the fraction of bone marrow (BM) cell subpopulations (A) and EVs (B) in irradiated mice compared to controls. Mice were irradiated with the indicated doses and 24 h after irradiation BM cells and EVs were isolated, labelled and analysed by flow cytometry as described in the Methods. Cell types (A) and EV types (B) are shown above the individual graphs. Error bars represent SD, p values are shown above the graphs.

yield and not loose part of the microvesicles. Differential centrifugation is the most accepted method for this purpose, which is an acceptable compromise between eliminating larger apoptotic bodies and maintaining EV yield as well. Although we recognise that DLS measurements can only give a rough estimate of EV size, still these data do not indicate an increase in the average size of EVs from the irradiated samples. Electron microscopy images and flow cytometry data further support our conclusion.

The aim of this work was to identify the main EV-releasing cell populations in the BM and to investigate how ionising radiation changes EV production by BM cells. Radiation-induced changes in the fraction of the major BM cell subpopulations and in the EV subgroups carrying BM cell surface markers of total-body irradiated mice were investigated by flow cytometry. Flow cytometry is a widely used and sensitive method for identifying and characterising subgroups in a mixed cell population. However, detection of single-vesicles below 1000 nm size is challenging due to their small size and difficulties in discriminating real particle events from background noise but also due to the low abundance of surface antigens present on EVs.²⁶ Introduction of calibration beads in the flow cytometric measurement for analysis of EVs can help in a better identification of real EV events.²⁷ In our study, determining the size gate for EV identification was performed by two types of calibration beads. First, we used a commercially available calibration bead mixture called Gigamix, which contains beads with different well-defined diameters ranging between 100 nm and 900 nm and inserted the gate in a way to identify particles ranging between 100 nm and 500 nm. This method is generally accepted,^{28,29} although recently its accuracy has been questioned,^{1,30} since synthetic solid beads and biological vesicles have different refractive indices (e.g., 200 nm

EVs scatter 40–300-fold less light than 200 nm polystyrene beads at a wavelength of 405 nm) for which reason polystyrene beads cannot be used directly for the sizing of EVs.^{31,32} Chandler et al. demonstrated that 400 nm polystyrene beads likely correlate with 1,000 nm microparticles.³¹ Erdbrugger et al have confirmed this using 200 nm liposomes and 200 nm polystyrene beads.³³ Therefore, beside Gigamix beads, we used the HOBs for EV gating. These beads have very similar light scattering properties to biological vesicles,²⁴ therefore they are superior to polystyrene beads in determining the size of EVs by flow cytometry. These findings are in agreement with our results, since we demonstrated that the gate/position on dot plot of 100 nm polystyrene beads corresponded to the largest particles in the 200–400 nm sized HOBs. This is also in accordance with our DLS and TEM measurements which indicated that the mean diameter of our EVs was below 300 nm.

As EVs carry surface markers reflecting their cellular origin,³⁴ identification of EVs released by BM cell populations was based on the presence of typical BM cell-specific antigens on the membrane surface of EVs. Due to characteristics of EV biogenesis, EVs originating from one particular cell type do not necessarily harbour all cell surface markers specific for that cell. Therefore, in contrast to our BM cell phenotyping approach, where identification of a cell subpopulation was done by a minimum of two or more cell surface markers, we used one-marker positivity to identify the cell of origin of EVs. This approach was used by others as well, for example EVs expressing either CD61, CD41 or CD235 and not necessarily all three markers were considered to be of platelet origin.^{32,35,36} Although by this approach, we cannot exclude the possibility that EVs carrying a particular surface marker originate from multiple cell types. For example,

several of these cell surface markers (e.g., CD29, CD44) are integrins primarily involved in cell-cell and cell-matrix communication within the BM niche and are not exclusively related to one cell type. The presence of integrins on EV surface is quite common since integrins have major role in EV binding and uptake by the target cells.^{6,37} This means that EVs carrying CD29 or CD44 cannot be attributed to MSCs only, despite the fact that MSCs are reported to be important EV producers.^{38,39} Similarly, both CD11b and Gr1 as single markers are present on different myeloid and granuloid cells in various stages of differentiation, not only on progenitor cells. Thus, CD11b+ or Gr1+ EVs indicate only their myeloid or granuloid origin. On the other hand, markers such as CD90.2 or c-kit are relatively specific for HSCs (c-kit) and lymphoid progenitors (CD90.2), thus EVs carrying these markers most probably originate from these cells.

BM cells are very radiosensitive and high-dose irradiation leads to massive cell death within BM stem and progenitor cells, as it was shown in our previous study in C57Bl/6 mice.¹¹ Here, we report very similar findings in CBA/Ca mice. Due to great differences in the radiosensitivity of BM cells and largely reduced cell numbers after irradiation with 3 Gy, we assumed altered EV secretion by BM cells of irradiated animals. Indeed, this was confirmed, since the distribution of EVs carrying different surface markers changed profoundly in the mice irradiated with 3 Gy compared to non-irradiated controls. A significant, approximately twofold reduction was seen in the fraction of EVs expressing the CD29 or CD44 integrins, which points to an ionising radiation-induced

alteration in integrin-mediated EV uptake. On the other hand, despite the fact that the pool of both HSCs and LPs significantly decreased after irradiation with 3 Gy, the fraction of EVs carrying HSC or LP markers did not change or even increased in these BM-derived EVs compared to control mice, indicating that EV release by HSCs and LPs increased after irradiation.

In conclusion, we showed that EVs carry markers characteristic for the different BM cell subpopulations but the presence of these markers allows identification of the cellular origin of the EVs only to a limited extent. High-dose ionising radiation induced a strong rearrangement in the pool of BM-derived EVs which were markedly different from BM cell pool rearrangements. Low-dose irradiation had very limited effect on EV secretion. These data indicate that EVs are among the major mediators of ionising radiation-induced signals in the BM beside other soluble molecules^{40,41} and a better understanding of the mechanisms of EV-mediated signalling could improve our understanding on how ionising radiation induces different BM pathologies.

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