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Use of a Humanized Mouse Model System in the Validation of Human Radiation Biodosimetry Standards

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Abstract

After a planned or unplanned radiation exposure, determination of absorbed dose has great clinical importance, informing treatment and triage decisions in the exposed individuals. Biodosimetry approaches allow for determination of dose in the absence of physical measurement apparatus. The current state-of-the-art biodosimetry method is based on the frequency of induced dicentric chromosomes in peripheral blood T cells, which is proportional to the absorbed radiation dose. Since dose-response curves used for obtaining absorbed dose for humans are based on data sourced from *in vitro* studies, a concerning discrepancy may be present in the reported dose. Specifically, T-cell survival after *in vitro* irradiation is much higher than that measured in humans *in vivo* and, in addition, is not dose dependent over some dose ranges. We hypothesized that these differences may lead to inappropriately inflated dicentric frequencies after *in vitro* irradiation when compared with *in vivo* irradiation of the same samples. This may lead to underestimation of the *in vivo* dose. To test this hypothesis, we employed the humanized mouse model, which allowed direct comparison of cell depletion and dicentric frequencies in human T cells irradiated *in vivo* and *in vitro*. The results showed similar dicentric chromosome induction frequencies measured *in vivo* and *in vitro* when assessed 24 h postirradiation despite the differences in cell survival. These results appear to validate the use of *in vitro* data for the estimation of the absorbed dose in human radiation biodosimetry.

INTRODUCTION

Radiation biodosimetry is an important part of dose determination strategies used in different radiation scenarios (1, 2). The most widely used source material in human biodosimetry is blood, from which resident cells can be withdrawn and assessed for radiation-induced damage using many different methodologies. The most precise of these methods is the estimation of frequency of dicentric chromosomes in peripheral blood T cells, which is proportional to the absorbed radiation dose (2–7). Despite the reliable results that can be acquired by this methodology, a potential problem is that the dose-response curves used when evaluating *in vivo* human radiation exposure are actually based on data sourced from *in vitro* studies. While response at the single cell level is agnostic to radiation insult alone, the *in vivo* response is systemic in nature. Any discrepancy in responses would impart an error in the doses reported for *in vivo* irradiation. There is some evidence to

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support a discrepancy (*ex vivo* vs. *in vivo*) in radiation response (8, 9). We and others have observed that the radiation-induced frequency of T-cell death *in vivo* is higher than that measured *in vitro*. Indeed, total-body irradiation (TBI) of patients leads to significant lymphocyte depletion, which is dose dependent (10, 11). In contrast, T-cell survival in irradiated samples of cultured T cells is higher (dose-to-dose comparison) and dose dependency in survival of *in vitro* is significantly reduced [(12, 13) and our results shown below]. Based on these observations, we considered that dicentric chromosome frequencies measured in *ex vivo* irradiated blood may be different from those acquired from *in vivo* irradiations where the overall survival outcomes for the cell is different. If true, the application of *ex vivo* generated curves to an *in vivo* process would result in underestimation of the predicted *in vivo* dose. Consequently, there is a need for an *in vivo* model that addresses this potential problem. We reasoned that a very good model in that respect might be the humanized mouse model, since it would permit the study of ionizing radiation effects in a circulating human T lymphocyte. This model has recently been validated for biodosimetry studies in a candidate protein marker study (14). Humanized mice are generated by starting with a genetically modified immunocompromised mouse, which is subsequently engrafted with a human hematopoietic system that populates the mouse bone marrow, spleen and peripheral blood with human lymphocytes. T cells can be found in significant numbers in the mouse spleen and blood of such humanized mice (15). After preliminary experiments with this system, we decided that in principle it can be used to characterize the *in vivo* radiation-induced dicentric chromosome frequencies in human cells present in the mice. Here we describe the results of a study in which the goals were: 1. To validate the humanized mouse model as an *in vivo* model for radiation-induced dicentric chromosome formation; and 2. To determine the validity of extrapolating radiation damage (via dicentric frequency expansion) from lymphocytes irradiated *ex vivo*.

A strategy comprised of three steps was employed to achieve these goals. The first step was to demonstrate radiation sensitivity equivalence between human T lymphocytes circulating in humans and comparable human T lymphocytes circulating in humanized mice. For this, we compared the DNA damage detection capacity of cells from these two sources using data obtained from γ -H2AX assay. The second step was to prove that the radiation-induced cell killing of human T cells from humanized mice resembled the observed differences for the same end point between *in vivo* and *in vitro* irradiations in humans. For this, we compared the radiation response of human cells from humanized mice and human donors for cell killing end points *in vitro* and *in vivo*. Finally, we sought to demonstrate that the increase of radiation-induced dicentric chromosome frequency with dose was equivalent in samples irradiated *ex vivo* and *in vivo*.

MATERIALS AND METHODS

Humanized Mice Generation

All animal husbandry and experimental procedures were conducted in accordance with applicable federal and state guidelines and approved by the Animal Care and Use Committee of Columbia University. Mice were housed at the Columbia University animal

facility (New York, NY) with a standard 12:12 h light-dark schedule and given water and regular rodent chow *ad libitum*.

Female immunodeficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice (Jackson Laboratory, Bar Harbor, ME), aged 6 to 8 weeks, were engrafted with commercially available human cord blood CD34⁺ cells (Cincinnati Children's Hospital Medical Center; Cincinnati, OH). For the engraftment, the NSG mice were exposed to 2.0 Gy of γ -rays, followed by injection of 200,000 human CD34⁺ into the mouse tail vein.

Sixteen weeks later, human cell engraftment was assessed in the mouse blood by flow cytometry, using antibodies specific to human blood cells including CD45 clone HI30 (leukocyte common antigen), CD3 clone UCHT1 (marker for T cells), CD19 clone 2H7 (marker for B cells), CD11b clone TCRF44 (marker for granulocytes, monocytes/macrophages) and mouse-specific CD45 antibody clone: 30-F11 (all from BioLegend® Inc., San Diego, CA).

Irradiations

All *in vivo* and *in vitro* irradiations were performed using an X-Rad 320 X-ray machine (Precision X-Ray Inc., North Branford, CT) operated at an energy of 320 kVp, a current of 12.5 mA and dose rate of 0.88 Gy/min. For *in vivo* irradiations, mice were placed in a specifically designed mouse irradiation holder (Precision X-Ray). Control mice were sham irradiated. The radiation dose was validated using a Radcal® ion chamber (Monrovia, CA) placed in the mouse holder. During the actual irradiations, the delivery dose was monitored by ion chamber placed in the mouse holder.

Blood Collection

Human donors.—Whole blood was collected from three healthy volunteers (age range of 29–48 years old), at Columbia University Medical Center. Informed consent was obtained from all volunteers according to IRB protocol. The donors were required to complete an anonymous questionnaire to ensure that they had not been exposed to ionizing radiation in the previous two years before the blood draw, thus minimizing the effect of radiation as a confounding factor. For each donor, 12 ml of blood was drawn by venipuncture into spray-coated sodium heparin Vacutainer® tubes (Becton Dickinson and Company, Franklin Lakes, NJ).

Humanized mice.—Mouse blood was obtained either from the tail artery for engraftment analysis (an average of 50 μ l) or by cardiac puncture for dicentric analysis after euthanization.

Gamma-H2AX Foci Frequency Estimation

Human T cells were separated from humanized mouse whole blood and blood from human donors employing a CD3-specific pluriBead® suspension according to manufacturer instructions (pluriSelect, San Diego, CA). Purified cells were washed and fixed by resuspension in Cytofix/CytoPerm reagent (Becton Dickinson, Floral Hill, NJ) for 15 min at room temperature. Fixed cells were washed with Perm/Wash™ reagent and permeabilized by

suspension in 0.1% IGEPAL® + 10% donkey serum in Perm/Wash reagent. Radiation-induced γ -H2AX foci were stained using a primary antibody [Anti-gamma H2A.X (phospho S140) antibody, clone 3F2; Abcam®, Cambridge, MA] at 1:500 in blocking solution for 1 h at room temperature before being washed in Perm/Wash reagent and stained with an Alexa Fluor® 488 conjugated anti mouse secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h at room temperature. Cells were washed with Perm/Wash reagent before final resuspension in phosphate buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS). The stained cells were transferred to glass bottom dishes (14-mm glass microwell; MatTek Corp., Ashland, MA), covered with glass coverslip and imaged by confocal microscopy after the cells settled to the bottom of the microwells.

The resulting confocal z-stacks were processed to maximum intensity projection and then counted directly from the screen. The advantage of this methodology is that using disaggregated, spherical cells permits exceptional spatial resolution, enhancing the accuracy of acquired foci counts.

Determination of Cell Counts and Viability

All cell counts shown in this study were obtained using a CytoFLEX (13 color flow cytometer with volumetric-based fluidics allowing the exact determination of the sample volume; Beckman Coulter® Inc., Brea, CA). Validation of cell numbers was performed using analyses of fluorescent bead suspensions with known concentrations per unit volume (SPHERO™ AccuCount Ultra Rainbow Fluorescent Particles, 5.0–5.9 μ m; Spherotech Inc., Lake Forest, IL).

Peripheral blood T-cell counts were measured in samples from human donors irradiated *ex vivo* and human T cells from humanized mice irradiated *ex vivo* and *in vivo* at 24 h postirradiation. For the *ex vivo* experiments, enriched fraction of lymphocytes was separated from the whole blood by a density gradient centrifugation method using Histopaque®–1083 (Sigma-Aldrich® LLC, St. Louis, MO) which, in mouse blood, delivered better separation of the human cells than Histopaque-1077 optimized for human blood separation. The separated cells were washed with PBS and diluted to 1×10^6 cells/ml with complete RPMI 1640 media (Life Technologies, Carlsbad, CA) supplemented with 20% FBS (Thermo Fisher Scientific™ Tnc., Waltham, MA) and antibiotics (100 TU/ml penicillin, 100 μ g/ml streptomycin). The samples were divided and portions irradiated with 1, 2, 3 and 6 Gy. Control cells were sham irradiated. After irradiation, the samples were incubated in a humidified chamber at 37°C in 5% CO₂ for 24 h. Cell counts obtained from the *in vivo* irradiated humanized mice were measured by staining of mouse blood with anti-human-specific CD45 and CD3 antibodies and compared to the T-cell counts of the same mice obtained 2–3 weeks prior to irradiation.

Human T-cell viability was analyzed using Annexin-V apoptosis detection kit (Biolegend) according to manufacturer instructions. For this assay, human peripheral blood mononuclear cells (PBMC) from human donors and humanized mice were purified, as described above. Human T cells were identified by staining with APC-labeled anti-human CD3 antibody

(clone UCHT1; Biolegend) allowing minimal interference with the FITC-labeled Annexin-V during flow cytometry.

Dicentric Chromosome Assay

Some of the characteristics of the humanized mice impose specific limitations on chromosome dicentric studies. For example, because of the limited number of T cells surviving the *in vivo* irradiations and the large number of cells needed for dicentric studies, we had to isolate human lymphocytes from both blood and spleen of the humanized mice. This approach was validated in preliminary comparisons, which show that the radiation-induced dicentric frequencies in human T cells isolated from mouse blood and spleen from the same mouse are identical (data not shown). In addition, in contrast to human blood, the heterologous circulating factors present in mouse blood are known to inhibit mouse and human PHA-induced T-cell mitotic stimulation and cell cycle progression *in vitro* (16). We were able to reproduce that finding and observed an almost complete inhibition of T-cell division in both cultured humanized mouse blood diluted to 1:10 in RPMI/20% FBS media and spleen cell suspension diluted to 1:10 in the same media. To circumvent this limitation, for all experiments using human cells from humanized mouse blood, PBMC purification was performed.

Some optimization of the cell isolation methodology was also performed. Specifically, we found that the most efficient way (in terms of cell yield) to isolate human lymphocytes from mouse blood was by using Histopaque gradient centrifugation (Histopaque-1083; Sigma-Aldrich), while the most efficient way to isolate human cells from mouse spleen was to use two rounds of RBC lysis according to Koprowski *et al.* (17). All isolated cells were cultured in RPMI 1640 media (Life Technologies) supplemented with 20% FBS (Thermo Fisher Scientific) and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin).

Experimental schemes, described below, were used for the dicentric frequency measurements.

Cells from humanized mice for *in vitro* irradiation.—This was comprised of blood draw, cell purification, irradiation, cell culture for 2 h, 4% PHA addition and colcemid addition 24 h after the culture was set up to arrest cells at first division.

Blood from donors.—This was comprised of blood draw, irradiation, blood incubation for 2 h, 1:10 sample dilution with culture media supplemented with 4% PHA, colcemid addition after 24 h after the culture was set up.

Cells from humanized mice irradiated *in vivo*.—This was comprised of blood draw 24 h postirradiation, cell purification, cell resuspension in culture media supplemented with 4% PHA, colcemid addition 24 h after the culture was set up. The 24 h blood collection time point was used after *in vivo* irradiation, because it is recommended to ensure homogeneous blood cell mixture in non-uniformly exposed individuals (4). However, dicentric chromosome frequencies are considered stable *in vivo* for up to few weeks after exposure.

All samples were processed for dicentric frequency estimation at 50 h after the culture was set up with hypotonic solution treatment (KCl 0.075 M; Life Technologies) and fixation with Carnoy's solution (methanol:glacial acetic acid, 3:1, v/v). Slides were stained with Leishman stain (Leishman eosin methylene blue solution modified; Sigma-Aldrich). This histological stain allows for the identification of asymmetrical chromosome aberrations, including dicentrics. Chromosome analyses were performed only in human metaphases containing 46 centromeres. This allowed efficient segregation of human cells from mouse cells. A minimum of 1,000 cells per condition were scored in sham-irradiated and 1 Gy irradiated samples and 500 cells in 2 and 3 Gy irradiated samples. Multicentric chromosomes (di-, tri-, tetra-...) were recorded only when the corresponding number of acentric fragments was present. Multicentrics were converted into the equivalent number of dicentrics as $(n - 1)$, where n is the number of centromeres.

Statistics

To determine if the distribution of dicentrics among cells followed a Poisson, the U test was used (18). U values outside the interval ± 1.96 indicate that the dicentric cell distribution does not follow a Poisson with a 5% level of significance. To assess the potential statistical significance of radiation effects in the different sources of human T cells on various end points, statistical comparisons between means were performed using Student's t tests. Unpaired tests were applied to data on independent samples (e.g., irradiated versus control mice), and paired ones were applied to situations where repeated measures were obtained (e.g., cell counts at different time points on the same mice).

RESULTS

Comparisons of the DNA Damage Detection Capacity of Human Cells Isolated from Human Donors and Humanized Mice

In these experiments, we compared the *ex vivo* radiation-induced γ -H2AX foci frequencies in cells isolated from human donors and humanized mice. We used cells from three donors per dose, and two doses of radiation. The foci counts were obtained from 50 cells per donor and condition.

The results (Fig. 1) show a similar pattern of distribution of the γ -H2AX foci in human T cells from human and mouse donors for both the 0.5 and 1 Gy radiation doses. No statistically significant interindividual differences were detected when the mean γ -H2AX foci frequencies from the donors were compared for each dose (Fig. 2). The average values $\pm$ standard deviation were obtained for each of the human donors, as follows: 8.92 ± 2.43 , 7.36 ± 1.79 and 7.54 ± 1.48 after 0.5 Gy irradiation; and 17.42 ± 2.73 , 15.94 ± 1.84 and 13.94 ± 2.86 after 1 Gy irradiation. Identical calculations performed for the samples from the humanized mice exhibited a similar result: 7.24 ± 1.41 , 7.68 ± 2.02 and 7.58 ± 1.33 for 0.5 Gy irradiation; and $(15.68 \pm 2.45, 13.84 \pm 2.71 \text{ and } 14.18 \pm 1.98)$ for 1 Gy irradiation (Fig. 2).

The differences between the γ -H2AX foci frequencies for the 0.5 Gy and 1 Gy radiation doses were statistically significant for both sample types ($P = 0.0067$ for humanized mice; $P = 0.0048$ for human donors).

Importantly, no statically significant difference in the mean foci frequencies was found between the cell from human donors and humanized mice ($P = 0.36$ for 0.5 Gy; $P = 0.37$ for 1 Gy).

Together, these findings indicate that the DNA damage repair capacity of human cells from humanized mice is similar to human cells isolated from human donors.

T-Cell Depletion after In Vivo and Ex Vivo Irradiation

The goal of these experiments was to determine if the differences in radiation-induced lymphocyte death, measured *in vivo* and *ex vivo*, in humans can be reproduced in the humanized mouse model. T-cell counts in blood were measured in samples from human donors irradiated *ex vivo* and compared to blood T-cell counts from humanized mice irradiated both *ex vivo* and *in vivo* 24 h after irradiation. As shown in Fig. 3, a substantial dose-dependent human T-cell depletion was observed after *in vivo* irradiation of humanized mice. The average *in vivo* human cell counts at 24 h postirradiation with 1, 2 and 3 Gy were $53.37 \pm 3.64\%$, $25.74 \pm 2.00\%$ and $13.87 \pm 2.42\%$, respectively. The *t* tests that we performed revealed statistically significant differences in the cell counts between controls and irradiated samples at all doses ($P = 0.000107$ at 1 Gy, $P = 3.33\text{E}-8$ at 2 Gy and $P = 1.85\text{E}-6$ at 3 Gy) and also in dose-to-dose comparisons ($P = 0.0009$ for comparison of 1 and 2 Gy; $P = 0.0047$ for comparison of 2 and 3 Gy). Mice were not irradiated with 6 Gy because the human T cells would not be expected to survive this condition.

The cell counts in the *in vitro* irradiated samples exhibited a different response. Specifically, human donor T-cell counts 24 h after irradiation were $93.99 \pm 6.08\%$ for 1 Gy and $80.38 \pm 8.24\%$ for 6 Gy. The results obtained from irradiated human T cells isolated from humanized mice were very similar. The performed *t* tests revealed no statistically significant differences in the cell counts between controls and all radiation doses (i.e., 1, 2, 3 and 6 Gy) in human cells *ex vivo* irradiated from humanized mice ($P = 0.439$ at 1 Gy, $P = 0.1$ at 2 Gy, $P = 0.132$ at 3 Gy and $P = 0.093$ at 6 Gy) and for human donors ($P = 0.288$ at 1 Gy, $P = 0.482$ at 2 Gy, $P = 0.254$ at 3 Gy and $P = 0.063$ at 6 Gy). Also, no statistically significant differences in cell counts were detected in any of the dose-to-dose comparisons. Although no significant dose dependence was detected, blood cell counts were reduced commensurate with radiation dose (Fig. 3). Together, these findings point to a significant difference in cell counts between the *in vivo* and *ex vivo* conditions. We hypothesize that the observed high cell counts in the *ex vivo* irradiated samples may be due to the presence of T lymphocytes that are engaged in apoptosis but have yet to be completely eliminated. To confirm this hypothesis, we performed an apoptosis assay based on Annexin-V staining at 24 h postirradiation.

As shown in Fig. 4A, the proportion of Annexin-V-positive cells in cultured human T cells from humanized mice varied from $18.3 \pm 1.67\%$ (sham-irradiated samples) to $25 \pm 1.53\%$ (6 Gy irradiated samples). Analogous values for human T cells from donors were $8.7 \pm 0.7\%$

and $23 \pm 0.58\%$. Importantly, even if the apoptotic cells are scored as dead cells and eliminated from the total cell count per condition, the cell counts after irradiation *in vitro* are still much higher than the cell counts after *in vivo* irradiation (dose-to-dose comparison).

Radiation-Induced Dicentric Chromosome Analysis

The results of the dicentric analysis of T cells from humanized mice irradiated *in vivo*, samples from humanized mice irradiated *ex vivo* and human donors irradiated *ex vivo* are shown in Table 1 and Fig. 5. As expected, the increase in frequency of dicentric chromosomes is dose dependent. Only one dicentric was scored from 1,000 analyzed cells in the sham-irradiated specimens from humanized mice and human blood samples. Remarkably, no statistically significant differences in the dicentric frequencies were detected between the samples irradiated *in vivo* and *ex vivo* at any of the doses. The actual dicentric frequency results are very close; for samples from humanized mice irradiated *in vivo* and *ex vivo*, and for human blood samples, the results are, respectively, 0.14, 0.15 and 0.15 for 1 Gy, 0.49, 0.47 and 0.45 for 2 Gy and 0.72, 0.72 and 0.7 for 3 Gy.

The compliance of dicentric frequency distribution with Poisson distribution was not rejected for most of the samples. A slight overdispersion was detected only in three cases, which are in samples from humanized mice irradiated *in vivo* with 2 Gy (U test value of 2.42), samples from humanized mice irradiated *in vivo* with 3 Gy (U test value of 2.75) and samples from humanized mice irradiated *ex vivo* with 3 Gy (U test value of 2.05) (Table 1).

DISCUSSION

The estimation of the dicentric chromosome frequencies in human blood T cells is considered the gold standard in human biodosimetry. However, over time, an important difference in the radiation-induced T-cell death after *in vivo* and *ex vivo* irradiation has been reported. Indeed, a clinically observed, dose-dependent depletion of lymphocyte cell counts after TBI is very reproducible (11). In contrast to the *in vivo* biology, reports from radiation studies show that lymphocyte cell depletion after *in vivo* irradiation is much higher than the cell depletion *ex vivo* after irradiation and that *ex vivo* radiation-induced lymphocyte apoptosis is not dose dependent for a range of doses (12, 19). The differences in cell counts may lead to inappropriately inflated dicentric frequencies after *in vitro* irradiation compared to *in vivo* irradiation of the same samples. This may lead to underestimation of the *in vivo* dose, if such *ex vivo* data are used as reference for the *in vivo* data.

Considering this possibility, it is clear that an *in vivo* model could help to resolve any discrepancy and validate the current approach, which underpins much human radiation biodosimetry. Laboratory mouse models fail in this regard, since radiation sensitivity among such strains varies widely and the fidelity of response assessed over various end points can be poor (20, 21). An obvious candidate for *in vivo* reference is the humanized mouse model in which an engrafted human hematopoietic system produces human lymphocytes that can be found in mouse blood and spleen. We decided to use this model, and the selected strategy included validation of the model based on: 1. Comparisons of the DNA damage detection capacity of human T cells (on which the dicentric chromosome assay is based) isolated from human donors and humanized mice; and 2. Demonstration that the differences between the

in vivo and *ex vivo* cell death described for human blood cells can be shown in the humanized mice.

A good approach for the estimation of the DNA damage detection capacity of given cells is the measurement of the dose-dependent frequency of radiation induced γ -H2AX foci (22, 23). The foci counts are dose dependent and are a result of the phosphorylation of the histone H2AX by a DNA repair complex that forms at sites of DNA doublestrand breaks. Correspondingly, this phosphorylation event may be used as a proxy for both effective DNA damage detection and initiation of the DNA repair process (24, 25).

The comparison of T cells from human donors and humanized mice performed here shows that there are no statistically significant differences in γ -H2AX foci counts after 0.5 and 1 Gy irradiation (dose-to-dose comparison). The main hypothesis of this work was that the differences in cell survival between *in vivo* and *in vitro* irradiated human T cells may translate into differences in the measured dicentric frequencies. An important part of the validation of the humanized mouse model was to prove that it can recapitulate those differences with good fidelity.

The results show significant depletion in the blood cell counts in *in vivo* irradiated humanized mice when T-cell counts were compared to preirradiation data from the same animals. These observations are in agreement with other published results in *in vivo* mammalian model systems (26–28). Non-human primate model systems of study also exhibit radiation-induced depletion. In particular, *Macaca fascicularis* can exhibit a 70% depletion of total lymphocytes 24 h after 2 and 4 Gy irradiation (29). These findings harmonize with depletion effects seen also in human subjects of study. Data from human patients with malignant hematological disorders who received TBT showed significant lymphocyte depletion after 2 and 4 Gy (11).

While *in vivo* irradiation of cells imparts significant cell death and depletion in lymphocyte pools, this is not so in dose-matched comparisons with *ex vivo* irradiations. We obtained very different cell depletion results when human lymphocytes from human donors and humanized mice were irradiated *ex vivo*. Cell survival was much higher and T-cell counts were not altered in a dose-dependent fashion. This discrepancy has been reported elsewhere (12, 19). We initially attributed this discrepancy to incomplete apoptosis in the *ex vivo* irradiated samples and chose to measure the percentage of apoptotic cells in these subcultures to verify this notion. The results showed a sizable apoptotic population in the *ex vivo* irradiated samples; however, this population could not account for the differences in cell counts between the two conditions. Overall, these results confirmed that more cells with damage survived in the *ex vivo* irradiated samples, which may influence the dicentric results.

Mechanistically, there are at least two possible explanations of the lower cell depletion frequencies observed in the *ex vivo* irradiated samples. One explanation is that the apoptosis of lymphocytes is a result of the systemic organism response that produces extracellular factors triggering or supporting apoptosis. The lack of such factors could suppress apoptosis in irradiated cells *ex vivo*. In addition it was shown that in a living organism, pre-apoptotic cells are phagocytized and eliminated from the system (12). These mechanisms cannot occur

under *ex vivo* conditions. Another explanation could be related to the cell signaling environment in cell cultures, which is very different from *in vivo* conditions. Cell culture media are specifically formulated to drive cell growth and survival, and commonly contain FBS (a source of growth factors and cytokines). This environment is, in principle, anti-apoptotic and may result in higher cell survival compared to the environment in a living organism after TBI.

Having experimental proof that the humanized mouse model reproduces the survival differences seen in *ex vivo* and *in vivo* conditions, we expected some differences to emerge in the dicentric frequency comparison. The data, however, show that despite the differences in cell death, the dicentric frequencies after *in vivo* and *ex vivo* irradiation are very similar. No statistically significant difference in the dicentric frequencies was found in any of the dose-to-dose comparisons of irradiated *in vivo* cells from humanized mice compared to cells from human donors and cells from humanized mice irradiated *ex vivo*. These results validate the use of *in vitro* reference data employing dicentric frequencies for human biodosimetry at least at the 24 h postirradiation time point.

The explanation of these particular results may be that the number of T cells with chromosomal damage that reach M phase in both *in vivo* and *ex vivo* samples is the same. These are cells that can be driven through cell cycle checkpoints to divide by PHA stimulation and presumably have damage that is permissive for the initiation of cell division. The cells in excess in the *ex vivo* condition clearly cannot divide or die during subsequent cell division and are practically eliminated from the dicentric assay.

Overall, we conclude that the humanized mouse serves as a good model for radiation-induced dicentric chromosome generation. The value of the humanized mouse model, however, goes beyond the validation in this work. It is well known that longer incubations of blood (more than 24 h) lead to artifacts. For studies probing extended times after irradiation, the humanized mouse can become an important model for generation reference data for dose estimations. In addition, the humanized mouse could be used in gene expression studies and developmental studies where the status of the human hematopoietic system can be monitored for long periods of time.

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FIG. 1.

Distribution of γ -H2AX foci in T cells isolated from blood of human donors (gray) and humanized mice (black). The cells were subsequently exposed to 0.5 Gy (panel A) and 1.0 Gy (panel B) X rays. The results from three different donors are shown with separate bars reporting the number of foci for each donor per bin in the histogram.

FIG. 2.

Comparison of radiation-induced γ -H2AX foci frequencies in human T cells from humanized mice (black) and human donors (white). The results from three different donors are shown. Error bars indicate standard deviation (n = 50 cells).

FIG. 3. Human T-cell counts at 24 h postirradiation in samples from humanized mice (*in vivo*: black; *ex vivo*: white) and in human samples (*ex vivo*: gray). The percentage human T-cell counts relative to the preirradiation counts and standard error is shown (n = 3 animals or human donors per condition). **Statistically significant differences.

FIG. 4. Annexin-V-positive human T cells in samples from humanized mice (panel A) or human donors (panel B) irradiated *ex vivo* (n = 3 animals or human donors per condition).

FIG. 5. Dicentric chromosome frequencies in samples from irradiated humanized mice (*in vivo*: black; *ex vivo*: white) and blood from human donors (*ex vivo*: gray). The frequency of dicentrics and standard error of the mean are shown (n = 3 animals/human donors per condition).

TABLE 1

Analyzed Cells and Dicentric Chromosome Distribution in Human Lymphocytes Isolated from Humanized Mice Irradiated *In Vivo*, Humanized Mice Irradiated *Ex Vivo* and Human Blood Samples Irradiated *Ex Vivo*

	Analyzed cells	Dicentric chromosome distribution							Total dicentrics	Y	±SE	DI	U
		0	1	2	3	4	5	6					
0 Gy Hu-ms <i>in vivo</i>	1,000	999	1	0	0	0	0	1	1	0.00	±0.001	1.00	-
0 Gy Hu-ms <i>ex vivo</i>	1,000	1,000	0	0	0	0	0	0	0	0.00	±0	-	-
0 Gy Human blood	1,000	999	1	0	0	0	0	1	1	0.00	±0.001	1.00	-
1 Gy Hu-ms <i>in vivo</i>	1,000	873	111	16	0	0	0	143	143	0.14	±0.012	1.08	1.84
1 Gy Hu-ms <i>ex vivo</i>	1,000	858	134	8	0	0	0	150	150	0.15	±0.012	0.96	-0.95
1 Gy Human blood	1,004	866	128	9	1	0	0	149	149	0.15	±0.012	1.01	0.31
2 Gy Hu-ms <i>in vivo</i>	500	324	115	52	9	0	0	246	246	0.49	±0.034	1.15	2.42
2 Gy Hu-ms <i>ex vivo</i>	503	324	127	45	7	0	0	238	238	0.47	±0.032	1.08	1.33
2 Gy Human blood	500	323	137	33	6	1	0	225	225	0.45	±0.031	1.07	1.15
3 Gy Hu-ms <i>in vivo</i>	480	261	116	83	16	4	0	346	346	0.72	±0.042	1.18	2.75
3 Gy Hu-ms <i>ex vivo</i>	504	263	149	66	23	3	0	362	362	0.72	±0.040	1.13	2.05
3 Gy Human blood	500	245	173	68	13	1	0	352	352	0.70	±0.036	0.94	-0.95

Notes. Hu-ms *in vivo* = samples from humanized mice irradiated *in vivo*; Hu-ms *ex vivo* = samples from humanized mice irradiated *ex vivo*; human blood = human blood samples irradiated *ex vivo*; Y = observed frequency of dicentrics; SE = standard error; DI = dispersion index (variance/mean); U = U test values.