Title: Evaluation of γ-H2AX foci distribution among different peripheral blood mononucleated cell subtypes

Authors: Juan S. López[a], Mònica Pujol-Canadell[a], Pedro Puig[b,c], Gemma Armengol[a], Joan Francesc Barquinero[a*]

Affiliations:

[a] Unitat d'Antropologia Biològica, Departament de Biologia Animal, Biologia Vegetal i Ecologia, Universitat Autònoma de Barcelona, E-08193, Bellaterra, Catalonia, Spain.

[b] Departament de Matemàtiques, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Catalonia, Spain

[c] Centre de Recerca Matemàtica, E-08193 Bellaterra, Catalonia, Spain.

*Corresponding author: Francesc.Barquinero@uab.cat

Unitat d'Antropologia Biològica, Departament de Biologia Animal, Biologia Vegetal i Ecologia, Universitat Autònoma de Barcelona, E-08193, Bellaterra, Catalonia, Spain

Keywords: γ-H2AX foci distribution, peripheral mononucleated cells, ionizing radiation.

Biographical Author Notes:

Juan S. López, Histocytologist, Ph.D. student, Departament de Biologia, Animal, Biologia Vegetal i Ecologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra (Cerdanyola del Vallès), Spain.

Mònica Pujol-Canadell, Ph.D., Biologist, Postdoctoral research scientist, Departament de Biologia, Animal, Biologia Vegetal i Ecologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra (Cerdanyola del Vallès), Spain.

Pedro Puig, Ph.D., Mathematician, Full Professor, Department de Matemàtiques and member of the Centre de Recerca Matemàtica, Facultat de Ciències, Universitat Autònoma de Barcelona, Bellaterra (Cerdanyola del Vallés), Spain.

Gemma Armengol, Ph.D., Biologist, Associate Professor, Departament de Biologia, Animal, Biologia Vegetal i Ecologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra (Cerdanyola del Vallès), Spain.

Joan-Francesc Barquinero, Ph.D., Biologist, Associate Professor, Departament de Biologia, Animal, Biologia Vegetal i Ecologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra (Cerdanyola del Vallès), Spain.

ORCID numbers

Juan S. López: 0000-0002-9384-7504
Mònica Pujol-Canadell: 0000-0002-7403-9683
Pedro Puig: 0000-0002-6607-9642
Gemma Armengol: 0000-0003-2345-1106
Joan-Francesc Barquinero: 0000-0003-0084-5268

Abstract:

Introduction:

The detection of γ-H2AX foci in peripheral blood mononucleated cells (PBMCs) has been incorporated as an early assay for biological dosimetry. However, overdispersion in the γ-H2AX foci distribution is generally reported. In a previous study from our group, it was suggested that overdispersion could be caused by the fact that when evaluating PBMCs, different cell subtypes are analyzed, and that these could differ in their radiosensitivity. This would cause a mixture of different frequencies that would result in the overdispersion observed.

Objectives:

The objective of this study was to evaluate both the possible differences in the radiosensitivities of the different cell subtypes present in the PBMCs and to evaluate the distribution of γ-H2AX foci in each cell subtype.

Materials and methods:

Peripheral blood samples from three healthy donors were obtained and total PBMCs, and $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells were separated. Cells were irradiated with 1 and 2 Gy and incubated at 37 °C for 1, 2, 4, and 24 h. Sham-irradiated cells were also analyzed. γ-H2AX foci were detected after immunofluorescence staining and analyzed automatically using a Metafer Scanning System. For each condition, 250 nuclei were considered.

Results:

When the results from each donor were compared, no observable significant differences between donors were observed. When the different cell subtypes were compared, $CD8^+$ cells showed the highest mean of γ-H2AX foci in all post-irradiation time points. The cell type that showed the lowest γ-H2AX foci frequency was $CD56^+$. The frequencies

observed in $CD4^+$ and $CD19^+$ cells fluctuated between $CD8^+$ and $CD56^+$ without any clear pattern. For all cell types evaluated, and at all post-irradiation times, overdispersion in γ-H2AX foci distribution was significant. Independent of the cell type evaluated the value of the variance was four times greater than that of the mean.

Conclusion

Although different PBMC subsets studied showed different radiation sensitivity, these differences did not explain the overdispersion observed in the γ-H2AX foci distribution after exposure to IR.

Introduction

The phosphorylation of the histone variant H2AX (γ-H2AX) is one of the earliest events observed in cells following exposure to DNA-damaging agents, such as ionizing radiation (IR) (Rogakou et al. 1998). It appears within minutes and reaches maximum levels after 30 min (Rogakou et al. 1999). By using specific antibodies against γ-H2AX, it is possible to detect its phosphorylation as microscopic foci, and its detection by immunofluorescence techniques has been incorporated as an assay to be used in biological dosimetry (Barnard et al. 2015; Roch-Lefévre et al. 2010; Rothkamm et al. 2013). Similar to other biomarkers of dose, pre-established calibration curves are needed for dose assessment. However, due to the fast repair of radiation-induced DNA damage, calibration curves have been established at specific post-irradiation times, usually between 0.5 and 48 h (Andrievski and Wilkins, 2009; Beels et al. 2010; Chaurasia et al. 2021; Horn et al. 2011; Lisowska et al. 2013; Moquet et al. 2014, 2017; Redon et al. 2010; Roch-Lefévre et al. 2010). Recently, our group has proposed a tri-dimensional model, where both time and foci counts are considered for dose assessment (López et al. 2022). The model allows evaluating blood samples at any post-irradiation time between 0.5 and 24 h without the need for multiple dose-effect curves.

Over the past decades, the dicentric chromosome assay has been considered the "gold standard" method in biological dosimetry. The dicentric assay allows to distinguish between whole-body homogeneous exposures and heterogeneous exposures (Edwards et al. 1979; Barquinero et al. 1997; Gruel et al. 2013; Pujol et al. 2016). The ability to distinguish between these two scenarios is due to the fact that dicentric cell distribution fits a Poisson distribution after a homogeneous exposure while overdispersion is observed in cases where non-homogeneous exposures toke place. Contrary to dicentrics, the γ-

H2AX foci distribution after homogeneous exposures is generally overdispersed (Ding et al. 2016; Einbeck et al. 2018; Kato et al. 2006; Lisowska et al. 2013; Lloyd-Smith, 2007; López et al. 2022; Martin et al. 2013; Rothkamm et al. 2007; Rothkamm and Löbrich, 2003; Rübe et al. 2008). This prevents the use of the properties of the Poisson distribution to estimate heterogeneous irradiations.

A difference between dicentric and γ-H2AX is that dicentrics are analyzed only in $CD3^+$ cells (T-lymphocytes) including $CD4^+$ (T helpers) and $CD8^+$ (cytotoxic T cells), whereas γ-H2AX foci are usually detected in peripheral blood mononucleated cells (PBMCs), which include different subsets of peripheral blood cells, such as $CD4^+$, $CD8^+$, $CD19^+$ (B cells), and $CD56^+$ (NK cells). In our previous study proposing the surface model (López et al. 2022), overdispersion of the γ-H2AX foci distribution was observed in all analyzed samples. In that paper, we suggested that this overdispersion may be caused by the fact that several blood cell types were analyzed, and they may differ in their radiation response. If we assumed that for each cell type, the distribution of foci per cell follows a Poisson distribution, the mixture of different subpopulations with different frequencies would result in overdispersion.

The present study aims to evaluate the radiation sensitivity of different PBMCs subtypes and to study the distribution of radiation-induced γ-H2AX foci in each cell type.

2. Material and methods

2.1 Cell isolation

Peripheral blood samples from a 56-year-old healthy male, a 44- and 34-year-old healthy females were collected in tubes with heparin. All three donors have no history of exposure

to clastogenic agents and previous informed consent was obtained for each donor. The study has followed institutional, national, and international Ethical guidelines. It has been approved by the Animal and Human Experimentation Ethics Committee from the Universitat Autònoma de Barcelona (Reference: 2624).

Cell isolation was performed as previously described by our group (López et al. 2022). After isolation, PBMCs were incubated in 15 mL tubes with 10 mL of RPMI-1640, supplemented with L-glutamine 2 mM (Biowest, Barcelona, Spain), 15% fetal bovine serum (Life Technologies, Madrid, Spain), and 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin (Biowest) until the irradiation took place.

To separate the different lymphocyte subpopulations, PBMCs were incubated with the following monoclonal antibodies: for $CD3^+$ cells PBMCs were incubated with UCHT1 clone (Biolegend, San Diego, CA); for $CD4^+$ cells, tA161A1 clone (Biolegend) was used; for $CD8^+$ cells, SK1 clone, (Biolegend); for $CD19^+$ cells, H1B19 clone (Biolegend); and for $CD56^+$ cells, tHCD56 clone (Biolegend). For the different cell types, 3,000,000 cells were sorted with BDFACSJazz flow cytometer (BD Biosciences, San Jose, CA) equipped with 488 nm and 635 nm lasers.

2.2 Irradiation

PBMCs as well as the sorted cells ($CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and $CD56^+$) were sham irradiated or irradiated at 1 or 2 Gy with a 137Cs source (IBL437C, CIS Biointernational, GIF Yvette, France) located at the Unitat Tècnica de Protecció Radiològica of the Universitat Autònoma de Barcelona, Spain. Before irradiation, cells were kept at 37 °C for 1 h. Immediately after irradiation, cells were placed on ice for transportation during

30 min. This was done to halt DNA repair. Once in the laboratory, cells were incubated at 37 °C for 1, 2, 4 and 24 h. The starting point of DNA repair was considered at the time of introducing the cells into the incubator.

2.3 Immunofluorescence staining and microscopic analysis

Immunofluorescence staining and microscopic analysis were performed following protocols described previously (López et al. 2022). Foci analysis was performed in a motorized z-stage Zeiss Axio Imager.Z2 microscope, coupled with a camera. Signals were captured with an automated scanning fluorescence microscope software (Metafer 4, Meta Systems, Altlussheim, Germany) and analysed with MetaCyte, version 3.10.2, (Meta Systems, Medford, MA, USA). The integration time of the classifier was set between 0.04 and 0.36 s. Foci intensity threshold was set to 30% with a maximum gain of 500%, and the area to capture nuclei was between 30 and 400 μm^2. Once images were captured, clustered or damaged nuclei were excluded. For each condition, 250 nuclei were considered.

2.4 Statistical analysis

To check if the foci distribution among cells agreed with the Poison distribution, the u-test was used; u values >1.96 were considered as significantly overdispersed. For each cell type and irradiation condition the Kruskal-Wallis test was used to check if there were differences between donors, whereas pairwise comparisons were done using Mann–Whitney U test. To compare the mean values of the three donors, one-way ANOVA with the Tukey multiple pairwise test was used. Finally, to compare the mean background foci values to those observed after 24 h post-irradiation, the student t-test was used. In all tests significant differences were considered when $p<0.05$.

3. Results

The proportion of each cell type in each donor was evaluated (Table 1).There were no significant differences between donors, and the proportion of the different leukocyte subsets agreed with those expected for healthy individuals (Oras et al. 2020). The background yields of γ-H2AX foci for each donor and each cell type were evaluated (Figure 1). Only the yields corresponding to $CD4^+$ cells exhibited significant differences between donors (Kruskal-Wallis test, $p<0.01$): donor 3 showed significantly high values (1.60±0.14, mean ± standard error of the mean, SEM) in comparison to donors 1 and 2, who had 1.19±0.12 and 1.11±0.10 foci per cell respectively (Mann-Whitney test, $p<0.04$ in both cases). When the background values of the three donors were considered together, the mean frequency ±SEM of γ-H2AX foci for each cell type ranged from 1.17±0.10 for $CD56^+$ cells to 1.46±0.11 for $CD8^+$ cells. Finally, yields of γ-H2AX foci obtained after 1 and 2 Gy irradiation were analyzed (Figures 2 and 3) for each donor and for the four post-irradiation times evaluated. Although some statistically significant differences between donors were observed for some cell types and time points, these differences were not consistent. For example, in $CD56^+$ cells irradiated at 1 Gy significant differences between donors were found after 2 and 4 h post-irradiation. However, after 2 h it was donor 2 which showed the highest frequency of γ-H2AX foci, after 4 h this donor showed the lowest frequency. Similarly, in $CD19^+$ cells irradiated at 1 Gy, donor 2 showed elevated foci frequencies after 2 and 4 h. After 2 Gy irradiation, and for the same cell subtype, donor 3 showed elevated frequencies at the same time points. Due to the lack of significant differences among donors for the studied cell subsets, results from the three donors were grouped.

To check if γ-H2AX foci distribution conforms to the Poisson distribution in PBMCs and in each cell type, the normalized unit of the dispersion index, the u-test, was used. In the sham irradiated samples as well as after 1 and 2 Gy irradiation, variances were always higher than the mean with dispersion indexes higher than 1. U values indicated that in all cases overdispersion was significant, with u values higher than 1.96. Figure 4 shows the correlation between the mean and the variance for each cell type. As can be seen in the figure, the variance has a tendency to increase as the frequency increases. When this data was adjusted to a linear function, slopes were close to 4.

The decay in the mean frequencies of γ-H2AX foci with post-irradiation time was analyzed for both irradiation doses and for each cell type (Figure 5). In all cases there were significant differences between the cell subtypes (One-way ANOVA, $p<0.001$), except at 24 h after 1 and 2 Gy irradiation. In general, $CD8^+$ cells showed the highest mean in all post-irradiation time points. For $CD8^+$ cells and after 1 Gy, the mean frequencies ±SEM ranged from 12.97±0.27 after 1 h to 2.46±0.20 after 24 h; and after 2 Gy, from 21.38±0.17 to 3.47±0.16. On the other hand, the cell type that in general showed the lowest γ-H2AX foci frequency was $CD56^+$. After 1 Gy the mean frequencies ±SEM ranged from 8.91±0.23 after 1 h to 1.71±0.10 after 24 h, and in 2 Gy, from 15.74±0.30 after 1 h to 3.01±0.11 after 24 h. When the mean frequencies of γ-H2AX foci in $CD8^+$ cells were compared to those observed in $CD56^+$, the differences were always significant (Tukey pairwise test $p<0.05$), except for 1 and 2 Gy after 24 h post-irradiation. The frequencies observed in $CD4^+$ and $CD19^+$ cell subtypes fluctuated between $CD8^+$ and $CD56^+$ values without any clear pattern. When $CD3^+$ cells were considered, for both doses and all post-irradiation times, the observed frequencies lay between $CD8^+$ and $CD4^+$ cell

values, except after 2 Gy and in the 4 h post-irradiation time. Similarly, the frequencies of γ-H2AX foci in PBMCs lay between $CD8^+$ and $CD3^+$ values. When background foci values were compared with the remaining ones after 24 h, after both irradiation doses (1 and 2 Gy), the observed foci values after 24 h post-irradiation were statistically higher than the sham irradiated values (Student's t test $p<0.02$, in all cases), except for $CD4^+$ cells irradiated at 1 Gy.

4. Discussion

In the present study, when all PBMCs subtypes were considered together, the background frequency of γ-HA2X foci was 1.25±0.11 close to the value observed in a previous study from our group, where the surface model was presented: 1.35±0.06 (López et al. 2022). In our previous study, it was indicated that, in addition to a certain yield of foci due to endogenous production of oxidative stress, as observed by other authors (Tanaka et al. 2006; Vilenchik and Knudson, 2006), PBMCs isolation procedure stressed the cells. However, in comparison with other reported background frequencies that range from 0.01 to 0.2 foci per cell (Chaurasia et al. 2021; Mandina et al. 2011; Roch-Lefévre et al. 2010), the background level in our studies is higher. The diversity in the scoring methodologies, manual versus semi-automatic or automatic scoring, as well as laboratory variations in the classifier set-up in the latter case, could explain the observed differences. When considering cell subtypes, only the differences between $CD8^+$ and $CD56^+$ were significant ($p<0.05$). This could indicate that both endogenous damage and stress caused by the isolation procedures affect the different cell subtypes in a slightly different way.

When the foci frequencies of each individual were observed separately in the different cell types, either at control or post-irradiation times, some statistically significant

differences were obtained. However, these differences were not consistent. In all cell types, no donor was observed to show significant differences at both doses and at all post-irradiation times. It should be noted that by analyzing three donors it is unlikely that inter-individual differences would be observed. A larger cohort should be studied to be more representative. In a French study, five of the 27 individuals evaluated showed background frequencies significantly higher with respect to the mean (Roch-Lefévre et al. 2010), and no correlation between the background frequency of γ-H2AX foci and age or gender was found. In addition, the same study reported that the interindividual variations of the γ-H2AX yield after irradiation were similar to the intraindividual variations, indicating a lower interindividual variability. Other authors have also reported no interindividual differences for both the background yield and the radiation-induced one (Andrievski and Wilkins, 2009; Chaurasia et al. 2021; Ismail et al. 2007; Lisowska et al. 2013; Mandina et al. 2011). However, in a large study including 94 individuals, it has been described that age, alcohol use, and ethnicity have an influence on the endogenous levels of γ-H2AX, and that ethnicity and alcohol use may have influence on the repair kinetics (Sharma et al. 2015).

Regarding radiation-induced foci, $CD8^+$ cells were the ones showing the highest values of γ-H2AX foci at almost all post-irradiation times, while $CD56^+$ cells were the ones showing the lowest. The frequencies observed for the $CD4^+$ and $CD19^+$ cell subtypes lay between $CD8^+$ and $CD56^+$ values. A previous report analyzed the induction and repair of radiation-induced γ-H2AX foci in different blood cell populations by microscopy (Heylmann et al., 2021). They described that at an early time, 1-4 h, $CD3^+$ cells ($CD4^+$ plus $CD8^+$) show higher values than $CD56^+$. At later times, the frequencies of these cell types are intermixed. Another report using flow cytometry described that T cells ($CD4^+$

and $CD8^+$) show a higher fluorescence intensity of γ-H2AX in comparison to B cells ($CD19^+$) (Andrievski and Wilkins, 2009). Finally, another study showed that when T cell subsets were studied in culture, no differences between $CD4^+$ and $CD8^+$ were observed (Hamasaki et al. 2007). In general, our results agree with the consensus that T lymphocytes are more radiosensitive than B lymphocytes and suggest that, among T cells, $CD8^+$ are more radiosensitive. Interestingly, the endogenous signals in $CD8^+$ was also higher with respect to the other cell subtypes, indicating that $CD8^+$ cells seem to be more sensitive to different oxidative stressors, not only radiation. Despite observing differences in radiosensitivity between leukocyte subsets, at 24 h post-irradiation, almost all cell subtypes still showed higher frequencies of γ-H2AX foci compared to sham irradiated control values. This agrees with what has been proposed by other authors, that it is not necessary to isolate a specific cell subtype to better detect exposure to IR (Andrievski and Wilkins, 2009; Heylmann et al. 2021; Horn et al. 2011).

The analysis of γ-H2AX foci is an assay currently accepted as an early indicator of exposure to IR (Kulka et al. 2017; Wojcik et al. 2017). In this sense, several interlaboratory comparisons have been performed to harmonize and validate this technique (Ainsbury et al. 2014; Barnard et al. 2015; Moquet et al. 2017; Rothkamm et al. 2013a, 2013b). However, as with other exposure biomarkers, it is important to determine which probability distribution best fits the γ-H2AX foci data. This will allow distinguishing between different exposure scenarios. For example, the zero-inflated Poisson model allows distinguishing between partial and homogeneous exposures if dicentric chromosomes are analyzed (IAEA, 2011). Although some authors have described that the distribution of γ-H2AX foci per nucleus follows a Poisson distribution, and have applied the zero-inflated method to estimate partial irradiations (Horn et al.

2011; Redon et al. 2010), most studies show that the distribution of foci per cell is generally overdispersed, showing a variance greater than the mean (Ding et al. 2016; Einbeck et al. 2018; Kato et al. 2006; Lloyd-Smith, 2007; López et al. 2022; Martin et al. 2013; Rübe et al. 2008). In a previous study (Młynarczyk et al., 2022), we suggested that the coexistence of different cell subpopulations with possible differences in their radiosensitivities would result in a mixture of different Poissons, and a bayesian model based on a mixture of four poisons was proposed to estimate whole-body exposures to IR. Data presented here indicates that overdispersion remains at similar values for each leukocyte subset. Therefore, the overdispersion observed when evaluating PBMCs cannot be explained by different radiosensitivities between cell subtypes, although there are some significant differences. We show that there is a strong tendency for the variance to grow as the mean increases, with a ratio close to 4, similar to the overall value found in our previous study (López et al. 2022), which was 4.9. This suggests a constant dispersion index close to 4 for the different irradiation doses and post-irradiation times. Einbeck et al. (2018) analyzed their data sets using a quasi-Poisson method that requires a constant dispersion index. Although the model consisting of four Poissons showed a good fit, another likelihood function to represent foci formation should be sought. The zero-inflated index has been explored as a valuable tool for distinguishing between distinct overdispersed count data distributions (Puig and Valero, 2006). In this regard, additional analysis will be carried out to determine which statistical model best fits the observed γ-H2AX foci distribution.

Conclusion

Although the different PBMCs subsets studied showed different radiosensitivity, these differences do not explain the overdispersion observed in γ-H2AX foci distribution after exposure to IR.

Acknowledgements

This work received financial support from the Spanish Consejo de Seguridad Nuclear (BOE-A-2019-311). MPC, PP, GA and JFB are part of a SGR recognized by the Generalitat de Catalunya (2017SGR0255).

Disclosure statement

No conflict of interest was reported by the authors.

Availability of data

The data that support the findings of this study are openly available in Dipòsit Digital de Documents de la UAB (DDD) at https://ddd.uab.cat/record/268847 with the identifier https://doi.org/10.5565/ddd.uab.cat/268847.

References

Ainsbury EA, Al-Hafidh J, Bajinskis A, Barnard S, Barquinero JF, Beinke C, de Gelder V, Gregoire E, Jaworska A, Lindholm C, Lloyd D, Moquet J, Nylund R, Oestreicher U, Roch-Lefévre S, Rothkamm K, Romm H, Scherthan H, Sommer S, … Wojcik A. 2014. Inter- and intra-laboratory comparison of a multibiodosimetric approach to triage in a simulated, large scale radiation emergency. Int J Radiat Biol. 90(2): 193–202.

Andrievski A and Wilkins RC. 2009. The response of γ-H2AX in human lymphocytes and lymphocytes subsets measured in whole blood cultures. Int J Radiat Biol. 85(4): 369–376.

Barnard S, Ainsbury EA, Al-hafidh J, Hadjidekova V, Hristova R, Lindholm C, Monteiro Gil O, Moquet J, Moreno M, Rossler U, Thierens H, Vandevoorde C, Vral A, Wojewodzka M, Rothkamm K. 2015. The first gamma-H2AX biodosimetry intercomparison exercise of the developing European biodosimetry network RENEB. Radiat Prot Dosimetry 164(3):265–270.

Barquinero JF, Barrios L, Caballín MR, Miró R, Ribas M, Egozcue J. 1997. Biological dosimetry in simulated in vitro partial irradiations. Int J Radiat Biol. 71(4):435-40.

Beels L, Werbrouck J, Thierens, H. 2010. Dose response and repair kinetics of γ-H2AX foci induced by in vitro irradiation of whole blood and T-lymphocytes with X- and γ-radiation. Int J Radiat Biol. 86(9): 760–768.

Chaurasia RK, Bhat NN, Gaur N, Shirsath KB, Desai UN, Sapra BK. 2021. Establishment and multiparametric-cytogenetic validation of 60Co-gamma-ray induced, phospho-gamma-H2AX calibration curve for rapid biodosimetry and triage management during radiological emergencies. Mutat Res Genet Toxicol Environ Mutagen. 866.503354.

Ding D, Zhang Y, Wang J, Wang X, Fan D, He L, Zhang X, Gao Y, Li Q, Chen H. 2016. γ-H2AX/53BP1/pKAP-1 foci and their linear tracks induced by in vitro exposure to radon and its progeny in human peripheral blood lymphocytes. Sci Rep. 6. 38295

Edwards AA, Lloyd DC, Purrott RJ. 1979. Radiation induced chromosome aberrations and the poisson distribution. Radiat Environ Biophys. 16(2):89-100.

Einbeck J, Ainsbury EA, Sales R, Barnard S, Kaestle F, Higueras M. 2018. A statistical framework for radiation dose estimation with uncertainty quantification from the γ-

H2AX assay. PLoS One.13(11):e0207464.

Gruel G, Grégoire E, Lecas S, Martin C, Roch-Lefévre S, Vaurijoux A, Voisin P, Voisin P, Barquinero JF. 2013. Biological Dosimetry by Automated Dicentric Scoring in a Simulated Emergency. Radiat. Res. 179(5): 557–569.

Hamasaki K, Imai K, Nakachi K, Takahashi N, Kodama Y, Kusunoki Y. 2007. Short-term culture and γH2AX flow cytometry determine differences in individual radiosensitivity in human peripheral T lymphocytes. Environ Mol Mutagen. 48(1): 38–47.

Heylmann D, Ponath V, Kindler T, Kaina B. 2021. Comparison of DNA repair and radiosensitivity of different blood cell populations. Sci Rep. 11(1): 2478.

Horn S, Barnard S, Rothkamm K. 2011. Gamma-H2AX-based dose estimation for whole and partial body radiation exposure. PLoS One.6(9):e25113.

IAEA. (2011). Cytogenetic Dosimetry : Applications in Preparedness for and Response to Radiation Emergencies. In Manual Series. International Atomic Energy.

Ismail IH, Wadhra TI, Hammarsten O. 2007. An optimized method for detecting gamma-H2AX in blood cells reveals a significant interindividual variation in the gamma-H2AX response among humans. Nucleic Acids Res. 35(5):e36.

Kato TA, Nagasawa H, Weil MM, Little JB, Bedford JS. 2006. Levels of γ-H2AX foci after low-dose-rate irradiation reveal a DNA DSB rejoining defect in cells from human ATM heterozygotes in two AT families and in another apparently normal individual. Radiat Res. 166(3): 443–453.

Kulka U, Abend M, Ainsbury E, Badie C, Barquinero JF, Barrios L, Beinke C, Bortolin E, Cucu A, de Amicis A, et al. 2017. RENEB–Running the European Network of biological dosimetry and physical retrospective dosimetry. Int J Radiat Biol. 93(1): 2–14.

Lisowska H, Wegierek-Ciuk A, Banasik-Nowak A, Braziewicz J, Wojewodzka M, Wojcik A, Lankoff A. 2013. The dose-response relationship for dicentric chromosomes and γ-H2AX foci in human peripheral blood lymphocytes: Influence of temperature during exposure and intra- and inter-individual variability of donors. Int J Radiat Biol. 89(3): 191–199.

Lloyd-Smith JO. 2007. Maximum likelihood estimation of the negative binomial

dispersion parameter for highly overdispersed data, with applications to infectious diseases. PLoS One. 2(2): e180.

López JS, Pujol-Canadell M, Puig P, Ribas M, Carrasco P, Armengol G, Barquinero JF. 2022. Establishment and validation of surface model for biodosimetry based on γ-H2AX foci detection. Int J Radiat Biol. 98(1): 1–10.

Mandina T, Roch-Lefévre S, Voisin P, González JE, Lamadrid AI, Romero I, García O, Voisin P, Roy L. 2011. Dose-response relationship of γ-H2AX foci induction in human lymphocytes after X-rays exposure. Radiat Meas. 46(9): 997–999.

Martin OA, Ivashkevich A, Choo S, Woodbine L, Jeggo PA, Martin RF, Lobachevsky P. 2013. Statistical analysis of kinetics, distribution and co-localisation of DNA repair foci in irradiated cells: Cell cycle effect and implications for prediction of radiosensitivity. DNA Repair (Amst.). 12(10): 844–855.

Młynarczyk D, Puig P, Armero C, Gómez-Rubio V, Barquinero JF, Pujol-Canadell M. 2022. Radiation dose estimation with time-since-exposure uncertainty using the γ-H2AX biomarker. Sci Rep. 12(1): 19877.

Moquet J, Barnard S, Rothkamm K. 2014. Gamma-H2AX biodosimetry for use in large scale radiation incidents: Comparison of a rapid “96 well lyse/fix” protocol with a routine method. PeerJ. 6;2:e282.

Moquet J, Barnard S, Staynova A, Lindholm C, Monteiro O, Martins V, Rößler U, Vral A, Vandevoorde C, Wojewódzka M, et al. 2017. The second gamma-H2AX assay inter-comparison exercise carried out in the framework of the European biodosimetry network (RENEB). Int J Radiat Biol. 93(1): 58–64.

Oras A, Quirant-Sanchez B, Popadic D, Thunberg S, Winqvist O, Heck S, Cwikowski, M., Riemann, D., Seliger, B., Martinez Caceres, et al. 2020. Comprehensive flow cytometric reference intervals of leukocyte subsets from six study centers across Europe. Clin Exp Immunol. 202(3): 363–378.

Puig P, Valero J. 2006. Count Data Distributions. J Am Stat Assoc 101(473): 332–340.

Pujol M, Barrios L, Puig P, Caballín MR, Barquinero JF. 2016. A New Model for Biological Dose Assessment in Cases of Heterogeneous Exposures to Ionizing Radiation. Radiat Res. 185(2): 151-162

Redon CE, Nakamura AJ, Gouliaeva K, Rahman A, Blakely WF, Bonner WM, 2010. The Use of Gamma-H2AX as a Biodosimeter for Total-Body Radiation Exposure in Non-Human Primates. PLoS One. 5(11): e15544.

Roch-Lefévre S, Mandina T, Voisin P, Gëatan G, González-Mesa JE, Valente M, Bonnesoeur P, García O, Voisin P, Roy L. 2010. Quantification of γ-H2AX Foci in human lymphocytes: A method for biological dosimetry after ionizing radiation exposure. Radiat Res. 174(2): 185–194.

Rogakou EP, Boon C, Redon C, Bonner WM. 1999. Megabase Chromatin Domains Involved in DNA Double-Strand Breaks in Vivo. J Cell Biol. 146(5): 905–916.

Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. 1998. DNA Double-stranded Breaks Induce Histone H2AX Phosphorylation on Serine 139. J Biol Chem. 273(10): 5858–5868.

Rothkamm K, Balroop S, Shekhdar J, Fernie P, Goh V. 2007. Leukocyte DNA damage after multi-detector row CT: A quantitative biomarker of low-level radiation exposure. Radiology. 242(1):244-251

Rothkamm K, Barnard S, Ainsbury EA, Al-hafidh J, Barquinero JF, Lindholm C, Moquet J, Perälä M, Roch-Lefèvre S, Scherthan H, et al. 2013a. Manual versus automated γ-H2AX foci analysis across five European laboratories: Can this assay be used for rapid biodosimetry in a large scale radiation accident Mutat Res Genet Toxicol Environ Mutagen. 756(1–2): 170–173.

Rothkamm K, Horn S, Scherthan H, Rößler U, de Amicis A, Barnard S, Kulka U, Lista F, Meineke V, Braselmann et al. 2013b. Laboratory Intercomparison on the γ-H2AX Foci Assay. Radiat Res. 180(2): 149-155.

Rothkamm K, Löbrich M. 2003. Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. Proc Natl Acad Sci U S A. 100(9): 5057–5062.

Rübe CE, Grudzenski S, Kühne M, Dong X, Rief N, Löbrich M, Rübe C. 2008. DNA double-strand break repair of blood lymphocytes and normal tissues analysed in a preclinical mouse model: Implications for radiosensitivity testing. Clin Cancer Res. 14(20): 6546–6555.

Sharma PM, Ponnaiya B, Taveras M, Shuryak I, Turner H, Brenner DJ. 2015. High

Throughput Measurement of γ-H2AX DSB Repair Kinetics in a Healthy Human Population. PLoS One. 10(3): e0121083.

Tanaka T, Halicka HD, Huang X, Traganos F, Darzynkiewicz Z. 2006. Constitutive Histone H2AX Phosphorylation and ATM Activation, the Reporters of DNA Damage by Endogenous Oxidants. Cell Cycle, 5(17): 1940–1945.

Vilenchik MM, Knudson AG. 2006. Radiation dose-rate effects, endogenous DNA damage, and signaling resonance. Proc Natl Acad Sci U S A. 103(47): 17874–17879.

Wojcik A, Oestreicher U, Barrios L, Vral A, Terzoudi G, Ainsbury E, Rothkamm K, Trompier F, Kulka U. 2017. The RENEB operational basis: complement of established biodosimetric assays. Int J Radiat Biol. 93(1): 15–19.

Table 1. Proportion of different peripheral blood lymphocytes subtypes for each donor

	$CD4^+$	$CD8^+$	$CD3^+$	$C56^+$	$CD19^+$
Donor1	52.11	21.63	73.92	13.93	12.33
Donor 2	55.90	26.32	81.37	11.40	6.37
Donor 3	47.62	26.19	74.15	13.07	13.12
Mean	51.52	25,17	76.52	12.50	10.20

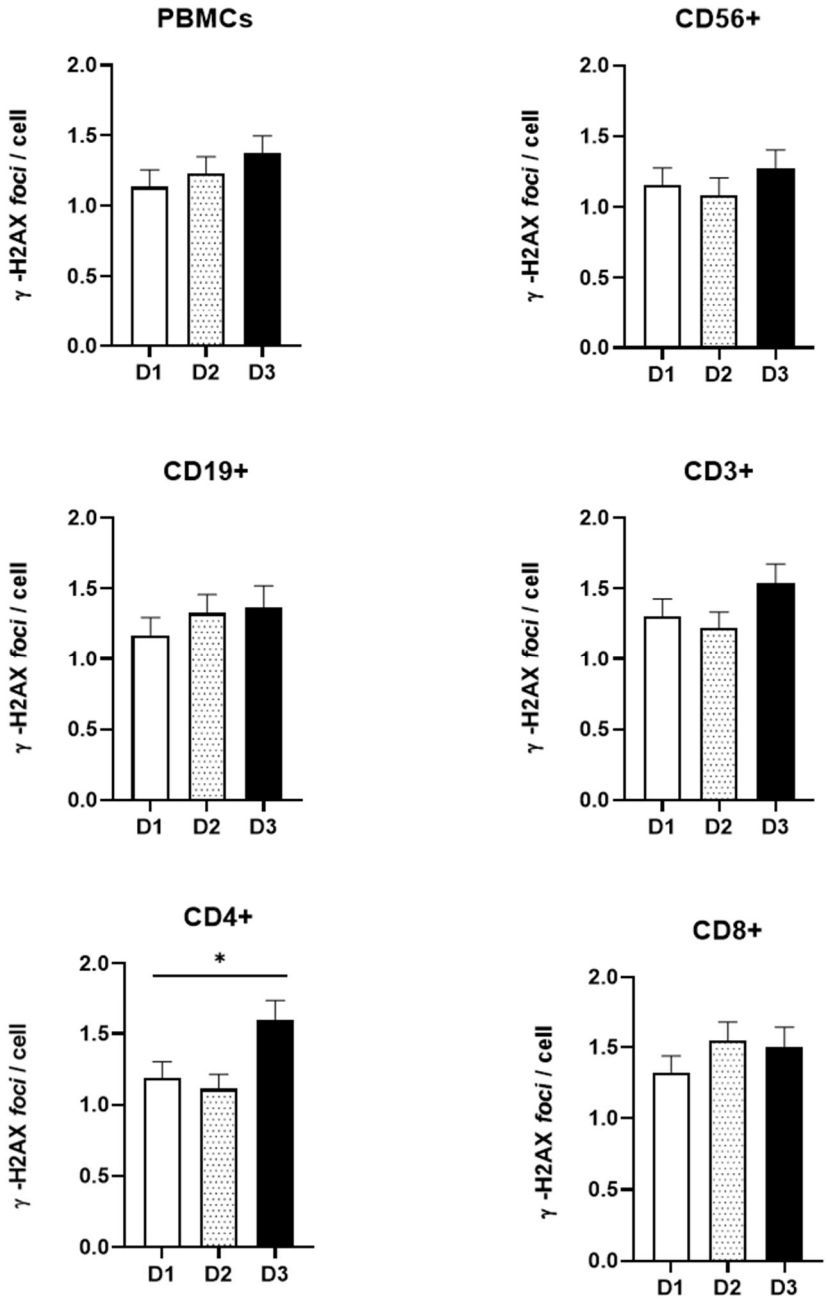

Figure 1. Background yields of γ-H2AX per cell for each donor and each cell type evaluated. D, donor. Barrs indicate the mean ± SEM. For $CD4^+$ cells, differences between donors were significant ($p<0.01$).

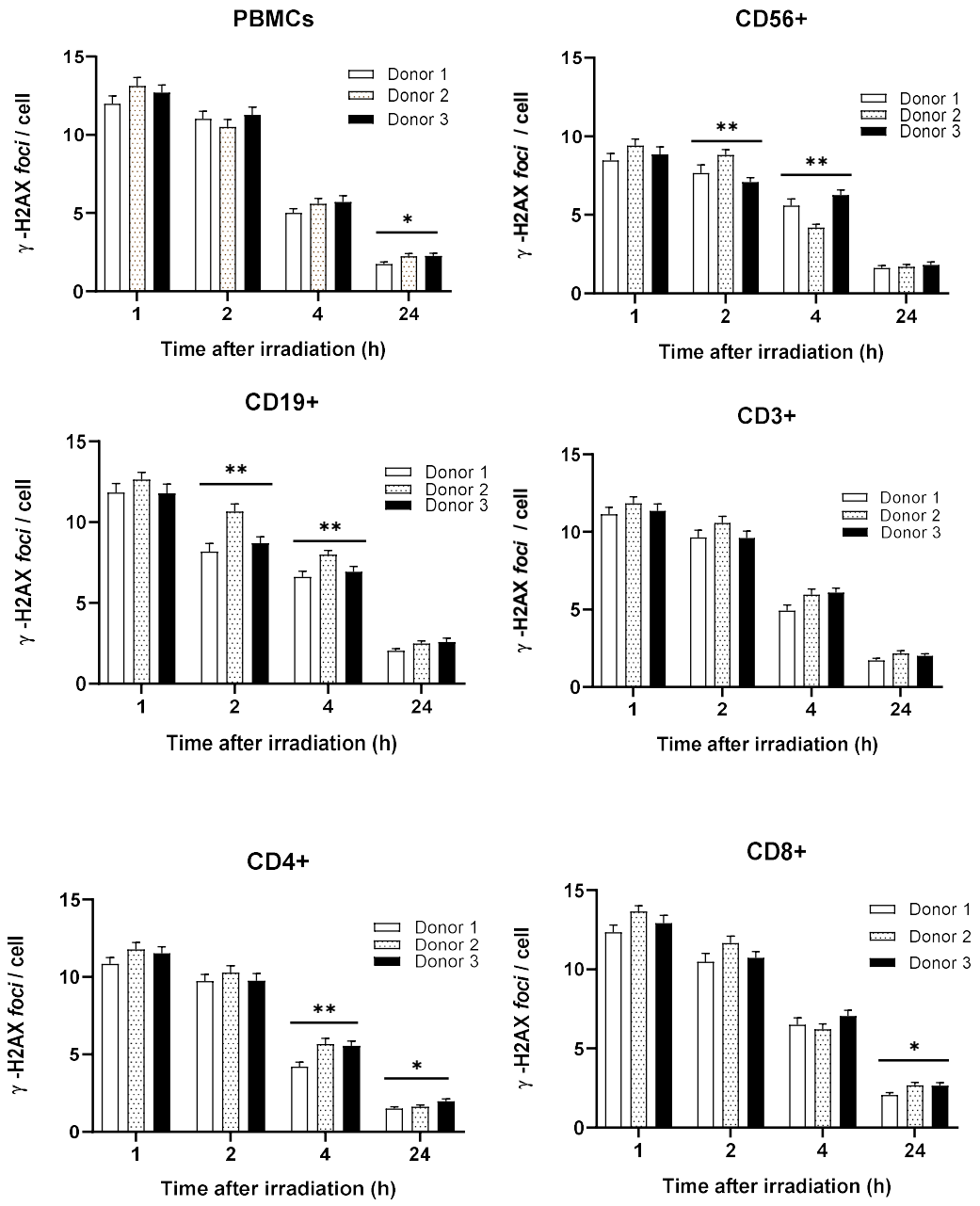

Figure 2. Yields of γ-H2AX foci per cell ± SEM after 1 Gy irradiation for each donor and for the four post-irradiation times evaluated. Significant differences between donors are inidicated with one ($p<0.05$) or two ($p<0.01$) asterisks.

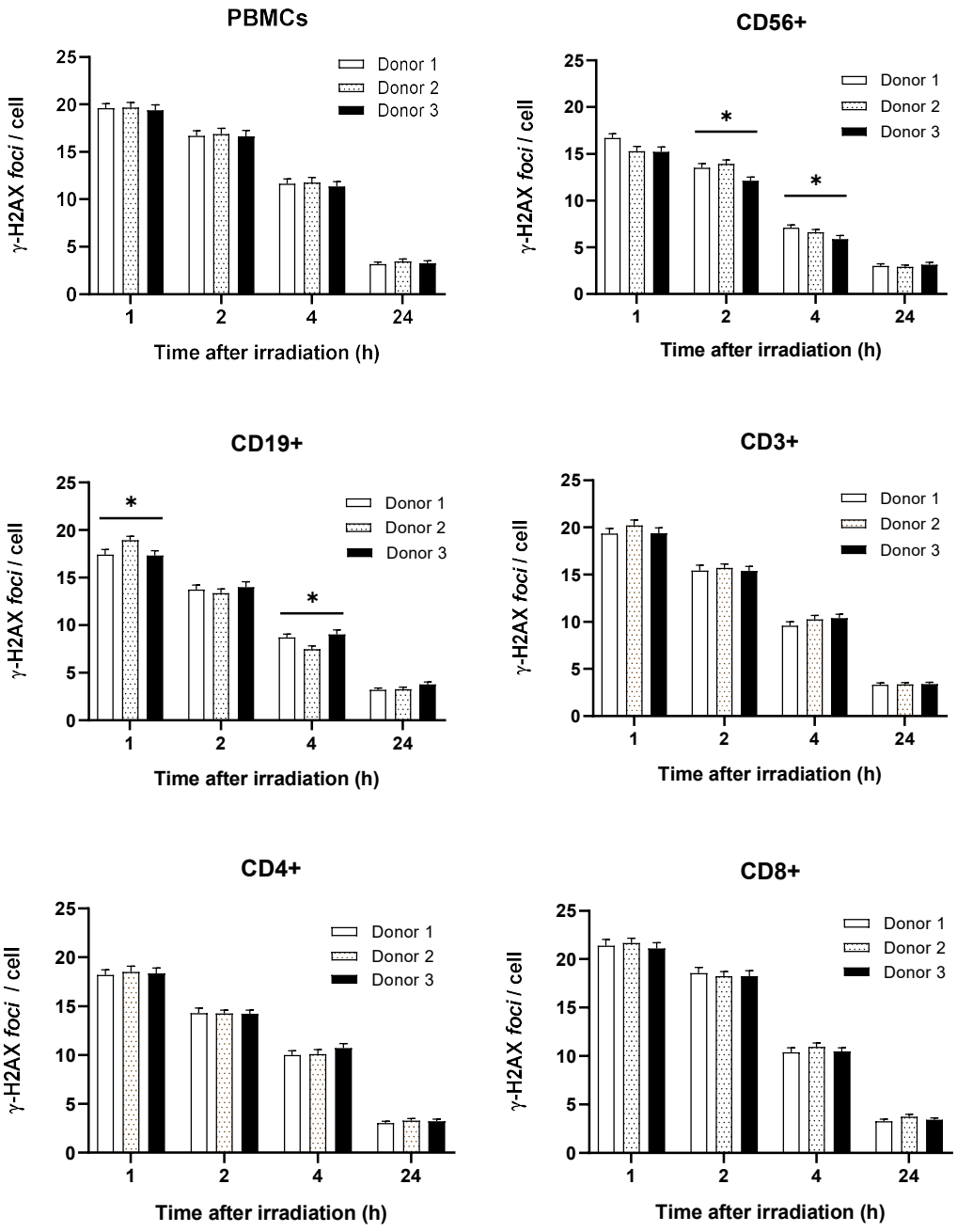

Figure 3. Yields of γ-H2AX foci per cell ± SEM after 2 Gy irradiation for each donor and for the four post-irradiation times evaluated. Significant differences between donors are inidicated with one asterisk ($p<0.05$).

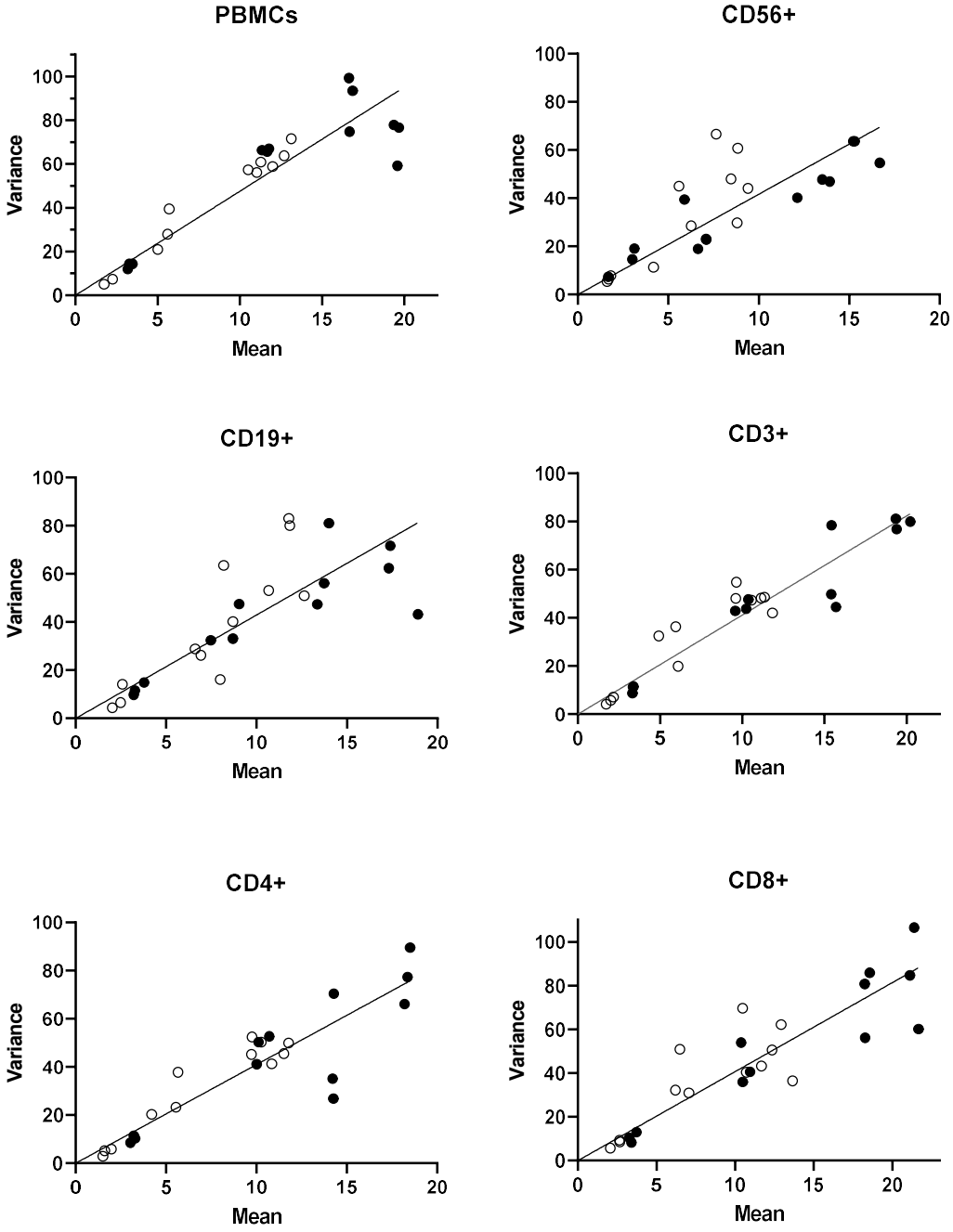

Figure 4. Correlation between the mean and the variance of the number of γ-H2AX foci per cell for each cell type. The slopes are: for PBMCs, 4.75±0.19; for $CD56^+$, 4.17 ±0.26; for $CD19^+$, 4.29±0.28; for $CD3^+$, 4.11±0.14; for $CD4^+$ 4.09±0.19; and for $CD8^+$, 4.07±0.20. Empty circles indicate the values obtained after 1Gy, and black circles the values obtained after 2 Gy.

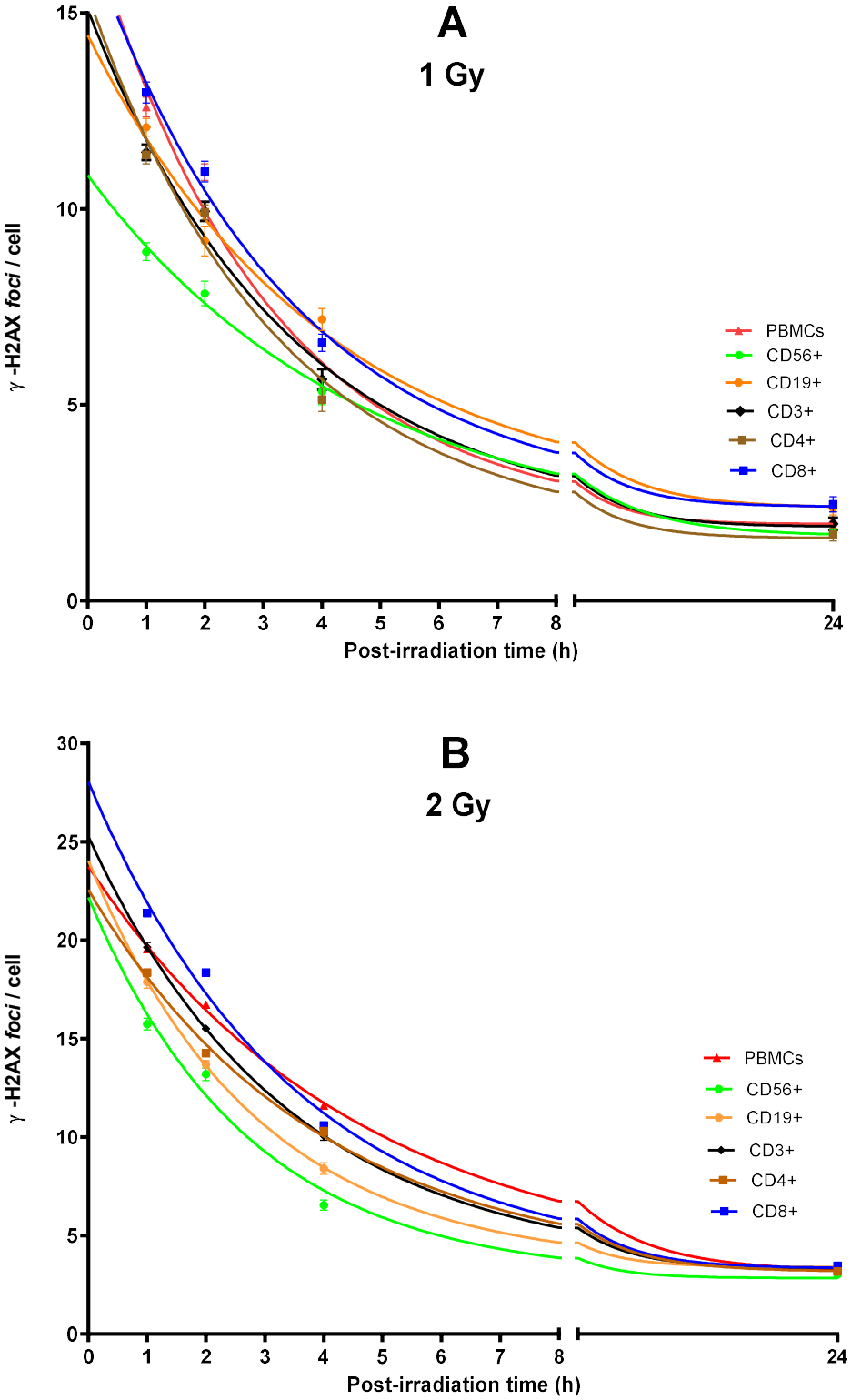

Figure 5. Decay in the γ-H2AX foci frequency with post-irradiation time for each cell subtype analyzed. A, frequency decay after 1 Gy irradiation. B, frequency decay after 2 Gy irradiation.