Human hematopoietic stem/progenitor cells display reactive oxygen species-dependent long-term hematopoietic defects after exposure to low doses of ionizing radiations

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Supplemental Methods

Primary cells

Cord Blood (CB) samples were collected from healthy infants with the informed written consent of the mothers based on the declaration of Helsinki. Samples were obtained in collaboration with the Clinique des Noriets, Vitry-sur-Seine and with the Cell Therapy Department in Hôpital Saint Louis, Paris, France. Samplings and experiments were acknowledged by the Institutional Review Board of INSERM (Opinion number 13-105-1, IRB00003888). $CD34^+$ cells were purified by immuno-magnetic selection using a CD34 MicroBeads kit (Miltenyi Biotec, Paris, France). For each experiment, we used a pool of $CD34^+$ cells from different healthy infants to diminish individual variability.

Flow cytometry

Femurs, tibias and hips were collected and BM cells were flushed in DMEM medium containing 10% FCS. Cells were labeled with specific anti-human antibodies in PBS buffer containing 2 mM EDTA and 0.5% BSA. In experiments in which the viability marker ZombiAqua (Biolegend) was used, cells were stained in PBS buffer only. Flow cytometry analyses were performed using BD-FACS-Canto (laser 488, 405 and 633, BD Bioscience), BD-LSR2 SORP (laser 405, 488, 561, and 633; BD Bioscience) and FACS-ARIA 2 (BD-Bioscience) cytometers. Results were analyzed using FlowJo Version 10.2 software (TreeStar).

Drug treatments

$CD34^+$ cells or HSPC were treated with NAC (50 μM, Sigma), Catalase (5 mg/mL, Sigma), SB203580 (p38MAPK inhibitor, 10 μM, Cell Signaling Technology) or Cysclosporine A (50 μg/mL, Sigma) for 1 hour at 37°C. Menadione (100 μM, Sigma) was used as a positive control of ROS production.

Transplantation assays

For primary transplantation, a pool of several samples of CB $CD34^+$ cells (10^5cells/mouse) were injected in NSG mice. Mice were sacrificed after 13 to 16 weeks post-transplantation. BM cells were recovered after flushing femurs, tibias and hips. Red cells were lysed using ACK lysing buffer (Gibco, LifeTechnologies). Engrafted human $CD45^+$ hematopoietic cells were measured

using specific anti-human CD45 antibodies. $CD45^+CD19^+$ B cells and $CD45^+CD14^+/CD15^+$ myeloid cells were quantified as previously done (Benyoucef et al., 2015). Serial transplantation assays were performed after phenotyping of BM cells from primary mice. Total BM cells were transplanted in new recipients with an equivalent of 5.10^4 $CD45^+$ $CD34^+$ $CD19^-$ human cells per mouse. In some experiments, human $CD34^+$ cells were isolated from mouse BM using CD34 microbeads (Miltenyi Biotech) and $CD34^+$ $CD38^{low}$ cells were purified by cell sorting then transplanted in secondary NSG mice ($1.6\ 10^4$ cells/mouse). Mice were sacrificed after 13 weeks post-transplantation.

Immunofluorescence

For DNA damage experiments, cell-sorted HSPC were irradiated and incubated 30 min, 1 hour or 3 hours at 37°C in MyeloCult medium(H5100, Stem Cell Technologies), fixed in paraformaldehyde 4% for 15 min at room temperature (RT) then washed in PBS. 2000 HSPC were coated on Poly-Prep slides (P0425-72EA, Sigma) overnight at 37°C then permeabilized with Triton™ X-100 (0.1%, 9002-93, Sigma) 10 min at RT. Cells were first incubated 1 hour with a saturation buffer containing 7.5% of goat serum and 7.5% of fetal bovine serum in PBS, then stained with anti-γH2AX (05-636, Merck Millipore) and anti-53BP1 (NB100_304, Novus Biologicals) overnight at 4°C, washed 3 times and finally stained with secondary antibodies, goat-anti-mouse-AF488 (A-11001) and goat-anti-rabbit AF-594 (A-11012, both from LifeTechnologies).

For assessment of 8-hydroxy-2'-deoxyguanisine (8-oxo-dG) lesions in DNA, sorted cells were irradiated at indicated doses or treated with H_2O_2 100 mM and cultured for 30 min in Myelocult H5100 (StemCell Technologies), then fixed in 1:1 methanol/acetone (-20°C) 5 min on ice. 10000 HSPC were coated on Poly-Prep slides (P0425-72EA, Sigma) at 4°C for at least 6 hours in PBS with calcium and magnesium ions. They were treated with HCl 2N (30 min), washed 3 times then neutralized with Tris-HCl pH 8,8 (5min). Cells were permeabilized using PBS + BSA 4 % + Triton 0,2% (15 min). Cells were first incubated 45 min with the saturation buffer (PBS + BSA 4% + Tween 0,2%) for 45 min, then stained with an anti-8-oxo-dG antibody (ab48508, AbCam) overnight at 4°C in the same buffer, washed 3 times and finally stained with goat-anti-mouse-AF647 (A-21235, LifeTechnologies) in PBS + Tween 0,2% for at least 1 hour.

For NRF2 translocation experiments, sorted cells were irradiated at indicated doses and

cultured for 2 hours in Myelocult (H5100, StemCell Technologies), then fixed in paraformaldehyde 4% 15 min at room temperature. 10000 HSPC were coated on Poly-Prep slides (P0425-72EA, Sigma) at 4°C for at least 6 hours in PBS with calcium and magnesium. Cells were permeabilized using PBS + BSA 4 % + Triton 0,2% (15 min). Cells were first incubated with the saturation buffer (PBS + BSA 4% + Tween 0,2%) for 45 min, then stained with an anti-NRF2 antibody (ab31163, AbCam) overnight at 4°C in the same buffer, washed 3 times and finally stained with goat-anti-rabbit-AF647 (A-21244, LifeTechnologies) in PBS + Tween 0,2% for at least 1 hour.

After 3 wash, staining with DAPI 1/2000 in PBS + Tween 0,2% was performed (5 min). Sealing of the slides was done using Fluoromount-G (Southern Biotech). Reading was done on the Leica TCS SP8 confocal microscope. Images were analyzed using ImageJ and CellProfiler softwares.

CFU-C assays

HSPC sorted from a pool of several samples of CB $CD34^+$, were seeded in complete methylcellulose medium (triplicates, 300 cells/mL, H4230, StemCell Technologies), supplemented with 1% L-glutamine, 1% penicillin/streptomycin, IL-3 (8 ng/mL), erythropoietin (2 IU/mL; Miltenyi Biotec), SCF (50 ng/mL), granulocyte colony stimulating factor (G-CSF; 25 ng/mL; Stem Cell Technologies) and granulocyte macrophage (GM) CSF (10 ng/mL; Stem Cell Technologies). Colonies were identified and scored after 12-14 days at 37°C. Depending on cord blood samples, between 60 to 80 colonies were generated from 300 sham or 20 mGy-irradiated HSPC. Serial plating was also performed: mixed or individual colonies were harvested in sterile 37°C pre-warmed PBS, and re-plated in complete methylcellulose medium for 12-14 additional days. In the case of mixed colonies, cells were counted then 2500 cells were plated in secondary cultured. When individual colonies were picked, they were re-plated in complete methylcellulose medium for 12-14 additional days in 24-well plate (10 cells/condition and / experiment). After 12-14 days, positive wells (wells with colonies) were numerated (Sup Figure 6).

Primary and extended Long-Term Culture Initiating Cell (LTC-IC) assays.

Limiting dilution allowed evaluating LTC-IC frequency within HSPC after exposure to different doses of irradiation. HSPC were seeded in 96-well plates pre-coated with MS5 stromal cells in

Myelocult medium (H5100, StemCell Technologies) (60, 20 or 6 cells per well) for 5 to 6 weeks. Each well was harvested and mixed with 500µL of complete methylcellulose (H4230, StemCell Technologies) before being plated in 24-well plates. LTC-IC-derived CFU-C were analyzed 12-14 days later. For extended LTC-IC assay, 3000 irradiated HSPC were seeded in 6-well plates pre-coated with MS5 stromal cells in MyeloCult medium (H5100, Stem Cell Technologies). Five weeks later $CD34^+$ expressing cells were cell sorted and re-seeded in limiting dilution (500, 100, 50, 25 and 10 cells per well in 96-well plates) with MS5 cells in MyeloCult medium for 5 additional weeks before to be tested for CFU-C during 12 to 14 days as described.

A positive well contained at least one colony. LTC-IC frequency was determined using L-CALC software (Stem Cell Technology).

CFSE staining

$3x10^5$ $CD34^+$ cells were labeled with CFSE (2.5 µM, sigma, France) and cultured ($5\text{-}10x10^3$/well) for 2 to 8 days in StemSpan medium (Stem Cell Technologies) supplemented with 1% L-glutamine, 1% penicillin/streptomycin, IL-3 (8 ng/mL), erythropoietin (2 IU/mL; Miltenyi Biotec), SCF (50 ng/mL), granulocyte colony stimulating factor (G-CSF; 25 ng/mL; Stem Cell Technologies) and granulocyte macrophage (GM) CSF (10 ng/mL; Stem Cell Technologies). Cell divisions were analyzed by flow cytometry.

Intracellular flow cytometry

Cells were first stained with antibodies directed against extracellular markers (CD34, CD90, CD38 and CD45). Then, cells were permeabilized and fixed using Cytoperm/cytofix and Perm wash buffer (BD Bioscience). Intracellular staining was performed for Ki67- FITC or PE kit (BD Biosciences) and Hoechst or cleaved-caspase3-PE kit (BD Biosciences) according to manufacturer instructions.

For phospho-P38MPAK, after extracellular staining, cells were first fixed with Phosflow Fix buffer I (BD Bioscience), then permeabilized using BD Phosflow Perm Buffer II following manufacturer procedures. Cells were then stained with anti-pP38-PE (clone 3D7, Cell signaling technology).

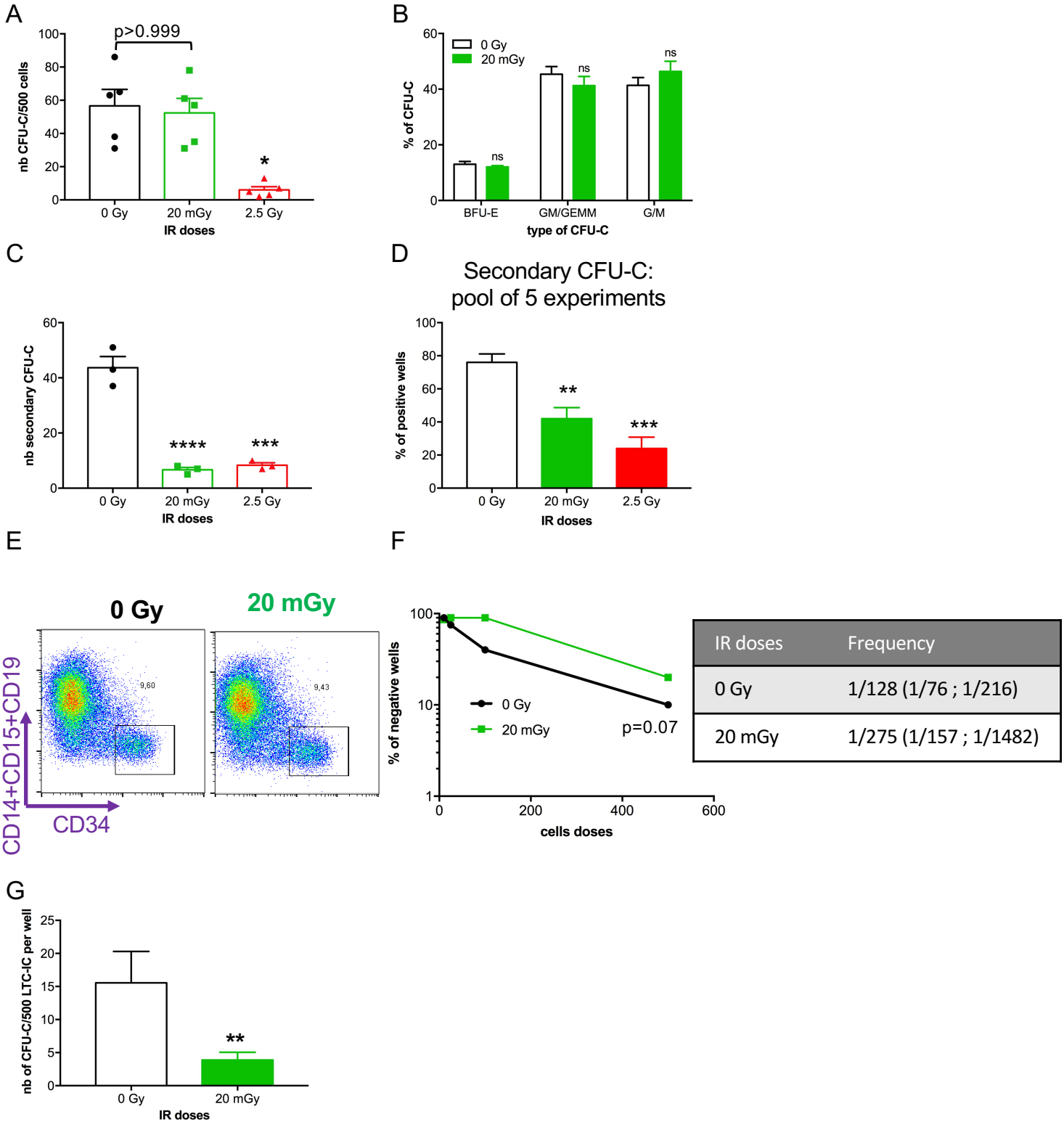
For phosphor-ATM (Ser1981), p53 and phospho-p53 (Ser 35), cells were fixed and permeabilized using Nuclear transcription factor buffer set (Biolegend) following manufacturer procedures.

Autophagy measurement

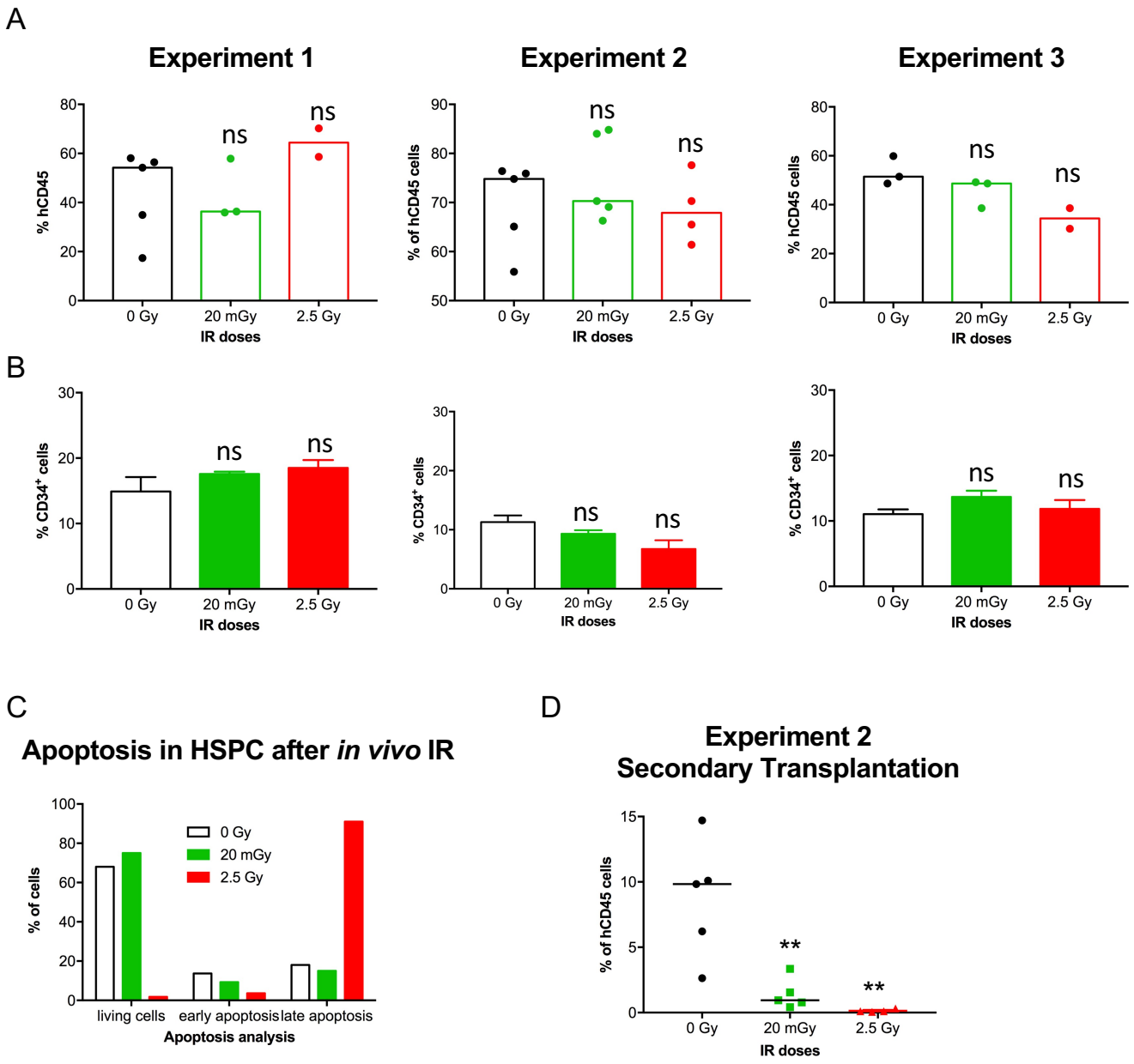
Cells were first stained with antibodies directed against extracellular markers (CD34, CD90, CD38 and CD45) at 37°C, then washed in PBS. Autophagy was measured using CytoID kit (Ref ENZ-51031-0050, Enzo Life Science) following manufacturer procedures.

Ab name	Clone	Fluorochrome	Reference	Brand
CD90	5E10	APC	559869	BD Bioscience
CD34	581	PE-Cy7	343516	Biolegend
CD34	581	APC	343510	Biolegend
CD38	HB-7	FITC	356610	Biolegend
CD45RA	HI100	PE	304108	Biolegend
CD45	HI30	BV421	304032	Biolegend
CD3	SK7	PeRCP-Cy5.5	344808	Biolegend
CD19	HIB19	APC-eF780	47-0199-42	ebioscience
CD19	HIB19	PE	302208	Biolegend
CD14	61D3	PE	12-0149-42	ebioscience
CD15	HI98	PE	301906	Biolegend
Phospho-ATM (Ser1981)	10H11.E12	PE	651203	Biolegend
p53	1C12	AFF488	2015	Cell Signaling
Phospho-p53 (Ser15)	16G8	AF647	8695	Cell Signaling
Cleaved-Caspase 3	C92-605	PE	561011	BD Bioscience
Phospho-p38MAPK (Thr130/Tyr182)	3D7	PE	6908S	Cell Signaling
8-Hydroxy-2'-deoxyguanosine (8oxo)	N45.1	purified	ab48508	abcam
phospho-Histone H2A.X (ser139) γH2AX	JBW301	Purified (mouse)	05-636	Merck Millipore
53BP1	polyclonal	Purified (rabbit)	NB100_304	Novus Biologicals
NRF2	polyclonal	Purified (rabbit)	ab31163	abcam
Goat-anti-mouse		AF488	A-11001	Life Technologies
Goat-anti-rabbit		AF594	A-11012	Life Technologies
Goat-anti-mouse		AF647	A-21235	Life Technologies

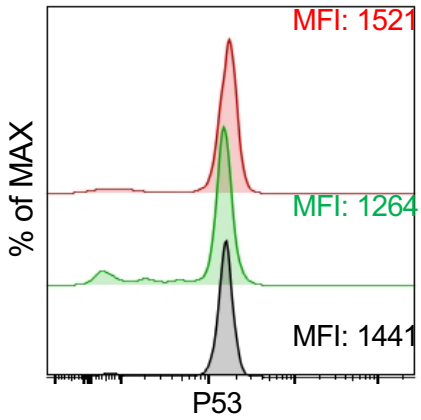
Sup table 1: Antibodies used for this study



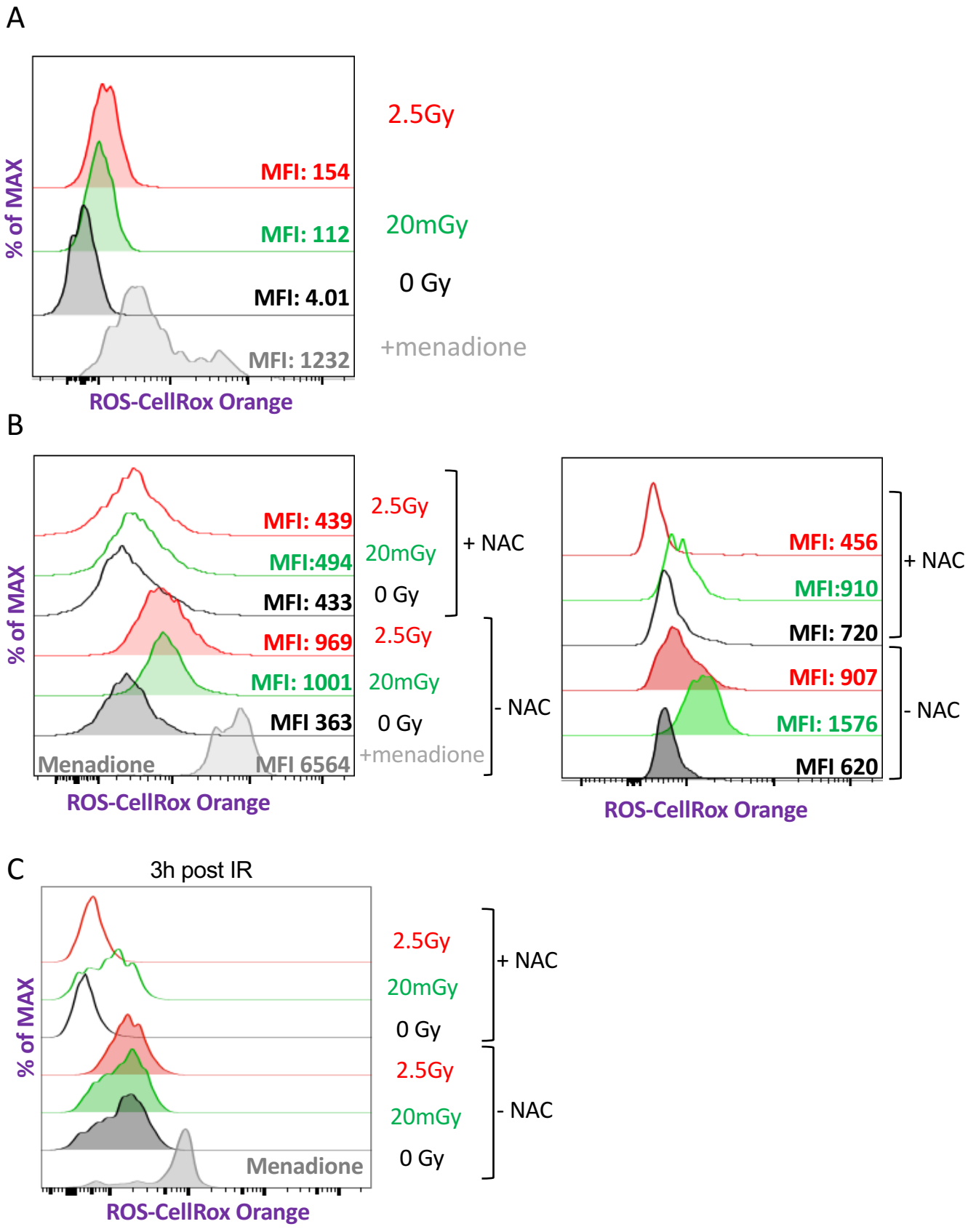
Sup Figure 1 related to Figure 1: (A) Cell-sorted HSPC were irradiated at indicated doses then culture for 12 days in CFU-C medium. CFU-C were then quantified. Primary CFU-C assay (cumulative results from 4 experiments with HSPC isolated from 4 independent pools of CB samples). HSPC (500 cells/plate) were plated in CFU-C conditions for 12 to 14 days and the CFU-C number was quantified. Raw data of normalized data shown in Figure 1B are represented here (B) Quantification of the different types of CFU generated (BFU-E, CFU-GEMM + CFU-GM and CFU-M+CFU-G) (C) Primary CFU-C were pooled and replated in methylcellulose for 12 to 14 days. Shown are the number of secondary CFU-C. Raw data of normalized data shown in Figure 1C are represented here (cumulative results from 3 experiments). **, $p<0.01$ (Mann and Whitney statistics) (D) CFU-GM colonies from primary cultures were picked and replated in methylcellulose for 12 more days in CFU-C condition in 24 wells plates. The percentage of wells with CFU-C is shown. (E-G) Cell-sorted HSPC were irradiated at indicated doses and cultured with MS5 stromal cells for 5 weeks in LTC-IC medium. Cells were thereafter recovered, stained with surface markers against CD14 CD15 CD19 (Ab conjugated to PE) and CD34 (Ab conjugate to APC). (E) shows gating strategy. Cultured $CD34^{hi}$ cells were sorted and re-seeded at limiting dilution on MS5 cells for 5 additional weeks. After 5 weeks, each single well was transferred in methylcellulose for 12 days. (F) Frequency of LTC-IC calculated using L-CALC software (G) Number of CFU-C obtained when 500 sorted $CD34^{hi}$ cells were cultured as described in (E).



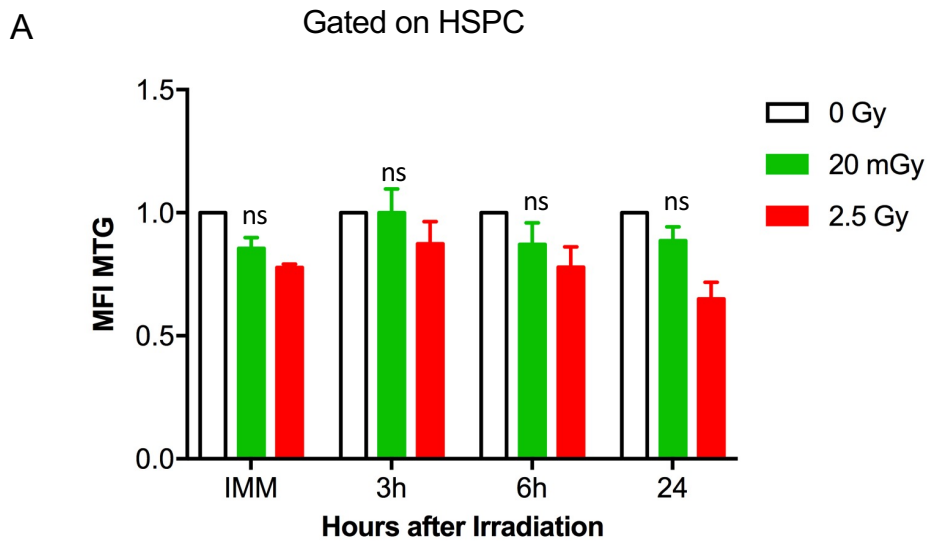
Sup Figure 2 related to Figure 2: Hematopoietic reconstitution capacities of human HSC after *in vivo* exposure to LDIR. NSG mice were grafted with 5.10^4 $CD34^+$ cells. Engrafted mice were irradiated 15 weeks post graft then sacrificed. 3 independent experiments. (A) Graphs represent human chimerism for each experiment just after NSG mouse irradiation and sacrifice. (B) Graphs represent the frequency of $CD34^+$ cells among $CD45^+$ compartment just after NSG mouse irradiation and sacrifice. (C) The apoptosis was measured in HSPC compartment after mouse exposure to irradiation. (D) BM cells were recovered from primary recipient mice and an equivalent of 5.10^4 $CD34^+$ $CD19^-$ BM cells were injected in secondary recipient mice. This graph represents the frequency of $hCD45^+$ cells in the BM of secondary NSG mice for experiment n°2. Statistical analysis : Anova one way 0Gy vs 20mGy p=0.0036 and 0 Gy vs 2.5 Gy p=0.0026.

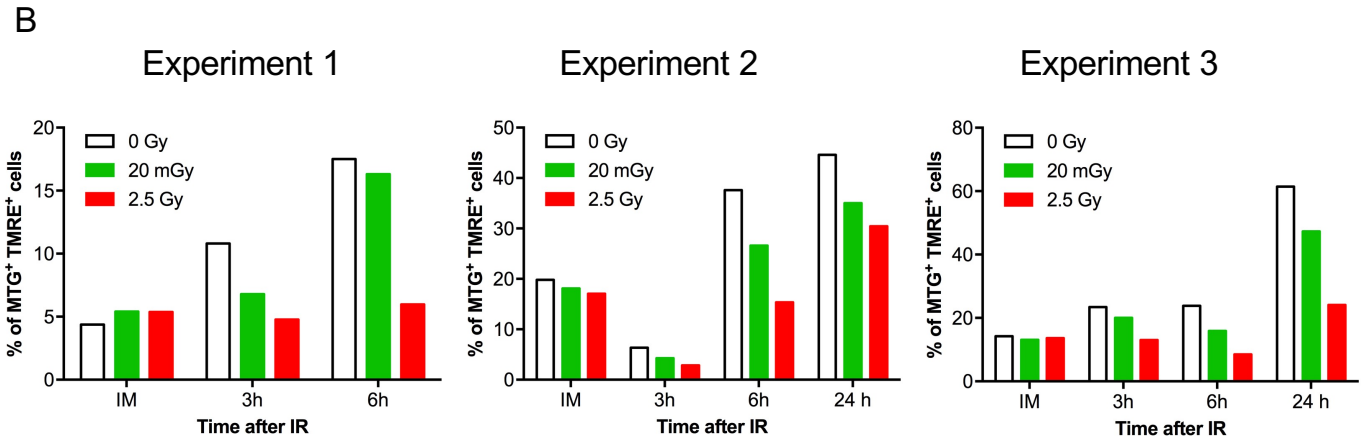


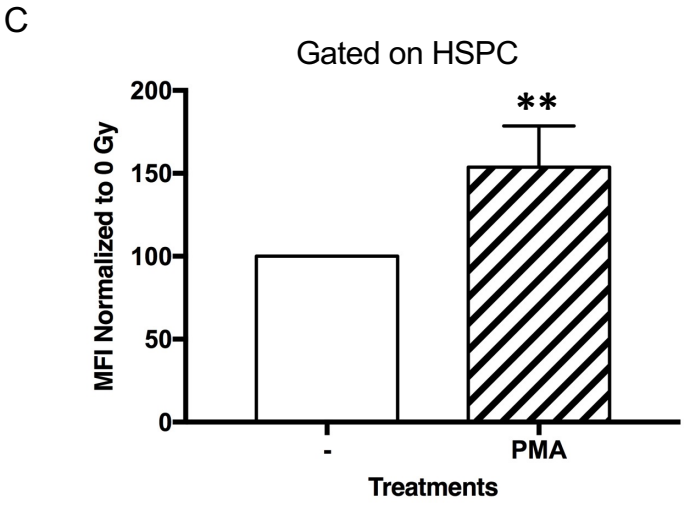
Sup Figure 3 related to Figure 4: $CD34^{+}$ cells were irradiated, cultured 3h at 37°C, stained for cell surface markers then fixed. Analysis p53 protein expression in $CD34^{+}$ $CD38^{low}$ $CD45RA^{-}$ $CD90^{+}$ HSPC by FACS 3 hours post IR (one representative experiment out of 4).



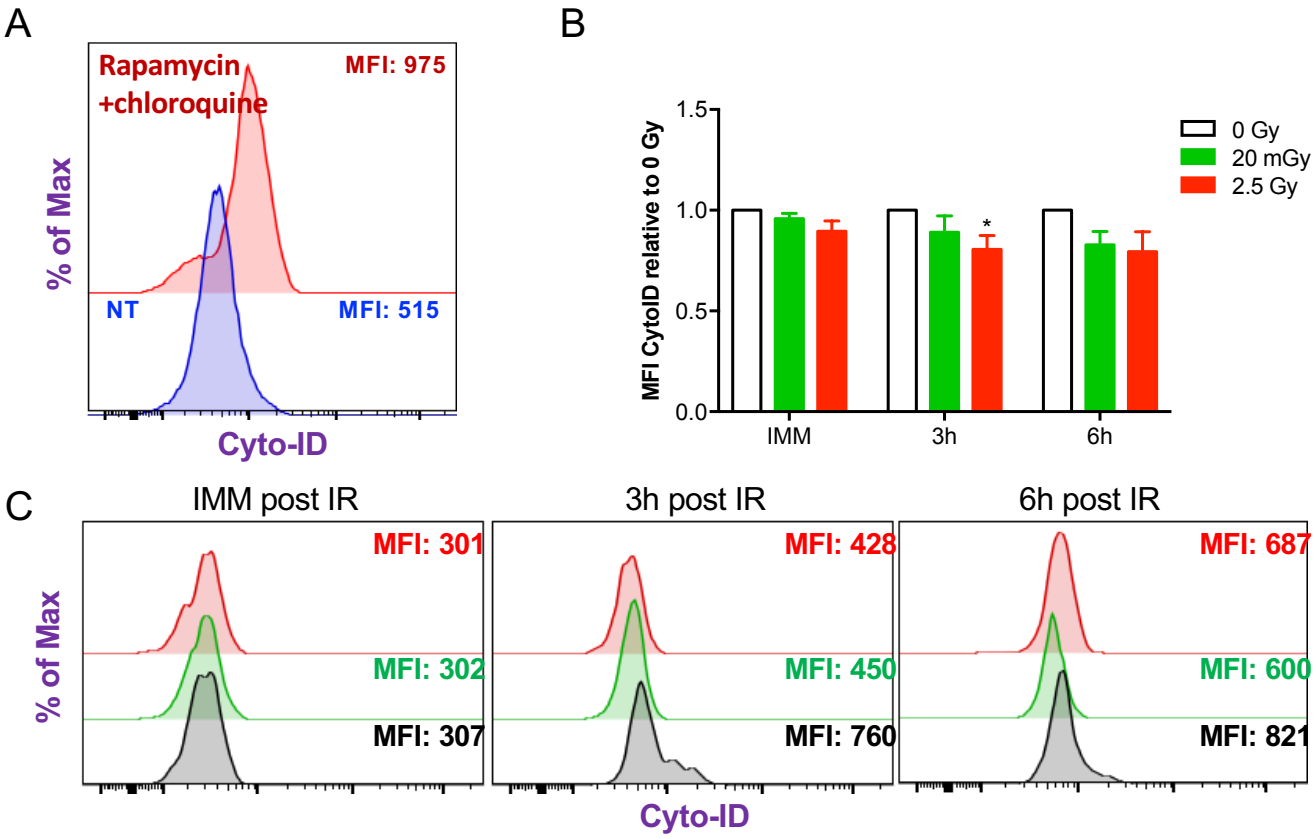
Sup Figure 4 related to Figure 5: Cell-sorted HSPC were treated or not with NAC, irradiated at indicated doses, then ROS levels were quantified immediately post IR on HSPC using CellRox Orange probe (3 experiments). (A) First experiment done with menadione (light grey) as positive control, full black histogram represents non-irradiated condition, green 20mGy-irradiated condition and red 2.5Gy-irradiated condition. (B) Second and third experiments made. Cell-sorted HSPC were treated or not with NAC. Full histogram represents without NAC treatment, and empty histogram represents after NAC treatment. (C) ROS levels were quantified in $CD34^{+}$ $CD38^{low}$ $CD45RA^{-}$ $CD90^{+}$ HSPC using CellRox Orange probe 3h post IR.



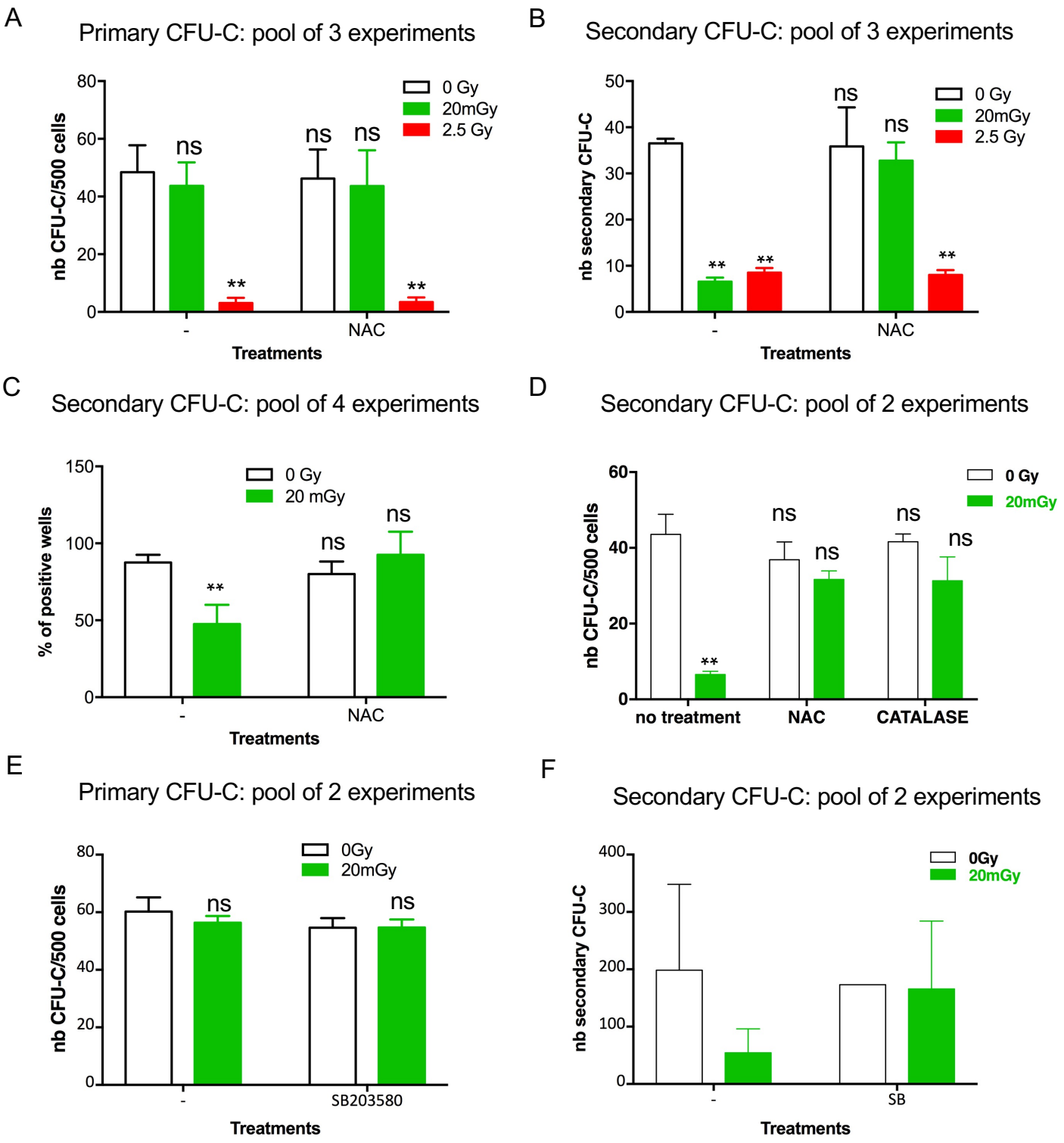




Sup Figure 5 related to Figure 5: (A-B) The mitochondrial activity was monitored over time by using MTG (Mitochondrial mass) and TMRE (membrane potential) probes in HSPC. (A) Shown is the mitochondria mass (MFI MTG) over time in culture in HSPC compartment (3 independent experiments) (B) Shown is the membrane potential (MFI TMRE) over time in culture in HSPC compartment for each experiment (C) $CD34^+$ cells were incubated at 37°C with PMA (positive control) for 30 min or remain untreated. Surface staining was made during incubation period. Then cells were fixed and permeabilized (see method) then stained with phospho-p38MAPK antibody. As expected increased p38MAPK phosphorylation is observed when HSPC are treated with PMA as shown by mean of fluorescence (MFI).



Sup Figure 6 related to Fig 5: (A) The autophagy activation was monitored over time by using the CytoID probe in HSPC. (A) As positive control of autophagy activation, $CD34^+$ cells were treated for 16h with chloroquine and rapamycin, known to induce autophagy, and thereafter incubated for 30 min with the CytoID probe. (B) $CD34^+$ cells were first irradiated then incubated with the cytoID probe at indicated time post IR. Shown is the autophagy activation (MFI CytoID) over time in culture in HSPC compartment (pool of 3 independent experiments) (C) Shown is the overlay histogram of the CytoID staining over time in culture in HSPC compartment for one representative experiment.



Sup Figure 7 related to Figure 6: Cell-sorted HSPC were treated or not with indicated drugs, irradiated at indicated doses and seeded in CFU-C conditions. CFU-C were then quantified. (A) Primary CFU-C assay (cumulative results from 3 experiments with HSPC isolated from 3 independent pools of CB samples). HSPC (500 cells/plate) were plated in CFU-C conditions for 12 to 14 days and the number of CFU-C was quantified. Raw data of normalized data shown in Figure 6A are represented here. (B) Primary CFU-C were pooled and replated in methylcellulose for 12 to 14 days. Shown are the number of secondary CFU-C. Raw data of normalized data shown in Figure 6B are represented here. (C) CFU-GM colonies were picked then cultured for 12 more days in CFU-C condition in 24 wells plates. The number of wells with CFU-C is shown. (D) Primary CFU-C untreated, NAC, or Catalase-treated were pooled and cultured in CFU-C conditions for 12 more days. The number of secondary CFU-C generated in each condition is shown. (E) Primary CFU-C assay (cumulative results from 2 experiments with HSPC). HSPC (500 cells/plate) were plated in CFU-C conditions for 12 to 14 days and the number of CFU-C was quantified. Raw data of normalized data shown in Figure 6C are represented here. (F) Primary CFU-C were pooled and replated in methylcellulose for 12 to 14 days. Shown are the number of secondary CFU-C. Raw data of normalized data shown in Figure 6D are represented here.