Mesenchymal Stromal Cells regulate human Hematopoietic Stem Cell survival and regeneration via cAMP/PKA pathway

Siva Sai Naga Anurag Muddineni[1,2], Chen Katz Even[1,2], Adi Zipin-Roitman[1,2], Katia Beider[3]

Yael Raz[4], Eviatar Weizman[5], Arnon Nagler[3], Michael Milyavsky[1,2]

Affiliations

[1]Department of Pathology, Tel Aviv University, Tel-Aviv, 69978, Israel.

[2]Sackler Faculty of Medicine, Tel Aviv University, Tel-Aviv, 69978, Israel.

[3]Hematology Division, Chaim Sheba Medical Center, Tel-Hashomer, Israel.

[4]Department of Obstetrics and Gynecology, Gynecologic Oncology Division, Lis Maternity Hospital, Tel Aviv Sourasky Medical Center, Tel-Aviv, 64239, Israel.

[5]G-INCPM, Weizmann Institute of Science, Rehovot 7610001, Israel

Corresponding author: Michael Milyavsky, mmilyavsky@post.tau.ac.il

Abstract

Ionizing radiation and chemotherapy suppress normal Hematopoietic Stem and Progenitor Cells (HSPCs) function and present an inherent risk of developing bone marrow failure and secondary leukemias. Mesenchymal Stromal Cells (MSCs) constitute an essential HSPC niche component by regulating HSPC quiescence, self-renewal, survival, and differentiation. Yet, the underlying pro-regenerative signaling pathways activated in HSPCs by MSCs remain poorly understood. Here, using co-culture system of bone marrow-derived MSCs and HSPCs we established that irradiated HSPCs co-cultured with MSCs underwent significantly less apoptosis and maintained their *in vivo* repopulating ability. We reveal that MSCs mediate protection by preserving pro-survival MCL1 protein levels in human HSPCs. Moreover, using unbiased HSPC transcriptomic analysis complemented with ex vivo and in vivo validation studies we discovered that MSCs activate cAMP/PKA/CREB signaling pathway in HSPCs to prevent MCL1 decline and apoptosis onset upon irradiation. Pharmacological activation of cAMP signaling pathway in human HSPCs replicated MSC mediated protection of HSPC functionality. Collectively, our results highlight the role of cAMP/PKA/CREB signaling pathway in MSC-HSPC cross-talk and its critical role in the regulation of survival and self-renewal ability of human HSPCs. Furthermore, we also revealed pharmacological agents that can mitigate radiation-induced hematological sequelae.

Introduction

Hematopoiesis is a highly regulated process that supplies mature blood cells of various lineages. In an adult human, 4-5 × 10^{11} blood cells are produced daily to ensure homeostasis[1]. Hematopoietic Stem and Progenitor Cells (HSPCs) are responsible for the hematopoietic homeostasis throughout our lifetime. HSPCs are endowed with the unique ability to produce more stem cells (a property defined as self-renewal), as well as, to give rise to progenitors with limited self-renewal capacity[2,3]. Ionizing radiation (IR), certain chemotherapies and repeated inflammatory exposure leads to HSPC apoptosis and impair their long-term regenerative capacity[4–7]. These HSPC-related dysfunctions may lead to myelosuppression, bone marrow failure and leukemic transformation[8]. It is therefore crucial to decipher the molecular mechanisms that regulate HSPC survival, maintenance and regeneration in order to ameliorate these sequelae.

HSPCs are regulated extrinsically by the bone marrow (BM) microenvironment referred to as 'hematopoietic stem cell niche'. The niche consists of the complex array of different cell types, including Mesenchymal Stromal Cells (MSCs), endothelial cells, osteoblasts, adipocytes, and sympathetic nerve cells amongst others[9].MSCs are multipotent stromal cells that can differentiate into osteoblasts, adipocytes and chondrocytes and support human HSPC long-term regenerative capacity ex vivo. MSC-HSPC co-culture experiments highlighted the importance of the cell contact-mediated HSPC-MSC interactions in regulating HSPC quiescence, self-renewal, survival and differentiation[10–12].

Detailed modeling of acute radiation response in murine hematopoietic system revealed a complex array of processes occurring in HSPCs, including apoptosis[13–15], senescence[6], precipitous differentiation[16], as well as dramatic changes in the supporting non-hematopoietic niche[17–21]. In parallel, several groups also identified factors produced by the niche capable of preventing DNA damage associated myelosuppression in mice (e.g. EGF[22], VEGF[23], Angiopoietin[24], Pleiotrophin[25], PGE2[26]. However, regarding factors able to mediate human HSPC protection and regeneration, data are scarce, and were obtained almost exclusively *in vitro* using bulk of CD34+ cells, but not HSPCs. Notably, recent studies revealed no protective effect of PGE2, EGF, TPO and FLT3L factors on human HSPCs subjected to sublethal irradiation *in vitro* and in xenotransplantation setting[27]. Thus, despite their ability to promote murine HSPCs regeneration, these molecules lack the same activity when tested on human HSPCs pointing to the existence of non-conserved pathways and/or unexpected toxicity and growth suppression (as was observed for PGE2[27]).

Thus, the mechanisms that underlie human HSPC regeneration are poorly understood and this gap in knowledge represents a barrier for developing targeted therapies that can

mitigate myelosuppression. Moreover, this gap in knowledge and observed differences with model organism studies, emphasize the need for a systematic analysis of the niche-producing factors regulating human HSPC survival and regeneration after DNA damage.

Our previous research established that compared to Progenitors, human HSPCs exhibit delayed DNA DSB rejoining, persistent DDR activation, and higher sensitivity to the cytotoxic effects of γ-radiation (IR)[4,5]. We demonstrated, that irradiated human HSPCs undergo rapid apoptosis via the p53/ASPP-1 pathway that can be blocked by the ectopic expression of pro-survival mitochondrial protein Bcl-2. Despite the vital role of MSCs in regulating HSPCs, the molecular mechanisms (and signaling pathways) of direct cell-to-cell communication in the hematopoietic microenvironment that govern HSPCs survival and long-term hematopoietic regeneration remain poorly understood.

Here, using co-culture system of MSCs and irradiated human HSPCs (young and adult) we established that irradiated HSPCs co-cultured with MSCs underwent significantly less apoptosis and maintained their in vivo repopulating ability. We found that MSC mediated protection is mediated by preserving pro-survival MCL1 levels in human HSPCs. Moreover, using unbiased transcriptomic analysis and ex vivo and in vivo validation we discovered that MSCs activate cAMP signaling pathway to prevent MCL1 decline and apoptosis onset upon irradiation. Importantly pharmacological activation of cAMP signaling pathway is able to replicate MSC mediated protection of HSPC functionality. Collectively, our results highlight the role of cAMP/PKA signaling pathway in MSC-HSPC cross-talk and its critical role in regulation of survival and self-renewal ability of human HSPCs.

Results

1) MSCs protect human HSPCs from DNA-damage induced apoptosis

Isolated human Hematopoietic Stem and Progenitor Cells (HSPCs) are exquisitely sensitive to Ionizing Radiation (IR) and lose their hematopoietic reconstitution potential[4,5,28]. HSPCs reside in a specialized bone marrow niche and are regulated by a variety of niche cells including Mesenchymal Stromal Cells (MSCs)[29,30]. To investigate how MSCs affect HSPC fate decisions after genotoxic stress we utilized HSPC-MSC co-culture system (Figure 1A). In this experimental system, human CD34+ cells cultured either in serum-free medium supplemented with cytokines (SCF, FLT-3 and TPO, thereafter SFT) or on top of MSCs were exposed to DNA damage (IR or chemotherapy). Then, apoptosis onset in HSPCs (defined as CD34+CD38-CD45RA-) was analyzed by flow cytometry using the gating strategy outlined in Supplementary Figure 1A. Of note, the same exogenous cytokine cocktail (SFT) was added to CD34+ cells cultured with MSC to minimize the number of experimental variables.

As we and others reported previously, irradiated human HSPCs underwent apoptosis at a high rate marked by a significant increase in the percentage of Annexin positive cells[4,5]. On the contrary, no apoptosis induction was observed in the irradiated HSPCs co-cultured with hBM-MSCs (Figure 1B).

The availability of human BM-derived MSCs is limited. To better understand the molecular mechanisms by which BM-MSCs protect human HSPCs from IR-induced apoptosis, we utilized murine OP9M2 MSCs, which were previously shown to support human hematopoiesis ex vivo[31]. To test whether OP9M2 could protect human HSPCs from cytotoxic stress similar to hBM-MSCs, we exposed CB-derived CD34+ cells to IR or Etoposide. We observed that both treatments resulted in a strong increase in the percentage of apoptotic HSPCs cultured in the presence of cytokines (SFT group). Strikingly no apoptosis induction was observed in HSPCs co-cultured with OP9M2 (OP9M2 group) even 48 hours post treatment (Figure 1B, Supplemental Figure 1B, 1C). To further substantiate these findings obtained with neonatal HSPCs, we next examined whether MSCs can protect adult human HSPCs isolated from healthy BM donors. We found no induction of apoptosis in irradiated BM HSPCs co-cultured with OP9M2 cells (Figure 1C). These data reveal that BM derived MSCs are capable of inhibiting DNA damage-induced apoptosis in human HSPCs. Furthermore, the apoptosis inhibiting properties is conserved between human and murine MSCs.

Blocking of IR-induced apoptosis in HSPCs can contribute to better regeneration. On the other hand, apoptosis-independent processes in injured HSPCs, including senescence and precocious differentiation can preclude their regeneration[13,32,33]. To quantitate the impact

of MSC co-culture on the functionality of irradiated HSPCs we employed long term culture initiating cell (LTC-IC) assay in vitro and SCID-repopulating cell (SRC) assay in vivo.

In LTC-IC limiting dilution analyses, we observed a massive drop in LTC-IC frequency of irradiated HSPCs cultured in SFT group, which was improved by 5-fold in irradiated HSPCs co-cultured with MSCs (Figure 1D). Similarly, we observed that irradiation impaired the SCID repopulating ability of HSPCs in SFT group (70% NT group vs 3% in IR group) while co-culture with MSCs led to a significant preservation of human functional HSPCs (50% NT group vs 16% IR group). (Figure 1E, Supplemental Figure 2A-C). Overall, these results suggest that BM-MSC relayed signals are capable of inhibiting genotoxic stress induced apoptosis of human HSPCs and preserving their functionality.

2) MSCs preserve anti-apoptotic MCL1 protein levels in irradiated human HSPCs

Alterations in the balance between the pro- and anti- apoptotic BCL-2 family proteins are implicated in the death of HSPCs after ionizing radiation[34]. BCL-2 family proteins, such as BCL-2, BCL-XL and MCL1, can antagonize pro-apoptotic proteins and protect cells from undergoing DNA damage-induced apoptosis[35]. However, the dynamics of these proteins in human HSPCs undergoing apoptosis is poorly defined. Thus, we measured the intracellular protein levels of BCL2, BCL-XL and MCL1 in human HSPCs cultured alone or on MSCs after irradiation using standardized and optimized dilutions of BCL2 family specific antibodies (Supplemental figure 3A-F). We observed that irradiation induced significant downregulation of MCL1 protein levels, but not BCL2 and BCL-XL proteins in HSPCs (Figure 2A-C; Supplemental Figure 4A). Strikingly, we found no change in MCL1 levels in irradiated HSPCs co-cultured with MSCs (Figure 2C). To further delineate the role of the specific BCL2 family members, in mediating MSC-relayed pro-survival signal we utilized BH-3 mimetics capable of neutralizing specifically BCL2, BCL-XL or MCL1. We observed that inhibition of both MCL-1 and BCL-XL proteins was required to block OP9M2-mediated protection of human HSPCs (Supplemental Figure 5B). Importantly, no changes in MSC viability were detected upon treatment of co-cultures with these drugs (data not shown). Collectively, these results reveal that downregulation of MCL1 is associated with cell-autonomous apoptosis onset in human HSPCs after IR. In co-culture conditions, MSCs maintain MCL1 levels in irradiated HSPCs and thus preserve the mitochondrial membrane integrity, essential for HSC survival and functionality.

3) HSPCs co-cultured with MSCs acquire stemness and anti-inflammatory gene signature

To elucidate the molecular programs that contribute to the enhanced survival and functionality of human HSPCs upon co-culture with MSCs we performed genome-wide RNA-sequencing of HSPCs cultured in SFT medium or on MSCs for 24 hours. Differential gene expression analysis revealed upregulation of genes related to AP1 Transcription

factor (FOSL2, FOS, FOSB, JUN), cellular metabolism (ALDOC, PFKFB4) and downregulated expression of pro-inflammatory genes (IL1B, STAT1 CCL1, CCL2) in HSPCs co-cultured with MSCs. Interestingly, these coordinated transcriptional programs were largely unmodified by IR. (Figure 3A, 3B). Gene Set Enrichment Analysis (GSEA) revealed that MSCs downregulated stress associated gene sets ("Apoptosis", "Unfolded Protein Response"), proliferation-related gene sets ("Myc targets V1", "Oxidative Phosphorylation") and pro-inflammatory gene sets ("Interferon Gamma response", "Interferon Alpha response") in HSPCs. On the other hand, gene sets primarily attributed to the most primitive HSC subsets, such as "Hypoxia", "Glycolysis" and LT-HSC stemness were enriched in HSPC-MSC co-cultures (Figure 3C,3D, E)[36–38]. To further characterize how MSCs contribute to the maintenance of stemness in human HSPCs and validate transcriptomic analyses, we measured endothelial protein C receptor (EPCR) expression, well known anti-inflammatory protein, that was recently shown to identify highly regenerative human HSC population[39]. Consistent with our GSEA analysis, we observed a significant upregulation of EPCR expression on human HSPCs cultured on MSCs relative to those cultured in SFT. Importantly, IR of HSPCs followed by co-culture with MSCs did not significantly impair EPCR induction or expansion of EPCR+ HSPCs (Figure 3F). Overall, transcriptomic analysis revealed a robust transcriptional reprogramming of HSPCs isolated from MSCs co-cultures. This reprogramming is associated with marked enrichment for stemness gene sets, expansion of regenerative subpopulations, and resilience to DNA damage.

4) MSCs activate cAMP/CREB signaling pathway to protect human HSPCs

To identify the upstream Transcription Factors (TFs) that alter gene expression in HSPCs upon interaction with MSCs, we performed GSEA using Transcription Factor Targets (TFT) gene sets collection. We observed that MSCs induced target genes of several TFs such as STAT5A, HNF3, FOXO4 and bZIP family of transcription factors like ATF1, AP1 & CREB1 in HSPCs (Supplemental Figure 6A; Figure 4A). Intriguingly, the DNA binding sites of CREB and AP1 TFs are strikingly similar, such that FOS and JUN (AP1 family members) can bind and transactivate cAMP-responsive elements (CREs) recognized by CREB[40]. Furthermore, cAMP dependent protein kinase A (PKA) strongly augments transcriptional activity of CREB, JUN and ATF1. Thus, we hypothesized that activation of PKA might be a key signaling event that control coordinated gene expression changes and pro-survival phenotype in HSPCs upon contact with MSCs. To test this hypothesis, we utilized a cAMP antagonist (Rp-8-Br-cAMPs) that prevents PKA activation[41]. We observed that MSCs were unable to protect HSPCs from IR-induced apoptosis when pretreated with Rp-8-Br-cAMPs (Figure 4B). These results strongly implicate PKA activation as a critical mediator of MSC mediated pro-survival phenotype in human HSPCs.

Amongst the different transcription factors activated in OP9M2 group, CREB1 (hereafter CREB) caught our attention, as CREB has been shown to regulate the induction of immediate early genes and has also been implicated in survival of human CD34+ cells[42]. Moreover, leading edge CREB target genes such as VGF, AREG, ID1, DUSP1 and JOSD1 which are upregulated upon co-culture with MSCs have been shown to play anti-apoptotic roles in different cellular contexts[43–47] (Supplemental Figure 6B). Thus, we decided to check whether OP9M2 MSCs protect human HSPCs by modulation of CREB signaling pathway

To check if CREB is activated upon co-culture with MSCs, we measured intracellular levels of Phospho-CREB (P-CREB S133) in human HSPCs under different culture conditions. We found that irradiation induced downregulation of P-CREB levels in HSPCs cultured in SFT group, while no change in P-CREB levels were observed in HSPCs cultured in OP9M2 group (Figure 4C). Moreover, addition of a cAMP antagonist, Rp-8-Br-cAMPS was able to abrogate MSC mediated CREB phosphorylation in irradiated HSPCs (Figure 4C). This observation suggests that CREB TF is activated upon co-culture with OP9M2 MSCs possibly in a cAMP/ PKA dependent manner (Figure 4A).

Previous studies have reported gap junction mediated transfer of cAMP between adjacent cells[48–50]. To test if OP9M2 MSCs transfer cAMP to HSPCs through gap junctions, we used Carbenoxolone – a gap junction inhibitor and GAP27 connexin mimetic peptide to inhibit gap junction activity. Neither carbenoxolone nor GAP27 abrogated OP9M2 mediated protection, suggesting that OP9M2 MSCs protect HSPCs in a gap junction independent manner (Supplemental Figure 6C, 6D). These results underscore the importance of cAMP/PKA/CREB signaling pathway activation in MSC-mediated protection of HSPCs.

5) Pharmacological activation of cAMP/CREB signaling pathway protects human HSPCs from IR induced apoptosis

Based on our findings which indicate interfering with cAMP activated signaling pathway results in the reversal of MSC mediated changes in HSPC gene expression and survival, we hypothesized that pharmacological stimulation of this pathway can recapitulate the MSC mediated pro-survival phenotype in HSPCs. To this end, we used a cocktail of Forskolin (adenylate cyclase activator) and IBMX (pan phosphodiesterase inhibitor) to raise cAMP levels and activate CREB signaling pathway. We found that treatment of HSPCs with Forskolin/IBMX cocktail induced expression of P-CREB levels as expected. Furthermore, elevation of cAMP levels prevented IR-induced loss of P-CREB in HSPCs (Figure 5A). Next, we examined whether activation of CREB signaling pathway by Forskolin/IBMX treatment could also protect HSPCs from irradiation-induced apoptosis. Remarkably, irradiated HSPCs pre-treated with Forskolin/IBMX underwent apoptosis at a significantly lower rate.

Treatment with cAMP antagonist, Rp-8-Br-cAMPs nullified the effect of Forskolin/IBMX treatment. (Figure 5B, Supplemental Figure 7A, 7B).

To test whether Forskolin/IBMX mediated protection from apoptosis was mediated by BCL2 family proteins, we measured intracellular levels of these proteins in HSPCs cultured in different conditions, 24 hrs after irradiation. We discovered that Forskolin/IBMX mediated protection of irradiated HSPCs is MCL1 dependent, similar to that mediated by OP9M2 MSCs (Figure 5D, Supplemental Figure 7C, 7D).

Similar to the results obtained with CB HSPCs, we found no induction of apoptosis in irradiated BM HSPCs treated with Forskolin/IBMX cocktail (Figure 5D). Overall, these results reveal strong involvement of CREB signaling pathway in DNA damage induced apoptosis of human HSPCs and further highlight its role in MSC mediated pro-survival phenotypes upon interaction with HSPCs.

6) Pharmacological activation of cAMP/CREB signaling pathway enhances engraftment and self-renewal ability of human HSPCs

To check if pharmacological elevation of cAMP signaling pathway enhances the repopulating ability of human HSPCs, we transplanted control- and Forskolin/IBMX-treated HSPCs cells into NSGW41 mice (Figure 6A). We observed a significant increase in human engraftment levels in mice BM that received Forskolin/IBMX treated CD34+ cells relative to mice injected with cytokine only treated cells. Of note, both groups demonstrated similar multilineage, peripheral blood and spleen engraftment (Figure 6B, 6D, Supplementary Figure 7E). Irradiated (3Gy) CD34+ cells cultured for 24hrs in the presence of SFT cytokines failed to establish human hematopoietic grafts in most of the recipients (4 out of 11) with mean engraftment level 0.5%. On the contrary, 9 out of 15 mice received FSKN/IBMX pretreated and irradiated CD34+ cells were engrafted. Mean engraftment level in this group was 3.3% which is 6.6-fold higher in comparison with the irradiated and cytokine treated group. The improved human chimerism exhibited by FSKN/IBMX treated HSPCs might be explained by modest increase in the proportion of Lin-CD34+ human cells detected in the engrafted mice 12 wks post transplantation (Figure 6C).

Serial transplantation is the gold-standard assay to measure the self-renewal ability of HSCs. To ascertain the effect of Forskolin/IBMX treatment on human HSCs self-renewal we collected the bone marrow of engrafted mice and retransplanted them into secondary recipients. We found low level of human chimerism (mean engraftment of 0.91%) in the BM of animals that received cytokine-only treated HSPCs. Strikingly, Forskolin/IBMX-exposed CD34+ cells produced high level of human bone marrow multilineage regeneration in the majority of secondary recipients (mean engraftment of 17.15%)

(Figure 6D). Moreover, transplanted irradiated Forskolin/IBMX treated HSPC grafts also reestablished human hematopoiesis in secondary mice BM and spleens (Figure 6E, 6F). Together, these long-term in vivo and in vitro data establish that pharmacological activation of cAMP pathway can mimic pro-survival phenomenon of MSC co-culture and offers significant radioprotection on human HSPCs. We also established that cAMP/PKA/CREB pathway regulates human HSPCs survival and self-renewal under homeostatic and genotoxic stress conditions and unveil the strong propensity of cAMP-elevating drugs to augment human HSC regeneration *in vivo*.

Discussion

Our results establish that BM-derived MSCs confer resistance to radiation- and chemotherapy-induced apoptosis in human HSPCs and maintain their BM repopulating ability. MSC-mediated protection from DNA damage-induced apoptosis relies on cAMP/PKA axis induction in HSPCs.

Mesenchymal stromal cells are widely studied as potential attenuators of DNA damage-induced toxicities[51,52]. Indeed, prior studies have demonstrated that 7-10 days of co-culture of BM-derived stromal cells[53] or primary endothelial cells[54] with human CD34+ cells exposed to sublethal radiation can increase their survival, promote expansion and recovery. We extend these reports by demonstrating that both murine and human BM MSCs effectively inhibit IR- and chemotherapy-induced apoptosis of more primitive CD34+CD38-CD45RA- HSPC compartment. However, in contrast to the studies above, we demonstrated that a brief co-culture period (18-24 hrs post-IR) is sufficient to rescue both LTC-IC and In Vivo repopulating ability. As human HSPCs do not undergo a first division during this time[55], we can attribute HSPC radioprotection in our experimental setting primarily to apoptosis inhibition rather than to ex vivo expansion of surviving HSPCs.

BCL-2 family proteins are master regulators of apoptosis, and their balanced regulation is crucial for the generation, maintenance, and functioning of the hematopoietic system[34]. DNA damaging agents such as irradiation and chemotherapy have been shown to induce apoptosis by neuralization of anti-apoptotic BCL2 family proteins[56–61]. Amongst the anti-apoptotic BCL-2 family proteins, MCL-1 has been reported to be rapidly downregulated in response to genotoxic stress[56,59]. Consistently, we observed a decline in MCL-1 protein levels, specifically in HSPCs exposed to irradiation. We observed no such decrease in MCL-1 in irradiated committed progenitors (CD34+CD38+), explaining their relative radio-resistance compared to their HSPC counterparts (data not shown)[4,5]. Strikingly, HSPCs in direct contact with MSCs attained a block in the decline of MCL1 levels in HSPCs. Combined inhibition of MCL-1 and BCL-XL using their respective inhibitors prevented MSC-mediated protection of irradiated HSPCs, strongly nominating these proteins as critical mediators of HSPCs survival in the niche context. Our findings extend the observations of Bohler and colleagues, who reported synthetic lethality between MCL-1 and Bcl-xL inhibitors on human CD34+ cells[62]. As both MCL-1 and Bcl-XL inhibitors are currently progressing through clinical trials to treat different malignancies, our results suggest possible harmful effect on the most primitive HSPCs, especially when given in this combination and in conjunction with DNA-damaging therapies. These results corroborate previous reports highlighting the importance of MCL1 and Bcl-XL in HSPC survival[62–65].

Previous studies that profiled the transcriptional response of murine HSPCs revealed a complex array of stress pathways affected by IR exposure[13,66–68]. However, the impact of MSCs on the transcriptome of human HSPCs exposed to IR is unique to our work. Strikingly, we observed that HSPC-MSC interaction itself resulted in profound transcriptome alterations in HSPCs that were refractory to further changes by IR. Some MSC-upregulated transcripts (e.g., FOS, JUN, and EGR1) participate in quiescence exit and resistance to stress pathways[69,70]. On the other hand, concurrent repression of the pro-inflammatory genes (e.g., STAT1, ISG15, CSF2, CCL1) might prevent pre-mature HSPC cycling driven by inflammatory- and TNF- signaling associated with DNA damage response. In addition, we observed a coordinated reduction in the magnitude of IR-induced p53 pro-apoptosis transcripts in HSPCs upon MSC interaction. These coordinated changes in MSC-contacting HSPCs are consistent with their higher resistance to general stress and DNA damage in particular.

Elevated activity of CREB and additional CRE-binding factors (e.g., ATF1, CREM1, and AP1 complex) in HSPCs from MSCs co-culture, as predicted by our transcriptome data and validated in HSPCs, provide a plausible molecular mechanism for quenching IR-induced apoptosis response. Indeed, efficient transcription of p53 target genes, as well as of CREB and NF-KB, are dependent on their binding to p300 and CBP histone acetyltransferases[71]. Given limited cellular amounts of p300/CBP, higher levels of active CREB, as indicated by Ser 133 phosphorylation in adherent HSPCs, might augment pro-survival, destabilize pro-apoptosis gene regulatory programs, and tilt HSPCs cell fate towards survival.

Although phosphorylation-dependent activation of CREB can be achieved via multiple cell signaling pathways[72], our study implicated cAMP/PKA activation in MSC-mediated protection of human HSPCs. Furthermore, recapitulation of MSC-mediated phenotypes, including inhibition of DNA damage-induced apoptosis and rescue of irradiated HSPCs regeneration, via direct simulation of HSPC intrinsic cAMP synthesis machinery by Forskolin/IBMX, provides additional support for the involvement of cAMP/PKA/CREB pathway in the MSC mediated pro-regenerative effects on HSPCs. In extension to the potent inhibition of DNA damage-induced apoptosis reported here, Negrotto et al. observed a decrease in nitric oxide and serum-deprivation induced apoptosis in CD34+ cells by cell membrane permeable cAMP analogs[73,74]. As the cAMP pathway and its downstream effectors can elicit both pro- and anti-apoptosis functions, depending on the cellular context and apoptosis stimuli examined, our results agree with the pro-survival functions of increased cAMP levels in quiescent human HSPCs.

How MSC contact leads to cAMP increase and PKA activation in HSPCs remains to be elucidated. However, we observed that it is independent of gap junction-mediated

communication implicated in cAMP exchange between contacting osteoblasts[75] and T regulatory cells[48,50].

In our view, the most intriguing findings are the greatly augmented long-term in vivo regenerating capabilities of human HSPCs by pharmacological upregulation of cAMP levels via Forskolin/IBMX treatment. Indeed, enhancing human hematopoiesis by employing controllable cAMP pathway modulation can provide enormous therapeutic opportunities in light of the extensive physiological and biochemical knowledge of the cAMP-dependent mechanisms. In this respect, treatment of murine HSPCs with prostaglandin E2 (PGE2), arguably the best-studied physiological cAMP elevating agent, resulted in the enhancement of HSPC function and radioprotection in vivo[26,66,67]. In contrast to the solid pro-survival function of this agent in mice, its radioprotective and pro-regeneration abilities in the human xenograft context were only marginal arguing for species-specific effects[27,76,77]. Our results also suggest that the magnitude of cAMP induction, signaling duration, and effectors mobilization, all of which might be different between Forskolin/IBMX and PGE2 treatments, can also account for this inconsistency[78,79].

Recent findings on the catecholaminergic modulation of murine HSCs and human CD34+ cells behavior directly or indirectly via the sympatho-adrenergic fibers present in the bone marrow niche, further raised the relevance of cAMP level regulation in control on HSPC functions[80–84].

Collectively, by molecular analysis of the HSPC-MSC co-culture system, we revealed that MSC pro-regenerative phenotypes depend on the induction of cAMP/PKA/CREB1 signal transduction pathway in human HSPCs. Pharmacological upregulation of cAMP levels in HSPCs significantly augmented their long-term *in vivo* regenerating capability under homeostatic and stress conditions. Therefore, we anticipate these findings will promote clinical prospects related to myelosuppression mitigation and HSPC transplantation strategies.

Materials and Methods

CD34+ HSPCs purification

Samples of cord blood (CB) were obtained according to procedures approved by the institutional review boards of the Sheba Medical Centre, Israel and Tel Aviv University. Informed consent was obtained from all subjects. CD34+ cells were enriched by positive selection with MACS CD34+ ultra-pure kit (Miltenyi Biotech) according to the manufacturer's instructions. Purified cells were stored in liquid nitrogen and used at later time points.

CD34+ HSPCs culture

Thawed CD34+ cells were cultured in StemSpan SFEM II serum-free medium (Stem cell technologies) supplemented with recombinant cytokines (Peprotech Asia): SCF (25ng/ml), FLT3L (25ng/ml), TPO (25ng/ml). To elevate cAMP levels, CD34+ cells were treated with Forskolin (10µM, Alomone labs) and IBMX (100µM, Sigma Aldrich). CD34+ cells were treated with following inhibitors– Etoposide (1µM, Sigma Aldrich) S63845 (0.1µM, Apex Biotech), A1155463 (0.1µM, Selleck Chem), Rp-8-Br-cAMPs (100µM, BioLog), Carbenoxolone (100µM, Sigma Aldrich), GAP27 (100µM, Med Chem Express).

CD34+ HSPCs and stroma co-culture

OP9M2 murine stromal cells were cultured in MEM-Alpha medium (Gibco, Life Technologies), supplemented with FBS (20%, Biological Industries), Pen/Strep (1%), L-glutamine (1%). Primary Human Bone Marrow stromal cells (BMSCs) were generated from bone marrow aspirate of healthy volunteers after signing an informed consent. BMSCs were isolated by plate adherence (2×10^4 cells per well in 24-well plates incubated overnight) and expanded for 48 hours.

For co-culture with CD34+ cells, 3*104 OP9M2 cells were plated in 24-well tissue culture treated plate (Greiner Bio-One) and incubated in the stroma medium (see above) for 24 hours. Following the incubation, medium was aspirated, and CD34+ enriched cells were plated on MSCs in StemSpan SFEM II serum-free medium supplemented with cytokines. Cells were maintained in a humidified incubator at 37 °C and CO2 (5%).

Annexin V apoptosis assay

To induce apoptosis, CD34+ cells cultured in different conditions were irradiated at 3 Gy using a Biobeam gamma-irradiator (Gamma service) or alternatively allowed to cycle for 24 hours and then treated with Etoposide (1µM) for an additional 48 hours. CD34+ cells were then washed and incubated for 30 mins with antibodies for surface markers – CD45RA BV605 (1:200), CD38 PE/Cy7 (1:100), and CD34 PE (1:100). CD34+ cells were then washed and stained with Annexin V- AF488 (Invitrogen) and Sytox blue dead cell stain (Invitrogen). Cells were analysed with Cytoflex flow cytometer (Beckman Coulter).

Intracellular flow cytometry

CD34+ cells were cultured in different conditions as indicated and stained with antibodies for HSPC surface markers - CD45RA BV605, CD38 PE/Cy7 and CD34 PE. Zombie NIR dye (Biolegend) was used to label dead cells. Cells were then fixed with 1.6% Paraformaldehyde and permeabilized in 90% ethanol followed by labelling with AF488-conjugated intracellular antibodies for BCL-2 (1:100), BCL-XL (1:100), MCL-1 (1:100) and P-CREB-Ser133 (1:100).

NOD/SCID repopulating cell assay

For primary transplantation, human CD34+ cells cultured in different conditions for 24 hours were injected into NSGW41 mice intravenously into the retrobulbar plexus. After 12 weeks, mice were euthanized and BM engraftment was analyzed by flow cytometry using hCD45, hCD33, hCD19 and hCD3 antibodies.

For secondary transplantation, the whole bone marrow of primary mice was reinjected into NSGW41 mice intravenously and the BM engraftment was analyzed by flow cytometry after additional 12 weeks.

Limiting dilution analysis using stroma-supported LTC-IC assay

96 well plates (Greiner Bio-One) were covered with 0.1% gelatin solution (Biological Industries) for 20 minutes, and dried open in a sterile hood for 120 minutes. Then, coated wells were seeded with MS5 stromal cells (7000 cells/ well) in 100µl of H5100 medium (STEM CELL technologies) supplemented with 1µM hydrocortisone (Sigma) + 1% P/S. MS-5 cells were irradiated (20 Gy) after seeding into 96 well plates. Twenty-four hours later 10/50/500 non irradiated $CD34^+$ cells or 1,500/7,500/15,000 irradiated (3Gy) $CD34^+$ cells per well were plated in 100 µl MyeloCult H5100 medium (STEM CELL technologies). Once a week half medium replacement was done. After 5 weeks the H5100 medium was replaced with Methocult H4434 Classic methylcellulose medium (100µl/ well, STEM CELL technologies). Wells that contained at least one colony after 10-14 days were considered positive.

RNA sequencing

CD34+ cells were cultured in different conditions, stained for HSPCs markers and sorted using BD FACS AriaIII cytometer. Total RNA was extracted using TRIZOL reagent (Invitrogen) and quality tested using a 2100 Bioanalyzer (Agilent technologies). Sequencing Libraries were prepared using MARSeq. Single reads were sequenced on 1 lane(s) of an Illumina NOvaSeq_sp. The output was ~15 million reads per sample.

Data analysis

Poly-A/T stretches and Illumina adapters were trimmed from the reads using cutadapt[85]; resulting reads shorter than 30bp were discarded. Reads were mapped to the H. sapiens

reference genome GRCh38 using STAR[86], supplied with gene annotations downloaded from Ensembl (and with EndToEnd option and outFilterMismatchNoverLmax was set to 0.04). Expression levels for each gene were quantified using htseq-count[87], using the gtf above. Differentially expressed genes were identified using DESeq2[88] with the betaPrior, cooksCutoff and independent filtering parameters set to False. Raw P values were adjusted for multiple testing using the procedure of Benjamini and Hochberg. The Pipeline was run using snakemake workflow engine[89].

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was done using the GSEA desktop application (Broad Institute). Genes were ranked by the DESeq2 statistic and pre-ranked GSEA was run using the GSEA hallmarks, Transcription Factor Targets (TFT) and other curated gene HSC gene sets from MSigDB database using standard settings. Heatmaps were created using Morpheus (Broad Institute).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 9, using student's t-test. Statistical significance is defined as $p<0.05$. Bars represent Mean ± Standard error of mean (SEM) of independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.

Data and materials availability

All raw sequencing data generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) and the accession number will be made available upon formal publication.

References

1. Kaushansky, K. Lineage-Specific Hematopoietic Growth Factors. *N. Engl. J. Med.* **354**, 2034–2045 (2006).

2. Orkin, S. H. & Zon, L. I. Hematopoiesis: An Evolving Paradigm for Stem Cell Biology. *Cell* **132**, 631–644 (2008).

3. Doulatov, S., Notta, F., Laurenti, E. & Dick, J. E. Hematopoiesis: A human perspective. *Cell Stem Cell* **10**, 120–136 (2012).

4. Milyavsky, M. *et al.* A Distinctive DNA damage response in human hematopoietic stem cells reveals an apoptosis-independent role for p53 in self-renewal. *Cell Stem Cell* **7**, 186–197 (2010).

5. Biechonski, S. *et al.* Attenuated DNA damage responses and increased apoptosis characterize human hematopoietic stem cells exposed to irradiation. *Sci. Rep.* **8**, (2018).

6. Shao, L. *et al.* Total body irradiation causes long-term mouse BM injury via induction of HSC premature senescence in an Ink4a- and Arf-independent manner. *Blood* **123**, 3105–3115 (2014).

7. Liu, J. *et al.* A synthetic double-stranded RNA, poly I: C, induces a rapid apoptosis of human CD34+ cells. *Exp. Hematol.* **40**, 330–341 (2012).

8. López, M. & Martín, M. Medical management of the acute radiation syndrome. *Reports Pract. Oncol. Radiother.* **16**, 138–146 (2011).

9. Durand, C., Charbord, P. & Jaffredo, T. The crosstalk between hematopoietic stem cells and their niches. *Curr. Opin. Hematol.* **25**, 285–289 (2018).

10. Méndez-Ferrer, S. *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466**, 829–834 (2010).

11. Greenbaum, A. *et al.* CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* **495**, 227–230 (2013).

12. Hanoun, M. & Frenette, P. S. This niche is a maze; An amazing niche. *Cell Stem Cell* **12**, 391–392 (2013).

13. Yamashita, M., Nitta, E. & Suda, T. Aspp1 Preserves Hematopoietic Stem Cell Pool Integrity and Prevents Malignant Transformation. *Cell Stem Cell* **17**, 23–34 (2015).

14. Labi, V. & Erlacher, M. How cell death shapes cancer. *Cell Death Dis.* **6**, 1–11 (2015).

15. Michalak, E. M. *et al.* Apoptosis-promoted tumorigenesis: γ-irradiation-induced thymic lymphomagenesis requires Puma-driven leukocyte death. *Genes Dev.* **24**, 1608–1613 (2010).

16. Wang, J. *et al.* A differentiation checkpoint limits hematopoietic stem cell self-renewal in response to DNA damage. *Cell* **148**, 1001–1014 (2012).

17. Si, S. *et al.* Hematopoietic insults damage bone marrow niche by activating p53 in vascular endothelial cells. *Exp. Hematol.* **63**, 41-51.e1 (2018).

18. Himburg, H. A. *et al.* Pleiotrophin mediates hematopoietic regeneration via activation of RAS. *J. Clin. Invest.* **124**, 4753–4758 (2014).

19. Doan, P. L. *et al.* Tie21 bone marrow endothelial cells regulate hematopoietic stem cell regeneration following radiation injury. *Stem Cells* **31**, 327–337 (2013).

20. Severe, N. *et al.* Stress-Induced Changes in Bone Marrow Stromal Cell Populations Revealed through Single-Cell Protein Expression Mapping. *Cell Stem Cell* **25**, 570-583.e7 (2019).

21. Golan, K. *et al.* Bone marrow regeneration requires mitochondrial transfer from donor Cx43-expressing hematopoietic progenitors to stroma. *Blood* **136**, 2607–2619 (2020).

22. Doan, P. L. *et al.* Epidermal growth factor regulates hematopoietic regeneration after radiation injury. *Nat. Med.* **19**, 295–304 (2013).

23. Katoh, O. *et al.* Vascular endothelial growth factor inhibits apoptotic death in hematopoietic cells after exposure to chemotherapeutic drugs by inducing MCL1 acting as an antiapoptotic factor. *Cancer Res.* **58**, 5565–5569 (1998).

24. Arai, F. *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**, 149–161 (2004).

25. Himburg, H. A. *et al.* Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. *Nat. Med.* **16**, 475–482 (2010).

26. Porter, R. L. *et al.* Prostaglandin E2 increases hematopoietic stem cell survival and accelerates hematopoietic recovery after radiation injury. *Stem Cells* **31**, 372–383 (2013).

27. Demmerath, E. M., Bohler, S., Kunze, M. & Erlacher, M. In vitro and in vivo evaluation of possible pro-survival activities of PGE2, EGF, TPO and FLT3L on human hematopoiesis. *Haematologica* **104**, 669–677 (2019).

28. Henry, E. *et al.* Human hematopoietic stem/progenitor cells display reactive oxygen species-dependent long-term hematopoietic defects after exposure to low doses of ionizing radiations. *Haematologica* **105**, 2044–2055 (2020).

29. Morrison, S. J. & Scadden, D. T. The bone marrow niche for haematopoietic stem cells. *Nature* **505**, 327–334 (2014).

30. Kumar, S. & Geiger, H. HSC Niche Biology and HSC Expansion Ex Vivo. *Trends Mol. Med.* **23**, 799–819 (2017).

31. Magnusson, M. *et al.* Expansion on Stromal Cells Preserves the Undifferentiated State of Human Hematopoietic Stem Cells Despite Compromised Reconstitution Ability. *PLoS One* **8**, (2013).

32. Biechonski, S., Yassin, M. & Milyavsky, M. DNA-damage response in hematopoietic stem cells: An evolutionary trade-off between blood regeneration and leukemia suppression. *Carcinogenesis* **38**, 367–377 (2017).

33. Shao, L., Luo, Y. & Zhou, D. Hematopoietic stem cell injury induced by ionizing radiation. *Antioxidants Redox Signal.* **20**, 1447–1462 (2014).

34. Kollek, M., Müller, A., Egle, A. & Erlacher, M. Bcl-2 proteins in development, health, and disease of the hematopoietic system. *FEBS J.* **283**, 2779–2810 (2016).

35. Czabotar, P. E., Lessene, G., Strasser, A. & Adams, J. M. Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. *Nat. Rev. Mol. Cell Biol.* **15**, 49–63 (2014).

36. Subramanian, A. *et al.* Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 15545–15550 (2005).

37. Eppert, K. *et al.* Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat. Med.* **17**, 1086–1094 (2011).

38. Liberzon, A. *et al.* The Molecular Signatures Database Hallmark Gene Set Collection. *Cell Syst.* **1**, 417–425 (2015).

39. Anjos-Afonso, F. *et al.* Single cell analyses identify a highly regenerative and homogenous human CD34+ hematopoietic stem cell population. *Nat. Commun.* **13**, 1–13 (2022).

40. Manna, P. R. & Stocco, D. M. Crosstalk of CREB and Fos/Jun on a single cis-element: Transcriptional repression of the steroidogenic acute regulatory protein gene. *J. Mol. Endocrinol.* **39**, 261–277 (2007).

41. Gjertsen, B. T. *et al.* Novel (Rp)-cAMPS analogs as tools for inhibition of cAMP-kinase in cell culture: Rasal cAMP-kinase activity modulates interleukin-1β action. *J. Biol. Chem.* **270**, 20599–20607 (1995).

42. Cheng, J. C. *et al.* CREB is a critical regulator of normal hematopoiesis and leukemogenesis. *Blood* **111**, 1182–1192 (2008).

43. Yang, L. H. *et al.* Neuronal survival factor VGF promotes chemoresistance and predicts poor prognosis in lung cancers with neuroendocrine feature. *Int. J. Cancer* 1611–1625 (2022) doi:10.1002/ijc.34193.

44. Busser, B. *et al.* Amphiregulin promotes BAX inhibition and resistance to gefitinib in non-small-cell lung cancers. *Mol. Ther.* **18**, 528–535 (2010).

45. Wong, Y. C., Wang, X. & Ling, M. T. Id-1 expression and cell survival. *Apoptosis* **9**, 279–289 (2004).

46. Kang, Y. *et al.* Adrenergic stimulation of DUSP1 impairs chemotherapy response in ovarian cancer. *Clin. Cancer Res.* **22**, 1713–1724 (2016).

47. Wu, X. *et al.* JOSD1 inhibits mitochondrial apoptotic signalling to drive acquired chemoresistance in gynaecological cancer by stabilizing MCL1. *Cell Death Differ.* **27**, 55–70 (2020).

48. Bopp, T. *et al.* Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J. Exp. Med.* **204**, 1303–1310 (2007).

49. Bedner, P. *et al.* Selective permeability of different connexin channels to the second messenger cyclic AMP. *J. Biol. Chem.* **281**, 6673–6681 (2006).

50. Liao, W. *et al.* Aged hematopoietic stem cells entrap regulatory T cells to create a prosurvival microenvironment. *Cell. Mol. Immunol.* (2023) doi:10.1038/s41423-023-01072-3.

51. Naji, A. *et al.* Biological functions of mesenchymal stem cells and clinical implications. *Cell. Mol. Life Sci.* **76**, 3323–3348 (2019).

52. Rühle, A. *et al.* The Therapeutic Potential of Mesenchymal Stromal Cells in the Treatment of Chemotherapy-Induced Tissue Damage. *Stem Cell Rev. Reports* 356–373 (2019) doi:10.1007/s12015-019-09886-3.

53. Drouet, M. *et al.* Mesenchymal stem cells rescue CD34+ cells from radiation-induced apoptosis and sustain hematopoietic reconstitution after coculture and cografting in lethally irradiated baboons: Is autologous stem cell therapy in nuclear accident settings hype or reality. *Bone Marrow Transplant.* **35**, 1201–1209 (2005).

54. Muramoto, G. G., Chen, B., Cui, X., Chao, N. J. & Chute, J. P. Vascular Endothelial Cells Produce Soluble Factors That Mediate the Recovery of Human Hematopoietic Stem Cells after Radiation Injury. *Biol. Blood Marrow Transplant.* **12**, 530–540 (2006).

55. Laurenti, E. *et al.* CDK6 levels regulate quiescence exit in human hematopoietic stem cells. *Cell Stem Cell* **16**, 302–313 (2015).

56. Nijhawan, D. *et al.* Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev.* **17**, 1475–1486 (2003).

57. Milosevic, J., Hoffarth, S., Huber, C. & Schuler, M. The DNA damage-induced decrease of Bcl-2 is secondary to the activation of apoptotic effector caspases. *Oncogene* **22**, 6852–6856 (2003).

58. Tichý, A. *et al.* Gamma irradiation of human leukaemic cells HL-60 and MOLT-4 induces decrease in Mcl-1 and Bid, release of cytochrome c, and activation of

caspase-8 and caspase-9. *Int. J. Radiat. Biol.* **84**, 523–530 (2008).

59. Li, X. H., Ha, C. T. & Xiao, M. MicroRNA-30 inhibits antiapoptotic factor Mcl-1 in mouse and human hematopoietic cells after radiation exposure. *Apoptosis* **21**, 708–720 (2016).

60. Trivigno, D., Bornes, L., Huber, S. M. & Rudner, J. Regulation of protein translation initiation in response to ionizing radiation. *Radiat. Oncol.* **8**, 1–12 (2013).

61. Zhou, T. *et al.* Downregulation of Mcl-1 through inhibition of translation contributes to benzyl isothiocyanateinduced cell cycle arrest and apoptosis in human leukemia cells. *Cell Death Dis.* **4**, 1–11 (2013).

62. Bohler, S. *et al.* Inhibition of the anti-apoptotic protein MCL-1 severely suppresses human hematopoiesis. *Haematologica* **106**, 3136–3148 (2021).

63. Campbell, C. J. V. *et al.* The human stem cell hierarchy is defined by a functional dependence on Mcl-1 for self-renewal capacity. *Blood* **116**, 1433–1442 (2010).

64. Delbridge, A. R. D., Opferman, J. T., Grabow, S. & Strasser, A. Antagonism between MCL-1 and PUMA governs stem/progenitor cell survival during hematopoietic recovery from stress. *Blood* **125**, 3273–3280 (2015).

65. Opferman, J. T. *et al.* Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science (80-.).* **307**, 1101–1104 (2005).

66. Liu, L. *et al.* Upregulation of SIRT1 Contributes to dmPGE2-dependent Radioprotection of Hematopoietic Stem Cells. *Stem Cell Rev. Reports* **18**, 1478–1494 (2022).

67. Patterson, A. M. *et al.* A Single Radioprotective Dose of Prostaglandin E2 Blocks Irradiation-Induced Apoptotic Signaling and Early Cycling of Hematopoietic Stem Cells. *Stem Cell Reports* **15**, 358–373 (2020).

68. Tung, L. T. *et al.* p53-dependent induction of P2X7 on hematopoietic stem and progenitor cells regulates hematopoietic response to genotoxic stress. *Cell Death Dis.* **12**, 1–13 (2021).

69. Hormaechea-Agulla, D. *et al.* Chronic infection drives Dnmt3a-loss-of-function clonal hematopoiesis via IFNγ signaling. *Cell Stem Cell* **28**, 1428-1442.e6 (2021).

70. Min, I. M. *et al.* The Transcription Factor EGR1 Controls Both the Proliferation and Localization of Hematopoietic Stem Cells. *Cell Stem Cell* **2**, 380–391 (2008).

71. Goodman, R. H. & Smolik, S. CBP/p300 in cell growth, transformation, and development. *Genes Dev.* **14**, 1553–1577 (2000).

72. Zhang, H., Kong, Q., Wang, J., Jiang, Y. & Hua, H. Complex roles of cAMP–PKA–CREB signaling in cancer. *Exp. Hematol. Oncol.* **9**, 1–13 (2020).

73. Negrotto, S. *et al.* Activation of cyclic AMP pathway prevents CD34+ cell

apoptosis. *Exp. Hematol.* **34**, 1420–1428 (2006).

74. Goichberg, P. *et al.* cAMP-induced PKCδ activation increases functional CXCR4 expression on human CD34+ hematopoietic progenitors. *Blood* **107**, 870–879 (2006).

75. Gupta, A. *et al.* Communication of cAMP by connexin43 gap junctions regulates osteoblast signaling and gene expression. *Cell. Signal.* **28**, 1048–1057 (2016).

76. Pelus, L. M., Hoggatt, J. & Singh, P. Pulse exposure of haematopoietic grafts to prostaglandin E2in vitro facilitates engraftment and recovery. *Cell Prolif.* **44**, 22–29 (2011).

77. Patterson, A., Orschell, C., Plett, A. & Sampson, C. 3161 – Cellular and Molecular Effects of $Dmpge_2$ Radiomitigation in Mouse and Human Bone Marrow Cells in Vivo Using C57Bl/6 and Humanized Nsg Mouse Models of Acute Radiation Syndrome. *Exp. Hematol.* **111**, S125 (2022).

78. Bodor, J., Spetz, A. L., Strominger, J. L. & Habener, J. F. cAMP inducibility of transcriptional repressor ICER in developing and mature human T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 3536–3541 (1996).

79. Jansen, S. R. *et al.* Prostaglandin E2 promotes MYCN non-amplified neuroblastoma cell survival via β-catenin stabilization. *J. Cell. Mol. Med.* **19**, 210–226 (2015).

80. Katayama, Y. *et al.* Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell* **124**, 407–421 (2006).

81. Liu, Y. *et al.* Dopamine signaling regulates hematopoietic stem and progenitor cell function. *Blood* **138**, 2051–2065 (2021).

82. Spiegel, A. *et al.* Catecholaminergic neurotransmitters regulate migration and repopulation of immature human CD34+ cells through Wnt signaling. *Nat. Immunol.* **8**, 1123–1131 (2007).

83. Maryanovich, M. *et al.* Adrenergic nerve degeneration in bone marrow drives aging of the hematopoietic stem cell niche. *Nat. Med.* **24**, 782–791 (2018).

84. Shao, L. *et al.* The neurotransmitter receptor Gabbr1 regulates proliferation and function of hematopoietic stem and progenitor cells. *Blood* **137**, 775–787 (2021).

85. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* **17**, 10 (2011).

86. Dobin, A. *et al.* STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).

87. Anders, S., Pyl, P. T. & Huber, W. HTSeq-A Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169 (2015).

88. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 1–21 (2014).

89. Köster, J. & Rahmann, S. Snakemake-a scalable bioinformatics workflow engine. *Bioinformatics* **28**, 2520–2522 (2012).

Figure legends

Figure 1. MSCs protect human HSPCs from DNA damage-induced apoptosis

A) Experimental design to study HSPC response and functionality upon co-culture with MSCs. **B)** Cord-Blood (CB) CD34+ cells were cultured in cytokine-supplemented medium, on top of human BM-derived MSCs or OP9M2 MSCs for 24 hours after 3Gy IR and analyzed for apoptosis in HSPCs using Annexin V staining (n=3). **C)** Human BM derived CD34+ cells were cultured in indicated conditions for 24 hours and analyzed for apoptosis in HSPCs using Annexin V staining (n=3). **D)** CB derived CD34+ cells were exposed to 3Gy IR prior to dividing to cytokine only or co-culture groups for 24 hrs followed by plating in LTC-IC assay for limiting dilution analysis. Five weeks later, the medium was replaced with methylcellulose and positive and negative wells were recorded based on colony formation potential 10-14 days later. The table summarizes LTC-IC frequency as calculated using L-CALC software. **E)** CB derived CD34+ cells were cultured in the indicated conditions for 24 hours and injected intravenously into NSGW41 mice. Human engraftment (hCD45+ cells, %, left panel) and lineage distribution (right panel) in the bone marrow of injected recipients were analyzed 15 weeks post-injection. Each symbol represents the engraftment level in one recipient. Horizontal lines represent the mean value for each group.

Figure 2. MSCs preserve anti-apoptotic MCL1 protein levels in irradiated human HSPCs.

CB derived CD34+ cells were cultured for 24 hours after irradiation in SFEM medium with cytokines or co-cultured with OP9M2 cells.

Levels of BCL2 **(A)**, BCL-XL (**B)** and of MCL-1 (C) in the different conditions were quantified using flow cytometry in HSPCs. Actual protein levels are shown in representative histograms (left) and the relative changes in expression are depicted as the ratio of MFI between IR and non-IR conditions. (right) (n=4 for BCL2), (n=4 for BCL-XL), (n=6 for MCL1).

Figure 3. HSPCs co-cultured with MSCs acquire stemness and anti-inflammatory gene signature.

CB derived CD34+ cells were cultured for 24 hours after irradiation in serum free medium with cytokines or co-cultured with OP9M2 cells. Cells stained and sorted using HSPC markers were subjected to RNA sequencing. **A)** Heatmap showing expression of differentially expressed genes upregulated upon co-culture with OP9M2 MSCs. **B)** Heatmap showing expression of differentially expressed genes downregulated upon co-culture with OP9M2 MSCs. **C) D) E)** GSEA pathway analysis of HSPCs cultured in cytokines vs co-cultured with MSCs **F)** EPCR expression quantified in CB CD34+ cells 96 hours after irradiation.

Figure 4. MSCs activate cAMP/CREB signaling pathway to protect human HSPCs.

A) GSEA enrichment plot of TAX-CREB target genes in HSPCs cultured in serum-free medium with cytokines or co-cultured with OP9M2 cells. **B)** CB CD34+ cells were cultured in indicated conditions for 24 hours and analyzed for apoptosis in HSPCs using Annexin V staining (n=6). **C)** CB CD34+ cells were cultured in indicated conditions for 24 hours and analyzed for expression of P-CREB levels by flow cytometry (n=3).

Figure 5. Pharmacological activation of cAMP/CREB signaling pathway protects human HSPCs from IR-induced apoptosis.

A) CB CD34+ cells were cultured in indicated conditions for 24 hours and analyzed for P-CREB levels (n=4). **B)** CB CD34+ cells were cultured in indicated conditions for 24 hours and analyzed for apoptosis in HSPCs using Annexin V staining (n=4). **C)** MCL-1 protein levels shown in histograms (left) and their expression quantified (right) (n=6) **D)** Human BM-derived CD34+ cells were cultured as indicated for 24 hours and analyzed for apoptosis in HSPCs using Annexin V staining (n=3).

Figure 6. Pharmacological activation of cAMP/CREB signaling pathway enhances engraftment and self-renewal ability of human HSPCs.

A) Experimental design to study HSPC functionality upon addition of Forskolin/ IBMX cocktail. **B)** Human chimerism and lineage distribution in the bone marrow of individual mice injected with control or Forskolin/IBMX treated hCD34+ cells (hCD45+, %). **C)** Immature cell quantitation in the bone marrow of mice injected with control or Forskolin/IBMX treated CD34+ cells (hCD34+ cell, %). **D)** Human chimerism in spleens of mice injected with control or Forskolin/IBMX treated hCD34+ cells (hCD45+, %). **E)** Human chimerism in the bone marrow of individual secondary mice injected with total BM of primary mice (hCD45+, %). **F)** Human chimerism in the spleens of individual secondary mice injected with total BM of primary mice (hCD45+, %). Each symbol represents the engraftment level in one recipient. Horizontal lines represent the mean value for each group.

Supplementary figure legends

Supplemental Figure 1

A) Flow cytometry gating strategy for In Vitro studies. **B)** Representative flow plot related to Figure 1C showing the percentage of Annexin + HSPCs. **C)** CB CD34+ cells were cultured for 24 hours in serum-free medium with SFT cytokines. After 24 hours, cells were treated with Etoposide (1µM) for 48 hours and analyzed for apoptosis using Annexin V (n=3).

Supplemental Figure 2

A) Flow cytometry gating strategy for In Vivo studies. **B)** Human CD45 chimerism in the peripheral blood of NSGW41 mice, 15 weeks after injection. **C)** Human CD45 chimerism in the spleen of NSGW41 mice, 15 weeks after injection.

Supplemental Figure 3

Validation and optimization of BCL2, BCL-XL and MCL1 antibodies for quantitation of anti-apoptotic proteins in human HSPCs. BCL2 antibody validation by Western blotting **A)** and Flow cytometry **B)** in leukemia cell lines that express variable levels of BCL2. Validation of BCL-XL antibody by Western blot **C)** and Flow cytometry **D)** in leukemia cell lines. Validation of MCL1 antibody by Western blot **E)** and Flow cytometry **F)** in leukemia cell lines.

Supplemental Figure 4

A) Flow cytometry gating strategy for In Vitro studies (Intracellular proteins).

Supplemental Figure 5

A) CB CD34+ cells were cultured in indicated conditions for up to 24 hours and analyzed for apoptosis in HSPCs using Annexin V staining (n=3).

Supplemental Figure 6

A) Gene Set Enrichment analysis showing significantly enriched transcription factor target genes in HSPCs co-cultured with OP9M2 MSCs versus HSPCs cultured in cytokine only media. **B)** Heatmap showing expression of Leading edge CREB1 target genes. **C) D)** CB CD34+ cells were cultured in indicated conditions for up to 24 hours and analyzed for apoptosis in HSPCs using Annexin V staining (n=3).

Supplemental Figure 7

A) CB CD34+ cells were cultured in indicated conditions for up to 72 hours and analyzed for apoptosis in HSPCs using Annexin V staining (n=3). CB CD34+ cells were cultured in indicated conditions for up to 24 hours and analyzed for **B)** CB CD34+ cells were cultured for 24 hours in serum-free media with cytokines. After 24 hours, cells were treated with Etoposide (1uM) for 48 hours and analyzed for apoptosis using Annexin V (n=3). **C)** BCL2 & **D)** BCL-XL expression in HSPCs (n=3). **E)** Human CD45 chimerism in peripheral blood of primary NSGW41 mice, 12 weeks after injection.

Antigen	Clone	Fluorophore	Dilution	Manufacturer	Catalog number
CD34	581	FITC	1-100	Biolegend	343504
	581	PE	1-100	Beckman Coulter	A07776
	8G12	APC	1-100	BD Bioscience	345804
CD38	HB7	PC7	1-100	Biolegend	356608
CD45RA	HI100	BV605	1-200	Biolegend	304134
CD201	RCR-401	PE	1-100	Biolegend	351904
	RCR-401	APC	1-100	Biolegend	351906
CD33	P67.6	PE	1-100	Biolegend	366608
CD19	SJ25C1	APC	1-100	Biolegend	363006
Lineage cocktail (CD3/14/16/19/20/56)		FITC	1-50	Biolegend	348801
CD45	30-F11	BV605	1-100	Biolegend	103140
CD3	REA613	FITC	1-100	Miltenyi Biotec	130-114-138
CD45	J33	PC7	1-100	Beckman Coulter	IM3548
IgG1	MOPC-21	AF488	1-400	Cell Signaling Technology	4878S
IgG1	DA1E	AF488	1-400/1-800	Cell Signaling Technology	2975S
BCL2	124	AF488	1-100	Cell Signaling Technology	59422S
BCL-XL	54H6	AF488	1-100	Cell Signaling Technology	2767S
MCL1	D2W9E	AF488	1-100	Cell Signaling Technology	58326S
P-CREB (S133)	87G3	AF488	1-200	Cell Signaling Technology	9187S
Survivin	71G4B7	AF488	1-200	Cell Signaling Technology	2810S
Annexin V		AF488	1-200	Invitrogen	A13201
		APC	1-200	Invitrogen	A35110
Sytox Blue			1-1000	Invitrogen	S34857

Zombie NiR			1-1000	Biolegend	423105

Acknowledgments

The authors thank Dr. H.K.A. Mikkola (University of California, Los Angeles, California, USA) for kindly providing the OP9M2 cell line. This work was partially supported by Israel Science Foundation (ISF 3480/19, ISF 1362/20) and Varda and Boaz Dotan Research Center in Hemato-Oncology grants (to MM). This work was performed in partial fulfillment of the requirements for PhD degrees of Siva Sai Muddineni and Chen Katz Even, the Dr. Miriam and Sheldon G. Adelson Graduate School of Medicine, Faculty of Medicine, Tel Aviv University, Israel. The authors would like to thank Dr. Irena Shur, Dr. Daria Makarovsky and Dr. Rami Khosravi for their technical assistance.

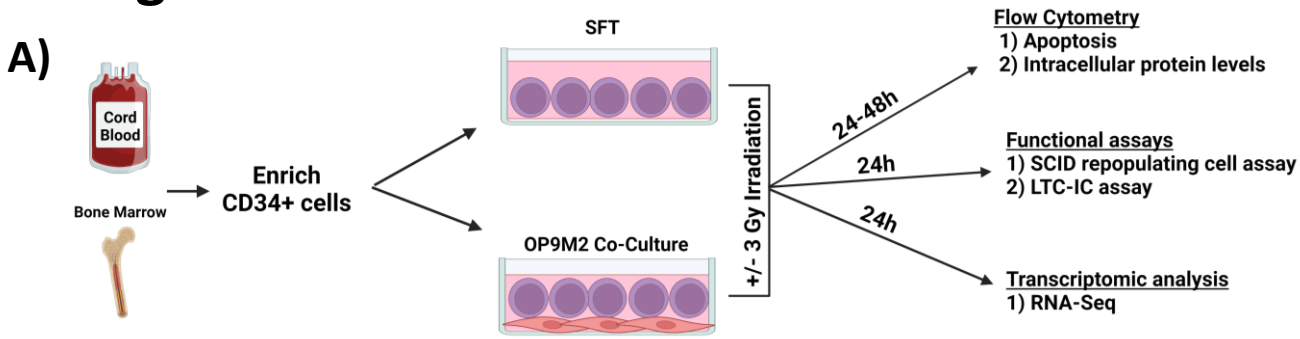
Author Contributions

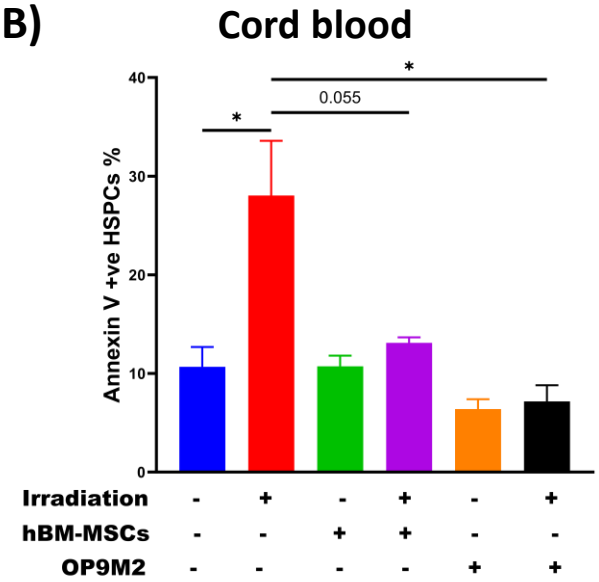
SSNAM, CKE and MM conceptualized the study, designed, performed and analyzed experiments. AZ, performed experiments; EW analyzed RNA-seq data; YR, AN and KB prepared and provided study material for experiments; SSNAM and MM wrote the original manuscript, MM provided financial support.

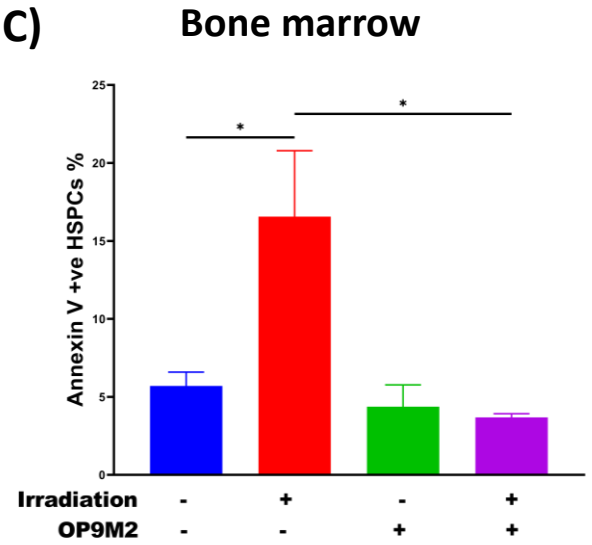
Competing interests

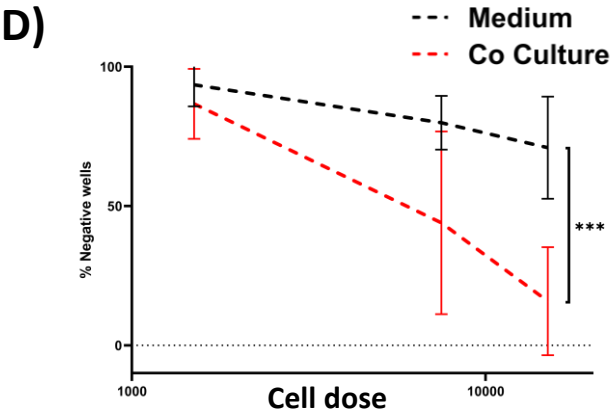
The authors have no conflict of interest to declare.

Figure 1

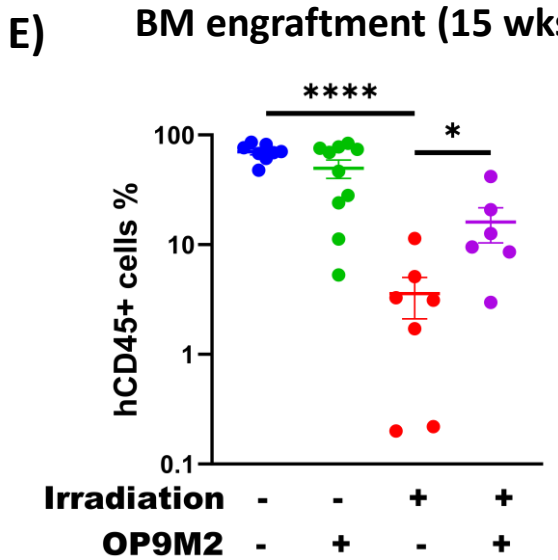








Culture conditions	IR dose (Gy)	Frequency	IC 95%
Medium	0	1/102	1/127 ; 1/81.2
	3	1/35685	1/46,864 ; 1/27,173
Co-Culture	0	1/127	1/157 ; 1/103
	3	1/7446	1/9501 ; 1/5835



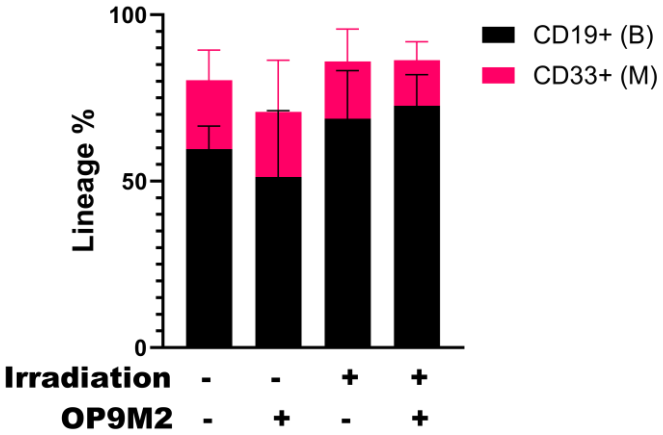
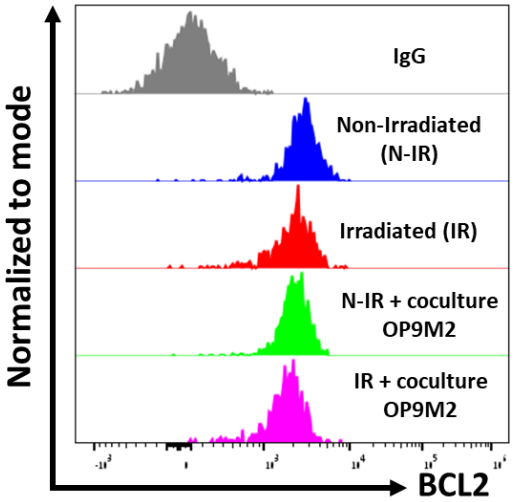
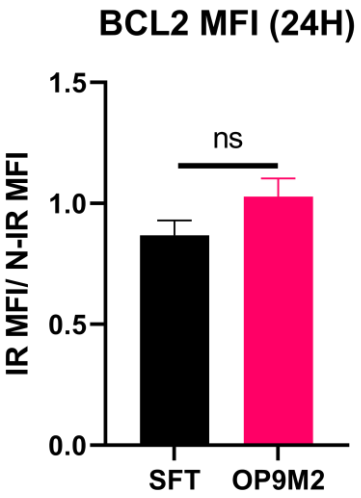


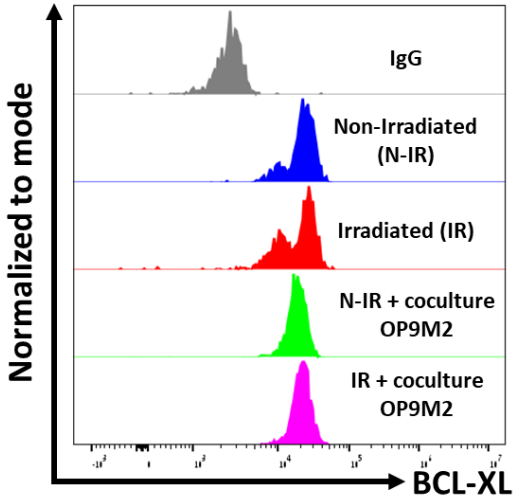
Figure 2

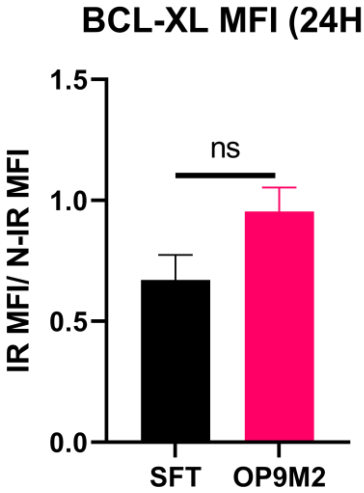
A)


Normalized to mode
IgG
Non-Irradiated (N-IR)
Irradiated (IR)
N-IR + coculture OP9M2
IR + coculture OP9M2
BCL2

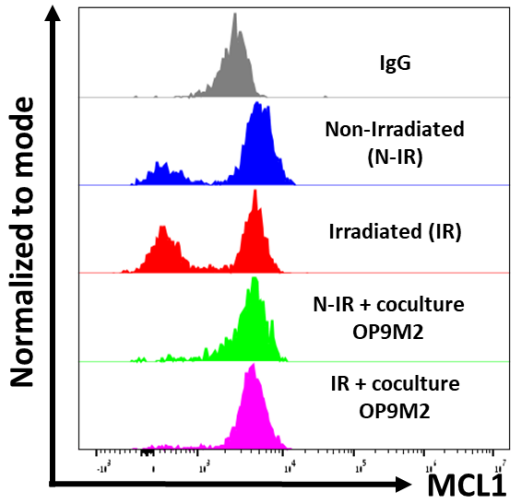

BCL2 MFI (24H)
IR MFI/ N-IR MFI
1.5
1.0
0.5
0.0
ns
SFT
OP9M2

B)


Normalized to mode
IgG
Non-Irradiated (N-IR)
Irradiated (IR)
N-IR + coculture OP9M2
IR + coculture OP9M2
BCL-XL


BCL-XL MFI (24H)
IR MFI/ N-IR MFI
1.5
1.0
0.5
0.0
ns
SFT
OP9M2

C)


Normalized to mode
IgG
Non-Irradiated (N-IR)
Irradiated (IR)
N-IR + coculture OP9M2
IR + coculture OP9M2
MCL1

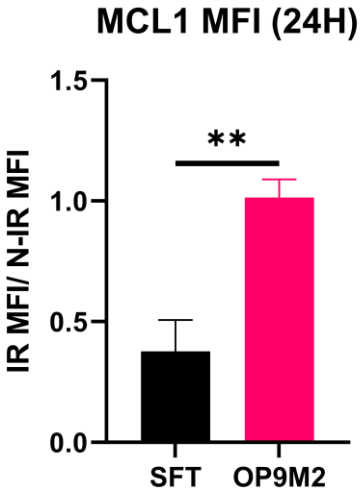
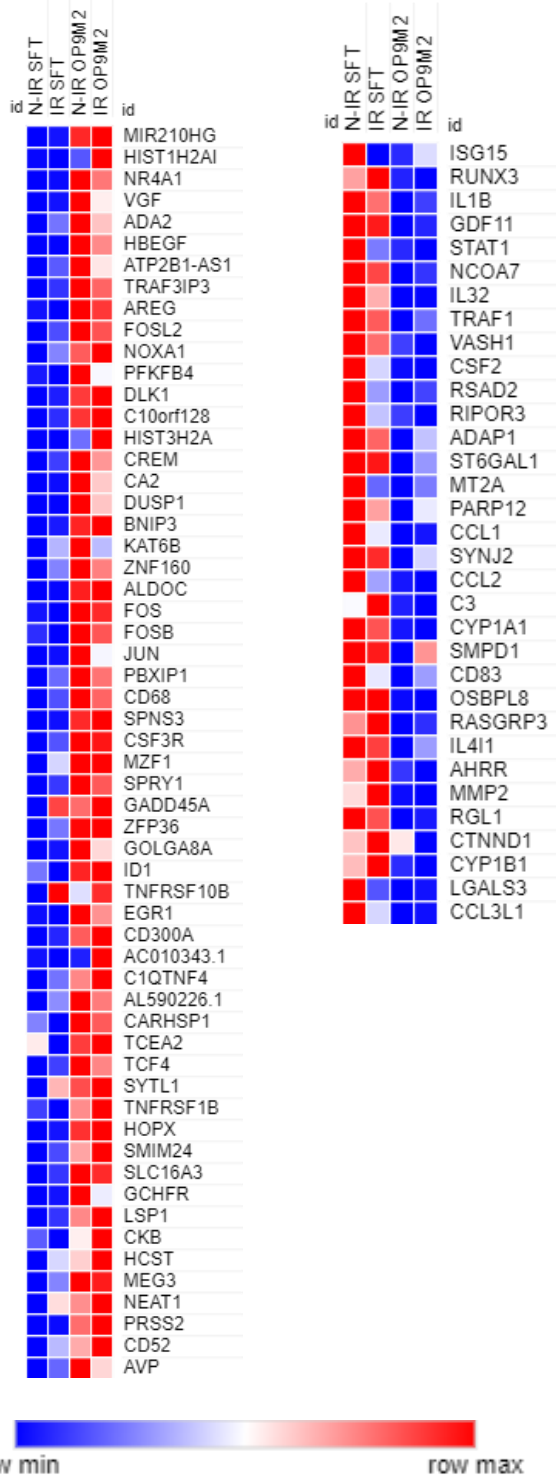

MCL1 MFI (24H)
IR MFI/ N-IR MFI
1.5
1.0
0.5
0.0
**
SFT
OP9M2

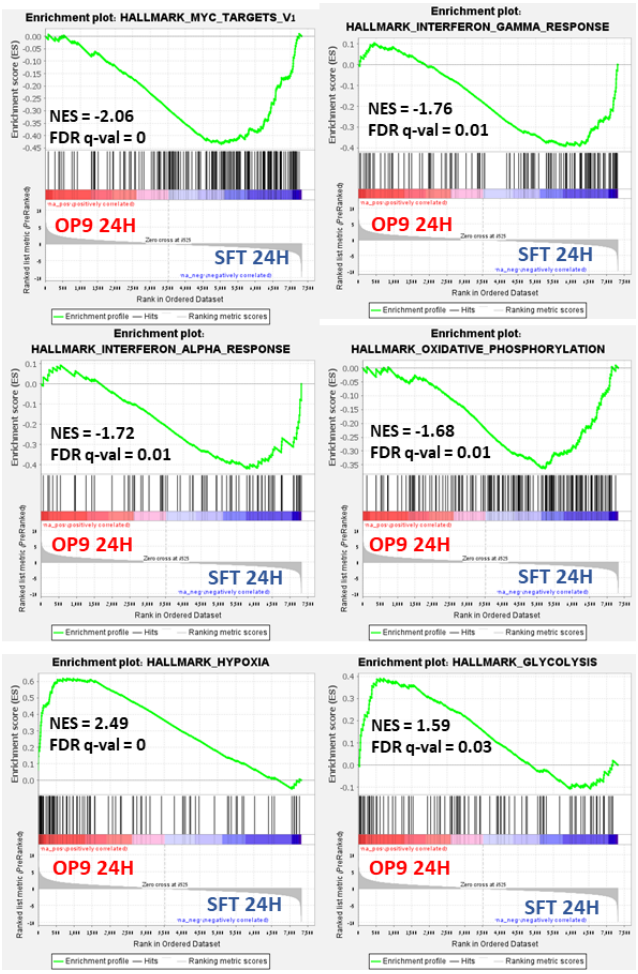
Figure 3

A) DE genes upregulated upon co-culture with OP9M2 MSCs

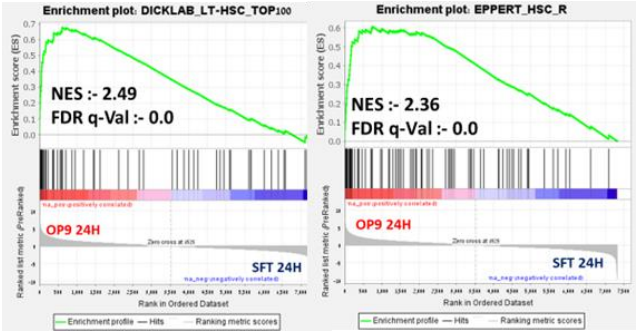
B) DE genes downregulated upon co-culture with OP9M2 MSCs



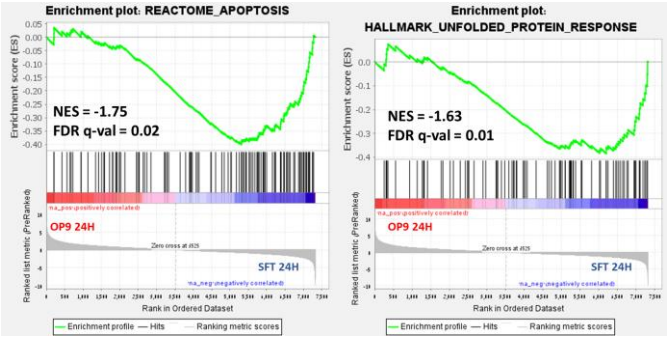
D)



E) HSC stemness associated gene sets



C)



F) EPCR expression (96 hrs)

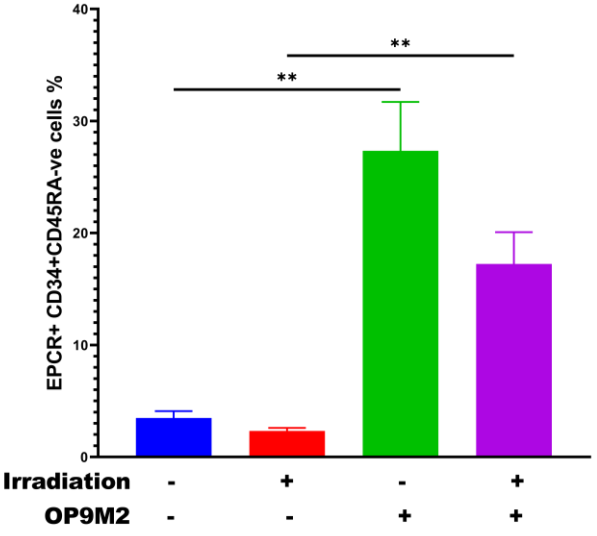
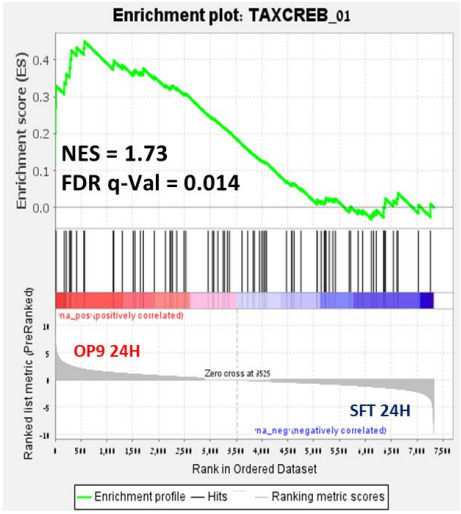
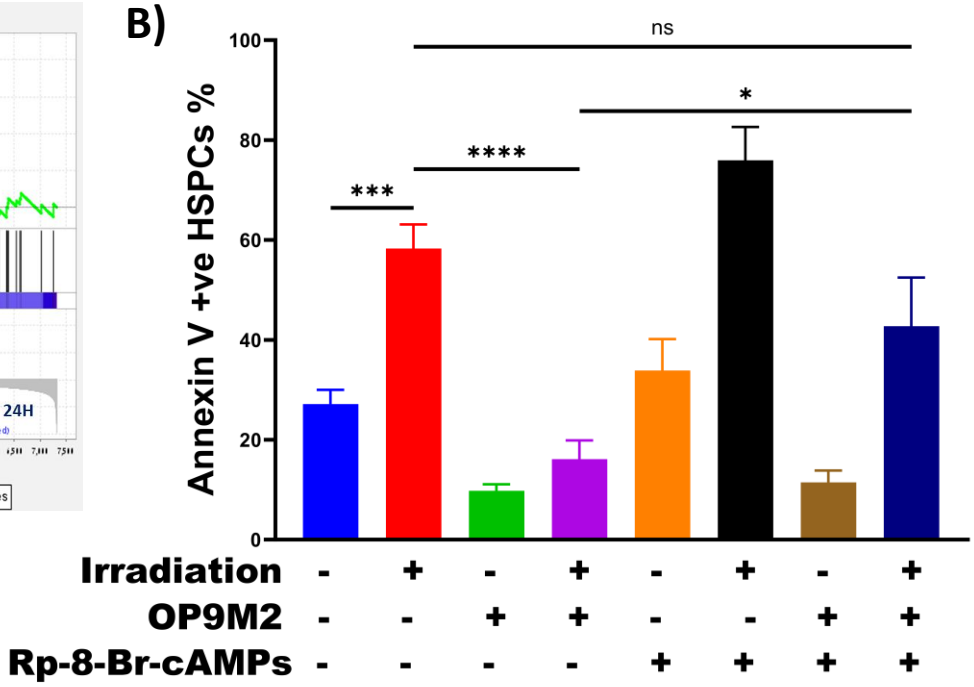


Figure 4

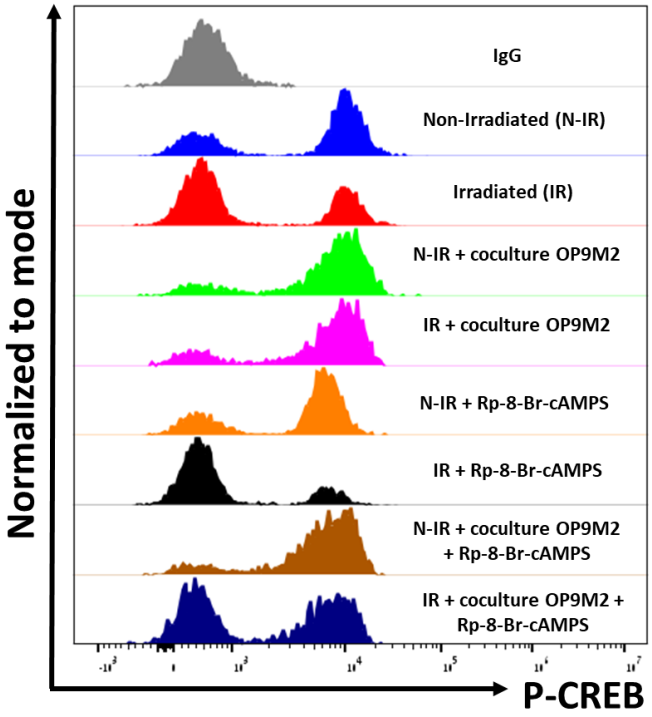
A)


Enrichment plot: TAXCREB_01
Enrichment score (ES)
NES = 1.73
FDR q-Val = 0.014
Ranked list metric (PreRanked)
OP9 24H
SFT 24H
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

B)


Annexin V +ve HSPCs %
ns
*
****
***
Irradiation - + - + - + - +
OP9M2 - - + + - - + +
Rp-8-Br-cAMPs - - - - + + + +

C)


Normalized to mode
IgG
Non-Irradiated (N-IR)
Irradiated (IR)
N-IR + coculture OP9M2
IR + coculture OP9M2
N-IR + Rp-8-Br-cAMPS
IR + Rp-8-Br-cAMPS
N-IR + coculture OP9M2 + Rp-8-Br-cAMPS
IR + coculture OP9M2 + Rp-8-Br-cAMPS
P-CREB

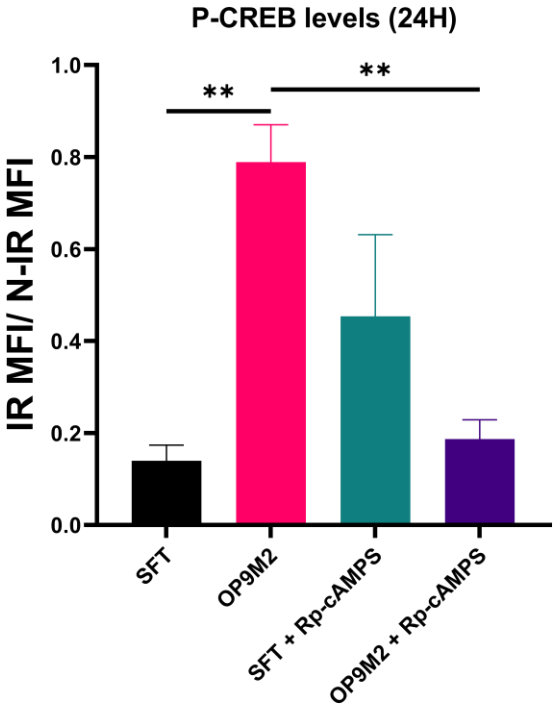
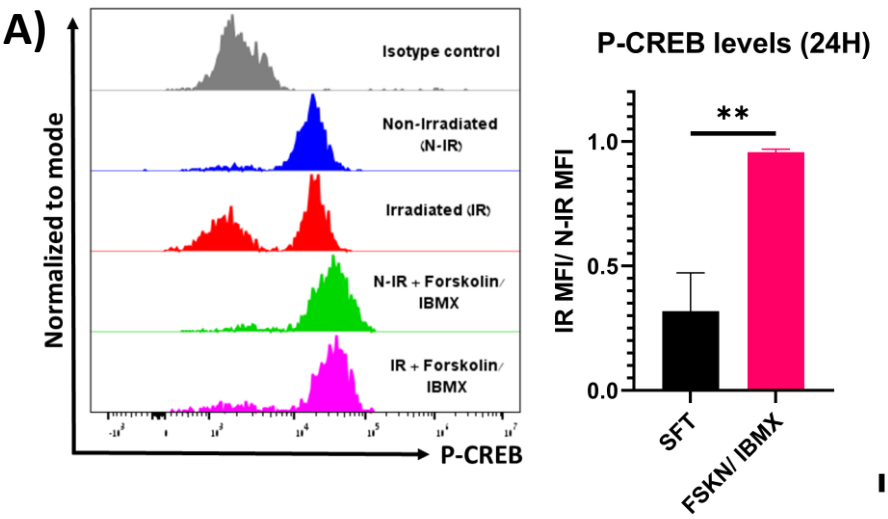
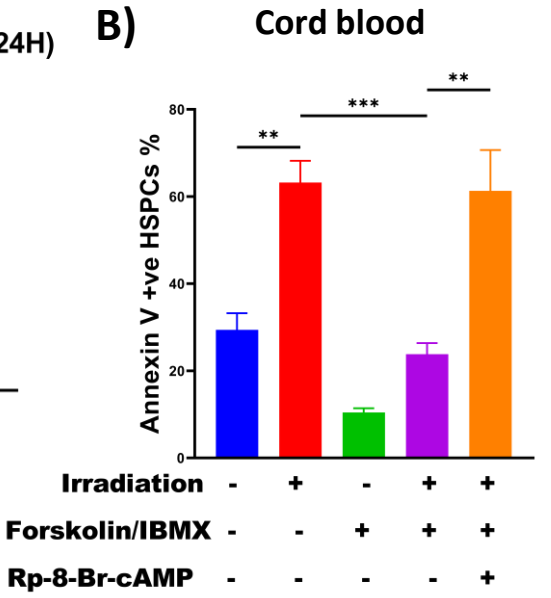
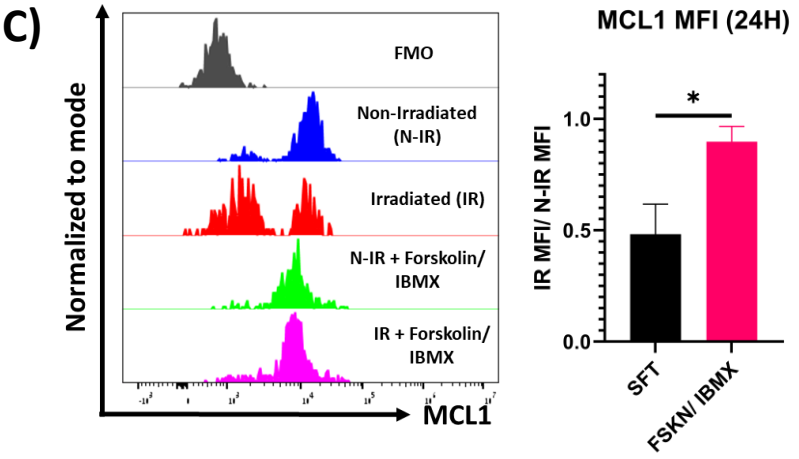

P-CREB levels (24H)
IR MFI/ N-IR MFI
**
**
SFT
OP9M2
SFT + Rp-cAMPS
OP9M2 + Rp-cAMPS

Figure 5


A)
Normalized to mode
Isotype control
Non-Irradiated (N-IR)
Irradiated (IR)
N-IR + Forskolin/ IBMX
IR + Forskolin/ IBMX
P-CREB
P-CREB levels (24H)
IR MFI/ N-IR MFI
1.0
0.5
0.0
**
SFT
FSKN/ IBMX


B)
Cord blood
Annexin V +ve HSPCs %
80
60
40
20
0
**
***
**
Irradiation - + - + +
Forskolin/IBMX - - + + +
Rp-8-Br-cAMP - - - - +


C)
Normalized to mode
FMO
Non-Irradiated (N-IR)
Irradiated (IR)
N-IR + Forskolin/ IBMX
IR + Forskolin/ IBMX
MCL1
MCL1 MFI (24H)
IR MFI/ N-IR MFI
1.0
0.5
0.0
*
SFT
FSKN/ IBMX

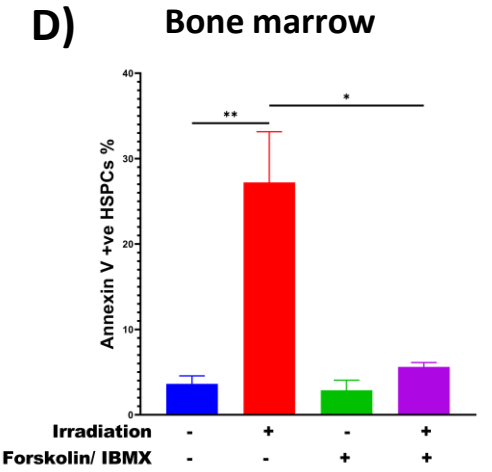
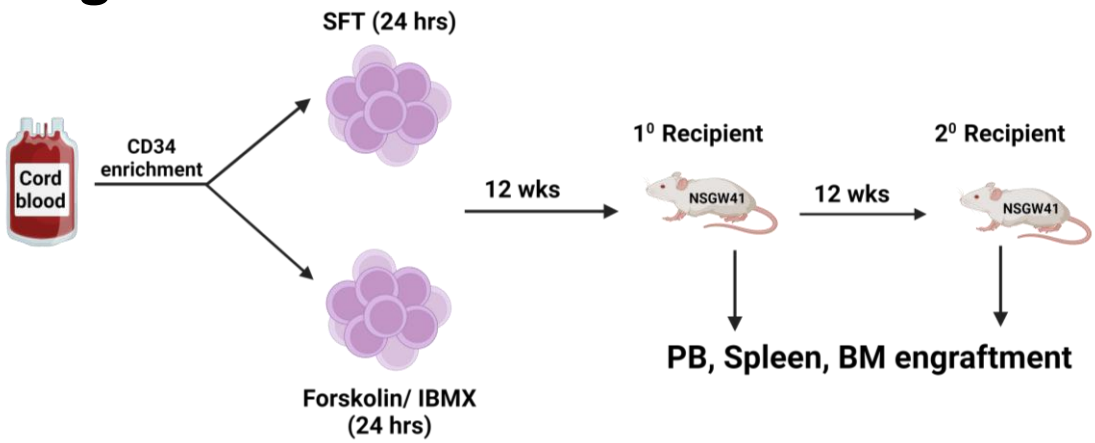
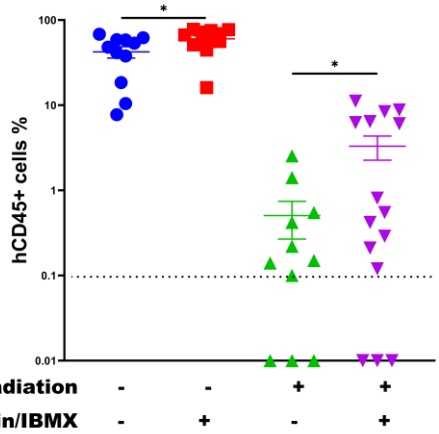

D)
Bone marrow
Annexin V +ve HSPCs %
40
30
20
10
0
**
*
Irradiation - + - +
Forskolin/ IBMX - - + +

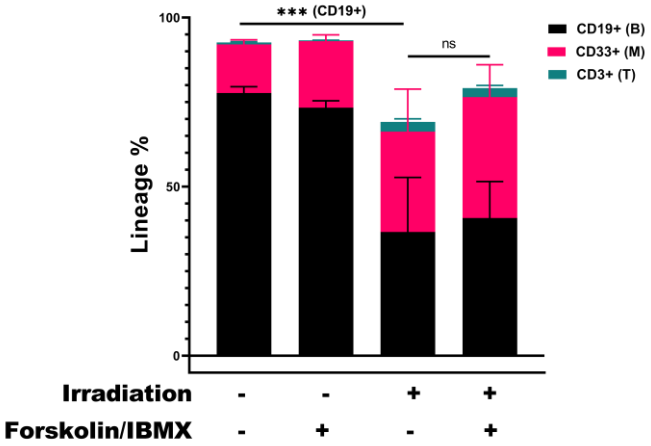
Figure 6

A)

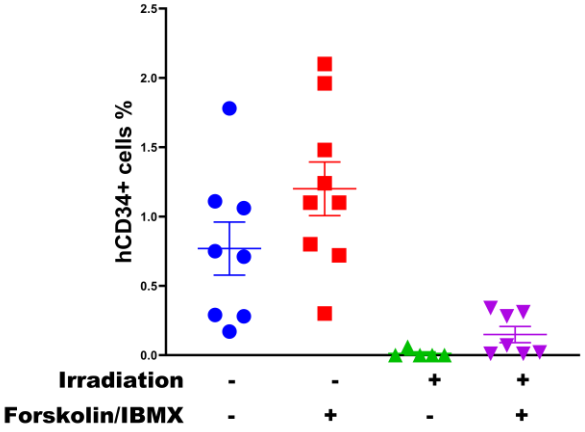


B)

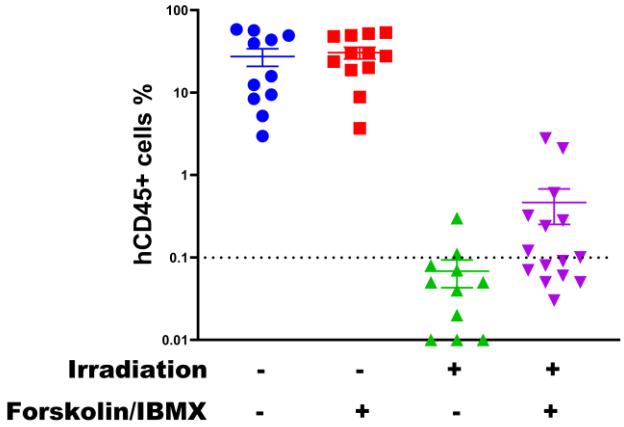




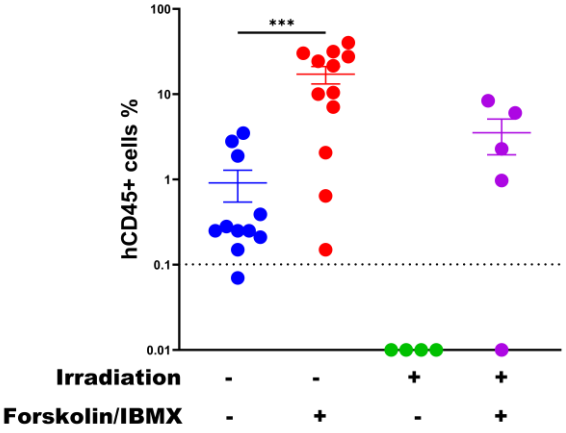
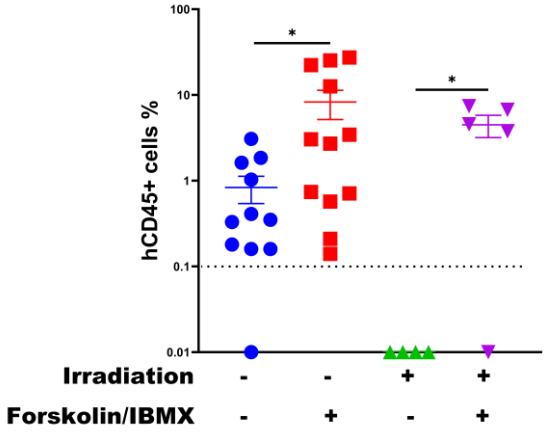
C)



D)



E)



F)