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TNFR2/p75 and TNFR1/p55 interactions in delayed non-targeted effects of radiation in bone marrow derives EPCs

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TNFR2/P75 and TNFR1/P55 INTERACTIONS IN DELAYED NON-TARGETED EFFECTS OF RADIATION IN BONE MARROW DERIVED EPCS

by

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ABSTRACT

The central dogma of radiation has been that all biological effects of ionizing radiation occur through direct damage of DNA in the nucleus. There is however evidence dating back to as early as 1950's which suggests that radiation can cause damage in unirradiated "bystander" cells through mechanisms currently unknown at low dose exposures (Parsons, 1954). This radiobiological bystander effect has since been observed in numerous studies involving microbeam experiments and media transfer experiments, where the observed DNA damage was significantly higher than the amount of cells directly traversed by radiation (Zhou, 2001; Mothersill, 1998). Elevation in the levels of IL-8, TNF-α, FASL, nitric oxide, and reactive oxygen species have been associated with low dose irradiation and are implicated as potential mediators of bystander effect (Narayanan, 1999; Burr, 2010; Rastogi, 2012). TNF-α has two types of receptors p55 and p75 that are part of the mechanism involving NF-κB gene transcription. In our study, we invested the role of TNF-α receptors in the

induction of p-H2AX foci (marker of DNA double strand breaks-DBS) in unirradiated mice endothelial progenitor cells (EPCs) post irradiation (IR). One set of EPCs were irradiated with low dose γ- radiation and the media of the cells transferred to corresponding unirradiated EPCs of wild type (WT), p55 knock out (KO), and p75KO mice. Our data showed that at 24 hours, control WT EPCs had the highest p-H2AX foci/cell and gradually decreased 3 and 5 days post media transfer. In contrast, p75KO and p55KO EPCs had lowest count of p-H2AX foci at 24 hours and increased at 3 days. At 5 days post conditioned media (CM) transfer, p55KO continued to have higher p-H2AX foci in an increasing trend whereas p75KO decreased in p-H2AX foci. Our findings showed that in comparison to WT EPCs, the expression of TNFR1/p55 and TNFR2/p75 greatly influenced the pattern of p-H2AX induction over time. ELISA analysis of the γ-irradiated WT and p55KO EPC conditioned media at different time points of the experiment showed that several cytokines and chemokines such as IL-1α, IL-1β, MCP-1, Rantes, and MIP-1α are associated with the increasing trend of p-H2AX foci in p55KO EPCs. Our in vitro mouse recombinant cytokine treatment of p55KO EPCs, using the concentrations of cytokines determined in the ELISA analysis confirmed the ability of TNF-α to induce p-H2AX foci and identified IL-1α as one of the main inducers.

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ABBREVIATIONS

γ-IR	γ-Irradiated
BM	Bone Marrow
BM-EPC	Bone Marrow Derived Endothelial Progenitor Cells
CHO	Chinese Hamster Ovary Cells
CM	Conditioned Medium
Cs-137	Cesium 137
DBS	Double Strand Breaks
DNA	Deoxyribonucleic Acid
EBM2	Ethylenediaminetetraacetic Acid
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EPC	Endothelial Progenitor Cells
FADD	Fas-Associated Death Domain
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FLICE	FADD-like ICE
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HPRT	Hypoxanthine-guanine Phosphoribosyltransferase
HSC	Hematopoetic Stem Cells

ICM	Irradiated Conditioned Medium
IGF-1	Insulin Growth Factor 1
IL-1	Interleukin-1
IL-1α	Interleukin-1α
IL-1β	Interleukin-1 Beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IR	Irradiated
IR-CM	Irradiated Control Medium
KO	Knock Out
LET	Linear Energy Transfer
MCP-1	Monocyte Chemoattractant Protein-1
MIP-1α	Macrophage Inflammatory Protein-1 Alpha
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NO	Nitric Oxide
NoIR	Non-irradiated
PBS	Phosphate-buffered Saline
PFA	Paraformaldehyde
p-H2AX	Phosphorylated-H2AX
PMA	Phorbol Myristate Acid
SCE	Sister Chromatid Exchanges
SCF	Stem Cell Factor

TNFR1	TNF-alpha Receptor I
TNFR2	TNF-alpha Receptor II
TNF-α	Tumor Necrosis Factor-α
TRADD	TNF-α Receptor-associated Death Domain
TRAF2	TNF Receptor-associated Factor
VEGF	Vascular Endothelial Growth Factor
WT	Wildtype

INTRODUCTION

Current understanding of radiation and its biological effects is that direct damage of DNA in the nucleus causes cell death and mutations. However there have been numerous studies, especially in the past two decades, which suggest that radiation can cause damage in unirradiated cells through radiobiological effects currently not fully understood. The term “bystander effect” was used to describe the ability of a cell, affected by an agent (e.g. radiation), to cause damage in other cells unaffected by the initial agent (Djordjevic, 2000). This radiobiological bystander effect was observed in radiation dosage as low as 0.31mGy in an experiment by Nagasawa and Little, where Chinese hamster ovary cells (CHO) were irradiated with α-particles in G1 phase and measured for the induction of sister chromatid exchanges (SCE) (1992). Results showed that 30% of cells had increased SCE even though only 1% of cell nuclei were traversed by α-particles.

Radiation causes genomic instability which is an all-encompassing term to describe the increased rate of alterations in DNA. It is delayed in effect and passed on to the progeny of irradiated cells (Wright, 1998). This instability can be measured as chromosomal alterations, changes in ploidy, micronucleus formation, mutations, microsatellite instabilities, and decreased plating efficiency (Morgan, 2012). One of the ways in which instability was observed in

radiobiological bystander effects is through the quantification of SCE, as mentioned in the experiment by Nagasawa and Little (Nagasawa and Little, 1992; Deshpande et al., 1996). Their results indicated a significant change in the genome of cells not traversed by radiation. In their further experimentation, wild-type Chinese hamster ovary (CHO) cells and xrs-5 cells were irradiated with alpha particles from 0.17 to 200 cGy. Xrs-5 cells are deficient in DNA double strand break repair and were found to have 15-fold increase in the number of cells with chromosomal aberrations than expected if the damage occurred only through irradiated cells, and wild type cells had a 4-fold increase in the number of cells with aberrations (Nagasawa and Little, 2002). Xrs-5 cells were significantly more sensitive to bystander effect in the range of 0.17-1.7cGy. Increase in the micronucleus formation was also noted by investigators of bystander effect (Azzam et al., 2001; Azzam et al., 1998). Cultured human fibroblasts AG1522, exposed to 1-10cGy, displayed higher than expected levels of DNA damage associated with elicitation of stress-inducing p21(also known as Waf1, Sdi1, Cip1) protein (Azzam et al., 2001). The frequency of micronuclei was measured 3 hours after exposure, and a 3-fold increase was noted in ranges of 1-3cGy while only a 4-fold increase was noted at 10cGy. DNA mutations were also observed by investigators by measuring the induction of Hypoxanthine-guanine phosphoribosyltransferase (HPRT) mutations in bystander cells (Nagasawa and Little, 1999). Non-irradiated bystander cells were again subjected to greater DNA damage than expected values.

In many radiation studies, the number of cells hit in the nucleus is estimated by statistical probability based on the dosage of radiation. With the development of the microbeam, it allowed investigators to accurately target the number of cells and the exact exposure to radiation. In studies by Zhou and colleagues, a precision microbeam was used to target the nucleus of 5, 10, 20, or 100% of CHO cells grown in culture with a single α-particle (Zhou et al., 2001). Gene mutations were measured by the yield of CD59- mutants induced in the population. The experimental results showed that in all percentages of cells irradiated, the induction of mutants was elevated above expected values. At 5% of cells irradiated, the mutant fraction was 58% what it was when all of the cells were irradiated. There was also little change going from 10% to 100% cells irradiated which indicated a plateau in bystander response. Chromatid breakage was also observed as another assessment of bystander effect. When 20% of the cells were irradiated with a single particle, data showed 36% of cells without chromatid breaks when the expected was 75%. Zhou's study also investigated the involvement of gap junctions in the transmission of bystander effect (Zhou et al., 1999; Zhou et al., 2001). Octanol, an inhibitor of gap junction communication, was used to treat cells in which 20% of the cell population was irradiated. The treatment was given 2 hours before irradiation and maintained 3 days after, and was found to drastically reduce the yield of CD59- mutants. Lindane, another

inhibitor of cell-cell communication, was used in a separate experiment that corroborates the results with octanol (Zhou et al., 1999).

The importance of cell-cell communication in non-targeted effects of radiation led investigators to postulate that soluble factors might mediate the signaling between cells. To test this, experiments were carried out in which the media of irradiated cells were transferred to unirradiated cells and observed for changes in damage and cell survival. In one study, human keratinocytes (HaCaT cells) were grown and irradiated with 0.5Gy and 5Gy γ-radiation either as single cells or microcolonies of 3-4 cells (Mothersill and Seymour, 1998). The irradiated cell medium (ICM) was then transferred to cell cultures unexposed to radiation. The findings showed a decrease in survival of cells receiving ICM and noted that the contact state of the ICM donors did not influence the degree of cell death. Removal of the ICM after 30 minutes also resulted in the same amount of cell survival as compared to cultures that were left in ICM for 9 days. To assess the nature of the signaling factor(s), the medium also was treated either by freezing in -20°C or heating to 70°C. It was found that the substance was stable at -20°C but destroyed at 70°C, concluded from the reduction of cell-killing in the ICM transfer. Tumor promoter phorbol myristate acid (PMA), which closes gap junctions, was also tested as a component of the experiment. However the results were contradictory to previous experiments with gap inhibitors as cell survival was reduced even with PMA in direct irradiation and ICM-treated cells. It

is suggested that either PMA has effects on other cellular responses, or possibly gap junctions are not entirely necessary for the propagation of bystander effects.

Dose response curves for γ-radiation has been found through medium transfer experiments using ranges of 0.01-5.0Gy on a human epithelial cell line. The degree of bystander effect seemed to saturate at ranges 0.03-0.05Gy as measured by clonogenic death (Mothersill and Seymour, 2000). In experiments using γ-radiation, a low-LET (linear energy transfer) radiation, it has been found that not all cell types have the same bystander response to radiation. In experiments with keratinocytes (epithelial cells), fibroblasts and radio-sensitive carcinoma cells, fibroblasts did not show induction of bystander effect through ICM transfer (Mothersill and Seymour, 1997; Mothersill and Seymour, 1998; Sowa et al., 2010). One possible explanation is that because cell communications are more advanced in epithelial cells compared to fibroblasts, that it affects the radiobiological bystander response *ex vivo*. It is also noted in the literature that the cellular response to radiation can vary between individuals, and genetic factors most likely to play a role in a cell's bystander effect as well (Mothersill and Seymour, 1999).

It is well studied in the literature that ionizing radiation at high doses can suppress the immune system; however the response at low doses is less well-known. Present risk assessment of high and low dose radiation is extrapolated

from epidemiological studies of atomic bomb survivors in Hiroshima and Nagasaki; however there is some uncertainty in the assessment of risk at lower doses (Pierce and Preston, 2000; Morgan, 2003). Current evidence suggests that the immune response is enhanced at lower doses and influences the production and the release of inflammatory cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interleukin-1-α (IL-1α), and others (Pandey et al., 2005). Full body low-dose radiation on mice has been found to enhance the function of macrophage (and CD8+ T cells) in several studies (Pandey et al., 2005; Lorimore et al., 2001; Wright, 2006). Macrophage, in addition to its ability to induce T-cell mediated immune response, has a significant role in the innate immune response. In the innate response, macrophages express cytotoxic effector mechanisms through soluble factors such as peroxidase, cytolytic proteases, nitric oxide (NO) and pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (Pandey et al., 2005). Macrophages are also responsible for phagocytic clearance of apoptotic cells.

TNF-α is thought to be an important factor in the immune response of non-irradiated cells. ELISA of lung fibroblasts irradiated with low doses of α-particles showed a dosage dependent increase in IL8 mRNA levels from 30 minutes to 24 hours post-irradiation (Narayanan et al., 1999). IL8 expression is under the control of cis-elements of nuclear factor NF-κB, which is associated with the major pro-inflammatory pathway for TNF-α. (Sethi et al., 2008). TNF-α was also

directly implicated as one of the damaging signals in *in vivo* bystander experiments, as well as Fas ligand (FasL), nitric oxide, and super oxide (Burr et al., 2010; Rastogi et al., 2012). In an experiment, hematopoietic cells from bone marrow were treated with radiation and measured for cells in sub-G0 region as a screening method for dead/damaged cells. The experiment showed an increasing trend in the number of sub-G0 cells in 1, 2, and 3 hour time points post-irradiation. When TNF-α neutralizing antibody was added to the irradiated bone marrow medium, the number of sub-G0 cells was significantly decreased in all three time points. Reduction was also noted for FasL neutralizing antibody, which might be another signal of interest.

Tumor necrosis factor (TNF-α) is a 17kDa pro-inflammatory cytokine discovered first through its anti-tumor properties. It binds to two different types of receptors: TNF-alpha receptor I (TNFR1) also known as p55 and TNF-alpha receptor II (TNFR2) also known as p75 (Sethi et al., 2008). The reason for having two different types of receptors isn't fully understood, but the binding affinity for TNF-α is similar in both receptors. Both p55 and p75 receptors are ubiquitously expressed on nearly all cell types, but the p55 receptor is predominantly expressed in immune cells, hematopoietic cells, and endothelial lineage cells such as endothelial progenitor cells (EPCs) (Ksontini et al., 2013). Current understanding of TNF receptors is that most biological signaling is done through TNFR1 p55, and the direct signaling role of TNFR2 p75 is unclear (Tartaglia et

al., 1991). Evidence suggests that the p55 receptor is pro-inflammatory and pro-apoptotic whereas p75 is proliferating and pro-angiogenic, but p75 has been known to regulate inflammatory signaling as well (Sethi et al., 2008; Tartaglia et al., 1991). p75 receptor has also been proposed to mediate the responses of p55 receptor to TNF through overlapping of intracellular signaling enhancing NF-κB-dependent gene transcription (Peschon et al., 1998; Weiss et al., 1997). Some studies have shown that both receptors can induce cytolysis in pathways distinct from one another (Grell et al., 1994). Experiments directly testing TNF receptors *in vivo* with baboons showed that administration of a p55 TNF-α receptor agonist resulted in tachycardia, hypotension, and granulocytosis (Van Zee et al., 1994). In contrast, p75 receptor agonist showed none of the mentioned responses, but the administration of TNF-α did produce a pyrexic response and shedding of p75 and p55 receptors.

The p55 TNF-α receptor has an intracellular domain called the death domain, which is absent in the p75 receptor (Ksontini et al., 2013; Sethi et al., 2008). When p55 receptor is triggered, TNF-α receptor-associated death domain (TRADD) is recruited along with Fas-associated death domain (FADD), and FADD-like ICE (FLICE), also known as caspase8, which induces apoptosis through caspase-3 activation. TNF receptor-associated factor (TRAF2) is also triggered by TRADD and is an intermediary for NF-κB, which as previously mentioned leads to the expression of inflammatory genes that can also suppress

apoptosis (Sethi et al., 2008). In contrast, p75 TNF-α receptor binds directly to TRAF1 and TRAF2 and is capable of activating NF-κB, JNK, p38 MAPK, and Erk1/2 (p42/p44). There is also evidence that p75 can cause apoptosis through pathway unrelated to TRADD, but it is currently unknown and requires further research (Haridas et al., 1998). Treatment of a mouse fibroblast cell line L929 with TNF-α was shown to induce DNA strand breaks at the single cell level (Fehsel et al., 1991). DNA strand breaks were observed 3-4 hours after treatment with TNF-α and cell death was observed in higher doses. At lower concentration treatments of TNF-α, induction of DNA damage was seen in cells without any loss through cell apoptosis. Many cytokines, chemokines, and inflammatory enzymes are implicated in mediating bystander effect in variety of cells. However the role of p75 or p55 TNF receptors in regulating radiobiological bystander effect is unknown in endothelial lineage cells, specifically in endothelial progenitor cells (EPC).

In this study, bone marrow (BM)-derived mouse endothelial progenitor cells (EPCs) were used to examine the roles of TNF receptors in bystander response of γ-irradiation. In previous experiments done by our laboratory, we have shown that TNFR2/p75 was required for proper neovascularization by endothelial progenitor cells in hindlimb ischemic tissue (Goukassian et al., 2007). In p75 knock-out (p75KO) mice, the induction of hindlimb ischemia increased apoptosis of endothelial cells and reduced levels of vascular endothelial growth

factors and fibroblast growth factor-2 mRNA transcripts. The mobilization of bone marrow derived endothelial progenitor cells (BM-EPCs) was also reduced by the loss of p75 receptor expression.

The hematopoietic EPCs originate from the bone marrow and are part of the subpopulation of hematopoietic stem cells (HSC), which have been shown to be sensitive to radiobiological bystander response (Burr et al.,2010; Lorimore et al., 2001; Wright, 1998). EPCs are recruited to areas of neovascularization in the process of vasculogenesis to repair tissues with ischemia and cardiovascular diseases (Asahara et al., 2011; Iwaguro et al., 2002; Ribatti, 2006). EPCs are critical also to endothelial maintenance, and thus it was suspected that radiation-induced EPC dysfunction could contribute to the pathogenesis of coronary and peripheral vascular diseases.

We hypothesize that inhibition of TNF-ligand-receptor interactions may alter TNF-mediated down-stream signaling thereby affecting regulation of inflammatory cytokines and chemokines which may augment non-targeted effects of non-irradiated cells.

METHODS

Animal Models

All mice [wild type (WT), TNFR2/p75 knockout (KO), and TNFR1/p55KO] used for this experiment were 8 to 12 weeks old and were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The WT mice were C57BL/6J-control of the mixed C57BL/6 and 129 background strains as defined by the vendor as N10F34. N10 represents that the strains have been backcrossed 10 times and F34 represents the number of inbreeding generations. The strain of mice for TNFR2/p75KO was B6.129S2-Tnfrsf1b<tm1Mwm>/J and the strain for TNFR1/p75KO was B6.129-Tnfrsf1a<tm1Mak>/J. The mice were housed in a temperature and light controlled (12 hours light and dark cycles) environment and fed a standard laboratory chow diet (Harland Teklad) with water access. The handling and maintenance of the animals were done in accordance with the guidelines set by the IACUC at Steward St. Elizabeth Medical Center (Boston, MA, USA).

Irradiation and Dosimetry

In the preparation of the mice for full-body irradiation, the animals were placed individually in rectangular polypropylene boxes with multiple air holes 3mm in diameter to provide a stress-free environment. The mice were then irradiated 8 at a time with γ-radiation and without anesthetics. All irradiation

experiments were done at Steward St. Elizabeth's Medical Center using a Cesium (Cs-137) source irradiator. A single dose for full-body irradiation experiments was 1 Gy at an average dose rate of 46.6 cGy per minute.

Full-Body γ-irradiation and *Ex Vivo* BM-derived EPC Culture

Young WT mice were exposed to 1Gy full-body γ-irradiation using the methods mentioned above. Using non-irradiated (NoIR) WT mice as the control, both NoIR WT and γ-irradiated (γ-IR) WT mice were euthanized at three time points 1 hour, 24 hours, and 7 days post irradiation. Immediately after each of the time points, the mice were overdosed with anesthesia and then euthanized using pre-filled CO2 chamber. Tubular bones of the animals were harvested for isolation of EPCs from the mononuclear fraction of bone marrow cells using a density gradient centrifugation. During the process, cells were suspended in sterile Dulbecco's phosphate-buffered saline (PBS; no calcium or magnesium) with 1% Ethylenediaminetetraacetic acid (EDTA) and treated with ammonium chloride for the hemolysis of red blood cells. The cells were then cultured in 6-well dishes (Corning Inc., Corning, NY, USA) on 22mm x 22mm square glass coverslips (Fisher HealthCare, Houston, TX, USA), that were coated with 0.2% gelatin (SIGMA, St. Louis, MO, USA) in a sterile hood a day before experimentation. EPCs were grown *ex vivo* in EC basal medium-2 (EBM2) (Lonza, Walkersville, PA, USA) with 10% fetal bovine serum (FBS), pinstripe antibiotics, and growth factor supplements as described in literature (Asahara et

al., 1999; Goukassian et al., 2007; Kalka et al., 2000). The culture media were then changed every 48 hours, and the cells reached 60-70% confluence 60 hours post-seeding. The cells on glass coverslips were then fixed in 4% paraformaldehyde (PFA, FD Neuro Technologies Inc., Columbia, MD, USA) and processed for immunostaining.

Media Transfer Experiments in WT BM-derived EPCs

Two identical sets of confluent *ex vivo* EPCs were prepared from the same NoIR WT mice. At 60-70% confluence, one of the sets of 6 well dishes was irradiated with 1Gy of γ-radiation. At 30 minutes, 1 hour, 5 hours, and 24 hours post-IR the media were collected and transferred to the second set of non-irradiated EPC cultures, which were the recipient EPCs for the media transfer study. Media was changed in all wells including the NoIR wells, controls of the experiment, on the day of the experiment and incubated in fresh media for 1 hour prior to irradiation. In the transfer process of the irradiated conditioned medium (IR-CM), it was filtered through a sterile 0.22µm membrane syringe filter (Corning Inc.) to prevent contamination of cells and 2 ml of IR-CM was transferred onto recipient EPCs 30 minutes, 1 hour, 5 hours, and 24 hours post-irradiation. At all four time points, the recipient EPCs were incubated 24 hours with IR-CM and then fixed and stained for immunostaining using an antibody against phosphorylated-H2AX (p-H2AX).

Media Transfer Experiments in WT, p55KO, and p75KO BM-derived EPCs

Two identical sets of sub-confluent *ex vivo* expanded EPCs were isolated for each of the WT, p55KO, and p75KO mice in the methods described above. The same animals were used corresponding to each set. At 60-70% confluence, one set of 6-well dishes from each WT, p55KO, and p75KO EPCs were irradiated with 1Gy γ-radiation and the IR-CM were collected at 1 hour, 5 hours, 24 hours, 3 days, and 5 days post-IR. The second set of No-IR WT, p55KO, p75KO EPCs were used as naïve recipients of the media transfer study. The media of all wells were changed with 3 ml of EBM2 on the day of the study and incubated for 1 hour before irradiation. Before the transfer, the IR-CM were filtered through sterile 0.22µm membrane syringe filter and 2 ml of IR-CM were transferred to recipient EPCs. In addition, both IR-CM and NoIR-CM were collected from WT, p55KO, p75KO EPCs and saved for ELISA profiling. EPC pellets were also harvested at each time point and snap-frozen in liquid nitrogen for future processing. After 24 hour incubation of the EPCs in IR-CM for each time point, EPCs on the coverslip were fixed and processed for p-H2AX immunostaining. All studies involved three biological replicates of WT, p75KO, and p55KO mice for each time point.

Phosphorylated-H2AX immunostaining, Imaging, and Analysis

Formation and decay of p-H2AX foci was observed through immunostaining in WT, p55KO, and p75KO EPCs treated with IR-CM. Glass

coverslips on which EPCs were grown were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT) and washed with ice-cold 1x PBS (MediaTech, Herdon, VA, USA) for 5 minutes. Permeabilization of the fixed cells were done using 0.1% Triton X-100 (SIGMA) for 15 minutes and washed with PBS for 5 minutes three times. Primary anti-p-H2AX rabbit monoclonal (Cat.9718S; Cell Signaling Technology, Danvers, MA, USA) and Alexa-488 goat anti-rabbit secondary antibodies (Cat.A11008; Life Technologies, Grand Island, NY, USA) were used to assess p-H2AX foci formation and decay. Topro-3 (Cat.T3605; Life Technologies) was used to visualize cell nuclei. Slides of the cells were imaged using a laser scanning confocal microscope (Meta510 ZEISS, Thornwood, NY, USA) under x100 magnification using immersion oil. Cells with apoptotic features or micronuclei were excluded in the p-H2AX analysis. Data was obtained from imaging three replicate samples of both WT, p55KO, p75KO EPCs treated with IR-CM and NoIR-CM, which totaled 300-400 cells in each of the time points. Using a computer assisted image analysis algorithm based on pixel and color distribution, p-H2AX foci were evaluated for quantification. Cells with ≥1 pH2AX foci were counted and plotted for mean foci/cell and percent of cells with an N number of p-H2AX foci.

Enzyme-linked Immunosorbent Assay (ELISA)

The Samples of IR-CM and NoIR-CM collected from WT and p55KO EPCs at 1 hour, 5 hours, 24 hours, 3 days, and 5 days post-IR were processed

for mouse multiplex cytokine ELISA according to manufacturer protocols (Signosis, Sunnyvale, CA, USA). Twelve cytokines, chemokines, and growth factors were analyzed for the concentrations in the media: Interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), Rantes, macrophage inflammatory protein-1 alpha (MIP-1α), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), epidermal growth factor (EGF), insulin growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), and stem cell factor (SCF). The plates were read using Tecan Spectra model 96 Well Microplate Reader (MTX Lab Systems, Vienna, VA, USA).

Cytokine Treatments Experiment in p55KO EPCs

Using the methods which were previously described, a set of sub-confluent *ex vivo* expanded EPCs were isolated from non-irradiated p55KO mice. At 60-70% confluence, the EPCs were treated with mouse recombinant proteins of cytokines that were observed to have significant increases in concentration from the ELISA analysis of post-IR p55KO EPC media. The recombinant proteins used in this experiment were IL-1α (Cat.D-61112; PromoKine, Heidelberg, Germany), MCP-1/CCL2 (Cat.D-64030; PromoKine, Heidelberg, Germany), and Rantes/CCL5 (Cat.D-64130; PromoKine, Heidelberg, Germany), which were available as a lyophilized powder and were reconstituted with sterile, ultra-pure water to a stock concentration of 0.1mg/ml and store in aliquots according to

manufacture specifications. TNF-α (Cat.34-8321-82; eBiosciences, San Diego, CA, USA) was available as 0.5mg/ml stock from the vendor. The cytokines were tested at various concentrations (IL-1α 290pg/ml, 580pg/ml; Rantes 600pg/ml, 1500pg/ml; MCP-1 580pg/ml, 1160pg/ml, 2900pg/ml, and TNF-α 100pg/ml, 1000pg/ml, 40000pg/ml) on separate EPC wells and incubated for 24 hours in normal growth conditions. The cells were then fixed and processed for p-H2AX immunostaining.

Statistical Analysis

All results are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA (StatView Software, SAS Institute Inc.; Gary, NC). Differences were considered significant at $p < 0.05$.

RESULTS

Full-body γ-irradiation and *Ex Vivo* BM-derived EPC Culture

In the preliminary experiment, we performed a low dose full-body γ-irradiation on WT mice from which BM-EPCs were harvested to observe the formation and decay of p-H2AX foci. p-H2AX foci as mentioned in the introduction have been proven to correlate well with the formation and decay of DNA double strand breaks repair (Rothkamm and Lobrich, 2003). IR EPCs were isolated from the mice at 1 hour, 24 hours, and 7 days post irradiation and compared with control NoIR EPCs collected at the same time points. In NoIR EPCs, we observed little change in the number of p-H2AX foci during the 7 day period. In **Figure 1**, the average number of foci per cell was 0.27±0.15, 0.27±0.15, and 0.29±0.08 at 1 hour, 24 hours, and 7 days respectively (p=NS, for all three time points). In comparison, IR EPCs had a greater number of foci/cell 5.79±1.18, 3.27±1.09, and 6.77±1.41 respectively at 1 hour, 24 hours, and 7 days. Between 1 hour and 24 hours there was an approximately 45% decrease in the number of foci (p=NS) and between 24 hours and day 7 there was more than a 200% increase ($p<0.006$). In addition, quantification of p-H2AX foci distribution for (N) number of foci/cell showed that at day 7 there was an increase in the number of cells with 12 or more p-H2AX foci/cell (**Figure 2**).

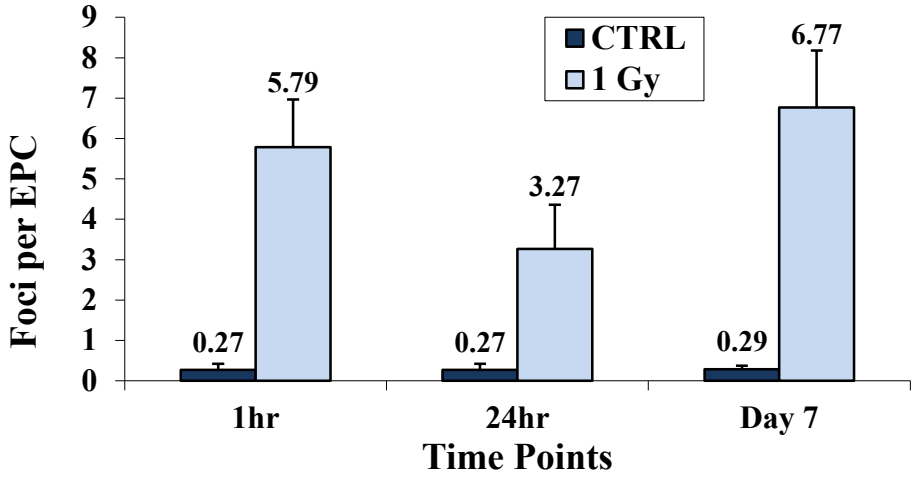

Figure 1. Mean number of foci per cell for control NoIR EPCs versus full body IR EPCs. The graph represents the foci/cell comparison between NoIR EPCs and IR EPCs at time points 1hr, 24hr, and 7day. The numbers on the top of the bars represents average foci/cell. The average foci per cell is significantly higher due to exposure to low dose radiation.

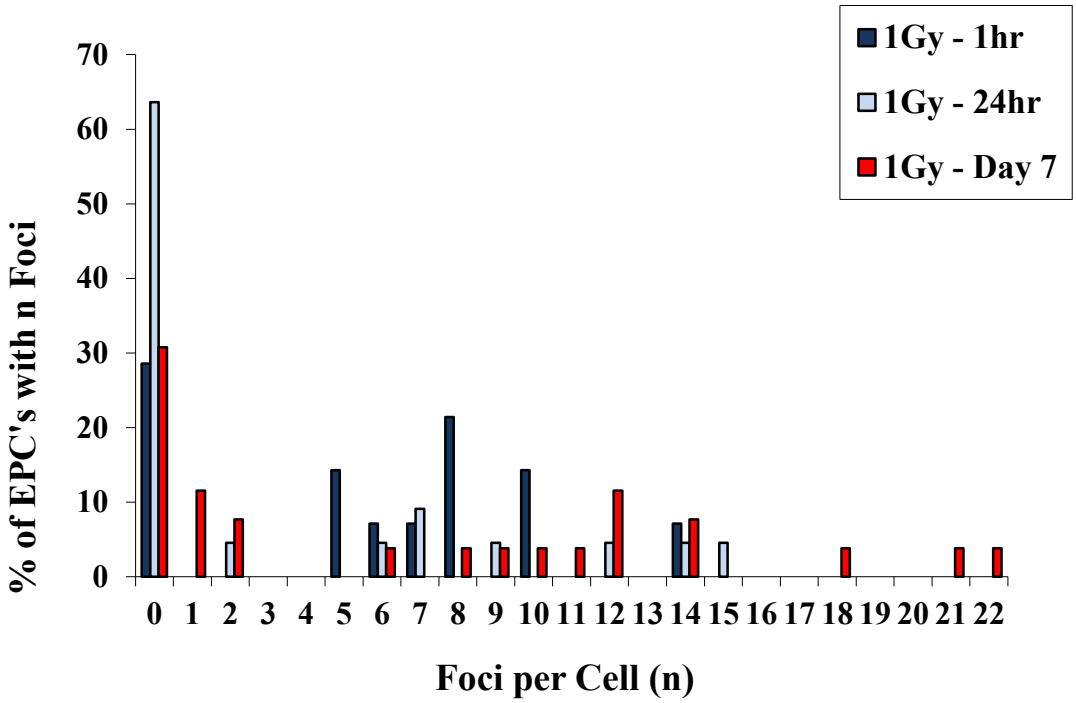

Figure 2. Distribution of EPCs with (N) foci at 1 Hour, 24 hours, and 7 days post full body IR. This graph represents the percentage of EPCs with (N) number of p-H2AX foci in a distribution from 0 to 22 foci/cell. There is a time-dependent increase in the maximum p-H2AX foci as well as an increase in the frequency of EPCs with a higher number foci/cell count.

The data indicates that the decay of p-H2AX for WT BM-derived EPCs between 1 hour and 24 hours represents a delayed or inefficient radiation-induced DNA repair. Furthermore, the significant increase in the number of foci/cell by day 7 suggests a significant radiobiological bystander response in BM-derived EPCs resulting in more DNA damage (Rothkamm and Lobrich, 2003; Sokolov et al., 2007).

Media Transfer Experiments in WT BM-derived EPCs

In order to determine that bystander responses can be induced in WT BM-derived EPCs through medium transfer experiments, one set of WT EPCs was exposed to γ-radiation *in vitro* and the IR conditioned medium (CM) was transferred to a second set of naïve recipient EPCs. In the control, NoIR-CM was transferred to the recipient EPC and after 24 hours post-treatment, they had negligible numbers of p-H2AX foci. Foci per cell was 0.71±0.12, 0.74±0.16, 0.79±0.17, and 0.82±0.20 respectively for 30 minutes, 1 hour, 5 hours, and 24 hours p = N.S (**Figure 3**). In the IR-CM treated EPCs the number of foci/cell was 0.99±0.19, 1.37±0.28, 1.39±0.29, and 3.16±0.46 for 30 minutes, 1 hour, 5 hour, and 24 hours respectively. Comparing 30 minutes to 24 hour time points, there was a significant 319% increase in the mean number of foci/cell ($p<0.0001$), and between 5 hour and 24 hours 227% increase was noted. In the quantification of p-H2AX foci distribution with (N) number of foci/cell for IR-CM EPCs, percent of

cells with more than 6 to 15-pH2AX foci/cell increased greatly at 24 hours suggesting a significant bystander effect (**Figure 4A, B**).

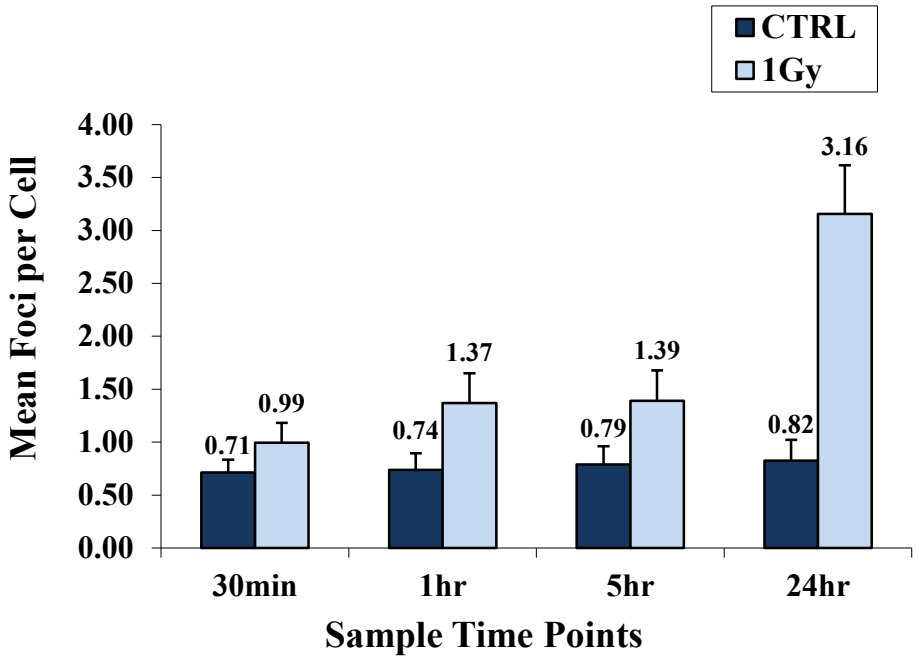

Figure 3. Mean p-H2AX foci per cell for EPCs post IR-CM treatment versus control. Graphical representation of mean foci at 30 minutes, 1 hour, 5 hour, 24 hours post conditioned media treatment. The numbers on the top of bars represent actual mean values.

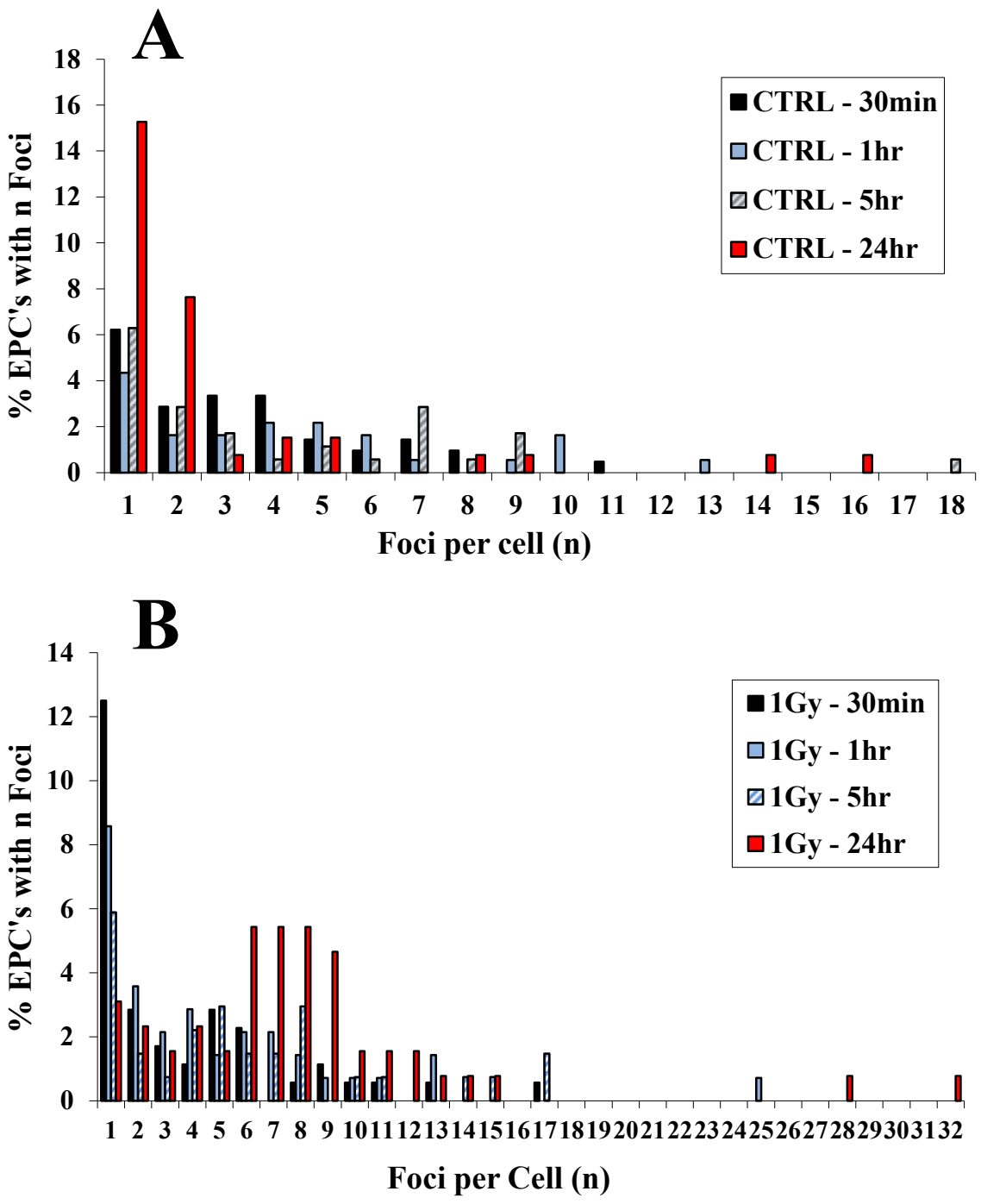

Figure 4. Distribution of percent EPCs with (N) Foci post CM treatment at time points 30 minutes, 1hour, 5hours, and 24 hours excluding 0 foci. (A) Distribution of (N) foci for Control NoIR-CM treated EPCs. Note this graph excludes 0 and the scale is from 1 to 18 foci/cell (B) Foci Distribution graph for IR-CM treated EPCs. Note that the scale is 1 to 32 foci/cell.

Media Transfer Experiments of WT, p55KO, and p75KO BM-derived EPCs

In order to examine the role of TNF-α receptors in the radiobiological bystander responses of EPCs, media transfer experiments were carried out in cells isolated from WT, p55KO, and p75KO mice. The data derived from three biological replicates for each of the genotypes and analysis of p-H2AX foci is summarized in **Table 1**. In the comparison of foci/cell in WT, p75KO, and p55KO EPCs treated with 1hour IR-CM (4.34±0.35, 6.86±0.97, and 8.48±0.89 respectively), both p75KO and p55KO EPCs showed a significant increase in the mean p-H2AX foci/cell over WT EPCs (WT vs. p75 $p<0.002$, WT vs. p55 $p<0.0001$) (**Figure5**). Treatment with 5hr IR-CM, interestingly showed similar foci/cell numbers between WT, p75KO, and p55KO EPCs with no significant difference (**Figure 5**). At 24 hours, there was a significant decrease in p-H2AX foci/cell in both p75KO and p55KO EPCs (3.72±0.46 and 4.77±0.57 respectively) compared to IR-CM treated WT EPCs (9.02±0.82), $p<0.0001$, in both comparisons (**Figure5**). Formation of p-H2AX foci/cell in medium transfer of day 3 IR-CM again showed no significant difference. The findings suggest that in 24 hours IR-CM, presence of both TNF receptors p55 and p75 is required to increase the formation of p-H2AX foci. **Figure 6** is another representation of the data from this experiment, but the time points of the x-axis are in a scale interval of hours.

Table 1. Mean foci data for media transfer experiments between WT, p75KO, and p55KO EPCs. Mean foci/cell data for WT, p75KO, and p55KO EPCs treated with IR-CM at time points 1 hour, 5 hours, 1 day, 3 days, and 5 days.

WT EPCs	Mean Foci	STDEV	SEM
CTRL	2.50	2.18	0.14
1Gy - 1hr	4.34	4.10	0.35
1Gy - 5hr	6.91	6.14	0.89
1Gy - Day 1	9.02	5.92	0.82
1Gy - Day 3	7.59	5.26	0.82
1Gy - Day 5	3.77	3.84	0.44

P75KO EPCs	Mean Foci	STDEV	SEM
CTRL	3.45	2.92	0.45
1Gy - 1hr	6.86	6.28	0.97
1Gy - 5hr	7.69	5.12	0.64
1Gy - Day 1	3.72	3.96	0.46
1Gy - Day 3	7.26	6.37	1.46
1Gy - Day 5	5.89	3.95	0.75

P55KO EPCs	Mean Foci	STDEV	SEM
CTRL	2.98	3.71	0.50
1Gy - 1hr	8.48	6.66	0.89
1Gy - 5hr	7.38	5.69	0.65
1Gy - Day 1	4.77	4.65	0.57
1Gy - Day 3	7.81	4.43	0.73
1Gy - Day 5	8.46	6.31	1.01

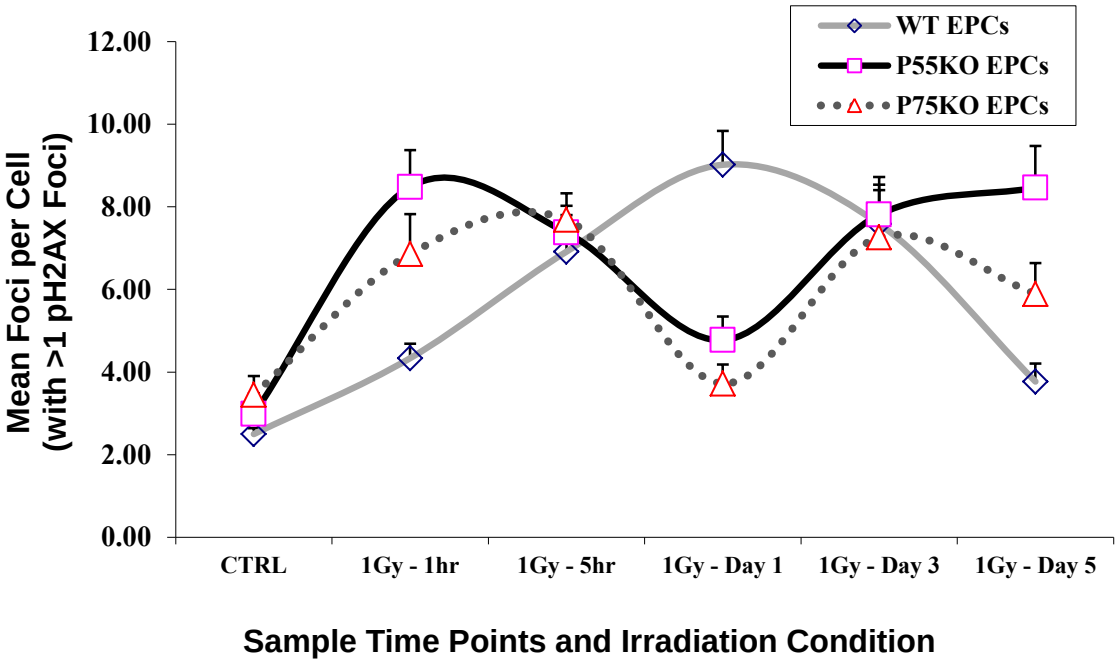

Figure 5. Comparison of p-H2AX foci formation and decay between WT, p55KO, and p75KO IR-CM EPCs (non-scaled). This figure is a graphical representation of the data in Table 1. Time points are not graphed in scale.

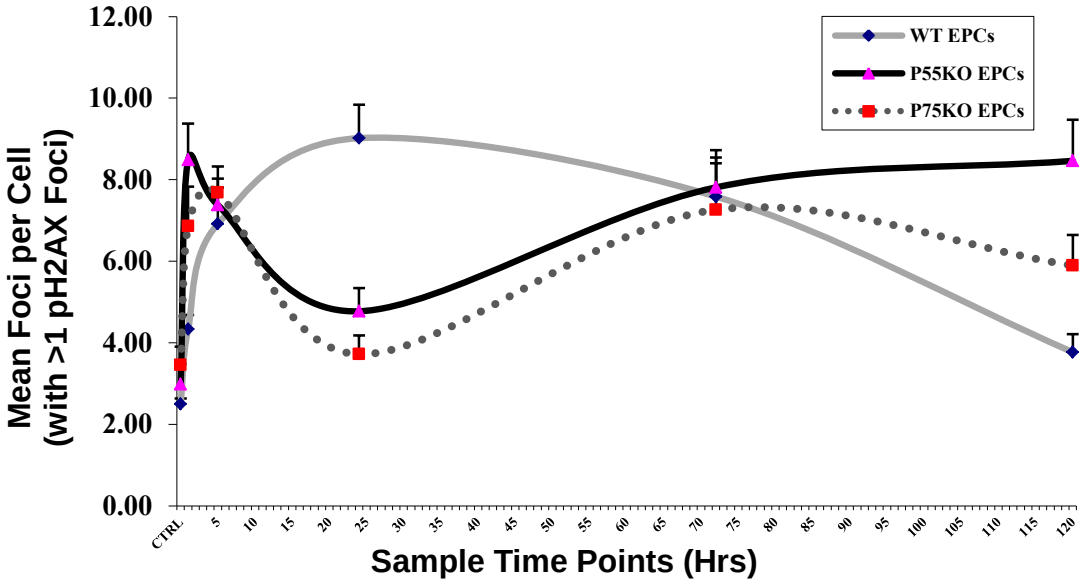

Figure 6. Comparison of p-H2AX foci formation and decay between WT, p55KO, p75KO IR-CM EPCs (scaled). This figure is a graphical representation of the data in Table 1. The graph is scaled to hours to accurately to portray the trend.

Day 5 results showed that WT continued its decline in the number of foci/cell from Day 1 to 3.77±0.44 foci/cell (WT day 3 vs. day 5: $p<0.0001$, day 1 vs. day 5: $p<0.0001$, and day 1 vs. day 3: p=NS) (**Figure 6**). In contrast, p55KO continued to increase in the foci/cell count of p-H2AX from day 1 to day 5, 8.46±1.01 (p55KO day 1 vs. day 3: $p<0.002$, day 1 vs. day 5: $p<0.0001$, and day 3 vs. day 5: p=NS). In p75KO EPCs, foci/cell count decreased from day 3 to 5.89±0.75 in day 5 (p75KO day 1 vs. day 3: $p<0.003$, day 1 vs. day 5: $p=0.04$, day 3 vs. day 5: p=NS). For both WT and p75KO EPCs treated with 5day IR-CM, there was a decreasing trend but a direct comparison of WT and p75KO EPCs showed a small significant difference in the number of foci/cell ($p<0.04$). In contrast, 5 day IR-CM treated p55KO EPCs had a significantly greater foci/cell count when compared to both WT and p75 EPCs at the same time point (p55KO vs. p75KO: $p<0.02$, p55 vs. WT: $p<0.0001$, and p75 vs. WT: $p<0.04$). Foci distribution of % EPCs with (n) number of foci is shown in **Figure 7 and 8.** Our findings suggests that a continuous increase in p-H2AX foci of p55KO EPCs indicates that signaling via p75 receptor plays a significant role in the delayed formation of p-H2AX foci.

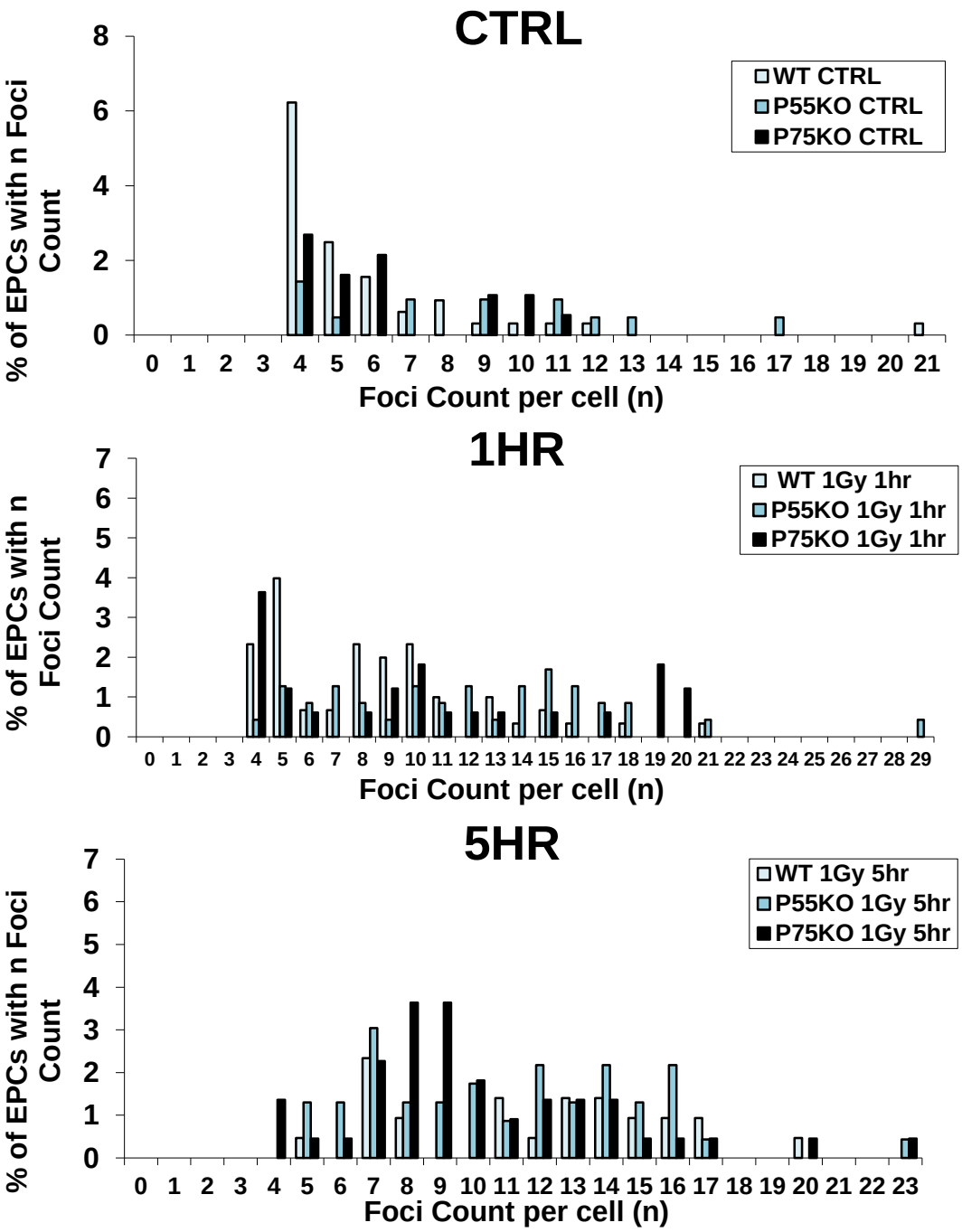

Figure 7. (n) Foci distribution of percent EPCs for WT, p55KO, p75KO for CTRL (NoIR), 1 hour, and 5 hour time points. Note that the scale for each graph is based on max number of foci observed in the analysis.

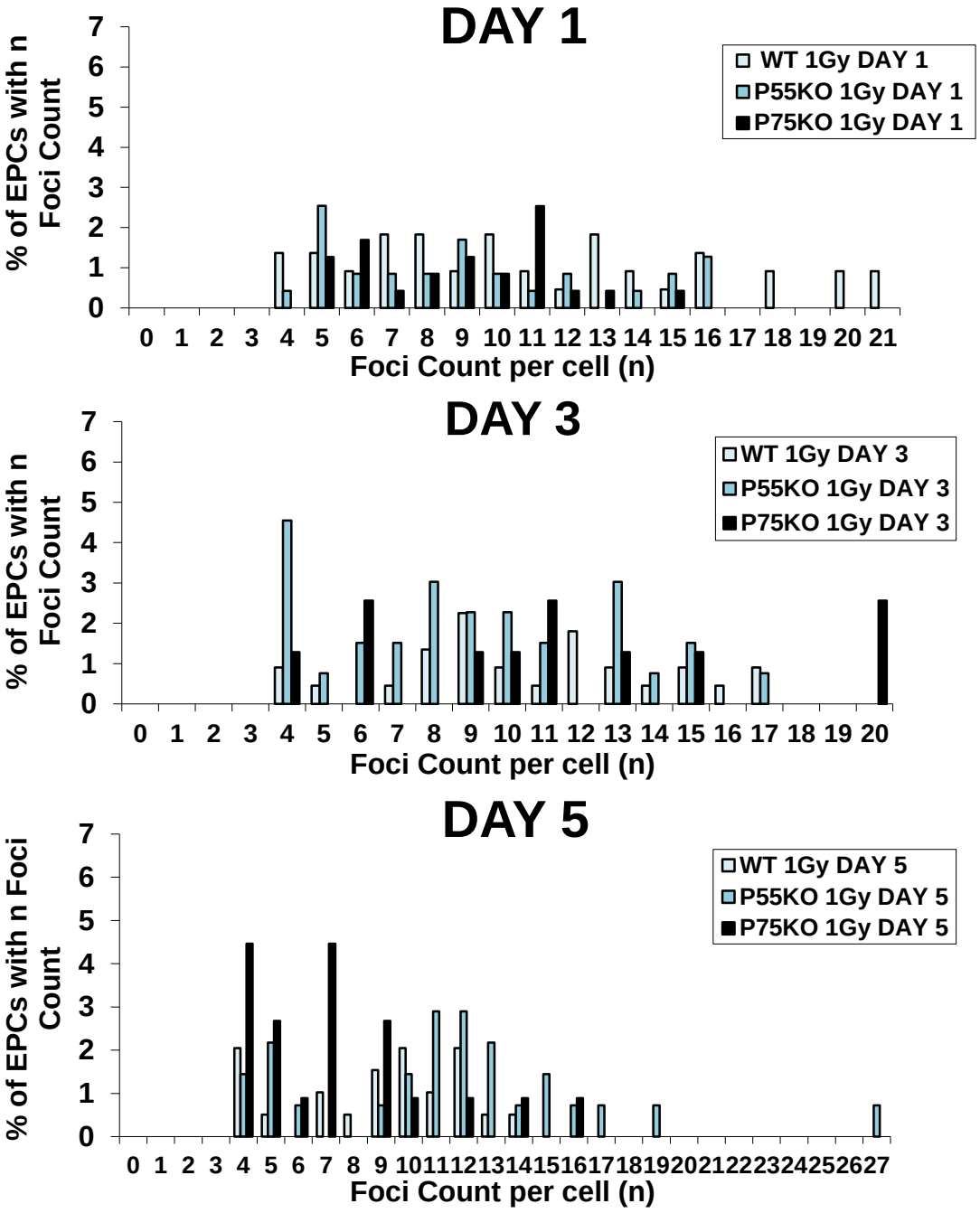

Figure 8. (n) Foci distribution of percent EPCs for WT, p55KO, p75KO for day 1, day 3, and day 5 time points. Significant divergence of the distribution was observed between the samples on day 1 and day 3, and p55KO EPCs continue an increasing trend in p-H2AX formation from day 1 to day 5. Note that the scale for each graph is based on the max number of foci observed in the analysis.

Enzyme-linked Immunosorbent Assay Analysis (ELISA) for WT versus P55KO EPCs

Based on our findings of a continuous increase in p-H2AX foci/cell of p55KO EPCs post IR-CM treatment, we focused the analysis on p55KO EPCs only. IR-CM media from WT and p55KO EPCs were collected at time points 1 hour, 5 hours, 1 day, 3 days, and 5 days during the media transfer process and the media were processed for multiplex (12-gene) ELISA to determine the production and accumulation of cytokines and growth factors into the media by γ-irradiation. The twelve factors analyzed were IL-1α, IL-1β, IL-6, MCP-1, Rantes, MIP-1α, G-CSF, GM-CSF, EGF, IGF1, VEFG, and SCF. In the comparison of p55KO IR-CM to WT IR-CM, the most significant increases (200-1600%) in concentrations were observed between days 1-5 in IL-1α, IL-1β, MCP-1, Rantes, MIP-1α, G-CSF, GM-CSF, EGF, and SCF (**Figure 9A-L**).

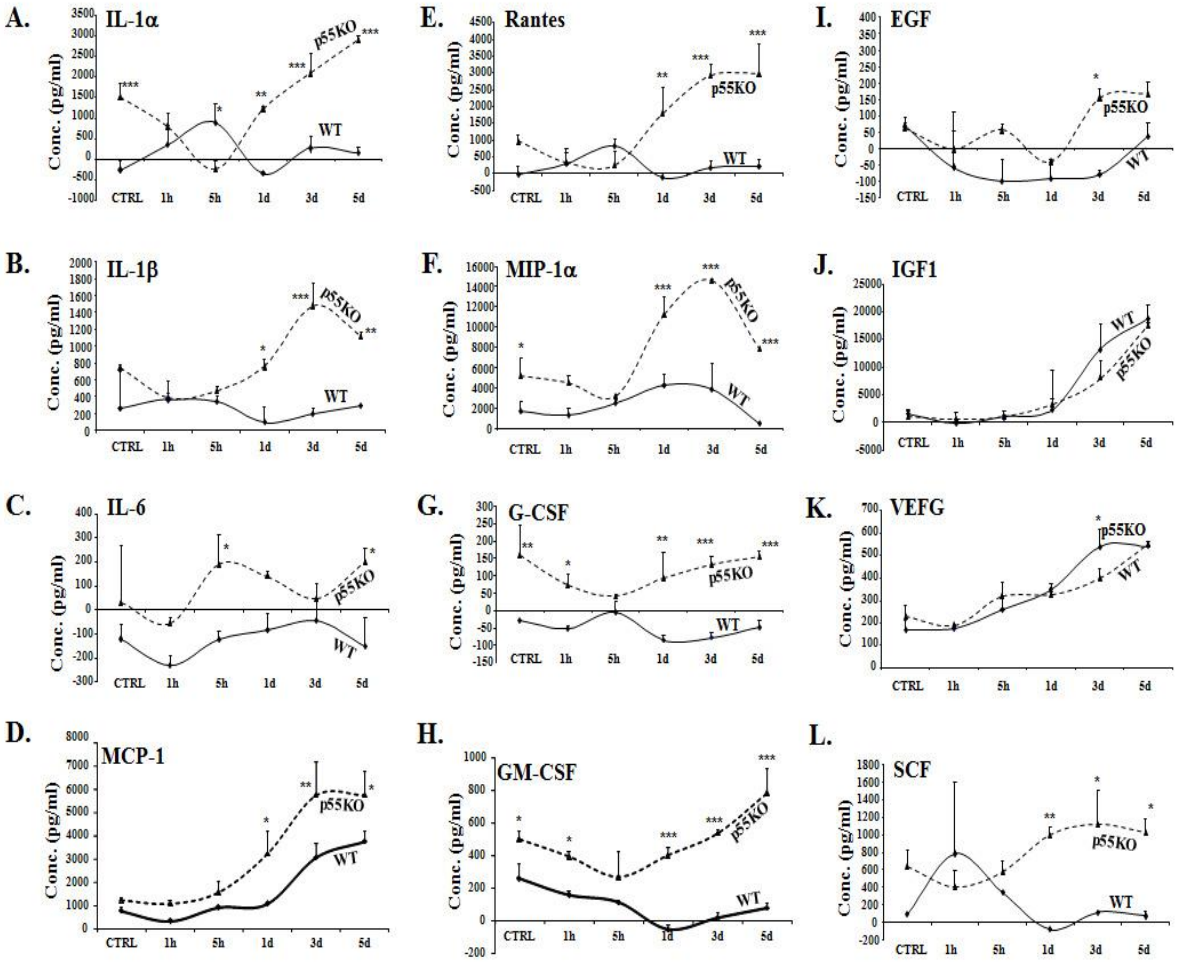

Figure 9. The results of the ELISA analysis on twelve cytokines, chemokines, and growth factors in irradiated p55KO EPC media compared to irradiated WT EPC media. The analysis was performed for time points 1 hour, 5 hour, 1 day, 3 day, and 5 days, same as the time points from the media transfer experiment. From this analysis, we observed significant increase in levels of IL-1α, IL-1β, MIP-1α, G-CSF, Rantes, and GM-CSF in p55KO EPC media compared to WT mainly between 1 and 5 days.

The increases suggests that the production and accumulation of these cytokines, chemokines, and growth factors are triggered by the role of p75 signaling as p55 receptor is knocked out in p55KO EPCs. In the case of IL-6 in **Figure 9C**, there is a significant bimodal increase in concentration at 5 hours and again at 5 days. It

suggests that the initial increase may be directly radiation-mediated whereas the second increase occurred long after the initial radiation, which may be the result of an inflammatory response. Comparing IR WT EPC media to NoIR WT EPC media, we noted significant increase in levels of IL-1α, MIP-1α, G-CSF, and GM-CSF ($p<0.0009$, $p<0.05$, $p<0.001$, and $p<0.05$, respectively) (**Figure 9A, F-H**). There was no significant difference between concentration of IGF1 and VEGF between WT IR-CM and p55KO IR-CM, except a small increase in VEGF concentration at day 3 in p55KO (**Figure 9J, K**). Our findings from multiplex ELISA studies strongly suggest that signaling through TNFR2/p75 in irradiated p55KO EPCs leads to significant increases in the production and accumulation of several cytokines such as IL-1α, IL-1β, Rantes, MCP-1, and MIP-1α (with the exception of MCP-1, there were also increases in WT IR EPCs versus WT NoIR EPCs) which are important in the processes that enhanced the delayed formation of p-H2AX observed in the media transfer experiment.

Cytokine Treatments Experiment in p55KO EPCs

From the results of our ELISA analysis, IL-1α, Rantes, and MCP-1 were selected for cytokine treatment in which non-irradiated “naïve” p55KO EPCs were exposed to mouse recombinant proteins. Evidence suggested that the production of these cytokines is elevated through TNFR2/p75 signaling and we sought to determine which cytokine(s) had a direct role in the induction of p-H2AX foci. Naïve p55KO EPCs were treated *ex vivo* with the following

concentrations determined from the ELISA analysis: IL-1α (290pg/ml, 580pg/ml), Rantes (600pg/ml, 1500pg/ml) and MCP-1 (580pg/ml, 1160pg/ml, 2900pg/ml). TNF-α was also tested at 100pg/ml, 1000pg/ml, 40000pg/ml to observe the effects of p75 signaling in p55KO EPCs. After 24 hours incubation with the cytokine treatments, the cells were stained and imaged for quantification. Results of the mean foci/cell count for each of the treatments is summarized in **Table 2.**

Table 2. Mean p-H2AX foci data for p55KO EPCs treated with cytokines IL-1α, Rantes, MCP-1, TNF-α. Data of mean p-H2AX foci of p55KO EPCs post 24 hour incubation with recombinant protein treatments.

p55KO EPC (Excluding 0 foci)	Mean Foci	STDEV	SEM
CTRL	2.18	2.28	0.22
IL1-α (580pg)	11.08	7.53	0.71
IL1-α (290pg)	10.07	9.80	0.88
Rantes (1500pg)	3.44	4.46	0.46
Rantes (600pg)	2.51	2.87	0.28
MCP-1 (2900pg)	2.60	2.74	0.22
MCP-1 (1160pg)	3.69	5.11	0.45
MCP-1 (580pg)	3.24	4.17	0.43
TNF-α (40000pg)	4.89	5.58	0.42
TNF-α (1000pg)	5.95	6.89	0.56
TNF-α (100pg)	3.95	3.59	0.25

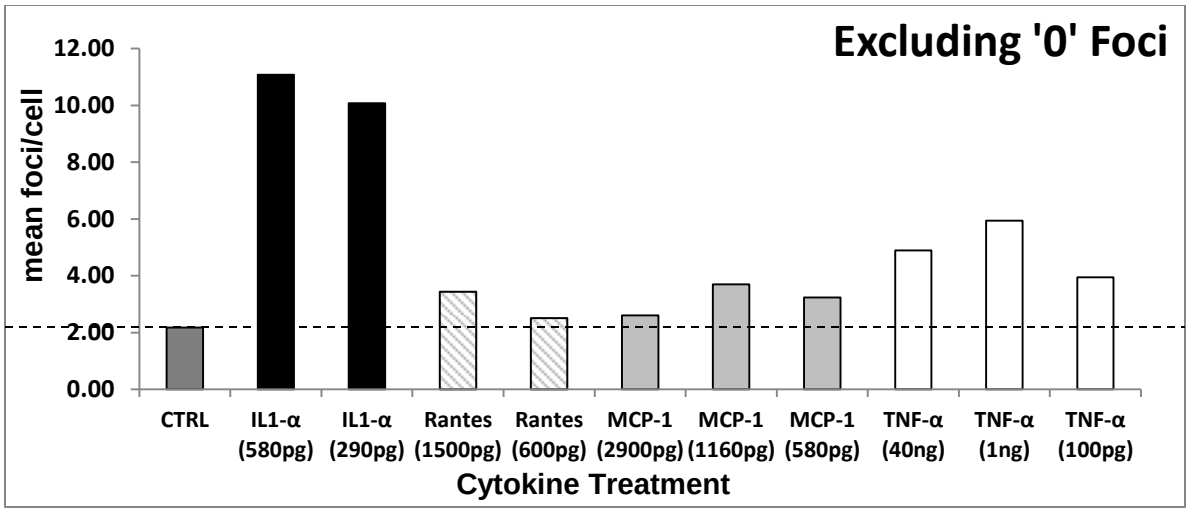

Figure 10. Mean p-H2AX foci data for p55KO EPCs treated with cytokines IL-1α, Rantes, MCP-1, TNF-α. Compared to the control, Rantes and MCP-1 did not have a significant difference in mean foci count, whereas TNF-α and especially IL-1α had significant increases in p-H2AX foci.

The data showed that the mean p-H2AX foci count of MCP-1 and Rantes were not significantly different from the control which had mean foci/cell count of 2.18±0.22, with the exception of MCP-1 at 1160pg/ml (mean foci count of 3.69±0.45, $p<0.03$) **Figure 10**. TNF-α treated naïve p55KO EPCs showed significant increases ($p<0.0001$) in the p-H2AX foci count at all concentration treatments ranging from 3.95±0.25 to 5.95±0.56 mean foci when compared to the control, MCP-1, and Rantes (**Table 2**). The p55KO EPCs treated with IL-1α showed the largest increase in the mean p-H2AX foci formation with 10.07±0.88 foci/cell at 290pg/ml and 11.08±0.71 at 580pg/ml ($p<0.0001$ significantly different from all treatments). Analysis of p-H2AX foci distribution of EPCs with 3 or more foci in **Figure 16** showed that in control p55KO EPCs, less than 1-1.3% of cells

had a maximum of 4-8 p-H2AX foci/cell and less than 0.5% of cells had 13-15 p-H2AX foci/cell . In comparison, TNF-α and IL-1α were observed to have greater than 2% and 4% of cell with 9-18 foci/cell (**Figure 16**). Remarkably foci/cell count as large as 19-31 were observed in 0.5-1% of cells treated with TNF-α, and ranges of 19-51 foci/cell were observed in p55KO EPCs treated with IL-1α (**Figure 13, 14**). These findings suggests that TNF-α and IL-1α but not MCP-1 or Rantes caused significant increases in the formation of p-H2AX foci in naïve BM-Derived TNFR1/p55KO EPCs.

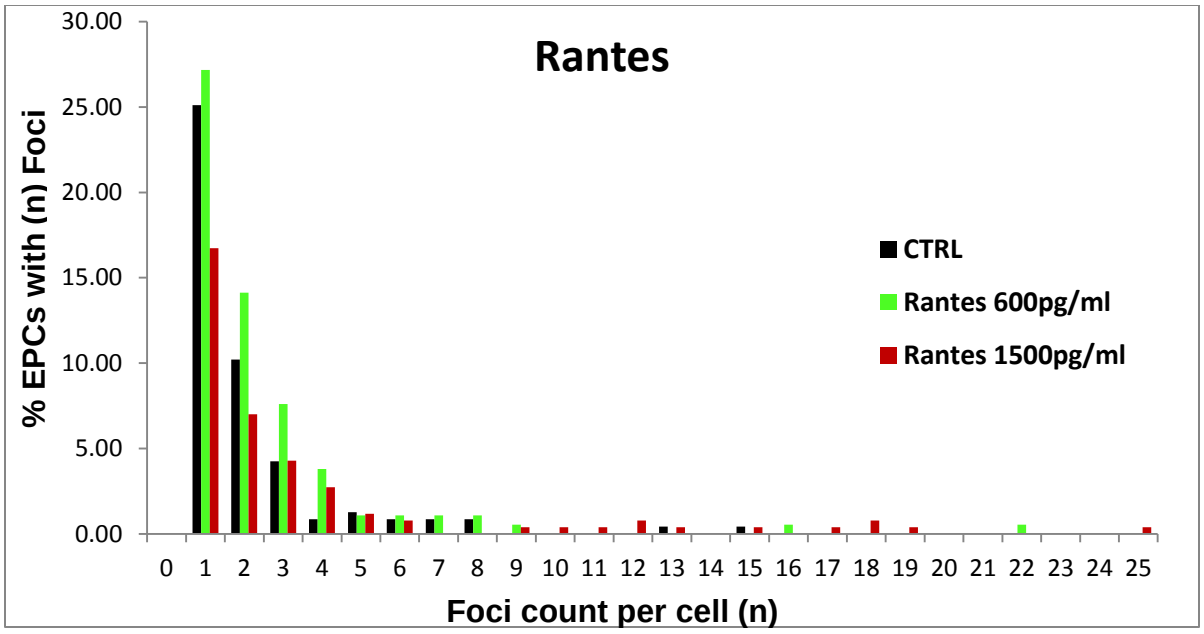

Figure 11. Percentage distribution of (n) foci for p55KOEPCs treated with Rantes. Comparing p-H2AX formation of Rantes treatments compared to the control, we did not notice a significant difference in distribution. This graph represent the distribution data excluding cells with zero foci.

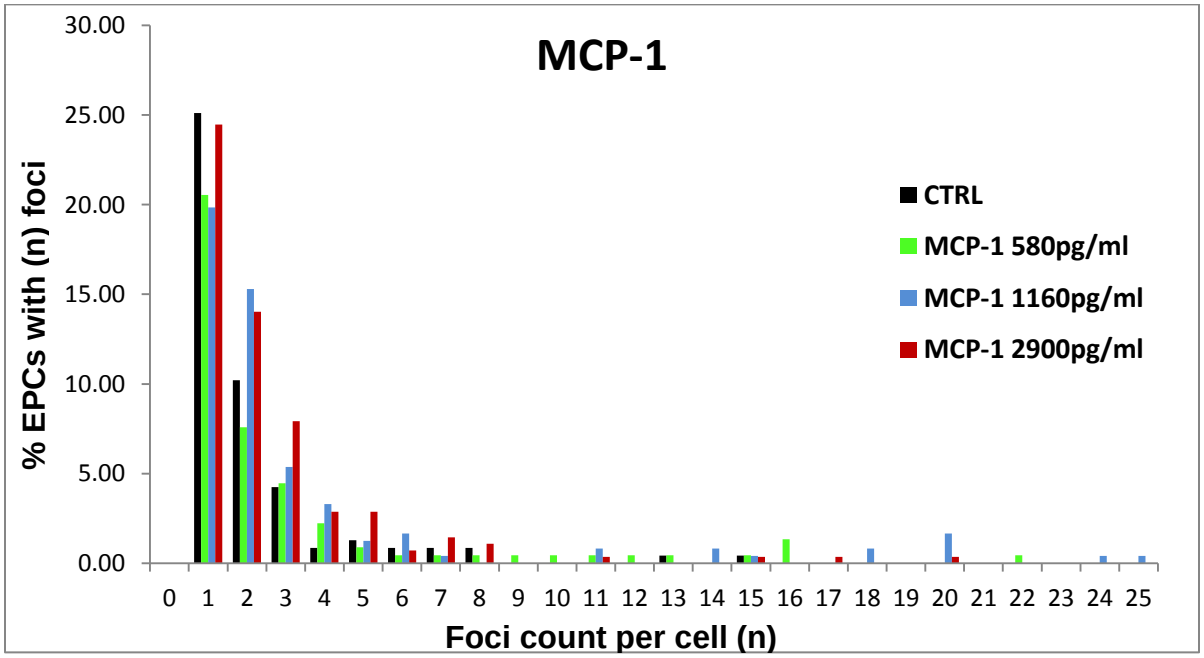

Figure 12. Percentage distribution of (n) foci for p55KOEPCs treated with MCP-1. The data for this graph excludes zero foci. MCP-1, same as Rantes did not produce a distribution of foci that were significantly different from the control

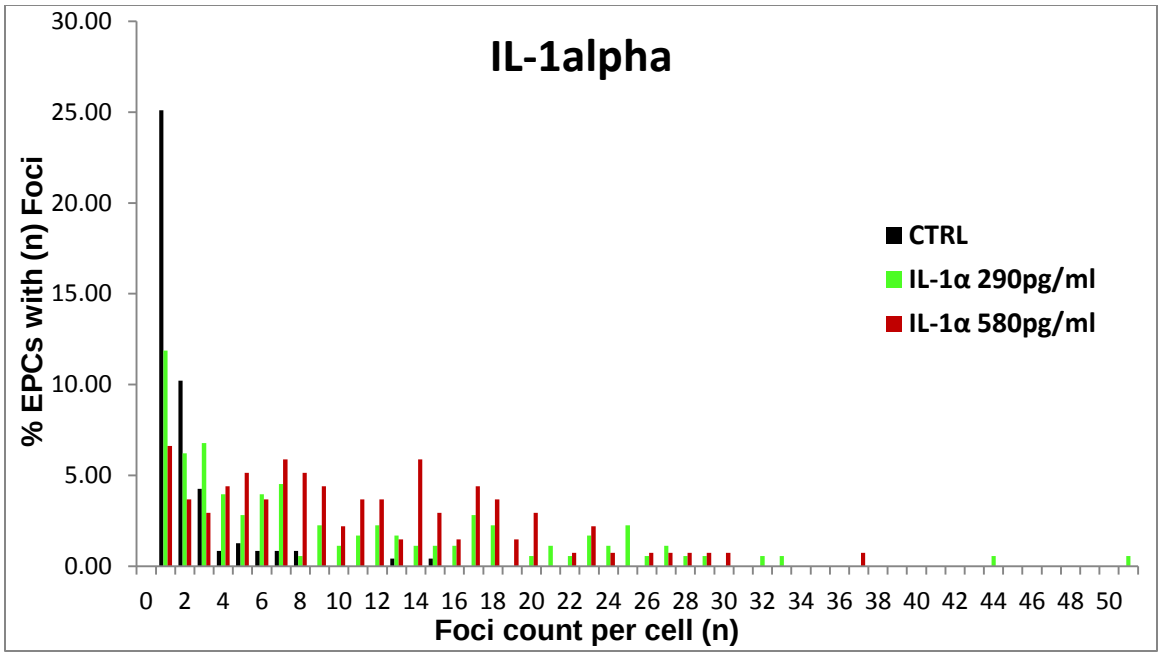

Figure 13. Percentage distribution of (n) foci for p55KOEPCs treated with IL1-α. The data for this graph excludes zero foci and the x-axis goes to 50 foci/cell. Significant dose-dependent increase in foci was observed for IL1-α foci distribution.

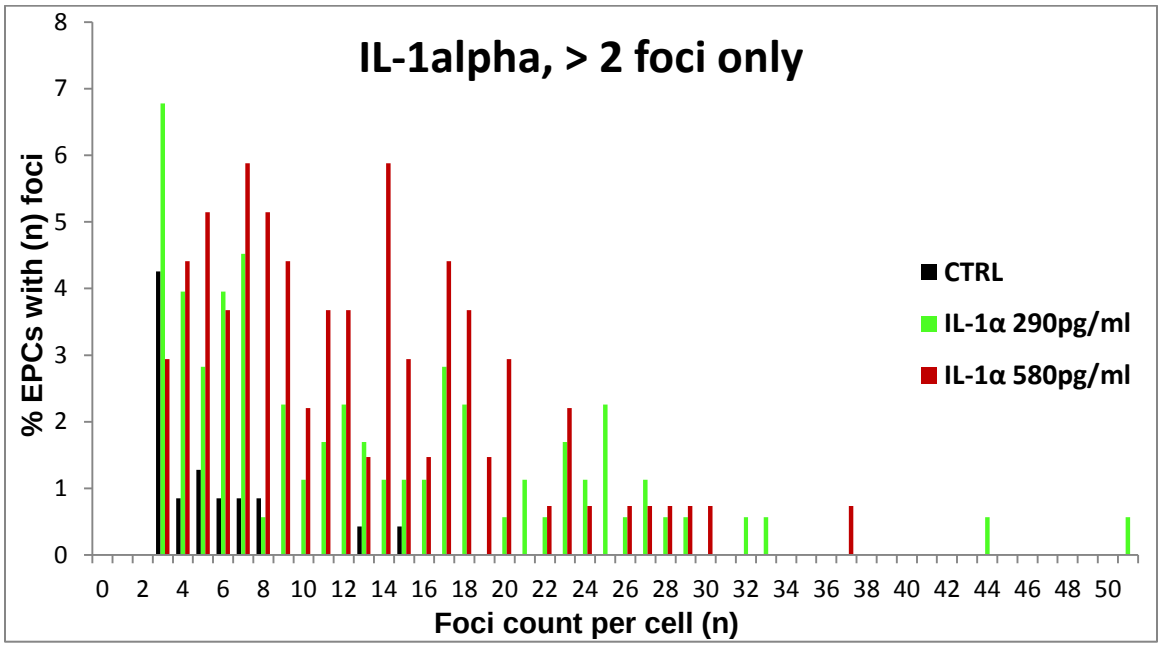

Figure 14. Percentage distribution of (n) foci for p55KOEPCs treated with IL-1α (>2 foci only). Based on this graph, IL-1α has significantly higher number of EPCs with higher number of foci count per cell as high as 50+. The data for this graph excludes 2 or less foci count to show the distribution at higher p-H2AX levels.

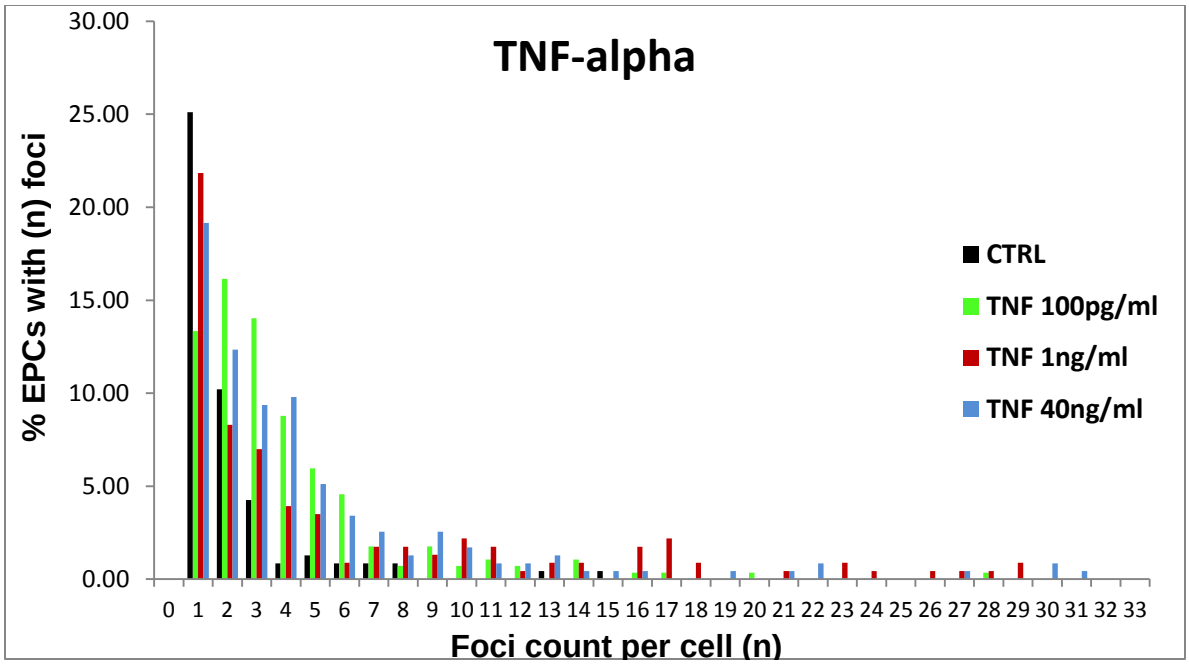

Figure 15. Percentage distribution of (n) foci for p55KOEPCs treated with TNF-α. The data for this graph excludes cells with 0 foci. The data from the cytokine treatments showed that TNF-α induced significantly higher levels of foci in all tested concentrations.

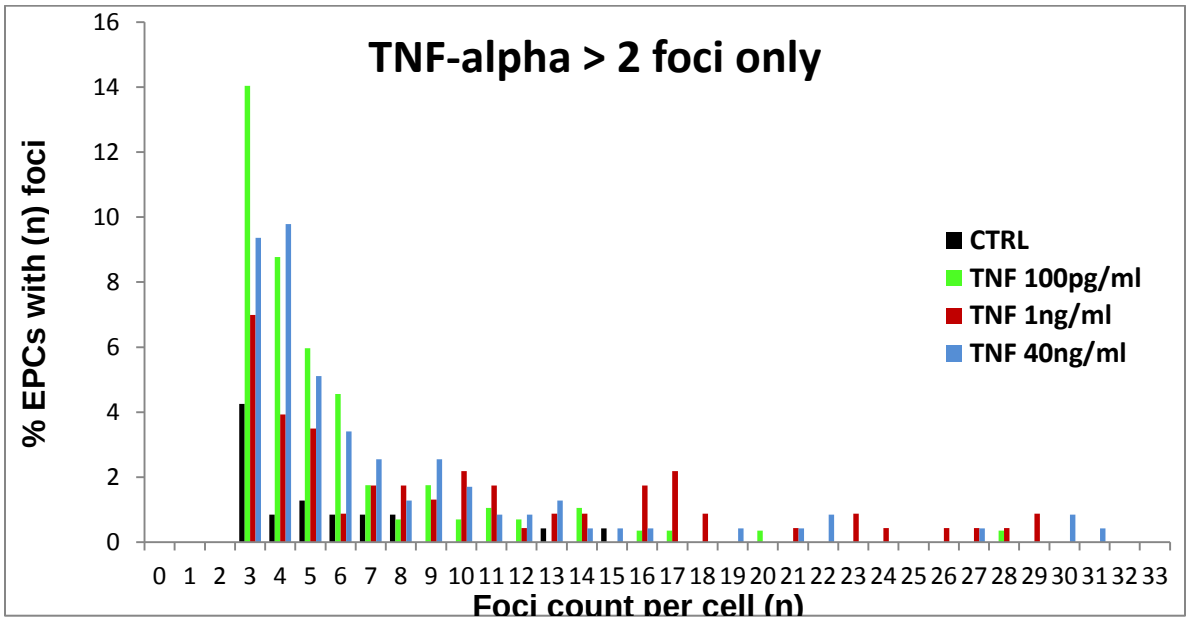

Figure 16. Percentage distribution of (n) foci for p55KOEPCs treated with TNF-α (> 2 foci only). The data for this graph excludes cells with ≤ 2 foci. Although not as drastic as the results of IL-1α, the percentage of cells with higher number of foci count increased.

DISCUSSION

The response of the immune system to low dose radiation is a subject of ongoing research. However, current evidence suggests that the immune response is enhanced, as observed through the activation of macrophage and CD8+ T cell responses (Pandey et al., 2005). Macrophage activation and neutrophil migration are normally part of a protective process at sites of tissue injury, but the cytotoxic mechanisms through soluble factors such as peroxidase, cytolytic protease, NO, TNF-α, and IL1 can be damaging, especially if they are prolonged in release (Wright and Coates, 2006). In our study we investigated TNF-α, specifically the receptors of TNF-α, in the role of non-targeted bystander effects. Experiments by our lab and others have shown that a significant age-associated decrease in TNFR2/p75 and increase in TNFR1/p55 occur in human and mice EPCs (Aggarwal et al.,1999; Goukassian et al., 2007). This age related disruption of TNF receptors was observed to cause impairment of angiogenesis and inhibition of EPC proliferation and function, which suggested the importance of TNF receptors to EPCs in radiobiological bystander effects.

In our media transfer experiment, the data at 24 hours showed a marked difference between WT and both p55KO and p75KO EPCs post IR-CM treatment. The number of p-H2AX foci for WT EPCs at 24 hours was at peak compared to all other observed time points, whereas p55KO and p75KO EPCs had the lowest

mean foci at this time. This suggested that the delayed response of bystander effects may be greatly enhanced by the interaction of both functional TNF receptors. Interestingly at 5 hours and 3 days the mean number of foci/cell was similar between all genotypes. However in the overall trend, p55KO EPCs were seen to continuously increase in mean foci/cell after the initial decrease at 24 hours. Although the exact trend of p75KO is not as clear, the difference in the trend of p55KO and p75KO EPCs compared to WT EPCs demonstrate the influence of TNF receptors in the development, decay, and delayed removal of p-H2AX foci of non-targeted effects.

Our ELISA analysis was directed on p55KO EPCs based on the increasing trend of p-H2AX foci/cell post IR-CM treatment and multiple cytokines, chemokines, and growth factors have been noted to increase in concentration. Of those cytokines, we choose to study IL-1α, Rantes, and MCP-1 and also tested TNF-α in our cytokine treatment experiment of naïve p55KO EPCs. The results showed that TNF-α and IL-1α both induced significant increases in p-H2AX foci formation with the greatest mean foci/cell being observed in IL-1α treatments. With IL-1α, we tested only two concentrations levels but the data suggested a possible dose-dependent relationship in the formation of p-H2AX foci, with higher concentration of IL-1α inducing a higher number of foci/cell (**Figure 10**). In TNF-α treatments the trend between the concentration and p-H2AX formation was unclear, which may be indicative of the dual function of p75 receptor signaling to

be pro-survival and inflammatory. Further experimentation would be required to find the dose-dependent relationship of these cytokines to induce p-H2AX foci, but it was observed that IL-1α and TNF-α induced significantly higher p-H2AX foci/cell. The data showed that MCP-1 and Rantes do not play a direct role in the induction of p-H2AX but may contribute indirectly in the immune responses of non-targeted bystander effect (Lee et al., 2000; Sica et al., 1990).

There are still many unanswered questions in current literature regarding the mechanism of low dose radiobiological effects. In several microbeam experiments, it was found that low-dose irradiation of the cytoplasm alone was able to induce significant bystander effects as measured by micronuclei and CD59 mutations (Shao et al., 2004; Wu et al., 1999). Experimentation on radio-resistant glioma cell population showed that 36% of micronuclei were increased when a single Helium ion was targeted through the cytoplasm (Shao et al., 2000). The analysis showed that nitric oxide (NO) played a role in the induction of micronuclei through the use of a NO scavenger and is among the identified soluble factors thought to be involved in bystander effects, along with reactive oxygen species and cytokines. The role of gap junctions is also an area for further study as there is contradicting evidence to its necessity in the propagation process, but hopefully it will be elucidated in the future with better understanding of the mechanisms behind bystander effects. It has been suggested that multiple

types of bystander effects might exist and a combination of processes are at play when a cell is expose to radiation (Morgan, 2012).

In our study, we investigated the role of TNF-α receptors in the induction of p-H2AX foci in EPCs post IR. The role of TNF receptors in normal cellular processes, especially p75 receptor, is not fully understood however there had been reports of a complex intracellular overlap in responses and mediation of p55 by p75 receptor in the enhancement NF-κB dependent gene transcription (Peschon et al., 1998; Weiss et al., 1997). A possibility for the next study would be to use a p75/TNFR2 neutralizing antibody on WT EPCs and observe the formation of p-H2AX foci post irradiation. Based on the results of this study, we hypothesize that the blocking of p75 receptor will reduce the damage from bystander effect of EPCs and potentially other cell types. Our understanding of radiobiological bystander effects has important implications for risk assessment of low dose radiation and possible development of countermeasures to reduce delayed harmful effects of ionizing radiation. Future findings could potentially further advances the knowledge in the field of nuclear imaging, radiotherapy for cancer treatment, space exploration, and many other fields where low dose radiation is a significant factor, which merits further experimentation.

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