

**The application of flow cytometry to examine damage clearance  
in stem cells from whole-body irradiated mice**

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## Executive Summary

The bone marrow contains many types of cells. Approximately 1-2% of these cells are critical for life, these are the so-called 'bone marrow stem cells' which divide indefinitely to produce platelets, red blood cells and white blood cells. Death of the bone marrow stem cells results in a diminished ability of the organism to make new blood cell components and can be fatal without medical intervention, such as a bone marrow transplant. Bone marrow stem cells are considered to be particularly sensitive to radiation injury. Therefore, it is important to understand how these cells response to total body radiation exposure and how these cells can be protected from radiation damage.

The aim of this project was to determine if these critical cells in the bone marrow are susceptible to short-term and long-term injury after a whole-body exposure to a sub-lethal low dose of ionizing radiation. The overall aims were to determine if the extent of injury produced by the sub-lethal radiation exposure would be cleared from the stem cells and therefore present no long-term genetic risk to the organism, or if the radiation injury persisted and had an adverse long-term consequences for the cell genome. This research question is of interest in order to define the risks to exposed persons after occupational, accidental or terrorism-related sub-lethal low-dose radiation exposures. The novel aspect of this project was the methodology used to obtain the bone marrow stem cell-like cells and examining the outcomes of sub-lethal low-dose radiation in a mammalian animal model. Four radiation treatments were used: single treatments of 0.01Gy, 0.1 Gy, 1 Gy and ten treatments of 0.1 Gy given over 10 days. Bone marrow stem cell-like cells were then harvested 6 hours, 24 hours and 24 days later.

The levels of radiation-induced cell death, damage to DNA and permanent changes to cellular DNA were measured in the isolated stem cell-like cells after each radiation treatment and time point and then the results were compared. We determined that the largest radiation dose produced the greatest damage but a linear relationship did not exist between cellular effects and radiation dose. The low dose exposures appeared to be more efficient at producing damage than the highest dose when normalized for the initial extent of damage. Additionally, we were able to demonstrate that immune stimulation given prior to radiation exposure can protect the critical bone marrow stem cell population from radiation injury. Based on this research we conclude that bone marrow stem-cell like cells response to radiation injury is dependent on the extent of the initial levels of damage and the effects of total-body low-dose exposures can not be predicted by extrapolating from high dose exposures.

The timeframe of the research project was extended to allow for additional experimental procedures to be conducted. These were needed to allow data generated at different treatment times and radiation doses to be compared across all experimental groups. However, this did not affect the budget of the funding program and the research was completed by the allocated budget. During the course of the research, the resolution of the experimental techniques was incrementally improved in order to measure the subtle low dose radiation effects in the stem cell-like cell populations. These enhanced assays are now being applied to additional research projects investigating other radiation effects and biological response mechanisms and new research initiatives for cancer research. The technical advances from this work are therefore widely applicable in other areas of science.

This research has provided new information about the radiation sensitivity of bone marrow stem cell-like cells following total-body exposures, and suggests that these critical cells might be more sensitive to radiation than more mature cells in the bone marrow. Further work is need with intermediate radiation doses to confirm this conclusion.

## Goals and objectives of project

### *Background*

Stem cells are defined by their ability to produce pools of precursor and differentiated functional cells, and as such are widely regarded as the most critical target of radiation-induced carcinogenesis. A small proportion of cells found in multiple tissues exhibit similar stem-cell like characteristics and are referred to as side population (SP) cells. These cells can be isolated by flow cytometry based upon their ability to rapidly efflux Hoechst 33342 dye and can be isolated from several tissues including bone marrow, liver, breast, jejunum and muscle (2-4; 8; 12; 13). Despite diverse tissue origins, side population cells share a similar surface marker profile. They are stem cell antigen-1 (Sca-1) and CD43 positive and lineage marker negative (12; 13; 16; 24; 33). Bone marrow-derived SP cells also express the endothelial marker CD31 (15; 24). These rare cells are highly enriched for stem cell activity and display a potent capacity for reconstituting locally-damaged tissue. For example, a small number of bone marrow-derived side population cells can fully reconstitute lethally-irradiated marrow in the mouse (11). Side population cells can also reconstitute tissue types distinct from their site of origin. Injected marrow SP cells can replace injured skeletal muscle and cardiac myocytes, whereas muscle SP cells can fully reconstitute irradiated bone marrow (13; 15). ***Because of the pluripotent nature of these cells, the characterization of their radiation response after low-dose exposure may be significant in understanding long-term low-dose radiation risks.*** To further investigate the involvement of SP cells in low-dose radiation exposure we designed our project with two aims:

1. To determine if DNA damage persists in bone marrow SP cells after low-dose radiation exposure.
2. To characterize whether immunological stress [treatment with bacterial lipopolysaccharide (LPS)] prior to radiation exposure could provide some amount of radioprotection for SP cells.

Based upon our previous work in low-dose hypersensitivity (19; 23), we hypothesized that low levels of radiation-induced DNA damage would evade detection mechanisms and persist in bone marrow SP cells leading to genomic instability. To test this hypothesis we adapted a pre-existing flow cytometry protocol from the Goodell lab to harvest bone marrow SP cells from *in vivo* irradiated C57/Bl/6 mice (0.01 Gy, 0.1 Gy, 1 Gy and 0.1 Gy x 10) (12). The animals were whole-body irradiated and allowed *in situ* repair periods of 30 minutes, 24 hours and 4 weeks following radiation exposure before the bone marrow was isolated. The SP cells were then sorted from the bulk of the bone marrow and examined using a series of high-resolution *in vitro* assays to investigate the mechanisms of DNA damage processing. The study was designed to examine three related endpoints in the SP cells after radiation exposure: DNA double-strand break repair, cell death by apoptosis, and genomic instability.

### *Isolation and staining of SP cells*

We encountered no difficulty in staining or sorting SP cells, although we did adopt a slightly different gating methodology that was better suited to our particular cell sorter. This was a very minor change however and did not affect the final outcome; it just improved our recovery

and ensured a more pure population for testing. The cell sorting process was considerably slower than anticipated due to the purchase of a BD FACSAria Special Order Flow Cytometer which replaced an older BD flow cytometry machine. This machine change had the potential to increase cell sorting speeds due to an increased fluidic pressure, but paradoxically we had to reduce sorting speeds to overcome cellular damage induced by the increased pressure. Moreover, this change necessitated that additional animals be included into the experimental design to serve as internal controls for the cell sorting process; with lengthened the overall duration of the project. However, once these difficulties were resolved the project proceeded as planned.

Based on our hypothesis, we expected to find increased damage (DNA double strand breaks) following low-dose whole-body exposures that remained undetected or unrepaired and eventually would translate in to epigenetic changes in the SP cellular DNA. The data described below lend support this hypothesis. All the DNA break aspects of the project described in our proposal were completed, but interpretation of the data was limited by the sensitivity of the assays available. In some instances, assays resolution prevented statistically significant conclusions from being made although clear trends are evident in the data.

Our efforts to determine if immunological stress could function as a modulator of *in vivo* radiation-induced DNA damage processing in SP cells were successful. In all instances, treatment with LPS was able to modulate the measured cellular response. This aspect of the study will be pursued in future studies which will involve LPS treatment in combination with radiation to better understand the interaction between the two treatments.

#### *DNA double strand breaks*

Measuring DNA double-strand breaks (DSBs) was chosen as the starting point for examination of radiation effects in side population cells because DNA DSBs are the most detrimental type of DNA damage produced by ionizing radiation. If left unrepaired, DNA double strand breaks can lead to chromosomal aberrations, genetic instability, cell-cycle arrest and cell death. In our project, DNA double-strand break repair fidelity was assayed by scoring the residual H2AX foci in side population cells after whole body irradiation. H2AX is a variant of histone H2A that is recruited to sites of DNA damage. It likely plays a role in recruitment of other repair proteins so its presence following radiation exposure can be used as a marker for DNA repair.

With minor changes to our existing H2AX protocol (20) we were able to complete this portion of the project as described originally; however the results were unexpected. Based upon our previous work and project hypotheses, we predicted that damage from low-dose radiation exposures (0.01 Gy and 0.1 Gy) would be undetected and remain unrepaired in SP cells resulting in H2AX foci that remain unresolved over time. This undetected DNA break damage would then lead to further downstream consequences such as increased apoptotic death or detrimental genetic changes, parameters also being measuring in the SP population.

SP cells exhibited a higher level of background H2AX foci than anticipated, so measuring radiation-induced damage after very low dose exposures (0.01 Gy and 0.1 Gy) was difficult (see Figures below). The high background in unirradiated SP cells was unexpected and

possibly the result of active V(D)J recombination in the immune system. Following 1.0 Gy, significant numbers of H2AX foci were observed and this number decreased over time post irradiation, with a rapid return to background levels, indicative of efficient repair. After immune stimulation with LPS, significantly fewer H2AX foci were evident at all doses and each dose exhibited a decrease in foci number over time, which would be expected if the cells were undergoing active DNA repair.

LPS-treated animals irradiated with 0.01 Gy exhibited normal repair as defined by H2AX disappearance. After LPS treatment DNA DSBs could be measured after 0.01 Gy but in the absence of LPS few breaks were detected. Given that LPS treatment produced this result in all of our single-dose groups, we are confident that ***LPS treatment was able to prime the response of the SP cells to DNA damage and provide some measure of radio-protection.*** While the data does not completely answer our original question of whether low-dose irradiated SP cells harbor damage, the effect of the LPS treatment cannot be disputed. An extended investigation into how and why immune stimulation was able to drastically modify the H2AX foci number in side population cells warrants further research.

### *Apoptosis*

Because one outcome of radiation-induced cellular damage could also be cell death by apoptosis, we also examined this process in the SP population. Apoptosis was of interest because it is a major mode of cell death for cells of lymphoid origin after DNA damage. Cell death was measured using a highly sensitive apoptosis assay that quantified the activation of Caspase-3/7, a key mediator of apoptotic cell death in mammals. This portion of the project differed from what was originally proposed due to cell number constraints in the SP cell population. The proposed apoptosis assay was changed to the Promega Apo-ONE® homogenous caspase-3/7 fluorescence plate assay due to the reported high sensitivity of this assay. We demonstrated that a more accurate measurement of caspase activity was produced by the Apo-ONE® assay compared to the original flow cytometry assay that was proposed.

Not unexpectedly, most changes in caspase activity occurred the day of irradiation not 24 hours post irradiation, even after immune stimulation by LPS. Following 0.01 Gy there was a significant decrease in the level of apoptosis on the day of irradiation which may indicate that normal apoptotic detection was not activated and that damaged cells that would normally be removed from the population were left unaffected. After LPS treatment, there was an increase in caspase activity at this dose, as predicted by our hypothesis. This increase implies that more cells exhibiting some sort of cellular damage (DNA DSBs) were being removed from the side population, which is undoubtedly beneficial in the long term and may indicate a slight radio-protective effect of LPS. Similar results were seen in animals treated with 1.0 Gy and LPS, where there was a significant increase in caspase-3/7 activity following LPS treatment. Interestingly, the opposite result was seen for animals treated with either 0.1 Gy or 0.1 Gy x 10, as treatment with LPS reduced the activity of the caspases, even 24 hours after irradiation. Why this effect would be dose specific but not exhibit a linear response remains unclear but may relate to low-dose hyper-radiosensitivity because the 0.1-0.2 Gy dose is often a transition dose point for the HRS-IRR responses. The validity of this theory cannot be proven directly by the H2AX data, but there is some support for it in the methylation data presented below. Regardless, it is evident

that ***LPS is able to modulate the side population cell death response to radiation, which may prove more significant than the effects of low-dose radiation overall.***

### *DNA methylation*

Because ionizing radiation is a well-known genotoxic and carcinogenic agent that may cause epigenetic changes in cellular DNA, genetic changes in side population cells was examined. Epigenetic changes are meiotically heritable and mitotically stable alterations in gene expression that include DNA methylation, RNA-associated silencing, and histone modification. For this project we choose to examine genomic instability as evaluated by examining DNA methylation in SP cells one month (28 days) after whole body irradiation.

This portion of the project was the least accomplished. Most issues arose from the external collaboration with Dr. Kovalchuk (University of Lethbridge, Canada). Little conclusive data has been produced by the collaboration. In non-LPS treated animals, we found greater unmethylation of DNA following higher doses of radiation, which would be expected, a greater increase in unmethylation (from control animals to irradiated animals) was seen following the lowest dose of radiation. This difference increased with decreasing dose. These data suggest that although a higher overall level of DNA unmethylation was observed following 1.0 Gy, a greater increase in unmethylation was seen following 0.01 Gy treated animals and their matched controls. ***This result confirms our hypothesis that undetected long-term damage following lower doses of radiation can lead to epigenetic changes such as decreased DNA methylation and possible genetic instability.*** However, due to the technical difficulties experienced by our collaborator associated with the extraction assay during sample DNA collection, we are unable to statistically compare these data with that obtained from immune stimulated animals. All of the samples from the LPS treated animals and all the fractionated dose samples provided very little DNA for methylation analysis and as such, likely resulted in inaccurate methylation analysis. From the data that was obtained, all the LPS treated animals except 0.1 Gy exhibit lower levels of unmethylation than their matched controls, which is what we would have expected if our hypothesis about LPS treatment was correct. However, all of these samples were also found to exhibit 90% + levels of unmethylation, which is unlikely, and so has lead us to question this particular data set. The exception of the 0.1 Gy dose to this trend also falls within our theory that low-dose HRS is playing a role in the response of SP cells to radiation, but poses the same problems in terms of the overall total level of unmethylation. If time and resources had allowed it, this portion of the project would have been repeated because it stands to provide the most valuable information. Regrettably this was possible, but we intend to investigate this matter further in future projects.

### **Summary of Project Activities**

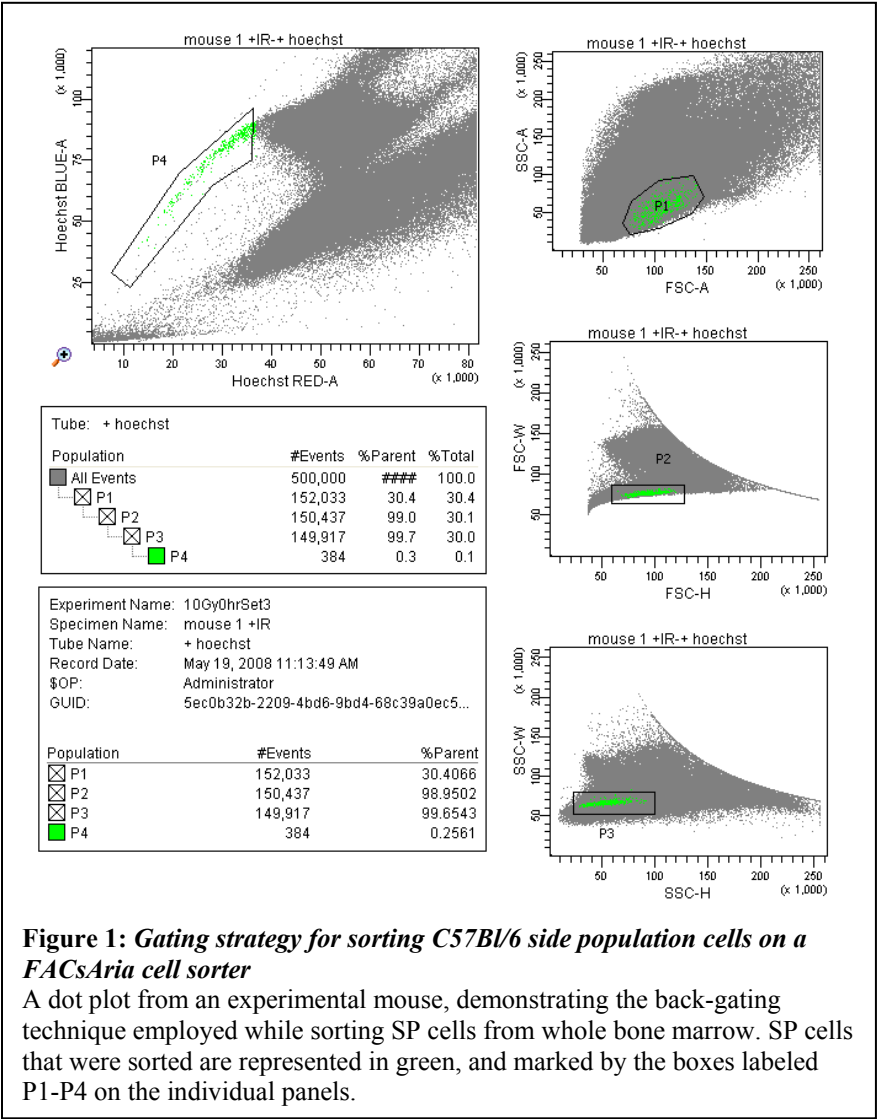
The goal of this proposal was to ascertain if radiation-induced DNA damage persists in stem cell populations after whole-body low-dose exposures and to characterize how immunological stress effects damage processing, both of which we accomplished to varying degrees. The experimental data for the cell sorting, DNA DSB assays, measure of apoptosis and DNA methylation are presented below. This data are obtained from n=4 animals per dose point

(0.01, 0.1 and 1 Gy as single doses and 10x0.1 Gy and the three time points (6 hours, 24 hours and 28 days).

Staining bone marrow for side population cells

At the close of this project we have successfully established within our laboratory a routine procedure for staining, identifying and sorting the SP cell population and testing the sorted cells for DNA double-strand breaks and cell death. We have continued to utilize the protocols designed for this project and have expanded their use to include examination of SP cells from cultured cell lines and tumor samples. However, several aspects of the project were altered from what was originally proposed due to limitations on the number of SP cells we were able to collect, including the protocol for staining H2AX and the method of caspase-3 detection.

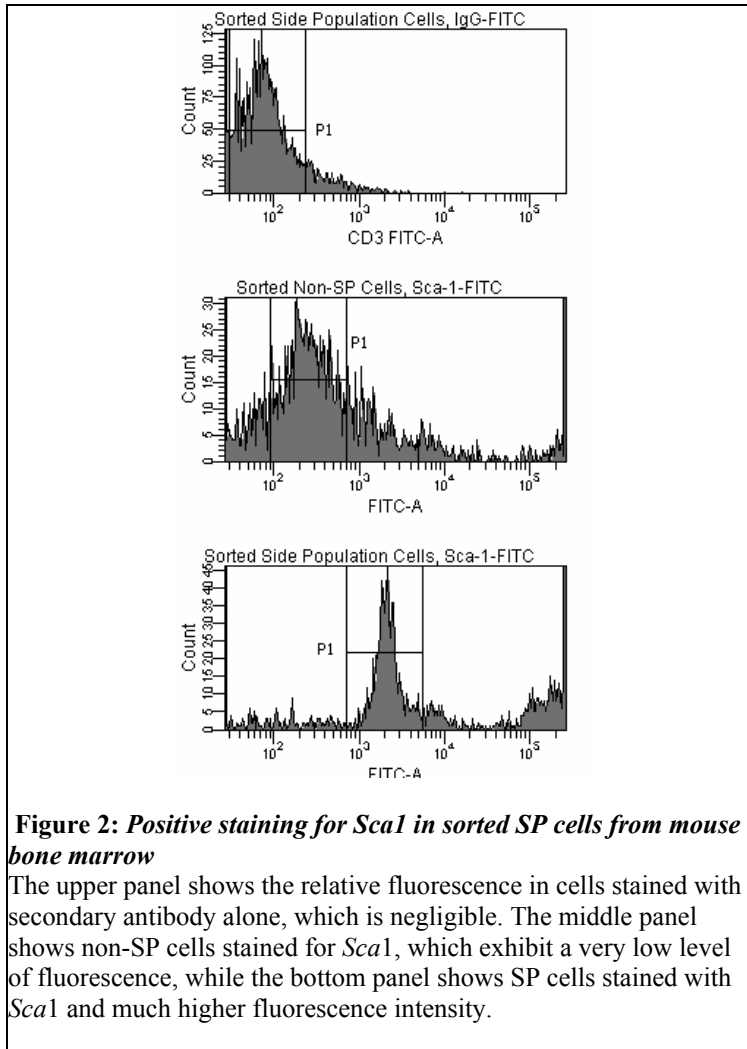
The staining procedure used was based upon the method developed by Goodell’s lab and remained relatively unchanged from what has been previously recorded (12). One modification adopted was a higher concentration of verapamil (50  $\mu$ M/mL was recommended, 100  $\mu$ M/mL was used) allowed us to better differentiate between the SP cells and non-SP cells when drawing gates for sorting. We also utilized a different gating technique that involved back-gating to produce a more pure population without doing a bulk sort or bead-based separation first (**Figure 1**).



**Figure 1: Gating strategy for sorting C57Bl/6 side population cells on a FACSAria cell sorter**

A dot plot from an experimental mouse, demonstrating the back-gating technique employed while sorting SP cells from whole bone marrow. SP cells that were sorted are represented in green, and marked by the boxes labeled P1-P4 on the individual panels.





The cells obtained from the sorted populations were confirmed with secondary antibody staining with markers for SP stem cells, including *Sca1*, CD34 and CD31. **Figure 2** shows an example of the *Sca1* positive staining (bottom panel) which is specific to an isolated population of SP cells from mouse bone marrow, but not non-SP cells (middle panel).

Based on previously published work, we anticipated being able to sort a relatively large number of SP cells from each the animal (greater than 100,000 cells), but we found in practice that smaller numbers than previously calculated were obtained in some animals.

There were differences in SP yields between mice that we could not predict and even some variation in day to day staining with Hoechst that made it challenging to consistently sort a large number of cells when each replicate consisted of only one mouse. Thus, we had to adapt our original assays to work

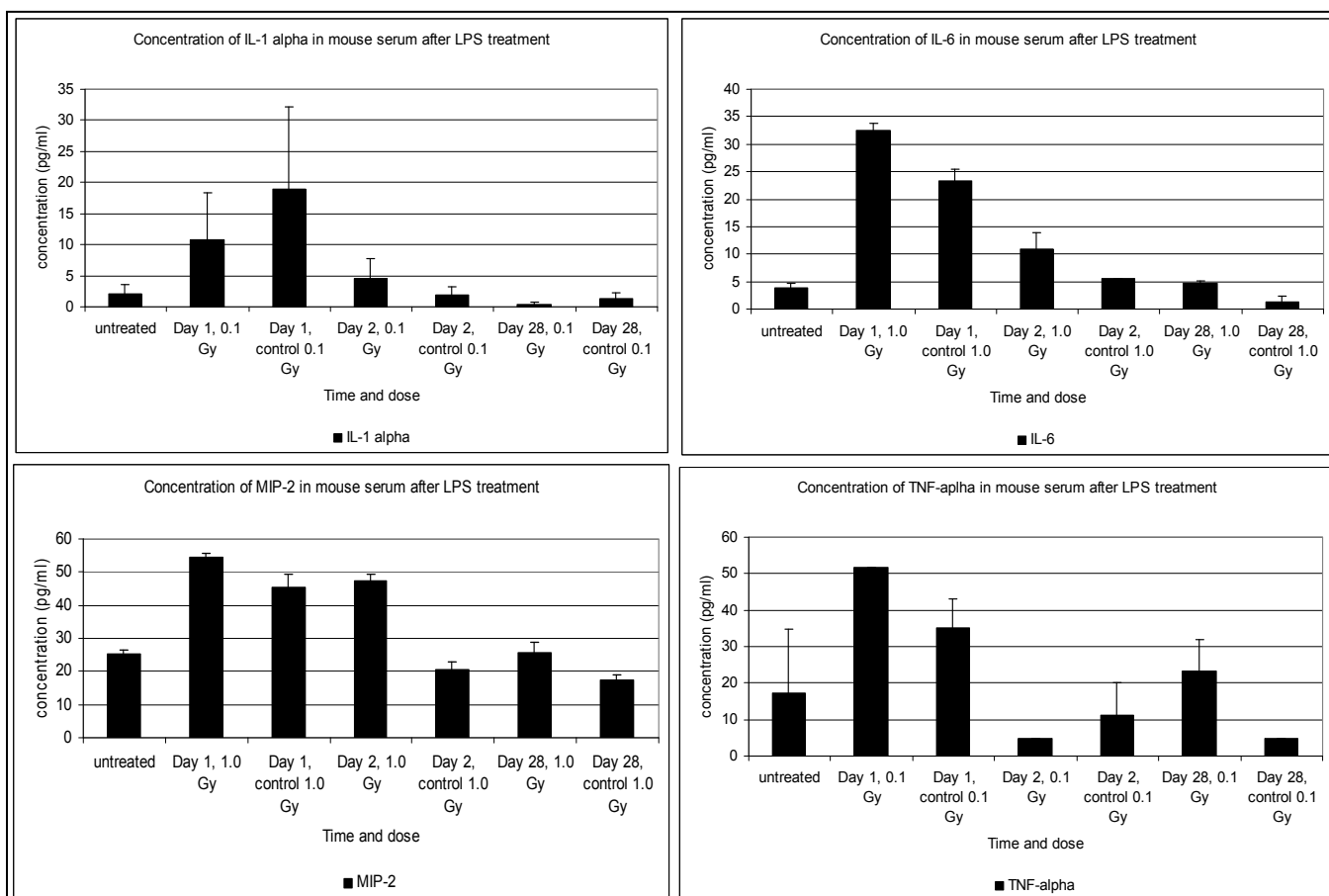
with a smaller number of SP cells than originally intended. For both the H2AX foci analysis and the caspase-3/7 activity assay, the number of cells was reduced to the absolute minimum, particularly for the 4 week time point in order to maximize the number of SP cells available for methylation analysis. Despite the lower number of SP cells obtained, we ultimately decided to forgo collection of more SP cells from other tissues such as muscle, due to the increased time that this would have added to the entire process. The timing of cell collection is very important for both measuring DNA damage and caspase activity so we instead focused on maximizing our use of the SP cells we were able to collect from bone marrow by adapting existing assays.

### *Effects of LPS treatment*

Pre-treatment with inflammatory agents like LPS can promote survival from otherwise lethal radiation doses with subsequent recovery of the hematopoietic system through interaction with host cells, particularly macrophages, and the release of cytokines and other inflammatory mediators (14; 30). Thus we proposed that immune stimulation would provide some measure of radio-protectiveness in the SP population, particularly after lower doses where we perceived the damage would be the greatest. The experimental plan necessitated the injection of animals with

lipopolysaccharide (LPS) to provide immune stimulation and our preliminary studies demonstrated a single *i.p.* injection of 0.1 mg/mL LPS (serotype 055B5, Sigma) was very well tolerated by the animal with no physical signs of toxicity, and no other detrimental effects when combined with radiation treatment, which was expected. A measurement of the actual immune stimulation produced was undertaken to ensure that any differences between the LPS treated and untreated mice could be attributed to the injection and subsequent immune response, not natural mouse to mouse variability.

In response to LPS, there is a temporal cascade of cytokines including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IFN- $\gamma$  and colony stimulating factors (CSF) that appear in the serum (22). Consequently cytokine levels were assessed as an indicator of LPS efficacy. Traditionally, this would be done by running multiple ELISAs with serum samples and examining each cytokine individually. However, we were able to utilize a magnetic bead-based multiplex assay which allowed for rapid



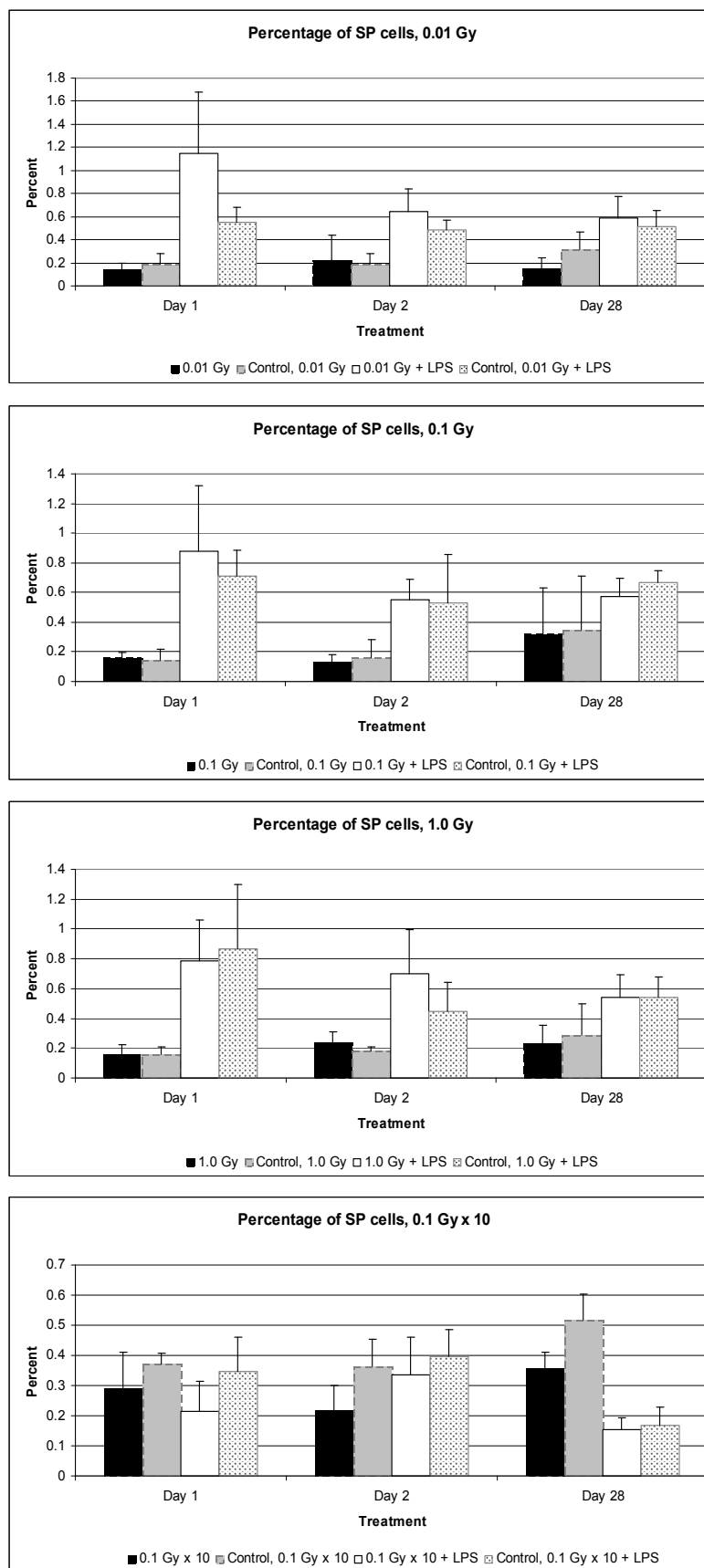
**Figure 3: Concentration of mouse cytokines after treatment with lipopolysaccharide (LPS)**

The graphs are representative of the changes exhibited by mouse cytokines in blood serum after LPS treatment. The black bars represent the concentration of IL-1 alpha, IL-6, MIP-2 and TNF-alpha in untreated mice and irradiated and unirradiated mice treated with LPS. All LPS treated mice were injected with a single *i.p.* injection of 0.1 mg/mL LPS (serotype 055B5) 48 hours prior to irradiation. As expected, the cytokine levels decreased over time in most animals, peaking at as much as 10 times the concentration found in non-immune stimulated animals. A similar trend was seen for the levels of IL-1 alpha (increase 1.5-2.5), IL-1 beta (increase 1.5-10 times), IL-6 (increase 2.5-10), MIP-2 (increase 1.5) and TNF-alpha (increase 1.5-2) for all doses except the fractionated dose for reasons discussed in the text.

detection of multiple cytokines in a single serum sample. We used a custom Bio-Plex Pro™

cytokine kit from Bio-Rad that included mouse IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF-  $\alpha$  and MIP-2, which is the analog for human IL-8. The results of these experiments showed elevated (1.5-10 times untreated controls) levels of all tested cytokines at 0.01, 0.1 and 1.0 Gy, particularly on the day of irradiation and 24 hours post irradiation which corresponds to 3 and 4 days after LPS injection respectively (**Figure 3**). We suspect that the cytokine levels in the fractionated-dose (10 x 0.1 Gy) animals had already dropped off significantly by the time of serum collection because of the 10 day irradiation period, which put them almost 2 weeks out from the initial injection. This does not mean that these animals did not exhibit similar elevated levels during the beginning of their radiation schedule, but rather that the immune stimulation had declined as expected over the 10 day irradiation time period. In future studies it may be worth considering a boost of LPS mid-way through the fractionated irradiation to ensure a consistent elevated immune response.

Unexpectedly, we found that LPS treatment significantly increased the number of SP cells detectable by Hoechst staining in both irradiated and control animals across all doses except the fractionated dose (Student's t-test,  $p < 0.05$ ) (**Figure 4**). The aberration in the fractionated animals is once again likely due to the increase in length of time from LPS injection to bone marrow collection (10 days of radiation). We currently are considering several hypotheses to explain this phenomenon and would like to investigate the effects of LPS on the side population in bone marrow in further detail, although it is outside the scope of this particular project. Nonetheless, it seems likely that immune stimulation causes an expansion in the SP cell compartment that may ultimately provide extra protection against radiation induced damage.



**Figure 4: Average percentage of SP cells following LPS treatment and radiation**

Treatment with LPS increases the number of SP cells stained regardless of dose. The percentages are likely unchanged in the fractionated studies because of the time lapse (10 days for irradiation) between treatment with and LPS and bone marrow collection.

### *DNA double strand breaks*

An early downstream target of the DNA damage response is the histone H2A variant, H2AX, which constitutes approximately 10% of the total histone H2A in mammalian cells (34). Subsequent to DNA damage, H2AX is rapidly phosphorylated at serine 139 (termed  $\gamma$ H2AX) in an ATM dependent manner (6; 9; 27). Phosphorylated H2AX is detectable as discrete foci in cells within minutes of radiation-induced DNA damage with peak induction at approximately 30-60 minutes post irradiation (5; 7). For this study, rapid measurement of H2AX was not feasible due to the Hoechst staining process, but every effort was made to keep cells from initiating repair (low temperature, kept in suspension) when possible.

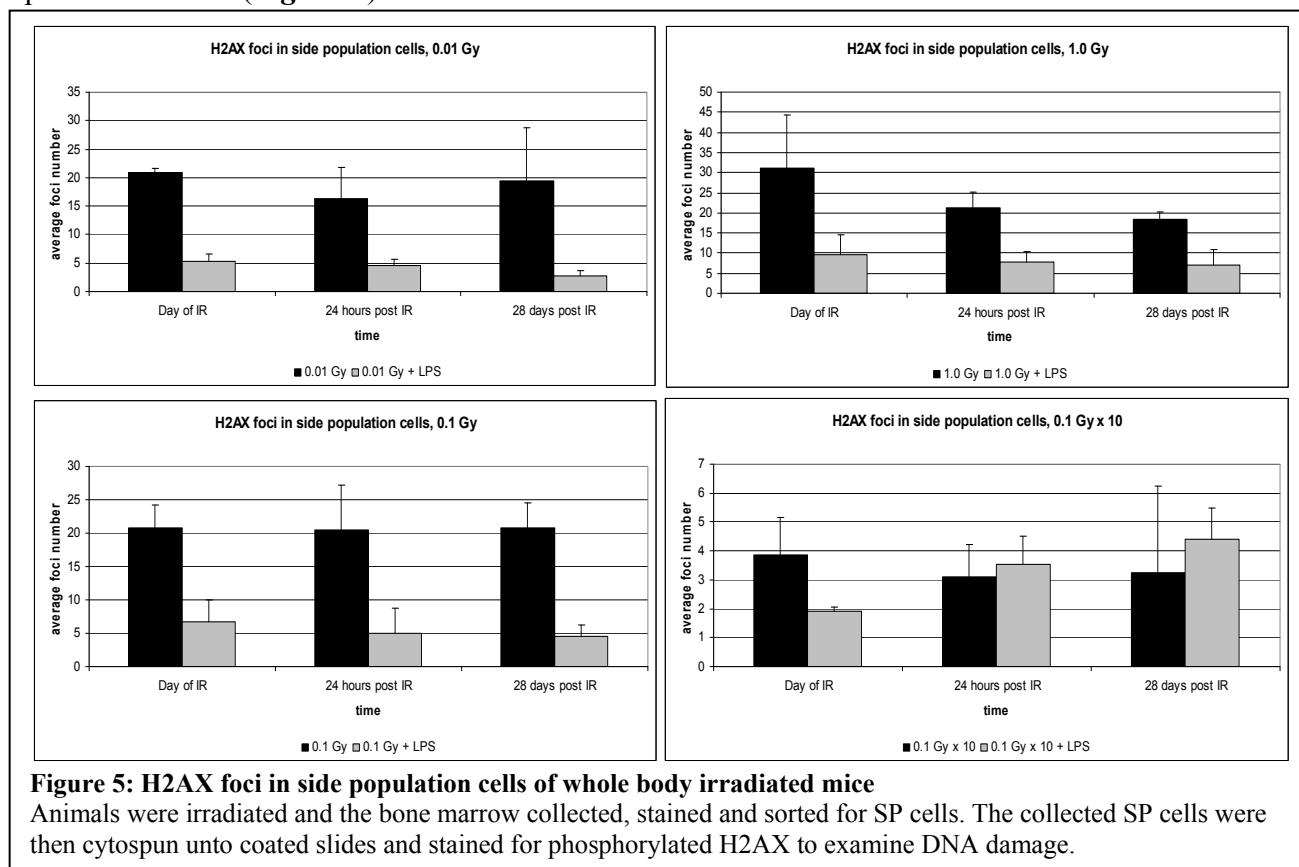
In non-immune stimulated animals we found high background levels of phosphorylated H2AX in both control and irradiated SP cells, which made it difficult to determine if there were differences in DNA double-strand breaks following irradiation at the lower doses (0.01 and 0.1 Gy). The seemingly high background levels of H2AX phosphorylation in SP cells may possibly be a result of active V(D)J recombination in the immune system, as H2AX is an active participant in this process. The interference of high levels of H2AX foci in control SP cells was unanticipated because we originally tested all the assays using the 1.0 Gy dose, where there are significant differences between the irradiated and control cells (Student's t-test,  $p = 0.1$ ).

There was also a significant drop in foci number over time following 1.0 Gy that was absent in the lower doses, which suggests that the high background H2AX levels were masking any effect of the radiation (Student's t-test,  $p = 0.1$ ). Interestingly, in the animals that received the fractionated dose, foci numbers were considerably lower overall, but remained consistent over time. This may indicate that the continued low dose of radiation (0.1 Gy) was not causing significant damage but was altering the normal levels of H2AX in the SP cells, creating a reduction in the overall number of foci. These changes in foci number after fractionation persisted 28 days after irradiation, demonstrating unexpected longevity.

Following LPS treatment, the level of background H2AX phosphorylation was considerably lower, similar to the animals that received a fractionated dose. At all single doses tested (0.01, 0.1 and 1.0 Gy) and all time points (day of irradiation, 24 hours post and 28 days post) there was significantly fewer H2AX foci in the LPS treated animals (Student's T-test  $p < 0.02$ ) (**Figure 5**). Additionally, there was an increase in foci number with dose and a decrease in foci number over time, which would be expected if the foci numbers were representative of radiation-induced DNA double strand breaks, as was seen following 1.0 Gy in the untreated animals. Thus, we hypothesize that the foci numbers seen in the immune stimulated animals represent the actual amount of damage induced by each radiation dose and the level of repair. We have not yet determined a plausible reason to explain why LPS treatment reduced the background number foci particularly since LPS alone has been found to increase recombination in immune cells. Regardless, it is clear that immune stimulation alone is enough to modify the H2AX phosphorylation of the SP population in a more significant manner than low-dose radiation.

In the fractionated dose treated animals, the results were more complicated. There were significantly less foci the day of irradiation in the LPS treated animals as compared to the

untreated animals, but the foci numbers were nearly equivalent the day after irradiation and 28 days post irradiation. Since we believe that the foci numbers in the LPS treated animals actually reflect the amount of DNA damage induced by the radiation treatment, this would suggest that during a fractionated dose schedule, immune stimulation does provide some measure of radio-protective effect (**Figure 5**).



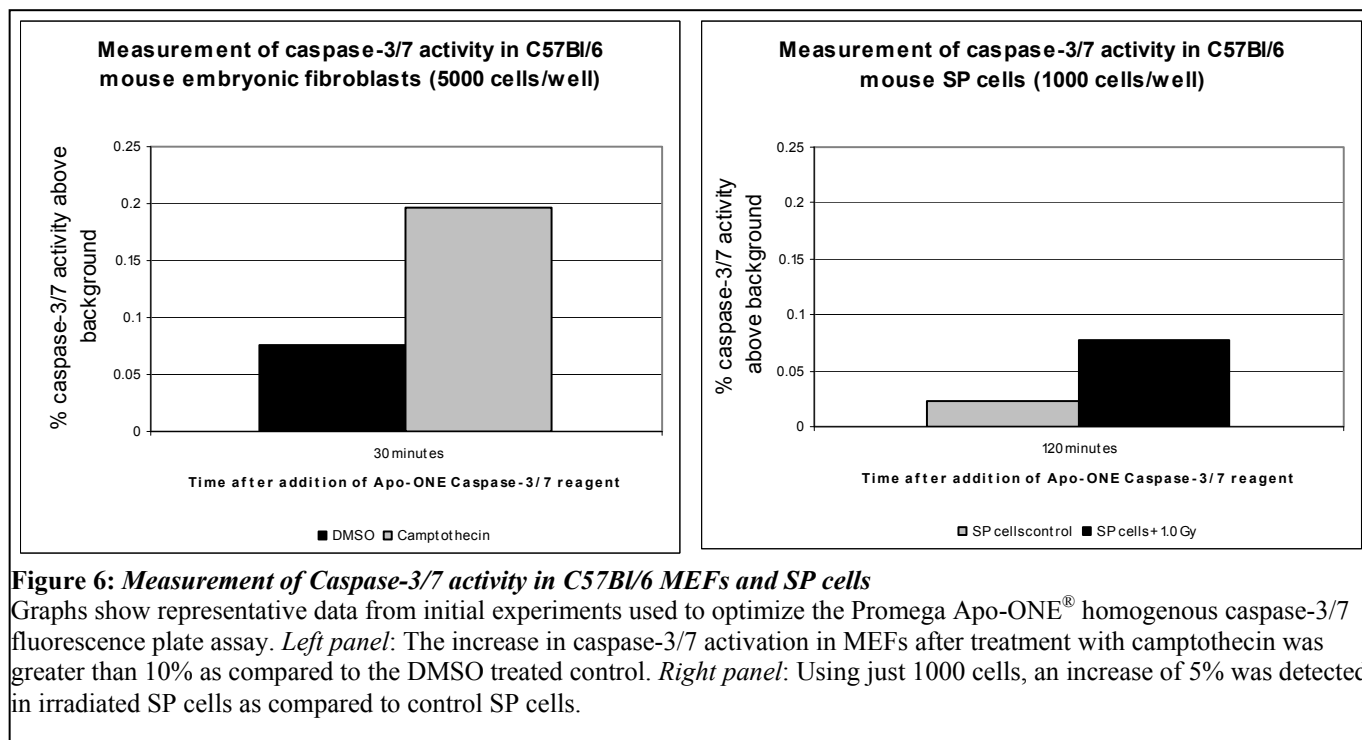
The sorting or staining process was not the cause of the increased H2AX phosphorylation (DNA damage) since low foci numbers were seen in the fractionated dose and LPS portions of the study which underwent identical treatment. Rather we suspect that the high level of H2AX phosphorylation in SP cells was typical, perhaps due to recombination or other chromatin conformations that are necessary in progenitor cells for rapid activation and differentiation when needed. We plan on investigating this matter further in future studies.

During the course of the project, changes were made in our H2AX foci experiments. It proved impractical and technically challenging to sort directly unto slides for the H2AX foci assay, so the cells were first sorted into small tubes and then cytopspun directly unto coated slides before fixation and staining, which allowed for greater retention of the cells on the slide. We ended up sorting 6000 cells per tube for this assay, instead of 10,000 as proposed to reduce the number of SP cells required per animal, which still provided more than enough cells for foci counting after fixation and staining. We also used an Alexa-Fluor<sup>®</sup> 488 secondary antibody instead of FITC because of its low background and increased brightness under the microscope.

### Caspase-3/7 activation

Apoptosis occurs through two main pathways, the extrinsic or cytoplasmic pathway triggered through the Fas death receptor and the intrinsic or mitochondrial pathway that is associated with the release of cytochrome-c from the mitochondria (35). Both the intrinsic and extrinsic apoptotic pathways converge and initiate the activation of caspase-3 (10; 29; 32), which cleaves the inhibitor of the caspase-activated deoxyribonuclease leading to nuclear apoptosis (32). Because apoptosis is the major mode of cell death for normal cells of lymphoid origin after DNA damage, we proposed to assess radiation-induced cell death of *in vivo* irradiated side population cells in an effort to measure their radiation sensitivity in the 0.05–1 Gy dose range (1; 21; 28).

In an effort to reduce the total number of SP cells required for each assay, a fluorescence based plate assay by Promega was utilized. The plate assay required a fewer cells to report apoptotic activity than our original plan of fixing and analyzing caspase activity via flow cytometry. In initial tests using C57BL/6 mouse embryonic fibroblasts (MEFs), we were able to track changes in caspase-3/7 activity using the Apo-ONE® homogenous Caspase-3/7 kit in as few as 5000 cells per well, and in tests using sorted SP cells from bone marrow, we saw differences with as few as 1000 cells per well (**Figure 6**). Ultimately 3000 SP cells were sorted directly into each well for our experiments, performed in triplicate.

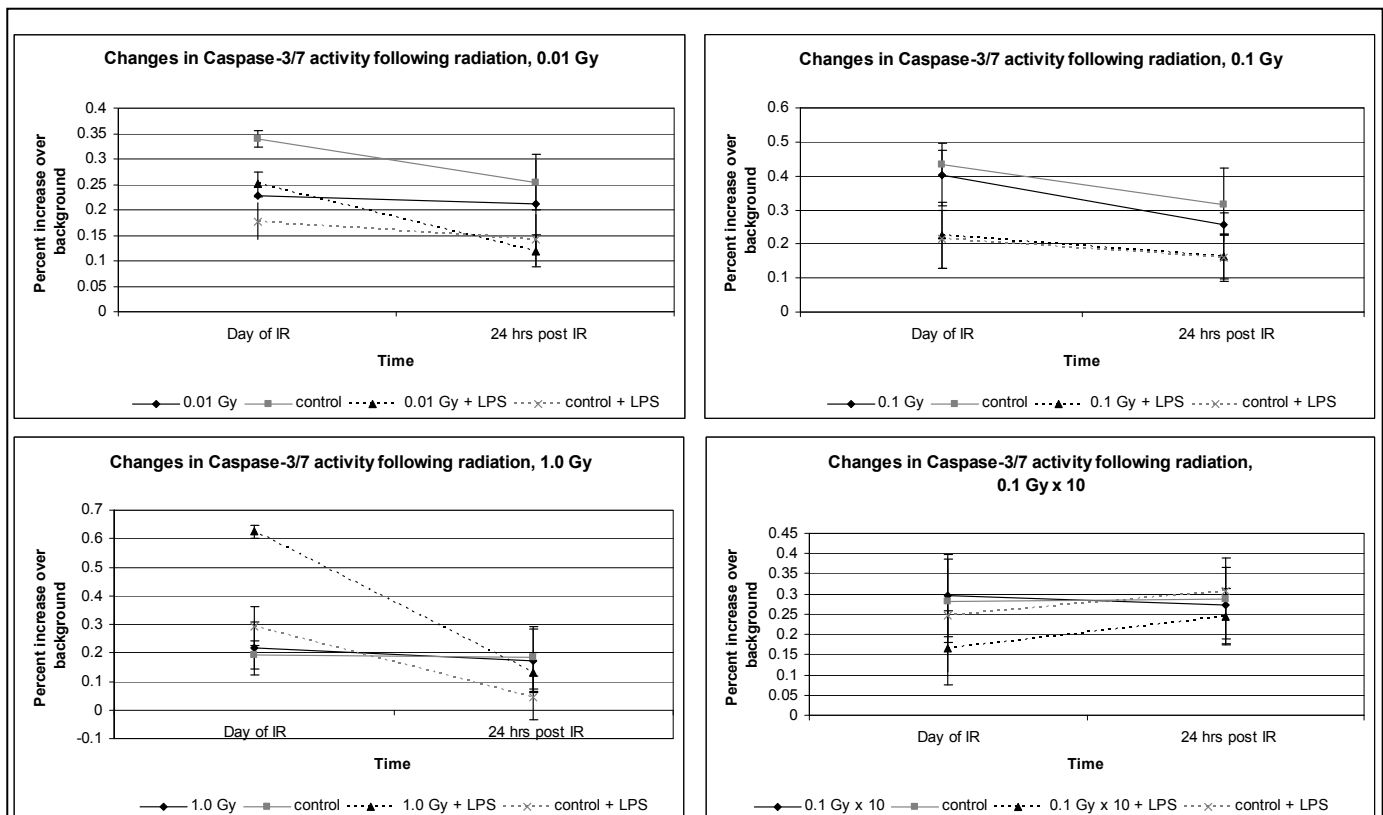


The data from the measurement of Caspase-3/7 activation were interesting. In non-LPS treated animals, irradiation with 0.01 Gy significantly reduced the level of caspase-3/7 activity compared to control on the day of irradiation but not 24 hours later (Student's t-test,  $p = 0.08$ ).

After treatment with LPS however, 0.01 Gy irradiated animals exhibited significantly higher levels of caspase activity as compared to the controls (Student's t-test,  $p = 0.02$ ). Also, when comparing the untreated and LPS treated animals that received radiation, the level of caspase-3/7 activity was higher following LPS treatment the day of irradiation but not 24 hours later (Student's t-test,  $p = 0.01$ ).

Following the 0.1 Gy dose, there was little difference between the irradiated animals and the controls, but treatment with LPS reduced the levels of activated caspase-3/7 significantly in the irradiated animals on both the day of irradiation and 24 hours post irradiation (Student's t-test,  $p = 0.07$ ,  $0.04$  respectively). After 1.0 Gy, the caspase activity was again similar for irradiated and control non-LPS animals, but significantly higher than controls following LPS treatment on both the day of irradiation and 24 hours post irradiation (Student's t-test,  $p = 0.03$ ,  $0.07$ ). There was also an increase in cleaved caspase-3/7 in the irradiated animals after LPS treatment the day of irradiation but not 24 hours post irradiation (Student's t-test,  $p = 0.0009$ ).

Animals treated with the fractionated dose exhibited a similar response to those irradiated with a single dose of 0.1 Gy in that treatment with LPS reduced the level of caspase-3/7 activity in the irradiated cells (Student's t-test,  $p = 0.06$ ,  $0.09$ ). Treatment with LPS did not affect the levels of caspase activity in the control cells, only the irradiated cells (**Figure 7**).



**Figure 7: Caspase-3/7 activation in side population cells after irradiation**

Animals were whole body irradiated and the bone marrow collected and stained for the side population. SP cells were then sorted into a 96-well plate and stained with the Apo-ONE<sup>®</sup> homogenous Caspase-3/7 kit from Promega. Caspase activity was measured using a fluorescent plate reader 3 hours after sorting when caspase activity had been found to peak.



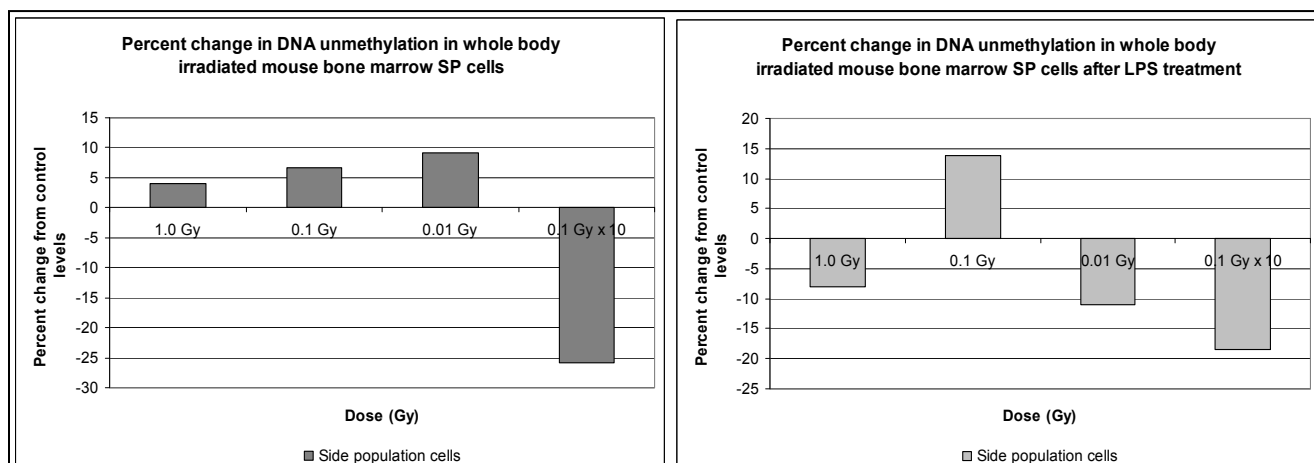
Based upon these results, we conclude ***that the effect of LPS on caspase activity after irradiation is dose dependent but not linear***. Within our tested dose range, LPS treatment induced higher levels of caspase activity after irradiation in animals treated with 0.01 Gy and 1.0 Gy but not 0.1 Gy or 0.1 Gy x 10. In the case of the fractionated study, treatment with LPS lowered the caspase activation to levels below that of the control animals which was unexpected. Why treatment with LPS would have such a marked effect in animals irradiated with single or multiple doses of 0.1 Gy is unclear, but may relate to the phenomena of low-dose hyper-radiosensitivity which often exhibits a dose transition point around 0.1 or 0.2 Gy. The complexities of the animal model make it difficult to determine outright if this is the case, but when one considers that the H2AX data supports the idea that the fractionated dose was not causing significant damage but still altering the levels of H2AX in the SP cells, the concept becomes more plausible. Thus, we suspect that the immune stimulation produced by LPS is radioprotective outside of the hyper-radiosensitivity range because a greater number of cells are marked for apoptotic death. The LPS immune stimulation also elevates the immune response, which may also allow for fewer chances that damaged cells evade detection due to 'hyper-diligent' detection. Additionally, an increased level of activation of caspase-3/7 after immune stimulation may also explain why we observed fewer foci in LPS treated animals- any damaged cells were rapidly cleared and little allowance or time for repair was made. In effect, there was shift towards removal of damaged cells rather than repair due to the stimulation of the bone marrow compartment. If there is in fact a relationship between radio-sensitivity and immune stimulation, further exploration of this relationship could prove beneficial in the future.

### *DNA methylation*

Ionizing radiation is a well-known genotoxic and carcinogenic agent and hematopoietic tissue is one of the main targets of radiation carcinogenesis. It has been shown previously to affect genome stability by altering DNA methylation patterns (17; 31) and radiation-induced global genome DNA hypomethylation has been linked to genome instability (18; 25; 26). In the project, we were interested in changes in DNA methylation patterns in side population cells isolated from the bone marrow. We anticipated finding the presence of radiation-induced epigenetic changes (DNA methylation) which would allow us to link to overall genetic change and the likelihood of a deleterious genetic event to low-dose whole body exposure.

Four weeks after irradiation, the bone marrow was collected and a small number of SP cells sorted for H2AX analysis while the bulk of the bone marrow was sorted for DNA extraction and subsequent methylation studies. These cells were snap frozen in liquid nitrogen and stored until a large batch was prepared to send to our collaborator in Canada. Upon arrival in their lab, the DNA was extracted and DNA methylation was determined by radiolabeled [3H]dCTP extension assay. Unfortunately we encountered the greatest number of difficulties with this portion of the project, and we regard the integrity of the later part of the data set with suspicion.

In non-LPS treated animals, increasing radiation dose produced a greater level of DNA unmethylation overall, up to 73% for the fractionated dose animals, which may indicate increased genomic instability. However, the difference between the control animals and the



**Figure 8: Changes in DNA methylation in mouse bone marrow side population cells**

Side population cells were sorted 4 weeks after whole body irradiation and then the level of DNA methylation was determined. Increasing dose produced higher levels of DNA unmethylation in non-LPS treated mice, but there were greater differences between control and irradiated cells at the lower doses. After LPS treatment, 0.01 Gy and 1.0 Gy exhibited increased amounts of DNA methylation which may indicate improved DNA stability. Animals treated with 0.1 Gy and 0.1 Gy x 10 exhibited an increase in DNA unmethylation, which may indicate increased genomic instability. In both LPS and non-treated animals, the fractionated dose schedule exhibited a higher level of DNA methylation that matched controls.

irradiated animals increased with decreasing dose, with the 0.01 Gy animals exhibiting the greatest increase in unmethylation at 9.1 % (**Figure 8**). Interestingly, the fractionated dose animals, which demonstrated higher levels of unmethylation overall, exhibited much lower levels than their matched control cells and so seemingly exhibited more methylation when compared to the other doses and their controls.

Following LPS treatment, the percent of unmethylation overall was much higher, above 90%, but for 0.01 Gy, 1.0 Gy and 0.1 Gy x 10 doses the levels were actually lower than the control cells and so can be considered to show less unmethylation. 0.1 Gy treated animals were the only to show higher levels of unmethylation than controls, which may indicate greater instability following this particular dose. These data are congruent with the previous data which demonstrated that 0.1 Gy doses exposures repeatedly produce unexpected or non-linear results in both DNA damage and apoptosis assays, and so may be further indication of the importance of this dose point in repair and recovery after radiation.

As discussed above, technical issues associated with the DNA methylation assays were experienced; these predominantly involved the isolation of DNA from bulk sorted SP cells for DNA methylation analysis. Validation samples were originally sent to our collaborators laboratory to determine if sufficient DNA could be isolated from small numbers of sorted SP cells, these preliminary experiments were successful and the methylation assay was completed. Therefore, the project proceeded as planned. Experimental samples were snap frozen and dispatched in two large batches for methylation analysis. From the first batch, adequate DNA was recovered and the level of unmethylation was as expected. However, the problems arose with the second batch of samples which included all of the LPS treated animals. Collecting sufficient DNA from these 'second batch' samples proved difficult despite the cell numbers being the same or greater than the first round, and as a result, obtaining accurate methylation

results became unfeasible. This may explain the high levels of unmethylation that was reported in these cells and described in the previous paragraphs. We were anticipating lower levels of DNA methylation and decreased genomic instability following LPS treatment, which can be supported (with the exception of the 0.1 Gy dose) by the caspase-3/78 and H2AX data, but is not very well described by the methylation assay data. Because the investment in this part of the project reflected over a year of work and a multitude of mice that were 4 weeks post irradiation, it could not be repeated within the grant time period. This is unfortunate because we are unable to determine how LPS treatment affected long-term DNA methylation and also unable to conclude how fractionation would contribute to epigenetic changes in SP cells. We are continuing to work with our collaborator to see if some amount of the data can be reanalyzed, but at the time of this report results were incomplete.

### *Conclusions*

The most significant observation from this study is the modulation (radioprotection) of radioresponse of SP cells induced by LPS treatment. Pre-treatment with LPS provided some measure of radioprotection to SP cells after low dose exposures and LPS was also found to significantly increase the percentage of SP cells within the bone marrow, which was an unexpected side effect of immune stimulation. Interesting aberrations in the animals dosed with 0.1 Gy suggest that low-dose hyper-radiosensitivity may play a role in the radiation response of SP bone marrow cells, although further study is also needed to confirm this. Additionally, the possibility that LPS treatment may reduce the amount of unmethylation induced by radiation needs to be clarified. Although this was not the initial goal of the project, the effect of LPS on SP cells is by far the most interesting outcome and will prompt continued investigations.

Overall, we believe that we succeeded in our goal of providing information on low-dose radiation risk and immune stimulation and that our use of an animal model for this study is unique. Information about the effect of low-dose whole-body exposures were obtained in a stem-cell like cell populations and measured for DNA breaks, apoptosis and overall genomic instability. Unexpectedly, for both DNA DSBs and apoptosis non-linear dose responses were evident which suggests a link to low-dose hyper-radiosensitivity. These observations are novel and the data are currently being prepared for publication.

The measurement of low-dose radiation responses after whole-body irradiation still remains difficult, but the results of this study will provide a basis for an expanded investigation into the interaction between the immune system and radiation damage response. In particular, because SP cells have been shown to act as effective progenitor cells, the ability to enhance the radioprotective niche of the bone marrow could prove beneficial in either treatment or disaster settings and that is where we intend to aim our future work.

## **Publications and conference papers, collaborations, techniques**

### *Publications*

Research papers are currently being prepared for publication detailing the significant findings from this project. Expected publication date summer, 2010.

### *Conference presentations and posters*

#### *Presentations:*

**Golden Horseshoe 2008: Radiation Symposium, Rochester, NY, USA. October 23<sup>rd</sup>-October 24<sup>th</sup>, 2008.**

*Damage clearance in mouse stems cells after whole-body irradiation.*

\*\*Sarah A. Krueger, Michele McGonagle, Olga Kovalchuk<sup>1</sup>, Alvaro Martinez, George D. Wilson and \*Brian Marples. William Beaumont Hospital, Royal Oak, MI, <sup>1</sup>The University of Lethbridge, Lethbridge, AB, Canada. \*\*Presenter, \*Primary Investigator.

**54<sup>th</sup> Annual Meeting of the Radiation Research Society, Boston, MA, USA. September 21<sup>st</sup>-24<sup>th</sup>, 2008.**

**Joint RRS/ASTRO Minisymposia: Stem Cells**

*Damage clearance in mouse stems cells after whole-body irradiation.*

\*\*Sarah A. Krueger, Michele McGonagle, Olga Kovalchuk<sup>1</sup>, Alvaro Martinez, George D. Wilson and \*Brian Marples. William Beaumont Hospital, Royal Oak, MI, <sup>1</sup>The University of Lethbridge, Lethbridge, AB, Canada. \*\*Presenter, \*Primary Investigator.

**William Beaumont Hospital Research Roundtable, Royal Oak, MI, USA. June 3<sup>rd</sup>, 2008.**

*The Application of Flow Cytometry to Examine Damage Clearance in Stem Cells from Whole-body Irradiated Mice.*

\*\*Sarah A. Krueger, Michele McGonagle, Olga Kovalchuk<sup>1</sup>, Alvaro Martinez, George D. Wilson and \*Brian Marples. William Beaumont Hospital, Royal Oak, MI, <sup>1</sup>The University of Lethbridge, Lethbridge, AB, Canada. \*\*Presenter, \*Primary Investigator.

#### *Posters:*

**55<sup>th</sup> Annual Meeting of the Radiation Research Society, Savannah, GA, USA. October 4<sup>th</sup>-7<sup>th</sup>, 2009.**

*Cellular damage and repair in hematopoietic stems cells from whole-body low-dose X-irradiated mice.*

\*\*Sarah A. Krueger, Michele McGonagle, Olga Kovalchuk<sup>1</sup>, Alvaro Martinez, George D. Wilson and \*Brian Marples. William Beaumont Hospital, Royal Oak, MI, <sup>1</sup>The University of Lethbridge, Lethbridge, AB, Canada. \*\*Presenter, \*Primary Investigator.

**DOE Low Dose Radiation Research Investigators Workshop VIII, Bethesda, MD, USA. April 6<sup>th</sup>-9<sup>th</sup>, 2009.**

*The application of flow cytometry to examine damage clearance in stem cells from whole-body irradiated mice.*

Sarah A. Krueger, Michele McGonagle, Olga Kovalchuk<sup>1</sup>, Alvaro Martinez, George D. Wilson and \*Brian Marples. William Beaumont Hospital, Royal Oak, MI, <sup>1</sup>The University of Lethbridge, Lethbridge, AB, Canada. \*Presenter, Primary Investigator.

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**DOE Low Dose Radiation Research Investigators Workshop VII, Washington D.C., USA. January 21<sup>st</sup>-23<sup>rd</sup>, 2008.**

*The application of flow cytometry to examine damage clearance in stem cells from whole-body irradiated mice.*

**\*\*Sarah A. Krueger, Michele McGonagle, Olga Kovalchuk<sup>1</sup>, Alvaro Martinez, George D. Wilson and \*Brian Marples.** William Beaumont Hospital, Royal Oak, MI, <sup>1</sup>The University of Lethbridge, Lethbridge, AB, Canada. **\*\*Presenter, \*Primary Investigator.**

*Collaborations*

William Beaumont Hospital Bio-Bank (Bio-Plex, FACs ARIA)

Olga Kovalchuk (DNA Methylation)

*Techniques*

Using whole body low-dose X-irradiated mice for:

- Flow cytometry based cell sorting of Hoechst stained SP populations
  - Caspase-3/7 assay from sorted SP cells
  - H2AX foci from sorted SP cells
  - DNA Methylation analysis from sorted SP cells
- Interleukin serum analysis from LPS injected mice using a bead-based multi-plex assay after irradiation

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