INVESTIGATION OF ABSCOPAL AND BYSTANDER EFFECTS IN IMMUNOCOMPROMISED MICE AFTER EXPOSURE TO PENCILBEAM AND MICROBEAM SYNCHROTRON RADIATION.

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Abstract

Out-of-field effects are of considerable interest in radiotherapy. The mechanisms are poorly understood but are thought to involve signalling processes, which induce responses in non-targeted cells and tissues. The immune response is thought to play a role. The goal of this research was to study the induction of abscopal effects in the bladders of NU-Foxn1^{nu} mice after irradiating their brains using pencil Beam (PB) or microbeam (MRT) irradiation at the European Synchrotron Radiation Facility (ESRF) in Grenoble France. Athymic nude mice injected with F98 glioma cells into their right cerebral hemisphere 7 days earlier were treated with either MRT or PB. After recovery times of 2, 12 and 48h, the urinary bladders were extracted and cultured as tissue explants for 24h. The growth medium containing the potential signalling factors was harvested, filtered and transferred to HaCaT reporter cells to assess their clonogenic survival and calcium signalling potential. The results show that in the tumour free-mice both treatment modalities produce strong bystander/abscopal signals using the clonogenic reporter assay, however the calcium data do not support a calcium channel mediated mechanism.

The presence of tumour reduces or reverses the effect. PB produced significantly stronger effects in the bladders of tumour bearing animals. We conclude that immunocompromised mice produce signals, which can alter the response of unirradiated reporter cells, however a novel mechanism appears to be involved.

Keywords: Synchrotron, Bystander, Abscopal, Athymic

Introduction

Current research on bystander effects i.e. effects detected in unirradiated cells receiving signals from irradiated cells is mainly conducted in vitro using two different methods; the microbeam irradiation of part of a cell culture or by allowing a non-irradiated culture to receive physical or medium borne signals from an irradiated culture (Mothersill and Seymour 1997; Prise 1998; Zhou et al. 2000). Apart from the important mechanistic questions relating to how these effects occur, the other key research area concerns potential impacts of these so-called *non-targeted* effects in radiotherapy (Burdak-Rothkamm and Prise 2009; Sun et al. 2014). Efforts to study pure bystander effects in vivo are complicated because irradiation of a part of the body does not preclude blood, endocrine and neural systems which receive irradiation from causing systemic effects confounding the mechanisms (Mancuso et al. 2008; Koturbash et al. 2011). There is a considerable old literature on abscopal or out-of-field effects in radiobiology and radiotherapy. These date back to the early 20th century (Murphy and Morton 1915; Mothersill and Seymour 2012) but were officially named by RH Mole who coined the term "abscopal" in 1953 (Mole 1953). In recent years the lines between in vivo bystander and abscopal effects have become blurred and the terms are often used interchangeably leading to assumptions that mechanisms identified in vitro may apply in vivo (MotherSill and Seymour 2012). A key system which is considered to be very relevant for such reactions is the immune system. This has been investigated following irradiation in partially exposed animals in vivo (Mancuso et al. 2008; Koturbash et al. 2008) and inflammatory responses in distant tissues have

been detected. It has also been investigated in mice, which received total body irradiation to ablate the bone marrow followed by injection with opposite sex bone marrow stem cells. The repopulating cells showed high levels of genomic instability which could only have come from signals in the irradiated microenvironment (Watson et al. 2000; Watson et al. 2001). In vitro work on components of the immune system confirm a role for the inflammatory *response* in what is described as a bystander effect, although it is not clear if signal production requires an intact immune response capability system (Holyoake et al. 2001). Also the question of whether a functional immune system is necessary to produce bystander/abscopal effects in vivo still requires clarification. To address some of these issues, we participated in experiments using athymic (Nude) mice at the European Synchrotron Research Facility (ESRF) microbeam facility in Grenoble in France. Synchrotron microbeam radiation has been employed in the development of innovative methods to treat brain cancer. Efforts are focused on improving the Microbeam Radiation Therapy (MRT) developed originally at the Brookhaven National Laboratory in New York. ESRF and other international facilities are focusing on this task. Teams of scientists consistently show that MRT yields a higher therapeutic index when compared with broad beam irradiation when treating aggressive tumours such as the intracerebral rat F98 glioma (Biston et al. 2004, Schültke et al. 2008), or 9L gliosarcoma (Laissue et al. 1998; Dilmanian et al. 2002; Bouchet et al. 2013). MRT has also been used for the palliation of mice bearing the aggressive murine squamous cell carcinomas VII (Miura et al. 2006) and the treatment of mammary tumours in mice (Crosbie et al 2010). MRT uses an array of

<100 μm wide quasi-parallel rectangular beamlets, created by a high flux of synchrotron X-rays that are spatially fractionated by the insertion of a multislit collimator (Slatkin et al. 1992). This configuration exposes the tissue to either peak-doses deposited by the photons of the microbeams or valley-doses resulting from scattered photons that hit the tissue between the microbeams (Blattmann et al. 2005). The dose variation between peaks and valleys depends on the configuration and size of the beam array but mostly range between 2-10% of the peak dose (Bräuer-Krisch et al. 2009). In terms of volume of tissue exposed, valley doses would be measured in between 4 to 8 fold more brain tissue than peak doses, thus explaining the high normal tissue tolerance observed by this treatment. Tissue tolerance is also expressed by the ratio between peak-to-valley doses or (PVDR), which is a critical factor in decreasing the dose to sensible areas, such as the hippocampus (Slatkin et al. 1992).

The efficacy of MRT over broad beam has been attributed to the effects of high valley doses given in a single fraction and reinforced by the peak doses. The valley dose -is sufficiently high to damage the tumour microvasculature but low enough to avoid great deleterious effects in the normal tissue counterpart (Bräuer-Krisch et al. 2010) . Recently, research has also shown that MRT seems to stimulate the immune system by regulating an early expression of a vast network of mediators such as growth factors, cytokines and lymphokines (Bouchet et al. 2013) . Furthermore, a comparison between MRT and broad beam revealed different molecular pathways involving the recruiting of tumour-associated immune cells (Yang et al. 2014). Dilmanian et al. (2007) have suggested that 6 hours after irradiation dying cells at

the beam's edge seem to signal neighbouring cells and promote the fast disappearance of hit cells and structural damage. Data from our team have shown that bystander signals seem to extend the area where development of γ-H2AX foci can be seen from the microbeam's edge into the valley dose areas after peak doses of 350Gy (Fernandez-Palomo et al. 2015). Also, abscopal or radiation-induced out-of-field tissue/organ effects have been investigated by our group. For instance, proteomic analysis of the unirradiated left brain hemisphere in normal Wistar rats suggests that the MRT-induced proteome may result in protective effects in the unirradiated brain (Smith et al. 2013) and unirradiated brain tissue and unirradiated urinary bladder tissue explants originated from right brain MRT-irradiated animals, induced bystander effects in our well established reported cell system (Fernandez-Palomo et al. 2013).

While MRT research has so far been focused on the treatment of primary brain tumours, research led by Schültke based on treatment technique called pencilbeam therapy (PB) aims also to treat secondary brain malignancies (Schültke et al. 2013) . The majority of the drugs typically used to treat metastatic brain tumors are not able to cross the blood-brain barrier (Pardridge 2005). Thus, patients with multiple brain metastases are often selected for whole brain radiotherapy (WBRT) simply because it is the only option available. However, the most common problem reported after WBRT are chronic changes in the white matter, which are associated with stroke-like migraines (Kerklaan et al. 2011; Black et al. 2013; Armstrong et al. 2014) , cognitive deficits (Shi et al. 2009; Peiffer et al. 2014; Forbes et al. 2014) and dementia (DeAngelis et al. 1989; D'Ambrosio et al. 2007; Tallet et al. 2012).

Following the principle of MRT, PB delivers extremely high doses of synchrotron X-rays along very narrow tracks to the tumour while sparing the normal tissue (Schültke et al. 2013). Since the tissue volume receiving peak doses is even smaller than with monoplanar MRT it is hoped that as a consequence the normal tissue tolerance is increased compared to monoplanar MRT. This could improve cancer therapy outcomes after whole brain exposure to PB. The work by Schültke et al. tested for motor and memory function after delivering PB to normal C57 BL/6J mice. Animals recovered well compared to controls after peak doses of 800 Gy and a peak center-to-center distance of 400 μm (Schültke et al. 2013). Also, in-situ brain studies using the γ-H2AX marker demonstrated that the geometry of PB was preserved after whole-brain irradiation and that DNA damage was correlated with the dose [Fernandez-Palomo et al, 2013a]. However, more preclinical studies are still needed to gauge the therapeutic ratio of PB and, in particular, to investigate mechanisms and to access the role of bystander effects in the tissue sparing seen with MRT and the newer PB.

Previous experiments using rats (Fernandez-Palomo et al. 2013a, 2015b) had shown that bystander effects could be detected in the contralateral brain and bladder of rats receiving right brain irradiation using MRT. It was also shown that the presence of a tumour experimentally introduced into the brain could reduce the strength of the signals measured as the ability of culture media from the tissue to reduce the cloning efficiency of a reporter cell line, and the ability to cause a calcium flux in reporter cells – both well established assays (Mothersill and Seymour 1998; F M Lyng, Seymour, and Mothersill 2000). In the current experiments we measured

signal strength from the bladder as a distant organ exposed to truly abscopal effects in nude mice receiving whole brain exposure to either MRT or PB irradiation from the synchrotron.

Materials and Methods

Animal Model**:**

10 weeks old young-adult male athymic nude mice (**Crl:NU-*Foxn1*^{nu}** Charles River, Germany) weighting 28-32 grams on arrival, were housed and cared for in the ESRF Animal Facility following a 12h light/dark cycle in agreement with French and Canadian Animal Care Protocols. The mice are immunodeficient because the absence of a thymus makes them poor in T-cells [Ikehara et al, 1984]. This strain originated in 1969, when Dr Rygaard paired the spontaneous hairless mutant "nude" mouse (which showed absence of thymus) with the NMRI strain (which had high viability and fertility) [Rygaard, 1969]. This finally creating a suitable mouse model for hosting tumour xenografts. According to the Charles River website "This immunodeficient nude mouse originated from NIH and was originally thought to be a BALB/c congenic. It was later determined that it was not inbred and is therefore maintained as an outbred. It is not associated with any stock or strain. The animal lacks a thymus, is unable to produce T cells, and is therefore immunodeficient."

Tumour Inoculation:

The tumour cell line selected was the F98 glioma, which shows mutant p53 and shares a wide range of characteristics with the glioblastoma multiforme (GBM)

(Barth and Kaur, 2009) . The F98 glioma cells have an infiltrative pattern of growth in the brain and they are weakly immunogenic (Tzeng et al. 1969) . This tumour model is often used in studies involving conventional radiotherapy and synchrotron radiation (Gil et al. 2011; Desmarais et al. 2012, 2015].

For these experiments, F98 cells were obtained from ATCC and grown in 75 cm^2 flasks containing 25 ml of Dulbecco's Modified Eagle Medium (GIBCO, France), supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. A 90% confluent flask was selected and cells were detached by incubation with 20 ml of calcium and magnesium-free Hank's Balanced Salt Solution (Gibco, France), during 20 minutes at 37°C in an atmosphere of 5% CO_2 in air. The cell suspension is centrifuged at 1000 rpm for 5 min, the pellet re-suspended in 1ml of fresh culture medium and counted using a haemocytometer. Athymic mice are anaesthetized using 1.5% isofluorane in 2 L/min compressed air, before and during the surgical procedure. A vertical incision of 1.5 to 2 cm is made on the skin following the sagittal plane. A hole is then drilled in the skull above the right hemisphere 3 mm to the right of the midline and 3 mm posterior to the coronal suture. Then a volume of 2 µl of culture medium containing 10,000 F98 cells is carefully inoculated 2.5 mm into the brain and the incision is closed 2 minutes after inoculation. Animals are supervised until they recovered and the housed for seven days to allow for tumour development.

Irradiations:

In preparation for the irradiations, mice were deeply anaesthetised using a cocktail of Ketamine/Xylazine (1 ml of 10% ketamine, 0,5ml of 2% xylazine, 8.5 ml non-saline solution) at 0.01 ml/g. Each irradiation group had 3 male mice and 1 female, which were individually positioned on the goniometer and received the radiation dose corresponding to its group. MRT and PB were delivered laterally to the whole brain in a right to left direction. Animals were allowed to recover for 2, 12 and 48 hours after irradiation and the urinary bladder was used for the study of abscopal effects while the brains served as material for other studies not shown in this paper. Animals were exposed to valley doses of 0.5 Gy and 2.5 Gy in a single treatment session. Peak entry doses were adjusted accordingly to achieve the valley doses. The array size of 8 mm high and 20 mm wide remained constant at all times. Animals irradiated with MRT were exposed to 22 Gy or 110 Gy skin-entry doses, while the animals receiving PB were exposed to 200Gy and 1000Gy respectively. The beam was set in a lateral view by locating its right side 4mm toward the nose from the eye, and from that point it extended 20 mm to the left (Figure 1). The peak entry doses correspond to the dose at 3 mm depth. They were calculated prior to the experiments using a solid water phantom (Gammex) and a pinpoint ion-chamber (PRW 31014). The peak entry and valley doses are then converted with Monte Carlo pre-calculated output factors into the desired beam size. Valley doses are also calculated using a Treatment Planning System based on CT data from previous the experiments on rats. Although benchmarking of the estimated doses is still in progress, Gafchromic film dosimetry agrees within 10% with the computed valley doses (Bartzsch and Tag, 2014) .

The array of multichromatic pencil beams was generated by a multislit collimator with a mean energy of 105 keV (Bräuer-Krisch et al. 2009). The typical dose rate during these experiments was ~14,000 Gy/sec. MRT was composed by rectangular quasi-parallel 50 μm wide microbeams with intermediate gaps of 400 μm on center. The PB array was formed by square quasi-parallel 50x50 μm microbeams, with 400 μm of intermediate distance on center. Untreated controls for both normal and tumour-bearing animals were included. HD-610 and MD-55 Gafchromic Films (ISP Advanced Materials) were used to verify all irradiation doses and modalities applied. After irradiation, mice were taken back to the ESRF animal facility for recovery in their correspondent treatment groups.

Previous studies indicated that scatter radiation does not play a role in the induction of abscopal and bystander effect after Synchrotron irradiation. However, we decided to control for scatter because this is the first time that bystander effects have been studied after PB. The scatter dose was measured at the level of the urinary bladder after 200Gy MRT, which is equivalent to 1000 Gy PB. The dose at the site was 1.36 mGy. Thus, an X-ray generator was used to deliver a whole-body dose of 1.36 mGy after adequate adjustment of the dose rate. All mice were moved back to the ESRF animal facility after irradiation. Untreated controls remained in the ESRF animal facility and never left the cage.

Sampling, explant tissue culture and culture medium harvest:

All animals received anaesthesia before euthanasia. Urinary bladders were extracted 2, 12 and 48 hours after irradiation. Immediately they were single-placed in 5 ml sterile tubes containing 1mL of Roswell Park Memorial Institute growth medium (RPMI 1640, Gibco, Canada). Supplemented with 10% FBS, 5ml of Penicillin-Streptomycin (Gibco, Canada), 5ml of L-glutamine (Gibco, Canada), 0.5 mg/ml of Hydrocortisone (Sigma-Aldrich, Canada), and 12.5 ml of 1M HEPES buffer solution (Gibco, Canada). Tissue samples were put on ice and immediately transported to the biosafety level 2 laboratory of the ESRF biomedical beamline.

Urinary bladders were cut into 3 equal-size pieces of approximately 2 mm^3 in a biosafety cabinet. The pieces were individually placed in the centre of a 25 cm^2 growth area flask (Falcon), containing 2 ml of the complete growth medium previously described. Flasks were then placed in an incubator set at 37°C, 5% CO_2 in air and left undisturbed during 24h to allow for the release of bystander signals. The irradiated-tissue conditioned medium (ITCM) was harvested at 24 hours, filtered using a sterile 0.22 μm filter (Acrodisc Syringe Filter with HT Tuffryn Membrane, Pall Life Sciences), and placed in a sterile 7mL tube. The collected media was kept at 4ºC in the dark and then transported to McMaster University for clonogenic reporter bioassays.

Clonogenic Reporter Cell Line:

The cell line selected as the reporter was the human epithelial HaCaT, which has been used by our group at McMaster University for several years due to the line's reliable and stable response to bystander signals (Mothersill and Seymour 1997).

The cell line was originally derived by Dr Petra Boukamp in Germany and was kindly given to us. The line was developed from normal human skin that surrounded a melanoma and became immortal spontaneously (Boukamp et al, 1999) . Although HaCaT cells have 3 p53 point mutations (Lehman et al. 1993), data show that the line remains functional with respect to inducing apoptosis and reproductive death and behaves as though wild-type p53 were present (Henseleit et al. 1997). In our laboratory HaCaTs also behave like wild-type cells in terms of the bystander effect response. The HaCaT cells were maintained in a 75 cm^2 grown area flask (Falcon) with RPMI 1640 supplemented as previously indicated. Cells 95% confluent were detached using 1:1 (v:v) solution of 0.02 % Trypsin/EDTA (1mM) (Gibco, Canada) and Dulbecco's Phosphate-Buffered Solution (1x) (Gibco, Canada). The number of cells was measured using an automatic cell counter (Beckman Coulter).

Clonogenic reporter bioassay:

Flasks containing 90-95% confluent HaCaT cells were selected for the experiments. Reporter flasks of 25 cm^2 growth area were seeded with 500 cells 6 hours before the medium transfer. Then the conditioned medium harvested in France was transferred into the reporter flasks. Plating efficiency and medium transfer controls were also set up. All flasks were then transferred into an incubator and left undisturbed for about 10-12 days to allow for colony formation using the technique developed by Puck and Marcus (1956) . Colonies were then stained using a 1:4 (v/v) solution of Carbol Fuchsin (Ricca Chemical Company) in water. Colonies with more than 50 cells were scored as survivors and the survival fraction was calculated using

the plating efficiency (PE) of the reporter cells as described in previous research (Fernandez-Palomo et al. 2011, 2015).

$$Plating\ Efficiency\ (PE) = \frac{\#\ of\ Colonies}{\#\ of\ Cells\ seeded} \times 100$$

Ratiometric calcium measurements

The following protocol was initially developed by Dr. Fiona Lyng, DIT, Dublin, Ireland (2000). 100,000 HaCaT cells were seeded in Glass Bottom Dishes (MatTek Corporation) containing 2 ml of cell culture media RPMI-1640 (Gibco, Oakville, Canada) supplemented with 10% FBS, and placed in a incubator at 37° C in an atmosphere of, 5% CO_2 in air for 24 hours. For calcium measurements, the culture medium was discarded and the cells were washed gently three times with Hank's Balanced Salt Solution (HBSS) with calcium and magnesium (Cat#: 14025-092, Gibco, Oakville, Canada), supplemented with 25 mM of HEPES (Gibco, Oakville, Canada). The HBSS was discarded and cells were loaded with 200 µl of 8.4 µM of Fura-2/AM (Sigma-Aldrich, Milwaukee, USA), for 1 hour at room temperature in the dark. This protocol avoided the compartmentalization of the dye within the cellular organelles, which was a problem if cells were placed in an incubator at 37° C. At the end of the loading time, the Fura-2/AM was discarded, the cells were washed three times with HBSS, and 300 µl of the same buffer was added to the dish. Cells were observed with a x40 oil objective on an Olympus inverted fluorescent microscope (Olympus Canada, Richmond Hill, Canada) and images were captured with a CCD Cool-Snap HQ camera (Photometrics, Tucson, Arizona). For the measurements, 100

μl of conditioned medium (or test medium) is added onto the cells 90 seconds after acquisition starts. Fura 2/AM emits light at 510 nm when is excited at 380 nm and 340 nm. The ratio of emissions between those wavelengths correlates with the calcium flux through the cellular membrane which we have shown to be the earliest measurable response to the presence of bystander signal in the test medium (Lyng et al, 2000). After obtaining the calcium concentrations plotted as a function of time post addition of the test medium, , the data from each of ten randomly selected cells were analyzed and the area under the curve was calculated for each cell and meaned to give an average result per sample. Each sample was repeated three times and there were 3 samples available to test from each bladder.

Statistical Analysis

Survival fractions are presented as a standard deviation of the mean. Significance between and within groups was determined using the Tukey multi-comparison test after a two-way ANOVA. Data was defined as significant when p values were ≤ 0.05. Graphs were plotted using the Prism 6.0 software.

RESULTS

Clonogenic assay

Figure 2 shows the clonogenic survival of reporter HaCaT cells grown in ITCM originated from the urinary bladder of nude mice. This figure focused exclusively on

comparing the effects of ITCM from normal and tumour-bearing mice. The data show that the presence of the F98 glioma in the right hemisphere, but in the absence of radiation, significantly increased the survival of the reporter cells compared to the control group (p-value = 0.0061). Similarly, clonogenic survival was also increased when ITCM originated from "scatter animals", which also had a tumour but were exposed to a whole body dose of 1.36 mGy (p-value = 0.0011). Comparing the Tumour Control and Scatter groups we can also observe that scatter radiation does not play a significant role modifying the clonogenic survival.

As a matter of comparison, figure 2 also includes reporter cells grown in ITCM originated from tumour free mice. ITCM from animal exposed to valley doses of 0.5 Gy, which correspond to 200 Gy for PB and 22 Gy for MRT, significantly decreased the survival of the reporter cells compared to the control group (p-value = < 0.0001 for PB; and p < 0.0001 for MRT).

Figure 3 shows the clonogenic survival of the reporter HaCaT cells receiving ITCM from urinary bladders harvested at 2, 12 and 48 hours after irradiation. The data generated 2 hours after irradiation (Fig 3A) indicate that ITCM from normal mice exposed to 200 Gy PB or 22 Gy MRT significantly reduced the survival of the reporter cells (p-value = < 0.0001 for PB; p= < 0.0001 for MRT). However, the difference between the two modalities was not significant in this case. When mice harbouring F98 tumours were irradiated with the same doses as above, ITCM from these animals did not significantly reduce the survival of the reporter cells. However, the difference between PB and MRT was now significant (p-value = 0.04)

with the PB inducing stronger signals. ITCM from animals exposed to 1000 Gy PB significantly reduced the clonogenic survival of the reporter cells (p-value = 0.01), while the 110 Gy MRT group did not. The PB and MRT modalities were also statistically different in this subgroup (p-value = 0.001).

Signals from bladders harvested 12 hours after irradiation (Fig 3B) did not induce any significant decrease in reporter survival. However, ITCM from mice dissected 48 h after irradiation (Fig 3C) significantly reduced the survival of the reporters compared to the control; regardless of the ITCM originating from animals exposed to 200 Gy PB (p-value < 0.0001) or 1000 Gy PB (p-value < 0.0001). Conversely, none of the MRT groups had an effect on the HaCaT cells. When comparing each valley dose sub-group, 200 Gy PB was significantly different to 22 Gy MRT (p-value < 0.0001), as well as for 1000 Gy PB and 110 Gy MRT (p-value < 0.0001).

Figure 4 shows the results for the calcium flux assay for the ITCM obtained from the bladder tissue of healthy control, the tumour control and the healthy animals whose brains were exposed to PB or an equivalent MRT dose. The data are presented as bar charts representing the total area under the curve for the flux from the point of addition of the ITCM to the return of the calcium spike to the baseline. While there are differences in area under the curve none of these are significant although the trend is similar to that seen in Figure 2 for the clonogenic endpoint. Figure 5 shows a comparison of the data for the PB and MRT irradiated tumour-bearing animals. These animals were sacrificed 2, 12 and 48hrs post irradiation of the brain. The signals from these bladders are very variable and show no significant effects or trends.

Discussion

The data presented in this paper result from an attempt to determine if the bystander/abscopal effects seen in rats could also be seen in athymic mice which have a compromised immune system. It is important to stress that NU-Foxn1^{nu} mice have an intact innate immune system and therefore the data presented could suggest that the abscopal response might not require an intact adaptive immunity or T cell response, but can still be operating through the innate immune response, via macrophages or neutrophils. Therefore the results support the hypothesis that the innate immune machinery would be a candidate for the abscopal effect - mediating a rapid reaction to damage or tumours and releasing cytokines into the circulation. These conclusions are supported by much of our data using fish models (Mothersill and Seymour 2009) which show that fish of at least 5 different species have very strong abscopal effects and can also communicate signals between irradiated and unirradiated animals. Fish are widely considered to be at an immune system transition point (Danilova 2006; Dzik 2010) relying mainly on innate immune response but having a very weak capacity for adaptive immunity (Watts, Munday, and Burke 2001).

A very intriguing finding was the influence of the presence of tumour on the response. The finding in this study, that the presence of tumour in the animal led to no or significantly weaker signals being “seen” by the reporter cells in

the clonogenic assay supports the observations in the rat experiments using F98 cells (Fernandez-Palomo et al. 2015). We are not aware of any other reports of bystander effects being weaker or ablated when tumour is present in the animal but very early work by our group (Mothersill and Seymour 1998) using human normal urothelium and urothelium from transitional cell carcinoma (TCC) patients did show a loss of signal or a switch to pro-survival signals in the irradiated tissues from the TCC group. It may be that the presence of a tumour in an organism means there is a systemic micro-environmental change leading to "pro-survival" rather than "pro-death" signals being produced. If this is so, it could have considerable implications for radiotherapy while also pointing to novel targets for improving outcomes.

In our previous studies, calcium flux was one of the most robust indicators of bystander signal production (Cristian Fernandez-Palomo, Bräuer-Krisch, et al. 2015; F. M. Lyng et al. 2006; Liu et al. 2006). In these experiments however, there appears to be a weaker association. The data suggest that in normal NU-Foxn1^{nu} mice, which did not have a tumour implanted in the brain, irradiation of the brain leads to the generation of signals capable of reducing the clonogenic survival in our reporter assay. However the calcium flux data, while supporting this trend, were not significant. This was mainly due to extremely high variability in the calcium flux response from cell to cell which made the errors very high. Such high variability, could suggest that the calcium flux pathway which we have established to be important in rodents,

fish and a wide variety of cell lines (Mothersill and Seymour 2012), may not be critical for the production of a bystander response in these immunocompromised mice. The fact that the clonogenic reporter cells did "see" a signal from the normal bladders suggests signal production occurred at some level but the transduction of the response might not depend on calcium. This would represent a novel signal transduction mechanism not reported before. Other candidate mechanisms could be those involving sodium channels. While these have not been studied directly in the bystander field, they are implicated in radiation induced neurological effects such as fatigue and are known to be involved in NOONOO cycle fatigue induced by radiation (Pall 2008; Song et al. 2009; Ghosh, Maurya, and Krishna 2008; Molenaar 2011) and NOS are candidate signalling molecules in the bystander field (He et al. 2012; Shao, Prise, and Folkard 2008). It is also possible that there is a "bypass" of the requirement for calcium channel fluxes. Recently our laboratory have shown that a UV signal from irradiated cells can trigger bystander effects in cells contained in flasks which do not share medium or receive any medium bourne signals (Le et al. 2015). At present however, we can only speculate on why calcium seems to be irrelevant for triggering the bystander signal production in the experiments reported here. In regard to the comparison between PB and MRT modalities, both had similar effects on normal tissue. This might be expected because the doses were calculated to irradiate similar tissue volumes and to lead to similar valley doses. However in the tumour bearing groups there was no bystander effect after MRT at any

time point post irradiation. When harvested after 48hrs the signal from the PB irradiated animals is significant however this trend was not seen at all in the 12hr group and was present but weaker at 2hrs post irradiation and only statistically significant following the high dose exposure.

To conclude, this study suggests that signals are produced by immunocompromised mice although the fact that their innate immune system is intact is probably important. The results suggest that the pathways involved in signal transduction may be different from those seen in immunocompetent animals. The question of whether abscopal effects following irradiation using PB or MRT in tumour bearing animals are beneficial or harmful remains open because the presence of tumour in the animal appears to weaken these signals even when these are measured in distant tissue without tumour.

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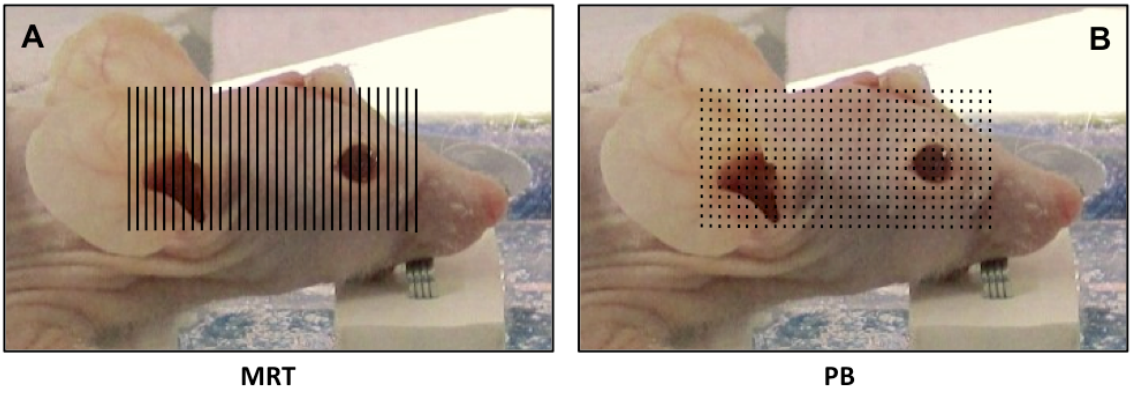

Figure 1. Schematic representation of the irradiation modalities.
This figure graphically shows the geometrical differences between the two arrays studied. Athymic Nude mice were exposed to whole brain irradiation of A) MRT or B) PB. The dimensions of actual beam delivered were 8 mm high & 20 mm wide. MRT: Microbeam Radiation Therapy; PB: Pencilbeam Therapy

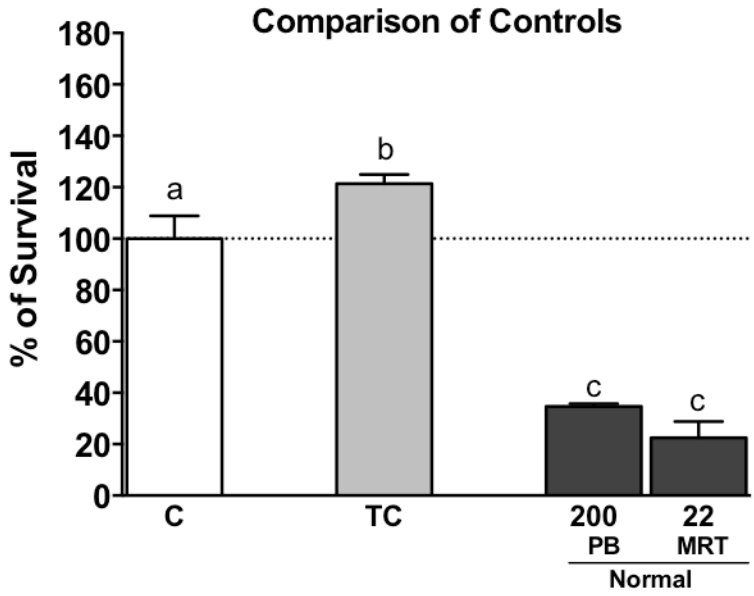

Figure 2. Comparison of clonogenic survival of HaCaT cells grown in ITCM from the control groups.
The figure shows the clonogenic survival of reporter HaCaT cells grown ITCM originated the urinary bladders of the exposed nude mice. Irradiated animals received a whole-brain exposure. The mice from the Tumour Control group were not irradiated. White bar = Normal animals. Gray bars = animals inoculated with F98 glioma cells. Black bars = Normal irradiated animals (22=22Gy; 200=200Gy). C=Normal Control, TC=Tumour control, PB=Pencil beam, MRT=Microbeam radiation

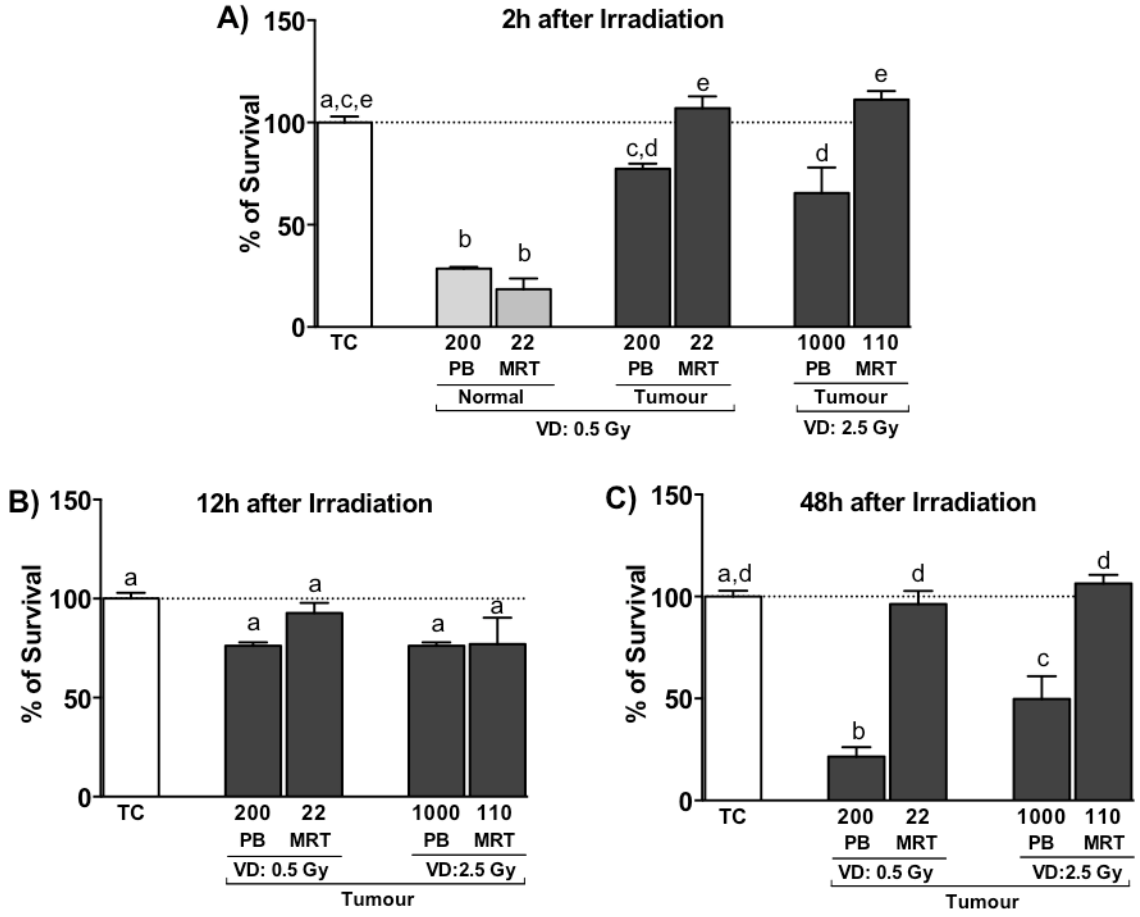

Figure 3. Clonogenic survival of HaCaT cells grown in ITCM from synchrotron irradiated nude mice.

ITCM was originated from the urinary bladders of the synchrotron irradiated nude mice. Animals received a whole-brain irradiation. Normal and tumour bearing mice were exposed to peak doses of 200Gy and 1000 Gy for PB and 22Gy and 110 Gy for MRT in order to achieve constant valley doses of 0.5Gy and 2.5 Gy respectively. White bar = Normal animals. Gray bars = tumour-free irradiated animals. Black bars = tumour-bearing irradiated animals. C=Control, TC=Tumour control, PB=Pencil beam, MRT=Microbeam radiation therapy, VD=Valley dose Letters a, b & c indicate significant differences between groups. Error bars indicate SEM.

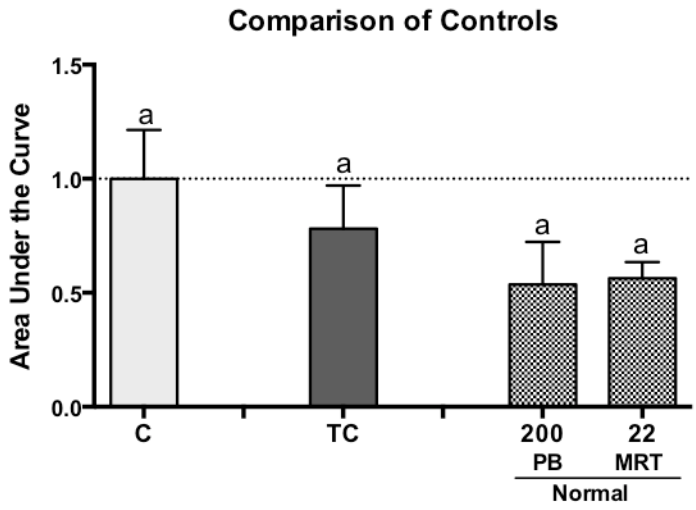

Figure 4. Comparison of Calcium Fluxes Between Controls.
The figure shows the calcium fluxes of reporter HaCaT cells exposed to ITCM originated from the urinary bladders of nude mice. Irradiated animals received a whole-brain exposure. C=Normal Control; TC=Tumour control (no radiation); Normal=Tumour-free irradiated animals (22=22Gy; 200=200Gy); PB=Pencil beam, MRT=Microbeam radiation, VD=Valley dose. Letters a, b & c indicate significant differences between groups. Error bars indicate SEM.

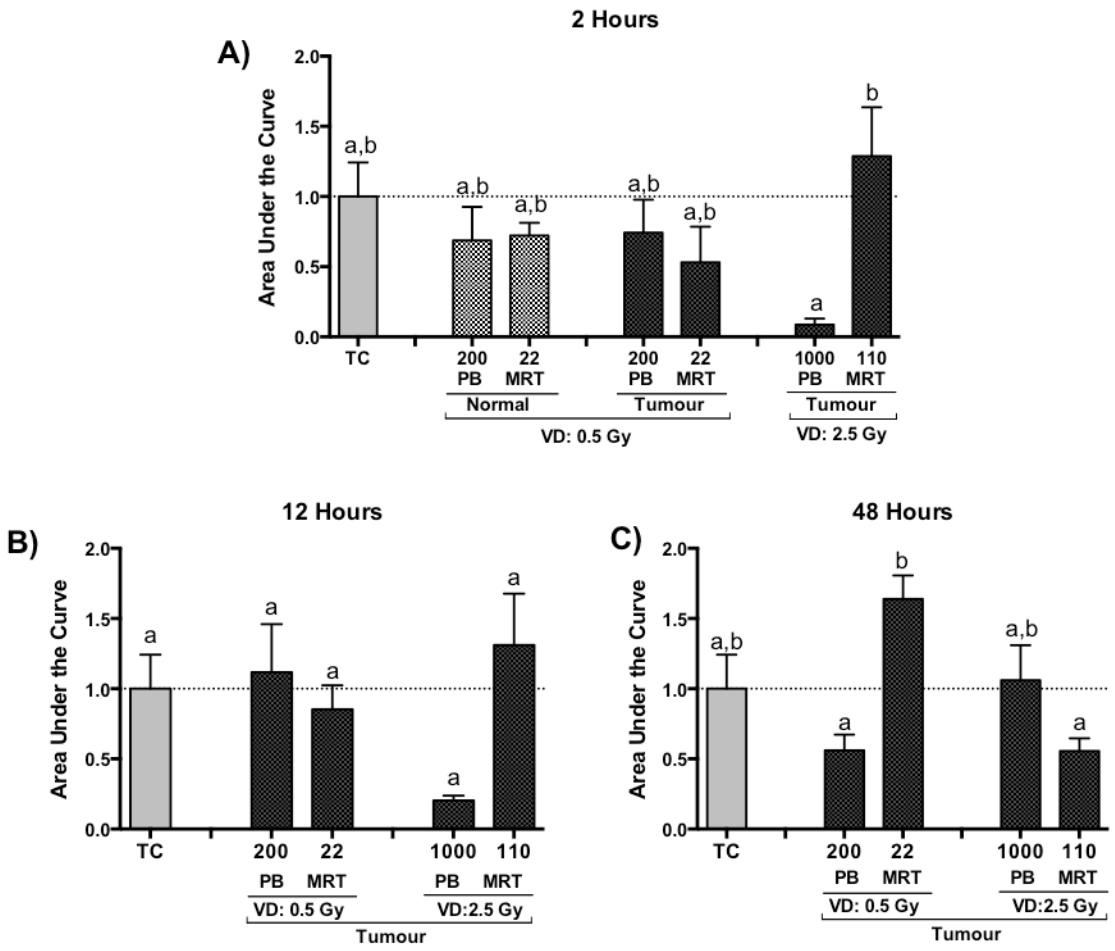

Figure 5. Calcium Fluxes of reporter HaCaT cells.
The figure shows the calcium fluxes of reporter HaCaT cells exposed to ITCM originated from the urinary bladders of nude mice. Irradiated animals received a whole-brain exposure. 200=200Gy; 1000=1000Gy; 22=22Gy; 110=110Gy. TC=Tumour control (no radiation); Normal=Tumour-free irradiated animals; Tumour=tumour-bearing irradiated animal, PB=Pencilbeam, MRT=Microbeam radiation**,** VD=Valley dose. Letters a, b & c indicate significant differences between groups. Error bars indicate SEM.