Chapter 5: Use of the adoptive transfer method to study bystander effects *in vivo*

Précis

Chapter Four described the development and optimisation of an adoptive transfer method in mice designed to deliver irradiated spleen cells into the spleens of unirradiated syngeneic hosts. This chapter now details the use of this adoptive transfer method to conduct a series of experiments aimed at detecting a radiation-induced bystander effect *in vivo* under a number of experimental conditions. The conditions tested included changing the number of irradiated cells, analysing the effects of the donor cells after a longer period *in vivo*, using a higher radiolabelling dose-rate and the use of an X-ray dose delivered *ex vivo* to the donor cells. The results of these experiments are presented here with discussion of the significance and implications of the findings to follow in Chapter Six.

Detection of bystander effects from chronically irradiated lymphocytes lodged in unirradiated mouse spleens

The aim of these first experiments was to use the newly developed adoptive transfer method to create a realistic bystander environment *in vivo*, based on published *in vitro* bystander data and *in vivo* responses to whole-body irradiations, and then monitor the response of the unirradiated cells in the recipient spleen tissue. This first set of three experiments, all performed under the same conditions, also served as the template for subsequent investigations. Conducting three repeat experiments allowed assessment of the reproducibility of the biological assays, and kept the technical difficulty of the method to a minimum during this initial phase of use. To allow data pooling, the parameters used on the three separate occasions were identical.

For each experiment, five donor C57BL/6J mice were euthanised to establish a radiolabelled donor cell culture. Donor T cells were radiolabelled for 18 h with 1.48 kBq.mL^{-1} of [methyl-^{3}H]-thymidine or sham-radiolabelled with the equivalent molar concentration of non-radioactive thymidine. By the end of the radiolabelling period, 69 ± 5% of cells had incorporated ^{3}H-thymidine into their DNA. Sham-radiolabelled donor cells were free of autoradiography grains. The mean incorporated radioactivity was 0.22 ± 0.02 mBq per donor cell; the adjusted mean radioactivity was 0.33 mBq per radiolabelled donor cell. The average radioactivity of the sham-radiolabelled donor cells was negligible at 0.35 ± 0.37 × 10^{-3} mBq per donor cell, and not significantly above zero (P = 0.24, *One-Sample T Test*). All donor cells, both radiolabelled and sham-radiolabelled, were labelled brightly with the CMRA fluorescent probe before adoptive transfer.

Over the three experiments, a total of fifteen C57BL/6J mice were injected with 5×10^5 radiolabelled donor T lymphocytes whilst a further thirteen received the same number of sham-radiolabelled donor cells (*Table 5.1*). Each recipient mouse was euthanised 22 h after adoptive transfer and their tissues isolated.

Table 5.1: Number of mice used in the initial three experiments using the adoptive transfer method

	Number of Mice Receiving	
	Radiolabelled Donor Cells	*Sham-Radiolabelled Donor Cells*
Experiment 1	5	6
Experiment 2	4	3
Experiment 3	6	4
TOTAL	15	13

The donor cell lodging frequencies in the spleen ranged from $0.46 – 3.3 \times 10^{-4}$ and were equivalent ($P = 0.36$) in the mice receiving radiolabelled cells ($1.4 \pm 0.6 \times 10^{-4}$) and those receiving sham-radiolabelled cells ($1.6 \pm 0.8 \times 10^{-4}$, *Figure 5.1*). There was no difference in the mean donor cell lodging frequency between the triplicate experiments ($P = 0.4$, ANOVA). Autoradiography in radiolabelled cell–recipients revealed that 64 ± 6% of donor cells identified in the four local screens were in fact radiolabelled (cf. 69 ± 5% labelling before adoptive transfer). Donor cells in spleen sections from mice receiving sham-radiolabelled cells were free from autoradiography grains.

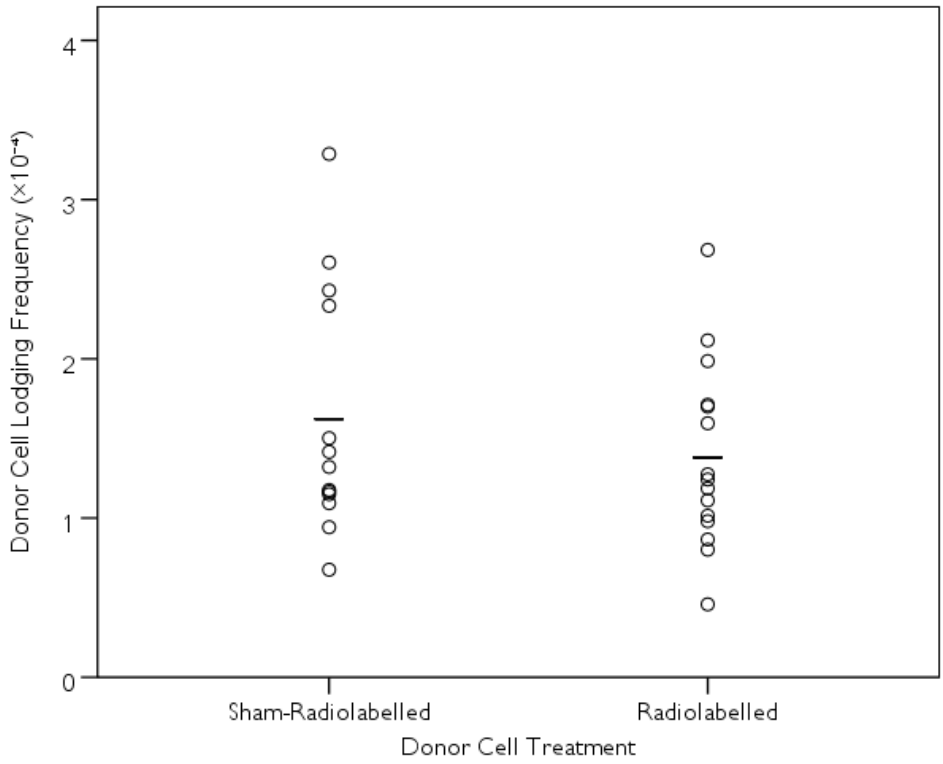

Figure 5.1: Donor cell lodging frequencies in the spleens of recipient mice

Mice were injected with 5 × 10^5 ^{3}H-radiolabelled or sham-radiolabelled splenic T lymphocytes and recipient mouse spleens were isolated 22 hours later. The donor cell lodging frequency in the spleen was determined for each mouse from the duplicate global screens on TUNEL- and Ki-67–stained tissue sections. Circles (O) show mean values for each mouse. Bars (—) show group means. *n* = 13 sham-radiolabelled, 15 radiolabelled.

The proportion of Ki-67–positive donor cells was the same in radiolabelled (56 ± 16%) and sham-radiolabelled (58 ± 11%) cell recipients after 22 h *in vivo* (*P* = 0.7) indicating similar levels of proliferation in both populations of donor cells. In the mice receiving radiolabelled donor cells, most lodged ^{3}H-positive donor cells were Ki-67–positive whilst the opposite was true for the lodged ^{3}H-negative donor cells (*Table 5.2*), consistent with the ^{3}H-negative donor cells representing those that did not proliferate to incorporate the radiolabel during the *ex vivo* culture.

Table 5.2: Ki-67 status of ^{3}H-positive and ^{3}H-negative lodged donor cells in mice receiving radiolabelled donor cell preparations

	^{3}H-positive	^{3}H-negative
Ki-67 negative	21%	81%
Ki-67 positive	79%	19%
	100%	100%

Results

There was no change in local apoptosis

There was no significant difference (P = 0.3) in the apoptosis levels of unirradiated cells that surrounded radiolabelled donor cells compared to those surrounding sham-radiolabelled donor cells (*Figure 5.2A* and *Table 5.3*). Initially an average 49 ± 14 fields per mouse were analysed in the local screen across the duplicate spleen sections analysed for apoptosis. However, following the *post hoc* exclusion of fields that did not contain ^{3}H-radiolabelled donor cells, the final mean number of fields analysed was reduced (31 ± 15) and the total number of bystander cells analysed per mouse was significantly lower for mice receiving radiolabelled donor cells compared to those receiving sham-radiolabelled cells (P = 0.01). The reduced number of fields analysed did not greatly reduce the accuracy of the measured apoptosis frequencies in mice receiving radiolabelled cells compared to sham-radiolabelled cells, i.e. there was only a small increase (23%) in the average standard error of the mean apoptosis frequency measured for each mouse.

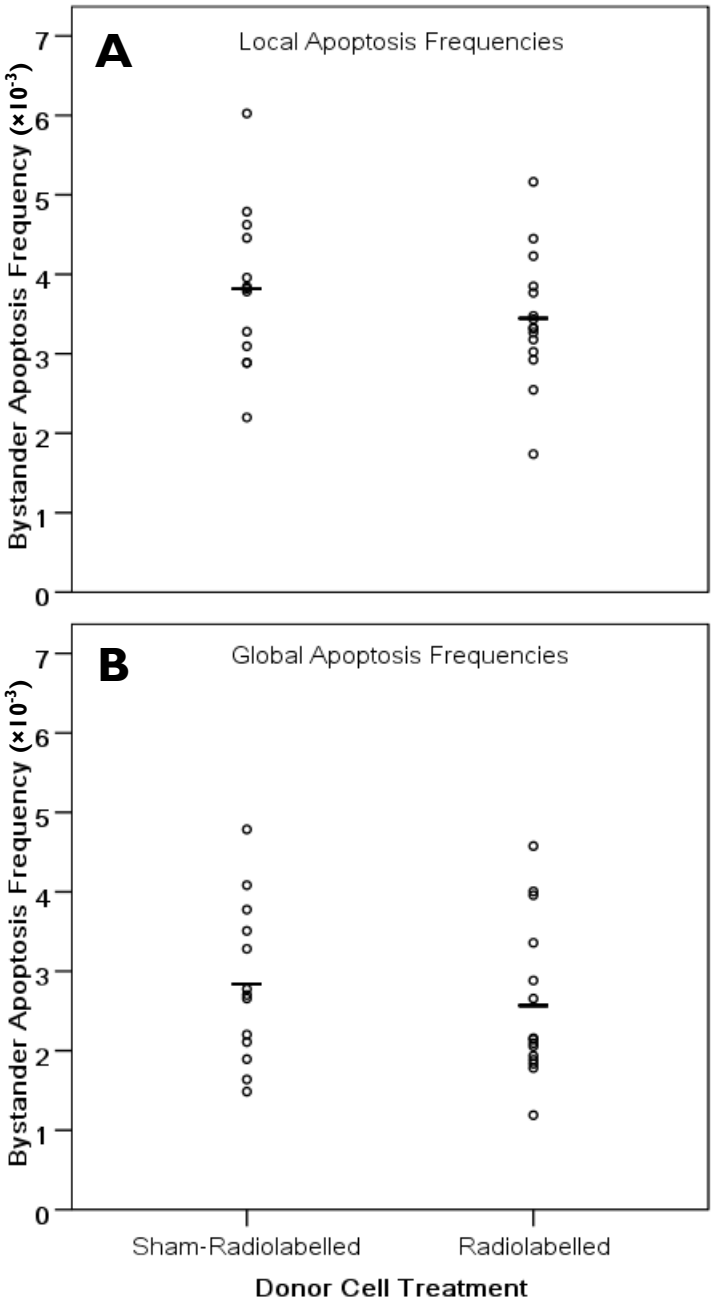

Figure 5.2: Local and global apoptosis frequencies in mice receiving sham-radiolabelled or radiolabelled donor cells

Mice were injected with 5 × 10^5 ^{3}H-radiolabelled or sham-radiolabelled splenic T lymphocytes and recipient mouse spleens were isolated 22 hours later. Levels of apoptosis in the recipient spleen cells were measured using the local screening method (A); or the global screening method (B). Circles (O) show the mean of two replicates for each mouse, bars (—) show group means, *n* = 13, 15 (sham-radiolabelled, radiolabelled).

Table 5.3: Summary of results from local and global screening of apoptosis

		Mean ± 95% Confidence Intervals		
		Donor Cells Received		T test
	Screen	*Sham-Radiolabelled*	*Radiolabelled*	*P*
Apoptosis Frequency	Local	$3.8 \pm 0.5 \times 10^{-3}$	$3.4 \pm 0.4 \times 10^{-3}$	0.3
	Global	$2.8 \pm 0.5 \times 10^{-3}$	$2.6 \pm 0.5 \times 10^{-3}$	0.5

There was no change in global apoptosis

There was no significant difference (P = 0.5) in the apoptosis frequency of unirradiated cells in the spleens of mice receiving radiolabelled donor cells compared to those receiving sham-radiolabelled donor cells (*Figure 5.2B* and *Table 5.3*). Since selection of global fields is random, the analysis included all fields irrespective of whether they contained radiolabelled donor cells, non-radiolabelled donor cells, or no donor cells at all. Thus, the mean number of fields scored was the same (77 ± 5 vs. 77 ± 3) and the number of cells analysed was equivalent in both groups of recipient mice (P = 0.71).

Local and global apoptosis frequencies were significantly different

Presuming no bystander effect, it was reasonable to expect that the levels of apoptosis should be the same in the area around radiolabelled donor cells and throughout the spleen as a whole. The data, however, showed a significant difference between the local and global apoptosis frequencies ($P < 10^{-6}$, *Paired-samples t test*) as can be seen in *Figure 5.2*, although the two measurements were highly correlated (*Paired sample correlation coefficient* = 0.717, $P < 10^{-4}$). This discrepancy was found for both recipient mouse groups, and thus was not due to an effect of the radiation. An explanation for this variance is the different set of cells surveyed with

each method, as the global screen is unbiased, and the local screen is limited to areas of the spleen where the donor T lymphocytes lodge. Since the areas and cell-types of the spleen differ in their levels of apoptosis (Sakaguchi *et al.*, 1995; Takahashi *et al.*, 2001b; reviewed in Elmore, 2006), this could explain the difference in bystander apoptosis measured using the two screening methods.

There was no change in local proliferation

There was no significant difference (P = 0.5) in the proliferation of bystander cells surrounding radiolabelled donor cells compared to bystander cells surrounding sham-radiolabelled donor cells (*Figure 5.3A* and *Table 5.4*). Initially, 52 ± 15 fields per mouse were analysed in the local screen across the duplicate spleen sections analysed for proliferation. However, following the *post hoc* exclusion of fields that did not contain ^{3}H-radiolabelled donor cells, the final mean number of fields analysed was reduced (38 ± 15), and the number of bystander cells analysed per mouse was significantly lower in mice receiving radiolabelled donor cells compared to those receiving sham-radiolabelled cells (P = 0.04). The reduced number of fields analysed per mouse did not reduce the accuracy of the measured proliferation indices for the mice receiving radiolabelled cells compared to sham-radiolabelled cells, i.e. there was no increase in the average standard error of the mean proliferation index measured for each mouse.

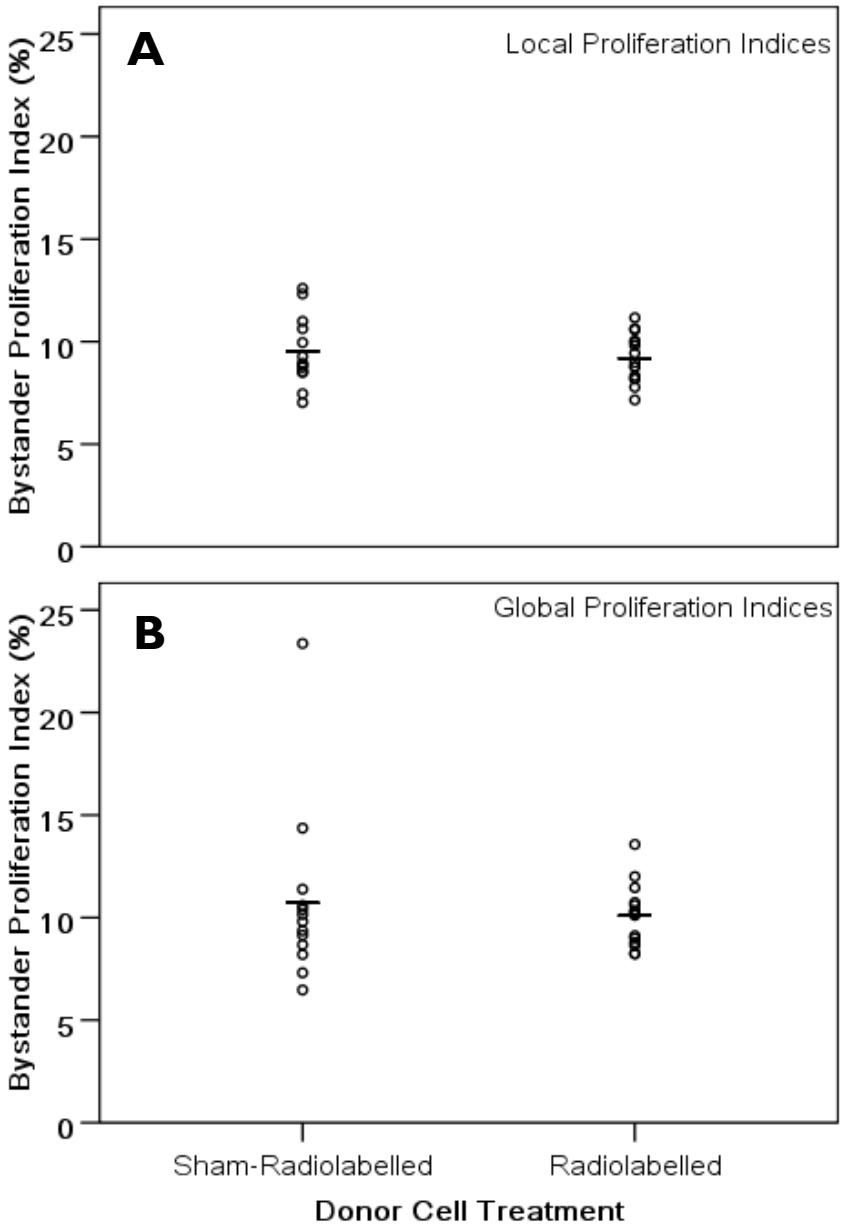

Figure 5.3: Local and global proliferation indices in mice receiving sham-radiolabelled or radiolabelled donor cells

Mice were injected with 5 × 10^5 ^{3}H-radiolabelled or sham-radiolabelled splenic T lymphocytes and recipient mouse spleens were isolated 22 hours later. Levels of proliferation in the recipient spleen cells were measured using the local screening method (A); or the global screening method (B). Circles (O) show the mean of two replicates for each mouse, bars (—) show group means, *n* = 13 sham-radiolabelled, 15 radiolabelled.

Table 5.4: Summary of results from local and global screening of proliferation

		Mean ± 95% Confidence Interval		
		Donor Cells Received		T test
	Screen	*Sham-Radiolabelled*	*Radiolabelled*	*P*
Proliferation Index	Local	9.5 ± 0.9%	9.2 ± 0.6%	0.5
	Global	10.7 ± 2.3%	10.1 ± 0.8%	0.6

There was no change in global proliferation

There was no significant difference ($P = 0.6$) in the proliferation of unirradiated cells in the spleens of mice receiving radiolabelled donor cells compared to those in control mice receiving sham-radiolabelled donor cells (*Figure 5.3B* and *Table 5.4*). Since selection of global fields is random, the analysis included all fields irrespective of whether they contained radiolabelled donor cells, non-radiolabelled donor cells, or no donor cells at all. Thus, the number of fields and the number of cells analysed was equivalent in both groups of recipient mice.

A single mouse (Mouse #44) that received sham-radiolabelled donor cells had a global proliferation index 119% above the group mean. When the same spleen sections were analysed using the local screening method, the local proliferation index was only 33% above the group mean. When the fields were examined manually to check for discrepancies (*Figure 5.4*), it was observed that large amounts of proliferation were occurring in follicular areas in both replicate sections (coefficient of variation = 0.4%).

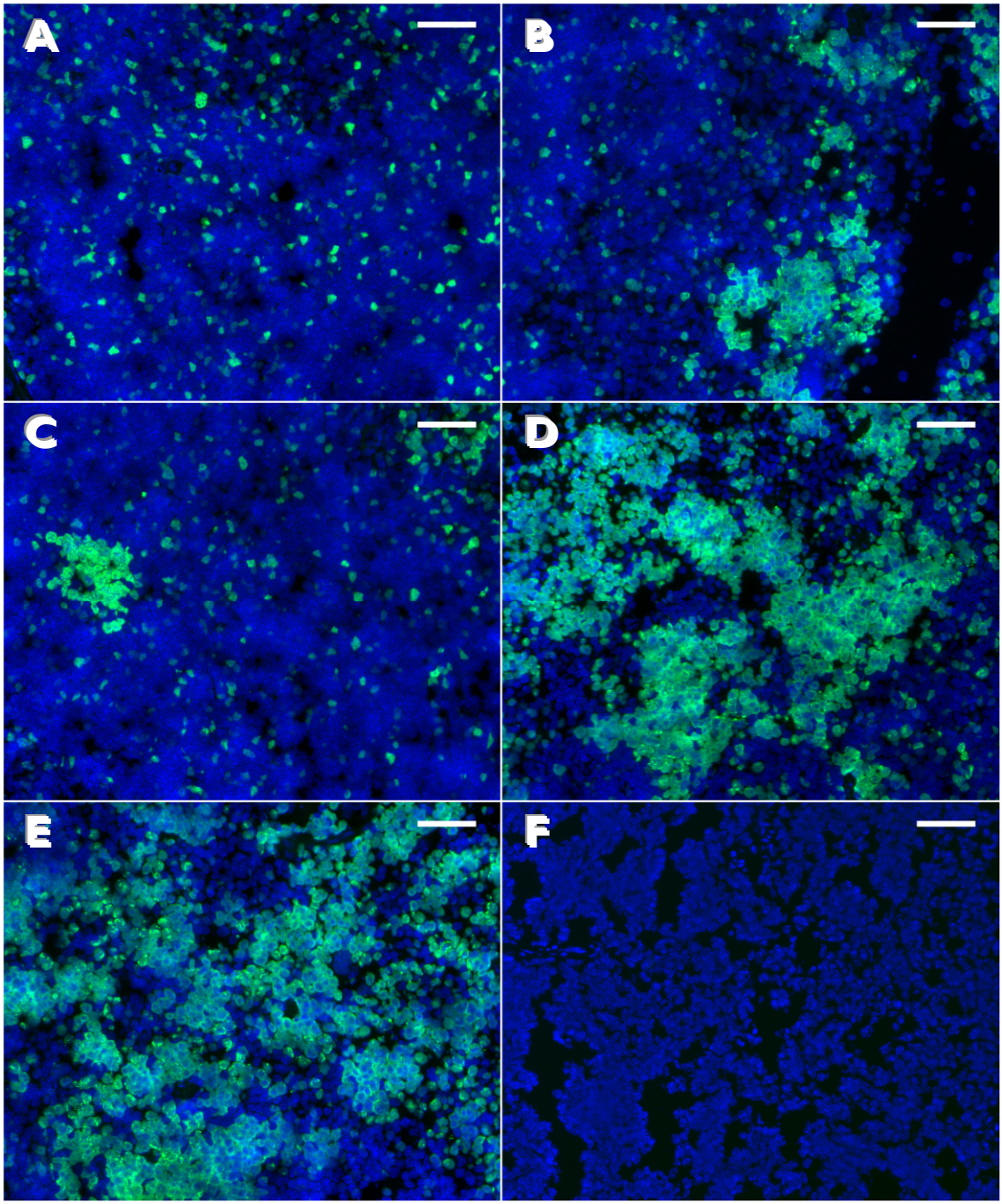

Figure 5.4: Very high levels of Ki-67 staining in Mouse #44

Above are six representative global proliferation images from a single mouse exhibiting very high levels of Ki-67 staining. Images are pseudo-coloured overlays of Ki-67 staining (green) and DAPI nuclear counterstain (blue). These fields are examples of sparse, low proliferation (A), and those also containing localised areas of proliferative activity (B & C) as usually observed in all recipient mice. Also shown are fields with large regions of intense proliferation (D & E) observed at high frequency in this mouse. Donor cells were rarely found in these areas and so the excessive proliferation was largely undetected by the local screening method. No staining was observed when Ki-67 antibody was omitted (F). Scale bars show 50 μm.

When the slides were analysed by the local screening method, the bias towards T cell areas where the donor cells tend to lodge, excluded most of the excessive proliferation. Although the reason for the outlier remained unexplained, the data were an accurate representation of the extent of proliferation in that mouse. This single outlier was the main cause of the increased standard deviation in the sham-radiolabelled recipient group.

Local and global proliferation indices were significantly different

In a similar manner to the apoptosis results, although highly correlated (*Paired sample correlation coefficient* = 0.643, $P < 10^{-3}$) there was a significant difference between the local and global proliferation indices ($P = 0.032$, *Paired-samples t test*) as seen in *Figure 5.3.* The variation is most likely due to real differences in proliferation between the total cell population (measured in the global screen) and donor T cell lodging areas (measured in the local screen), as seen in the example of the outlier described above. Since the correlation between the two measurements is strong, and holds for both the radiolabelled and sham-radiolabelled cell recipients, the level of proliferation in the area surrounding lodged donor cells reflects the amount of proliferation throughout the spleen. Thus, the difference between the proliferation measured using the two screening methods was not due to the radiation dose.

The levels of apoptosis or proliferation for mice receiving radiolabelled donor cells, did not change whether a donor cell was ^{3}H-positive

Within the mice receiving radiolabelled donor cells, the apoptosis frequency ($P = 0.66$, *Figure 5.5A*) and proliferation index ($P = 0.22$, *Figure 5.5B*) in the local fields did not change whether or not the donor cell(s) was radiolabelled, consistent with

expectations assuming no bystander effect. There was also no correlation between the number of radiolabelled donor cells in a local field and either the apoptosis frequency (P = 0.35, Spearman's rho) or the proliferation index (P = 0.06, Spearman's rho) in that field.

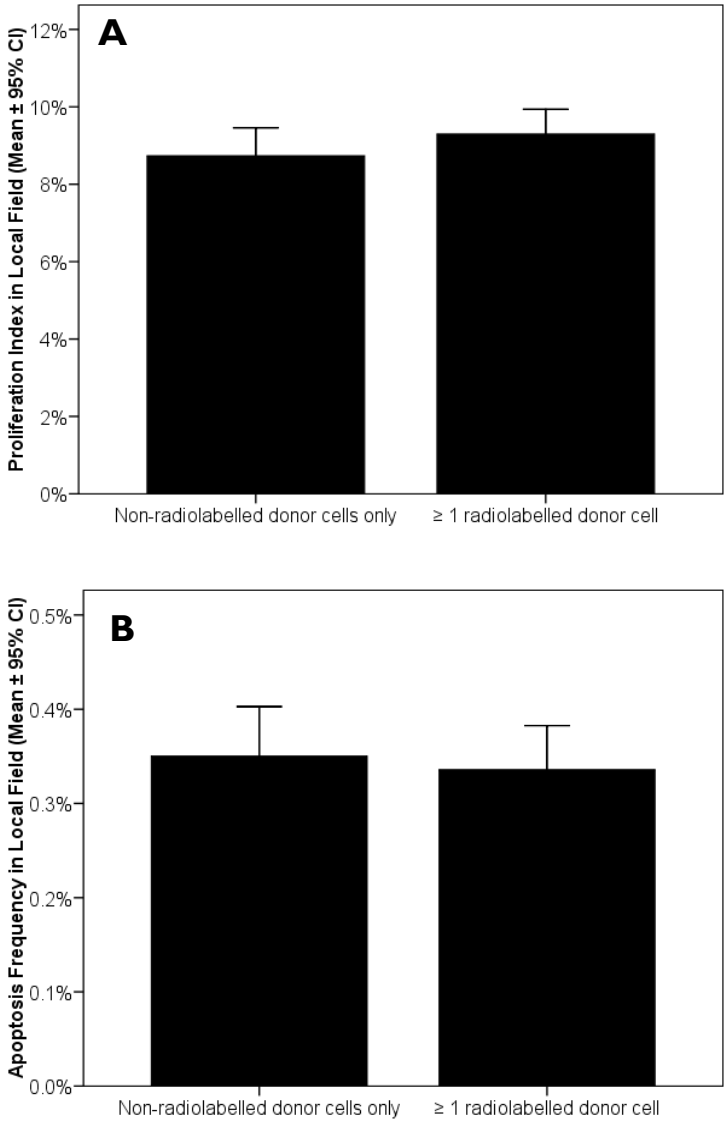

Figure 5.5: Apoptosis and proliferation within mice receiving radiolabelled donor cells in local fields with only ^{3}H-negative donor cells, versus fields with confirmed ^{3}H-positive donor cells

Within mice receiving radiolabelled donor cells (n =15), the mean apoptosis frequency (A) and the proliferation index (B) for each mouse was calculated separately in the local fields containing only non-radiolabelled donor cells and those fields containing at least one radiolabelled donor cell. The mean and upper 95% confidence interval for all the mice are shown for each category.

No evidence for significant effects from possible confounders

Whether analysing pooled data from the three experiments or each individually, there were no significant correlations between the sex of the mice, or the time order of the tail vein injections, and apoptosis or proliferation measured using either screening method ($P > 0.05$, Spearman's rho). Neither were there correlations between a mouse's apoptosis frequency or proliferation index (using either screening method) and their donor cell lodging frequency ($P > 0.05$, Paired sample correlations). In a comparison of the three experiments, there was a significant difference in the mean global apoptosis frequency (in both treatment groups) due to a higher frequency in the first of the three experiments ($P < 0.001$, ANOVA). Re-examination of the global screens for the first and second experiments (conducted within the same week) for any inconsistencies confirmed that the TUNEL-positive scores assigned were representative of the extent of apoptosis in the photographed fields.

Although the trend for greater apoptosis in the first experiment was the same, there was no significant difference between the repeat experiments using the local apoptosis screen to examine the same spleen sections. If the three experiments were analysed separately (instead of analysing the pooled data), the results of the hypothesis tests to detect a bystander effect were still not significant.

Under this first set of experimental conditions tested, the introduction of irradiated cells into the spleen did not alter the apoptotic or proliferative responses of unirradiated bystander cells and thus supported the null hypothesis of no bystander effect.

Detection of bystander effects from increased numbers of chronically irradiated lymphocytes

Results from a number of studies suggest that the induction of bystander effects may have a lower-threshold for irradiated cell numbers. For example, ^{3}H radiolabelling induced a proliferation bystander effect in multicellular clusters only after the ratio of irradiated to unirradiated cells reached 1:1 (Gerashchenko and Howell, 2005). Some bystander studies have reported significant effects after irradiating only a single cell (Shao *et al.*, 2003d); some, show bystander effects increasing with irradiated cell number (Mothersill and Seymour, 1997), whilst others, show bystander effects independent of irradiated cell numbers (Xue *et al.*, 2002). Thus, as the data can currently support any of these possibilities, the prerequisite of a minimum number or proportion of irradiated cells for the induction of a bystander effect *in vivo* is still unclear. However, since a dependence on irradiated cell numbers does exist in some experimental systems, it is possible that such dependence will also exist using the adoptive transfer method described here. Thus, having seen no change in apoptosis or proliferation in the unirradiated bystander cells in initial experiments, this next investigation repeated the previous experiments using the same parameters, except for a tenfold increase in the number of cells injected per mouse. The aim of this experiment was to create a realistic bystander environment *in vivo*, now with a higher ratio of irradiated-to-unirradiated cells, and then monitor the response of the unirradiated cells in the recipient spleen tissue.

The use of pKZ1 transgenic recipient mice in this experiment allowed the quantification of chromosomal inversions in the bystander spleen cells. Fifteen $pKZ1^{-/-}$ donor mice were used to prepare the radiolabelled donor cell culture. Donor cells were radiolabelled for 18 h with 1.48 $kBq.mL^{-1}$ of [methyl-^{3}H]-thymidine or

sham-radiolabelled with the equivalent molar concentration of non-radioactive thymidine. By the end of the radiolabelling period, 81% of cells had incorporated ^{3}H-thymidine into their DNA. Sham-radiolabelled cells were free of autoradiography grains. The incorporated radioactivity was 0.19 mBq per donor cell; the adjusted radioactivity was 0.23 mBq per radiolabelled donor cell. The radioactivity of the sham-radiolabelled donor cells was negligible. All donor cells (radiolabelled and sham-radiolabelled) were labelled brightly with the CMRA fluorescent probe before adoptive transfer.

Six pKZ1$^{+/-}$ mice were injected with 5×10^{6} radiolabelled donor T lymphocytes whilst a further five received the same number of sham-radiolabelled donor cells; each were euthanised 22 h after adoptive transfer. The donor cell lodging frequencies in the spleen were equivalent in the mice receiving radiolabelled cells ($2.6 \pm 0.9 \times 10^{-3}$) and those receiving sham-radiolabelled cells ($1.8 \pm 1.1 \times 10^{-3}$; $P = 0.24$). The lodging frequencies were an average 13.4-fold higher after the injection of ten times more donor cells than used in the initial experiments, suggesting that the lodging sites in the spleen for donor cells were not yet saturated (*Figure 5.6*). Only global screens for apoptosis and proliferation were performed, as it was not possible to photograph individual donor cells in isolation due to the large number of cells lodged in the spleen. Autoradiography in radiolabelled cell–recipients revealed that 66 ± 7% of donor cells surveyed across the four local screens were in fact radiolabelled (lower than before the adoptive transfer); donor cells in spleen sections from sham-radiolabelled cell-recipients were free from autoradiography grains.

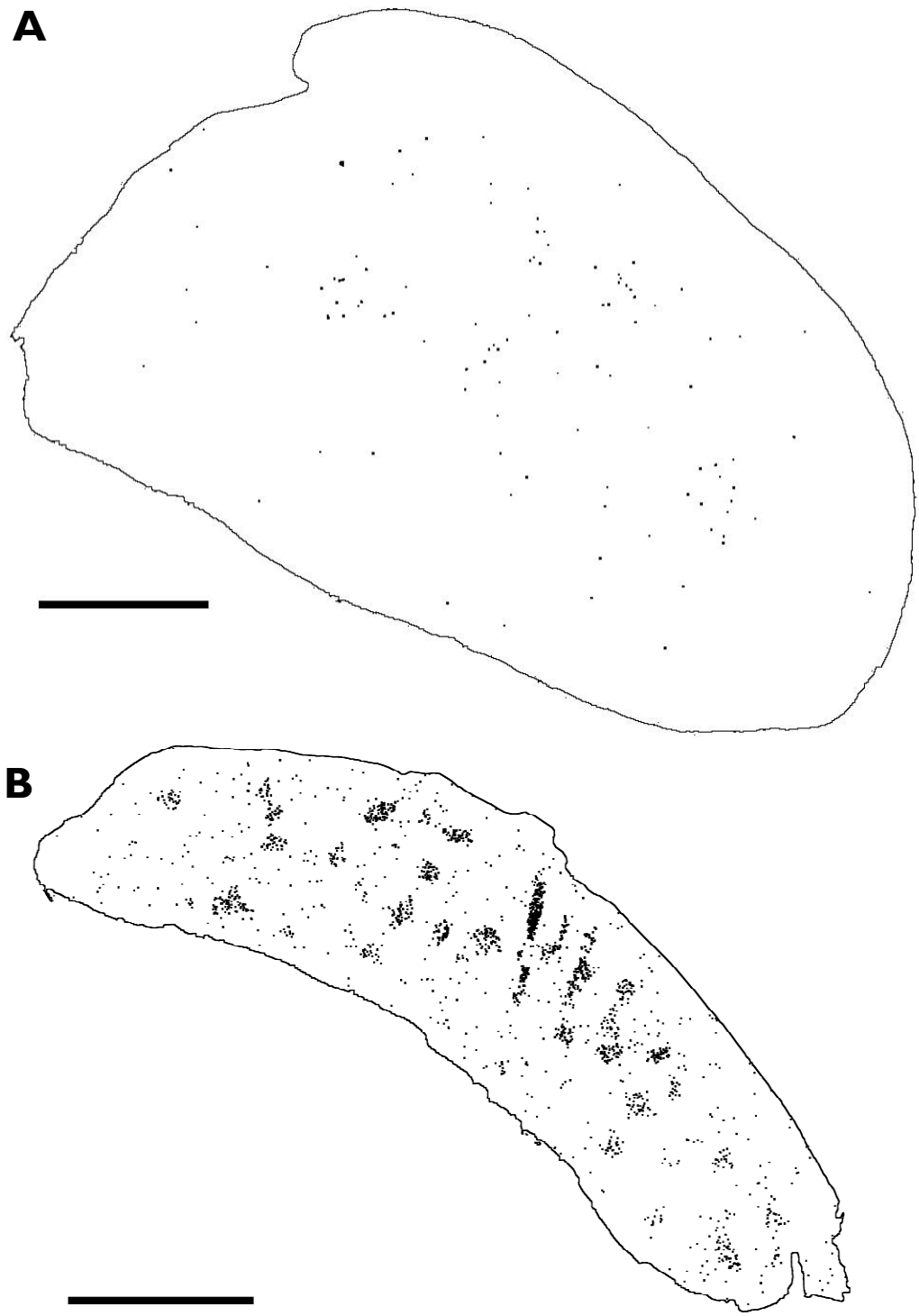

Figure 5.6: Lodging patterns in spleens of mice receiving 5 or 50 × 10^5 donor cells

A single spleen section from a mouse receiving 5 × 10^5 sham-radiolabelled donor cells (A) or 5 × 10^6 sham-radiolabelled donor cells (B) was photographed in a series of overlapping microscope images at 40× magnification, on the DAPI and CMRA fluorescence channels. The outline of the DAPI-stained spleen section is shown and the locations of lodged CMRA-positive donor cells are marked (not to scale, inflated ≈ 5 fold for visual clarity). Scale bars show 1 mm. The difference in the size of the two spleen cross-sections is due to the plane of the cutting through the spleen and is not representative of a difference in actual spleen size.

Results

There were no changes in apoptosis, proliferation or pKZ1 chromosomal inversions

There was no difference in global apoptosis frequency (P = 0.6) or global proliferation index (P = 0.25) between mice receiving radiolabelled donor cells and those receiving sham-radiolabelled cells (*Table 5.5*). The pKZ1 inversion frequency was not significantly different between treatment groups (*Table 5.5*) although there was a trend towards a reduction in pKZ1 inversion frequency (P = 0.075, *Figure 5.7*) in mice receiving radiolabelled cells. The large standard deviation in both groups was due to single mice with pKZ1 inversion frequencies divergent from the rest of the group. The outlier in the sham-radiolabelled donor cell–recipients was 58% below the group mean, although the variation between the triplicate assays was high ($1.4 \pm 1.3 \times 10^{-4}$). The outlier in the radiolabelled donor cell–recipients was 2.6-fold higher than the mean; however, the high inversion frequency in this mouse was remarkably consistent across the triplicate sections scored ($4.5 \pm 0.2 \times 10^{-4}$). Except for these two mice, a robust decrease in inversions might have been observed; however, to confirm this trend, the experiment would need to be repeated to increase the sample size.

Table 5.5: Summary of results from global screening of apoptosis, proliferation and pKZ1 inversions

		Mean ± 95% Confidence Interval		
		Donor Cells Received		T-test
	Screen	*Sham-Radiolabelled*	*Radiolabelled*	*P*
Apoptosis Frequency	Global	$5.0 \pm 0.6 \times 10^{-3}$	$5.5 \pm 1.3 \times 10^{-3}$	0.6
Proliferation Index	Global	11.6 ± 2.6%	14.68 ± 4.0%	0.25
pKZ1 Inversion Frequency	Global [a]	$3.3 \pm 1.1 \times 10^{-4}$	$1.7 \pm 1.4 \times 10^{-4}$	0.075

[a] pKZ1 inversions were screened using a modified global screening protocol.

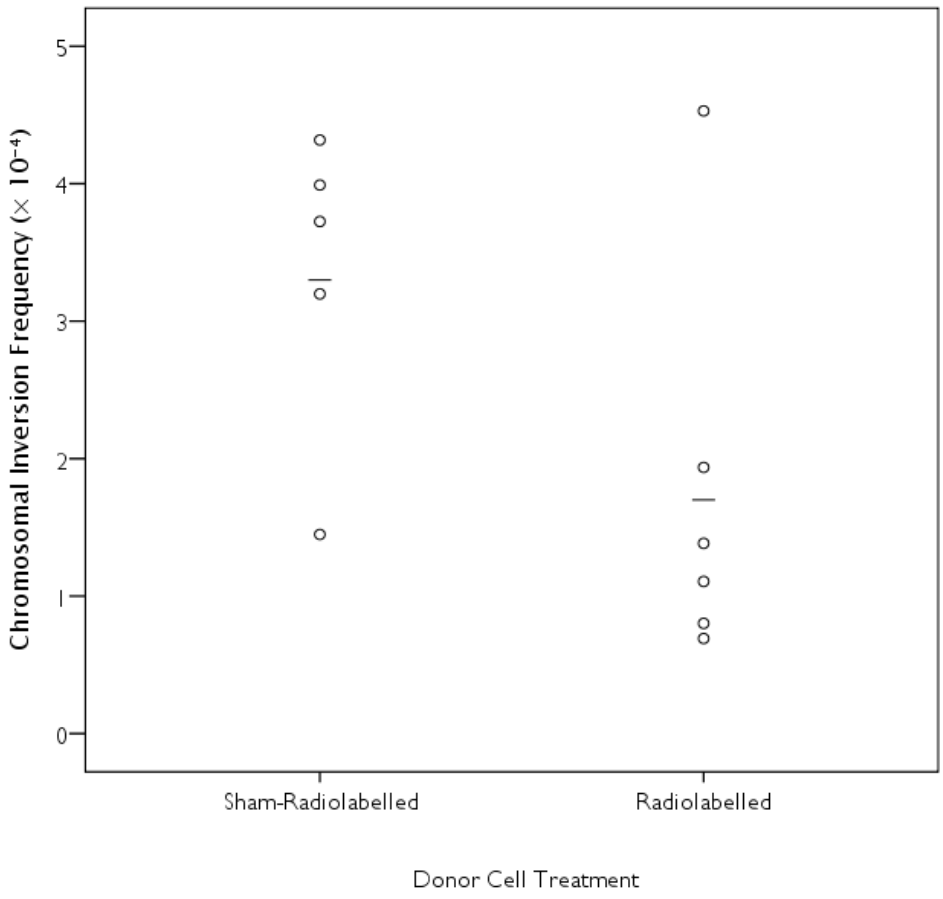

Figure 5.7: pKZ1 chromosomal inversion frequencies in mice receiving sham-radiolabelled or radiolabelled donor cells

Mice were injected with 5 × 10^6 ^{3}H-radiolabelled or sham-radiolabelled splenic T lymphocytes and recipient mouse spleens were isolated 22 hours later. Chromosomal inversions in the recipient spleen cells were measured using the modified global screening method. Circles (O) show the mean of three replicates for each mouse, bars (—) show group means, *n* = 5 sham-radiolabelled, 6 radiolabelled.

Proliferation index correlates with donor cell density in global fields

There was a significant negative correlation ($P = 10^{-6}$, Spearman's rho) between the number of donor cells lodged in a global field and the proliferation index measured for that same field in mice receiving sham-radiolabelled or radiolabelled donor cells (*Figure 5.8*).

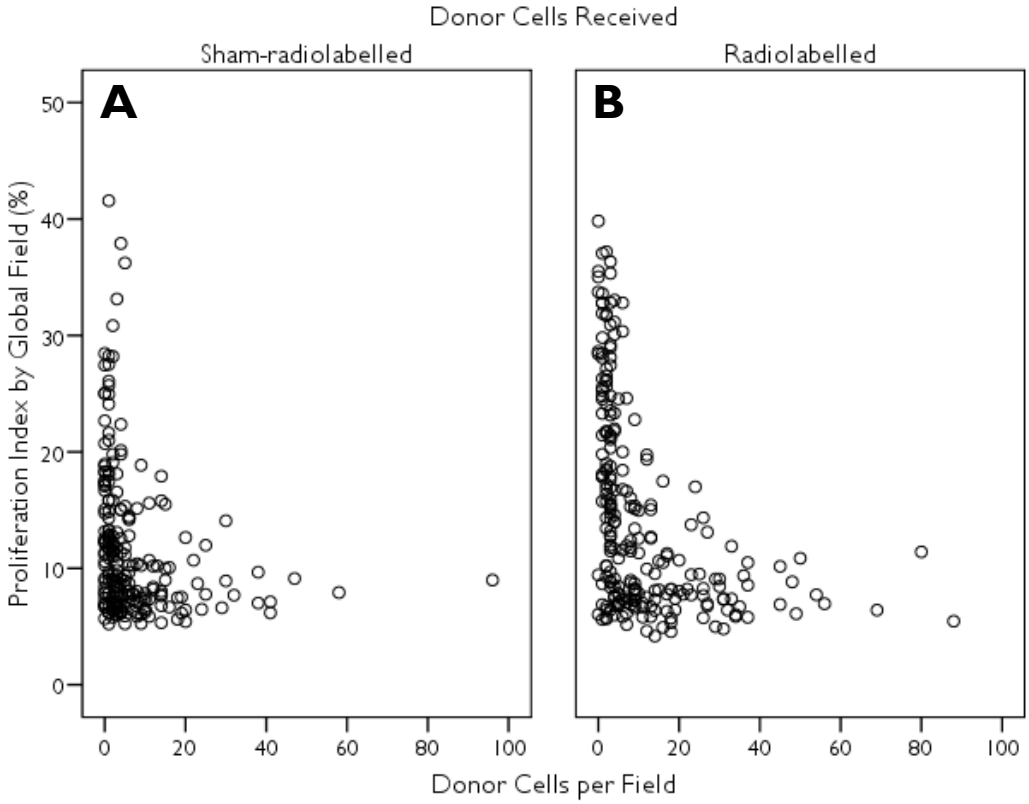

Figure 5.8: Effect of donor cell lodging density on proliferation index in global fields

The level of proliferation was assessed for each global field and was plotted here against the number of lodged donor cells appearing in the field. Results are shown separately for fields from mice receiving sham-radiolabelled (A) and radiolabelled donor cells (B).

This likely reflects a tendency for donor cells to lodge in non-proliferative T cell areas rather than in the highly proliferative B lymphocyte rich areas (a similar trend, although not significant was observed when injecting 5×10^5 donor cells in the initial experiments). There was no significant correlation between the number of donor cells lodged in a field and the apoptosis frequency measured for that same field. There were no correlations between a mouse's apoptosis frequency, proliferation index or pKZ1 inversion frequency, and their degree of donor cell lodging ($P > 0.2$, Paired Sample Correlation) for either recipient mouse group.

Thus, even increasing the ratio of irradiated-to-unirradiated cells by greater than 13-fold compared to the previous experiments, did not induce a bystander effect for the three endpoints (apoptosis, proliferation and pKZ1 chromosomal inversions) used here.

Detection of bystander effects from chronically irradiated lymphocytes after longer-term lodging *in vivo*

After high-dose whole-body irradiation, the peak of splenic apoptosis in mice is within the first 12 h after exposure (Takahashi *et al.*, 2001b; Takahashi *et al.*, 2003). However, between 8 h and 24 h after irradiation with 8 Gy, apoptosis in mouse spleens goes from being predominantly in the white-pulp to occurring within the red-pulp (Komarova *et al.*, 2000) suggesting both rapid and delayed induction of apoptosis. Similar timing of the anti-proliferative response to radiation is observed in spleen (Komarova *et al.*, 2000). Examining spleen tissues for bystander effects after 22 h in the initial experiments, was aimed at allowing time for the donor cells to transit to the spleen, lodge and initiate a signal, and then time for bystander cells to respond. It should be noted, that since the radiolabelled cells receive continual, repeated irradiations, 22 h after injection does not equate to 22 h post-irradiation.

Even though ICCM can induce early events in the apoptosis cascade in bystander cells as rapidly as 30 s after medium-transfer (Lyng *et al.*, 2000), it can also induce prolonged increases in apoptosis in bystander cells out to 48 h after exposure (Mothersill and Seymour, 1997; Lyng *et al.*, 2000). A sustained increase in bystander apoptosis days after exposure to irradiated cells or conditioned medium suggests either a delayed/long-term production of the bystander signal from the irradiated cell(s), or a delayed response in the unirradiated bystander cells. Evidence for the former comes from experiments showing that medium collected from irradiated cultures up to 18 days after irradiation is able to induce a toxic bystander signal (Lewis *et al.*, 2001), even following continued replenishment with fresh medium. Reactive oxygen species levels are increased out to 60 h after direct irradiation and out to 30 h in co-cultured bystander cells (Yang *et al.*, 2005). Irradiating only a

fraction of cells with α-particles induces bystander apoptosis in fibroblasts after three days (Belyakov *et al.*, 2001) and in bystander explant outgrowths from three to seven days after irradiation (Belyakov *et al.*, 2002; Belyakov *et al.*, 2003, 2006).

Evidence for long-term bystander proliferation is more difficult to discern from published studies since most assays only quantify relative increases in the final cell number which cannot distinguish between early increases of short duration or sustained, low-level mitogenic responses (Gerashchenko *et al.*, 2004). Data suggestive of sustained proliferation responses in bystander cells include increased proliferation during a 24-h co-culture with carbon-ion irradiated cells, which continued when the bystander cells were removed, replated and subcultured for a further 84 h (Shao *et al.*, 2003a). Furthermore, expression of the cell-cycle dependent proliferation marker proliferating-cell nuclear antigen (PCNA) increased twofold in bystander cells 24 h after exposure to medium from α-particle irradiated cells (Iyer *et al.*, 2000).

The time of analysis for most bystander endpoints is dictated either by the experimental design or is chosen based on a temporal study to find the time of peak induction. Since bystander apoptosis and proliferation have both been observed up to several days after exposure, and in some cases, not observed at earlier time points, it was possible that the same delayed timing was occurring in the *in vivo* bystander system. Having observed no bystander effects after one day of lodging *in vivo*, the aim of these experiments was to create a realistic bystander environment *in vivo* and monitor the response of the unirradiated cells in the recipient spleen tissue over a longer period. Thus, this investigation repeated the initial experiments, except

allowing three days for the donor cells to remain lodged in the spleen tissue before collecting tissues for analysis.

For each of two replicate experiments, five pKZ1$^{-/-}$ donor mice were used to prepare the radiolabelled donor cell culture. Donor cells were radiolabelled for 18 h with 1.48 kBq.mL^{-1} of [methyl-^{3}H]-thymidine or sham-radiolabelled with the equivalent molar concentration of non-radioactive thymidine. By the end of the radiolabelling period, 77.2 ± 0.1% of cells had incorporated ^{3}H-thymidine into their DNA. The incorporated radioactivity for the first experiment was 0.23 mBq per donor cell; the adjusted radioactivity was 0.31 mBq per radiolabelled donor cell. Radiolabelling dosimetry was not independently confirmed for the second experiment, however, identical radiolabelling conditions were used, incorporation rates were near identical to the first experiment (77.1% vs. 77.2%) and autoradiography confirmed similar grain densities (*Figure 5.9*). The average radioactivity of the sham-radiolabelled donor cells, measured for both trials, was negligible (0.6 ± 6.9 × 10^{-5} mBq per cell). Sham-radiolabelled cells were free of autoradiography grains in both trials. All donor cells (radiolabelled and sham-radiolabelled) were labelled brightly with the CMRA fluorescent probe before adoptive transfer.

Over the two replicate experiments, sixteen mice (14 pKZ1$^{+/-}$ and 2 pKZ1$^{-/-}$) were injected with 5 × 10^{5} radiolabelled donor T lymphocytes, whilst a further seventeen mice (16 pKZ1$^{+/-}$ and 1 pKZ1$^{-/-}$) received sham-radiolabelled donor cells (Experiment One: 10 sham, 8 radiolabelled; Experiment Two: 7 sham, 8 radiolabelled). In addition, during the second experiment, three pKZ1$^{-/-}$ mice received vehicle-only tail vein injections (200 µL PBS) and two pKZ1$^{-/-}$ mice were

placed under the anaesthetic but were not injected. Each recipient mouse was euthanised 72 h after adoptive transfer.

The donor cell lodging frequencies in the spleen were equivalent in the mice receiving radiolabelled cells ($9.1 \pm 5.2 \times 10^{-5}$) and those receiving sham-radiolabelled cells ($9.0 \pm 6.0 \times 10^{-5}$; $P = 0.9$). Autoradiography in radiolabelled cell-recipients revealed that 21 ± 8% of donor cells identified in the local screens were in fact radiolabelled (cf. 77.2 ± 0.1% labelling before adoptive transfer); donor cells in spleen sections from sham-radiolabelled cell-recipients were free from autoradiography grains. The proportion of Ki-67–positive donor cells was <3% in either recipient mouse group 72 h after adoptive transfer, suggesting that the effect of the mitogen had dissipated *in vivo*.

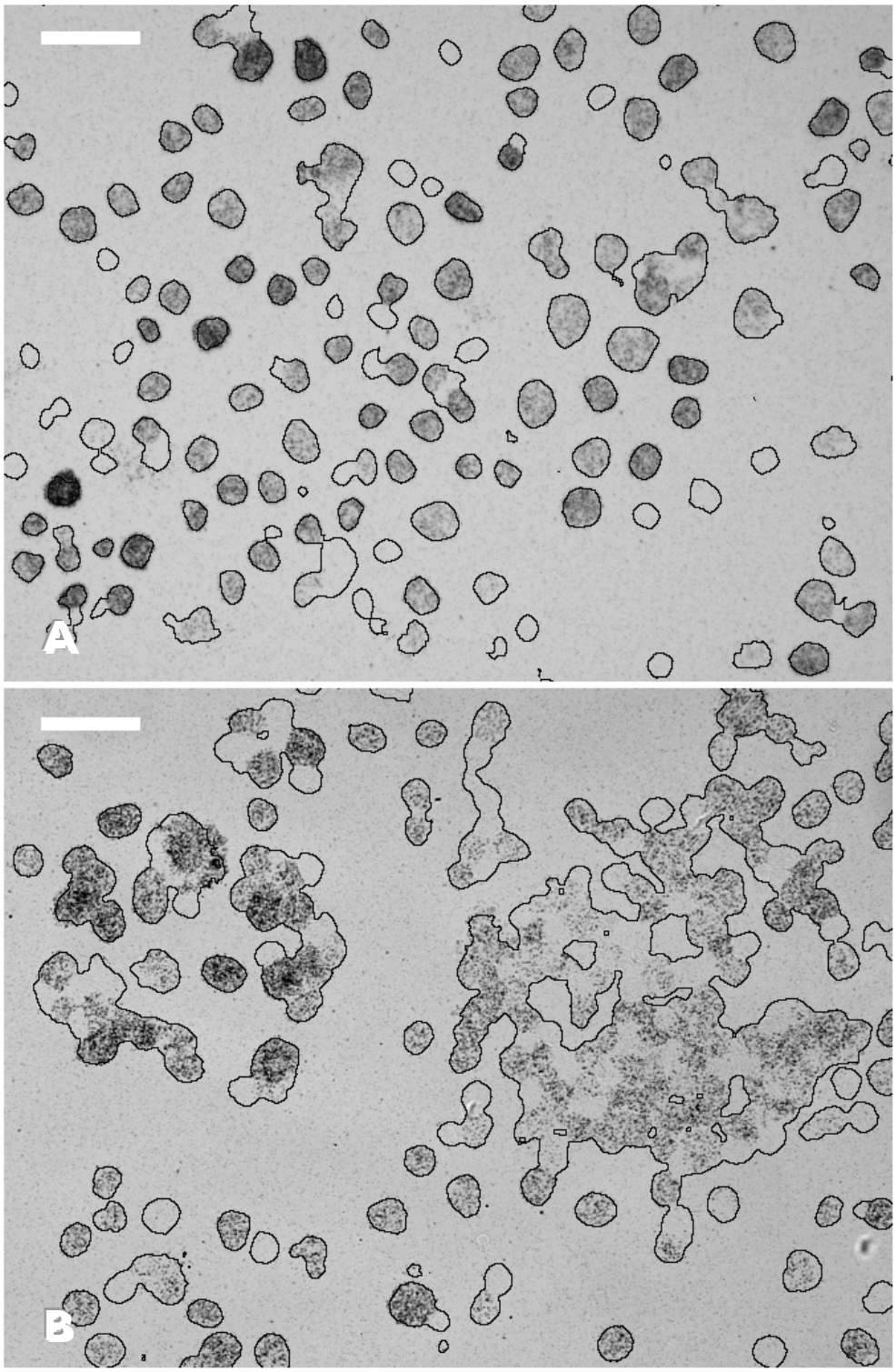

Figure 5.9: Equivalent donor cell radiolabelling in duplicate trials

Radiolabelled donor cells were sampled at the end of the 18-h radiolabel incorporation period and processed for autoradiography. Brightfield images (226 µm × 173 µm) of radiolabelled donor cell autoradiogram from Experiment 1 (A) and Experiment 2 (B) are shown. Images show deposited autoradiography silver grains and outlines of cell nuclei (detected by DAPI counterstain). Both autoradiograms were exposed for 168 h, scale bars show 25 µm.

Results

No changes in apoptosis or proliferation

No changes in apoptosis or proliferation were observed in the area surrounding lodged donor cells using the local screening method, or across the spleen using the global screening method (*Table 5.6*). There was no significant difference in the rank order between the mice receiving PBS only, the mice that were anaesthetised without injection, and the mice receiving sham-radiolabelled cells for either apoptosis ($P > 0.105$, Mann Whitney U-test) or proliferation ($P > 0.57$, Mann Whitney U-test).

As in the previous experiments, there was a significant negative correlation between the number of donor cells lodged in a field and the proliferation index measured for that same field (*Table 5.7*). There was a weak, but significant positive correlation (Correlation Co-efficient = 0.08–0.12, $P < 0.035$, *Spearman's rho*) between the number of donor cells lodged in a global field and the apoptosis frequency measured for that same field, however, this effect was similar in both recipient mouse groups (*Table 5.7*). The same correlation was only observed for mice receiving sham-radiolabelled donor cells in local fields (*Table 5.7*), possibly a result of the reduced number of local fields examined when fields containing non-radiolabelled cells were excluded.

Table 5.6: Summary of local and global screening results for apoptosis and proliferation

		Mean ± 95% Confidence Interval		
		Donor Cells Received		T test
	Screen	*Sham-Radiolabelled*	*Radiolabelled*	*P*
Apoptosis Frequency	Local	$6.3 \pm 1.2 \times 10^{-3}$	$6.4 \pm 1.8 \times 10^{-3}$	0.8
	Global	$5.2 \pm 0.8 \times 10^{-3}$	$5.1 \pm 1.0 \times 10^{-3}$	0.8
Proliferation Index	Local	9.3 ± 0.9%	9.4 ± 1.9%	0.95
	Global	11.9 ± 2.5%	13.4 ± 2.8%	0.4

Table 5.7: Correlations between number of donor cells and proliferation/apoptosis in local and global fields

		Spearman's rho correlation probability (*P*)	
		Donor Cells Received	
	Screen	*Sham-Radiolabelled*	*Radiolabelled*
Proliferation in field *versus* Donor cells per field	Local	0.012	0.000001
	Global	0.00005	0.02
Apoptosis in field *versus* Donor cells per field	Local	0.000002	0.22
	Global	0.001	0.035

Comparing the averaged data for each mouse, a similar positive correlation was observed for the apoptosis frequency and mean donor cell lodging frequency within mice (*Table 5.8* and *Figure 5.10*). This correlation was not observed in the initial experiments of this study, where spleen tissues were analysed ≈ 1 day after adoptive transfer (*Figure 5.10*).

Table 5.8: Correlations between donor cell lodging frequency and global and local apoptosis frequencies

Apoptosis Frequency *versus* **Donor cell lodging frequency**	**Paired-sample correlation probability (*P*)**	
	Donor Cells Received	
Screen	*Sham-Radiolabelled*	*Radiolabelled*
Local	0.001	0.008
Global	0.019	0.045

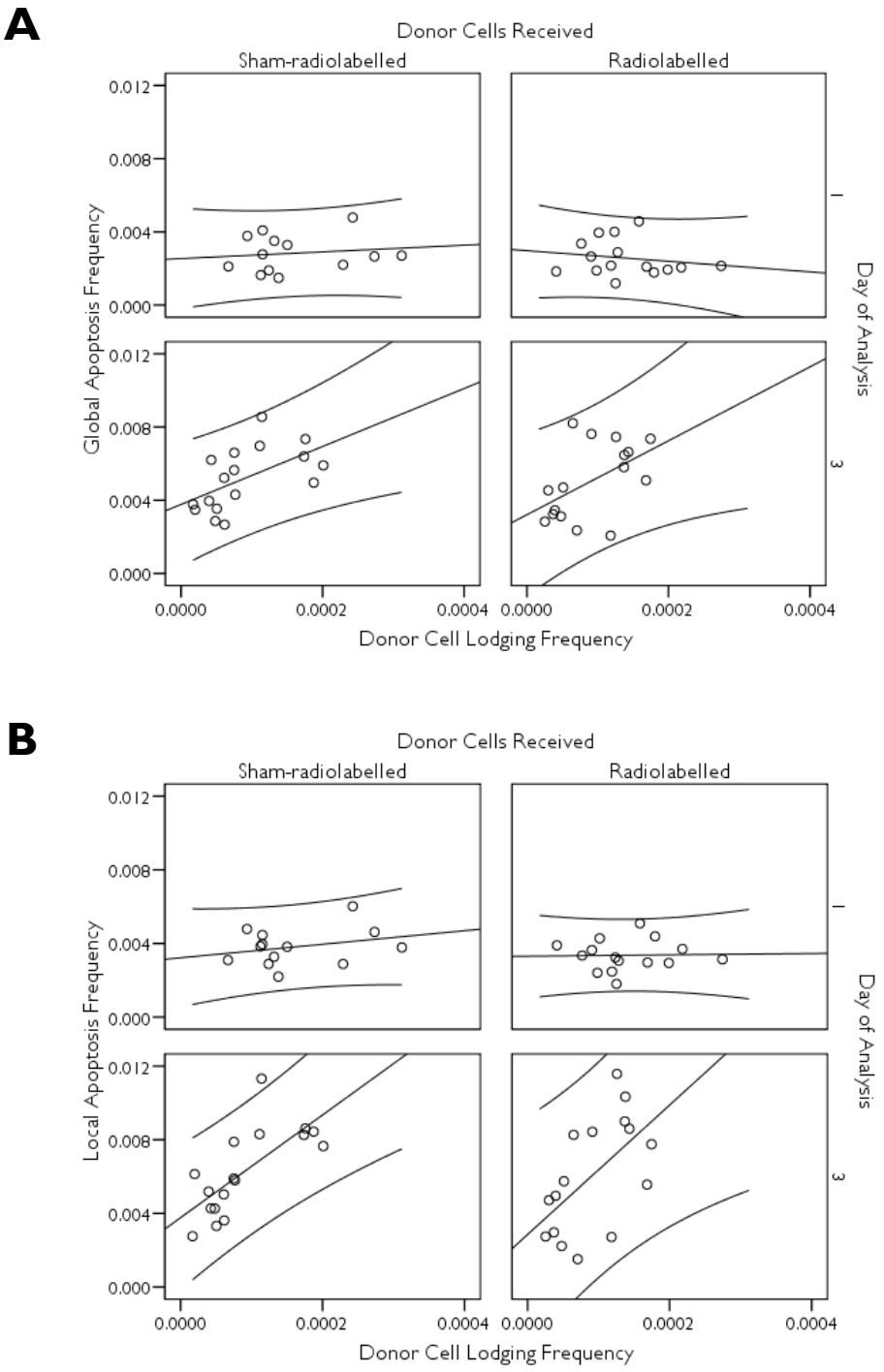

Figure 5.10: Correlation between apoptosis frequency and donor cell lodging frequency for analysis of spleen tissues at one or three days

The global (A) and local (B) apoptosis frequencies and donor cell lodging frequencies were determined for mice receiving 5 × 10^5 sham-radiolabelled or radiolabelled donor cells with tissues analysed after one day (initial experiments conducted under the original conditions) or three days lodging *in vivo (*the current set of experiments). The mean value for each mouse is shown (O), along with a line of best fit (least squares linear regression) and 95% confidence intervals for the fit.

In the studies of Schwulst *et al.* (2007), mice adoptively transferred with 7×10^7 uninjured splenocytes induced apoptosis in recipient spleen cells after 24 h; an effect further exacerbated by injecting dying cells. A similar radiation-independent bystander effect of transplanted cells cannot be excluded in this case, although, the cell numbers injected in this study were two orders-of-magnitude lower and the effect was not observed after 22 h, only after 72 h. There was not enough data to test the hypothesis of a causative relationship between donor cell lodging (irradiated or unirradiated) and delayed apoptosis in recipient spleen cells. There were no correlations ($P > 0.05$) between proliferation index and the degree of donor cell lodging within mice for either recipient mouse group or screening method.

Thus, even allowing the donor cells to interact with the unirradiated recipient cells for three days did not induce a bystander effect for apoptosis or proliferation using the adoptive transfer model. These results support the null hypothesis of no bystander effect. A positive correlation between the number of donor cells lodged in a field and the level of apoptosis in the field was found for both recipient mouse groups (sham-radiolabelled and radiolabelled); a similar correlation was found between the global apoptosis frequency and the donor cell lodging frequency within mice.

Detection of bystander effects from high dose-rate chronically irradiated lymphocytes

The question of dose-dependency for the induction or magnitude of bystander effects has no doubt received the most research attention since the identification of the bystander phenomenon. The research has proceeded from two directions; the first has focussed on the higher ranges as relevant to radiotherapy (relating more to abscopal effects), the second, aims to lower the experimental doses towards the range of radiation protection limits with the aim of identifying a threshold-dose. In the former, bystander experiments are often conducted using only one acute dose, for example, from 10 Gy (Kanasugi *et al.*, 2007) up to 15 Gy (Gaugler *et al.*, 2007), compared to an unirradiated control group. Other investigators use lethal chronic irradiation with dose-rates >2-fold higher than required to kill the cells (Xue *et al.*, 2002; Kishikawa *et al.*, 2006).

Experiments conducted specifically to determine a dose-response include ranges of: 1–7 Gy X-rays (Lewis *et al.*, 2001), 0.05–3 Gy X-rays (Schettino *et al.*, 2003; Schettino *et al.*, 2005), 1–10 Gy γ-rays (Baskar *et al.*, 2007), 0.005–5 Gy γ-rays (Seymour and Mothersill, 2000; Mothersill and Seymour, 2002) and 0.25–2.5 Gy of α-particles (Baskar *et al.*, 2007). Whilst some experiments show an effect of dose on the level of bystander responses (Seymour and Mothersill, 2000; Baskar *et al.*, 2007), most do not produce a predictable dose-response relationship, and many show no dependence on dose at all (Lewis *et al.*, 2001; Yang *et al.*, 2005).

A number of studies have been conducted only with doses below 1 Gy (Ermakov *et al.*, 2005; Mothersill *et al.*, 2005b; Shankar *et al.*, 2006; Ermakov *et al.*, 2007), or including one high and one low dose (Lyng *et al.*, 2000, 2002b;

Mothersill *et al.*, 2005b). Together, the results of these dose-response experiments do not allow a prediction one way or the other, for whether a bystander effect *in vivo* would be dependent on dose. However, since some experimental systems demonstrate a dose-dependency, it is possible that the doses used in the previous adoptive transfer experiments were below such a dose-threshold. Thus, this experiment aimed to create a bystander environment *in vivo* with irradiated cells receiving a high dose-rate and high cumulative dose, and then monitor the response of the unirradiated cells in the recipient spleen tissue. This experiment repeated the conditions of the original experiments, but this time increasing the ^{3}H activity in the radiolabelling medium by 50-fold.

Spleens from eight donor pKZ1$^{-/-}$ mice were used to establish the radiolabelled donor cell culture. Donor T cells were radiolabelled for 18 h with 74 kBq.mL^{-1} of [methyl-^{3}H]-thymidine or sham-radiolabelled with the equivalent molar concentration of non-radioactive thymidine (23.25 nM). By the end of the radiolabelling period, 51% of cells had incorporated ^{3}H-thymidine into their DNA. Sham-radiolabelled cells were free of autoradiography grains. The incorporated radioactivity was 14.05 mBq per donor cell; the adjusted radioactivity was 27.5 mBq per radiolabelled donor cell (≈ 100-fold higher dose rate than the previous experiments). The average radioactivity of the sham-radiolabelled donor cells was negligible. All donor cells (radiolabelled and sham-radiolabelled) were labelled brightly with the CMRA fluorescent probe before adoptive transfer.

Five pKZ1$^{+/-}$ mice were injected with 5×10^{5} radiolabelled donor T lymphocytes whilst a further six received the same number of sham-radiolabelled donor cells. Each recipient mouse was euthanised 22 h after adoptive transfer and their tissues

isolated. The donor cell lodging frequencies in the spleen were significantly lower ($P = 10^{-7}$) in the mice receiving radiolabelled cells ($0.6 \pm 0.2 \times 10^{-4}$) compared to those receiving sham-radiolabelled control cells ($2.2 \pm 0.2 \times 10^{-4}$). Autoradiography in radiolabelled cell-recipients revealed that only 5 ± 4% of donor cells identified in the local screens were in fact radiolabelled (cf. 51% labelling before adoptive transfer). Donor cells in spleen sections from sham-radiolabelled cell-recipients were free from autoradiography grains. The reduction in the proportion of radiolabelled donor cells was further evidenced by the significant difference in the proportion of Ki-67–positive donor cells ($P = 7 \times 10^{-8}$) between the mice receiving sham-radiolabelled donor cells (39.6 ± 2%) and those receiving radiolabelled donor cells (10.6 ± 4%).

Results

There were no changes in apoptosis or proliferation

There was no change in global apoptosis frequency ($P = 0.7$) or the proliferation index ($P = 0.99$) in the spleen between mice receiving radiolabelled donor cells and those receiving sham-radiolabelled cells (*Table 5.9*). Because only 5% of the lodged donor cells remaining after 22 h *in vivo* were radiolabelled, there were too few fields to examine the radiolabelled fields alone. However, since the donor cells are known to lodge non-randomly, examination of the areas around the surviving lodged donor cells was likely to survey the areas where the radiolabelled donors were lodged shortly after adoptive transfer. Thus, the local screening was still performed on the spleen tissues without excluding fields where the donor cells were not radiolabelled.

Table 5.9: Summary of global screening results for apoptosis and proliferation

		Mean ± 95% Confidence Interval		T-test
		Donor Cells Received		
	Screen	*Sham-Radiolabelled*	*Radiolabelled*	*P*
Apoptosis Frequency	Global	$6.5 \pm 1.5 \times 10^{-3}$	$6.8 \pm 0.6 \times 10^{-3}$	0.7
Proliferation Index	Global	10.8 ± 3.4%	10.9 ± 3.5%	0.99

Similar to the results using the global screening method, there was no change in apoptosis frequency (P = 0.9) between mice receiving radiolabelled donor cells (8.6 ± 1.4 × 10^{-3}, ± 95% CI) and those receiving sham-radiolabelled cells (8.6 ± 1.5 × 10^{-3}, ± 95% CI) using the local screening method (without excluding unlabelled donor cells). Similarly, there was no change in the proliferation index in the spleen (P = 0.66) between mice receiving radiolabelled donor cells (9.0 ± 2.3%, ± 95% CI) and those receiving sham-radiolabelled cells (8.6 ± 1.7%, 95% CI) when examining all the local fields. There were no significant correlations with donor cell density within fields or the donor cell lodging frequency within mice, for either apoptosis or proliferation (P > 0.05).

Thus, even increasing the dose-rate of the irradiated donor cells by ≈ 100-fold did not induce a bystander effect for apoptosis or proliferation using the adoptive transfer model. These results support the null hypothesis of no bystander effect.

Detection of bystander effects from splenocytes exposed *ex vivo* to X-radiation

Bystander effects from low-LET radiations have previously been studied *in vitro* using either β-particles (Gerashchenko and Howell, 2004; Persaud *et al.*, 2005; Boyd *et al.*, 2006; Persaud *et al.*, 2007) or photons (Mothersill and Seymour, 1997; Seymour and Mothersill, 2000; Gerashchenko and Howell, 2003a; Liu *et al.*, 2004; Schettino *et al.*, 2005). Experiments labelling with β-particle emitting radionuclides produce a much lower dose-rate in the irradiated cells than those applying a single X- or γ-ray dose. Differences in the levels of damage produced (Stenerlöw *et al.*, 2002) and any interfering effects of prolonged radiation exposure (Amundson *et al.*, 2003) could alter the response of the irradiated cells. For example, in a direct comparison between photons and β-particles, bystander cytotoxicity saturated above 2 Gy γ-rays whilst increasing uptake of ^{131}I induced dose-dependent reductions in bystander cell survival (Boyd *et al.*, 2006).

In order to determine whether an acute X-ray dose would induce a bystander effect in the adoptive transfer system, where ^{3}H-radiolabelling had not, the aim of this experiment was to create a bystander environment *in vivo* using donor cells exposed *ex vivo* to a single dose of X-radiation, and monitor the response of the unirradiated cells in the recipient spleen tissue. Two radiation doses were chosen; 0.1 Gy, a lower dose similar to that applied in the chronic radiolabelling experiments, and 1 Gy, a higher dose in the range commonly used in bystander experiments conducted *in vitro*.

Five donor C57BL/6J mice were euthanised to establish the acute irradiated donor cell culture. Donor splenocytes were irradiated with 0.1 Gy, 1 Gy or were sham-

irradiated. Six C57BL/6J mice were injected with 5×10^5 0.1 Gy-irradiated donor splenocytes, six received 5×10^5 1 Gy-irradiated donor splenocytes whilst a further eight received the same number of sham-irradiated donor cells. Each recipient mouse was euthanised 22 h after adoptive transfer and their tissues isolated. The donor cell lodging frequencies in the spleen were significantly affected by treatment group (P = 0.025, ANOVA) across mice receiving 1 Gy irradiated cells ($3.6 \pm 0.6 \times 10^{-5}$), 0.1 Gy irradiated cells ($6.2 \pm 1.9 \times 10^{-5}$) or sham-irradiated cells ($5.8 \pm 2.3 \times 10^{-5}$) (*Table 5.10* and *Figure 5.11*). *Post-hoc* multiple comparisons showed a significant difference only between the 0.1 and 1 Gy groups (P = 0.037, *Bonferroni correction*). As expected, since no mitogen was added, the proportion of Ki-67–positive donor cells was <5% in each of the mouse groups.

Table 5.10: Summary of local and global screening results for apoptosis and proliferation

		Mean ± 95% Confidence Interval			
		Donor Cells Received			ANOVA
	Screen	*0 Gy*	*0.1 Gy*	*1 Gy*	*P*
Apoptosis Frequency	Local	$4.1 \pm 0.8 \times 10^{-3}$	$4.4 \pm 0.9 \times 10^{-3}$	$4.2 \pm 0.8 \times 10^{-3}$	0.9
	Global	$3.0 \pm 0.3 \times 10^{-3}$	$3.3 \pm 0.5 \times 10^{-3}$	$3.3 \pm 0.5 \times 10^{-3}$	0.5
Proliferation Index	Local	10.9 ± 1.4%	11.2 ± 1.4%	9.9 ± 1.0%	0.3
	Global	11.3 ± 1.2%	12.6 ± 2.0%	11.1 ± 1.4%	0.4

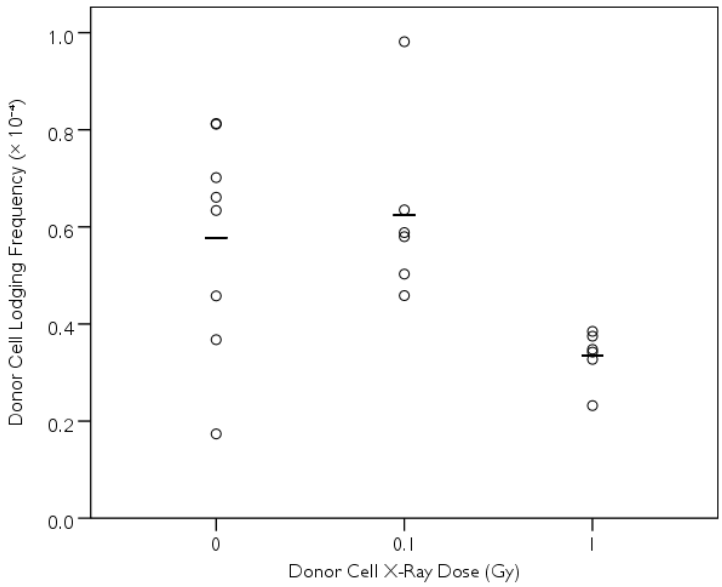

Figure 5.11: Donor cell lodging frequencies in the spleens of recipient mice

The donor cell lodging frequency was determined for each mouse from the duplicate global screens on TUNEL- and Ki-67–stained spleen sections. Circles (O) show mean values for each mouse, bars (—) show group means, *n* = 8, 6, 6 (0 Gy, 0.1 Gy, 1 Gy).

Results

There were no changes in apoptosis or proliferation

There was no effect of treatment group on apoptosis frequency or the proliferation index in bystander cells between mice receiving 0, 0.1 or 1 Gy-irradiated cells *(Table 5.10*) using either screening method ($P > 0.3$, ANOVA). There were no significant correlations with donor cell density within fields, or the donor cell lodging frequency within mice, for either apoptosis or proliferation ($P > 0.05$).

Again, these results failed to meet the criterion set for the detection of a bystander effect *in vivo*. Thus, donor cells irradiated with either 0.1 or 1 Gy did not induce a bystander effect using the adoptive transfer model.

Summary

This chapter described a series of investigations to detect radiation-induced bystander effects *in vivo*, examining a range of experimental conditions as summarised in *Table 5.11*. In each of the experiments detailed here, there were no significant differences in apoptosis or proliferation between mice receiving irradiated donor cells and those mice receiving sham-irradiated control cells, for either the local or global screening methods (*Table 5.12*). As such, no evidence was found for a radiation-induced bystander effect in this *in vivo* system. Similarly, when pKZ1 inversions were analysed in spleen tissues from transgenic pKZ1$^{+/-}$ recipient mice in the increased cell numbers experiment, no significant difference between the treatments groups was found; although, the trend towards a decreased inversion frequency was the largest effect size observed out of the three endpoints examined (48.5% decrease). The frequency of pKZ1 inversions can now be determined in the recipient mouse spleens from the longer-term lodging and high dose-rate experiments (which also included pKZ1$^{+/-}$ transgenic recipient mice) to investigate if a similar effect is observed. The performance of the adoptive transfer method as well as the significance and implications of the results described in this chapter will be discussed in the Chapter Six.

Table 5.11: Summary of experimental conditions

Experiment	Irradiation	Dose-rate ($mGy.h^{-1}$)	Maximum Accumulated dose (mGy)	Number of cells adoptively transferred	Lodging time *in vivo* *(h)*
Initial experiments	^{3}H	3.1	96	500,000	22
Increased donor cells experiment	^{3}H	2.16	67	5,000,000	22
Longer-term lodging experiments	^{3}H	2.91	236	500,000	72
High dose-rate experiment	^{3}H	258	7998	500,000	22
Acute X-ray experiment	X-rays	300,000	100	500,000	22
	X-rays	300,000	1000	500,000	22

Table 5.12: Effect sizes for apoptosis and proliferation across the series of experiments conducted to detect a radiation-induced bystander effect *in vivo*

Experiment	Apoptosis		Proliferation	
	Screening Method		*Screening Method*	
	Local	*Global*	*Local*	*Global*
Initial Experiments	-10.5%	-7.1%	-3.2%	-5.6%
Increased cell numbers experiment	ND	+10%	ND	+27%
Longer-term lodging experiments	+1.6%	-1.9%	+1.0%	+12.6%
High dose-rate experiment	0% [a]	+4.6%	-4.5% [a]	+0.9%
Acute X-ray experiment: 0.1 Gy	+2.4%	+10%	+2.8%	+11.5%
Acute X-ray experiment: 1 Gy	+7.3%	+10%	-9.2%	-1.8%

Effects sizes are calculated as the mean apoptosis frequency/proliferation index in the mice receiving radiolabelled/irradiated donor cells divided by the levels in the mice receiving sham-radiolabelled/sham-irradiated donor cells, minus 100%. Positive effect sizes indicate increases above the control, negative effect sizes represent decreases compared to the control mice.

ND- Local screening method not used

a Local screening method performed on all fields without excluding non-radiolabelled donor cells.