Chapter 3: Research intent and design

Précis

This chapter outlines which of the identified priorities for bystander research, described in the previous chapter, were included in the study's objectives as related to the overall study aim. It explains the series of specific research questions posed in the study and the hypotheses that were tested.

Research questions

The previous experimental findings on bystander effects *in vitro* and the radiation protection community's need for *in vivo* validation of these findings highlights the severe knowledge gap that exists in the bystander field – one of *in vivo* translation and relevance. This investigation aimed to determine whether intercellular communication of radiation effects between irradiated and unirradiated cells exists *in vivo*, and if so, if it could potentially alter the risk of radiation-induced cancer from a linear dependence on dose in the low-dose range. To this end, the following questions were posed:

1. Do radiation-induced bystander effects of the nature seen in cell culture investigations occur *in vivo*; if so,
2. Do biological effects induced in unhit cells change the fate of those cells, so as to potentially result in an altered risk of developing cancer; and,
3. Can such bystander effects be triggered at the low radiation doses required to result in non-uniform dose exposure?

Research method and hypotheses

(1) Do radiation-induced bystander effects of the nature seen in cell culture investigations occur in vivo?

The technical obstacles to irradiating specific cells *in situ* within a living animal necessitated the development of a novel method to answer this first question. With current technology, the most practical means to deliver a known dose to remote, specific cells within an animal is to isolate cells, irradiate or radiolabel them *ex vivo* and return them to the animal with a means to track their destination. Such an adoptive transfer method allows the construction of an experimental model with a small subset of identifiable, recently (or continually) irradiated cells sparsely located within an unirradiated host tissue. Parallel hosts that receive equivalent donor cells omitting only the radiation dose, control for the intrusion of the transferred cells and any signalling triggered by their isolation and *ex vivo* manipulation. After allowing time for the sending and receiving of any putative bystander signals, and for any effects to occur, the unirradiated host tissues of interest are isolated and examined.

Similar biological responses in hosts receiving irradiated or unirradiated cells, would support the null hypothesis – no bystander effect; a statistically significant difference between the groups (for any biological endpoint) would cause rejection of the null hypothesis and would provide evidence for the communication of effects from irradiated to unirradiated cells. In the event of failure to observe a bystander effect with initial experimental conditions, it would then be necessary to alter the experimental system to accommodate the factors previously found to be important in the communication of bystander effects *in vitro.* Only after comprehensively exploring the range of critical *in vitro* parameters, such as: radiation type, LET, dose-rate, cell-type, the timing of analysis and the ratio of irradiated-to-bystander cells,

would it be possible to conclude that *bystander effects of the nature seen in cell culture investigations* did not occur *in vivo*. This distinction is important. The broad nature of the bystander effect definition (as seen in the large variety of endpoints propounded as evidence for bystander effects) results in a problem for invalidating it. A formal, logical disproof is impossible; any change, in any endpoint, under any circumstances could corroborate the bystander hypothesis. It is necessary to accept instead, that for the discovery of an *in vivo* bystander effect to be of any value to estimating carcinogenic risks after low-dose radiation exposures, it must occur under realistic conditions, relevant to that field. Such an approach limits the burden of proof required to contradict the bystander effect theory and allows for testable and falsifiable hypotheses. Failure to do so permits the misidentification of experimental artefacts or completely distinct phenomena (e.g. abscopal effects) as bystander effects, simply by virtue of their meeting vague, ill-defined criteria. Thus, this study is limited to conditions facing members of the public exposed to low dose-rate radiation, and explores the first research question in this context.

(2) Do biological effects induced in unhit cells change the fate of those cells, so as to potentially result in an altered risk of developing cancer?

In line with the first question's limits for disproving the bystander theory, the second narrows the criteria for proving the effect. This study aimed to determine whether intercellular communication between irradiated and unirradiated cells occurs *in vivo, and if so,* if it would alter current estimations of cancer risk following low-dose radiation exposures. Under the current broad definition, transmission of any *effect* from irradiated to unirradiated cells would validate the bystander hypothesis. However, the existence of bystander effects after low-dose radiation exposures is of interest to radiation risk assessors specifically because of the implications of altering

carcinogenic risk estimates. The induction of a biological endpoint that ultimately, does not significantly alter the risk of tumour development in bystander cells may be a curious phenomenon, but would fall outside the scope of interest for protection against the carcinogenic potential of radiation.

Monitoring bystander cells to evaluate carcinogenesis directly was beyond the scope of this project, requiring prohibitively large numbers of animals to have sufficient statistical power (even using cancer-prone animals). Therefore, in order to evaluate the contribution of bystander effects to carcinogenesis, the analyses were restricted to biological endpoints expected to affect the initiation, promotion, or eradication of neoplastic cells – termed cell fate. Thus, similar bystander cell fates in hosts receiving either irradiated or unirradiated cells, would support the null hypothesis – no contribution of bystander effects to cancer risk; whilst a statistically significant change in bystander cell fate between the groups would cause rejection of the null hypothesis in favour of the alternate hypothesis – that bystander effects may influence cancer risk. Immediate translation from effects on surrogate markers of carcinogenesis, or short-term cell fate, to altered cancer risk is not possible. However, demonstrating a perturbation of key stages in cancer development would allow further investigations to determine the magnitude of the effect and any possible counteractions in order to evaluate whether such an effect results in a meaningful change in cancer risk.

(3) Can such bystander effects be triggered at the low radiation dose-rates required to result in non-uniform dose exposure?

Whereas the first research question limits the field of the search for bystander effects, the second and third questions narrow the goal. Whether a bystander effect produced

in laboratory conditions, even one with a direct link to a cancer endpoint, alters carcinogenic risk after exposure to radiation is perhaps the most pertinent question of all. A cell nucleus irradiated with 10 Gy may indeed signal to its neighbours; however, it is difficult to imagine such a cell surrounded by unirradiated bystander cells, except under experimental conditions. Likewise, if cells have mechanisms for dealing with bystander exposure conditions it is reasonable to presume that they developed in the context of very low fluence high-LET particles and/or a low flux of photons that create rare ionisations, isolated in both time and space. The third research question ensures that any finding of a bystander effect, even one changing endpoints likely to alter carcinogenic risk, also meets the criteria of being inducible by realistic and relevant conditions.

Summary

This thesis thus describes the development and validation of an experimental method, and the use of this method to answer the three questions posed here. The success of these investigations, and the implications and significance of the results to the field of bystander effect research, is then discussed.

Chapter 4: Development of the adoptive transfer method for detecting bystander effects *in vivo*

Précis

This chapter details the stages of selection, planning, testing and optimisation of the techniques used in developing the adoptive transfer method designed to test the research questions outlined in Chapter 3 and describes the experimental method in detail. The results of pilot experiments to validate the method are also presented.

Initial decisions in the development of the *in vivo* bystander method

Use of animals

Alternatives to the use of animals in testing the research hypotheses were considered. Much work has been done in traditional mammalian cell culture systems (Mothersill *et al.*, 2006), three-dimensional cell culture (Gerashchenko and Howell, 2003b), artificial human tissue constructions (Belyakov *et al.*, 2005) and primary cells from human donors (Marozik *et al.*, 2007). Each of these methods has their advantages and disadvantages. Regulators, however, have communicated the need to demonstrate bystander effects *in vivo* before they can be taken into account when considering cancer risk estimation (ICRP: Publication 99, 2006). *In silico* models of low-dose radiation responses have been developed incorporating various detrimental and protective bystander effects (Khvostunov and Nikjoo, 2002; Nikjoo and Khvostunov, 2003; Ballarini *et al.*, 2006; Liu *et al.*, 2007; Schöllnberger *et al.*, 2007); these models are nevertheless based on the *in vitro* experimental findings and still require *in vivo* validation. It was thus necessary to answer the research questions

in the context of a whole-animal system. The Flinders University Animal Welfare Committee approved and monitored all the experiments involving the use of animals.

Choice of species

The adoptive transfer method was developed in mice for a number of reasons. Although the majority of research groups conduct bystander effect studies *in vitro* using human cell lines (usually lung epithelial cells in order to simulate domestic/occupational exposure to inhaled radon progeny), most perform *in vivo* studies in laboratory rodents (Watson *et al.*, 2000; Xue *et al.*, 2002; Lorimore *et al.*, 2005; Mothersill *et al.*, 2005b; Kishikawa *et al.*, 2006). Rodents are also the model of choice for adoptive transfer experiments – with only a few large-mammal studies (ovine and porcine) previously conducted (reviewed in Pabst and Binns, 1989). Experimentation in rodents is cheaper, quicker and easier than large-mammal studies. Since adoptively transferred allogenic cells are eliminated from the circulation within hours, inbred strains are needed (readily available for rodents) to avoid the need for autologous transfer (Butcher and Ford, 1986). Previous studies have investigated bystander effects *in vivo* (Watson *et al.*, 2000; Xue *et al.*, 2002; Mothersill *et al.*, 2005b), and low-dose radiation effects in general (Lorimore *et al.*, 2001; Coates *et al.*, 2003; Hooker *et al.*, 2004b) in mice. Thus, mice were considered the ideal species for establishing the adoptive transfer method. There is limited evidence that there are sex-specific responses to low-dose radiation exposure (Raiche *et al.*, 2004; Cassie *et al.*, 2006) and the induction of radiation-induced phenomena such as abscopal effects (Pogribny et al., 2004; Koturbash et al., 2008a; Koturbash et al., 2008b). This study used male and female mice in mixed groups and noted their responses, although it was not designed with the statistical power to analyse sex-specific responses.

Choice of mouse strain

The experiments in this investigation were performed in the C57BL/6 mouse strain based on data regarding their response to radiation and their proven responses to bystander stimuli. The C57BL/6 mouse is generally considered a comparatively radioresistant strain in terms of deterministic and stochastic effects (ICRP: Publication 99, 2006, pp. 81-2) and is commonly used in radiobiology studies. Experiments investigating the effect of strain differences on the bystander response show C57BL/6 but not CBA/Ca mouse-induced bystander apoptosis in a bladder epithelium explant model (Mothersill *et al.*, 2005b). C57BL/6 mice are more resistant than BALB/c strains to tumour formation after whole-body irradiations (Ullrich *et al.*, 1996; Ponnaiya *et al.*, 1997) and show over twofold higher apoptosis in the spleen and higher levels of stabilised TP53 than the DBA/2 strain (Coates *et al.*, 2003). Studies of ionising radiation-induced genomic instability have shown C57BL/6 strains to be 'relatively resistant' compared with CBA/Ca mice (Gowans *et al.*, 2005). In addition to a radiation response free from inherited DNA-repair defects identified in other strains, the relative radioresistance of C57BL/6 mice suggests a possible role for low dose protective/defence mechanisms conferred by bystander signalling.

Some experiments in this study used pKZ1 transgenic mice (originally described by Matsuoka *et al.*, 1991) to provide a DNA-level endpoint: chromosomal inversions. pKZ1 mice irradiated with low doses of X-rays (5 - 100 µGy) where the electron track density in the tissue is less than one track per cell, show an induction of chromosomal inversions in spleen and prostate (Hooker *et al.*, 2004b; Zeng *et al.*, 2006). The energy deposited at these low doses suggests that the chromosomal inversions represent a marker of changed DNA-maintenance and/or repair rather than

direct DNA damage. The pKZ1 assay also displays a non-linear radiation dose-response with doses of 1 and 10 mGy reducing the inversion frequency below the unirradiated control levels, and adaptive responses of equal magnitude after conditioning doses between 1 µGy and 10 mGy (Day *et al.*, 2006). Together, these results suggest that bystander signalling within the spleen tissue controls the response to radiation in the very low dose range. Thus, later experiments with this adoptive transfer model were performed in pKZ1 mice to allow the assessment of chromosomal inversions in bystander cells.

The pKZ1 mouse (bred on a C57BL/6J background) is hemizygous for a transgene encoding the *Escherichia coli* gene *lacZ*. The transgenic construct contains the *E. coli lacZ* gene flanked by recombination signal sequences from the Vκ21c and Jκ5 gene segments of the mouse immunoglobulin light chain gene. The recombination signal sequences normally facilitate the formation of secondary structures in the DNA of the immunoglobulin light chain gene that are recognised by the recombination activating gene products RAG1 and RAG2 during immunoglobulin gene rearrangement in early B cells. Enzymatic processing of the secondary structures by RAG1 and RAG2 normally results in deletion of the intervening sequence and rejoining to form a contiguous V–J rearrangement. The resolution of such hairpin secondary structures specifically by RAG1/RAG2 or non-specifically by alternate DNA-repair pathways can sometimes result in the correct rejoining of the intervening sequence (reverting to the original sequence) or rejoining in the reverse orientation, giving rise to an inversion of the DNA sequence.

The pKZ1 transgene construct encodes the *lacZ* gene in the antisense transcriptional orientation with respect to the upstream constitutive enhancer promoter complex

from the chicken β-actin gene; preventing expression in its original form. However, in the rare event of an inversion of the sequence between the pair of recombination signal sequences, the *lacZ* sequence is now in the sense orientation in an open reading frame and thus transcription can occur. The *lacZ* gene product is the enzyme β-galactosidase. The pKZ1 assay enables the detection of *E. coli* β-galactosidase activity in cells using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). The pKZ1 mice used here were sourced from a colony maintained at the Flinders Medical Centre animal house facility, originating from founder stock described in Matsuoka *et al.* (1991).

Pure C57BL/6J mice and C57BL/6J mice hemizygous and nullizygous for the pKZ1 transgene were group-housed (littermates) in microisolator cages (12 h light/dark cycles) under Physical Containment Level 2. Mice were fed on joint stock ration (Ridley Agriproducts, SA, Australia) and sterile water *ad libitum*. All mice used in the experiments were <5 months old. Full quality control for viruses, parasites, and bacteriology was performed on a quarterly basis and all mice tested had a negative status. Transgenic status for each mouse from the pKZ1 colony was determined by PCR amplification of the transgene sequence from DNA extracted from a tail clipping (method described in detail in Day *et al.*, 2007a).

Euthanasia method

Mice were euthanised by carbon dioxide asphyxiation following the guidelines published by the *International Consensus Meeting on Carbon Dioxide Euthanasia of Laboratory Animals* (Hawkins *et al.*, 2006), in compliance with *The Australian and New Zealand Council for the Care of Animals in Research and Teaching* recommendations (Marshall *et al.*, 1994). Briefly, mice were removed from their

cages and placed into a sealed chamber that was then flooded with carbon dioxide (at less than twenty per cent of chamber volume per minute) for at least six minutes, before isolating tissues.

Choice of cells and tissues

For this study, lymphocytes were chosen as the target irradiated cells and the spleen as the initial bystander organ for study (although liver, lung and bone marrow samples were collected in parallel for future study). Development of the adoptive transfer method involved creating a bystander environment by the transfer of *ex vivo*-irradiated cells into unirradiated host tissues to achieve homogeneity of the donor cells and host tissue. Although the intricacies of splenic tissue architecture are often underestimated, the spleen does provide a receptive environment where adoptively transferred lymphocytes from a syngeneic donor can not only lodge, but also immediately begin to participate in the functioning of the organ (Manfra *et al.*, 2001). The fluid nature of splenic lymphoid tissue allows the donor cells to interact with the indigenous cell population within a normal *in vivo* microenvironment, unlike with transplanted tumours or tissue engraftments. Unlike in previous bone marrow transplantation models for studying bystander effects (Watson *et al.*, 2000; Lorimore *et al.*, 2005), adoptively transferred lymphocytes will lodge and remain in the spleen without the need for high-dose irradiation of the recipient mice.

The spleen has previously been identified as an ‘important radiation target organ’ (Koturbash *et al.*, 2007) and has been implicated in generating abscopal effects after high-dose irradiations (Pogribny *et al.*, 2004; Raiche *et al.*, 2004). The sensitivity of the haematopoetic system to deterministic effects of high-dose radiation has also resulted in the radiation responses of circulating lymphocytes and lymphoid tissues

to be closely examined (Fisher and Harty, 1982; Olivieri *et al.*, 1984; Fenech and Morley, 1986; Shadley *et al.*, 1987; Wiencke *et al.*, 1992; Radford, 1994; Shankar *et al.*, 1999). It was important that donor cells should not accumulate in the tissue under investigation to the point where they disrupted organ function. Donor cells which are lodged sparsely throughout a tissue allow the examination of regions of host tissue under the influence of single irradiated cells, useful for studying local signalling. Adoptive transfer of lymphocytes offers great flexibility in altering donor cell burden, with highly predictable localisation patterns.

Adoptive transfer of splenocytes

The potential for administering competent immune cells for therapy has driven research over the past decades into the behaviour of adoptively transferred lymphoid cells (reviewed by Sprent and Miller, 1973). Our understanding of trafficking and localisation of lymphoid cells in recipient hosts has developed from the idea of a non-specific entrapment of cells in tissues with large vascular catchments (Sprent, 1973; Sprent and Basten, 1973) to one of highly targeted mobilisation and recruitment of circulating lymphocytes (McEvoy *et al.*, 1997; Tang *et al.*, 1998). The adoptive transfer method developed in this study was based on a large body of data in the literature on splenic lymphocyte localization, which is reviewed below. The studies described below were all conducted in mice, however, porcine studies have largely reported similar results (Pabst and Binns, 1989).

The earliest studies demonstrated that intravenously transferred splenic lymphocytes showed highly reproducible patterns of tissue localisation within the first hour after injection, with a gradual redistribution from the spleen (maximum uptake after 1 h) to lymph nodes (maximum uptake after 18–24 h) (Butcher and Ford, 1986; Pabst and

Binns, 1989). Pabst and Binns (1989) reviewed the early characterisations of the behaviour of adoptively transferred lymphocytes showing that:

- Upon entering the spleen, both B and T lymphocytes leave the blood in the marginal zones where B cells remain whilst T cells home to the periarteriolar lymphatic sheath.
- Homing of lymphocytes to the spleen is unaffected by donor cell treatment with trypsin, dextran sulphate or low temperatures during *in vitro* culture; but,
- Concanavalin A or corticosteroid treatment do result in reduced splenic localisation; and,
- Dead or dying donor cells show no propensity for lodging in the spleen.

Unstimulated (G0/G1 and early S-phase) lymphocytes predominantly localise in the liver, lung and then spleen whereas Concanavalin A-stimulated (late S and G2+M-phase) lymphoblasts derived from any lymphoid tissue home to liver, small intestine, spleen, lung, large intestine and then bone marrow (Tseng *et al.*, 1989). Mechanisms for the specificity of lymphocyte localisation were later identified, and confirmed with adoptive transfer experiments. The cell surface marker CD43 mediates splenic T cell homing to the recipient spleen; when donor CD43 is blocked, transferred T cells accumulate in the blood in the first few hours and localisation in the spleen only returns to normal gradually over at least forty-eight hours (McEvoy *et al.*, 1997). Short and long-term homing of transferred T and B lymphocyte subsets to non-splenic lymphoid organs is dependent on L-selectin expression (Tang *et al.*, 1998). When equivalent numbers of normal B and T cells are transferred, the donor cells found in the spleen after one or forty-eight hours are predominantly T cells (~10% of

the total T cells injected) and increasing the number of injected cells above twenty million does not affect the relative tissue migration frequencies (Tang *et al.*, 1998).

T-memory and T-naïve donor cell subsets can be identified in the spleen up to two weeks after injecting immature T lymphocytes (Manfra *et al.*, 2001), proving that adoptively transferred lymphocytes can participate in host immune reactions and that lodged cells exhibit normal behaviour *in situ*. Together, these results confirmed the suitability of T lymphocytes as donor cells for the *in vivo* bystander effect model. The cells were expected to lodge within the spleen shortly after injection (1–2 hours: Albright *et al.*, 1998; Manfra *et al.*, 2001; Moeller *et al.*, 2003), and to localise to specific areas within the white pulp. The rapid clearance of the donor cells from the bloodstream and the gradual redistribution of transferred T cells from the spleen to the lymph nodes increased the chances that identified donor cells would have remained *in situ* since initial lodging rather than having recently migrated to the spleen. Changes in lymphocyte localisation patterns in the spleen have been observed between old and young recipients. Experiments demonstrated reduced acquisition of donor cells in the spleen of older recipients (>26 months) regardless of the age of the donor mouse (Albright *et al.*, 1998), attributed to a deterioration of the splenic microenvironment with age. For this reason, all experiments were conducted in young mice < 5 months old.

Summary

Based on the requirements of the *in vivo* bystander effect model, the adoptive transfer model was established in male and female C57BL/6J mice (and mice containing the pKZ1 transgene) using splenic lymphocytes lodged in the spleens of host mice.

Donor splenocytes: Chronic radiolabelling experiments

Method outline

The adoptive transfer method for detecting radiation-induced bystander effects *in vivo* was based on the premise of obtaining splenocytes from donor mice, introducing a stable, radioactive source into the donor cells, labelling the cells to allow them to be identified later and then re-introducing them into recipient mice (*Figure 4.1*). This section outlines and justifies the decisions that were made in the development and optimisation of the donor cell radiolabelling protocol, including the results of preliminary experiments. It details the final methods that were chosen, and the results of experiments performed to validate the protocols.

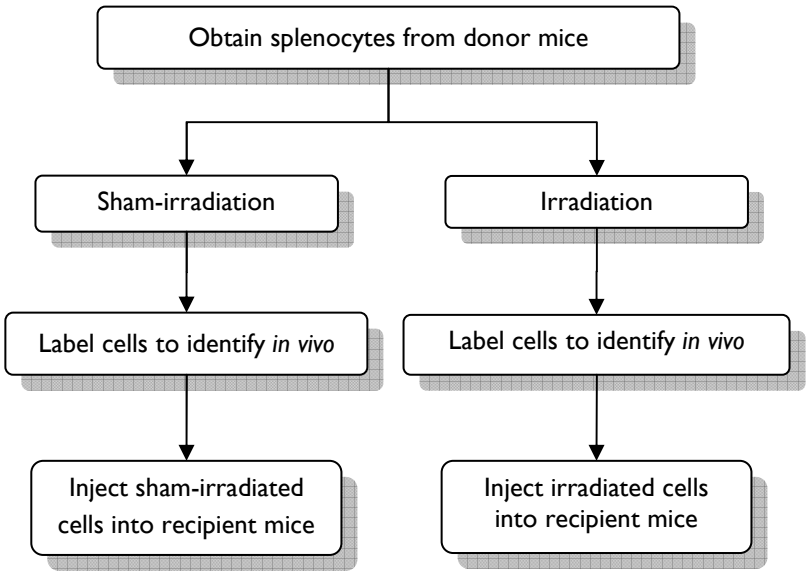

Figure 4.1: Starting method for chronic radiolabelling experiments

The steps in the initial strategy for the chronic radiolabelling adoptive transfer method are shown.

Selecting a technique to irradiate donor cells

Choosing a radionuclide

For the chronic irradiation scenario, the choice of radiation source was based on three factors. The ideal radionuclide would:

- emit β-particles with sub-cellular path-lengths without producing penetrating gamma photons, to confine energy deposition to target cells;
- deliver an absorbed dose per disintegration low enough to allow chronic sub-lethal irradiation (< 10 $mGy.Bq^{-1}$); and
- be incorporated into a stable, localisable biological molecule.

The radioactive hydrogen isotope tritium (^{3}H) was the only radionuclide that could fulfil each of these prerequisites, and followed by the Auger electron emitting isotopes of iodine, is the radionuclide used most extensively to self-irradiate individual cells (Hofer and Hughes, 1971; Bishayee *et al.*, 1999; Bishayee *et al.*, 2000; Howell and Bishayee, 2002; Persaud *et al.*, 2005; Boyd *et al.*, 2006). Tritium is a low energy β-emitter with average decay energy of 5.7 keV (maximum 18.6 keV) and a half-life of 12.33 years (reviewed in Fairlie, 2003). The mean path-length of tritium's low energy β-particles in water is 1 µm with a maximum of 7 µm (reviewed in International Commission on Radiation Units and Measurements, 1970).

A β-particle travelling through tissue loses energy by ionising and exciting other molecules as it passes, and as a result, it gradually slows down. Towards the end of its track, the β-particle (now with less velocity) can interact with more molecules, producing more ionisations and slowing exponentially. Thus, each β-particle deposits a majority of its energy in a small area at the terminus of its track with the more

isolated ionisation events along the particle's initial path estimated to be of 'relatively little biological significance' (Nikjoo and Goodhead, 1991).

The radiation weighting factor of tritium is currently set at unity (ICRP: Publication 60, 1991). Experimentally however, tritium's relative biological effectiveness depends largely on:

- its form (i.e. tritiated water or bound to organic molecules);
- its distribution (whether distributed evenly in body water or concentrated by its incorporation into bio-molecules such as DNA or proteins); and,
- the cell-type and biological endpoint in question.

Choosing a radiolabelling molecule

In order to confine the energy deposition within the cells, it was necessary to contain the tritium atoms themselves within the target cells, and since tritiated water distributes evenly within body water (reviewed in Fairlie, 2003) the tritium atoms needed to be incorporated into a stable organic molecule. Although tritium can be substituted for hydrogen in amino acids, lipids or other cellular precursors, the radiolabelling of nucleosides – primarily thymidine as the only nucleoside unique to DNA – is the most common method, and the method used by *in vitro* bystander experiments examining chronic self-irradiation (Bishayee *et al.*, 1999; Bishayee *et al.*, 2001; Persaud *et al.*, 2005; Pinto *et al.*, 2006).

The need to assign increased risk for tritium incorporated into DNA molecules is still a matter of debate and formed a key deliberation of the United Kingdom's *Committee Examining Radiation Effects of Internal Emitters*. On one hand, the fact that the mean electron path length of tritium and the diameter of a chromosome are

on the same scale (~1 µm) immediately suggests the damaging possibility of high-density ionisations at electron track ends intersecting with DNA (Day, 2003). Conversely, since relative biological effectiveness is simply an experimental observation of biological impact per unit dose with respect to a reference radiation – usually 250 kVp X-rays or γ-rays from ^{60}Co or ^{137}Cs (ICRP: Publication 92, 2003) – any additional risk should be evident upon examination (Harrison, 2003). *In vivo* comparisons show the relative biological effectiveness of tritium to vary widely (between 1 and 3.4) whether tritiated water or tritiated thymidine are used (Straume, 1991), and reveal no consistent elevation of risk from DNA-incorporated ^{3}H. This may be due to the counteractive increase in relative biological effectiveness of tritiated water due to its concentration in the hydration shell of DNA – caused by the increased hydrogen bond strength between ^{3}H and ^{1}H (Mathur-De Vre *et al.*, 1982).

As such, the proximity of incorporated tritiated thymidine to DNA is taken into account in microdosimetric calculations of absorbed dose; however, no relative increase in biological effect is factored into estimations of the dose equivalent (ICRP: Publication 92, 2003). This disjunction between observed relative biological effectiveness and prescribed radiation weighting factors is at the heart of current controversy surrounding qualitative and quantitative differences in radiation quality – as can be seen in the dissenting views in the final report of the *Committee Examining Radiation Effects of Internal Emitters* (2004, p. 5). Radiolabelling the DNA of cultured cells is a simple matter of adding exogenous ^{3}H-thymidine to the culture medium prior to or during DNA replication; since in proliferating cells, ^{3}H-thymidine is phosphorylated to form ^{3}H-thymidine monophosphate and thus enters the nucleotide pool used for DNA synthesis (Cleaver, 1967).

Preliminary results with donor cell radiolabelling

Since cells must undergo DNA synthesis in order to incorporate ^{3}H-thymidine into their DNA, donor splenocytes needed to be cultured *ex vivo* to facilitate radiolabelling. The culture conditions needed here were optimised to ensure the maximum proliferation possible, whilst reducing the total time *ex vivo* and keeping manipulation of the cells to a minimum. In preliminary experiments, primary splenic mononuclear cells, isolated using standard density-gradient centrifugation techniques, were established in culture. To stimulate DNA synthesis, the cells were exposed to the polyclonal mitogen concanavalin A (Sigma Aldrich Corp., MO, USA). Despite attempts to improve the proportion of donor cells proliferating during the culture period, the splenocytes showed a poor response to mitogen stimulation (<10% of cells in S-phase from 0–40 h) until the third day in culture (data not shown). A possible explanation was that the concanavalin A was primarily stimulating the T lymphocytes that represented only a minority of the cells in the splenocyte cultures (Piguet *et al.*, 1976). This suspicion was confirmed when the cultured donor splenocytes showed significantly enhanced proliferation when co-stimulated with *E. coli* lipopolysaccharide, a known B lymphocyte mitogen (data not shown). However, the use of lipopolysaccharide to stimulate the donor cells in the adoptive transfer method would have been impractical since the endotoxin would have induced an immune response when the cells were adoptively transferred (Peavy *et al.*, 1978; Groeneveld and van Rooijen, 1985).

Although concanavalin A does stimulate both B and T lymphocytes, the response of the B cells is a secondary response dependent on the presence of co-stimulated T cells (Mugraby *et al.*, 1975) and is delayed compared to the T cell response (Boldt *et al.*, 1975). Stimulation of isolated splenic T lymphocytes with concanavalin

A produces a more robust proliferative response (Mugraby *et al.*, 1975). This finding was confirmed here in the mouse primary splenocyte cultures by separating the B and T cell fractions by antibody labelling and separation using paramagnetic beads (*Pan T Cell Isolation Kit*, Miltenyi Biotec GmbH, Germany, method detailed below). The T cell fraction showed an earlier proliferative response to concanavalin A compared to the unseparated spleen cells (*Figure 4.2*) with the peak after 40 h in culture; the unseparated cells didn't reach the same level even after 90 h. From this point forward, the donor cell isolation, *ex vivo* culture and mitogen stimulation were optimised specifically for splenic T lymphocytes.

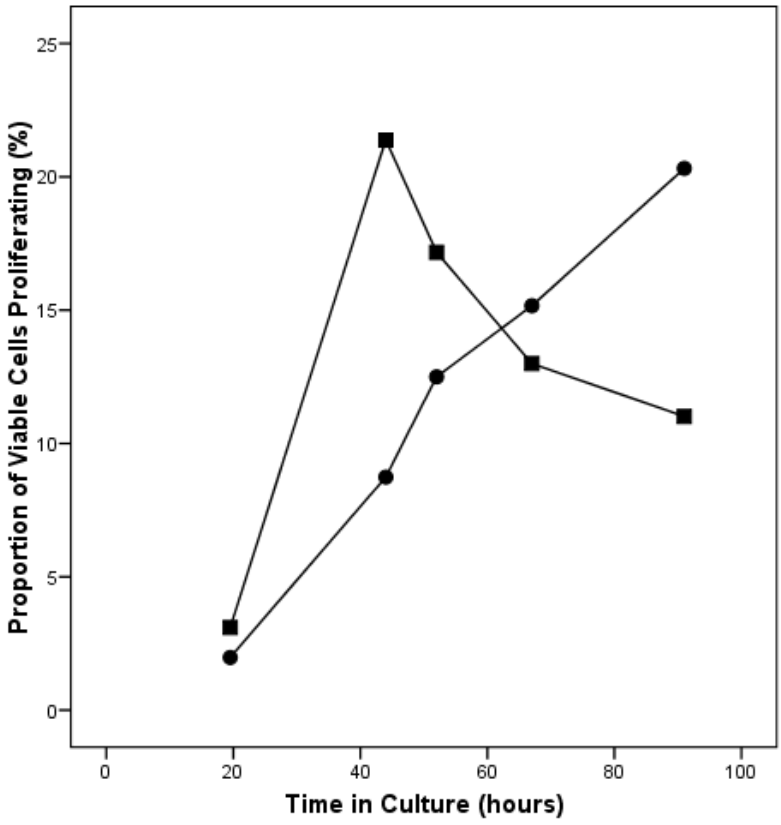

Figure 4.2: Proliferative response to concanavalin A in donor splenocytes

Splenocytes prepared by density gradient centrifugation (circles) or T lymphocytes further isolated by magnetic separation (squares) were stimulated with 5 $\mu g.mL^{-1}$ concanavalin A and cultured for 95 h. At various times, cells were sampled and the DNA was stained with propidium iodide. The DNA staining of each cell was measured by flow cytometry and those with DNA content between 2N and 4N were counted as proliferating cells. The proportion of viable cells proliferating (%) is shown for both populations of cells.

Isolation of splenocytes and preparation of donor cell cultures

Mononuclear cells were isolated from donor mouse spleens using a standard density-gradient centrifugation technique before T lymphocytes were separated by negative-selection with antibodies bound to paramagnetic beads. The use of magnetic cell-separation techniques is an efficient and highly specific method for isolating target cells suitable for injection into recipient animals (Kamath *et al.*, 2004; Skokos *et al.*, 2007). Cells can be isolated using a positive (Martin-Fontecha *et al.*, 2003) or negative selection (Kamath *et al.*, 2004) regimen or a combination of both (Leithauser *et al.*, 2001). Using a negative-selection system allows the cells of interest to pass through the column without antibody binding while the unwanted cells are captured. The *Pan T Cell Isolation Kit*, which uses a range of antibodies directed against non-T lymphocyte cell-surface markers, has been used previously to isolate untouched splenic T lymphocytes for adoptive transfer (Sun *et al.*, 2005). Pre-isolation of mononuclear cells with density-gradient separation, although not necessary, further increased the yield of the target cells by removing non-target cells (data not shown). The *Pan T Cell Isolation Kit* worked as follows: a cocktail of biotinylated antibodies with affinity for non-T lymphocyte cell-surface markers (CD11b, CD45R (B220), DX5 and Ter-119) was added to the cell suspension, which bound to their respective cell-types. Paramagnetic beads coated with streptavidin were then added which bound to the biotin molecules on the antibodies. By passing the cell suspension through a column in a magnetic field, those cells bound to the antibody-biotin – streptavidin-bead complexes were captured while the unlabelled cells passed through.

The other common method of concentrating T lymphocytes from spleen cell suspensions (Lee *et al.*, 2004; Igari *et al.*, 2007) involves passing the cells through a

nylon wool column and collecting the non-adherent cells (Julius *et al.*, 1973). The negative-selection strategy offered by the magnetic separation technique was chosen as it gives a more pure T cell isolate than nylon wool adherence (Callahan and Moynihan, 2002; Thomas *et al.*, 2003; Reynolds *et al.*, 2007).

Method:

For each experiment at least five mice were euthanised, their spleens removed and placed into ice-cold cell culture medium (RPMI 1640 [CSL Limited, VIC, Australia] supplemented with 5% v/v foetal bovine serum [Trace Scientific Ltd., VIC, Australia], 0.29 $g.L^{-1}$ L-glutamine [Trace Scientific Ltd.], 50 IU/mL penicillin [Trace Scientific Ltd.], 50 µg/mL streptomycin [Trace Scientific Ltd.] and buffered with 0.21% w/v Sodium Bicarbonate); hereafter 'RPMI medium'). The splenic capsule was gently perfused with RPMI medium and massaged to expel splenocytes into the medium. A syringe with 21-gauge needle was used to prepare a single-cell suspension. The cell suspension was then under-laid with an equal volume of polysucrose/sodium ditrizoate solution (*Histopaque® 1083*, Sigma Aldrich Corp.) at a density of 1.083 $g.mL^{-1}$ (designed for the isolation of murine mononuclear cells) before high-speed centrifugation at 1050 *g* for 10 min. The mononuclear cells which collected at the medium-*Histopaque®* interface were transferred to fresh RPMI medium. The collected cells were centrifuged (377 *g* for 15 min) and the supernatant was aspirated and replaced with fresh RPMI medium, twice, to remove any remaining separation medium. Splenic T lymphocytes were then isolated using the *Pan T Cell Isolation Kit* following the manufacturer's instructions.

Briefly, isolated splenic mononuclear cells were resuspended in 40 µL of *MACS cell separation buffer* (phosphate buffered saline (PBS) supplemented with 0.5% foetal bovine serum and 2 mM EDTA, freshly prepared and 0.45 µm filter sterilised, Millex-GS

disposable filter, Millipore Corp., MA, USA) per 10^7 cells. *Biotin-Antibody Cocktail* was added at 10 µL per 10^7 cells. Cells were incubated for 10 min at 4°C before adding 30 µL of MACS buffer and 20 µL of *Anti-Biotin Microbeads* per 10^7 cells. Cells were incubated for 15 min at 4°C before adding 10 × labelling volume of MACS buffer. Cells were centrifuged at 170 *g* for 10 min and then the supernatant was aspirated. The cell pellet was resuspended in 500 µL of MACS buffer per separation column to be used. The *MS Columns* were placed in the magnetic field and rinsed with 500 µL of MACS buffer. For each column, the 500 µL of cell suspension was applied, followed by 3 × 500 µL washes with MACS buffer, collecting the isolated T cell suspension in the effluent. The recovered viable cells (that excluded trypan blue) were counted using a haemocytometer.

The effectiveness of the T cell isolation procedure on two independent spleens was assessed by labelling the positive- (magnetic bead–bound non-T cells) and negative-fractions (untouched T cells) with fluorescently-tagged antibodies against the pan T cell marker CD90 (*anti-CD90–FITC*, Miltenyi Biotec GmbH) and biotin (*anti-Biotin–PE*, Miltenyi Biotec GmbH). Counting 15,000–30,000 cells by flow cytometry (*FACScan*®, Beckman Coulter, Inc., CA, USA) showed that 92.5 ± 2.3%[1] of the T cell fraction was CD90+ and less than 0.1% were labelled with biotinylated antibodies. The yield was assessed by comparing the number of CD90+ cells in the unseparated and recovered T cell fractions revealing a recovery of 97% of the CD90+ cells, an average of 17 ± 6% of the total number of cells. The mean number of total splenocytes isolated per spleen for the 23 donor mice used in the pilot experiments was 6.8 ± 0.7 × 10^7, equating to approximately 1.2 × 10^7 T lymphocytes that could be obtained per donor mouse.

[1] All values shown in this thesis are the mean ± 1 standard deviation unless otherwise indicated.

Ex vivo culture of donor splenic T lymphocytes

Designing a complete culture medium

Few adoptive transfer experiments include an *ex vivo* culture period of the donor cells on which a radiolabelling protocol could be modelled. Thus, the protocol for establishing the T lymphocytes in culture prior to injection was largely based on one adoptive transfer study that did include a radiolabelling culture period (Tseng *et al.*, 1989) as well as considering methods from several other studies that cultured splenic T cells not destined for transplantation (Ben-Efraim *et al.*, 1976; Mulligan *et al.*, 1998; Frasca *et al.*, 1999; Manfra *et al.*, 2001). Although minor variations were seen in the published splenic T cell culture protocols, a basic repertoire was identified. The common conditions included incubation in RPMI 1640 cell culture medium supplemented with 200 mM $_{L}$-glutamine, antibiotic(s), 5–10% foetal bovine serum and 0–50 µM β-mercaptoethanol. Cell densities ranged from 10^5 to 10^7 cells per millilitre in 200 µL volumes. Cultures were incubated at 37°C in a humidified atmosphere of 5–10% CO_2 in air. The consistent use of these culture conditions left a number of decisions: whether to add β-mercaptoethanol, and the choice of serum levels; and, a number of parameters to be optimised experimentally: cell densities and CO_2 concentrations.

In tissue culture medium, β-mercaptoethanol acts as a reduction/oxidation regulator maintaining glutathione levels as a donor of thiol groups, protecting cells from oxidative damage – a role usually performed by macrophages and monocytes *in vivo* (Pruett *et al.*, 1989). Additionally, β-mercaptoethanol facilitates cellular uptake of cysteine – a precursor of the amino acid cystine, necessary for protein synthesis – by forming a mixed disulphide that can be transported into the cell where it dissociates;

the β-mercaptoethanol returning to the medium to continue the cycle (Ishii *et al.*, 1981a; Ishii *et al.*, 1981b). The addition of β-mercaptoethanol to lectin-stimulated rat lymphocytes has been shown to increase proliferation and a range of metabolic enzyme activities (Aidoo *et al.*, 1996). Since robust proliferation was the goal of the donor cell culturing, 50 µM β-mercaptoethanol was included in the splenocyte culture medium, as it was in the adoptive transfer pre-cultures used by Tseng *et al.* (1989). Using a serum concentration of 10% v/v was chosen for similar reasons, since the higher serum level is routinely chosen when inducing proliferation for *in vitro* assays (Mulligan *et al.*, 1998) and when culturing T lymphocytes in the absence of cell-conditioned medium (Hu *et al.*, 1992). In one study, splenic T cells did not respond optimally to mitogen until the serum level reached 10% (Mohapatra *et al.*, 2001). It was also the level used in the adoptive transfer pre-culturing/isolation protocols (Tseng *et al.*, 1989; Manfra *et al.*, 2001).

Stimulating proliferation of donor cells

Mouse splenocytes established in *ex vivo* culture do not proliferate without mitogenic stimulation (Piguet *et al.*, 1976). The most common mitogens used are *E. coli* lipopolysaccharide (LPS), phytohaemagglutinin (PHA), concanavalin A (ConA), or lethally irradiated feeder cells. Concanavalin A, used in the preliminary experiments with unseparated splenocytes, was chosen as the mitogen to stimulate the proliferation of the donor T lymphocytes. The use of irradiated feeder cells was immediately excluded due to potential interference from signals released into the culture in response to the high-dose irradiation, and, LPS was avoided due to the potential for toxic effects from the endotoxin when transferred into the recipient mice. In every study of lectin-stimulation examined, ConA was found to be more suited than PHA for use with mouse cells, with spleen cells, and with T lymphocytes

in particular (Boldt *et al.*, 1975; Piguet *et al.*, 1976; Dumont and Barrois, 1977; Tseng, 1982; Hu *et al.*, 1992; Fasanmade and Jusko, 1995). In addition, the adoptive transfer experiment that formed the basis of this method used concanavalin A–stimulated lymphocytes, and as such, their tissue-lodging patterns had already been characterised (Tseng *et al.*, 1989).

The range of ConA concentrations normally used in the stimulation of mouse splenic T cells is from 0.1–20 $\mu g.mL^{-1}$ (Dumont and Barrois, 1977; Fasanmade and Jusko, 1995). The adoptive transfer experiments in Tseng *et al.* (1989) stimulated the donor T lymphocytes with ConA from 0.2–4 $\mu g.mL^{-1}$. Since the purity and potency of lectin batches vary, the donor T cells were stimulated with a range of ConA concentrations from 0.1–10 $\mu g.mL^{-1}$ using the culture medium described above; concentrations below 1 $\mu g.mL^{-1}$ were found to be sub-mitogenic, whilst 10 $\mu g.mL^{-1}$ was cytotoxic (*Figure 4.3*). The distribution throughout the cell-cycle was analysed in these same cultures by staining the DNA with propidium iodide and measuring the DNA content of 30,000 cells by flow cytometry (proliferating cells were defined as those with DNA content between 2N and 4N, indicative of incomplete DNA replication). The most potent induction of proliferation was seen with 5 $\mu g.mL^{-1}$ ConA (*Figure 4.4*).

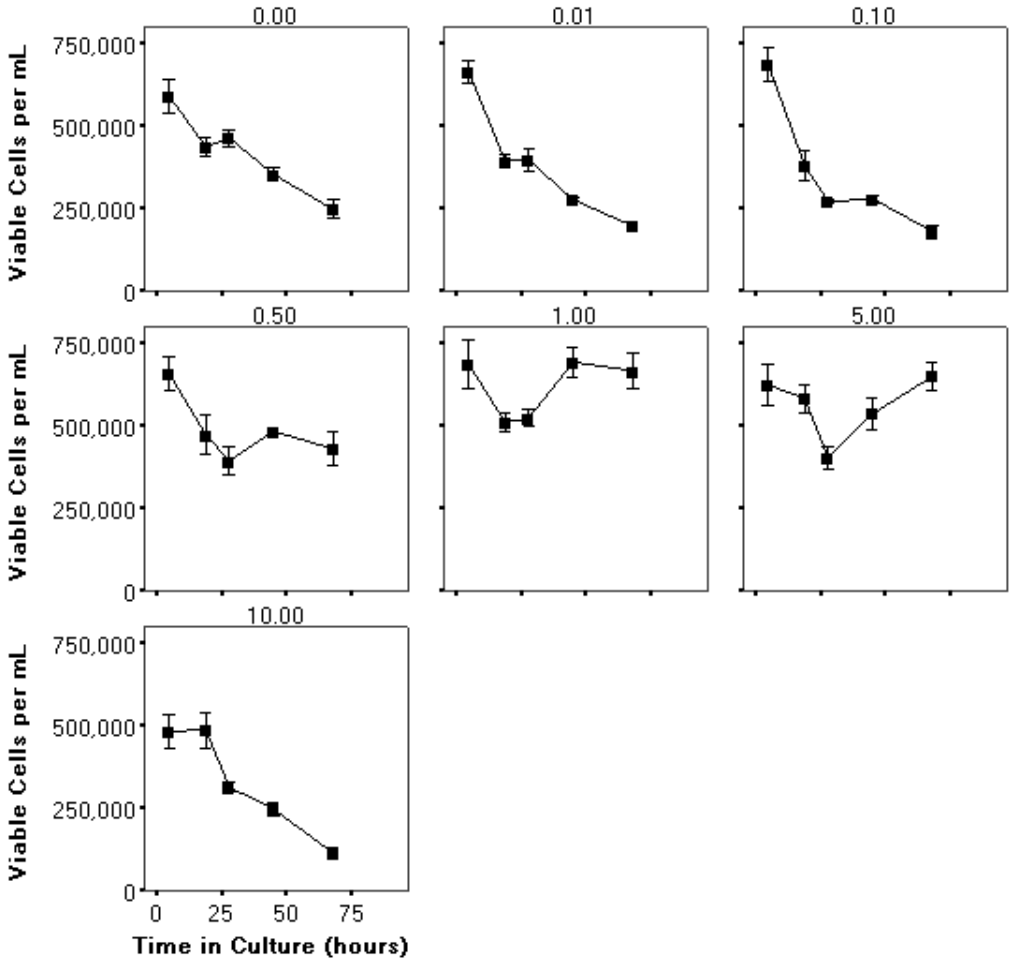

Figure 4.3: Viable cell numbers of donor T lymphocytes after ConA stimulation

Donor T lymphocytes established at 10^6 cells.mL^{-1} were cultured in splenocyte culture medium with the addition of concanavalin A at varying concentrations (shown in panels, μg.mL^{-1}). The numbers of viable cells per mL (mean ± SD), recorded at various times over the following 75 h, are shown (*n*=3).

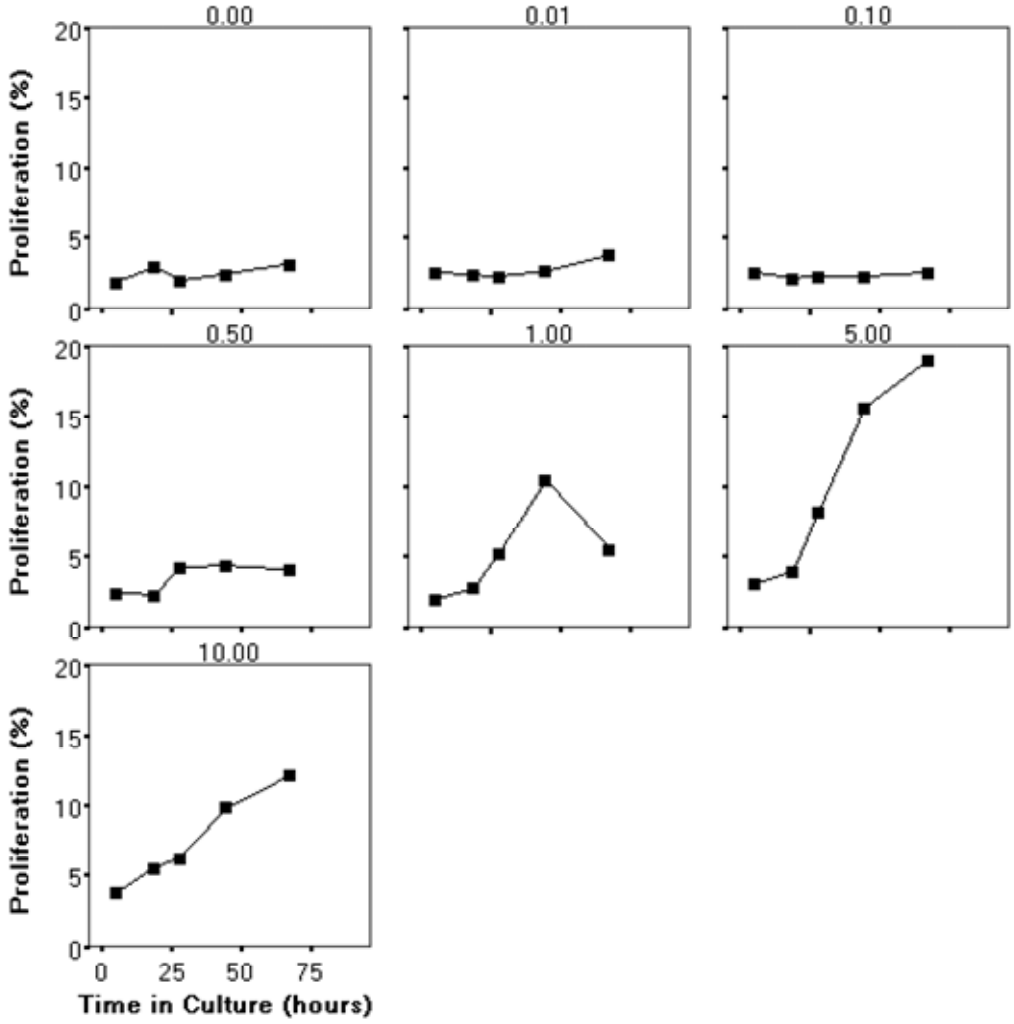

Figure 4.4: Cell-cycle progression of donor T lymphocytes after ConA stimulation

Donor T lymphocytes established at 10^6 cells.mL^{-1} were cultured in splenocyte culture medium with the addition of concanavalin A at varying concentrations (shown in panels, μg.mL^{-1}). The proportion of viable cells proliferating (mean of pooled triplicate samples) was measured by DNA content analysis at various times over the following 75 h, is shown.

The effect of initial cell density was tested over the range of 10^5–10^7 cells per mL in 200 µL (96-well U-bottom plate), 1 mL (6-well plate) and 5 mL (25 cm^2 flask) volumes. As expected from the published studies on splenic T cell culture, cells only grew in the micro-cultures established in 96-well plates (data not shown). The optimal initial cell density was found to be 1×10^6 cells.mL^{-1} (*Figure 4.5*). Increasing the atmospheric CO_2 from 5% to 10% during incubation also improved donor cell growth (*Figure 4.6*).

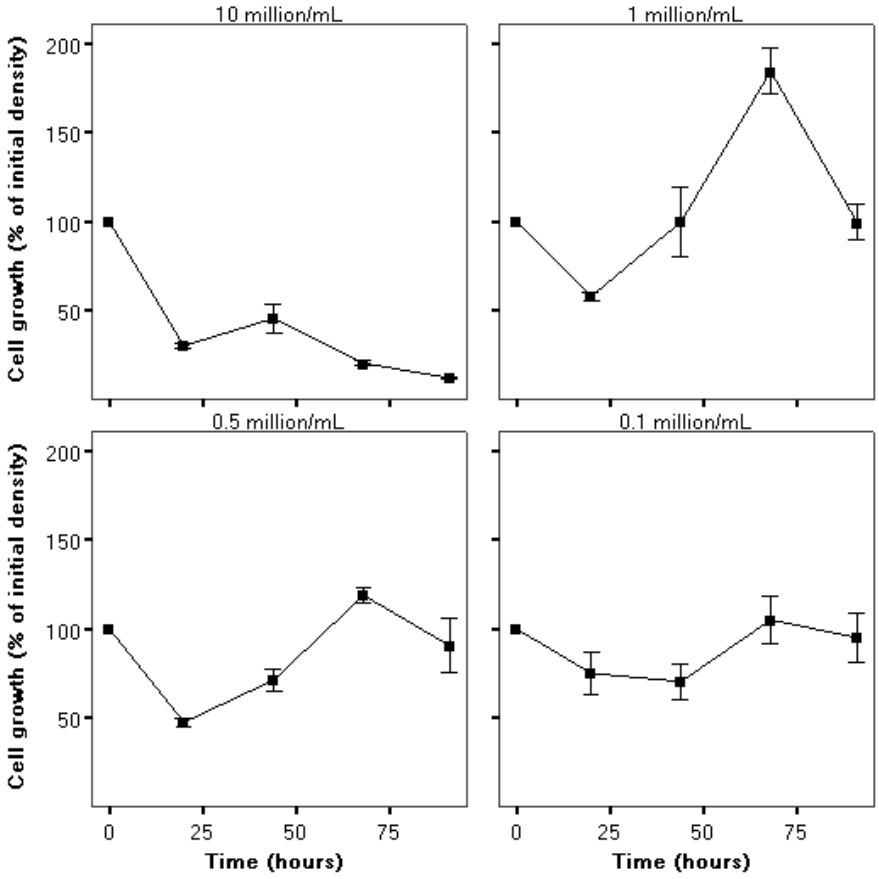

Figure 4.5: Effect of cell density on donor cell growth in culture

Donor T lymphocytes established at varying cell densities (shown in panels) were cultured in splenocyte culture medium with the addition of 5 µg.mL^{-1} concanavalin A. The numbers of viable cells per mL (mean ± SD), recorded at various times over the following 75 h, are shown (n = 3).

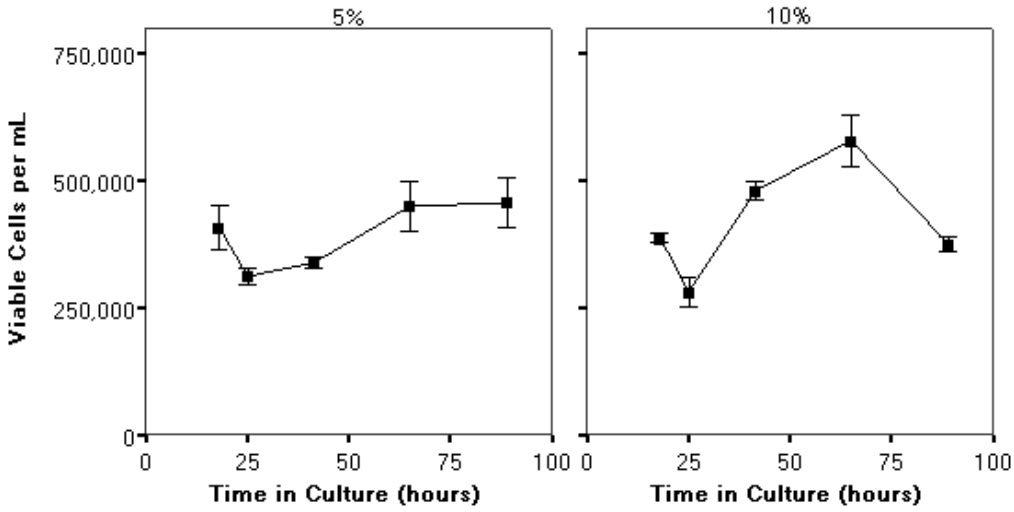

Figure 4.6: Effect of atmospheric CO_2 on donor cell growth in culture

Donor T lymphocytes established at 10^6 cells.mL^{-1} were cultured in splenocyte culture medium with the addition of 5 µg.mL^{-1} concanavalin A in either 5% (left panel) or 10% CO_2 in air (right panel). The numbers of viable cells per mL (mean ± SD), recorded at various times over the following 95 h, are shown (n = 3).

Final Culture Conditions

The final culture conditions, based on the results of the experiments optimising the proliferation of the donor T cells, were as follows.

Method:

Splenocyte culture medium (SCM) was prepared from the base RPMI medium by the addition of fetal bovine serum to a final concentration of 10% v/v, 50 µM β-mercaptoethanol and 5 µg.mL^{-1} of concanavalin A. Freshly prepared SCM was filter-sterilised (0.45 µm, Millex-GS disposable filter) and pre-warmed to 37°C. The isolated T cell population from the magnetic cell separation was centrifuged at 240 *g* for 5 min. The supernatant was vacuum aspirated and the cell pellet gently resuspended at 10^6 cells.mL^{-1} in SCM. Using a multi-channel pipette, 200 µL of cell suspension was added per well into 96-well U-bottom tissue culture plates. Cells were cultured at 37°C in a humidified atmosphere containing 10% CO_2 in air.

To test the performance of the final culture conditions, spleens were isolated from two donor mice and the T lymphocytes were isolated, pooled, and cultured as described above. The number of viable cells per millilitre was recorded over the following 66 h in culture (*Figure 4.7*). The nadir of growth, as seen in the previous optimisation experiments was after 20–25 h that then steadily increased over the following 40 h.

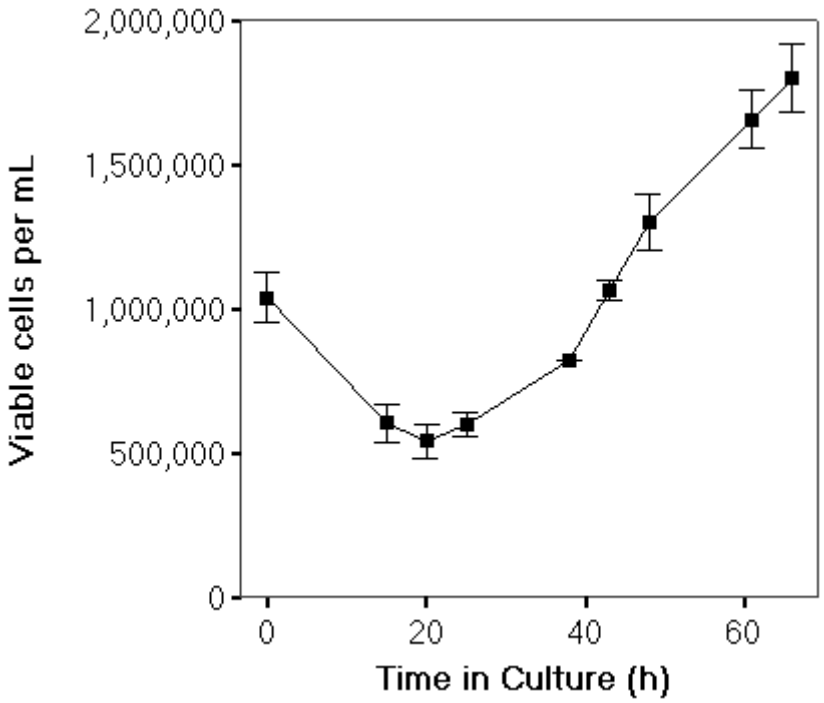

Figure 4.7: Representative growth of donor cells in culture

Donor T lymphocytes established at 10^6 cells.mL^{-1} were cultured in splenocyte culture medium with the addition of 5 µg.mL^{-1} concanavalin A. The numbers of viable cells per mL, recorded at various times over the following 65 h, are shown. Markers show mean ± SD of three replicates.

Incorporation of ^{3}H-thymidine radiolabel into donor cells

In order to allow the maximum proportion of donor cells to undergo DNA synthesis in the presence of the radiolabel, the ^{3}H-thymidine incorporation time for the mitogen-stimulated primary spleen cells used in this model needed to be extended well over the time used in traditional pulse-labelling of synchronised cell cultures (usually 1 h). *In vitro* bystander experiments using DNA-incorporated ^{3}H-thymidine to chronically irradiate target cells, have previously used labelling periods between 12 h (Bishayee *et al.*, 2001; Persaud *et al.*, 2005; Persaud *et al.*, 2007) and 18 h (Gerashchenko and Howell, 2004). The longer, 18-hour incubation period was chosen for this model, to maximise the proportion of cells synthesising DNA in the presence of the radiolabelled thymidine but without extending the time that the cells were in culture beyond that used in similar *in vitro* experiments. Significant, even lethal, inter-nucleosomal cleavage of DNA from tritium disintegrations has been reported for cells cultured for long periods (2 days) in the presence of ^{3}H-thymidine (0.2–2 μCi.mL^{-1}) (Solary *et al.*, 1992); however, this type of DNA-damage was unlikely to pose a difficulty in these experiments which used a 5- to 50-fold lower radioactivity and radiolabelled for less than one day.

To assess the proportion of donor cells undergoing DNA synthesis during the *ex vivo* culture period, the incorporation of non-radioactive bromodeoxyuridine (BrdU, BD Biosciences, CA, USA) over an 18-h period was measured. The initial use of a non-radioactive label in these optimisation experiments provided a quick method for detecting labelled cells and measured the labelling of the cells in the absence of any inhibitory effect that might have occurred due to the incorporated radionuclide. Following an initial period in culture (20, 25, 43 or 48 h) BrdU was added to donor T cell cultures to a final concentration of 10 mM, and after 18 h, the cells were washed

and fixed onto slides. Incorporated BrdU was detected using a biotinylated anti-BrdU antibody, followed by applying streptavidin–horse radish peroxidase and DAB chromogenic substrate following the manufacturer's supplied protocol (*BrdU In-situ detection kit*, BD Biosciences). The degree of labelling increased, the later the label was added to the cells, however, all four incubation periods showed strong BrdU labelling (>80% of donor cells).

Given that the aim was to reduce the time the donor cells were in culture, the 25–43 h incubation time was selected for further testing. This incubation period was ideal as it began shortly after the nadir in viable cell numbers when the first round of proliferation would begin, such that the proliferating cells would be pseudo-synchronised, producing an even dose-distribution. In further experiments to confirm the suitability of the radiolabelled period chosen, two independent donor cell cultures were incubated for 18 h with BrdU after an initial 25 h in culture. The BrdU labelling of the two donor cell cultures was 88% and 87% (*Figure 4.8*) confirming that the labelling duration and timing was appropriate for radiolabelling of the donor cells without extensive time in culture.

The range of radioactivity considered for the radiolabelled donor cells was bound by the minimum possible dose-rate (effectively one ^{3}H disintegration during the *in vivo* period) and the maximum tolerable dose-rate (the dose-rate that causes 100% lethality). Over a 24 h *in vivo* lodging period, a mean of 1 accumulated disintegration is equivalent to a mean radioactivity of 0.012 mBq per cell.

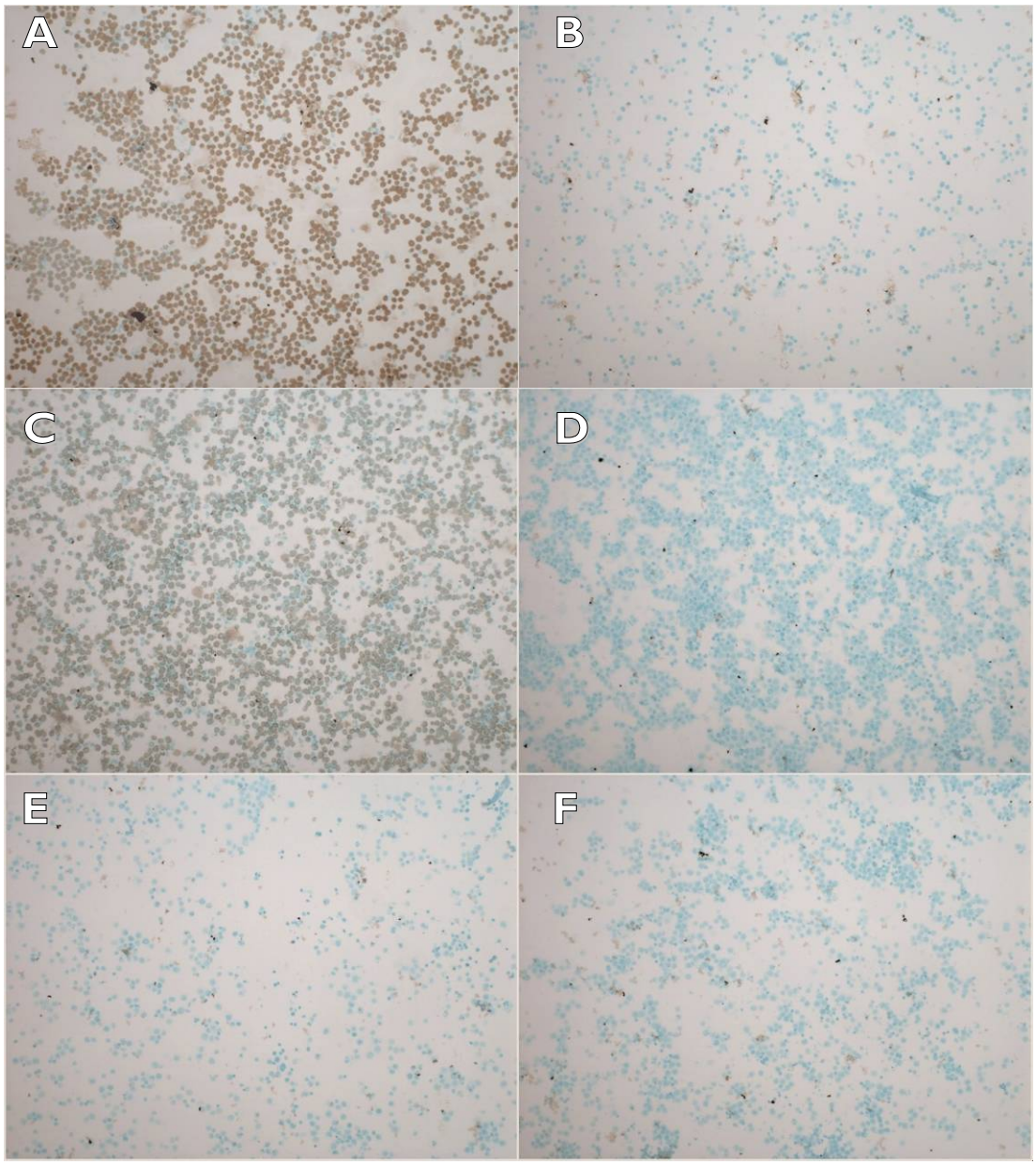

Figure 4.8: Incorporation of BrdU into donor T cells

Two donor cell cultures were established using the finalised donor cell radiolabelling protocol. After 25 hours in culture, 10 mM BrdU was added for an 18-h period before fixing the cells, staining with anti-BrdU antibody (brown) and counterstaining (blue). Photographs (magnification 200×) are shown of culture 1 (A) and its control with antibody omitted (B), culture 2 (C) and controls with antibody omitted (D), BrdU omitted (E) or BrdU and antibody omitted (F). Nuclear staining with anti-BrdU antibody is seen in 88% (A) and 87% of donor cells (C) respectively.

In fibroblasts radiolabelled with ^{3}H-thymidine, ≈2600 ^{3}H disintegrations accumulated over a ten-day period resulted in 1% survival (Bishayee *et al.*, 2001); an equivalent dose delivered over a 24 h *in vivo* lodging period would be equivalent to a radioactivity of ≈ 30 mBq per cell. The initial radioactivity chosen for study here was approximately one tritium disintegration per hour (0.28 mBq) in each radiolabelled cell. In the work of Gerashchenko & Howell (2004) the radioactivity incorporated into radiolabelled cells increased linearly with the ^{3}H-thymidine concentration in the medium. From these data, 1.48 kBq.mL^{-1} was estimated as the radioactivity of the labelling medium likely to result in incorporation of 0.2–0.3 mBq.cell^{-1}. The high specific activity of the ^{3}H-thymidine used (3.18–3.33 TBq/mmole) resulted in relatively low thymidine concentrations (0.465 nM) required to deliver the desired dose; however, the same molar concentration of non-radioactive thymidine was added to the sham-treated cells to control for any possible disruption to the intracellular nucleotide pool (Cleaver, 1967).

Method:

Stock [methyl-^{3}H]-thymidine (37 MBq.mL^{-1}, specific activity 3.18 TBq.mmol-1, GE Co., GE Healthcare, Waukesha, WI) was serially diluted in splenocyte culture medium (SCM) to 59.2 kBq.mL^{-1}. After 25 h in culture, 5 µL of the 59.2 kBq.mL^{-1} [methyl-^{3}H]-thymidine was added to each 200 µL well for radiolabelled donor cells (final radioactivity 1.48 kBq.mL^{-1}, final thymidine concentration 0.465 nM). The cell culture plates were returned to the incubator for a further 18 h. For sham-radiolabelled donor cells, 0.0186 µM nonradioactive thymidine (Sigma Aldrich Corp.) was prepared in SCM, and 5 µL was added per well (final concentration 0.465 nM).

Assessing ^{3}H-thymidine incorporation into donor cells by autoradiography

Autoradiography was used to confirm the incorporation of ^{3}H-thymidine into the radiolabelled cells and to determine the proportion of labelling (*Figure 4.9-A*) along with the sham-radiolabelled control cells (*Figure 4.9-B*). Cells in the radiolabelled cultures showed one of three levels of autoradiography grains deposited over the nuclei: moderate incorporation, heavy incorporation (indicative of two rounds of proliferation in culture), or no incorporation (cells that did not replicate DNA during the radiolabelling period).

Method:

At the conclusion of the radiolabelling period, culture medium was removed from sample wells of the radiolabelled and sham-radiolabelled donor cell populations, and the cells were pulse-chased for 1 h with fresh SCM containing 0.465 nM non-radioactive thymidine. The cells in each well were then resuspended and the 200 µL volume was centrifuged (700 rpm, 5 min: *Shandon Cytospin II*, Thermo Fisher Scientific, MA, USA) onto positively charged glass microscope slides. Slides were fixed in 4% formaldehyde (from paraformaldehyde in PBS, Sigma Aldrich Co.) for 5 min, washed twice in PBS for 2 min, and allowed to dry. Slides were dehydrated through 70%, 90% then 100% ethanol and again allowed to dry. Under darkroom conditions, *EM-1 Hypercoat™ emulsion* (GE Co., GE Healthcare) was melted in a 43°C water-bath for 30 min before the liquid emulsion was poured into a dipping chamber. Each slide was dipped vertically into the emulsion for 5 s, slowly withdrawn, and stood upright for at least 10 min to begin setting. Slides were placed in a slide rack, sealed in a lightproof box with anhydrous silica gel added in the base, and exposed for 7 days.

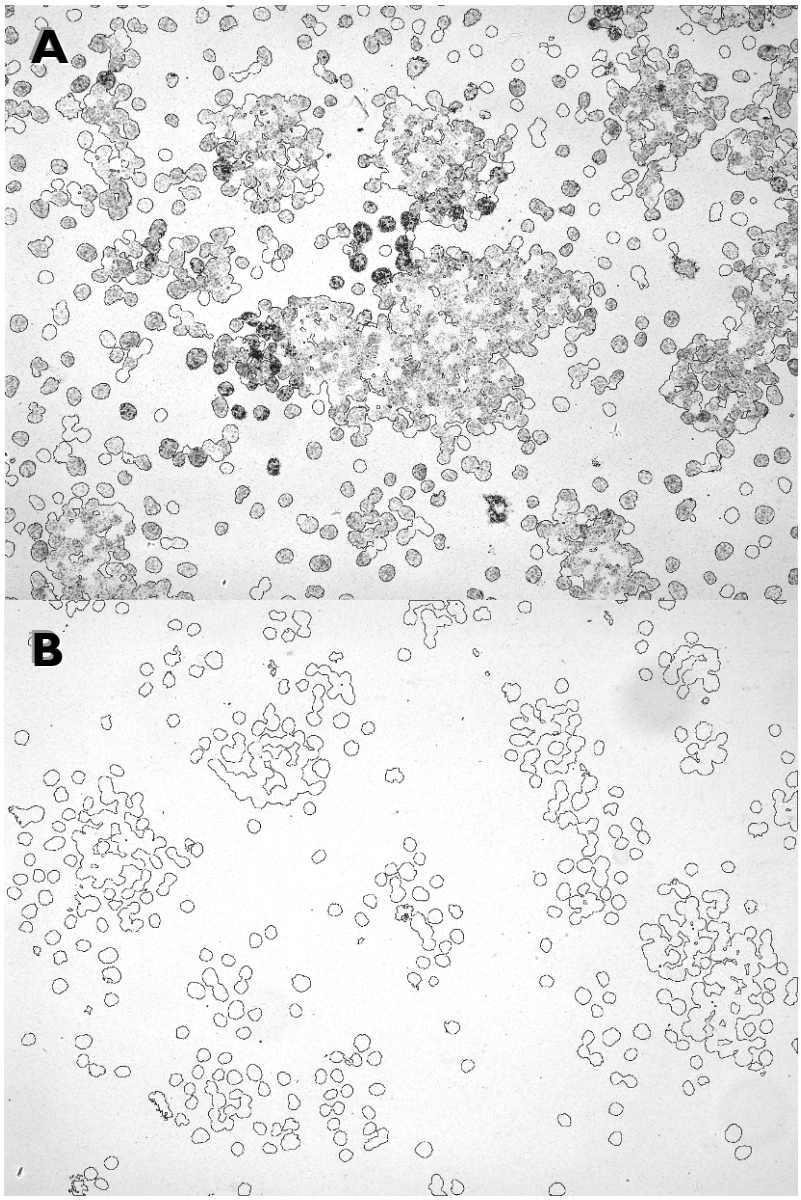

Figure 4.9: ^{3}H-thymidine incorporation into donor cells

Donor T lymphocytes were radiolabelled (A) or sham-radiolabelled (B) for 18 h, according to the final culture protocol. A sample of donor cells were assessed for radiolabelling by autoradiography as described. Photographs of the cells and any deposited silver grains (black) are shown (20× objective lens). The outlines show the computer-detected edges of cell nuclei based on the intensity of DAPI staining relative to background.

Exposed slides were placed in photographic developer (*Developer D-19*, Eastman KODAK Co., NY, USA) for 2 min without agitation, the reaction was stopped by placing slides in 1% acetic acid for 10 s, then the emulsion was cleared for 5 min (*KODAK Fixer*, Eastman KODAK Co.). Developed slides were washed in running tap water for 15 min, and then allowed to dry. Glass coverslips were mounted over the cells using *Vectashield ® with DAPI* (Vector Laboratories, CA, USA) containing the DNA counterstain 4',6-diamidino-2-phenylindole (DAPI). The slides were examined using combined fluorescence and brightfield microscopy to photograph DAPI-stained nuclei and silver-grains deposited during autoradiography.

Determining mean radioactivity per cell

The degree of incorporation of the ^{3}H-thymidine into the donor cell DNA was assessed by measuring the mean radioactivity per cell at the end of the 18-hour incubation period by liquid scintillation counting (according to the method of Gerashchenko and Howell, 2004).

Method:

At the end of the ^{3}H-thymidine incubation period, the cells in each 200 µL well were resuspended and pooled. Triplicate 50 µL samples were taken from the pooled cell populations (radiolabelled and sham-radiolabelled), added to 50 µL of trypan blue and counted for viable cells using a haemocytometer.

Triplicate 200 µL samples were then taken from the pooled cell suspensions and each was added to 5 mL of scintillation cocktail (*ReadySafe*, Beckman Coulter, Inc.) in a 6 mL plastic scintillation vial (*PolyQ*, Beckman Coulter, Inc.). The pooled cell suspensions were then centrifuged (5 min, 240 *g*) to pellet the cells. Triplicate 200 µL samples of the

supernatant were taken and added to 5 mL scintillation cocktail, as above. In a further eight vials containing 5 mL scintillation cocktail, a standard curve was prepared in duplicate for ^{3}H-thymidine activities of 0, 3.7, 0.37 and 0.037 kBq.

The vials were mixed by inversion and the counts per minute were read on a LS3801 Liquid Scintillation Counter (Beckman Coulter, Inc.) using the ^{3}H counting window for 2 min or, until the 2σ error was < 2.0 (whichever was the earlier). The counts per minute were converted to radioactivity (in becquerel) by comparison with the counts from the prepared standard curves, which routinely gave counting efficiencies of 0.51 ± 0.06. The mean radioactivity per cell was calculated by subtracting the mean total radioactivity for the supernatants from those of the cell suspensions, with the remaining cell-associated radioactivity divided by the number of cells estimated to be in each 200 µL aliquot based on the viable cell count performed at the time of sampling.

The mean radioactivity per cell represents the average of the radioactivity in the radiolabelled and non-radiolabelled cells present within the donor cell culture; dividing this value by the proportion of radiolabelled cells as determined by autoradiography gave the adjusted mean radioactivity per radiolabelled cell.

Fluorescent labelling of donor cells for i*n vivo* tracking

Fluorescent labelling method

In order to detect biological effects in proximity to the adoptively transferred donor cells, the donor cells needed to be labelled so that they could be specifically identified within the recipient tissues. The use of a fluorescent labelling method allowed the donor cells to be visualised independently of the biological endpoint being tested and vice versa, yet enabled simultaneous imaging. Pre-staining with a fluorescent dye eliminated the need to develop the reaction, as is required with antigen labelling e.g. BrdU incorporation, and did not require the use of transgenic cells e.g. cells expressing green fluorescent protein (Leithauser *et al.*, 2001). A pre-staining method for the adoptive transfer protocol needed a fluorescent dye that could be easily loaded into the donor cells and yet stably retained.

Candidates that met these desired properties were the class of membrane-permeable, intracellular-bound fluorescent dyes known as *CellTracker*™ probes (Invitrogen Corp., CA, USA). The *CellTracker*™ molecules contain a chloromethyl moiety which allows the dye molecules to easily pass across the cell membrane when the thiol-reactive probes then undergo a reaction with cysteine residues in proteins or glutathione to form adducts which can no longer pass through the cell membrane. The protein-dye adducts can then be fixed for histology with aldehyde-based fixation protocols (West *et al.*, 2001). In order to be compatible with multi-colour fluorescence microscopy with fluorescein-based staining methods, a dye fluorescing within the Orange/Red filterset *Chroma 31002* (Chroma Technologies) was required (i.e. Excitation 515–550 nm & Emission 575–615 nm).

CellTracker™ Orange CMTMR (5- and 6-(4-chloromethyl)benzoylamino-tetramethylrhodamine) (Invitrogen Corp.) is an orange fluorescent dye used extensively in adoptive transfer methods, including the labelling of mouse splenic lymphocytes for *in vivo* tracking after injection into the tail vein (McEvoy *et al.*, 1997; Miller *et al.*, 2002). Cell tracking probes such as CMTMR have been gradually replacing the use of older dyes such as DiI due to their low toxicity (Tuorto *et al.*, 2003) and long-term tracking ability i.e. > 1 year *in vivo* (Goolsby *et al.*, 2003). In adoptive transfer experiments with mouse splenocytes either expressing transgene-encoded green fluorescent protein or labelled *ex vivo* with 5 µM CMTMR, no difference in blood clearance over 24 hours or donor cell accumulation in spleen, liver, lung and kidney after 24 hours was observed (Moeller *et al.*, 2003).

For use in this project, the closely related *CellTracker™ Orange CMRA* (Invitrogen Corp.) dye was chosen. CMRA is brighter with an improved cytoplasmic staining pattern compared to CMTMR and is compatible with standard orange/red fluorescence filtersets. Its use in mouse splenic lymphocyte adoptive transfer experiments has recently been published (Mitoma *et al.*, 2007; Skokos *et al.*, 2007). Protocols labelling the donor cells with the fluorescent probe during, or following, the radiolabelling period were tested. Adding the CMRA probe along with the ^{3}H-thymidine/thymidine removed several washing and processing steps, reduced the time between the end of the radiolabelling period and the adoptive transfer, and resulted in brighter labelled cells (data not shown).

Method:

A 1 mM working stock of *CellTracker™ Orange CMRA* was freshly prepared by resuspending the contents of a vial (50 µg) in 90 µL of pure DMSO. During the preparation of the radiolabelling/sham-radiolabelling solution, CMRA working stock was added (10% v/v, 0.1 mM) such that the final concentration in the cell culture medium was 2.5 µM.

Confirming CMRA fluorescent labelling

Donor cells were examined using fluorescence microscopy to ensure that they were brightly labelled with the CMRA fluorescent probe before proceeding with the adoptive transfer. Donor cells were routinely 100% labelled with the CMRA probe (*Figure 4.10*); thus, if no CMRA-positive cells were identified in the spleen sections, it could be assured that the cells had been appropriately labelled before injection.

Method:

The cells in a sample well (one from both the radiolabelled and sham-radiolabelled cell populations) were resuspended, and then the 200 µL volume centrifuged onto an positively charged glass microscope slide (700 rpm, 5 min, *Shandon Cytospin II*) at the end of the 18-h radiolabelling period. The slides were fixed in 4% formaldehyde (from paraformaldehyde in PBS) for 5 min, rinsed twice in PBS for 2 min, and a glass coverslip was mounted with *Vectashield® with DAPI*. The slide was examined on an *Olympus Ax70* epifluorescent microscope using a filtercube covering the range of CMRA excitation/emission. Bright CMRA-labelling in both donor cell populations (radiolabelled and sham-radiolabelled) was confirmed before proceeding with the adoptive transfer.

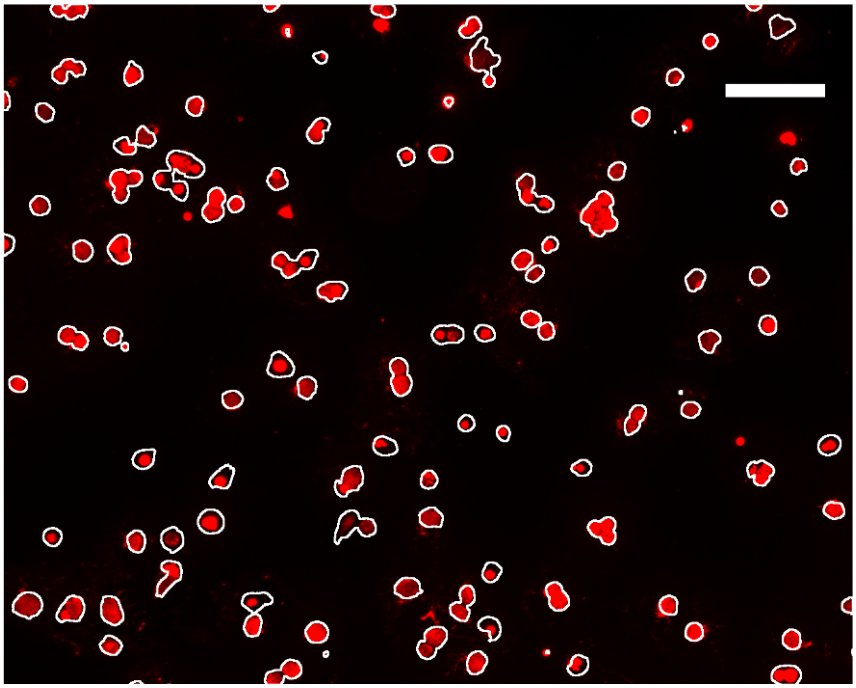

Figure 4.10: CMRA labelling of donor cells

Donor T lymphocytes were isolated, cultured for 25 h, then radiolabelled and incubated with 2.5μM CMRA for 18 h, according to the final culture protocol. A sample of donor cells was assessed for CMRA labelling as described. The outlines (white) show the locations of cell nuclei determined by the areas of DAPI staining. Each donor cell is stained with the CMRA fluorescent probe (red). Image was photographed with 10× objective lens, scale bar shows 100 μm.

Preparation of donor cells for adoptive transfer

The radiolabelled/sham-radiolabelled and fluorescently labelled donor cells were washed, equilibrated in PBS without serum and resuspended to a concentration appropriate for the adoptive transfer experiment.

Method:

After the triplicate samples of the supernatant were taken from the donor cell cultures for measuring the donor cell radioactivity, the remaining supernatant was vacuum aspirated. The cells were resuspended in 10 mL of fresh SCM and incubated for a further 30 min (37°C, 10% CO_2 in air) before the cell suspensions were centrifuged (5 min, 241 *g*), and the supernatant vacuum aspirated. The cells were resuspended in 20

mL filter-sterilised PBS (0.22 µm, Millex-GS disposable filter) and centrifuged again (5 min, 241 *g*) with the supernatant vacuum aspirated. Based on the total cell numbers determined from the triplicate cell counts taken during the radioactivity measurements, the donor cells were resuspended in filter-sterilised PBS at 2.5 – 25 × 10^6 cells.mL^{-1}. The cell suspensions were then taken to the Flinders Medical Centre Animal House Facility ready for adoptive transfer.

Summary

The final protocol for the chronic radiolabelling of donor splenic T lymphocytes is outlined in *Figure 4.11*. Splenic T cells were isolated from a pooled spleen cell suspension by density gradient centrifugation followed by antibody labelling and magnetic cell sorting. The donor T lymphocytes were established in culture and stimulated with concanavalin A. After 25 h, ^{3}H-thymidine (or thymidine for the controls) was added to the culture medium, along with the CMRA fluorescent probe. Following the 18-h radiolabelling period, samples were taken for radioactivity measurements (liquid scintillation counting), radiolabelling assessments (microautoradiography) and confirming the fluorescent labelling (fluorescence microscopy). The remaining donor cells were washed and resuspended in PBS ready for adoptive transfer.

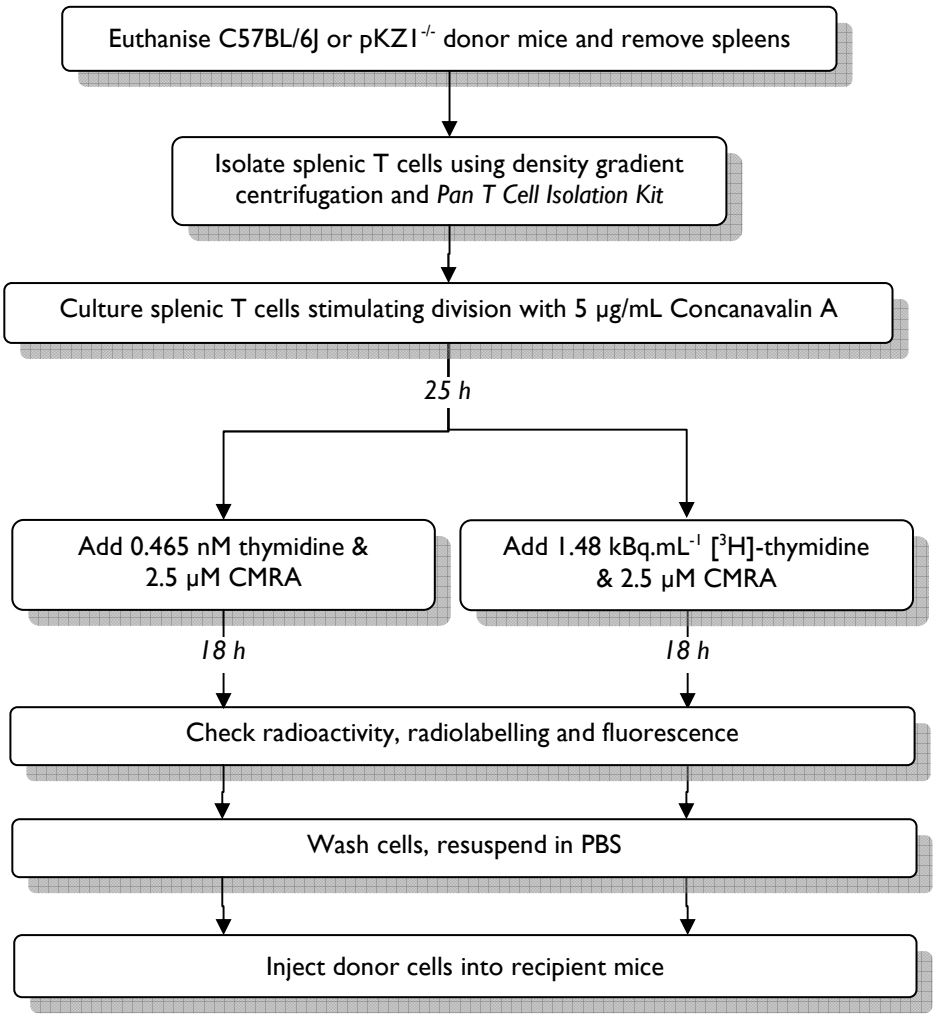

Figure 4.11: Final protocol for the chronic radiolabelling adoptive transfer method

Donor splenocytes: Acute X-irradiation

Method outline

In an experiment to detect bystander effects induced by acutely irradiated cells, the adoptive transfer method developed for chronic radiolabelling was modified to accommodate *ex vivo* irradiation (*Figure 4.12*). This section describes the variations in the methods for donor cell isolation, preparation, fluorescent labelling, and irradiation.

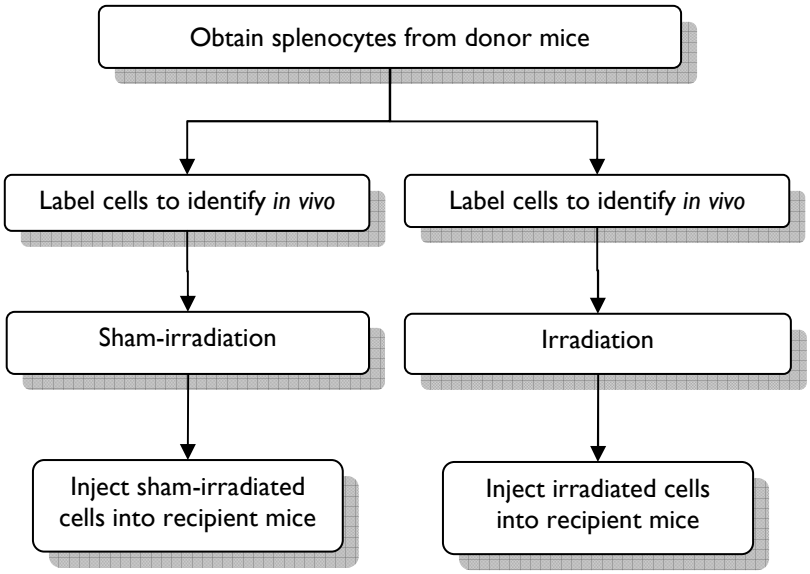

Figure 4.12: Initial strategy for the acute irradiation adoptive transfer method

Selecting a technique to irradiate donor cells

Choosing a radiation source

In vitro bystander experiments using low-LET photon sources have been performed using γ-sources (Seymour and Mothersill, 2000; Lorimore *et al.*, 2005), soft X-ray microbeams (<5 keV) (Kashino *et al.*, 2004; Schettino *et al.*, 2005; Hill *et al.*, 2006) and diagnostic X-ray linear accelerators (250 kVp) (Lewis *et al.*, 2001; Yang *et al.*, 2005; Kadhim *et al.*, 2006). In the experiments performed here, the doses required (0.1 – 1 Gy) were too high for the operational maximum of the diagnostic X-ray linear accelerators available (250 kVp) in our facility. Instead, a *Siemens Primus* 6 MV linear accelerator was chosen to irradiate the donor cells. The monoenergetic photons produced by the 6 MV linear accelerator are of much higher energy than those in comparable 250 kVp X-ray irradiations; however, 30–50% of the absorbed dose of hard X-rays is from interactions of the low-energy secondary electrons (Nikjoo, 1991). Since higher energy photons travel faster through biological matter they deliver less energy along their path and it is thought that for both the 6 MV or 250 kVp photon spectra, the majority of the biological effects arise from the lower energy secondary electron tracks in the ultrasoft X-ray range (Hill *et al.*, 2006).

Isolation of splenocytes and preparation of donor cell cultures

The decision to use splenic T lymphocytes for the chronic radiolabelling experiments was based on the need to effectively stimulate the cells to divide and thus incorporate the tritiated thymidine. In the case of the acute *ex vivo* X-irradiations, the isolation of T lymphocytes to allow potent mitogen stimulation was not required. Isolation of the T lymphocyte compartment of the spleen reduced the number of donor cells to ≈17% of the initial yield. A mixed splenocyte population was used here as it increased the

number of cells available while using less donor mice. Differences in the expected lodging patterns of B and T lymphocytes after adoptive transfer are discussed above. The mixed splenocyte population used as target cells in the acute X-irradiation experiments, was not established in culture as proliferation was not required to incorporate a radiolabel, so labelling with the CMRA fluorescent probe was performed in a short, 1 h incubation step.

Method:

Three mice were euthanised, and splenic mononuclear cells were prepared as for the chronic radiolabelling method. The donor cells were then transferred to fresh RPMI medium containing 10 μM CMRA. The cells were pooled, incubated for 1 h (37°C, 5% CO_2 in air), then centrifuged (10 min, 170 *g*) and the supernatant aspirated and replaced with a modified PBS (adjusted to match the normal osmolarity of mouse serum (333 $mmol.kg^{-1}$) to avoid lysis of the target cells, (Sheridan and Finlay-Jones, 1977)). The cells were washed twice more in the modified PBS, then resuspended at 2.5×10^6 $cells.mL^{-1}$ ready for irradiation and adoptive transfer.

Exposure of donor cells to X-radiation

Donor cells were given an acute dose of X-rays using the *Siemens Primus* 6 MV linear accelerator (Siemens Corporation, NY, USA) at the Adelaide Radiotherapy Centre (Flinders Private Hospital).

Method:

The donor cells were placed in a plastic tissue culture flask to a depth of 5.5 mm (corresponding to a volume of 5.88 mL on a 10.7 cm^2 surface-area). To account for the lack of build-up above the surface of the cell suspension, a build-up layer of 1.4 cm

depth of water equivalent (RW3) was placed above the flask, which was placed within a field size of 12 cm × 12 cm at a source-to-surface distance of 100 cm. In prior dosimetry calibrations, *Tissue Maximum Ratios* were used to determine the monitor units required to be delivered to the liquid at the bottom of the flask to achieve the desired dose and this was confirmed by MOSFET dosimeters placed in the culture medium. MOSFET dosimeter calibrations reported dose error of ± 3% (95% confidence). The donor cells were irradiated at 5 $Gy.min^{-1}$ with 0.1 or 1 Gy (or, the beam was not energised for sham-irradiated cells).

Summary

The final protocol for the acute irradiation of donor splenocytes is outlined in *Figure 4.13*. Splenocytes were isolated from a pooled spleen cell suspension by density gradient centrifugation. The donor cells were labelled for 1 h with the CMRA fluorescent probe. The cells were washed thrice and resuspended in modified PBS, before irradiation with 0.1 Gy, 1 Gy or sham-irradiation just prior to adoptive transfer. A sample of cells was taken to confirm the fluorescent labelling (fluorescence microscopy) and perform viability measurements.

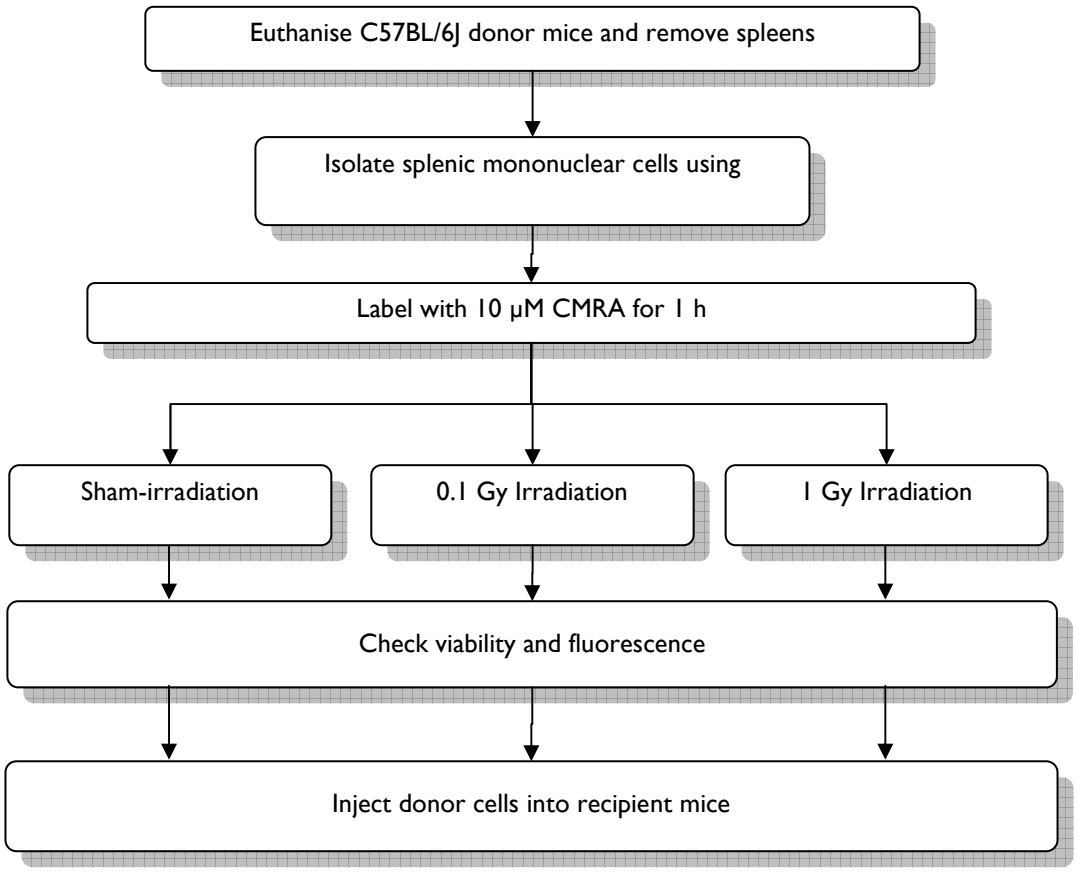

Figure 4.13: Final protocol for the acute irradiation adoptive transfer method