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A Lower Dose Threshold for the *in vivo* Protective Adaptive Response to Radiation. Tumorigenesis in Chronically Exposed Normal and *Trp53* Heterozygous C57BL/6 Mice

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Low doses of ionizing radiation to cells and animals may induce adaptive responses that reduce the risk of cancer. However, there are upper dose thresholds above which these protective adaptive responses do not occur. We have now tested the hypothesis that there are similar lower dose thresholds that must be exceeded in order to induce protective effects *in vivo*. We examined the effects of low dose/low dose rate fractionated exposures on cancer formation in *Trp53* normal or cancer-prone *Trp53* heterozygous female C57BL/6 mice. Beginning at 6 weeks of age, mice were exposed 5 days/week to single daily doses (0.33 mGy, 0.7 mGy/h) totaling 48, 97 or 146 mGy over 30, 60 or 90 weeks. The exposures for shorter times (up to 60 weeks) appeared to be below the level necessary to induce overall protective adaptive responses in *Trp53* normal mice, and detrimental effects (shortened lifespan, increased frequency) evident for only specific tumor types (B- and T-cell lymphomas), were produced. Only when the exposures were continued for 90 weeks did the dose become sufficient to induce protective adaptive responses, balancing the detrimental effects for these specific cancers, and reducing the risk level back to that of the unexposed animals. Detrimental effects were not seen for other tumor types, and a protective effect was seen for sarcomas after 60 weeks of exposure, which was then lost when the exposure continued for 90 weeks. As previously shown for the upper dose threshold for protection by low doses, the lower dose boundary between protection and harm was influenced by *Trp53* functionality. Neither protection nor harm was observed in exposed *Trp53* heterozygous

mice, indicating that reduced *Trp53* function raises the lower dose/dose rate threshold for both detrimental and protective tumorigenic effects.

INTRODUCTION

The Linear No-Threshold hypothesis, which assumes that risk is directly proportional to dose without a threshold, forms the basis for all human and environmental radiation protection practices (1). However, the ability of cells and animals to adapt to low doses of ionizing radiation and lessen the detrimental effects of further radiation or of other stressors, as well as spontaneous events, is well documented (3-16). The adaptive response is an evolutionarily conserved response, and is seen in all forms of life from single cell prokaryotes and eukaryotes to multi-cellular organisms, including both adult and fetal mammals (17, 18). The impact of these universal, adaptive, and hence inherently non-linear responses on human and environmental radiation protection principles and practices is being considered and debated (19-30).

The adaptive response to radiation is a response to low doses and dose rates (31). We have previously shown that in mice *in vivo,* such low doses induce an adaptive response that increases tumor latency, restoring a portion of the lifespan that would otherwise have been lost (32, 33). However, there are upper dose thresholds, above which the protective response does not occur (34), and similar observations have been made for human cells in culture (35). The dose at which these upper thresholds occur is tissue type specific, and is influenced by *Trp53* functionality (32-34). Low levels of other stressors, like heat or certain chemicals, when given independently can also induce the adaptive response to radiation (32). However, combined exposure to two or more different stressors, each given at a level

that independently would induce an adaptive response to radiation, can exceed the upper "stress" threshold, and prevent this protective response (36).

The existence of upper dose (stress) thresholds for the adaptive response then raises the question as to whether there is a similar lower dose threshold, below which the level of damage or stress is too low to induce a protective adaptive response. The existence of such a lower dose threshold for protection would have profound implications for radiation protection practices and principles, since most occupational and environmental radiation doses and dose rates are below those typically investigated for the induction of adaptive responses.

In contrast to upper dose thresholds, there is only sparse evidence for the potential existence of lower dose thresholds for the induction of the adaptive response to radiation, and most reports measure either molecular or cellular endpoints. For example, normal human fibroblasts in culture produced an adaptive response for increased chromosomal break repair capacity after a single dose of 1 mGy but not after 0.1 mGy (33). Lower dose thresholds (at high dose rate) have also been reported for cell killing in human cells in tissue culture (37) and for chromosomal inversions measured at the cellular level in mice *in vivo* (38,39).

Reports investigating the existence of lower dose thresholds for protective endpoints directly measuring risk *in vivo* are even more sparse. One study examined the effect of continuous low dose rate exposure on immune-compromised MRL-*lpr/lpr* mice, and showed that lifetime exposure at 0.35 mGy/h or 1.2 mGy/h prolonged lifespan, but the lifespan extension at the lower dose rate was less effective than at the higher dose rate (and hence

dose). Likewise, continuous exposure that stopped after 5 weeks at either dose rate was less effective than the lifetime exposure (40, 41). Although the authors did not directly test the possibility, those results implied that there could be a threshold at some even lower dose and/or dose rate, below which the protective effects could disappear. Recently, we have reported that for chronic ulcerative dermatitis, a spontaneous, autoimmune-type age-related disease in C57BL/6 mice, very low dose, fractionated exposures can induce a protective adaptive response in both *Trp53* normal and cancer-prone heterozygous mice, but that a lower threshold level of exposure, similar in both cases, must first be passed. In the mice with reduced *Trp53* functionality, doses below the threshold produced detrimental effects for that disease, but no detrimental effects were seen in mice with normal *Trp53* function (42).

As in the latter non-cancer disease study, the work presented here also focuses on fractionated radiation exposures given to normal and *Trp53* heterozygous C57BL/6 mice, delivered over increasing portions of the animal's lifespan on a schedule and pattern typical of potential human occupational exposures. This fractionated and chronic dose pattern was meant to represent an occupational exposure pattern of a very low dose given during only a portion of each day of a typical Monday to Friday work schedule. The study described here however, now examines effects on cancer risk.

Heterozygosity for *Trp53* is known to make mice prone to spontaneous cancer development (43, 44), but it is also known to influence most aspects of the adaptive response in mice (45), including the frequency and latency of both spontaneous and radiation induced cancer in adult mice (32, 33) and the sensitivity of fetal mice to teratogenesis induced by high

doses of radiation (46). In the current report we examine the influence of this low, "occupation-like" dose regimen on *in vivo* cancer risk in C57BL/6 mice with either normal or reduced *Trp53* functionality, and assess the observations in terms of low dose thresholds for protection by adaptive responses.

MATERIALS AND METHODS

Breeding and Genotyping

Male mice carrying a single defective copy of the *Trp53* gene (B6.129S2-*Trp53*^{tm1Tyj}/$^{+}$) were obtained from the Jackson Laboratory (Bar Harbor ME) and were crossed with 129X1/SvJ female mice (*Trp53* $^{+/+}$), also obtained from the Jackson Laboratory. The resulting F1 female progeny were genotyped prior to exposure or assignment to the unexposed control groups. *Trp53* heterozygous (+/-) and normal (+/+) females were retained for the radiation exposed groups and unexposed controls.

Determination of the *Trp53* genotypes of the F1 female mice was accomplished by PCR analysis of DNA extracted from a small tail clip, as previously described (34).

Animal housing

Female F1 *Trp53* heterozygous (+/-) or normal (+/+) mice were randomly assigned to groups, each with between 188 and 258 animals. All mice were housed in a specific pathogen free animal facility utilizing filter top cages in ventilated cage racks with automatic watering and were examined daily. Food was supplied *ad libitum*. Temperature and

ventilation were computer controlled at each rack. Temperature was maintained at 23 +/- 2ºC and a 12 h light/dark cycle was used.

All housing, handling and experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and with the pre-approval of the local Animal Care Committee. Mice were kept for either the duration of their natural lifespan or until euthanization was required according to the guidelines of the Canadian Council on Animal Care.

Irradiation

The chronic radiation exposure was designed to simulate a potential occupational exposure. A fractionated low dose, low dose rate exposure was given once per day, 5 days per week, for increasing fractions of the lifespan of the mice. The unrestrained mice in their plastic cages were exposed, beginning at about 6 weeks of age, to ^{60}Co-γ-irradiation at low dose rate from an open beam source (GammaBeam 150, Atomic Energy of Canada Limited). Animals were exposed daily to about 0.33 mGy doses of γ-radiation delivered at low dose rate (0.7 mGy/h), and the exposure was repeated 5 days/week (Monday to Friday). Animals were exposed for 30 weeks (to 257 days of age), 60 weeks (to 467 days of age) or 90 weeks (to 676 days of age). Source decay over this extended period was accommodated by regularly adjusting the distance between the source and the animals, in order to maintain the stated daily dose and dose rate. The total dose received by the animals that lived to the end of each exposure was 48, 97 or 146 mGy. Animals that died prior to the end of the assigned exposure received proportionally less total dose. Control animals that received no exposure

(above natural background) were handled in the same way and at the same time as the exposed animals, but were held in a shielded portion of the exposure facility during the time the test animals were exposed.

Tumor analysis

All malignant tumors were diagnosed by post-mortem gross and histological examination. In any animal where two instances of one tumor type were found, this was scored as one tumor of that type, since it was not always possible to distinguish between a primary tumor and a metastasis. If one animal contained two or more tumors that were of different tumor types, then one tumor of each type was scored.

During the course of their normal lifespan, some animals developed paralysis coincident with spinal osteosarcoma. Using this endpoint, we have previously reported an increase in spinal osteosarcoma latency when *Trp53* heterozygous mice received a single 10 mGy adapting dose (33). The analysis reported in the work presented here also included an assessment of tumor latency in those heterozygous animals where spinal osteosarcoma was identified in association with paralysis.

Statistical analyses

Statistical tests were conducted using the software program SigmaStat 3.01 (Systat Software Inc. Richmond CA.). Specific types of (or total) malignant tumor frequencies and the frequencies of mice with malignant tumors were compared using Chi Squared analyses. Survival probability was analyzed using a Kaplan Meier analysis and differences were tested

for significance using a Log Rank analysis. Differences with $p \leq 0.05$ were considered significant.

Since the different chronic exposures extended over significant and variable portions of the animal's lifespans, in some analyses of the *Trp53* normal mice we tested the possibility that the effects were age dependent, by restricting the datasets to mice alive at 676 days of age. This allowed a comparison of the effects of the various exposures in older mice that had all been exposed to the full 48 mGy (257 days of age), 97 mGy (467 days of age) or 146 mGy (676 days of age) dose. Since for the *Trp53* heterozygous mice, essentially all mice were dead by 676 days of age, we did the comparable analysis using 467 days of age, in which case the mice had all been exposed to the full 48 mGy (257 days of age) or 97 mGy (467 days of age).

In other analyses we tested the possibility that mice dying during the course of the chronic exposure influenced the outcome. The survival analysis of those mice was accordingly restricted to only those mice alive at the time they had completed their respective full dose exposures of either 48, 97 or 146 mGy. Each of these groups of mice was compared to the comparable group of unexposed mice, defined as only those mice alive at the age when each exposed group finished its respective chronic exposure.

RESULTS

Survival of unexposed Trp53 normal and heterozygous mice

Figure 1 shows the survival probabilities of the unexposed *Trp53* normal and heterozygous mice, relative to the ending times of the three different chronic, fractionated exposure periods. The numbers of mice in each test or control group are given in Table 1. Kaplan Meier survival analysis indicated significantly shorter survival of the unexposed *Trp53* heterozygous mice compared to the unexposed *Trp53* normal mice (Fig. 1, $P<0.001$). Because of this difference in lifespans, the 30, 60 or 90-week exposures spanned relatively different fractions of the animals normal lifespans. For example, the longest (90-week) exposure spanned virtually the entire lifespan of all the *Trp53* heterozygous mice, while about 60% of the *Trp53* normal mice remained alive at the end of that exposure.

***Trp53*$^{+/+}$ (normal) mice**

a) All Trp53$^{+/+}$ mice

Survival probability analysis of all *Trp53* normal animals in the exposed and unexposed groups showed that the 30 week exposures that stopped at 257 days of age (48 mGy maximum dose) significantly decreased survival (median lifespan 676 days) when compared to the unexposed animals (median lifespan 704 days, $P<0.04$) or when compared to the animals that continued to be exposed for 90 weeks, to 676 days of age (146 mGy maximum dose, median lifespan 700 days, $P<0.005$). Continuing the exposure for 60 weeks (467 days of age, 97 mGy maximum dose) or 90 weeks restored the lifespan (median lifespan 684 and 700 days respectively) such that the survival probabilities were not significantly different from that of the unexposed animals (median lifespan 704 days). This

result indicated that the lowest exposure increased the risk of lifespan loss, but that increasing the dose, by extending the exposure, restored that lifespan and eliminated the increased risk of the shorter exposure.

b) *Normal (Trp53$^{+/+}$) mice with malignant tumors*

Chi-square analyses showed no significant difference, as compared to the unexposed mice, in either the proportion of the *Trp53* normal mice with malignant tumors or in the incidence of malignant tumors (Table 1).

Survival probability analysis of only those *Trp53*$^{+/+}$ mice with malignant tumors indicated that the exposures that stopped after 30 weeks (at 257 days of age, 48 mGy) significantly decreased survival when compared to the unexposed animals ($P<0.003$). As was the case when all mice were considered, this decreased survival probability was reversed as the exposure time (and dose) increased. Continuing the exposure for 60 weeks or for 90 weeks eliminated this increased risk from the 30-week exposure, and returned the survival probability to values not significantly different from that of the unexposed animals. Figure 2 shows the median lifespans of the unexposed mice and mice exposed for the 30, 60 or 90 week periods.

We tested the possibility that mice bearing malignant tumors that were dying during the course of the chronic exposure influenced the outcome. The results of the comparisons for significant differences in survival probability noted above for all the mice with malignant tumors did not change, indicating that early death did not influence the outcomes.

We also tested the possibility that the response may be dependent on age and exposure to the full 30, 60 or 90-week dose, so the survival analysis was further restricted to mice with malignant tumors alive at 676 days of age. Again, the exposure that stopped after 30 weeks significantly shortened lifespan (median lifespan 772 days) when compared to the unexposed mice (median lifespan 808 days, $P<0.02$). As was the case when all the mice with malignant tumors were considered, extending the exposure for 60 or 90 weeks eliminated this increase in risk and restored the lifespans of those older exposed mice (median lifespans 785 and 827 days respectively) such that they were not different from the unexposed mice (median lifespan 808 days).

These results indicated that the increased risk of lifespan loss seen in all the animals exposed to the lowest dose was due to a loss of lifespan in animals with malignant tumors, and that the elimination of that increased risk, by extending the exposure and increasing the dose, was due to a regain of normal lifespan in the mice with malignant tumors. These effects were not influenced by animal age or early death.

c) Normal (Trp53$^{+/+}$) mice without malignant tumors

Survival probability analysis of all the *Trp53*$^{+/+}$ mice without malignant tumors indicated that none of the exposures significantly altered lifespan, when compared to the unexposed mice. However, restricting the analysis to older mice without malignant tumors (those alive at 676 days of age) indicated that the 90-week exposure significantly increased median lifespan, when compared to the unexposed mice (to 834 days from 784 days). As previously

reported, this protective effect resulted largely from a reduction in the incidence and severity of acute ulcerative dermatitis, a spontaneous, autoimmune and age related non-malignant skin disease in older C57BL/6 mice (42).

d) $Trp53^{+/+}$ mice malignant tumor type

Since the above analysis indicated that the increased risk from the lower (shorter) exposures was associated with mice with malignant tumors, the data (Table 1) were examined further to identify the nature of the tumors responsible for this response. The data set of mice with malignant tumors was sub-divided into two sets, mice with lymphomas or mice with all other malignant tumor types together. Lymphomas of B- or T-cell types were further considered separately.

1) All Lymphomas in $Trp53^{+/+}$ Mice

Kaplan Meier analysis indicated that the radiation exposures that stopped after 30 weeks reduced the survival probability of mice with B- or T-cell lymphomas, compared to unexposed mice ($P<0.001$). Continuing the exposure for 60 weeks also significantly reduced the survival probability of mice with lymphomas ($P<0.007$), but the observed reduction was less than in the mice exposed for 30 weeks ($P<0.05$). However, when the exposure was extended to 90 weeks the survival probability was not significantly different from that of the unexposed mice, but was significantly greater than in the group of mice only exposed for 30 weeks ($P<0.001$) (Fig. 3).

We tested the possibility that mice dying during the course of the chronic exposure influenced the outcome. The results of the comparisons for significant differences in survival

probability noted above for all the mice with lymphomas did not change, indicating that early death did not influence the outcomes.

The survival analysis of mice with lymphomas was further restricted to those alive at 676 days of age. Again, the exposures that stopped after 30 weeks (48 mGy) or 60 weeks (97 mGy) significantly shortened lifespan (median lifespan 814 and 823 days respectively) when compared to the unexposed mice (median lifespan 880 days, $P<0.001$for both comparisons). However, the mice that received the full 90 weeks of exposure (146 mGy) had a survival probability (median survival 902 days) not significantly different from the unexposed mice (median survival 880 days). These results indicated that the outcome of the exposures in the *Trp53* normal mice did not depend on the age of the mice.

Chi-square analyses of the proportion of mice with lymphomas (B- plus T-cell types together (Table 1) showed that the 30-week exposure of the *Trp53* normal animals increased the proportion of the mice with lymphomas, as compared to the unexposed mice ($P=0.007$). Increasing exposure time (and therefore dose) to 60 weeks reduced this excess to a marginally non-significant trend ($P=0.053$) and after 90 weeks of exposure the proportion of mice with lymphomas was not different from the unexposed control. The appearance of lymphomas with time in the control and exposed groups of *Trp53* normal animals is shown in Fig. 4.

Taken together, these results indicated that the increase in the risk of loss of lifespan associated with mice with malignant tumors and exposed to the lowest (30-week, 48 mGy)

dose was further associated specifically with mice that had lymphomas. This lifespan loss at the lowest 48 mGy dose (and marginally also with the 97 mGy dose) correlated with an increase in lymphoma frequency. Furthermore, the increased risk of lifespan loss could also be detected after the 60-week, 97 mGy dose. As for all mice, and for all mice with malignant tumors, the increased risk of lifespan loss and increased lymphoma frequency was eliminated by continuing the exposure to 90-weeks, for a total dose of 146 mGy.

2) B-Cell Lymphomas in Trp53$^{+/+}$ Mice

In both the exposed and unexposed *Trp53*$^{+/+}$ mice, most of the lymphomas were B-cell type (Table 1). Chi-square analyses of the proportion of mice with B-cell lymphomas showed that none of the exposures of the *Trp53* normal animals significantly altered the proportion of the mice with B-cell lymphomas, as compared to the unexposed mice. However, Kaplan Meier survival analysis indicated that exposure for 30 weeks reduced the survival probability of mice with B-cell lymphomas (median survival 815 days), compared to the unexposed group (median survival 880 days, $P<0.002$). Increasing the exposure duration to 60 or 90 weeks returned the survival probability to values (median survival 837 and 900 days, respectively) not significantly different from that of the unexposed animals (median survival 880 days).

As noted above for the mice with all lymphomas, we tested the influence of early death and old age on the significance of the survival probability outcome for B-cell lymphomas, and again found no influence on the outcome.

These results show that the 30-week exposure increased risk by reducing B-cell tumor latency but not by increasing B-cell tumor frequency, and that the higher doses from the 60 or 90-week exposures increased the B-cell latency to that of the spontaneous B-cell tumor latency of unexposed mice.

3) T-Cell Lymphomas in Trp53$^{+/+}$ Mice

T-cell lymphomas were rare in unexposed *Trp53*$^{+/+}$ mice (Table 1) but exposure for 30 or 60 weeks significantly increased the proportion of mice with this tumor type, as compared to unexposed mice (P=0.001, P<0.02, respectively). However, continuing the exposure for 90 weeks prevented that increase, and the proportion of these animals with T-cell lymphomas was not different from that of the unexposed mice, but was significantly lower than either the 30 or 60 week exposed groups (P=0.004 and P=0.032 respectively). Kaplan Meier survival analysis (Fig. 5) indicated that the survival probability of the *Trp53* normal mice with T-cell lymphomas was also significantly decreased by exposures for 30 or 60 weeks (P<0.001, P<0.004, respectively) but after 90 weeks of exposure, survival probability was no longer different from that of unexposed animals. As for all lymphomas and for B-cell lymphomas, restricting the T-cell data to test for effects due to early death or old age did not influence the significance of the outcome.

Considering both the B-cell and T-cell lymphoma data, the results indicate that the increased risk of lifespan loss resulting from the 30-week (48 mGy) and 60-week (97 mGy) exposures, and associated with mice with lymphomas, was due to an increased risk from both lymphoma cell types. The elimination of that increase in risk (back to the level of

unexposed mice) by increasing the exposure to 90-weeks (146 mGy) was also associated with both lymphoma types.

4) $Trp53^{+/+}$ Mice with malignant tumors other than lymphomas

The above analyses suggested that the chronic fractionated exposures were influencing the risk of lymphomas. To test whether the exposures were also influencing the risk of other tumors (Table 1), the data sets of mice with all types of malignant tumors were restricted by removing those mice with lymphomas. Kaplan Meier analysis indicated no significant influence of any exposure on the survival probability of mice with non-lymphoma malignant tumors, in comparison to the unexposed mice. Median survivals of the exposed and unexposed control mice are shown in Fig. 6, and although the data is suggestive of an increased survival after 60 weeks of exposure, the large uncertainties preclude that conclusion. Restricting the analysis to only those older animals that had received the full 48, 97 or 146 mGy dose did not alter this result. Chi-squared analysis showed no significant change in the frequency of all other malignant tumors (other than lymphomas) taken together. These results indicate that the chronic fractionated exposures influenced the risk of lymphomas, but had no detectible influence on other tumor types taken together in *Trp53* normal mice.

Because of the suggestive nature of the data in Fig. 6, the analysis was further narrowed by separately considering the two other major tumor types, carcinomas and sarcomas (Table 1). For mice with carcinomas, Kaplan-Meier analysis indicated no significant influence of any exposure on the survival probability, in comparison to the unexposed mice,

and Chi-squared analysis showed no significant change in the carcinoma frequency (Table 1). Analyses of sarcoma type tumors however, showed a significant protective effect by the 60-week exposure, with sarcoma frequency (Table 1) significantly reduced when compared to either the unexposed animals (P=0.005), or to the animals exposed for 30 weeks (P=0.05) or for 90 weeks (P=0.006). However, this protective effect appeared to be lost if the exposure was continued for 90 weeks since the sarcoma frequency at that point was not different from that of the unexposed animals. Similarly, Kaplan Meier analysis indicated a significant increase in survival probability from the 60-week exposure in comparison to the unexposed mice (P=0.002) or to the mice exposed for 30 week (P=0.002) or 90 weeks (P=0.004). Again, the analysis showed that this protective effect of the 60-week exposure appeared to be lost when the exposure was continued for 90 weeks, where survival probability was not different from that of the unexposed mice. Median survivals of the exposed and unexposed control mice are shown in Fig. 7. These results indicate that exposure had to be continued for 60 weeks in order to induce significant protective effects, again suggesting that a lower dose threshold had to be first exceeded. Conversely, extending the exposure to 90 weeks apparently exceeded the upper dose threshold for protective effects in this specific tissue type, and precluded that induction. We have previously reported observations showing that upper dose thresholds for protective responses vary with the tissue type in mice (33).

Cancer-Prone Trp53$^{+/-}$ Mice

The frequency of unexposed *Trp53* heterozygous mice with spontaneous malignant tumors was about double that observed in the wild type *Trp53* mice ($P<0.001$), as was the total frequency of spontaneous tumors (Table 1, $P<0.001$).

a) All cancer-prone Trp53 heterozygous mice

Survival probability analysis of all *Trp53* heterozygous animals in the exposed and unexposed groups showed that neither the 30 week, 60 week nor 90 week exposures had any influence on the survival of the exposed mice, as compared to the unexposed *Trp53* heterozygous mice.

b) Trp53 heterozygous mice with malignant tumors

Chi-square analyses showed no significant difference, when compared to the unexposed mice, in either the proportion of the *Trp53* heterozygous mice with malignant tumors or in the incidence of malignant tumors (Table 1).

Survival probability analysis of the $Trp53^{+/-}$ mice with malignant tumors indicated that none of the exposures (30, 60 or 90 week) influenced survival when compared to the unexposed $Trp53^{+/-}$ mice. The median survival times for the unexposed animals, and animals exposed for 30, 60 or 90 weeks were 504, 523, 485 and 505 days respectively.

As for the *Trp53* normal mice, we tested the possibility that the response of the heterozygous mice may be age dependent. Survival probability analysis of the mice alive at

467 days of age showed no significant effect of any of the 30, 60 or 90-week exposures, compared to the unexposed mice.

c) Lymphomas in Trp53$^{+/-}$ Mice

As in the *Trp53* normal mice, most of the lymphomas in the exposed and unexposed *Trp53*$^{+/-}$ mice were B-cell lymphomas, and T-cell type lymphomas were less frequent (Table 1). However, unlike the *Trp53* normal mice, Kaplan Meier analysis of the cancer prone *Trp53* heterozygous mice indicated that, compared to unexposed mice, the radiation exposures that stopped after either 30, 60 or 90 weeks had no influence on the survival probability of mice with B and T-cell lymphomas, either taken together (median survival times 630, 625, 645 and 651 days respectively) or analyzed for B- or T-cell lymphomas separately. Also unlike the *Trp53* normal mice, none of the radiation exposures had any influence on the frequency of T-cell lymphomas in the *Trp53* heterozygous mice. The appearance of lymphomas with time in the control and exposed groups of *Trp53* heterozygous mice is shown in Fig. 8.

As in the case of all mice with malignant tumors, we tested the possibility that age influenced the survival probability of exposed mice with lymphomas. Restricted to older mice, those alive at 467 days of age, the analyses again showed that the radiation exposures that stopped after either 30, 60 or 90 weeks had no influence on the survival probability of mice with B and T-cell lymphomas.

d) Trp53$^{+/-}$ Mice with malignant tumors other than lymphomas

Kaplan Meier survival analysis of those mice with all types of malignant tumors but without lymphomas indicated no significant influence on survival probability after any of the exposures. The survival analysis of these mice with malignant tumors but without lymphomas was further restricted to older mice, those alive at 467 days of age. Kaplan Meier analysis again indicated no significant influence on survival probability after any of the exposures, as compared to the unexposed mice.

We have previously reported that a single low dose influenced the latency of spontaneous spinal osteosarcomas associated with paralysis in C57BL/6 *Trp53* heterozygous mice (33). We therefore examined the current data for mice with the same condition. Compared to the unexposed mice, there was no effect of any of the radiation exposures on survival probability or the frequency of spinal osteosarcomas associated with paralysis.

e) *Heterozygous ($Trp53^{+/-}$) mice without malignant tumors*

Survival probability analysis of all the $Trp53^{+/-}$ mice without malignant tumors indicated that none of the exposures significantly altered lifespan, when compared to the unexposed mice, and restricting the analysis to older mice without malignant tumors (those alive at 467 days of age) also indicated no effect of exposure on lifespan of older animals.

DISCUSSION

National radiation protection regulations and practices as applied to both people and the environment are generally based on the recommendations of the ICRP, which assume a

linear no-threshold relationship between risk and dose (1). There is a large body of evidence that indicates that this assumption does not hold at low doses, and that low doses can actually decrease risk by inducing protective adaptive responses. We have previously reported that there are tissue specific upper dose thresholds for these protective responses against radiation-induced and spontaneous cancer, and that *Trp53* functionality influences adaption and this protective threshold (33, 34). In the work presented here we have tested the possibility that a lower dose threshold also exists for protective effects against cancer *in vivo*. For this test, we have used a dose regimen meant to be relevant to radiation protection and which represented a typical occupational exposure pattern. Using this same exposure regimen we have previously reported that low dose thresholds appear to exist for protection against chronic ulcerative dermatitis, a spontaneous, autoimmune-type age-related disease in C57BL/6 mice (42). For that non-cancer disease, these very low dose fractionated exposures induced a protective adaptive response in both *Trp53* normal and heterozygous mice, but a lower threshold level of exposure, similar in both cases, had to be exceeded. In cancer-prone mice with reduced *Trp53* functionality (*Trp53* heterozygotes), doses below the threshold produced detrimental effects.

From the work presented here it is clear that there is also a lower dose threshold for induced protective responses against spontaneous cancer, but that a low dose below that threshold, delivered in an "occupational-like" exposure pattern could increase cancer risk in C57BL/6 *Trp53* normal mice. This increased risk was evident as an overall decreased lifespan (decreased tumor latency) and increased frequency of lymphomas in mice exposed for 30 or 60 weeks. The increase in cancer risk was specific to lymphomas, both B and T-cell

type, with the B-cell tumors showing decreased latency and the T-cell tumors increased frequency. Likewise, exceeding that dose threshold and inducing protective effects that reduced the risk back to that of unexposed animals, by continuing the exposure for 90 weeks, was also due to a reduction in the risk of mice with B or T-cell lymphomas, returning the tumor frequency and latency back to the values seen for spontaneous lymphomas in unexposed mice. In addition to the effects on lymphomas, these exposures also influenced the risk of sarcomas, another tumor type that occurs spontaneously with relatively high frequency in these mice. Exposures of 60 weeks duration were required to exceed the lower dose threshold for protection for this tumor type, and unlike lymphomas, doses below that threshold did not increase sarcoma risk. However, continuing the exposure to 90 weeks apparently exceeded the upper dose threshold for induction of protective effects and the protective effects were lost. These results again reinforce the concept that the same exposure can produce fundamentally different responses in the various individual tissue types in the same animal. The results, along with our earlier cancer and non-cancer data (33, 34, 42) also suggest that all tissues show both upper and lower thresholds for induction of protective adaptive responses.

T-cell type lymphomas are a relatively infrequent spontaneous tumor type in *Trp53* normal C57BL/6 mice (Table 1), but are commonly induced in mice by high doses of radiation (34). Adapting doses can decrease the frequency and increase the latency of these tumors (10, 34). Such T-cell lymphomas arise with a higher spontaneous rate in cancer-prone *Trp53* heterozygous C57BL/6 mice and again single low adapting doses reduce this risk (33, 34). The 30 and 60-week chronic fractionated dose schedule employed in the work presented

here appeared to have had a detrimental effect in the *Trp53* normal mice, similar to that seen after a high acute dose, and continuing the exposure for 90 weeks resulted in a protective effect similar to that seen when such high doses are preceded by a low adapting dose (34). However, since the extension of the exposure to 90 weeks necessarily occurs after the 30 or 60-week exposure, and after the high-dose-like effect seen from those lower exposures, the protection afforded by the 90-week exposure functionally resembles the induced protection reported by Day et al. (47) where an initial high dose was followed by the protective low adapting dose.

In contrast to the *Trp53* normal mice, the *Trp53* heterozygous mice showed no influence of any of the exposures on overall lifespan in mice with or without tumors, or on the frequency or latency of cancers of any tumor type (including B or T-cell lymphomas). These results indicate that the elevated risk seen in the *Trp53* normal mice exposed to the lower chronic fractionated doses was completely dependent on full *Trp53* gene function.

CONCLUSIONS

The work presented here, using direct measures of radiation risk *in vivo*, tests the hypothesis that lower dose thresholds exist for radiation-induced protective adaptive responses against cancer. This current work on cancer risk in C57BL/6 mice, and our previous report (42) on a non-cancer disease (chronic ulcerative dermatitis) both indicate that lower dose thresholds for protective adaptive responses exist *in vivo*. Chronic “occupation-like” exposures that were below this dose threshold did not induce protective responses for either measure of risk. Furthermore, detrimental effects from the exposures that were below

the adaptive threshold could be observed for some tumor types, depending on the level of *Trp53* functionality of the mice. Reduced *Trp53* functionality, which elevates spontaneous cancer risk, eliminated all effects of these exposures on cancer risk in the cancer prone mice, indicating that the lower dose threshold for both protective and detrimental effects is raised in mice with reduced *Trp53* functionality. For both measures of risk *in vivo* (cancer or non-cancer disease), increasing the total dose by continuing to expose the mice with this chronic fractionated regimen resulted in induction of adaptive responses that protected against the detrimental effects of the lower, sub-threshold doses. Coupled with earlier cellular observations of lower dose thresholds for protective effects (31, 37-39), the observations that these thresholds also exist for both cancer and non-cancer diseases *in vivo* suggest that this may be a general phenomenon.

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FIGURE LEGENDS

Figure 1. Survival probability of unexposed *Trp53* normal (closed circles) and *Trp53* heterozygous (open squares) C57BL/6 mice. The vertical lines show the end of the 30-week (dotted line), 60-week (dashed line) and 90-week exposures (solid line) relative to the normal lifespan of the animals.

Figure 2. Median survival (+/- SE) of the *Trp53* normal mice with malignant tumors that were either unexposed or exposed to the chronic fractionated doses for 30, 60 or 90 weeks. Kaplan Meier survival analyses were used to test for significant differences. ** indicates $P<0.01$ compared to the unexposed mice.

Figure 3. Median survival (+/- SE) of the *Trp53* normal mice with lymphomas that were either unexposed or exposed to the chronic fractionated doses for 30, 60 or 90 weeks. Kaplan Meier survival analyses were used to test for significant differences. *** indicates $P<0.001$ and ** indicates $P<0.01$ compared to the unexposed mice.

Figure 4. Increase in lymphoma frequency with time in *Trp53* normal mice that were either unexposed (closed circles) or exposed to the chronic fractionated doses for 30 weeks (open circles), 60 weeks (open triangles) or 90 weeks (open squares). The vertical lines indicate the end of the 30-week (dotted line), 60-week (dashed line) and 90-week exposures (solid line).

Figure 5. Survival probability of *Trp53* normal mice with T-cell lymphomas that were unexposed (heavy solid line) or exposed to the chronic fractionated doses for 30-weeks (light

solid line), 60 weeks (dashed and dotted line) or 90 weeks (dashed line). Kaplan Meier survival analyses were used to test for significant differences. *** indicates P<0.001 and ** indicates P<0.01 compared to the unexposed mice or to the 90-week exposed mice.

Figure 6. Median survival (+/- SE) of the *Trp53* normal mice with all malignant tumors other than lymphomas, that were either unexposed or exposed to the chronic fractionated doses for 30, 60 or 90 weeks. Kaplan Meier survival analyses were used to test for significant differences. There was no significant change in survival from any exposure, compared to the unexposed mice.

Figure 7. Median survival (+/- SE) of the *Trp53* normal mice with sarcomas, that were either unexposed or exposed to the chronic fractionated doses for 30, 60 or 90 weeks. Kaplan Meier survival analyses were used to test for significant differences. ** indicates P<0.01 compared to the unexposed mice.

Figure 8. Increase in lymphoma frequency with time in the cancer-prone *Trp53* heterozygous mice that were either unexposed (closed circles) or exposed to the chronic fractionated doses for 30 weeks (open circles), 60 weeks (open triangles) or 90 weeks (open squares). The vertical lines indicate the end of the 30-week (dotted line), 60-week (dashed line) and 90-week exposures (solid line).

Table 1. Malignant tumors in control and exposed *Trp53* $^{+/+}$ and *Trp53*$^{+/-}$ mice

Exposure Group	*Trp53* Genotype	Number of Animals	Total number of Malignant Tumors (Per animal)	Lymphomas (B-cell: T-cell)	Osteosarcomas	Sarcomas	Carcinomas	Other
Control	+/+	223	99 (0.44)	39 (37:2)	3	47	10	0
30 Weeks	+/+	209	115 (0.55)	68 (51:17)	3	36	8	0
60 Weeks	+/+	232	106 (0.46)	64 (51:13)	3	22	17	0
90 Weeks	+/+	188			0	40	8	0

			92 (0.49)	44 (42:2)				
Control	+/-	230	215 (0.94)	48 (36:12)	89	61	11	6
30 Weeks	+/-	258	220 (0.85)	48 (39:9)	95	61	16	0
60 Weeks	+/-	233	196 (0.84)	49 (40:9)	84	49	12	2
90 Weeks	+/-	251	228 (0.91)	42 (34:8)	98	63	22	3

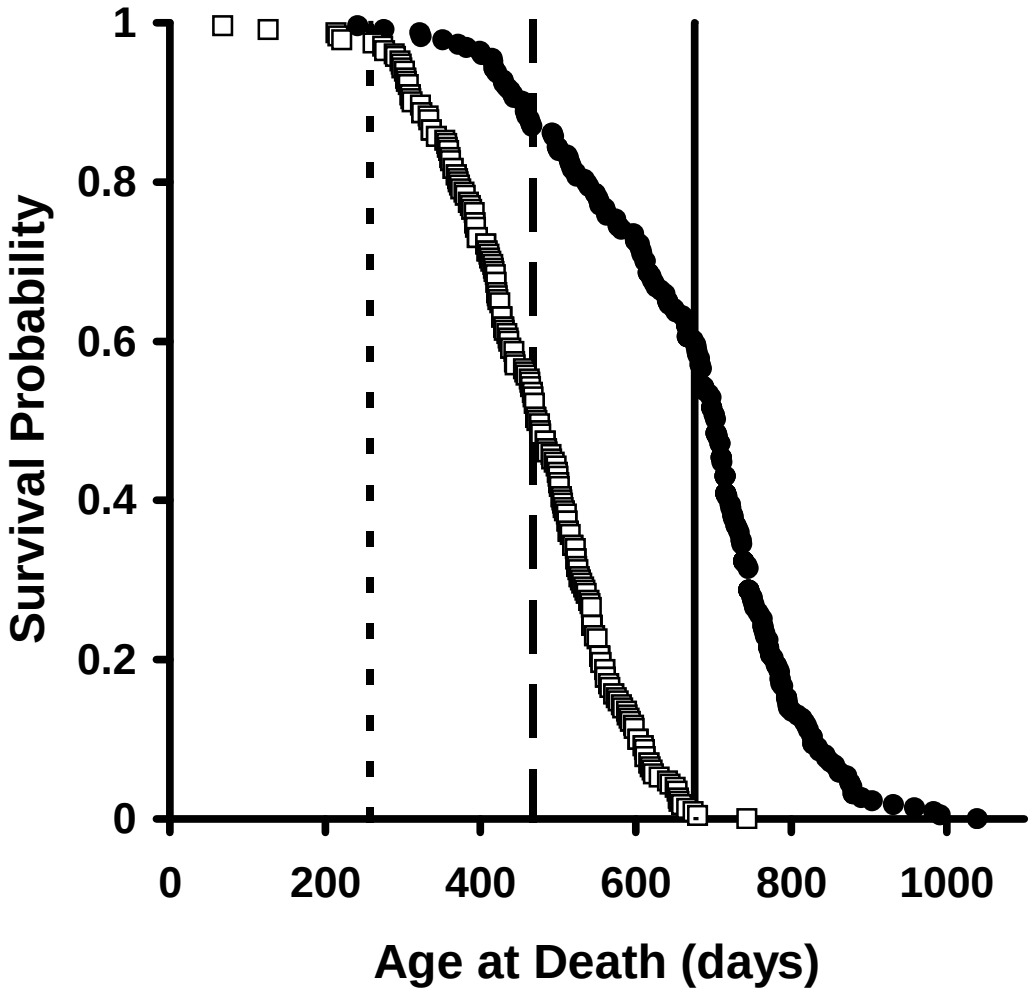
Survival Probability
1
0.8
0.6
0.4
0.2
0
0
200
400
600
800
1000
Age at Death (days)

Figure 1

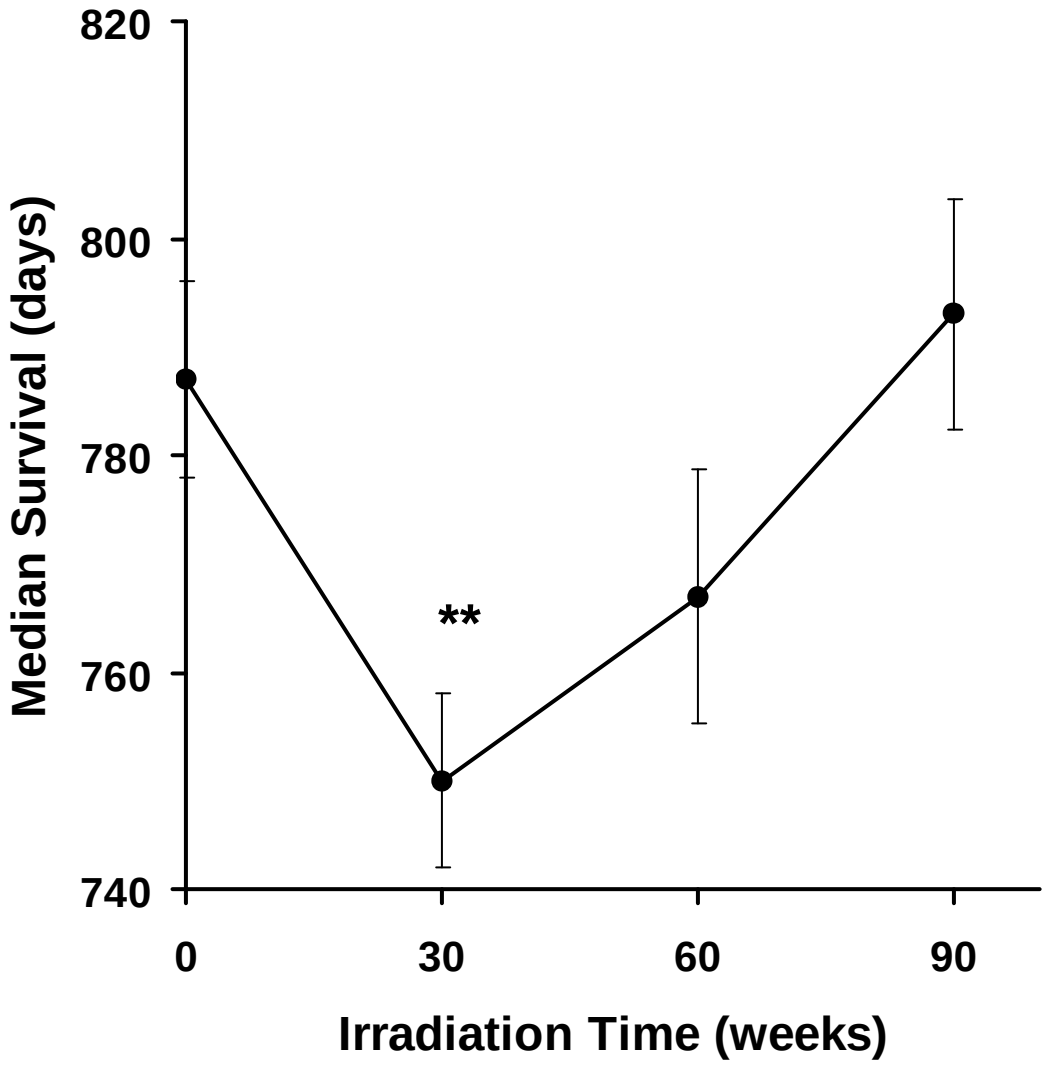
820
800
780
760
740
Median Survival (days)
**
0
30
60
90
Irradiation Time (weeks)

Figure 2

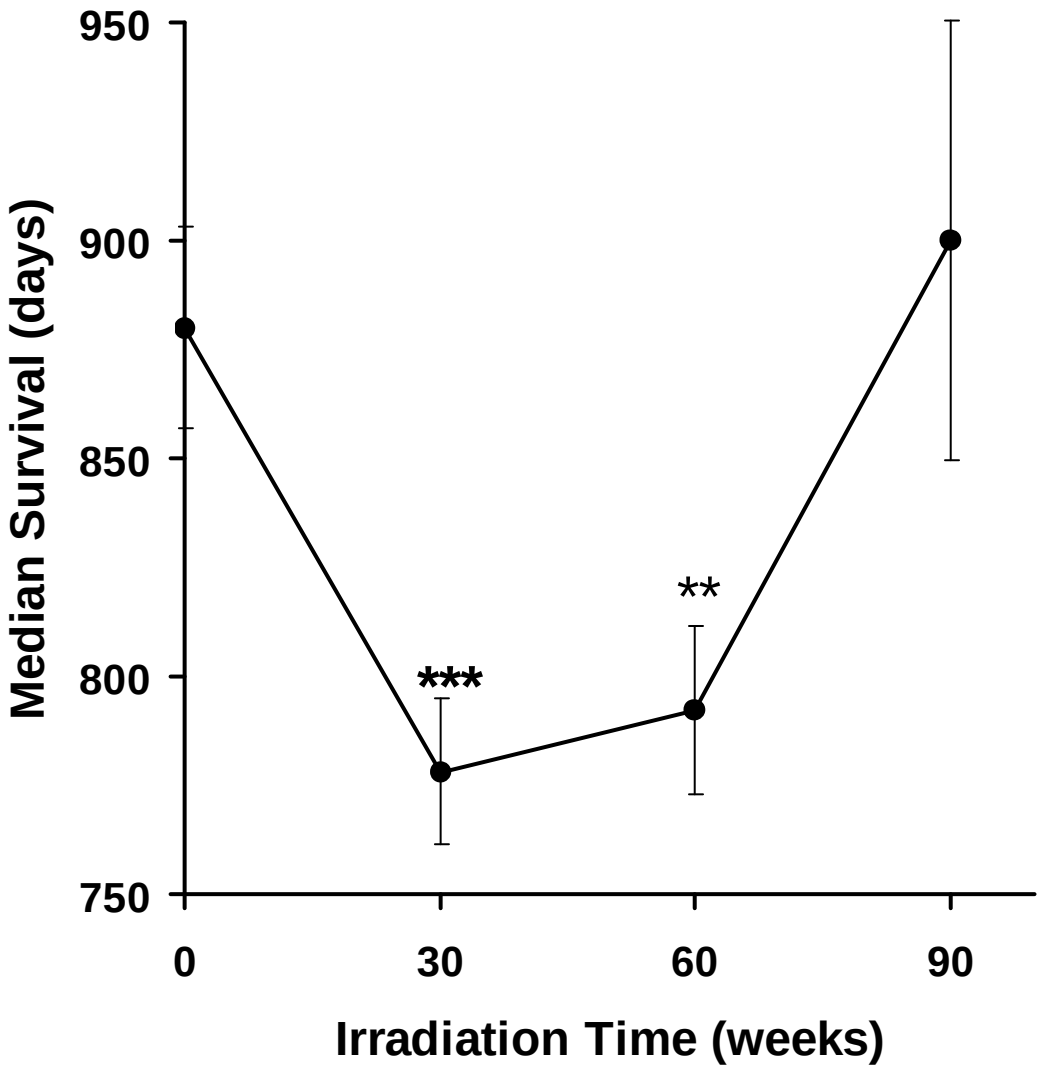
950
900
850
800
750
Median Survival (days)
***
**
0
30
60
90
Irradiation Time (weeks)

Figure 3

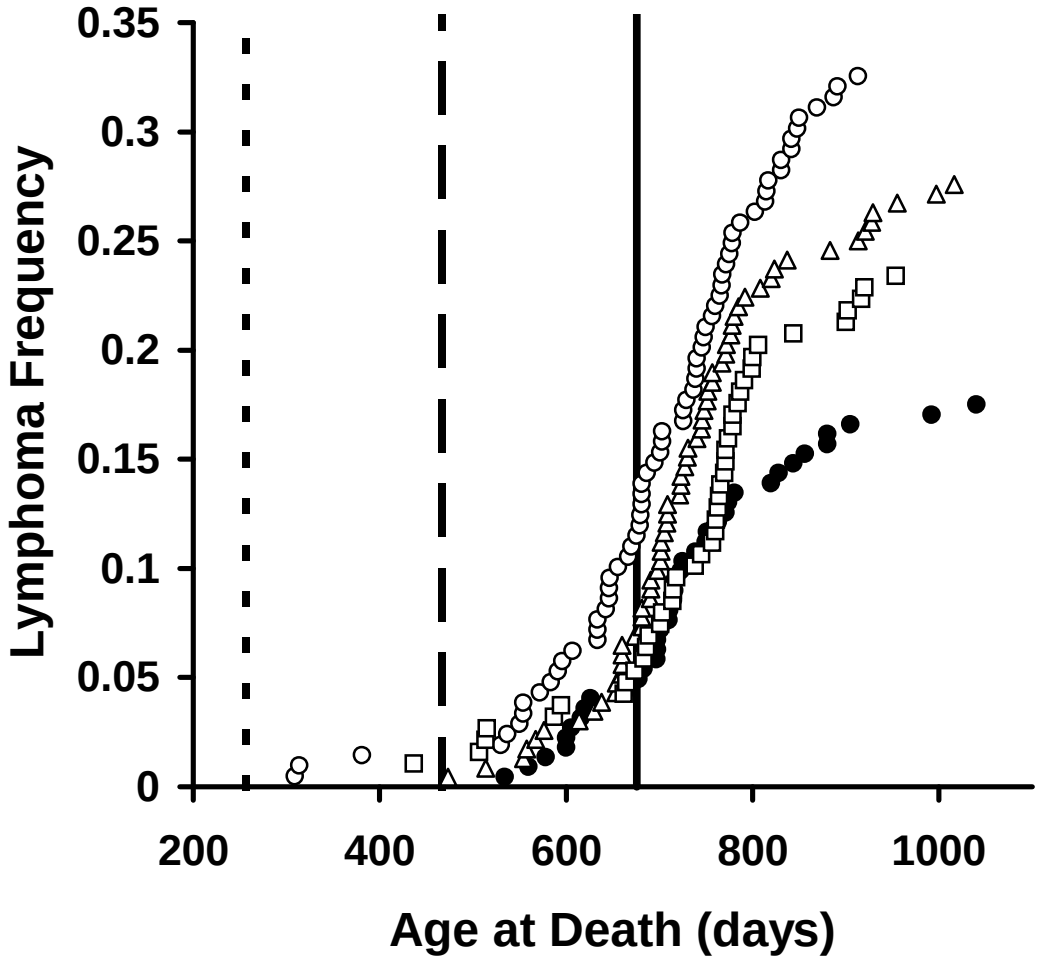
Lymphoma Frequency
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
200
400
600
800
1000
Age at Death (days)

Figure 4

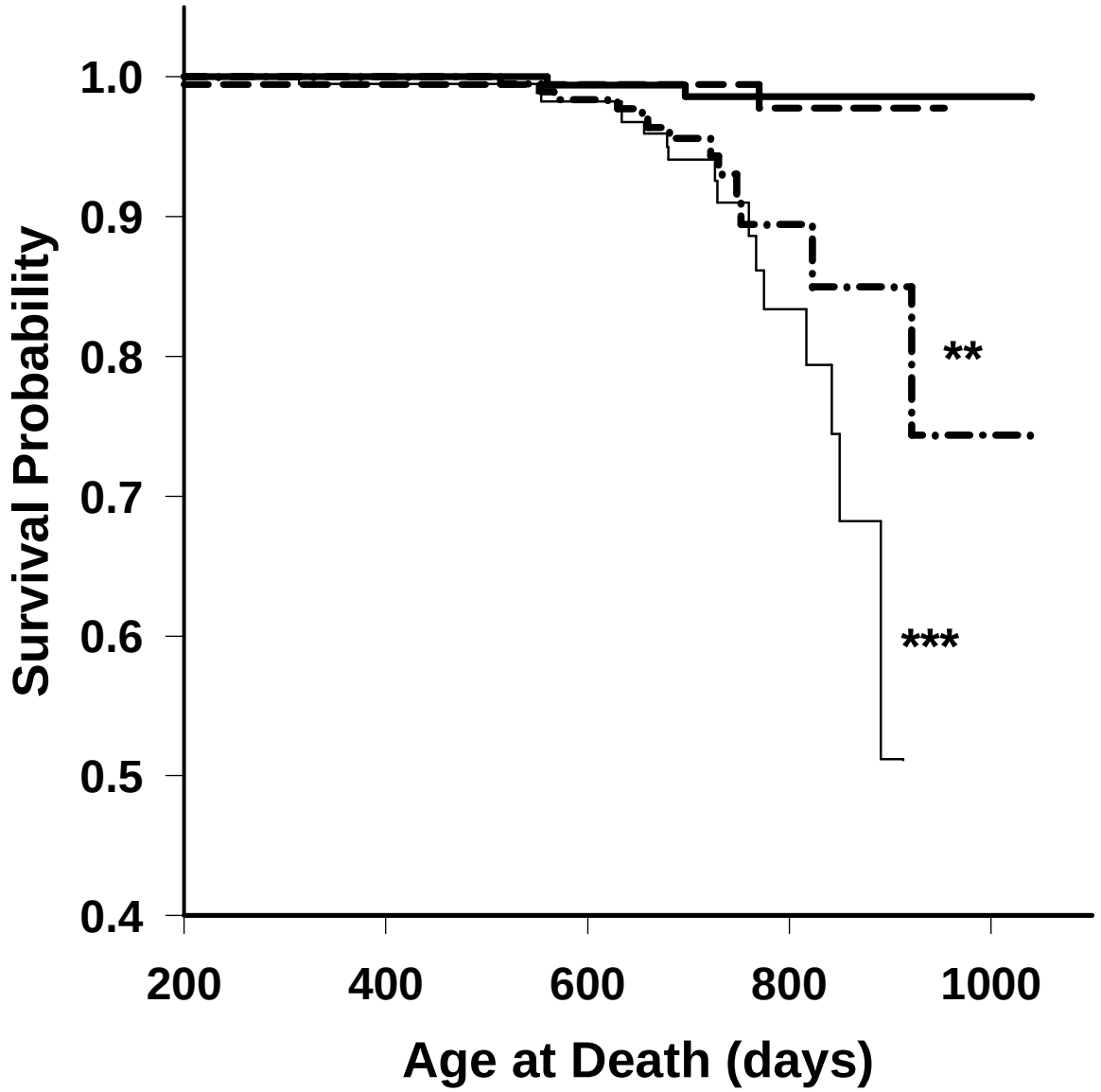
1.0
0.9
0.8
0.7
0.6
0.5
0.4
Survival Probability
**
***
200
400
600
800
1000
Age at Death (days)

Figure 5

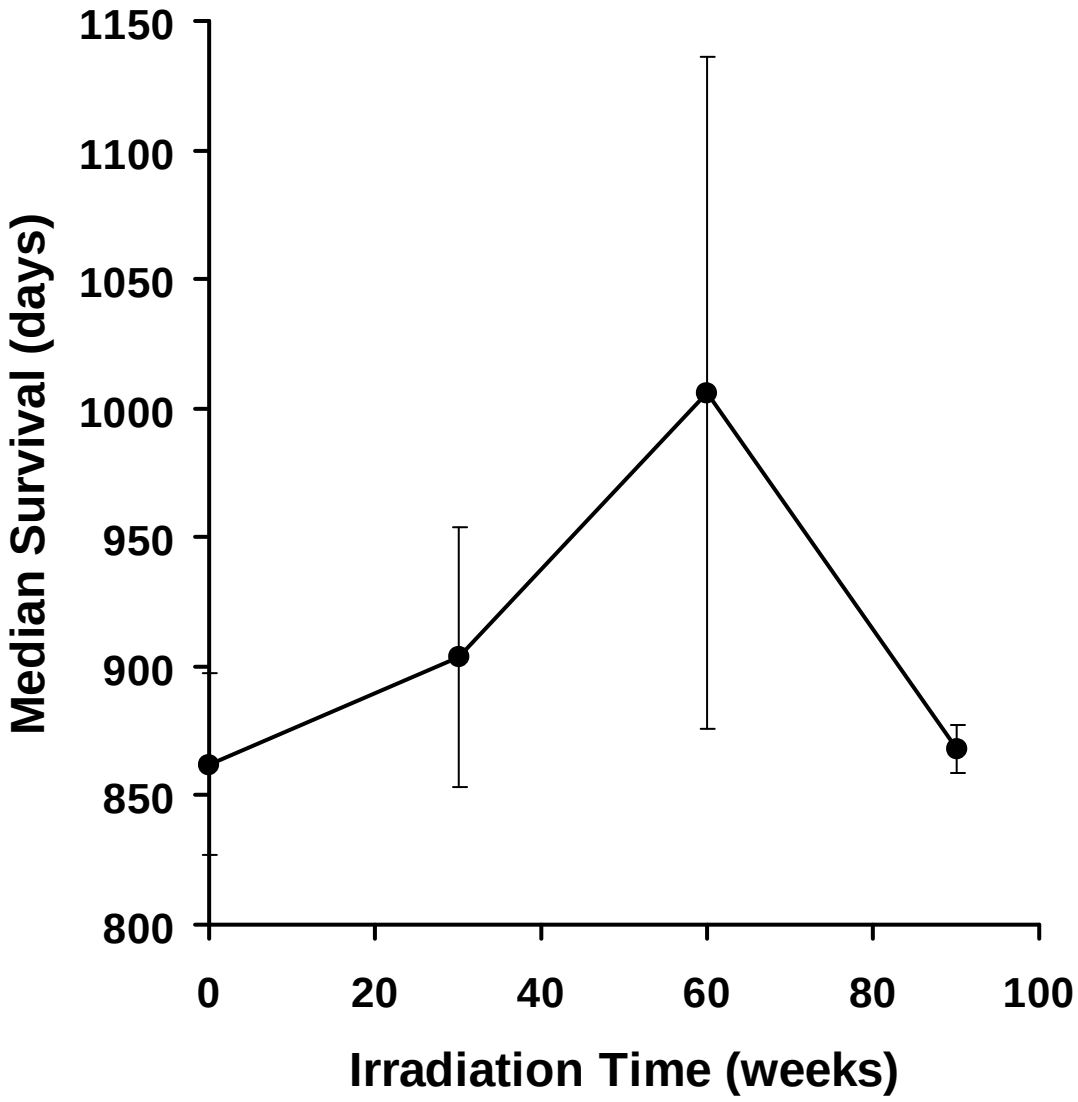
1150
1100
1050
1000
950
900
850
800
Median Survival (days)
0
20
40
60
80
100
Irradiation Time (weeks)

Figure 6

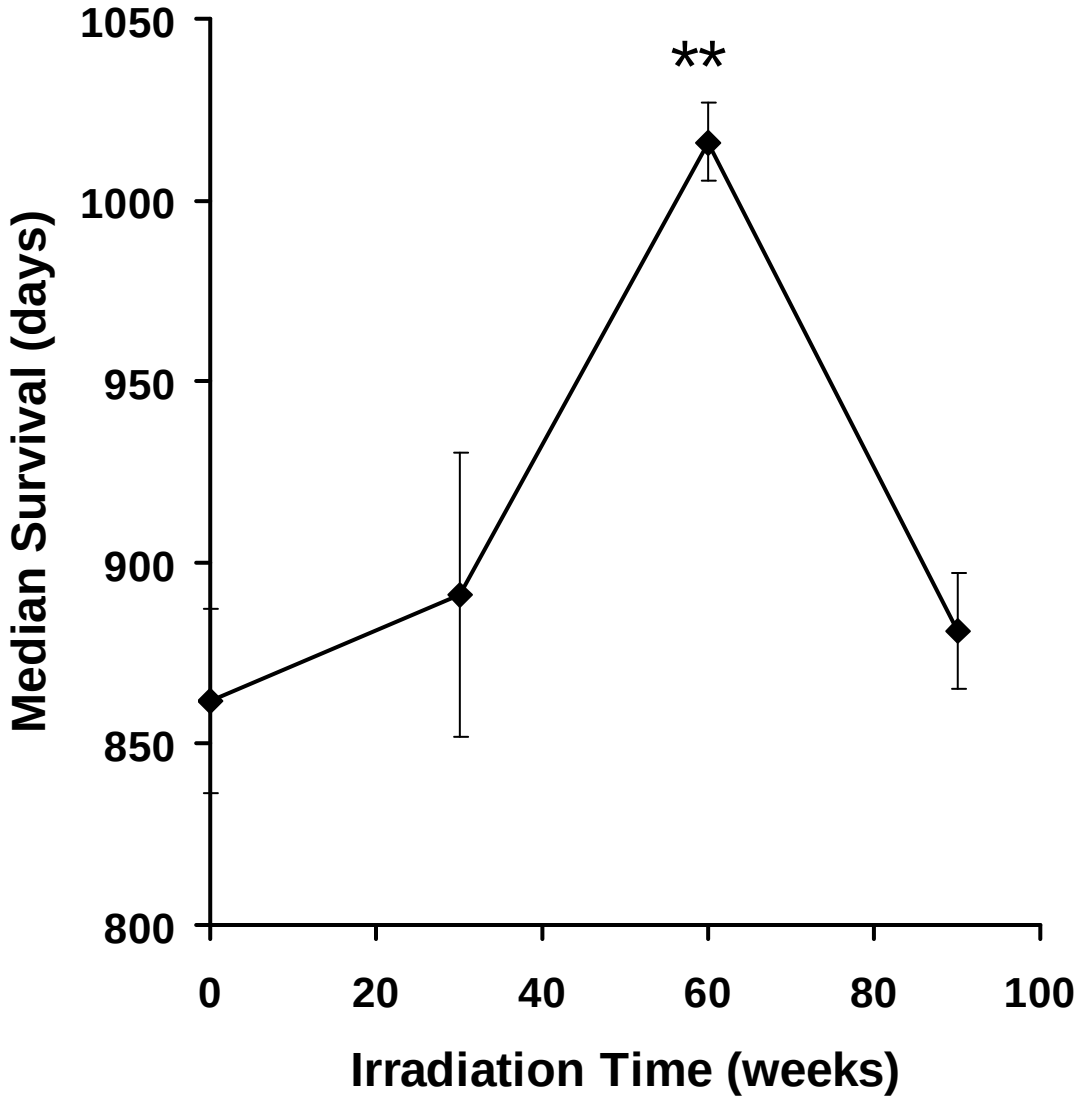
1050
1000
950
900
850
800
**
Median Survival (days)
0
20
40
60
80
100
Irradiation Time (weeks)

Figure 7

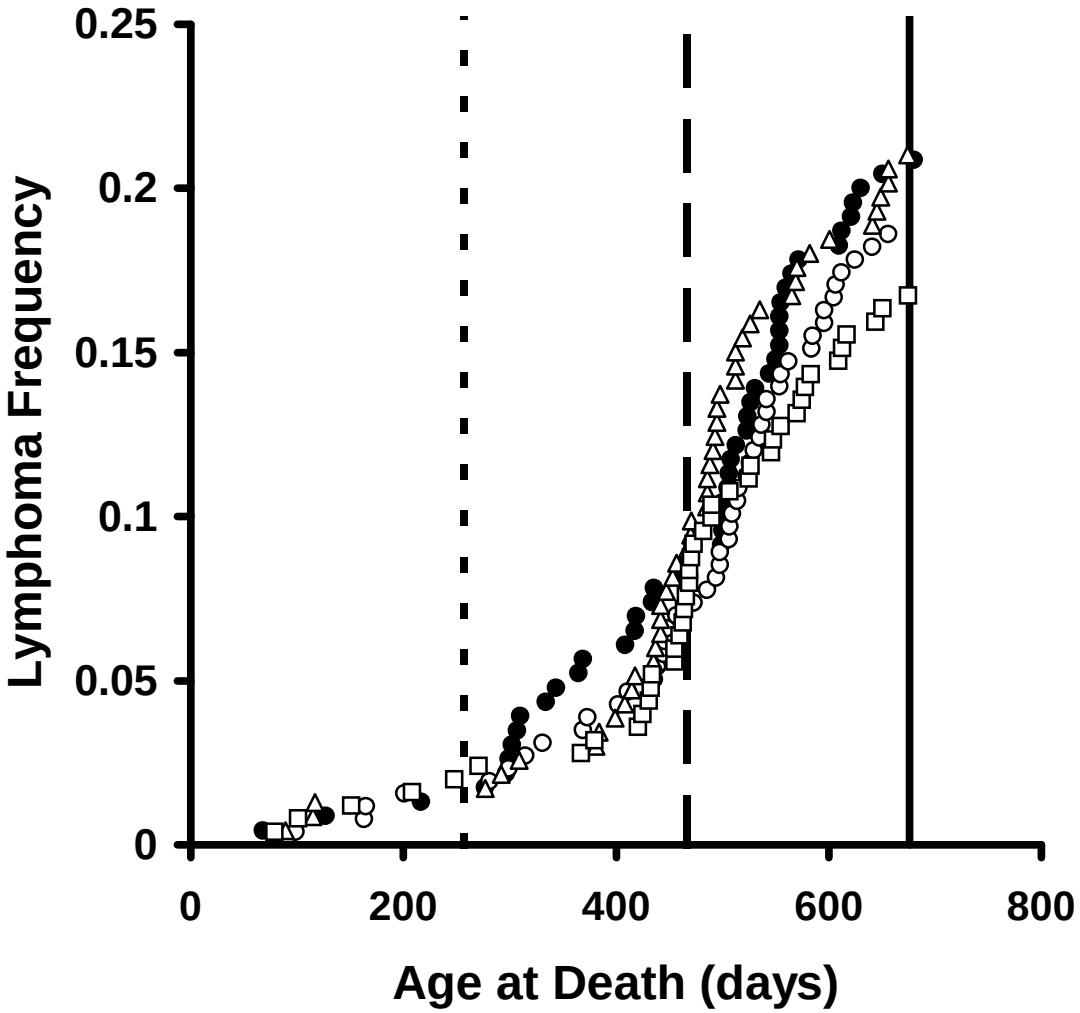
0.25
0.2
0.15
0.1
0.05
0
Lymphoma Frequency
0
200
400
600
800
Age at Death (days)

Figure 8