A comparative analysis of Rag2/Ii2rg (R2G2) and NSG™ radiosensitivity.

Introduction

The increasing variety of immunodeficient mouse strains that are available can make choosing an appropriate model difficult and confusing. This is particularly relevant to researchers employing radiation in their studies - whether as a pre-conditioning step (e.g. myeloablation) or testing the efficacy of radiation therapy - since different models display inherent differences in their sensitivities to radiation.

Highly immunodeficient strains that carry multiple genetic alterations have been invaluable to many areas of research. Relevant to this paper are the models completely lacking natural killer (NK) cells, which were generated via deletion or truncation of the common gamma chain/Ii2rg (Cao, 1995; DiSanto, 1995; Ohbo, 1996). Since Ii2rg is required to mediate the effects of multiple cytokines including IL-2, IL-4, IL-7, IL-9, and IL-15, disruption of the Ii2rg gene leads to major defects in lymphocyte and lymphoid tissue development. Mice carrying the Ii2rg null allele or truncated mutant have been bred into the non-obese diabetic-severe combined immunodeficiency (NOD.SCID) model to generate the highly immunodeficient NSG™ (Shultz, 2005) and NOG (Ito, 2002) mice, respectively. An equally powerful model is the Rag2/Ii2rg double knockout (R2G2) immunodeficient mouse (Goldman, 1998; Mazurier, 1999), a model that is now available from Envigo. In addition to comparable defects in B-, T-, and NK-cells with NSG™/NOG mice, the presence of the Rag2 mutation in place of scid has the benefit of providing a radioresistant phenotype, which is an important feature for a variety of research applications.

In addition to providing a comparative summary of Rag2/- and SCID models, this paper presents the results of a radiosensitivity study which compared the growth and survival of Envigo’s Rag2/Ii2rg (R2G2) model to the NSG™ model using three different doses of radiation. With this knowledge in hand, scientists can make better informed decisions when choosing an immunodeficient model for their studies.
Comparison of Rag2<sup>−/−</sup> and SCID models

SCID mice and their multigenic counterparts are the most commonly used immunodeficient mouse strains. First identified in a colony of C.B-17 mice (Bosma, 1983), SCID mice harbor a spontaneous mutation in the Prkdc gene. Prkdc encodes the catalytic subunit of the DNA-dependent protein kinase (DNA-PK) and is required for non-homologous end joining (NHEJ). Since NHEJ is essential for V(D)J recombination, a process which gives rise to immunoglobulin and T-cell receptor diversity, the mutation of Prkdc results in failed B- and T-cell maturation (Belizario, 2009).

Unlike the spontaneous mutation carried by SCID mice, Rag2<sup>−/−</sup> animals were engineered to harbor a germline deletion of the recombination-activating gene 2 (Rag2) (Mombaerts, 1992; Shinkai, 1992). Since the enzyme product encoded by this Rag gene serves to ensure proper V(D)J recombination, its deletion also leads to B- and T-cell deficiencies. Notably, the Rag2<sup>−/−</sup> strain serves as the breeding partner for Il2rg<sup>−/−</sup> mice to create the Rag2<sub>2</sub>Il2rg double knockout (R2G2).

Although SCID and Rag2<sup>−/−</sup> mice are often described as exhibiting similar immune dysfunction, the level of B- and T-cell function differs between the two models. Indeed, SCID mice are known to become “leaky” with age, such that virtually all mice more than one-year old contain detectable levels of B and T lymphocytes (Nonoyama, 1993), which can inhibit xenograft growth. In contrast, Rag2<sup>−/−</sup> mice do not display a “leaky” phenotype and are thus considered more immunodeficient than SCID mice. This feature of Rag2<sup>−/−</sup> mice also makes them better suited for long-term in vivo assays.

Despite some obvious advantages of the Rag2<sup>−/−</sup> model over the SCID model, the SCID model remains more heavily used by researchers. One potential reason for this discrepancy is the fact that the SCID strain was identified in 1983, almost a full decade before advances in recombinant DNA technology led to the generation of Rag2<sup>−/−</sup> animals. During this time, SCID mice were made widely available to researchers around the world who, in turn, generated reliable data regarding the immunological properties of the model and optimization of protocols for human cell transplantation. Nevertheless, as the interest and use of immunodeficient mice continues to rise, scientists are becoming more aware of the advantages offered by other models, including Rag2<sup>−/−</sup> mice.

Radiosensitivities of Rag2<sup>−/−</sup> and SCID models

In addition to having a “leaky” immunodeficient phenotype, the SCID mouse is known for being hypersensitive to ionizing radiation (Biedermann, 1991; Fulop, 1990). As noted earlier, NHEJ plays multiple roles in cells. It is required for V(D)J recombination, as well as for proper DNA repair. Consequently, the disruption of NHEJ through the mutation of the Prkdc gene renders SCID mice sensitive to DNA damage induced by radiation exposure. Furthermore, as the mutation is present in every cell, all tissues have the potential to be affected. In contrast, the radiosensitivity of Rag2 mice is comparable to wild-type mice, as the Rag2 enzyme is only required for V(D)J recombination. Several studies have tested the radiosensitivity of immunodeficient mouse strains (e.g. Biedermann, 1991; Fulop, 1990; Goldman, 1998; Shultz, 2000; Shultz, 2005). Overall, the data support the view that Rag2 mice are more radioresistant than SCID mouse strains, and help to establish the advantage of the Rag2Il2rg model for studies requiring radiation, especially those studies that may require higher doses of radiation exposure.

There are two main types of studies in which the radiosensitivities of immunodeficient mice require careful consideration. First, whole-body irradiation is often carried out as a pre-conditioning myeloablative step to enhance the engraftment of transplanted hematopoietic cells (e.g., Goldman, 1998). For example, this is a critical step prior to generating “humanized” immune system mice. Sub-lethal irradiation has been shown to stimulate the bone marrow production of stem cell factor (SCF) production and facilitate hematopoiesis (Broudy, 1997). Irradiation also serves to eliminate endogenous HSCs and other residual immune cells to make room for engrafted cells. Notably, the higher the radiation dose, the greater the likelihood of achieving more complete myeloablation. For instance, Down et al. used murine bone marrow chimera models to evaluate the efficacy of host total body irradiation given at different doses for engraftment of syngeneic and allogeneic bone marrow. While partial engraftment of syngeneic marrow was seen at single doses as low as 2Gy, the donor component became increasingly more prominent with increasing radiation dose (100% was achieved at 7Gy). Importantly, resistance of the host appeared to prevent allogeneic engraftment until 5.5Gy, and the authors noted that there was a steep radiation dose response observed after that level, so that the level of chimerism ≥6Gy became comparable with syngeneic engraftment (Down, 1991).

Of course, investigators must balance the benefits of achieving higher myeloablation with the toxic side effects of radiation by considering factors such as the strain and age of recipient mice. Second, the radiosensitivities of immunodeficient mice are also an important factor to consider for oncology studies. Along with chemotherapy, radiation therapy remains an integral part of anti-cancer treatment regimens with up to 75% of patients requiring some form of radiation during the course of treatment (Kahn, 2012). In addition to the direct effects of radiation on the tumor, there are also bystander effects (also known as abscopal effects) induced by radiation (mostly at higher doses). These effects are thought to be immune-mediated and their implications for cancer therapy are only beginning to be unraveled (Sologuren, 2014; Demaria, 2004).

On this note, in a study by Shiraishi et al., it was shown that the administration of a chemokine after local tumor site irradiation (using a dose of 6Gy) prolonged survival of the mice. Indeed, the tumor was completely eradicated in about 50% of the animals with daily administration of the chemokine, and importantly, tumor growth at the non-irradiated site was inhibited, suggesting the chemokine enhanced the bystander effect of radiation (Shiraishi, 2008). While mouse-based radiation therapy studies certainly
exist in the literature (e.g., Speers, 2016; Karnak, 2014; Shiraishi, 2008), advances in technology, such as small animal image-guided radiation platforms (Butterworth, 2015), will undoubtedly contribute to a rise in the use of radiation therapy in pre-clinical studies in the near future. The availability of radioresistant mice including the R2G2 model will allow scientists to implement these advanced technologies into their research.

Overall, researchers need to carefully consider immunodeficient model selection for their studies.

This can be a complex decision, as many factors are involved and many strains of immunodeficient mice are available. When a model with enhanced radioresistance is warranted, it is advisable to choose a Rag-based model, such as the highly immunodeficient R2G2, which is a suitable host for xenografts using conventional immortalized cancer cell lines and patient-derived tissues or cells, and is also ideal for studies examining the efficacy of radiation as part of a cancer treatment regimen.

In the following section we show that Envigo’s R2G2 is more radioresistant than the NSG mouse, using three different doses of radiation.

**Radiosensitivity study**

A study was conducted using Envigo’s R2G2 model (B6;129-Rag2<sup>2<sub>mgf<sub>tm1Fwa<sub>/I2rg<sub>tm1Rsky<sub>/DwlHsd) to empirically characterize its radiosensitivity. For comparative purposes, NSG™ (NOD. Cg-Prkdc<sup>scid<sub>Il2rg<sub>tm1Wjl<sub>/SzJ) mice were also included, and this model has been shown to tolerate radiation doses of up to 4Gy (Shultz, 2005). A summary of the methods and results of the study are presented below.

**Materials and methods**

On study day 1, groups of R2G2 and NSG™ mice (n=5 per group) aged 7-8 weeks were exposed to one of three doses of whole body irradiation: 2Gy, 4Gy or 6Gy. Mice were irradiated using a RadSource RS-2000, which employs a 160 kV, 4.2 kW x-ray source. After receiving the radiation dose, animals were monitored daily for changes in body weight and survival for up to 30 days. Animals were euthanized when moribund (e.g. >20% body weight loss, loss of righting reflex). All animals were maintained on Teklad Global Rodent Diet 2918 (18% protein).

**Results**

The effect of varying doses of radiation on the survival and body weight of R2G2 and NSG™ mice was evaluated. Groups of mice were irradiated with radiation doses of 2Gy, 4Gy, or 6Gy and monitored for 29 days. As shown in Figures 1A and 2A, no meaningful differences in survival or body weight change were observed between the R2G2 and NSG™ models at the lowest dose of radiation (2Gy). Survivability was 100% for both models at 29 days (Figure 1A), and after an initial small drop in body weight in both models, after 29 days the R2G2 and NSG™ mice had gained an average of 5.1% and 8.8%, respectively (Figure 2A). In contrast, at a radiation dose of 4Gy, NSG™ mice experienced 100% mortality by post-irradiation day 8 (Figure 1B), and showed a drastic loss of body weight (Figure 2B). Meanwhile, R2G2 mice displayed 100% survivability (Figure 1B), and robust body weight gain showing a similar trend to that of the 2Gy radiation dose (Figure 2B). At the highest radiation dose of 6Gy, approximately 50% of the R2G2 mice survived for 14 days, but by the 17-day timepoint, there was 100% mortality (Figure 1C). For the NSG™ mice, these animals experienced 100% mortality by the 5-day timepoint (Figure 1C). Thus, with regard to survivability, the R2G2 mice survived approximately three-fold longer than the NSG™ mice following a radiation dose of 6Gy. As for body weight, both models exhibited reduced body weight after the 6Gy dose of radiation, albeit the body weight loss of the R2G2 mice was gradual, in contrast to the rapid body weight loss seen in the NSG™ mice (Figure 2C).

Overall, the results of the radiosensitivity study demonstrate that R2G2 mice are less radiosensitive at radiation doses of 4Gy and 6Gy, relative to NSG™ mice. Indeed, even at the highest dose of radiation tested (6Gy), R2G2 mice showed 50% survivability at 14 days, and only a gradual loss of body weight over the time-period leading up to 100% mortality of these animals. This contrasts with the 100% mortality observed for NSG™ mice after only five days, which was accompanied by dramatic body weight loss.
Figure 1. Percent Survival of Envigo’s R2G2 model and NSG™ mice irradiated at 2Gy (A), 4Gy (B), and 6Gy (C) (Green = R2G2; Dark grey = NSG™).

Figure 1A: Percent Survival of Envigo’s R2G2 model and NSG™ mice irradiated at 2Gy

Radiation dose given on study day 1
Maintained on Teklad Global Rodent Diet 2918 (18% Protein)

Figure 1B: Percent Survival of Envigo’s R2G2 model and NSG™ mice irradiated at 4Gy

Radiation dose given on study day 1
Maintained on Teklad Global Rodent Diet 2918 (18% Protein)
Figure 1C: Percent Survival of Envigo's R2G2 model and NSG™ mice irradiated at 6Gy

- Radiation dose given on study day 1
- Maintained on Teklad Global Rodent Diet 2918 (18% Protein)
Figure 2. Percent change in body weight of Envigo’s R2G2 model and NSG™ mice irradiated at 2Gy (A), 4Gy (B), and 6Gy (C) (Green = R2G2; Dark grey = NSG™).

Figure 2A: Percent change in body weight of Envigo’s R2G2 model and NSG™ mice irradiated at 2Gy

Figure 2B: Percent change in body weight of Envigo’s R2G2 model and NSG™ mice irradiated at 4Gy
Conclusions

The development of highly immunodeficient strains of mice has ushered in an era of advanced research models. However, as touched upon in this paper, not all immunodeficient mice are equal, and understanding the inherent differences associated with each strain is critical when planning a study, especially those requiring irradiation. The results of an internal study comparing the radiosensitivity of Envigo’s Rag2/Il2rg double knockout (R2G2) model to the NSG™ model, confirm that R2G2 mice are less radiosensitive than NSG™ mice, while both are highly immunodeficient. The R2G2 mouse features a higher degree of immunodeficiency than many models (e.g. athymic nude), and greater radioresistance than SCID strains. Thus, for scientists pursuing studies that require an immunodeficient model and exposure to higher doses of irradiation, the R2G2 model is the superior choice.

A wide variety of options are available when it comes to choosing an immunodeficient mouse, and model selection is not always straightforward. Discussions with Envigo personnel when planning a project can help save time, effort, and resources.
Enhancement of Lymphoid Small animal image-NOD/LtSz-Rag1null mice: an