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Humanized mouse model: technical insight and applications

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ABSTRACT

Humanized mouse models have emerged as an important tool for cancer research. These models enable the grafting of human immune and cancer cells, allowing the study of the complex interaction between the immune system and cancer. The work presented in this thesis has been developed in the animal facility at the department of Surgery, Oncology and Gastroenterology of the University of Padova, housed at the “Istituto Oncologico Veneto" (IOV-IRCCS). It describes the practical work done to establish a humanised mouse model based on the immunodeficient strain NOD scid gamma (NSG). This model has been subsequently used to perform studies aimed at the treatment of Graft-versus-Host-Disease (GvHD). The techniques used in this work include animal irradiation with gamma rays to create a suitable environment for the engraftment of human cells, intravenous injection of peripheral blood mononuclear cells (PBMCs) to reconstitute the human immune system, and the assessment of animal weight and general health status through the study. To evaluate the humanization rate and the composition of the engrafted human cells, blood samples were periodically collected by puncturing of the submandibular vein, stained with a cocktail of antibodies and analysed by flow cytometry. The results demonstrated successful engraftment of human immune cells, with humanization rates and animal survival varying in accordance with gamma radiation exposure and the number of cells injected. The engrafted human immune cells were predominantly T cells, which is consistent with the expected reconstitution patterns following injection of the PBMCs. The knowledge obtained from this study was further employed to investigate innovative strategies for the management of GvHD, hopefully leading to an improvement in the prognosis of bone marrow transplantation in patients with hematologic malignancies.

1. INTRODUCTION

1.1 Role of the Immune System in Cancer and the Risks of Bone Marrow Transplantation

The immune system plays a crucial role in recognising and eliminating harmful pathogens, abnormal cells, and foreign substances, helping to preserve the integrity of the body's tissues. A well-regulated immune response is essential for combating diseases such as infections and cancer. However, when the immune system becomes dysregulated, it can lead to serious health issues, as seen in conditions like graft-versus-host disease (GvHD), which occurs in patients undergoing bone marrow transplantation for haematological malignancies.

The immune system constantly monitors the body for abnormal cells, including cancerous ones. This surveillance is performed by various immune cells that identify and destroy cells exhibiting mutations or abnormal behaviour. Key players in this process include:

- **T-cells**, which identify and eliminate infected or abnormal cells through direct killing or by activating other immune components.
- **Natural Killer (NK) cells**, which rapidly target and kill cells lacking appropriate markers, a feature often associated with cancer cells.
- **Dendritic cells**, which present antigens from abnormal cells to T-cells, activating the adaptive immune response.
- **B-cells**, which produce antibodies that bind to abnormal cells, marking them for destruction.
- **Macrophages**, which play a dual role by phagocytosing (engulfing and digesting) abnormal cells and presenting their antigens to T-cells. They also secrete various cytokines that help to recruit and activate other immune cells, thereby enhancing the immune response against tumours.

Haematological malignancies, such as leukaemia and lymphoma, are distinct types of cancer because the cancerous cells arise from the immune system itself. Treatment options for patients with these immunological tumours often involve the destruction of the patient’s immune system, which is the source of the tumour, through radio- and/or chemotherapy, followed by bone marrow transplantation. Transplanted bone marrow replaces the patient's destroyed immune system with healthy stem cells from a donor. While this treatment can be life-saving, it also carries significant risks, particularly GvHD, which will be discussed later in this thesis.

1.2 Immunodeficient Mouse Models in Research

The immune system is a complex network of various cell types that communicate through a wide array of cytokines. When this intricate system is dysregulated or certain components malfunction, it can result in inappropriate immune responses and/or immunodeficiency. The development of induced mutant and genetically engineered mouse models has been pivotal in advancing our understanding of molecular and gene-driven mechanisms underlying immune disorders. The ability to manipulate the mouse genome through the introduction of specific mutations has enabled researchers to generate mouse models that mimic certain immunodeficiency conditions observed in humans[1].

Immunodeficient mice are defined by defects in one or more components of their immune system, such as T, B, or NK cells[2]. Today, genetically engineered immunodeficient mouse models are essential tools in biomedical research, particularly in the fields of oncology, immunology, infectious diseases, and stem cell biology. Their capacity to accept human cells and tissues allows researchers to study biological processes and human pathologies that are difficult to investigate in other systems, aiding in the development of novel therapies[3].

In cancer research, these mouse models have proven especially valuable, as human tumour cells can be implanted into them to study tumour growth and evaluate potential treatments in vivo. These mice, which carry human immune cells, closely mimic human tumour development. It is now possible to grow nearly all types of primary human tumours in vivo, including most solid tumours and haematological malignancies[4]. Beyond cancer research, immunodeficient mouse models have been engrafted with human cells to study autoimmune diseases such as systemic lupus erythematosus and multiple sclerosis, as well as viral infections like HIV/AIDS. These models are also invaluable for testing the pharmacokinetics, pharmacodynamics, and toxicity of new drugs intended to treat human immune diseases, given their lack of a complete immune response.

The first genetic mouse model of immunodeficiency was discovered in 1962. It was characterised by a spontaneous deletion of the *Foxn1* gene, which regulates thymus development. Mutations in this gene result in T-cell immunodeficiency and congenital alopecia[5]. These mice, which display a "nude" phenotype, are athymic and lack mature T cells.

Several years later, the non-obese diabetic (NOD)/ShiLTJ mouse model was developed as a model for autoimmune type 1 diabetes, featuring defects in antigen presentation, NK cell function, and the T lymphocyte repertoire. In 1985, the severe combined immunodeficiency (SCID) model was generated, characterised by non-functional T and B cells. However, the presence of high numbers of murine NK

cells reduced the efficiency of engraftment in SCID mice, limiting their utility in research. The transfer of SCID mutations to the NOD background resulted in the NOD-SCID model. These mice exhibit defects in both innate and adaptive immunity inherited from their parental strains[6].

In 1997, a mouse model with a mutation in the *IL-2rγ* gene was developed. This mutation leads to a deficiency in functional NK cells. A further refinement combined the SCID mutation with a complete null allele of the *IL-2rγ* gene, leading to the development of one of the most immunodeficient and widely used mouse models in research today: the NSG™ mouse, introduced in the early 2000s[7].

These models have provided significant opportunities for medical research, with the potential to drive the development of therapies for immune diseases, which continue to present substantial challenges to human health.

1.3 The Development of Humanized Mouse Models

The understanding of various biological processes in humans has significantly advanced through experimental studies on animal models, particularly rodents. In the field of tumours immunology, the advancements made using mouse models have been invaluable. However, human and rodent biology do not completely overlap, and differences in immune system function, tumour microenvironment, and drug metabolism can limit the translatability of findings from these models to human cancer biology.

For example, the cell surface marker CD28, which plays a crucial role in T-cell activation and signalling, is expressed on all $CD4^+$ and $CD8^+$ T cells in mice but only on approximately half of $CD8^+$ T cells in humans[8]. An illustrative case is the drug fialuridine (TGN1412), initially developed as a treatment for hepatitis B and targeting CD28, an essential marker for T-cell activation. This drug was tested in several animal models, including mice, and was deemed safe. However, when administered to humans at just 1/500th of the dose considered safe in animals, it caused severe organ failure. This adverse outcome is likely due to the differing expression of CD28 on T cells between species[9].

Consequently, the complexities of human immune responses and tumours heterogeneity necessitate complementary approaches, such as humanized mouse models, organoids, and clinical studies, to more accurately predict therapeutic efficacy and safety in humans.

In particular, the use of humanized mouse models, which simulate human biology at the cellular and molecular levels[10], plays a fundamental and increasingly important role in understanding immune

responses to cancer development and treatment, enabling a more precise evaluation of therapeutic strategies.

Technically, a humanized mouse model consists of a genetically modified mouse engineered to accept human cells or tissues, develop a human immune system, or express human gene products[11]. These models are generated by transplanting human components into immunodeficient mice, such as the aforementioned NSG strain, or by introducing human gene sequences into the mouse genome. They are used to study complex human-specific biological processes that cannot be adequately represented by *in vitro* or *in silico* models, particularly regarding the intricate activities of the immune system in fields like oncology and transplantation[12].

Thus, the process of humanization was introduced to study complex human pathologies that can only be analysed in a living organism. Humanization allows researchers to closely represent human physiology. The absence of mouse immune components permits the engraftment of various human cells without triggering rejection, thus avoiding the need to use murine cell lines, which have their limitations[13].

Several mechanisms underlying human diseases remain poorly understood, and humanized mouse models may facilitate the exploration of various disorders. For instance, human immunodeficiency viruses, such as HIV type 1 (HIV-1) and HIV type 2 (HIV-2), which are responsible for AIDS, have been successfully studied using these models. Other human pathogens, including *Mycobacterium tuberculosis*, *Borrelia hermsii*, and the Dengue virus (DENV), as well as autoimmune diseases, have also been investigated[8].

The development of a humanized mouse model involves several steps aimed at generating a mouse with a functional human immune system or specific humanized organs. This process begins with selecting an appropriate immunodeficient mouse strain based on the specific requirements of the study. Following strain selection, the mice may undergo irradiation using gamma rays or X-rays to deplete the existing bone marrow and other immune cells that could mediate engraftment rejection. This step creates space in the bone marrow niche for the efficient engraftment of human cells[14]. The goal is to reconstitute the mouse's immune system with human cells, such as hematopoietic stem cells (HSCs), progenitor cells (HSPCs), or peripheral blood mononuclear cells (PBMCs), which are previously isolated from human donors. These cells can be injected via various routes, with intravenous injection being the most commonly used. Once inside the mouse, the human cells migrate to target tissues and

organs, where they engraft and begin to interact with the mouse environment. Over time, the mouse becomes “humanized” as these human cells develop and function within the host. The extent and functionality of humanization can vary depending on the chosen model and experimental conditions.

1.4 NOD scid gamma Mouse Model

The NOD scid gamma mouse (strain name: NOD.Cg-Prkdc^{scid} Il2r^{gtm1Wjl}/SzJ) is an immunodeficient mouse model developed by Dr. Leonard Schultz at Jackson Laboratory, commonly referred to by its branded name, NSG. NSG mice are regarded as one of the most immunodeficient mouse models available, allowing for the engraftment of a broad range of primary human cells[15]. The genetic background of NSG mice is derived from the inbred NOD mouse strain, NOD/ShiLtJ, which contributes to a reduction in the innate branch of the immune system, resulting in defective macrophages and dendritic cells[15].

The term "SCID" (severe combined immunodeficiency) refers to a loss-of-function mutation in the *Prkdc* gene, which impairs the development of T and B cells, leading to their near absence in the body. The NSG mouse strain, which includes the SCID mutation, is highly sensitive to irradiation due to its compromised DNA repair mechanisms. This sensitivity, combined with additional mutations, makes the NSG mouse an exceptional model for the engraftment of human cells and tissues[16].

The *Prkdc* gene encodes a DNA-dependent protein kinase that is crucial not only for immune cell development but also for repairing DNA double-strand breaks. DNA damage frequently arises from normal cellular processes, and this protein plays a fundamental role in maintaining genomic integrity by repairing double-strand breaks. Consequently, mice carrying the mutated *Prkdc* gene exhibit increased sensitivity to physical and chemical agents that can damage DNA, such as irradiation. This characteristic results in a reduced need for irradiation compared to other engineered mouse[17].

The gamma chain of the interleukin-2 receptor (IL2_{rg}) is a component of cell surface receptors that bind and transduce signals from six different interleukins (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21), which play essential roles in the development, proliferation, and survival of various immune cell types[18]. NSG mice carry a null mutation of this gene, resulting in severely compromised development of T and B lymphocytes, as well as NK cells. While myeloid components such as granulocytes, monocytes, and macrophages are present in NSG mice, the disruption of the adaptive immune system significantly impacts the overall immune response, including the function of the innate

immune system[11]. The SCID mutation destabilizes the hematopoietic cells in these mice, rendering the bone marrow stem cell niche available for potential engraftment.

Beyond cancer research, many other fields have benefited from the development of the NSG mouse strain. Hematopoietic cancers, for instance, engraft better in NSG mice compared to other engineered models, facilitating more efficient studies of leukaemia cell lines. This advantage extends to Patient-Derived Xenografts (PDX), where tumours tissue from patients is implanted into NSG mice, preserving critical cell-cell interactions and the tumours microenvironment. The PDX model has also demonstrated advantages as a preclinical model in drug screening and biomarker development[19]. Furthermore, the benefits of the NSG mouse model extend to the field of infectious diseases, including HIV and AIDS. These mice are also utilized in gene therapy research aimed at correcting genetic defects.

1.5 Importance of NSG Mice in the Study of Graft-versus-Host Disease

GvHD is a significant complication that can arise following allogeneic bone marrow transplantation, where hematopoietic stem cells or bone marrow cells are transplanted from a donor into a recipient's body. In this context, the term "graft" refers to the transplanted cells, while "host" refers to the recipient's tissues[20]. he transplanted stem cells mature into blood cells, theoretically establishing a new lineage of blood cells within the recipient. Hematopoietic or bone marrow cell transplantation is often utilized in patients with specific oncological conditions related to blood cancers, such as leukaemia and lymphoma, as well as bone marrow diseases like aplastic anaemia. This procedure aims to administer healthy hematopoietic cells to patients with dysfunctional or depleted bone marrow[21].

GvHD results from a complex interplay of immunological factors during allogeneic transplantation[22] (The occurrence and severity of GvHD are influenced by several factors, including the source of the stem cells, the age of the patient, the conditioning regimen, and the anti-GvHD prophylaxis employed[23].

Under normal circumstances, immune system cells combat pathogens, such as bacteria and viruses, and protect the body from the formation and proliferation of potentially cancerous cells. However, after bone marrow transplantation, donor cells may recognize the recipient's body as foreign, triggering a robust immune response against it. Lymphocytes, particularly cytotoxic T cells, play a crucial role in this response. Upon activation, these T cells proliferate and target the host tissues, exacerbated by the

release of inflammatory mediators that amplify the immune response and contribute to extensive tissue damage. This cascade can result in severe organ dysfunction and significant morbidity for the recipient[23].

GvHD can be classified into two types based on clinical manifestations: acute and chronic[24]. Three primary organs are commonly affected: the skin (manifesting as rash/dermatitis), the liver (leading to hepatitis/jaundice), and the gastrointestinal tract (resulting in abdominal pain/diarrhea). Acute GvHD is graded on a scale from 0 to 4, depending on the number and extent of organs involved. In contrast, chronic GvHD can affect additional areas, including the oral cavity, esophagus, musculoskeletal system, eyes, and genitals[24].

To date, GvHD remains the leading cause of failure in hematopoietic stem cell transplantation from compatible donors. The development of humanized mouse models has been instrumental in advancing research in this area, particularly the NSG model, which is widely utilized to study GvHD. NSG mice demonstrate rapid disease progression and significant human T-cell activity, making them valuable for testing new treatments and understanding the mechanisms of GvHD. This research aims to identify potential new therapies for clinical trials, ultimately improving recovery rates in patients with haematological malignancies and reducing the impact of GvHD.

1.6 Animal Well-Being

The development and use of laboratory animals raise several ethical questions central to the debate on animal research. The primary ethical concerns revolve around the sentience of animals and the potential harm inflicted upon them. These concerns extend to the justification of such harm, questioning its ethical acceptability[25].

In recent years, the protection of animal rights and the pursuit of animal welfare have become increasingly prominent themes in society and politics, leading to a growing demand for transparency in animal research. Many individuals feel uncomfortable with the necessity of experiments involving laboratory animals, yet there are compelling reasons for their continued use in research aimed at developing new therapies. Most people may not realize that the use of animals would be willingly avoided if current technologies allowed for viable alternatives.

A complete organism is often essential to accurately reproduce the pathology under investigation, as certain therapeutic effects manifest only within a whole organism equipped with all necessary organs

that can receive and modify the therapy itself. Such effects may not be observable in isolated cells, typically employed in the initial stages of laboratory research.

While humans differ from animals in various aspects, they share a significant portion of evolutionary history, including many conserved molecules and functional pathways. These shared biological features, coupled with the complexity of interactions among different organs, compartments, and cell types, underscore the need for animal models in research. Consequently, the use of animals in research has led to the development of important national and international regulations and guidelines designed to ensure their safety and well-being while minimizing waste and suffering.

In the European Union (EU), animal research is regulated under Directive 2010/63/EU, which protects animals used for scientific purposes. This directive applies to all live, non-human vertebrates, as well as cephalopods, and provides comprehensive guidelines on various aspects of animal use in research. These guidelines encompass housing and care conditions, procedures to minimize pain and distress, and requirements for ethical authorization of research projects involving animals. The primary aim of the directive is to uphold high standards of welfare for animals in scientific research while promoting advancements in research methodologies that can reduce or eliminate the need for animal use.

A key aspect of Directive 2010/63/EU is the principles of the 3Rs—Replacement, Reduction, and Refinement. Developed over five decades ago by scholars Russell and Burch from the Universities Federation for Animal Welfare (UFAW), the 3Rs provide a framework for humane animal research[25]. The principles are defined as follows:

- **Replacement**: It focuses on seeking alternative methods that do not involve animals, utilizing techniques such as cell cultures, computer simulations, or lower organisms (e.g., bacteria or plants) that do not experience pain. The directive encourages the development of new methods to replace animal testing.
- **Reduction**: This principle advocates for the use of fewer animals in experiments. Researchers should design their experiments to require the minimum number of animals necessary to achieve reliable results. This can be accomplished through improved experimental designs, appropriate statistical methods, and data sharing to prevent unnecessary repetition of experiments.
- **Refinement**: It focuses on minimizing suffering in animals. This includes identifying ways to reduce pain, stress, and discomfort during experiments and improving the living conditions of

the animals. Researchers are also encouraged to establish clear guidelines for when to terminate an experiment early if an animal experiences excessive suffering.

Animal research is regulated not only for ethical reasons but also for scientific integrity. The overall health of the animals significantly impacts the reliability of experimental results. If animals are unhealthy, stressed, or in pain, the data obtained may not accurately reflect the intended study outcomes. This consideration is particularly crucial when using mouse models to investigate tumours or other diseases, which already induce discomfort and pain.

Maintaining animal health is also closely related to the principle of reduction. Reproducible experiments yield consistent results that can be repeated with fewer animals, thereby reducing variability and the need for larger sample sizes to achieve statistically significant outcomes.

Before any animal research project can begin, it must undergo review and approval by an ethics committee, with a project authorization granted by the competent authorities. Applications for project authorization must include a detailed project proposal, a non-technical summary for public understanding, and a scientific justification for the necessity of animal research. Additionally, proposals must specify the number of animals involved and the expected level of suffering. Each proposal is evaluated through a harm-benefit analysis by the competent authority in each EU Member State, which ultimately decides whether to grant the license.

In Italy, prior to approval by the Ministry of Health, the protocol must be assessed by the Committee for Animal Care (Organismo preposto al Benessere Animale: OpBA). This committee ensures the protection of the rights, safety, and well-being of the animals involved in the protocol. The OpBA conducts a preliminary review, which is subsequently evaluated by the committee's scientific representative. The OpBA communicates directly with the requesting researcher to approve the protocol for further submission to the Ministry of Health or request modifications for improvement. Upon submission, the Ministry may request additional documentation or corrections before ultimately approving or denying the authorization.

Once all authorizations have been granted and the study protocol is ready to start, significant efforts are required to optimize and ensure the welfare of laboratory animals. Throughout the observation period, animals are maintained under standard housing conditions that comply with legal requirements, including unrestricted access to water and food, environmental enrichment, and nesting opportunities.

Respecting the social nature of animals is also crucial; for instance, mice should not be isolated from their peers except for specific and authorized reasons.

Animal conditions are closely monitored by animal care staff and research personnel throughout the study, assessing various physical and behavioural parameters, such as weight loss, fur condition, unprovoked behaviour, response to handling, and overall body functions. A health scoring system guides necessary actions to alleviate animal suffering. If an animal exhibits signs of distress during examinations, measures to relieve discomfort are determined in consultation with the designated veterinarian. Should the distress be severe and unmanageable, euthanasia is performed as a humane end-point.

The humane endpoint is a critical consideration in laboratory settings, defined as the earliest point at which an animal's distress necessitates its removal from the study to prevent or minimize pain[26]. This endpoint is assessed not only through clinical signs but, when possible, also by measuring molecular biomarkers indicative of pain, such as elevated levels of catecholamines, cortisol, and neuropeptides like substance P, which is involved in pain transmission[27]. It is vital to ensure that humane endpoints align with scientific objectives and do not exceed what is necessary for the research, even if some distress is intrinsic to specific experimental models, such as the weight loss experienced by mice with induced GvHD.

Scientific research relies on animals to fulfil its goals, which requires a continuous responsibility and ethical obligation to ensure their welfare. This commitment to improving care practices extends beyond mere regulatory compliance; it constitutes a moral imperative. Looking forward, there is hope that advancements will lead to viable alternatives to animal use. Significant progress has already been made, with many studies utilizing surrogate systems such as organoids, spheroids, three-dimensional cultures, and organ-on-chip models. Nevertheless, fields like oncology and immunology still necessitate the use of complex organisms. Until such time that animals can be entirely replaced, upholding their dignity and welfare remains crucial for the ethical advancement of scientific knowledge.

2. AIM OF THE THESIS

GvHD often arises following allogeneic bone marrow transplants and significantly contributes to poor patient prognosis; in severe cases, it can lead to patient mortality. There is a pressing medical need for fast and effective treatments for GvHD, and further research is essential to identify viable therapies. To explore therapeutic approaches for treating GvHD, an appropriate animal model is required.

This thesis outlines the necessary steps to successfully establish a humanised mouse model that is suitable for investigating potential treatments within the context of a larger project focused on developing innovative strategies for GvHD management. The steps involved include acquiring various animal handling techniques and executing experimental procedures, such as intravenous injection, blood sampling, and ear marking. Given the aggressive nature of this disease, constant monitoring of the animals and their health status, along with learning appropriate interventions to minimise pain, distress, and discomfort, were integral components of this work.

In conclusion, this thesis addresses three fundamental aspects of in vivo experimentation:

1. Animal care, welfare, and ethical regulations
2. Animal handling and procedural performance
3. Data analysis and interpretation of results

3. MATERIAL AND METHODS

3.1 Animal Facility

The generation of a humanised mouse model requires many steps, beginning with experimentation design and documents preparation to obtain the approval from the OpBA and then authorisation from the Ministry of Health. The experimentation procedures I participated in were conducted in an animal facility which adheres to high cleanliness standards. Entry to this area is permitted only after wearing the required personal protective equipment (PPE). The experimental area of the facility is equipped with all necessary materials and equipment, in order to allow the researcher to perform their work without having to access other parts of the facilities such as the breeding rooms.

The NSG mice used in this work are highly immunodeficient and are irradiated, making them very susceptible to infections. For this reason, these animals are housed in Individually Ventilated Caging Systems (IVCs) and their manipulation is conducted under a biosafety cabinet that provide protection against possible pathogens. The animal facility where the experiments detailed in this thesis were conducted is part of the Dipartimento di Scienze Chirurgiche Oncologiche e Gastroenterologiche at the University of Padova. It can accommodate approximately 6,000 mice for experimentation and breeding and includes a P3 room equipped with a Microcage isolation device for pathogen containment, as well as instruments for embryo transfer techniques.

The facility features advanced imaging platforms for small animals, including the Explore Optics (GE), a computed tomography instrument (Explore Locus, GE), a bioluminescence device (Lumina II, Perkin-Elmer), an in vivo imaging platform (IVIS Spectrum, Perkin Elmer), and a trimodal PET/SPECT/CT instrument (MiLABS).

The animal facility is classified as specific-pathogen free (SPF), meaning it provides housing, breeding, and operational areas free from a defined list of pathogens[28]. SPF animals are subject to rigorous monitoring, with the majority being bred and raised within the facility itself, ensuring they remain free from specific pathogens. In some cases, animals are sourced from specialised companies, which are required to supply animals with certified health conditions that meet SPF standards. When animals are acquired from external animal facilities, they must be accompanied by proper certification and undergo a quarantine period before being housed with the resident animals. These procedures are implemented to maintain the facility's status as pathogen-free and ensure the continued health and well-being of the animals. The importance of maintaining SPF animals within the facility is primarily related to the

consistency and reliability of experimental outcomes. Ensuring that experimental results are attributed to the variables under investigation, rather than unforeseen infections, is critical for accurate data interpretation. Moreover, the absence of pathogens makes it easier to replicate studies in other facilities, increasing the likelihood that findings are valid and reproducible. Finally, healthier animals are less prone to developing diseases that could potentially interfere with the interpretation of results, further enhancing the quality of the research[29].

A specific section of the facility is classified as biosafety level 3 (BL3), which requires strict monitoring of laboratory personnel and adherence to specific safety protocols. This includes an entry sequence through an anteroom for donning personal protective equipment (PPE) and proper disposal of waste. The BL3 area is designated for research involving pathogens capable of causing serious or life-threatening diseases through inhalation, such as *Mycobacterium tuberculosis* and *Rickettsia rickettsii*.

3.2 Irradiation

Irradiation was performed in a dedicated area by authorised personnel. NSG mice were exposed to radiation levels of 2 or 4 Gy using a radioactive caesium source.

3.3 Intravenous Injection

Four hours after irradiation, NSG mice were intravenously injected with varying amounts of peripheral blood mononuclear cells (PBMCs), ranging from 10 to 30 × 10^6 cells, to reconstitute the human immune system in the murine recipients. The injection was performed in the most lateral caudal vein. To enhance blood flow and vein visibility, the tail was warmed using an infrared lamp, reaching a maximum temperature of 37–39°C, and the puncture site was disinfected. For safety, the mice were restrained in a mouse restrainer to prevent movement and facilitate tail extension for injection. A small needle (25G) was then carefully inserted into the vein, and the PBMC solution was slowly injected to minimise harm to the animal. The needle was gently removed afterward.

3.4 Application of the Ear Tag

Ear tagging of the mice was conducted to ensure accurate record-keeping, especially when different mice exhibited varying characteristics, such as differing radiation exposure or PBMC quantities. A

small, numbered metal tag was applied to the ear using a special applicator. The mouse was gently restrained to minimise movement, and the applicator pierced the ear at a designated location. Due to the size of the tags and the rapid growth of mouse ears, this method is suitable only for weaning and older mice. Both the applicator tip and the ear tags were disinfected before use. Although ear tags can cause discomfort and may occasionally fall out, proper positioning in the inner lower third of the pinnae, avoiding capillaries, is crucial. If positioned correctly, the tag should hang from the ear adjacent to the face[30].

3.5 Mice Health Assessment

It is important to assess animal welfare after each procedure. Healthy animals ensure the progress and success of the experiment and help limit suffering. Mice that have been inoculated and irradiated were weighed twice a week to monitor weight fluctuations, which are indicators of clinical deterioration. Weight loss is a primary symptom of GvHD, and while some weight loss was anticipated, excessive weight loss, defined as a 25% reduction of the mouse's starting body weight, indicates that intervention is necessary to prevent further distress[31].

In addition to weight, daily health assessments were conducted to evaluate the animals' overall condition. Both animal-based measures (such as signs of distress) and resource-based measures were employed to provide a comprehensive understanding of their welfare. Indicators of distress included sagging skin and altered resting posture, while the quality of fur and teeth also reflected welfare status. Mice typically groom themselves, maintaining clean fur; dental issues can arise if they do not have adequate means to gnaw on food or environmental enrichment. Therefore, daily health assessments are vital for ensuring the well-being of the mice and the integrity of the research outcomes[32].

3.6 Blood Sampling

To evaluate the humanisation rate and the composition of the engrafted human cells, blood samples were periodically collected by puncturing the submandibular vein. This method allows for a sample volume with minimal trauma to the animal[33]. Blood sampling was performed under anaesthesia using isoflurane. The submandibular vein was punctured with a 25-gauge needle or a 3 mm lancet, collecting a maximum of 150 µl of blood in a 1.5 ml tube containing 30 µl of heparin to prevent clotting.

To draw the blood, the personnel needs to restrain the animal with the non-dominant hand by grasping the loose skin over the shoulders[34]. The puncture site for submandibular blood collection in mice is located behind the mandible. Only the tip of the needle or lancet should penetrate the vessel, and with gentle pressure, the change in consistency should be felt to confirm entry into the vein. Once the required amount of blood is collected, the hand restraining the animal can be gradually loosened. This relaxation of the skin will naturally stop the bleeding, although gentle pressure with gauze can be applied if needed to ensure hemostasis.

Following the procedure, the animal is returned to its home cage and monitored until it has fully recovered from anesthesia. To avoid any harm to the animal, correct localization of the puncture site is essential. As illustrated in Fig.1, the hairless spot along the jawline should first be identified (Fig.1A). The puncture site is approximately 0.5 cm superior and lateral towards the shoulder (Fig.1B). A representation of the mandibular plexus (Fig.1C) and an example of successful blood collection (Fig.1D) are also shown for reference[34].

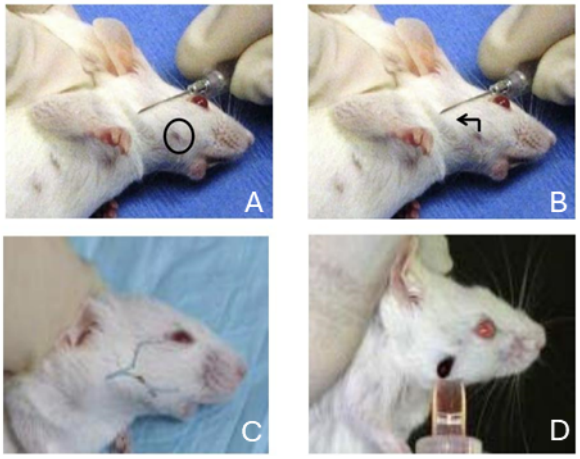

Figure 1: Representation of the corrrct procedure to identify the mandibular plexus and perform sub-mandibular blood withdrawal.

3.7 Red Blood Cell (RBC) Lysis

Before the blood can be stained to perform flow cytometry analysis, red blood cells (RBC) need to be removed. The blood is transferred to a 5 ml tube and incubated for 15 minutes at room temperature with 2 ml of 1x RBC Lysis Buffer (eBioscience). After incubation, the solution is diluted adding 2 ml of PBS to stop the lysis, and centrifuged for 6 minutes at 300g at 4°C. If no blood cells are present or are in small amounts the samples are washed again with 2 ml of PBS to eliminate residual lysis buffer. In case of strong presence of residual red cells, the lysis step is repeated.

3.8 Flow Cytometry

Once RBCs were removed from the samples, cells were stained with a viability marker and antibodies specific to mouse immune cells, human immune cells, and human T cells. The anti-human CD45 antibody was used to identify human immune cells, while anti-mouse CD45 identified mouse cells. CD45 is a marker expressed on all immune cells in both species, with slight structural differences allowing for discrimination. T lymphocytes among human CD45-positive cells were identified using the anti-CD3 antibody, and lymphocytes were further classified as cytotoxic or helper T cells using CD8 and CD4 markers, respectively. The antibodies used were sourced from BD Biosciences: PerCP-Cy5.5 anti-human CD45, BV786 anti-mouse CD45, FITC anti-human CD3, PE anti-human CD8, and APC anti-human CD4. To distinguish dead cells from live ones, the Live/Dead fixable stain (Invitrogen) was used. Cells were incubated with antibodies and the viability stain for 20 minutes at 4°C, then washed with PBS to remove unbound antibodies and suspended in 300 µl of PBS for reading. The samples were acquired on a LSRFortessa (BD Biosciences) equipped with five lasers.

4. RESULTS

4.1 Determination of the Amount of Irradiation and PBMCs Required to Obtain a Murine Model of GvHD

To establish a GvHD model for studying experimental therapies, it is crucial to create a humanized animal in which donor immune cells can proliferate and attack the host, initiating inflammation and tissue damage typical of this disease. Flow cytometry was employed to assess the degree of humanization (Fig.2A). Quantitative parameters, such as weight loss, along with qualitative assessments of the animal's health based on physical and behavioural observations, were utilized to evaluate disease progression. The presence of human cells in the blood of irradiated NSG animals injected with PBMCs was clearly distinguishable from a control NSG animal that was irradiated but not injected with human cells (Fig.2B).

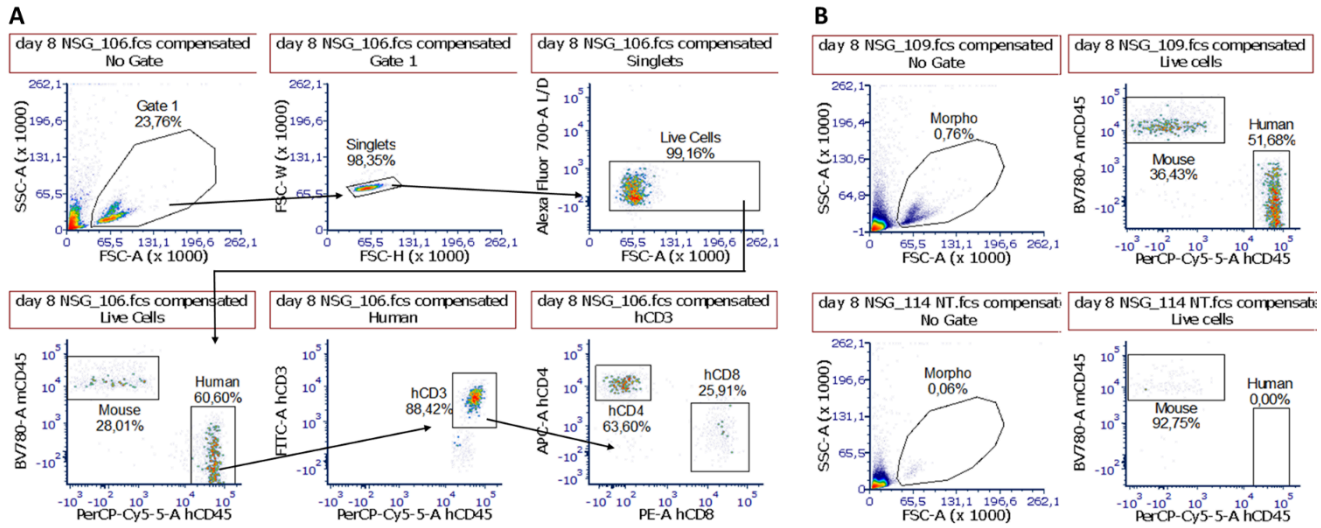

Figure 2: (A) Flow cytometry gating strategy for determining the percentage of human immune cells (hCD45) in the blood of NSG mice after irradiation and PBMC transfer. (B) Comparison between the blood of an NSG mouse irradiated and injected with PBMCs from a human donor (+human PBMCs) or not (NO human PBMCs).

Based on prior studies, the animals underwent sub-lethal irradiation at 4 Gy, followed by the injection of 30×10^6 PBMCs isolated from healthy donors provided by a collaborating group from Vicenza Hospital ULSS-8 Berica. Animals irradiated at 4 Gy and injected with 30×10^6 PBMCs displayed a rapid onset of GvHD, with human cell engraftment ranging from 29% to 80.3% (mean 59.13 ± 19.4%) at day 8, and approximately 90% (mean 89.7 ± 7.4%) at day 12 (Fig.3A). However, this rapid

engraftment was accompanied by early mortality (Fig.3C). Weight loss served as an indicator for assessing the humane endpoint, set at 25% loss from the animal's initial weight (Fig.3B). The first death occurred on day 8, with all animals succumbing by day 15 post-PBMC injection.

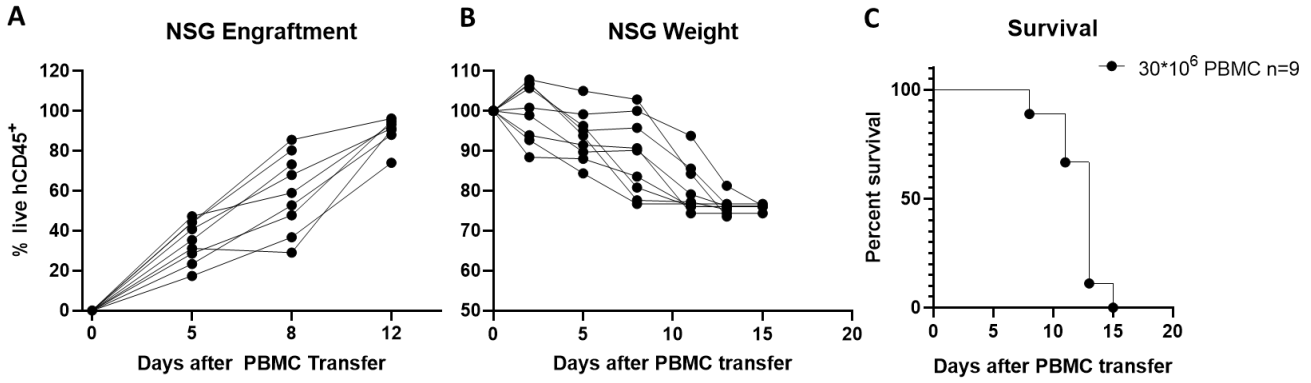

Figure 3: (A) Percentage of human CD45 (hCD45) cells in the blood of NSG animals at different time points after PBMC transfer, each line representing a single NSG animal. (B) Percentage of weight loss compared to initial weight; each line represents a single NSG animal. (C) Survival analysis (n=9).

4.2 Development of a Usable Time Window for Therapeutic Intervention in the GvHD Humanized Model

Although a successful GvHD model was established, the duration from disease onset to animal death was insufficient for therapeutic testing. Different combinations of irradiation and PBMC doses were therefore explored, as these factors significantly influence disease onset and progression.

Attempts were made to decrease the number of injected PBMCs from 30×10^6 to 15 or 10×10^6 cells while maintaining a constant irradiation dose of 4 Gy. Additionally, a combination of 10×10^6 PBMCs with a lower irradiation dose of 2 Gy was tested (Fig.4A-B).

Under these conditions, injecting 15×10^6 cells post-irradiation at 4 Gy resulted in a similar time frame for death as seen with the 30×10^6 PBMC group. Deaths began occurring after day 10, compared to the initial trial, where mice required euthanasia as early as day 8. In contrast, administering 10×10^6 cells with 4 Gy irradiation also led to mortality beginning on day 10, but only half the animals (4 out of 8) required euthanasia by day 26, with the rest needing it before day 33 (Fig.4A-B). Engraftment patterns were similar to the 15×10^6 cell injection, with human CD45 cells appearing and propagating between days 3 and 6 (Fig.4A).

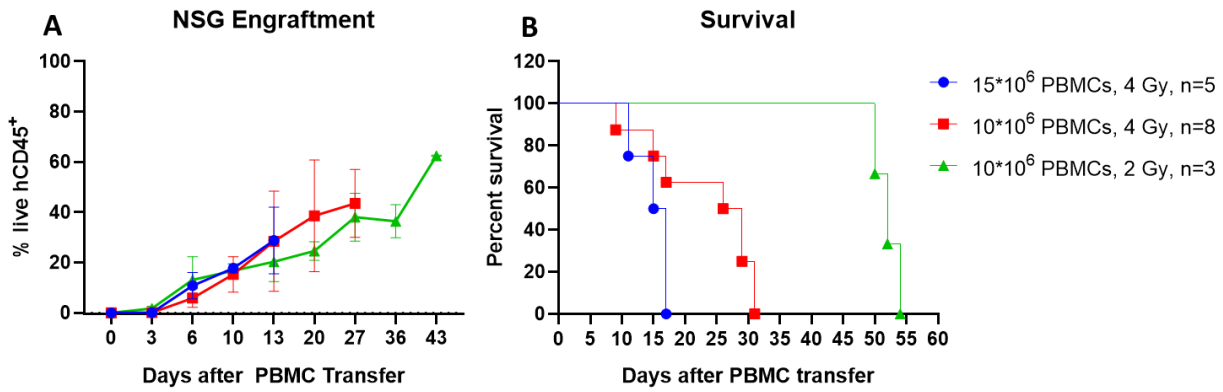

Figure 4: (A) Percentage of hCD45 cells in the blood of NSG animals at different time points after transfer of 15×10^6 PBMCs with 4 Gy irradiation (blue line); 10×10^6 PBMCs with 4 Gy irradiation (red line); and 10×10^6 PBMCs with 2 Gy irradiation (green line), each line representing a single NSG animal. (B) Survival analysis.

Despite similar engraftment capacity and initial human cell propagation between days 3 and 6, the onset of GvHD was significantly delayed when injecting 10×10^6 PBMCs after 2 Gy irradiation. This delay may be attributed to reduced inflammation from lower irradiation or the presence of murine cells mitigating human T cell activation. Consequently, all animals survived until day 50, requiring euthanasia before day 60 (Fig.4A-B). This extended timeline aligns more with chronic GvHD than acute GvHD, complicating the identification of treatment initiation points. Thus, we decided to conduct further experiments using 4 Gy irradiation with 10×10^6 PBMC injection, as this combination provided a more suitable timeframe for initiating treatment before disease onset.

4.3 Identification of a Possible Time Window to Initiate Treatment

After establishing the model with 4 Gy irradiation and 10×10^6 PBMC injection, the experiment was repeated to evaluate engraftment efficiency and variability. Consistent conditions are essential for reliable results during treatment assessment. Human cell expansion was similar across all animals up to day 10 (Fig.5A). Post-day 10, some animals exhibited faster PBMC expansion than others, resulting in increased variability (Fig.5B).

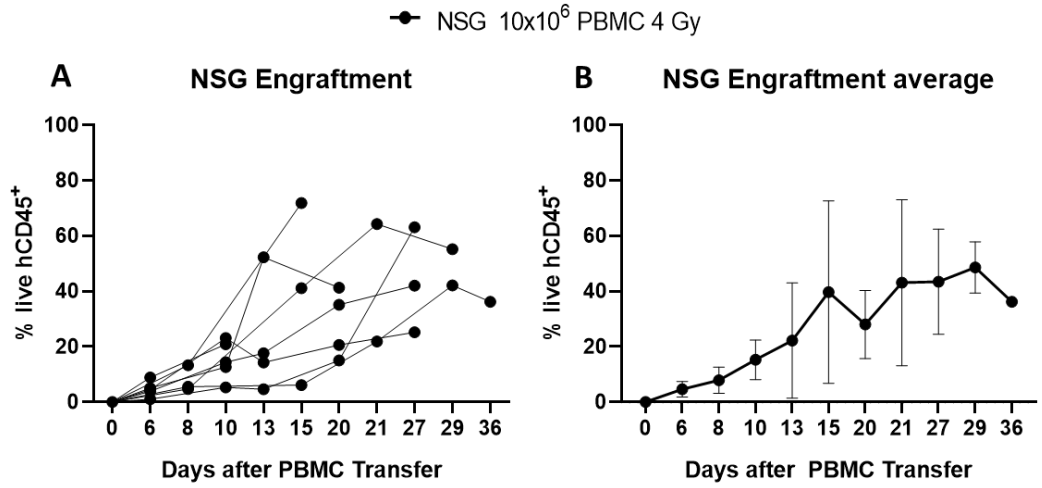

Figure 5: (A) Percentage of hCD45 cells in the blood of NSG animals at different time points after transfer of 10×10^6 PBMCs and 4 Gy irradiation, each line representing a single animal (n=8). (B) Percentage of hCD45 cells from animals shown in panel A, represented as average ± SD.

Based on these observations, we identified a possible treatment initiation window between days 6 and 10. During this period, all mice exhibited similar levels of PBMC expansion, and their health remained stable, showing no significant signs of advanced GvHD pathology. These conditions are optimal for conducting studies aimed at understanding how potential treatments can mitigate the onset of GvHD, a serious complication of allogeneic hematopoietic stem cell transplantation for hematologic malignancies.

This series of experiments provided a foundation for testing the efficacy of therapeutic agents in delaying the onset of GvHD or alleviating its symptoms. However, the primary focus of this thesis was on observing and participating in the daily work performed by research and animal care personnel in the animal facility. This work encompasses not only technical aspects, such as experimental procedures like ear tagging and intravenous injection, but also emphasizes the importance of assessing animal welfare and implementing practices to ensure the health and well-being of the animals throughout their stay.

5. DISCUSSION AND CONCLUSIONS

The establishment of a humanized mouse model for studying GvHD represents an important step in medical research, as it allows for the testing of new treatments in a controlled environment. The study demonstrates that using a combination of sub-lethal irradiation (4 Gy) and the injection of 30×10^6 peripheral blood mononuclear cells (PBMCs) successfully leads to a high level of human immune cell engraftment. This engraftment is vital for triggering a GvHD response, which closely resembles what occurs in humans after a stem cell transplant. However, the quick onset of mortality, where all the animals died by day 15, presents a significant challenge for testing potential therapies.

The examination of different doses of PBMCs revealed that reducing the number of injected cells to 15×10^6 did not significantly increase survival rates. This suggests that the original dose of 30×10^6 PBMCs may be optimal for creating a strong immune response necessary for GvHD. Interestingly, when a lower number of PBMCs (10×10^6) was combined with a reduced irradiation dose of 2 Gy, a significant delay in mortality was observed, allowing some mice to survive until day 50. This indicates that lower irradiation can help manage the inflammatory response, which is key to prolonging disease progression in the model. However, this extended timeline can complicate the identification of the optimal time to initiate treatment, as the onset of GvHD may be marked by subtler symptoms that are difficult to detect in the early stages and may appear over more prolonged time periods

Importantly, the study identified a possible treatment window between days 6 and 10 after PBMC injection, during which the mice showed stable health and consistent levels of human immune cell expansion. This period is crucial for testing new therapies, as it allows researchers to intervene before the symptoms of GvHD become severe. Exploring various treatments during this time could lead to a better understanding of how to delay the onset of GvHD or reduce its severity in patients receiving stem cell transplants.

In summary, this study successfully developed a humanized mouse model for GvHD, which is useful for testing new treatments. After conducting experiments to determine the optimal dosage of PBMCs for injection and appropriate levels of irradiation (measured in Gy) to establish a therapeutic window, further experiments, beyond the scope of this thesis, have been conducted to explore novel therapeutic options.

This humanized mouse model was developed in collaboration with Vicenza Hospital ULSS Berica, with the primary aim of testing innovative strategies to reduce the inflammatory response that drives

GvHD. This approach involves the use of a monoclonal antibody designed to limit the infiltration of cytotoxic T lymphocytes into the gastrointestinal tract and the use of mesenchymal stem cells (MSCs) to modulate inflammation through immunosuppression. This humanized mouse model has been instrumental in this research for two key reasons: i) it offers a better simulation of human GvHD pathology, and ii) it enables the use of the monoclonal antibody, which targets a human-specific antigen that does not cross-react with the corresponding mouse antigen.

The work conducted in this thesis has provided the laboratory with an optimized humanized mouse model that has since been successfully used in further experiments. These ongoing studies aim to determine the efficacy, optimal administration schedule, and dosage of both the monoclonal antibody and MSCs, used either alone or in combination, offering new opportunities for therapeutic intervention.

The time spent working side by side with the researchers of the immunology and tumor microenvironment laboratory and with the animal care staff has significantly increased my understanding of the scientific process. Collaborating closely with the research and animal care teams provided me with the knowledge necessary to monitor animal health through diligent observation and daily assessments. This practical experience emphasised the significant impact that careful monitoring can have on both animal well-being and the overall success of research projects. The relationship between animal welfare and research integrity cannot be overstated. Maintaining high standards of care is not merely an ethical obligation; it is essential for advancing scientific knowledge and ensuring the reliability of results. Researchers and animal care staff work together to ensure that animals remain in the best possible health throughout the course of experiments, prioritising their welfare and well-being at all times. This experience allowed me to understand the critical importance of animal welfare in achieving valid and reproducible experimental outcomes. Ensuring animal well-being not only enhances their quality of life but also contributes to the integrity of the data collected, ultimately leading to reliable and meaningful results.

In a project that involves studying GvHD, some degree of pain and distress may be unavoidable. For this reason, researchers, in collaboration with the designated veterinarian, analysed any possible sources of suffering and developed an intervention plan designed to minimise pain and discomfort, alongside establishing clear criteria for humane euthanasia.

For example, if a mouse shows signs of severe weight loss or excessive lethargy, researchers may decide to perform humane euthanasia, as these symptoms are indicative of a non-treatable stage of

GvHD. This is an example of animals that start experiencing suffering that cannot be reversed, in this case the established humane euthanasia criteria ensure that decisions are made promptly and ethically to prevent further distress.

To ensure animal well-being, health monitoring is not the only practice required. Performing procedures in the best possible manner is equally important. For instance, I developed skills in blood sampling, which is essential for evaluating the health status and humanization rates of the mice. Understanding proper methodologies for blood collection, such as selecting appropriate veins, preparing the site with antiseptic, and employing techniques that minimise discomfort, enhanced my knowledge of humane practices in animal research.

Additionally, I was also taught how to perform intravenous injections and ear tagging for identification purposes. Throughout this process, I learned to conduct all procedures in accordance with ethical guidelines, which emphasised the importance of maintaining humane treatment of the animals involved.

In summary, this thesis details the development of a humanized mouse model, which serves as a valuable tool for studying GvHD, providing a more accurate representation of human immune responses. Upholding high standards of animal welfare is essential to balance scientific progress with ethical responsibilities, and it was critical to the successful establishment of this model. By prioritising ethical considerations alongside rigorous scientific methodologies, researchers can ensure that their work not only advances knowledge but also respects the lives of the animals involved.

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