Animal Models for Preclinical Development of Allogeneic Hematopoietic Cell Transplantation

Scott S. Graves, Maura H. Parker, and Rainer Storb

Scott S. Graves is a staff scientist in the Transplantation Biology Program at the Clinical Research Division of the Fred Hutchinson Cancer Research Center in Seattle, Washington. Maura H. Parker is also a staff scientist in the Transplantation Biology Program at the Clinical Research Division of the Fred Hutchinson Cancer Research Center in Seattle, Washington. Rainer Storb is a full member and head of the Transplantation Biology Program at the Clinical Research Division of the Fred Hutchinson Cancer Research Center in Seattle, Washington as well as a professor of Medicine, Oncology at the Department of Medicine, University of Washington in Seattle, Washington

Address correspondence and reprint requests to Rainer Storb, MD, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N, D1-100, PO Box 19024, Seattle, WA 98109-1024 or email: rstorb@fredhutch.org. You may also contact Dr. Graves at sgraves@fredhutch.org

Abstract

Since its inception in the 1950s, hematopoietic cell transplantation (HCT) has become a highly effective clinical treatment for malignant and nonmalignant hematological disorders. This milestone in cancer therapy was only possible through decades of intensive research using murine and canine animal models that overcame what appeared in the early days to be insurmountable obstacles. Conditioning protocols for tumor ablation and immunosuppression of the recipient using irradiation and chemotherapeutic drugs were developed in mouse and dog models as well as postgrafting immunosuppression methods essential for dependable donor cell engraftment. The random-bred canine was particularly important in defining the role of histocompatibility barriers and the development of the nonmyeloablative transplantation procedure, making HCT available to elderly patients with comorbidities. Two complications limit the success of HCT: disease relapse and graft versus host disease. Studies in both mice and dogs have made significant progress toward reducing and to some degree eliminating patient morbidity and mortality associated with both disease relapse and graft versus host disease. However, more investigation is needed to make HCT more effective, safer, and available as a treatment modality for other non-life-threatening diseases such as autoimmune disorders. Here, we focus our review on the contributions made by both the murine and canine models for the successful past and future development of HCT.

Key words: canine; cell; hematopoietic; model; murine; preclinical; transplantation

Introduction

Hematopoietic cell transplantation (HCT) is a widely used therapy for malignant and nonmalignant hematological disorders.^{1,2} Hematopoietic stem cells correct nonmalignant hematopoietic disorders such as immunodeficiency diseases and anemias as well as allow clinicians to use more aggressive marrow-toxic irradiation protocols for the treatment of hematological malignancies. More

than 70 years of studies in animal models have been essential for achieving success in human patients and will be necessary to refine the procedure to minimize toxicity and improve outcomes.

Researchers concluded from early experiments that protection from lethal irradiation was due to humoral factors rather than engraftment of donor cells.^{3–6} In 1956, three independent groups (Rijswijk Radiobiology Lab, the Netherlands; Harwell

Radiochemistry Labs, UK; and Oakridge National Labs, USA) provided clear evidence that a cellular mechanism was responsible for the rescue of mice from the lethal effects of irradiation of irradiation.⁷ The cellular hypothesis gained indisputable acceptance following a series of critical studies showing that recipient mice given stem allogeneic marrow were protected from lethal irradiation and were tolerant to donor skin grafts.^{8,9} These studies clearly indicated that living cells and not humoral factors are responsible for recovery following lethal irradiation. These early experiments laid the groundwork for therapeutic HCT.

Further experiments using mice with established leukemia showed that mice survived longer after irradiation when given an injection of homologous (allogenic) versus isologous bone marrow.^{10,11} This was the first demonstration of an immune-mediated control of hematopoietic malignancy known as the graft-versus-leukemia (GVL) or graft-versus-tumor (GVT) effect. However, animals receiving allogeneic HCT (allo-HCT) eventually succumbed to a wasting disease, presumed at the time to be malnourishment from radiation-damaged intestinal tissue.^{11,12}

Translation of initial HCT studies in mice to human patients was disappointingly unsuccessful. Although an initial human study of six cases demonstrated the safety of marrow transplantation, only transient engraftment was observed and only in one patient.¹³ HCT in patients with refractory leukemia, in which the donor was an identical twin, resulted in successful donor hematopoietic cell engraftment; however, the patients ultimately relapsed, indicating a lack of GVL response.¹⁴ These studies were a clear indication that matching of donor and recipients was necessary for engraftment, yet some genetic difference was required for donor targeting of tumor cells. The first persistent allograft was described in 1965 in a patient with leukemia; however, the patient eventually died from secondary syndrome, which was most likely chronic graft-versus-host disease (GVHD).¹⁵

Clinical trials for patients with hematological malignancies were failing due to premature application of results from murine studies to human clinical trials. In 1970, this opinion was further substantiated in a report covering 200 patients treated with HCT for hematological diseases in which all recipients had died of either graft failure, GVHD, infections, or disease relapse.¹⁶ More knowledge surrounding what was required for successful engraftment and tumor targeting was necessary.

In subsequent years, researchers directed their attention toward identifying alternative animal models that would better predict results in the clinic. Research using large animal models, primarily dogs, supplemented research using inbred mice. The focus was on conditioning regimens, matching of donor and recipient pairs, and GVHD prophylaxis. Studies in these large animal preclinical models were successfully translated to the clinic as effective treatment protocols for malignant and nonmalignant hematopoietic diseases.

Future studies are especially required to address two major problems: disease relapse and nonrelapse mortality. Once these issues are firmly under control, allo-HCT may result in better survival of patients with hematological disorders and be of medical benefit for less life-threatening diseases such as autoimmune disorders and solid organ/tissue transplantation.¹⁷ The purpose of the current report is to describe the role of animal models, primarily the mouse and dog, in the successful development of allo-HCT protocols and their potential in future research efforts towards unresolved issues.

Current Clinical Need

Traditional myeloablative pretransplant conditioning involves high-dose total body irradiation (TBI) and/or chemotherapy to reduce the patient's tumor burden before HCT and suppress the host immune system to prevent rejection of the donor graft. These myeloablative protocols are generally only acceptable in younger patients or older patients with limited comorbidities. This is particularly confounding, since hematological malignancies often occur in older patients; the median age for acute myelogenous leukemia and non-Hodgkin lymphoma range from 65 to 75 years. Studies in the dog model established a regimen of reduced-intensity, non-myeloablative conditioning and postgrafting immunosuppression that reduces allo-transplant-related toxicities and maintains donor cell engraftment.¹⁸

A recent review of 1092 patients with advanced hematologic malignancies given non-myeloablative conditioning with fludarabine and 2 cGy TBI followed by a human leukocyte antigen (HLA)-matched related or unrelated HCT showed an overall 5-year relapse mortality rate of 18% to 60%, depending on risk of relapse. Most relapse occurs within the first 2 years and depends on the disease and disease burden.^{19,20} A combined analysis of other reduced intensity conditioning (RIC) and non-myeloablative regimens yielded similar results, with a 43% average rate of relapse (range, 22–65%). A phase III trial by the Blood and Marrow Transplant Clinical Trials Network comparing myeloablative and RIC regimens showed that progression-free survival was significantly lower in the RIC arm of the study (47.3% vs. 67.8% in myeloablative arm).²¹ These data suggest that reduced intensity regimens may not be sufficient in all patients for achieving disease remission. Increasing the intensity of the conditioning regimen to reduce the incidence of relapse would be too toxic for patients with high comorbidities and unnecessary for a majority of the patients. Therefore, less toxic means for reducing relapse need to be identified.

In the Blood and Marrow Transplant Clinical Trials Network study described above, the primary cause of death in the myeloablative arm was GVHD (52%). In the analysis described by Storb et al.,^{19,20} 43% of related recipients and 59% of unrelated recipients developed grade II–IV acute GVHD, and the cumulative incidence of both acute and chronic GVHD was 75%. Overall, the 5-year nonrelapse mortality was 24%, and the majority (20.2%) were associated with GVHD. Specifically, 16% of patients died from complications related to either acute GVHD alone or from acute GVHD progressing to chronic GVHD, and an additional 4.2% died with de novo chronic GVHD. In this analysis, acute GVHD conveyed no additional beneficial GVL/GVT effect but significantly increased the hazard ratios for both nonrelapse mortality and the incidence of chronic GVHD, which increased from 25% to close to 50%.²⁰

A number of reports showed significantly higher rates of chronic GVHD with peripheral blood HCT compared to marrow transplant, most likely due to higher numbers of lymphocytes contained in the peripheral blood graft.^{22–29} Conversely, when the number of transplanted lymphocytes was reduced by *in vitro* or *in vivo* T-cell depletion (anti-thymocyte gamma globulin, alemtuzumab, post-HCT cyclophosphamide),^{30,31} the rates of chronic GVHD declined significantly. However, the benefit from decreasing the incidence of chronic GVHD is offset by significantly increased relapse rates in patients with hematological malignancies.

Reduced-intensity regimens have the benefit of lower toxicity and are appropriate for a greater number of patients. However, the risk of relapse is significant, and therefore,

reduced-intensity regimens need to be improved to effectively treat the malignancy and prevent relapse without increasing the risk of acute or chronic GVHD. However, myeloablative approaches remain effective in a number of situations, thereby necessitating new approaches to prevent and treat GVHD.

Animal Models

Once HCT was successful in the first human patients, a long history ensued in which studies in preclinical animal models played a key role in reducing toxicity and improving patient outcomes. Studies in mice and canines focused on MHC typing³²⁻³⁷ and pretransplant conditioning regimens such as TBI,³⁸⁻⁴⁵ chemical immunosuppression,⁴⁶⁻⁴⁸ and radioimmunological ablation.^{49,50} Other critical studies evaluated hematopoietic stem cell dosage,⁵¹⁻⁵⁴ post-transplant immunosuppression,^{18,55-61} prevention of graft failure and disease relapse,⁶²⁻⁶⁵ and nonrelapse mortality, primarily acute and chronic GVHD.^{66,67}

Mice are unquestionably the most cost-effective research animal model. Mice require little laboratory space, are easily maintained and handled, and antibodies specific to a wide variety of cellular antigens are abundantly available. Mice are small and, therefore, efficiently dosed on a mg/kg basis with costly early phase development drugs and biologicals. Mice are genetically well defined and are ideal research animals for genetic manipulation to study pathways and mechanisms. Mice also facilitate study of a wide variety of hematopoietic disease models, including murine hematopoietic tumors and human tumor xenografts in immune-deficient models.

Weaknesses of the murine HCT model are well documented.⁶⁸ Early HCT studies in mice did not translate well to the clinic. Mice have a short life span, making long-term engraftment studies impossible. Although specific strains of mice can mimic various individual aspects of the GVHD syndromes seen in humans, GVHD studies in mice fail to reproduce the full spectrum of the disease (see below). A single stem cell can repopulate a mouse immune system,⁶⁹ but repopulation of human and dog hematopoietic systems has always been polyclonal.⁷⁰ Mice are generally maintained under gnotobiotic conditions, and thus, the impact of complex microbiota on the murine immune system is largely absent.

Large animals, primarily dogs, and to some lesser extent nonhuman primates, have been used to supplement studies using inbred strains of mice. Dogs are superior to mouse models for HCT for several reasons. Dogs are particularly well-suited due to their mixed genetic background as a result of long-term random breeding in the laboratory setting. Dogs display phenotypic diversity and longer life spans compared to mice.⁷¹ Dogs used in experimental research are not raised under gnotobiotic conditions, and therefore they are subject to similar immune conditions imposed by intestinal microbiota following HCT as are humans.^{72,73} Dogs also possess a relatively short gestation period (average 63 days) and have large-sized litters, allowing for successful studies evaluating matching for MHC antigens, known as dog leukocyte antigens (DLA). Importantly, DLA class I and II genes simulate HLA matching for donor and recipient pairs.^{74,75}

Canine and human CD34+ marrow cells possess similar *in vitro* and *in vivo* characteristics.⁷⁶ Although nonhuman primates are also similar to human in many respects, dogs are easier to handle, less expensive to purchase and maintain, and lack virulent primate pathogens transmissible to humans. Importantly, successful canine HCT studies in DLA-matched donor and recipient pairs with postgrafting immunosuppression reestablished a

sense of confidence that HCT for aplastic anemia and hematological malignancies could be successful in patients.^{34,77}

However, the dog model does have limitations. Antibodies directed against dog hematopoietic cell antigens are not as common as those for mice and nonhuman primates. Dogs are more expensive to purchase and house compared to mice but are less expensive than nonhuman primates. Currently, dogs are not as amenable to knock in/knock out studies as are mice. However, both dogs and primates have been used effectively in stem cell gene therapy.⁷⁰ Additional comparisons between the three species are described by Stolfi et al.⁷⁸ Table 1 lists the suitability of mouse, dog, and nonhuman primate for a variety of HCT characteristics described above.

Disease Relapse

Disease relapse, or progression of the underlying malignancy, remains a critical cause for failure of allogeneic HCT in the clinical setting.¹⁹ GVL and GVT effects are the result of an active immune process involving donor T cells and, likely, donor NK cells. Postgrafting immune suppression for GVHD prevention and slow development of the donor immune system contribute to limited donor GVL/GVT activity early after transplant.²⁰ Increasing the intensity of the conditioning regimen to reduce tumor burden would increase regimen-related toxicity in medically infirm patients. New approaches to reduce tumor burden and boost donor immune cell function are needed to overcome the problem of relapse.

Animal Models

Mouse models for disease relapse after HCT have the advantage that a large number of transplantable tumors exist that are specific to various strains of mice. These tumors often show genetic instability and can be injected into mice with intact immune systems before HCT. However, mouse tumor models often do not share characteristics with human tumors such as latency period, biology of metastasis, or clinical outcome to new therapies.⁷⁹ Many of the murine hematological tumor cell lines have been extensively cultured *in vitro* and have become highly sensitive to chemotherapy and alloreactive T cell targeting.⁷⁸ In contrast, most human tumors have adopted means of avoiding immune recognition and resisting chemotherapy. Spontaneous murine hematological tumors have been developed, such as murine chronic myelogenous leukemia after transfecting marrow cells with BCR/ABL,⁸⁰ and can be viewed as a more appropriate model for development of therapeutic interventions for disease relapse.

Dogs develop lymphomas, sarcomas, and melanomas.⁷⁸ Cancers in companion dogs share histological features with human tumors such as tumor growth over time, tumor heterogeneity, disease relapse, and metastatic microenvironment characteristics.⁸¹ The obvious deficiency in using animals with spontaneous tumors is the difficulty in conducting controlled randomized studies in a timely manner. However, several reports have been published describing HCT treatment of lymphoma in companion dogs.⁸²⁻⁸⁴

Genetic modification of canine hematopoietic stem cells prior to transplant opens up the possibility of generating leukemia *in vivo*. Two dogs transplanted with autologous genetically modified donor CD34+ hematopoietic stem cells overexpressing HOXB4 developed myeloid leukemia within approximately 2 years.⁸⁵ Moreover, accidental transfusion of trace numbers of cells from one of these dogs into two immune-suppressed dogs

Table 1 Comparison of suitable features for animal model HCT development

	Mouse	Canine	Nonhuman primate
Early contributions to HCT	3	3	1
Comparable cell surface receptors (mAb)	1	1	2
Reduced-intensity conditioning	1	3	1
Multilineage chimerism	1	3	3
Clinical translation	1	3	3
Genetic relevance	1	2	3
Metabolic similarity	1	2	3
Genetic manipulation studies ^a	3	2	2
Acute GVHD modeling	3	3	1
Chronic GVHD modeling	1	3	1
Cost	3	1	1
Accessibility	3	2	1

Suitability is based on the overall contribution of the species to each criterion and is on a scale of 1 to 3, with 3 being the greatest.

^aIncludes knock in-out studies and gene transfer experiments.

resulted in development of myeloid leukemia in both animals.⁸⁶ These studies indicate that generating canine models of leukemia are possible and may provide a model for investigating GVL and relapse.

Cell-Based Targeting of Tumors

One approach to treating disease relapse with low risk of toxicity is with post-transplant donor lymphocyte infusion (DLI) and/or cytokine therapy to boost the anti-tumor effect of donor immune cells.⁸⁷ Weiss and colleagues used co-infusion of BCL1 cells, a B-cell leukemia cell line, with T-cell-depleted donor bone marrow after lethal irradiation to establish a model of minimal residual disease for evaluating the effect of DLI and cytokine therapy.⁸⁸ A low level of disease (10^4 BCL1 cells) was eliminated with IL-2 alone or cells alone, yet a higher level of disease (10^5 BCL1 cells) required DLI and IL-2 to eliminate the leukemia.

In a mouse model of acute myeloid leukemia, mice were infused with leukemia cells prior to irradiation and transplant with bone marrow and spleen cells from syngeneic, congenic, and allogeneic donors.⁸⁹ At a tumor burden of 10^5 mouse model of acute myeloid leukemia cells, transplant with allogeneic cells was curative, yet congenic transplant was unsuccessful. Mice receiving post-transplant IL-2 in addition to congenic transplant were able to eliminate the tumor, suggesting cytokine therapy after reduced intensity or non-myeloablative transplant could stimulate the GVL/GVT effect without increasing GVHD.

In dogs, non-myeloablative preconditioning followed by a DLA-identical marrow transplant and postgrafting immune suppression results in stable mixed hematopoietic chimerism.¹⁸ Targeting host hematopoietic cells with the aim to increase donor chimerism can act as a surrogate for studying GVL activity and prevention of disease relapse. Infusion of donor lymphocytes after establishing stable mixed chimerism in dogs failed to increase donor chimerism, even with the addition of a 2-week course of IL-2 to stimulate proliferation.^{90,91} If the donors were first sensitized to minor histocompatibility antigens by a recipient-to-donor skin graft, DLI resulted in a rapid shift to full donor chimerism, clearly demonstrating a graft-versus-host effect.^{90,92} Recently,⁹³ sensitized female dogs to male antigens using adenovirus constructs encoding sections of SMCY and the entire SRY genes. After female-to-male non-myeloablative transplants, male antigen-sensitized DLI

from the female donor caused a shift in donor chimerism in two of three male recipients.

Adoptive immunotherapy with natural killer (NK) cells has the potential to reduce tumor burden without increasing the risk of GVHD. Transplant of expanded donor NK cells in addition to marrow and spleen cells in a mouse model of leukemia improved survival compared to controls and resulted in less severe GVHD, suggesting NK cells may be able to reduce tumor burden.⁹⁴ A trial in human patients in which a single dose of NK cells is added to non-myeloablative haploidentical transplant with post-transplant cyclophosphamide showed potential for improved progression-free survival at 2 years, indicating further study and optimization is merited.

Chimeric antigen receptor (CAR) engineered T cells have yielded impressive results in the treatment of B cell malignancies and afford a novel approach towards preventing disease relapse. These results have been achieved using CD19-specific,^{95–97} CD20-specific,⁹⁸ or CD30-specific⁹⁹ CAR-T cells for treating B cell malignancies. In a study of 30 children and adults diagnosed with ALL, 90% achieved complete remission of their disease.⁹⁶ Development of this technology depended on validation in primarily murine tumor models. Long-term survival was established in nude mice bearing NIH3T3 cells expressing human ERBB2 antigen.¹⁰⁰ Second- and third-generation CAR constructs containing costimulatory molecule signaling domains were also validated in murine models.^{101,102}

Validation of CAR-T cell efficacy is also possible in dog spontaneous tumor models. Canine T cells can be expanded ex vivo, transfected with a CD20- ζ targeting domain, and used to treat dogs with relapsed B cell lymphoma. In a study by,¹⁰³ three injections of CAR-T cells into a dog with relapsed B cell lymphoma were safely tolerated and led to transient anti-tumor effects. The advantage of using the dog as a model for CAR-T cell development/validation is that a spontaneous tumor replicates the complexities of the tumor microenvironment of human B-cell neoplasia and is considered a relevant and predictive model for the development of therapies for the treatment of non-Hodgkin lymphoma.^{104–106}

Antibody-Based Targeting of Tumors

Antibody-radionuclide conjugates can specifically target toxic radiation to the tumor and reduce off-target effects of TBI. Historically, iodine-131 and yttrium-90, which are both β -particle-emitting isotopes, have been used in the majority of

radioimmunotherapy preclinical and clinical studies.¹⁰⁷ Radioimmunotherapy using yttrium-90-anti-CD22 in conjunction with unconjugated anti-CD20 IgG successfully cured 80% of nude mice grafted with the human B-cell lymphoma, Ramos.¹⁰⁸ Use of both isotopes has been described in hundreds of clinical trials that attest to their efficacy for the treatment of hematological and solid malignancies. One potential advantage of using beta- and gamma-emitting radionuclides is that the path length of radiation is long (0.8–11.3 mm, respectively), resulting in a “cross-fire” effect against non-antigen-bearing tumor cells. However, the long path length may also result in targeting of nonmalignant “bystander cells.” Moreover, iodine-131 and yttrium-90 both have a long half-life of 2.5 and 8 days, respectively, and low energy emissions of 0.7 and 2.3 MeV, respectively.

Alternatively, radioimmunoconjugates using alpha emitters such as astatine-211 and bismuth-213 possess a short half-life (7.2 hours and 1 hour, respectively) and a limited path length (0.04–0.06 mm), thereby limiting off-target effects. Moreover, they have a high energy transfer (5.9 MeV and 8 MeV, respectively) and are expected to more effectively eliminate target cells. A bismuth-213-labeled antibody specific to Thy-1.2 specifically eliminated a Thy-1.2 + EL-4 murine tumor cell line in vivo.¹⁰⁹ A bismuth-213-labeled anti-CD45 antibody administered at 3.3 mCi/kg provided sufficient preconditioning for stable donor hematopoietic engraftment in a DLA-identical canine model of allo-HCT.¹¹⁰ However, the widespread use of bismuth-213 is precluded by high cost and limited availability.

Orozco and colleagues¹¹¹ showed that astatine-211 was able to substitute for bismuth, showing that astatine-labeled anti-CD45 antibody improved the median survival time of mice bearing leukemic cells in a dose-dependent manner, indicating the potential in reducing tumor burden prior to transplant. Similar to the results seen with bismuth-213, astatine-211-labeled anti-CD45 was able to substitute for TBI conditioning in a DLA-identical HCT model.¹¹² Seven of eight dogs conditioned for transplantation with 155 to 165 μ Ci/kg of the astatine-211 immunoconjugate developed long-term donor chimerism. Collectively, these studies suggest that administering radioimmunoconjugates may be appropriate for reducing tumor burden and improving relapse rates without adding toxicity or impairing donor immune recovery.

GVHD

In human patients GVHD is broadly defined as either acute, occurring within 100 days, or chronic, which develops 100 days and beyond after transplantation, with the disease lasting up to several years. The two syndromes differ in their clinical presentation. Acute GVHD typically manifests with a systemic syndrome of weight loss, diarrhea, skin rash, and high mortality. Up to 80% of patients given HLA-identical allo-HCT develop acute GVHD.⁶⁶ Chronic GVHD presents as an autoimmune condition with systemic fibrosis and the production of auto-antibodies.¹¹³ Both diseases are induced by donor T cells, and the targeted tissues are primarily the skin, intestinal tract, lung (chronic GVHD), and liver.

GVHD was first described as “secondary disease,” a wasting syndrome that occurred following transplant. Decades of preclinical work in both mice and canine models clearly demonstrated that this syndrome is the result of donor T cells attacking host tissues.^{114–124} The ability to match donors and recipients based on HLA typing reduces the risk of GVHD, yet

fatal GVHD can develop still develop, presumably as a result of minor histocompatibility antigen mismatches.^{34,125–131}

Animal Models of Acute GVHD

Mouse models of acute GVHD generally involve myeloablative conditioning using a lethal dose of irradiation (600–1300 cGy, depending on the strain), followed by transplant of H-2 incompatible bone marrow supplemented with donor lymphocytes, either splenocytes or lymph node T cells. The result is a systemic disease that normally affects the GI tract, liver, and skin and is lethal between 10 and 30 days after transplant. Sensitivity to radiation dose is dependent on the strain such that B6 mice are more resistant to radiation than BALB/c, and F1 progeny are more resistant than parental strains.¹³²

Murine models of acute GVHD include MHC-mismatched models, minor histocompatibility antigen (miHA)-mismatched models, and xenogenic models. The most common MHC-mismatched model of acute GVHD is a transplant from C57/Bl6 (H^{2b}) donors to Balb/c (H^{2d}) recipients. Parent-to-F1 transplants using C57Bl/6 parental donors also generate acute GVHD; however, not all parental strains are able to induce acute GVHD, and disease development depends on irradiation. Interestingly, transplant from C57/Bl6 (H^{2b}) parental donors to recipients with mutations in MHC I (B6.C-H2^{bm1}) and/or MHC II (B6.C-H2^{bm12}) demonstrated that a mismatch in both MHC class I and class II is required for development of acute GVHD, suggesting that both CD4+ and CD8+ T cells are involved in disease induction.¹³³

The miHA-mismatched models also rely on pretransplant irradiation, typically ranging from 600 to 1000 cGy. Either CD4+ or CD8+ T cells can contribute to disease pathology in the miHA-mismatch setting. Many of the models display systemic disease, but there is variation. For example, a transplant from a B10 (H^{2b}) donor to a BALB.b (H^{2b}) recipient generates acute GVHD without any skin involvement. Xenotransplant of human peripheral blood mononuclear cells into immune-deficient mice results in systemic disease. Immune-deficient mice require a lower dose of irradiation, typically 200 to 300 cGy depending on the strain. It is a CD4+ T cell-dependent model, as human APCs are required to process mouse antigens and T cell recognition of MHC molecules is restricted by species.

Mouse models of acute GVHD represent a controlled experimental system that allows analysis of single variables. However, humans exhibit genetic and phenotypic diversity, varied exposure to microorganisms, and variation in health status that all can affect outcomes. Mice are generally housed in specific-pathogen-free conditions; however, the microbiome has the potential to contribute to the generation of intestinal GVHD and may determine severity.

Exposure to a radioactive source, such as ¹³⁷Cs, is typically used as conditioning in mouse models of HCT. For human patients, a linear accelerator is typically used to generate and emit high-energy x-rays, which penetrate deeper into tissue for TBI. Moreover, unlike in mouse models, conditioning in human patients varies in intensity from non-myeloablative to myeloablative, depending on age, disease status, and comorbidities, and may involve chemotherapy and/or TBI. Moreover, postgrafting immune suppression as GVHD prophylaxis is rarely used in mouse models yet is standard of care in human patients. Postgrafting immune suppression will impact onset of GVHD and tumor progression.

The dog model uses more clinically relevant conditioning regimens^{46–48,134} and postgrafting immune suppression with such

individual agents as methotrexate (MTX), cyclosporine (CSP) azathioprine, succinyl acetone, and tacrolimus alone^{56,58–60,135} or in combination.^{56,60,136,137} Studies in the nonhuman primate HCT model generally rely on conditioning and postgrafting immunosuppression.^{138–140} Early studies in dogs demonstrated that successful marrow grafts in lethally irradiated dogs results in graft versus host reactions.¹⁴¹ Once serotyping was established to determine histocompatibility, consistent GVHD is induced in dogs using myeloablative TBI followed by transplant of marrow from mismatched and unrelated dogs.¹⁴² The tissues targeted and the resulting pathology closely resemble human acute GVHD.^{143,144}

Preventing and Treating Acute GVHD

Dogs given CSP after a DLA-mismatched and unrelated transplant had a lower incidence of GVHD, yet a significant number failed to engraft.¹⁴⁵ The combination of MTX and CSP was most effective for delaying onset of acute GVHD and permitted stable engraftment.^{145,146} A similar synergism was observed when MTX was combined with another calcineurin inhibitor, tacrolimus.⁶⁰ The effectiveness of the combination of CSP and MTX was confirmed in randomized, phase III studies in humans.^{147,148}

Mycophenolate mofetil (MMF), an inhibitor of DNA synthesis, appeared no better than MTX, CSP, or tacrolimus for preventing GVHD in dogs.¹³⁷ However, MMF showed synergism when combined with CSP and proved very effective both in enhancing engraftment after non-myeloablative conditioning and in controlling acute GVHD.^{18,137} A further canine study showed that rapamycin (sirolimus) could be substituted for MMF in combination with CSP.¹⁴⁹ MMF/CSP or MMF/tacrolimus is now widely used for human allogeneic HCT after reduced-intensity conditioning regimens and represents the current state-of-the-art in that setting.¹⁵⁰

If acute GVHD develops, corticosteroids are the first and most effective treatment option.¹⁵¹ However, steroids have a number of undesirable side effects, and GVHD can become steroid-refractory, prompting a number of studies to find new means of treating the disease. Studies in mouse models showed that IL-11, IL-1 β antagonists, TNF α antagonists, and IL-6 antagonists successfully treat acute GVHD.^{152–158} However, clinical trials in humans were unsuccessful and, in the case of IL-11 therapy, generated adverse side effects that resulted in unexpectedly high mortality.^{159–164} In these cases, the mouse models of acute GVHD were unable to predict outcomes in human patients. More recent studies in mouse models have identified HDAC inhibitors and JAK1/2 inhibitors as potential treatment options.^{165–167} Studies in human patients are promising. Vorinostat, an HDAC inhibitor, reduces the severity of acute GVHD,^{168,169} and ruxolitinib was able to achieve a complete response rate of 46.3% in patients with steroid-refractory acute GVHD.¹⁷⁰

Costimulatory Blockade

Blocking costimulatory molecules required for activation and expansion of T cells is predicted to be effective in abrogating pathogenic T cell responses after allo-HCT. Costimulatory molecule blockade has been investigated as a means to prevent or treat GVHD in mouse models.^{171–175} Following myeloablative allo-HCT, selectively blocking CD28 and ICOS, but not CTLA4, prevented acute GVHD in mice more effectively than blocking either CD28 or ICOS alone.

Blocking the CD28 costimulatory signal in the dog model with human CTLA4-Ig, in combination with MTX/CSP immunosuppression, increased survival and resulted in a lower

incidence of GVHD.¹⁷⁶ Currently, abatacept (human CTLA4-Ig) is in clinical trials as GVHD prophylaxis. Directly targeting CD28 with an anti-CD28 mAb while leaving the coinhibitory pathway of CTLA-4 intact has been proposed.¹⁷⁷ Our lab produced an anti-CD28 mAb with in vitro antagonistic activity.¹⁷⁸ However, injection of the anti-CD28 mAb into normal dogs produced a “cytokine storm” analogous to that seen in human volunteers, suggesting that anti-CD28 Fab or Fab constructs without cross-linking ability may be superior for human applications.¹⁷⁹

Animal Models of Chronic GVHD

The murine models used to study chronic GVHD have shorter disease onset periods of 14 to 49 days, and disease manifestations are restricted in organ involvement. It is rare to recapitulate all chronic GVHD disease manifestations in a single mouse model. Transplant from C57/Bl6 (H^{2b}) donors to recipients with mutations in MHC I (B6.C-H2bm1) resulted in mild chronic GVHD, whereas transplant into mice with mutations in MHC II (B6.C-H2bm12) resulted in severe systemic chronic GVHD,^{133,180} suggesting that CD4⁺ T cells are the main contributors to disease pathology.

Mouse models of chronic GVHD are varied. The sclerodermatous models typically involve lethal irradiation followed by an miHA-mismatched transplant, and the resulting disease is primarily T_H2-dependent fibrosis of the skin.¹⁸¹ The autoantibody/lupus-like models are generally parent-to-F1 MHC-mismatched transplants and may or may not involve TBI. The most common model is a DBA/2 (H^{2d}) to B6D2F1 (H^{2b/d}) transplant, which results in lymphadenopathy, splenomegaly, and autoantibody production.

Some models claim multi-organ involvement; however, the disease is normally restricted to a small number of tissues. A DBA/2 (H^{2d}) to BALB/c (H^{2d}) transplant results in production of autoantibodies and scleroderma.¹⁸² A C57/B6 (H^{2b}) to B10.Br (H^{2b}) transplant results mainly in bronchiolitis obliterans, but mild pathology was detected in oral mucosa and autoantibodies detected in the liver.¹⁸³

In contrast, fibrosis in human chronic GVHD can be systemic or pleiotropic, the repertoire of autoantibodies is more diverse, and lymphadenopathy and splenomegaly do not occur. Nephritis in human GVHD is rare but common in mouse model. Therefore, murine models of chronic GVHD do not adequately recapitulate the human disease.

Dogs conditioned with 8.5 to 9.2 Gy of total body irradiation followed by infusion of marrow from DLA-nonidentical littermates and postgrafting immunosuppression with MTX developed two distinct clinical forms of GVHD.¹⁴³ The median onset for acute GVHD was 13 days after transplant, while the chronic form developed at a median of 124 days after transplant. This temporal relationship recapitulates the human clinical condition better than does the mouse model.

The canine chronic GVHD model was not pursued at this time because investigators believed that solutions to treating the disease would be identified clinically. This assumption did not materialize, and chronic GVHD has remained a major problem in humans that is difficult to treat. Recently, we described a protocol in which dogs, conditioned with 9.2 Gy TBI and transplanted with DLA-mismatched unrelated marrow and buffy coat cells followed by postgrafting immunosuppression with MTX and CSP, developed de novo chronic GVHD, the clinical course of which resembled chronic GVHD seen in human.¹⁸⁴ Moreover, the target organs in the canine model (skin, liver,

gastrointestinal tract, and lungs) exhibited the same pathology as that observed in the human condition.¹⁸⁵

Treating Chronic GVHD

Compared to acute GVHD, the mouse model of chronic GVHD has provided few insights into treatment options.⁶⁷ The recent characterization of the canine model of chronic GVHD opens up the possibility of testing new drugs and biomolecules and investigating the role of the costimulatory pathways. Specifically, ICOS was upregulated on CD3+ cells within the blood, lymph nodes, and spleen in dogs affected by chronic GVHD, providing a potential target for therapy.¹⁸⁶ Administration of an antibody specific to canine ICOS resulted in a temporary remission of chronic GVHD symptoms and a significant prolongation in survival from the onset of the disease compared to control dogs.¹¹⁹

Accessibility and Acceptability

Mice are more accessible for study than dogs. Small animal vivaria are common at most major research institutions and large academic institutions. Canine and nonhuman primate vivaria have limited access. Special needs must be met for large animals such as treatment rooms, surgical suite(s), and kennels designed to humanely serve the dog's or the primate's needs. Sterile technique, surgical methods, and anesthesia are on par with that used in hospitals. For total body irradiation, a costly linear accelerator or linear accelerator is commonly used for an external beam radiation source for large animals, while for mouse irradiation, a far less expensive cesium irradiator in a shielded container can easily suffice.

A veterinarian is required for both small and large animal care, as are highly trained technical staff for caring, handling, and treating large animals. Protocols for experimental testing for large animals are exceedingly detailed and require investigators comply with strict regulations that ensure humane care and treatment of the animals. A great deal of the investigators' time is required to comply with the institutional animal care and use committee forms and validation processes. These issues have been recently examined and a need for consolidation and revamping of current practices suggested.¹⁸⁷ Overall, the cost and space required have a great impact on the accessibility of conducting large animal studies versus small animal studies.

Acceptability is an issue for any animal experimentation required in the testing of new therapeutic approaches for HCT. There are levels of pain that must be addressed and mitigated with the proper anesthesia and analgesia so that suffering is eliminated. The dog models have special concerns to researchers and the public, as dogs are companion animals while mice are generally not. It is important to note that what has been learned in the laboratory for HCT using the canine model has been returned to general dog healthcare by making available to dog owners the procedures for treating canine hematological and solid malignancies.^{82–84,188,189} Further education of the public with regards to the benefit of the appropriate use of animal models is essential for acceptability.

Overview and Next Steps

There are limitations to any animal model used for evaluating new drugs and protocols for clinical translation. The complexity of the MHC of different species and how it relates to environmental conditions, tumor heterogeneity, drug pharmacokinetics,

and intensity of conditioning regimens all play a part in translation of therapies to the clinic. Nevertheless, animal models provide a critical role in drug and protocol development between *in vitro* testing and clinical application from perspectives of toxicity, pharmacology, and efficacy. As in all studies, investigators should keep in mind that selection of the appropriate animal model for studies in HCT should be based on past performance of the model and not on availability/accessibility or lowest cost.

Both the murine and canine models have been invaluable in making HCT a highly successful therapy for the treatment of malignant and nonmalignant hematopoietic disorders. Despite tremendous success, two important areas of concern remain: disease relapse and GVHD. Undoubtedly, both mouse and dog will continue to contribute toward elucidation of these two problems.

In regards to disease relapse, the murine model will have significant impact in the development of next-generation CAR-T cells using human tumor xenografts in NOD/SCID or humanized mice. Dog HCT models with spontaneous or HOXB4-transformed hematopoietic cells⁸⁵ can better replicate the human condition for testing new CAR-T cell safety and efficacy. Natural killer cells, used to supplement/replace T cells in HCT or in adoptive immunotherapy following relapse, require further vetting in mouse and dog HCT models for their attractive GVT effects without inducing GVHD.^{190,191} Again, the canine model can be used provided the appropriate mAbs are developed for NK cell selection and expansion *ex vivo*.

GVHD also remains a significant complication to successful HCT. Only recently has there been reported a protocol for reliably inducing chronic GVHD in dogs that recapitulates all the manifestations of the disease seen in the human setting. Continued investigation into the application of costimulatory molecule blockade to chronic GVHD and especially steroid refractory GVHD needs to be tested in the canine HCT model.

Another important factor in looking forward is to extend to the animal models proteomics and genomic approaches that are widely applied to human systems. Identification and function of candidate molecules that are under investigation should bear close similarity between the two species so that mechanistic analyses and translations to the clinic can be properly made.^{192–194}

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