HHS Public Access

Author manuscript

Vet Comp Oncol. Author manuscript; available in PMC 2022 April 27.

Published in final edited form as:

Vet Comp Oncol. 2020 December ; 18(4): 471–483. doi:10.1111/vco.12608.

Developments and Translational Relevance for the Canine Hematopoietic Cell Transplantation Preclinical Model

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Abstract

The development of safe and reliable hematopoietic cell transplantation (HCT) protocols to treat human patients with malignant and non-malignant blood disorders was highly influenced by preclinical studies obtained in random-bred canines. The surmounted barriers included recognizing the crucial importance of histocompatibility matching, establishing long-term donor hematopoietic cell engraftment, preventing graft-versus-host disease, and advancing effective conditioning and postgrafting immunosuppression protocols, all of which were evaluated in canines. Recent studies have applied the tolerance inducing potential of HCT to solid organ and vascularized composite tissue transplantation. Several advances in HCT and tolerance induction that were first developed in the canine preclinical model and subsequently applied to human patients are now being recruited into veterinary practice for the treatment of malignant and nonmalignant disorders in companion dogs. Here we review recent HCT advancements attained in the canine model during the past 15 years.

Keywords

Large-animal; marrow; conditioning; irradiation; hematological; GVHD

1. INTRODUCTION

Hematopoietic cell transplantation (HCT) is a routinely used therapy for the clinical treatment of both malignant and nonmalignant hematological diseases.[1–4] The tolerogenic properties that develop as a result of HCT have been applied towards the treatment of autoimmune diseases and solid organ transplantation to eliminate the need of life-long immunosuppression for the prevention of graft rejection and disease progression respectively.[5,6] Decades of research in rodent and large animal models have led to these developments. In retrospect, it is difficult to imagine the successful development of HCT without the significant contributions made by the canine preclinical animal model.[7,8]

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Conflicts of Interest: There are no conflicts of interest to report.

The dog has been particularly well-suited as an animal model for HCT for several reasons. Dogs, like humans, have a great deal of phenotypic diversity and a well-mixed gene pool due to centuries of random breeding.[9] Also, dogs are not raised under gnotobiotic conditions, resulting in the development of a complex immune system responsive to microorganisms and common pathogens that contribute to a diverse intestinal flora analogous to the human condition.[10] Importantly, similarly to humans, dogs have major histocompatibility complex (MHC) antigens, defined as dog leukocyte antigens (DLA) coding for class I and class II genes. The development of the dog model for HCT was critically dependent on the characterization of these polymorphic loci.[11] In general, dogs have reasonably large liters, thus allowing intra-family matching of DLA class I and II genes for successful HCT outcome.

In 2007, our laboratory reviewed the successful development of HCT in context with the numerous contributions made using the preclinical dog model over several decades of research.[7] Since then, new studies have been reported that are worth noting in this present review. Here, we reveal that the dog remains a translationally relevant animal model for the continued development of the safety and success of HCT in both the patient clinic and veterinary practice settings.

1.1. Dog leukocyte antigen typing

In 1958, Van Rood and colleagues reported that during pregnancy about one third of women generate antibodies against foreign HLA antigens.[12] In the same year, Dausset identified the Rh (D) antigen on leukocytes that in part led to the awarding of the Noel Prize in 1980 for his work defining the importance of genetically determined cell surface structures that regulate immunological reactions.[13] These discoveries formed the cornerstone for studies defining the genetics of human leukocyte antigens and the eventual identification of the importance of HLA typing in transplantation. Development of an outbred canine model that could predict the outcome for HCT in human patients depended on a credible degree of matching of the MHC genes of man and dog. Initially, MHC typing of littermate pairs for HCT relied upon a battery of anti-leukocyte sera, obtained by cross-immunizing littermate pairs with buffy coat blood cells. Anti-sera plus complement and mixed leukocyte reactions using dog leukocytes were routinely performed to select appropriate matched or mismatched littermates for HCT studies.[14–18] Using these typing methods, an HCT study comprised of 16 serotype-matched donor and recipient pairs showed that all recipients engrafted after receiving 1500–1580 Rads of total body irradiation. Six dogs died of GVHD, 3 died of pneumonia, and 7 survived long-term without GVHD.[18] Although serological typing method were sufficient for identifying what was to become known as dog leukocyte antigen (DLA)-matched littermates for HCT studies, it was insufficient and cumbersome for identifying DLA-matched unrelated pairs. Nevertheless, the results proved that histocompatibility antigens played a crucial role in the successful engraftment of donor hematopoietic cells. Later, characterization of the molecular basis of DLA led to the identification of the polymorphic class I and II genes and resulted in a robust method to accurately genetically type related and unrelated dogs.[11,19–22]

Recently, we developed an improved method for typing DLA class I loci that obviated the need for radioactivity and single-stranded conformation polymorphism gel electrophoresis. Application of this method led to the identification of several new DLA-88 alleles.[23,24] To date, approximately 72 DLA-88 alleles have been published.[24] A most recent, and as yet unpublished, study of DLA-88 using next generation sequencing has identified 52 new DLA-88 allele candidates in a random population of 288 dogs (Harkey personal communication). This rate of new allele discovery indicates that a larger pool of yet undiscovered DLA-88 alleles exists in the domestic dog population.

In addition to DLA-88, a divergent class I gene, DLA-79 was examined for possible involvement in transplantation when mismatches between donor and recipient at this locus were considered.[25] In this retrospective study the investigators evaluated stored samples of peripheral blood mononuclear cell (PBMC) DNA collected from 108 transplanted dogs. No statistically significant correlation between DLA-79 disparity and graft rejection or graft-versus-host disease (GVHD) in the recipients was found. Furthermore, based on typing 407 dogs, the initial studies suggested that DLA-79 gene was not highly polymorphic, with only 4 alleles identified to date.[25] At this point, the exact function of DLA-79 is unknown.

Although DLA-88 is considered the most polymorphic class I gene, a recent study indicated that of 49 breeds genotyped using RT-PCR-based sequencing, DLA-12 and DLA-64 were more polymorphic than previously believed.[26] In addition, the genomic organization of DLA-88 and DLA-12 haplotype structures has been described, as well as the identification of several new alleles for DLA-88, DLA-12, and DLA-64. To fully molecularly characterize DLA-88, Barth et al. performed peptide binding analyses on DLA-88*50101 and HLA-A*02:01.[27] Remarkably, the results showed that DLA-88*5010-derived peptides bound to HLA-A*–02:01, suggesting that T cell-based immunotherapies, including minor antigen vaccines, may be developed in the canine as have been done in humans. Towards this end, a pilot study in canines using minor histocompatibility antigens as vaccines was conducted for the purpose of breaking tolerance in mixed chimeric recipients.[28] Success in this model could help separate the graft-versus-tumor effect from GVHD. In this study, one of three male, mixed chimeric dogs that had been transplanted with DLA-identical female marrow underwent a shift from mixed to nearly complete donor chimerism when given donor leukocyte infusions from the female donors after being sensitized using constructs expressing male-specific gene disparities as immunogens. No GVHD was observed in the responding dogs. The study suggests the possibility of using minor antigen vaccines to safely vaccinate a stem cell donor against tumor specific antigens.

1.2. Monoclonal antibodies

One limitation of performing preclinical studies in dogs, compared to mice or primate species, is the comparatively fewer monoclonal antibodies (mAb) directed against antigens on canine hematopoietic cells.[8] Nevertheless, several mAb and fusion proteins cross-reactive against or specific to canine lymphocytes have been described over the last several years. As to the former, Schuberth et al. published a particularly useful panel of cross-reactive mAb specific for canine leukocytes, platelets and erythrocytes.[29] Screening anti-human mAbs specific for CD154 led to our identification of the anti-human CD154 mAb, 5c8, as

an antibody that bound to canine lymphocytes and blocked alloimmune reactions of dog PBMC *in vitro*.[30] *In vivo* studies were done to determine whether the 5c8 mAb could induce host-versus-donor hyporesponsiveness in dogs conditioned with 100 cGy TBI and given DLA-identical marrow transplantation followed by postgrafting immunosuppression with CSP and MMF. All 6 dogs engrafted with 3 showing sustained engraftment for periods > 26 weeks, while control dogs not given 5c8 uniformly rejected their grafts given this transplant protocol.

In 2006, the Sandmaier group reported that the mAb, S5, specific to canine CD44, sensitized natural killer (NK) cells to irradiation and facilitated marrow engraftment in DLA haploidentical-recipients when given in conjunction with 200 cGy total body irradiation (TBI) before and postgrafting immunosuppression consisting of CSP and MMF after HCT.[31,32] This nonmyeloablative protocol was effective in establishing uniform engraftment across a DLA-haplotype mismatched barrier, but sustained engraftment occurred in only 43% of transplants. In a recent study, Thakar and colleagues posed the question whether the conditioning regimen of 200 cGy TBI plus the mAb S5 would increase engraftment in dogs given DLA-haploidentical HCT followed by postgrafting immunosuppression regimen of MMF and CSP with addition of methotrexate.[33] The investigators showed that the addition of low-dose MTX (50 mg/m^2) to the regimen improved time to rejection but failed to influence rates of rejection compared to controls. Dogs administered higher doses of MTX developed increased levels of lymphopenia following treatment with S5 mAb.

NK cells have been reported to demonstrate antiviral activity and have been used in immunotherapy to treat cancer,[34,35] but studies using NK cells in the preclinical canine HCT model are few. This area of study requires development of mAb against canine cell surface markers and mechanistic studies of NK cell activation and function that are translatable to human NK cells. To date, few mAb against canine NK cells have been described. Monoclonal antibodies reacting to canine NK cells include anti-CD44,[36] anti-CD5,[37] and anti-NCR1.[38] Recently, we produced an anti-canine CD94 mAb that is expressed on NK and NKT cells.[39] The mAb proved effective in enriching NK/NKT cells from dog PBMC. Flow cytometry indicated anti-CD94 bound to cultured and in vitro expanded cells that retained potent cytolytic activity when tested against the canine cell line CTAC. In general, the mechanistic functions of canine cell surface receptors on NK cells is not well understood. In humans, CD94 can up- or downregulate NK cell function depending on which NKG2 receptor the molecule interacts.[40] The role CD94 plays, if any at all, in canine NK cell activity remains to be determined. We hypothesize that anti-canine CD94 may be useful in expanding cytolytic NK cells for immunotherapy and improvement of HCT engraftment with reduced incidence of GVHD. Studies are underway addressing this question.

Over the past decade, our laboratory has focused on producing canine-specific monoclonal antibodies and fusion proteins designed to block costimulatory molecules for improved donor cell engraftment, reduced conditioning toxicity, or treatment of GVHD.[41–44] The CD28/CTLA-4:B7 pathway is perhaps the most well-studied mechanism of the costimulatory molecule blockade mechanism.[45] A CTLA4-Ig fusion protein can bind B7 thus block interaction of both CD28 and CTLA-4 with B7. Previous studies showed that an anti-human CTLA4-Ig (abatacept) cross-reacted with canine lymphocytes and was effective

in reducing TBI to 100 cGy for successful DLA-identical HCT and effective in reducing GVHD.[46,47] Working on the premise that a canine specific CTLA4-Ig would bind with greater affinity and thus be more effective in costimulatory blockade, we designed and produced a canine-specific CTLA4-Ig fusion protein composed of canine CTLA-4 and canine Ig for improved pharmacokinetics and to avoid an immune response. *In vivo* studies indicated that canine CTLA4-Ig effectively blocked an IgG response to multiple sheep red blood cell vaccinations.[41] However, *in vitro* studies showed that the canine CTLA4-Ig fusion protein was merely equivalent to the human version, abatacept, in blocking a canine mixed leukocyte reaction, thus indicating further characterization of a canine CTLA4-Ig fusion protein irrelevant at the time.

Alternatively, we took the approach that an antibody directed against CD28 would prevent the agonistic effects of CD28:B7 pathway on T-cell activation while leaving the downregulatory CTLA-4:B7 pathway intact.[43] Canine CD28 was cloned, mice immunized with a CD28 murine Ig fusion protein, and mAb producing clones evaluated for binding to T-cells. One antibody clone was antagonistic when tested in soluble form *in vitro* in mixed leukocyte reactions. Scale-up efforts of this clone produced sufficient quantities of the anti-CD28 mAb for toxicity evaluation in dogs.[44] Surprisingly, following IV infusion of the antibody, the dogs rapidly developed a toxicity profile consistent with a cytokine storm as that observed in human volunteers given the anti-human CD28.[48] This result was not predicted from nonhuman primate studies done in preclinical development of anti-human CD28 mAb. These findings along with ours strongly suggest that whole antibodies, capable of cross-linking CD28 molecules and thereby resulting in activation of the CD28 pathway, should be judiciously avoided and indicate that anti-CD28 single chain Fv and the like are preferred.

Inducible costimulatory (ICOS) was initially identified on resting memory and activated T lymphocytes.[49] Subsequent findings indicated the ICOS-B7RP-1 pathway functions to promote expansion, differentiation and survival of T cells.[50] ICOS activation also results in release of IL-10, an important cytokine in T-regulatory cell function. Based on these premises, we speculated ICOS blockade could be effective in the prevention and treatment of acute and chronic GVHD respectively. In 2004 our lab sequenced canine ICOS.[42] To this end, we produced a mAb specific to ICOS on canine lymphocytes. Initial investigations revealed that ICOS was upregulated on the surface of lymphocytes isolated from dogs with chronic GVHD.[51]

1.3. Conditioning regimens for hematopoietic cell transplantation engraftment

Conditioning the recipient before allogeneic HCT suppresses the immune response against the hematopoietic cell graft and, in the case of hematological malignancies, limits the progression of the fundamental disease. High-dose or myeloablative conditioning regimens using TBI, chemotherapeutics or a combination of both were later replaced with lesser intensity regimens, making HCT available to both the very young and the elderly patients and those with serious co-morbidities. Antibodies and mAb were in part responsible for this paradigm shift. For example, treatment of recipients with anti-thymocyte serum (ATS) for the elimination of the host T cell response was initially tested in canines before entering

the clinic. Protocols for the clinic were often first developed and tested in the canine model (reviewed in Storb et al.[52])

1.3.1. Total body irradiation—Early canine HCT investigations in our laboratory showed that myeloablative doses of total body irradiation (TBI) of 920 Gy could be replaced with a nonmyelablative dose of 200 cGy TBI when followed by infusion of DLA-identical marrow stem cells and pharmacological postgrafting immunosuppression consisting of mycophenolate mofetil (MMF) and cyclosporine (CSP), given for 28 and 35 days respectively.[53] Efforts to further reduce the dose of TBI to 100 cGy using granulocyte-colony stimulating factor (G-CSF)-mobilized PBMC with or without marrow failed to consistently result in long-term mixed chimerism in dogs given hematopoietic cells from DLA-identical littermates.[54,55] Substituting granulocyte-colony stimulating factor (G-CSF)-mobilized PBMC for marrow and extending postgrafting immunosuppression from 35 to 100 days prolonged stable donor engraftment.[55] Taking the approach of inducing donor-specific tolerance in the DLA-identical transplant setting, the Storb Lab attained stable hematopoietic chimerism in 4 of 6 evaluable dogs that were tolerized against donor PBMC using CTLA-4 Ig before 100 cGy TBI conditioning followed by HCT and postgrafting immunosuppression with CSP and MMF.[46]

Studies in canines over the past decade have continued to test the efficacy of TBI dose reduction on engraftment. Lange et al. attempted to increase engraftment efficiency in DLA-identical setting following 100 cGy TBI by vaccinating donors with repetitive injections of recipient leukocyte lysates or by augmenting the hematopoietic cell allograft with donor monocyte-derived dendritic cells.[56] The modifications were well tolerated, however, neither approach resulted in stable long-term engraftment.

Mielcarek and colleagues attempted to reduce TBI dose to 50 cGy by tolerizing the host to donor minor antigens by first activating recipient T cells before HCT with repeated infusions of donor PBMC followed by injections of methotrexate (MTX), CTLA4-Ig, denileukin diftitox (IL-2/diphtheria fusion protein), CTLA4-Ig + MTX, or anti-CD154 + MTX.[57] A second approach was taken to enhance the graft versus host activity of the donor cells by expanding recipient dendritic cells with infusions of Flt3 ligand in vivo pre- or post-HCT. Their results for both approaches showed that dogs engrafted, but sustained engraftment was observed in only 14% of all dogs in the combined experimental groups, an outcome not significantly different as that observed in dogs given 100 cGy TBI.

Recipient dogs treated with CTLA4-Ig and donor PBMC and 200 cGy before receiving DLA-haploidentical littermate HCT and post-grafting immunosuppression demonstrated in vivo and in vitro hyporesponsiveness and initially engrafted with mixed chimerism. However, graft rejection occurred after 12 to 22 weeks. Although no diminution in NK cell function was observed, neither did GVHD develop. The absence of reduced NK cell function was suspected as a reason for graft rejection.[58]

Extracorporeal photopheresis (ECP) alone or in combination with pentostatin, a purine analog administered before 100 cGy TBI, failed to prevent graft rejection in a DLA-identical HCT setting.[59] Additionally, we posed the question whether intensified postgrafting

immunosuppression could translate to stable long-term engraftment of DLA-identical HCT following low dose TBI (ranging between 50 to 300 cGy).[60] Various three-drug combinations of CSP, MMF, sirolimus, and methotrexate were well tolerated, and recipient percentages of CD3, CD4, and CD8 in peripheral blood, spleen and lymph node decreased in an irradiation dose-dependent manner. Nonetheless, graft rejection occurred in 13 of 16 recipients at 100 cGy TBI. In the 100 cGy TBI conditioning model, when CTLA4-Ig was replaced with methotrexate on days −5 and −3 with day 0 being the day of DLA-identical HCT, only 2 of 6 dogs engrafted longer than 26 weeks.[61] These results suggest that the ability of MTX to induce host-versus-graft hyporesponsiveness may be less than what can be accomplished using costimultory molecule blockade.

Early studies indicated dose rate was a factor in DLA-HCT engraftment. While engraftment hematopoietic cell engraftment occurred in dogs at TBI doses of 900 cGy given at 7 cGy/min, long term engraftment was seen at a fractionated dose of 450 cGy when the dose rate was increased to 70 cGy/min.[62,63] Applying the logic that high TBI dose rate increases the conditioning efficacy, DLA-identical HCT was performed at 100 cGy TBI, at dose rates of 7 or 70 cGy/min followed by postgrafting immunosuppression with MMF and CSP.[64] Donor chimerism in dogs given 100 cGy TBI at the 70 cGy/min rate showed a significantly extended engraftment period over that observed in recipients given the same TBI dose at 7 cGy/min; however, graft rejection was the ultimate result at both dose rates.

In a separate study, we evaluated the effect of delay of HCT following successively longer periods of time. Engraftment was the readout and no effort was made to prevent GVHD in this model by not using postgrafting immunosuppression. HCT donors were DLA-haploidentical littermates. The doses of TBI ranged from 450 to 920 cGy with delays between TBI and HCT of 0, 4, 8, 10 days. There was a positive correlation between radiation dose and HCT delay; specifically, the longer the delay between TBI and HCT, the higher the TBI dose required for successful engraftment.[65] The results of these studies could have implications in the event of a radiation exposure accident in which rapid identification of potential donor siblings for rescue HCT is feasible.

1.3.2. Radioimmunotherapy—Canine studies aimed at improving radiation conditioning measures for HCT have also focused on RIT as a replacement for external beam TBI or total lymphoid irradiation (TLI). In contrast to external beam TBI and TLI, radioimmunotherapy consists of conjugating a radionuclide to a mAb or other highly specific targeting moiety to target radionuclides.[66–68] Such a methodology has the advantage of delivering radiation to the target cell by the inherent nature of antibody-antigen specificity. Early RIT studies evaluated the anti-tumor effects of antibodies conjugated to β-emitting radionuclides, yttrium-90 (^{90}Y), rhenium-188 (^{188}Re) or iodine-131 (^{131}I).[69–73] Radioimmunoconjugates using β-emitters have been used clinically for the treatment of acute myeloid leukemia, myelodysplastic syndrome and non-Hodgkin lymphoma.[74–78]

Radioimmunotherapy developments in canines over the past decade have shifted from β-emitting to α-emitting radionuclides such as bismuth-213 (^{213}Bi) and astatine-211 (^{211}At). There are important reasons for this preference. Whereas β-emitting radionuclides have a long pathlength (0.8–4 mm), a property ideal for solid tumors, the α-emitters have a

relatively shorter path length (40–80 um) which limits the crossfire effect and damage to normal tissues when used to treat hematological diseases. The α-emitters have a higher linear energy transfer (LET) with a range of 60–230 keV/uM compared to β-emitters with a LET ranging between 0.1–1.0 Kev/uM, thus providing greater cytotoxicity, and more specific delivery to tumor cells.[79] Finally, the α-emitter ^{211}At has a relatively short half-life (T½) of 7.2 hours, while the β-emmitter ^{131}I half-life is 7 days which adds to nonspecific toxicity.

Early canine studies explored targeted radioimmunotherapy with ^{213} Bi in conditioned recipients for allogeneic HCT from DLA-identical littermates. To this end, ^{213}Bi was coupled to an anti-CD45 mAb. Encouragingly, despite ^{213}Bi's short half-life, consistent engraftment was seen.[80] In further studies, an antibody that targeted the T-cell receptor α/β, when coupled to ^{213}Bi also facilitated stable engraftment in dogs.[81] The short half-life of ^{213}Bi (T½ =45.6 min) and its cost limits its practicality and has resulted in a shift towards using the more readily available and longer lived ^{211}At (T½ = 7.21 hr).

CD45 is an appropriate immunotherapeutic target antigen for use in patients with malignant and nonmalignant blood disorders. A mouse anti-canine mAb specific to CD45 was conjugated to ^{211}At and tested as a conditioning reagent in a dose finding study.[67] Dogs given doses of ^{211}At-labeled CD45 at less than or equal to 405 μCi/kg without HCT rescue showed myelosuppression followed by autologous cell recovery and transient liver toxicity. A second group of dogs was treated with the radioimmunoconjugate at levels of 155 to 625 μCi/kg before allogeneic HCT and followed with postgrafting immunosuppression. Seven of eight dogs developed long-term donor mononuclear cell chimerism (19%–58%). Similarly, the anti-CD45-^{211}At radioimmunoconjugate was used to assess the uptake and tissue accumulation with either 0.75 or 1.0 mg/kg dose of ^{211}At in dogs receiving autologous HCT.[68] The highest uptake of ^{211}At was the spleen, followed by liver and lymph nodes. Lymphocytes in the blood and marrow were also efficiently targeted. Liver toxicity was transient as indicated by an increase in serum liver enzyme levels. Importantly, the absorbed doses of radiation in the blood, marrow and lymph nodes averaged 297 cGy/166 MBq, indicating that localization was sufficient for successful HCT conditioning.

These canine studies served as the basis for three ongoing clinical trials at the Seattle Cancer Care Alliance. Two of these trials involve older patients with myeloid malignancies undergoing either HLA-matched related or unrelated HCT or related HLA-haploidentical HCT. The purpose of adding targeted radioimmunotherapy in these two trials is to reduce the tumor burden before HCT and thereby reduce the risk of disease relapse after HCT. The third clinical trial involves patients with serious non-malignant blood diseases and will evaluate whether anti-CD45 antibody ^{211}At conjugate can be substituted for some of the currently used systemic chemotherapy.

1.3.3. Total body irradiation and mesenchymal stromal cells—Mesenchymal stromal cells, also called stem cells (MSC), have been described as multipotent nonhematopoietic progenitor cells with the capacity to differentiate into a variety of cell types including osteoblasts, neuroectoderm, mesoderm, and mature marrow stromal cells.[82,83] MSC have potent immunological properties and offer the potential to repair

and revitalize damaged tissues. However, despite a large body of evidence supporting their use, several questions remain to be answered regarding pluripotency, self-renewal, in vivo differentiation, and mechanism of action.[84–86] MSC-mediated enhanced repair of a variety of degenerative conditions has been examined in canines. These studies include successful recovery of damaged spinal cord neurons,[87] induction of osteochondrogenic activity,[88,89] increased tubular epithelial cell proliferation in cisplatin-induced kidney damage,[90] successful treatment of osteonecrosis in a canine femoral head model,[91] repair of infarcted myocardial tissue,[92] and suppression of inflammation of ruptured crucial ligament.[93] However, MSC-loaded microcarriers failed to regenerate structure when implanted within degenerated intervertebral discs.[88,94]

The use of canine MSC for improving HCT outcomes has not met with similar success. Based on the immunosuppressive properties of MSC, we posed the question whether marrow-derived MSC could enhance marrow engraftment in a DLA-identical HCT recipient conditioned with 100 cGy TBI. MSC were cultured from marrow obtained from the HCT donor. Cultured MSC expressed CD10, CD13, CD29, CD44, CD73/SH-3, CD90/Thy-1, and CD106/VCAM-1. Cultured canine MSC suppressed alloreactive lymphocyte proliferation in vitro in a dose-dependent manner. However, following administration of 100 cGy TBI, intravenous infusions of MSC along with the marrow graft (day = 0) and on day 35 failed to prevent marrow graft rejection in a period not significantly different from control dogs not given MSC.[95]

Kornblit and colleagues addressed the question whether MSC engraft after transplantation in a DLA-identical or unrelated transplant setting.[96] Following conditioning with 920 cGy TBI, dogs transplanted with marrow and given canine MSC transfected with the GFP-retrovirus, pOT-24, revealed that labeled MSC were found in marrow, spleen and liver in 75% and 50% of transplanted dogs given DLA-identical or unrelated MSC, respectively. Taken together, these studies indicate that MSC can be safely administered to dogs during HCT but fail to alter the course of graft rejection or GVHD. In retrospect, a possible explanation is that the immunosuppressive properties of MSC when administered intravenously fail to sufficiently localize to affect the broadly systemic conditions of graft rejection or GVHD. However, it has been shown in an irradiated mouse model that immortalized or primary MSCs can exert regenerative properties to the marrow without direct cell-cell interaction.[97]

1.4. Prevention and treatment of graft-versus-host disease—The DLA-mismatched dog model has a long history of developing regimens for the prevention and treatment of GVHD, serving as the basis for the successful translation of these methods to the clinic. Observations in 1968 revealed that fatal GVHD in dogs could result when HCT was performed across minor histocompatibility barriers.[16] The immunosuppressive drug methotrexate (MTX) was first used above for prevention of GVHD.[98] Later, canine studies found synergism between MTX and calcineurin inhibitors.[99,100] These canine results were successfully translated for GVHD prevention in human patients.[101–104] Until now, the combination of a calcineurin inhibitor with MTX has been the most wildly used immunosuppressive regimen used clinically.

Post-grafting immunosuppression, as developed to a large extent in dogs, has been effective in specifically treating acute GVHD in the clinic, a disease affecting primarily skin, gut, and liver of the patient and generally presenting within 100 days after transplantation. Description of putative acute GVHD was reported in 95 dogs following HCT in 1979.[105] Acute GVHD was described again in a beagle following nonmyeloablative HCT and allogeneic HCT from a DLA-identical littermate. In this dog, GVHD, characterized by conjunctivitis, skin erythema and lesions, developed at day 52 and was treated with methylprednisolone and cyclosporine A. After two relapses, the dog was euthanized.[106] Anti-thymocyte globulin (ATG) was first reported to be effective in treating acute GVHD in the canine model.[107] These canine results were subsequently confirmed in a prospective trial in human patients.[108] In the 1990s, experiments using canines were initiated with MMF, a then novel immunosuppressive agent that had the potential to limit lymphocyte proliferation after allogeneic HCT and thereby control GVHD. On its own, MMF appeared disappointing and worse than methotrexate, CSP or tacrolimus in preventing GVHD in dogs.[109,110] However, when combined with CSP, MMF was synergistic and proved effective towards enhancing hematopoietic engraftment after a nonmyeloablative conditioning regimen and preventing acute GVHD.[53,109] This canine work resulted in the development of nonmyeloablative conditioning regimens for HLA-matched related and unrelated HCT in older or medically infirm patients with malignancies.[108,111,112] Rapamycin (sirolimus) was also shown to be effective when substituted for MMF in combination with CSP.[113]

Strategies for preventing acute GVHD in the canine model over the last decade have included a combination of extracorporeal photopheresis (ECP) and pentostatin for dogs given the unrelated DLA-mismatched HCT following conditioning with 920 cGy TBI.[114] Of the nine dogs given ECP alone or ECP with pentostatin after HCT, seven dogs achieved engraftment, and 6 of those developed acute GVHD. Compared to control dogs, ECP with or without pentostatin failed to impact the outcome of acute GVHD.

In humans, mice and dogs, MSC have been shown to have an immunosuppressive effect in vitro in a MHC-unrestricted manner.[115–117] The effect of MSC on GVHD in humans is both positive and null.[118–120] Mielcarek and colleagues set out to determine in the canine HCT model whether the immunosuppressive effects of MSC on GVHD can be reproduced.[116] Dogs were given allogeneic marrow following 920 cGy TBI without postgrafting immunosuppression for the prevention of GVHD. MSC cell lines or primary third party MSCs were injected intravenously for 3 days on the first week and for 2 days for the second week after HCT. Prior to administration, MSC cell lines and primary MSCs demonstrated in vitro suppression of T-cell proliferation in a dose-dependent manner. However, dogs given MSC either rejected their grafts or developed GVHD in a course not different from controls not given MSC.

Chronic GVHD was first reported in the 1970s and occurs in approximately 50% of patients undergoing HCT.[121,122] Chronic GVHD generally develops de novo or out of acute GVHD and usually beyond 100 days post-transplantation. Chronic GVHD has characteristics similar to an autoimmune disease and generally affects the skin, liver, eyes (keratoconjunctivitis sicca), gingiva, salivary glands, lung (bronchiolitis obliterans) and esophagus.[106,123,124] Chronic GVHD studies in canines were first described in 1982,[125] but

thereafter received little attention until recently since researchers expected solutions towards treating the syndrome would be first identified in the clinic.

While prednisone was introduced in 1981 as having benefit in treating chronic GVHD in human patients,[126] no further significant improvement in therapy of chronic GVHD has been reported since then despite numerous prospective trials. Given the disappointing lack of progress in clinical trials, we re-established a model of chronic GVHD in canines.[124] To this end, we used DLA-mismatched unrelated marrow grafts infused into recipients following 920 cGy TBI which were given before postgrafting immunosuppression consisting of MTX (days 1, 3, 6, 11) and CSP for 80 days.[124] Chronic GVHD, similar in scope and tempo to that seen in the clinic, was observed in 8 of 10 dogs over a period of 43 to 164 days. Surface expression of CD28 and inducible costimulator (ICOS) molecules were elevated on PBMC identified in dogs with clinical signs of chronic GVHD.[51] The ability to track ICOS upregulation was based on previous research in which we first reported on the cloning and characterization of ICOS expressed on canine lymphocytes in 2004.[42] Accordingly, we carried out a study in which we administered pharmacological doses of anti-canine ICOS mAb at the time of clinical diagnosis of chronic GVHD and found significant prolongation of survival in the treated dogs compared to control dogs not given anti-ICOS mAb.[127] The results of this study were promising and supported the hypothesis that targeting ICOS, a costimulatory molecule upregulated on activated lymphocytes, is an effective approach to down-regulating effector cells specifically involved in GVHD.

1.5. Hematopoietic cell transplantation for canine malignant diseases—

Canines are highly susceptible to many of the malignant diseases observed in mankind and as such provide an insightful model towards the investigation of therapies to treat cancer. However, treatment of canine hematological malignancies is generally not successful following radiation and chemotherapy alone.[128] Lack of success can be attributed to onset of multi-drug resistance and disseminated micro metastasis.[129] The preclinical development of HCT in canines for treatment of malignant disorders in humans can be adapted for the treatment of hematological diseases in companion animals (for review see Lupu and Storb[7]).

Early studies by the Thomas Lab showed that approximately 30% of dogs diagnosed with spontaneous malignant lymphoma while in chemotherapy-induced remission, progressed to long-term remission following treatment with HCT with autologous PBMC.[130,131] Recently, several studies have affirmed the approach of using autologous HCT following high-dose cyclophosphamide with high-dose TBI for the treatment of spontaneous T- and B-cell lymphomas. A 5-drug chemotherapy trial ending with high dose cyclophosphamide and autologous marrow transplantation for dogs with lymphoma was conducted to determine the maximum tolerated dose of cyclophosphamide. Dogs with the lowest stage disease and receiving the highest tolerated dose (500mg/m^2) of cyclophosphamide had the best median survival time of 139 weeks.[132] In the case of B-cell lymphoma, autologous engraftment was achieved in 87.5% of client-owned dogs treated with cyclophosphamide and recombinant human G-CSF-mobilized PBMC collected by aphaeresis before 1000 cGy TBI. The median disease-free survival for all dogs was 271 days, and 33% transplanted before they relapsed remained disease free for a median overall survival of 524 days.[133] The same treatment regimen was carried out on 15 client-owned dogs diagnosed with T-cell lymphoma.

Thirteen of 15 dogs engrafted after high-dose cyclophosphamide, G-CSF mobilized PBMC therapy and varying doses of TBI. Two of 13 dogs survived for 741 and 772 days after transplantation.[134]

In the canine allogeneic transplantation setting, identification of prospective donors for the treatment of the spontaneous generation of hematological malignancies has relied on DLA typing for MHC matching, a process not commonly available to most veterinary practices. In 2006, our lab reported on the successful transplantation of a golden retriever diagnosed with T-cell lymphoma. Following administration of 2 doses of 4 Gy TBI and canine G-CSF mobilized leukapheresis product from a DLA-matched second cousin donor, full donor chimerism was achieved and the recipient remained in complete remission.[135] Suter and colleagues reported that a dog with acute large granular lymphocytic leukemia responded well to chemotherapy and administration of DLA-matched CD34+ cells obtained from a sibling donor. Stable full donor chimerism was established for a period greater than 2 years.[136]

The role of dogs for treatment of hematological malignancies has been made more attractive following the development of a canine leukemia model. Retroviral vectors expressing growth-promoting genes such as HOXB4 can be overexpressed in transduced CD34+ stem cells. Dogs transplanted with autologous HOXB4-transduced CD34+ stem cells developed myeloid leukemia.[137] Infusion of trace numbers of HOXB4 overexpressing CD34+ cells from a third- party dog during the post-grafting immunosuppression stage of nonmyeloablative HCT resulted in myeloid leukemia in two mixed chimeric dogs given DLA-haploidentical transplants.[138] This finding suggested that the canine qualifies as a clinically relevant large animal model for studying the biology of leukemia and the evaluation of novel approaches towards its treatment when dogs are infused with HOXB4 overexpressing CD34+ stem cells.

1.6. Hematopoietic cell transplantation for canine nonmalignant diseases

Allogeneic DLA-identical HCT has been examined as a therapeutic application for a wide variety of canine nonmalignant diseases including enzymatic, anemias, and storage disorders.[139–141] In addition to these previously described disorders,[7] Duchenne muscular dystrophy (DMD), the most common form of muscular dystrophy, is a genetic disease that can be manifested in dogs. In both humans and dogs, DMD is a X-linked recessive disorder resulting from mutations in the dystrophin gene. Progressive muscle weakness begins at ages 3–5 years, with eventual death in the third decade of life due to cardiac and respiratory failure. Transplantation of wild-type muscle stem cells has been proposed as a therapy for this disease.

In view of this hypothesis, we failed to confirm previously published murine data that hematopoietic stem cells led to repair of diseased muscle in DMD dogs.[142] Moreover, direct injection of dog leukocyte identical hematopoietic cells into muscle of dystrophic dogs failed to establish normal satellite cells and dystrophin expression.[143] We hypothesized that immune tolerance would permit successful engraftment of allogeneic muscle progenitor cells and evaluated whether immune tolerance would enable engraftment of donor muscle satellite cells injected intramuscularly following establishment of donor hematopoietic cell

chimerism.[144] Here, DLA-identical littermate donors were matched for DMD affected recipients. Following administration of 200 or 920 cGy TBI to recipients, donor marrow and G-CSF mobilized PBMC were injected and stable chimerism was observed. Marrow donors provided muscle biopsies from which muscle stem cells were cultured over a 14-day period. Injection of cultured cells restored low but significant levels of dystrophin expression at the sites of injection. No immune cell infiltrate was observed at the injection sites. Greater dystrophin expression will require improving donor wild-type dystrophin expression in a ubiquitous manner.

Leukocyte adhesion deficiency (LAD) is a genetic disorder due to a mutation within the CD18 gene.[145] The disease is characterized by severe leukocytosis and neutrophil adhesion defects. The canine analog of LAD, termed CLAD, is characterized by the same phenotype and results from a defect in CD18.[146] The primary therapy for both LAD and CLAD is prophylactic antibiotic therapy. However, nonmyeloablative marrow transplantation has been used to treat and reverse the disease of CLAD affected pups.[147] In this report, two dogs were transplanted with marrow from DLA-identical littermates. Long-term mixed chimerism ensued with CD18 expression detected on leukocytes using flow cytometry. Similar results have been reported for CLAD therapy using nonmyeloablative HCT and busulfan as a conditioning agent[148] and CD34+ and hematopoietic stem cells transduced with a foamy virus vector expressing canine CD18.[149–151]

Gene therapy combined with HCT has proven to be a therapeutic and effective approach to treating additional heritable diseases in canines as a model for the human conditions. Early studies using gene transfer to hematopoietic stem cells (HSC) had to overcome several hurdles such as efficient mobilization of HSC, appropriate cell culture conditions, transduction efficiency, and optimum viral vector constructs.[152] Efficient gene therapy has been obtained with gamma, lentivirus or foamy virus viral vectors.[152] Kennedy and colleagues[153] compared standard cytokine cocktail for retroviral transduction culture over periods of 4.5 days or 18 hours for CD34+ cell reconstitution of x-SCID dogs. While culturing cells for 4.5 days resulted in decreased engraftment, the 18-hour culture period resulted in T-cell reconstitution similar to that seen with fresh CD34+ cells but with impaired thymopoiesis. The results suggest culture conditions for CD34+ cell viral transduction are critical for optimum reconstitution. In the canine pyruvate kinase deficiency (PKD) model, a dog received transplantation of autologous CD34+-selected HSC transduced ex vivo with a foamy viral vector containing EGFP, pyruvate kinase, and MGMTP140K following 920cGy TBI. EGFP expression was 3.5% to 33% of myeloid cells, giving a functional cure.[154] Recent genotoxicity studies indicate transduction of HSC with foamy viral vectors is safe and leads to extended periods of polyclonal expansion of hematopoietic progenitor cells. As has been done in dogs receiving retroviral gene transfer,[155] long-term follow-up preclinical studies are needed for proper safety evaluation of foamy viral vectors for treating a variety of hematological diseases.[151]

1.7. Tolerance solid organ transplantation and vascularized composite allografts

The establishment of immune tolerance in the patient towards the donor antigens of the transplanted tissues has long been a primary goal in the field of transplantation.

Hematopoietic cell chimerism following HCT has shown to be an effective method for inducing immune tolerance and preventing rejection of solid organ transplantation (SOT). Several solid organs, including skin, gut, heart and lung, have been successfully transplanted into canines following induced tolerance using HCT. [156–162] In nephrectomized marrow transplanted recipients, allografted kidneys from respective marrow donors were accepted in 5 of 5 dogs for periods greater than one year without additional immunosuppression.[163] In control dogs receiving an identical protocol without HSC infusion, all showed signs of acute rejection after transplant at a mean of 24 days. A 5-year follow-up study of the chimeric hematopoietic cells and kidney recipients revealed normal kidney function with no histological evidence of graft rejection. However, donor-specific tolerance did not extend to skin as 2 of 4 dogs given skin grafts from respective marrow-kidney donors were rejected.[164] In a similar study, conditioning dogs with 200 cGy TBI followed by HCT and kidney allograft transplantation with transient post grafting immunosuppression enabled stable kidney engraftment in both the DLA-identical and DLA-haploidentical settings.[165]

Establishing donor mixed hematopoietic chimerism has the potential of leading to GVHD. To mitigate this outcome, we posed the question whether donor hematopoietic cell chimerism can be eliminated after kidney engraftment while preserving tolerance towards the kidney allograft.[166] To this end, we administered a second dose of 200 cGy TBI to the recipient dogs followed by an infusion of G-CSF-mobilized recipient PBMC that had been collected and frozen before HCT. The recipient dogs rejected donor hematopoietic cell chimerism yet maintained their kidney allografts without signs of rejection for the duration of the period, which was greater than one year.

Vascularized composite allografts (VCA) is a transplantation procedure to reconstruct a patient’s face or hands for restoration of both form and function. The allograft is composed of muscle, fat, connective tissue, nerves, vasculature, and skin. Traditionally, intensive immunosuppression has been used to prevent graft rejection, but immune tolerance through HCT offers a superior alternative for preventing rejection. To validate this approach, methods were recently developed to successfully transplant VCA in dogs.[156] VCA were transplanted either coincidently with donor marrow with grafts accepted for periods > 62 weeks or transplanted several months after DLA-identical HCT and grafts accepted for periods ranging between 52 and 90 weeks.[167,168] In both cases, marrow donors not receiving HCT showed clinical signs of VCA rejection within 30 days after transplant. In the DLA-haploidentical HCT setting, mixed chimerism was established in dogs given 450 cGy and HCT using either marrow or G-CSF-mobilized peripheral blood stem cells and transplanted VCA from the respective stem cell donors. Postgrafting immunosuppression consisted of MMF and CSP.[169] One dog receiving marrow accepted the marrow graft and VCA long-term while 3 dogs rejected both marrow transplant and VCA within 5–7 weeks after transplantation. All 4 dogs given G-mobilized blood HSC accepted their stem cell and VCA grafts, but 3 of the 4 developed GVHD. Importantly, 1 dog rejected the stem cell graft by week 15, while the VCA, including the skin component, was accepted long-term (>90 weeks). These results offer proof of principle that HCT can induce immune tolerance towards all components of a VCA allograft, including highly immunogenic skin. Yet, uniform positive results will require further development of conditioning or postgrafting immunosuppression regimens.

1.8. Canine studies for future developments towards improving hematopoietic cell transplantation

Reducing TBI doses below 200 cGy, and prevention or treatment of acute and chronic GVHD, respectively, remain important goals in the efforts to reduce toxicity and improve the use of HCT for treatment of hematological disorders. Achieving these goals has the added benefit on rendering HCT safe as a tolerogenic procedure for tissue transplantation, thus avoiding of life-long immunosuppression. Recent studies in dogs have eliminated protocols with *a priori* potential success while pointing to new directions of possible opportunity. Effective use of costimulatory molecule blockade and the induction of immune tolerance in the recipient, together with depletion of recipient NK cell function, may play well in future studies. Radioimmunotherapy targeting CD45+ cells with a mAb coupled to a short-lived high energy α-emitting radionuclide, as exemplified by ^{211}At, bears further investigation. Work needs to continue that leads to the development of new mAbs and fusion proteins that are specific to and agonistic towards costimulatory molecule activation pathways. A single chain anti-CD28 mAb with sufficient affinity is a possible valuable reagent towards this goal. These biologicals need to have the appropriate human analog and, if possible, be of canine origin in the case of fusion proteins to allow for multiple dosing and to avoid immune recognition by the host. Gene therapy for the treatment of a variety of inborn errors of metabolism and defects has been successfully examined in canines. This is a burgeoning area of genetic manipulation of CD34+ HCT in canines and one that is appropriate for proof of principal and safety studies in the future.

Acknowledgements

The authors wish to thank Alix McPhearson, Michele Spector, DVM and the Fred Hutchinson Cancer Research Center's animal technicians for their assistance in these studies. Stacy Zelmer and Debe Higginbotham for DLA-typing and chimerism analysis; and Helen Crawford for manuscript preparation.

Funding:

This work was supported by grants P30 CA015704 and P01 CA078902 from the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health, which had no involvement in the study design; the collection, analysis and interpretation of data; the writing of the report; nor in the decision to submit the article for publication.

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