Published in final edited form as:

Exp Hematol. 2009 October ; 37(10): 1157–1166. doi:10.1016/j.exphem.2009.07.004.

Transmission and expansion of HOXB4-induced leukemia in two immunosuppressed dogs: Implications for a new canine leukemia model

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Abstract

Objective—There are currently no large animal models to study the biology of leukemia and the development of novel anti-leukemic therapies. We have previously shown that dogs transplanted with homeobox B4 (HOXB4)-transduced autologous CD34⁺ cells developed myeloid leukemia associated with HOXB4 overexpression. Here, we describe the transmission, engraftment, and expansion of these canine leukemia cells into two genetically unrelated, immunosuppressed dogs.

Methods—Two dogs immunosuppressed after MHC-haploidentical hematopoietic cell transplantation (HCT) and exhibiting mixed donor-host chimerism were infused trace amounts of HOXB4-overexpressing leukemia cells from a third-party dog.

Results—Six weeks after infusion of HOXB4-overexpressing leukemia cells, these two dogs rapidly developed myeloid leukemia consisting of marrow and organ infiltration, circulating blasts, and in one dog, chloromatous masses. Despite neither of these dogs sharing any dog leukocyte antigen (DLA)-haplotypes with the sentinel case, the HOXB4-transduced clones engrafted and proliferated without difficulty in the presence of immunosuppression. Chimerism studies in both dogs confirmed that donor and, in one case even, host hematopoietic cell engraftment was lost and replaced by third party HOXB4 cells.

Conclusions—The engraftment and expansion of these leukemia cells in dogs will allow studies into the biology of leukemia and the development and evaluation of novel anti-leukemia therapies in a clinically relevant large animal model.

Keywords

HOXB4; leukemia; canine model

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Conflicts of Interest Disclosure

INTRODUCTION

The use of basic science approaches to the study of cancer biology and treatment have yielded significant advances in the field. Yet, it is the application of this knowledge to animal models that has furthered our understanding of cancer growth and development and has revolutionized our ability to test new drugs and cellular therapies before entry into clinical trials. The murine model has frequently been used as the animal system of choice due to its low cost, easy handling, and ability to maintain xenografts of human cancers. However, there are limitations to using mice. Genetically-engineered mice, which frequently take advantage of germ-line mutations to promote cancer growth, may not effectively represent most human leukemias (with the exception of some pediatric leukemias), which mainly develop from acquired rather than inherited mutations [1]. Likewise, the use of human xenografts has unique constraints due to their need to be implanted into NOD/SCID mice which lack innate immune systems, limiting their ability to mount anti-tumor responses and making a realistic model of cancer development challenging. Contrary to classical small animal models, a large animal system such as the out-bred dog offers several advantages including similar immune systems [2], propensity toward the same genetic diseases [3], and homology of genes [4] compared to humans. There has also been great precedence for translating canine studies to the clinical setting, as has been seen in hematopoietic cell transplantation (HCT) [5], Duchenne muscular dystrophy [6], and hemophilia [7]. However, there is currently no canine leukemia model in place to take advantage of the unique benefits this large animal species can provide to clinical research. Recently, we have shown that retroviral vectors expressing growth-promoting genes such as HOXB4 can induce leukemia in the large animal model, in contrast to similar retroviral backbones without HOXB4 which have shown no adverse events. In particular, Zhang et al. have reported on two dogs transplanted with autologous HOXB4-transduced CD34+ cells that developed myeloid leukemic clones early after transplant. However, progression to overt leukemia did not occur until two years later [8], suggesting that HOXB4 must work in collaboration with multiple cooperative mutagenic events to promote leukemogenesis. Such a delay could limit the time-feasible propagation of this model. Here, we report on the rapid expansion of canine leukemia in vivo when two dogs that underwent DLA-haploidentical HCT and were receiving post-grafting immunosuppression were accidentally infused with HOXB4 leukemia cells and quickly developed fulminant myeloid leukemia.

MATERIALS AND METHODS

Laboratory animals, hematopoietic cell transplantation studies, and inoculation of HOXB4 clone

G490 and G542 were two dogs enrolled on an experimental study evaluating the safety and efficacy of using a novel conditioning and immunosuppressive regimen for DLA-haploidentical HCT. Non-myeloablative conditioning was performed as previously described [9] followed by infusion of recombinant canine-granulocyte colony stimulating factor-derived peripheral blood stem cells (PBSC) on day 0. Post-transplant immunosuppression consisted of single-dose methotrexate of 100 mg/m², prolonged CSP dosed at 30 mg/kg/day, and MMF dosed at 20 mg/kg/day through days +210 and +180 after transplant (including taper), respectively. G374 was enrolled on another study evaluating the efficacy of transplanting autologous CD34+ cells transduced with a gammaretroviral vector containing HOXB4 tagged with green fluorescent protein (GFP) into a lethally irradiated dog as previously published [8]. All dogs underwent DLA analysis using family studies to determine highly polymorphic MHC class I and class II by microsatellite markers [10,11], with DLA-DRB1 alleles determined by direct sequencing [12]. Both studies were approved by the Institutional Animal Care and Use Committee at FHCRC, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Supportive care in the form of

antibiotics, blood transfusions, and supplemental fluids were administered as needed. During a several-week period, all three dogs were receiving aggressive IV fluid support either due to post-transplant complications (G490 and G542) or progressive leukemia (G374). It was suspected that during this time, inadvertent contamination of a common IV fluid line with minute traces of peripheral blood accidentally transferred the HOXB4-containing leukemia cells from G374 to G490 and G542 (Fig. 1).

Post-transplant Evaluations

Cell subsets were verified with flow cytometry using canine monoclonal antibodies either directly conjugated to PE [CD34 (1H6, IgG₁; BD Biosciences Pharmingen, San Diego, CA)], or biotinylated antibodies with streptavidin-PE [CD3 (CA17.6F9, IgG_{2b}) [13], TCR $\alpha\beta$ (CA15.9D5, IgG₁) [14], CD21 (CA2.1D6, IgG₁) [15], canine myeloid cells (DM5, IgG₁) [16], and CD14 (TUK4, IgG_{2a}; DAKO, Carpinteria, CA)]. CA17.6F9 was kindly provided by Dr. Peter Moore (School of Veterinary Medicine, University of California, Davis). Chimerism studies quantified the contributions of recipient, donor, and 3rd party cells in peripheral blood and other tissues using variable number tandem repeat (VNTR) analysis, and data was analyzed with ABI Prism 310 Genetic Analyzer and Gene Scan 3.1 Software (Applied Biosystems, Foster City, CA). Mixed leukocyte reactions (MLR) were performed as previously described [17] to determine the degree of host lymphocyte proliferation in response to self (negative control), donor, 3rd party unrelated donor, and concanavalin A (ConA) (latter two being positive controls). A natural killer (NK) cell functional assay was performed to determine the degree of cytotoxic killing post-transplant using canine thyroid adenocarcinoma (CTAC) as the target as previously described [18]. Percent specific lysis was expressed as: [(experimental CPM – spontaneous CPM) / maximum release CPM – spontaneous release CPM] $\times$ 100, where CPM represents the mean of triplicate counts per minute. MLR and NK functional assays were used in G490 and G542 to evaluate immune reconstitution after HCT. Additionally, complete blood counts were followed regularly to ascertain white blood cell counts, in addition to subsets such as monocytes, in all three dogs. Evaluation of HOXB4 cell populations by clone tracking PCR and Southern blot was performed as previously described [8]. Bone marrow aspirate, biopsy, and tissue from organs were prepared for morphological examination. Glass slides were stained with Wright-Giemsa stain.

RESULTS

Transmission, engraftment, and expansion of HOXB4 leukemia in two immunosuppressed dogs

Approximately 6 weeks after presumed inoculation of trace amounts of blood containing HOXB4 leukemia cells (40–60% monocytosis), both G490 and G542 began showing emergence of proliferating leukemia in peripheral blood (Fig. 1). Specifically, the HOXB4 clone was first detectable by VNTR analysis in peripheral blood mononuclear cells (MNC) of G490 and G542 at 70 and 91 days after non-myeloablative, DLA-haploidentical HCT, respectively (Fig. 2). In G490's case, the HOXB4 clone rapidly grew and quickly overtook the stable mixed donor-host chimerism that developed after transplant, rapidly converting all of the peripheral blood MNC chimerism to that of G374's within a 10-day period. In the case of G542, the emergence of G374's MNC chimerism was of a slower kinetics, and over the course of 7 weeks, the HOXB4 clone expanded and completely overtook donor chimerism. In both dogs, flow cytometry studies of the blood demonstrated the presence of differentiated GFP⁺ cells of a myeloid origin, representing the GFP tag on the HOXB4 vector, and subsequent expansion of these cells in the dogs. Additionally, flow cytometry performed on marrow taken from G542 on the day of euthanasia showed that all CD34⁺ progenitors were marked with GFP (Fig. 3).

Clinical and morphological presentation of HOXB4-derived myeloid leukemia similar to human acute myeloid leukemia

The sentinel case was G374, a dog that had received CD34+ cells transduced with a gammaretroviral vector containing HOXB4 and eventually developed acute myelomonocytic leukemia as previously reported [8]. Based on retrospective Southern blot analysis, the leukemic clone, with at least two provirus integration sites, was found to be present one month after transplant, although transformation to overt leukemia was noted 17 months later with the emergence of monocytosis. Significant monocytosis in the range of 20–70,000/mm³ did not occur until 18 months after transplant of the HOXB4-transduced cells (Fig. 4). In contrast, both G490 and G542 showed rapid expansion of leukemia. G490 developed a quickly growing rectal chloroma 7 weeks after inoculation with G374's leukemia, after which a mild monocytosis (between 800–5000/mm³) appeared in the peripheral blood prior to euthanasia. On necropsy, tissue from the rectal chloroma was analyzed using VNTR with results confirming triple chimerism, with the major contribution coming from G374 (47%) (Fig. 5). The marrow was packed with immature cells with morphology consistent with both myeloblasts and myelomonoblasts. Additionally, leukemic infiltrates were seen in other organ tissues, including kidney, liver, and spleen on histopathology. No infiltrates were seen in sampled lymph nodes, which retained a mixed chimerism (93% recipient, 7% donor) with no contribution from G374 by VNTR analysis. In the case of G542, progressive peripheral monocytosis (between 2000–6000/mm³) emerged 2 months after the initial exposure and lasted until the dog was euthanized approximately 1 month later. No chloromas were discovered on necropsy. Similar to G490, the marrow was also packed with immature cells consisting morphologically of both myeloblasts and myelomonoblasts (Fig. 6). Leukemic organ infiltration was also discovered in the spleen, liver, lung, as well as lymph nodes in the cervical and mesenteric regions on histopathology. On VNTR analysis, the lymph nodes demonstrated a triple chimerism consisting of host 89%, donor 4%, and G374 7%.

Identical integration site of HOXB4 verified using clone tracking PCR

To verify that the myeloid leukemia discovered in both G490 and G542 emerged from G374, the same HOXB4 clone was identified at 209 bp in all three samples by clone-tracking PCR. Furthermore, for G542, Southern blot analysis was performed confirming that G542 had identical integration sites of HOXB4 compared to G374. For this assay, DNA isolated from peripheral blood was digested with BgIII, which cuts inside the vector once and once in the genome relative to the site of provirus integration and released a unique band for both integrants (Fig. 7).

Engraftment and expansion of HOXB4 leukemia occurred in the presence of persistent T and NK cell function

Both G490 and G542 were heavily immunosuppressed after undergoing nonmyeloablative, DLA-haploidentical HCT and were on full dose CSP and MMF when inoculated with the HOXB4 leukemia cells. As part of routine immune surveillance testing performed on these dogs for their primary HCT study, T and NK cell function were evaluated at selected time points after transplant. This testing showed that during the period of presumed inoculation, cells from both dogs still maintained immune function as they could mount appropriate in vitro responses despite in vivo exposure to immunosuppression. Fig. 8 shows representative MLR and NK cell assays for G542, which were performed at day +98 after DLA-haploidentical HCT (approximately 9 weeks after HOXB4 transmission) during a period of time when the HOXB4 clone was steadily expanding. At this time point, there still existed the capability of G542's T cells to proliferate on an in vitro MLR assay in response to both 3rd party cells and ConA, as exhibited by uptake of ³H in the proliferating cells due to allogeneic and mitogenic stimuli, respectively. Additionally, the presence of NK cytolytic activity at this time point demonstrated

an intact ability to engage in MHC non-specific killing, as evidenced by a specific lysis of 62% using an effector to target ratio of 30:1 (compared to normal dogs G457 and G566 having a specific lysis of 34% and 26%, respectively).

DLA typing shows disparate haplotypes in each of the three dogs

Family studies evaluating ancestry of each dog found that G374 and G542 had no relationship to each other. There was a distant relationship between G374 and G490 (G374's great-grandmother was the sister of G490's grandfather). Despite this distant family relationship, MHC class I and II antigens were unique based on VNTR analyses. Furthermore, using direct sequencing methods, the DLA DRB1 alleles on class II were sequenced for each dog revealing unique haplotypes. Specifically, the DRB1 haplotypes for G374 consisted of *00601/*01501, while G490 had *00201/*00501 and G542 had *00901/*02201.

DISCUSSION

Here we demonstrate the transmission and expansion of a HOXB4-mediated leukemia to immunosuppressed dogs. These circulating leukemia cells were able home to the marrow, undergo clonal expansion and metastatic spread typical of an acute leukemia, and engraft across MHC barriers in the presence of immunosuppression. These observations suggest that these clonal cells overexpressing HOXB4 can be used to study the biology of leukemia and develop novel treatment approaches in the canine model.

The transmission is thought to have occurred accidentally by way of a contaminated fluid line containing minute traces of peripheral blood from G374. Other modes of transmission were considered and were ruled out. Specifically, during the time that G374 was alive, neither G490 nor G542 received any blood transfusions, had any open wounds, underwent any procedures (other than IV fluid replacement) requiring sterile equipment, and had no social interactions with G374 as both G490 and G542 were males and thus separated from G374, who was female. The most likely explanation is contamination of an IV fluid line during a period of time when both the sentinel case and two infected dogs were receiving frequent fluid support due to complications of proliferating leukemia and HCT, respectively. For multiple weeks predating the transfer, G374 had mild monocytosis. However, during the presumed time that the cells were transferred, G374 exhibited profuse monocytosis in the 40–60% range which defined this as a proliferating leukemia. Despite the initial HOXB4 clone taking almost 19 months to emerge as an acute leukemia, the transmitted leukemia progressed very rapidly in the inoculated dogs. Specifically, after an incubation period of approximately 6 weeks, during which time the trace amounts of leukemia homed to the marrow and underwent clonal expansion, it only took 10 days for G490 and 7 weeks for G542 to present with overt leukemic burden requiring euthanasia. In G490, both host and donor engraftment were lost and replaced by HOXB4 leukemia engraftment. In contrast, only donor engraftment was lost in G542 while host engraftment persisted during leukemia growth. This may be a reflection of modifications in immunosuppression, as MMF incidentally had been increased at day +57 due to improved gastrointestinal tolerance. The initial identification of G374's leukemia clone was by GFP expression, which by flow cytometry appeared as a unique GFP+ population in G490's and G542's unstained peripheral myeloid and marrow stem cells. This was later confirmed by molecular analysis identifying the unique provirus integration site in both dogs. Clone tracking PCR (in addition to Southern blot for G542) using primers specific to this same integration site was seen in all three dogs confirming the existence of an identical clone.

All three dogs presented with manifestations of myeloid leukemia. The index case, G374, developed an insidious myelomonocytic leukemia that eventually manifested as extensive peripheral monocytosis. Similarly, both G490 and G542 presented with myelomonocytic leukemia, peripheral blood monocytosis, and additionally in the case of G490, a massive rectal

chloroma. These manifestations reflect some of the same heterogeneous clinical presentations of human acute leukemias. However, these varied presentations could also be a reflection of other factors, including unequal numbers of infused HOXB4 over-expressing leukemia cells and different biological environments into which the leukemia was infused. For example, G490 was relatively early in the transplant course, with the transmission likely occurring within the first 3–4 weeks after transplant during a period of time when donor tolerance was developing. On the other hand, G542 was further along after transplant (6–7 weeks) which may have contributed to a different presentation.

Similar to HOXB4 over-expression in canine cells, human acute leukemias can develop when a molecular mutation gives rise to a clonal population having a growth advantage over normal cells. Although pediatric patients can develop germ line mutations leading to leukemia development, molecular mutations are frequently acquired during the lifetime of a person. Once this clonal population develops a growth advantage, these cells can infiltrate the marrow space, outcompete normal hematopoietic cell development, and eventually spread hematogenously to organs or collect as chloromatous masses. In the case of this canine leukemia, the HOXB4 gene similarly provided an acquired growth advantage to allow this clonal population to undergo unchecked proliferation in these dogs. To this end, there is a growing wealth of literature indicating that homeobox genes are aberrantly expressed in human leukemias. Scholl et al. report that homeobox transcription factors CDX2 and CDX4 are expressed in 90% and 25% of leukemia cells obtained from acute myeloid leukemia patients, respectively [19,20]. Additionally, Ferrando et al. describe HOX gene dysregulation as an important feature of MLL-rearranged T- and B-cell acute lymphoid leukemias [21].

Although both G490 and G542 were immunocompromised after DLA-haploidentical HCT and on concurrent immunosuppression, T and NK cell functional assays established that these cells could maintain immune function when removed from their environment. In particular, the MLR showed the ability of cells taken from these two dogs to proliferate in response to allogeneic and mitogenic stimulation similar to controls. Additionally, the NK cell assays showed a higher degree of lysis when compared to two normal controls. This increased cytolytic activity is typically observed in our HCT model and is reflective of robust NK cell recovery after MHC-haploidentical HCT. In this case, this cytolytic activity does not appear to be hampered by contaminating leukemia cells. Both in vitro assays are taken with the caveat that, although MNC have been systemically exposed to immunosuppressive drugs, these cells were isolated from Ficoll blood and have been washed of any residual CSP or MMF before use in the assays. Thus, when removing these immunosuppressive agents, these cells have the ability to function normally unlike cells taken from xenograft models such as NOD/SCID mice. This suggests that despite relative immune competence of T and NK cells, the HOXB4 clone could proliferate and out-compete innate and adaptive immune systems to overtake donor, and in the case of G490, even host engraftment, when immune suppression is present.

Based on VNTR and direct sequencing of Class I and II alleles, respectively, the leukemia cells overexpressing HOXB4 from G374 shared no genetic similarities with either G490 or G542. Despite the immense immunological barriers present in both cases, cells overexpressing HOXB4 could readily engraft and expand across MHC barriers in the presence of immune suppression. These observations imply the ability to generate and propagate a large animal model using genetically-unique HOXB4 clones.

We have now established a cell line from this clone which is actively being investigated. The availability of this canine leukemia cell line would allow us to study biological questions such as the role of genetic and molecular mechanisms in the development of leukemia stem cells. Such cell lines could also supply the nidus for the development of a large animal leukemia model by hastening the generation of canine leukemia hosts in an economically-feasible

manner. When developing this model, it would be important to investigate whether these clonal leukemia cells could engraft and proliferate without the presence of immune suppression, or whether immune suppression could be weaned once engraftment has been established. This would not only justify the proliferative engraftment advantage HOXB4 cells have over normal host cells, but would also provide a more realistic translational leukemia model (i.e., one with an intact immune system that would be able to modulate normal graft-versus-leukemia responses). We have previously shown that spontaneously occurring canine solid tumors can be transplanted subcutaneously into normal dogs given CSP, but when immunosuppression is removed, these tumors regress and become necrotic [22]. However, another study has shown that canine transmissible venereal tumor, a naturally occurring histiocytic tumor, can proliferate in a clonal manner in the absence of immunosuppression [23]. Once this large animal model has been developed, we would then have the capability of studying novel drugs or adoptive immunotherapy strategies to eradicate leukemia. Some platforms of investigation could include adoptive immunotherapy with NK cells [24] or antigen specific immunotherapy with CD8+ T cells [25]. With initial work directed at identifying potential antigens of target in HOXB4 cell lines, HOXB4 dog models could then be used to develop translational immunotherapies for the clinic.

CONCLUSION

The unique ability of animal models to serve as the interface between basic science research and clinical trials has allowed great advances in the field of oncology. We have presented our analysis of a viable canine myeloid leukemia model that presents similarly to human myeloid leukemias, is clonal in nature, develops in short dormancy, and engrafts over MHC barriers. As HCT studies performed in the canine model have a strong past precedence of being translatable to the clinical arena [5,26,27], this model would have great potential impact in the field.

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

ACKNOWLEDGEMENTS

We thank Michele Spector, DVM, the canine facilities staff, and weekend investigators for their care of dogs on this study and Helen Crawford, Bonnie Larson, and Sue Carbonneau for help with manuscript preparation. This study was supported by grant HL36444 from National Heart, Lung, and Blood Institute.

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Figure 1.

Timeline of events showing the course of transmission and expansion of HOXB4-overexpressing leukemia clones.

Figure 2.

Peripheral blood mononuclear cell (MNC) chimerism studies demonstrate the rapid emergence and expansion of the 3rd party HOXB4 leukemia in the DLA-haploidentical HCT model. MNC were isolated and chimerism was determined by VNTR analysis weekly after DLA-haploidentical HCT showing the ability of HOXB4 cells to engraft and overcome mixed donor-host chimerism.

A) In G490, the expansion of the HOXB4 clone was rapid. Over the course of 10 days, this dog completely lost both host and donor grafts. **B)** In G542, the expansion of HOXB4 took place over 7 weeks. The dog maintained persistence of host engraftment but eventually lost its donor graft.

Figure 3.

Blood and marrow necropsy samples from G542 demonstrate GFP+ cells of a myeloid lineage by FACS. **A)** Unstained peripheral blood showed the presence of two distinct populations. Because the vector expressed GFP, HOXB4 clones could be detectable within the FL1-FITC window. When peripheral blood was stained with biotinylated MABs and incubated with streptavidin-PE, double-staining (GFP+/SAPE+) demonstrated that cells of a myeloid origin (CD14, DM5) contained the HOXB4 clone in 9% and 50% of its cells, respectively. Cells of a lymphoid origin (CD3, TCR $\alpha\beta$, and CD21) contained only minute numbers of cells that were GFP+/SAPE+.

B) Marrow was stained with CD34-PE and demonstrated that all CD34+ progenitor cells expressed the GFP vector (1.5%).

Figure 4.

The kinetics of A) white blood cell (WBC) and B) absolute monocyte counts (AMC) in relation to time to euthanasia indicate different trends of leukocytosis and monocytosis with leukemia progression in all 3 dogs (# indicates presumed time period G490 was infected with G374's cells; * indicates presumed time period G542 was infected with G374's cells) G374 exhibited a rapid burst then progressive leukocytosis and monocytosis approximately 50 days prior to euthanasia. G490 had stable counts that began to rise 2 weeks, then more rapidly several days, prior to euthanasia. G542 demonstrated a progressive rise in leukocytosis and monocytosis over the course of one month prior to euthanasia.

Figure 5.

G490 demonstrated clinical manifestations of canine myeloid leukemia similar to human acute myeloid leukemia. **A)** G490 was euthanized on Day +80 after DLA-haploidentical HCT when a rapidly emerging rectal chloroma presented over the course of several days. **B)**

Histopathological review of the chloroma demonstrated proliferating cells with morphology consistent with myelomonocytic leukemia. The chloroma was analyzed for chimerism using VNTR, demonstrating contributions from donor (37%), recipient (16%), and G374 (47%).

C) Typical of clinical acute leukemias, there was hematogenous spread and presence of leukemic infiltrates within several organs, including liver and spleen (not shown). Here, leukemic infiltrates are seen invading the renal cortex.

Figure 6. Bone marrow morphology from both (A) G490 and (B) G542 show myelomonocytic leukemia. Bone marrow biopsies taken at the time of necropsy show diffuse sheets of monomorphic blasts having abundant cytoplasm and active mitoses, with morphology consistent with myelomonocytic leukemia (high magnification).

Figure 7.

A) Identical integration site is verified in G542, G490 and G374 by clone tracking PCR. All three dogs demonstrate the HOXB4 clone at 209 bp. G450 is a known negative control. **B)** In G542, Southern blot demonstrates the same two integration sites from the HOXB4 clone as seen in G374. When cut with BglII, 2.7K (c-myb site) and 3.76K (PRDM16 site) are seen in both dogs.

Figure 8.

A) MLR demonstrates presence of T cell function after inoculation with HOXB4 clone. Prior to HCT, T cells are able to proliferate normally when exposed to allogeneic (G457, donor; G566, unrelated 3rd party) and mitogenic (ConA) stimuli similar to controls. Two weeks after HCT, G542 has a suppressed reaction to the donor cells as tolerance to G457 emerges early after HCT but has a continued strong proliferative activity against 3rd party and ConA as is normally seen. However, by Day +98, approximately 7 weeks after G542 was inoculated with the HOXB4 clone, T cells taken for G542 still show high degrees of proliferation not only to 3rd party and ConA, but also to autologous cells and donor. The T cells thus maintain an ability to proliferate, but this dysregulated response is likely a result of contaminating HOXB4 cells.

B) NK functional assays demonstrate ongoing robust activity after inoculation with HOXB4 clone. Prior to HCT, G542 had similar baseline NK functional activity as donor (G457) and unrelated 3rd party (G566). By Day +15, as is normally seen early after DLA-haploidentical HCT, NK activity is more robust than baseline controls and is a reflection of rapid recovery of donor NK cells after HCT. By Day +98 after HCT, approximately 7 weeks after inoculation

with HOXB4, there remains similar robust cytolytic activity of NK cells despite contamination with HOXB4 clones.