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Effective Multi-lineage Engraftment in a Mouse Model of Fanconi Anemia Using Non-genotoxic Antibody-Based Conditioning

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Conditioning chemotherapy is used to deplete hematopoietic stem cells in the recipient's marrow, facilitating donor cell engraftment. Although effective, a major issue with chemotherapy is the systemic genotoxicity that increases the risk for secondary malignancies. Antibody conjugates targeting hematopoietic cells are an emerging non-genotoxic method of opening the marrow niche and promoting engraftment of transplanted cells while maintaining intact marrow cellularity. Specifically, this platform would be useful in diseases associated with DNA damage or cancer predisposition, such as dyskeratosis congenita, Schwachman-Diamond syndrome, and Fanconi anemia (FA). Our approach utilizes antibody-drug conjugates (ADC) as an alternative conditioning regimen in an FA mouse model of autologous transplantation. Antibodies targeting either CD45 or CD117 were conjugated to saporin (SAP), a ribosomal toxin. *FANCA* knockout mice were conditioned with either CD45-SAP or CD117-SAP prior to receiving whole marrow from a heterozygous healthy donor. Bone marrow and peripheral blood analysis revealed equivalent levels of donor engraftment, with minimal toxicity in ADC-treated groups as compared with cyclophosphamide-treated controls. Our findings suggest ADCs may be an effective conditioning strategy in stem cell transplantation not only for diseases where traditional chemotherapy is not tolerated, but also more broadly for the field of blood and marrow transplantation.

INTRODUCTION

Fanconi anemia (FA) is an inherited bone marrow failure disorder resulting from an intrinsic defect in DNA repair, which leads to an increased risk for cancers such as acute myeloid leukemia and squamous cell carcinoma.[1] Currently, 22 genes are implicated in the pathogenesis of FA.[2] The only effective treatment for the hematologic complications of FA is an allogeneic hematopoietic stem cell transplant (HSCT).[3,4] Conditioning prior to hematopoietic stem cell (HSC) infusion is used to create space in the marrow niche and immunosuppression to prevent graft rejection. Initial conditioning regimens for FA included the alkylating agent cyclophosphamide (Cy) alone at doses used for aplastic anemia (100–200 mg/kg); however, significant mortality and morbidity from cytotoxicity was observed.[5–7] Subsequently, *in vitro* studies were done demonstrating increased chromosomal breakage when FA lymphocytes were exposed to Cy.[8,9] Lower doses of Cy combined with total body irradiation (TBI) resulted in fewer regimen-related toxicities, although issues with acute (25%–40%) and chronic (up to 40%) graft versus host disease (GvHD) were observed.[10] Reduced-intensity conditioning (RIC) regimens are now used for FA patients and utilize low-dose Cy (20–40 mg/kg), fludarabine, and anti-thymocyte globulin (ATG).[11] Although overall survival for allogeneic transplantation in younger FA patients with bone marrow failure is greater than 90% when using RIC, late complications that include GvHD, mixed chimerism, and the development of secondary malignancies[1] continue to be an issue.[12]

Gene therapy provides an alternative approach by introducing a corrected *FANCA* gene into autologous cells, eliminating GvHD risks and associated complications.[13] Current FA trials use purified $CD34^+$ HSCs that undergo *ex vivo* gene transfer and subsequent reinfusion without prior conditioning. Although limited successes have been obtained regarding the persistence and expansion of gene-modified cells, there remains the concern of ongoing residual FA hematopoiesis that can potentially result in clonal evolution and leukemogenesis. Our group has demonstrated in our FA mouse model[14] that Cy is both an effective conditioning and post-transplantation selection agent that facilitates the engraftment of gene-modified cells and elimination of residual host hematopoiesis.[14] However, because of its genotoxicity, Cy or

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other such agents should be avoided in diseases associated with DNA repair defects, such as FA. Antibody drug conjugates (ADCs) that target HSCs provide a promising nongenotoxic alternative of preparing the marrow prior to cell infusion that addresses the issues of persistent host hematopoiesis, conditioning toxicity, and low levels of engraftment extant in transplantation and gene therapy for FA patients. CD117 (c-kit)[15,16] and CD45[17,18] have been targets for unconjugated blocking antibodies. CD117 blockade alone failed to achieve engraftment in immunocompetent mice, requiring the addition of radiation or CD47 blockade.[19] Better efficacy with CD117 monoclonal antibody was observed in a FA mouse model that was thought to be secondary to increased c-kit signaling in FA HSCs but with high levels of residual hematopoiesis and low levels of donor engraftment likely secondary to minor histocompatibility mismatches between wild-type (WT) donors and FA recipients.[15] Other studies have shown the effective use of ADCs using a CD45 conjugated to the ribosomal toxin saporin (SAP) to achieve HSC depletion, donor engraftment, and immune reconstitution without toxicity in normal mice.[20] Recently, effective depletion of the HSC niche and subsequent engraftment of donor cells was achieved in C57BL/6J mice using CD117-SAP.[21] Furthermore, combination of CD117-SAP and CD45-SAP conditioning was able to successfully engraft gene-modified cells in a mouse model of hemophilia.[22] In this study, we evaluated the ability of CD45-SAP and CD117-SAP to deplete HSCs in bone marrow using the well-established *Fanca*$^{-/-}$ mouse model with the goal of facilitating donor engraftment using much-reduced cell doses than have been previously described.[15] Toxicity and efficacy of these ADCs were compared with Cy, because it is used in many transplantation protocols[11,12] and has been proved to be particularly toxic to FA cells *in vitro*.[8,9] We have also previously shown that *Fanca*$^{-/-}$ mouse bone marrow is particularly sensitive to Cy when administered intraperitoneally and therefore is an effective conditioning agent for gene-modified cells.[14,23] We showed substantial HSC depletion by CD45-SAP and CD117-SAP that was similar to Cy treatment but with substantially less toxicity. These ADCs also facilitated the engraftment of FA-heterozygous cells at levels that were at least comparable with Cy conditioning, demonstrating the efficacy of this non-genotoxic conditioning platform for potential clinical translation to FA patients in both the allogeneic transplantation and gene therapy settings.

RESULTS

ADC-Based Conditioning Efficiently Depletes HSCs while Preserving Bone Marrow Cellularity in *Fanca*$^{-/-}$ Mice

To understand the effects of ADC conditioning on resident bone marrow HSCs, we performed necroscopy on *Fanca*$^{-/-}$ mice after a single dose of either CD45-SAP or CD117-SAP and assessed them by histopathology and flow cytometry. Cy conditioning was used as a positive control because *Fanca*$^{-/-}$ mice recapitulate the unique marrow sensitivity to alkylating and cross-linking agents, which is characteristic of FA patients. In comparison with Cy-treated mice that exhibited notable marrow aplasia, mice receiving either CD45-SAP or CD117-SAP demonstrated preserved cellularity, similar to mock animals (no treatment) (Figure 1A), as quantified by unbiased computer analysis (HALO) (Figure 1B; Figure S1) and veterinary pathology review. We then analyzed the ability of these ADCs to specifically deplete HSCs and progenitor cells, as determined by the LSK phenotype (lineage$^-$sca1$^+$c-kit$^+$) (Figure 1C). Although Cy and CD117-SAP performed equally well in depleting the LSK population, CD45-SAP treatment resulted in significantly more residual LSK cells (Figure 1D). When evaluating the more primitive long-term HSC compartment (the LSK/SLAM population, which is denoted as lineage$^-$sca1$^+$c-kit$^+$CD150$^+$CD48$^-$) (Figure S2), both ADCs demonstrated robust LSK/SLAM depletion that was more effective than Cy treatment (Figure 1E). Peripheral blood counts were also measured pre- and post-conditioning (Figure S3). There was no significant decrease in total white blood count, hemoglobin, absolute lymphocyte count, or absolute neutrophil count in ADC-treated mice as compared with untreated animals. Furthermore, post-conditioning total white blood counts in CD117-SAP- and CD45-SAP-treated animals were significantly higher than Cy animals. CD117-SAP-conditioned animals did demonstrate thrombocytopenia after conditioning ($p < 0.005$), which correlated to a relative decrease in megakaryocytes noted on H&E staining of the bone marrow.

Comparable and Multi-lineage Engraftment in ADC- and Cy-Treated *Fanca*$^{-/-}$ Mice

To assess the ability of ADCs to facilitate engraftment of donor cells, we conditioned *Fanca*$^{-/-}$ mice with either CD45-SAP or CD117-SAP 1 week prior to infusion of 1×10^6 whole marrow cells from healthy *Fanca*$^{+/-}$ heterozygote littermates. *Fanca*$^{+/-}$ littermates were selected as donors rather than WT controls to avoid any histocompatibility mismatch discrepancies that could affect engraftment and to also mimic an optimized gene therapy cell product where all infused cells express one functional copy of the *FANCA* gene. Donor marrow was transduced with a lentiviral vector coding for GFP, enabling easy tracking by flow cytometry. Peripheral engraftment was observed as early as 4 weeks post-transplant and persisted for the entire duration of the study (Figure 2A). Interestingly, average GFP expression in the blood of Cy-conditioned animals was higher than in the ADC-treated groups at the 4-week time point but reached statistical significance only in the CD117-SAP group (Cy: 13.74 ± 2.52, n = 4; CD117-SAP: 5.26 ± 0.77, n = 8; $p = 0.0019$). Starting at 8 weeks post-transplantation there was no difference in engraftment between Cy- and ADC-treated animals. By 6 months, average GFP expression in the peripheral blood remained similar between groups, CD45-SAP (14.1% ± 7.44%, n = 8), CD117-SAP (18.7% ± 10.16%, n = 8), and Cy controls (22.0% ± 3.44%, n = 4). In agreement with these results, the frequency of GFP$^+$ HSCs (GFP$^+$LSK cells) in the bone marrow at necropsy was not significantly different among the Cy, CD45-SAP, and CD117-SAP groups (Figure 2B). Engrafted cells possessed multi-lineage differentiation potential in all transplanted mice, as evidenced by GFP$^+$ cells in both the myeloid and lymphoid lineages in the peripheral blood starting at 12 weeks post-transplantation (Figure 2C). By 24 weeks, there was no significant difference in total lymphocytes, granulocytes, and monocytes between mock, Cy-, and ADC-treated animals. Furthermore, there was no significant difference in the fraction of GFP$^+$ cells in these different lineages between the Cy and ADC cohorts (Figures S4 and S5) except for slightly more GFP$^+$ lymphocytes in the Cy cohort as compared with CD45-SAP.

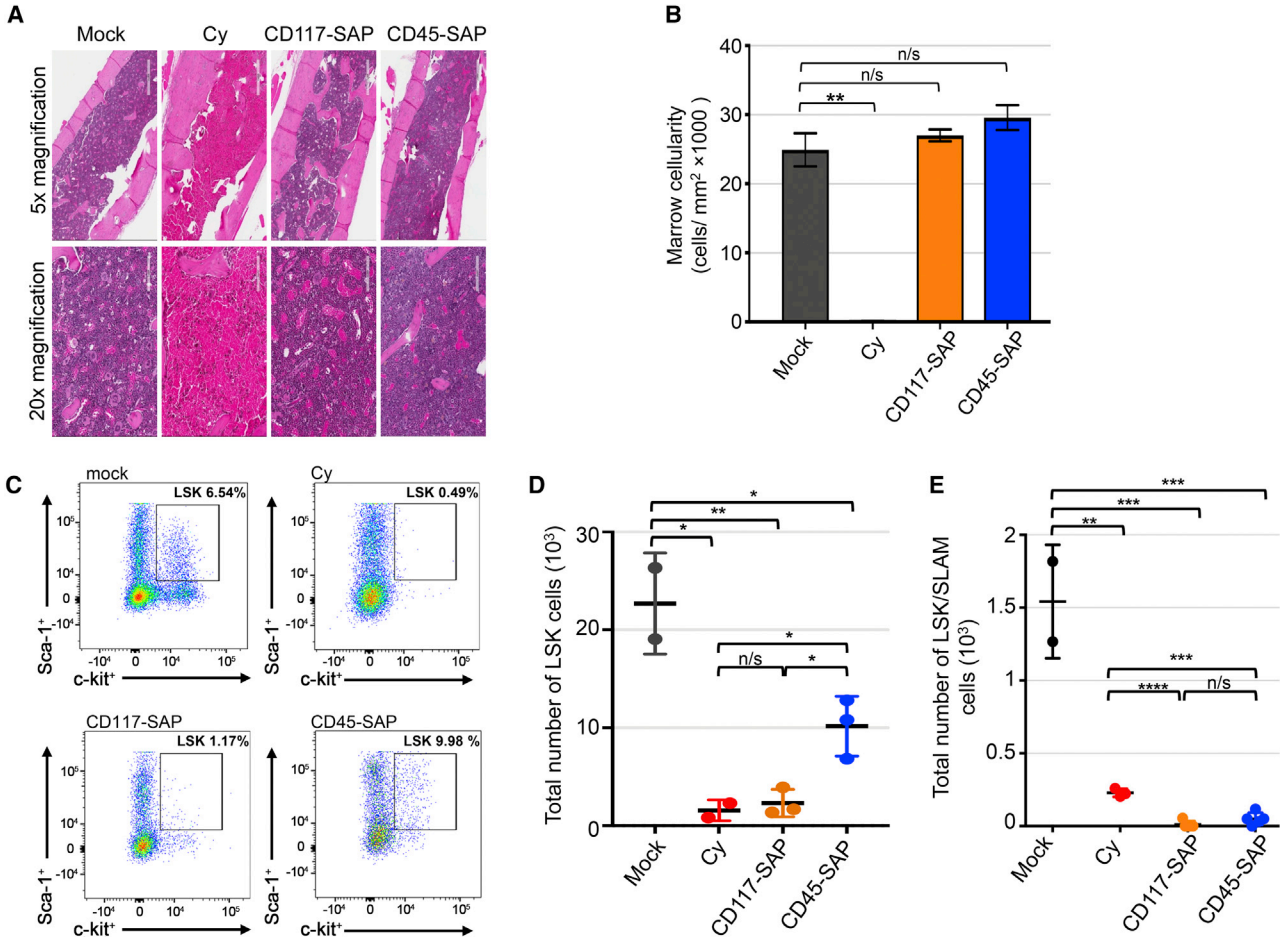

Figure 1. CD117-SAP and CD45-SAP Deplete the HSC Population in *Fanca*$^{-/-}$ Mouse Bone Marrow without Myeloablation

(A) Representative hematoxylin and eosin (H&E) staining images from *Fanca*$^{-/-}$ mouse bone marrow collected on the day of transplant after treatment with Cy, CD117-SAP, or CD45-SAP. Mock animals received no treatment. Bone marrow assessed at ×5 and ×20 original magnification. (B) Quantitation of marrow cellularity using HALO software analysis of H&E slide images assessing equivalent areas (mm^2) per slide. (C) Representative flow cytometry plots showing frequency of Lin$^-$Sca-1$^+$c-kit$^+$ (LSK) populations in the bone marrow of untreated (mock) versus conditioned animals. (D) Calculated number of LSK cells per animal based on frequencies determined by flow cytometry (C) for mock (n = 2), Cy (n = 2), CD117-SAP (n = 3), and CD45 (n = 3). (E) Calculated number of LSK/SLAM cells assessed by flow cytometry for mock (n = 2), Cy (n = 3), CD117-SAP (n = 5), and CD45-SAP (n = 5). *p < 0.05; **p < 0.01; ****p < 0.0001; n/s, non-significant. See also Figures S1–S3 and S9.

Total LSK numbers post-transplantation in experimental cohorts almost returned to each cohort's pre-transplantation levels (Figure S6), although the CD117-SAP cohort still had an average LSK population lower than the mock group.

Improved Engraftment of a Reduced Number of Donor Cells in ADC-Conditioned *Fanca*$^{-/-}$ Mice

To recapitulate the clinical scenario in FA gene therapy where the number of cells to be infused is generally limited, we then conducted studies with a reduced donor cell dose for infusion to determine whether ADC conditioning would still be effective in this setting. ADC groups receiving lower cell doses (0.5×10^6) had comparable levels of peripheral engraftment to the Cy cohort, which received twice as many cells (1×10^6) (Figure 3A). Consistent with our prior observation, Cy-conditioned mice generally had higher levels of engraftment at the 4-week time point compared with the ADC-treated groups, with the exception of the CD45-SAP cohort, which received 10×10^6 cells (Figure 3A). However, by 8 weeks, ADC-conditioned animals had equivalent, if not superior, engraftment relative to Cy-treated animals, and by 24 weeks, ADC-treated mice that had received 10×10^6 cells had significantly higher levels of engraftment compared with Cy. Moreover, there was no significant difference in engraftment when comparing the rest of the ADC-conditioned animals, including animals receiving 0.5×10^6 cells, with Cy-treated mice. Although there was a wide range of engraftment, all ADC-treated cohorts demonstrated at least equivalent, if not superior, engraftment of GFP$^+$ LSK cells relative to Cy-conditioned animals (Figure 3B). Two animals in the low-cell-dose cohort conditioned with CD45-SAP did not engraft; however, the other two animals showed robust levels of GFP expression. Multi-lineage engraftment in the peripheral blood

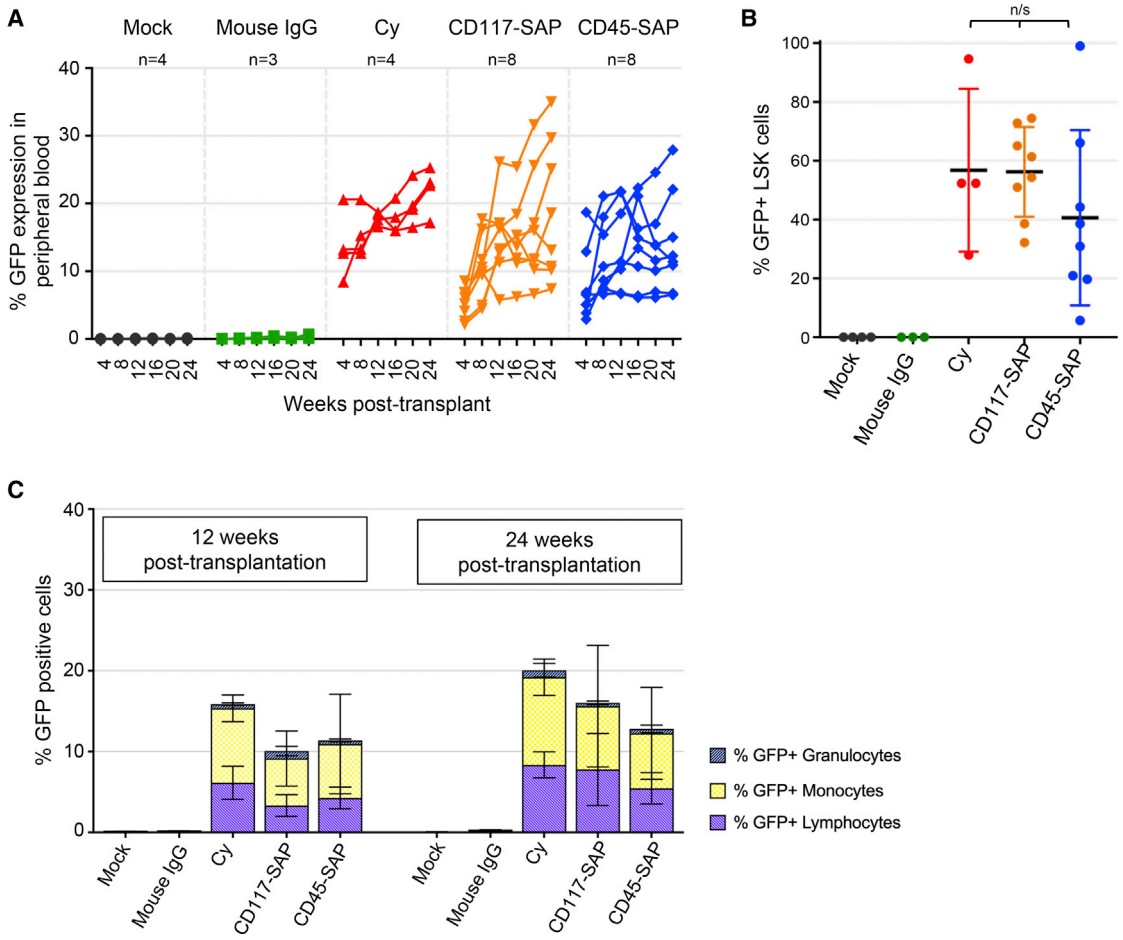

Figure 2. Multi-lineage Peripheral Blood and Bone Marrow Engraftment of *Fanca*$^{-/-}$ Mice Treated with Cy or ADC Conditioning

CD117-SAP and CD45-SAP facilitate engraftment of 1 × 10^6 donor cells from heterozygote *Fanca* mice. (A) Measurement of GFP expression as surrogate for donor cells in the peripheral blood of transplanted animals. Each individual line represents a single mouse tracked over 24 weeks as indicated on the x-axis with number of mice in each group indicated at the top. (B) Frequency of donor-derived (GFP$^+$) LSK cells in the bone marrow at necropsy (24 weeks post-transplantation) in each transplanted animal. (C) Donor derived (GFP$^+$) in the indicated lineage subsets in the peripheral blood at 12 and 24 weeks post-transplantation. All animals survived the 24-week period. n/s, non-sigificant. See also Figures S4–S6.

was also observed in all experimental groups, with a dose-dependent pattern noted in the CD117-SAP group (Figure 3C). Lineage analysis indicated no significant difference in total lymphocyte, granulocyte, and monocyte populations among the mock, Cy, and ADC groups (Figure S7). Furthermore, no significant difference in GFP engraftment was noted in B-lymphocytes (CD3$^-$CD19$^+$) or in CD4$^+$ or CD8$^+$ T-lymphocytes when comparing Cy- with ADC-treated animals (Figure S8). Generally, a positive correlation could be drawn between the number of infused cells and engraftment efficiency, particularly for CD117-SAP-treated animals (R^2 = 0.9773, 24-week engraftment for the CD117-SAP cohort).

Functional Correction of the FA Phenotype in Engrafted *Fanca*$^{-/-}$ Animals

At the time of necropsy, bone marrow cells obtained from experimental animals were plated into colony-forming assays in the presence or absence of the DNA alkylating agent mitomycin C (MMC) to verify functional correction of the FA phenotype. Colonies were picked and individually genotyped to determine the percentage of the bone marrow derived from donor versus host. Colonies from CD45-SAP and CD117-SAP animals were almost 100% donor derived at both 0 and 10 nM MMC, indicating that the majority of resident HSCs with colony-forming potential were donor-derived (i.e., heterozygous cells) (Figure 4A). In contrast, the frequency of donor-derived colonies was lower in the Cy group but increased when plated in 10 nM MMC, consistent with a selective advantage for heterozygous cells that exhibit resistance to oxidative stress as compared with FA cells. As expected, colonies grown in 10 nM MMC from the treated groups were comparable in size and morphology with 0 nM MMC, in contrast with small and atrophic colonies developing in 10 nM MMC media from the mock animals (Figure 4B).

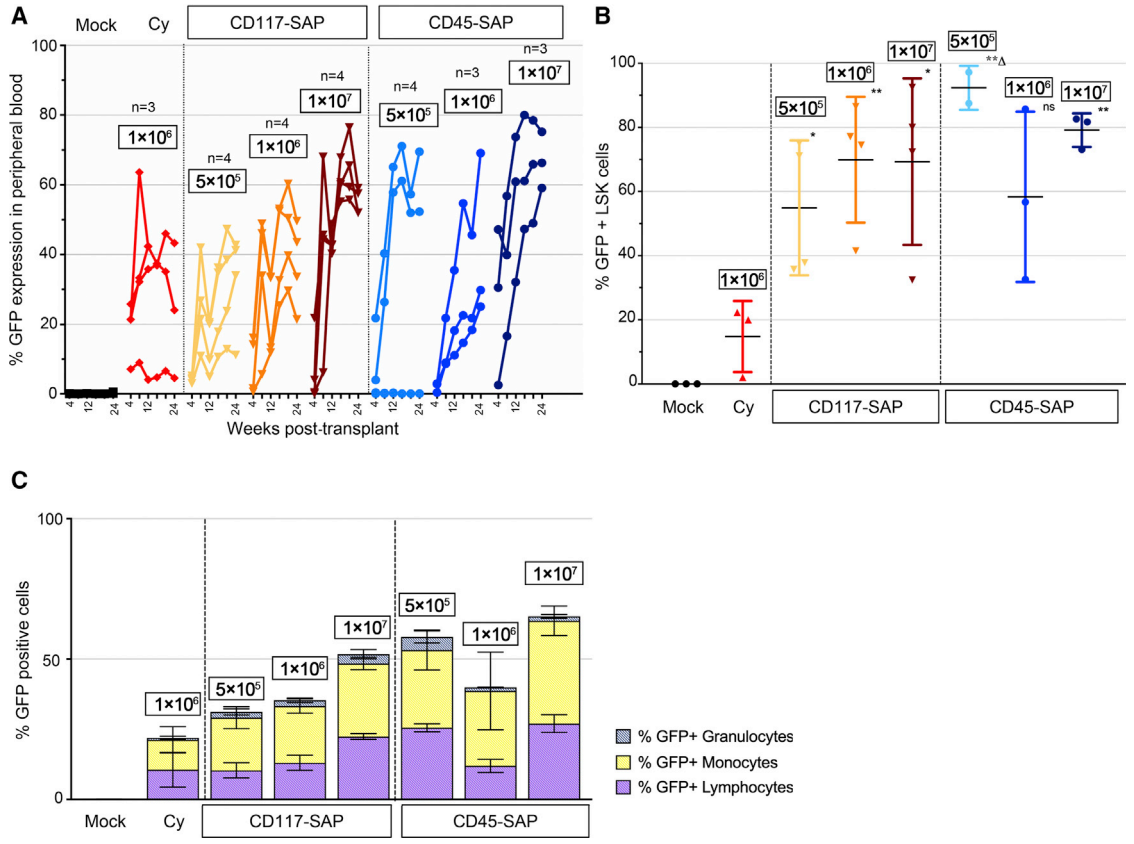

Figure 3. Peripheral Blood and Bone Marrow Engraftment of *Fanca*$^{-/-}$ Mice Receiving Variable Doses of Donor Cells after Cy or ADC Conditioning

Animals conditioned with CD117-SAP or CD45-SAP were given 0.5×10^6, 1×10^6, or 10×10^6 donor *Fanca*$^{+/-}$ cells transduced with a GFP lentiviral vector. Cy-conditioned animals received 1×10^6 cells. (A) GFP expression measured by flow cytometry in the peripheral blood over a 24-week period. Each individual line represents a single mouse. Cell dose and number of mice in each group are indicated at the top. (B) Frequency of donor-derived (GFP$^+$) LSK cells in the bone marrow at necropsy (24 weeks post-transplantation) in each transplanted animal. (C) Donor derived (GFP$^+$) in the indicated lineage subsets in the peripheral blood at necropsy. All animals survived the 24-week period except for one Cy-treated animal. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, n/s, non-sigificant. $^{\Delta}$Animals (n = 2) that did not engraft were excluded from this graph. See also Figures S7 and S8.

Reduced Toxicity in *Fanca*$^{-/-}$ Mice Treated with ADCs as Compared with Cy

During post-transplantation monitoring, Cy mice were observed to have diarrhea and weight loss, which was not present in ADC-conditioned animals. Cy-treated animals were noted to have significant weight loss compared with ADC and untreated animals starting at 5 weeks post-transplantation ($p = 0.0336$). These animals eventually demonstrated improvement in weight by 16 weeks post-transplantation. In contrast, there was no significant difference in weight in the ADC-treated group as compared with the mock group (Figure 5A). Further toxicity studies were performed on a separate cohort of animals that received a single dose of conditioning and were necropsied on the equivalent day of transplantation. No evidence of intestinal or colonic pathology after Cy treatment was observed by vet pathology review of slides. There was patchy hepatic inflammation on H&E staining for ADC animals only at high magnification (Figure 5B). However, there was no significant difference in liver function testing between ADC-treated animals in comparison with mock controls, whereas Cy-treated animals showed evidence of hepatic inflammation specifically with elevated AST and bilirubin but did not translate to any histopathologic changes on H&E staining (Figure 5C). There was no evidence of cardiac, small-intestinal, or renal toxicities by histopathology in any of the treated animals as assessed on the day of transplantation.

DISCUSSION

Our studies demonstrate here that individual treatment with CD45-SAP or CD117-SAP facilitates HSC elimination and engraftment of donor cells at least as efficiently as Cy, a well-established HSC-depleting chemotherapeutic agent in FA but limited by substantial genotoxicity.[8,9] We show that CD45-SAP and CD117-SAP efficiently deplete the LSK and SLAM HSC populations with repopulating potential. Our observation that CD117-SAP is more effective than CD45-SAP at depleting LSK cells is likely secondary to increased c-kit signaling in FA HSCs, which has been shown previously.[15] Furthermore, total lineage$^-$ c-kit$^+$ populations were eliminated by

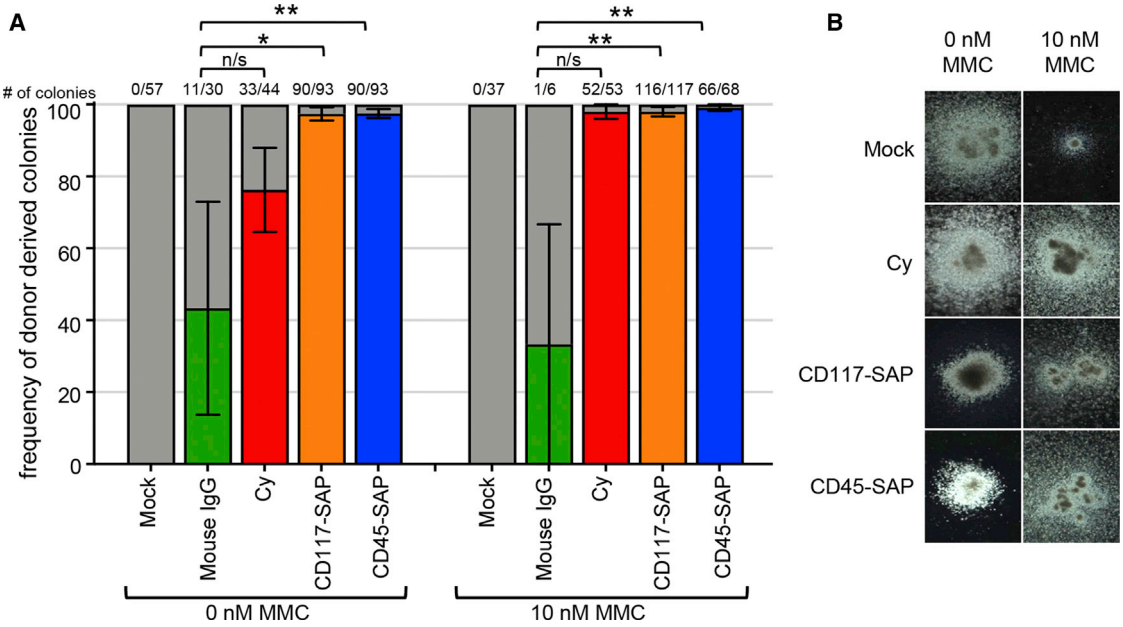

Figure 4. Colony-Forming Unit Assay to Measure Functional Correction in *Fanca*$^{-/-}$-Transplanted Animals Receiving 1 × 10^6 Cells

(A) Frequency of donor-derived colonies as determined by PCR analysis of single colonies grown on methylcellulose assays containing 0 (left) and 10 nM MMC (right). Gray portions represent residual percent host-derived colonies, and colored portions represent donor-derived colonies. The total number of colonies analyzed is listed on top of each bar as a proportion of donor:total. (B) Representative images of colonies growing in 0 and 10 nM media from each treatment group. All error bars represent standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$. n/s, non-significant.

CD117-SAP, which targets the same epitope as our c-kit fluorescent antibody, with comparable results to Cy and CD45-SAP (Figure S9). Our collaborators have demonstrated CD117-SAP clearance after 7 days; therefore, it is unlikely that this ADC interfered with our assessments of the LSK HSC pool. Both ADCs are more effective at eliminating SLAM HSCs and may spare progenitor cells, in contrast with Cy, which appears to mainly target progenitors. This finding is similar to prior studies using either CD45-SAP or CD117-SAP in C57BL/6J mice where SLAM HSCs were eliminated while sparing progenitor populations.[20,21] The ability of ADCs to selectively remove more quiescent HSCs, whereas Cy depletes progenitor cells, could explain our observations of superior short-term engraftment in Cy-conditioned animals at the 4-week time point post-transplantation in comparison with ADC-treated animals. Furthermore, engraftment in Cy animals eventually leveled off, whereas engraftment in ADC animals appeared to increase over 24 weeks, likely secondary to superior long-term HSC engraftment in these animals. A similar phenomenon was demonstrated in prior studies using CD117-SAP, as well as CD117-SAP and CD45-SAP in combination, when compared with sublethal TBI where superior engraftment was initially observed.[21,22]

Importantly, both ADCs selectively depleted the HSC population without seemingly affecting the bone marrow cellularity, in contrast with Cy, which induced widespread bone marrow aplasia. These results are consistent with findings from C57BL/6 mice where CD45-SAP treatment was associated with a decrease in marrow cellularity by 48 hours post-treatment that recovered by 7 days.[20] Similarly, CD117-SAP treatment in normal mice does not appear to have an effect on bone marrow cellularity by 7 days post-treatment.[21] We surmised that preserving the bone marrow cellularity would be particularly relevant for clinical translation, because ADC conditioning would not cause significant cytopenias secondary to marrow aplasia and, therefore, would be a safer alternative than myeloablative regimens. The peripheral blood counts obtained from animals pre- and post-conditioning did not show significant anemia or leukopenia with either ADC conditioning. Post-conditioning complete blood counts (CBCs) were performed on the day of transplantation; therefore, the nadir in peripheral blood counts may have occurred at a later time point. However, other groups studying CD117- and CD45-SAP conjugates in normal mice have not observed peripheral count suppression when measured at other time points post-conditioning; therefore, any possible cytopenias induced by ADC conditioning are likely mild and transient. There was thrombocytopenia secondary to CD117-SAP that also corresponded to histopathology findings of decreased megakaryocytes in the bone marrow by H&E staining. Thrombocytopenia was resolved after repeat CBC was performed 2 weeks after transplantation of donor cells. Although CD117 is noted to be expressed on megakaryocyte progenitors, other groups have not demonstrated thrombocytopenia specifically in normal C57BL/6J mice when evaluated 8 days after CD117-SAP treatment.[21] Interestingly, when CD117-SAP and CD45-SAP were used as pre-conditioning for platelet-specific factor VIII gene therapy, there were higher levels of platelet chimerisms detected than leukocyte chimerisms, which is likely secondary to CD117-SAP targeting megakaryocytes

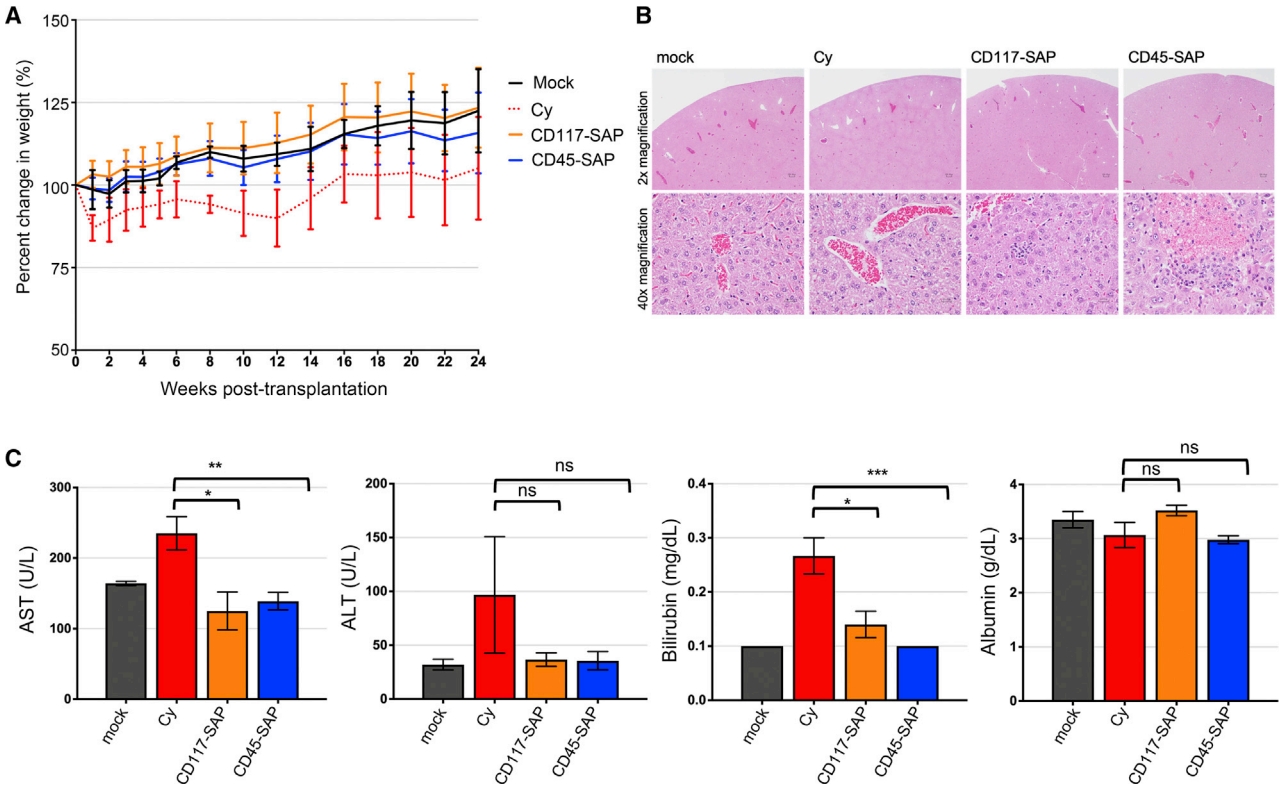

Figure 5. Comparison of Cy- and ADC-Induced Toxicity in Treated *Fanca*$^{-/-}$ Animals

(A) Percent change in weight in animal cohorts during 24-week post-transplantation monitoring. (B) Representative hematoxylin and eosin (H&E) staining images from *Fanca*$^{-/-}$ mice after treatment with Cy, CD117-SAP, or CD45-SAP. (C) Evaluation of hepatic toxicity as determined by liver function testing including aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, and albumin after a single dose of Cy, CD117-SAP, and CD45-SAP. Blood was drawn on the equivalent day of transplantation (12 days after CD117-SAP, 7 days after CD45-SAP, and 2 days after Cy). Mock animals received no treatment. All error bars represent standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$. n/s, non-significant.

and their progenitors.[22] Furthermore, the hemophilia mouse model used has both C57BL/6 and 129S background strains. Our FA mice have a 129S1/SvlmJ background and had CBCs drawn 12 days after CD117-SAP treatment, which may explain the difference in these findings compared with the C57BL/6J mice. Surprisingly, CD45-SAP conditioning did not result in neutropenia, but did have comparable absolute lymphopenia similar to Cy, although this was not significantly different from untreated animals.

Our initial studies showed that ADC conditioning is as effective as Cy in facilitating engraftment and long-term persistence of donor cells in *Fanca*$^{-/-}$ animals (Figure 2A). We also demonstrated multi-lineage engraftment starting at 12 weeks with no difference between engrafted granulocytes, lymphocytes, and monocytes when comparing Cy and ADC cohorts (Figure 2C). Although we generally found that an increased number of infused donor cells improved engraftment efficiency, this was particularly profound with CD117-SAP, similar to previous findings in normal mice.[21] Furthermore, CD45-SAP had variable engraftment at lower cell doses, where half of the animals engrafted with approximately 60% GFP expression in the peripheral blood and the other half did not engraft. Furthermore, because CD34$^+$ HSCs purified from FA patients in gene therapy clinical trials are limited in numbers due to low levels of CD34 expression,[24] we recapitulated these conditions in our experiments. The average total cell dose given to patients in the ongoing FA trial is 4.8×10^6 total cells/kg, whereas the average CD34$^+$ count is 7.0×10^5 CD34$^+$ cells/kg. In our studies, mice were approximately 0.3 kg and received a range of $0.5–10 \times 10^6$ cells, which is $1.6–33.3 \times 10^6$ total cells/kg. We found higher levels of engraftment with ADCs at lower cell doses, particularly in the bone marrow HSC population, in comparison with Cy. The robust engraftment observed at these lower cell doses may be secondary to ADC conditioning preserving the bone marrow cellularity, including supportive cells in the microenvironment (Figures 3A and 3B). Variability in lot-to-lot potency of SAP as established by manufacturer guidelines could also have contributed to differences in engraftment observed with ADCs between experiments. Importantly, cell doses used in our experiments are at least 10-fold less than used in similar studies,[16,23] recapitulating the clinical challenge in gene therapy studies for FA. Moreover, we demonstrated superior HSC depletion of donor cell engraftment using our ADC platform in comparison with previous studies using CD117 monoclonal antibodies in FA mice.[16]

We confirmed correction of the FA phenotype in transplanted animals through functional studies using colony outgrowth assays to assess MMC resistance. MMC increased the fraction of donor-derived, *FANCA*-expressing colonies, demonstrating resistance to oxidative stress and functional correction of the FA defect. In the absence of MMC, the majority of colonies formed from the ADC-treated animals expressed a functional copy of the *FANCA* gene, unlike Cy-treated animals where a portion of colonies lacked a functional *FANCA* gene, indicative of residual host hematopoiesis (Figure 4A). However, Cy-treated animals still were found to have a portion of colonies without a functional *FANCA* gene, suggesting the presence of residual host hematopoiesis that also correlates to our data from Figure 1E that Cy treatment does not fully eliminate HSCs in the *Fanca*$^{-/-}$ mice. These data are relevant for clinical translation, because the presence of host hematopoiesis in FA poses the persistent risk for leukemogenesis. Current gene therapy trials for FA do not use conditioning; therefore, host hematopoiesis is ongoing after engraftment of gene-modified cells. Although no patients have reported to develop hematologic malignancies, the addition of non-genotoxic conditioning could improve long-term outcomes.

Our novel findings suggest CD45-SAP and CD117-SAP could provide a key component in cellular therapies for patients unable to tolerate genotoxic conditioning regimens. A CD117 monoclonal antibody is currently being evaluated as a sole conditioning agent for severe combined immunodeficiency (ClinicalTrials.org: NCT02963064). Using an ADC as opposed to monoclonal antibody could enhance target effects without relying on antibody-mediated cytotoxicity. CD117-SAP has a potential role in the gene therapy setting, because this ADC is clearly able to engraft a low dose of infused cells with a predictable cell dose response. CD45-SAP may be more applicable in the allogeneic transplantation setting because it could provide an additional immunosuppressive benefit. Although our studies were performed in FA, this conditioning method could be broadly applicable to numerous other diseases, particularly those with intrinsic DNA repair defects.

MATERIALS AND METHODS

Mice

Fanca$^{-/-}$ mice have been previously described.[23,25] Mice were bred, housed, and maintained at Fred Hutchinson Cancer Research Center (Fred Hutch) under Institutional Animal Care and Use Committee (IACUC)-approved protocol 1456 and in pathogen-limited housing conditions.

Conditioning and Transplant

Adult female heterozygote *Fanca*$^{+/-}$ bone marrow was passed through 70-μm filters, hemolyzed, and transduced using a second-generation GFP lentiviral vector at a multiplicity of infection (MOI) of 10 as previous described, except granulocyte colony-stimulating factor (G-CSF) was not used in this study.[23] The HIV-1-based pRRL.sincPPT.PGK.GFP.wpre used is a self-inactivating lentivirus vector containing a human phosphoglycerate kinase promoter (PGK) driving the expression of GFP. The next day, cells were infused in 200 μL PBS containing 1% heparin via tail vein injection. Recipient mice received 120 mg/kg Cy (equivalent to 9.7 mg/kg adult human dose[26]) in 100 μL via intraperitoneal injection 48 h before transplant or a single dose of either CD45-SAP (3 mg/kg total complex) or CD117-SAP (1.5 mg/kg total complex) via tail vein injection 1 week prior to receiving 1×10^6 donor cells. CD45 (clone 104), CD117 (clone 2B8), and SAP were provided by collaborators at Magenta Therapeutics. Doses were based on HSC clearance and pharmacokinetic data performed by Magenta Therapeutics. For HSC depletion, toxicity studies, and cell dose modification experiments (Figures 1, 3, and 5), similar dosing was performed for CD45-SAP and Cy, whereas CD117-SAP was given 12 days prior to necropsy per recommendations from Magenta based on additional pharmacokinetic studies done on C57BL/6 mice that demonstrated clearance of CD45-SAP within 24 h and clearance of CD117-SAP after 7 days. Animals in the toxicity and HSC depletion cohort were euthanized, and necropsies were performed on the same day (i.e., equivalent day of transplantation) that animals in the transplantation cohorts received donor cells. CBC analysis was performed on peripheral blood obtained through retro-orbital puncture prior to necropsy.

Sample Analysis

Blood collection began 4 weeks post-transplant through retro-orbital puncture, collected into EDTA Microtainers (BD Biosciences, San Jose, CA, USA), and stained as previously described.[27] Antibodies used were mouse CD45.1/CD45.2-V500 (30-F11), CD3-Pacific Blue (17A2), CD4-allophycocyanin (APC) (RM4-5), CD8-peridinin-chlorophyll-protein (PerCP) (53-6.7), CD14-phycoerythrin (PE) (rmC5-3), CD19-APC-H7 (1D3), Lineage-V450, c-Kit-APC (2B8), Sca1-PE-Cy7 (D7), CD150-PerCP/Cy5.5 (TC15-12F12.2), and CD48-APC-Cy7 (HM48-1) (BD Biosciences, BioLegend [San Diego, CA, USA], eBioscience [Waltham, MA, USA]). Lineage subsets were gated based on side scatter with CD45 on the x axis (Figure S4). Lymphocyte subsets were further analyzed based on surface markers (Figure S4). DNA was extracted (QIAGEN, Hilden, Germany) and analyzed as previously described.[23]

Methylcellulose Assays

Hemolyzed marrow was plated at a concentration of 20–50 $\times$ 10^4 cells and maintained as previously described.[23] Colonies were imaged using an EVOS Flc (Life Technologies, Carlsbad, CA, USA). Individual colonies were genotyped by PCR as previously described.[28] MMC was incorporated into methylcellulose colony media as a selecting agent for *FANCA*-expressing colonies.

Histopathology

Bones and peripheral tissues were fixed in 10% neutral-buffered formalin for 5 days and embedded, sectioned, and stained by the Experimental Histopathology Core at Fred Hutch. Tissues were evaluated by veterinarian pathologist for evidence of organ toxicity. Marrow cellularity was assessed by veterinarian pathologist and quantified by HALO Image Analysis version 2 (Indica Labs, Corrales, NM, USA). HALO analysis was performed on H&E slides, and the total area analyzed for cellularity was the same for each sample (0.144 mm^2) with unbiased parameters.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.02.001.

AUTHOR CONTRIBUTIONS

H.-P.K. is the principal investigator of the study and coordinated the overall execution of the projects. H.-P.K., M.A.S., and K.G.H. conceived the study, and designed and coordinated the experiments. Y.S.R. and M.A.S. prepared the ADC conjugates prior to their injection. M.A.S. and C.I. processed mouse samples, performed necropsy and tissue collection, and conducted flow cytometry and colony PCR. D.T.S. developed the CD117-SAP conjugate. R.P., A.E.B., and M.P.C. provided reagents and input for experimental design and analysis. M.A.S., O.H., and K.G.H. wrote the manuscript, which was critically reviewed by B.R.B., D.T.S., and H.-P.K.

CONFLICTS OF INTEREST

H.-P.K. is a consultant for Rocket Pharma and Homology Medicine. A.E.B., R.P., and M.P.C. are full-time salaried employees of Magenta Therapeutics. D.T.S. and B.R.B. are members of the Scientific Advisory Board of Magenta. B.R.B. receives remuneration as an advisor to Kamon Pharmaceuticals, Inc., Five Prime Therapeutics Inc., Regeneron Pharmaceuticals, Magenta Therapeutics, and BlueRock Therapeutics; receives research support from Fate Therapeutics, RXi Pharmaceuticals, Alpine Immune Sciences, Inc., Abbvie Inc., BlueRock Therapeutics Leukemia and Lymphoma Society, Childrens' Cancer Research Fund, and KidsFirst Fund; and is a co-founder of Tmunity.

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