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CD37 in acute myeloid leukemia: a novel surface target for drug delivery

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Abstract:
Acute myeloid leukemia (AML) is the most common and lethal leukemia in adults. AML consists of many genetic subtypes which limits broad applicability of targeted therapy. We discovered that the hematopoietic restricted tetraspanin CD37 is expressed on all primary AML blasts and thus may represent a common therapeutic target for AML regardless of subtype. We demonstrate that the internalization properties of CD37 are distinct in AML blasts when compared to normal blood cells, and that CD37 rapidly accumulates inside AML blasts via dynamin-dependent endocytosis. Our work revealed that the clinically relevant anti-CD37 antibody drug conjugate (ADC) Debio 1562 (αCD37-DM1) is highly cytotoxic to AML blasts, but not normal hematopoietic stem cells. We found that αCD37-DM1 improved clinical outcomes and overall survival in multiple in vivo models of AML. Together, these data demonstrate that targeting CD37 with an ADC such as αCD37-DM1 is a feasible and promising therapeutic option for the treatment of AML.

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CD37 in acute myeloid leukemia: a novel surface target for drug delivery

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Key Points

- CD37 is a surface receptor present on most AML blasts which has unique internalization properties when compared to normal blood cells.
- Treatment of AML with anti-CD37 ADC demonstrates specific cytotoxicity in vitro and improved overall survival in vivo.

Text (3895 words). **Abstract** (153 words). **Figures** (5). **Tables** (2). **References** (42).

Abstract

Acute myeloid leukemia (AML) is the most common and lethal leukemia in adults. AML consists of many genetic subtypes which limits broad applicability of targeted therapy. We discovered that the hematopoietic restricted tetraspanin CD37 is expressed on all primary AML blasts and thus may represent a common therapeutic target for AML regardless of subtype. We demonstrate that the internalization properties of CD37 are distinct in AML blasts when compared to normal blood cells, and that CD37 rapidly accumulates inside AML blasts via dynamin-dependent endocytosis. Our work revealed that the clinically relevant anti-CD37 antibody drug conjugate (ADC) Debio 1562 (αCD37-DM1) is highly cytotoxic to AML blasts, but not normal hematopoietic stem cells. We found that αCD37-DM1 improved clinical outcomes and overall survival in multiple in vivo models of AML. Together, these data demonstrate that targeting CD37 with an ADC such as αCD37-DM1 is a feasible and promising therapeutic option for the treatment of AML.

Introduction

Acute myeloid leukemia (AML) is the most lethal and commonly diagnosed acute leukemia in adults and is associated with accumulation of undifferentiated blasts[1–5]. These blasts disrupt normal hematopoiesis and promote profound immune suppression[1,6,7]. Effective therapeutic strategies for AML have been hampered in part to the heterogeneous nature of AML as a disease and the similarity of AML blasts and normal hematopoietic stem cells [5,8]. The standard of care 7 + 3 induction chemotherapy is limited to younger patients with adequate organ function and results in prolonged hospitalizations and morbidity[9–11]. Furthermore, patients who achieve a complete remission still require additional consolidative therapy, and in the absence of allogeneic stem cell transplant the majority of patients will relapse and die from disease.

The increased understanding of the complex network of chromosomal rearrangements, mutations, epigenetic changes, antigen expression and immune dysregulation in AML has led to the pursuit of novel targeted therapeutics with notable successes toward targets such as FLT3,

IDH1, and IDH2 mutated disease[5,12,13]. However, these three abnormalities represent only a small fraction of patients with AML. The bcl-2 mimetic venetoclax in combination with the hypomethylating agent azacitidine has allowed for broader targeting and has shown significant clinical activity across most AML subtypes in elderly patients not eligible for chemotherapy; however, significant myelosuppression and immune suppression accompany this therapeutic strategy suggesting cross reactivity with normal hematopoiesis[14,15]. Antibody drug conjugates (ADCs) are highly attractive therapeutic strategies as they specifically target and kill cells expressing a unique antigen reducing off-target side effects. To date, there has only been one successful ADC in AML, the FDA approved anti-CD33 ADC Gemtuzumab ozogamicin (GO); however, the role of this ADC has been limited to the subset of CD33 expressing AML[16–18] . While other ADCs are currently being investigated for the treatment of AML[19,20], success has been limited due to the lack of an ideal target, which is traditionally defined as a target with ubiquitous expression among multiple subtypes of AML yet absent on normal hematopoietic cells.

CD37, a heavily glycosylated hematopoietic tetraspanin most commonly expressed in mature B-cells, was recently reported to be expressed in multiple subtypes of AML at a transcriptional and protein level[21–28]. This expression profile in combination with the safety profile of CD37 ADCs in humans suggest CD37 targeting may represent a safe and effective therapeutic strategy for the treatment of AML[26,28,29]. In this report we build upon previous works to demonstrate that CD37 is a shared surface receptor among most primary AML blasts and demonstrate for the first time that CD37 has unique internalization properties in leukemic blasts when compared to normal blood cells. Furthermore, we show that targeting CD37 in AML with the representative anti-CD37 ADC Naratuximab emtansine (αCD37-DM1) results in specific cytotoxicity of AML blasts while preserving normal hematopoietic cells. Thus, our work sets the stage for a promising anti-leukemic therapeutic strategy.

Methods

Cells and Cell lines: All AML specimens were obtained by protocols approved by The Ohio State University Institutional Review Board (protocol #2009C0019). AML cell lines were purchased from ATCC and DSMZ and cultured in Gibco RPMI Medium 1640 (Life Technologies, Carlsbad, California), supplemented with FBS (VWR, Radnor, PA), penicillin G (56 U/ml, Life Technologies, Carlsbad, CA) and streptomycin (56 ug/ml, Life Technologies, Carlsbad, California). Primary AML cells were cultured in 10% FBS medium enriched with 20 ng/ml FLT-3 ligand, IL-3, hSCF, and GM-CSF (PeproTech, East Windsor, New Jersey).

Flow Cytometry: Experiments were analyzed with Gallios Flow Cytometer (Beckman Coulter, Pasadena, California) or LSR Fortessa (BD, Franklin Lakes, New Jersey) followed by Kaluza v 2.1 analysis (Beckman Coulter, Pasadena, California). Prior to staining with antibodies, cells were Fc blocked with a mixture of 50% human serum/PBS or with 100 ug/ml human IgG on ice for 30 minutes (Athens Research, Athens, Georgia). Antibodies used for human immunophenotype analyses: Annexin-FITC (BD Pharminogen), Annexin-PeCy7 (Biolegend), CD2-BV421 (TS 1/8, Biolegend), CD3-FITC (UCHT1, BD Biosciences), CD19-PeCy7 (SJ25C1, Biolegend and J3-119 Beckman Coulter), CD37-PE/AF647 (DebioPharm/Invitrogen), CD45-FITC (5B1, Miltenyi), Caspase-3-AF700 (C92-605, BD), Live/Dead NIR/Aqua (Invitrogen), PARP-1-BV421 (F21-852, BD), PI (Leinco Technologies), Transferrin-AF647 (Invitrogen). Antibodies used for murine immunophenotype analyses: CD37-AF647 (DebioPharm/Invitrogen), CD3-FITC (145-2C11, BD), Ly6C-PE (HK1.4, eBio), CD19-PeCy7 (6D5, BioLegend), NK1.1-AF700 (PK136, BD), CD11b-PerCP-Cy5.5 (M1/70, Biolegend), CD4-BV650 (RM4-5, Biolegend), CD45-BUV395 (30-F11, BD), Ly6G/C-BV510 (RB6-8C5, Biolegend), CD8a-BV421 (53-6.7, BioLegend). Additional information can be found in the appendix.

αCD37-DM1 (Naratuximab emtansine, Debio 1562, formerly IMGN 529): Debio 1562, an anti-CD37 antibody drug conjugate owned by Debiopharm, was kindly supplied by Debiopharm (Debiopharm, Lausanne, Switzerland[30]) as part of a materials transfer agreement.

Annexin V/Propidium Iodide Assays: Cells were treated with 0.125 ug/ml αCD37-DM1 or the nonbinding isotype huIgG-SMCC-DM1 (Iso-DM1) for 72 hours, and subsequently stained with Annexin V and Propidium iodide (see above) according to manufacturer's instructions (Leinco Technologies, Fenton, Missouri). Cells were then analyzed by flow cytometry as described above.

Antibody conjugation: The anti-CD37 antibody K7153a was conjugated to the fluorochromes PE and AF647 per labeling and detecting kit instructions provided by Invitrogen (Invitrogen, Waltham, Massachusetts).

Colony Forming Unit Assays: PBMCs isolated from primary AML samples, HSCs from bone marrow (BM) aspirates and from GCSF stimulated stem-cell enriched products obtained through OSU-CCC were cultured as described above, rested overnight, then treated with 2.5 ug/ml αCD37-DM1, Iso-DM1 or PBS for 36 hours. After 36 hours, 2 x 10^4 cells were mixed with Methocult Medium Optimum without EPO (Stemcell Technologies, Vancouver, Canada), plated in triplicates in 6 well plates and incubated in 1.5% O_2. Colonies were then counted following the guidelines outlined in the technical manual titled "Human Colony-Forming Unit (CFU) Assays Using MethoCult" provided from StemCell Technologies (Document # #28404) with an Olympus microscope (Olympus, Center Valley, Pennsylvania).

Cell Viability Assays: Leukemic cells were seeded at 1.5 x 10^4 viable cells in 100 ul/well in triplicates in 96 well culture plates. MTS tetrazolium and phenazine methosulfate (Sigma-Aldrich, St. Louis, Missouri) were added to plates at 24, 48 and 72 hours and analyzed at 492 nm optical density with the Synergy/HTX plate reader (Biotek, Winooski, Vermont). Subsequent analysis was conducted in GraphPad Prism 7 (Dotmatics, San Diego, California).

Internalization Assays: Internalization assays were modified from reference provided as detailed in the appendix[31]. In brief, cells were thawed, counted and rested overnight in appropriate growth medium. The following day, cells were washed and resuspended in serum-free media at

a density of 1e6 cells/ml and incubated at 37C for 1h. After 1 hour, cells were aliquoted into flow tubes, washed with PBS and Fc blocked for 30 min on ice. Next, CD37-AF647 or transferrin-AF647 were added to appropriate tubes and incubated at 37C for 2h (surface + internal CD37) or on ice for 2h (surface CD37). After the 2 hour incubation, cells were washed, and stained for flow cytometry as described previously. The fold change in CD37-AF647 was calculated using the following equation (CD37 MFI at 2h 37C – CD37 MFI at 2h ice)/CD37 MFI 2h ice to determine the number of times CD37 internalized over the course of two hours.

Fluorescent Microscopy: Fluorescent microscopy was performed on fixed AML cell lines and primary AML samples (described above) as previously described {PMID: 26583570}. Briefly, samples (100 µl) were deposited on ultra-thin 8 well imaging plates (Lab-Tek, Thermo Fisher Scientific, Waltham, MA) at a density of 5 x 10^5 cells/ml and allowed to settle for 20 minutes. Images were collected at room temperature using an ANDOR EMCCD camera (Andor Techology Ltd, Belfast, Northern Ireland, United Kingdom) on an automated Nikon TiE microscope (Nikon, Shinagawa, Tokyo, Japan) under DIC imaging and epifluorescence imaging conditions with 640 nm excitation for AlexaFlour647-anti-CD37 antibodies.

Animal Studies: NOD scid gamma (NSG) mice and NOD.Rag1^{null} IL2rg^{null} SGM3 (NRGS) mice purchased through Jackson Laboratory (Jackson Laboratory, Bar Harbor, Maine) were treated with IP injections of 25 mg/ml busulfan (Gland Pharma Limited, India) 24 hours prior to engraftment with leukemic cells **(Table 2).** In all models, mice were bled weekly to assess for disease burden by flow cytometry and sacrificed upon meeting early removal criteria (ERC) or at the end of the study. All animal experiments adhered to guidelines of IACUC of The Ohio State University (2015A00000043-R2). Additional study details can be found in appendix.

Creation of humanized CD37 knock-in transgenic mouse: The humanized CD37 mice (hCD37) were produced collaboratively by the corresponding authors and by Biocytogen (Beijing, China) using CRISPR/Cas9 to insert Human EGE-DJH-019 flanked by 1.5 kb arms of homology

in between the murine 5'UTR and 3'UTR regions of EGE-DJH-019 on chromosome 7 of C57BL/6J mice. Male and female hCD37 heterozygous mice were intercrossed to breed wild-type, heterozygous and homozygous progenies. Expression of CD37 was established in relevant cell types previously described **(Figure 4B**)[32].

Statistical analysis: Data analysis were performed in SAS 9.4 (SAS Institute, Cary, NC). For experiments using 2 matched samples, paired t-tests were used. Two-sample t-tests were used to compare 2 independent groups, while ANOVA was conducted for experiments when more than 2 groups were involved. For the *in vivo* experiments with longitudinal measures and the *in vitro* experiments using primary cells, mixed effect modeling was performed, incorporating observational dependencies for each subject[33]. Holm's procedure was used to adjust multiplicities[34]. The association between CD37 expression and sensitivity to αCD37-DM1 was evaluated using Spearman correlation method. Survival probabilities were compared using a log-rank test[35].

Results

CD37 is a shared surface receptor among all primary AML blasts

CD37 expression of normal CD34+ CD38- hematopoietic stem cells (HSCs), normal peripheral blood mononuclear cells (PBMCs), primary AML blasts and AML cell lines was assessed using the CD37 targeting antibody K7153a conjugated to the fluorochrome PE (αCD37-PE). Staining and analysis of cryopreserved HSCs, PBMCs and primary AML blasts revealed that surface expression of CD37 is most prevalent on B cells, with comparably dim expression on all other blood cell subsets tested **(Figure 1A).** Using the gating strategy depicted in **Figure S1A**, we identified surface expression of CD37 on all primary AML blasts **(Figure 1A).** All AML cell lines evaluated expressed CD37 except KG1a (n=8/9) (data not shown). Unexpectedly, we discovered that CD37 surface expression is dynamic and sensitive to laboratory manipulations such as Ficoll Hypaque cell separation and cryopreservation **(Figure S2).** This previously unrecognized phenomenon may explain the lack of description of CD37 on AML cells with inherently lower levels of expression. Next, we sought to determine whether the level of surface CD37 on primary AML blasts correlated to known AML classifiers and clinical outcomes. Our data revealed CD37 expression did not correlate to 2017 European LeukemiaNet (ELN) classification[36] **(Figure 1B),** mutational status, immunophenotype or overall survival **(Figure 1C)**, a finding not surprising given virtual uniform expression across AML subtypes.

Rapid CD37 receptor internalization properties is unique to AML blasts

Having demonstrated that virtually all AML cell lines and primary AML blasts express CD37**,** we aimed to determine the potential of this receptor as a therapeutic target for anti-CD37 ADCs in AML. First, we sought to understand the internalization properties of CD37. We cultured AML blasts and normal donor B cells and monocytes with K7153a conjugated to the fluorochrome AlexaFluor 647 (αCD37-AF647). Cells were incubated with αCD37-AF647 for two hours on ice to

quantify CD37 surface expression and for two hours at 37C to quantify both surface and intracellular CD37 **(Figure 2A).** The calculated fold change in CD37-AF647 represents the number of times each CD37 molecule internalized over the course of two hours. We found that AML blasts internalize CD37 significantly more frequently than normal B cells (p=0.001) and monocytes (p=0.01) **(Figure 2B).** Surprisingly, the frequency of CD37 internalization was significantly greater in AML blasts, which have dim CD37 expression, when compared to B cells (p=0.001), which have the highest surface expression of CD37 among all hematopoietic cells **(Figure 2B, Figure 1A).** Thus, CD37 receptor internalization is independent of surface expression making CD37 a unique target for ADCs. Interestingly, our data revealed two distinct cohorts of primary AML samples based on frequency of CD37 internalization. We discovered that primary AML samples harboring activating signaling mutations such as FLT3, MLL and KRAS internalized CD37 with a trend toward increased frequency (p= 0.053) than those without signaling mutations[8,37]**(Figure 2C).** Having discovered AML blasts have unique CD37 internalization properties, we next sought to determine the mechanism of internalization in AML blasts. AML cell lines were treated with the dynamin inhibitor Dyngo4a[38] and constitutive internalization inhibitor Amiloride[39] followed by incubation with αCD37-AF647 with subsequent analysis by flow cytometry and epifluorescence and confocal microscopy. Only dynamin inhibition substantially decreased αCD37-AF647 fluorescence **(Figure S3A)** and prevented localization of fluorescence within the cell **(Figures S3B,C).**

To confirm our findings were due to specific binding of αCD37-AF647 to CD37 and subsequent internalization, we generated three CD37 KO AML cell lines, MV-411 KO, THP-1 KO and OCI-AML3 KO using CRISPR-cas9[40] **(Figure S4A-C)**. Incubation of the CD37+ parental cell lines and their KO counterparts with αCD37-AF647 revealed selective uptake and accumulation of AF647 in the CD37+ parental cell lines **(Figure 2D, Figure S4D).** Confirming our findings, epifluorescence imaging revealed localization of AF647 within vesicles of CD37+ AML cell lines

with no uptake or localization in the CD37 KO cell line **(Figure 2E).** Collectively, our data demonstrate αCD37-AF647 is specific for CD37 and is frequently internalized by dynamin dependent endocytosis upon binding.

αCD37-DM1 induces apoptosis and cell death in AML cell lines and primary AML blasts while sparing normal HSCs

Our findings of rapid internalization kinetics of CD37 in AML blasts and high specificity of αCD37-AF647 for CD37 led us to hypothesize that treatment of AML blasts with an αCD37 ADC would result in specific and significant cellular cytotoxicity. To test our hypothesis, we used αCD37-DM1, K7153a conjugated to the maytansinoid DM1 via a succinimidyl-4-(N-maleimidomethyl) (SMCC) linker, and its isotype, chkti-SMCC-DM1 (Iso-DM1)[30]. To determine the specificity and cytotoxicity of αCD37-DM1 in AML blasts we treated OCI-AML3 and OCI-AML3 KO with αCD37-DM1 and Iso-DM1. We found that treatment with αCD37-DM1 resulted in significant dose-dependent cytotoxicity in OCI-AML3 but had no effect on OCI-AML3 KO **(Figure 3A).** Next, we aimed to show that αCD37-DM1 induced cell death occurred due to delivery of the payload DM1. Because DM1 is known to induce cell death through activation of the apoptotic cascade [41,42], we assessed the frequency of AML blasts undergoing apoptosis in response αCD37-DM1. First, we assessed apoptosis of CD37+ and KO AML cell lines treated with αCD37-DM1 and Iso-DM1 using Annexin V/PI staining. αCD37-DM1 significantly increased the frequency of Annexin+ PI+ blasts in the CD37+ AML cell lines when compared to Iso-DM1 **(Figure 3B).** As expected, αCD37-DM1 had no effect on CD37 KO AML cell lines. Confirming our findings of αCD37-DM1 induced apoptosis we found elevated levels of cleaved PARP-1, a key substrate in caspase 9 activation, in AML cell lines treated with αCD37-DM1 **(Figure 3C).** Finally, we assessed the efficacy of αCD37-DM1 in untreated newly diagnosed and relapsed primary AML samples and normal HSCs in colony forming assays. Primary AML blasts treated with αCD37-DM1 produced significantly fewer colonies when compared to Iso-DM1 control **(Figure 3D).** In contrast, αCD37-DM1 had no effect

on colony formation of normal HSCs **(Figure 3E,F).** Serial replating of these HSC samples confirmed αCD37-DM1 is not cytotoxic in normal HSCs in vitro as HSCs treated with αCD37-DM1 maintained colony forming potential upon serial replating **(Figure 3E,F).** Thus, our data show αCD37-DM1 is uniquely cytotoxic to AML blasts and induces its effects via DM1 activation of the apoptotic cascade.

Treatment with αCD37-DM1 results in minimal toxicities in vivo in novel human CD37 knock in mouse

As we demonstrated αCD37-DM1 was cytotoxic to AML blasts while sparing normal HSCs in vitro, we next sought to determine the safety profile of αCD37-DM1. We generated a humanized knock-in CD37 (hCD37) mouse to understand how αCD37-DM1 would affect normal HSCs in vivo. To recapitulate the native levels of expression in our hCD37 mouse model, mouse CD37 in all cell lineages was replaced by human CD37 but retained endogenous mouse promoters. Homozygous and heterozygous hCD37 mice were treated with αCD37-DM1, Iso-DM1 or vehicle followed by serial bleeds to assess changes in complete blood counts **(Figure 4A).** First, we sought to confirm that CD37 expression in this mouse model recapitulated what is found in humans**.** We found that CD37 was highly expressed on B cells with variably dim expression on other cell subsets, such as T cells, monocytes and neutrophils, mirroring what is seen in humans **(Figure 4B).** Next, we assessed the effect of αCD37-DM1 on total WBC counts. We found that total WBC counts decreased noticeably in both the heterozygous hCD37 and homozygous hCD37 mice that was almost entirely due to on target B-lymphocyte reduction, with sparing of both T and NK cell subsets **(Figure 4C, 4D).** Additionally, we found that organ function was unaffected by αCD37-DM1 **(Figure S5)** and that bone marrow architecture from αCD37-DM1 and Iso-DM1 treated mice were normal **(Figure 4E).** Thus, αCD37-DM1 results in no notable myelosuppression, organ toxicity or impaired organ function in this model suggesting αCD37-DM1 may represent a unique

therapeutic option for treatment of AML as it results in high cytotoxicity of AML blasts while sparing normal hematopoietic cells.

αCD37-DM1 improves overall survival and decreases AML burden in vivo in multiple models

To test the efficacy of αCD37-DM1 in vivo, we used several different AML mouse models. In all studies, mice were randomly assigned to the αCD37-DM1, Iso-DM1 or vehicle cohort and assessed for disease burden by flow cytometry **(Figure 5A).** In the AML THP-1 cell line xenograft, treatment with 10 mg/kg αCD37-DM1 significantly improved clinical outcomes and overall survival **(Figures 5B, 5C).** Because 10 mg/kg αCD37-DM1 resulted in eradication of disease, we conducted a pilot study to determine if a lower dose of αCD37-DM1 could be used. Our study showed that 2.5 mg/kg provided complete receptor occupancy for more than 7 days, leading us to use 2.5 mg/kg in subsequent PDX models (data not shown). Mice engrafted with a primary AML sample (AML 065) were monitored until they demonstrated circulating disease >/= 2.5% and were then randomized to a treatment group (PDX 1). Those treated with αCD37-DM1 had no detectible disease in circulation at end of study (10 weeks) and analysis of bone marrow tissue showed a small population of leukemia (15.77% +/- 5.82% CD45+ blasts) in some of the αCD37-DM1 treated mice **(Figure 5D, 5E)**. To determine the effect of initiating treatment earlier in the disease course, we engrafted mice with a primary AML sample (AML 066) and initiated treatment 4 weeks post-engraftment with subsequent monitoring for overall survival (PDX 2). Mice treated with αCD37-DM1 had no detectable circulating disease **(Figure 5F)** and significantly prolonged overall survival **(Figure 5G).** Our in vivo studies demonstrate that αCD37-DM1 can significantly decrease disease in primary human AML models.

Discussion

Herein, we explored the potential of the hematopoietic restricted tetraspanin CD37 as a potential therapeutic target for anti-CD37 ADCs for the treatment of AML. We show in a large cohort of untreated newly diagnosed and relapsed primary AML samples that CD37 is found on most primary AML blasts and is independent of subtype. Thus, CD37 may serve as a promising therapeutic target for AML. Recent works have demonstrated that level of CD37 transcript of AML blasts correlates with shorter overall survival and that surface expression correlates to ELN classification[25,28]. Within our cohort of 55 untreated newly diagnosed or relapsed refractory AML samples, we did not observe a correlation between CD37 expression and overall survival or any other clinical classifiers. Variations in CD37 expression in response to laboratory manipulations such as cryopreservation and cellular isolation is one possible explanation for these findings.

Despite low levels of surface expression, AML blasts were capable of frequent internalization of CD37 upon binding of αCD37. Surprisingly, we found that the frequency of CD37 internalization is independent of surface expression of this antigen. B cells display the highest level of CD37 surface expression among all hematopoietic cells yet internalize CD37 significantly less frequently than AML blasts. Our data revealed that the internalization properties of AML blasts are significantly different from those of normal monocytes suggesting that the frequent internalization of CD37 is a phenomenon unique to AML blasts. Our data suggests that frequent internalization of CD37 on AML blasts allows for high efficacy of the anti-CD37 ADC αCD37-DM1. We show that despite dim CD37 surface expression on these transformed myeloid cells, αCD37-DM1 is highly cytotoxic to AML cell lines and primary AML blasts both in vitro and in vivo. Upon binding to CD37, αCD37-DM1 is internalized in AML blasts resulting in subsequent activation of the apoptotic cascade due to delivery of the ADC payload DM1. Using CD37 KO AML cells lines, we demonstrate the high specificity of αCD37-DM1 for its target CD37. Future studies in which CD37 is restored in the CD37 KO AML cell lines with subsequent cytotoxicity assessment could further support the specificity documented within our studies. Most importantly, we demonstrate in

multiple xenograft models of cell lines and patient derived AML cells that single agent treatment with αCD37-DM1 significantly decreases leukemic burden and improves overall survival. Collectively, our data show that the unique internalization properties of CD37 in AML blasts allows for high efficacy of αCD37-DM1 despite low levels of surface expression. Thus, CD37 surface expression need not be an inclusion criterion for treatment with αCD37 ADCs.

As CD37 is not restricted to AML blasts but is found on other hematopoietic cell subsets, we assessed the potential toxicity of αCD37-DM1 in hematopoietic cells, including hematopoietic stem cells (HSCs). We generated a humanized CD37 knock-in mouse model and performed serial colony forming unit assays with normal CD34+ CD38- HSCs to address the possibility that αCD37-DM1 could be cytotoxic to normal HSCs. Our colony forming unit assays showed that HSCs, both from G-CSF mobilized donors and from unstimulated healthy bone marrow aspirates, are not significantly affected by αCD37-DM1 treatment as the majority of samples formed colonies in response to treatment with αCD37-DM1 and continued through serial replating. Furthermore, treatment of our humanized CD37 knock-in mice with αCD37-DM1 resulted in on target B lymphocyte reduction but no myelosuppression, decreased marrow cellularity or aplasia. These data are consistent with what has been documented with this ADC in the clinical setting, in which therapy with αCD37-DM1 causes moderate cytopenias in a minority of patients[29]. Thus, our work with this ADC suggests that αCD37-DM1 preferentially targets and kills leukemic blasts and largely spares normal hematopoietic cells, providing hope that we have finally identified a therapeutic target in AML which will result in cytotoxicity of AML blasts without major myelosuppression.

In summary, our results demonstrate that CD37 is ubiquitously expressed on AML blasts and that treatment with an anti-CD37 ADC provides an effective, swift and potentially safer option for patients as it lacks the profound myelotoxicity of most of the current up front treatment options. Somewhat uncommon to antibody directed therapy, CD37 surface expression is not correlated

with efficacy which may be due to the unique kinetics of membrane trafficking of CD37 in AML blasts. While we were unable to associate pathologic characteristics to membrane trafficking and cytotoxic activity within the cohort of samples evaluated within our studies, additional correlative studies within a larger cohort would be instrumental in identifying a biomarker for cytotoxic activity. Overall, we have demonstrated that targeting CD37 is a novel and highly promising approach for AML and that further evaluation of CD37 targeting agents such as αCD37-DM1 in clinical trials is warranted. The presence of CD37 on the majority of AML blasts provides evidence that this antigen might serve relevance for other directed immune therapies such as bispecific antibodies and chimeric antigen receptor T-cells.

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Authorship Contributions: E.J. performed experiments, analyzed results, interpreted data and wrote the manuscript. E.A., C.C, and D.E. performed experiments and analyzed results. K.Z., R.W., B.H., and A.M. set-up, performed and performed histopathology for mouse studies. P.B., P.H., C.C., C.L. and N.J.V. performed the confocal and epifluorescent imaging. S.O., K.W., Z.H., T.C., and S.E. provided guidance and materials for genetic editing experiments and sequencing analyses. C.M. performed FACS sorting. R.L., N.G., E.H., N.M., and B.L.M provided editorial comments. X.M. performed statistical analyses. K.T.L. and J.C.B designed the studies, supervised the studies, interpreted data and provided editorial comments. K.T.L. and J.C.B. are co-corresponding authors and contributed equally to this work.

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Table 1A: Primary AML samples used for *in vitro* assays

Table 1B: Primary AML samples used for *in vitro* assays

Table 2: Leukemic cells used in *in vivo* studies.

Table Legends:

Table 1. Primary AML samples used for *in vitro* assays. (A) Molecular mutations, ELN classification, CD37 expression (expression of cryopreserved samples after resting 12-16 hours overnight),and fold change αCD37-AF647 (fluorescence αCD37-AF647 2h at 37C – surface αCD37-AF647)/surface αCD37-AF647) on primary AML samples used in internalization assays. **(B)** Molecular mutations, ELN classification, CD37 expression (expression of cryopreserved samples after resting 12-16 hours overnight), and number of colonies formed in response to treatment with αCD37-DM1 normalized to negative control displayed for primary samples used in colony forming unit assays.

Table 2. Leukemic cells used in *in vivo* studies. Cytogenetics, molecular mutations, immunophenotype, 2017 ELN classification and expression of CD37 (ΔMFI CD37) listed for the THP-1 AML cell line and primary AML samples (AML 065 and AML 066) used for *in vivo* models of AML.

Sample ID	Cytogenetics	Molecular Mutations	ELN classification	CD37 Expression (ΔMFI)	Fold change αCD37-AF647
AML 060	Normal	CEBPA, JAK3, NRAS	Favorable	0.08	3
AML 062	t(8;21)	FLT3-TKD, PTEN, SF3B1	Favorable	0.1	1.3
AML 072	Complex	CEBPA, NOTCH1, TP53	Adverse	0.16	0.5
AML 065	No dividing cells	ASXL1, NPM1, TET2	Favorable	0.2	2.5
AML 004	Normal	FLT3-ITD, NPM1	Intermediate	0.26	3.2
AML 047	del 13q	DNMT3A, MPL, GATA2	Intermediate	0.3	16.2
AML 067	Normal	NA	Intermediate	0.43	7
AML 068	Normal	NPM1	Favorable	0.85	6.9
AML 006	NA	NA	NA	0.94	12.3
AML 066	t(6;11)	FLT3-TKD	Adverse	1.11	8.2
AML 059	Normal	ASXL1, IDH2, KRAS, NRAS, SRSF2	Adverse	1.67	9.1
AML 079	Complex	FLT3-ITD, NPM1, DNMT3A	Adverse	2.61	7.6
AML 045	Complex	DNMT3A, FLT3-ITD, NPM1	Adverse	3.24	10.7
AML 013	Normal	NRAS, KMT2D, DNMT3A, RAD21	Favorable	4.78	1.7
AML 043	del 9q, trisomy 21	CEBPA	Favorable	6.65	3.9
AML 032	Complex	TP53	Adverse	17.65	2.2
AML 097	Complex	NA	NA	NA	7.79
AML 098	Normal	FLT3-ITD	Adverse	NA	5.64
AML 099	Trisomy 11	FLT3-ITD	Adverse	NA	3.66
AML 064	Normal	ASXL1, CEBPA, IDH2, KRAS, SRSF2	Intermediate	NA	2.5

AML 100	t(9;22)	NPM1	Favorable	NA	1.1
AML 101	Normal	FLT3-ITD	Adverse	NA	1

Sample ID	Molecular Mutations	ELN Classification	CD37 Expression (ΔMFI)	Colonies Formed (Normalized to Negative Control)
AML 001	No data	Favorable	0.18	0.7
AML 002	No data	Adverse	2.68	0.9
AML 004	No data	Intermediate	0.26	0.5
AML 006	IDH-1, FLT3-ITD	NA	0.94	0.1
AML 011	FLT3-ITD	Favorable	0.15	0.8
AML 013	None found	Favorable	4.78	0.8
AML 015	FLT3-ITD, NPM1	Intermediate	0.14	0.2
AML 025	No data	Adverse	3.33	0.9
AML 046	TP53	Adverse	0.28	0.8
AML 048	NPM1, IDH1, DNMT3A	Favorable	0.21	0.3
AML 062	FLT3-TKD, PTEN, SF3B1	Favorable	0.1	0.0
AML 065	NPM1, TET2	Favorable	0.2	0.0

Sample ID	CD37 Expression (ΔMFI)	Reduction in absolute blasts (Normalized to Isotype control)
AML 001	0.18	0.5
AML 002	2.68	0.9
AML 003	ND	0.5
AML 004	0.26	1.0
AML 005	0.34	0.9
AML 006	0.94	0.9
AML 013	4.78	1.0
AML 024	ND	0.5
AML 025	3.33	1.3
AML 047	0.3	1.1
AML 049	0.11	0.4
AML 060	0.08	1.0
AML 062	0.1	0.1
AML 067	0.43	0.8
AML 068	0.85	1.0
AML 069	ND	0.5
AML 070	ND	0.9
AML 071	ND	0.5

	THP-1	AML 065	AML 066
Cytogenetics	Complex	Insufficient Metaphases	t(6;11)
Molecular Mutations	NRAS, TP53	NPM1, TET2	FLT3-TKD
Immunophenotype	$CD34^-$, $CD33^+$, $HLA\text{-}DR^+$	$CD34^-$, $CD33^+$, $CD117^-$, $CD11b^+$, $CD14^-$, $HLA\text{-}DR^-$	$CD34^-$, $CD33^+$, $CD117^-$, $CD11b^+$, $CD14^-$, $HLA\text{-}DR^+$
ELN	Adverse	Favorable	Adverse
ΔMFI CD37	6.86	0.2	1.11

Figure Legends

Figure 1. CD37 is expressed on the surface all primary AML blasts. (A) CD37 surface expression on normal hematopoietic stem cells (HSCs), normal donor B cells, T cells, NK cells, monocytes and AML. HSCS n=5; B cells n=3; T cells n=3; NK cells n=3, Monocytes n=3. AML = 55. Mean ± SEM reported. **(B)** CD37 expression on primary AML blasts grouped by 2017 ELN classification. Favorable n = 17. Intermediate n= 13. Adverse n = 23. Mean ± SEM reported. **(C)** Oncoprint for a cohort of 55 cryopreserved untreated, newly diagnosed AML samples in which CD37 expression was assessed 12h post-cryopreservation.

Figure 2. CD37 rapidly internalizes in AML blasts. **(A)** Schematic of internalization assays in which the fold change in αCD37-AF647 expression over the course of 2 hours was determined (fluorescence αCD37-AF647 2h at 37C – surface αCD37-AF647)/surface αCD37-AF647) **(B)** Fold change in αCD37-AF647 of primary AML blasts, normal donor B cells and normal donor monocytes. AML n=21; B cells n=4; monocytes n=4. Mean ± SEM reported. **(C)** Fold change in αCD37-AF647 of primary AML blasts with and without mutations in activated signaling genes. AS mut n= 13; Other n= 6. Mean ± SEM reported. **(D)** Fold change in αCD37-AF647 in AML cell lines. MV-411 n=3; THP-1 n=3; OCI-AML3 n=3; MV-411 KO n=3; THP-1 KO n=3; OCI-AML3 KO n=3. *$p < 0.05$, ** $p < 0.01$. Mean ± SEM reported. **(E)** Representative epifluorescent images of CD37+ and CD37- AML cell lines after incubation with αCD37-AF647 at 37C over the course of 2 hours. Scale bar is 10 um.

Figure 3. αCD37-DM1 is cytotoxic to AML cell lines and primary AML blasts but not HSCs in vitro. **(A)** CD37+ and CD37- AML cell line response to Iso-DM1 (blue) and αCD37-DM1 (red) treatment assessed by mitochondrial activity. OCI-AML3 n=3; OCI-AML3 KO n =5; $p < 0.0001$ for OCI-AML3 at concentrations greater than 0.125 ug/ml. Mean ± SEM reported. **(B)** Percent annexin+ PI+ cells in response to 0.125ug/ml Iso-DM1 and αCD37-DM1 treatment for 72 hours

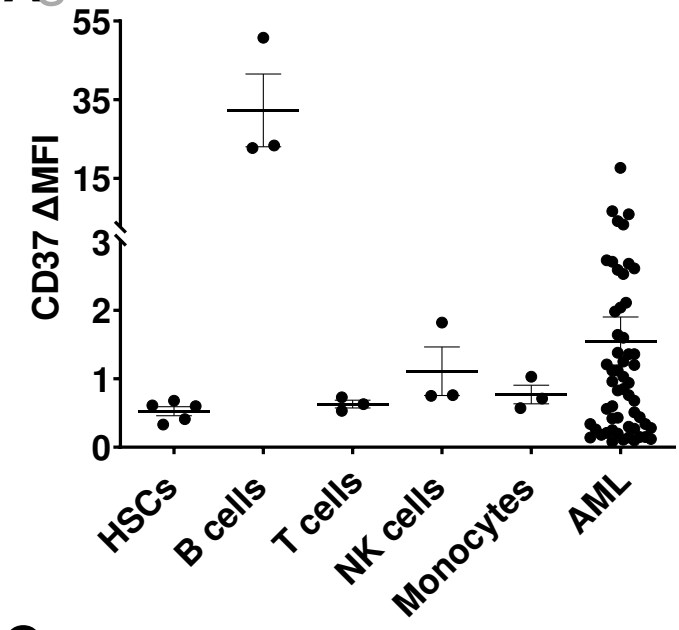
in CD37+ and CD37- AML cell lines. MV411 n=3; THP-1 n=3; OCI-AML3 n=3; MV-411 KO n=3; THP-1 KO n=3; OCI-AML3 KO n=3; **p < 0.01. Mean ± SEM reported. **(C)** Frequency of live cells staining positively for PARP-1 in response to treatment with 1.25 ug/ml Iso-DM1 and αCD37-DM1 for 48 hours. MV-411 n=3; OCI-AML3 n=4; THP-1 n=4; OCI-AML3 KO n=3. **p < 0.01. Mean ± SEM reported. **(D)** Number of colonies formed per 1000 leukemic stem cells plated for primary AML samples treated with Iso-DM1 and αCD37-DM1. n=13. **p < 0.01. Mean ± SEM reported. **(E-F)** Number of colonies formed in unstimulated normal donor bone marrow (BM) aspirates and G-CSF mobilized CD34+ CD38- hematopoietic stem cells (G-CSF mobilized HSCs) treated with vehicle, Iso-DM1 and αCD37-DM1. First plating BM aspirates n=4. G-CSF mobilized HSCs n=5. Mean ± SEM reported. Second plating BM aspirates n=3, G-CSF mobilized HSCs n=2. Mean ± SEM reported. Third plating BM aspirates n=3, G-CSF mobilized HSCs n=2. Mean ± SEM reported

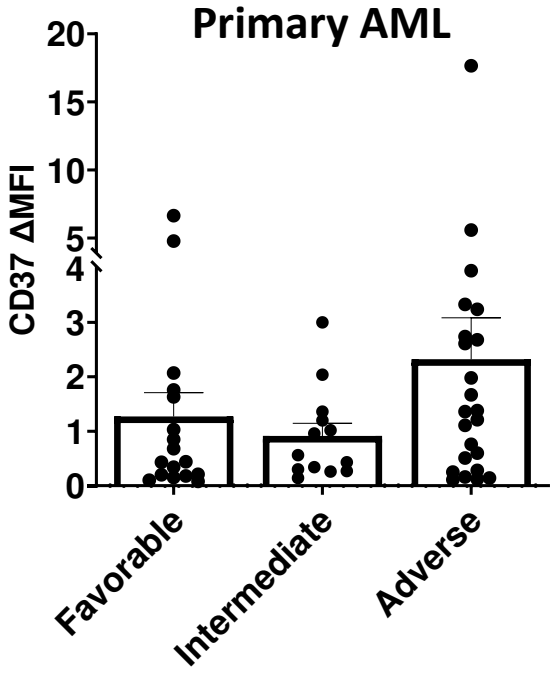
Figure 4. αCD37-DM1 shows few on-target hematopoietic toxicities in humanized CD37 mouse model. (A). Schematic of experimental design. **(B)** Baseline CD37 expression on B cells, T cells, monocytes and neutrophils from a male, heterozygous humanized CD37 knock-in mouse**.** **(C)** Complete blood counts for homozygous and heterozygous humanized CD37 mice treated with αCD37-DM1 and Iso-DM1. $hCD37^{+/+}$, Iso-DM1 n= 5; $hCD37^{+/+}$, αCD37-DM1 n= 5; $hCD37^{-/-}$, Iso-DM1 n=5; $hCD37^{-/-}$, αCD37-DM1 n= 5. Error bars represent ± SEM. **(D)** Proportion of lymphocytes of total CD45+ live cells. **(E)** Histopathology images performed on bone marrow specimens from humanized CD37 knock-in mice treated with αCD37-DM1 , Iso-DM1 and vehicle treated mice at 40x magnification.

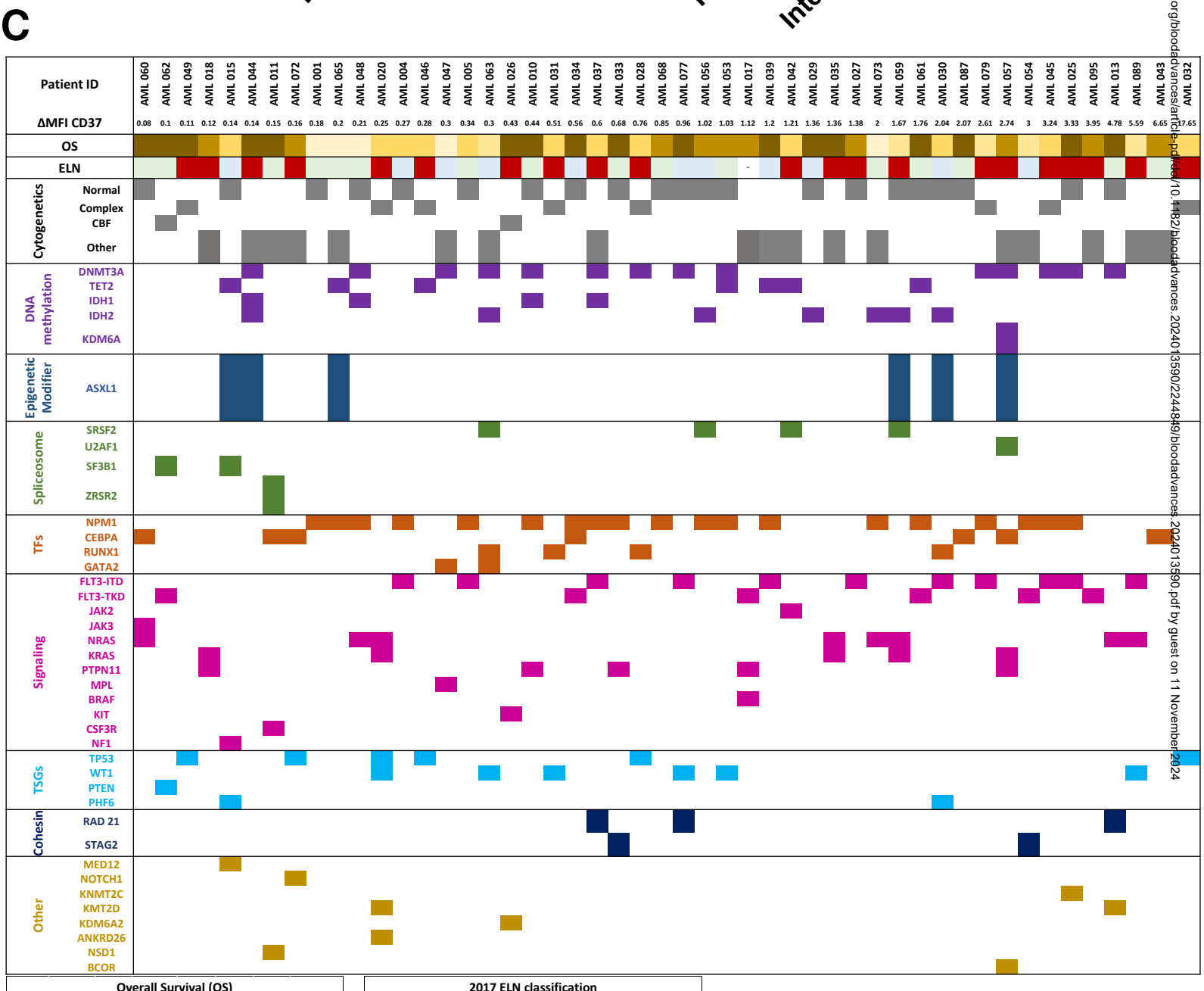
Figure 5. αCD37-DM1 improves overall survival, decreases circulating disease and decreases leukemic burden *in vivo.* (A) Schematic of *in vivo* studies with αCD37-DM1. **(B)** Overall survival for THP-1 xenograft. n=10. **p < 0.01, **** p < 0.0001. Mean ± SEM reported. **(C)** Percent hCD33+ leukemic cells present in liver, bone marrow and spleen in THP-1 xenograft.

p < 0.01, ***p < 0.001, ****p < 0.0001. Mean ± SEM reported. **(D) Percent circulating hCD45+ leukemic cells in PDX 1. Mean ± SEM reported. **(E)** Percent hCD45+ leukemic cells present in liver, bone marrow and spleen in PDX-1. ***p < 0.001, **** p < 0.0001**.** Mean ± SEM reported. **(F)** Percent circulating hCD45+ leukemic cells in PDX-2. Vehicle n=3; Iso-DM1 n=4; αCD37-DM1 n=3. **(G)** Overall survival for PDX-2. Vehicle n=3; Iso-DM-1 n=4; αCD37-DM1 n=3. *p < 0.05. Mean ± SEM reported.

Figure 1







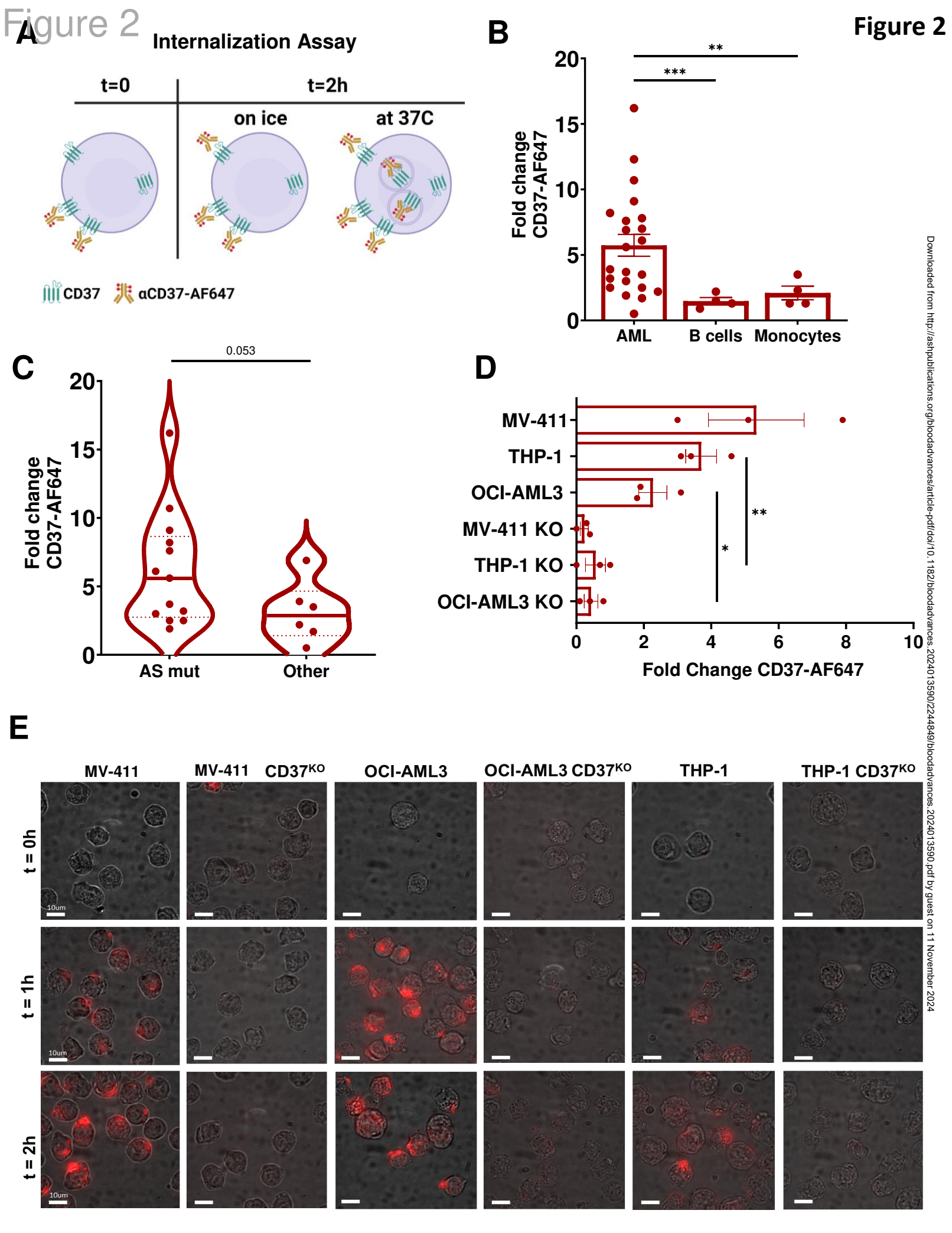
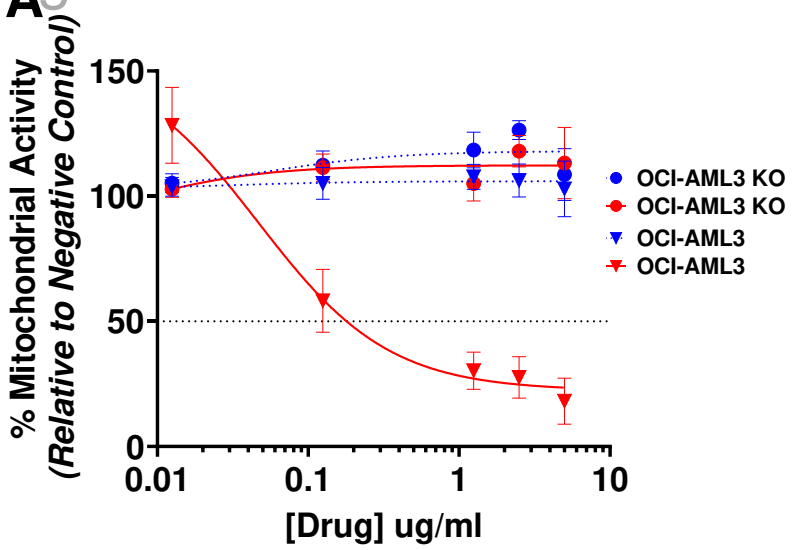
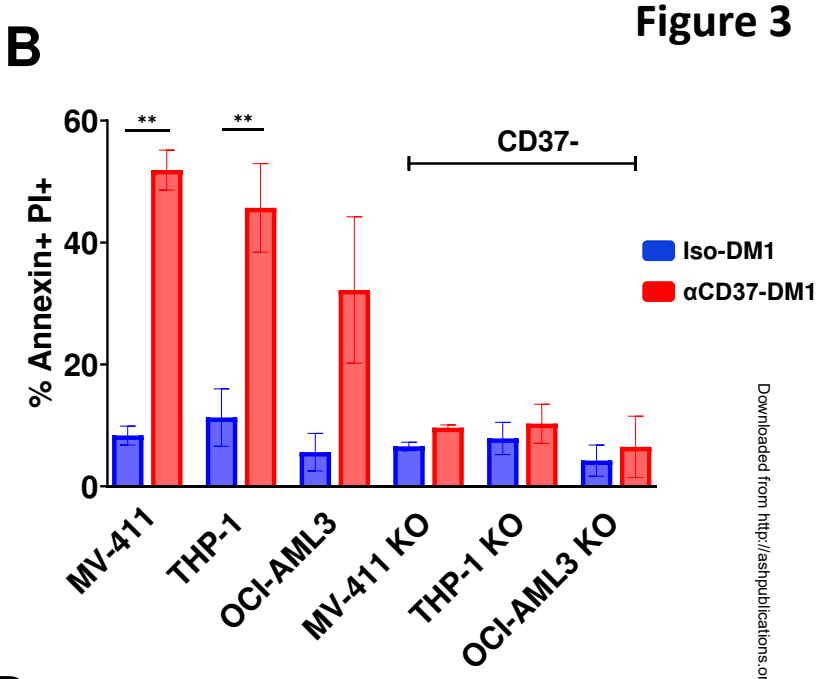
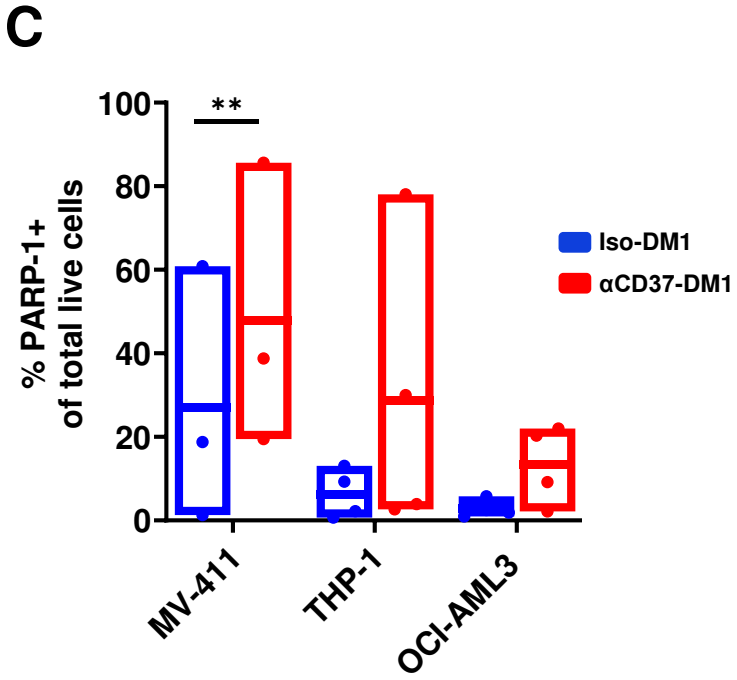
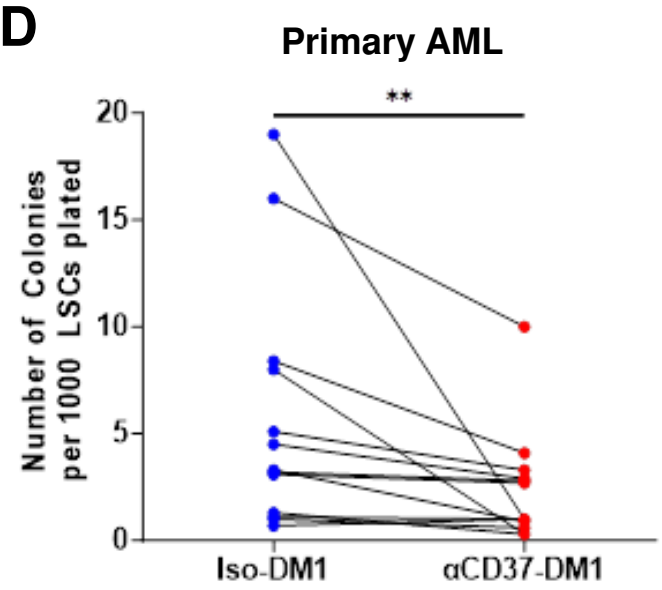


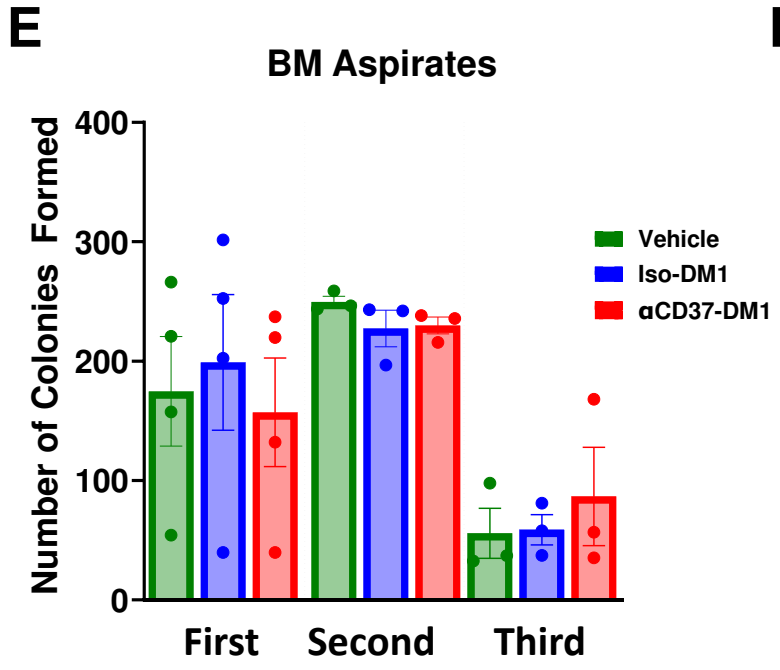
Figure 3











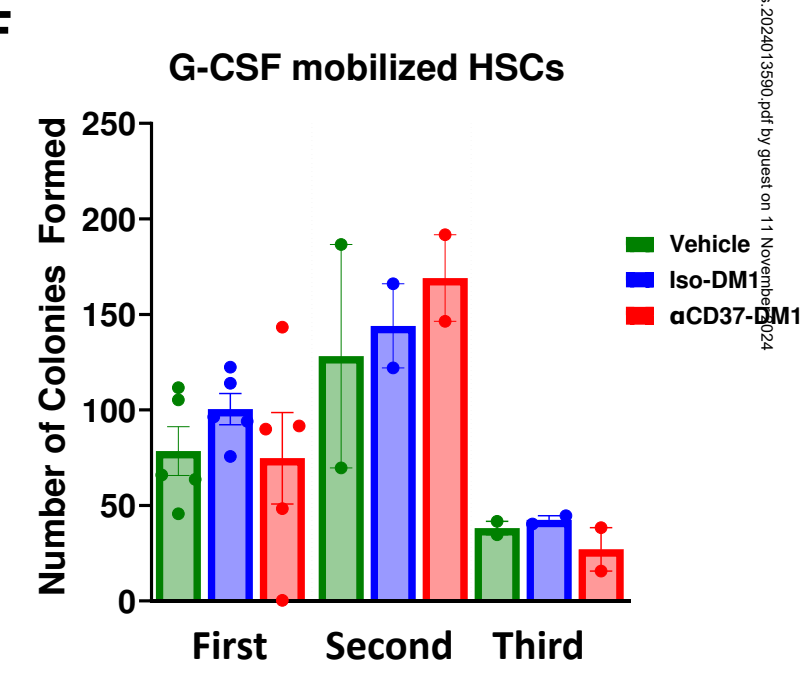
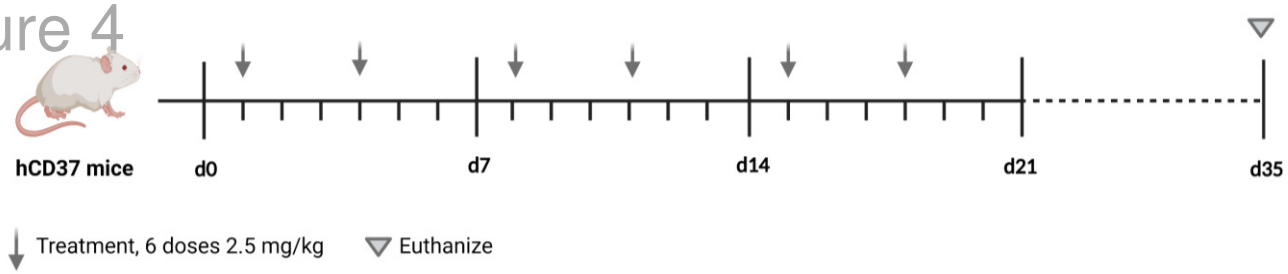


Figure 4

A



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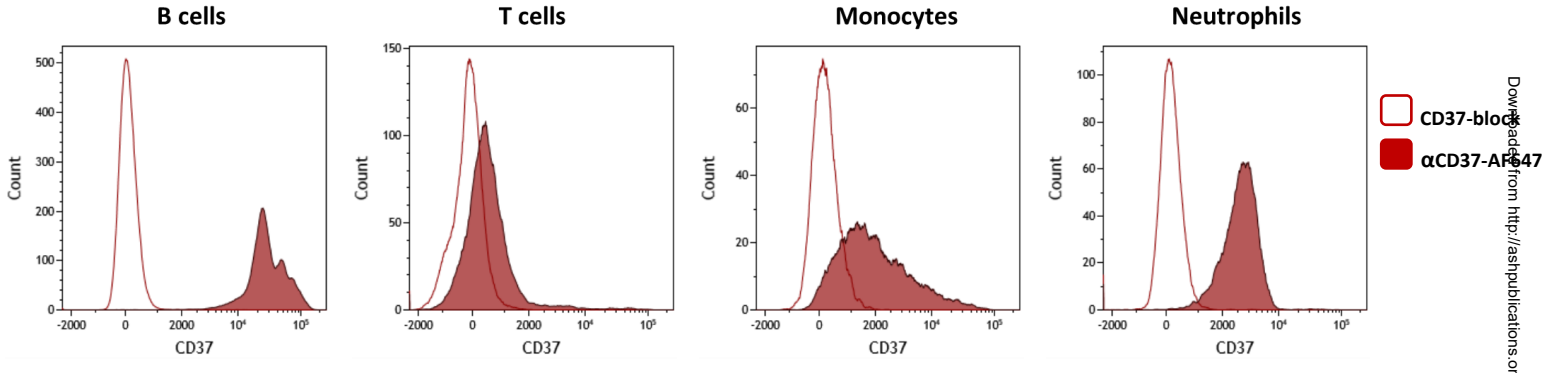

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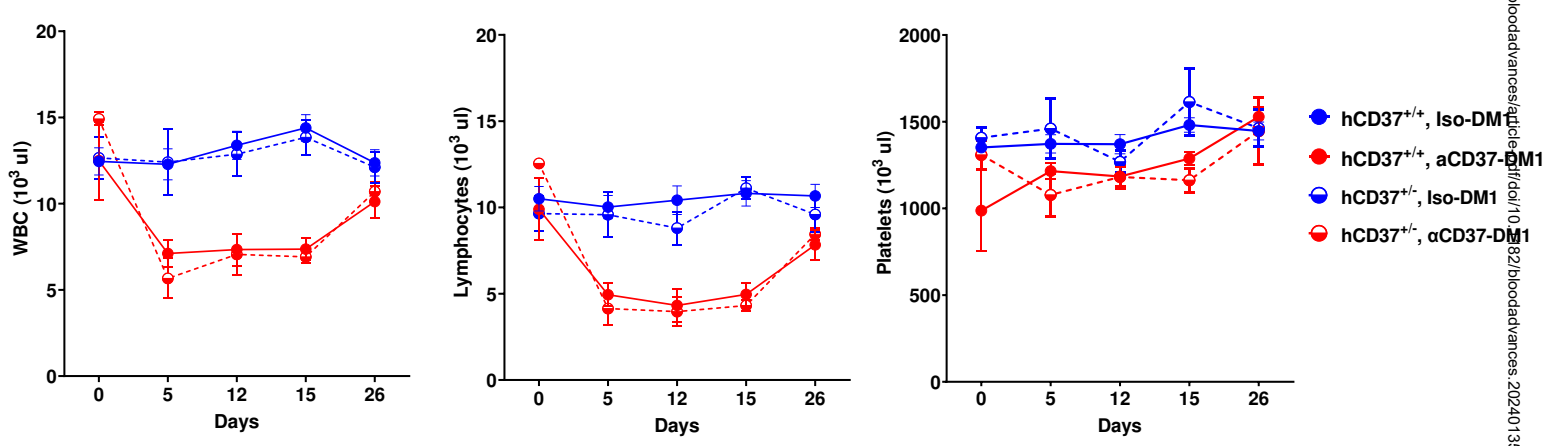

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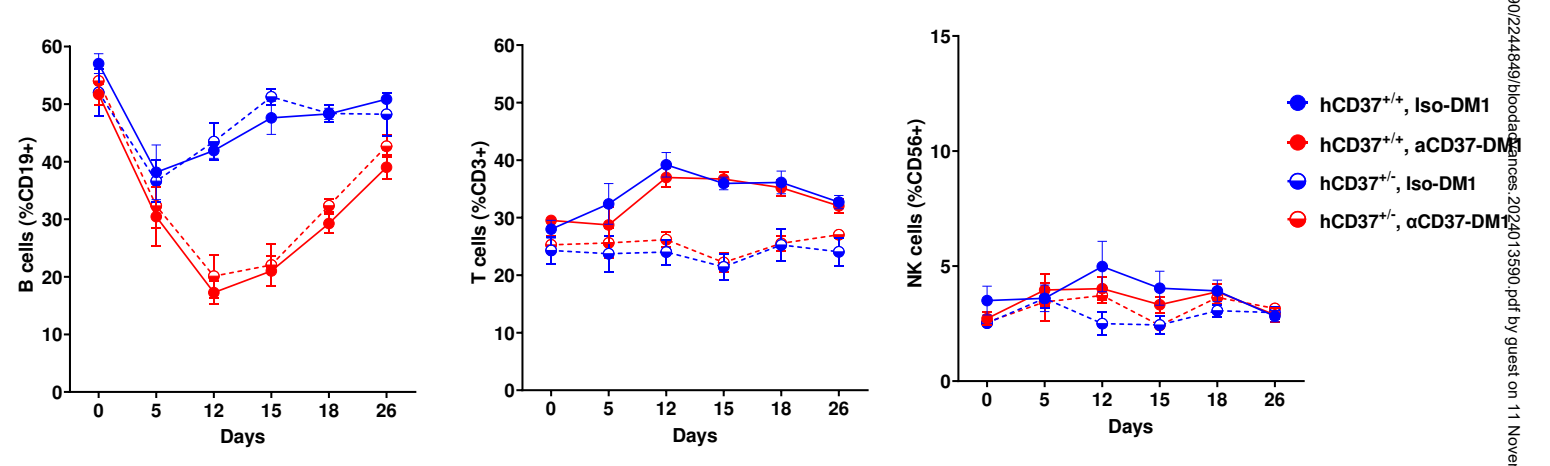

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