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Targeting the 5T4 oncofoetal glycoprotein with an antibody drug conjugate (A1mcmmaf) improves survival in patient derived xenograft models of acute lymphoblastic leukemia

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Running Title: **Targeting residual ALL subclones**

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Abstract

Outcome in childhood acute lymphoblastic leukemia is prognosticated on levels of minimal residual disease after remission induction therapy. Higher minimal residual disease levels are associated with inferior results even with intensification of therapy, and suggest identification and targeting of minimal residual disease cells as a therapeutic strategy. Here we identify high expression of 5T4 in subclonal populations of patient derived xenografts from patients with high post induction minimal residual disease levels. 5T4 positive cells showed preferential ability to overcome the NOD-*scid*IL2R γ^{null} mouse xenograft barrier; migrated *in vitro* on a CXCL12 gradient; preferentially localised to bone marrow *in vivo* and displayed ability to reconstitute the original clonal composition on limited dilution engraftment. Treatment with A1mcMMAF (5T4-Antibody Drug conjugate) significantly improved survival without overt toxicity in mice engrafted with a 5T4 positive acute lymphoblastic leukemia cell line. Mice engrafted with 5T4 positive patient derived xenograft cells, were treated with combination chemotherapy or dexamethasone alone and then given A1mcMMAF in the minimal residual disease setting. Combination chemotherapy was toxic to NOD-*scid*IL2R γ^{null} mice. While dexamethasone or A1mcMMAF alone improved outcomes, the sequential administration of dexamethasone and A1mcMMAF significantly improved survival ($p=0.0006$) over either monotherapy. These data show specifically targeting minimal residual disease cells improved outcomes and support further investigation of A1mcMMAF in high risk B-cell precursor acute lymphoblastic leukemia patients identified by 5T4 expression at diagnosis.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer of childhood. Intensive combination chemotherapy produces cure rates ~90% but is associated with considerable morbidity. Recent high throughput genetic analyses have successfully identified new genetic subtypes^{1, 2} as well as aberrant epigenetic³ and signalling pathways^{4, 5} that are potential targets for precision therapy⁶. The paradigm for targeted treatment is the subset of children with a BCR-ABL1 fusion where the addition of the tyrosine kinase inhibitor imatinib to intensive chemotherapy improved outcomes significantly^{7, 8}. More recently, immunological therapy targeting antigens expressed by B-cells using monoclonal antibodies with or without payloads⁹ and/or activating cytotoxic T-cells are showing great promise¹⁰. Thus we are now on the cusp of a change from iteratively derived non-specific chemotherapy to a designed targeted approach.

We recently reported that the 5T4 oncofoetal glycoprotein (also known as trophoblast glycoprotein (TPBG) and WNT-activated inhibitory factor 1 (WAIF1) is upregulated in high-risk cytogenetic subgroups and overexpressed on the plasma membrane of lymphoblasts obtained at relapse, in patients with B-cell precursor (BCP) ALL¹¹. 5T4 is a 72-kDa N-glycosylated transmembrane protein expressed by syncytiotrophoblasts in the placenta. Most mature cells including lymphoid cells do not express it. 5T4 is associated with differentiating embryonic stem cells^{12, 13}, and mechanistically associated with the directional movement of cells through the regulation of epithelial mesenchymal transition¹²⁻¹⁴, facilitation of CXCL12/CXCR4

chemotaxis^{15, 16} and favouring non-canonical over canonical WNT/ β -catenin pathway signalling^{17, 18}. 5T4 is expressed by tumour initiating cells in human non-small cell carcinomas¹⁹ and expressed by a number of carcinomas²⁰. The selective pattern of 5T4 tumour expression, its association with a tumour-initiating phenotype plus a mechanistic involvement with cancer spread has stimulated the development of 5T4 vaccine, 5T4 antibody targeted-superantigen and 5T4 antibody-drug conjugate (ADC) therapies through preclinical and into clinical studies^{21, 22}. The ADC is a 5T4 humanized monoclonal antibody (A1) linked by sulfydryl-based conjugation delivering a microtubule-disrupting agent, monomethyl auristatin F (MMAF) via a maleimidocaproyl linker. A1mcMMAF has shown potent activity in a variety of solid tumour models, with induction of long-term regression after the last dose and no significant toxicity in a simian model²³ and tolerable toxicity in patients with solid tumours²⁴.

Murine models of childhood ALL suggest that minimal residual disease (MRD) after therapy is represented by a rare cell population that combines the phenotypes of bone marrow microenvironment-mediated dormancy, stemness, and *in vivo* drug resistance²⁵. We previously reported that a BCP-ALL cell line had a subpopulation of cells that expressed 5T4 (5T4+) and these cells showed migration on a CXCL12 axis and a differential dissemination and infiltration in a mouse model when compared to the 5T4 negative (5T4-) subpopulation. A 5T4 mouse antibody targeted superantigen combined with human peripheral blood mononuclear cells showed activity *in vitro* and *in vivo*¹¹. Here we report that 5T4+ subclones are present in expanded numbers in patient derived xenografts (PDX) samples obtained

from patients with high levels of post induction minimal residual disease (MRD). When used in combination with chemotherapy, A1mcMMAF showed specific activity in 5T4+ PDX models of ALL.

Methods

BCP-ALL Patient Derived Xenografts (PDX) samples

Two categories of PDX samples were assessed, based on the reported post induction MRD levels in the patients from which they were derived. For the purposes of this paper, those with MRD levels $\leq 10^{-4}$ (SR03, SR014, SR_M1 and SR_M2) were classified as standard risk (SR) and those with higher levels (HR08, VHR03, HR_M1 and HR_M2) as high risk (HR) (Supplementary Table 1) ²⁶. $1-2 \times 10^6$, or fewer, PDX cells obtained from splenic fractions at were transplanted intravenously (iv) into non-irradiated 6-10 week old NOD-*scid*IL2R γ^{null} (NSG) mice (group size 6 unless stated). Engraftment was assessed in tail vein blood samples using flow cytometry. Comparative engraftment rates, sites of engraftment and tumour load were assessed by harvesting animals at various time points. Relative clonogenicity was determined by limiting dilution engraftment analysis in NSG mice as previously described ²⁷. The proportions of 5T4 leukaemic blasts were quantified by flow cytometry. Studies were approved by Thameside and Glossop Research Ethics Committees (Manchester, UK: Reference 07/Q1402/56). All procedures conformed to the regulations of the UK Animal Licence Act.

Flow cytometry

Individual or multiplex flow cytometry for 5T4¹¹ and human specific CD45 (CD45 APC-Cy7 or PE-Cy7), CD34 (FITC or PE-Cy7), all eBioscience; Hatfield, UK), were performed with directly conjugated isotope and appropriate controls for individual and multiplex analyses; cross channel

fluorescence was compensated using the FlowJo matrix software (FlowJo LLC, Oregon, USA). *In vivo* leukemia engraftment was analysed using 25 µl of heparinised peripheral blood after red blood cells lysis (eBioscience) by human CD45 flow cytometry. The overall disease burden was determined by expression of the ratio of human to mouse CD45 positive blasts per sample. Peripheral blood cellular component analyses were performed using a XE-2100 automated haematology system (Sysmex, Milton Keynes, UK).

Migration Assays

Were performed as previously described ¹¹.

5T4 depletion

Depletion and enrichment of 5T4 positive blasts from PDX samples was performed using Magnetic-Activated Cell Sorting (MACS) microbeads and columns from Miltenyi Biotec (Surrey, UK), and a 5T4 specific monoclonal antibody (mAb) ¹¹ conjugated to a PE fluorochrome using the EasyLink R-Phycoerythrin Conjugation Kit from Abcam (Cambridge, UK).

Antibody-drug conjugate therapy

Sup5T4 Lenti/Luc/mCherry leukemia cells ¹¹ (5×10^6) were given ip and different BCP-ALL PDX samples at various doses were given iv to NSG mice. Mice were treated with either A1-mcMMAF or control-ADC (Neg-8-8-hG1mcMMAF) at 5mg/kg intraperitoneally (ip) beginning 7 days after tumour challenge with a cycle of 3 or 4 doses of ADC given at 4 day intervals (treatment block of 12-16 days) and in some cases further ADC cycles were

repeated after a gap of one week, or mice were given no therapy²³ (Supplemental Table 2). Sup5T4 B-ALL ip challenge was monitored by IVIS¹¹. For other PDX samples tumour engraftment was assessed by peripheral blood monitoring and at an appropriate point, comparison of tumour burden assessed by flow cytometry in cell suspensions from spleen and bone marrow. Two weeks of a vincristine (V), dexamethasone (X) and asparaginase (L), dosing protocol established in NSG mice²⁸, was followed by various cycles of ADC starting at different times after HR08 primagraft challenge. A similar protocol using dexamethasone (DEX) alone followed by A1mcMMAF was also tested. Efficacy was determined by measurement of BCP-ALL engraftment and survival.

Statistical Analysis

With the exception of long-term survival analyses the data presented are representative of three or more experiments. Graph-Pad Prism software was used for individual or multiple group comparisons by 2- tailed Student's t-test or ANOVA-Tukey; survival analysis used Kaplan-Meier plots, Log-Rank Mantel-Cox and regression analysis for hazard ratios (HZR).

Results

5T4 expressing PDX have an aggressive phenotype in NSG mice

Four SR and 4 HR BCP-ALL PDX samples were analysed for 5T4 cell surface expression by flow cytometry. All 4 HR B-ALLs had significant (20-80%) proportions of 5T4+ cells while 5T4 was undetectable in all 4 SR PDX samples (Figure 1A). The overall leukaemogenicity was generally reflected in the rate of tumour engraftment and time to morbidity as HR primgrafts with > 50% 5T4 positivity showed earlier engraftment and death than SR primgrafts (Figure 1B). When pooling the data, 5T4+ HR PDX showed a significant faster engraftment time when compared to 5T4 negative SR PDX, with median survivals of 71 versus 280 days respectively ($P < 0.0001$).

5T4 expression correlates with leukemia engraftment and time to morbidity *in vivo*

In this study all 5T4+ PDX belonged to the MRD positive HR group. The ability of a high MRD phenotype in overcoming the xenograft barrier²⁶ leading to shorter times to leukemia in NSG mice have been previously described²⁹. To further investigate leukaemic engraftment according to the 5T4 phenotype, depletion and enrichment fractionations using a MACs system and a 5T4 specific monoclonal antibody were performed on HR08 PDX cells. The efficacy of MACs separation of 5T4 positive blasts is illustrated by Figure 2 A. From a starting population of 76.8% 5T4+ leukaemic cells, there was $\geq 96\%$ enrichment or depletion. Transplantation of 2×10^6 mock, 5T4 depleted or enriched cells led to a significant delay ($P = < 0.05$) in engraftment kinetics for 5T4 depleted blasts (Figure 2B). At morbidity, post mortem analyses of

splenic content revealed that all animals, independent of fractionation, exhibited recapitulation of the parental heterogeneous HR08 phenotype, with approximately 75% of the blasts expressing 5T4 (Figure S1). The delay in engraftment did not correlate with improved animal survival (Figure 2C). Such subclonal recapitulation of the original leukaemic population with similar survival times has been previously described, reflecting the plasticity of the BCP-ALL cell ³⁰. It is also possible that MACS separation was incomplete and up to 80,000 residual 5T4 positive blasts persisted in the depleted challenge doses. A limited dilution experiment performed using either a 1000 or 100 cell challenge comparing mock versus 5T4 depleted engraftment showed a significant impact of 5T4 depletion on engraftment (Figure 3A). This also translated into significantly improved survival (Figure 3B). Thus in the HR08 BCP-ALL PDX 5T4 positive blasts were the most clonogenic of a heterogeneous leukaemic population.

Chemotaxis of 5T4 positive PDX cells on a CXCL12 gradient

In normal culture, SupB15 BCP-ALL 5T4+ cells differentiate to produce 5T4 negative cells with concurrent loss of CD34 expression and a more mature phenotype ¹¹. These changes were concordant with reduced CXCL12 mediated chemotaxis, lower production of matrix metalloproteases and modulation of the activation state of integrins, consistent with a reduced capacity to populate extra-medullary sites ¹¹. The responses of PDX cells to a CXCL12 chemokine gradient or a positive control gradient of foetal calf serum (FCS) were examined. All PDX samples migrated actively along a FCS gradient, confirming the functional viability of the cells (Figure 4 A). HR but not

SR PDX cells were chemotactically responsive to CXCL12 and this was inhibited by pre-incubation with 5T4-specific monoclonal antibody but not the IgG1 control (Figure 4 B). Further evidence that 5T4 positive blasts were the predominant responders to the chemokine gradient was provided by preferential accumulation of 5T4 expressing PDX cells in the chemoattractant chamber in response to CXCL12 but not FCS (Figure S2 A). NSG mice have high expression of CXCL12 in the bone marrow³¹. In the HR08 PDX model, peripheral blood and harvested spleens of NSG mice were composed of approximately 75% 5T4 positive blasts, while >95% of the HR08 cells recovered from the femoral flushes expressed 5T4 (Figure S2 B). Thus both *ex vivo* and *in vivo* evidence suggest that 5T4 positive blasts respond to a CXCL12 gradient.

***In vivo* A1mcMMAF therapy of Sup5T4 BCP-ALL cell line**

To assess the efficacy of the A1mcMMAF, mice were engrafted intraperitoneally with Sup5T4 Lenti/Luc/mCherry¹¹ (5×10^6) and treatment initiated one week later with control-ADC or A1mcMMAF. Bioluminescent imaging used to monitor leukemic engraftment showed that both one and two cycles of A1mcMMAF were effective in limiting tumour growth compared to control-ADC (Figure 5 A-B). Control-ADC had some impact in comparison to the untreated tumours (Figure 5 B), possibly through Fc binding on the blasts in the peritoneal cavity (untreated versus 1 cycle or 2 cycle control-ADC; $p < 0.05$ and $p < 0.01$). 5T4 targeted ADC treatments significantly prolonged survival when compared to untreated or control-ADC ($p = 0.04$) (Figure 5 C). Though median survival was 133 days in those who received two cycles of

A1mcMMAF, compared to 113 days in those who received one, this was not statistically significant ($p = 0.25$). Cessation of the administration of A1mcMMAF (day 37 for second cycle) correlated with increased tumour growth and mCherry positive leukemia cells isolated post therapy from the ovary, a common extra medullary site of Sup5T4 tumour spread¹¹, retained the parental 5T4 phenotype (Figure S3). This suggests the potential for continuing multiple cycles of therapy.

In vivo A1mcMMAF therapy of B-ALL PDX

A significant therapeutic effect of A1mcMMAF on a leukaemic cell line that uniformly expresses 5T4 may not be as effective in leukemia with heterogeneous 5T4 expression. To test this, mice were challenged iv with 2×10^6 HR08 (~75% 5T4 positive) cells, and one week later either remained untreated or received three cycles (4 doses every 4 days, repeated after 1 week, last dose on day 61) of control or A1mcMMAF. The efficacy of therapy was monitored by the weekly analysis of peripheral blood for the presence of human tumour cells as a measure of leukemia engraftment. A1mcMMAF administration significantly delayed engraftment (day 66; $p=0.001$), but did not influence overall survival (Figure S4 A-B). Similar results were seen with VHR03 PDX (~56% 5T4 positive) given 2×10^6 iv followed by 2 cycles of ADC (Figure S5). Postulating that this could be due to an excess of tumour cells injected, the experiment was repeated with transplantation of 2×10^3 HR08 cells, followed a week later by three cycles of therapy. At this transplantation dose level, A1mcMMAF treatment significantly reduced engraftment ($p = 0.02$)

and prolonged survival ($p = 0.0014$) (Figure 6 A-B). No effects of the Control-ADC were observed in the PDX challenged mice.

A1mcMMAF in combination with chemotherapy for B-ALL primagraft treatment

Next, the impact of A1mcMMAF in the context of VXL therapy was investigated. Unexpectedly, animals transplanted with 1×10^6 HR08 cells and treated four weeks later suffered rapid and unpredicted adverse reactions after the first course of VXL, prior to the administration of A1mcMMAF, requiring immediate cessation of the experiment (Figure S6 A). Upon autopsy organs displayed signs consistent with tumour lysis syndrome³², and so a subsequent experiment delivered VXL therapy one week after challenge to reduce the target tumour load. Two weeks of VXL therapy was followed by 3 cycles (4 doses every 4 days, last day of treatment day 75) of control or A1mcMMAF treatment. At day 66, animals in the untreated group had a mean tumour engraftment of 69.2% +/- 3.1 while all treated groups exhibited little if any evidence of leukemia in the peripheral blood (Figure S6 B). Leukaemic cells were present in peripheral blood samples by day 85 in both VXL and VXL/control-ADC treated animals with mean engraftments of 18.7% +/- 7.2 and 7.9% +/- 4.8 respectively. No circulating blasts were detected in the peripheral blood of the combination therapy group mice (Figure S6 C). Despite the apparent success of these therapies in reducing leukemia engraftment, no significant impact on overall survival was observed in any of the treated groups (Figure S6 D). Although animals in treated groups reached morbidity at the same time as untreated animals, they did so for very different

reasons. The most typical post mortem sign of leukemia engraftment, marked splenic enlargement, was only evident in animals that had remained untreated. All animals that received VXL therapy reached morbidity with normal or only marginally enlarged spleens, indicating greatly reduced tumour load. Indeed by the termination of the experiment no signs of leukemia had been detected in the marrow, spleens or peripheral blood of any animal that had received VXL therapy and reached morbidity prematurely.

Dexamethasone and A1mcMMAF treatment

To minimise the adverse reactions seen with VXL, an alternative combination protocol of dexamethasone (DEX) followed by 4 cycles of control or A1mcMMAF was tested. Furthermore, general indicators of normal haematopoiesis i.e. haemoglobin, total red and white blood cell and reticulocyte count were measured. Accordingly, 2×10^3 HR08 PDX cells were transplanted. A week later, dexamethasone was given daily (Mon-Fri) for two weeks, followed by four cycles of control or A1mcMMAF (4 doses every 4 days, last dose day 96). No toxicity or myelosuppression was observed during the course of this experiment (Figure S7). Engraftment was noted at days 75 and 100 in untreated and DEX treated mice respectively and at day 108 all groups receiving DEX had significantly less circulating blasts in peripheral blood samples compared to the untreated group (Figure 7 A). By day 129, engraftment in the DEX only, and DEX followed by control-ADC groups had increased and were no longer statistically different to the untreated group while the A1mcMMAF / DEX combination therapy animals peripheral blood remained tumour free (Figure 7 B). By day ~170 engraftment levels in the

DEX and untreated groups were similar, ~ 90% compared to < 20% in the A1mcMMAF/DEX combination (Figure 8 A). The median survival times of untreated, control-ADC/DEX, DEX and A1mcMMAF/DEX animals were 169.5, 144.5, 220 and > 350 days respectively. While DEX treatment was able to confer a significant survival advantage ($p = 0.006$) when employed as a monotherapy, the greatest impact on survival was observed in combination with A1mcMMAF ($p = 0.0006$). (Figure 8 B). The outcomes of DEX/control-ADC treated mice appeared to be inferior to untreated animals ($p = 0.012$). We speculate this to be a consequence of DEX induced upregulation of Fc receptors³³ mediating a non-specific uptake of the microtubule-disrupting agent, leading to increased toxicity and death. The specificity of the A1mcMMAF targets the drug to leukaemic cells presumably avoiding such toxicity in the DEX and A1mcMMAF treated animals.

Discussion

Our data show that development of leukemia was faster with 5T4 enriched cells suggesting that cells that express this protein are better adapted to overcome the xenograft barrier. 5T4+ ALL blasts migrate on a CXCL12 axis and favour migration to the bone marrow environment. The haematopoietic stem cell compartment in NSG mice is thought to facilitate donor over host cell engraftment ³⁴, a potential explanation for the rapid engraftment of 5T4+ cells. In murine xenotransplantation models of childhood ALL, reconstitution of leukemia recapitulates the clinical manifestations of the disease with more rapid engraftment times correlating with a higher risk of therapeutic failure ^{26, 29}. Though our sample size is small, in the model described here, PDX selection was based on the MRD response to therapy. Subclonal populations expressing significant amounts of 5T4 were seen only in the 4 MRD^{hi} PDX's and our previous studies link 5T4 expression to high risk of relapse in paediatric BCP-ALL patients ¹¹. The bone marrow microenvironment provides a protective niche for ALL cells ^{25, 35-38} and in the context of this paper suggests that 5T4+ cells in the protective bone marrow microenvironment niche may survive chemotherapy and significantly contribute to the MRD population that has recently been described ²⁵. Using the MLL primagraft with the highest proportion of 5T4 positive blasts, 5T4 specific antibody/magnetic bead depletion and limiting dilution challenge in NSG mice clearly demonstrated that 5T4 positive blasts are the most clonogenic *in vivo* and consistent with the concept of a leukaemia initiating cell ³⁰. Concomitant with earlier reports of ALL xenografts, limiting dilution studies show that 5T4+ cells are able to recapitulate the original leukemic population demonstrating the

considerable plasticity of the ALL cell ^{26, 27}. In the context of MRD then residual 5T4+ cells could give rise to disease recurrence in patients.

Current treatment strategies for ALL use non-specific cytotoxic drugs. Recently immunological therapy targeting antigens expressed specifically on the surface of the B-cell have generated considerable interest. The target in the majority of these trials has been CD19, expressed on malignant and non-malignant B-cells ³⁹⁻⁴¹. As the antigens are expressed on non-malignant cells, they are also associated with prolonged B-cell suppression as well as the emergence of escape clones no longer expressing CD19. Ideally the target should be expressed selectively by leukemic subclone(s) that remain post-therapy and give rise to recurrences. The problem is not a great deal is known about surface antigens associated with a resistant phenotype. Recently, a chimeric antigen receptor T-cell (CAR-T) approach was shown to be effective in targeting the thymic stromal lymphopoietic receptor (TLSPR) ⁴². Like 5T4, TLSPR is expressed primarily by malignant subclones that persist after therapy. In this paper, we modelled this by first transplanting 5T4+ PDX, treating with chemotherapy and then in the MRD setting where blasts were not detectable in peripheral tail vein bleeds, administered A1mcMMAF. A1mcMMAF significantly delayed the emergence of leukaemic blasts in both VXL and D treated mice. Although we have previously reported HR08 to be resistant to steroids *ex vivo* ²⁶, Dex treated PDX mice showed an initial response, followed by disease kinetics similar to untreated mice. In contrast, mice receiving 4 weeks of 5T4 showed a significantly longer latency in disease recurrence and a significant survival advantage, suggesting there is

benefit of using repetitive cycles. In fact a mcMMAF conjugated anti-CD19 (Denintuzomab Mafodotin) ADC, given at 3-weekly frequency has produced remission rates of 35% with acceptable toxicities in an ongoing phase I study⁴³.

While NOD-SCID and NSG mice are now the xenografts of choice for leukemia PDX models, our results suggest that there are drawbacks that may require some caution in the interpretation of the effects of drugs. An increased tumour load in NSGs led to tumour lysis, requiring an altered cell dose and treatment schedule. Clearly engraftment kinetics differs with patient samples and passages, and need to be established before the efficacy of therapy can be assessed. In leukemia–engrafted NSG mice, treatment with VXL therapy was associated with mortality despite absence of leukemia post mortem. In contrast to previous reports²⁸, morbidity and mortality in these mice was neither due to CNS infiltration nor attributable to impaired murine haematopoiesis. In general our experience is that NSG mice are less tolerant of physical handling compared to the NOD-SCID strain. Children treated with combination chemotherapy for ALL require supportive therapy to avoid treatment related mortality. We speculate that a combination of frequent intraperitoneal administrations, influences of multiple cytotoxic drugs, the aggressive expansion and subsequent necrosis of malignant cells combine to produce the morbidity observed.

5T4 positive blasts home toward CXCL12 *in vitro* and we speculate that this is a contributing factor to their engraftment capacity, as demonstrated by the

enrichment of 5T4 positive blasts in NSG mouse femurs. Furthermore, a specific monoclonal antibody to 5T4 was shown to interfere with CXCL12 chemotaxis of HR B-ALL patient derived primagraft cells. This may be of clinical relevance when considering ways to increase the exposure of leukaemia cells to cytotoxic drugs. A CXCR4 inhibitor, AMD3100, has been used as a means to mobilise leukaemic blasts from the bone marrow systemically to increase the relative bioavailability of chemotherapy ⁴⁴. A limitation of such therapy is that CXCR4 is a chemokine receptor widely expressed by many cell lineages. Since normal tissue levels of 5T4 are low, if its influence on chemotaxis could be specifically targeted it might allow a disruption of CXCR4 function more specifically to malignant haematopoietic cells.

The bulk of ALL cells are chemosensitive and morphological remission is achieved using a combination of 3-4 drugs in ~98%. Current strategy for childhood ALL consists of intensifying therapy for those with high MRD levels, continuing treatment for 2-3 years and for some allogeneic stem cell transplantation. Thus conventional therapy, though effective, is complicated, expensive and toxic. CD19 targeted therapy already been shown to work optimally in the MRD setting ⁴³, as treating overt disease with targeted immunological agents is associated with toxicity. Ideally such therapy should be specific to the cells that comprise the MRD population to avoid unwanted side effects. Our experiments provide evidence that immunological targeting of antigens specific to resistant leukemic subclones in the MRD setting offers a novel adjunct to current therapeutic strategies. It is possible that

combinations of antibodies along with other targeted approaches may gradually change the way we treat ALL ⁴⁵. We now have an extensive understanding of CD antigens expressed on the B-cell plasma membrane ⁴⁶, this now needs to be extended to biologically relevant markers expressed by resistant leukemic blasts. As Denintuzomab Mafodotin which uses the same payload is already showing promising results, our data suggest that A1mcMMAF could be safely and efficaciously employed in either induction or consolidation therapy regimens in BCP-ALL patients identified to be 5T4+ by flow cytometry prior to starting induction chemotherapy.

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Figure Legends

Figure 1: Engraftment kinetics of BCP-ALL PDX in NSG mice according to 5T4 phenotype

(A) The expression of 5T4 at the surface of 4 HR (blue symbols) and 4 SR (red symbols) or relapse primagrafts was performed by flow cytometry and representative histograms plotted using FlowJo. The mean percentages of 5T4 positive blasts from 3 separate experiments (\pm SEM) were plotted to compare expression across risk stratification (unpaired, 2-tailed t-test; $p < 0.0001$). (B) Kaplan Meier plots of the individual HR (28 mice) versus SR (20 mice) transplanted animals. When the data from SR and HR challenged mice are pooled the median survival was 71 versus 280 days respectively ($p < 0.0001$); HZR and (95% CI) = 8.84 (4.2-18.5).

Figure 2: Engraftment of 5T4 depleted and enriched HR08 blasts in NSG mice

(A) HR08 blasts were separated by surface expression of 5T4 and resultant populations were determined to be 97% depleted and 96% enriched respectively. (B) Depleted, enriched and mock depleted populations of HR08 (1×10^6) were transplanted into NSG mice. The rate of engraftment monitored by the detection of hCD45 cells in the peripheral blood demonstrated a significant impact of 5T4 depletion on engraftment (ANOVA-Tukey; $p < 0.05$). (C) Kaplan-Meier plots showed no significant differences in time to morbidity of the groups receiving the different fractionated leukemia blasts.

Figure 3: Engraftment of 5T4- and mock- depleted HR08 blasts in NSG mice

Depleted and mock-depleted populations of HR08 (1000 and 100 cells) were transplanted into NSG mice. (A) Shows the significant impact of 5T4 specific depletion on engraftment (hCD45 cells in the peripheral blood) with both challenge doses (ANOVA/Tukey; $p < 0.001$). (B) Kaplan-Meier plots of time to morbidity for each fractionated population. 5T4 depleted compared to mock treated cells showed significant differences in survival for both doses of tumour (Log-Rank Mantel-Cox; for 1000 cells: $p = 0.001$; HZR and (95% CI) = 20.1 (3.4-120.8). For 100 cells: $p = 0.003$; HZR = 17.3 (2.7-110.4). Key for symbol/line colour is given as insert in 3A

Figure 4: CXCL12 chemotaxis of BCP-ALL PDX cells.

Transwell migration of 2 SR and 2 HR PDX samples in response to 10% FCS gradient or 12.5nM CXCL12 was monitored kinetically using a modified Boyden chamber system. (NG= no gradient). (A) All PDX samples were responsive to the serum gradient (blue lines) compared to no gradient (NG; black lines) (2 tailed t-test; $p < 0.001$) but (B) only HR primagrafts responded to CXCL12 (2 tailed t-test; $p < 0.05$); this chemotaxis was blocked by pre-incubating cells with a mAb to 5T4 (red lines) but not an isotype IgG control (blue lines) or with NG (black lines)

Figure 5: A1mcMMAF monotherapy of Sup5T4 cells *in vivo*

Animals were challenged with Sup5T4 cells ip at day 0 and received either no treatment (black circles/line) or one (light blue squares/line) or two (dark blue triangles/line) cycles of A1mcMMAF or one (red triangles/line) or two (purple diamonds/line) cycles of control-ADC treatment starting after one week. (A) IVIS images of tumour growth at day 43. Growth of tumours was quantified using log radiance (photons/sec/cm²/sr) =photons. A1mcMMAF shows significant growth control: ANOVA-Tukey: Untreated versus 1 cycle or 2 cycle A1mcMMAF; $p < 0.0001$; Control-ADC 1 or 2 cycles versus A1mcMMAF 1 or 2 cycles respectively: $p < 0.05$ and $p < 0.01$. (C) Kaplan-Meier plots show that only A1mcMMAF (one or 2 cycles) but not the control-ADC treatments influence the overall survival. Log-Rank Mantel-Cox shows significant affects compared to untreated animals of one and two cycle of A1mcMMAF respectively ($p = 0.04$; HZR: 6.3 (1.08-36.52) and $p = 0.002$; HZR: 24.14 (3.36-173.4) and no significant differences of control-ADC treatments. Dotted vertical lines represent timing of doses of ADC therapy (see supplemental Table 2)

Figure 6: A1mcMMAF monotherapy of HR08 B-ALL PDX challenge

(A) At a lower tumour challenge of 2000 HR08 cells only A1mcMMAF significantly reduces engraftment (ANOVA/Tukey; $p = 0.02$) which corresponds with (B) a significant improvement in overall survival with A1mcMMAF treatment (Log-Rank Mantel-Cox; $p = 0.0014$, HZR: 21.55 (CI 3.73-124.5). Dotted vertical lines represent timing of doses of ADC therapy (see

supplemental Table 2). Untreated, black symbols/line; control-ADC, red symbols/line and 5T4-ADC blue symbols/line.

Figure 7: Combination DEX chemotherapy and A1mcMMAF treatment of HR08 PDX: early engraftment.

(A) Percentage of peripheral blood blasts is significantly reduced at day 108 in 2×10^4 HR08 cells engrafted NSG mice administered DEX therapy 1 week after transplantation of (ANOVA-Tukey; $p < 0.05$ for DEX and DEX/CTRL-ADC, $p < 0.001$ for DEX/A1mcMMAF). (B) At day 129 engraftment of HR08 blasts had increased in the DEX and DEX-control treated groups and were comparable to the untreated group. There was significantly less engraftment in the DEX/A1mcMMAF group when compared to the untreated group ($p < 0.001$), and the other DEX treated groups ($p < 0.001$ vs DEX and $p < 0.05$ vs DEX/CTRL-ADC).

Figure 8: Combination DEX chemotherapy and A1mcMMAF treatment of HR08 PDX: engraftment kinetics and survival.

(A) The engraftment of HR08 blasts remained significantly lower ($p < 0.001$) in the DEX/A1mcMMAF therapy group than in all other groups for at least 172 days. (B) A significant improvement in overall survival compared to untreated animals (Log-Rank Mantel-Cox) is seen with DEX: $p = 0.006$; HZR: 9.6 (1.9-47.6); DEX/A1mcMMAF $p = 0.0006$; HZR: 21.55 (3.7-124.5). DEX/control-ADC treatment shows slightly poorer survival than untreated animals: $p = 0.012$; HZR: 0.13 (0.03-0.63). Hatched areas and dotted vertical lines represent

timing of chemotherapy and doses of ADC therapy respectively (see supplemental Table 2). Black circles/line, untreated; purple triangles/line, DEX alone; red diamonds/line DEX-control-ADC; blue circles/line DEX=5T4-ADC.

Figure 1

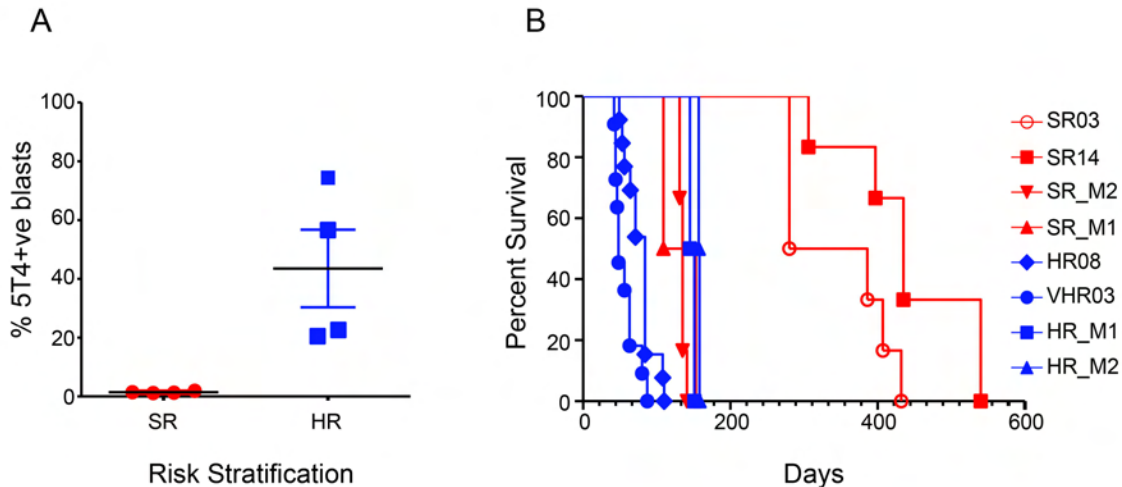


Figure 2

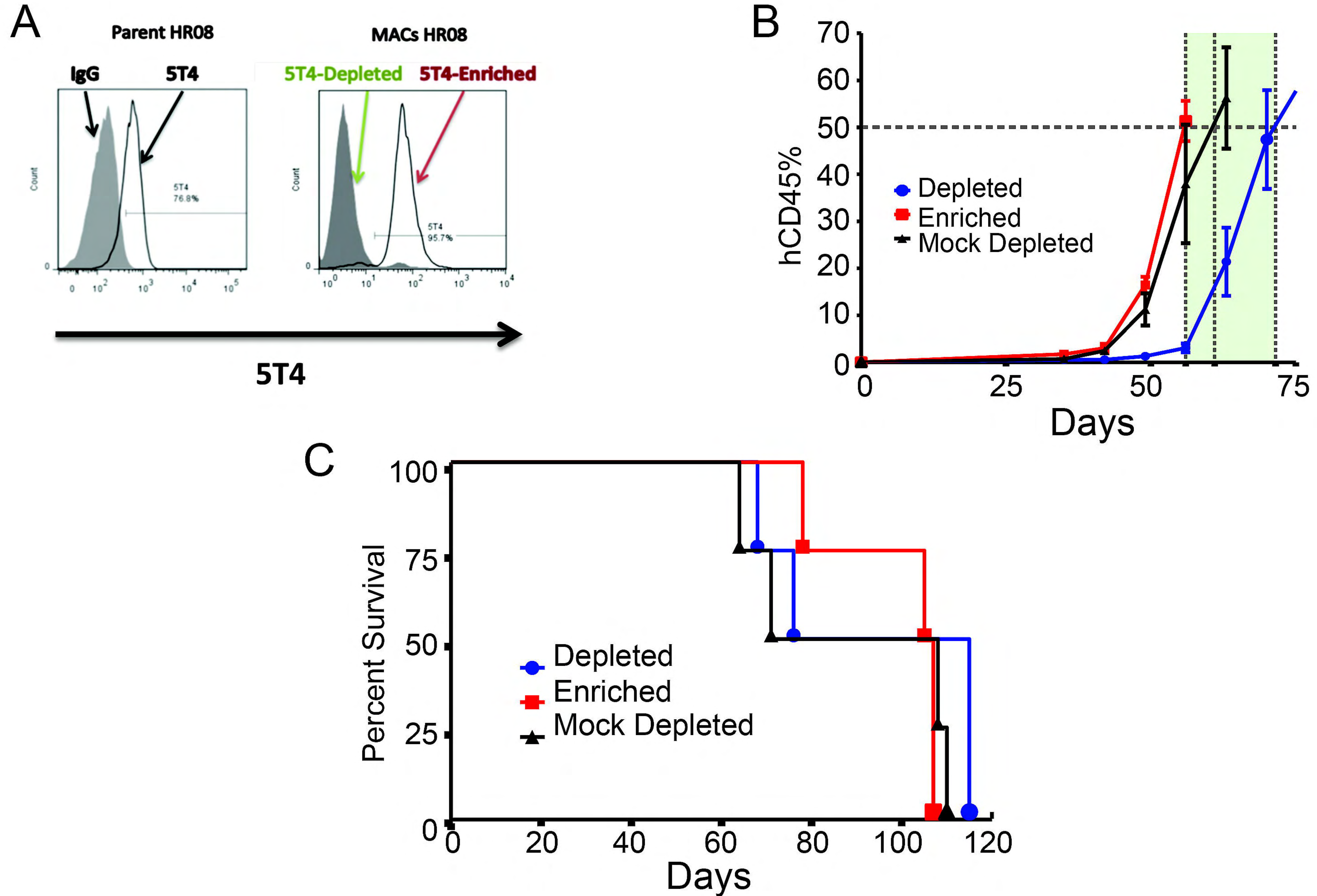


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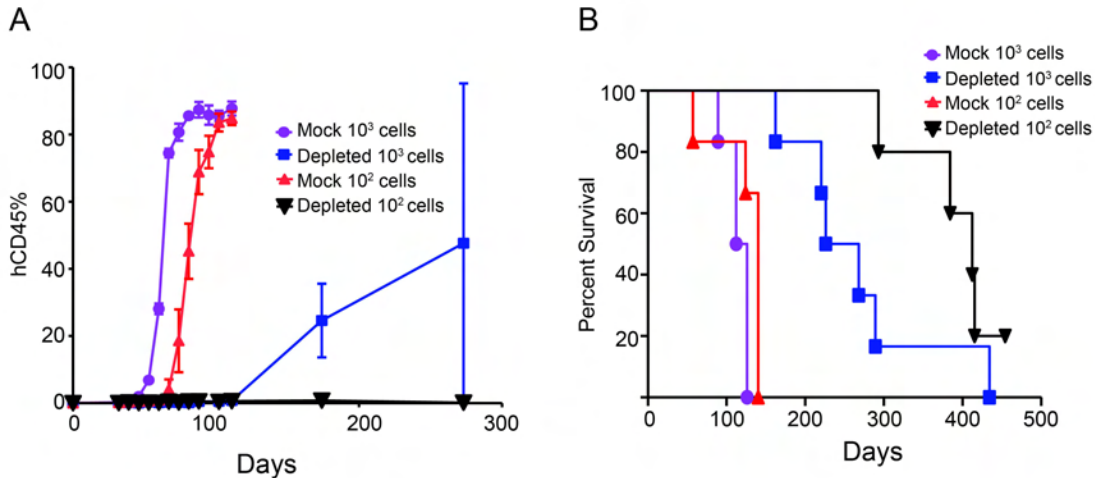
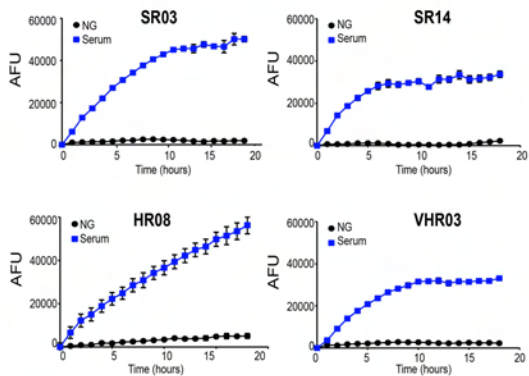


Figure 4

A



B

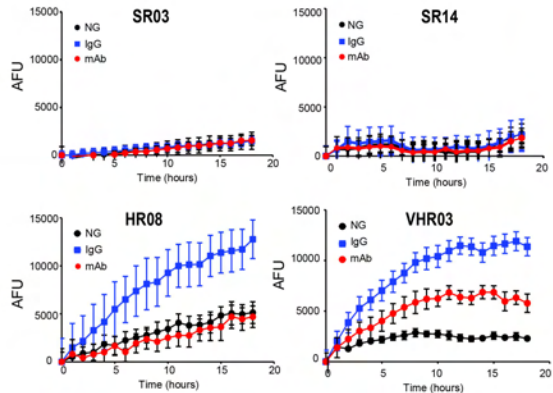


Figure 5

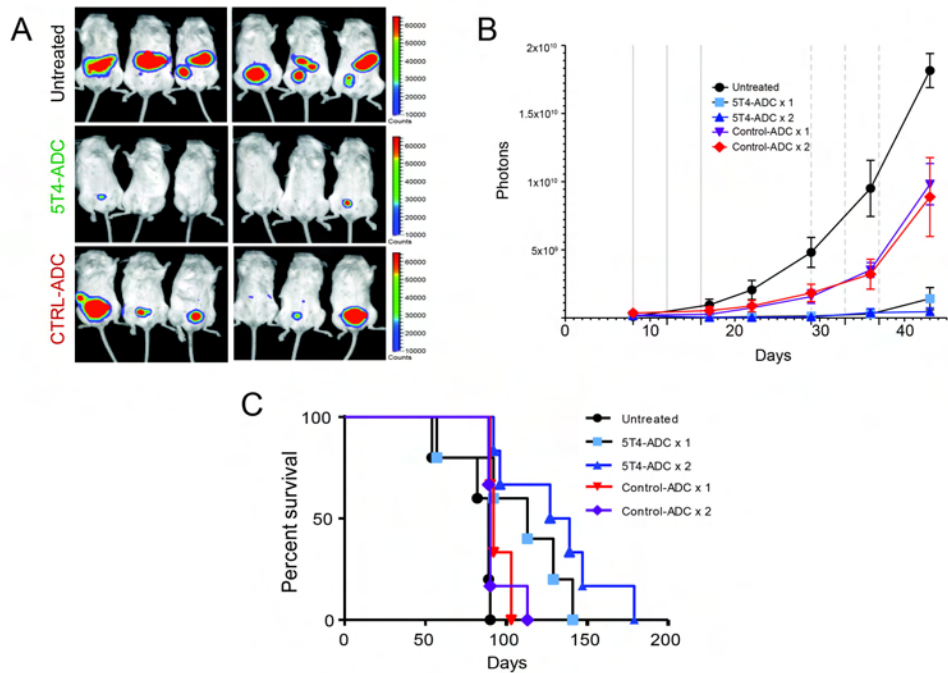
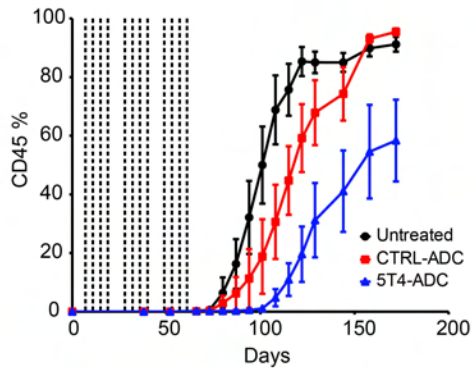


Figure 6

A



B

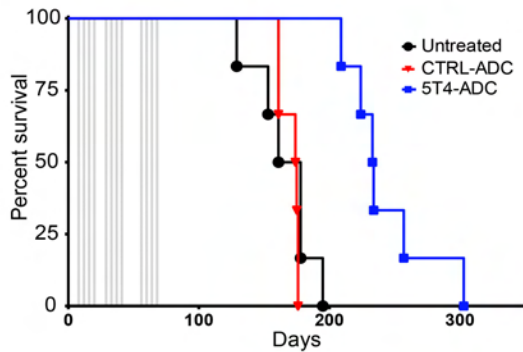
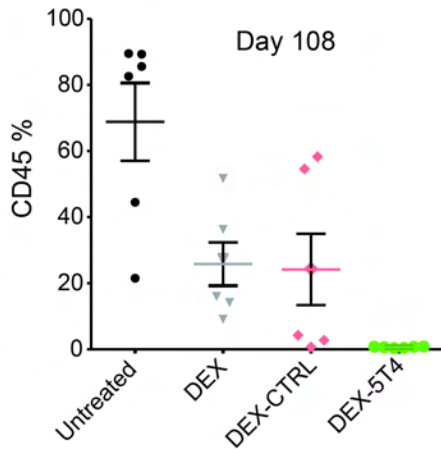


Figure 7

A



B

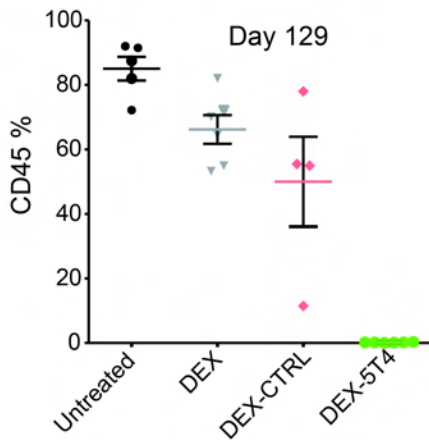
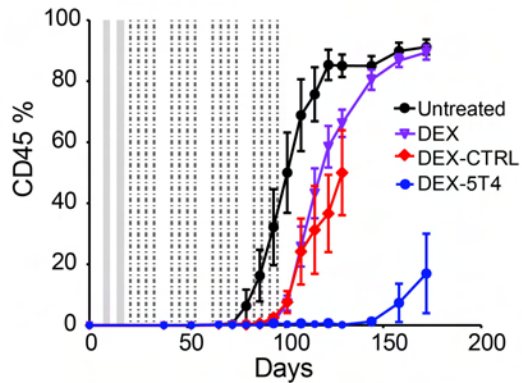
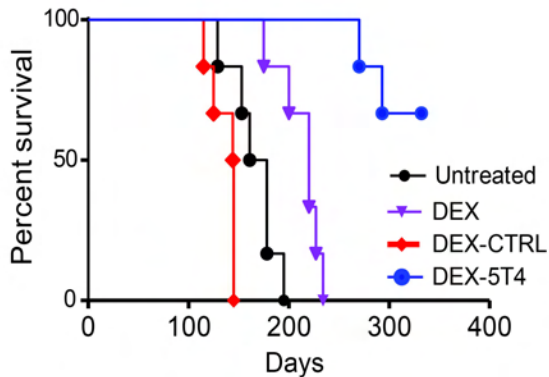


Figure 8

A



B



Supplemental Table 1

UPN	MRD Risk	Cytogenetics	Sex	Age (Years)
SR03	SR	NA	F	3.4
SR14	SR	ETV6-RUNX1	M	5.8
SR_M1	SR	ETV6-RUNX1	M	11.6
SR_M2	SR	High Hyperdiploid	M	6.1
VHR03	HR	NA	M	17.1
HR08	HR	MLL	F	0.3
HR_M1	HR	High Hyperdiploid	M	11.5
HR_M2	HR	t(1;9)	F	15

List of patient samples engrafted into NSG mice to create PDX models. MRD Risk, SR = MRD 10^{-4}; HR = MRD $\geq 10^{-4}$ detected post induction. Details on SR03, SR14, VHR03 and HR08 have been previously reported¹.

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Supplemental Table 2

Dosing schedules for immunotherapy experiments

Figure	Tumour	Dose/ Route Day 0	Chemo	ADC (start)	**ADC Doses per Cycle	No. of cycles	Monitoring
5	Sup5T4	5x10 ⁶ ip	-	Untreated	-	-	IVIS weekly
			-	5T4-ADC (day 7)	3	2	
			-	Control-ADC (day 7)	3	2	
6	HR08	2x10 ⁶ iv & 2x10 ³ iv	-	Untreated	-	-	PBL flow cytometry
			-	5T4-ADC (day 7)	4	3	
			-	Control-ADC (day 7)	4	3	
S1	VHR08	2x10 ⁶ iv	-	Untreated	-	-	PBL flow cytometry
			-	5T4-ADC (day 7)	4	2	
			-	Control-ADC (day 7)	4	2	
7	HR08	1x10 ⁶ iv	-	Untreated	-	-	PBL flow cytometry
			VXL*	Untreated	-	-	
			VXL	5T4-ADC (day 21)	4	3	
			VXL	Control-ADC (day 21)	4	3	
8	HR08	2x10 ³ iv	-	Untreated	-	-	PBL flow cytometry
			DEX***	Untreated	-	-	
			DEX	5T4-ADC (day 21)	4	4	
			DEX	Control-ADC (day 21)	4	4	

*VXL given on weekdays of weeks 2 and 3 after tumour initiation

** ADC given 3 or 4 times at 4 day intervals and cycle repeated after a week.

*** DEX given on weekdays of weeks 2 and 3 after tumour initiation

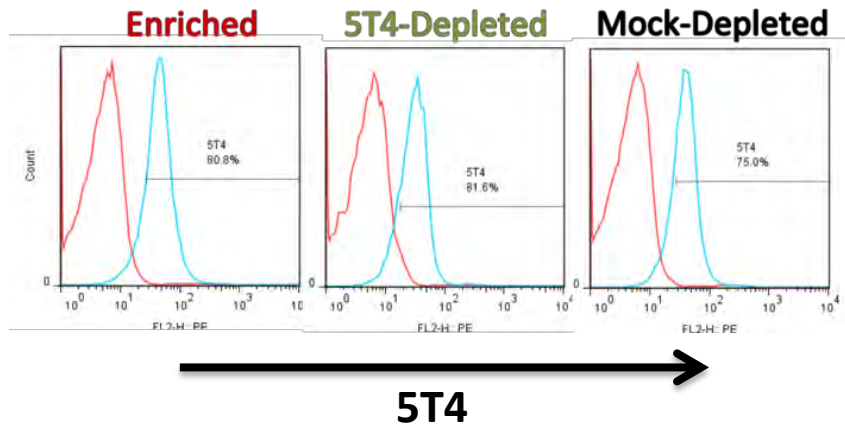


Figure S1. Engraftment of 5T4 depleted and enriched HR08 blasts in NSG mice

At termination, spleens were harvested from all animals. Flow cytometry of splenic cells showed recapitulation of the parental phenotype, ~75% 5T4 positive blasts, in all groups independent of fractionation.

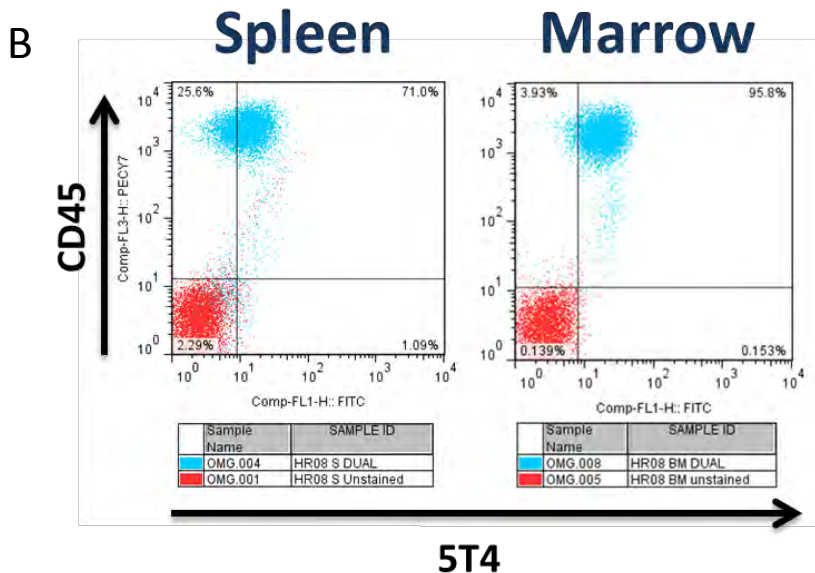
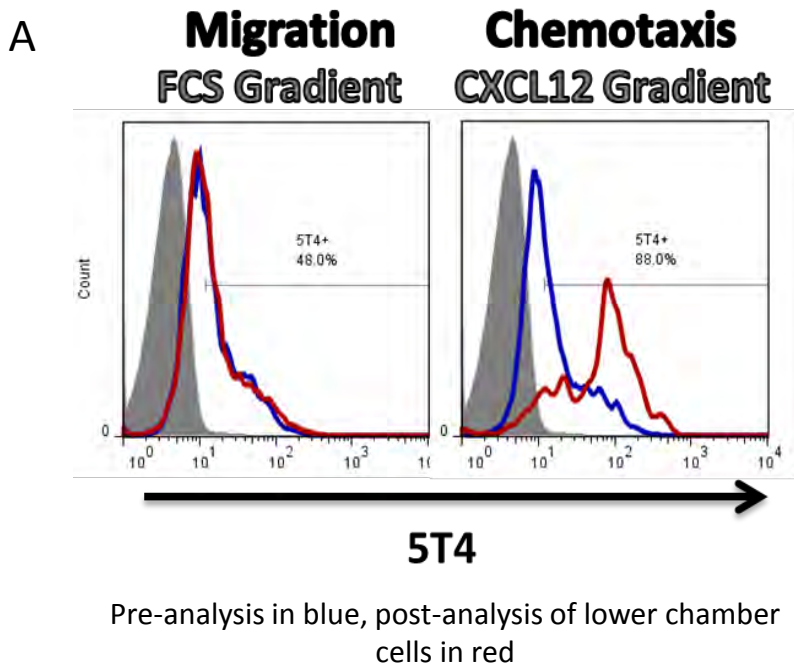


Figure S2. CXCL12 chemotaxis of BCP-ALL PDX cells.

(A) 5T4 positive blasts are the predominant responders to the CXCL12 gradient as seen by preferential accumulation of 5T4 expressing PDX cells in the chemoattractant chamber in response to CXCL12 but not FCS.

(B) Enrichment of 5T4 positive blasts seen in HR08 blasts in the bone marrow of NSG hind limb femurs. Leukaemic blasts in BM are >95% 5T4 positive compared to spleen levels (71%).

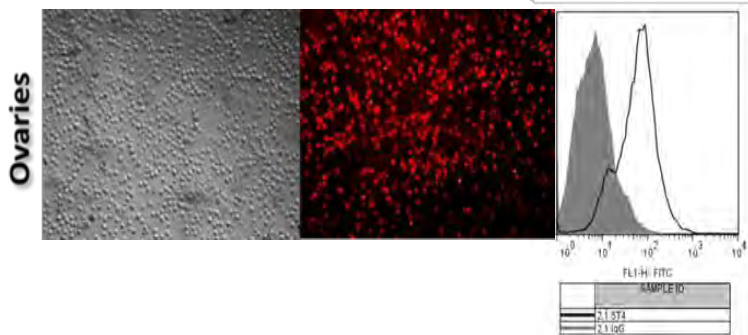


Figure S3. A1mcMMAF monotherapy of Sup5T4 cells *in vivo*. Ovaries were disaggregated at end of the experiment to identify Sup5T4 cells (mCherry) and then analysed by flow cytometry. Recurrent leukaemia, post A1mcMMAF treatment, retains a 5T4 positive phenotype.

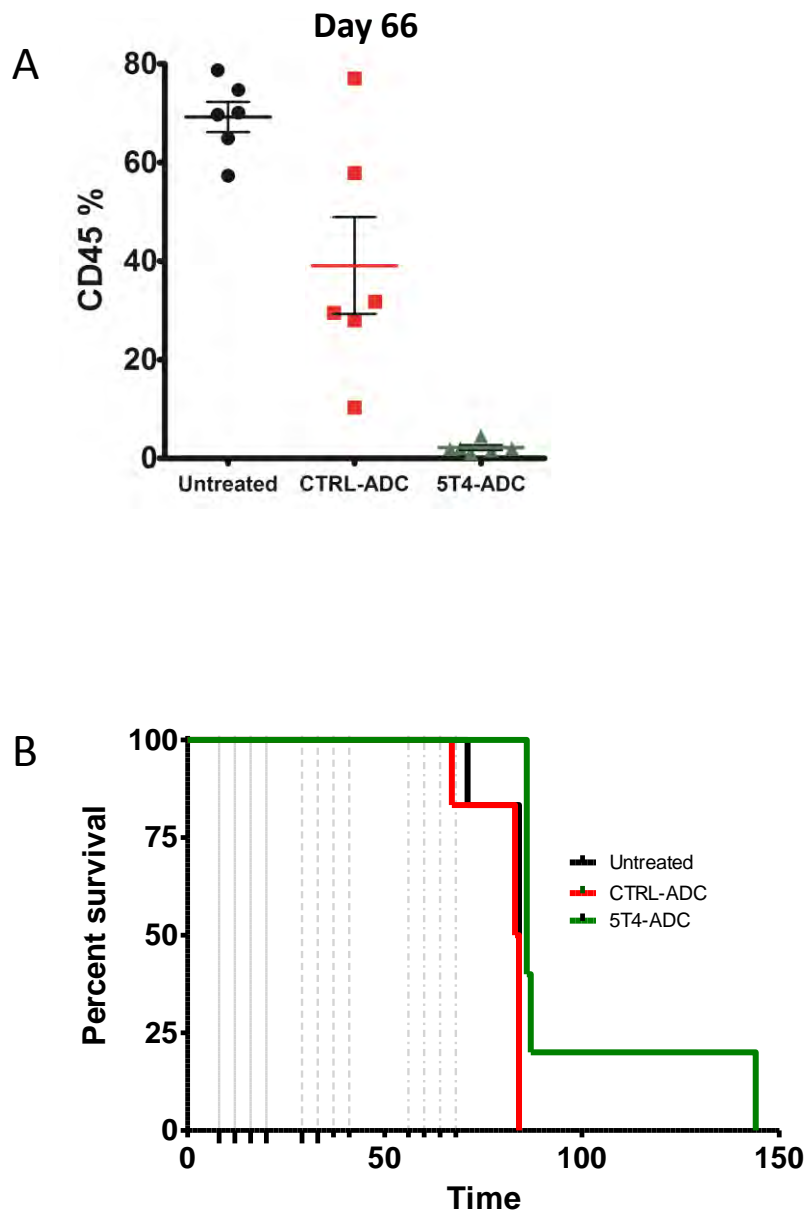
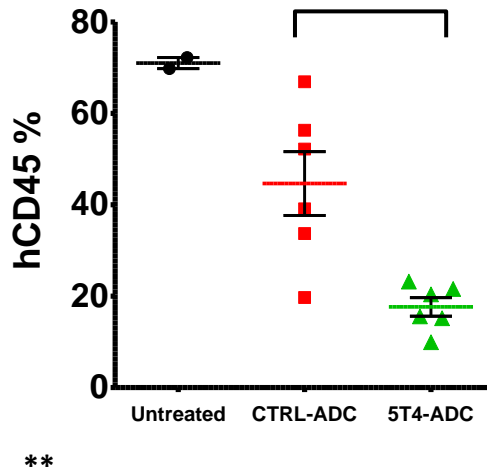


Figure S4. A1mcMMAF monotherapy of HR08 B-ALL PDX challenge (A) A1mcMMAF significantly reduces the engraftment of 1×10^6 HR08 cells in NSG mice at day 66 (ANOVA/Tukey; $p < 0.0001$) but (B) had no impact on the overall survival. Dotted vertical lines represent timing of doses of ADC therapy (see supplemental Table 2)

A



B

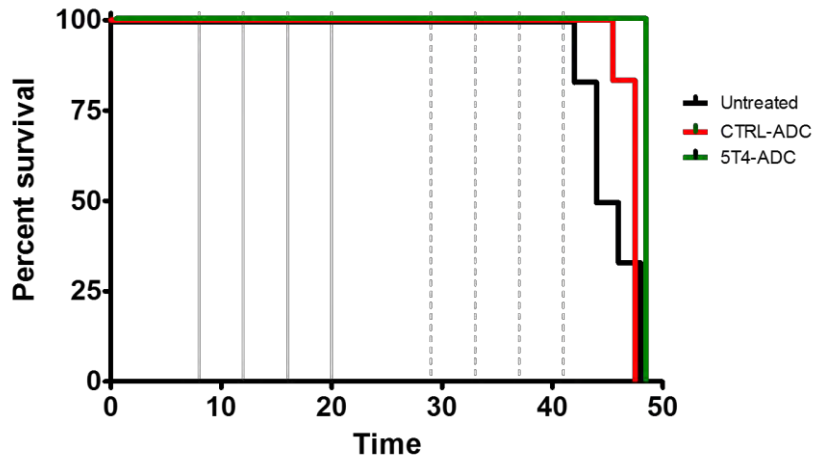


Figure S5: Engraftment and survival of VHR03 primagrafts given ADC therapy

(A) Compared to control therapy, 5T4-ADC significantly reduces the engraftment of 2×10^6 VHR03 cells in NSG mice at day 42 (unpaired t test; $p=0.004$) but (B) had no impact on the overall survival

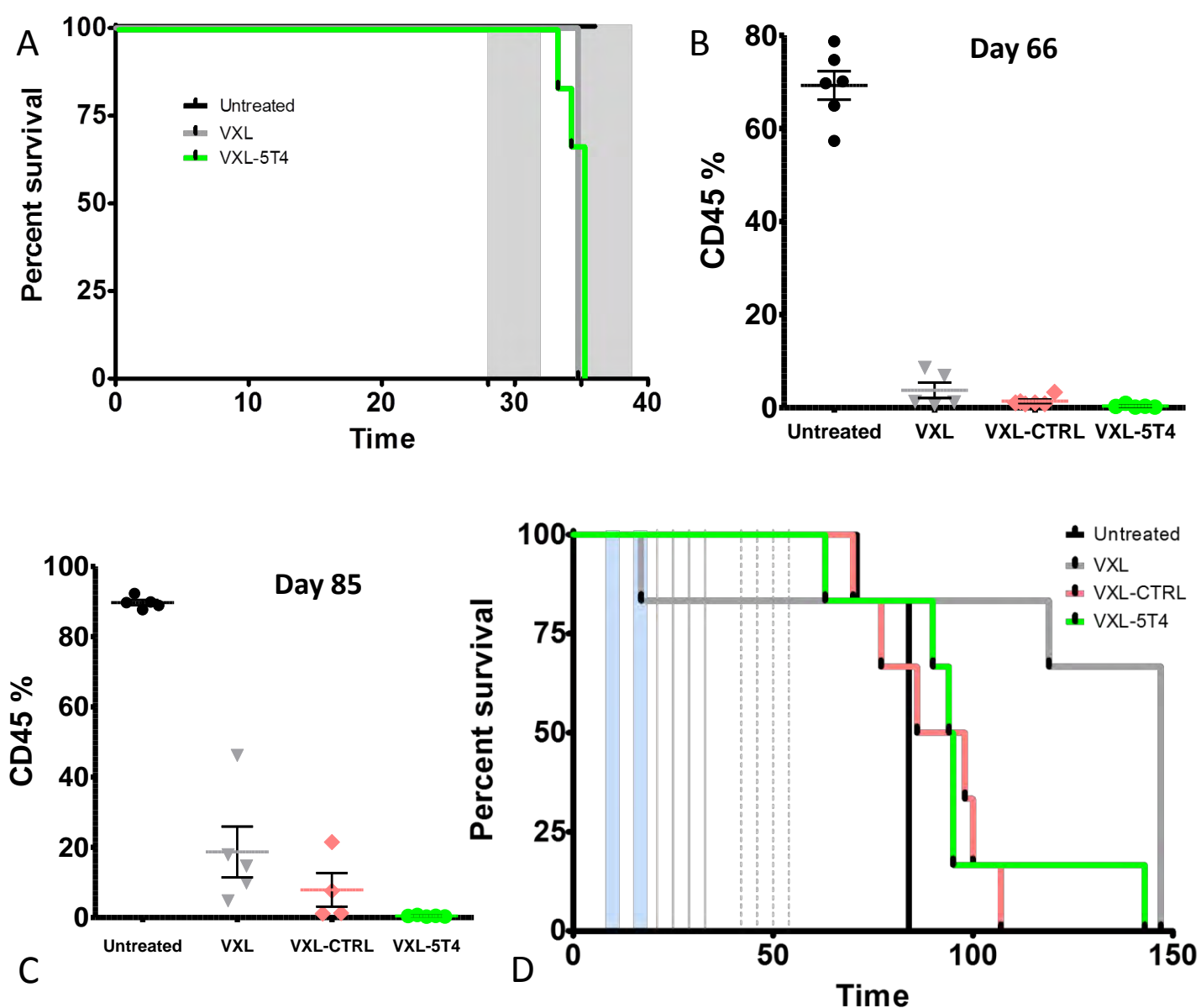


Figure S6: Combination VXL chemotherapy and ADC treatment of HR08 PDX

(A) Kaplan-Meier plots show that when VXL therapy is administered 4 weeks after transplantation of 1×10^6 HR08 cells all animals reached morbidity due to therapeutic challenge, within the first VXL cycle of drugs and before A1mcMMAF administration.

(B) VXL therapy administered 1 week after transplantation of 1×10^6 HR08 cells significantly reduced engraftment for all groups in NSG mice at day 66 (ANOVA-Tukey; $p < 0.0001$).

(C) At day 85 engraftment of HR08 blasts had increased in the VXL and VXL-control treated groups, while the A1mcMMAF combination therapy group were significantly less engrafted ANOVA-Tukey; $p < 0.05$.

(D) Kaplan-Meier plots show no significant impact in overall survival between treatment groups. Hatched areas and dotted vertical lines represent timing of chemotherapy and doses of ADC therapy respectively (see supplemental Table 2)

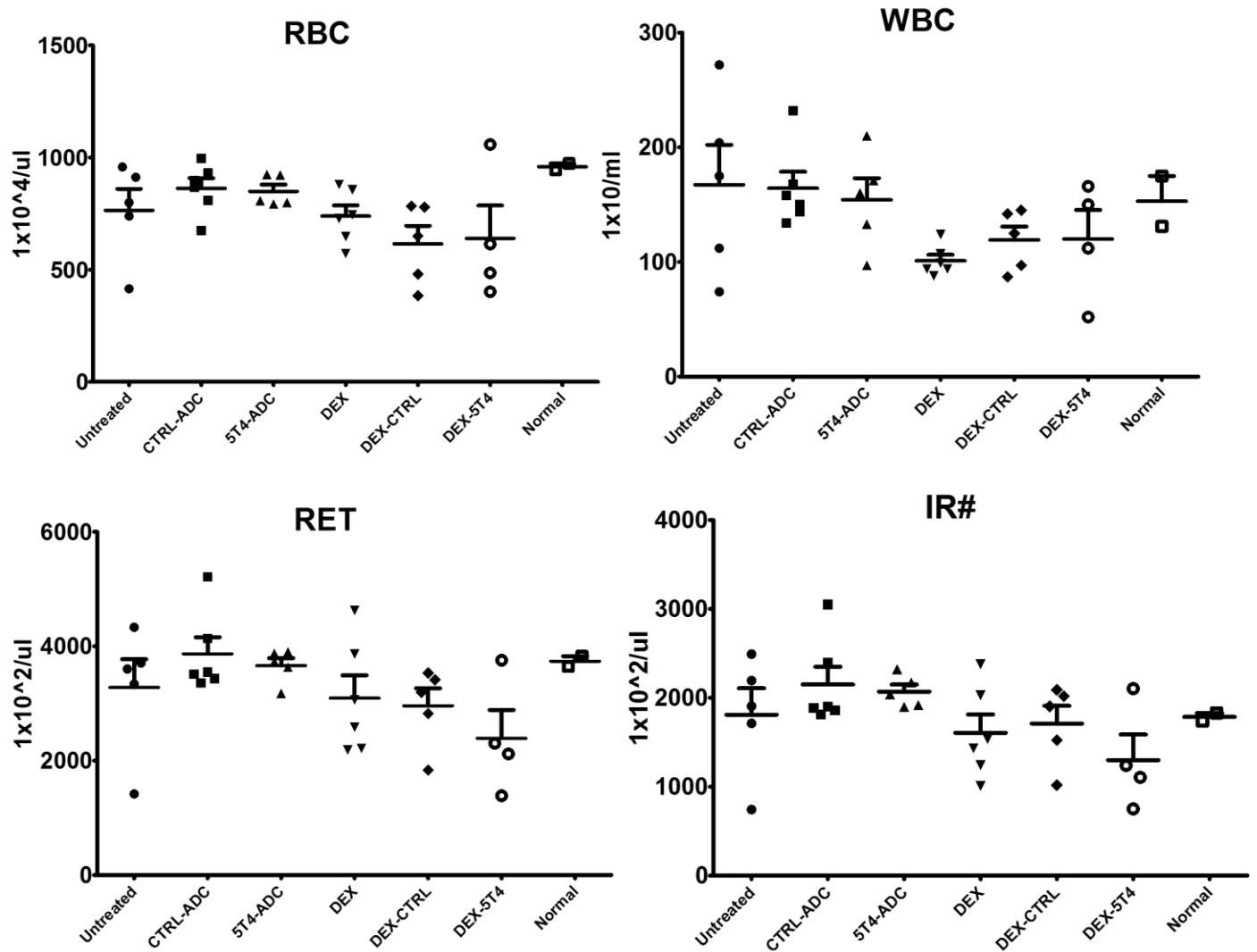


Figure S7: Monitoring in vivo hematopoiesis

Anaemia is one of the most frequent side effects of anticancer treatment, it is also caused by disease itself. Effective erythropoiesis can be monitored by quantitative measurement of reticulocytes, the amount of RNA in these cells can be assessed by flow cytometry and divided into low- (LFR), middle- (MFR) and high-fluorescence reticulocytes (HFR), this distribution is correlated with their maturation (Luczynski et al 2006; Adv. Med Sci 51: 188-90). To assess this we used the Sysmex XT-2000iV system. Analyses of murine peripheral blood from day 42 of 5T4-ADC monotherapy and combination therapy with DEX. No significant differences were observed across any groups, including additional controls of un-transplanted mice, in red (RBC) or white blood cell (WBC) count, overall reticulocyte number (RET) or immature reticulocyte fraction (IR#).