ARTICLE

Targeting the 5T4 oncofetal glycoprotein with an antibody drug conjugate (A1mcMMAF) improves survival in patient-derived xenograft models of acute lymphoblastic leukemia

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ABSTRACT

Outcome in childhood acute lymphoblastic leukemia is prognosticated from levels of minimal residual disease after remission induction therapy. Higher levels of minimal residual disease are associated with inferior results even with intensification of therapy, thus suggesting that identification and targeting of minimal residual disease cells could be a therapeutic strategy. Here we identify high expression of 5T4 in subclonal populations of patient-derived xenografts from patients with high, post-induction levels of minimal residual disease. 5T4-positive cells showed preferential ability to overcome the NOD-*scid*IL2Rγ^{null} mouse xenograft barrier, migrated *in vitro* on a CXCL12 gradient, preferentially localized to bone marrow *in vivo* and displayed the ability to reconstitute the original clonal composition on limited dilution engraftment. Treatment with A1mcMMAF (a 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*IL2Rγ^{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 that specifically targeting minimal residual disease cells improved outcomes and support further investigation of A1mcMMAF in patients with high-risk B-cell precursor acute lymphoblastic leukemia identified by 5T4 expression at diagnosis.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer of childhood. Intensive combination chemotherapy produces cure rates of ~90% but is associated with considerable morbidity. Recent high throughput genetic analyses have successfully identified new genetic subtypes[1,2] as well as aberrant epigenetic[3] and signaling pathways[4,5] that are potential targets for precision therapy.[6] The paradigm for targeted treatment is the subset of children with a *BCR-ABL1* fusion in whom 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 payloads[9] and/or activating cytotoxic T cells, is showing great promise.[10] Thus we are now on the cusp of a change from iteratively derived non-specific chemotherapy to a designed, targeted approach.

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We recently reported that the 5T4 oncofetal 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) ALL.[11] 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 transition,[12-14] facilitation of CXCL12/CXCR4 chemotaxis[15,16] and favoring non-canonical over canonical WNT/β–catenin pathway signaling.[17,18] 5T4 is expressed by tumor-initiating cells in human non-small cell carcinomas[19] and by a number of carcinomas.[20] The selective pattern of 5T4 tumor expression, its association with a tumor-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 (mc) linker. A1mcMMAF has shown potent activity in a variety of solid tumor models, with induction of long-term regression after the last dose and no significant toxicity in a simian model[23] and tolerable toxicity in patients with solid tumors[24]

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 resistance.[25] 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 xenograft (PDX) samples obtained from patients with high levels of post-induction MRD. When used in combination with chemotherapy, A1mcMMAF showed specific activity in $5T4^+$ PDX models of ALL.

Methods

B-cell precursor acute lymphoblastic leukemia patient-derived xenograft samples

Two categories of PDX samples were assessed, based on the reported post-induction MRD levels in the patients from whom they were derived. For the purposes of this paper, those with MRD levels ≤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) (*Online Supplementary Table S1*).[26] PDX cells (1-2x10^6, or fewer) obtained from splenic fractions were transplanted intravenously into non-irradiated 6- to 10-week old NOD-*scid*IL2Rγ^{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 tumor 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 leukemic 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 with the regulations of the UK Animal License Act.

Flow cytometry

Individual or multiplex flow cytometry studies for 5T4[11] and human specific CD45 (CD45 APC-Cy7 or PE-Cy7) and CD34 (FITC or PE-Cy7) (all from 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, OR, USA). *In vivo* leukemia engraftment was analyzed by human CD45 flow cytometry using 25 μL of heparinized peripheral blood after lysis of the red blood cells (eBioscience). The overall disease burden was determined by expression of the ratio of human to mouse $CD45^+$ blasts per sample. Analyses of peripheral blood cellular components were performed using an XE-2100 automated hematology system (Sysmex, Milton Keynes, UK).

Migration assays

The migration assays were performed as previously described.[11]

5T4 depletion

Depletion and enrichment of $5T4^+$ 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[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] (5x10^6) were given intraperitoneally and different BCP-ALL PDX samples at various doses were given intravenously to NSG mice. Mice were treated with either A1mcMMAF or control-ADC (Neg-8-8-hG1mcMMAF) at a dose of 5 mg/kg intraperitoneally beginning 7 days after tumor challenge with a cycle of three or four 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 1 week, or mice were given no therapy[23] (*Online Supplementary Table S2*). Sup5T4 B-ALL intraperitoneal challenge was monitored by IVIS.[11] For other PDX samples tumor engraftment was assessed by peripheral blood monitoring and, at an appropriate point, comparison of tumor burden assessed by flow cytometry in cell suspensions from spleen and bone marrow. Two weeks of a vincristine, dexamethasone and asparaginase (VXL) protocol, established in NSG mice,[28] was followed by various cycles of ADC starting at different times after HR08 primagraft challenge. A similar protocol using dexamethasone 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 a two-tailed Student *t*-test or ANOVA-Tukey; survival

analysis was based on Kaplan-Meier plots, log-rank Mantel-Cox and regression analysis for hazard ratios.

Results

5T4-expressing patient-derived xenografts have an aggressive phenotype in NSG mice

Four SR and four HR BCP-ALL PDX samples were analyzed by flow cytometry for cell surface expression of 5T4. All four HR B-ALL had significant (20-80%) proportions of 5T4$^+$ cells while 5T4 was undetectable in all four SR PDX samples (Figure 1A). The overall leukemogenicity was generally reflected in the rate of tumor engraftment and time to morbidity as recipients of HR primagrafts with >50% 5T4 positivity showed earlier engraftment and death than those which received SR primagrafts (Figure 1B). When pooling the data, the engraftment of 5T4$^+$ HR PDX was significantly faster than that of 5T4$^-$ 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 high MRD to overcome the xenograft barrier,[26] leading to shorter times to leukemia in NSG mice, has already been described.[29] To further investigate leukemic engraftment according to the 5T4 phenotype, depletion and enrichment of 5T4 were performed on HR08 PDX cells using a MACS system and a 5T4-specific monoclonal antibody. The efficacy of MACS separation of 5T4$^+$ blasts is illustrated in Figure 2A. From a starting population of 76.8% 5T4$^+$ leukemic cells, there was ≥96% enrichment or depletion. Transplantation of 2x10^6 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, independently of fractionation, exhibited recapitulation of the parental heterogeneous HR08 phenotype, with approximately 75% of the blasts expressing 5T4 (*Online Supplementary Figure S1*). The delay in engraftment did not correlate with improved survival of the animals (Figure 2C). Such subclonal recapitulation of the original leukemic 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$^+$ blasts persisted in the depleted challenge doses. A limited dilution experiment performed using either a 1,000- 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$^+$ blasts were the most clonogenic of a heterogeneous leukemic population.

Chemotaxis of 5T4-positive patient-derived xenograft cells on a CXCL12 gradient

In normal culture, SupB15 BCP-ALL 5T4$^+$ cells differentiate to produce 5T4$^-$cells with concurrent loss of CD34 expression and a more mature phenotype.[11] These changes are 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 extramedullary sites.[11] The responses of PDX cells to a CXCL12 chemokine gradient or a positive control gradient of fetal calf serum were examined. All PDX samples migrated actively along a fetal calf serum gradient, confirming the functional viability of the cells (Figure 4A). HR but not SR PDX cells were chemotactically responsive to CXCL12; this responsiveness was inhibited by pre-incubation with 5T4-specific monoclonal antibody but not the IgG1 control (Figure 4B). Further evidence that 5T4$^+$ 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 fetal calf serum (*Online Supplementary Figure S2A*). NSG

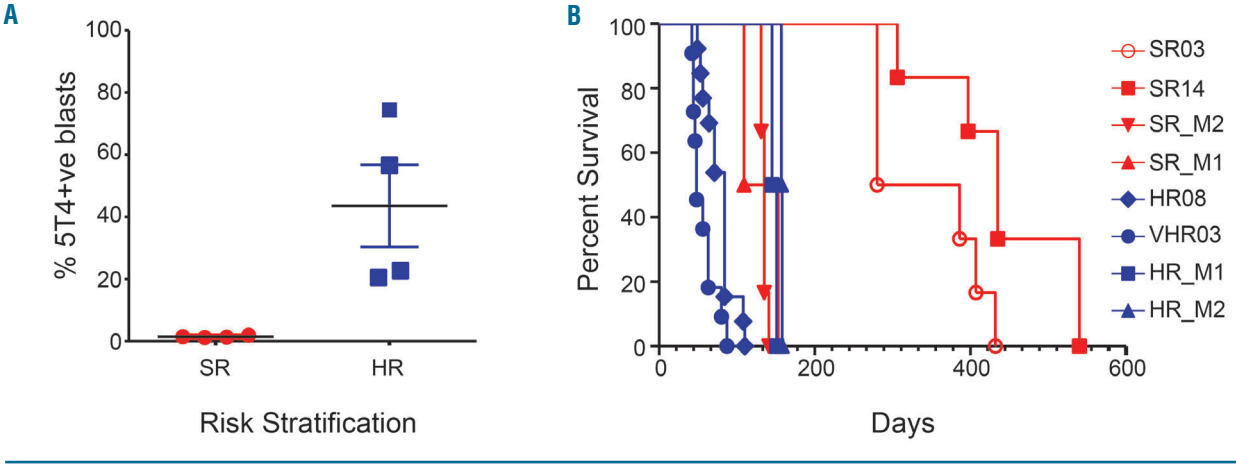

Figure 1. Engraftment kinetics of B-cell precursor acute lymphoblastic leukemia patient-derived xenograft mice according to 5T4 phenotype. (A) The expression of 5T4 at the surface of four HR (blue symbols) and four SR (red symbols) or relapse primagrafts was determined by flow cytometry and representative histograms plotted using FlowJo. The mean percentages of 5T4$^+$ blasts from three separate experiments (± 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 were pooled the median survival was 71 *versus* 280 days, respectively (P<0.0001); hazard ratio (95% confidence interval) = 8.84 (4.2-18.5).

mice have high expression of CXCL12 in the bone marrow.[31] In the HR08 PDX model, peripheral blood and harvested spleens of NSG mice were composed of approximately 75% 5T4$^+$ blasts, while >95% of the HR08 cells recovered from femoral flushes expressed 5T4 (*Online Supplementary Figure S2B*). Thus, there is both *ex vivo* and *in vivo* evidence suggesting that 5T4$^+$ blasts respond to a CXCL12 gradient.

In vivo A1mcMMAF therapy of the Sup5T4 B-cell precursor acute lymphoblastic leukemia cell line

To assess the efficacy of A1mcMMAF, mice were engrafted intraperitoneally with Sup5T4 Lenti/Luc/mCherry[11] (5x10^6 cells) and treatment initiated 1 week later with control-ADC or A1mcMMAF. Bioluminescent imaging used to monitor leukemic engraftment showed that both one and two cycles of

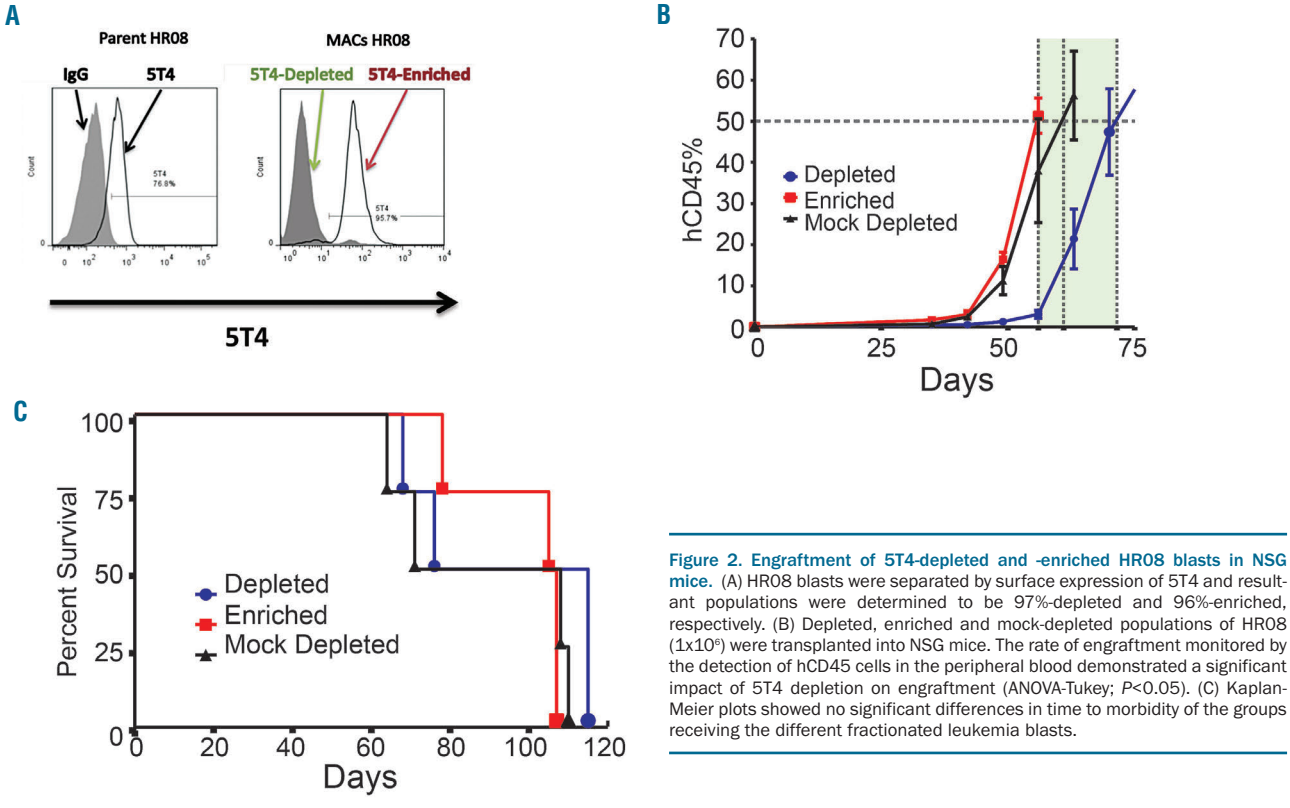

Figure 2. Engraftment of 5T4-depleted and -enriched HR08 blasts in NSG mice. (A) HR08 blasts were separated by surface expression of 5T4 and resultant populations were determined to be 97%-depleted and 96%-enriched, respectively. (B) Depleted, enriched and mock-depleted populations of HR08 (1x10^6) were 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.

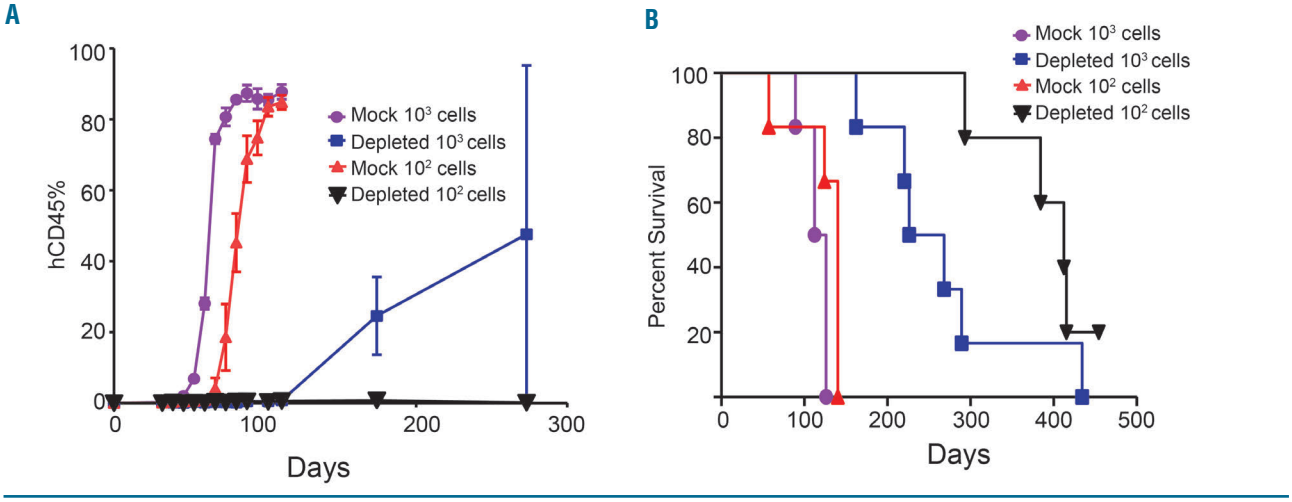

Figure 3. Engraftment of 5T4- and mock-depleted HR08 blasts in NSG mice. 5T4-depleted and mock-depleted populations of HR08 (1000 and 100 cells) were transplanted into NSG mice. (A) 5T4-specific depletion had a significant impact on engraftment (hCD45 cells in the peripheral blood) with both challenge 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 tumor (log-rank Mantel-Cox; for 1000 cells: P=0.001; hazard ratio (95% confidence interval) = 20.1 (3.4-120.8); for 100 cells: P=0.003; hazard ratio = 17.3 (2.7-110.4).

A1mcMMAF were effective in limiting tumor growth compared to control-ADC (Figure 5A,B). Control-ADC had some impact in comparison to the untreated tumors (Figure 5B), possibly through Fc binding on the blasts in the peritoneal cavity (untreated *versus* 1 cycle or 2 cycle control-ADC; $P<0.05$ and $P<0.01$, respectively). 5T4-targeted ADC treatments significantly prolonged survival when compared to untreated or control-ADC ($P=0.04$) (Figure 5C). Although the median survival of animals that received two cycles of A1mcMMAF was 133 days, compared to 113 days in those that received one cycle, this difference was not statistically significant ($P=0.25$). Cessation of the administration of A1mcMMAF (day 37 for second cycle) correlated with increased tumor growth and mCherry-positive leukemia cells isolated after therapy from the ovary, a common extramedullary site of Sup5T4 tumor spread,[11] retained the parental 5T4 phenotype (*Online Supplementary Figure S3*). This suggests the potential for continuing multiple cycles of therapy.

In vivo A1mcMMAF therapy of B-cell acute lymphoblastic leukemia patient-derived xenografts

A significant therapeutic effect of A1mcMMAF on a leukemic cell line that uniformly expresses 5T4 may not be as effective in leukemia with heterogeneous 5T4 expression. To test this, mice were challenged intra-

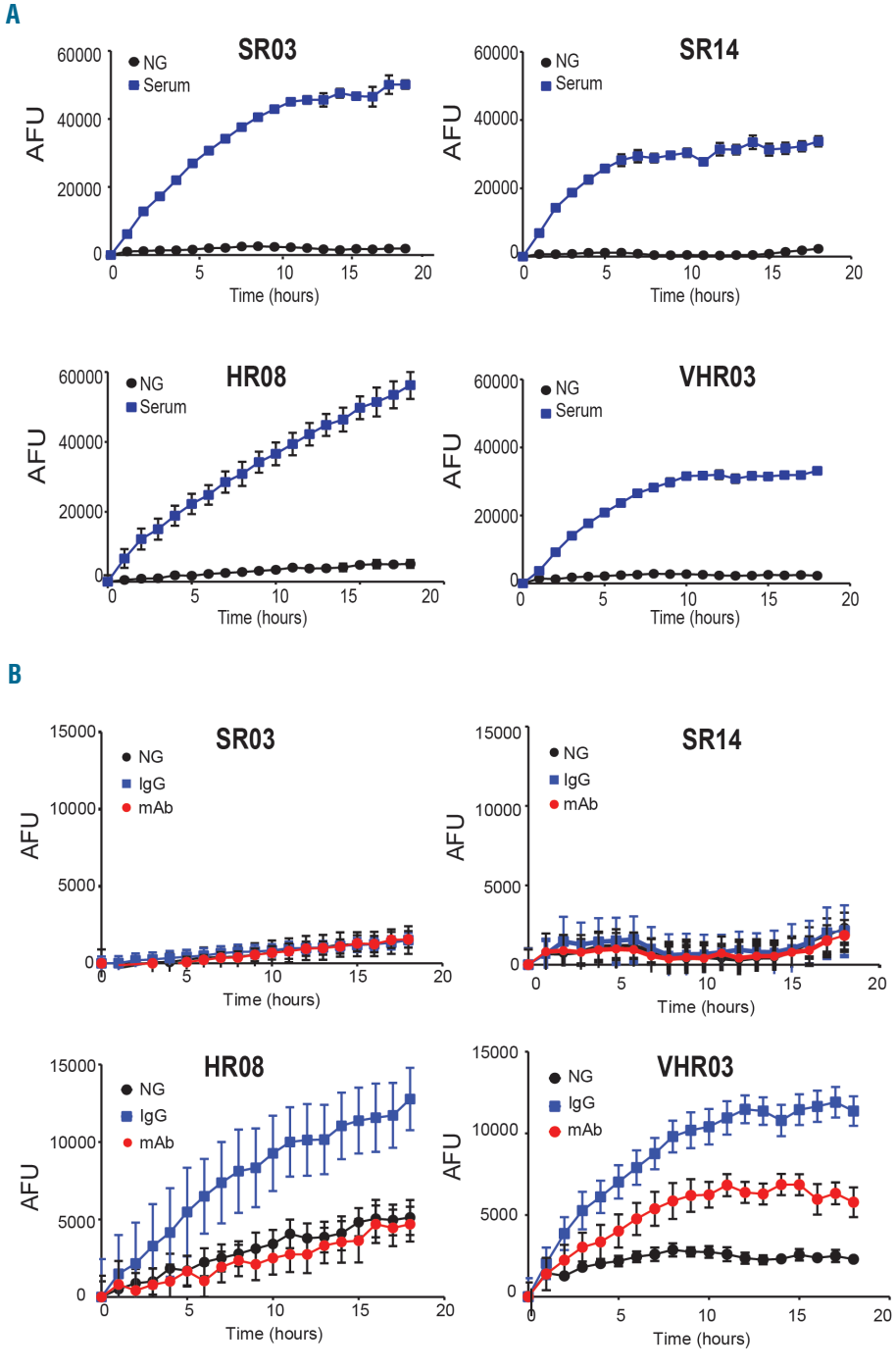

Figure 4. CXCL12 chemotaxis of B-cell precursor acute lymphoblastic leukemia patient-derived xenograft cells. Transwell migration of two SR and two HR PDX samples in response to a 10% fetal calf serum gradient or 12.5 nM CXCL12 was monitored kinetically using a modified Boyden chamber system. (NG= no 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 monoclonal antiboby (mAb) to 5T4 (red lines) but not an isotype IgG control (blue lines) or with NG (black lines).

venously with 2x10^6 HR08 (~75% 5T4$^+$) cells, and 1 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 weekly analysis of peripheral blood for the presence of human tumor cells as a measure of leukemia engraftment. A1mcMMAF administration significantly delayed engraftment (day 66; *P*=0.001), but did not influence overall survival (*Online Supplementary Figure S4A,B*). Similar results were seen with VHR03 PDX (~56% 5T4$^+$) given at a dose of 2x10^6 intravenously followed by two cycles of ADC (*Online Supplementary Figure S5*). Postulating that this could be due to an excess of tumor cells injected, the experiment was repeated with transplantation of 2x10^3 HR08 cells, followed 1 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 6A,B). No effects of the control-ADC were observed in the PDX-challenged mice.

A1mcMMAF in combination with chemotherapy for B-cell acute lymphoblastic leukemia primagraft treatment

Next, we investigated the impact of A1mcMMAF in the context of VXL therapy. Unexpectedly, animals transplanted with 1x10^6 HR08 cells and treated 4 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 (*Online Supplementary Figure S6A*). Upon autopsy organs displayed signs consistent with tumor lysis syndrome,[32] and so in a subsequent experiment VXL therapy was delivered 1 week after challenge to reduce the target tumor load. Two weeks of VXL therapy was followed by three 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 tumor engraftment of 69.2% ± 3.1 while all treated groups exhibited little if any evidence of leukemia in the peripheral blood (*Online Supplementary Figure S6B*). Leukemic cells were present in peripheral blood samples by day 85 in both VXL and VXL/control-ADC treated animals with mean engraftment of 18.7% ± 7.2 and 7.9% ± 4.8, respectively. No circulating blasts were detected in the peripheral blood of the mice treated with the combination therapy (*Online Supplementary Figure S6C*). 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 (*Online Supplementary Figure S6D*). 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

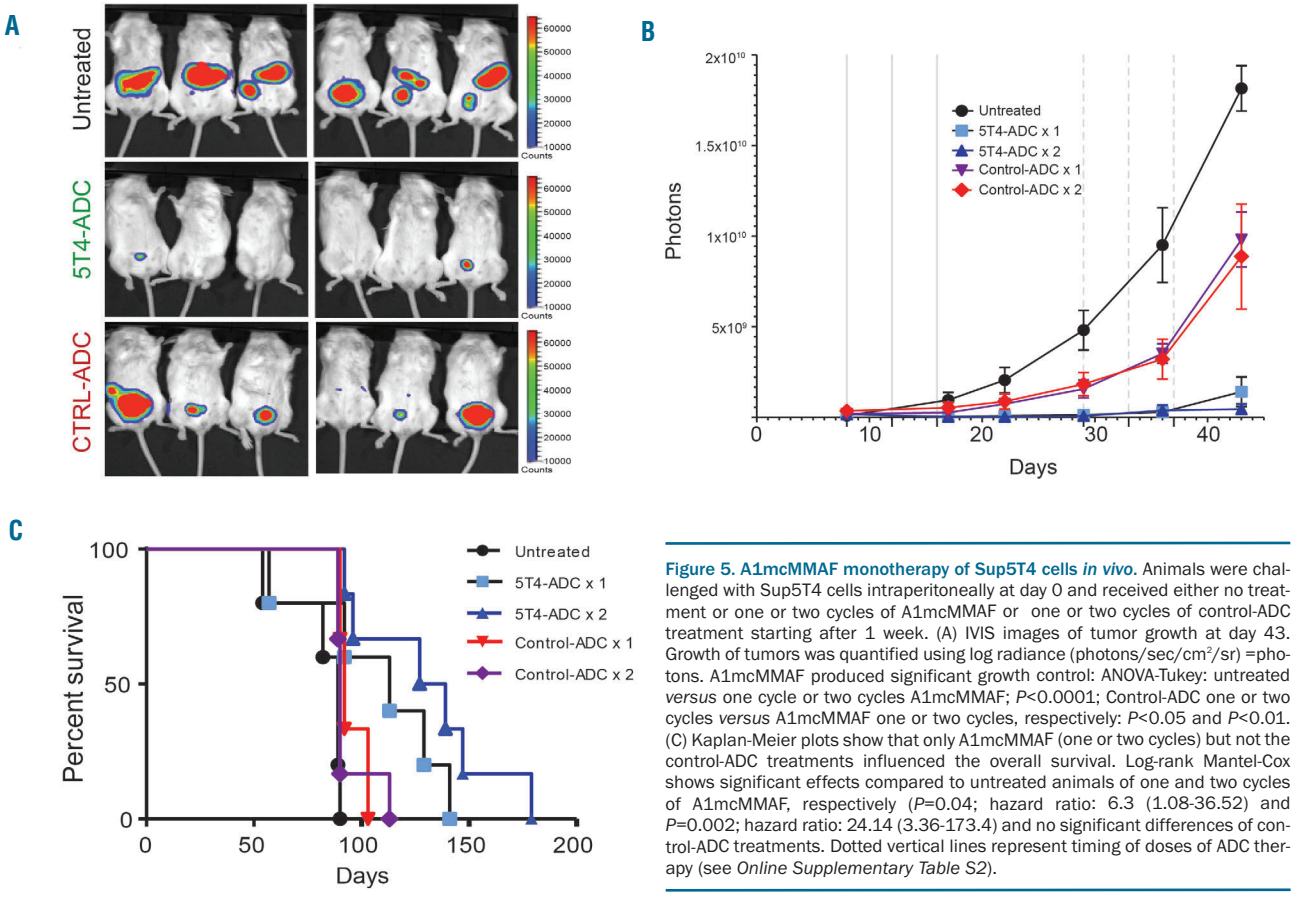

Figure 5. A1mcMMAF monotherapy of Sup5T4 cells *in vivo*. Animals were challenged with Sup5T4 cells intraperitoneally at day 0 and received either no treatment or one or two cycles of A1mcMMAF or one or two cycles of control-ADC treatment starting after 1 week. (A) IVIS images of tumor growth at day 43. Growth of tumors was quantified using log radiance (photons/sec/cm^2/sr) =photons. A1mcMMAF produced significant growth control: ANOVA-Tukey: untreated *versus* one cycle or two cycles A1mcMMAF; *P*<0.0001; Control-ADC one or two cycles *versus* A1mcMMAF one or two cycles, respectively: *P*<0.05 and *P*<0.01. (C) Kaplan-Meier plots show that only A1mcMMAF (one or two cycles) but not the control-ADC treatments influenced the overall survival. Log-rank Mantel-Cox shows significant effects compared to untreated animals of one and two cycles of A1mcMMAF, respectively (*P*=0.04; hazard ratio: 6.3 (1.08-36.52) and *P*=0.002; hazard ratio: 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 *Online Supplementary Table S2*).

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 tumor load. Indeed by the termination of the experiment no signs of leukemia had been detected in the marrow, spleen or peripheral blood of any animal that had received VXL therapy and reached morbidity prematurely.

Dexamethasone and A1mcMMAF treatment

To minimize the adverse reactions seen with VXL, an alternative combination protocol of dexamethasone followed by four cycles of control or A1mcMMAF was tested. Furthermore, general indicators of normal hematopoiesis, i.e. hemoglobin, total red and white blood cell and reticulocyte counts, were measured. Accordingly, $2x10^5$ HR08 PDX cells were transplanted. A week later, dexamethasone was given daily (Monday-Friday) for 2 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 (*Online Supplementary Figure S7*). Engraftment was noted at days 75 and 100 in untreated and dexamethasone-treated mice, respectively, and at day 108 all groups receiving dexamethasone had significantly fewer circulating blasts in peripheral blood samples compared to the untreated group (Figure 7A). By day 129, engraftment in the dexamethasone only, and dexamethasone followed by control-ADC groups had increased and was no longer statistically different from that in the untreated group while the peripheral blood of the animals treated with A1mcMMAF/dexamethasone combination therapy remained tumor-free (Figure 7B). By day ~170 engraftment levels in the dexamethasone and untreated groups were similar, being ~90%, compared to <20% in the group treated with the A1mcMMAF/dexamethasone combination (Figure 8A). The median survival times of untreated, control-ADC/dexamethasone-, dexamethasone- and A1mcMMAF/dexamethasone-treated animals were 169.5, 144.5, 220 and >350 days, respectively. While dexamethasone treatment was able to confer a significant survival advantage (P=0.006) when employed as monotherapy, the greatest impact on survival was observed when it was given in combination with A1mcMMAF (P=0.0006)

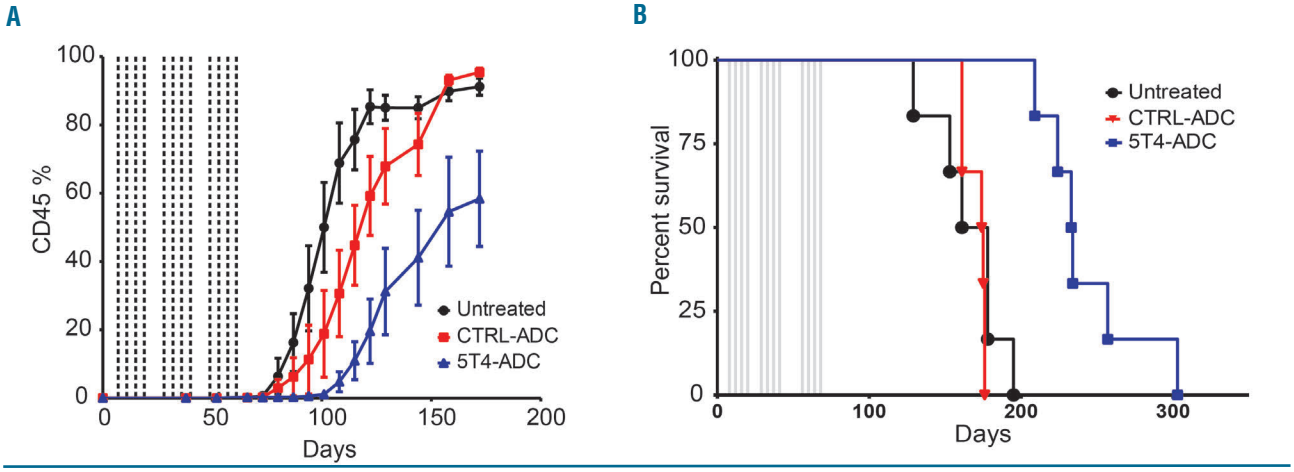

Figure 6. A1mcMMAF monotherapy of HR08 B-cell acute lymphoblastic leukemia patient-derived xenotransplant challenge. (A) At a lower tumor challenge of 2000 HR08 cells only A1mcMMAF significantly reduced engraftment (ANOVA/Tukey; P=0.02) which corresponded with (B) a significant improvement in overall survival with A1mcMMAF treatment (log-rank Mantel-Cox; P=0.0014, hazard ratio: 21.55 (95% confidence interval 3.73-124.5). Dotted vertical lines represent timing of doses of ADC therapy (see *Online Supplementary Table S2*). Untreated, black symbols/line; control-ADC, red symbols/line and 5T4-ADC blue symbols/line.

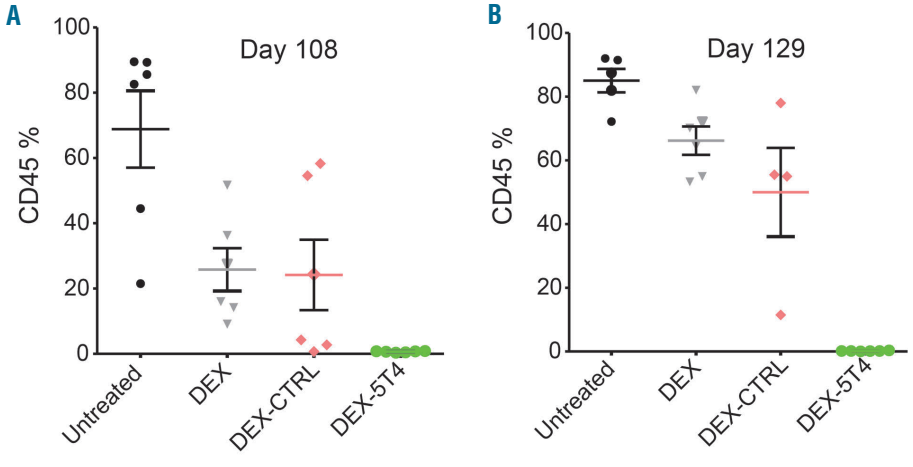

Figure 7. Combination dexamethasone chemotherapy and A1mcMMAF treatment of HR08 patient-derived xenograft: early engraftment. (A) The percentage of peripheral blood blasts was significantly reduced at day 108 in NSG mice engrafted with $2x10^4$ HR08 cells administered dexamethasone (DEX) therapy 1 week after transplantation (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 that of 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 8B). The outcomes of dexamethasone/control-ADC-treated mice appeared to be inferior to those of untreated animals (P=0.012). We speculate that this is a consequence of dexamethasone-induced upregulation of Fc receptors[33] mediating a non-specific uptake of the microtubule-disrupting agent, leading to increased toxicity and death. The specificity of A1mcMMAF targets the drug to leukemic cells, presumably avoiding such toxicity in the animals treated with dexamethasone and A1mcMMAF.

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 favor migration to the bone marrow environment. The hematopoietic 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] Although our sample size was 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 four MRD^{hi} PDX and our previous studies linked 5T4 expression to high risk of relapse in pediatric BCP-ALL patients.[11] The bone marrow microenvironment provides a protective niche for ALL cells[25,35-38] and in the context of this study suggests that 5T4$^+$ cells in the protective bone marrow microenvironment niche may survive chemotherapy and contribute significantly to the MRD population that has recently been described.[25] Using the MLL primagraft with the highest proportion of 5T4$^+$ blasts, 5T4-specific antibody/magnetic bead depletion and limiting dilution challenge in NSG mice clearly demonstrated that 5T4$^+$ blasts are the most clonogenic *in vivo* and consistent with the concept of a leukemia-initiating cell.[30] Concomitant with earlier reports on 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, residual 5T4$^+$ cells could therefore 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 after therapy and give rise to recurrences. The problem is that not a great deal is known about surface antigens associated with a resistant phenotype. Recently, a chimeric antigen receptor T-cell 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 study, we modeled this by first transplanting 5T4$^+$ PDX, treating with chemotherapy and then, in the MRD setting in which blasts were not detectable in peripheral tail vein bleeds, administering A1mcMMAF. A1mcMMAF significantly delayed the emergence of leukemic blasts in both VXL- and dexamethasone-treated mice. Although we previously reported that HR08 is resistant to steroids *ex vivo*,[26] dexamethasone-treated PDX mice showed an initial response, followed by disease kinetics similar to that of 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 from using

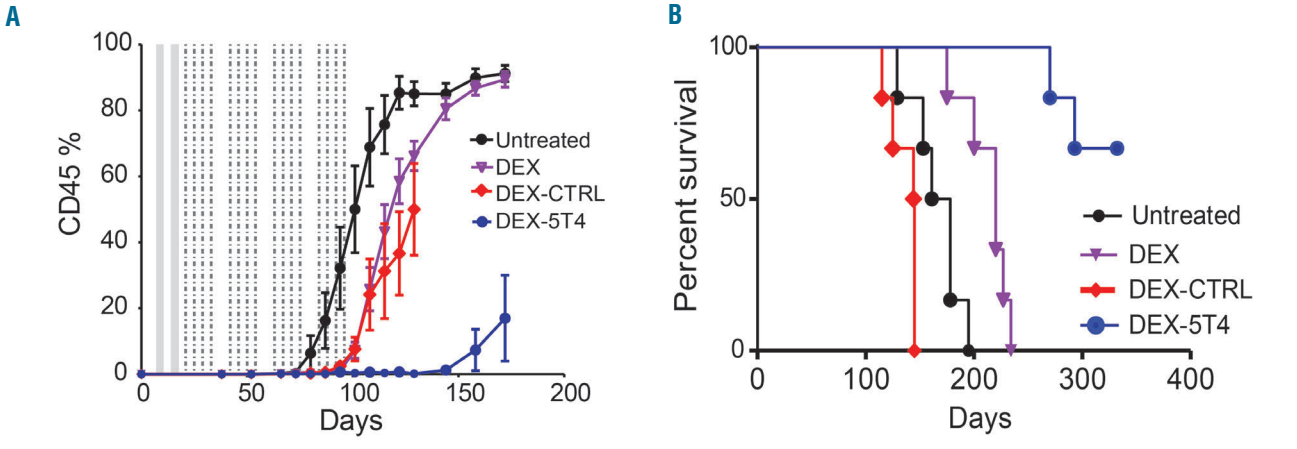

Figure 8. Combination dexamethasone chemotherapy and A1mcMMAF treatment of HR08 patient-derived xenograft: engraftment kinetics and survival. (A) The engraftment of HR08 blasts remained significantly lower (P<0.001) in the group treated with dexamethasone (DEX)/A1mcMMAF than in all other groups for at least 172 days. (B) A significant improvement in overall survival compared to untreated animals (log-rank Mantel-Cox) was seen with DEX: P=0.006; hazard ratio (95% confidence interval): 9.6 (1.9-47.6); DEX/A1mcMMAF P=0.0006; hazard ratio (95% confidence interval): 21.55 (3.7-124.5). DEX/control-ADC treated animals showed slightly poorer survival than untreated animals: P=0.012; hazard ratio (95% confidence interval): 0.13 (0.03-0.63). Hatched areas and dotted vertical lines represent timing of chemotherapy and doses of ADC therapy, respectively (see *Online Supplementary Table S2*). Black circles/line, untreated; purple triangles/line, DEX alone; red diamonds/line DEX-control-ADC; blue circles/line DEX-5T4-ADC.

repeated cycles. In fact a mcMMAF-conjugated anti-CD19 (denintuzomab mafodotin) ADC, given at 3-week intervals has produced remission rates of 35% with acceptable toxicities in an ongoing phase I study.[43]

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 tumor load in NSG mice led to tumor lysis, requiring an altered cell dose and treatment schedule. Clearly engraftment kinetics differs with patients' samples and passages, and needs 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 post-mortem leukemia. In contrast to previous reports,[28] morbidity and mortality in these mice was neither due to central nervous system 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$^+$ 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$^+$ blasts in NSG mouse femora. 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 leukemia cells to cytotoxic drugs. A CXCR4 inhibitor, AMD3100, has been used as a means of mobilizing leukemic 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 disruption of CXCR4 function confined more specifically to malignant hematopoietic cells.

The bulk of ALL cells are chemosensitive and morphological remission is achieved using a combination of three or four drugs in ~98% of cases. The current strategy for childhood ALL consists of intensifying therapy for those with high MRD levels, continuing treatment for 2-3 years and, in some cases, performing allogeneic stem cell transplantation. Thus conventional therapy, though effective, is complicated, expensive and toxic. CD19-targeted therapy has 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] Now that we have an extensive understanding of CD antigens expressed on the B-cell plasma membrane,[46] this 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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