Original Article

Low Dose Total Body Irradiation Combined With Recombinant CD19-Ligand × Soluble TRAIL Fusion Protein is Highly Effective Against Radiation-resistant B-precursor Acute Lymphoblastic Leukemia in Mice ☆

Fatih M. Uckun [a,b,c,*], Dorothea E. Myers [a], Hong Ma [a], Rebecca Rose [d], Sanjive Qazi [a,e]

[a] Children's Center for Cancer and Blood Diseases, Children's Hospital Los Angeles (CHLA), Los Angeles, CA 90027, USA
[b] Division of Hematology–Oncology, Department of Pediatrics, University of Southern California Keck School of Medicine (USC KSOM), Los Angeles, CA 90027, USA
[c] Norris Comprehensive Cancer Center, University of Southern California Keck School of Medicine (USC KSOM), Los Angeles, CA 90027, USA
[d] Rose Pathology Services, LLC, St. Paul, MN 55104, USA
[e] Bioinformatics Program, Gustavus Adolphus College, 800 W College Avenue, St. Peter, MN 56082, USA

ABSTRACT

In high-risk remission B-precursor acute lymphoblastic leukemia (BPL) patients, relapse rates have remained high post-hematopoietic stem cell transplantation (HSCT) even after the use of very intensive total body irradiation (TBI)-based conditioning regimens, especially in patients with a high "minimal residual disease" (MRD) burden. New agents capable of killing radiation-resistant BPL cells and selectively augmenting their radiation sensitivity are therefore urgently needed. We report preclinical proof-of-principle that the potency of radiation therapy against BPL can be augmented by combining radiation with recombinant human CD19-Ligand × soluble TRAIL ("CD19L–sTRAIL") fusion protein. CD19L–sTRAIL consistently killed radiation-resistant primary leukemia cells from BPL patients as well as BPL xenograft cells and their leukemia-initiating in vivo clonogenic fraction. Low dose total body irradiation (TBI) combined with CD19L–sTRAIL was highly effective against (1) xenografted CD19$^+$ radiochemotherapy-resistant human BPL in NOD/SCID (NS) mice challenged with an otherwise invariably fatal dose of xenograft cells derived from relapsed BPL patients as well as (2) radiation-resistant advanced stage CD19$^+$ murine BPL with lymphomatous features in CD22ΔE12xBCR-ABL double transgenic mice. We hypothesize that the incorporation of CD19L–sTRAIL into the pre-transplant TBI regimens of patients with very high-risk BPL will improve their survival outcome after HSCT.

1. Introduction

B-precursor acute lymphoblastic leukemia (BPL) is the most common childhood cancer and a major cause of cancer-related mortality in children and adolescents (Asselin et al., 2013). Total body irradiation (TBI)-based myeloablative pretransplant conditioning regimens are frequently used in hematopoietic stem cell transplantation (HSCT) for high-risk remission BPL (Bar et al., 2014; Bachanova et al., 2012; Bernard et al., 2014; Kalaycio et al., 2011; Pulsipher et al., 2009; Balduzzi et al., 2014; Tracey et al., 2013; Locatelli et al., 2012; Mori et al., 2012; Gaynon et al., 2006; Marks et al., 2006). Although TBI-

containing contemporary conditioning regimens are more effective than conditioning regimens without TBI (Kalaycio et al., 2011; Marks et al., 2006), relapse rates have remained high post-HSCT even after the use of very intensive TBI-based conditioning regimens, especially in patients with a high minimal residual disease (MRD) burden (Uckun et al., 1993a; Pulsipher et al., 2009; Balduzzi et al., 2014; Bar et al., 2014; Bachanova et al., 2012; Asselin et al., 2013), which is in agreement with our published data demonstrating that BPL cells are radiation-resistant (Uckun et al., 1993b, 2010a,b,c, 2013, 2015a,b). New agents capable of killing radiation-resistant BPL cells and selectively augmenting their radiation sensitivity are therefore urgently needed. Such new agents could provide the basis for innovative and highly effective conditioning regimens that employ reduced intensity TBI with substantially reduced toxicity for organs at risk.

Ionizing radiation has been shown to activate the Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) signaling pathway in BPL cell lines, which has been proposed to cooperate synergistically with the cytotoxic effect of radiation (Kruyt, 2008, Gong et al., 2000).

Inducing TRAIL signaling with a soluble TRAIL protein has been shown to amplify the potency of ionizing radiation against BPL cell lines (Wissink et al., 2006). Furthermore, the anti-cancer activity of sTRAIL, as well as agonistic anti-TRAIL-R antibodies, is potentiated by irradiation (Fox et al., 2010; Dimberg et al., 2013; Bremer, 2013; Merino et al., 2007). The anti-cancer activity of recombinant human sTRAIL in preclinical models was more impressive when it was combined with chemotherapeutic drugs or radiation (Fox et al., 2010; Dimberg et al., 2013; Bremer, 2013; Merino et al., 2007). We recently developed the recombinant human CD19L–sTRAIL fusion protein as an apoptosis-inducing anti-leukemic biotherapeutic agent with favorable safety and pharmacokinetic features as well as potent single agent anti-BPL activity in vivo (Uckun et al., 2015a). The purpose of the present study was to examine the ability of CD19L–sTRAIL, a potent and selective inducer of pro-apoptotic TRAIL signaling in CD19-positive BPL cells, to augment the anti-leukemic potency of low dose TBI in NS mouse xenograft models and CD22ΔE12xBCR double-transgenic (Tg) mouse models of radiation-resistant BPL. Our study provides preclinical proof of principle that the anti-leukemic potency of TBI regimens can be significantly augmented by CD19L–sTRAIL at non-toxic dose levels. Further development of CD19L–sTRAIL plus reduced intensity TBI as a new conditioning regimen has the potential to significantly improve the event-free survival (EFS) outcome and long-term health status of high-risk remission BPL patients undergoing HSCT.

2. Materials and Methods

2.1. Recombinant Human CD19L–sTRAIL Fusion Protein

A stepwise molecular cloning strategy was employed using the commercially available pFUSE-hIgG1-Fc2 plasmid (Invivogen, CA) as the backbone vector to construct an expression cassette for CD19L–sTRAIL (Uckun et al., 2015a). CD19L–sTRAIL is a fusion protein of sTRAIL connected to the C-terminus of the CD19L protein through a flexible linker (Fig. S1A). It was produced in a mammalian expression system, as recently described in detail (Uckun et al., 2015a). CD19L–sTRAIL was purified using anion exchange (AIE) chromatography and size exclusion (SE) chromatography (Fig. S1B1). The presence of the CD19L and sTRAIL domains was documented by Western blot analysis (Fig. S1B2 & B3). The CD19-specific immunoreactivity of CD19L–sTRAIL was confirmed by demonstrating that fluorescent-labeled CD19L–sTRAIL binds to the surface membrane of $CD19^+$ human leukemia cells, but not to CD19 receptor-negative human 293T cells (Uckun et al., 2015a). The binding of CD19L–sTRAIL to $CD19^+$ human leukemia cells was mediated via its CD19L domain which was directed to the CD19 extracellular domain, because it could be blocked by an IgG1-Fc fusion protein of recombinant human CD19L used as a CD19 receptor-specific competitor as well as by a recombinant CD19 extracellular domain protein (but not by a recombinant CD19 intracellular domain protein) (Uckun et al., 2015a). CD19L–sTRAIL caused apoptosis in the CD19 receptor positive ALL-1 cell line in a concentration-dependent fashion with ~90% apoptosis at 210 fM and >99% apoptosis at 2.1 pM (Uckun et al., 2015a). CD19L–sTRAIL induced apoptosis of BPL cells was mediated by the CD19-specific binding of its CD19L-domain to BPL cells because (i) the presence of a 20-fold molar excess of soluble $CD19^{ECD}$ protein that specifically binds the CD19L domain could significantly block the anti-leukemic action of CD19L–sTRAIL, whereas a 20-fold molar excess of soluble $CD19^{ICD}$ protein that was included as a control protein did not affect its action and (ii) sTRAIL alone did not cause apoptosis in ALL-1 cells even at a 100 nM concentration (Uckun et al., 2015a). NHS-Fluorescein (NHSF) (5/6-carboxyfluorescein succinimidyl ester; excitation wavelength: 494 nm, emission wavelength: 518 nm — both identical to the same parameters for FITC; Thermo Scientific, Waltham, MA) was used for fluorescent labeling of CD19L–sTRAIL via its primary amines according to the

manufacturer's recommendations, as previously described (Uckun et al., 2015a).

2.2. Leukemia Cells

Primary leukemia cells from 17 BPL patients and 10 BPL xenograft clones derived from spleen specimens of xenografted NS mice inoculated with primary leukemia cells from 5 pediatric BPL patients (4 in relapse, 1 newly diagnosed) were used in the described experiments. The secondary use of leukemic cells for subsequent molecular studies did not meet the definition of human subject research per 45 CFR 46.102 (d and f) since it did not include identifiable private information, and it was approved by the IRB (CCI) at the Children's Hospital Los Angeles (CHLA). We also used $CD19^+$ primary mouse BPL cells from CD22ΔE12-transgenic (Tg) and CD22ΔE12xBCR-ABL double-Tg mice.

2.3. Flow Cytometry, Immunofluorescent Staining, Western Blot Analyses, and Apoptosis Assays

Immunofluorescent staining of cells, flow cytometry, immunoblotting using the ECL detection system (Amersham Pharmacia Biotech), and apoptosis assays were performed, as described in detail in previous publications (Uckun et al., 2010a,b, 2011a,b, 2012, 2013, 2015a,b).

2.4. Evaluation of the In Vitro Anti-leukemic Potency of CD19L–sTRAIL Plus Radiation Using Apoptosis Assays

We used primary leukemia cells from 17 patients with BPL as well as 10 ALL xenograft clones derived from 5 BPL patients to examine the in vitro anti-leukemic potency of CD19L–sTRAIL + radiation. Cells were analyzed for apoptosis at 48 h after the initiation of treatment using the standard quantitative flow cytometric apoptosis assay with the Annexin V-FITC Apoptosis Detection Kit from Sigma (St. Louis, MO), as previously reported (Uckun et al., 2015a; Uckun et al., 2011b, 2013). The labeled cells were analyzed on a LSR II flow cytometer (Becton Dickinson, Lakes, NJ). The in vitro anti-leukemic potency of CD19L–sTRAIL plus radiation was documented by comparing the numbers of residual viable lymphoid cells in the 48 h cultures of untreated control cells vs. cells exposed to the test treatments. Specifically, the number of viable cells was determined by first determining in each sample the number of lymphoid cells remaining in the P1 lymphoid window of the FSC vs. SSC light scatter plots using the formula: N(P1) = 10,000 (i.e. total number of cells analyzed) × % of cells in P1. Then, we determined the % of viable cells in the P1-window as the % of Annexin V-FITC$^-$ PI$^-$ cells in the left lower quadrant of the Annexin V-FITC vs. PI fluorescence intensity dot plots. The number of viable lymphoid cells (N[viable]) was then determined using the formula: N(P1) × % Viable Cells in P1/100. The percent apoptosis (%A) was calculated using the formula: 100 − N[viable for Test sample] / N[viable for Control sample] × 100. We constructed a Mixed Model ANOVA with one factor that identified the treatment effect for either primary leukemia cells from BPL patients or BPL xenograft cells for the analysis of the apoptosis data. The statistical model included a fixed effect ("treatment") and a random effect ("case") that controlled for multiple measurements taken from a BPL xenograft sample or primary BPL cells. The least squares method was used to fit the parameters for the general linear model and these parameters were utilized to generate prediction equations and best-fit lines were visualized by plotting leverage graphs using standard coding procedures. We examined the distribution of the residuals of the model for equal dispersion around the line of best fit to assess the effect of the boundary values of 0 and 100 on the model. Effect sizes from differences between treatment least square mean estimates were used in the design of planned linear contrasts to determine significant effects (two-tailed $P < 0.05$ deemed significant calculated in JMP software [SAS, Cary, NC]). The standard error

estimate (Root Mean square Error term in the model) used for testing differences between group means was obtained from the residuals of the linear fit for all the data and this minimized the effect of skewed standard deviation estimates from measurements close to the boundary values. To compare group means between 2 treatments within a single level of a factor, the linear contrast utilized the standard error derived from the model and the comparison group means were coded with linear parameter values of 1 and −1 to calculate effect size between the 2 treatments. Linear contrasts compared treatment of: CD19L–sTRAIL alone with each combination of CD19L–sTRAIL plus 50, 100 or 200 cGy dose of radiation; radiation alone (50, 100 or 200 cGy) with each combination of CD19L–sTRAIL plus 50, 100 or 200 cGy dose of radiation; and, 50 cGy with 200 cGy dose of radiation.

2.5. Evaluation of the Effects of In Vitro CD19L–sTRAIL + Radiation Treatments on Leukemia-initiating Cells (LICs) in BPL Xenograft Samples

In experiments aimed at evaluating the effects of CD19L–sTRAIL + radiation (RAD) on the LICs (i.e. putative leukemic stem cell fractions capable of engrafting and causing overt leukemia in NS mice) in the BPL xenograft samples, leukemia cells (cell density: 2×10^6 cells per mL) isolated from the spleens of xenografted mice challenged with primary leukemic cells from a pediatric BPL patient were (i) irradiated with 2 Gy γ-rays (N = 11), (ii) treated for 24 h at 37 °C with CD19L–sTRAIL at a concentration of 2.1 pM (N = 14), (iii) treated with 2 Gy γ-rays + 2.1 pM CD19L–sTRAIL (24 h incubation at 37 °C) (N = 4), or (iv) left untreated for 24 h at 37 °C and then reinjected (N = 12). We compared the effects of CD19L–sTRAIL plus radiation vs. other treatments on the ability of the LICs in BPL xenograft specimens to engraft and cause fatal leukemia in NS mice, as previously described (Uckun et al., 2013, 2015a). For the analysis of the in vitro potency of various treatments against the LICs in xenograft specimens, we compared the mean spleen size, spleen cellularity as well as absolute lymphocyte counts (ALCs) of mice inoculated with xenograft cells that were subjected to these treatments prior to injection (radiation: 24 h culture at 37 °C followed by 2 Gy γ-ray exposure immediately prior to injection; CD19L–sTRAIL: 24 h incubation at 37 °C prior to injection; radiation + CD19L–sTRAIL: 24 h incubation with CD19L–sTRAIL followed by 2 Gy γ-ray exposure immediately prior to injection). For statistical analysis of the data, we employed both Independent T-tests with correction for unequal variance (Microsoft Excel) where appropriate and Linear Contrasts that were constructed from Mixed Model ANOVA for spleen size and nucleated spleen cell counts (Log$_{10}$ transformed). P-values less than 0.05 were deemed significant if the False Discovery Rate was less than 10%.

2.6. Transgenic (Tg) Mice

The CD22ΔE12-transgenic (Tg) mouse model of BPL was recently described (Uckun et al., 2015b). CD22ΔE12xBCR-ABL double-Tg mice were established by breeding commercially obtained male BCR-ABL (p190) Tg founder mice (B6.Cg-Tg (BCR-ABL) 623Hkp/J, Jackson Labs) with female CD22ΔE12-Tg mice. Pups were screened for the presence of the BCR-ABL and CD22ΔE12 transgenes by PCR analysis of tail DNA: the CD22ΔE12-Tg amplicon of 391 bp was detected as previously described (Uckun et al., 2014, 2015b). DNA was also amplified with a BCR-ABL Tg forward primer agagatcaaacaccctaacct and a BCR-ABL Tg reverse primer ccaaagccatactccaaatgc for an expected amplicon of 417 bp. Double-Tg mice were identified by PCR detection of both transgenes in their genomic DNA samples (Fig. S2).

2.7. Standard Chemotherapy Drugs

We used the standard chemotherapy drugs commonly used in B-precursor ALL therapy, including Vincristine (Lot #: X067139A;

Manufacturer: Hospira Inc., Lake Forest, IL), PEG-Asparaginase (Oncospar; Lot #: 0009A; Manufacturer: Enzon Pharmaceuticals Inc., Bridgewater, NJ); as well as Dexamethasone (Cat. #: D9184-100MG; Sigma, Saint Louis, MO) as controls. Imatinib was obtained from the LC Labs (Cat# BCK-104; Woburn, MA). The chemotherapy drugs were obtained from the Pharmacy of the Children's Hospital Los Angeles.

2.8. Irradiation of Cells and Mice

NS mice and Tg mice with advanced leukemia were placed in autoclaved 2 L Pyrex Griffin glass beakers (VWR, Radnor, PA) and irradiated with single dose TBI (2 Gy for NS mice/4 Gy for transgenic leukemic C57BL/6 mice) delivered at 106 cGy per min using a self-shielded Cs-137 irradiator (Mark I Irradiator-68A, JL Sheperd & Associates, San Fernando, CA), as previously reported (Uckun et al., 2010c). Cells were irradiated with 50–200 cGy γ-rays in a single exposure using the Mark I Cs-137 irradiator (Uckun et al., 2010c, 2013).

2.9. Evaluation of the Safety and Efficacy of Systemic CD19L–sTRAIL + Low Dose TBI Regimen in a NS Mouse Model of Relapsed BPL
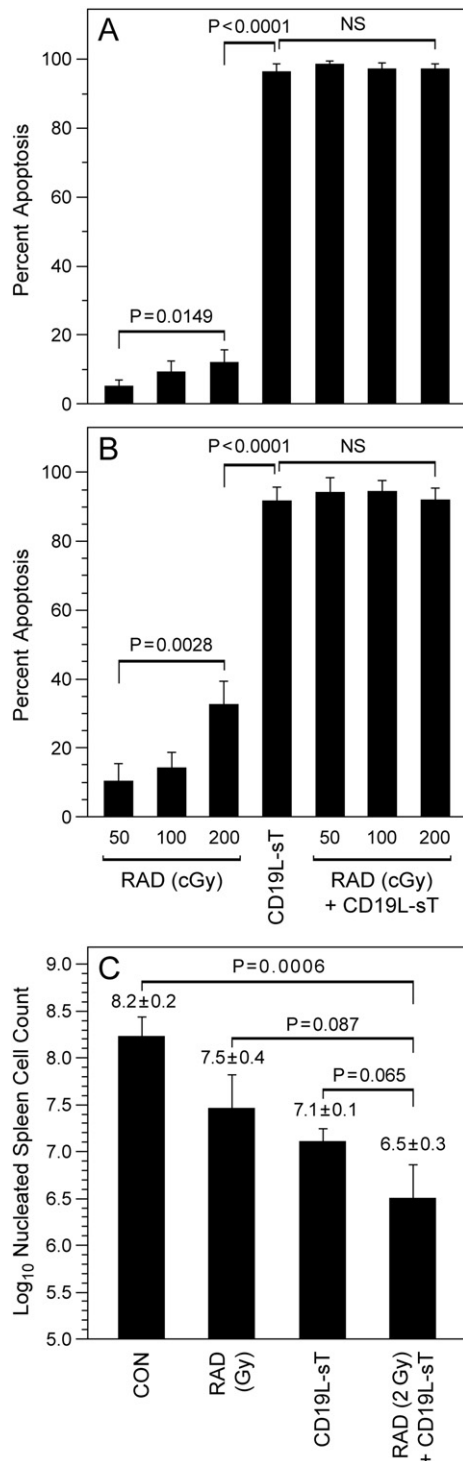
The anti-leukemic activity and toxicity of CD19L–sTRAIL + TBI combination therapy were studied in a NS mouse model of human BPL (Uckun et al., 2013, 2015a). NS mice (NOD.CB17-Prkdc^{scid} J; 4–6 weeks of age at the time of purchase, female) were obtained from the Jackson Laboratory (Sacramento, CA). The research was conducted according to Institutional Animal Care and Use Committee (IACUC) Protocol # 280-12 that was approved on 7-10-2012. All animal care procedures conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington DC 1996, USA). The specific pathogen-free (SPF) environment for immuno-deficient NS mice was ensured by the use of Micro-Isolator cages, which were autoclaved, complete with rodent chow and hardwood Sani-Chips for bedding. Water was provided ad libidum and was also autoclaved as well as supplemented with Bactrim or Septra (0.89 mg per mL sulfa-methoxazole, 0.18 mg per mL trimethoprime) by adding 22.75 mL of Bactrim or Septra to each liter of water once per week as prophylaxis. A light and dark cycle of 12 h each was strictly adhered to, as was a room temperature of 70–75 °F. Animals remained within the confines of the Micro-Isolators except for scheduled cage changes and treatments, which were performed in a laminar flow hood. Ibuprofen was used as a pain reliever to reduce the discomfort associated with treatment or inoculation of leukemia cells. The first 2 independent experiments (20 mice per experiment) were aimed at evaluating the safety of the CD19L–sTRAIL + TBI combined immunoradiotherapy regimen and its ability to prevent leukemia initiating cell (LICs) fractions of the i.v. inoculated xenograft clones from causing fatal leukemia in NS mice. These xenograft clones were derived from primary leukemia cells of two pediatric BPL patients in relapse. NS mice (6–8 week old, female, same age in all cohorts in each independent experiment) were inoculated intravenously (iv) with ALL xenograft cells (2×10^6 leukemia cells in 0.2 mL PBS) via tail vein injection with a 27-gauge needle. All NOD/SCID mice were genetically identical, of the same age, and in each experiment all mice were inoculated with the same number of BPL cells from the identical BPL xenograft clone. This statistical equivalency of mice allowed the use of a pseudo-randomization convenience allocation to assign mice to identified cages. For random treatment allocation, cages were randomly selected to receive one of the specified treatments. Mice were treated with 2 Gy TBI alone, CD19L–sTRAIL alone (17 fmol per kg per day i.v. on days 1 and 2), or with CD19L–sTRAIL + 2 Gy TBI (CD19L–sTRAIL on days 1 and 2 at 17 fmol per kg per day i.v. plus 2 Gy TBI on day 2, 1 h after CD19L–sTRAIL injection). Untreated mice challenged with the same number of leukemia cells were included as controls (CON). Mice were monitored daily and electively euthanized by CO$_2$ asphyxia when any mouse developed morbidity. Blood was collected by cardiac puncture after euthanasia

for (i) complete blood counts (CBCs) on a Bayer Advia 120 (San Diego, CA) blood analyzer and (ii) standard serum chemistry tests using an Olympus AU5400 (Sacramento, CA) instrument. Bone marrow function was assessed by complete blood counts (CBCs) as well as microscopic bone marrow examinations. Liver toxicity was assessed with measurements of serum levels of ALT, alkaline phosphatase (Alk. Ptase.), total bilirubin, and albumin. Pancreas toxicity was assessed with measurements of serum amylase and lipase levels. Renal toxicity was assessed with measurements of serum creatinine and BUN levels. Two-sample Student's T-tests (degrees of freedom adjusted for unequal variances) were performed to assess the significance of the differences in spleen counts, CBCs or blood chemistry values between treatment groups. P-values of less than 0.05 were deemed significant and not corrected for multiple comparisons if the false discovery rate was less than 10% for the number of comparisons that were performed across all the pairwise treatment groups and CBCs and Blood Chemistry measurements. Peripheral smears were stained with Wright–Giemsa and examined microscopically for the presence of circulating leukemia cells. Images were taken with an EVOS XL Core Light Microscope (AMG Bothel, WA) using a 40× objective. The spleens of mice were removed and measured, and cell suspensions were prepared for determination of mononuclear cell counts. At the time of necropsy, 19 different tissues (bone, bone marrow, brain, spinal cord, uterus, ovary, heart, thyroid gland, large intestine, small intestine, kidney, liver, lymph node, lungs, pancreas, peripheral nerve, skeletal muscle, thymus, urinary bladder) were collected within 15 min after sacrifice. Organs were preserved in 10% neutral phosphate buffered formalin and processed for histologic sectioning. For histopathologic studies in 20 mice (5 mice per treatment group), formalin fixed tissues were dehydrated and embedded in paraffin by routine methods. Glass slides with affixed 4–5 micron tissue sections were prepared and stained with Hematoxylin and Eosin (H&E). The brain, liver, kidney, lymph nodes, and bone marrow were examined for their leukemic involvement. Organs were examined for toxic lesions. Images were taken with an EVOS XL Core Light Microscope (AMG Bothel, WA) using 20× and 40× objectives or a Nikon Eclipse Ci camera (Melville, NY) equipped with a Nikon Digital Sight DS-U3 microscope camera controller and Nikon's advanced imaging software suite NIS-Elements. We next examined the effects of the CD19L–sTRAIL + TBI regimen on the survival outcome of xenografted NS mice with advanced and symptomatic leukemia. NS mice (6–8 week old, female, same age in all cohorts in each independent experiment) were inoculated intravenously (iv) with ALL xenograft cells derived from a relapsed pediatric BPL patient (2×10^6 leukemia cells in 0.2 mL PBS) via tail vein injection with a 27-gauge needle. Treatments were initiated on day 10 after the inoculation of xenograft cells unless mice became symptomatic from their leukemia earlier. Our previous studies using our NS mouse model of aggressive BPL (Uckun et al., 2015a) exhibited a median survival time of 17 days (standard deviation = 2 days). A prospective power analysis (2 sample means; JMP, SAS, Cary, NC) testing for a treatment-induced doubling of the median survival time required a total sample size of 6 mice (viz.: 3 mice per group comparing 2 groups) at a 1% significance level and 95% power. A two-sample, one-sided prospective power analysis (exact binomial distribution) was performed to calculate the sample size required per group in a pair-wise comparison to document a statistically significant increase in 60-day survival outcome from 5% (single modality) to 60% (combination modality). This analysis showed that 8 mice per group would be required to document this difference at a 5% significance level and 80% power. All NOD/SCID mice were genetically identical, of the same age, and in each experiment all mice were inoculated with the same number of BPL cells from the identical BPL xenograft clone. This statistical equivalency of mice allowed the use of a pseudo-randomization convenience allocation to assign mice to identified cages. For random treatment allocation, cages were randomly selected to receive one of the specified treatments based on the results of the power analyses regarding the required minimum sample sizes for each treatment group. We allocated a larger than

minimally required number of mice to the CD19L–sTRAIL and CD19L–sTRAIL + TBI treatment groups for more accurate determination of the potential toxicities and tissue collection at the time of death for future molecular biomarker discovery research. We applied concealment of treatment allocation and blind outcome assessment to reduce the risk of bias in our conclusions. Daily health care assessments were performed by animal care technicians not involved in the treatment assignments or treatments who also made the determinations about which of the mice needed to be electively sacrificed to meet the humane endpoints criteria in laboratory animal experimentation. Investigators did not participate in individual health status or outcome assessments. Altogether, 11 CON mice were left untreated. Twenty-three mice with symptomatic xenografted BPL were treated with CD19L–sTRAIL as a single agent (24 fmol per kg over 2 days with commencement on day 6 (N = 10) or 51 fmol per kg over 3 days with commencement on day 10 (N = 13)). Eleven mice received 2 Gy TBI either on day 7 (N = 5) or day 11 (N = 6). Seven mice were treated with chemotherapy administered as a cocktail on day 10: Vincristine (0.05 mg per kg) + Dexamethasone (2 mg per kg) + PEG-Asparaginase (850 IU per kg). Sixteen test mice with symptomatic xenografted leukemia were treated with CD19L–sTRAIL (24 fmol per kg over 2 days with commencement on day 6 plus 2 Gy TBI on day 7 (N = 5) or 51 fmol per kg CD19L–sTRAIL over 3 days with commencement on day 10 plus 2 Gy TBI on day 11 (N = 11). For the analysis of the NS mouse xenograft data on the in vivo potency of CD19L–sTRAIL + low dose TBI vs. other treatments, event-free survival (EFS) times were measured from the day of inoculation of xenograft cells to the day of death or killing of the test mice treated with CD19L–sTRAIL + TBI or other treatments. The probability of survival was determined and the event-free interval curves were generated using the Kaplan–Meier product limit method, as in previous studies (Uckun et al., 2013, 2015a,b). Log-rank tests were performed to compare differences in median survival estimates between all groups and pairwise comparison of the individual treatment groups.

2.10. Evaluation of the Efficacy of the Systemic CD19L–sTRAIL + Low Dose TBI regimen in CD22ΔE12xBCR-ABL Double Transgenic Mice with Advanced BPL

CD22ΔE12xBCR-ABL double-Tg mice spontaneously develop fatal BPL with lymphomatous features at a median of 78 days (Fig. S2). For mice randomly assigned to this project, a prospective power analysis (One-sample proportions using the exact method based on a binomial distribution, One-sided test; JMP, SAS, Cary, NC) showed that a sample size of 6 mice per treatment group would be required to demonstrate an increase in the proportion of CD22ΔE12xBCR-ABL double-Tg mice surviving at 4 weeks from the onset of symptomatic leukemia from a baseline of 0% to 40% with a specific treatment at a 5% significance level and 95% power. Therefore, it was determined that using a group size of 6 mice would make a comparative study sufficiently powered to detect treatment-related large effect sizes. We also performed a two-sample, one-sided prospective power analysis (exact binomial distribution) to calculate the sample size required for each treatment group in a pairwise comparison to observe an increase in 4-week survival outcome from 10% (single modality) to 65% (combination modality). This analysis showed that a sample size of 9 mice per group (18 in total) would be required to detect a change in 4-week survival from 10% to 65% with 5% significance at 80% power. We allocated a larger than minimally required number of mice to the CD19L–sTRAIL and CD19L–sTRAIL + TBI treatment groups (i.e. 10 mice/group) for more accurate determination of the potential toxicities of the combined modality and tissue collection at the time of death for future molecular biomarker discovery research. Leukemic double-Tg mice were randomly assigned an identifying number and a random sequence of mouse numbers was determined by picking out index cards without replacement on days multiple mice developed leukemia. Each leukemic mouse in sequence was

For example, whereas a Group 4 mouse starting on therapy on Monday–Wednesday received 3 daily treatments of CD19L–sTRAIL, a Group 4 mouse starting on therapy on Friday could only receive one dose of CD19L–sTRAIL. Of the 11 mice in Group 4, 5 received a single dose of CD19L–sTRAIL followed by 4 Gy TBI and 6 received 3 doses of CD19L–sTRAIL plus 4 Gy TBI administered on day 1 at 1 h post CD19L–sTRAIL. After the completion of the planned allocations, 6 additional mice were randomized to receive CD19L–sTRAIL alone or Imatinib alone by random assignment of these 2 treatment groups using index cards picked out of a container with replacement. The treatment assignments were performed to obtain CD19L–sTRAIL-resistant and Imatinib-resistant BPL cells for future molecular biomarker discovery research. No objective responses were observed in either group. The data from the CD19L–sTRAIL treated mice were combined with the data from the other CD19L–sTRAIL treated mice in Group 3 to eliminate any bias in effect size assessment. Overall, 7 mice received 3 doses and 1 mouse received 2 doses of CD19L–sTRAIL as a single agent. We used this randomization combined with concealment of treatment allocation and blind outcome assessment to reduce the risk of bias in our conclusions. Animal care technicians not involved in the treatment assignments or treatments performed daily health care assessments. Investigators did not participate in individual health status or outcome assessments. In addition, any unintended intergroup differences in initial tumor size were formally excluded by statistical comparisons. The initial tumor size was comparable among groups, as measured by the longest diameter: 1.0 ± 0.1 cm for CON (N = 8), 1.0 ± 0.1 cm for TBI (N = 5), 1.2 ± 0.2 cm for CD19L–sTRAIL (N = 8), and 1.1 ± 0.1 cm for CD19L–sTRAIL + TBI (N = 11) (One-way ANOVA, P = 0.5). Statistical analyses were performed by a bioinformatics expert (S.Q.) who was not involved in treatment assignments, treatments, or outcome assessments. The masses were photographed using an iPhone 4S (Los Angeles, CA) equipped with an 8-megapixel iSight camera and the dimensions were measured at indicated time points in order to determine the effect of the treatments on disease progression. The tumor-free survival (TFS) (duration of tumor-free interval), progression-free survival (PFS) (time from initiation of therapy to 10% increase in the longest diameter of tumor mass), and overall survival (OS) (time from onset of symptomatic leukemia to the day of death or killing) were determined for each treatment group. Significance of pairwise differences in median values between treatment groups for TFS and PFS times was assessed using Non-parametric Wilcoxon Tests (JMP Software v10.02, SAS, Cary, NC). The probability of OS was determined and the event-free interval curves were generated using the Kaplan–Meier product limit method, as in previous studies (Uckun et al., 2013, 2015a,b). Log-rank tests were performed to compare differences in median survival estimates between all groups and pairwise comparison of the individual treatment groups. Post-treatment tumor size (longest diameter) was normalized to day 1 measurements. Tumor growth profiles of control mice and mice treated with the CD19L–sTRAIL + low dose TBI regimen were compared using a repeated measures analysis of covariance controlling for

Fig. 1. In vitro anti-leukemic potency of CD19L–sTRAIL and CD19L–sTRAIL + low dose radiation against primary BPL cells and ALL xenograft clones. [A & B] The bar charts depict the mean ($\pm$SEM) percentage of apoptosis in human BPL cells treated with ionizing radiation (RAD) (dose levels: 50, 100, or 200 cGy), CD19L–sTRAIL (2.1 pM) or RAD + CD19L–sTRAIL. The percentage of apoptosis was determined as described in the Materials and Methods section. Panel A depicts the results obtained using BPL xenograft clones. Panel B shows the cumulative data on xenograft clones plus primary leukemia cells from BPL patients. [C] CD19L–sTRAIL was examined for its ability to abrogate the ability of leukemia-initiating BPL xenograft cells derived from BPL patients to cause leukemia in NS mice. ALL xenograft cells (2×10^6 cells/sample) were treated with 2.1 pM CD19L–sTRAIL for 24 h at 37 °C and then irradiated with 2 Gy γ-rays. Controls included untreated cells as well as cells that were cultured for 24 h at 37 °C without the addition of CD19L–sTRAIL and then irradiated with 2 Gy γ-rays. Cells were washed in PBS, resuspended in PBS and inoculated into NS mice intravenously via tail vein injections in a 200 μL total volume. Mice were monitored for signs of leukemia and all mice in a given experiment were sacrificed when any mouse developed morbidity. The bar graphs show for each treatment group the average (mean $\pm$ SEM) Log_{10}-transformed nucleated spleen cell count. CD19L–sT: CD19L–sTRAIL.

assigned to one of the 4 treatment groups by randomly picked index cards with replacement designating each of the 4 treatment groups (Group 1: No treatment; Group 2: TBI alone; Group 3: CD19L–sTRAIL alone at 17 fmol per kg per day i.v. for 1–3 days; Group 4: CD19L–sTRAIL at 17 fmol per kg per day i.v. for 1–3 days + 4 Gy TBI at 1 h post the 1st CD19L–sTRAIL injection) for random assignment of treatment groups. Treatments were initiated within 2 days after the onset of symptomatic leukemia or detection of measurable tumor masses. The number of CD19L–sTRAIL treatments in Groups 3 and 4 depended on the day therapy was initiated as treatments could only be given during weekdays.

heterogeneity between mice (REML method to partition experimental and mice variance components; JMP Software v10.02, SAS, Cary, NC). The model was comprised of a fixed factor ("treatment"), time co-variate ("day"), interaction term ("treatment × day") and a random factor (Mouse ID). F-test comparing the least square means for control versus CD19L–sTRAIL + TBI means calculated in the fixed factor was utilized to assess the significance of overall remission or reduction of tumor size (P-values < 0.05 deemed significant). The probability of survival (OS) was determined and the event-free interval curves were generated using the Kaplan–Meier product limit method, as in previous studies (Uckun et al., 2013, 2015a,b). Log-rank tests were performed to compare differences in median survival estimates between all groups and pairwise comparison of the individual treatment groups.

2.11. Statistics

Standard methods were used for statistical analysis of data (Uckun et al., 2013, 2015a,b). In particular, a linear model was used for the analysis of the apoptosis data. For the evaluation of the in vivo anti-leukemic activity of CD19L–sTRAIL, the probability of survival was determined and the event-free interval curves were generated using the Kaplan–Meier product limit method. Log-rank tests were performed to compare differences in median survival estimates between all groups and pairwise comparison of pooled controls vs. test mice treated with CD19L–sTRAIL, TBI, or CD19L–sTRAIL + TBI. For the analysis of the in vitro potency of CD19L–sTRAIL against leukemic stem cells in xenograft specimens, two-tailed T-tests with correction for unequal variance (Microsoft Excel) were performed comparing various parameters of leukemic burden of the NS mice that were inoculated with the treated vs. untreated xenograft cells, including the mean spleen size and cellularity for the various treatments.

2.12. Study Approval

The animal research in mice was conducted according to Institutional Animal Care and Use Committee (IACUC) Protocols 280-12 and 293-10 that were approved by the IACUC of CHLA. All animal care procedures conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington DC 1996, USA). Leukemia cells isolated from deidentified patient specimens were used in the described experiments. The secondary use of leukemia cells for subsequent laboratory studies did not meet the definition of human subject research per 45 CFR 46.102 (d and f) since it did not include identifiable private information, and the corresponding research protocol CCI-10-00141 was approved by the CHLA IRB (CCI) (Human Subject Assurance Number: FWA0001914).

3. Results

3.1. CD19L–sTRAIL Plus Low Dose Radiation Induces Apoptosis in Radiation-resistant Primary Human BPL Cells and BPL Xenograft Clones

We used a quantitative flow cytometric apoptosis assay to examine the ability of CD19L–sTRAIL (2.1 pM) plus low dose radiation with 50–200 cGy (i.e., 0.5–2 Gy) γ-rays to cause apoptotic death in freshly obtained primary leukemic cells from patients with BPL (N = 17) as well as very aggressive in vivo clonogenic human leukemic B-cell precursors isolated from the spleens of xenografted NS mice that developed overt leukemia after inoculation with primary leukemic blast cells from patients with BPL (N = 10) in side by side comparison with 50–200 cGy γ-rays administered in a single dose. Although we observed a dose-dependent apoptosis in irradiated samples of BPL xenograft cells (P = 0.0149 for 5.35 ± 1.59% apoptosis at 50 cGy vs. 12.14 ± 3.46% apoptosis at 200 cGy) (Fig. 1A) as well as primary BPL cells (combined dataset: P-value = 0.0028 for 10.4 ± 4.9% apoptosis at 50 cGy vs. 32.9 ± 6.6%

apoptosis at 200 cGy), even 200 cGy γ-rays killed only 45 ± 9% of primary BPL cells (N = 17) and 12 ± 3% of BPL xenograft cells (N = 10). The average percentage of apoptotic leukemia cells after 200 cGy γ-rays was 33 ± 7% for all 27 samples combined (Fig. 1B). These results are in accord with previous reports and confirm that BPL cells are radiation-resistant with SF2 (surviving fraction at 2 Gy) values > 0.5 (Uckun et al., 1993a,b, 2013). CD19L–sTRAIL (2.1 pM) consistently caused apoptosis in these radiation-resistant human BPL cell populations with 89 ± 6% (N = 17) apoptosis for primary BPL cells, 97 ± 2% (N = 10) for BPL xenograft cells, and 92 ± 4% (N = 27) for the entire sample size (P < 0.0001 vs. 200 cGy γ-rays). The in vitro potency of the CD19L–sTRAIL fusion protein and the limited sensitivity of the apoptosis assay platform prevented an accurate assessment of the potential of the combination therapy vs. CD19L–sTRAIL alone using these in vitro tests (Fig. 1B).

3.2. CD19L–sTRAIL Plus Low Dose Radiation Kills Leukemia-initiating Cells (LICs) in BPL Xenograft Samples

We compared the effects of CD19L–sTRAIL (2.1 pM × 24 h) (N = 14), ionizing radiation (2 Gy) (N = 11), and CD19L–sTRAIL + ionizing radiation (N = 4) on the ability of LICs representing the putative leukemic stem cell subpopulation of radiation-resistant BPL xenograft clones to initiate leukemia in immunodeficient NS mice. Controls included 12 mice inoculated with untreated xenograft cells. Whereas all 12 control mice receiving untreated BPL xenograft cells developed leukemia, only 5 of 11 mice receiving irradiated BPL xenograft cells and only 2 of the 14 mice receiving CD19L–sTRAIL treated BPL xenograft cells developed leukemia (P = 0.0008 for radiation vs. CON; P < 0.0001 for CD19L–sTRAIL vs. CON). Although none of the 4 mice injected with xenograft cells exposed to a combination of CD19L–sTRAIL + radiation developed leukemia, the in vitro potency of the fusion protein prevented an accurate assessment of the potential of the combination therapy. Spleen counts were significantly reduced with CD19L–sTRAIL + 2 Gy radiation compared to control (Independent T-test, Df = 14, T value = 4.415, P = 0.0006; Linear Contrast, P = 0.0004). Strong trends were observed for the reduction in spleen count comparing radiation alone (Independent T-test, Df = 9.22, T value = 1.917, P = 0.087; Linear Contrast, P = 0.041) or CD19L–sTRAIL alone (Independent T-test, Df = 16, T value = 1.984, P = 0.065; Linear Contrast, P = 0.17) vs. combination of radiation + CD19L–sTRAIL. The combination group had the lowest (albeit not significantly lower than the radiation or CD19L–sTRAIL groups) average leukemia burden (6.8 ± 3.4 × 10^6 cells per spleen). The measured values for other groups were 347.8 ± 106.1 × 10^6 cells per spleen for the CON group (P = 0.0004 vs. the combination group), 213.7 ± 128.8 × 10^6 cells per spleen for the radiation group (P = 0.041 vs. the combination group), and 26.3 ± 12.9 × 10^6 cells per spleen for the CD19L–sTRAIL group (P = 0.0006 vs. CON, P = 0.065 vs. the combination group) (Fig. 1C).

3.3. CD19L–sTRAIL Plus Low Dose TBI is Safe and Prevents the Development of Fatal Leukemia in a NS Mouse Model of Relapsed BPL

The limited sensitivity of the in vitro apoptosis assays and the remarkable single agent anti-leukemic potency of CD19L–sTRAIL did not allow an accurate assessment of potential additive or synergistic effects between CD19L–sTRAIL and radiation in vitro. Therefore, we next set out to determine if CD19L–sTRAIL can augment the anti-leukemic potency of TBI against BPL cells in vivo. We first examined the in vivo toxicity and short-term anti-leukemic activity of CD19L–sTRAIL plus low dose TBI (2 Gy) in a feasibility study employing 2 separate NS xenograft models of relapsed human BPL. In both models, xenografted human BPL has a very aggressive course and progresses rapidly. CD19L–sTRAIL was administered i.v. for 2 consecutive days (days 1 and 2), at a non-toxic total dose level of 17 fmol per kg per day after the administration of an invariably fatal dose (2 × 10^6 cells per
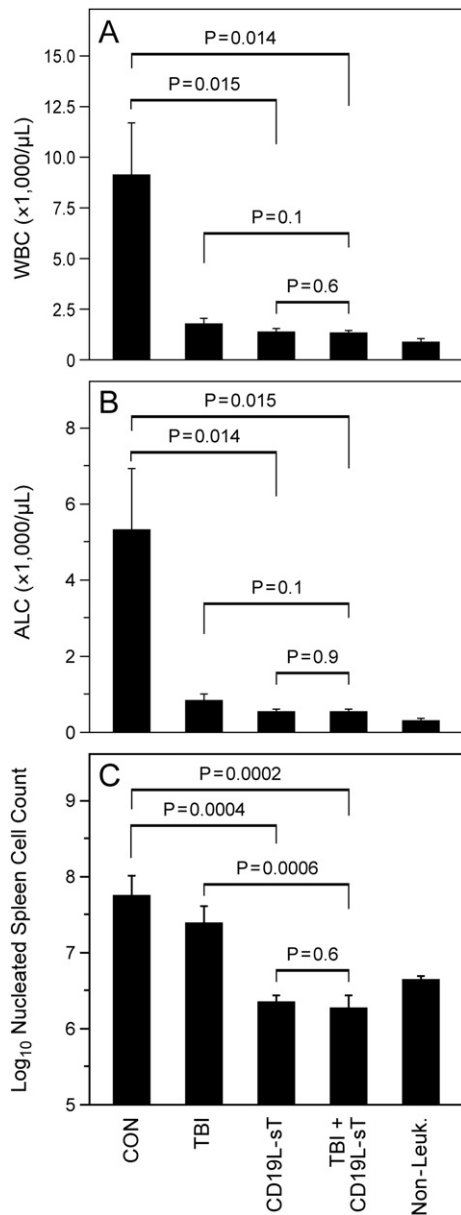
Fig. 2. In vivo anti-leukemic activity and toxicity of CD19L–sTRAIL alone or in combination with low dose TBI in NS mouse models of relapsed BPL. NS mice (5 per group per xenograft model × 2 models; 10 mice per treatment group cumulatively) were inoculated with 2×10^6 xenograft cells derived from 2 relapsed BPL patients. CD19L–sTRAIL was administered intravenously for 2 days (days 1 and 2) at a non-toxic daily dose level of 17 fmol per kg per day. One of the treatment groups received TBI (2 Gy, day 2) and one group received CD19L–sTRAIL (17 fmol per kg per day × 2 days, days 1 and 2) + 2 Gy TBI on day 2. The experiment was terminated by euthanasia of all mice in all treatment groups when untreated control mice (CON) showed signs of morbidity. Depicted are the bar graphs showing the mean values for the nucleated spleen cell count and absolute lymphocyte count (ALC) as surrogate measures of the leukemia burden. Pairwise Independent Student's T-tests (unequal variance correction) were performed to calculate P-values comparing CD19L–sTRAIL and CD19L–sTRAIL + low dose TBI groups with other treatment groups.

mouse) of ALL xenograft cells derived from two relapsed BPL patients. All untreated control mice (N = 10) as well as all low dose TBI-treated mice (N = 10) – but none of the 10 CD19L–sTRAIL + low dose TBI treated mice – developed severe morbidity due to overt leukemia by day 19 (Fisher's exact, two-tailed, P < 0.0001). Likewise, none of the mice treated with CD19L–sTRAIL alone developed morbidity due to treatment toxicity or overt leukemia. CD19L–sTRAIL plus low dose TBI

exhibited potent anti-leukemic activity, as measured by normal ALCs values with the absence of circulating leukemia cells (Fig. 2A & B) as well as normal nucleated spleen cell counts (Fig. 2C). The average ALCs was $0.31 \pm 0.08 \times 10^3$ per μL (WBC: $0.9 \pm 0.2 \times 10^3$ per μL) for non-leukemic control NS mice that were not inoculated with human leukemia cells. By comparison, the average ALCs of untreated CON NS mice that were inoculated with ALL xenograft cells was $5.3 \pm 1.6 \times 10^3$ per μL (WBC: $9.1 \pm 2.6 \times 10^3$ per μL) due to circulating leukemia cells. Likewise, TBI-treated mice had high ALCs values (albeit lower than those in untreated controls) due to circulating leukemia cells. In contrast to the CON or low dose TBI alone groups of mice, test mice treated with CD19L–sTRAIL + low dose TBI had ALCs values ($0.56 \pm 0.06 \times 10^3$ per μL, P = 0.0146) and WBC ($1.3 \pm 0.1 \times 10^3$ per μL, P = 0.0144) values that were within the normal range (Fig. 2A & B). Control mice developed overt disseminated leukemia with massive splenomegaly and an average nucleated spleen cell count of $178 \pm 60 \times 10^6$ (Log_{10} count: 7.8 ± 0.3) that was significantly higher than the nucleated spleen counts of non-leukemic control NS mice ($4.6 \pm 0.4 \times 10^6$, Log_{10} count: 6.6 ± 0.4, P < 0.0001) (Fig. 2C). In mice treated with CD19L–sTRAIL + low dose TBI, the nucleated spleen cell count was only $3.3 \pm 1.0 \times 10^6$ (Log_{10} count: 6.3 ± 0.9), which was significantly lower than the spleen cell counts of CON mice (P = 0.0002) or mice treated with low dose TBI alone (57.1 ± 17.0, Log_{10} count: 7.4 ± 0.2, P = 0.0006) (Fig. 2C). The ALCs (P = 0.9) as well as nucleated spleen cell counts (P = 0.7) of mice treated with CD19L–sTRAIL alone were similar to those of mice treated with CD19L–sTRAIL + low dose TBI (Fig. 2).

There was no clinical or laboratory evidence of moderate-severe acute toxicity associated with the CD19L–sTRAIL + low dose TBI treatments (Fig. 3, Table S1). We performed detailed histopathological examination of multiple tissues from each of 20 NS mice inoculated with ALL xenograft cells derived from a relapsed BPL patient (5 untreated, 5 TBI-treated, 5 CD19L–sTRAIL treated and 5 CD19L–sTRAIL + TBI treated mice). All of the untreated but none of the CD19L–sTRAIL + TBI treated mice showed microscopic evidence of disseminated leukemia involving their bone marrow and at least one of the other 4 target organ sites examined (viz., lymph node, thymus, brain, spinal cord) (2 Tailed Fisher's exact, P < 0.0001). Disseminated leukemia was also found in 4 of 5 TBI-treated mice, as documented by thymic infiltration. No toxic lesions suggestive of significant parenchymal organ damage were detected in any of the 18 organs examined in CD19L–sTRAIL + TBI treated mice (Table S1). Mild bile duct proliferation was found in one of the CD19L–sTRAIL treated mice but not in any of the other mice (Fig. S3). One of 5 mice treated with CD19L–sTRAIL + TBI had minimal hepatocellular degeneration and necrosis in the perivenular location (Fig. S3).

3.4. CD19L–sTRAIL Plus Low Dose TBI Improves the Event-free Survival Outcome in a NS Mouse Model of Advanced Relapsed BPL

Intravenous inoculation of NS mice with 2×10^6 cells of the xenograft UCN3 derived from a relapsed BPL patient causes rapidly progressive fatal BPL with diffuse bone marrow involvement becoming evident within 7 days. Treatments were initiated on day 10 after the inoculation of 2×10^6 xenograft cells unless mice developed signs of leukemia-associated morbidity. The median EFS for this CD19L–sTRAIL + low dose TBI group was 72 days, which was significantly longer than the EFS for the untreated control group CON (17 days, P < 0.0001), TBI alone group (64 days, P = 0.0014), CD19L–sTRAIL alone group (20 days, P = 0.0022), or standard VDL chemotherapy group treated with a combination of Vincristine (0.05 mg per kg) + Dexamethasone (2 mg per kg) + PEG-Asparaginase (850 IU per kg) (17 days, P = 0.0033) (Fig. 4). No mouse in the CD19L–sTRAIL plus TBI group experienced a toxic death or signs of treatment related toxicity. At 64 days, the EFS rate was $0 \pm 0\%$ of mice in the CON or VDL groups, $18 \pm 12\%$ of mice in the TBI alone group and $6 \pm 6\%$ of mice in the CD19L–sTRAIL alone group. In contrast, the EFS rate was $63 \pm 18\%$ (95% confidence
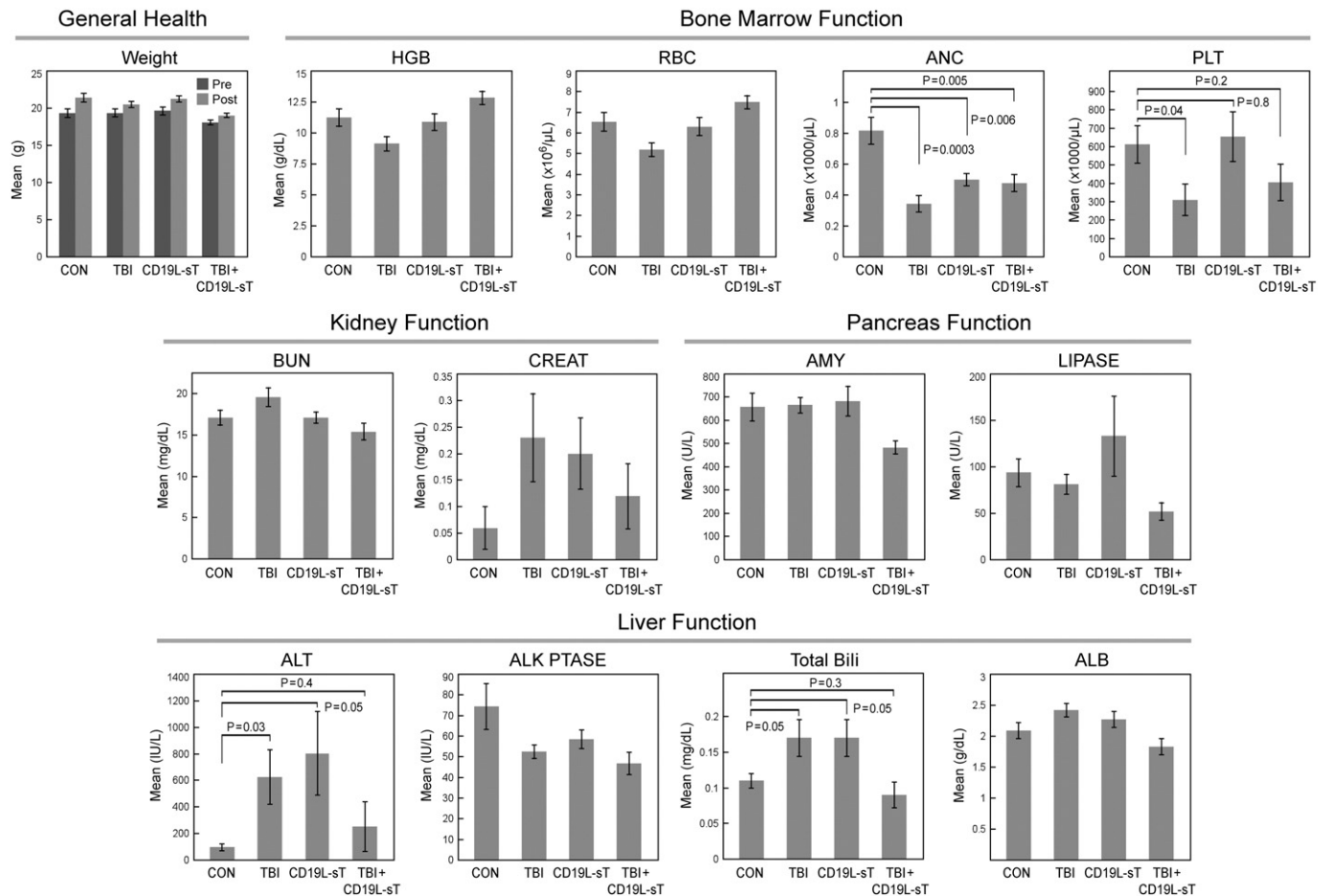
Fig. 3. In vivo safety of CD19L–sTRAIL alone or in combination with low dose TBI in NS mouse models of relapsed BPL. NS mice (5/group/xenograft model × 2 models; 10 mice/treatment group cumulatively) were inoculated with 2×10^6 xenograft cells derived from 2 relapsed BPL patients. CD19L–sTRAIL was administered intravenously for 2 days (days 1 and 2) at a nontoxic daily dose level of 17 fmol per kg per day. One of the treatment groups received TBI (2 Gy, day 2) and one group received CD19L–sTRAIL (17 fmol per kg per day × 2 days, days 1 and 2) + 2 Gy TBI on day 2. The experiment was terminated by euthanasia of all mice in all treatment groups when untreated control mice (CON) showed signs of morbidity. Depicted are the bar graphs showing the mean values for each organ function parameter determined. Pairwise Independent Student's T-tests (unequal variance correction) were performed to calculate P-values comparing CD19L–sTRAIL and CD19L–sTRAIL + low dose TBI groups with other treatment groups. CD19L–sT: CD19L–sTRAIL.

interval = 26–89%) for mice treated with CD19L–sTRAIL + low dose TBI, which was superior to the EFS outcome in any other group (Log rank test, CD19L–sTRAIL + TBI versus all other groups combined, P = 0.0001). These results provided the preclinical proof-of-concept that CD19L–sTRAIL plus low dose TBI would be a safe and effective treatment modality for the treatment of relapsed BPL. The combination treatment was able to overcome the radiation resistance of the leukemia cells and it was more effective than the VDL combination chemotherapy in this model of advanced stage relapsed BPL. We hypothesize that the incorporation of CD19L–sTRAIL into the pre-transplant TBI regimens of patients with relapsed BPL will help improve their survival outcome after hematopoietic stem cell HSCT.

3.5. CD19L–sTRAIL Plus Low Dose TBI Improves the Survival Outcome in a CD22ΔE12xBCR-ABL Double Transgenic Model of Advanced Murine BPL

There is a 57.5% identity of the extracellular domain of the mouse and human CD19 proteins. CD19L–sTRAIL showed significant binding to the surface of highly radiation-resistant CD19$^+$ murine BPL cells from CD22ΔE12xBCR-ABL double-Tg mice (Fig. S2C) and induced 42–66% apoptosis within 24 h (Fig. S2D). CD19L–sTRAIL + low dose (2 Gy) radiation in vitro was markedly more effective than CD19L–sTRAIL alone or low dose radiation alone and caused 88–94% apoptosis (Fig. S2D). Therefore, the CD22ΔE12xBCR-ABL double-Tg mouse model provides a unique opportunity to study the clinical potential of CD19L–sTRAIL plus low dose TBI as a new treatment modality for radiation-resistant BPL in a host with a healthy immune system devoid of the inherent shortcomings of the NS mouse xenograft models. Furthermore, murine BPL cells are DR4$^-$ and show weaker binding to CD19L–sTRAIL than human BPL cells. Therefore, this murine BPL model also provides an opportunity to search for combination regimens that would overcome CD19L–sTRAIL resistance in clinical settings due to reduced expression levels of DR4 and CD19. We randomly assigned mice to one of 4 treatment protocols when they developed symptomatic leukemia or measurable tumor masses. As shown in Fig. 5, the combination of CD19L–sTRAIL (17 fmol per kg per day × 3 days or 17 fmol per kg per day single dose) with 4 Gy TBI yielded PFS and TFS outcomes significantly superior to those of untreated control mice (CON) or mice treated with TBI alone or CD19L–sTRAIL alone.

The comparison of the cumulative tumor progression curves for the CON group vs. CD19L–sTRAIL + TBI group showed a rapid regression of tumor size and a markedly favorable alteration of the disease course over a period of 14 days (Repeated Measures ANCOVA, treatment effect, $F_{1,10.8} = 153.7$, P < 0.0001). We observed a greater than 1.5-fold increase of tumor size in CON mice within 4 days, whereas the tumors of the mice in the CD19L–sTRAIL + TBI group rapidly regressed within 4 days and remained undetectable for 14 days after each course of treatment (Fig. 5B). The mean ± SE values for PFS were 24.0 ± 4.0 days for CD19L–sTRAIL + TBI, but only 0 ± 0 days for CON (Non-parametric Wilcoxon test, P < 0.0001), 3.4 ± 0.9 days for CD19L–sTRAIL alone (P = 0.0003), and 9.0 ± 4.6 days for TBI (P = 0.020) (Fig. 5C). Five of 5 mice treated with a single dose of CD19L–sTRAIL + single dose TBI
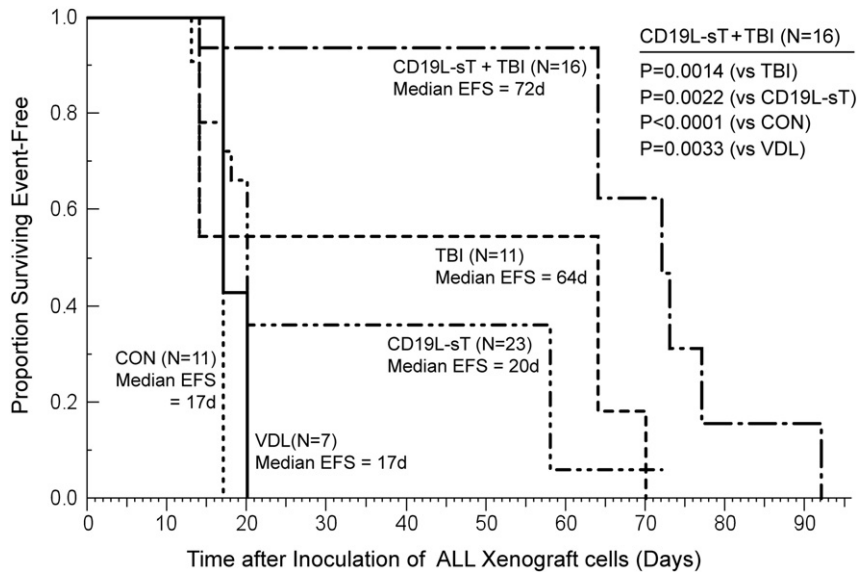
Fig. 4. CD19L–sTRAIL plus low dose TBI against chemotherapy-resistant BPL. Depicted are the EFS curves of NS mice that were inoculated i.v. with xenograft cells (2×10^6 cells per mouse) derived from primary leukemia cells of a relapsed BPL patient. Sixteen test mice with symptomatic xenografted BPL were treated with CD19L–sTRAIL + TBI (Schedule 1: CD19L–sTRAIL on days 10,11 and 12; TBI on day 11, N = 6; Schedule 2: CD19L–sTRAIL on day 6; TBI on day 7, N = 5; Schedule 3: CD19L–sTRAIL on days 6 and 7; TBI on day 7, N = 5). Besides untreated mice (N = 11), additional controls included mice treated with CD19L–sTRAIL alone (N = 23), TBI alone (N = 11), or 3-drug combination chemotherapy VDL (Vincristine + Dexamethasone + PEG-Asparaginase) (N = 7). The probability of survival was determined and the event-free interval curves were generated using the Kaplan–Meier product limit method. Log-rank tests were performed to compare differences in median survival estimates between all groups and pairwise comparisons. P-values for pairwise comparisons for the CD19L–sTRAIL + TBI group versus each of the other 4 groups exhibited significant increases in survival times for all the comparisons. CD19L–sT: CD19L–sTRAIL.

and 6 of 6 mice treated with 3 doses of CD19L–sTRAIL + single dose TBI rapidly achieved a remission (time to remission: 3.3 ± 0.5 days). By comparison, only 3 of 6 mice treated with TBI alone (Fisher's exact test, P = 0.03) and none of the 8 mice treated with CD19L–sTRAIL alone achieved remission (P < 0.0001). The duration of remission as measured by the TFS times were 21.7 ± 3.9 days for CD19L–sTRAIL + TBI, but only 7.2 ± 3.9 days for TBI alone (Non-parametric Wilcoxon test, P = 0.0172) and 0.0 ± 0.0 days for CON (P < 0.0001) or CD19L–sTRAIL alone (P = 0.0002) (Fig. 5C). Two mice on the combination regimen relapsed and achieved a second remission after a single dose of CD19L–sTRAIL + TBI (Mouse #1: Remission 1: 19 days, Remission 2: 13 days; Mouse #2: Remission 1: 18 days, Remission 2: 12 days). Due to the consistent remission induction and prolonged remissions, the overall survival (OS) time was much longer for the CD19L–sTRAIL + TBI group than for the other groups (Fig. 5D). The survival outcome as measured by the number of days the mice remained alive after the onset of symptomatic leukemia/1st day of treatment showed a markedly improved outcome after CD19L–sTRAIL + TBI vs. TBI alone or CD19L–sTRAIL alone. The median OS times were 29 days for CD19L–sTRAIL + TBI but only 4 days for CON (Log-rank test, P < 0.0001), 7.5 days for CD19L–sTRAIL alone (P < 0.0001), and 13.5 days for TBI alone (P = 0.0021). We also treated 3 leukemic mice with Imatinib daily for 3 consecutive days (daily i.v. dose: 120 mg per kg in PBS = the mouse equivalent dose for the 340 mg per m² per day dose level used in recent studies on Ph⁺ ALL patients) as an additional control treatment group: There were no remissions and no meaningful prolongation of PFS (mean ± SE = 3.0 ± 0.0 days) or OS (median survival: 8 days) in the Imatinib group (Fig. 5). These results extended the data obtained in the NS mouse xenograft model and provided further preclinical proof-of-concept that CD19L–sTRAIL plus low dose TBI would be a safe and effective treatment modality in the treatment of radiation-resistant BPL.

4. Discussion

TBI-based conditioning regimens have not prevented leukemic relapses post-HSCT (Uckun et al., 1993a; Pulsipher et al., 2009; Balduzzi et al., 2014; Bar et al., 2014; Bachanova et al., 2012). Therefore, the identification of new drugs that can help overcome the radiation resistance

of BPL cells would be an important step forward in efforts aimed at improving the post-HSCT outcomes. We hypothesize that the incorporation of CD19L–sTRAIL into the pre-transplant TBI regimens of patients with recurrent or high-risk BPL will help overcome the radiochemotherapy resistance of their leukemia cells and thereby improve their treatment response and survival outcome after HSCT. In the present study, the combination of CD19L–sTRAIL with 2 Gy TBI in NS mice or 4 Gy TBI in CD22ΔE12xBCR-ABL double-Tg mice was very well tolerated without any treatment related morbidity or toxic deaths. In xenografted NS mice treated with CD19L–sTRAIL + 2 Gy TBI, there were no treatment related deaths, and blood tests did not reveal changes suggestive of significant hematologic, renal, pancreatic, or hepatic toxicity. Furthermore, histopathological evaluations did not show treatment-related toxic lesions suggestive of significant parenchymal organ damage in any of the organs examined. Therefore, it should be feasible to use this CD19L–sTRAIL + 2 Gy TBI module in place of 2 Gy in fractionated TBI regimens.

Monoclonal antibodies and their fragments can cause potentially life-threatening immunotoxicity, including cytokine storms, anaphylaxis and anaphylactoid reactions, as well as autoimmunity. CD19L–sTRAIL is not a monoclonal antibody-based therapeutic agent but it is plausible that it could also cause similar side effects in clinical settings due to its immunogenicity as a fusion protein. Therefore, it will be important to carefully examine its potential immunotoxicological effects in pharmacologically relevant animal species prior to a first-in-human clinical trial. Furthermore, biotherapeutic agents can sometimes trigger activation of the innate immune system and a toll-like-receptor response or cause a potentially fatal inflammatory response with a "cytokine storm". The use of CD19L–sTRAIL higher dose levels and prolonged schedules may cause unexpected side effects alone and/or in combination with low dose TBI in clinical settings.

The demonstrated ability of CD19L–sTRAIL to markedly augment the anti-leukemic potency of low dose TBI, induce remissions and improve the EFS outcome in two separate models of radiation-resistant BPL without serious added toxicity provides the preclinical proof of concept for CD19L–sTRAIL plus reduced intensity TBI with total doses as low as 6 Gy as a new conditioning regimen that has the potential to significantly improve the EFS outcome and long-term health status of high-risk remission BPL patients undergoing HSCT.
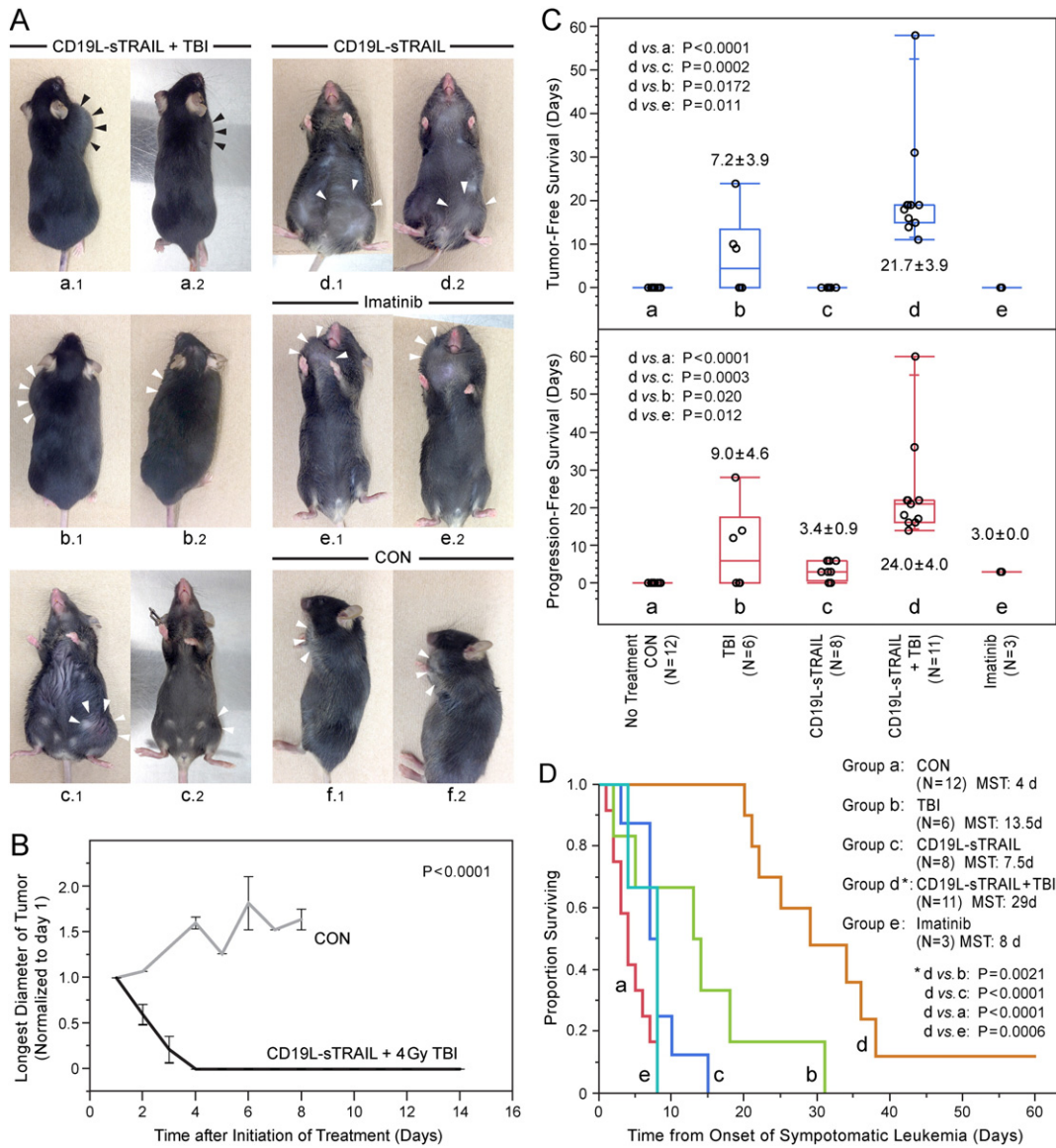
Fig. 5. Anti-leukemic activity of CD19L–sTRAIL plus low dose TBI against murine B-precursor leukemia in CD22ΔE12xBCR-ABL double-Tg mice. The CD19L–sTRAIL + TBI (N = 11) test group (Group d) was compared to untreated control mice (N = 12) (Group a). Additional controls included mice treated with CD19L–sTRAIL alone (N = 8) (Group c), TBI alone (N = 6) (Group b), or Imatinib alone (N = 3) (Group e). [A] Depicted are the day 1 vs. day 7 images of representative CD22ΔE12xBCR-ABL double-Tg mice assigned to different treatment protocols. [B] Tumor sizes of untreated control mice vs. mice treated with CD19L–sTRAIL (17 fmol per kg per day × 3 days or 17 fmol per kg per day single dose) + 4 Gy TBI were determined using the longest diameter measurement of each tumor in the mice and normalized to the pre-treatment tumor size on day 1. Depicted are the values for normalized tumor size during the first 14 days after the initiation of treatment. The repeated measures analysis of co-variance model explained significant proportion of the variance in the growth profiles with a highly significant difference between CON and CD19L–sTRAIL + TBI groups (72% of the variance explained, $F_{1,10.8} = 153.7$, $P < 0.0001$). [C] Tumor-free survival (C) (in days) and progression-free survival (D) (in days) distributions are depicted using Quantile dot plots (box represents 25th, median and 75th percentiles, and the whiskers represent the minimum, 10th, 90th and maximum values). Pair-wise differences in median survival times were calculated using Non-parametric Wilcoxon tests. [D] The survival outcome as measured by the number of days the mice remained alive after the onset of symptomatic leukemia is depicted by interval curves generated using the Kaplan–Meier product limit method and the P-values were calculated using Log-rank tests to take into account censored data in the CD19L–sTRAIL + TBI arm and the 3 animals censored at 18, 25 and 60 days were included in the curve.

The main focus of our study was to design a potent reduced intensity TBI-based pre-transplant conditioning regimen for BPL. However, CD19L–sTRAIL + TBI may also serve as a major component of pre-transplant conditioning strategies for other forms of poor prognostic B-lineage lymphoid malignancies owing to the abundant expression of CD19 on neoplastic cells from non-Hodgkin's lymphoma patients and other forms of B-lineage leukemias as well (Uckun et al., 1988; D'Cruz and Uckun, 2013).

Author Contributions

All authors have made significant and substantive contributions to the study. All authors reviewed and revised the paper. F.M.U. was the

NIH-funded Principal Investigator who designed, directed and supervised this study and wrote the initial draft of the manuscript. S.Q. performed the statistical analyses and PK parameter determinations. R.R. (DVM, PhD, Diplomate ACVP) performed the histopathological examination of the mouse tissues. D.E.M. purified CD19L–sTRAIL and Fc-tagged CD19L proteins; she also performed the fluorescent labeling of CD19L–sTRAIL and sTRAIL. H.M. prepared the CD19L–sTRAIL plasmid and coordinated its expression.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.02.008.

References

Asselin, B.L., Gaynon, P., Whitlock, J.A., 2013. Recent advances in acute lymphoblastic leukemia in children and adolescents: an expert panel discussion. Curr. Opin. Oncol. (Suppl. 3), S1–S13 http://dx.doi.org/10.1097/CCO.0000000000000017 (quiz S14-6).

Bachanova, V., Burke, M.J., Yohe, S., Cao, Q., Sandhu, K., Singleton, T.P., Brunstein, C.G., Wagner, J.E., Verneris, M.R., Weisdorf, D.J., 2012. Unrelated cord blood transplantation in adult and pediatric acute lymphoblastic leukemia: effect of minimal residual disease on relapse and survival. Biol. Blood Marrow Transplant. 18, 963–968.

Balduzzi, A., Di Maio, L., Silvestri, D., Songia, S., Bonanomi, S., Rovelli, A., Conter, V., Biondi, A., Cazzaniga, G., Valsecchi, M.G., 2014. Minimal residual disease before and after transplantation for childhood acute lymphoblastic leukemia: is there any room for intervention. Br. J. Haematol. 164, 396–408.

Bar, M., Wood, B.L., Radich, J.P., Doney, K.C., Woolfrey, A.E., Delaney, C., Appelbaum, F.R., Gooley, T.A., 2014. Impact of minimal residual disease, detected by flow cytometry, on outcome of myeloablative hematopoietic cell transplantation for acute lymphoblastic leukemia. Leukemia Res.Treat. http://dx.doi.org/10.1155/2014/421723 (Article ID: 421723).

Bernard, F., Auquier, P., Herrmann, I1., Contet, A., Poiree, M., Demeocq, F., Plantaz, D., Galambrun, C., Barlogis, V., Berbis, J., Garnier, F., Sirvent, N., Kanold, J., Chastagner, P., Chambost, H., Michel, G., 2014. Health status of childhood leukemia survivors who received hematopoietic cell transplantation after BU or TBI: an LEA study. Bone Marrow Transplant. 49, 709–716. http://dx.doi.org/10.1038/bmt.2014.3 (Epub 2014 Feb 17).

Bremer, E., 2013. Targeting of the tumor necrosis receptor superfamily for cancer immunotherapy.. (Review Article), ISRN Oncology. http://dx.doi.org/10.1155/2013/371854 (Article ID: 371854).

D'Cruz, O.J., Uckun, F.M., 2013. Novel mAb-based therapies for leukemia. In: Uckun, F.M. (Ed.), Monoclonal Antibodies in Oncology. Future Medicine. Future Medicine Ltd, London, pp. 54–77 http://dx.doi.org/10.2217/ebo.13.218.

Dimberg, L.Y., Anderson, C.K., Camidge, R., Behbakht, K., Thorbum, A., Ford, H.L., 2013. On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics. Oncogene 32 (11), 1341–1350. http://dx.doi.org/10.1038/onc.2012.164.

Fox, N.L., Humphreys, R., Luster, T.A., Klein, J., Gallant, G., 2010. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-1 and receptor-2 agonists for cancer therapy. Expert. Opin. Biol. Ther. 10 (1), 1–18. http://dx.doi.org/10.1038/sj.bjc.6605987.

Gaynon, P.S., Harris, R.E., Altman, A.J., Bostrom, B.C., Brenneman, J.C., Hawks, R., Steele, D., Zipf, T., Stram, D.O., Villaluna, D., Trigg, M.E., 2006. Bone marrow transplantation versus prolonged intensive chemotherapy for children with acute lymphoblastic leukemia and an initial bone marrow relapse within 12 months of the completion of primary therapy: Children's Oncology Group Study CCG-1941. J. Clin. Oncol. 24, 3150–3156.

Gong, B., Almasan, A., 2000. Apo2 ligand/TNF-related apoptosis-inducing ligand and death receptor 5 mediate the apoptotic signaling induced by ionizing radiation in leukemic cells. Cancer Res. 60 (20), 5754-60.

Kalaycio, M., Bolwell, B., Rybicki, L., Absi, A., Andresen, S., Pohlman, B., Dean, R., Sobecks, R., Copelan, E., 2011. BU- vs TBI-based conditioning for adult patients with ALL. Bone Marrow Transplant. 46, 1413–1417.

Kruyt, F.A.E., 2008. TRAIL and cancer therapy (Mini-review). Cancer Lett. 263, 14–25.

Locatelli, F., Schrappe, M., Bernardo, M.E., Rutella, S., 2012. How I treat relapsed childhood acute lymphoblastic leukemia. Blood 120, 2807–2816. http://dx.doi.org/10.1182/blood-2012-02-265884.

Marks, D.I., Forman, S.J., Blume, K.G., et al., 2006. A comparison of cyclophosphamide and total body irradiation with etoposide and total body irradiation as conditioning regimens for patients undergoing sibling allografting for acute lymphoblastic leukemia in first or second complete remission. Biol. Blood Marrow Transplant. 12, 438–453.

Merino, D., Lalaoui, N., Morizot, A., Solary, E., Micheau, O., 2007. TRAIL in cancer therapy: present and future challenges. Expert Opin. Ther. Targets 11, 1299–1314.

Mori, T., Aisa, Y., Kato, J., Yamane, A., Nakazato, T., Shigematsu, N., Okamoto, S., 2012. Safety and efficacy of total body irradiation, cyclophosphamide, and cytarabine as a conditioning regimen for allogeneic hematopoietic stem cell transplantation in patients with acute lymphoblastic leukemia. Am. J. Hematol. 87, 349–353.

Pulsipher, M.A., Bader, P., Klingebiel, T., Cooper, L.J., 2009. Allogeneic transplantation for pediatric acute lymphoblastic leukemia: the emerging role of peritransplantation minimal residual disease/chimerism monitoring and novel chemotherapeutic, molecular, and immune approaches aimed at preventing relapse. Biol. Blood Marrow Transplant. 15, 62–71.

Tracey, J., Zhang, M.J., Thiel, E., Sobocinski, K.A., Eapen, M., 2013. Transplantation conditioning regimens and outcomes after allogeneic hematopoietic cell transplantation in children and adolescents with acute lymphoblastic leukemia. Biol. Blood Marrow Transplant. 19, 255–259.

Uckun, F.M., Jaszcz, W., Ambrus, J.L., Fauci, A.S., Gajl-Peczalska, K., Song, C.W., Wick, M.R., Myers, D.E., Waddick, K., Ledbetter, J.A., 1988. Detailed studies on expression and function of CD19 surface determinant by using B43 monoclonal antibody and the clinical potential of anti-CD19 immunotoxins. Blood 71, 13–29.

Uckun, F.M., Jaszcz, W., Chandan-Langlie, M., Waddick, K.G., Gajl-Peczalska, K., Song, C.W., 1993a. Intrinsic radiation resistance of primary clonogenic blasts from children with newly diagnosed B-cell precursor acute lymphoblastic leukemia. J. Clin. Invest. 91, 1044–1051.

Uckun, F.M., Kersey, J.H., Haake, R., Weisdorf, D., Nesbit, M.E., Ramsay, N.K., 1993b. Pretransplantation burden of leukemic progenitor cells as a predictor of relapse after bone marrow transplantation for acute lymphoblastic leukemia. N. Engl. J. Med. 329 (18), 1296–1301.

Uckun, F.M., Qazi, S., Ma, H., Tuel-Ahlgren, L., Ozer, Z., 2010a. STAT3 is a substrate of SYK tyrosine kinase in B-lineage leukemia/lymphoma cells exposed to oxidative stress. Proc. Natl. Acad. Sci. U. S. A. 107 (7), 2902–2907.

Uckun, F.M., Goodman, P., Ma, H., Dibirdik, I., Qazi, S., 2010b. CD22 exon 12 deletion as a novel pathogenic mechanism of human B-precursor leukemia. Proc. Natl. Acad. Sci. U. S. A. 107, 16852–16857.

Uckun, F.M., Dibirdik, I., Qazi, S., 2010c. Augmentation of the anti-leukemic potency of total body irradiation (TBI) by a novel P-site inhibitor of spleen tyrosine kinase (SYK). Radiat. Res. 174, 526–531.

Uckun, F.M., Sun, L., Qazi, S., Ma, H., Ozer, Z., 2011a. Recombinant human CD19-ligand protein as a potent anti-leukemic agent. Br. J. Haematol. 153, 15–23.

Uckun, F.M., Qazi, S., Ozer, Z., Garner, A.L., Pitt, J., Ma, H., Janda, K.D., 2011b. Inducing apoptosis in chemotherapy-resistant B-lineage acute lymphoblastic leukaemia cells by targeting HSPA5, a master regulator of the anti-apoptotic unfolded protein response signalling network. Br. J. Haematol. 153, 741–752.

Uckun, F.M., Ma, H., Zhang, J., Ozer, Z., Dovat, S., Mao, C., Ishkhanian, R., Goodman, P., Qazi, S., 2012. Serine phosphorylation by SYK is critical for nuclear localization and transcription factor function of Ikaros. Proc. Natl. Acad. Sci. U. S. A. 109, 18072–18077.

Uckun, F.M., Qazi, S., Cely, I., Sahin, K., Shahidzadeh, A., Ozercan, I., Yin, Q., Gaynon, P., Termuhlen, A., Cheng, J., 2013. Nanoscale liposomal formulation of a SYK P-site inhibitor against B-precursor leukemia. Blood 121, 4348–4354.

Uckun, F.M., Qazi, S., Ma, H., Yin, L., Cheng, J., 2014. A rationally designed CD22ΔE12-siRNA nanoparticle for RNAi therapy in B-lineage lymphoid malignancies. EBioMed. 1, 141–155.

Uckun, F.M., Myers, D.E., Qazi, S., Ozer, Z., Rose, R., D'Cruz, O., Ma, H., 2015a. Recombinant human CD19L–sTRAIL effectively targets B-cell precursor acute lymphoblastic leukemia. J. Clin. Invest. http://dx.doi.org/10.1172/JCI76610.

Uckun, F.M., Ma, H., Cheng, J., Myers, D.E., Qazi, S., 2015b. CD22ΔE12 as a molecular target for RNAi therapy. Br. J. Haematol. http://dx.doi.org/10.1111/bjh.13306.

Wissink, E.H., Verbrugge, I., Vink, S.R., Schader, M.B., Schaefer, U., Walczak, H., Borst, J., Verheij, M., 2006. TRAIL enhances efficacy of radiotherapy in a p53 mutant, Bcl-2 overexpressing lymphoid malignancy. Radiother. Oncol. 80, 214–222.