SUPPLEMENTARY METHODS

Expression and purification of ABL602 bispecific antibodies

The ExpiFectamine™ 293 transfection kit (Thermo Fisher Scientific, A14524) and Expi293F™ cells (Thermo Fisher Scientific, A39242) were used to transiently express ABL602 bispecific antibodies. The Expi293F™ cells of 3.0×10^6 cells/mL with higher than 95% viability in 300 mL of cell culture media and the linearized plasmid DNAs containing a CMV promoter with a heavy chain and a light chain inserted respectively encoding ABL602 bispecific antibodies were prepared. The prepared plasmid DNAs at an optimized ratio and the ExpiFectamine™ 293 reagent were mixed and added to the cell culture. The cell culture was incubated on a shaker with orbital shaking at 150 rpm. The temperature was maintained at 37 °C while the CO_2 level was at 8%. After 6 days of incubation, the cells were pelleted by centrifugation at 4,000 rpm and 25 °C for 10 min, and the supernatant was collected for purification. For purification, a protein A column pre-packed with MabSelect Sure™ (Cytiva, 29-0491-04) was equilibrated with 0.1 M Tris, pH 7.0 buffer before loading onto the cell culture fluid. Following loading, the column was washed with 0.1 M Tris (pH 7.0), followed by elution using 0.1 M citrate (pH 3.5). The elution was then neutralized by adding 0.1 M Tris, pH 9.0 buffer. The samples were dialyzed in PBS buffer (Sangon Biotech, B548117-0500) and purified by fractionation using Superdex™ 200 (Cytiva, 17-5175-01) to remove product-related impurities and increase purity. The final samples were filtered with 0.2 μm filters.

Binding affinity to human CLL-1 and human CD3 by Surface Plasmon Resonance

To measure the human CLL-1 binding affinity, each BsAb was captured on the surface of a Protein A chip (Cytiva, 29127556) at approximately 125 RU (response unit). Recombinant human CLL-1 proteins (Sino Biological, 11896-H07H) using 1x HBS-EP buffer (Cytiva, BR100669) ranging from 50 nM to 1.5625 nM with 2-fold serial dilution, including 0 nM,

and were injected into the flow cells. The association phase was set to 60 s under 30 µL/min and the dissociation phase was 180 s under 30 µL/min. At the end of the dissociation phase, the 10 mM Glycine-HCl pH 1.5 (Cytiva, BR100354) was injected for 30 s under 30 µL/min to regenerate the chip surface.

To measure human CD3 binding affinity, recombinant human CD3 (Sino Biological, CT026H0323H) with Acetate pH 4.0 (Cytiva, BR100349) was immobilized on a CM5 chip (Cytiva, B100399) to reach 50 RU using an Amine Coupling Kit (Cytiva, BR100633). Each BsAb candidate was diluted with 1xHBS-EP buffer ranging from 200–12.5 nM as a 2-fold serial dilution including 0 nM. The diluted BsAbs were injected into the flow cells. The association phase was set to 60 s and the dissociation phase was set to 180 s. At the end of the dissociation phase, the 10 mM Glycine-HCl pH2.0 (Cytiva, BR100355) was injected for 30 s under 30 µL/min to regenerate the chip surface.

The binding kinetics of each BsAb to human CLL-1 and CD3 were measured using a BiacoreTM T200 system (Cytiva). The sensorgram data were processed by subtracting the signals obtained from uncaptured or unimmobilized flow cells at 0 nM. Kinetic parameters were calculated from a 1:1 binding model using the BiacoreTM T200 evaluation software version.01 (Cytiva). All experiments were conducted at 25 ℃.

Binding assays to normal peripheral blood

To determine the binding profile of ABL602 to normal peripheral blood cells, hIgG1 isotype control (SIGMA, I5154) and ABL602 were labeled with Alexa Fluor 555 using an AF555 antibody labeling kit (Invitrogen, A20187) per the manufacturer's guide. Peripheral blood mononuclear cells (PBMCs) and neutrophils were isolated by ficoll density gradient centrifugation from healthy donors and incubated with 200 nM and 50 nM of Alexa Fluor-labeled ABL602 for T cells and other immune cells, respectively. Antibodies used for staining

immune cells are as follows: anti-human CD33-BB515, anti-human CD45-BV786, anti-human CD3-V500, anti-human CD56-BB515, anti-human CD8-BV650 (BD Horizon), anti-human CD11c-APC, anti-human CD14-PECy7, anti-human CD4-APC Cy7, anti-human CD19-AF700, anti-human HLADR-BV510, anti-human CD16-APC, anti-human CD15-PECy7 (BioLegend). Samples were acquired on LSRfortessa (BD) flow cytometer and data were analyzed using FlowJo.

Granzyme B and perforin analysis

U937 or HL60 cells of 2 x 10^4 were incubated with purified T cells (1 x 10^5, n=4) and 1 nM of ABL602 or control bsAb in a 96-well plate for 24 h, followed by the addition of Golgi Plug Transport inhibitor (BD) for 5 h. To facilitate the intracellular granzyme B and perforin staining, the cells were initially stained with anti-human CD45, CD3, CD8, and CD4 antibodies, and then fixed and permeabilized using the BD Fix/Perm kit. Permeabilized cells were stained with antibodies against granzyme b (R&D systems) and perforin (BioLegend) for 30 min at 4 °C.

Cytokine analysis

Human purified T cells (1×10^5) from three healthy donors were cultured with ABL602 2+1 or MCLA-117 analogs at the maximum killing dose (approximately EC_{90}) for each antibody in the presence or absence of U937 cell line in 96 well-U bottom plates. After 48 h, the supernatant was harvested to measure the human cytokine levels. The TNF-α, IL-2, INF-γ, and IL-6 concentrations in cell culture supernatants were determined by ELISA (R&D systems) kit per the manufacturer's protocol.

SUPPLEMENTARY TABLE

Supplementary Table 1. Surface Plasma Resonance binding assay

Coated Antigen	Clone	Ka (1/Ms)	Kd (1/s)	K_D (M)
CLL-1	**ABL602 1+1**	$4.01x10^{5}$	$1.60x10^{-3}$	$3.95x10^{-9}$
	ABL602 2+1	$7.01x10^{5}$	$2.63x10^{-3}$	$3.75x10^{-9}$
CD3	**ABL602 1+1**	$4.54x10^{5}$	$3.78x10^{-2}$	$84x10^{-9}$
	ABL602 2+1	$3.54x10^{5}$	$4.89x10^{-2}$	$138x10^{-9}$

SUPPLEMENTARY FIGURE LEGENDS

Supplemental Figure 1 Schematic diagram of antibody binding to CLL-1-coated beads and T cells. Beads with or without CLL-1 coating were incubated with antibodies, followed by incubation with T cells. Antibodies bound to T cells were detected. The figure was drawn using Biorender (https://biorender.com/).

Supplemental Figure 2 In vitro binding of ABL602 2+1 to human peripheral blood cells. Histograms showing purified human PBMC and PMN from a representative donor stained with an APC-labeled IgG1 isotype control antibody (gray) or APC-labeled ABL602 2+1 (red). The gating strategies for the subsets analyzed were granulocytes based on SSC-FSC; NK cells: $CD3^{-}CD56^{+}$; B cells: $CD19^{+}$; monocytes: $CD14^{+}CD33^{+}$; myeloid dendritic cells: $BDCA1^{+}CD19^{-}CD4^{-}CD11c^{+}$; CD4 T cells: $CD3^{+}CD4^{+}$; CD8 T cells: $CD3^{+}CD8^{+}$ in PBMC; and neutrophils: $CD16^{+}CD15^{+}$ in PMN.

Supplemental Figure 3 ABL602 2+1 activates $CD4^+$ or $CD8^+$ T cells in a CLL-1 expression-dependent manner, as indicated by the expression of CD69, Granzyme B, and Perforin. (A) Purified T cells were incubated with various cancer cell lines at an E:T ratio of 5:1. The proportion of $CD4^+CD69^+$ T cells among $CD4^+$ T cells was determined using flow cytometry. (B) Primary $CD3^+$ T cells were isolated from PBMCs by negative selection. T cells were incubated with AML cells (E:T ratio of 5:1) and 1nM ABL602 2+1 for 24 h. Intracellular staining for granzyme B and perforin was performed using a Golgi plug and the Fixation/Permeabilization Solution kit. The Granzyme B and perforin expressions were determined as the percentage of live $CD8^+$ cells stained for Granzyme B or perforin using flow cytometry.

Supplemental Figure 4 Gating strategy for AML blasts and T cells. Singlets were selected from the FSC-A versus FSC-W and SSC-A versus SSC-W dot plots, and dead cells were excluded using a fixable viability kit. Within live cells, $CD45^{dim}SSC^{low}$ and $CD45^{high}$ cells were defined as AML blasts and leukocytes, respectively. AML blasts were further analyzed for CLL-1 and CD33 expression. T cells were defined as $CD14^-CD3^+$ leukocytes and further characterized as $CD4^+$ or $CD8^+$ T cells.

Supplemental Figure 5 Both ABL602 and MCLA-117 induce the cytokine release in the presence of U937 but not in the absence of U937. Purified T cells were cultured with corresponding EC_{90} doses of ABL602 2+1 or MCLA-117 analog in the presence or absence of U937 cancer cells for 48 h. The supernatant was analyzed for the indicated cytokines by ELISA.