IGLV3-21^{R110}-directed bispecific antibodies activate T cells and promote killing in a high-risk subset of chronic lymphocytic leukemia

Claudia Fischer,[1,2] Shih-Shih Chen,[3] Johanna Nimmerfroh,[1,4] Anne Eugster,[1,2] Simon Stücheli,[1,2] Christoph Schultheiß,[1,2] Corinne Widmer,[5] Dominik Heim,[5] Benjamin Kasenda,[1] Jakob Passweg,[5] Sebastian Kobold,[6-8] Lukas Egli,[9] Nicolò Coianiz,[9] Obinna Chijioke,[9,10] Nicholas Chiorazzi,[3] Marie Follo,[11] Heinz Läubli,[1,4] Matthias Peipp[12] and Mascha Binder[1,2]

[1]Division of Medical Oncology, University Hospital Basel, Basel, Switzerland; [2]Laboratory of Translational Immuno-Oncology, Department of Biomedicine, University of Basel and University Hospital Basel, Basel, Switzerland; [3]Institute of Molecular Medicine, The Feinstein Institutes for Medical Research, Northwell Health, Manhasset, NY, USA; [4]Laboratory of Cancer Immunotherapy, Department of Biomedicine, University of Basel and University Hospital Basel, Basel, Switzerland; [5]Division of Hematology, University Hospital Basel, Basel, Switzerland; [6]Division of Clinical Pharmacology, Klinikum der Universität München, Munich, Germany; [7]German Cancer Consortium (DKTK), Partner Site Munich, Munich, Germany; [8]Einheit für Klinische Pharmakologie (EKLiP), Helmholtz Munich, Research Center for Environmental Health (HMGU), Neuherberg, Germany; [9]Cellular Immunotherapy, Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland; [10]Institute of Pathology and Medical Genetics, University Hospital Basel, Basel, Switzerland; [11]Lighthouse Core Facility, Department of Medicine I, Medical Center of the University of Freiburg, Freiburg, Germany and [12]Division of Antibody-Based Immunotherapy, Department of Medicine II, Kiel University, Kiel, Germany

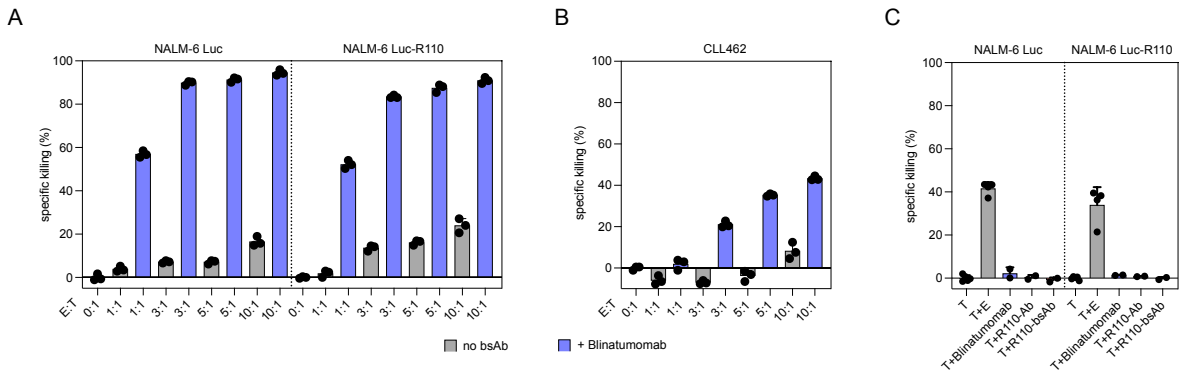
Correspondence: M. Binder
mascha.binder@usb.ch

Received: February 27, 2025.
Accepted: September 1, 2025.
Early view: September 11, 2025.

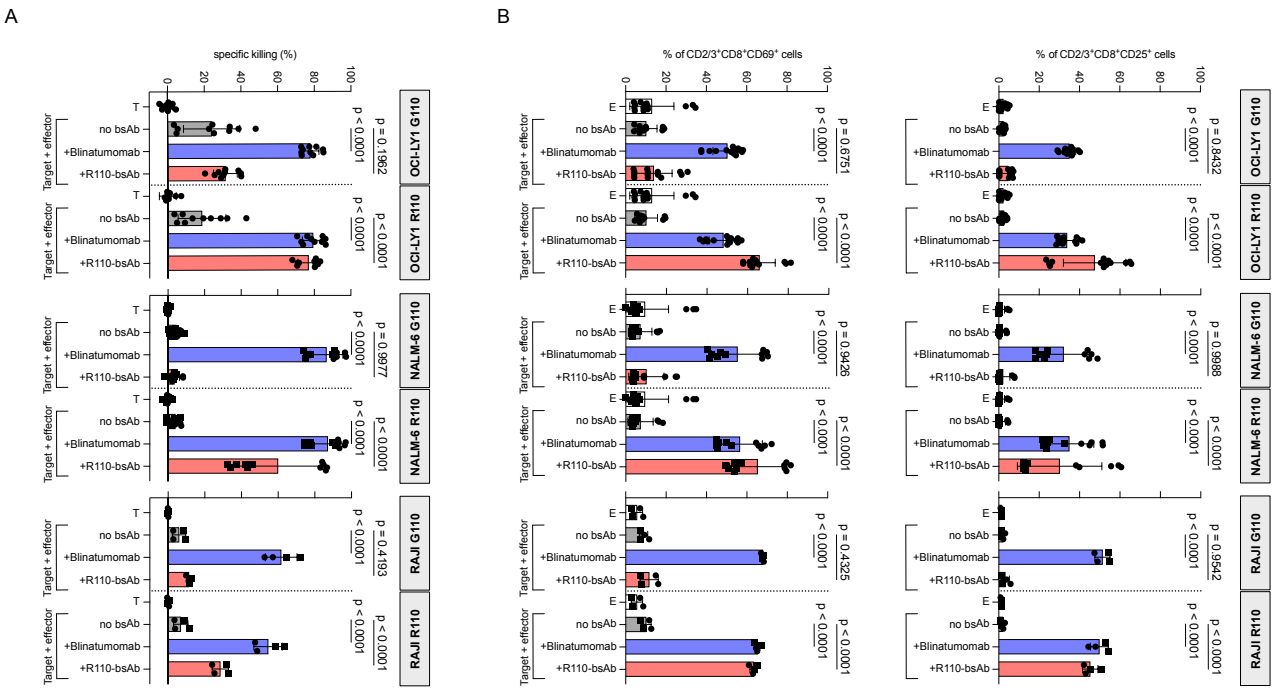
https://doi.org/10.3324/haematol.2025.287697

©2026 Ferrata Storti Foundation
Published under a CC BY-NC license

Online Supplementary Appendix

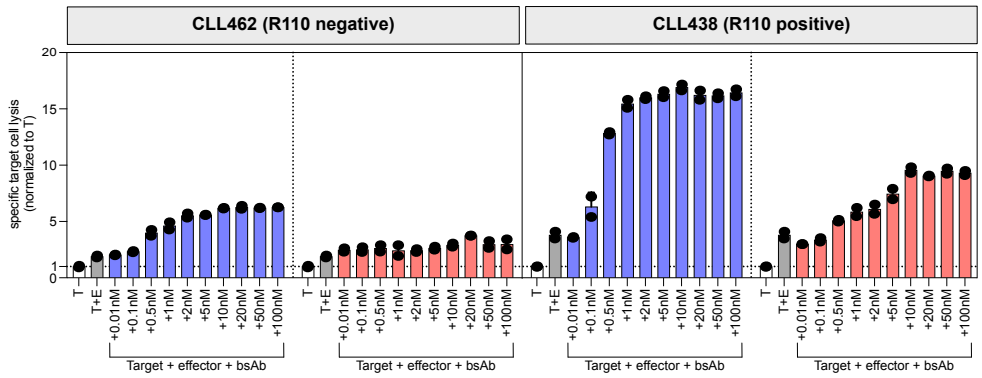


Supplementary Figure 1. Determination of suitable E:T ratios for co-culture experiments. A Specific killing of NALM-6 Luc and NALM-6 Luc-R110 cells incubated at different E:T ratios (0:1, 1:1, 3:1, 5:1, 10:1) with healthy donor (HD) T cells and 2 nM Blinatumomab. **B** Specific killing of primary CLL cells with HD T cells in different E:T ratios (0:1, 1:1, 3:1, 5:1, 10:1) and 5 nM of Blinatumomab. Each bar plot represents the mean of three technical replicates with error bars as SD. **C** Specific killing of NALM-6 Luc and NALM-6 Luc-R110 cells with an E:T ratio of 5:1 after 24 h of incubation with Blinatumomab, the monospecific R110-Ab or R110-bsAb. Each bar plot represents the mean of at least two technical replicates with error bars as SD. E: effector cells, T: target cells, bsAb: bispecific antibody.



Supplementary Figure 2. Efficacy of R110-bsAb in R110 positive cell line models.

A Specific killing of OCI-LY1 G110/R110, NALM-6 G110/R110 and RAJI G110/R110 cells incubated for 24 h with healthy donor (HD) T cells in a 5:1 ratio and 2 nM bispecific antibodies. **B** Percentage of activation marker expressing $CD8^+$ HD T cells after 24 h of co-culture with different target cells and treated with 2 nM Blinatumomab or R110-bsAb. Dots and squares are technical replicates representative for two different healthy donors used derived from N = 4 (OCI-LY1 G110/R110), N = 3 (NALM-6 G110/R110) or N = 1 (RAJI G110/R110) independent experiments and error bars as SD. Statistical significances were determined by ordinary one-way ANOVA combined with a Šidák`s multiple comparisons test. T: target cells, E: effector cells, bsAb: bispecific antibody.



Supplementary Figure 3. Efficacy of R110-bsAb in primary CLL. Specific cell lysis of CLL462 and CL438 target cells with healthy donor (HD) T cells in a 5:1 E:T ratio incubated for 48 h with a non-serial dilution of Blinatumomab or R110-bsAb. Cell Lysis was normalized to the cell lysis of target cells without effector cells or bispecific antibody. Each bar plot represents the mean of two technical replicates with error bars as SD. T: target cells, E: effector cells, bsAb: bispecific antibody.

Supplementary Table 1: Clinical characteristics of CLL patients.

Patient	Sex	Age range	Previously treated at time of sampling	IGLV3-21 R110 status
CLL424	f	50-60	yes	positive
CLL438	m	80-90	yes	positive
CLL472	m	79-80	no	positive
CLL462	f	60-70	no	negative
CLL477	m	80-90	no	negative
CLL479	m	undetermined	no	negative

METHODS

Cell lines, primary CLL and healthy donor blood cells

Cell lines (DMSZ) and IGLV3-21^{R110} or IGLV3-21^{G110} light chain expressing variants thereof were generated as previously described (1). Suspension-adapted Chinese hamster ovary (CHO)-S cells purchased from Thermo Fisher Scientific were cultivated in CD CHO-Medium (Thermo Fisher Scientific) with 1% HT Supplement (Thermo Fisher Scientific) and 1% GlutaMax-I (200 mM L-Ala-L-Gln, Thermo Fisher Scientific) added to the medium. For antibody production, they were kept in CD OptiCHO supplemented with 1% GlutaMax-I, 1% Poloxamer 188, and 1% HAT-Supplement (CHO production medium, Thermo Fisher Scientific).

Blood samples from CLL patients were collected after informed consent as approved by the ethics committees of the Universities of Hamburg–Eppendorf, Halle-Wittenberg and Basel. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (Cytiva). If necessary, Pan T cells, Pan B cells or CD34^{+} hematopoietic stem cells were additionally isolated via magnetic-activated cell sorting (MACS, Miltenyi Biotec). PBMCs and T cells were resuspended in FCS + 10% Dimethyl sulfoxide (DMSO) and cryopreserved in liquid nitrogen. IGLV3-21^{R110} expression was characterized by next-generation sequencing (NGS) of the light chain loci as previously described (2-11).

Bispecific antibody constructs

The bispecific antibody construct is derived from the humanized antigen-binding fragment (Fab) of the IGLV3-21^{R110}-specific antibody from AVA Lifescience GmbH (Denzlingen, Germany; patent EP 4 227 322 A1). The R110 bispecific antibody (R110-bsAb) was designed as a heterodimeric IgG1-based antibody consisting of a fragment

crystallizable region (Fc) attached to either an anti-IGLV3-21^{R110} Fab or an anti-CD3 (UCHT1) single chain variable fragment (anti-CD3 scFv). Its light and heavy chain fragments were cloned into the pcDNA3.1(+) vector containing a CMV promotor for antibody expression in CHO-S cells. Knob-into-whole mutations (S354C, T366W vs. Y349C, T366S, L368A, Y407V) in the constant fragments (Fc) of its heavy chains facilitate heterodimerization (12). L234A and L235A point mutations were induced to reduce unspecific Fc-FcR interactions (13).

Bispecific antibody production and purification

Antibody production was performed using the MaxCyte STX Scalable Transfection Systems (14). In brief, CHO-S cells were cultivated at 37°C, 6% CO_2, 145 rpm in serum free CD-CHO medium for at least 2 weeks before being transfected via the MaxCyte STX Scalable Transfection System (MaxCyte, (15)). Transfected cells were further incubated at 32°C, 6% CO_2, 145 rpm for 24 h before sodium butyrate (Sigma) was added. During the following days, feed stock solution, which contained 70% CHO CD Efficient Feed A Stock Solution (Thermo Fisher Scientific), 14% Yeastolate TC UF (Becton Dickinson), 3.5% GlutaMax-I(200 mM)

and 12.5% Glucose (450 g/L, Sigma), was added to the cells until the cell viability dropped below 50%. Supernatant containing the antibody was filtered. For purification, antibody was isolated with the Capture Select^{TM} CH1 Affinity Matrix (Thermo Fisher Scientific) following the manufacturer's instructions. Multimers were excluded via size exclusion chromatography using the Äkta Chromatography System (Cytiva). The commercial bispecific antibody Blinatumomab (anti-CD19/anti-CD3, Blincyto®, Amgen) was used as positive control (16).

In vitro cytotoxicity assay and cytokine quantification

For in vitro cytotoxicity assays using NALM-6 Luc (-R110), NALM-6, RAJI or OCI-LY1 (G110/R110) cells, $2x10^4$ target cells were seeded with effector cells in a 96-well plate and co-incubated in complete media (RPMI 1640 Medium, GlutaMAX™ Supplement, 100 U/mL penicillin and 100 mg/mL streptomycin, 50 µM beta-Mercaptoethanol, Thermo Fisher Scientific; 10% Human Serum) at 37°C and 5% CO_2. For primary CLL target cells, $4x10^4$ cells were seeded. The different E:T ratios as well as the bsAb concentrations used for experiments are indicated in the respective figure descriptions. Cell lysis of target cells was determined after 24 hours of co-culture via flow cytometry using a CytoFLEX™ (Beckman Coulter). Deviating incubation periods are indicated in respective figure descriptions. CD22 PE/Dazzle 594 (HIB22), CD2 FITC (TS1/8) and CD3 APC-Cy7 (SK7, BioLegend) or CD3 FITC (SK7, BD Biosciences) as well as DAPI (Miltenyi) were used for differentiation of effector and target cells or live and dead cells. bsAb-dependent T cell activation was determined using CD8 FITC (SK1), CD25 PE (M-A251, BD Biosciences), CD8 BV650 (SK1), CD4 BV605 (SK3), CD25 BV510 (M-A251) and CD69 APC (FN50, BioLegend) antibodies. The FlowJo™ Software (v.10.10.0) was used for flow cytometric analysis. The percentage of specific killing was determined by the following formula:

$$\%_{\text{specific killing}} = 100 \times ((\%\text{Viability}_{\text{untreated cells}} - \%\text{Viability}_{\text{treated cells}}) / \%\text{Vaibility}_{\text{untreated cells}})$$

Specific target cell lysis was calculated by division of the untreated target cell lysis mean and the sample cell lysis with cell lysis being defined as the loss of $CD22^+$ cells. Fold-change of activation marker-expressing effector cells was equally calculated by the division of the percentage of untreated $CD69^+/CD25^+$ effector cells and the percentage of activation marker positive, treated samples. The amount of released

IFN-γ in the supernatant of the 24 h co-culture was quantified using the LEGENDplex bead-based immunoassay (Biolegend) according to the manufacturer`s instructions. For fluorescence imaging, RAJI R110 cells were seeded at twice the usual quantity and incubated for 20 hours at 37°C and 5% CO_2. Following Hoechst and Nile Red staining (1:100, Sigma-Aldrich), images were captured using the Zeiss LSM 710 confocal microscope and the 20x/0.8 Plan-Apochromat objective.

In vivo killing assays

For cell line-based xenograft assays, healthy donor T cells were activated with 25 µL/mL Immunocult Human CD3/CD28/CD2 T cell activator (STEMCELL) and expanded for 9 days in culture in Prime-XV T cell CDM supplemented with 10 ng/ml IL-7 and IL-15. A total of 10 NSG (NOD/SCID/IL2rγnull) mice were injected subcutaneously (s.c.) into the right flank with $2x10^6$ NALM-6 R110 lymphoma cells suspended in Corning® Matrigel® Matrix High Concentration Phenol-Red-Free diluted 1:1 in phenol red-free DMEM without additives. On day 7, $3x10^6$ expanded healthy donor T cells were injected intravenously either alone or with R110-bsAb (0.5 mg/kg/dose). Afterwards cells were treated biweekly with R110-bsAb for a total of 5 times. Tumor volume was measured every 2 to 3 days starting on day 10 and calculated according to the formula: D/2 x d x d, with D and d being the longest and shortest tumor diameter in mm, respectively.

Patient-derived xenograft assays were conducted as described previously (17). For each mouse, $20x10^6$ PBMC were combined with $0.5x10^6$ of pre-activated T cells from the same CLL patient in a 40:1 ratio. T cell activation was achieved via CD3/CD28 dynabeads and recombinant IL-2 (R&D Systems) treatment over a period of 7 days.

The cell mixture was then injected intravenously in a total of 15 NSG mice. After 10 days, the mice were divided equally into three groups (n = 5 per group) and treated intravenously with either PBS, 0.25 µg/g R110-bsAb or 0.25 µg/g Blinatumomab. The bispecific antibodies were further readministered biweekly. After three weeks, the mice were sacrificed and the spleens were harvested. T and B cell populations were quantified by flow cytometry using anti-human CD19 PE-CF594 (HIB19) and anti-human CD3 PE-Cy7 antibodies (SK7, BD Biosciences). Animal studies were performed in compliance with experimental protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute for Medical Research.

Lastly, the effect of R110-bsAb on healthy, polyclonal PBMC, was analyzed using 3–5 month old NFA2 (NOD.Cg-*Rag1*^{tm1Mom} *Flt3*^{tm1Irl} *Mcph1*$^{Tg(HLA-A2.1)1Enge}$ *Il2rg*^{tm1Wjl}/J) mice that were injected intraperitoneally (i.p.) with 2.5×10^6 PKH26-labelled PBMCs originating from two different donors, either alone or in the presence of 0.25 μg/g of R110-bsAb or Blinatumomab (in total n = 18). NFA2 mice were housed under a 12 h light/12 h dark cycle (lights on: 6 am, lights off: 6 pm) at temperatures from 21–24 °C with 35–70% humidity. After 16 hours, the mice were sacrificed, and peritoneal cells were collected via lavage and analyzed by flow cytometry. Mice in which PBMC cells could not be detected by flow cytometry were excluded from the dataset to control for unsuccessful i.p. injections. The antibodies anti-human CD19-BUV661 (HIB19, BD Biosciences) and Zombie NIR™ Fixable Viability Kit (Biolegend) were used for the analysis.

Statistical analysis

Ordinary one-way Analysis of Variance (ANOVA) or 2way ANOVA combined with a Šidák`s multiple comparisons testing was used for the comparison of cell lysis of target cells and activation marker expression on effector cells in the absence or presence of bsAb. For *in vivo* anaylsis, the non-parametric Kruskal-Wallis test was used. Non-linear regression models were further utilized to highlight the dose-dependent decrease of target cell viability and increase of T cell activation marker expressing effector cells. For statistical analyses, the GraphPad Prism software (v10.3.0 (461)) was used.

1. Märkl F, Schultheiß C, Ali M, et al. Mutation-specific CAR T cells as precision therapy for IGLV3-21R110 expressing high-risk chronic lymphocytic leukemia. Nat Commun. 2024;15(1):993.

2. Anna O, Anna B, Minna V, et al. Monitoring multiple myeloma by next-generation sequencing of V(D)J rearrangements from circulating myeloma cells and cell-free myeloma DNA. Haematologica. 2017;102(6):1105-11.

3. Paschold L, Simnica D, Brito RB, et al. Subclonal heterogeneity sheds light on the transformation trajectory in IGLV3-21R110 chronic lymphocytic leukemia. Blood Cancer J. 2022;12(3):49.

4. Paschold L, Simnica D, Willscher E, et al. SARS-CoV-2–specific antibody rearrangements in prepandemic immune repertoires of risk cohorts and patients with COVID-19. J Clin Invest. 2021;131(1).

5. Paschold L, Willscher E, Bein J, et al. Evolutionary clonal trajectories in nodular lymphocyte-predominant Hodgkin lymphoma with high risk of transformation. Haematologica. 2021;106(10):2654-66.

6. Schieferdecker A, Oberle A, Thiele B, et al. A transplant “immunome” screening platform defines a targetable epitope fingerprint of multiple myeloma. Blood. 2016;127(25):3202-14.

7. Schliffke S, Akyüz N, Ford CT, et al. Clinical response to ibrutinib is accompanied by normalization of the T-cell environment in CLL-related autoimmune cytopenia. Leukemia. 2016;30(11):2232-4.

8. Schultheiß C, Paschold L, Simnica D, et al. Next-Generation Sequencing of T and B Cell Receptor Repertoires from COVID-19 Patients Showed Signatures Associated with Severity of Disease. Immunity. 2020;53(2):442-55.e4.

9. Schultheiß C, Simnica D, Willscher E, et al. Next-Generation Immunosequencing Reveals Pathological T-Cell Architecture in Autoimmune Hepatitis. Hepatology. 2021;73(4):1436-48.

10. Simnica D, Ittrich H, Bockemeyer C, Stein A, Binder M. Targeting the Mutational Landscape of Bystander Cells: Drug-Promoted Blood Cancer From High-Prevalence Pre-neoplasias in Patients on BRAF Inhibitors. Front Oncol. 2020;10.

11. Thiele B, Kloster M, Alawi M, et al. Next-generation sequencing of peripheral B-lineage cells pinpoints the circulating clonotypic cell pool in multiple myeloma. Blood. 2014;123(23):3618-21.

12. Merchant AM, Zhu Z, Yuan JQ, et al. An efficient route to human bispecific IgG. Nat Biotechnol. 1998;16(7):677-81.

13. Pejchal R, Cooper AB, Brown ME, Vásquez M, Krauland EM. Profiling the Biophysical Developability Properties of Common IgG1 Fc Effector Silencing Variants. Antibodies. 2023;12(3):54.

14. Lutz S, Klausz K, Albici A-M, et al. Novel NKG2D-directed bispecific antibodies enhance antibody-mediated killing of malignant B cells by NK cells and T cells. Front Immunol. 2023;14.

15. Steger K, Brady J, Wang W, et al. CHO-S Antibody Titers >1 Gram/Liter Using Flow Electroporation-Mediated Transient Gene Expression followed by Rapid Migration to High-Yield Stable Cell Lines. SLAS Discov. 2015;20(4):545-51.

16. Löffler A, Gruen M, Wuchter C, et al. Efficient elimination of chronic lymphocytic leukaemia B cells by autologous T cells with a bispecific anti-CD19/anti-CD3 single-chain antibody construct. Leukemia. 2003;17(5):900-9.

17. Patten PEM, Ferrer G, Chen S-S, et al. A Detailed Analysis of Parameters Supporting the Engraftment and Growth of Chronic Lymphocytic Leukemia Cells in Immune-Deficient Mice. Front Immunol. 2021;12.