Preclinical development of a humanized chimeric antigen receptor against B-cell maturation antigen for multiple myeloma

Lorena Perez-Amill,[1] Guillermo Suñe,[1] Asier Antoñana-Vildosola,[1] Maria Castella,[1] Amer Najjar,[2] Jaume Bonet,[3] Narcis Fernández-Fuentes,[4] Susana Inogés,[5] Ascensión López,[5] Clara Bueno,[6] Manel Juan,[7] Alvaro Urbano-Ispizua[1,8,9] and Beatriz Martín-Antonio[1,8]

[1]Department of Hematology, Hospital Clinic, IDIBAPS, Barcelona, Spain; [2]Department of Pediatrics - Research, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA; [3]Laboratory of Protein Design and Immunoengineering, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland; [4]Department of Biosciences, Faculty of Sciences and Technology, Universitat de Vic - Universitat Central de Catalunya, Vic, Spain; [5]Department of Hematology and Cell Therapy Area and Department of Immunology and Immunotherapy, Clinic University of Navarra, Navarra, Spain; [6]Josep Carreras Leukemia Research Institute and Cell Therapy Program of the School of Medicine, University of Barcelona. Barcelona, Spain; [7]Department of Immunotherapy, Hospital Clinic, IDIBAPS, Barcelona, Spain; [8]Josep Carreras Leukemia Research Institute, Barcelona, Spain and [9]Department of Hematology, University of Barcelona, Barcelona, Spain

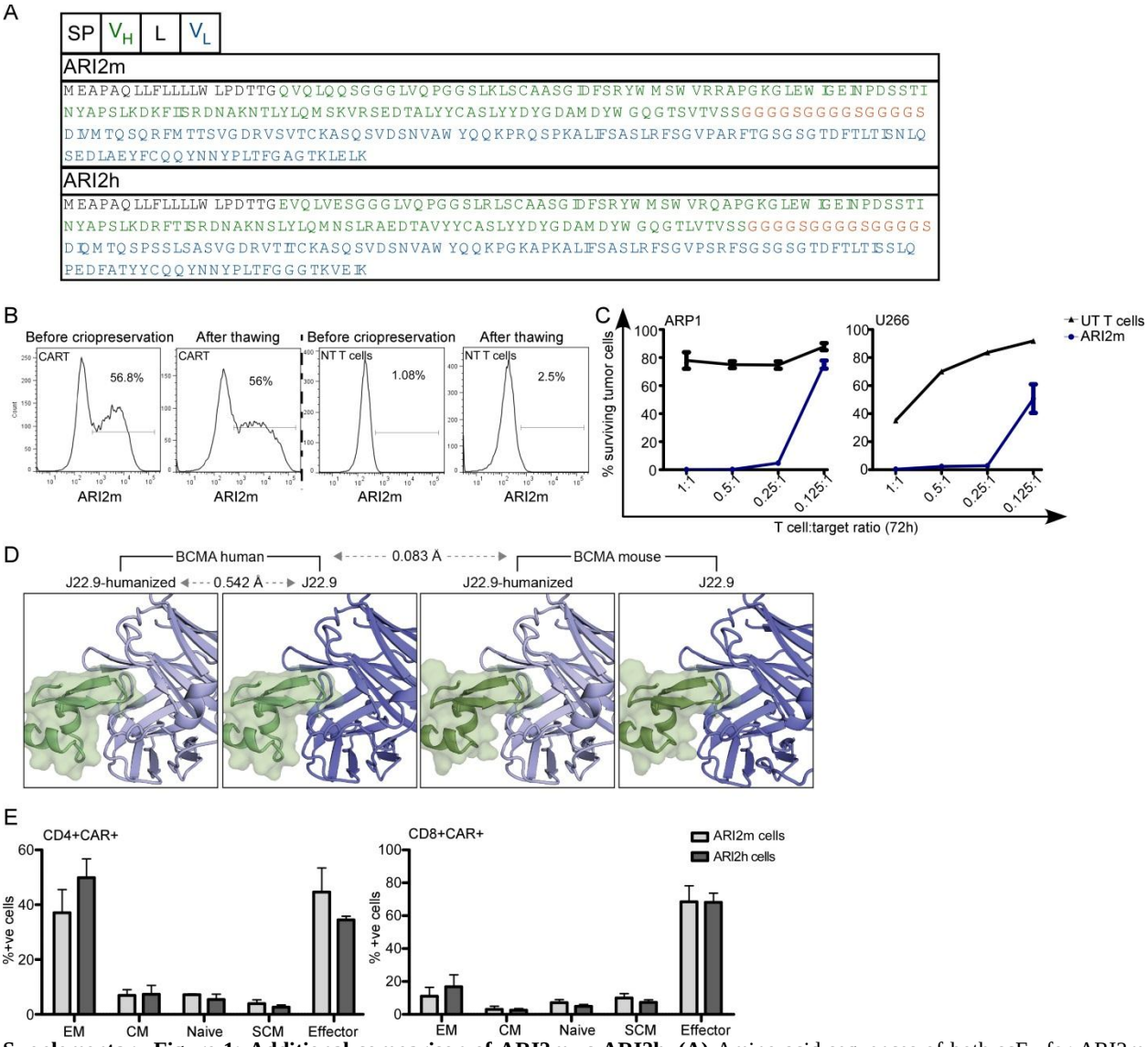
 doi:10.3324/haematol.2019.228577

Received: June 3, 2019.

Accepted: January 3, 2020.

Pre-published: January 9, 2020.

Correspondence: *BEATRIZ MARTIN-ANTONIO* - bmartina@clinic.cat



Supplementary Figure 1: Additional comparison of ARI2m *vs* ARI2h. (A) Amino acid sequences of both scFv for ARI2m and ARI2h. **(B)** Percentage of ARI2m cells before and after cryopreservation. UT T cells are shown as control. **(C)** Limiting dilution cytotoxicity assay of ARI2m and UT T cells *vs* MM cells (ARP1 and U266). (**D**). Protein-protein interfaces between the two scFv variants (J22.9 and J22.9h) and the two BCMA homologues. Root Mean Square Deviation (RMSD) between variants is annotated. (**E**). Phenotype characterization of T cells transduced with either ARI2m or ARI2h CAR construct and after being expanded for 7 days (n=3). EM (effector memory), CM (central memory), SCM (stem cell memory).

Supplementary Movie 1: live time lapse images of ARI2m cells stained in blue (CMAC) and RPMI-MM cells overexpressing BCMA fused to green fluorescent protein (GFP). Images were acquired every 20 seconds using a Leica SP5 confocal microscopy.

Supplementary Movie 2: live time lapse images of ARI2m cells stained in blue (CMAC) and ARP1-MM cells overexpressing BCMA fused to green fluorescent protein (GFP). Images were acquired every 20 seconds using a Leica SP5 confocal microscopy.

Supplementary methods:

Cell cultures: RPMI8226, U266 and K562 cell lines were purchased from American Tissue Culture Collection (ATCC, Manassas, VA). ARP1 cell line was kindly provided by Multiple Myeloma Research Center (Little Rock, AK, USA). Cell lines K562, RPMI8226 and ARP1 were cultured in RPMI with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/streptomycin (Pen/Strep) and U266 with 15% FBS. HEK293-T cells were cultured with DMEM with 10% FBS and 1% Pen/Strep.

Virus production and T cell transfection: HEK293-T cells were transfected with lentiviral vectors (pCCL-EF1α-BCMA, pREV-REV, pMDLg/pRRE and pCMV-VSV-G). Lentiviral supernatant was collected 48 hours later and concentrated with LentiX-Concentrator (Clontech, Takara) following the manufacturer's protocol. Concentrated lentivirus was kept at -80ºC until use. T cells from healthy donors were activated on day 0 with CD3/CD28 Dynabeads and transduced with concentrated lentivirus on day 2 in media containing Polybrene (Merck Millipore). Spinoculation was performed using centrifugation at 2,000 rpm for 1hour.

CART cell expansion: Lymphocytes were obtained by Ficoll and magnetic T cell depletion (Miltenyi Biotec). T cells were expanded in Click's media (50% RPMI, 50% Click's (Irvine Scientific), 5% human Serum and 1% Pen/strep), activated with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific) and IL-2 (100 IU/mL) every other day. Experiments were performed after 8-10 days of T cell expansion.

Flow cytometry: CAR-BCMA was detected with a recombinant BCMA-Fc protein (Enzo Life Sciences) and a secondary antibody anti-human IgG Fc conjugated to Brilliant Violet (BV)-421 (Biolegend). Antibodies used were CD3-APC, CD8-PeCy7 and CD4-APCH7 (Becton Dickinson). Memory subsets were identified with CD45Ra-APC (Biolegend), CCR7-BV510 and CXCR3-Alx-488 (BD biosciences). MM cells were stained with CD138-BV421 (Becton Dickinson) and BCMA-APC (Biolegend). Flow cytometry analysis was performed using FlowJo software.

Cytotoxicity: Assays were performed using different effector:target ratios and at different time-points. Target cells used in these assays had been previously modified with a lentiviral vector to over-express GFP-firefly luciferase (GFP-ffLuc). Percentage of remaining live GFP+ tumor cells was analyzed by flow cytometry according to the following formula: % of live cell=100x(N. of GFP+ cells with T cells at time x /N of GFP+ cells alone at time x).

Immunogenicity prediction: NetMHC uses an approximation algorithm that reliably predicts the affinity of peptides of lengths 8, 10 and 11, for which affinity data for training are rare. The method uses predictors trained on peptides of length 9 to successfully extrapolate to other lengths. In short, the method approximates each peptide of any length to a number of 9-mers, by inserting X (for 8-mers) or deleting amino acid(s) (for 10- and 11-mers) and set the final prediction to an average of the 9-mer predictions. Bind Level indicates if the peptide is predicted to bind stronger than a certain threshold. Predicted affinities weaker than 500 nM or lower than the 1% percentile score have no indications [1-3].

***In vivo* myeloma murine model**: 8-12 weeks old NOD/SCID IL-2Rcnull (NSG) mice were irradiated at 2Gy at day -1. At day 0, mice received i.v. 1-1.5 $x10^6$ GFP-ffLuc-ARP-1 cells. Either at day 6 or day 14, depending on the disease model, mice received i.v. either untransduced (UT) T cells or CART cells. Mice were subjected to weekly bioluminescence imaging (BLI) using a Hamamatsu color CDD camera (Hamamatsu Photonics Systems, Bridgewater, NJ) following IP injection of D-luciferin (20 mg/mL PBS). Signal was quantified using Image J software.

Proliferation assays: CART cells were stained with CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Thermo Fisher Scientific) before being co-cultured under different conditions and cell lines for 96 hours. Proliferation was analyzed by Flow cytometry.

Cytokine production and sBCMA: IFN-γ, TNF-α, IL-6, IL-1β cytokines were quantified by ELISAs (ELISA MAX™ Deluxe Set, Biolegend) following manufacturer's protocol. Soluble BCMA was detected by ELISA (Human BCMA/TNFRSF17 DuoSet ELISA, R&D systems) following manufacturer's protocol.

Macrophage differentiation: After performing Ficoll, monocytes were isolated with RosetteSep Human Monocyte Enrichment Cocktail (Stem Cell Technologies) and macrophages were differentiated from monocytes after 1 week expansion with RPMI 10% FBS and 0.1mg/ml M-CSF (Thermo Fisher Scientific).

Confocal microscopy: RPMI cell line overexpressing BCMA fused to green fluorescent protein (GFP) was generated and then co-cultured with CART cells stained with Cell Tracker™ Blue CMAC Dye (Thermo Fisher Scientific). BCMA was also detected by confocal fluorescence microscopy with monoclonal anti-TNRSF17 mouse antibody (Sigma-Aldrich) and secondary anti-mouse IgG Alexa 647 (Cell signaling Technologies). Images were acquired using a Leica SP5 microscope. 405, 488 and 633 lasers were used for excitation. For time lapse experiments, *in vivo* image acquisitions were performed every 20 seconds.

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