Development and manufacture of novel locally produced anti-BCMA CAR T cells for the treatment of relapsed/refractory multiple myeloma: results from a phase I clinical trial

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SUPPLEMENTARY MATERIALS AND METHODS

HBI0101 CAR structure and generation of clinical grade HBI0101 retroviral bank.

The design of HBI0101 CAR construct, has been previously described in [1]. Briefly, it consists of a heavy chain connected by a linker to a light chain, both derived from the C11D5.3 antibody [2]; CD8α-derived hinge and transmembrane (TM) domains; co-stimulatory 4-1BB and CD3ζ domains (Supplementary Figure S1). The full construct was inserted into an MSGV1 retroviral plasmid (kindly gifted by Dr. Steven Rosenberg, NCI). PG13 HBI0101 were generated by transiently transfecting Phoenix-ECO cells (ATCC) with the plasmid encoding the gamma-retroviral vector MSGV1-HBI0101 using JetPrime reagent (Tamar) and subsequently transducing PG13 cells (ATCC) with HBI0101-Phoenix-ECO cell-free vector supernatants. PG13 transduced population was subsequently sub-cloned by limiting dilution, and the PG13-HBI0101 expanded to generate a seed bank. The certified PG13 seed bank was sent to the Indiana University Vector Production Facility (IU-VPF) in Indianapolis that has generated a master cell bank (MCB) and a GMP-certified HBI0101 clinical grade retroviral supernatant for the transduction of MM patients' autologous T-cells.

HBI0101 cells clinical grade production.

Leucocytes are collected at day -10 by leukapheresis, using the Spectra Optia apheresis system, and then transferred to the Facility for Advanced Cellular Therapy-Hadassah (FACT-H). Cells are separated to peripheral mono-nuclear cells (PBMCS) by Ficoll gradient and T-cell stimulated using anti-CD3 (OKT3; Miltenyi) and IL-2 (Proleukine; Novartis) for two days (Supplementary Figure S4). At day -8, stimulated T cells are transduced with 1/25 or 1/50 diluted HBI0101 retroviral supernatant overnight. Then, cells are seeded into GRex100 devices filled with AIM-V medium (Gibco) supplemented with 5% human AB serum (Access Cell Culture), 1% Glutamax (Gibco) and 300 IU/mL IL-2 for seven days of expansion (Supplementary Figure S4). Medium and IL-2 replenishment was performed every 2-3 days. At the day of patient's infusion, cells are washed three times with saline with 1% human albumin (Kedrion), and then formulated into the final drug product (DP) at the concentration of $15x10^6$ CART cells/mL in saline with 2.5% human albumin. DP infusion volume varied according to cell doses.

Outline of QC and sterility tests for HBI0101 production.

Quality control testing of in-process (IP) and end-of-product (EOP) HBI0101 cells are performed along the manufacturing process as detailed in Supplementary Figure S4A, and include: i) determination of the percent of transduction, assessed by flow cytometry using BCMA-FITC recombinant protein (ACROBiosystems), and performed at days -7, -2 and 0; ii) *in-vitro* efficacy of CART cells, assessed by the release of interferon-γ by ELISA (R&D) following stimulation with myeloma cell line, and performed at day -2; iii) determination of the vector copy number (CPN) by real-time (RT) PCR of the transduced cells' genomic DNA at day -2; iv) the absence of replication competent retrovirus (RCR) at day -2 is confirmed by PCR analysis of the transduced cells' genomic DNA using GALV primers set, as detailed in [3]; v) the characterization of the different cellular subsets was performed at day 0 by flow cytometry, using antibodies mixture as follows: anti-CD3 (Beckman Coulter), anti-CD4

(Biolegend), anti-CD8 (BD), anti-CD56 (Biolegend), anti-CD19 (Biolegend), and anti-CD14 (Beckman Coulter) ; vi) pH of the DP was assessed at day 0. All the tested parameters represent release criteria of the final DP, which specifications are further detailed in Supplementary Table S4.

In addition, and in compliance with ANNEX 1 guidelines for Advanced Therapy Medicinal Products (ATMPs), IP and EOP HBI0101 cells are tested for sterility according to the timeline detailed in Supplementary Figure S4B. Sterility testing were performed by an outsourced GMP-accredited institution (HyLabs).

HBI0101 CART *in vivo* detection.

Blood was collected from MM patients, prior to and following HBI0101 cells infusion, at the designated times. Genomic DNA was extracted and purified, using the Qiagen QIAamp DNA Blood Mini Kit. CAR copy number was determined by quantitative real-time PCR using the Taqman-based primers as follows: MSGV1 primers (Forward: CGGCAGCCTACCAAGAACA; reverse primer: TGTGTCGCCGACTCGGTAA; probe: CGGTGGTACCTCACC), and the TaqMan Fast advance Master Mix (Applied Biosystems). Standard curves of MSGV1 plasmid (ranging from $10^{\wedge 7}$-$10^{\wedge 0}$ copies) was generated by serial dilution of the MSGV1 plasmid. The number of circulating CART in 1mL of blood was then calculated by extrapolating the number of retroviral copies inserted into each CART cell.

BM immunophenotyping.

Bone marrow (BM) aspirates were collected from patients before and a month after HBI0101 infusion, labeled with α-hCD38, α-hCD138 (Beckman Coulter) and α-hBCMA (Biolegend), and further analyzed using the 10-colors Navios flow cytometer.

Soluble BCMA (sBCMA) quantification.

Serum BCMA was analyzed by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (R&D). Assay Range: 15.6-2000 pg/mL; Assay sensitivity validated by the user: 8.28 pg/mL.

Multiplex Analysis.

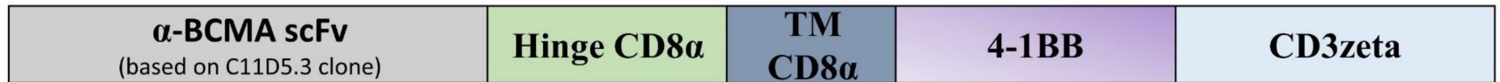
Sera from MM patients at baseline (day -10) and at T_{max} (day of CART peak concentration (C_{max})) were processed with the Luminex Performance Human XL Cytokine Magnetic Panel (25-Plex) FCSTM18-26 (R&D), and further assessed by Luminex MAGPIX Instrument with xPONENT 4.2. The multiplex panel included the following cytokines/chemokines: CCL2, CCL3, CCL4, CXCL10, IL-8, G-CSF, GM-CSF, IFN-α, IFN-γ, IL-10, IL-12, IL-13, IL-15, IL-17, IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, CCL5, TNF-α, VEGF and FGF. Precision and sensitivity data for these parameters is reported in the manufacturer's manual. Data was analyzed using MILLIPLEX® Analyst 5.1 Software.

Since we were unable to assess the concentration of IFN-γ, TNF-α and IL-2 using the multiplex array, we determined these cytokines' concentration by ELISA (R&D).

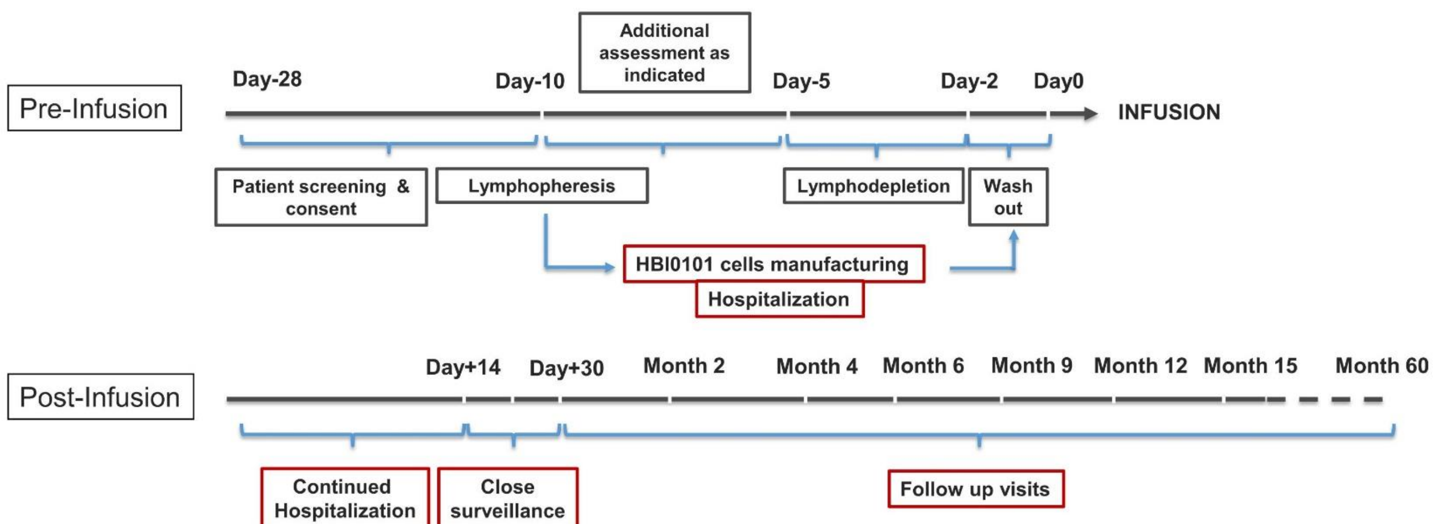
Statistical analysis.

A sample size of 20 patients was planned for the purpose of comparison between the different HBI0101 doses for both safety and short-term efficacy. Descriptive statistics was performed using median with range for continuous variables, and counts and percentages for categorical variables. OS was defined as the time from randomization until death from any cause and is measured in the intent-to-treat. PFS was defined as the time from CART infusion until disease progression or death from any cause. OS and PFS were estimated using the Kaplan-Meier method. Overall response rate (ORR) was defined as the proportion of patients achieving either a stringent complete response (sCR), a complete response (CR), a very good partial response (VGPR) or a partial response (PR). Descriptive statistics were performed using percentages. For the purpose of this phase I data analysis, data was collected until February 3rd 2022. Data were compiled using Excel software and all statistical analyses were performed using GraphPad Prism (v9.2.0) software. Appropriate statistical methods were used to calculate significance, as described in figure legends (two-way ANOVA and paired or unpaired *t*-test).

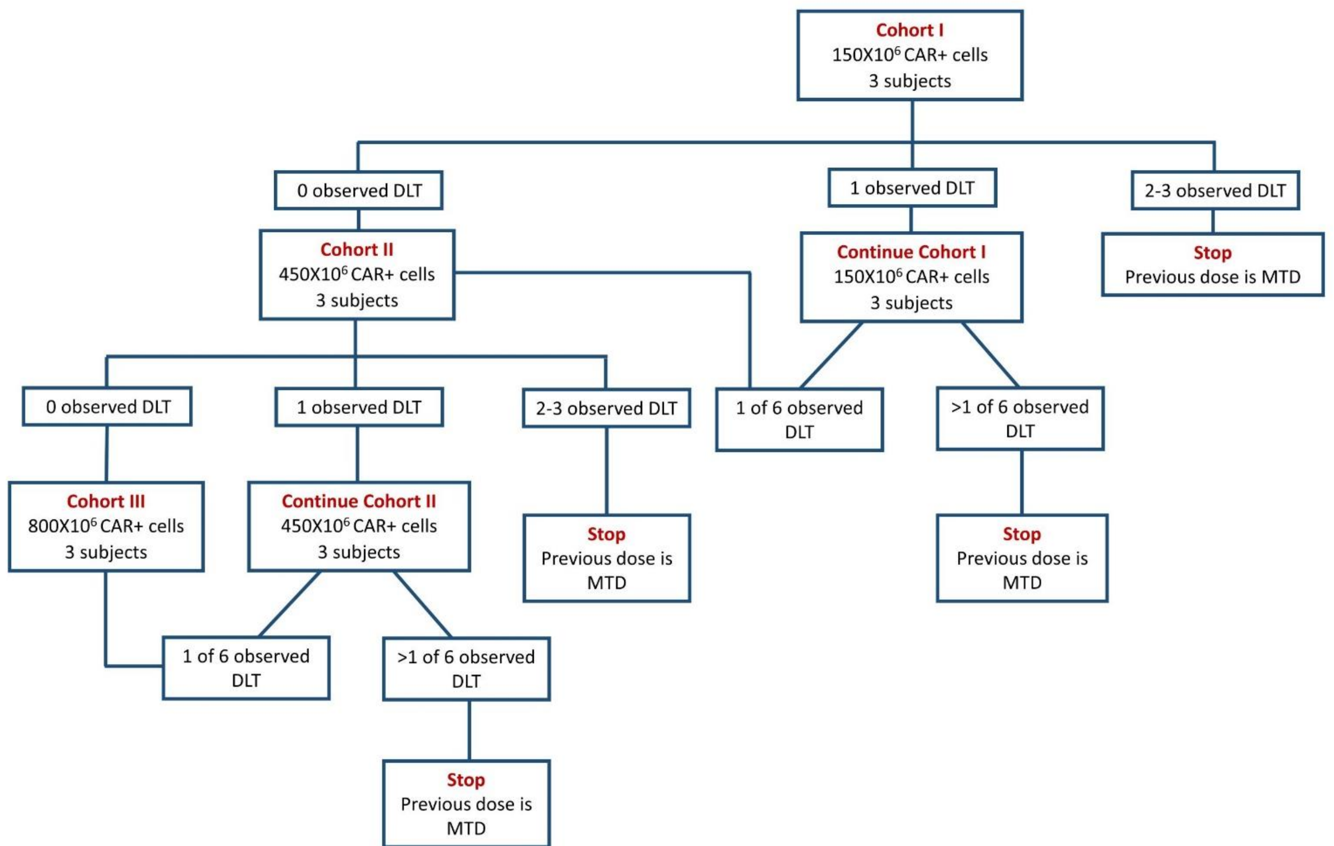
SUPPLEMENTARY FIGURES



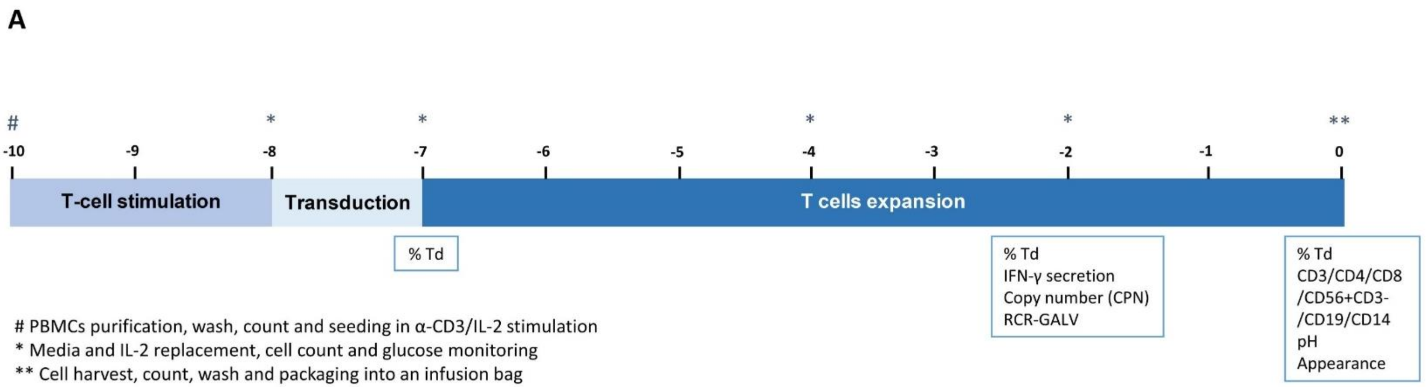
Supplementary Figure S1. Schematic of HBI0101 CAR construct. CAR sequence consists of a C11D5-3 anti-BCMA single chain variable fragment (scFv), CD8α hinge and transmembrane regions, the cytoplasmic portion of the 4-1BB costimulatory molecule, and the CD3ζ T-cell activation domain.

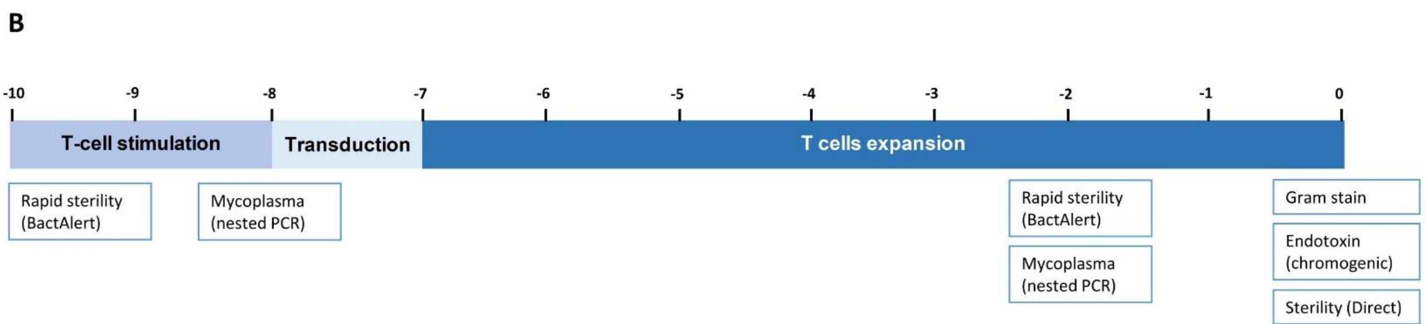


Supplementary Figure S2. HBI0101-Clinical study outline. Before enrolment, candidates are screened for their eligibility. At day -10 prior to infusion, lymphocytes are collected using the Sprectra Optia apheresis instrument. Patients are then hospitalized for baseline assessment, and HBI0101 production from the fresh/frozen apheresis is initiated. On days -5 to -2, patients are T-cells depleted with fludarabine and cyclophosphamide, and after two days of "wash-out" from lymphodepletion, infused with the manufactured HBI0101 (day 0). After infusion, patients are hospitalized for two weeks for safety follow up. Routine follow-up at the indicated time points (1, 2, 4, 6, 9, 12, 15…60 months after HBI0101 infusion) is performed for five years or until patient's discontinuation from the study.

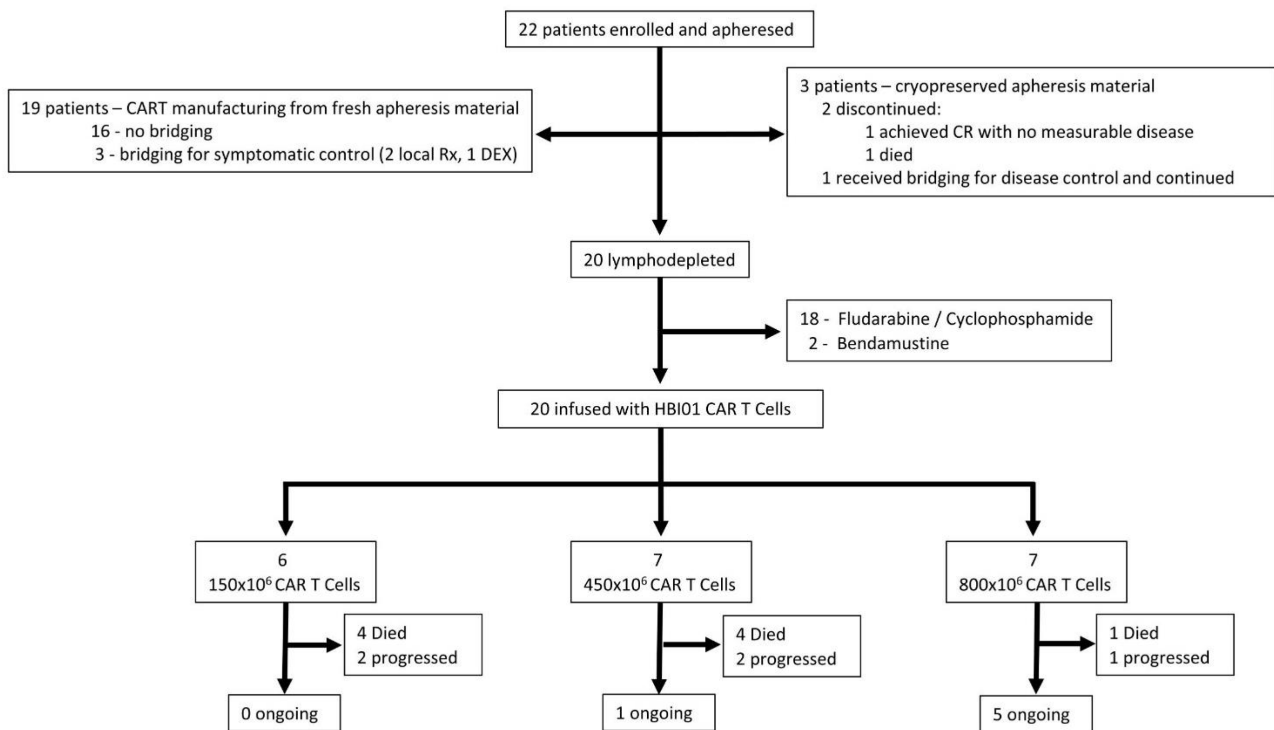


Supplementary Figure S3. The "3 + 3" dose escalation study design. DLT, dose limiting toxicity; MTD, maximum tolerable dose.

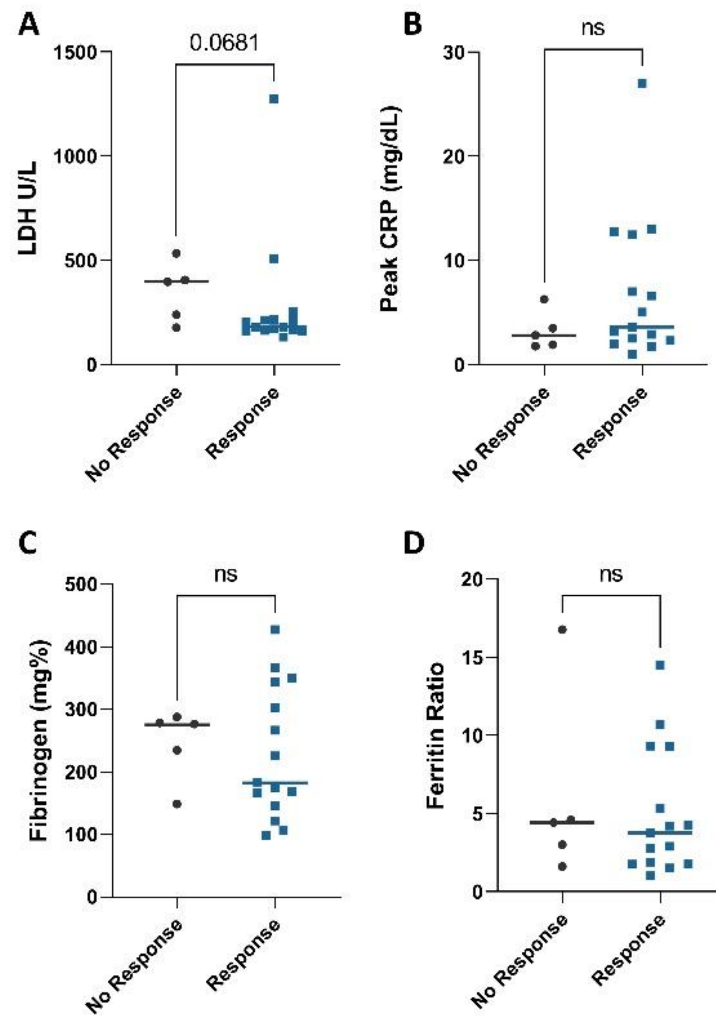




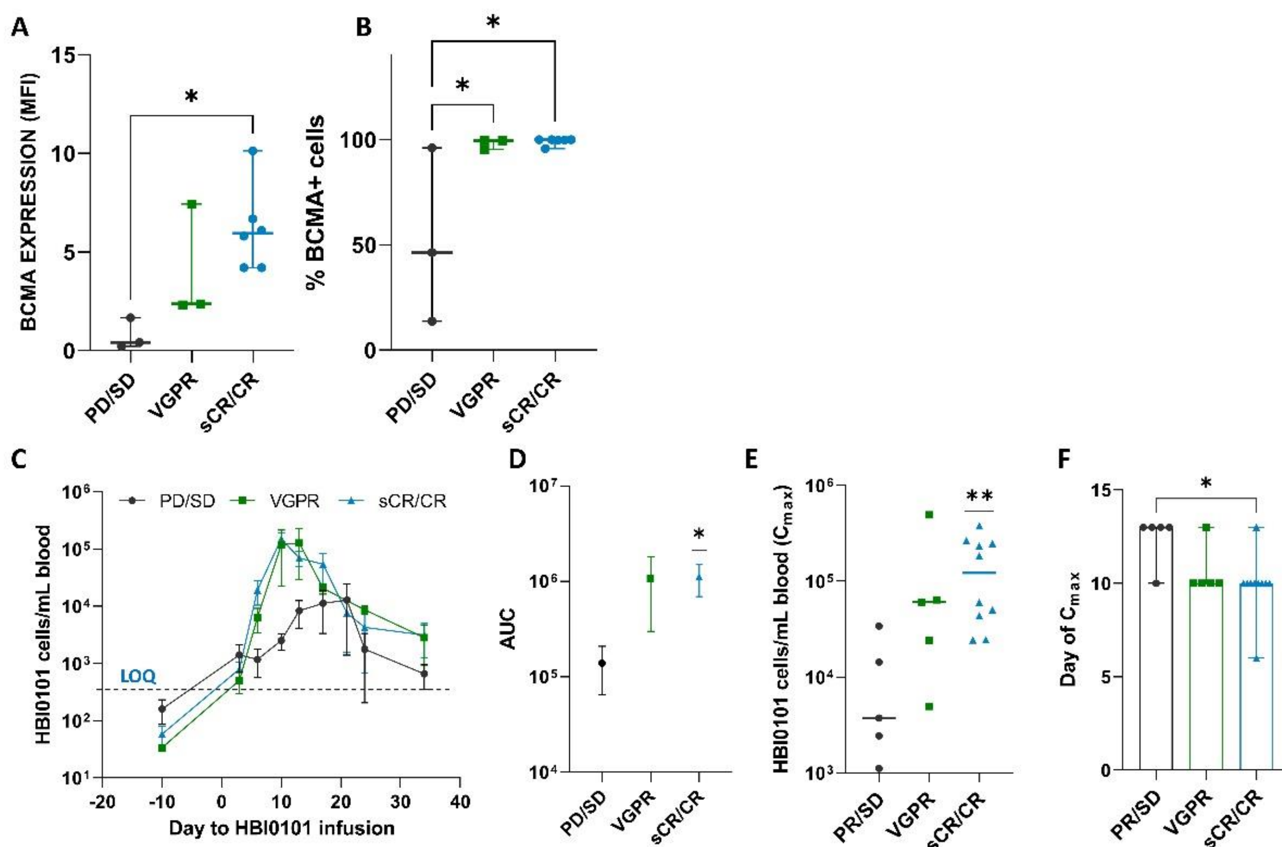
Supplementary Figure S4. QC and sterility testing along HBI0101 manufacture. HBI0101 culture samples were collected at the indicated time points for QC (A) and sterility (B) in-process (IE) and end-of-process (EOP) control. Td, transduction, CPN, copy number.



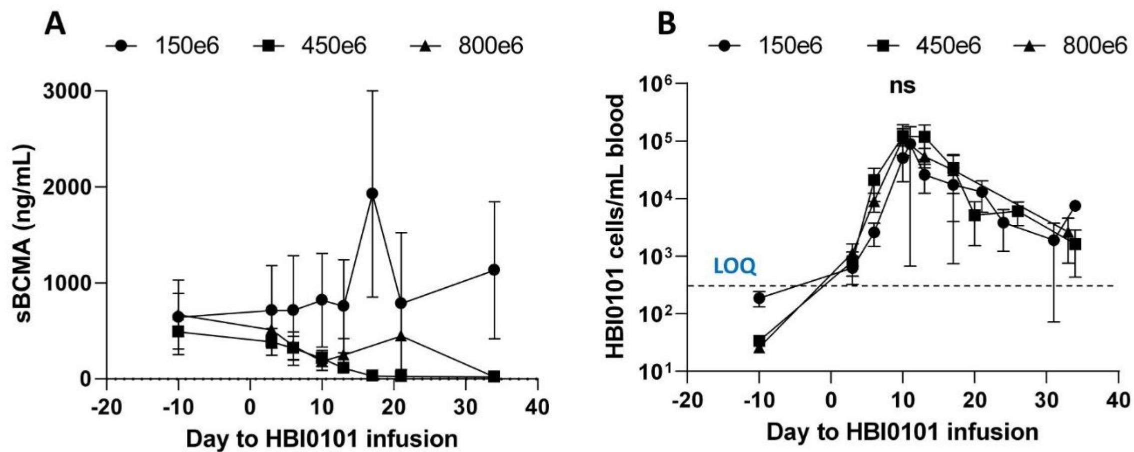
Supplementary Figure S5. Consort diagram. A total of twenty MM patients, out of the 22 patients that were enrolled and leukapheresed, underwent lymphodepletion, and were treated according to a "3+3" dose escalation study design: cohort 1 was administered with 150x10^6 CAR+ cells (N=6), cohort 2 with 450x10^6 CAR+ (N=7) and cohort 3 with 800x10^6 CAR+ (N=7). DEX, dexamethasone; Rx, radiation.



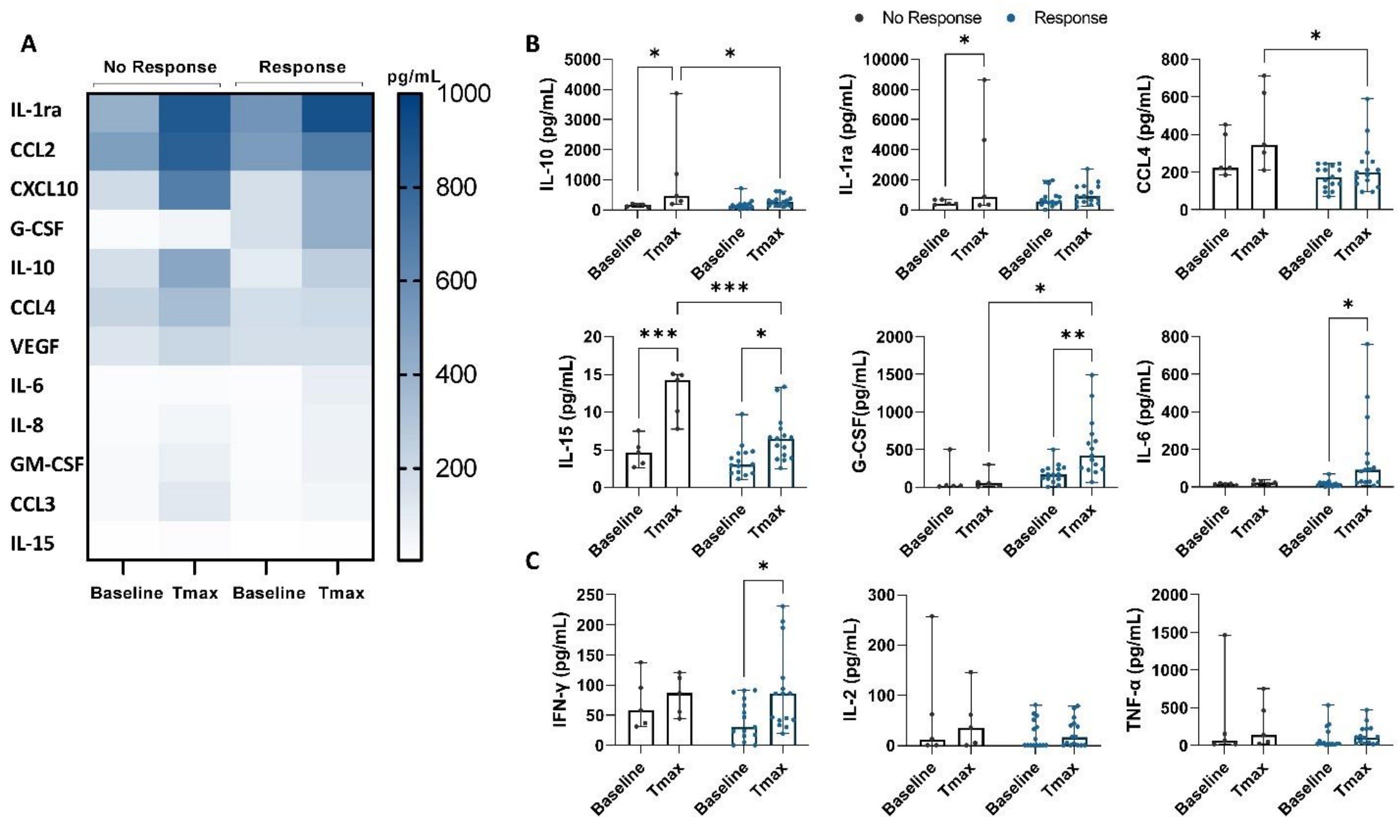
Supplementary Figure S6. Correlation between laboratory parameters and response to HBI0101. Lactate dehydrogenase (LDH) (A), Peak C-reactive protein (CRP) (B), Fibrinogen (C) and Ferritin ratio (D) levels were assessed at day 14 post HBI0101 infusion. Bars represent the median.



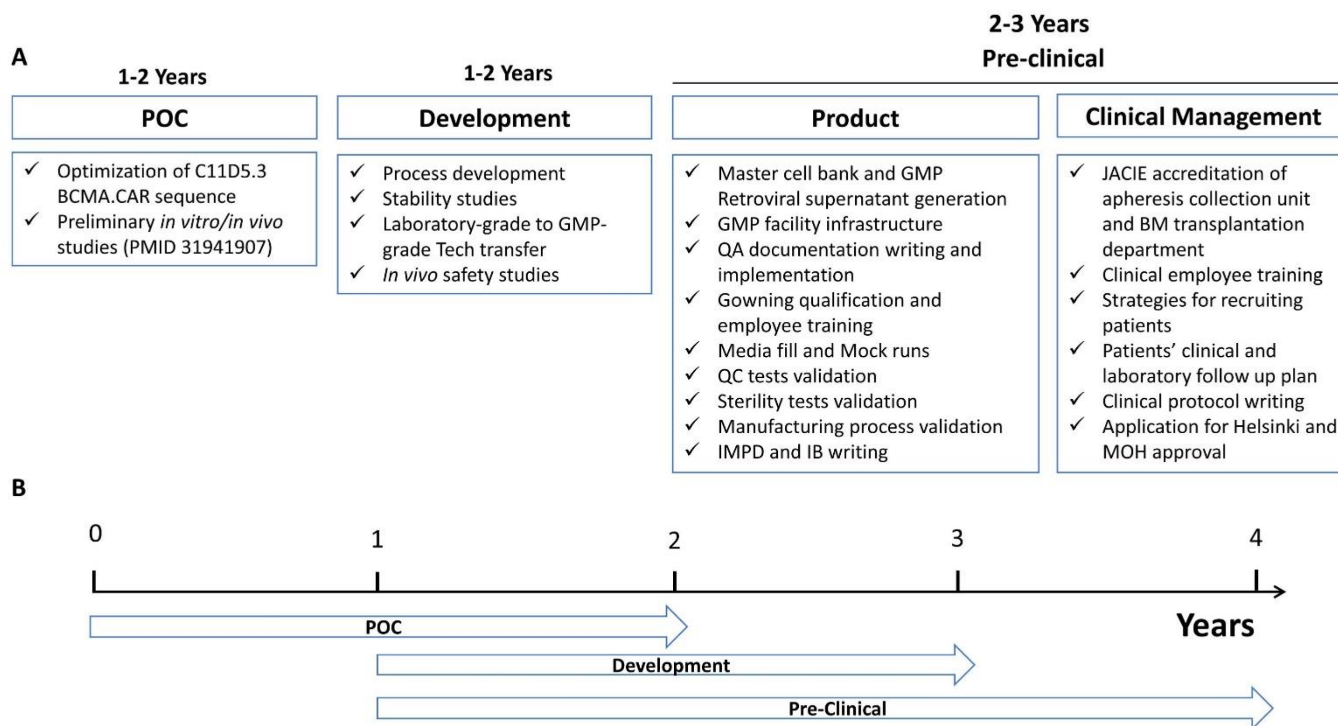
Supplementary Figure S7. (A-B) Analysis of BCMA expression on MM-PCs. HBI0101-treated MM patients were classified according to SD/PD (black dots, N=3)), VGPR (green squares, N=3)) and sCR/CR (blue circles, N=6) groups. The mean fluorescence intensity (MFI) (A) and the percent of BCMA-positive PCs (B) were determined by flow cytometry. (C-F) HBI0101 CART in vivo kinetics in MM patients treated with HBI0101 and classified by SD/PD (black dots, N=5), VGPR (green squares, N=5) and sCR/CR (blue circles, N=10). (C) The median number of HBI0101-CART cells per 1mL blood in the SD/PD *vs.* VGPR and sCR/CR groups was determined by quantification of CAR transgene levels by qRT-PCR method following CART infusion at the indicated times and further adjusted to the copy numbers per transduced cell at the day of CART infusion. The limit of quantitation (LOQ) was 500 CART/mL blood. (D) HBI0101 CART cell overall expansion in the first month of CART therapy. Area under the curve (AUC) as a measure of CART overall expansion was calculated with Prism software (GraphPad). (E) HBI0101 cells *in-vivo* median concentration at peak (C_{max}) in the SD/PD (black dots) *vs.* VGPR (green squares) and sCR/CR (blue triangles) groups. (F) Median time to C_{max} (T_{max}) in the SD/PD *vs.* VGPR and sCR/CR groups. Upper and lower bars Ɪ represent the maximal and minimal values, respectively. SD, stable disease; PD, progressive disease; VGPR, very good partial response; (s)CR, (stringent) complete response. * $p<0.05$, **$p<0.01$, by unpaired *t*-test.



Supplementary Figure S8. Analysis of HBI0101 cells persistence and sBCMA levels by dose-cohort. (A) Soluble BCMA (sBCMA) levels prior to and following CART infusion determined by ELISA in the different dose-cohorts. Legend as follows: black circles, 150x10^6 CAR+; black squares, 450x10^6 CAR+ and black triangles, 800x10^6 CAR+ cells. Statistical analysis by 2-way ANOVA. (B) HBI0101 *in-vivo* kinetics according to HBI0101 dose. The number of HBI0101 CART per 1mL blood was determined by quantification of CAR transgene levels by qRT-PCR method following CART infusion at the indicated times, and further adjusted to the CPN of HBI0101 cells before infusion. The limit of quantitation (LOQ) was 500 CART/mL blood.



Supplementary Figure S9. Comparison of Cytokine profile for Predicting Response to HBI0101-treatment. (A) Heat map analysis of 12 cytokine detected in serum samples of "response" patients (N=15) *vs.* "no response" (N=5) patients, at baseline (at day -10) and at T_{max} following HBI0101-treatment. Rows represent the cytokines that were affected by HBI0101-treatment in the, while columns represent the median of the cytokines values of the patients of the "response" and "no response" groups, "before" and "after" HBI0101-infusion. Cytokines values at day of C_{max} following CART infusion in the "response" group were used as reference values. Key color legend is indicated, with intense dark colors for high cytokine values, and light color for low cytokine values. Median and range values of the represented cytokines are detailed in Supplementary Table S5. (B-C) Graphical representation of the cytokines that were significantly differentially expressed between the "response" and "no response" groups, prior to and following HBI0101 infusion. Black lines represent the median, upper and lower bars I represent the maximal and minimal values, respectively. R, response; NR, no response. Statistical analysis by 2-way ANOVA.



Supplementary Figure S10. Main steps in the bench-to-bedside translation of HBI0101 CART-based therapy. POC, proof-of-concept; GMP, Good Manufacturing Practice; IMPD, Investigational Medicinal Product Dossier; IB, Investigator's Brochure; JACIE, The Joint Accreditation Committee ISCT-Europe & EBMT; MOH, Ministry of Health, QA, quality assurance; QC, quality control.

SUPPLEMENTARY TABLES

Supplementary Table S1: Inclusion and exclusion criteria

	Inclusion criteria	Exclusion criteria
Age	≥ 18 years	
ECOG	0 – 2	
Disease	Relapsed / refractory	
	Measurable disease - at least one: - Serum M-protein ≥ 0.5 g/dL - Urine M-protein ≥ 200 mg/24 h - involved FLC ≥ 5 mg/dL (50 mg/L) provided serum FLC ratio is abnormal - A biopsy-proven evaluable plasmacytoma - Bone marrow plasma cells > 20% of total bone marrow cells - Non secretory disease provided there is a measurable disease by PET-CT or bone marrow	
	last line refractory/responsive	
	BCMA levels on plasma cell prior to treatment is NOT an inclusion/ exclusion criterion.	
		Known central nervous system disease
Anti-myeloma therapy	≥ 3 different lines including: PI, IMiD, Antibody	Systemic therapy for MM within 14 days prior to leukapheresis
	Bridging local radiation therapy allowed	Systemic therapy for MM within 14 days prior to the start of lymphodepletion
		Investigational cellular therapies within 8 weeks prior to the start of lymphodepletion
Medications	Physiological replacement doses of steroids up to: - 40 mg/day hydrocortisone - 10 mg/day prednisone - 1.5mg/ day dexamethasone	Therapeutic doses of steroids within 3 days prior to leukapheresis
		- Long-acting growth factors within 14 days prior to leukapheresis - Short-acting growth factors within 5 days prior to leukapheresis
WCBP	Negative serum pregnancy test	
Sexually active male/female	Use effective birth control	
Prior toxicity	Recovery to ≤ Grade 2 or baseline of any non-hematologic toxicities	
Lab results and Organ function		Inadequate hepatic function: - AST and/or ALT > 2.5 x ULN - direct bilirubin > 2 x ULN

		Inadequate renal function: Serum creatinine clearance /estimated clearance of <20 (ml/min).
		INR or PTT > 1.5 x ULN, unless on a stable dose of anticoagulant for a thromboembolic event
		CBC: - ANC < 1000 cells/mm3 - platelet count < 30,000 mm3 - hemoglobin < 8 g/dL (Blood transfusion allowed) - ALC < 500 cells/mm3
		Left ventricular ejection fraction < 40%
Prior medical conditions	Relapsed MM after allogenic SCT without evidence of GVHD after cessation of any IST for at least one month before recruitment to the study	Ongoing treatment with chronic IST
		Presence of active infection within 72 hours prior to lymphodepletion
		Significant co-morbid condition
		Known human immunodeficiency virus (HIV) positivity
		Active Hepatitis B or Hepatitis C infection
		History of stroke, unstable angina, myocardial infarction, ventricular arrhythmia requiring medication within 3 months
	acute line insertion induced thrombosis allowed	Venous thromboembolic event requiring anticoagulation and any of the following: - stable dose of anticoagulation for < 1 month - Grade 2, 3, or 4 hemorrhage in the last 30 days - continued symptoms
	Second malignancies in addition to myeloma providing it has not required therapy in the last 2 years and in remission or it is a successfully treated non-metastatic basal cell or squamous cell skin carcinoma, or prostate cancer that does not require therapy	Second malignancies in addition to myeloma, if the second malignancy has required therapy in the last 2 years or is not in complete remission;
	Pregnant or lactating women	

ECOG: Eastern Cooperative Oncology Group Performance Status ; BCMA: B-cell maturation antigen ; PI: Proteasome Inhibitor ; ImiD: Immunomodulatory Drug ; WCBP: women of childbearing potential ; AST: Aspartate Aminotransferase ; ALT: Alanine transaminase ; ULN: Upper Limit of Normal ; ANC: Absolute Neutrophil Count ; ALC: Absolute Lymphocyte Count ; CBC: Complete Blood Count ; INR: International ratio ; PTT: partial thromboplastin time ; GVHD: Graft versus Host Disease ; IST: Immune suppressive Therapy.

Supplementary Table S2: Patients' previous treatments: exposures and refractoriness.

	Total N=20	Cohort 1 n=6	Cohort 2 n=7	Cohort 3 n=7
PROTEASOME INHIBITOR				
BORTEZOMIB				
EXPOSED	19(95%)	6(30%)	6(30%)	7(35%)
REFRACTORY	17(85%)	6(30%)	4(20%)	7(35%)
CARFILZOMIB				
EXPOSED	11(55%)	4(20%)	5(25%)	2(10%)
REFRACTORY	10(50%)	4(20%)	5(25%)	1(5%)
IXAZOMIB				
EXPOSED	2(10%)	0(0%)	2(10%)	0(0%)
REFRACTORY	2(10%)	0(0%)	2(10%)	0(0%)
IMIDS				
LENALIDOMIDE				
EXPOSED	18(80%)	6(30%)	5(25%)	7(35%)
REFRACTORY	18(80%)	6(30%)	5(25%)	7(35%)
THALIDOMIDE				
EXPOSED	1(5%)	0(0%)	19(5%)	0(0%)
REFRACTORY	1(5%)	0(0%)	1(5%)	0(0%)
POMALIDOMIDE				
EXPOSED	17(85%)	5(25%)	5(25%)	7(35%)
REFRACTORY	17(85%)	5(25%)	5(25%)	7(35%)
ANTI-CD38				
DARATUMUMAB				
EXPOSED	19(95%)	6(30%)	6(30%)	7(35%)
REFRACTORY	19(95%)	6(30%)	6(30%)	7(35%)
ISATUXIMAB				
EXPOSED	1(5%)	0(0%)	1(5%)	0(0%)
REFRACTORY	1(5%)	0(0%)	1(5%)	0(0%)
ANTI-BCMA (BELANTAMAB MAFODOTIN)				
EXPOSED	9(45%)	2(10%)	5(25%)	2(10%)
REFRACTORY	9(45%)	2(10%)	5(25%)	2(10%)

All data are presented as number of patients, percent of patients (no., %).

Supplementary Table S3: HBI0101 final product characteristics and release criteria.

	No Response	Response	Release criteria
DP Identity			
% Viability	94.2±1.3	96.2±0.3	≥ 70.0%
CAR+CD3+ cells (%)	99.7±0.0	99.6±0.1	≥ 70.0%
CAR+CD3+CD4+ cells (%)	31.6±9.1	16.7±3.1	ND*
CAR+CD3+CD8+ cells (%)	66.6±8.5	81.4±2.9	ND*
DP Potency			
CAR+ cells (%)	47.8±2.7	52.8±2.8	≥ 10.0%
CAR+CD3+CD4+CD56+ cells (%)	11.4±5.3	19.3±4.3	ND*
CAR+CD3+CD8+CD56+ cells (%)	39.0±10.8	51.8±4.6	ND*
IFN-γ secretion (fg/CAR+ cell)	246.2±54.1	336.1±49.9	2.0-1000.0 fg**/CAR+ cell
CPN/Td cell	2.4±0.2	2.9±0.2	ND*
DP Impurity			
CD19 (%)	0.0±0.0	0.0±0.0	≤ 1.00%
CD14 (%)	0.0±0.0	0.0±0.0	≤ 1.00%
CD56+CD3- (%)	0.5±0.2	0.8±0.1	ND*

The production process lasts 10 days for all the patients. HBI0101 drug products (DPs) at day of infusion (day 0) was characterized as follows: *DP Identity*. The percent of CD3+, CD4+ and CD8+ cells (gated on live CAR+ cells) was determined by flow cytometry. *DP Potency*. The percent of BCMA.CAR+ cells (gated on live cells) was determined by flow cytometry using human recombinant BCMA protein. The percent of activated cytotoxic T cells (CD3+CD56+) was determined by flow cytometry (gated either on CD4+ or CD8+ T cells). The secretion of IFN-γ by each HBI0101 transduced cell, was assessed by ELISA at day -2. IFN-γ was released into the co-culture supernatant of HBI0101-transduced cells with NCI-H929 (1:1 E:T ratio) following an overnight incubation. The total amount of IFN-γ in each culture well was quantified and then divided by the number of transduced cells introduced into the well. The copy number (CPN) of HBI0101 inserts into every CAR transduced cell was quantified by qRT-PCR as described in Materials and Methods and further adjusted to transduced cell. Release criteria specifications are described in the right column of the table. No statistical significance was observed in none of the released parameters, attesting to the robustness of the production process. *DP Impurity*. The percent of "cell impurities", as to CD19+, CD14+ and CD3-CD56+ (gated on live cells) was determined by flow cytometry, and do not exceed 1% each in all twenty batches. * ND, not determined. ** fg, femtogram.

Supplementary Table S4: CAR-T associated CRS and ICANS

CRS (Grade 0-2)				
	All (N=20)	Cohort 1 (n=6)	Cohort 2 (n=7)	Cohort 3 (n=7)
Start day [a]	1 (0-21)	6 (0-21)	0 (0-1)	1 (0-3)
Duration days [a]	2 (1-5)	3 (0-5)	2 (1-3)	2 (1-5)
Tocilizumab given [b]	8 (40%)	1 (17%)	4 (57%)	3 (43%)
Grade 2 events [b]	18 (90%)	1 (17%)	4 (57%)	3 (43%)
ICANS (Any Grade)				
Events	0	0	0	0

[a] Median (range); [b] number of patients (range).

Supplementary Table S5: Cytokine profiling of MM patients

pg/mL	No Response		Response	
	Baseline	T_{max}	Baseline	T_{max}
IL-1ra*	412 (329-683)	873 (306-8624)	558 (7-1960)	907 (229-2706)
CCL2	502 (441-768)	830 (424-1796)	516 (13-832)	690 (423-1608)
CXCL10	189 (150-502)	672 (301-2234)	168 (3-500)	421 (190-1490)
G-CSF	21 (8-502)	52 (10-301)	168 (5-500)	421 (67-1490)
IL-10*	169 (88-201)	462 (193-3864)	107 (14-709)	255 (101-618)
CCL4*	223 (186-452)	347 (210-713)	175 (70-246)	199 (95-590)
VEGF	137 (52-1218)	211 (113-1265)	168 (4-535)	166 (59-871)
IL-6*	16 (8-21)	23 (10-38)	14 (1-70)	92 (7-758)
IL-8	21 (14-37)	50 (30-110)	23 (1-119)	70 (30-165)
GM-CSF	32 (23-64)	77 (44-145)	22 (10-61)	68 (24-117)
CCL3	34 (19-148)	119 (84-137)	28 (10-135)	47 (11-774)
IL-15*	5 (3-7)	14 (8-15)	3 (1-10)	6 (3-13)
IFN-γ*	58 (31-137)	87 (44-121)	30 (0-91)	86 (20-231)
IL-2	12 (0-257)	0 (0-80)	35 (0-146)	17 (0-79)
TNF-α	62 (15-1463)	137 (20-751)	22 (7-535)	105 (15-468)

Concentrations are in pg/mL. * Cytokines that were significantly differently expressed in the different groups (see Figure 6B-C). Baseline cytokine concentration was determined at day -10 prior to CART administration. T_{max}, time of concentration of CART cells at peak.

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