Chimeric antigen receptor T cell immunotherapy in multiple myeloma

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Chimeric antigen receptor T cell immunotherapy in multiple myeloma

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ABBREVIATIONS AND ACRONYMS

ADA: Anti-drug antibody

ADC: Antibody-dependent cytotoxicity

ADCC: Antibody-dependent cellular cytotoxicity

AEMPS: Spanish Agency of Medicines and Medical Devices

AlloSCT: Allogeneic stem cell transplantation

ANC: Absolute neutrophil count

ASCT: Autologous stem cell transplantation

ASTCT: American Society for Transplantation and Cellular Therapy

Axi-cel: Axicabtagene ciloleucel

B-ALL: B-cell acute lymphoblastic leukemia

BCMA: B cell maturation antigen

Bid: Twice a day

BsTCE: Bispecific T cell engager

CAR: Chimeric antigen receptor

CAR-T: Chimeric antigen receptor T cell

CI: Confidence interval

Cilta-cel: Ciltacabtagene autoleucel

CLL: Chronic lymphocytic leukemia

CMV: Cytomegalovirus

CNS: Central nervous system

CR: Complete response

CRAB: Hypercalcemia, renal failure, anemia and ostelolytic bone lesions

CSF: Cerebrospinal fluid

DLBCL: Diffuse large B-cell lymphoma

DOR: Duration of response

D-VTD: Daratumumab plus bortezomib plus thalidomide plus dexamethasone

EMA: European Medicines Agency

FDA: Food and Drug Administration

FL: Follicular lymphoma

FLC: Free-light chain

FP: Final product

G-CSF: Granulocyte colony-stimulating factor

GMP: Good manufacturing practice

GPRC5D: G protein-coupled receptor class C group 5 member D

GSI: Gamma-secretase inhibitor

HLH: Hemophagocytic lymphohistiocytosis

HR: Hazard ratio

IEC-HS: Immune effector cell-associated hemophagocytic lymphohistiocytosis-like syndrome

IFNγ: Interferon-γ

KD: Carfilzomib plus dexamethasone

KRD: Carfilzomib plus lenalidomide plus dexamethasone

ICAHT: Immune effector cell-associated hematotoxicity

ICE: Immune effector cell encephalopathy

ICI: Immune checkpoint inhibitor

Ide-cel: Idecabtagene vicleucel

IL: interleukin

IMID: Immunomodulating drug

IMWG: International Myeloma Working Group

IMWG-FI: International Myeloma Working Group – frailty index

ISS: International Staging System

LAG3: Lymphocyte activation gene 3

LD: Lymphodepletion

MM: Multiple myeloma

MMAF: Monomethyl auristatin F

MNT: Movement and neurocognitive treatment-emergent adverse events

MRD: Measurable residual disease

MRI: Magnetic resonance imaging

NGF: Next-generation flow

NGS: Next-generation sequencing

NHL: non-Hodgkin lymphoma

NK: Natural killer

ORR: Overall response rate

OS: Overall survival

PD1: Programmed death 1

PFS: Progression-free survival

PI: Proteasome inhibitor

PVD: Pomalidomide plus bortezomib plus dexamethasone

RBC: Red blood cell

RD: Lenalidomide plus dexamethasone

R-ISS: Revised International Staging System

R/R: Relapsed/refractory

sBCMA: Soluble B cell maturation antigen

sCR: Stringent complete response

SOC: Standard of care

TCE: T cell engager

TCR: T cell receptor

TIGIT: T cell immunoreceptor with Ig and ITIM domains

TIM3: T cell immunoglobulin and mucin domain 3

Tisa-cel: Tisagenlecleucel

TME: Tumor microenvironment

TPO: Thrombopoietin

US: United States

VMP: Bortezomib, melphalan and dexamethasone

VRD: Bortezomib plus lenalidomide plus dexamethasone

VTD: Bortezomib plus thalidomide plus dexamethasone

LIST OF ARTICLES IN THE THESIS

Thesis in compendium of publications format.

The thesis consists of 3 objectives and 3 articles:

- **Oliver-Caldés A**, González-Calle V, Cabañas V, Español-Rego M, Rodríguez-Otero P, Reguera JL, López-Corral L, Martin-Antonio B, Zabaleta A, Inogés S, Varea S, Rosiñol L, López-Díaz de Cerio A, Tovar N, Jiménez R, López-Parra M, Rodríguez-Lobato LG, Sánchez-Salinas A, Olesti E, Calvo-Orteu M, Delgado J, Pérez-Simón JA, Paiva B, Prósper F, Sáez-Peñataro J, Juan M, Moraleda JM, Mateos MV, Pascal M, Urbano-Ispizua A, Fernández de Larrea C. *Fractionated initial infusion and booster dose of ARI0002h, a humanised, BCMA-directed CAR T-cell therapy, for patients with relapsed or refractory multiple myeloma (CARTBCMA-HCB-01): a single-arm, multicentre, academic pilot study.* Lancet Oncol. 2023 Aug;24(8):913-924.

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- **Oliver-Caldés A,** Battram AM, Mañé-Pujol J, Pérez-Amill L, Bachiller M, Calderon H, Castella M, Carpio J, Salsench SV, Bosch M, Tovar N, Cardus O, Urbano-Ispizua A, Moreno DF, Rodríguez-Lobato LG, Lozano E, Rosinol L, Juan M, Martin-Antonio B, Fernández de Larrea C. *Different approaches to block TIGIT as a single target in the academic BCMA-CART ARI0002h fail to achieve a significant improvement in a model of multiple myeloma.*

  Submitted for publication.

THESIS SUMMARY (Spanish)

LA INMUNOTERAPIA CON CÉLULAS CAR-T EN EL MIELOMA MÚLTIPLE

INTRODUCCIÓN

A pesar de los avances terapéuticos de las últimas décadas, el mieloma múltiple (MM) sigue siendo incurable. Los pacientes con enfermedad avanzada desarrollan multiresistencia a tratamientos. La inmunoterapia con células CAR-T modifica genéticamente linfocitos para que reconozcan un antígeno diana en la célula tumoral y la eliminen. El antígeno de maduración de linfocitos B (del inglés, BCMA) es una diana para inmunoterapia en MM. Existen dos CAR-T aprobados contra BCMA, que han demostrado superioridad respecto al tratamiento estándar en pacientes con enfermedad avanzada. Sin embargo, la mayoría de pacientes acaban recayendo. Por ello, identificar biomarcadores de respuesta y comprender mecanismos de resistencia, permitirá mejorar esta terapia.

Otras limitaciones de esta terapia incluyen su elevado coste y la disponibilidad limitada de espacios de producción, que dificultan el acceso a la misma. Los CAR-T académicos producidos con el modelo "*point-of-care"* tienen un coste menor y facilitan el acceso a la terapia. En este sentido, el Hospital Clínic de Barcelona, ha desarrollado dos CAR-T académicos, dirigidos contra CD19, ARI-0001, y BCMA, ARI0002h, para el tratamiento de hemopatías CD19-positivas y MM, respectivamente. ARI0002h mostró resultados preclínicos prometedores, que condujeron al diseño de un ensayo clínico (EC), CARTBCMA-HCB-01, para evaluar ARI0002h en pacientes con MM recaído/refractario (R/R).

HIPÓTESIS

1. La producción a escala humana de ARI0002h con linfocitos de pacientes con MM R/R es factible, obteniendo un producto eficaz y seguro.
2. Características biológicas como la expresión de BCMA en células de MM, la presencia de BCMA soluble en suero, las subpoblaciones de células T y sus receptores de puntos de control inmune, la cinética de ARI0002h, características clínicas basales (enfermedad extramedular, citogenética de

alto riesgo), o el desarrollo de anticuerpos contra ARI0002h, se correlacionan con la eficacia y seguridad de ARI0002h en MM.

3. Inhibir los mecanismos de agotamiento de ARI0002h mediante el bloqueo del receptor TIGIT mediante diferentes estrategias, mejora su eficacia.

OBJETIVOS

1. Analizar la producción, eficacia y seguridad de ARI0002h en MM R/R en el EC CARTBCMA-HCB-01.
2. Determinar biomarcadores de eficacia y seguridad en muestras de pacientes tratados con ARI0002h en el EC CARTBCMA-HCB-01.
3. Inhibir los mecanismos de agotamiento de ARI0002h mediante el bloqueo del receptor TIGIT y evaluar su impacto a nivel preclínico.

MÉTODOS

Trabajo 1

CARTBCMA-HCB-01 es un EC piloto, multicéntrico, que evaluó ARI0002h en 30 pacientes con MM. La producción de ARI0002h se realizó en dos centros académicos. Los pacientes recibieron una infusión inicial fraccionada y una dosis de refuerzo, al menos 3 meses tras la primera infusión. Los objetivos principales fueron la respuesta global (RG), tasa de desarrollo de síndrome de liberación de citoquinas (SLC) y eventos neurológicos.

Trabajo 2

Se obtuvieron resultados de 60 pacientes incluidos en CARTBCMA-HCB-01, con mayor seguimiento que en la primera cohorte reportada en el trabajo 1. En estos, se evaluó la expresión de BCMA, BCMA soluble, subpoblaciones de células T, cinética de ARI0002h, desarrollo de anticuerpos anti-CAR-T, aplasia de células B, citoquinas y enfermedad residual, y se correlacionaron con los resultados clínicos.

Trabajo 3

Se utilizaron tres estrategias para bloquear TIGIT en ARI0002 y evaluar la eficacia de cada una *in vitro* e *in vivo*: (i) Adición de un anticuerpo bloqueante, (ii) Transformar ARI0002h en un CAR-T de cuarta generación que secreta un bloqueante de TIGIT, (iii) Eliminar TIGIT de ARI0002h.

PRINCIPALES RESULTADOS

Trabajo 1

Todos los productos se obtuvieron satisfactoriamente. 47% de los pacientes tenían plasmocitomas (20% localización extramedular). Con un seguimiento medio de 18 meses la RG fue del 100% (67% respuestas completas (RC)). Se observó SLC en 80% de los pacientes (grados 1-2). No se observó neurotoxicidad. La mediana de supervivencia libre de progresión (SLP) fue 14,5 meses.

Trabajo 2

Un 50% de los pacientes tenían plasmocitomas (18% localización extramedular). A una media de 23,1 meses, la tasa de RG fue del 95%. El 90% de los pacientes presentaron SLC (5% grados>3) y 2 pacientes neurotoxicidad grado 1. La mediana de SLP fue de 15,8 meses. La expresión de BCMA no predijo la respuesta, pero el BCMA soluble se correlacionó con peor pronóstico y gravedad del SLC. El marcador de activación HLA-DR en la aféresis se asoció con mayor SLP y se observó un aumento de receptores de punto de control inmune.

Trabajo 3

Agregar un anticuerpo bloqueante de TIGIT a ARI0002h mejoró su eficacia *in vitro*, pero no aumentó la supervivencia *in vivo*. El CAR-T de cuarta generación, ARITIGIT, tampoco mejoró la supervivencia. En un modelo de recaída, ARITIGIT prolongó la supervivencia sin alcanzar significación estadística. La eliminación de TIGIT en ARI0002h (TIGIT KO-ARI0002h) utilizando CRISPR/Cas9 no mostró diferencias *in vitro*. En un modelo *in vivo* de estrés donde ARI0002h no mejoró la supervivencia, TIGIT KO-ARI0002h la mejoró, pero sin ser significativa comparado a ARI0002h.

CONCLUSIONES

1. ARI0002h ha demostrado eficacia y seguridad en pacientes con MM R/R incluidos en el EC CARTBCMA-HCB-01.
2. La fabricación de ARI0002h en dos centros académicos fue factible, mostrando el éxito del modelo “point-of-care” de fabricación para aumentar el acceso a esta terapia.
3. La administración fraccionada de CAR-T puede contribuir a una reducción en la incidencia de eventos adversos inmunes graves y neurotoxicidad.
4. La administración de una dosis de refuerzo de ARI0002h en pacientes con respuesta inicial fue segura y podría mejorar la eficacia al tratamiento.
5. Los pacientes con MM y plasmocitomas extramedulares pueden beneficiarse de ARI0002h; sin embargo, su SLP puede ser inferior a pacientes sin afectación de partes blandas o con plasmocitomas paraesqueléticos.
6. Una carga tumoral elevada, medida por proteína monoclonal sérica y BCMA soluble, se correlacionó con peores resultados, mientras que una mayor expresión de HLA-DR en la aféresis predijo una mayor SLP.
7. La pérdida del antígeno diana es un mecanismo infrecuente de resistencia a ARI0002h, mientras que el agotamiento de células CAR-T, indicado por aumento de expresión de TIGIT y PD-1, puede influir en la recaída tras tratamiento.
8. Bloquear TIGIT en ARI0002h con distintas estrategias ha mostrado mejoras limitadas en un modelo murino.

INTRODUCTION

Multiple myeloma

1. Definition and diagnosis

Multiple myeloma (MM) is a clonal plasma cell dyscrasia that accounts for 1-1.8% of all cancers and 10% of all hematologic malignancies, being the second most common(1). The estimated incidence in Europe is 4.5-6.0 cases/100.000/year(2).

The diagnosis of MM requires the presence of ≥ 10% bone marrow plasma cells or biopsy-proven paraskeletal or extramedullary plasmacytomas plus evidence of end-organ damage attributable to the disease, commonly referred as CRAB symptoms: hypercalcemia, renal failure, anemia and osteolytic bone lesions. In 2014, the International Myeloma Working Group (IMWG) revised the diagnostic criteria, allowing the use of specific biomarkers to also define the disease, which included the presence of ≥ 60% bone marrow plasma cells, an involved/ uninvolved serum free light chain (FLC) ratio ≥ 100 (the involved FLC level must be ≥ 100mg/L) or the presence of more than one focal lesion on magnetic resonance imaging (MRI), of at least 5mm in size(3,4).

2. Prognosis and response

Response to treatment and prognosis are influenced by various factors. In 1975, the Durie-Salmon staging system was introduced and consisted of commonly available clinical parameters which predicted myeloma tumor burden, and it was widely adopted as the standard for prognostication in MM(5). This system included observer-dependent variables such as the number of lytic lesions on the skeletal survey. An effort to ensure a more objective pretreatment classification of patients led to the emergence of the currently used International Staging System (ISS), based on serum β_2-microglobulin and albumin, and the revised-ISS (R-ISS), which combines the previous parameters with the presence of chromosomal abnormalities detected by fluorescent in situ hybridation (del(17p) and/or t(4;14) and/or t(14:16)), associated with adverse prognosis, and serum lactate dehydrogenase (6,7). Also, the presence of extramedullary

plasmacytomas in the context of MM is correlated to a more aggressive disease behavior and poorer prognosis(8).

Besides baseline features, the depth and duration of response after initial treatment are strong predictors of long-term outcomes. Response is assessed according to the IMWG criteria(9). Measurable residual disease (MRD) evaluation using sensitive techniques such as next-generation sequencing (NGS) or flow cytometry (NGF) provides valuable prognostic information, since MRD negativity is associated with improved progression-free survival (PFS) and overall survival (OS)(10,11). Moreover, MRD has been recently accepted by the Food and Drug Administration (FDA) Oncologic Drugs Advisory Committee as a viable accelerated approval end point in myeloma trials.

Multiple myeloma remains an incurable disease. Despite significant improvement in patients' survival over the past two decades, only 10%-15% of patients with MM achieve or surpass expected survival compared to the general population(2). Consequently, the exploration of novel therapeutic approaches becomes of utmost importance.

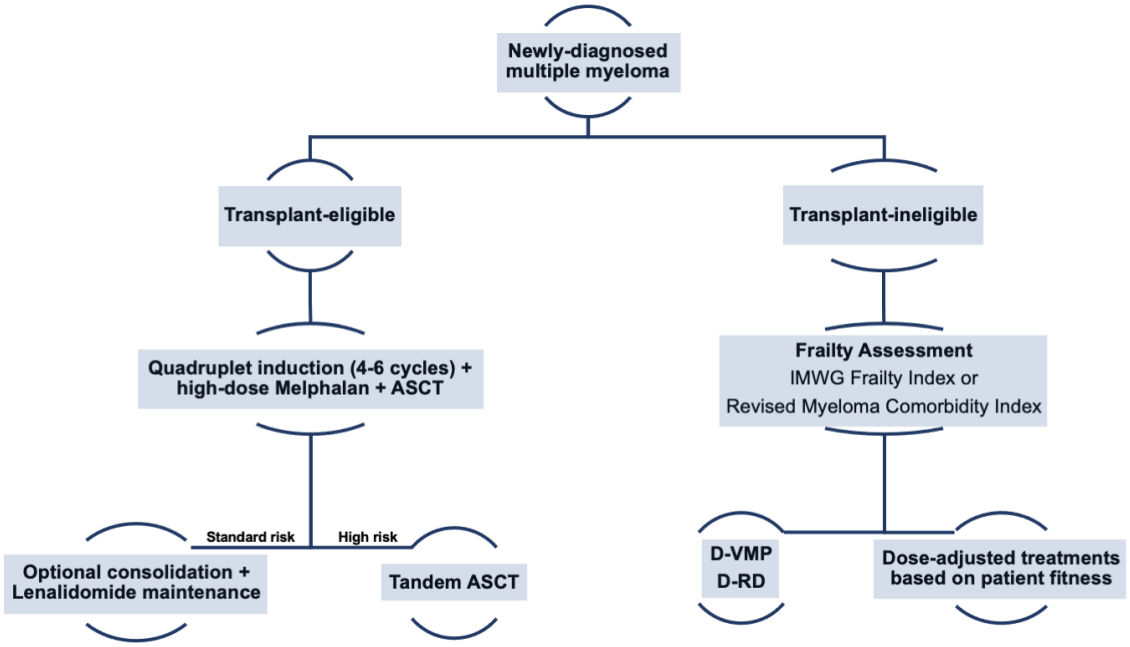
3. Treatment overview

Treatment for MM has evolved significantly from the first documented case in 1844(1). After the initial use of chemotherapy combinations containing melphalan and corticosteroids, high dose melphalan in the form of an autologous stem cell transplantation (ASCT) became the standard of care in the first line(12,13). Increased understanding of the microenvironmental interactions between malignant plasma cells and the bone marrow niche, led to the development of novel agents such as immunomodulating drugs (IMID) (thalidomide, lenalidomide, pomalidomide)(14–16), proteasome inhibitors (PI) (bortezomib, carfilzomib, ixazomib)(17–20) and monoclonal antibodies targeting CD38 (daratumumab, isatuximab)(21–24) or SLAMF7 (elotuzumab)(25,26), which have represented a major advance in MM treatment.

3.1. Newly diagnosed

Treatment for newly diagnosed MM depends on patients' eligibility for ASCT according to age and co-morbidities (Figure 1).

Figure 1. Treatment algorithm in newly diagnosed multiple myeloma patients(27,28)**.**



3.1.1. Transplant-eligible patients

In transplant eligible patients, until recent years, an induction therapy with the triplet bortezomib, thalidomide/lenalidomide and dexamethasone (VTD/VRD) was the preferred option(2,27). The addition of daratumumab to VTD as induction and consolidation in the randomized CASSIOPEIA trail significantly improved overall response rates (ORR) and MRD negativity rates, which translated into a prolonged PFS(29). Based on these results, daratumumab-VTD (D-VTD) induction regimen was approved by the FDA and European Medicines Agency (EMA) and a quadruplet induction, followed by high-dose melphalan and ASCT, would be the preferred option nowadays. Only D-VTD is approved to date, but there is evidence that the replacement of thalidomide by lenalidomide, and even bortezomib for carfilzomib may improve response depth(30–32).

After ASCT, consolidation treatment would be optional, while lenalidomide maintenance is strongly recommended. The EMN02/HOVON-95 study randomized patients to receive bortezomib, lenalidomide and dexamethasone (VRD) consolidation or not, followed by lenalidomide maintenance. Although median PFS was longer in the VRD consolidation group (58.9 vs. 45.5 months), this was not translated into a superior OS(33). In the BMT CTN 0702 STAMINA study, patients were randomized to receive either no consolidation, 4 cycles of VRD, or a second ASCT, followed by lenalidomide maintenance. Both PFS and OS were comparable between groups(34). Interestingly, a benefit towards consolidation was observed in high-risk cytogenetic patients in both studies, for which tandem ASCT is recommended.

Lenalidomide maintenance has been extensively investigated in randomized trials(35–37). A meta-analysis including 1208 patients showed a PFS benefit of 2 years (52.8 vs. 23.5 months) and 2.5 years of OS benefit with lenalidomide compared to placebo/observation(38). Optimal duration of lenalidomide maintenance is a topic of debate. After achievement of sustained disease control, continuous maintenance therapy may have drawbacks in terms of adverse events and quality of life. Results from two studies with identical schemes except for lenalidomide maintenance, which was administered for 1 year in one study and continuous in the other study, showed a 20.2-month longer PFS in favor of the continuous therapy(39,40). A retrospective post-hoc analysis of the Myeloma XI phase 3 trial suggested that maintenance beyond 3 years is associated with improved PFS, especially in patients with positive MRD prior to maintenance start. Despite that, at a certain time between 4 to 5 years after ASCT, maintenance may no longer offer a benefit compared to observation. Interestingly, in MRD-negative patients, maintenance continuation beyond 3 years seemed to be of limited value(41). Another study evaluating the role of ixazomib added to lenalidomide plus dexamethasone as a maintenance strategy, showed that lenalidomide discontinuation could be evaluated in MRD-negative patients after 2 years (42), which may be a safe approach while awaiting results of randomized trials in MRD-negative patients. Importantly, technical issues regarding MRD measurements, such as false-negative results in the context of

patchy disease or bone marrow hemodilution need to be considered when using MRD as a tool for discontinuation(43).

3.1.2. Transplant-ineligible patients

For transplant-ineligible patients, bortezomib, melphalan plus prednisone (VMP), and lenalidomide plus dexamethasone (RD) were the standards of care(2,28). A randomized phase 3 trial comparing VRD to RD showed the superiority of VRD in terms of PFS (41 vs. 29 months; p=0.003) and OS (not reached vs. 69 months; p=0.011), although 43% of the patients included in this study were younger than 65 years of age(44). Based on this results, EMA approved VRD for newly diagnosed patients not eligible for ASCT. The ENDURANCE trial failed to proof superiority of carfilzomib, lenalidomide and dexamethasone (KRD) vs. VRD in this subset of patients(45). On the other hand, the ALCYONE(46) and MAIA(47,48) phase 3 trials analyzed the addition of daratumumab to the standard regimens VMP and RD, respectively, and a benefit in the daratumumab groups was observed in PFS and OS, becoming these regimens the new standard of care.

Since MM has a growing incidence with age, approximately one third of patients are frail at diagnosis(2). The IMWG Frailty Index (IMWG-FI) based on age, instrumental activities of daily living and co-morbidities, identifies intermediate and frail patients with inferior PFS and OS due to an increased incidence of toxicity and early discontinuation(49). The Revised Myeloma Comorbidity Index (R-MCI) evaluates age, organ function, frailty, performance status and cytogenetics, and was validated in a cohort of 801 patients(50). Despite growing efforts to define frailty, these patients are underrepresented in studies and there is a lack of frailty-adapted clinical trials to address treatment choice. Most recommendations agree to dose-adjusted treatments based on patient fitness, according to expert-opinion guidelines(51–53).

3.2. Relapsed or refractory disease

3.2.1. Treatment after first relapse

Despite treatment advances, MM remains an incurable disease and initial remissions will be followed by relapses requiring therapy. Resistance to major drug classes used in the first line remains the most critical factor for the choice of the subsequent treatment. Patient-related characteristics should also be considered(2,54,55). Due to the recent introduction of different treatment combinations and modalities such as consolidation and maintenance to the first line, an individual evaluation should guide second-line treatment in each patient. For lenalidomide-refractory patients, although class switch is important, pomalidomide may be active. A longer duration of lenalidomide therapy before progression and a longer IMID-free period are associated with better outcomes when receiving pomalidomide(56,57). The currently approved regimens for lenalidomide-resistant patients based on phase 3 clinical trial data are monoclonal antibody-based or PI-based and are summarized in Table 1 (16,18,21,23,24,26,58–60).

Patients who are refractory to both lenalidomide and bortezomib due to an early progression to initial therapy are particularly challenging and have fewer options at relapse. Monoclonal antibody regimens combined with pomalidomide or carfilzomib could be useful(61,62). For patients who are not refractory to lenalidomide, a second line may include a RD-based regimen (19,20,22,25). In this group, patients who received a fixed duration of a bortezomib-based treatment that ended at least 6 months before relapse, could benefit from a PI-regimen as second line(18,21,24,59).

Daratumumab-refractory patients will become an increasing issue due to the recent approval of this drug in the first line for both ASCT eligible and ineligible patients(29,46,47). There is limited data on the outcomes of patients relapsing after first-line daratumumab-based therapy. For patients not exposed to lenalidomide, such as those treated with D-VTD or D-VMP, a combination of lenalidomide with a PI, or a different class of monoclonal antibody, should be

considered(19,20,25). For lenalidomide-refractory patients, in addition to daratumumab refractoriness, PI combinations such as carfilzomib plus dexamethasone (KD) or pomalidomide, bortezomib and dexamethasone (PVD) are reasonable options. To date, CD38-antibody resistance mechanisms are not fully understood. Therefore, the reversibility of this resistance and the plausibility of CD38-antibody retreatment are yet to be elucidated(63,64). Salvage ASCT could be considered in patients who deferred the procedure at the time of first remission and those with a progression-free interval after front-line ASCT above the median expected PFS, which would be 3 to 4 years in most studies(38,65).

Table 1. Approved regimens for lenalidomide-resistant patients based on phase 3 clinical trial results(55).

	Regimen	Median prior lines	Len-ref (%)	mPFS, months (all)	mPFS, months (Len-ref)	Approval
CASTOR	Dara-Vd	2 (1-10)	60 (24)	16.7	7.8	YES (1 prior line)
	Vd		81 (33)	7.1	4.9	
CANDOR	Dara-Kd56	2 (1-3)	99 (32)	NE	NE	YES (FDA: 1-3; EMA: 1 prior line)
	Kd56		55 (36)	15.8	11.1	
IKEMA	Isa-Kd56	2 (1-3)	57 (32)	NE	NE	YES (FDA:1-3; EMA:1 prior line)
	Kd56		42 (34)	19.15	15.7	
APOLLO	Dara-Pd	2 (1-5)	120 (79)	12.4	9.9	YES
	Pd		122 (80)	6.9	6.5	
ICARIA	Isa-Pd	3 (2-11)	144 (94)	11.5	11.4	YES (2 prior lines)
	Pd		140 (92)	6.5	5.59	
ELOQUENT-3	Elo-Pd	3 (2-8)	59 (98)	10.3	NR	YES (2 prior lines)
	Pd		55 (96)	4.7	NR	
ENDEAVOR	Kd	2 (1-3)	113 (24)	18.7	8.6	YES (1 prior line)
	Vd		122 (26)	9.4	6.6	
OPTIMISMM	VPd	2 (1-3)	200 (71)	11.2	NR	YES (EMA: 1 prior line)
	Pd		191 (69)	7.1	NR	
BOSTON	Sel-Vd	1 (1-3)	77 (39)	13.93	9.59	YES (FDA: 1prior line)
	Vd		77 (37)	9.46	7.23	

Abbreviations: *Dara: daratumumab; d: dexamethasone; Elo: elotuzumab; Isa: isatuximab: K: carfilzomib; Len-ref: lenalidomide refractory; mPFS: median progression-free survival; P: pomalidomide; Sel: selinexor; V: bortezomib.*

3.2.2. Treatment after two or more prior lines

Treatment of relapsed patients after 2 prior lines is challenging, since most patients have often received all major drug classes. Prior exposure and refractoriness to IMID, PI and CD38 antibodies will determine drug selection at this point. The prospective LocoMMotion study analyzed the real-life standard of care (SOC) outcomes in triple-class exposed R/R MM patients (prior exposure to at least a PI, IMID and anti-CD38 antibody), with ≥3 prior lines or double refractoriness to a PI and IMID. In this heavily pretreated group, ORR was 29.8%, median PFS and OS were 4.6 and 12.4 months and, interestingly, up to 92 different regimens were administered to these patients, reflecting a lack of clear standard of care in patients with triple-class exposed MM in the real-world practice(66).

Selinexor (an oral selective inhibitor of a nuclear export small molecule that inhibits exportin 1 function) and belantamab mafadotin (a humanized anti-B cell maturation antigen (BCMA) IgG1 monoclonal antibody conjugated to the microtubule disrupting agent monomethyl auristatin F (MMAF)) could be considered in the relapsed/refractory (R/R) setting, since their mechanisms of action differ from those of the traditional drug families and have shown activity in monotherapy. Still, the optimal timing of use and treatment combinations of both agents in the context of additional immunotherapeutics entering the MM landscape requires further study(67–70). A new generation of IMID (or cereblon modulators), such as iberdomide or mezigdomide, have shown activity in heavily pretreated patients with manageable toxicity and are being explored in combinations with several other agents (PIs, anti-CD38 monoclonal antibodies)(71,72). However, their regulatory approval requires substantially more data.

Immune system and multiple myeloma

The ability of malignant plasma cells to evade and suppress immune surveillance is a hallmark of cancer. Normal plasma cells grow within a supportive niche in the bone marrow, surrounded by stromal and immune cells. Malignant plasma cells

tend to retain these features and depend on the interactions with the microenvironment to survive. The capacity of these cells to grow outside the bone marrow niche is associated with the development of extramedullary plasmacytomas and plasma cell leukemia, which indicate a more aggressive disease(73).

Myeloma cells are also surrounded by T and NK cells, which should exert a cytotoxic effect on tumor cells. This immune control is evaded via a complex interplay between myeloma and immune cells, that translates into an exhaustion state of lymphocytes. Exhaustion is defined as the progressive loss of functionalities and a reduced proliferative capability of lymphocytes(74). Mechanisms of exhaustion involve the upregulation of immune checkpoint receptors (programmed death-1 (PD1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), lymphocyte activation gene 3 (LAG3), T cell immunoglobulin and mucin domain 3 (TIM3)) on lymphocytes, and their ligands on tumor cells, as well as a dysregulation of stromal cells. Strategies to reverse the resultant immune tolerance and restore antimyeloma immunity are a major research focus(73).

Immunotherapy refers to the use of strategies that enhance the immune response to prevent or treat a disease. The first immunotherapeutic approach in MM was the use of allogeneic stem cell transplantation and its graft-versus-myeloma effect(75,76). Over the past decades, immunotherapy strategies for MM have continued to evolve, with the inclusion of IMIDs and CD38-directed antibodies in the myeloma therapy repertoire.

The use of immune checkpoint inhibitors (ICI), particularly PD1-blocking agents, is widely spread in solid tumors and lymphoid malignancies other than MM. Despite strong preclinical evidence of a potential benefit of blocking the PD-1 pathway in MM(77,78), single-agent results from the clinical trials were disappointing(79). In contrast, significant efficacy was observed in a phase 1 trial that combined pembrolizumab and lenalidomide in R/R patients(80). However, two phase 3 clinical trials combining pembrolizumab with either lenalidomide (KEYNOTE-185) or pomalidomide (KEYNOTE-183) were halted due to

unexpected safety findings(81–83). TIGIT, an inhibitory receptor expressed on T-cells and natural killer (NK)-cells, has emerged as an immunotherapy target in cancer, including MM. TIGIT interacts with its main ligand, poliovirus receptor (PVR), and nectin-2 (N2), located on antigen-presenting cells and tumor cells. The TIGIT/CD226 pathway is constituted by different inhibitory (TIGIT, CD96, and CD112R) and activating (CD226) receptors that bind to their ligands (PVR, N2 and nectin-3) with different affinities(84,85); TIGIT binds PVR with the highest affinity, which is lower for N2 and nectin-3. While nectin-3 is restricted to non-hematopoietic tissues, PVR and N2 are expressed in hematopoietic cells and over-expressed in many human malignancies(86,87), including MM(88). Preclinical studies have suggested that TIGIT blockade successfully reinvigorates T-cell function against MM(89,90). The ClinicalTrials.gov database reports 65 trials using an anti-TIGIT Ab to treat solid and hematologic malignancies(91), with three active trials for MM.

More recently, cellular immunotherapies such as chimeric antigen receptor T cells (CAR-T) and bispecific T cell engagers (TCE) have dramatically improved outcomes of MM patients.

Cellular immunotherapies for hematological malignancies

1. Chimeric antigen receptor T cells

1.1. Definition and history of CAR T cell development

CAR-T cell therapy involves genetically modifying T cells to express a synthetic protein called chimeric antigen receptor (CAR) on their surface. The CAR protein enables specific targeting of antigens present on the surface of tumor cells, which provides a highly specific approach for cancer treatment(92).

The concept of CAR-T cell therapy was first proposed in 1898 by Dr. Zelig Eshhar who demonstrated the feasibility of redirecting T cells to a specific antigen *in vitro*(93). Initial preclinical and clinical studies performed with first-generation CAR-T, failed to prove a significant benefit. Efforts to optimize their design led to the realization that coupling CD3ζ to a co-stimulatory domain, the so-called

second-generation CAR-T, was crucial to sustain proliferation and cytotoxic responses(94). In 2010 and 2013, the first case reports of a chronic lymphocytic leukemia (CLL) patient(95) and two pediatric patients with B-cell acute lymphoblastic leukemia (B-ALL)(96) treated with CD19-CART with remarkable success were published. From that moment, growing research in the field led to the regulatory approvals of the first two CAR-T therapies in 2017, tisagenlecleucel (tisa-cel) and axicabtagene ciloleucel (axi-cel) for pediatric B-ALL and non-Hodgkin lymphoma (NHL)(97–99). Currently, CAR-T design is in continuous improvement, new target antigens have emerged, the manufacturing process has been optimized and indications have broadened to other hematological diseases(100,101).

1.2. CAR-T structure

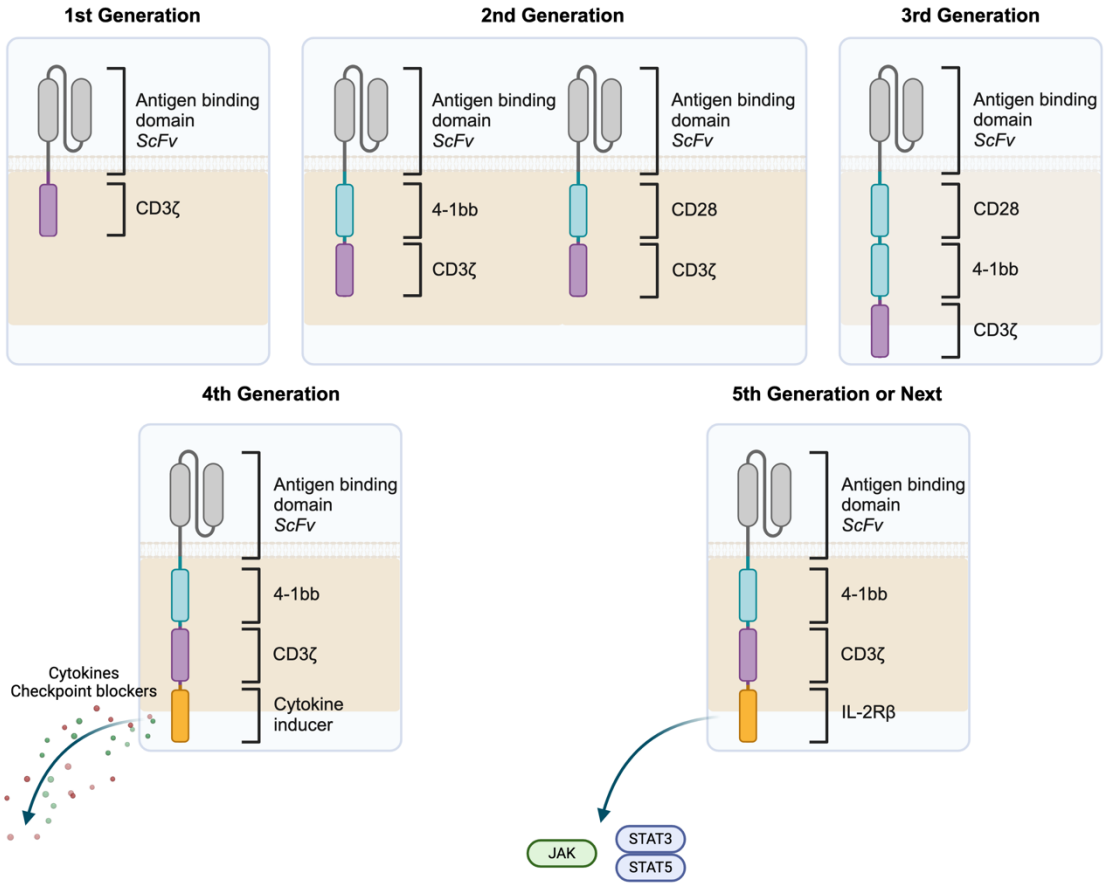
The CAR protein is composed of four parts(102,103):

- **Extracellular antigen-binding domain:** derived from a single-chain variable fragment (scFv) of an antibody, which is responsible for antigen recognition on the target cell. The antibody of origin may be from a species other than human (e.g., murine, camelid).
- **Hinge:** extracellular structural region that extends the binding units from the transmembrane domain. It provides flexibility and contributes to the length that allows for the interaction between the antigen-binding domain and the targeted epitope.
- **Transmembrane domain:** responsible for anchoring the CAR to the T cell membrane.
- **Intracellular signaling domain:** it is constituted by components of the T cell receptor (TCR) signaling pathway such as CD3ζ, which can be coupled to one or more co-stimulatory domains (e.g. CD28, 4-1BB). It provides activation and proliferation signals to the T cell upon antigen recognition.

The structure of the CAR has evolved over the years to improve its function, which led to different generations of CARs (Figure 2)(104). First generation CARs

consist of a single signaling domain, usually the CD3ζ chain, lacking co-stimulatory signals. Second-generation CARs incorporate an additional co-stimulatory domain such as CD28 or 4-1BB coupled to the CD3ζ chain. Third generation CARs incorporate two additional co-stimulatory domains. Fourth generation CARs or armored CARs are second generation CARs that incorporate genetic modifications beyond co-stimulatory signals, which may include genes encoding cytokine secretion or immune checkpoint inhibitors to improve function and counteract tumor-induced immunosuppression. The fifth, or 'next generation' of CARs, also based on second generation CARs, contain a truncated cytoplasmic interleukin (IL) 2 receptor β-chain domain with a binding site for the transcription factor STAT3. The antigen-specific activation of this receptor simultaneously triggers TCR (through the CD3ζ domains), co-stimulatory, and cytokine (JAK–STAT3/5) signaling, which effectively provides all three synergistic signals required physiologically to drive full T cell activation and proliferation.

Figure 2. Generations of CAR-T cells(104). Created with BioRender.com.

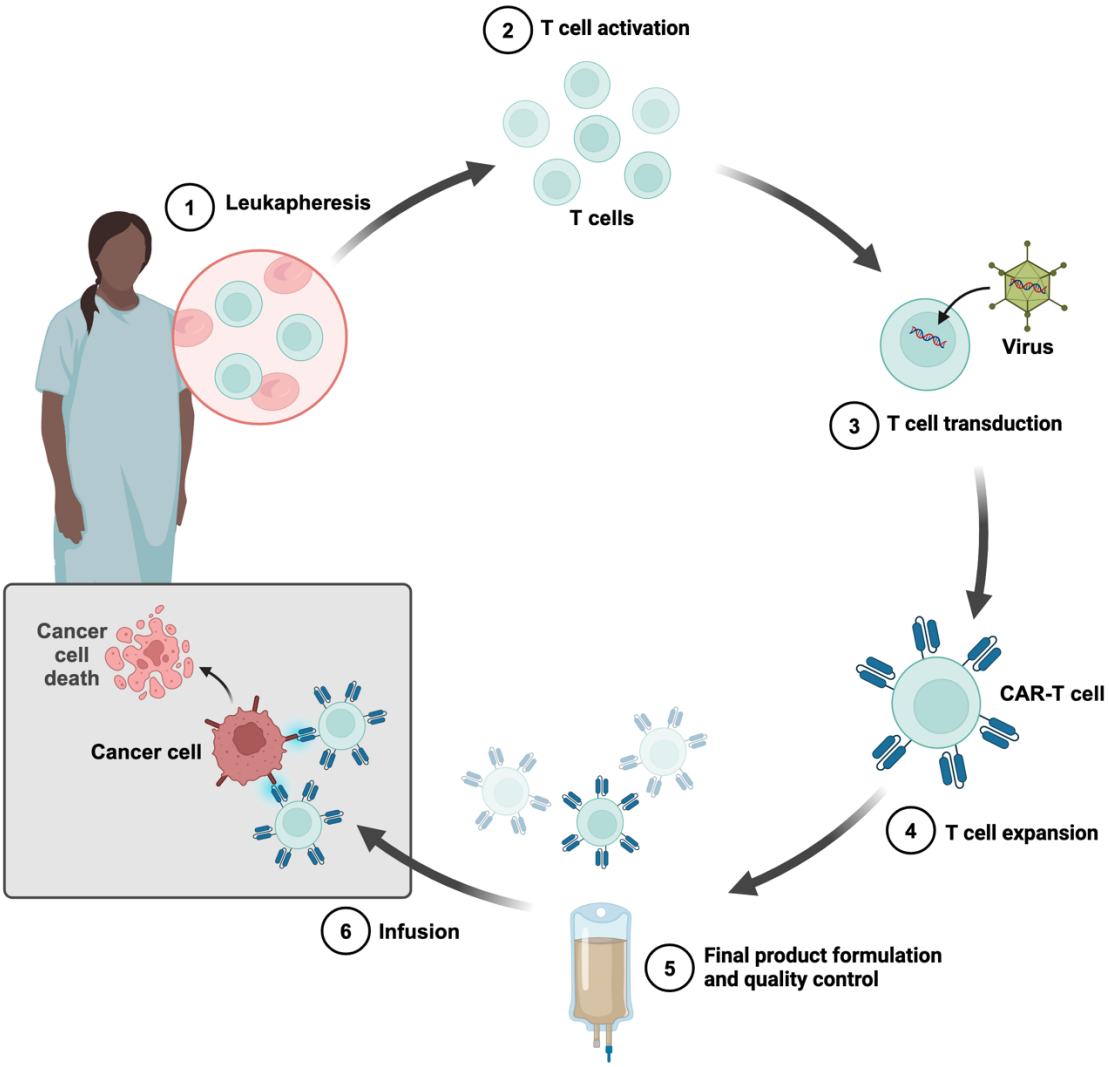


CAR-T cells for cancer are designed to recognize and bind to a specific surface antigen expressed on tumor cells. Adequate antigen selection is critical for therapy success through its role in determining specificity, selectivity, efficacy and safety. Ideally, the targeted antigen should be invariably and uniformly expressed on the surface of the tumor cells of interest, while absent in healthy tissues, to minimize on-target off-tumor effects. Antigens essential for tumor cell growth and survival will be more likely consistently expressed(105).

1.3. Manufacturing and administration

Human-scale manufacturing of CAR-T cells starts with the obtention of T-cells by leukapheresis from an autologous (patient) or allogeneic (healthy donor) source. The collected T cells are activated using a combination of co-stimulatory molecules (e.g., CD3/CD28 beads) and cytokines. Activation promotes T cell proliferation and enhances the cell susceptibility to genetic alterations. At this point, T cells are genetically modified to express the CAR. Several methodologies are available, one of which is transduction with viral vectors (e.g., lentivirus, gamma-retrovirus), the most extended option. Other options for genome editing include transposon systems, RNA transfection, and CRISPR/Cas9. Following genetic modification, T cells are cultured with appropriate growth factors and cytokines to promote their expansion, a process that occurs over several days(106). Once the desired CAR-T cell number has been reached, quality controls are performed, and cells are harvested and formulated into a final product (FP) suitable for patient infusion, which may include a cryopreservation step. Rigorous quality control criteria regarding purity, cell viability, sterility, absence of replication-competent lentivirus, if applicable, and potency, must be met to successfully release the product for patient administration (Figure 3). Importantly, the CAR-T manufacturing procedure is a highly specialized process performed in compliance with good manufacturing practice (GMP) conditions(102).

Figure 3. Vein-to vein process including leukapheresis, manufacturing and infusion(106). Created with BioRender.com.



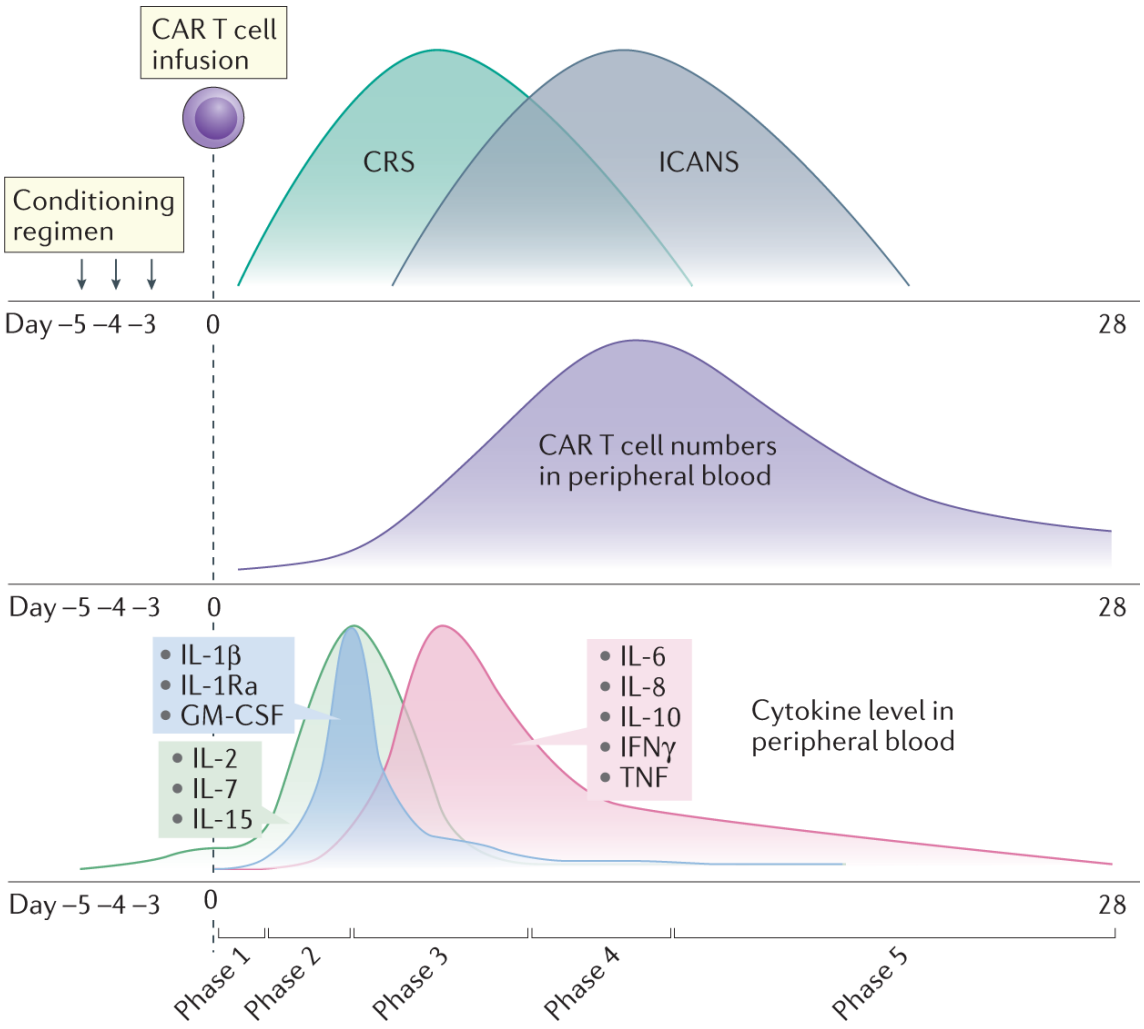
When product release is confirmed and the patient is ready to receive the CAR-T, the product is thawed (only when previously cryopreserved) and infused, generally following a lymphodepleting chemotherapy (LD) that creates a favorable immune environment for the CAR-T to engraft. The cytotoxic function that infused CAR-T cells exert on cells expressing the target antigen generates a systemic immune response for which patients are closely monitored to detect and treat potential side effects.

1.4. Treatment-related adverse events

The main immune adverse events related to CAR-T therapy are cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome

(ICANS). Both are potentially life-threatening systemic inflammatory responses triggered by the *in vivo* activation and proliferation of CAR-T and the subsequent increased levels of cytokines such as IL-1, IL-6, and interferon γ (IFNγ).

Figure 4. Pathophysiology of CRS and ICANS(107)**.** A relative timeline for the onset and duration of CRS and ICANS is depicted.



The pathophysiology of CAR-T immune-related side effects can be divided into five phases (Figure 4): (1) Trafficking of CAR-T cells to the tumor site and antigen recognition. (2) CAR-T proliferation and cytokine production by CAR-T cells, which will activate other immune cells of the tumor microenvironment (TME), such as macrophages, leading to the onset of CRS. (3) Increase in cytokine levels and expansion of CAR-T in the peripheral blood, starting the systemic inflammatory response, which can cause endothelial injury and vascular leakage of multiple

tissues. Associated effects include hypoxia, hypotension, and organ damage. (4) The breakdown of the blood-brain barrier can favor the diffusion of cytokines and migration of CAR-T cells into the cerebrospinal fluid (CSF), coinciding with the onset of ICANS. (5) Activation-induced cell death of T cells following eradication of tumor cells results in a reduction of the systemic immune response, with the consequent disappearance of CRS and/or ICANS symptoms and a potential persistence of long-term memory CAR-T cells(107).

CRS is characterized by the onset of fever and may associate hypotension and end-organ damage. ICANS has a later onset and is often associated to CRS. Manifestations include confusion, delirium, aphasia, tremor, and seizures, although any neurologic symptom can be present. The onset may be vague and gradual, therefore, close monitoring of the patient vital signs, and the use of neurologic evaluation scales (e.g., Immune effector cell encephalopathy (ICE) score) is important to allow for early detection(108).

Management of CRS should be provided, according to the grading system proposed by the American Society for Transplantation and Cellular Therapy (ASTCT) consensus, with antipyretics, supportive care, tocilizumab (an IL-6 receptor antagonist), and corticosteroids. Concurrent causes of fever, such as infections, should be ruled out, and empiric antibiotics should be administrated. For ICANS, brain MRI and CSF evaluation are rarely helpful but can be used to rule out alternative diagnoses. Prompt treatment with corticosteroids and supportive measures should be started. Whereas tocilizumab is very effective for the management of CRS, its utility in ICANS is controversial due to a potential increase in serum levels of IL-6, due to the receptor blockade that the drug exerts on peripheral tissues(108–113).

Initially, the use of tocilizumab and corticosteroids was reserved for the treatment of severe CRS/ICANS. To prevent the onset of more severe manifestations, early treatment, or even prophylactic strategies using tocilizumab and/or steroids, have been proposed and are under evaluation. Still, caution remains because of the concern that these interventions could affect long-term CAR-T efficacy. In some studies, the use of prophylactic tocilizumab in CD19-CART treated NHL

decreased the incidence of severe CRS, but its effect in the occurrence of ICANS is disputed. Prophylactic levetiracetam and corticosteroids in patients with lymphoma treated with axi-cel in the ZUMA-1 study cohort 6 revealed a potential benefit in decreasing high-grade CRS/ICANS(114). Also, the use of levetiracetam prophylaxis and early corticosteroids and tocilizumab in ZUMA-1 cohort 4, showed similar results, although differences in the main characteristics in respect of cohorts 1 and 2, including tumor burden, had to be balanced by propensity score matching(115). Overall, a trend in clinical practice moves towards early intervention in the lower grades of CRS/ICANS. Even though the incorporation of prophylactic corticosteroids seems promising, further studies are needed before its systematical use in CRS/ICANS management.

In refractory patients, retrospective studies have suggested that anakinra could be beneficial, but results of prospective clinical trials for both treatment and prophylaxis are pending(116). Siltuximab (IL-6 antagonist) and emapalumab (IFNγ-blocking antibody) have also been used, with limited data available. Anti-CAR-T-directed strategies are the last resort in severe cases. These interventions may include chemotherapy, tyrosine kinase inhibitors, or T-cell directed strategies (e.g., antithymocyte globulin, alemtuzumab). Experience is limited and the risk of infections may be particularly high(117,118).

Other side effects of CAR-T therapy include tumor lysis syndrome, immune effector cell-associated hematotoxicity (ICAHT) and infections. Tumor lysis syndrome is caused by rapid tumor cell death, which leads to the release of intracellular components to the bloodstream, causing dyselectrolytemia and urate deposition in the kidneys, potentially causing renal dysfunction. Prophylactic measures, including hydration and pharmacologic agents to decrease uric acid levels, may be used according to risk stratification(119).

ICAHT is the most frequent adverse event after CAR-T therapy. Cytopenias can be profound and long-lasting, and add to the immunosuppression already conferred by B-cell aplasia and the consequent hypogammaglobulinemia, developed as an undesired on-target off-tumor effect of CD19- and BCMA-directed CAR-T. In a joint effort between the European Society for Blood and

Marrow Transplantation (EBMT) and the European Hematology Association (EHA), a consensus on grading and management of ICAHT was established (120). The ICAHT grading system was developed based on neutropenia, since the main concern relates to profound and prolonged neutropenia, and isolated anemia or thrombocytopenia are rare. Risk stratification can be performed using a combination of the HEMATOTOX score(121,122), measured before lymphodepletion chemotherapy, and the ICAHT grading system. Prophylactic granulocyte colony-stimulating factor (G-CSF) can be considered in high-risk patients from day +2 after infusion. Besides general management with red blood cell (RBC) and platelet transfusion in the early period, management of established cytopenias includes G-CSF treatment, thrombopoietin (TPO) agonists, autologous or allogeneic stem cell boost, if available, and allogeneic stem cell transplantation as a last option(123).

Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory condition resulting from an abnormal immune activation, particularly of macrophages, that presents with fever, hyperferritinemia, cytopenias and, eventually, multiorgan failure. In the setting of CAR-T cell therapy, up to 3.4% of patients develop a condition with overlapping clinical features and shared underlying pathophysiology termed by the ASTCT consensus group as immune effector cell-associated hemophagocytic lymphohistiocytosis-like syndrome (IEC-HS), which characteristically manifests after traditional CRS has resolved(124,125). IEC-HS is defined as the development of a pathological and biochemical hyperinflammatory syndrome independent from CRS and ICANS that manifests with features of macrophage activation/HLH, but is attributable to immune effector cell therapy, and is associated with a progression or new onset (beyond CRS/ICANS) of cytopenias, hyperferritinemia, coagulopathy with hypofibrinogenemia, and/or transaminitis. The underlying biology is not fully understood, although some features, such as baseline inflammation, tumor burden and CAR T cell construct, can influence the development of IEC-HS. Although IEC-HS can be fatal and life-threatening, there are some patients with clear evidence of hyperinflammatory features who remain clinically well and in whom resolution occurs without intervention. Due to a lack of prospective studies and limited literature regarding HLH-like manifestations following CAR-T, the

Introduction

ASTCT consensus in grading and management of IEC-HS is based on opinion-expert recommendations and relies on available evidence from small CRS/IEC-HS cohorts and treatment of classical HLH(126). In asymptomatic patients, or those with mild symptoms, intervention is not indicated. For patients who require treatment, the consecutive use of anakinra, corticosteroids and ruxolitinib is recommended. For refractory cases, etoposide and emapalumab could be considered.

CAR-T recipients are at increased risk of infections related to lymphodepletion chemotherapy, immunosuppression and ICAHT, added to the underlying disease-related predisposition. Although the highest risk appears early after CAR-T infusion, infections can occur weeks to months after treatment, especially in those patients with prolonged cytopenias. The cause can be bacterial, viral or fungal, and particular attention should be paid to opportunistic infections. Respiratory viruses (e.g., influenza, respiratory syncytial virus) and cytomegalovirus (CMV) are significant concerns, as well as invasive candidiasis or aspergillosis, particularly in patients with prolonged neutropenia and disruption of the mucosal barrier. Most early infections (before day +30) are bacterial or respiratory viruses, while beyond day +30 viral infections predominate(127,128). Current EHA/EBMT guidelines recommend the use of prophylaxis until immune reconstitution (Table 2). If hypogammaglobulinemia is present (serum IgG levels below 4 g/L), intravenous (IV) immunoglobulin (Ig) is recommended in pediatric patients and is to be considered in adults, especially if serious or recurrent infections(110,129).

Table 2. Prophylaxis recommendations in CAR-T recipients(110)**.**

Prophylaxis	EBMT/EHA recommendation	Comments
Antibacterial	Not routinely recommended.	Can be considered in case of prolonged neutropenia and should be based on local guidelines, e.g., with levofloxacin or ciprofloxacin.
Anti-viral	Valacyclovir 500 mg bid or acyclovir 800 mg bid.	Start from LD chemotherapy until 1-year post-CAR-T cell infusion and until CD4+ count >0.2 x 10^9/L.
Anti-pneumocystis	Co-trimoxazole 480 mg once daily or 960 mg three times each week. Start from LD chemotherapy until 1-year post-	Can be started later depending on center guidelines. In case of co-trimoxazole allergy (or cytopenias precluding use of co-trimoxazole), pentamidine inhalation (300 mg

	CAR-T cell infusion and until CD4+ count >0.2 x 10^9/L. If prolonged myelosuppression, postpone start after ANC >0.5 x 10^9/L.	once every month), dapsone 100 mg daily or atovaquone 1500 mg once daily can be considered.
Anti-fungal	Not recommended routinely; consider posaconazole (300 mg/ day) or fluconazole (200 mg/day) or micafungin (50 mg IV/day) in patients with severe (ANC <0.5 x 10^9 /l) or prolonged (>14 days) neutropenia and/or in patients on long-term or high-dose (>72 h) corticosteroids or in patients post-alloSCT.	In patients with prior alloSCT, prior invasive aspergillosis and those receiving corticosteroids, posaconazole prophylaxis should be considered.
Immunoglobulins	IV immunoglobulins (0.4 g/kg) and SC immunoglobulins (0.1-0.15 g/kg) should be administered 3-6 weekly or weekly, respectively.	Routine in children. Consider in adults with serious/recurrent infections with encapsulated organisms and hypogammaglobulinemia (<4 g/l).

Abbreviations: *AlloSCT: allogeneic stem cell transplantation; ANC: absolute neutrophil count; Bid: twice a day; CAR-T: chimeric antigen receptor T cell; CRS: cytokine release syndrome; EBMT: European Society for Blood and Marrow Transplantation; EHA: European Haematology Association; G-CSF: granulocyte colony-stimulating factor; ICANS: immune effector cell-associated neurotoxicity syndrome; IV: intravenous; LD: lymphodepletion; SC: subcutaneous.*

2. Bispecific T cell engagers

Bispecific TCE (BsTCE) are molecules that simultaneously engage CD3 on the surface of T cells and the target of interest on the surface of tumor cells. Their structure derives from different combinations of the Fc, Fab and Fv portions of a monoclonal antibody. Although the goal of monoclonal antibodies is to route physiologic effector functions such as antibody-dependent cytotoxicity (ADC) and antibody-dependent cellular cytotoxicity (ADCC) towards the tumor cell, in BsTCE the lysis of the target cell, CD3, is not desired, and the aim is to engage the T cell into direct cytotoxicity. For this purpose, the Fc region is either genetically-edited or removed. In the latter situation, the molecular weight of the protein is significantly reduced, shortening its half-life, which may require administration by continuous infusion. To avoid that, preserving the size and critical portions of the Fc region could improve pharmacokinetics. Another relevant feature of BsTCE is that a monovalent binding to the immune cell is required, otherwise, bivalent binding of CD3 can induce crosslinking between T cells, inducing T cell lysis. Therefore, the ideal combination would consist of a high-affinity binding site to the tumor antigen and a low-affinity binding site to

CD3. Finally, replacing immunogenic regions with human-homolog sequences could diminish immunogenicity and the formation of antidrug antibodies (ADA)(130,131).

3. Benefits and drawbacks of chimeric antigen receptor T cells and bispecific T cell engagers

CAR-T and BsTCE are immunotherapy strategies that have changed the treatment of cancer patients over the past decade. Both strategies require hospitalization (for step-up dosing in cycle 1 in the case of BsTCE) and are subject to similar adverse events, including CRS, ICANS and infections. Still, many distinctive factors should be considered to choose the most suitable treatment for a specific patient(132–135). A general overview of the benefits and drawbacks regarding the use of CAR-T versus BsTCE is provided in Table 3.

Table 3. Benefits and drawbacks of the use of CAR-T vs. BsTCE(135)**.**

	Chimeric antigen receptor T cells	**Bispecific T cell engagers**
Availability	Individualized manufacturing is required for autologous CAR-T cells, therefore the time to obtain a commercial manufacturing slot, to perform leukapheresis and a manufacturing period, need to be considered.	Off-the-shelf. Ready-to-use, allowing for immediate treatment.
Manufacturing failure	Low, but possible	Does not apply
Lymphodepletion chemotherapy	Yes	No
Pharmacokinetics	“One and done” approach, with a subsequent treatment-free interval.	Short half-life requiring frequent or continuous administration.
Dose adjustment	In general, one single non-modifiable full dose.	Flexible dosing regimens, allowing dose and schedule adjustment to minimize toxicity.
Tumor penetration	Enhanced migration and penetration in tumor sites.	Limited tumor penetration.
Immune toxicity	Less favorable safety profile in terms of CRS and ICANS.	More favorable safety profile in terms of CRS and ICANS.
Infections	60-70% (~20% grade grade ≥3)	76% (~45% grade grade ≥3)
Immunogenicity	Development of ADA has been reported although seems to be less frequent.	Multiple administrations increase the development of ADA, which could neutralize the BsTCE, limiting its efficacy.

Abbreviations: *Anti-drug antibodies: ADA; BsTCE: bispecific T cell engager; CAR-T: chimeric antigen receptor T cell; CRS: cytokine release syndrome; ICANS: immune effector cell-associated neurotoxicity syndrome.*

Immunotherapy for multiple myeloma

1. Chimeric antigen receptor T cells

1.1. Target antigens

Although several antigens have been investigated and proposed, BCMA is currently the most developed target in CAR-T cells for multiple myeloma. BCMA is a transmembrane glycoprotein expressed on the surface of malignant and normal plasma cells, some subsets of B cells, and certain bone marrow stromal cells. Importantly, it is typically low or absent in normal tissues. BCMA plays a critical role in survival and proliferation of myeloma cells by binding to its ligands, such as B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), leading to activation of signaling pathways that promote cell growth and survival. Interestingly, membrane-bound BCMA can undergo γ-secretase-mediated shedding, leading to serum circulation of soluble BCMA (sBCMA)(105,136,137).

GPRC5D is a cell surface protein present on plasma cells, which is believed to play a role in promoting proliferation and survival, although its mechanisms of action are not fully understood. Its expression in other tissues is limited or absent, but its presence has been reported in epithelial structures of the skin, nails, tongue, hair follicles and eccrine glands. Low mRNA levels have also been detected in the motor neurons of the inferior olivary nucleus of the brainstem in the central nervous system (CNS)(138).

CD38-targeted therapies have been widely used in the setting of MM. CD38 is a transmembrane glycoprotein involved in cell adhesion, signal transduction and immunomodulation functions. It is highly expressed on myeloma plasma cells and other hematopoietic cells such as B cells, T cells, NK cells and myeloid cells. Its expression in non-hematopoietic tissues is limited or absent(139). Alternative targets under investigation include CD138, SLAMF7 and CD229, amongst others(140).

1.2. Approved CAR-T for multiple myeloma

To date, two CAR-T cell constructs have been approved by FDA and EMA for the treatment of MM: idecabtagene vicleucel (ide-cel) and ciltacabtagene autoleucel (cilta-cel).

1.2.1. Idecabtagene vicleucel

Idecabtagene vicleucel is an autologous, second-generation 4-1BB-based CAR-T with a murine scFv directed against BCMA. The pivotal KarMMA trial reported an ORR of 73%, with 33% of patients achieving complete response (CR) (26% stringent CR (sCR)) and a median PFS of 8.8 months (95% confidence interval (CI) 5.6–11.6), after a median follow-up of 13.3 months. Some expected CAR-T cell-related adverse events were CRS in 84% (5% grade ≥3), ICANS 18% (3% grade ≥3), and cytopenias (neutropenia 91%, anemia 70% and thrombocytopenia 63%)(141). The KarMMA-3 phase 3 trial randomized R/R MM patients 2:1 to receive either ide-cel or one of five standard regimens. A total of 386 patients were randomized (254 allocated to ide-cel and 132 to SOC). At a median follow-up of 18.6 months, median PFS was 13.3 months in the ide-cel group compared to 4.4. months in the SOC group (hazard ratio (HR) 0.49 95%CI 0.38-0.65; p<0.001). Regarding safety, CRS occurred in 88% of patients (5% grade ≥3) and neurotoxic events in 15% (3% grade ≥3)(142).

The previous data paved the way for FDA and EMA conditional approval in 2021 of ide-cel for the treatment of adult patients with relapsed/refractory multiple myeloma who had received at least four and three, respectively, prior lines of therapy, including an immunomodulatory agent, a proteasome inhibitor, and an anti-CD38 monoclonal antibody. Recently, an expanded indication after 2 prior lines of therapy was granted by both regulatory agencies.

A retrospective study with real-word data in the United States (US) included 159 of 196 leukapheresed patients who ended up receiving ide-cel as SOC; 120 patients would have been ineligible for KarMMa mainly because of comorbidities.

ORR and CR rates were 84% and 42%, respectively. At a median follow-up of 6.1 months from infusion, median PFS was 8.5 months and median OS was 12.5 months. CRS was observed in 82% (3% grade ≥3) and ICANS in 18% (6% grade ≥3)(143).

1.2.2. Ciltacabtagene autoleucel

Ciltacabtagene autoleucel is an autologous, second generation 4-1BB-based CAR-T, with two camelid (llama) heavy chains that bind to two different epitopes of BCMA. The pivotal CARTITUDE-1 trial reported an ORR of 97.9%, with 82.5% of patients achieving a CR. Median PFS and OS were not reached after 27.7 months of follow-up, with 27 months PFS and OS rates of 54.9% (95%CI 44.0-64.6) and 70.4% (95%CI 60.1-78.6). Interestingly, a shorter duration of response (DOR), PFS and OS were reported in patients with high-risk cytogenetics, ISS III, high tumor burden or extramedullary plasmacytomas. In terms of safety, CRS occurred in 95% of patients and cytopenias were present in 96% with neutropenia, 81% with anemia and 79% thrombocytopenia(144,145). Besides ICANS, other treatment-related neurotoxicities, including movement and neurocognitive treatment-emergent adverse events (MNT) were reported in this study. MNT referred to cluster of movement and cognitive and personality changes, while other neurotoxicities included cranial nerve palsy, sensory loss, peripheral neuropathy, altered mental status and nystagmus. A total of 21 patients (21.7%) developed any kind of neurotoxicity. ICANS and other neurotoxicities (including MNTs) were not mutually exclusive; 17 (18%) patients developed ICANS and 13 (13%) patients other neurotoxicities, of which 9 (9%) presented both; MNTs were reported in 6 of these patients (6%). Patients who experienced MNT were more frequently presenting with high tumor burden, grade ≥2 CRS or any grade ICANS, and high CAR-T cell expansion/persistence. Strategies to mitigate these effects were implemented across all CARTITUDE trials, which included an enhanced bridging therapy to reduce baseline tumor burden, early aggressive treatment of CRS and ICANS, and extended monitoring and reporting time for neurotoxicity beyond 100 days post-infusion(146).

Introduction

The CARTITUDE-4 phase 3 trial randomized lenalidomide-refractory MM patients to receive either cilta-cel or physicians' choice of SOC. A total of 419 patients were randomized (208 allocated to ide-cel and 211 to SOC). At a median follow-up of 15.9 months, median PFS was not reached in the cilta-cel group compared to 11.8 months in the SOC group (hazard ratio (HR) 0.26 95%CI 0.18-0.38; p<0.001). The proportion of patients achieving an overall response, CR or MRD negativity was significantly higher in the cilta-cel group. In terms of safety, 76% of patients in the CAR-T group experienced CRS (1.1% grade ≥3), ICANS in 4.5% (all grades 1-2), 1 patient developed MNT (grade 1), 9.1% cranial nerve palsy (1.1% grade ≥3), and 2.8% had CAR-T-related peripheral neuropathy (0.6% grade ≥3)(147).

The previous data led to FDA and EMA approval in 2022 of cilta-cel for the treatment of adult patients with relapsed/refractory multiple myeloma who had received at least four and three, respectively, prior lines of therapy, including an immunomodulatory agent, a proteasome inhibitor, and an anti-CD38 monoclonal antibody. The FDA recently granted an expanded indication of this product and cilta-cel has become the first BCMA-CART approved after only one line of therapy, including a PI and refractoriness to lenalidomide. The same expanded full approval has been recommended by the Comittee for Medicinal Products for Human use, but EMA approval is pending.

2. Bispecific T cell engagers

Several BsTCE are under clinical investigation for MM. To date, two BsTCE targeting BCMA, Teclistamab and Elranatamab, and one targeting G protein-coupled receptor class C group 5 member D (GPRC5D), Talquetamab, have received regulatory approval for triple-exposed R/R MM patients who have progressed to four prior lines of therapy(148–150). A subcutaneous route of administration is recommended for all agents. Also, escalating "step-up" doses and hospital admission are recommended for the first 2 to 4 administrations to monitor short-term adverse events such as CRS and ICANS. Administration is continuous until disease progression. Teclistamab is administered weekly, and Elranatamab and Talquetamab are administered weekly first, and biweekly

afterwards in persistent responders, although further de-escalation approaches are under investigation. The phase 1/2 clinical trials that led to their approval included elderly patients (over 80 years of age), patients with high-risk cytogenetics and extramedullary disease. A summary of the patient characteristics, efficacy and safety of the pivotal clinical trials is provided in Table 3(135).

Table 3. Baseline patient characteristics, efficacy and safety of multiple myeloma patients included in the phase 1 and 2 clinical trials of the approved bispecific T cell engagers(135)**.**

	Teclistamab	**Elranatamab**	**Talquetamab**[2]
Trial	MajesTEC-1	MagnetisMM-3	MonumenTAL-1
Target antigen	BCMA	BCMA	GPRC5D
Number of patients	165	123	130
Median age, years (range)	64 (33–84)	68 (36–89)	64 (39–84)
Median time since diagnosis, years (range)	6.0 (0.8–22.7)	Not reported	6.6 (0.9–27.0)
Median prior lines of therapy, n (range)	5 (2–14)	5 (2–22)	6 (2–17)
ECOG ≥1 (%)	66.7%	63.4%	Not reported
High-risk cytogenetics[1] (%)	25.7%	25.2%	16%
Extramedullary plasmacytomas (%)	17%	31.7%	15%
Triple class refractory (%)	77.6%	96.7%	75%
Penta-drug refractory (%)	30.3%	42.3%	25%
Overall response rate (%)	63%	61%	68%[3]
Median PFS, months (range)	11.3 (8.8–17.1)	NR (9.9–NR)	Not reported
Median follow-up, months (range)	14.1 (0.3–24.4)	14.7 (0.2–25.1)	7.7 (0.7–24.0)
CRS, (%) (grade 3-4)	72.1% (0.6%)	57.7% (0%)	78.4% (1.4%)
ICANS, (%) (grade 3-4)	3% (0%)	3.4% (0%)	6.8% (0%)
Infections, (%) (grade 3-4)	76.4% (44.8%)	69.9% (39.8%)	39.2% (6.8%)

[1]del(17p), t(4;14), t(14;16). [2]Only data from the subcutaneous treatment cohort are shown. Adverse event data refer only to the 405 μg weekly and 800 μg every 2 weeks dose cohorts. [3]Response data are for the 108 patients treated with the "most active" subcutaneous dose (defined as 135, 405, and 800 μg/kg weekly or 800 and 1,200 μg/kg every other week).

Abbreviations: *BCMA: B cell maturation antigen; CRS: cytokine release syndrome; ECOG: eastern cooperative oncology group; GPRC5D: G protein-coupled receptor class C group 5 member D; ICANS: immune effector cell-associated neurotoxicity syndrome.*

Sequencing of immunotherapies

Integration of the different immunotherapies into the therapeutic journey of patients remains a field of uncertainty. The lower response rates and durations observed after sequencing of immunotherapies may be related to target antigen

resistance mechanisms, T-cell fitness/exhaustion or simply a reflection of more heavily pretreated patients(135).

Immunotherapies with different mechanisms of action may share the same target antigen, but most CAR-T clinical trials with available results have tended to exclude patients who had received a prior therapy targeting the same antigen. The CARTITUDE-2 cohort C study enrolled R/R MM patients with a prior non-cellular BCMA therapy to receive cilta-cel. In this subset of 20 patients, ORR was 60% and median PFS of only 9.1 months, results that were significantly lower compared to the BCMA-naïve cohort of CARTITUDE-1(151). Similarly, commercial ide-cel real-world data reported inferior outcomes in patients with prior exposure to BCMA-directed therapies, with a median PFS of 3.2 months (prior BCMA treatment) compared to 9.0 months (BCMA-naïve) (p=0.0002)(152). Interestingly, the MejesTEC-1 cohort C study, which enrolled 40 patients with prior exposure to BCMA-directed therapies, reported an ORR of 53% for the whole cohort, but also for those patients with prior BCMA-CART exposure (n=15)(153). Patients enrolled in a clinical trial receiving elranatamab, with prior BCMA-treatment exposure (n=87), showed an ORR of 46% and a median PFS of 5.5 months, while in the 36 patients with a prior BCMA-CART, ORR was 53% and median PFS was 10 months(154). The lack of randomized studies prevents from drawing definite conclusions, but a sequence involving the administration of CAR-T cells first, followed by bispecific TCE, may yield superior outcomes compared to the reverse order.

Verification of the persistent expression of the target antigen holds significance in situations where administering a therapy directed towards a previously treated target is considered. A limitation arises from the potential unavailability of standardized tests to assess certain antigens across all medical centers. With the presumable introduction of different target antigens in the future immunotherapy approvals, a target switch should be considered, when possible. This could potentially facilitate a re-upregulation of the previously targeted antigen(135).

Access to therapy and equity in the real-world setting

Equity and access to treatment is a major concern regarding cellular immunotherapies. The complexity of manufacturing, research costs, and the perceived value and innovation provided by these therapies, have established high prices that build financial barriers to access. Country differences in healthcare funding, infrastructure, and reimbursement policies cause geographic disparities(155).

The availability of commercial slots for CAR-T manufacturing is another challenge that limits equal widespread of this therapy(156,157). The manufacturing capacity of commercial products does not efficiently cover the needs of MM patients(158). A study reported that up to 40% of MM patients did not receive a CAR-T, despite being eligible. In the remaining 60%, median time from consultation to leukapheresis was 38 days (range 8-703) and vein-to-vein time was 42 days (34-132)(159).

An academic point-of-care manufacturing model has been proposed as a potential solution, addressing key barriers to broad implementation of CAR-T therapy. Decentralizing CAR-T production could reduce costs and enhance flexibility, minimizing logistical challenges and allowing for rapid production and delivery of CAR-T cells. However, ensuring compliance with regulatory standards, maintaining consistent product quality and the investing in adequate infrastructures to meet GMP requirements, can be limiting factors(160).

The cohabitation of both academic and commercial CART products is probably inevitable. Most academic CAR-T-based therapies are in phase 1 clinical trials, but it is very tough for these centers to complete phase 2 or 3 clinical trials, making centralized authorization unaffordable. Moreover, pharmaceutical companies are usually more reluctant to develop drugs for pediatric or rare diseases. In that field, academic centers could be in the vanguard, employing the innovations and improved skills learned from the industry, to benefit these minorities(161).

Development of academic CAR-T in Barcelona: the ARI project

Hospital Clinic of Barcelona and other academic centers worldwide are focused on advancing CAR-T therapy development, exploring novel approaches in translational research and clinical trials, always within the academic setting. The ARI project was initiated by Ariana Benedé, a pediatric patient who was diagnosed with B-ALL at the age of 13. Ariana, along with her family and friends, organized numerous charitable initiatives aimed at fundraising to bring CAR-T therapy to Spain, which was only available in the United States then. The project was financially possible thanks to the support of public authorities and civil society, with donations made by nearly 1500 individuals, 23 associations and foundations, and 56 companies.

The first milestone of the project was the preclinical development and validation of ARI-0001, a 41BB-based autologous second-generation CD19-CART for the treatment of CD19-positive malignancies, entirely developed at Hospital Clinic of Barcelona(162,163). The CART19-BE-01 clinical trial evaluated ARI-0001 in patients with R/R B-ALL, diffuse large B cell lymphoma (DLBCL), primary mediastinal B cell lymphoma, follicular lymphoma (FL), and chronic lymphocytic leukemia (CLL)(164,165). Efficacy and safety results were comparable to other commercial or academic available products. As a result, ARI-0001 received authorization from the Spanish Agency of Medicines and Medical Devices (AEMPS) under the hospital exemption approval pathway, to treat adult patients (> 25 years) with R/R B-ALL.

The hospital exemption pathway is a regulatory framework that allows healthcare institutions to administer unapproved advanced therapy medicinal products to patients under certain circumstances. These circumstances usually involve life-threatening conditions for which no alternative therapy is available. The product must comply with the required quality controls, traceability, and pharmacovigilance regulations. Its use is limited to the country where it was developed; in the case of ARI-0001, national legislation only grants the license to the hospital that developed the product. A conditional license was granted for

three years, and it is subject to a follow-up annual report, fulfillment of specific regulatory obligations, and data re-evaluation to obtain a further 5-year renewal. These requirements aim to increase efficacy and safety evidence, in the absence of evidence coming from a phase 3 trial. This is in opposition to the conditional authorization given to centrally approved products for an indefinite period, which are only subject to the provision of additional data. Hospital exemption allows for advanced therapy medicinal products development in close contact with clinical practice and rapid access by patients at a lower cost, compared to regular market authorization(166).

Meanwhile, an alternative construct targeting BCMA, termed ARI0002h, was designed and validated *in vitro* and *in vivo*. ARI0002h is humanized a 4-1BB-based BCMA-CART, genetically transduced on autologous T cells using a lentiviral vector(162). Encouraging results from preclinical investigations led to the development of a pivotal, single-arm, open-label, multicenter clinical trial, CARTBCMA-HCB-01, for the treatment of patients with R/R MM.

HYPOTHESIS

1. Human-scale academic manufacturing of ARI0002h with autologous lymphocytes from patients with relapsed/refractory multiple myeloma is feasible: ARI0002h is active against this disease, with an acceptable safety profile.

2. Some biological characteristics, such as surface BCMA expression in myeloma cells, the presence of serum soluble BCMA, T cell subpopulations (including exhaustion markers), ARI0002h kinetics, clinical baseline features (such as the presence of extramedullary disease or high-risk cytogenetics), or the development of anti-CAR-T antibodies, correlate with efficacy and safety after ARI0002h infusion in patients with multiple myeloma.

3. Targeting CAR-T exhaustion through a TIGIT blockade on ARI0002h using different approaches enhances its efficacy in both preclinical in vitro and in vivo studies, allowing for the translation of these advancements into the clinical setting.

OBJECTIVES

1. Analyze and describe the manufacturing feasibility, efficacy, and safety of ARI0002h, an academic autologous CAR-T directed against BCMA, for the treatment of relapsed/refractory multiple myeloma in the CARTBCMA-HCB-01 clinical trial.

2. Collect and analyze samples obtained from patients with multiple myeloma included in the CARTBCMA-HCB-01 clinical trial to determine biomarkers of efficacy and safety after the infusion of ARI0002h.

3. Target CAR-T cell exhaustion by blocking TIGIT on ARI0002h using three different approaches: (i) The addition of an external anti-TIGIT antibody, (ii) Transforming ARI0002h into a 4^{th} generation CAR-T capable of secreting a TIGIT-blocker and, (iii) Knock-out of TIGIT on CAR-T cells.

MATERIAL, METHODS AND RESULTS

Article 1

Fractionated initial infusion and booster dose of ARI0002h, a humanised, BCMA-directed CAR T-cell therapy, for patients with relapsed or refractory multiple myeloma (CARTBCMA-HCB-01): a single-arm, multicentre, academic pilot study

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Summary

Background Chimeric antigen receptor (CAR) T-cell therapy is a promising option for patients with heavily treated multiple myeloma. Point-of-care manufacturing can increase the availability of these treatments worldwide. We aimed to assess the safety and activity of ARI0002h, a BCMA-targeted CAR T-cell therapy developed by academia, in patients with relapsed or refractory multiple myeloma.

Methods CARTBCMA-HCB-01 is a single-arm, multicentre study done in five academic centres in Spain. Eligible patients had relapsed or refractory multiple myeloma and were aged 18–75 years; with an Eastern Cooperative Oncology Group performance status of 0–2; two or more previous lines of therapy including a proteasome inhibitor, an immunomodulatory agent, and an anti-CD38 antibody; refractoriness to the last line of therapy; and measurable disease according to the International Myeloma Working Group criteria. Patients received an initial fractionated infusion of 3×10^6 CAR T cells per kg bodyweight in three aliquots (0·3, 0·9, and $1·8\times10^6$ CAR-positive cells per kg intravenously on days 0, 3, and 7) and a non-fractionated booster dose of up to 3×10^6 CAR T cells per kg bodyweight, at least 100 days after the first infusion. The primary endpoints were overall response rate 100 days after first infusion and the proportion of patients developing cytokine-release syndrome or neurotoxic events in the first 30 days after receiving treatment. Here, we present an interim analysis of the ongoing trial; enrolment has ended. This study is registered with ClinicalTrials.gov, NCT04309981, and EudraCT, 2019-001472-11.

Findings Between June 2, 2020, and Feb 24, 2021, 44 patients were assessed for eligibility, of whom 35 (80%) were enrolled. 30 (86%) of 35 patients received ARI0002h (median age 61 years [IQR 53–65], 12 [40%] were female, and 18 [60%] were male). At the planned interim analysis (cutoff date Oct 20, 2021), with a median follow-up of 12·1 months (IQR 9·1–13·5), overall response during the first 100 days from infusion was 100%, including 24 (80%) of 30 patients with a very good partial response or better (15 [50%] with complete response, nine [30%] with very good partial response, and six [20%] with partial response). Cytokine-release syndrome was observed in 24 (80%) of 30 patients (all grade 1–2). No cases of neurotoxic events were observed. Persistent grade 3–4 cytopenias were observed in 20 (67%) patients. Infections were reported in 20 (67%) patients. Three patients died: one because of progression, one because of a head injury, and one due to COVID-19.

Interpretation ARI0002h administered in a fractioned manner with a booster dose after 3 months can provide deep and sustained responses in patients with relapsed or refractory multiple myeloma, with a low toxicity, especially in terms of neurological events, and with the possibility of a point-of-care approach.

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Introduction

The survival of patients with multiple myeloma has improved as a result of the incorporation of the combination of proteasome inhibitors, immunomodulating agents, and anti-CD38 monoclonal antibodies into the standard of care since 2008. Still, a large proportion of patients continue to relapse and multidrug resistance remains an important challenge leading to poor outcomes in patients with relapsed or refractory multiple myeloma.[1,2]

Chimeric antigen receptor (CAR) T-cell therapy has emerged as a promising option for relapsed or refractory multiple myeloma. Several CAR T-cell products targeting BCMA using different approaches in terms of origin of

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Research in context

Evidence before this study

Effective therapies with acceptable safety profiles are needed to improve outcomes of patients with relapsed or refractory multiple myeloma, particularly for patients with disease that is refractory to immunomodulatory drugs, proteasome inhibitors, and anti-CD38 monoclonal antibodies, who have few treatment options available. We searched PubMed on Feb 15, 2023, for publications in English, with no date restriction, using the keywords "myeloma", "B-cell maturation antigen", "chimeric antigen receptor T (CART)" AND "fractionated dose" OR "booster dose". Although multiple trials assessing BCMA-targeted chimeric antigen receptor (CAR) T-cell therapies and related adoptive cellular approaches are available, we found no published studies that used a fractionated dose or a booster dose.

Added value of this study

In this single-arm, multicentre study using ARI0002h (an autologous CAR T-cell product targeting BCMA that is under development at Hospital Clinic de Barcelona, an academic institution in Spain), a fractionated dose of ARI0002h followed by a booster dose 100 days later led to early, deep, and durable responses in adult patients with relapsed or refractory multiple myeloma who had received at least two previous treatment regimens, including a proteasome inhibitor, an immunomodulatory drug, and an anti-CD38 monoclonal antibody. This trial reports novel findings in the field of immunotherapy for multiple myeloma. First, we reduced the potential immunogenicity of the CAR by using a humanised single-chain variable fragment for the recognition of BCMA, instead of one of full animal origin (mouse or llama), as is the case for the already approved commercial CAR T cells. Second, we lowered toxicity by using a fractionated administration scheme— no cases of neurotoxicity were observed. Third, a second dose was planned after 100 days in patients with response and no relevant toxicity, providing fresh and active CAR T cells to consolidate or deepen the response. Finally, this trial incorporated the concept of point-of-care treatment in cellular therapy, showing that a strategy based on two production sites and five treating hospitals is possible in a public health system.

Implications of all the available evidence

ARI0002h is a viable treatment option for patients with relapsed or refractory multiple myeloma, with an activity and safety profile comparable to approved CAR T-cell products. A point-of-care strategy to facilitate real access and administration of the product is crucial, given limited patient access to these treatments in the USA and Europe because of the lack of manufacturing capacity and reimbursement.

the antigen-recognition domain (murine, humanised, human, or llama), co-stimulatory domain (4-1BB and CD28), and transduction method (lentiviral, retroviral, or transposon)[3] are under clinical investigation. Nevertheless, only two BCMA CAR T-cell therapies, idecabtagene vicleucel and ciltacabtagene autoleucel, have been approved by the US Food and Drug Administration after at least four previous lines of therapy and the European Medicines Agency after at least three for the treatment of multiple myeloma, including proteasome inhibitors, immunomodulating agents, and anti-CD38 monoclonal antibodies.[4,5] The idecabtagene vicleucel study reported an overall response rate of 73%, with 33% of patients having complete responses (26% stringent complete responses), with a median progression-free survival of 8·8 months (95% CI 5·6–11·6) and some expected CAR T cell-related adverse events: cytokine-release syndrome in 107 (84%) of 128 patients (5% grade ≥3), immune effector cell-associated neurotoxicity syndrome (ICANS) in 23 (18%; 3% grade ≥3), and cytopenias (neutropenia 117 [91%], anaemia 89 [70%], and thrombocytopenia 81 [63%]).[6] In a phase 3 clinical trial, idecabtagene vicleucel showed improved responses and progression-free survival compared with standard care in patients exposed to three families of drugs: proteasome inhibitors, immunomodulating agents, and anti-CD38 antibodies.[7] The 2-year update[8] of the ciltacabtagene autoleucel phase 3 trial showed an overall response rate of 97·9%, with 82·5% being stringent complete responses in 97 patients with multiple myeloma treated with $0{\cdot}75\times10^6$ CAR T cells per kg bodyweight. Median progression-free survival and overall survival were not reached after a median follow-up of 27·7 months. Cytokine-release syndrome was reported in 92 (95%) of 97 patients (4% grade ≥3) and neurotoxicity in 20 (21%; nine [9%] grade ≥3), including both ICANS and other neurotoxicities. Parkinsonism symptoms occurred in six (6%) patients, with one related death and two deaths due to other causes. Parkinsonism-like neurotoxicity decreased to less than 1% after patient management strategies.[9] A study[10] of the first allogeneic BCMA-CAR T-cell therapy (ALLO-715) reported an overall response rate of 71%, including 25% or more with complete response, with a very short median time (5 days [range 0–20]) from enrolment to lymphodepletion.

Our academic institution developed two CAR T-cell constructs; one directed against CD19 (ARI-0001; varnimcabtagene autoleucel) and another against BCMA (ARI0002h). The CART19-BE-01 multicentre clinical trial with ARI-0001 for adult and paediatric CD19-positive malignancies led to its approval as hospital exemption in Spain in February, 2021, making it the first academic CAR T-cell therapy in clinical use in Europe to our knowledge.[11–13] From our previous experience treating CD19 malignancies with ARI-0001, we observed how administering the initial dose in a fractionated manner might diminish severity of

immune-related side-effects without reducing efficacy, although that study was not specifically designed to compare outcomes of a fractionated versus non-fractionated infusion.[12,14]

ARI0002h is a humanised 4-1BB-based BCMA-CAR T-cell therapy, lentivirally transduced on autologous T cells obtained by peripheral blood leukapheresis, that has proven antitumour activity in preclinical in vitro and in vivo approaches.[15] We aimed to investigate the safety and activity of ARI0002h in patients with relapsed or refractory multiple myeloma after an initial fractionated infusion and a non-fractionated booster dose, administered at least 3 months after the first infusion in patients with some degree of response and no limiting side-effects.

Methods

Study design and participants

CARTBCMA-HCB-01 is a single-arm, open-label study done in five academic centres in Spain. Eligible patients had relapsed or refractory multiple myeloma; were aged 18–75 years; had an Eastern Cooperative Oncology Group performance status of 0–2; two or more previous lines of therapy including a proteasome inhibitor, an immunomodulating agent, and an anti-CD38 antibody; refractoriness to the last line of therapy; and measurable disease (serum and urine monoclonal protein >10 g/L or 200 mg/24 h and involved free light chain >100 mg/L) according to the International Myeloma Working Group (IMWG) criteria;[16] and a life expectancy of more than 3 months. Exclusion criteria included previous BCMA-directed therapy and a non-adequate organ system function including an estimated glomerular filtration rate below 50 mL/min. The full list of exclusion criteria, study protocol, and participating sites are summarised in the appendix (pp 2–5).

Patients provided written informed consent. The study protocol was approved by the Ethics Committee of Hospital Clínic de Barcelona and was done in accordance with the Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects.

Procedures

Clinical coordination and vector viral production were done in Hospital Clínic de Barcelona (Barcelona, Spain). Two centres were responsible for CAR T-cell production: Hospital Clínic de Barcelona and Clínica Universidad de Navarra (Pamplona, Spain; appendix p 6). Three CAR T development strategies were adopted to improve outcomes. Firstly, the murine single-chain variable fragment (scFv), obtained from the J22.9 antibody, was humanised to reduce immunogenicity. We did preclinical experiments to show non-inferiority between constructs containing the humanised scFv versus murine scFv.[15] Second, the first dose was fractioned into 3 aliquots to reduce toxicity. Finally, we planned a second infusion (booster dose) of ARI0002h at least 3 months after the first infusion, as an experimental attempt to improve CAR T-cell persistence and response. Patient characteristics, including sex, were defined by electronic medical records. Race and ethnicity data were not collected. We assessed patients for eligibility and proceeded to leukapheresis in their respective centres. Fresh apheresis products were sent to one of the two production centres. The target dose was 3×10^6 CAR-positive cells per kg bodyweight for the first infusion and a second dose of up to 3×10^6 CAR-positive cells per kg was also obtained, when possible. The final product was cryopreserved.

Bridging therapy was allowed in the period between apheresis and lymphodepletion according to investigator's choice. Lymphodepletion was administered intravenously on days –6 to –4 (before infusion) and included fludarabine (30 mg/m²/day; total dose 90 mg/m²) and cyclophosphamide (300 mg/m²/day; total dose 900 mg/m²). The first CAR T infusion was split into three administrations of 0·3 (10%), 0·9 (30%), and $1{\cdot}8\times10^6$ (60%) CAR-positive cells per kg intravenously on days 0, 3, and 7, with at least 24 h between doses in all cases. If adverse events occurred between administrations, remaining doses were adjusted until resolution. The booster dose of up to 3×10^6 CAR-positive cells per kg was administered in a single intravenous infusion after day 100 in patients with some degree of response and no limiting side-effects after the first dose, including grade 3–4 cytokine-release syndrome or ICANS and other adverse events of interest (persistent cytopenias or macrophage activation syndrome). Lymphodepletion was readministered with the same scheme only in patients without CAR T-cell persistence in peripheral blood.

We followed up patients for 36 months, or until progression or death. Patients could be removed from the study on the basis of patient decision. Laboratory monitoring was planned in the following days and months from infusion: days 1, 10, 14, 21, 28, 35, 42, 56, 70, 84, and 100; and months 4, 6, 7, 8, 9, 10, 11, 12, 15, 18, 21, 24, 27, 30, 33, and 36. Bone marrow aspirates assessing measurable residual disease by next generation flow cytometry at a sensitivity of 1×10^{-6} were planned on days 28 and 100 and months 6, 12, 18, and 24. Assessment was done using two-tube eight-colour flow cytometry according to the EuroFlow platform, using a FACSCanto (BD Biosciences, [San Jose, CA, USA]) flow cytometer and Infinicyt 2.0 software (Cytognos SL, Salamanca, Spain; appendix p 7). We considered any detectable level of measurable residual disease greater than 1×10^{-6} to be positive. PET-CT scans were planned at screening and on day 100 and month 12 to assess plasmacytomas. Multiple myeloma disease evaluation was planned on days 28, 56, and 100; and months 4–12, 15, 18, 21, 24, 27, 30, 33, and 36. Responses were assessed according to IMWG criteria (appendix pp 6–7).[17] Adverse events were monitored in all follow-up visits, including a daily monitoring from day of infusion until hospital discharge. Cytokine-release

See Online for appendix

syndrome and ICANS were assessed according to the American Society for Transplantation and Cellular Therapy consensus.[18] Intravenous immunoglobulins could be administered according to local guidelines when IgG concentrations were below 400 mg/dL.

Samples were obtained from peripheral blood and bone marrow for correlative studies (appendix p 10). BCMA expression on bone marrow plasma cells was measured by flow cytometry (PE anti-human CD269 [BCMA] antibody; Biolegend [San Diego, CA, USA]) at baseline and in measurable residual disease-positive disease. Molecules of BCMA were quantified using the BD QuantiBRITE Beads (BD Biosciences; molecules/cell). The kinetics of ARI0002h in the peripheral blood were measured by quantitative PCR (qPCR) assessing the time course of vector transgene Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element to elucidate the number of ARI0002h copies per cell. Inmunogenicity against ARI0002h was assessed by flow cytometry on Attune Next (Invitrogen, Thermofisher Scientific [Waltham, MA, USA]) flow cytometer to assess the presence of human anti-human antibodies in the peripheral blood (appendix pp 8–9).

Outcomes

The primary safety endpoint was the proportion of patients who developed cytokine-release syndrome, ICANS, or both in the first 30 days after ARI0002h administration. The primary activity endpoint was overall response rate in the first 100 days of infusion, defined as achievement of at least partial response.[17] Secondary endpoints were complete response rates at 100 days and 6 months from infusion, overall response at 6 and 12 months, time to best response and time to complete response, measurable residual disease negativity rates at day 100 and month 6, plasmacytoma assessment by PET-CT at day 100, duration of response, progression-free survival, progression-free survival at 12 months, overall survival, presence of infusion reactions, tumour lysis syndrome, neurotoxicity besides ICANS, prolonged cytopenias defined as the reduction of neutrophil or platelet peripheral blood counts, grade 3 or 4 after 4 weeks from infusion, ARI0002h persistence, BCMA expression, and soluble BCMA. Quality-of-life assessment was also prespecified in the protocol but results are not provided since data are not available. Duration of response was defined as the time between first response and disease progression. Progression-free survival was defined as the time between infusion of ARI0002h and disease progression or death. Overall survival was defined as the time between infusion of ARI0002h and death due to any cause. Time to best response was defined as time between infusion and deepest response achieved according to IMWG response criteria. Time to complete response was defined as time between infusion and achievement of complete response in applicable patients.

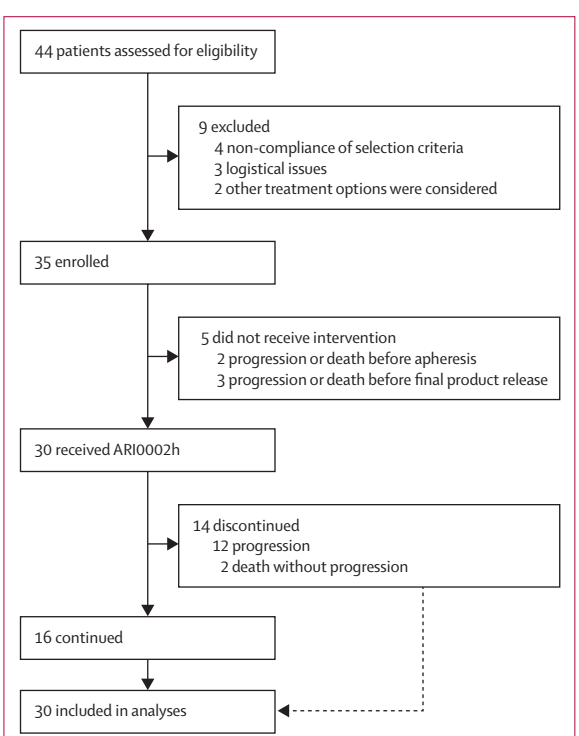

Figure 1: **Trial profile**

Statistical analysis

We initially proposed to recruit patients with the objective to treat 30 patients. We assumed that some patients would not be treated with ARI0002h because of early progression before or after apheresis. We estimated a pre-treatment patient loss of 20%; therefore, we reasoned that 36 patients would be necessary to achieve the 30 administrations expected. Sample size calculation was not done. Response analyses were done in all patients who received at least one fraction of ARI0002h. We had two hypotheses: (1) overall response rate in the first 100 days would be at least 80%, which would allow us to rule out with 95% confidence that the real overall response rate is below 65% in a population similar to the one studied; and (2) overall response rate in the first 100 days would be at least 70%, which would allow us to rule out with 95% confidence that the real overall response rate is below 54% in a population similar to the one studied.

The analysis population for safety was patients who received at least one cycle of lymphodepletion.

Here, we present results of the planned interim analysis with a cut-off date of Oct 20, 2021 to initiate regulatory review by the Spanish National Competent Authority (SNCA) and thus receive authorisation for hospital exemption. Interim data is provided to the Spanish National Competent Authority for the rolling review on a periodic basis, including information regarding safety and activity after the administration of the booster dose. At the cut-off date of the planned

	Patients treated with ARI0002h (n=30)
Age, years	61 (53–65)
Sex	
Female	12 (40%)
Male	18 (60%)
Median time since diagnosis, years	4·7 (3·7–9·1)
Heavy chain isotype	
IgG	14 (47%)
IgA	8 (27%)
Bence Jones	7 (23%)
IgD	1 (3%)
Light chain isotype	
κ	15 (50%)
λ	15 (50%)
ISS stage	
I	5/25 (20%)
II	8/25 (32%)
III	12/25 (48%)
ECOG performance status	
0	18/29 (62%)
1	9/29 (31%)
2	2/29 (7%)
Plasmacytomas	14 (47%)
Extramedullary plasmacytomas	6 (20%)
High-risk cytogenetics*	10 (33%)
TP53 alterations	7 (23%)
t(4;14)	4 (13%)
t(14;16)	1 (3%)
Number of previous lines	3·5 (2·8–5·0)
Triple exposed†	30 (100%)
Triple refractory†	20 (67%)
Penta exposed‡	11 (37%)
Penta refractory‡	7 (23%)
(Table 1 continues in next column)	
(Continued from previous column)	
Refractory to the last line	30 (100%)
Previous drug exposure	
Bortezomib	30 (100%)
Carfilzomib	15 (50%)
Ixazomib	2 (7%)
Lenalidomide	30 (100%)
Lenalidomide refractory	22 (73%)
Thalidomide	17 (57%)
Pomalidomide	17 (57%)
Daratumumab	30 (100%)
Daratumumab refractory	27 (90%)
Previous autologous stem-cell transplantation	28 (93%)
Previous allogeneic stem-cell transplantation	4 (13%)
Previous autologous and allogeneic stem-cell transplantation	4 (13%)
Bone marrow plasma cells	11·0% (1·0–32·5)
Serum monoclonal protein, g/L	12·5 (0–30·4)
Urine monoclonal protein, g/24 h	0·08 (0–1·08)
Differential sFLC, mg/L	443·2 (154·9–1144·7)

Data are n (%), n/N (%), or median (IQR). ECOG=Eastern Cooperative Oncology Group. ISS=international staging system. sFLC=serum free light chain. *Some patients had more than one high-risk cytogenetic abnormality: one patient had del(17p) plus t(4;14) and another patient had del(17p) plus t(14;16). †To a proteasome inhibitor (bortezomib or carfilzomib), an immunomodulatory drug (lenalidomide or pomalidomide), and an anti-CD38 monoclonal antibody (daratumumab). ‡To bortezomib, carfilzomib, lenalidomide, pomalidomide, and an anti-CD38 monoclonal antibody.

Table 1: **Baseline characteristics of patients treated with ARI0002h**

interim analysis, general follow-up and follow-up after the booster dose was short. To provide scientifically relevant data after a longer follow-up of patients, we did also a post-hoc analysis with a cut-off date of May 15, 2022.

Duration of response, progression-free survival, overall survival, time to best response, and time to complete response were analysed using the Kaplan-Meier method. Survival calculations between independent groups were tested with the log-rank test. When appropriate (eg, for response), 95% CIs were calculated using the Wilson score method. For survival times, 95% CIs were calculated with the log method. Reasons for censoring were last follow-up without progression or death without progression for duration of response, last follow-up without progression or death for progression-free survival, and last follow-up without death for overall survival.

Post-hoc analyses of vein-to-vein time according to receipt of bridging therapy (yes or no), response and progression-free survival according to receipt of the full first dose of ARI0002h (yes or no), expansion of CAR T-cells in peripheral blood after booster dose according to receipt of lymphodepletion (yes or no), duration of response and progression-free survival according to the achievement of complete response, progression-free survival and overall survival according to the presence of plasmacytomas were done.

p values from post hoc comparisons are provided, which should be considered informative and descriptive, but not conclusive. We calculated p values of categorical variables with χ^2 or Fisher's exact test according to sample size. We calculated p values of continuous variables, including paired sample comparisons, with the Student's *t* test, Wilcoxon–Mann–Whitney, or Wilcoxon signed-rank test according to their adherence to the Gaussian distribution tested with the Saphiro–Wilk test. If appropriate, p values were adjusted for multiple comparisons with the Benjamini–Hochberg method. A p value of less than 0·05 was considered statistically significant.

Statistical analyses were done with SAS System (version 9.4) and R (version 4.3.0).

This study is registered with ClinicalTrials.gov, NCT04309981, and EudraCT, 2019-001472-11.

	Grade 1	Grade 2	Grade 3–4
Cytokine release syndrome	15/24 (63%)	9/24 (38%)	0
Immune effector cell-associated neurotoxicity syndrome	0	0	0
Infusion reaction	1/30 (3%)	0	0
Tumour lysis syndrome	0	1/30 (3%)	0
Persistent cytopenias	0	0	20/30 (67%)

Data are n (%). Adverse events of special interest are depicted per MedDRA preferred term.

Table 2: **Adverse events of special interest**

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Between June 2, 2020, and Feb 24, 2021, we assessed 44 patients for eligibility (figure 1). We enrolled 35 patients, of whom 33 (94%) underwent leukapheresis and 30 (86%) received ARI0002h (figure 1). The median manufacturing time of ARI0002h was 10 days (IQR 9–10), with a mean transduction rate of 56% (SD 25). All final products for the first infusion were successfully obtained at the first attempt except one, which was produced with a second apheresis. Median turnaround time, defined as days from apheresis reception to product liberation, was 30 days (IQR 26–36; range 19–45). In a patient requiring urgent treatment, the product was released in as fast as 19 days. The median age of patients who received ARI0002h was 61 years (IQR 53–65), 12 (40%) were female, and 18 (60%) were male (table 1). 14 (47%) of 30 patients presented with plasmacytomas at inclusion, with six (20%) presenting with a true extramedullary, non-paraskeletal location (table 1). Assessment of disease before and after bridging therapy showed either no change or progression of the serum M-protein or free light chain in six patients (43%); in eight (57%) a decrease was observed, with no patients having a complete response (appendix p 11).

All 30 patients received fractions 1 and 2. Five (17%) patients did not receive the third fraction of the first dose of ARI0002h because of cytokine-release syndrome. 24 (86%) of 28 eligible patients received the second administration of ARI0002h (booster dose) in a single infusion of 100% of the dose. Two patients were not eligible for the booster dose because of extramedullary progression on day 100 (n=1) and death (cranial injury) in month 4 (n=1). Four (14%) of the 28 eligible patients did not receive the booster dose because of prolonged cytopenias, diagnosis of a secondary neoplasm, macrophage activation syndrome, and lymphocytosis due to CAR T-cell expansion (n=1 each; appendix p 12). In 19 (79%) of the 24 patients who were reinfused, 3×10^6 CAR-positive cells per kg were available for the booster dose; $1{\cdot}8\times10^6$ CAR-positive cells per kg were available for three patients and $1{\cdot}2\times10^6$ CAR-positive cells per kg were available for two patients (appendix p 12). Median time to booster dose was 4 months (IQR 3–5; range 3–7). Eight (33%) of 24 patients received a second lymphodepletion regimen before the booster dose, based on CAR T-cell detection in peripheral blood.

At the interim analysis (data cutoff Oct 20, 2021; median follow-up 12·1 months [IQR 9·1–13·5]), 10 (33%) of 30 (95% CI 19–51) patients had discontinued: eight patients developed disease progression and two died without progression (one after a cranial injury in month 4 and one after severe SARS-CoV-2 pneumonia in month 9). No discontinuations occurred due to manufacturing failures. An additional death occurred in a patient with disease progression. No deaths occurred within the first month of treatment.

Cytokine-release syndrome was observed in 24 (80%) of 30 patients, in each case after receiving at least the second fraction of 30% (total dose 40%), with no grade 3 or higher events (15 [63%] grade 1 and nine [38%] grade 2; table 2). Median time to onset of cytokine release syndrome was 7 days (IQR 5–8) from the first fraction (10%), with a median duration of symptoms of 2 days (0–14). Tocilizumab was administered in 19 (63%) of 30 patients, mainly for persistent grade 1 cytokine-release syndrome, and three (10%) patients received steroids. Because of cytokine-release syndrome, a clinical decision was made that five patients would not receive the third fraction of $1{\cdot}8\times10^6$ CAR-positive cells per kg. No cases of ICANS or late neurologic events were observed. None of the patients who received the booster dose had cytokine-release syndrome, ICANS, or any adverse events of special interest after the booster dose.

One mild infusion reaction and one moderate tumour lysis syndrome were reported (table 2). Prolonged cytopenias were reported in 20 (67%) of 30 patients (table 2). The median duration of grade 4 neutropenia was 35 days (95% CI 26–44) and time to complete resolution of cytopenias was 4 months (95% CI 3–5) for neutropenia, 12 months (6–18) for thrombocytopenia, and 3 months (1–13) for anaemia. All patients recovered without requiring a stem-cell boost. The overall safety profile is shown in table 3.

Infections were reported in 20 (67%) of 30 patients. 45 infectious episodes (seven [16%] grade ≥3) were reported, with most patients experiencing an average of 1–2 infection episodes (appendix p 13). Respiratory tract infections were the most common, with 24 (53%) episodes, including three (10%) of 30 patients having SARS-CoV-2 infections. Other relevant adverse events were a new diagnosis of colon adenocarcinoma, considered non-related to ARI0002h, and one reactivation of hepatitis B virus, considered related to ARI0002h. Three cases of macrophage activation syndrome occurred, two of them in combination with a grade 1 cytokine-release syndrome; all cases resolved completely.

Overall response rate during the first 100 days from infusion was 100%, including 24 (80%) of 30 patients with a very good partial response or better (15 [50%] with complete response, nine [30%] with very good partial response, and six [20%] with partial response). Median time to complete response was 3·8 months (IQR 1·0–11·6). On day 28, measurable residual disease by next generation flow cytometry was evaluable in 22 (73%) of 30 samples, with 21 (95% [95% CI 78–99]) patients presenting a negative result (appendix p 14). On day 100, 24 (92% [95% CI 76–98]) of 26 evaluable samples were negative (appendix p 14).

In a post-hoc analysis using a data cutoff date of May 15, 2022 (median follow-up 18 months [IQR 15–20]), the overall response rate was 100% (95% CI 89–100) with 20 patients with complete response (67% [49–81]), eight with very good partial response (27% [14–44]), and two with partial response (7% [2–21]; figure 2A). 18 (60%) of 20 patients with complete response had a stringent complete response. Responses at month 6 were 14 (47% [95% CI 30–64]) of 30 patients with complete response, 11 (37% [22–55]) with very good partial response, two (7% [2–21]) with partial response, two (7% [2–21]) with progressive disease, and one (3% [1–17]) death without relapse. Responses at different timepoints are shown in the appendix (pp 15–16). Median time to best response was 3·3 months (95% CI 1·0–3·4), with some patients improving responses after 100 days (figure 2B). The differences in overall response rate at 100 days and at this follow-up (median 18 months) show that responses deepen over time, with five (25%) of 20 patients having a complete response after month 3 (100 days), and five (17%) of 30 patients having their best response after 6 months (one converted to very good partial response and four converted to complete response; figure 2B). 20 (80%) of 25 evaluable patients were negative for measurable residual disease at 6 months, and 16 (80%) evaluable patients were negative for measurable residual disease at 12 months (appendix p 14).

14 (58% [95% CI 30–76]) of 24 patients who received the booster dose had a stringent complete response before reinfusion, six (25% [12–45]) patients maintained the response after reinfusion (all very good partial response) and four (17% [7–36]) patients had an improved response after the booster dose, two patients with very good partial response and two with partial response converted to complete response (figure 2C).

At the May 15, 2022, data cutoff date, median duration of response was not reached (95% CI 12·9–not reached; figure 3A) and median overall survival was not reached (8·0–not reached; figure 3B). Two patients died in response in months 4 and 9. 12-month overall survival was 86·5% (95% CI 75·1–99·7). Median progression-free survival was 14·5 months (95% CI 12·8–not reached; figure 3B). 14 (47%) patients had a progression-free survival event: 12 developed disease progression and two died without progression. 12-month progression-free survival was 70% (95% CI 55–89).

	Grade 1–2	Grade 3	Grade 4	Grade 5
Blood and lymphatic system disorders				
Anaemia	16 (53%)	2 (7%)	1 (3%)	0
Neutropenia	9 (30%)	8 (27%)	13 (43%)	0
Thrombocytopenia	13 (43%)	8 (27%)	6 (20%)	0
Asthenia	9 (30%)	0	0	0
Pyrexia	7 (23%)	2 (7%)	0	0
Oedema peripheral	3 (10%)	0	0	0
Febrile neutropenia	..	1 (3%)	0	0
Lymphocytosis	..	0	1 (3%)	0
Lymphopenia	..	0	1 (3%)	0
Gastrointestinal and hepatobiliary disorders				
Diarrhoea	5 (17%)	1 (3%)	0	0
Hepatobiliary disorders				
Hepatotoxicity	3 (10%)	0	0	0
Immune system disorders				
Cytokine release syndrome	24 (80%)	0	0	0
Haemophagocytic lymphohistiocytosis	3 (10%)	0	0	0
Respiratory tract infection	3 (10%)	0	0	0
Upper respiratory tract infection	6 (20%)	0	0	0
Investigations				
Hypocalcaemia	7 (23%)	0	0	0
Hypomagnesaemia	6 (20%)	0	0	0
Headache	5 (17%)	0	0	0
Back pain	4 (13%)	0	0	0
Alanine aminotransferase increased	4 (13%)	1 (3%)	0	0
Aspartate aminotransferase increased	3 (10%)	1 (3%)	0	0
Blood lactate dehydrogenase increased	3 (10%)	0	0	0
Gamma-glutamyltransferase increased	3 (10%)	0	0	0
Hypokalaemia	3 (10%)	0	0	0
Psychiatric disorders				
Anxiety	3 (10%)	0	0	0
Infections and infestations				
COVID-19	..	0	0	1 (3%)
Leishmaniasis	..	1 (3%)	0	0
Rhinovirus infection	..	1 (3%)	0	0
Septic shock	..	1 (3%)	0	0
Severe acute respiratory syndrome	..	1 (3%)	0	0
Staphylococcal bacteraemia	..	1 (3%)	0	0
Injury; poisoning and procedural complications				
Head injury	..	0	0	1 (3%)
Nervous system disorders				
Seizure	..	0	1 (3%)	0
Renal and urinary disorders				
Acute kidney injury	..	1 (3%)	0	0
Vascular disorders				
Hypertension	..	1 (3%)	0	0

For grades 1–2, only adverse events with an occurrence of 10% or more are shown. For grades 3–5, all adverse events are shown. Adverse events are reported according to Common Terminology Criteria of Adverse Events (version 5.0).

Table 3: **Adverse events (n=30)**

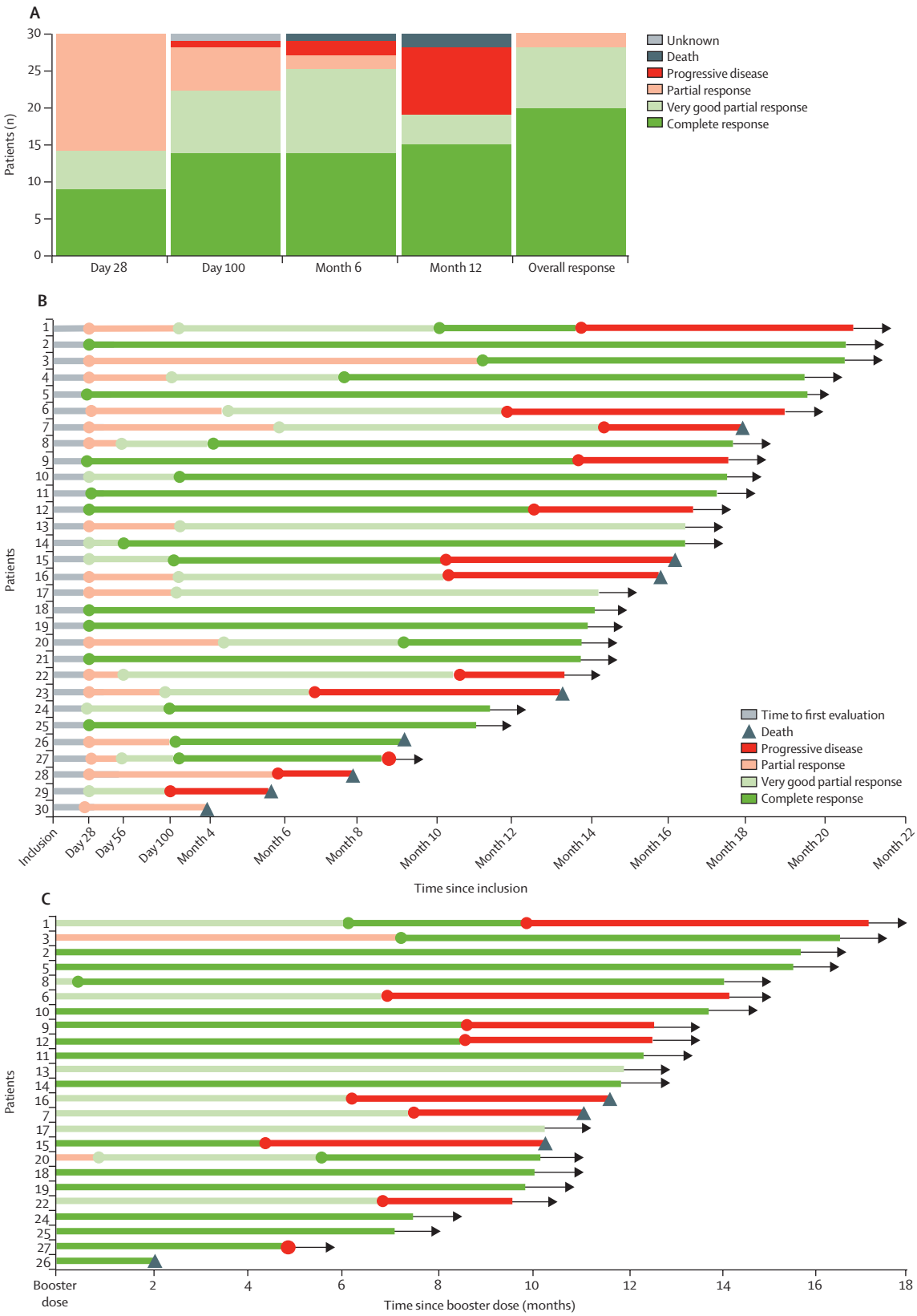

Figure 2: **Activity of ARI0002h**
(A) Overall response rate and response evaluation at consecutive timepoints. (B) Swimmer plot with the response of each individual patient after first infusion (n=30). Data cutoff was May 15, 2022. (C) Swimmer plot with the response of each patient after booster dose (n=24).

A PET-CT scan at day 100 showed a metabolic response in 13 (93% [95% CI 69–98) of 14 patients with a plasmacytoma at baseline. The six patients with plasmacytomas who had disease progression died, and all patients without plasmacytomas who had disease progression were alive at data cutoff.

ARI0002h detection in peripheral blood by PCR showed a median persistence of 5·0 months (95% CI 3·8–6·2). 15 (52%) of 29 patients with available samples on day 100, seven (28%) of 25 with available samples at month 6, and four (20%) of 20 with available samples at month 12 had detectable CAR T cells in peripheral blood (appendix p 19). The peak of expansion was observed on day 14 for most patients (range 7 days to 6 months; appendix p 20). Mean copies per genome at the peak of expansion were 11·1 (SD 14·2). Nine (75%) of 12 patients with an available sample at relapse, three (33%) still had detectable CAR T cells in peripheral blood.

After the booster dose, 12 (50%) of 24 patients presented a low-grade expansion of CAR T cells immediately after administration, with a mean peak of 4·0 copies per genome (SD 9·2; appendix p 20). In a post-hoc analysis, we found no correlation between previous lymphodepletion and expansion of CAR T cells (three [38%] of eight patients receiving lymphodepletion expanded *vs* nine [56%] of 16 without lymphodepletion; p=0·67).

The mean number of BCMA molecules per cell on malignant bone marrow plasma cells by flow cytometry at inclusion was 1306·5 (SD 889·1). The change in the number of BCMA molecules per cell in six paired samples available at relapse showed a decrease from mean 1784 (1229·8) molecules per cell to 1001·5 (916·1) molecules per cell (p=0·054). None of the six samples had a complete loss of BCMA expression. Soluble BCMA was detectable in peripheral blood of all patients at inclusion with a mean of 89·3 ng/mL (SD 124·6). A significant decrease was observed in all patients on days 28 and 100 (p<0·0025 at both timepoints), and the 12 patients who relapsed had detectable soluble BCMA at the end-of-treatment sample (appendix p 21).

Positivity of human anti-human antibodies was not sustained for each individual patient (appendix p 22). We detected human anti-human antibodies in 21 (70%) of 30 patients (appendix p 22). Eight (25%) of 12 patients who relapsed had available human anti-human antibody measurements, of whom only two (25%) were positive.

In a post-hoc analysis, median vein-to-vein time was 43 days (IQR 35–54), with differences among patients who did or did not receive bridging therapy (54 days [IQR 44–58] *vs* 36 days [IQR 30–43]; p=0·0006). We found no differences in response rates or progression-free survival between patients who received only the first two fractions of the first dose versus those who received the full first dose (complete response: three [60%] of five *vs* 17 [68%] of 25; p=0·73; median progression-free survival: 14·5 months [95% CI 12·8–not reached] *vs* not reached [12·1–not reached]; p=0·83; post-hoc analysis). In a post-hoc

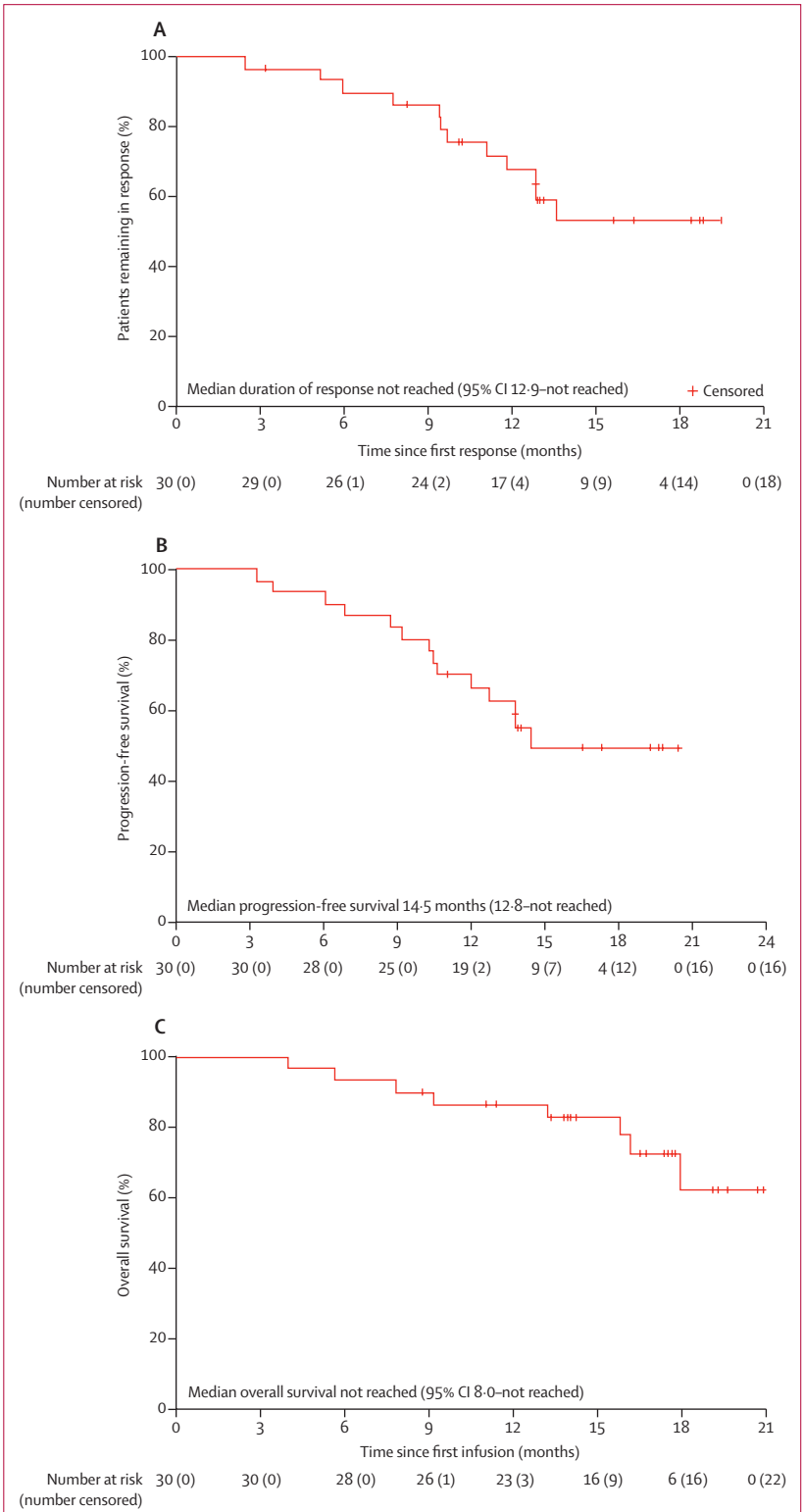

***Figure 3*: Duration of response and survival of patients treated with ARI0002h**
(A) Duration of response (n=30). (B) Progression-free survival. (C) Overall survival. Median follow-up for survival was 18 months (IQR 15–20). Data cutoff was May 15, 2022.

analysis, median duration of response in patients who had a complete response was not reached (95% CI not reached–not reached) versus 9·7 months (6·0–not reached) in those who did not have a complete response (p=0·0041; appendix p 17). No differences in terms of progression-free survival or overall survival were detected according to the presence or absence of plasmacytomas at inclusion (post-hoc analysis; appendix p 18).

Discussion

CAR T-cell therapy is a promising option for patients with heavily treated relapsed or refractory multiple myeloma. Here, we showed that ARI0002h, a CAR T-cell therapy developed in academia and administered in a fractioned manner with the option of adding a booster dose after day 100, can provide deep and sustained responses with low-grade toxicity in patients with a poor prognosis. In this study, all treated patients had at least a partial response, with good results in terms of measurable residual disease negativity as early as day 28 after treatment. The development of cytokine release syndrome with ARI0002h was similar to that of other BCMA-CAR T-cell therapies, showing a lower grade of severity, with none grade 3 or above and no cases of ICANS or late neurotoxicity. Therefore, ARI0002h might be a reasonable alternative to other BCMA CAR T-cell constructs,[3] including the already approved idecabtagene vicleucel and ciltacabtagene autoleucel. Still, this study has methodological limitations in terms of design and analysis, including the small sample size, leading to wide 95% CIs.

We attribute the lower incidence of immune-related side-effects, especially severe cases, to the fractionation of the first dose, as previously observed with ARI0001 in CD19-positive malignancies.[12] Other factors, such as construct features, manufacturing process, and total cell-dose administered might also play an important role in safety. Patients who received only two fractions of the first dose showed similar outcomes compared with those receiving the full dose. Patients who received only two fractions of the first dose developed an early cytokine-release syndrome, probably reflecting a fast in vivo expansion of the CAR T-cell therapy, which might explain the similar outcome to that of those who received the full first dose. Prolonged cytopenias and infections were a common adverse event, with a similar profile to that reported in other BCMA-CAR T-cell studies.[6,9] A study[9] of ciltacabtagene autoleucel reported infections in 56 (58%) of 97 patients and a retrospective analysis showed 47 infection events in 29 (53%) of 55 patients after BCMA CAR T-cell therapy.[19]

Some patients with multiple myeloma might have soft-tissue involvement in the form of paraskeletal or extramedullary plasmacytomas. The treatment of these patients is a highly unmet need because available treatments have poor efficacy.[20] The progression-free survival of patients treated with ARI0002h according to soft-tissue involvement at inclusion did not differ significantly, highlighting the positive effect that CAR T-cell therapy might have in this subgroup of patients with multiple myeloma.[6,21]

The incorporation of a second infusion of ARI0002h 100 days after the first administration was meant to deepen and lengthen responses. No relevant side-effects were observed and patients were rapidly discharged; therefore, an outpatient or at-home management could be evaluated.[22,23] In terms of activity, four out of ten patients with measurable disease had an improved response after the booster dose. Because there was no comparator group in this trial, we cannot affirm that these results are only attributable to the second infusion. In other BCMA-CAR T-cell trials,[6,9] a deepening of responses over time has been reported. However, median time to best response reported in the ciltacabtagene autoleucel study was 2·6 months,[9] suggesting that most patients had the deepest response within the first 3 months. Although the booster dose might play a role in improving and lengthening responses, this association is difficult to establish in this non-randomised trial, especially as 14 of 24 patients already had complete responses at the time they received the booster dose. The feasibility in terms of manufacturing, the potential benefits in response depth and the absence of related side-effects warrants further investigation of the booster dose. An earlier administration and a randomised study exploring this feature could be helpful to clarify the efficacy of this approach.

Despite promising results, patients continued to relapse after treatment with ARI0002h; therefore, strategies to overcome relapses are of pertinent interest. Correlative studies done on patient samples highlight different mechanisms that might be responsible for relapse after ARI0002h treatment. A median persistence of CAR T cells in peripheral blood of 5 months, although similar to that of other available BCMA-CAR T cells, is still short (idecabtagene vicleucel reported persistence in 59% of patients at 6 months and 36% at 12 months and most ciltacabtagene autoleucel patients had transgene concentrations below the threshold of quantification at 6 months).[6,9] The short duration of CAR T-cell persistence in peripheral blood contrasts with long response durations in terms of progression-free survival. BCMA quantification on malignant plasma cells decreased from baseline levels in most patients, but no patients became BCMA negative and soluble BCMA was positive in all patients at relapse. Finally, several patients had detectable CAR T cells at relapse, suggesting that CAR T-cell exhaustion with increase of some surface markers[24] could play a role in shortening duration of responses.

Demand for CAR T-cell therapy is increasing, and it will presumably be introduced into earlier lines of treatment.[25] However, high costs, manufacturing delays, and potential bottlenecks of centralised, industry-driven CAR T-cell production can limit access to this therapy.[26–28] Additionally, despite the European Commission authorisation for

idecabtagene vicleucel and ciltacabtagene autoleucel, the products are not available in Spain and several other European countries because no reimbursement agreement exists. ARI0002h is an academic CAR T-cell therapy that has been manufactured in two facilities in Spain, being a real point-of-care manufacturing approach in these two centres (Barcelona and Pamplona). This approach can increase the number of slots available for production, allowing a fast release when required by the clinical situation of the patient; in our study, this was as short as 19 days. ARI0002h can be manufactured at up to a quarter of the cost of that of commercial CAR T cells. For these reasons, we believe that CAR T cells produced at academic institutions will become essential to improve access around the world.

Contributors

AO-C, BM-A, LR, NT, RJ, SV, JS-P, JD, MJ, MP, AU-I, and CFdL participated in conception and design of the work. AO-C and ME-R participated in the writing of the manuscript draft. AO-C, VG-C, VC, PR-O, JLR, LL-C, AZ, BP, SI, AL-DdC, ML-P, AS-S, JAP-S, FP, JMM, and M-VM provided data and figures. AO-C, VG-C, VC, PR-O, JLR, L-LC, AZ, BP, SI, AL-DdC, ML-P, and CFdL contributed to data collection. AO-C, ME-R, AZ, BP, SI, AL-DdC, LGR-L, SV, and JS-P performed data analysis and interpretation. AOC and CFdL have verified the data. All authors revised the article and gave approval of the final version to be published.

Declaration of interests

AO-C declares support for attending meetings from Janssen. VG-C declares receiving honoraria from Janssen, Pfizer, Bristol Myers Squibb (BMS)/Celgene, and GSK; support for attending meetings or travel from Janssen and GSK; and participation on data safety monitoring or advisory board from Janssen. VC declares receiving honoraria from Janssen, BMS, Sanofi, GSK, and Amgen; support for attending meetings or travel from Janssen; and participation on data safety monitoring or advisory board from Janssen, Sanofi, and Amgen. PR-O declares receiving honoraria from consulting activities from BMS, Janssen, Sanofi, Abbvie, Pfizer, Roche, and GSK; honoraria from lectures from BMS, Janssen, Sanofi and GSK; support for attending meetings or travel from Abbvie; and participation on data safety monitoring or advisory board from Janssen. JLR declares receiving consulting fees from Janssen; honoraria from Janssen, Amgen, Sanofi, Kite/Gilead, Novartis, and BMS; support for attending meetings or travel from Kite/Gilead; and participation on data safety monitoring or advisory board from Janssen, BMS, and Novartis. LL-C declares receiving honoraria from Kite/Gilead, Celgene, Janssen, and Novartis; support for attending meetings or travel from Kite/Gilead, Celgene, Janssen, and Novartis; and participation on data safety monitoring or advisory board from Janssen. BM-A declares to be co-inventor in the patent of ARI0002. LR declares honoraria from Janssen, BMS/Celgene, Amgen, Takeda, Sanofi, and GSK; participation on data safety monitoring or advisory board of Janssen, BMS-Celgene, Amgen, Takeda, Sanofi, and GSK. ML-P declares receiving consulting fees from Celgene/BMS; honoraria from Janssen and Kite/Gilead; and participation on data safety monitoring or advisory board from Celgene/BMS and Novartis. LGR-L declares honoraria from Janssen, GSK, Sanofi, and BMS; travel grants from Janssen, Amgen, GSK, Pfizer and Sanofi; and participation on data safety monitoring or advisory board from GSK and Sanofi. AS-S declares receiving travel grants from Jazz Pharmaceuticals, Pfizer, and MSD. JAP-S declares research and travel support Takeda, Abbvie, Gilead, AMGEN, Jazz, Alexion, Pierre Fabre and Beigene; educational activities, speaker, and advisory fees with Gilead, Jazz, Alexion, AMGEN, Novartis, Janssen, BMS, and MSD; and participation on data safety monitoring or advisory board from Gilead, Jazz, Alexion, AMGEN, Novartis, Janssen, BMS and MSD. BP reports research funding from BMS, GSK, Roche, Beigene, and Sanofi; consultancy fees from BMS, GSK, Janssen, Sanofi and Takeda; honoraria from Adaptive, Amgen, Becton Dickinson, BMS/Celgene, GSK, Janssen, Sanofi, and Roche; and support for attending meetings from GSK. FP declares receiving grants from the Spanish Ministry of Health (ISCIII) and the Government of Navarra; honoraria from Janssen, Oryzon, Dialectica, Novartis, Instituto Roche, Servier, ViviaBiotech, and Techspert; support for attending meetings or travel from Gilead, Celgene, and Janssen; and a leadership or fiduciary role in RICORS Terav and IDISNA. MJ declares receiving research or travel support by Fundació Bancaria la Caixa, ISCIII, and CellNex Teleom; honoraria from educational activities, speaker, and advisory roles with Miltenyi and indirectly with sponsors of congresses; and participation on data safety monitoring or advisory boards with MAB Gyala. JMM declares receiving honoriaria from Gilead/Kite, Novartis, BMS/Celgene, and Roche; travel grants, accommodation, and expenses from Jazz Pharma, Gilead-Kite, Janssen, and Sandoz; and consulting or advisory board fees in Jazz Pharma, Novartis, and Sandoz. M-VM declares honoraria derived from lectures and participation in advisory boards from Janssen, BMS/Celgene, Takeda, Amgen, GSK, Pfizer, Regeneron, Roche, and Sanofi. MP declares receiving honoraria from Thermofisher Scientific and LetiPharma. AU-I declares receiving grants from the Spanish Ministry of Health (ISCIII); honoraria for lectures from BMS and Gilead; support for attending meetings from Amgen; 23% of participation in ARI-002 patent ARI-001 patent in preparation; advisory board fees and participation in Miltenyi biomedicine; being coordinator of the Spanish group of CAR T-cell therapy. CFL declares receiving grants through his institution from BMS, Janssen, and Amgen; honoraria from Amgen, Janssen, BMS, GSK, and Sanofi; support for attending meetings or travel from Janssen, BMS, GSK, and Amgen; and participation in data safety monitoring or advisory boards with Janssen, BMS, Amgen, Pfizer, and Sanofi. All other authors declare no competing interests.

Data sharing

Individual, de-identified participant data and data dictionary can be made available, with publication, at the request of qualified investigators whose proposed use of the data has been approved by IDIBAPS, after a data access agreement is signed. Requests can be made to the corresponding author. Additional related documents are available at https://clinicaltrials.gov/ct2/show/NCT04309981.

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Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Oliver-Caldés A, González-Calle V, Cabañas V, et al. Fractionated initial infusion and booster dose of ARI0002h, a humanised, BCMA-directed CAR T-cell therapy, for patients with relapsed or refractory multiple myeloma (CARTBCMA-HCB-01): a single-arm, multicentre, academic pilot study. *Lancet Oncol* 2023; published online July 3. https://doi.org/10.1016/S1470-2045(23)00222-X.

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Summary of the study protocol and eligibility criteria

<table>
<tr><td colspan="2">Sponsor’s Name:
Institut D’Investigacions Biomèdiques Agustí Pi i Sunyer (IDIBAPS).
C/Rosselló 149-153 08036 Barcelona, Spain.
Telephone number: 93 2275400.</td></tr>
<tr><td colspan="2">Final product name:
ARI0002h cells. CARTBCMA_J22.9-h:CD8TM:4-1BB:CD3.</td></tr>
<tr><td colspan="2">Investigational Medicinal Product (IMP)
ARI0002h cells. CARTBCMA_J22.9-h:CD8TM:4-1BB:CD3.
Adult differentiated autologous T-cells from peripheral blood, expanded and transduced with a lentivirus to express a chimeric antigen receptor with anti-BCMA (TNFRSF17) specificity conjugated to the 4-1BB co-stimulatory region and signal-transduction CD3z that has been humanized.</td></tr>
<tr><td>Study title:</td><td>Pilot study of the infusion of differentiates autologous T-cells from peripheral blood, expanded and transduced with a lentivirus to express a chimeric antigen receptor with anti-BCMA (TNFRSF17) specificity humanized conjugated with the co-stimulatory region 4-1BB and signal-transduction CD3z (ARI0002h) in patients with relapsed/refractory multiple myeloma with at least two prior line including proteasome inhibitor, immunomodulatory drug and anti-CD38 monoclonal antibody.</td></tr>
<tr><td>Investigator’s team:</td><td>1-Chief investigator (coordinator):
Carlos Fernández de Larrea
2-Principal investigators at participating sites (Cohort 1):
Carlos Fernández de Larrea - Hospital Clínic de Barcelona
Paula Rodríguez Otero- Clínica Universitaria de Navarra
Juan Luis Reguera Ortega- Hospital Virgen de Rocío
Mª Victoria Mateos- Instituto de investigación Biomédica de Salamanca
José Mª Moraleda- Hospital U. Virgen de la Arrixaca</td></tr>
<tr><td>Participating sites:</td><td>1. Hospital Clínic de Barcelona c/ Villarroel, 170 08036 Barcelona
2. Clínica Universidad de Navarra Av. De Pío XII, 36 31008 Pamplona
3. Hospital Virgen del Rocío, Av. Manuel Siurot, s/n 41013 Sevilla
4. Instituto de investigación Biomédica de Salamanca Paseo de San Vicente, 58-182 37007, Salamanca
5. Hospital Universitario Virgen de la Arrixaca, Crta. Madrid-Cartagena, s/n, 30120 El Palmar, Murcia</td></tr>
<tr><td colspan="2">Study population:
Patients with relapsed/refractory multiple myeloma who have received treatment with at least 2 prior lines including proteasome inhibitor, immunomodulatory drug and anti-CD38 monoclonal antibody.</td></tr>
<tr><td colspan="2">Phase and clinical trial design:
First-in-human, pilot, open, non-randomised, single-arm, prospective, national, multicentre, clinical trial.</td></tr>
<tr><td colspan="2">Primary Objective:</td></tr>
</table>

To assess the safety and efficacy of CARTBCMA ARI0002h in patients with relapsed/refractory multiple myeloma who have received treatment with at least two prior lines including proteasome inhibitor, immunomodulatory drug and anti-CD38 monoclonal antibody.

Secondary objectives:

- To evaluate the effectiveness of ARI0002h
- To assess the duration of the response after the administration of ARI0002h
- To evaluate the overall survival after the administration of ARI0002h
- To evaluate the persistence of ARI0002h cells in peripheral blood after administration
- To assess the effect of ARI0002h treatment on the quality of life of patients
- To evaluate the adverse events occurring at 3 months and at one year

Primary Endpoints:

- **Efficacy primary endpoint:**

Overall response rate (ORR) in the first 3 months of the first infusion (defined as at least achieve partial response according to the criteria of the International Myeloma Working Group).

- **Safety primary endpoint:**

Rate of patients who develop a cytokine release syndrome and/or neurological toxicity in the first 30 days after the administration of ARI0002h, according to the criteria and gradation defined in the international consensus (Lee, Santomasso et al., 2019).*

Secondary Endpoints:

- Duration of the response (see criteria for second dose of ARI0002h)
- Response rate over the first year
- Complete response rate (CR) at 3 and 6 months after the first infusion.
- Overall response rate (ORR) at 6 months after the first infusion.
- Time to complete response.
- Time to better response.
- Negative measurable residual disease (MRD) rate in bone marrow at 3 and 6 months.
- Response rate of extramedullary disease by PET-TC at 3 months.
- Progression free survival (PFS), defined as the time elapsed between the administration of ARI0002h and the progression of the disease or death. Patients who are alive and in complete remission will be censored at the time of the last follow-up.
- Progression free survival at 12 months after the first infusion, defined as the time elapsed between the administration of ARI0002h and the progression of the disease or death. Patients who are alive and in complete remission at 12 months will be censored at the time of the last follow-up.
- Overall survival (OS) defined as the time elapsed between the infusion of ARI0002h and the death of the patient from any cause. Live patients will be censored at the time of the last follow-up.
- Presence of infusion reactions.
- Tumour lysis syndrome at any time after the administration of the treatment.
- Cytokine release syndrome, according to the criteria and grades defined in the international consensus (Lee, Santomasso et al., 2019).
- Neurological toxicity.
- Presence of prolonged cytopenia, defined as the reduction of neutrophil or platelet peripheral blood counts, grade 3 or 4 for more than 4 weeks after infusion.
- Persistence of CART BCMA ARI0002 in peripheral blood, which will be determined by flow cytometry and quantitative PCR of the transgene with monthly periodicity in the first 6 months and subsequently quarterly until 2 years after infusion.
- Expression of BCMA at diagnosis and at relapse, evaluated by multiparameter flow cytometry in bone marrow aspirate samples.
- Levels of soluble BCMA in serum pre-treatment and during treatment and its correlation with the degree of response of multiple myeloma.
- Quality of life at baseline, monthly basis the first 6 months and quarterly until 2 years from

infusion.

Inclusion Criteria (main):

1. Patients between the age of 18 and 75 years with diagnosis of multiple myeloma.
2. Disease measurable** by monoclonal component in serum and/or urine or by free light chains in serum according to the eligibility criteria for clinical trials of the International Myeloma Working Group.
3. Previous two or more lines of treatment. Patients must have received at least a proteasome inhibitor (such as bortezomib or carfilzomib), an immunomodulatory drug (lenalidomide or pomalidomide) and an anti-CD38 monoclonal antibody (such as daratumumab).
4. Refractory to the last line of treatment.
5. ECOG functional status ranging from 0 to 2.
6. Life expectancy over 3 months.
7. Patients who, after being informed, give their consent by signing the Informed Consent document.

Exclusion Criteria (main):

1. Previous allogeneic transplant in the prior 6 months to inclusion or GVHD that requires active systemic immunosuppressive treatment.
2. Previous treatment with CAR T-cell therapy or BCMA directed therapy.
3. Absolute lymphocyte count $<0.1x10^9$/L.
4. Previous neoplasia, except if patients have been in complete remission >3 years, except for cutaneous carcinoma (non-melanoma).
5. Active infection that requires treatment.
6. Active infection by HIV, HBV or HCV.
7. Uncontrolled medical disease.
8. Severe organic condition that meets any of the following criteria: EF 3 times normal value (except Gilbert syndrome).
9. Previous diagnosis of symptomatic AL amyloidosis.
10. Pregnant or lactating women. Women of childbearing age should have a negative pregnancy test in the screening phase.
11. Women of childbearing age, including those whose last menstrual cycle was in the year prior to screening, who cannot or do not wish to use highly effective contraceptive methods from the beginning until the end of the study.
12. Men who cannot or do not wish to use highly effective contraceptive methods from the beginning to the end of the study.
13. Contraindication to receive conditioning chemotherapy.

*The following safety endpoints were considered adverse events of special interest (AESI): CRS, ICANS, infusion reaction, tumour lysis syndrome and persistent cytopenia.

**Measurable disease: serum (10 g/L) or urine (200mg/24h) monoclonal protein, or free light chain (FLC) (100 mg/L) of the involved FLC.

Table 1. List of sites from which patients were recruited, names of the principle investigators responsible for each site and number of patients which were recruited from that particular site.

Centre	**Principal Investigator**	**Number of patients treated**
Hospital Clínic de Barcelona	Carlos Fernández de Larrea	12/30
Instituto de investigación Biomédica de Salamanca	Mª Victoria Mateos	8/30
Hospital Universitario Virgen de la Arrixaca	José Mª Moraleda	5/30
Clínica Universidad de Navarra	Paula Rodríguez Otero	3/30
Hospital Virgen del Rocío	Juan Luis Reguera Ortega	2/30

Supplementary methods

Study procedures:

To undergo apheresis a minimal peripheral blood lymphocyte absolute number of $0{\cdot}1x10^9$/L was required.

Bridging therapy was allowed in the period between apheresis and LD in patients with a clinical requirement for treatment and was chosen according to the previous treatments and local centre criteria. Forty-seven percent (n=14) received bridging therapy mostly based on carfilzomib (n=5; 36%) and pomalidomide (n=4; 29%). Other regimens included cyclophosphamide (n=1), polychemotherapy (n=2), lenalidomide (n=1) and lenalidomide plus carfilzomib (n=1).

ARI0002h cells were manufactured using the CliniMACS Prodigy system (Miltenyi Biotec). Approximately 200×10^6 T-cells were activated with anti-CD3 and anti-CD28 antibodies (MACS GMP T Cell TransAct) together with IL-7 and IL-15. After 24-48 hours of T-cell stimulation, cells were transduced with a lentiviral vector containing the ARI0002h CAR gene construct at a multiplicity of infection of ≈ 10. The cells are expanded in a period that varies between 7 and 12 days. In the end, the final product is washed, cryopreserved and tested for identity, potency, sterility, and adventitious agents to ensure it fulfils the required specifications.

Endpoints:

Responses were assessed according to IMWG criteria as follows:

Standarg IMWG response criteria	
Stringent complete response	Complete response as defined below plus normal FLC ratio and absence of clonal cells in bone marrow biopsy by immunohistochemistry (κ/λ ratio ≤4:1 or ≥1:2 for κ and λ patients, respectively, after counting ≥100 plasma cells)
Complete response	Negative immunofixation on the serum and urine and disappearance of any soft tissue plasmacytomas and **<**5% plasma cells in bone marrow aspirates
Very good partial response	Serum and urine M-protein detectable by immunofixation but not on electrophoresis or ≥90% reduction in serum M-protein plus urine M-protein level
Partial response	≥50% reduction of serum M-protein plus reduction in 24 h urinary M-protein by ≥90% or to <200 mg per 24 h; If the serum and urine M-protein are unmeasurable, a ≥50% decrease in the difference between involved and uninvolved FLC levels is required in place of the M-protein criteria; If serum and urine M-protein are unmeasurable, and serum-free light assay is also unmeasurable, ≥50% reduction in plasma cells is required in place of M-protein, provided baseline bone marrow plasma-cell percentage was ≥30%. In addition to these criteria, if present at baseline, a ≥50% reduction in the size (SPD) of soft tissue plasmacytomas is also required
Minimal response	≥25% but ≤49% reduction of serum M-protein and reduction in 24-h urine M-protein by 50–89%. In addition to the above listed criteria, if present at baseline, a ≥50% reduction in the size (SPD) of soft tissue plasmacytomas is also required
Stable disease	Not recommended for use as an indicator of response; stability of disease is best described by providing the time-to-progression estimates. Not meeting criteria for complete response, very good partial response, partial response, minimal response, or progressive disease
Progressive disease	Any one or more of the following criteria: Increase of 25% from lowest confirmed response value in one or more of the following criteria: Serum M-protein (absolute increase must be ≥0·5 g/dL); Serum M-protein increase ≥1 g/dL, if the lowest M component was ≥5 g/dL; Urine M-protein (absolute increase must be ≥200 mg/24 h); In patients without measurable serum and urine M-protein levels, the difference between involved and uninvolved FLC levels (absolute increase must be >10 mg/dL); In patients without measurable serum and urine M-protein levels and without measurable involved

	FLC levels, bone marrow plasma-cell percentage irrespective of baseline status (absolute increase must be ≥10%); Appearance of a new lesion(s), ≥50% increase from nadir in SPD of >1 lesion, or ≥50% increase in the longest diameter of a previous lesion >1 cm in short axis; ≥50% increase in circulating plasma cells (minimum of 200 cells per μL) if this is the only measure of disease
Clinical relapse	Clinical relapse requires one or more of the following criteria: Direct indicators of increasing disease and/or end organ dysfunction (CRAB features) related to the underlying clonal plasma-cell proliferative disorder. It is not used in calculation of time to progression or progression-free survival but is listed as something that can be reported optionally or for use in clinical practice; Development of new soft tissue plasmacytomas or bone lesions (osteoporotic fractures do not constitute progression); Definite increase in the size of existing plasmacytomas or bone lesions. A definite increase is defined as a 50% (and ≥1 cm) increase as measured serially by the SPD of the measurable lesion; Hypercalcaemia (>11 mg/dL); Decrease in haemoglobin of ≥2 g/dL not related to therapy or other non-myeloma-related conditions; Rise in serum creatinine by 2 mg/dL or more from the start of the therapy and attributable to myeloma; Hyperviscosity related to serum paraprotein

*Adapted from Kumar S, Paiva B, Anderson KC, et al. International Myeloma Working Group consensus criteria for response and minimal residual disease assessment in multiple myeloma. Lancet Oncol 2016; **17**: e328–46.*

Sample processing techniques:

Measurable residual disease (MRD)

Measurable residual disease (MRD) was assessed at baseline, day 28, day 100, months 6, 12, 18 and 24 by Next Generation Flow (NGF). Evaluation was performed using the 2-tub 8-color according to the EuroFlow platform (complete protocol can be found in the public protocol section from euroflow webpage: app.euroflow.org/downloads/public - protocol 1·3), using a FACSCanto^{TM} (BD Biosciences, La Jolla, CA, United States) flow cytometer and Infinicyt 2.0 software (Cytognos SL, Salamanca, Spain). Tube 1 included CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-PerCPCy5.5, CD19-PECy7, CD117-APC, and CD81-APCH7 monoclonal antibodies. In tube 2, the CyIgKappa-APC and CyIgLambda-APCH7 were included instead of CD117-APC and CD81-APCH, respectively. In those samples with MRD positive status, BCMA expression on MM tumour cells was reevaluated by the replacement of CD56-PE by BCMA-PE. The limit of detection of NGF is close to 10^{-6}, while the limit of quantification is of $<5 \times 10^{-6}$.

BCMA expression and soluble BCMA

BCMA expression on bone marrow plasma cells was measured by flow cytometry (PE anti-human CD269 (BCMA) Antibody; Cat #: 357504, Biolegend) at baseline and in MRD positive disease. Molecules of BCMA were quantified using the BD QuantiBRITE^{TM} Beads (molecules/cell).

The presence of soluble BCMA was determined longitudinally on serum samples by enzyme-linked immunosorbent assay (ELISA) from R&D Systems, Minneapolis, MN, USA (catalogue # DY193E), as previously described. This ELISA-based method has a sensitivity 93.2% (95% confidence interval [CI]: 84−100%), specificity of 76.4% (95% CI: 67.4−85.3%), and an area under the curve of 0.8805 [15]. Serum samples were thawed and diluted 1:200. The ELISA assay was carried out according to the manufacturer's protocol and the plates were analysed using an EPOCH microplate spectrophonometer (Bio-tek Industries, Winooski, VT, USA) plate reader set to450 nm with BioTek Gen5 Data Analysis Software.

Peak and persistence of ARI0002h

Kinetics of ARI0002h in the peripheral blood was measured by quantitative polymerase chain reaction (qPCR) determining the time course of vector transgene WPRE to elucidate the amount of ARI0002h copies per cell. Blood samples were processed to obtain genomic DNA using MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche, catalogue # 03 730 964 001) on the MagNA Pure Compact System (Roche). Number of transgene copies per cell was determined by quantitative real-time PCR using Light

Cycler® 480 SYBRGreen® I Master (Roche, catalogue # 04707516001). Pairs of primers were designed against the GATA2 gene (control) and WPRE sequence (part of the transgene).

Primer sequences are as follows: GATA2_F: 5'tggcgcacaactacatggaa 3'; GATA2_R: 5'cgagtcgaggtgattgaagaaga 3'; WPRE_F: 5'gtcctttccatggctgctc 3'; WPRE_R: 5'ccgaagggacgtagcaga 3'. Absolute quantification method was used to determine copy number. Standard curves were prepared using 1:10 serial dilutions of plasmids containing GATA2 or transgene. Final number of molecules in the reaction ranged from 108 to 102 molecules. For GATA2 quantification, GATA2 cDNA was cloned in a pCRII-Topo vector (Invitrogen). pCCL-CAR19 vector was used in the same way to quantify transgene copy number. The following PCR program was used: 1) Initial denaturalization: 95oC, 5'; 2) 40 cycles of: 95oC, 10''; 58oC, 10''; 72oC, 5''; 3) Melting curve.

Kinetics of ARI0002h in the bone marrow was measured by flow cytometry. Briefly, five 8-color combinations of mAbs were designed aiming at quantifying different subsets of CAR-T cells, subsetting them according to maturation stages, and determining the percentage of activation, cytotoxic and inhibitory receptors as described in supplementary table X. BCMA positive CAR-T cells were identified with a Biotin-SP-conjugated AffiniPure Goat anti-mouse IgG, F(ab´)2 fragment specific (Jackson Immunoreserach Laboratories) and BV421 Streptaividin antibody (BD).

Human anti-human antibodies (HAHA)

Inmunogenicity against ARI0002h was evaluated by flow cytometry on Attune Next Flow Cytometer (Invitrogen, Thermofisher Scientific) to determine the presence of human anti-human antibodies (HAHAs).
HEK293T cell line were transduced with a lentivirus containing ARI0002h plasmid (MOI 2) for 48 hours at 37^{o}C in DMEM-10% FBS supplemented with polybrene (Millipore TR-1003-G). ARI0002h expression was checked by flow cytometry using human BCMA protein fused with human Fc (Enzo, ALX-522-026-C050) and anti-human IgG-BV421 (Biolegends, catalog #409318). These HEK293T transduced cells are incubated with patient's serum samples and the presence of HAHAs is revealed with anti-human IgG-FITC antibody (Life technology, H10101C). The threshold to determine the presence of HAHAs is 20%.

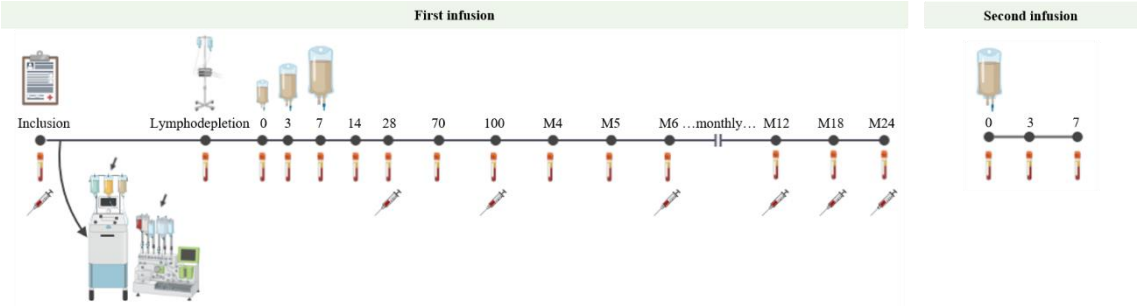
Panel	Antigen	Label	Clone	Ref. #	Company
Quantification	CAR-T	BV421			
	CD45	OC515	GA90	CYT-45OC	Cytognos
	CD25	FITC	2A3	345796	BD
	CD127	PE	A019D5	351304	Biolegend
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	TCRgd	PECy7	11F2	655410	BD
	CD19	PECy7	J3-119	IM3628	BC
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Subsetting	CAR-T	BV421			
	CD45	OC515	GA90	CYT-45OC	Cytognos
	CD62L	FITC	SK11	347443	BD
	CD27	PE	L128	340425	BD
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	CD45RA	PECy7	HI100	304126	Biolegend
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Inhibition	CAR-T	BV421			
	CD45	OC515	GA90	CYT-45OC	Cytognos
	TIM3	FITC	F38-2E2	345022	Biolegend
	TIGIT	PE	741182	FAB7898P	R&D
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	PD-1	PECy7	PD1.3	A78885	BC
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Cytotoxicity	CAR-T	BV421			

	CD45	OC515	GA90	CYT-45OC	Cytognos
	CD57	FITC	HNK-1	333169	BD
	cyGrzmB	PE	GB11	561142	BD
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	CD56	PECy7	NCAM16.2	335826	BD
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Activation/Inhibition	CAR-T	BV421			
	CD45	OC515	GA90	CYT-45OC	Cytognos
	HLADR	FITC	L243	347400	BD
	LAG3	PE	T47-530	565616	BD
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	CD39	PECy7	A1	328212	Biolegend
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD

Statistical analysis:

Descriptive statistics that accompanied all analyses were calculated according to standard methods: continuous variables (mean, SD, minimum, P25, median, P75, maximum, valid n), categorical variables (total column %, each category n), ordinal variables with few categories (less than 10) will be described using two tables: one including continuous variables descriptive parameters (whenever that their interpretation is reasonable) and the other including categorical variables descriptive parameters, ordinal variables with >10 categories, the same approximation used for continuous variables will be applied.

Figure 1. Sample extraction timeline to perform correlative studies. Peripheral blood (PB) samples are obtained at inclusion, lymphodepletion, days 0, 3, 7, 14, 28, 70, 100, months 4, 5, 6, 7, 8, 9, 10, 11, 12, 18 and 24. Extra PB samples are obtained at days 0, 3 and 7 after second infusion, if administered. Bone marrow samples are obtained at inclusion, day 28, day 100, month 6, month 12, month 18 and month 24**.**



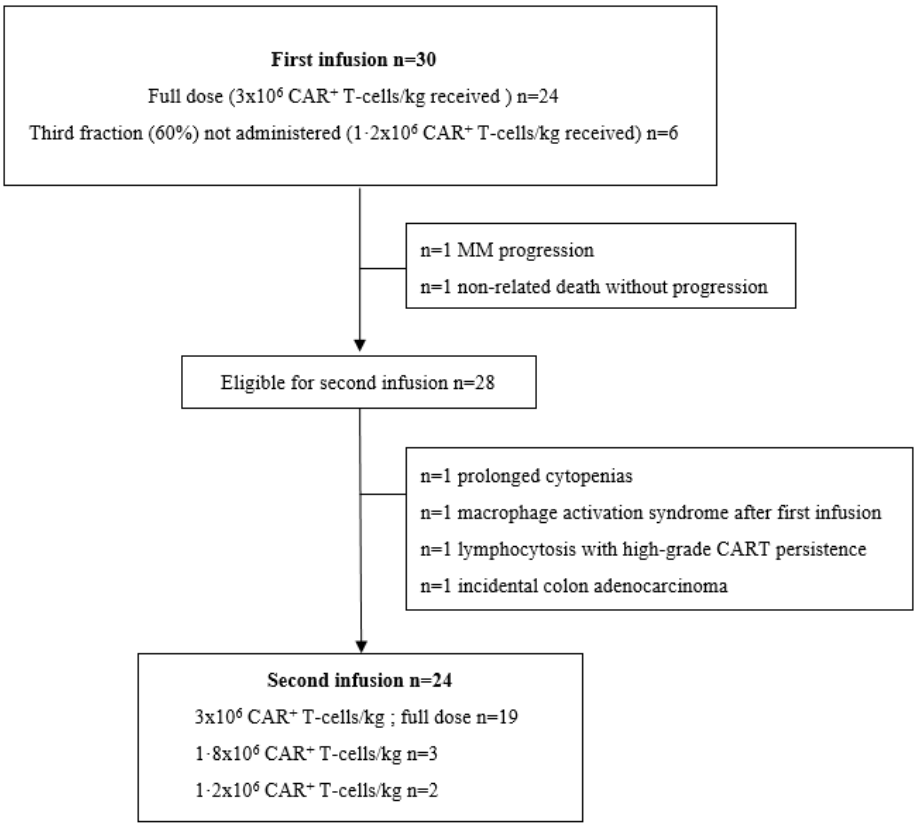
Created with biorender.com.

Table 2. Laboratory parameters before and after bridging therapy (BT) of each individual patient.

Patient code	Laboratory parameter	Assessment before BT	Assessment after BT
Patient 3	Serum M protein (g/L)	0	0
	Involved FLC (mg/L)	760	405
Patient 5	Serum M protein (g/L)	20·8	8·4
	Involved FLC (mg/L)	8·07	0·28
Patient 9	Serum M protein (g/L)	93·2	39·1
	Involved FLC (mg/L)	1250	136
Patient 12	Serum M protein (g/L)	11·1	0
	Involved FLC (mg/L)	3270	1720
Patient 13	Serum M protein (g/L)	71·1	5·1
	Involved FLC (mg/L)	1690	258
Patient 14	Serum M protein (g/L)	18·0	11·0
	Involved FLC (mg/L)	7326	2885
Patient 15	Serum M protein (g/L)	0	0
	Involved FLC (mg/L)	617	1497
Patient 16	Serum M protein (g/L)	33·4	41·0
	Involved FLC (mg/L)	5·2	4·34
Patient 18	Serum M protein (g/L)	0	0
	Involved FLC (mg/L)	139	178
Patient 19	Serum M protein (g/L)	33·2	43·4
	Involved FLC (mg/L)	418	335
Patient 20	Serum M protein (g/L)	0	0
	Involved FLC (mg/L)	900	431
Patient 22	Serum M protein (g/L)	0	0
	Involved FLC (mg/L)	614	433
Patient 23	Serum M protein (g/L)	0	0
	Involved FLC (mg/L)	543	687
Patient 26	Serum M protein (g/L)	52·8	62·8
	Involved FLC (mg/L)	3373	4665

Abbreviations: BT: bridging therapy; FLC: free light-chain.

Figure 2. First and second infusion flowchart.



Four patients did not receive the booster dose for the following reasons: second lymphodepletion and booster dose were contraindicated in a patient due to a persistent grade 4 thrombocytopenia without CART persistence, a patient developed a macrophage activation syndrome after first infusion and booster dose was contraindicated, a patient was diagnosed with colon adenocarcinoma with a substantial delay of the booster dose that ended in relapse, and a patient had a persistent lymphocytosis with a high PB CART detection and the clinical decision was not to administer the booster dose.

Table 3. Infections and infestations.

Infections episodes (per PT)	Occurrences/n patients	%	Intensity
Acinetobacter infection	1 / 1	3.3%	Moderate
Bacteraemia	2 / 1	3.3%	Moderate
Bronchitis	2 / 2	6.7%	Mild
COVID-19	2 / 2	6.7%	Mild
	1 / 1	3.3%	Intense
Campylobacter colitis	1 / 1	3.3%	Moderate
Candida infection	1 / 1	3.3%	Moderate
Clostridium difficile infection	1 / 1	3.3%	Mild
	1 / 1	3.3%	Moderate
Cytomegalovirus infection	1 / 1	3.3%	Moderate
Enterococcal infection	1 / 1	3.3%	Moderate
Gastroenteritis	1 / 1	3.3%	Moderate
Klebsiella infection	1 / 1	3.3%	Moderate
Legionella infection	1 / 1	3.3%	Mild
Leishmaniasis	1 / 1	3.3%	Intense
Lower respiratory tract infection	1 / 1	3.3%	Mild
Oral candidiasis	2 / 1	3.3%	Mild
Pharyngitis	1 / 1	3.3%	Mild
Pneumonia	1 / 1	3.3%	Intense
Pneumonia klebsiella	1 / 1	3.3%	Moderate
Pseudomonas infection	1 / 1	3.3%	Moderate
Respiratory tract infection	1 / 1	3.3%	Mild
	4 / 3	10.0%	Moderate
Rhinovirus infection	1 / 1	3.3%	Intense
Septic shock	1 / 1	3.3%	Intense
Severe acute respiratory syndrome	1 / 1	3.3%	Intense
Staphylococcal bacteraemia	1 / 1	3.3%	Intense
Tooth abscess	1 / 1	3.3%	Mild
Upper respiratory tract infection	4 / 3	10.0%	Mild
	4 / 4	13.0%	Moderate
Urinary tract infection	2 / 2	6.7%	Moderate

Reported infection events as per MedDRA System Organ Class (SOC) “Infections and infestations” and corresponding preferred term (PT). Intensity is noted for each reported event.

Figure 3. Measurable residual disease assessment by Next Generation Flow in bone marrow at different time points (sensitivity $1x10^{-6}$). Samples classified as not evaluable where due to bone marrow hemodilution. Samples classified as no data were missing samples.

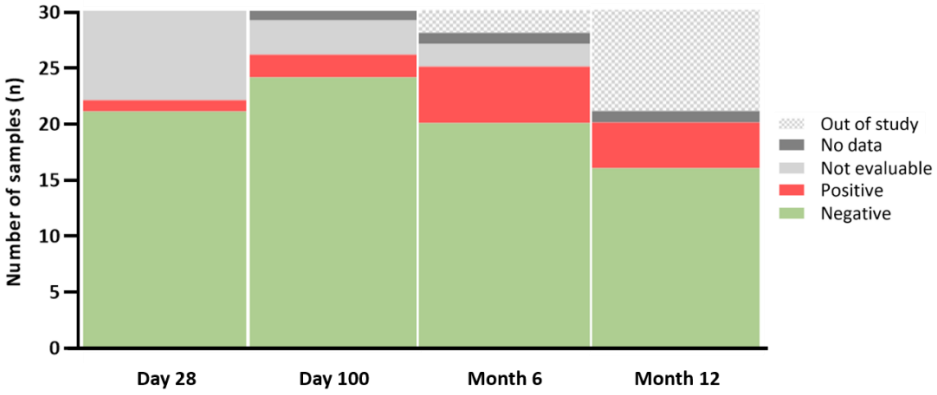


Table 4. Overall response rate and responses at different time points.

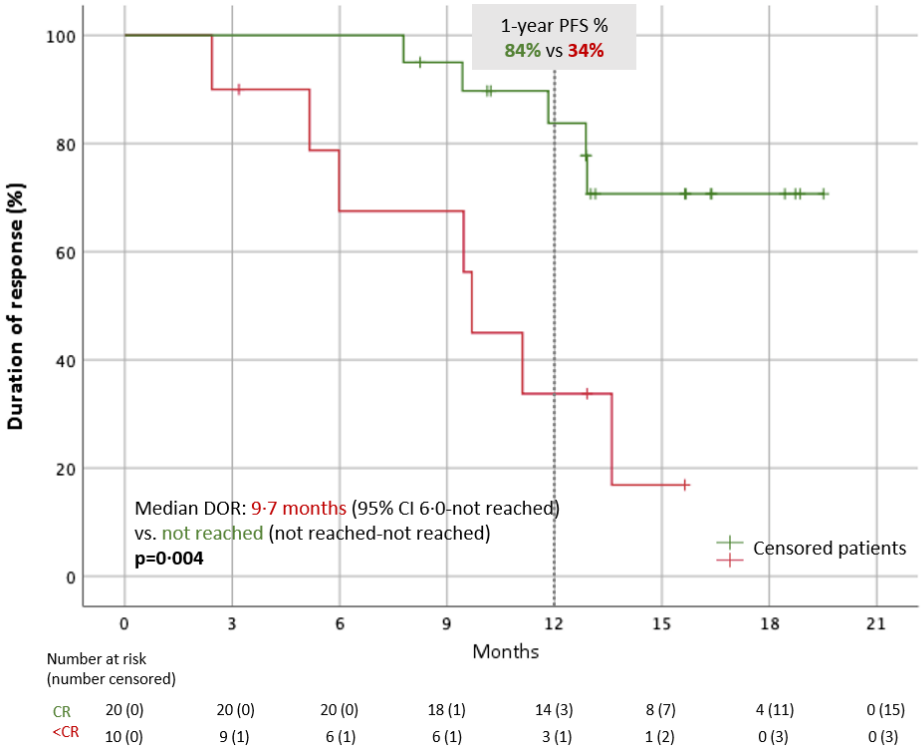
	ORR in the first 3 months	Day 28	Day 100	Month 6*	Month 12*	ORR*
Complete response	15/30 (50%)	9/30 (30%)	14/30 (47%)	14/30 (47%)	15/30 (50%)	20/30 (67%)
Very good partial response	9/30 (30%)	5/30 (17%)	8/30 (27%)	11/30 (37%)	4/30 (13%)	8/30 (27%)
Partial response	6/30 (20%)	16/30 (53%)	6/30 (20%)	2/30 (7%)	0	2/30 (7%)
Progressive disease	0	0	1/30 (3%)	2/30 (7%)	9/30 (30%)	0
Death without progression	-	0	0	1/30 (3%)	2/30 (7%)	-
Unknown status	-	0	1/30 (3%)	0	0	-
Best response achieved*	-	11/30 (37%)	11/30 (37%)	2/30 (7%)	6/30 (20%)	-
Best response achieved* (cumulative)	-	11/30 (37%)	22/30 (73%)	24/30 (80%)	30/30 (100%)	-
Patients enrolled	30/30 (100%)	30/30 (100%)	30/30 (100%)	27/30 (90%)	19/30 (63%)	30/30 (100%)
Patients out-of-study**	-	0	0	3/30 (10%)	11/30 (37%)	-

Abbreviations: ORR: overall response rate.
*Post-hoc analysis (median follow-up time of 18 months).
**Due to progression or death.

Results – Efficacy: Relapse or death for each individual patient.

Twelve patients developed disease progression at the following timepoints (months): 3·3, 6·1, 6·9, 8·8, 10·4, 10·4, 10·6, 12·1, 12·8, 13·8, 13·8, 14·5. Additionally, two patients died in response in months 4 and 9·1.

Figure 4. Duration of response (DOR) and overall survival (OS) according to the achievement of complete response: green = complete response, red = less than complete response.



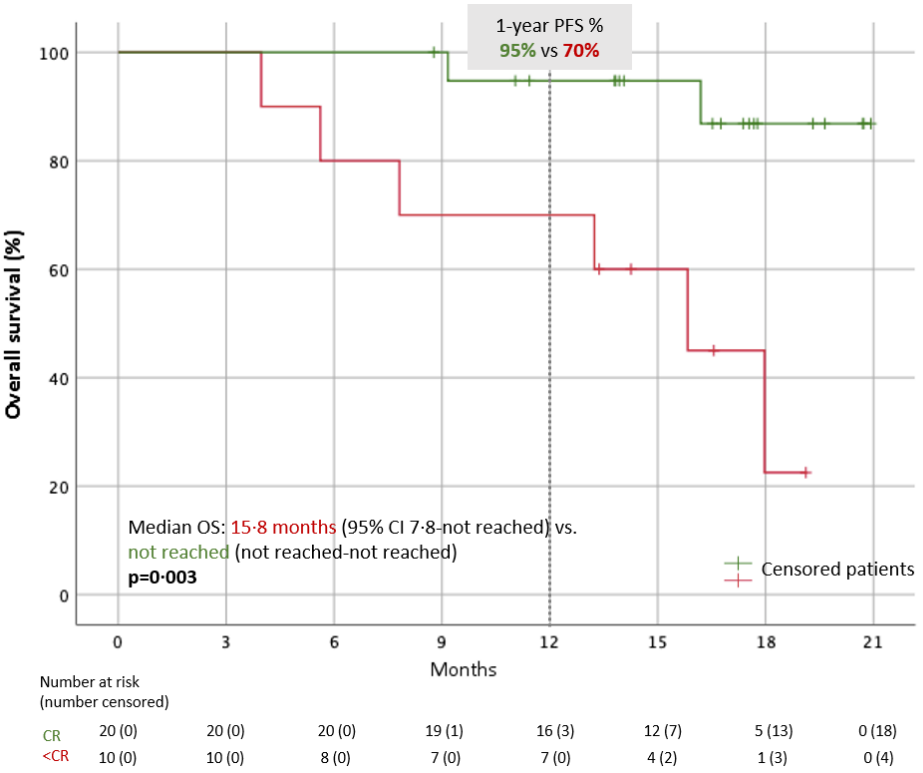
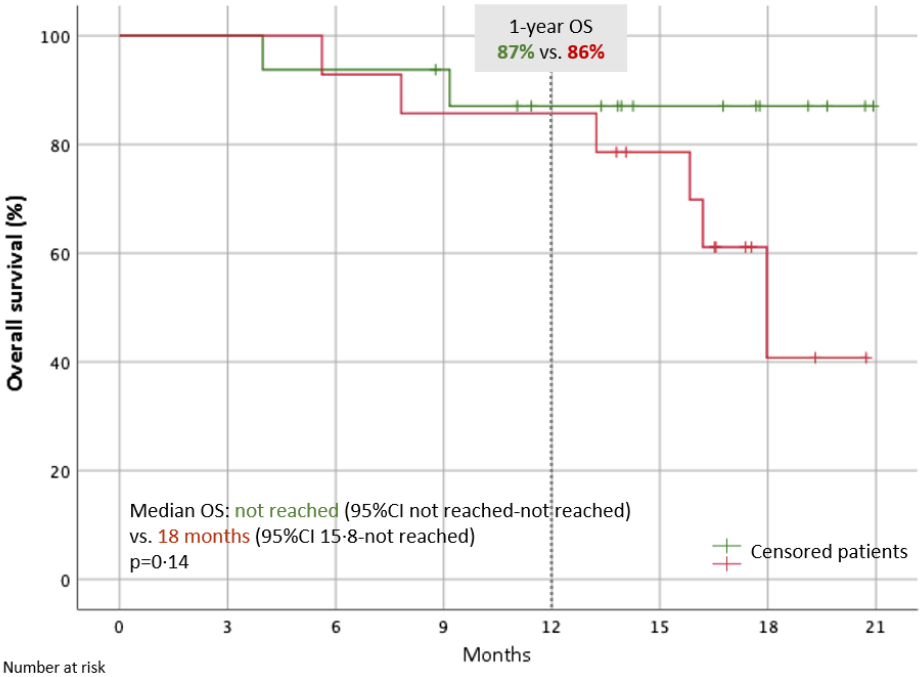


Figure 5. Progression-free survival (PFS) and overall survival (OS) according to the presence or absence of plasmacytomas at inclusion: red = presence, green = absence.



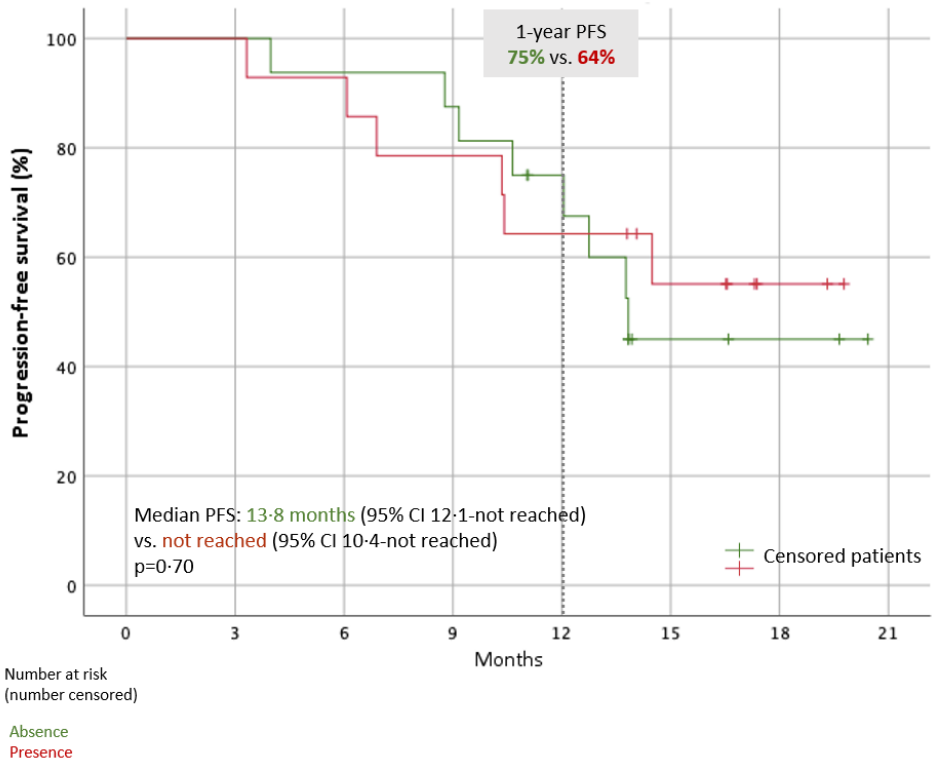


Table 5. Number (n) and percentage (%) of patients with detection of ARI0002h in peripheral blood over time.

	D+3	D+7	D+14	D+28	D+70	D+100	M4	M5	M6	M7	M8	M9	M10	M11	M12
n*	6/27	14/29	27/27	29/29	20/27	15/29	15/25	11/23	7/25	9/25	5/25	5/23	5/21	5/22	4/20
%	22	48	100	100	74	52	60	48	28	36	20	22	24	23	20

*Numbers in the denominator refer to patients with available and evaluable samples.

Figure 6. Peak and persistence of ARI0002h in peripheral blood measured by polymerase chain reaction (PCR). (**A**) ARI0002h detection after initial infusion. In red (n=6): data of the n=4 patients who did not receive a booster dose for clinical reasons and n=2 additional patients who died or relapsed prior to booster dose. In blue (n=24) data of persistence until booster dose of patients who received it. (**B**) Expansion and persistence of ARI0002h in patients who received the booster dose.

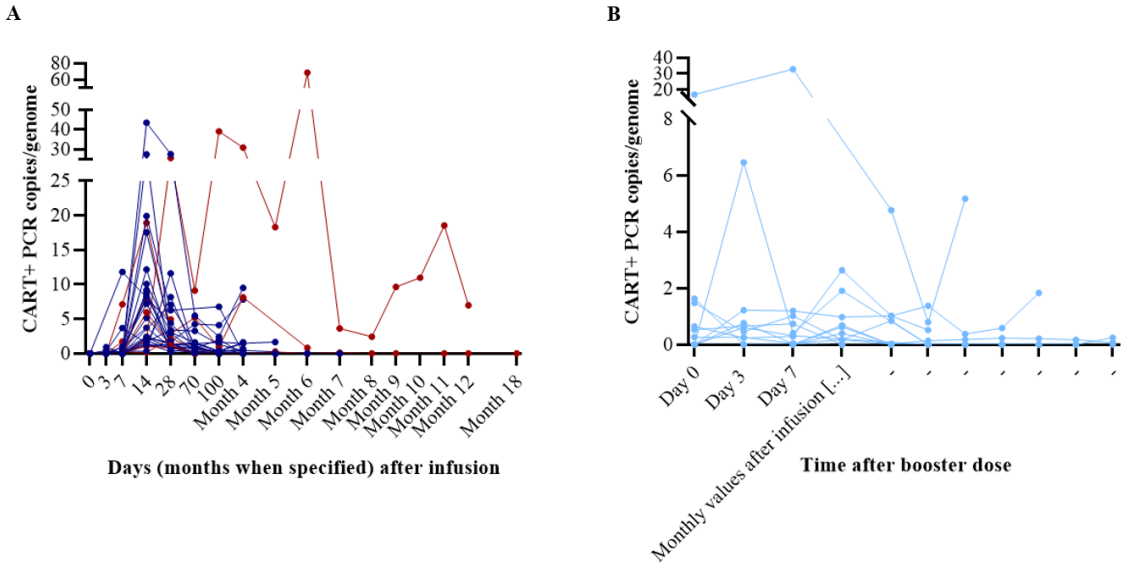


Figure 7. B-cell maturation antigen (BCMA) correlative data. (**A**) BCMA expression on malignant plasma cells in the bone marrow at inclusion and in the end-of-treatment visit of relapsed patients. (**B**) Soluble BCMA in peripheral blood at different time points, including data of relapsed patients in the end-of-treatment visit.

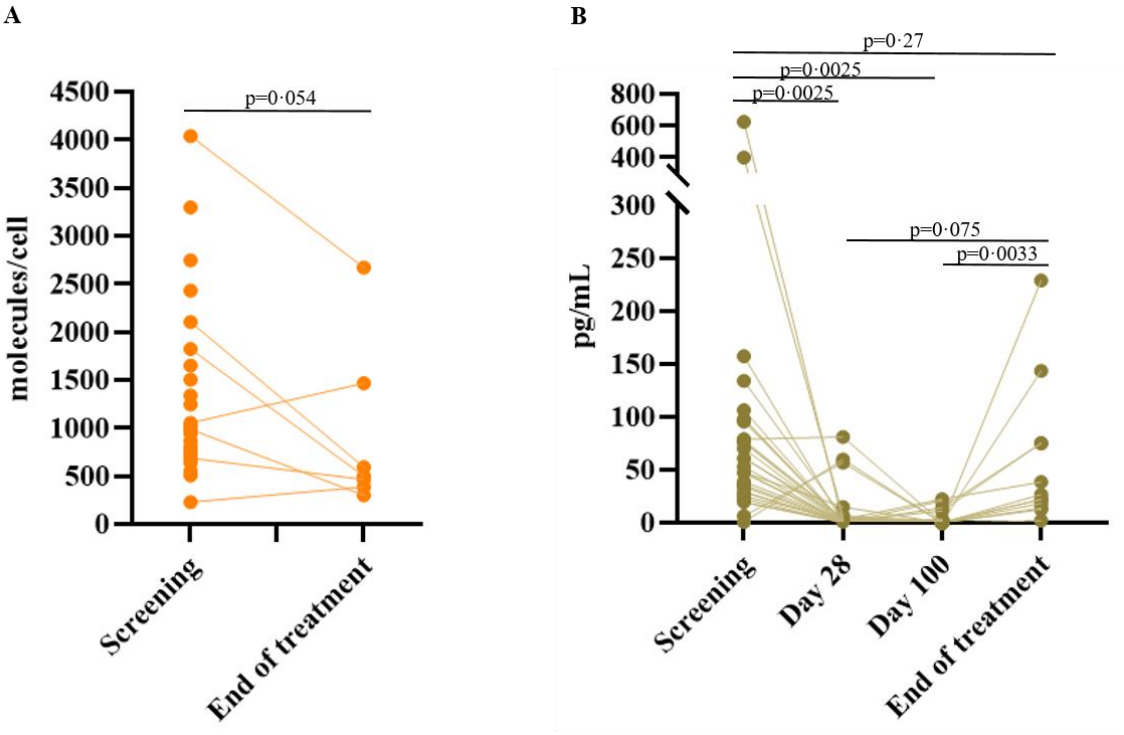


Table 6. Human anti-human antibody positivity in different timepoints.

	At some point	Day 28	Day 100	Month 6	Month 12
Positive HAHA n (%)	21/30 (70%)	2/29 (6·9%)	1/27 (3·7%)	3/25 (12%)	6/16 (37·5%)
Patients with results	30	29	27	25	16
Patients out-of-study	-	0	0	3	11
Result not available	-	1	3	2	3

Abbreviations: HAHA: human anti-human antibody.

Data sharing statement

Individual, de-identified participant data and data dictionary can be made available, with publication, at the request of qualified investigators whose proposed use of the data has been approved by IDIBAPS, after a data access agreement is signed. Requests can be made to the corresponding author (cfernan1@clinic.cat). Additional related documents are available at: https://clinicaltrials.gov/ct2/show/NCT04309981.

Article 2

Biomarkers of Efficacy and Safety of the Academic BCMA-CART ARI0002h for the Treatment of Refractory Multiple Myeloma

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ABSTRACT

Purpose: B-cell maturation antigen (BCMA)-chimeric antigen receptor T-cells (CART) improve results obtained with conventional therapy in the treatment of relapsed/refractory multiple myeloma. However, the high demand and expensive costs associated with CART therapy might prove unsustainable for health systems. Academic CARTs could potentially overcome these issues. Moreover, response biomarkers and resistance mechanisms need to be identified and addressed to improve efficacy and patient selection. Here, we present clinical and ancillary results of the 60 patients treated with the academic BCMA-CART, ARI0002h, in the CARTBCMA-HCB-01 trial.

Patients and Methods: We collected apheresis, final product, peripheral blood and bone marrow samples before and after infusion. We assessed BCMA, T-cell subsets, CART kinetics and antibodies, B-cell aplasia, cytokines, and measurable residual disease by next-generation flow cytometry, and correlated these to clinical outcomes.

Results: At cut-off date March 17, 2023, with a median follow-up of 23.1 months (95% CI, 9.2–37.1), overall response rate in the first 3 months was 95% [95% confidence interval (CI), 89.5–100]; cytokine release syndrome (CRS) was observed in 90% of patients (5% grades ≥3) and grade 1 immune effector cell-associated neurotoxicity syndrome was reported in 2 patients (3%). Median progression-free survival was 15.8 months (95% CI, 11.5–22.4). Surface BCMA was not predictive of response or survival, but soluble BCMA correlated with worse clinical outcomes and CRS severity. Activation marker HLA-DR in the apheresis was associated with longer progression-free survival and increased exhaustion markers correlated with poorer outcomes. ARI0002h kinetics and loss of B-cell aplasia were not predictive of relapse.

Conclusions: Despite deep and sustained responses achieved with ARI0002h, we identified several biomarkers that correlate with poor outcomes.

Introduction

The introduction of B-cell maturation antigen (BCMA) directed chimeric antigen receptor T-cell (CART) therapy to the treatment of relapsed/refractory (R/R) multiple myeloma (MM) is undoubtedly revolutionary, with the achievement of high-response rates and median survivals that overcome those achieved with conventional therapy in this setting (1–3). These outstanding results led to the approval of idecabtagene vicleucel (ide-cel; ref. 4) and ciltacabtagene autoleucel (cilta-cel; ref. 5), and results of several CARTs targeting BCMA for R/R MM have been reported so far, including different single-chain variable fragment (scFv) origins (mouse, llama, humanized, and human) and T-cell origin (autologous vs. allogeneic; refs. 6–8). Also, GPRC5D has emerged as an active immunotherapeutic target with promising results (9). Consequently, the demand for this therapy overcomes availability and, in some countries and health systems, the costs of this therapy are unaffordable. These unmet needs could be improved by academic CARTs.

Our institution developed ARI0002h, a second-generation 4–1BB-based CAR, with a humanized scFv directed against BCMA (10). An academic, pivotal, single-arm, open label clinical trial (CARTBCMA-HCB-01) for the treatment of R/R MM patients was started. Results of

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Translational Relevance

There is an increase in demand for CART therapy, which will presumably grow even more with CARTs moving to earlier lines of treatment. Demand overcomes availability, and the costs of CART therapy are unaffordable for some countries and public health systems. In this sense, academic CARTs could be a promising option to palliate these problems, allowing a strategy of point-of-care that could be implemented worldwide.

Despite impressive outcomes observed with BCMA-CART in patients with heavily pretreated multiple myeloma, this therapy is not curative and patients with myeloma continue to relapse. Understanding the mechanisms of failure of CART therapy and finding biomarkers of response that could be addressed to improve efficacy is of main importance.

the first cohort of 30 patients showed an overall response rate (ORR) of 100%, with 67% achieving complete responses (CR). After a median follow-up of 18 months, progression-free survival (PFS) was 14.5 months and overall survival (OS) was not reached, with a PFS rate at 12 months of 70%. Cytopenias were the most common side effect and although 80% of patients developed cytokine release syndrome (CRS), all cases were grades 1 to 2. No cases of neurotoxicity or late neurologic events were observed (11).

Despite the clinical outcomes obtained with BCMA-CARTs, patients continue to relapse and the absence of a plateau in the survival curves reported by different constructs does not suggest a definitive cure. This, added to the cost that CART therapy signifies in the health care systems of many countries, emphasizes the importance of finding predictive factors and mechanisms of resistance to guide patient selection. Some of the proposed features that could impact CART function are BCMA antigen density, soluble BCMA levels (including the possibility of a BCMA antigen escape), T-cell subsets (stem cell memory vs. effector), T-cell exhaustion, and the effect of nonhuman scFv leading to anti-CAR antibodies (12).

Here, we report the clinical results from a planned interim analysis with cut-off date March 17, 2023, of 60 patients treated with ARI0002h, including longer follow-up of the initial cohort and 30 additional patients from cohort 2. We also describe ancillary studies performed on patients samples evaluating potential mechanisms of efficacy and resistance to ARI0002h.

Patients and Methods

Study design and subjects

CARTBCMA-HCB-01 is a pilot, single-arm, open-label study performed in seven Spanish centers. Patients ages 18 to 75 years old with RRMM were eligible if they had measurable disease, as assessed by M-protein (serum >10 g/L or urine >200 mg/24 hours) or serum free light chain levels (>100 mg/L), received ≥2 prior regimens, including a proteasome inhibitor, an immunomodulatory drug and an anti-CD38 antibody, and were refractory to the last line of treatment. Prior BCMA-directed treatment was an exclusion criterion. ARI0002h was lentivirally transduced on autologous T cells. Bridging therapy was allowed after apheresis according to investigator discretion. Lymphodepletion (LD) included cyclophosphamide (900 mg/m^2 total dose) and fludarabine (90 mg/m^2 total dose). The target dose (3×10^6/kg CAR$^+$ cells) was administered in a fractionated manner (10%/30%/60%), with at least 24 hours between infusions. A booster dose of up to 3×10^6 CAR$^+$ cells/kg was planned at least 3 months after the first dose in patients with any kind of response and no limiting side effects. Prior to the booster dose, LD was repeated following the same scheme only when CART cell persistence was ruled out (Supplementary Fig. S1). The study was registered with EudraCT (2019–001472–11) and ClinicalTrials.gov (NCT04309981). The study was performed in accordance with the Declaration of Helsinki Ethical Principles for Medical Research involving Human Subjects and the protocol was approved by the Ethics Committee of Hospital Clinic de Barcelona. All subjects provided written informed consent. Information on representativeness of study participants is provided in Supplementary Table S1.

Samples

Apheresis and final product (FP) samples were obtained and analyzed. Peripheral blood (PB) and bone marrow (BM) samples were obtained at the established timepoints; PB at inclusion, prior to LD, on days 0, 3, 7, 14, 28, 70, 100 and months 4, 5, 6, 7, 8, 9, 10, 11, 12, 18, 24; BM at inclusion, days 28 and 100, and months 6, 12, 18, 24. When patients were withdrawn from the study, end-of-treatment (EOT) samples from the PB and BM, if possible, were collected. Potency was analyzed in the FP by flow cytometry, assessing killing capacity of CAR-T cells against the U266 tumor cell line (RRID: CVCL_0566). Measurable residual disease (MRD) was assessed by next-generation flow. Kinetics of ARI0002h were measured by qPCR in the PB and by flow cytometry in the BM. B-cell kinetics were measured by flow cytometry. BCMA molecules/cell on plasma cells (PC) were quantified by flow cytometry. Soluble BCMA was quantified in serum by ELISA. T-cell subsets were analyzed by flow cytometry. Serum levels of cytokines were measured by ELISA. Anti-CAR antibodies were determined by flow cytometry (**Fig. 1**). Detailed information is described in the Supplementary Materials and Methods.

Clinical results

Primary endpoints were overall response rate (ORR) within the first 3 months and rate of CRS and/or neurotoxicity in the first 30 days. Response was assessed as per International Myeloma Working Group (IMWG) criteria (13) and BM minimal residual disease (MRD; ref. 14) was analyzed by next-generation flow. Adverse events (AE) were graded using CTCAE v5.0. CRS and neurotoxicity were graded according to the American Society for Transplantation and Cellular Therapy (ASTCT) criteria (15). Duration of response (DOR) was defined as time between first response and progression (patients who were evaluable for response but died without progression were censored at the last follow-up). PFS was defined as time between infusion and progression or death and OS was defined as time between infusion and death.

Statistical analysis

Categorical variables were reported in number or proportions and continuous variables were reported as mean and SD or median and interquartile range. Comparisons between categorical values were performed with χ^2 or Fisher exact test. The Student t test or the Wilcoxon test (when appropriate) were used to analyze the continuous variables. The log-rank test was used to analyze the differences in survival curves, and the Kaplan–Meier method to graphically depict the PFS and OS. A Cox proportional hazards model was used to calculate the HR. Pearson or Spearman correlation tests were used when appropriate. A two-sided P value of <0.05 was considered statistically significant. Statistical analysis was performed with SAS

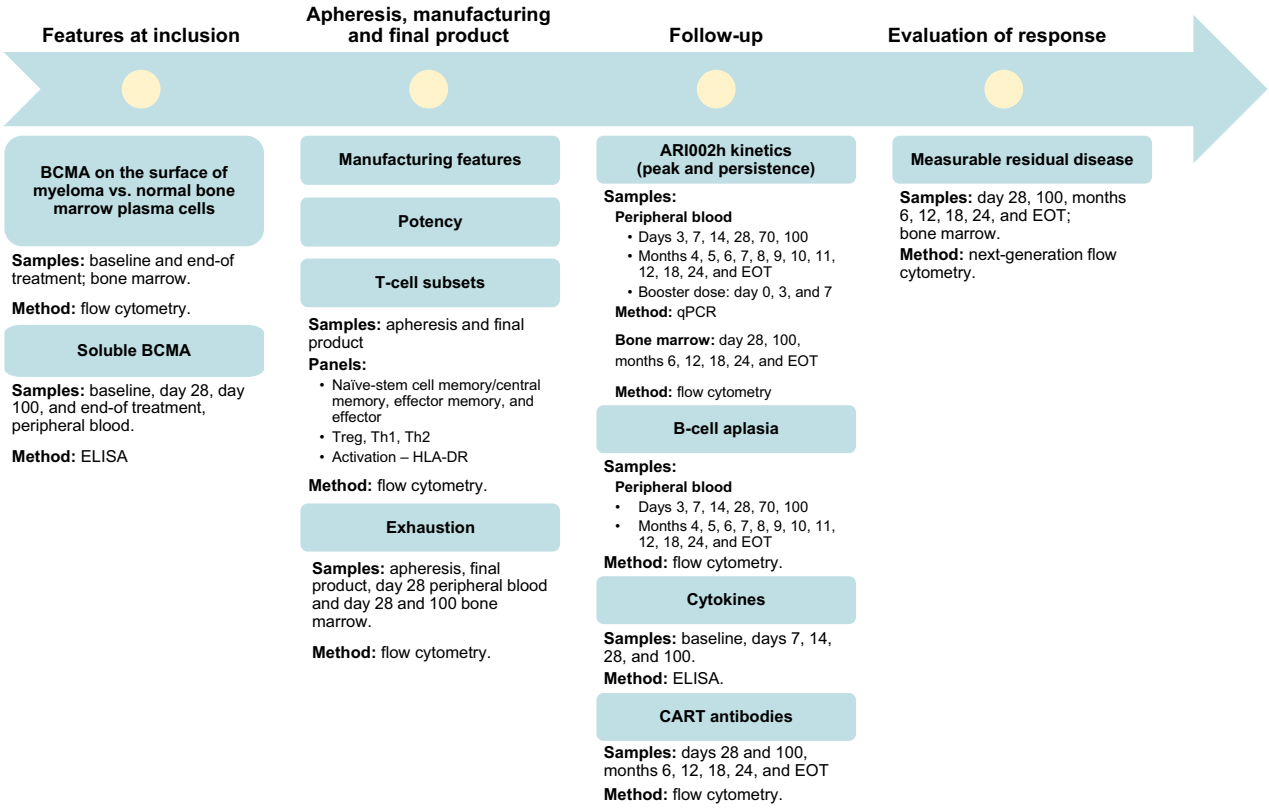

Figure 1.
Sample assessment workflow. Summary of biomarker analysis performed on samples according to its order of appearance: (i) features at inclusion; (ii) apheresis, manufacturing, and final product analysis; (iii) follow-up findings; and (iv) evaluation of response.

System (v9.4), GraphPad Prism (v10.0.2; RRID: SCR_002798), and IBM SPSS Statistics (v29.0; RRID: SCR_002865).

Data availability

All materials, data, and protocols described in the manuscript will be made available upon request, if the request is made within 6 years of publication. Data are not publicly available due to patient's privacy concerns. A summary of the study protocol is included in the Supplementary Data of this article.

Results

Clinical outcomes of ARI0002h

As of March 17, 2023, 72 patients with R/R MM were screened, of which 69 underwent apheresis and 61 received LD, with 60 patients finally receiving ARI0002h (**Fig. 2A**). This is the population that was evaluated in the studies described herein. The main patient characteristics and response data are described in **Table 1**. Bridging therapy was administered to 48% of patients. The ORR in the first 3 months was 95% [≥very good partial response (VGPR) in 77%]. Median time to first response was 1 month. Responses deepened over time, with 58% achieving CR [55% stringent CR (sCR)], 30% VGPR, 7% partial response. Only 1 patient (2%) was refractory, and 2 patients (3%) died prior to first evaluation [septic shock and macrophage activation syndrome (MAS)].

With a median follow-up of 23.1 months [95% confidence interval (CI), 9.2–37.1], estimated median PFS was 15.8 months (95% CI, 11.5–22.4; **Fig. 2B**). Median OS was not reached with OS rates at 12 and 18 months of 81% and 69%, respectively (**Fig. 2C**). Eighteen of 60 (30%) patients died. Causes of death were disease progression ($n = 13$), COVID19 ($n = 2$), cranial trauma, septic shock, and MAS ($n = 1$ each).

CRS was observed in 90% with 5% grades ≥3. Median time to CRS was 7 days (range 1–14) with a median duration of 4.5 days. Eight (13%) patients did not receive the third fraction due to CRS. ICANS grade 1 was reported in only 2 patients (3%) with no late neurologic events. Six patients (10%) developed a MAS (four grades 1–2, one grade 3, one death). Tocilizumab and steroids were administered in 68% (mainly for persistent grade 1 CRS) and 30% of patients, respectively.

The booster dose had been administered to 44 of 55 eligible patients (80%), with no CRS, ICANS, or MAS or other toxicities. Median time after first infusion was 4.4 months (IQR, 4.0–5.1); 34% received a second LD regimen. Response was evaluable in 42 patients; 45% ($n = 19$) were already in sCR, 29% ($n = 12$) maintained the previously achieved response, and 26% ($n = 11$) improved their response. Six patients (10%) developed neoplasias after ARI0002h infusion. Detailed information is provided in Supplementary Table S2.

B-cell maturation antigen role in efficacy

BCMA data were obtained from two sources: (i) quantification of BCMA molecules on the surface of myeloma versus normal PC in the BM and (ii) measurement of soluble BCMA in the serum.

Mean BCMA molecules on the surface of myeloma PC at inclusion was 1,261 molecules/cell (SD 838, range 225–4,040). A decrease in

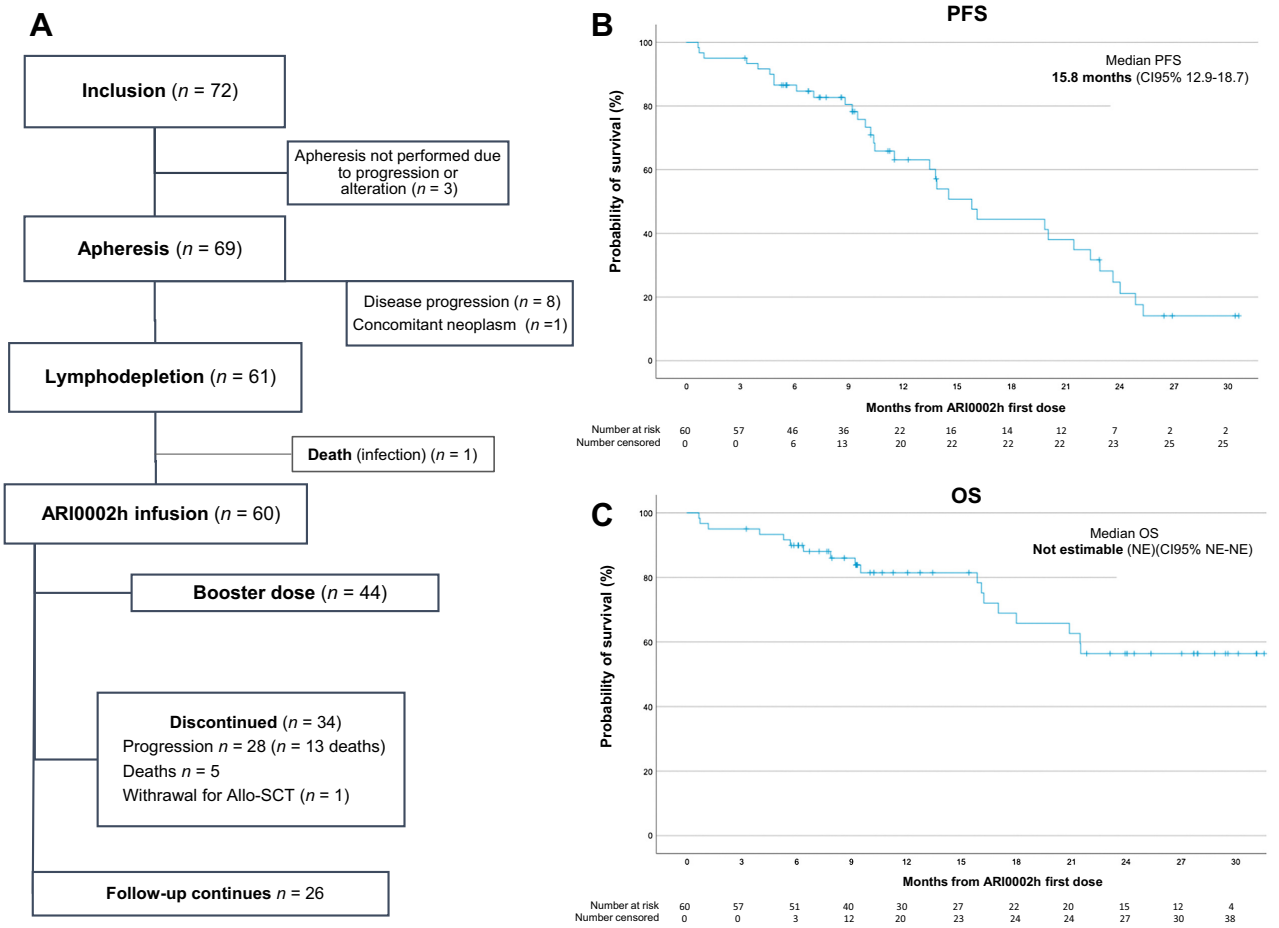

Figure 2.

ARI0002h clinical outcomes ($n = 60$). **A,** CONSORT diagram of CARTBCMA-HCB-01 at cutoff date March 17, 2023. **B,** PFS and **(C)** OS of the 60 patients treated with ARI0002h with a median follow-up of 23.1 months (95% CI, 9.2–37.1).

number was observed between inclusion and relapse in myeloma PC (17 samples available; $P = 0.019$; **Fig. 3A**), although none of the patients became BCMA negative in the BM. Conversely, BCMA in normal PC decreased only in 1 of 4 patients with paired samples available. We found elevated sBCMA in all patients at both inclusion (252,661 pg/mL; SD 411,464) and relapse (142,887; SD 402,826), except for a patient. However, BCMA was detected in the bone marrow PC of this subject. The only patient who was primarily refractory to ARI0002h showed an increase in sBCMA between baseline and day 28 (**Fig. 3B**).

The amount of BCMA molecules on the surface of myeloma PC was equivalent between patients who did or did not achieve a CR on days 28, 100 or as their best response (**Fig. 3C**); survival (PFS and OS) was not correlated to the quantity of BCMA either. In terms of sBCMA, baseline values were lower in patients who achieved CR (days 28, 100 or CR as best response), although differences were not statistically significant. Interestingly, sBCMA measurements on day 28 were predictive of CR achievement at all three timepoints (**Fig. 3D**). In addition, comparing baseline and day 28 sBCMA with the best response achieved, without segregating into CR/<CR, an association was observed (**Fig. 3D**). Moreover, a higher sBCMA at baseline was correlated with a shorter time to event in DOR ($P < 0.001$), PFS ($P = 0.003$), and OS ($P = 0.008$). Differences in baseline sBCMA were observed between patients who developed CRS grades 1 to 2 versus higher grade CRS ($P = 0.041$; **Fig. 3E**). sBCMA levels did not differ according to the presence of plasmacytomas. The number of BCMA molecules on myeloma PC or the quantity of sBCMA did not affect ARI0002h persistence or peak magnitude.

Manufacturing and potency

All products were obtained at first attempt but two. In one the apheresis was repeated and the other one was manufactured using cryopreserved T cells that were available. Both products were successfully manufactured in the end. Mean T-cell transduction was 76%; dose was calculated on the basis of CAR+ cells, therefore all patients received the same quantity of CARTs and all patients had 3×10^6 CAR^+ cells/kg available for the first infusion. In the case of the booster dose, 38/44 (86%) received 3×10^6 CAR^+ cells/kg in a unique infusion ($n = 2$ received 1.2×10^6 CAR^+ cells/kg and $n = 4$ received 1.8×10^6 CAR^+ cells/kg; which was the dose available). Median CART production and turnaround times were 9 days (IQR, 8–10) and 32 days (IQR, 27–37), respectively. Median vein-to-vein time was 41 days (IQR, 34–51), with differences between patients who did or did not receive bridging therapy (48 days vs. 36 days; $P = 0.004$). No impact on

Table 1. Main features of patients treated with ARI0002h.

Features	ARI0002-treated patients ($n = 60$)
Age (years); median (range)	58 (36–74)
Sex (F/M)	26/34
Heavy chain isotype IgG/IgA/IgD/Only light chain (%)	52/27/3/18
Light chain isotype kappa/lambda (%)	55/45
ISS stage at baseline I/II/III (%)	49/23/28
Plasmacytomas (%)	50
Extramedullary location	18
Serum M protein (g/L); mean (range)	10.5 (0–90)
Bone marrow PCs (%); median (IQR)	13 (2–31)
High-risk cytogenetics (%)[a]	28
Number of previous lines; median (range)	3 (2–10)
Prior autologous stem cell transplantation (%)	90
Prior allogeneic stem cell transplantation (%)	8
Prior drug exposure (%) / refractoriness (%)	
Bortezomib	100/52
Lenalidomide	100/78
Anti-CD38 mAb	100/93
Carfilzomib	57/45
Pomalidomide	52/48
Bridging therapy (%)	48
Overall response in the first 100 days (%)	95
≥Complete response (stringent CR)	44(40)
Very good partial response	33
Partial response	18
Refractory	2
Death prior to evaluation	3
Measurable residual disease negative; *n* negative/*n* evaluable (%) in total; % of 10^{-6}, 10^{-5}, 10^{-4}	
Day 28	40/41 (98%)[b]; 47.5, 42.5, 10
Day 100	49/51 (96%)[b]; 55.1, 34.7, 10.2
CRS; *n* (%)	54 (90)
Grade 1–2	51 (85)
Grade ≥3	3 (5)
Neurotoxicity; *n* (%)	
Immune-effector cell associated neurotoxicity syndrome	2 (3)
Late neurologic events	0

[a]del(17p), t(4;14), t(14;16).
[b]Three of the unavailable samples were due to: 1 patient was primary refractory and 2 patients died before first evaluation.

PFS and OS was observed according to manufacturing time. *In vitro* killing capacity of the CAR T cells was tested in all FP, but no correlation was found between potency and clinical outcomes, in line with previously reported data (16–18).

T-cell subsets in the apheresis and final product

T-cell populations in the apheresis and the final product (FP; in CAR^+ and CAR^- cells) are depicted in **Fig. 4A**. We found an inverted CD4/CD8 ratio in the apheresis and FP, but greater expansion of CD4 cells during manufacturing was observed, with an increased CD4/CD8 ratio in the FP compared to the apheresis (0.83 vs. 0.43; $P = 0.0011$). The FP was enriched in central memory cells (15.5% vs. 3.7%; $P < 0.001$), while naïve plus stem cell memory T cells were increased in CD8 (25.5% vs. 5.7%; $P < 0.0001$) but decreased in CD4 (10.1% vs. 15.9%; $P < 0.001$). After cell expansion during manufacturing, the proportion of Th1 (20.9% vs. 11.8%; $P < 0.0001$) and Treg (11.9% vs. 1.5%; $P < 0.0001$) populations were also increased, accompanied by a large increase in cells expressing the activation marker HLA-DR (CD8: apheresis 15.2% vs. FP 72%, $P < 0.0001$; CD4: apheresis 9.3% vs. FP 68.2%, $P < 0.0001$).

Regarding the association of the analyzed T-cell subsets and clinical outcomes, a higher proportion of CD8 central memory cells in the FP (but not in the apheresis) was correlated with the achievement of CR on day 100 ($P = 0.045$). Several populations in the apheresis showed a mild-intermediate correlation with the proportion of CD8 central memory cells in the FP (Supplementary Table S3), suggesting that a starting material enriched in CD4 naive and CD4 CM cells, could have a roll helping CAR^- T central memory differentiation during cell culture. We could not find a correlation with Tregs or other T-cell subsets, although the CAR^- population of the FP was enriched in Treg (30%), compared with the CAR^+ fraction (11.9%). In contrast, a higher percentage of the activation marker HLA-DR in the apheresis (but not in the FP) was associated with an increased PFS (CD8: HR, 1.050; $P = 0.046$; CD4: HR, 1.063; $P = 0.041$); for OS only a trend towards significance was found (CD8: HR, 1.056; $P = 0.084$; CD4: HR, 1.055; $P = 0.050$).

Exhaustion markers

We analyzed exhaustion markers (PD-1, TIGIT, TIM-3, LAG-3) in the apheresis, FP, in the PB on day 28, and in the BM on days 28, 100, and EOT. We observed a marked increase in PD-1 and TIGIT from the FP to days 28 and 100 in the BM ($P < 0.001$), and in PD-1 from the FP to day 28 in the PB ($P < 0.001$; **Fig. 4B**). TIM-3 and LAG-3 expression increased from apheresis to the FP ($P < 0.001$), but a decrease was observed in the PB and BM on day 28 ($P < 0.001$). From day 28, TIM-3 started to increase in CAR^+ cells of the BM, but LAG-3 expression in the BM remained low.

In terms of impact on efficacy, patients who achieved CR on day 28, 100, and as best response had a lower expression of TIGIT in the BM on day 28 ($P = 0.011$, $P = 0.013$, $P = 0.011$, respectively; **Fig. 4C**). We did not find a correlation between PD-1 expression and response. Higher levels of PD-1 and TIGIT in the apheresis, TIM-3 and TIGIT in CAR^+ CD8 T cells in the PB on day 28 and TIM-3 in CAR^+ CD4 T cells in the PB on day 28 correlated with shorter PFS and OS (Supplementary Table S4; Supplementary Fig.2).

Kinetics of ARI0002h

Persistence of ARI0002h was measured in both PB and BM. Of note, BM samples were obtained less frequently, potentially introducing a sampling bias. Median persistence of ARI0002h in the PB and BM was 4.9 (95% CI, 3.8–5.9) and 5.6 months (95% CI, 3.6–7.7), respectively. The mean peak was 10.4 copies/genome (SD, 11.4; range: 0.68–68.8; **Fig. 5A**) and occurred on day 14 and 28 in 70% and in 18% of patients, respectively. Persistence in the PB at each timepoint is depicted in Supplementary Fig.3. We analyzed persistence and the magnitude of the peak according to baseline tumor burden, measured as proportion of BM PC and baseline sBCMA, with no significant differences (Supplementary Figs. S4 and S5).

Of the 44 patients who received the booster dose, expansion data were evaluable from 39. Expansion in the PB was observed in 61.5% of patients ($n = 24$), not observed in 35.9% and the result was uncertain in 1 patient. Median persistence after booster dose in patients who expanded was 3.7 months (95% CI, 1.4–6.0). Half of the expansions (12/24) occurred within the first 7 days after the booster dose. The peak observed after the booster dose was lower (mean 2.74 copies/genome;

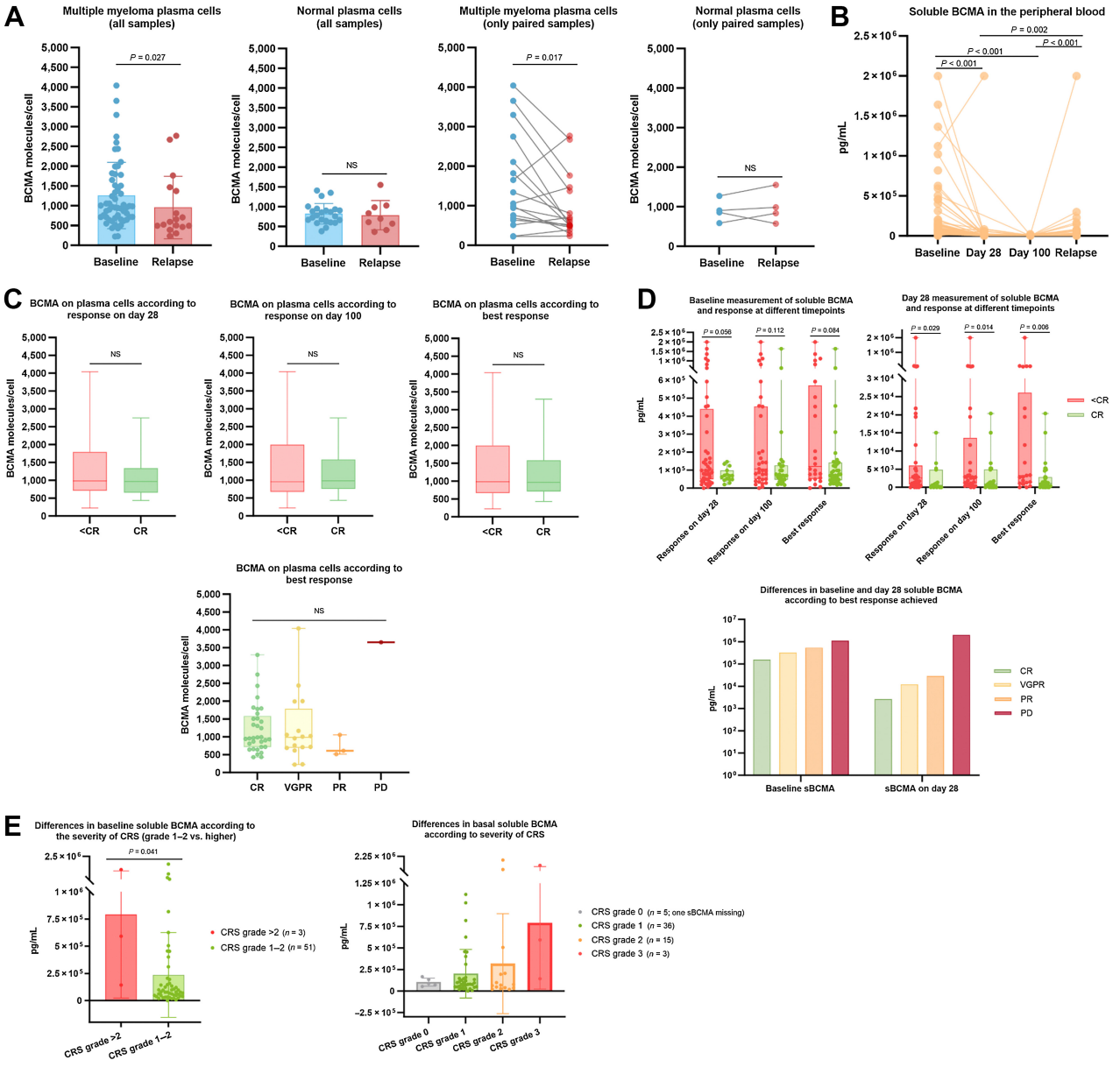

Figure 3.

BCMA behavior in ARI0002h-treated patients. **A,** BCMA molecules/cell on myeloma and normal PCs at baseline and relapse in all patients (left) and only in paired samples (right). **B,** Soluble BCMA (sBCMA) levels at different timepoints. A decrease is observed in all responding patients, with an increase at relapse. The only patient who was refractory developed an increase of sBCMA levels from baseline to day 28. **C,** The density of BCMA molecules on myeloma PCs was not different in complete responders versus patients who achieved less than a complete response. **D,** Baseline sBCMA was lower in complete responders versus less than a complete response on days 28 and 100, and in best response achieved, although differences were not statistically significant. sBCMA levels on day 28 were lower in complete responders at all three timepoints. When analyzing sBCMA at baseline and day 28, we found a correlation with the response achieved. **E,** sBCMA levels in patients according to severity of cytokine release syndrome.

SD, 6.6) than after the initial expansion (**Fig. 5B**), although samples after 14 days (median time to peak observed after the first infusion) were not available. Second LD was administered to 6 of 14 patients in the nonexpansion group and to 5 of 24 of the expansion group ($P = 0.266$). In 6 patients, a CART expansion in the PB was observed prior to MM relapse, including 2 patients who simultaneously became MRD positive. ARI0002h was detectable in 32.1% (9/28) of the patients with samples at relapse (**Fig. 5C**).

To establish the potential impact of PB CART persistence in DOR, PFS, and OS, we performed a survival analysis setting a landmark time at 3 and 4.9 months (the median persistence of this cohort) from infusion. A log-rank comparison categorizing persistence at both timepoints showed no differences (Supplementary Fig. S6). Triple- or penta-refractory patients had a similar persistence of ARI0002h. For patients with prior allogeneic stem cell transplantation (alloHSCT; $n = 5$), ARI0002h persistence was high, but was not statistically longer

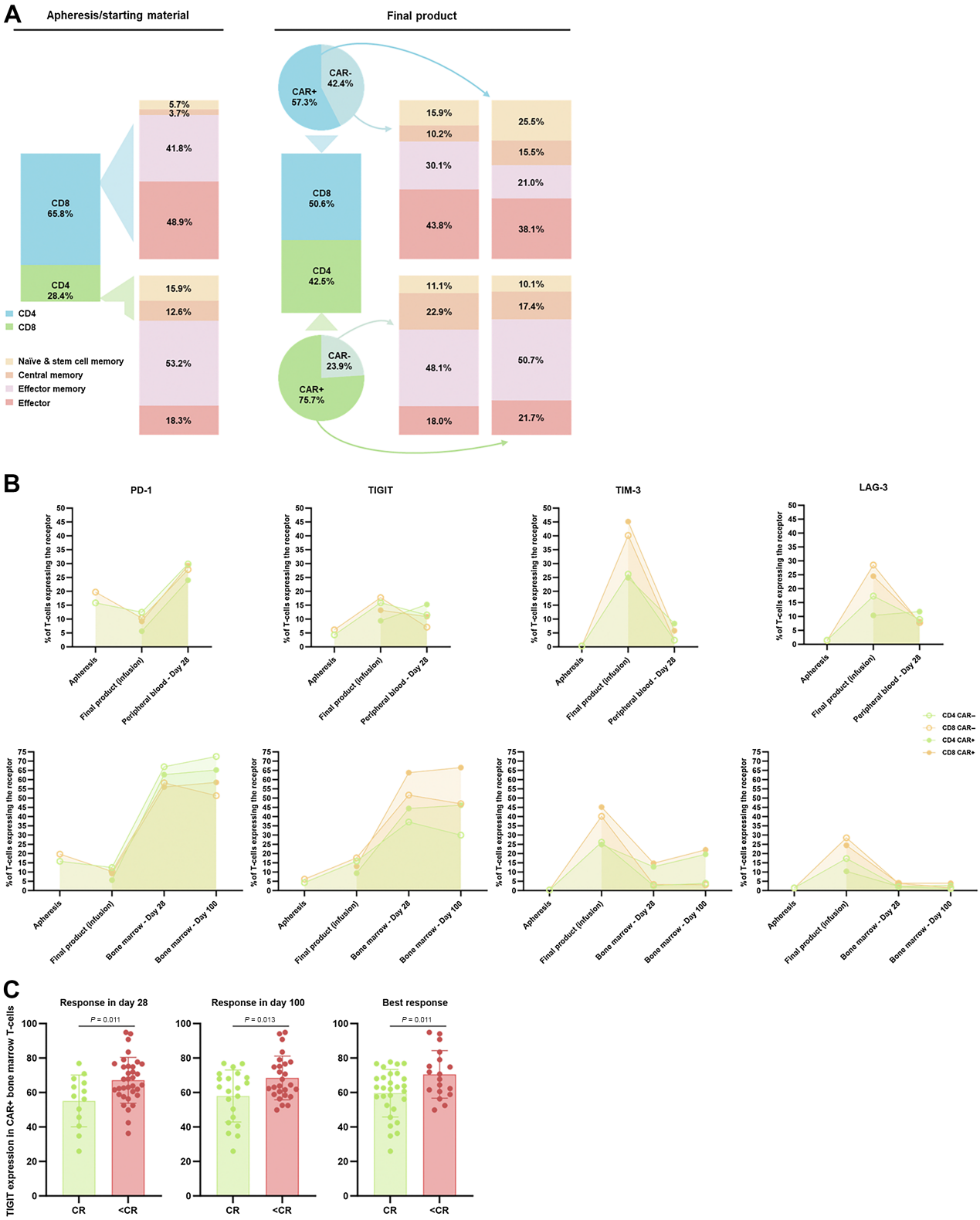

Figure 4.

T-cell subpopulations in the apheresis, final product, and in the peripheral blood and bone marrow at different timepoints after infusion of ARI0002h. **A,** Changes in T-cell subsets (naïve, central memory, effector memory and effector cells in CD4, CD8, CAR^+, and CAR^-) between the apheresis and final product. Gating was performed on $CD3^+$ cells, $CD8^-CD4^-$ cells and $CD8^+CD4^+$ cells were not included in the graph. **B,** Changes in exhaustion markers (PD-1, TIGIT, TIM-3, LAG-3) on CD8 and CD4 CAR^+ and CAR^- cells between the apheresis, final product, and peripheral blood on day 28 (top graphs), and bone marrow on days 28 and 100 (bottom graphs). **C,** Differences in TIGIT expression between patients who did or did not achieve a complete response on days 28, 100, and as best response.

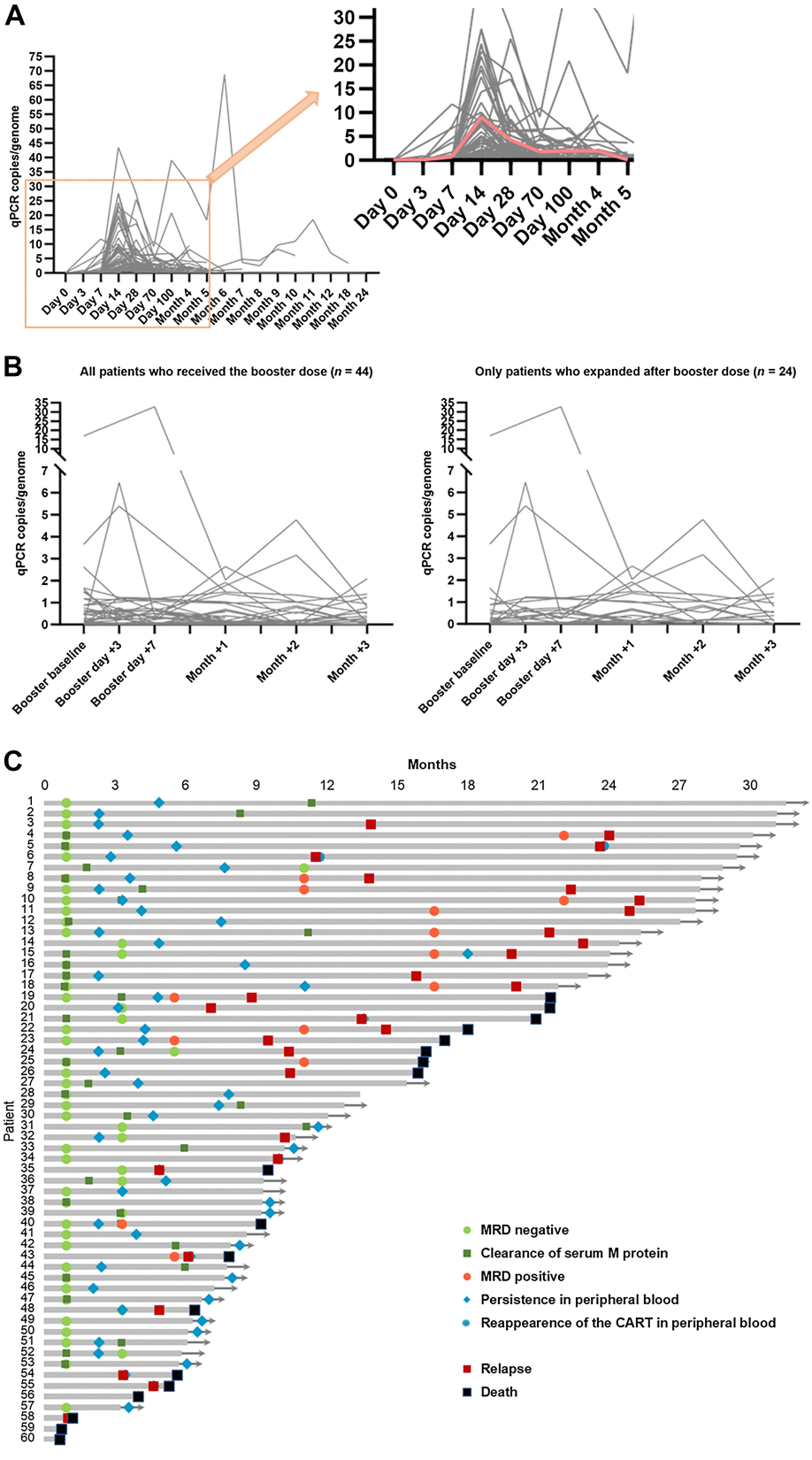

Figure 5.

Kinetics of ARI0002h and B cells. **A,** ARI0002h kinetics of individual subjects, from infusion to booster dose administration (measurements after booster dose of each individual patient are not depicted in this graph). Most patients present the peak of expansion on day 14. The red line represents the mean levels of ARI0002h. **B,** ARI0002h kinetics after booster dose in all reinfused patients ($n = 44$) and only in those in which ARI0002h expanded ($n = 24$). **C,** Swimmer plot showing the timing of MRD negativity, M protein clearance, MRD positivity, relapse and death for each patient. Persistence of the CART and reappearance (if occurred) are also depicted. PFS in patients with detectable CART in the end-of-treatment sample was (in months): 1 (refractory patient), 3.4, 4.6, 4.9, 6.1, 9.9, 11.5, 13.5, and 23.6.

compared with those who had not undergone alloHSCT in either the PB (11.1 months vs. 4.9 months; $P = 0.417$) or BM (11.1 months vs. 5.4 months; $P = 0.315$; Supplementary Fig. S7).

The magnitude of the peak of expansion had no impact on PFS or OS, but did correlate with the probability of CRS occurring (CRS vs. no CRS: 11.1 vs. 3.7 copies/genome; $P = 0.030$). A trend to higher proportion of CD4 T cells on peak day was observed in patients who were in CR on day 100 (CR, 19.0 vs. <CR, 12.5; $P = 0.046$) and who achieved a CR as best response (CR, 25.7 vs. <CR, 18.8; $P = 0.056$).

B-cell aplasia

The disappearance of normal B cells is a well-known on-target/off-tumor effect of BCMA targeted CARTs. All 60 patients treated with ARI0002h had B cells in the PB at inclusion (mean, 8.1%; SD, 12.2%) and all of them eventually developed B-cell aplasia. On the day of infusion, 31.6% ($n = 12/38$) of patients lacked B cells, probably due to the LD regimen, although in four of these cases we observed a brief recovery. Median time from infusion to the start of B-cell aplasia was 3 days (IQR, 12; range: 0–29) and the median duration was 3.3 months (95% CI, 2.6–3.9). Among the 28 patients who developed disease progression, 5 (17.9%) maintained a continuous B-cell aplasia in the EOT samples; 1 patient was not evaluable and in the remaining 22 patients median time from B-cell recovery to relapse was 10.3 months (IQR, 12.6; range: 0.5–24.3; Supplementary Fig. S8). Of the 9 patients who relapsed with CART in PB, B cells were undetectable in only 4.

Cytokines

Cytokine results are only available for the first cohort of 30 patients. Serum quantities of different cytokines are depicted in Supplementary Fig. 9A. See also Supplementary Table S5 and Supplementary Fig. S10. Patients who experienced CRS of any grade, compared to those who did not, showed significant differences in baseline IFNγ (CRS 4.1 vs. no CRS 8.4 pg/mL; $P = 0.029$), granzyme B on day 14 (CRS, 365.9 pg/mL vs. no CRS, 39.6 pg/mL; $P = 0.040$), IL6 on day 14 (CRS, 370.8 pg/mL vs. no CRS, 8.1 pg/mL; $P = 0.014$) and IL6 on day 28 (CRS, 310.2 pg/mL vs. no CRS, 5.5 pg/mL; $P = 0.020$; Supplementary Fig. S9B).

CART antibodies

We detected antibodies against the CART in 60% ($n = 36/60$) of patients. Although we detected antibodies in consecutive samples in few patients, in most of them, anti-CAR antibodies were not sustained over time. The detection of anti-CAR antibodies at each timepoint is summarized in the Supplementary Table S6. Ten patients had a positive antibody measurement prior to the booster dose, and of those, 5 (50%) expanded, 3 did not expand (30%), and in 2 expansion was not evaluable. We analyzed survival setting a landmark time at 3 months, comparing patients with or without antibodies at that timepoint, with no differences in PFS ($P = 0.435$) or OS ($P = 0.824$). Anti-CAR antibodies were detected in the EOT sample of 25% (7 of 28) of patients (Supplementary Table S7), but they were also detected in the 5 patients who have not relapsed after more than 20 months (Supplementary Table S8).

Measurable residual disease by next-generation flow cytometry

MRD analysis results obtained for the 60 patients at different timepoints are shown in Supplementary Table S9. MDR-negative rates in evaluable samples on days 28 and 100 were 98% and 96%, respectively. Of note, 24% ($n = 13/54$) of the samples were not evaluable on day 28 due to hemodilution. MRD was evaluable at the expected sensitivity of 10^{-6} in approximately half of the samples in the initial timepoints (days 28 and 100) and in 60% to 75% of samples at later timepoints (months 6–24). A sensitivity of at least 10^{-5} was achieved in 87.5% to 100% of all samples at all timepoints. No differences were observed in PFS according to MRD-negative sensitivity (Supplementary Fig. S11).

In 12 of 28 patients (43%) who developed disease progression, MRD positivity was observed early, prior to overt relapse (**Fig. 5C**). This premature detection is probably underestimated due to the frequency of BM sampling. In 2 patients, the positivity was evident at a sensitivity of 10^{-4}, in 8 patients at 10^{-5}, and in 2 at 10^{-6}. Median time from MRD positivity to relapse was 3.1 months (IQR, 3.4; Supplementary Fig. S12). When analyzing MRD in 19 available end-of-treatment (EOT) samples, 3 were MRD negative: 2 had extramedullary disease progressions and 1 patient withdrew for alloHSCT.

Other clinical correlations

When analyzing tumor burden markers other than sBCMA, we found that M-protein correlated with shorter PFS ($P = 0.013$), whereas the proportion of BM PC did not. No differences in terms of PFS were observed according to the presence of triple-refractoriness, high-risk cytogenetics or extramedullary disease, when accounting for all plasmacytomas. There was a trend to shorter PFS when plasmacytomas were extramedullary (Supplementary Figs. S13A–S13D). Interestingly, International Staging System (ISS) was a better predictor calculated at baseline rather than at diagnosis of MM (Supplementary Figs. S13E and S13F). In a multivariate analysis, all three variables, sBCMA, M-protein and ISS at baseline retained statistical significance (Supplementary Table S10).

The impact of MRD in PFS could not be analyzed in this study since almost all patients were MRD negative on days 28 and 100. An involved serum-free light chain below the lower limit of normality was found in 80% and 84.3% of patients on days 28 and 100 and a correlation with PFS was observed ($P = 0.004$) with day 100 measurements (Supplementary Fig. S14).

Discussion

In this study, previously reported ARI0002h preliminary outcomes (11) were confirmed, with 30 additional patients and longer follow-up. One distinctive feature of this study was the booster dose. Both expansion and an improvement of responses were observed after booster dose administration. It is unknown whether patients who received the booster dose in sCR (45%) had a longer DOR. Expansion results were similar between patients receiving or not a second LD, which suggests that it may not be mandatory. Still, a bias is present, because LD was administered according to CART persistence prior to booster dose, and therefore groups were not balanced. Because the study lacks a randomized comparison group, it is uncertain whether the booster dose is fully responsible for the benefits in terms of efficacy. Despite that, we believe that the feasibility of the booster dose manufacturing, administration and the lack of side effects warrant further investigation of this strategy.

The fractionated administration of ARI0002h may have contributed to a slightly delayed profile of CART kinetics and cytokine expression, compared with other reported BCMA-CART that are administered in a single infusion (4,8,19). In ARI0002h, the peak of expansion was mainly observed in days 14 to 28. The highest levels of IL6 and granzyme B were observed also from day 7, onwards. This behavior might have a different effect on immunologic parameters and could explain the lower neurologic events observed in ARI0002h, although these observations can only be confirmed with a randomized study.

MAS/hemophagocytic lymphohistiocytosis (HLH) related deaths have been reported after cilta-cel and ide-cel (5,20). In another study (21), 21.8% of patients treated with BCMA-CART met criteria for MAS/HLH. In our study, MAS/HLH was screened along follow-up, especially because 1 patient died due to MAS. All cases who met any of the available criteria, even if only laboratory criteria were found, were reported as MAS. All cases presented a previous CRS (4 grades 1 and 2 grades 2).

Factors associated with the duration of response in patients with MM treated with CARTs against BCMA can be related to the host (age, preforming status), baseline disease (tumor burden, cytogenetics, extramedullary disease), and with the CART itself.

In our study, the ISS calculated at baseline rather than at diagnosis was a better predictor of outcomes. The finding that an involved serum FLC below the limit of normality in day 100 was predictive of longer PFS could be useful in patients who achieve a negative MRD or present hemodiluted BM samples.

The quantity of BCMA molecules on the surface of PCs did not impact response or survival in this cohort. However, sBCMA levels at baseline were lower in complete responders and seemed to have a response prediction capacity when measured on day 28. Of note, higher levels were also associated with CRS grade of severity, which would be inconsistent considering that no effector activity is performed against sBCMA. For this reason, we believe that the worse outcomes of high sBCMA levels found in this study in terms of both efficacy and toxicity have to do with sBCMA as a surrogate marker of tumor burden, rather than a sink for ARI0002h, although clinical data have reported a benefit in blocking cleavage of BCMA (22,23). Also, our data suggest that sBCMA levels could be used as a tumor marker in BCMA-positive patients, as suggested by several other studies (24–29). We did not find any evidence of BCMA negative relapse in this series. Because the expression was significantly lower at relapse, a mechanism associated with this low BCMA reservoir population cannot be discarded.

Reported data on T-cell subpopulations in (30–34) treated with CART suggest that long-lived self-renewing early memory phenotypes are of vital importance for CART expansion and promote profound and sustained tumor responses. Most studies are focused on the apheresis product, but a few studies that analyzed the FP report similar findings (19,35,36). ARI0002h T-cell subpopulation distribution showed that CART cells in the final product were not terminally differentiated, retaining effector functions while maintaining memory phenotypes with proliferation ability. In our cohort, only CD8 central memory cells in the FP correlated with response on day 100. We did not find clinical correlations regarding regulatory T cells, apheresis subpopulations, or populations other than CD8 central memory cells in the FP. In one study, a lower expression of HLA-DR was observed in nonresponders to CART for acute lymphoblastic leukemia (32). In our data, the activation marker HLA-DR in both CD8 and CD4 correlated with longer PFS.

As previously observed for cilta-cel and ide-cel (37,38), the persistence of ARI0002h cells seems to be unrelated to the probability of progression. In this study, median persistence of the CART was 4.9 months, whereas median PFS was 15.8 months. Similarly, the loss of B-cell aplasia occurred soon before relapse in the majority of patients. In contrast, more than 30% of patients had detectable levels of ARI0002h at relapse. We observed a clear increase in the expression of exhaustion markers, particularly PD-1 and TIGIT in the BM, but also TIM-3 from day 28, and markers remained high in the two BM samples at relapse with detectable CART. Moreover, in some patients, the detection of CARTs at relapse did not coexist with a B-cell aplasia. All of this highlights the role that exhaustion may play in ARI0002h cells. Mechanisms to overcome exhaustion could include obtaining a higher percentage of long-lasting cells in the FP, such as central memory T cells (39), and perpetuating T-cell activity with immune checkpoint blockade. A booster dose was administered with the aim of improving efficacy, with CARTs not exposed to MM, and therefore not exhausted, but based on exhaustion markers kinetics observed, we could consider an earlier administration.

Despite the humanized nature of the scFv, the development of anti-CAR antibodies was observed. Yet, their neutralizing ability and the potential deleterious impact on ARI0002h efficacy remains unclear and further studies are ongoing. The antibody detection at several time-points in 5 patients who remain in response after more than 20 months of follow-up, the CART expansion observed after the booster dose in patients with anti-CAR antibodies, and the lack of impact in terms of PFS and OS in patients with a positive detection at 3 months, supports the idea that these antibodies may not have a clinical impact.

MRD negativity was achieved in the first 100 days by almost all patients treated with ARI0002h, which makes comparisons between MRD status and PFS or OS unfeasible. Despite that, the MRD negativity rates in the BM were higher compared to the CR rates determined by the classic IMWG criteria (14) that include serum M protein and free light chain evaluation. Therefore, the use of NGF or other high-sensitivity techniques to analyze MRD could detect responders faster as reported elsewhere (40,41). In addition, MRD positivity was observed in some patients prior to relapse.

One limitation of the study is that the follow-up of cohort 2 is relatively short. Furthermore, we were not able to perform genomic studies on the samples, although mechanisms of CART resistance mediated by genomic alterations are infrequent, unlike for T-cell enhancers (42).

In summary, ARI0002h preliminary outcomes were confirmed. An increase in exhaustion markers in the BM was observed and correlated with poor outcomes, together with low HLA-DR marker, high tumor burden and sBCMA.

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Note

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Supplemental methods

Study design

CARTBCMA-HCB-01 is a pilot, single-arm, open-label study held in 7 Spanish centers. Patients aged 18-75 years old with RRMM were eligible if they had measurable disease, as assessed by M-protein (serum>10g/L or urine>200mg/24h) or serum free light chain levels (>100mg/L), received ≥2 prior regimens, including a proteasome inhibitor, an immunomodulatory drug and an anti-CD38 antibody, and were refractory to the last line of treatment. Prior BCMA-directed treatment was an exclusion criterion. ARI0002h was lentivirally transduced on autologous T-cells. Bridging therapy was allowed after apheresis according to investigator discretion. Lymphodepletion (LD) included cyclophosphamide (900mg/m^2 total dose) and fludarabine (90mg/m^2 total dose). The target dose ($3x10^6$/kg CAR+cells) was administered in a fractionated manner (10%/30%/60%), with at least 24h between infusions.

Samples

Patients' samples were obtained and processed at the following timepoints:

- **Apheresis**
- **Final product (FP)**
- **Peripheral blood:** baseline, prior lo LD, in days 0, 3, 7, 14, 28, 70, 100 and months 4, 5, 6, 7, 8, 9, 10, 11, 12, 18, and 24.
- **Bone marrow:** baseline, days 28 and 100, and months 6, 12, 18, and 24.

The following table shows an overview of the analysis performed in each sample according to the compartment:

Apheresis		**Final product**	
Analysis	**Technique**	**Analysis**	**Technique**
T-cell subsets	Flow cytometry	T-cell subsets	Flow cytometry
-	-	Potency	Flow cytometry

Peripheral blood		**Bone marrow**	
Analysis	**Technique**	**Analysis**	**Technique**
Kinetics of ARI0002h	Quantitative polymerase chain reaction (qPCR)	Kinetics ARI0002h	Flow cytometry
-	-	Measurable residual disease detection	Next generation flow cytometry
Soluble BCMA	Enzyme-linked immunosorbent assay (ELISA)	BCMA quantification on the surface of plasma cells	Flow cytometry
T-cell subsets	Flow cytometry	T-cell subsets	Flow cytometry

B-cell kinetics	Flow cytometry	-	-
Anti-CAR antibodies	Flow cytometry	-	-
Cytokine measurement	Enzyme-linked immunosorbent assay (ELISA)	-	-

A FACSCanto (BD Biosciences, New Jersey, USA) flow cytometer and Infinicyt 2.0 software (Cytognos S.L., Salamanca, Spain) were used to perform flow cytometry analysis in BM samples. An Attune Next Flow flow cytometer (Thermofisher Scientific, Massachusetts, USA), the Attune Cytometric Software and FlowJo (V10.7.1) (RRID:SCR_008520) were used to analyze PB samples.

Specific methods used for each analysis are specified in the following paragraphs:

Potency

ARI0002h transduced T cells obtained after the manufacturing process (final product, FP) are challenged with the u266 multiple myeloma cell line (cells previously transduced with GFP) at a target/effector (tumor cell/T-cell) ratio of 2.5:1 in RPMI medium with 10% FBS, 1% glutamine and 1% penicillin-streptomycin. After 24 hours of co-culture, the quantity of live tumor cells was determined by flow cytometry and the percentage of T-cell mediated killing is calculated with respect to tumor cells alone, setup in the same assay, at the same quantity but without T-cell co-culture.

BCMA evaluation: soluble BCMA and plasma cell surface BCMA

After serum sample thawing and dilution (1:200), an enzyme-linked immunosorbent assay (ELISA) from (R&D Systems, Minneapolis, USA) was conducted according to the manufacturer's protocol. ELISA plates were analyzed using an EPOCH microplate spectrophotometer plate reader (BioTek Industries, Winooski, Vermont, USA) set to 450 nm. Data were processed with the BioTek Gen5 Data Analysis Software. The detection range was 2.62–20.000 pg/mL.

Plasma cell surface BCMA was measured by flow cytometry at baseline and in MRD positive disease. A PE anti-human CD269 (BCMA) antibody (Cat. number: 357504; Biolegend, San Diego, USA) was used for staining and the BD QuantiBRITE™ Beads kit (BD Biosciences, New Jersey, USA) enabled molecule quantification (molecules/cell). In samples with positive measurable residual disease, BCMA molecules were stained by replacing CD56-PE with BCMA-PE in the EuroFlow protocol tube 1 (see above). For baseline measurements, in patients receiving bridging therapy, a second baseline sample was obtained after treatment to do the measurements.

T-cell subsets

Antibody panels applied to analyze T-cell subpopulations are shown in the following table:

Panel	Antigen	Label	Clone	Catalog number	Company

Quantification	CAR-T	BV421			
	CD45	OC515	GA90	CYT-45OC	Cytognos
	CD25	FITC	2A3	345796	BD
	CD127	PE	A019D5	351304	Biolegend
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	TCRgd	PECy7	11F2	655410	BD
	CD19	PECy7	J3-119	IM3628	BC
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Subsets	CAR-T	BV421	M1310G	410704	Biolegend
	CD45	OC515	GA90	CYT-45OC	Cytognos
	CD62L	FITC	SK11	347443	BD
	CD27	PE	L128	340425	BD
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	CD45RA	PECy7	HI100	304126	Biolegend
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Exhaustion	CAR-T	BV421	M1310G	410704	Biolegend
	CD45	OC515	GA90	CYT-45OC	Cytognos
	TIM3	FITC	F38-2E2	345022	Biolegend
	TIGIT	PE	741182	FAB7898P	R&D
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	PD-1	PECy7	PD1.3	A78885	BC
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Cytotoxicity	CAR-T	BV421	M1310G	410704	Biolegend
	CD45	OC515	GA90	CYT-45OC	Cytognos
	CD57	FITC	HNK-1	333169	BD
	cyGrzmB	PE	GB11	561142	BD
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	CD56	PECy7	NCAM16.2	335826	BD
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD
Activation/ Inhibition	CAR-T	BV421	M1310G	410704	BD
	CD45	OC515	GA90	CYT-45OC	Cytognos
	HLADR	FITC	L243	347400	BD
	LAG3	PE	T47-530	565616	BD
	CD8	PerCP-Cy5.5	SK-1	341050	BD
	CD39	PECy7	A1	328212	Biolegend
	CD4	APC	SK3	345771	BD
	CD3	APCH7	SK7	641415	BD

Kinetics of ARI0002h

In peripheral blood samples, genomic DNA was acquired with the MagNA Pure Compact Nucleic Acid Isolation Kit I on the MagNA Pure Compact System. Primers were designed against the vector transgene WPRE sequence (WPRE_F: 5'gtcctttccatggctgctc 3'; WPRE_R: 5'ccgaagggacgtagcaga 3') and the GATA2 gene – control (GATA2_F: 5'tggcgcacaactacatggaa 3'; GATA2_R: 5'cgagtcgaggtgattgaagaaga 3'). The number of transgene copies/cell was determined by quantitative real-time PCR using Light Cycler® 480 SYBRGreen® I Master (Roche, Basel, Switzerland).

In bone marrow samples, five 8-color combinations of monoclonal antibodies allowed T-cell subset determination (see T-cell subset section), including a panel with a Biotin-conjugated goat anti-mouse IgG F(ab')2 primary antibody (Jackson ImmunoResearch,

Cambridge, UK) plus a BV421-Streptavidin secondary antibody, both used for CAR staining prior to flow cytometry analysis using a FACSCanto cytometer and the Infinicyt 2.0 software.

ARI0002h persistence was defined as the time between infusion and the first sample in which ARI0002h was no longer detected (qPCR result below 0.1 copies/genome in PB samples, and a negative result by flow cytometry in BM samples). For PB samples, at least two consecutive samples had to be reported as negative to confirm loss of persistence.

B-cell kinetics

The antibody panels applied to determine T-cell subsets include a *Quantification* panel, which contains a PE-Cy7 anti-CD19 antibody (Beckman Coulter, California, USA) that allows B-cell detection in the peripheral blood (see above).

Cytokine measurement

For cytokine analysis, serum levels of 14 cytokines were measured at 5 different timepoints (screening, day 7, 14, 28 and 100 after CART infusion). An enzyme-linked immunosorbent assay (ELISA) was performed using the commercially available Simple Plex assay from ProteinSimple (Biotechne, R&D Systems, Minneapolis, USA) according to manufacturer's protocol. The detection range for used immunoassays was as follows: IFN-g 0.17 – 4000 pg/mL, IL-1b 0.4 - 1,530 pg/mL, IL-2 0.54 - 2,050 pg/mL, IL-15 0.51 - 1,950 pg/mL, CCL3 1.05 - 4,000 pg/mL, granzyme B 1.31 - 5,000 pg/mL, IL-4 0.52 - 2,020 pg/mL, IL-6 0.28 - 2,652 pg/mL, IL-12 0.62 - 5,890 pg/mL, IL-10 0.58 - 2,212 pg/mL, IL-17a 1.05 - 10,000 pg/mL, CXCL10 0.6 - 920 pg/mL, IL-8 0.19 - 1,804 pg/mL and TNFa 0.3 - 1,160 pg/mL. The results were analyzed using the automated benchtop ELISA platform ELLA™ (Biotechne, R&D Systems, Minneapolis, USA). No levels of IL-4 were detected, therefore no information is reported regarding this cytokine.

Anti-CAR antibodies

To detect the presence of anti-CAR antibodies, a HEK-293T cell line (RRID:CVCL_0063) was transduced with the ARI0002h lentivirus using a multiplicity of infection of 2 for 48 hours at 37ºC in DMEM-10% FBS supplemented with polybrene (Cat. number: TR-1003-G; Merck Millipore, Massachusetts, USA). ARI0002h expression was confirmed using a recombinant BCMA protein fused with a human Fc (Cat. number: ALX-522-026-C050; Enzo Life Sciences, New York, USA) and a BV421-anti-human IgG antibody (Cat. number: 409318; Biolegend, San Diego, USA). ARI0002h expressing HEK-293T cells were incubated with patient's serum and a FITC-anti-human IgG antibody (Cat. number: H10101C; Biolegend, San Diego, USA) was used to determine the presence of anti-CAR antibodies. A threshold of 20% was considered positive.

Measurable residual disease (MRD) by next generation flow (NGF) flow cytometry

Measurable residual disease (MRD) was assessed by 2-tube 8-colour next generation flow cytometry in accordance with the EuroFlow platform (see full protocol on the EuroFlow website: app.euroflow.org/downloads/public - protocol 1·3):

- **Tube 1:** CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-PerCPCy5.5, CD19-PECy7, CD117-APC, and CD81-APCH7.
- **Tube 2:** as tube 1, but CyIgKappa-APC and CyIgLambda-APCH7 were included instead of CD117-APC and CD81-APCH7, respectively.

Limit of detection: 10^{-6}. Limit of quantification: $<5 \times 10^{-6}$.
To reach a sensitivity of 10^{-5}, 2 million cells were required. To reach a sensitivity of 10^{-6}, 10 million cells were required. Besides that, to consider a sample as evaluable, the presence of erythroblasts, B-precursor cells and mastocytes was also a requirement, since these populations are only found in the bone marrow. An absence of these populations suggests that the sample is hemodiluted. Anyway, the presence of clonal plasma cells, even in a hemodiluted sample, was given as positive MRD, since the expected number of clonal plasma cells would be higher if the sample was correct.

Clinical results

Duration of response (DOR) was defined as time between first response and progression (patients who were evaluable for response but died without progression were censored at the last follow-up). Progression-free survival (PFS) was defined as time between infusion and progression or death and overall survival (OS) was defined as time between infusion and death.

Statistical analysis

A two-sided p-value of <0.05 was considered statistically significant. Statistical analysis was performed with SAS System (v9.4), GraphPad Prism (v10.0.2) (RRID: SCR_002798) and IBM SPSS Statistics (v29.0) (RRID: SCR_002865).

Summary of the study protocol

Sponsor's Name:

Institut D'Investigacions Biomèdiques Agustí Pi i Sunyer (IDIBAPS).
C/Rosselló 149-153 08036 Barcelona, Spain.
Telephone number: 93 2275400.

Final product name:

ARI0002h cells. CARTBCMA_J22.9-h:CD8TM:4-1BB:CD3.

Investigational Medicinal Product (IMP)

ARI0002h cells. CARTBCMA_J22.9-h:CD8TM:4-1BB:CD3.
Adult differentiated autologous T-cells from peripheral blood, expanded and transduced with a lentivirus to express a chimeric antigen receptor with anti-BCMA (TNFRSF17) specificity conjugated to the 4-1BB co-stimulatory region and signal-transduction CD3z that has been humanized.

Study title:

Pilot study of the infusion of differentiates autologous T-cells from peripheral blood, expanded and transduced with a lentivirus to express a chimeric antigen receptor with anti-BCMA (TNFRSF17) specificity humanized conjugated with the co-stimulatory region 4-1BB and signal-transduction CD3z (ARI0002h) in patients with relapsed/refractory multiple myeloma with at least two prior line including proteasome inhibitor, immunomodulatory drug and anti-CD38 monoclonal antibody.

Investigator's team:

1. **Chief investigator (coordinator):**
   Carlos Fernández de Larrea
2. **Principal investigators at participating sites (Cohort 1):**
   - Carlos Fernández de Larrea - Hospital Clínic de Barcelona
   - Paula Rodríguez Otero - Clínica Universitaria de Navarra
   - Juan Luis Reguera Ortega - Hospital Virgen de Rocío
   - Mª Victoria Mateos - Instituto de investigación Biomédica de Salamanca
   - José Mª Moraleda- Hospital U. Virgen de la Arrixaca
3. **Principal investigators at participating sites (added to Cohort 2):**
   - Joaquín Martínez López - Hospital Universitario 12 de Octubre
   - Marta Sonia González Pérez – Complejo Hospitalario Universitario de Santiago

Study population:
Patients with relapsed/refractory multiple myeloma who have received treatment with at least 2 prior lines including proteasome inhibitor, immunomodulatory drug and anti-CD38 monoclonal antibody.

Phase and clinical trial design:
First-in-human, pilot, open, non-randomised, single-arm, prospective, national, multicentre, clinical trial.

Primary Objective:
To assess the safety and efficacy of CARTBCMA ARI0002h in patients with relapsed/refractory multiple myeloma who have received treatment with at least two prior lines including proteasome inhibitor, immunomodulatory drug and anti-CD38 monoclonal antibody.

Secondary objectives:

- To evaluate the effectiveness of ARI0002h
- To assess the duration of the response after the administration of ARI0002h
- To evaluate the overall survival after the administration of ARI0002h
- To evaluate the persistence of ARI0002h cells in peripheral blood after administration
- To assess the effect of ARI0002h treatment on the quality of life of patients
- To evaluate the adverse events occurring at 3 months and at one year

Primary Endpoints:

- **Efficacy primary endpoint:**

Overall response rate (ORR) in the first 3 months of the first infusion (defined as at least achieve partial response according to the criteria of the International Myeloma Working Group).

- **Safety primary endpoint:**

Rate of patients who develop a cytokine release syndrome and/or neurological toxicity in the first 30 days after the administration of ARI0002h, according to the criteria and gradation defined in the international consensus (Lee, Santomasso et al., 2019).*

Secondary Endpoints:

- Duration of the response (see criteria for second dose of ARI0002h)

- Response rate over the first year
- Complete response rate (CR) at 3 and 6 months after the first infusion.
- Overall response rate (ORR) at 6 months after the first infusion.
- Time to complete response.
- Time to better response.
- Negative measurable residual disease (MRD) rate in bone marrow at 3 and 6 months.
- Response rate of extramedullary disease by PET-TC at 3 months.
- Progression free survival (PFS), defined as the time elapsed between the administration of ARI0002h and the progression of the disease or death. Patients who are alive and in complete remission will be censored at the time of the last follow-up.
- Progression free survival at 12 months after the first infusion, defined as the time elapsed between the administration of ARI0002h and the progression of the disease or death. Patients who are alive and in complete remission at 12 months will be censored at the time of the last follow-up.
- Overall survival (OS) defined as the time elapsed between the infusion of ARI0002h and the death of the patient from any cause. Live patients will be censored at the time of the last follow-up.
- Presence of infusion reactions.
- Tumour lysis syndrome at any time after the administration of the treatment.
- Cytokine release syndrome, according to the criteria and grades defined in the international consensus (Lee, Santomasso et al., 2019).
- Neurological toxicity.
- Presence of prolonged cytopenia, defined as the reduction of neutrophil or platelet peripheral blood counts, grade 3 or 4 for more than 4 weeks after infusion.
- Persistence of CART BCMA ARI0002 in peripheral blood, which will be determined by flow cytometry and quantitative PCR of the transgene with monthly periodicity in the first 6 months and subsequently quarterly until 2 years after infusion.
- Expression of BCMA at diagnosis and at relapse, evaluated by multiparameter flow cytometry in bone marrow aspirate samples.
- Levels of soluble BCMA in serum pre-treatment and during treatment and its correlation with the degree of response of multiple myeloma.
- Quality of life at baseline, monthly basis the first 6 months and quarterly until 2 years from infusion.

Inclusion Criteria (main):

1. Patients between the age of 18 and 75 years with diagnosis of multiple myeloma.
2. Disease measurable** by monoclonal component in serum and/or urine or by free light chains in serum according to the eligibility criteria for clinical trials of the

International Myeloma Working Group.

3. Previous two or more lines of treatment. Patients must have received at least a proteasome inhibitor (such as bortezomib or carfilzomib), an immunomodulatory drug (lenalidomide or pomalidomide) and an anti-CD38 monoclonal antibody (such as daratumumab).
4. Refractory to the last line of treatment.
5. ECOG functional status ranging from 0 to 2.
6. Life expectancy over 3 months.
7. Patients who, after being informed, give their consent by signing the Informed Consent document.

Exclusion Criteria (main):

1. Previous allogeneic transplant in the prior 6 months to inclusion or GVHD that requires active systemic immunosuppressive treatment.
2. Previous treatment with CAR T-cell therapy or BCMA directed therapy.
3. Absolute lymphocyte count $<0.1x10^9$/L.
4. Previous neoplasia, except if patients have been in complete remission >3 years, except for cutaneous carcinoma (non-melanoma).
5. Active infection that requires treatment.
6. Active infection by HIV, HBV or HCV.
7. Uncontrolled medical disease.
8. Severe organic condition that meets any of the following criteria: EF 3 times normal value (except Gilbert syndrome).
9. Previous diagnosis of symptomatic AL amyloidosis.
10. Pregnant or lactating women. Women of childbearing age should have a negative pregnancy test in the screening phase.
11. Women of childbearing age, including those whose last menstrual cycle was in the year prior to screening, who cannot or do not wish to use highly effective contraceptive methods from the beginning until the end of the study.
12. Men who cannot or do not wish to use highly effective contraceptive methods from the beginning to the end of the study.
13. Contraindication to receive conditioning chemotherapy.

Representativeness of Study Participants

Cancer type(s)/subtype(s)/stage(s)/condition	Relapsed/refractory multiple myeloma (RRMM)
Considerations related to:	
Sex	In the United States (US) throughout 2017, 17,490 men and 12,790 women were diagnosed of MM, with a male/female ratio of 1.5. From patients treated with CAR-T cells (ide-cel) in real life world data in the US reported in 2023, 57% were male.
Age	In a recent survey in two regions of Spain (1994-2016), patients with MM were diagnosed at a median age of 72 years. However, the median age of the patients receiving ide-cel in the US was 64 years.
Race/ethnicity	There is a marked racial disparity in the incidence of MM and asymptomatic monoclonal gammopathies, with a two to threefold increased risk in blacks compared with whites, after adjusting for socioeconomic and other risk factors, suggesting a genetic predisposition.
Geography	The estimated newly diagnosed MM cases in the US throughout 2017 is 30,280, representing 1.8% of all new cancer, with an incidence rate (per 100,000 inhabitants and age-standardized to the 2000 United States standard population) of 8 and 5.2 respectively. Similar or slightly lower age-standardized incidence rates can be found in European countries. Conversely, in Asian countries, the incidence is particularly low. The incidence rates of MM in Spain, adjusted to the European population were 3.54 cases by 100.000/year for men and 2.54 cases by 100.000/year for women in 2005.
Overall representativeness of this study	The age and sex distribution of our study is similar to the average distribution of MMRR treated with CAR-T cells in real life. We do not have information about race/ethnicity in our trial in Spain.

Table 1. Patients diagnosed with a neoplasia.

	Neoplasia	Time from ARI002h infusion to diagnosis (months)
1	Skin – Basal cell carcinoma	4.2
2	Skin – Squamous cell carcinoma	4.9
3	Skin – In situ melanoma	9.2
4	Breast carcinoma	11.3
5	Papillary thyroid microcarcinoma	20.5
6	Colon adenocarcinoma	3.9

One additional patient was diagnosed with lung adenocarcinoma after inclusion and apheresis, but prior to lymphodepletion, and was withdrawn of the study.

Table 2. Correlation between T-cell subsets in the apheresis with the proportion of CD8 central memory cells in the final product (FP).

T-cell subset in the apheresis	P value	Spearman ρ (ro)
CD8	0.154	-
CD4	0.121	-
Naïve CD8	0.943	-
Central memory CD8	0.098	-0.26
Effector memory CD8	0.053	-0.3
Effector CD8	**0.029**	**0.34**
Naïve CD4	**0.007**	**0.41**
Central memory CD4	**0.045**	**0.31**
Effector memory CD4	**0.023**	**-0.35**
Effector CD4	**0.082**	**-0.27**

Table 3. Cox regression analysis of the impact of the expression of exhaustion markers in progression-free (PFS) and overall survival (OS).

Exhaustion marker	Sample, day & cell	Survival	B	Exp(B) (95%CI)	p value
PD-1	Apheresis	PFS	0.060	1.061 (1.024-1.100)	0.0010
		OS	0.052	1.053 (1.005-1.103)	0.030
TIGIT	Apheresis	PFS	0.223	1.25 (1.087-1.437)	0.0018
		OS	0.200	1.221 (1.009-1.478)	0.040
TIGIT	Day 28 PB CD8 CAR+	PFS	0.092	1.097 (1.038-1.159)	0.00099
		OS	0.089	1.094 (1.016-1.177)	0.017
TIM-3	Day 28 PB CD8 CAR+	PFS	0.137	1.147 (1.047-1.257)	0.0033
		OS	0.169	1.184 (1.057-1.326)	0.0036
TIM-3	Day 28 PB CD4 CAR+	PFS	0.064	1.066 (1.022-1.112)	0.0028
		OS	0.075	1.078 (1.020-1.138)	0.0077

Figure 1. Design of the study: timeline of lymphodepletion, ARI0002h fractionated infusion and booster dose.

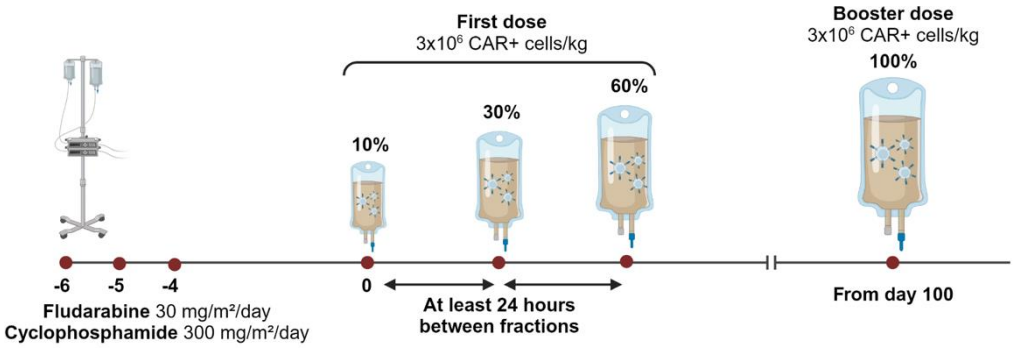


Figure 2. PD-1 and TIGIT expression in two patients (“A” and “B”), with available bone marrow samples across follow-up, including the end-of-treatment (EOT) sample. Of the 9 patients who relapsed with detectable CARTs in the peripheral blood, bone marrow EOT samples were available in these 2. Both patients had a high expression of both exhaustion markers, especially in CAR+ T-cells at all timepoints, including at relapse, which highlights the negative impact that exhaustion receptors may have in the efficacy of ARI0002h.

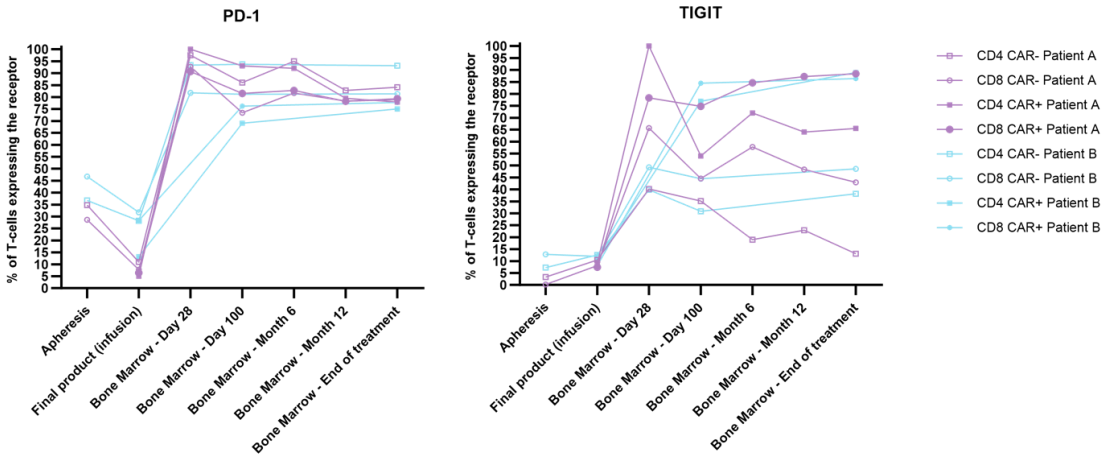
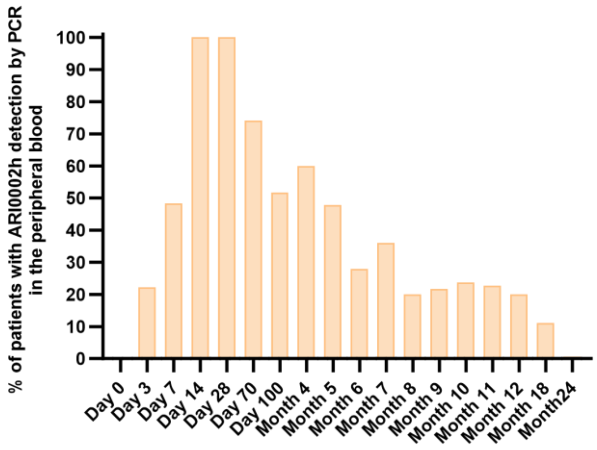


Figure 3. Persistence of ARI0002h in the peripheral blood at each timepoint. The table shows the number of positive / available samples at each timepoint.



	D3	D7	D14	D28	D70	D100	M4	M5	M6	M7	M8	M9	M10	M11	M12	M18	M24
Positive	6	14	27	29	20	15	15	11	7	9	5	5	5	5	4	1	0
Available samples	27	29	27	29	27	29	25	23	25	25	25	23	21	22	20	9	8
%	22,2	48,3	100,0	100,0	74,1	51,7	60,0	47,8	28,0	36,0	20,0	21,7	23,8	22,7	20,0	11,1	0,0

Figure 4. ARI0002h peak magnitude according to baseline tumor burden measured as proportion of bone marrow plasma cells and soluble BCMA. Baseline tumor burden was classified into low, medium, high and very high in the following manner: proportion of bone marrow plasma cells (low <10%, medium 11-20%, high 21-50%, very high > 50%) and levels of soluble BCMA (low <50.000pg/ml, medium 50.001-100.000pg/ml, high 100.001-500.000pg/ml, very high > 500.000pg/ml). No differences were observed either in terms of serum M protein (data not shown); 29 of 60 patients had less than 10 g/L and were included based on serum free-light chain. NS = non-significant.

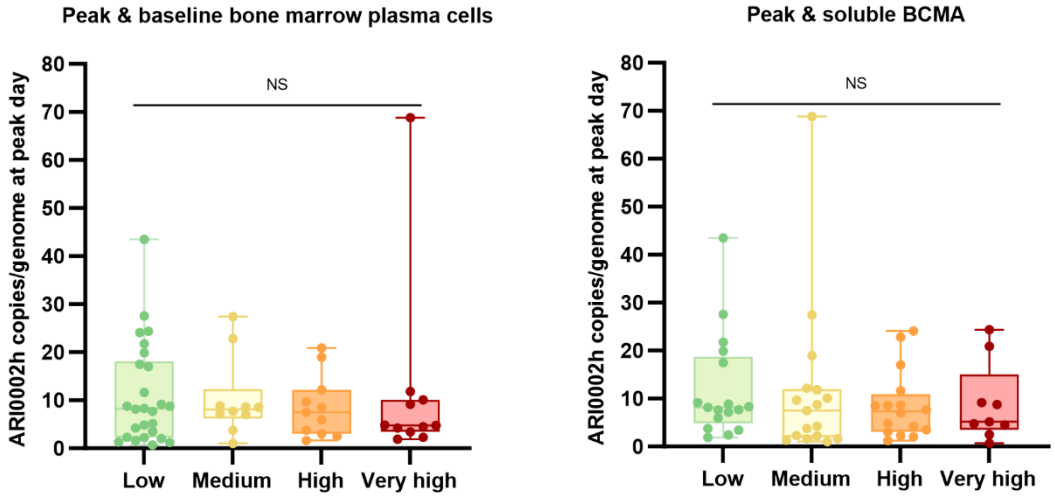


Figure 5. ARI0002h persistence according to baseline tumor burden measured as proportion of bone marrow plasma cells and soluble BCMA. Baseline tumor burden was classified into low, medium, high and very high in the following manner: proportion of bone marrow plasma cells (low <10%, medium 11-20%, high 21-50%, very high > 50%) and levels of soluble BCMA (low <50.000pg/ml, medium 50.001-100.000pg/ml, high 100.001-500.000pg/ml, very high > 500.000pg/ml). No differences were observed either in terms of serum M protein (data not shown). Low = green; medium = yellow; high = orange; very high = red. NE = not estimable.

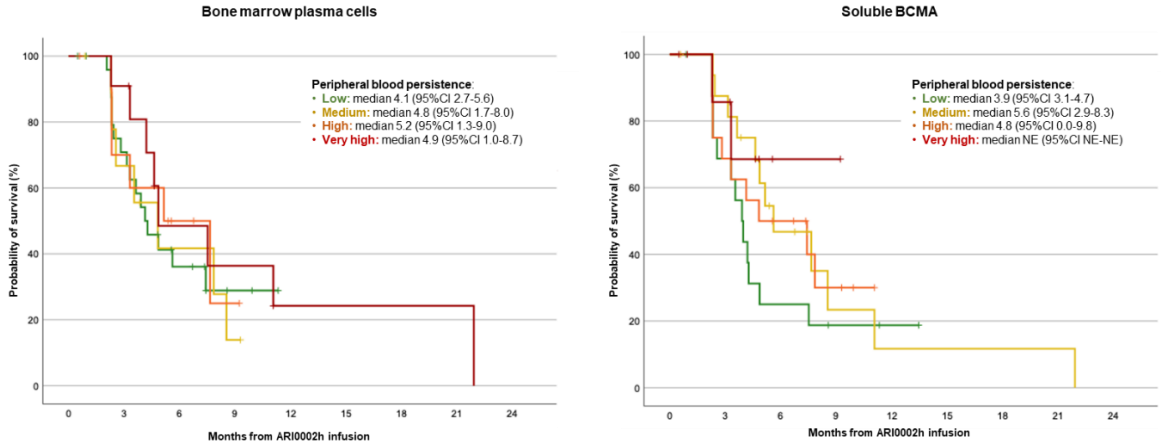
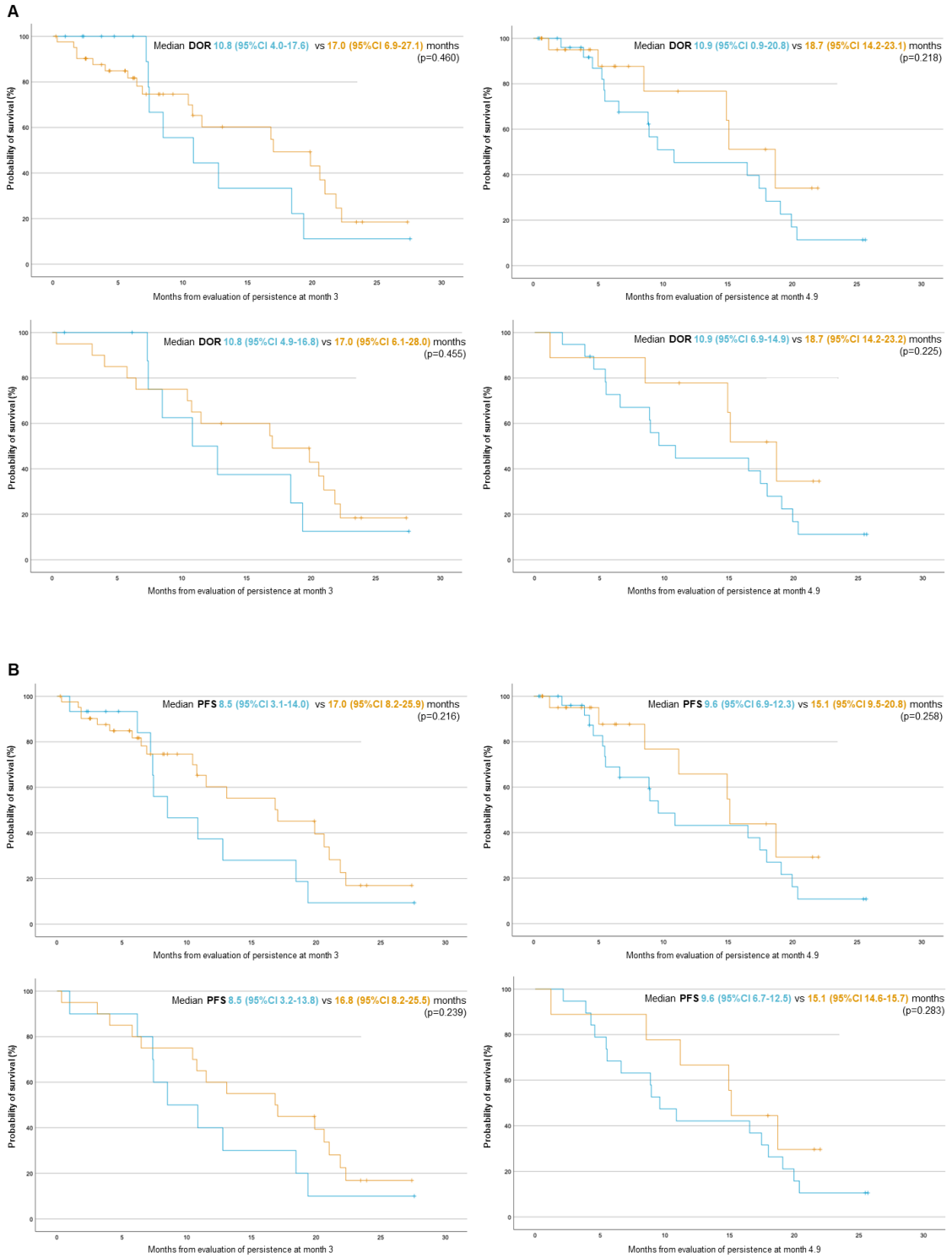


Figure 6. Impact of ARI0002h persistence in months 3 and 4.9 on **(A)** duration of response (DOR), **(B)** progression-free survival (PFS) and **(C)** overall survival (OS) in all patients (upper graphs) and in only cohort 1 (lower graphs). Patients with ARI0002h persistence (orange) vs. no persistence (blue).



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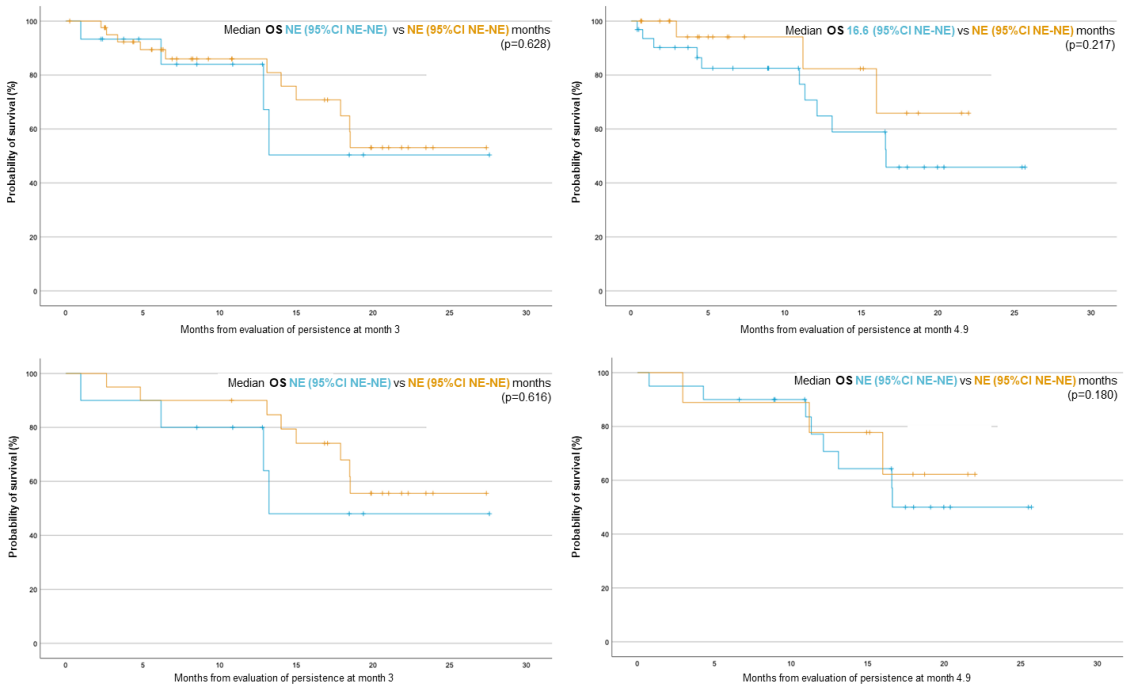

Events occurred before 3 month landmark			
	DOR	**PFS**	**OS**
Refractory	1	1	1
Progression	-	-	-
Death without progression	2	2	2
Not reached	-	-	-
Total missed events	3	3	3
Total included events	57	57	57
Events occurred before 4.9 month landmark			
	DOR	**PFS**	**OS**
Refractory	1	1	1
Progression	4	4	-
Death without progression	3	3	3
Not reached	1	1	1
Total missed events	9	9	5
Total included events	51	51	55

Figure 7. Persistence of ARI0002h in the peripheral blood according to **(A)** triple- and **(B)** penta-refractoriness (yes = orange, no = blue), and **(C)** prior allogeneic stem cell transplantation (yes = orange, no = blue).

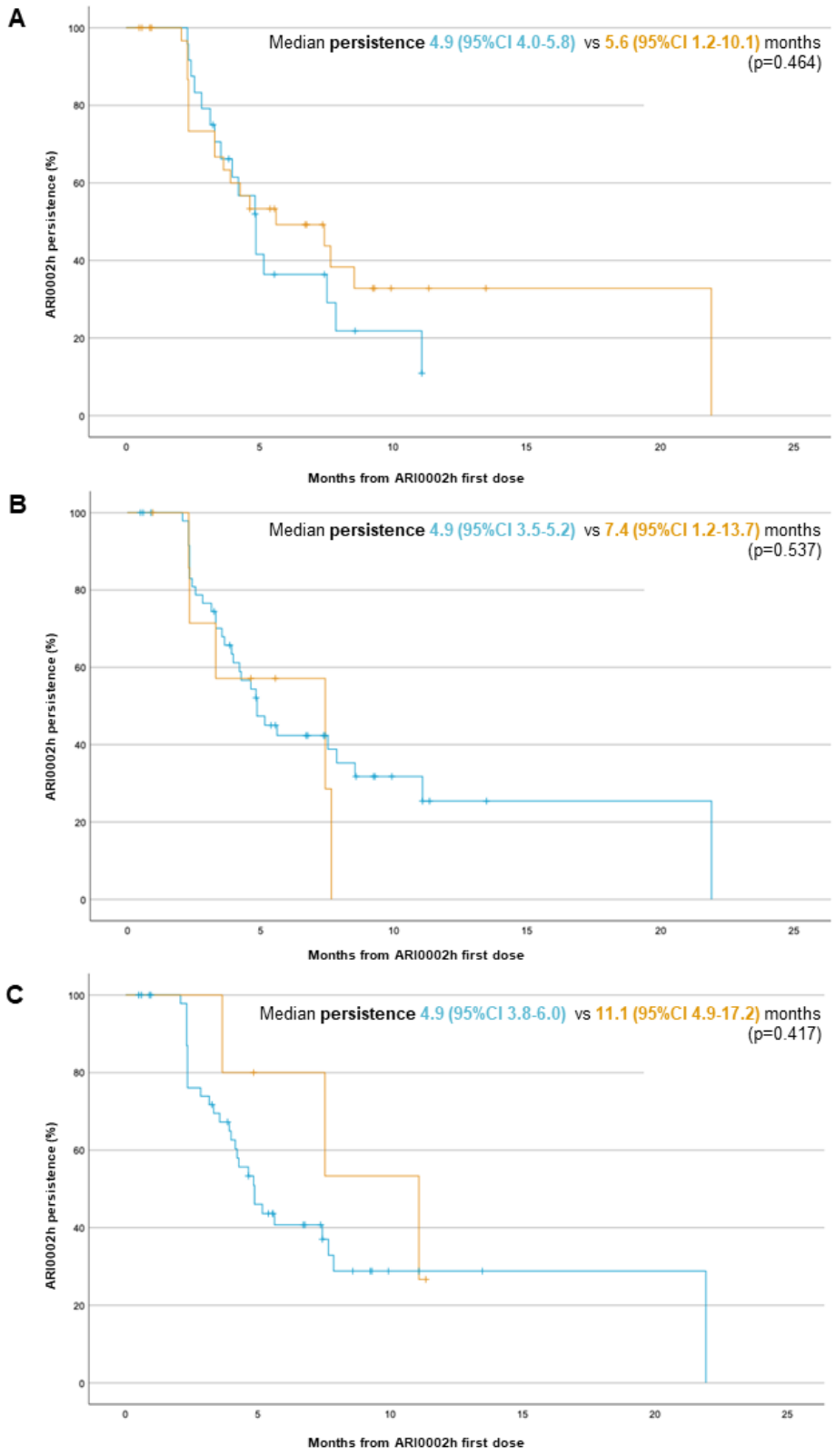

Figure 8. B-cell kinetics after ARI0002h infusion until study withdrawal. In the right-hand graph, B-cell levels are depicted only until booster dose, together with ARI0002h levels.

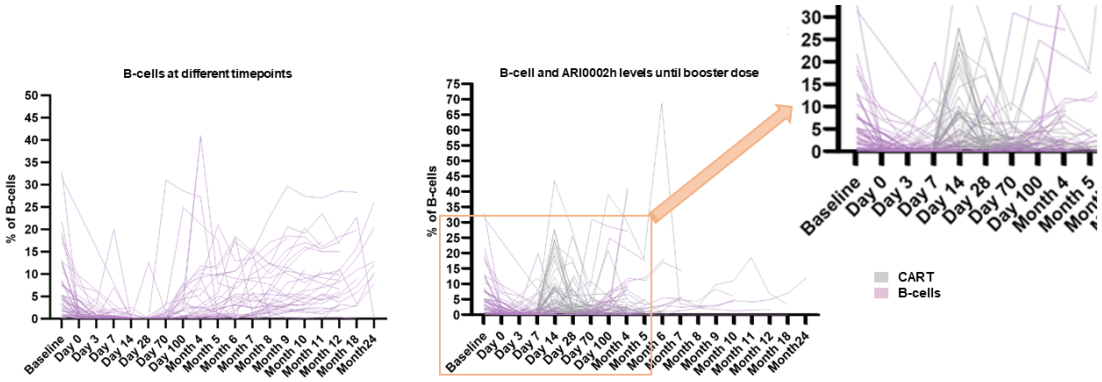

Figure 9. Cytokine levels in the peripheral blood of patients treated with ARI0002h. (A) Levels of cytokines and granzyme B at different timepoints, from baseline to day 100. (B) Differences in IFN-γ, granzyme B and IL-6 levels according to the development of CRS and CRS intensity (absence, grade 1 and grade 2).

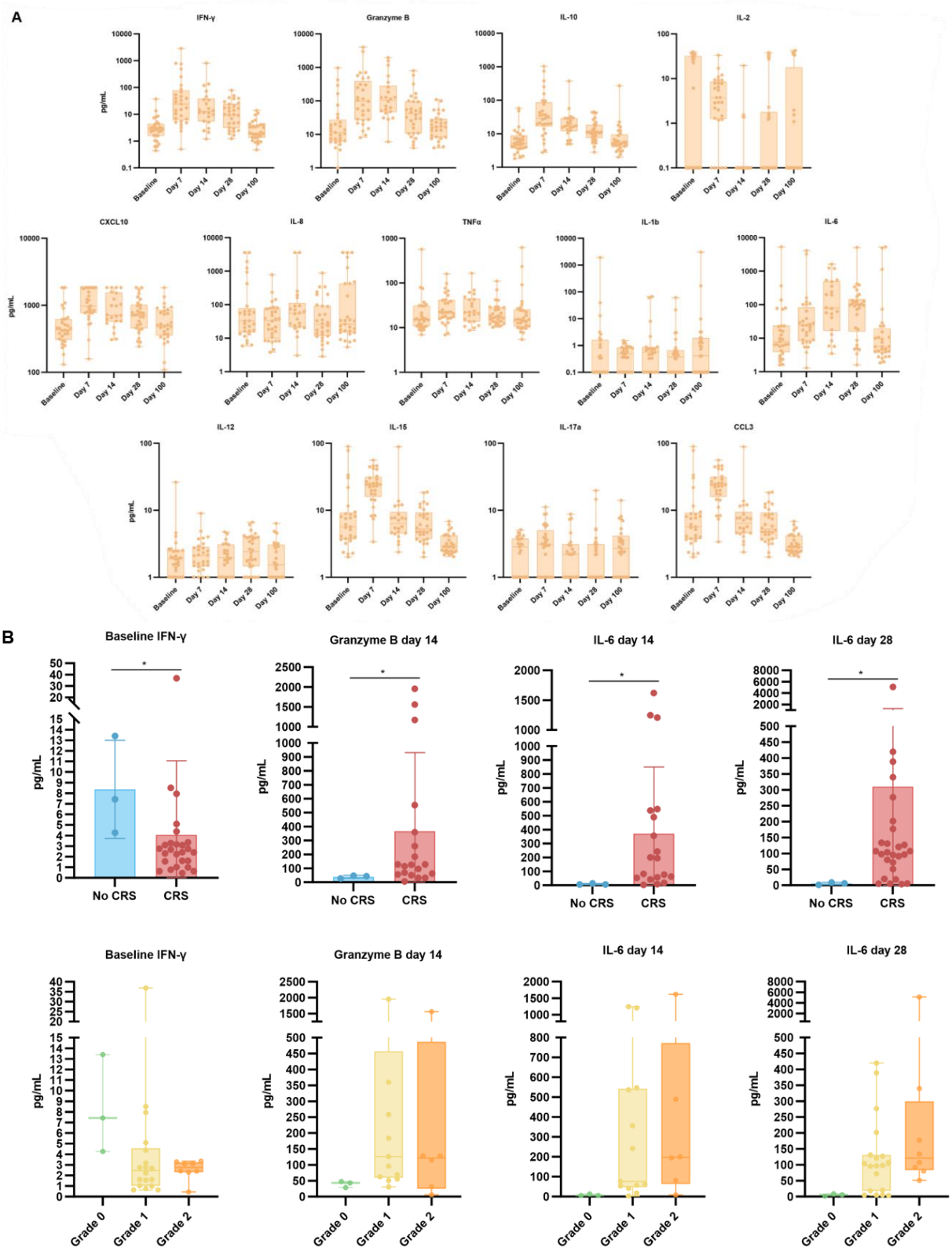

Table 4. Cox regression analysis of the impact of the levels of cytokines in progression-free (PFS) and overall survival (OS). Higher IFNγ, granzyme B and IL-10 on day 7, and IL-2 levels on day 100, were correlated with worse progression-free and overall survival. CXCL10 and IL-8 on day 7 were positively correlated with worse overall survival.

Cytokine	Survival	B	Exp(B) (95%CI)	p value
IFN-γ day 7	PFS	0.001	1.001 (1.00026-1.0018)	0.009
	OS	0.001	1.001 (1.00025-1.00183)	0.010
Granzyme day 7	PFS	0.00050	1.0005 (1.000072-1.00092)	0.022
	OS	0.00053	1.00053 (1.000092-1.000097)	0.018
IL-10 day 7	PFS	0.003	1.003 (1.001-1.005)	0.002
	OS	0.005	1.005 (1.002-1.008)	0.00057
IL-2 day 100	PFS	0.035	1.035 (1.009-1.063)	0.009
	OS	0.033	1.034 (1.004-1.065)	0.029
CXCL10 day 7	PFS	0.00073	1.00073 (0.999-1.0015)	NS 0.072
	OS	0.00124	1.00124 (1.000064-1.00241)	0.039
IL-8 day 7	PFS	0.0030	1.0030 (0.999-1.006)	NS 0.074
	OS	0.004	1.004 (1.001-1.007)	0.025

Figure 10. (A) Progression-free survival (PFS) and **(B)** overall survival (OS) curves obtained after dichotomizing the values of each cytokine (IL-10 on day 7, Granzyme on day 7, IFN-γ on day 7, IL-2 on day 100) according to the mean (lower=blue vs. higher=orange) in the study population at the selected time after infusion. To elucidate whether the elevation of these cytokines was caused by a higher tumor burden, we performed a correlation analysis with serum M protein or the percentage of BM PC that was not significant; sBCMA showed a positive correlation only with IL-2 levels on day 100 (r_s=0.418; p=0.024).

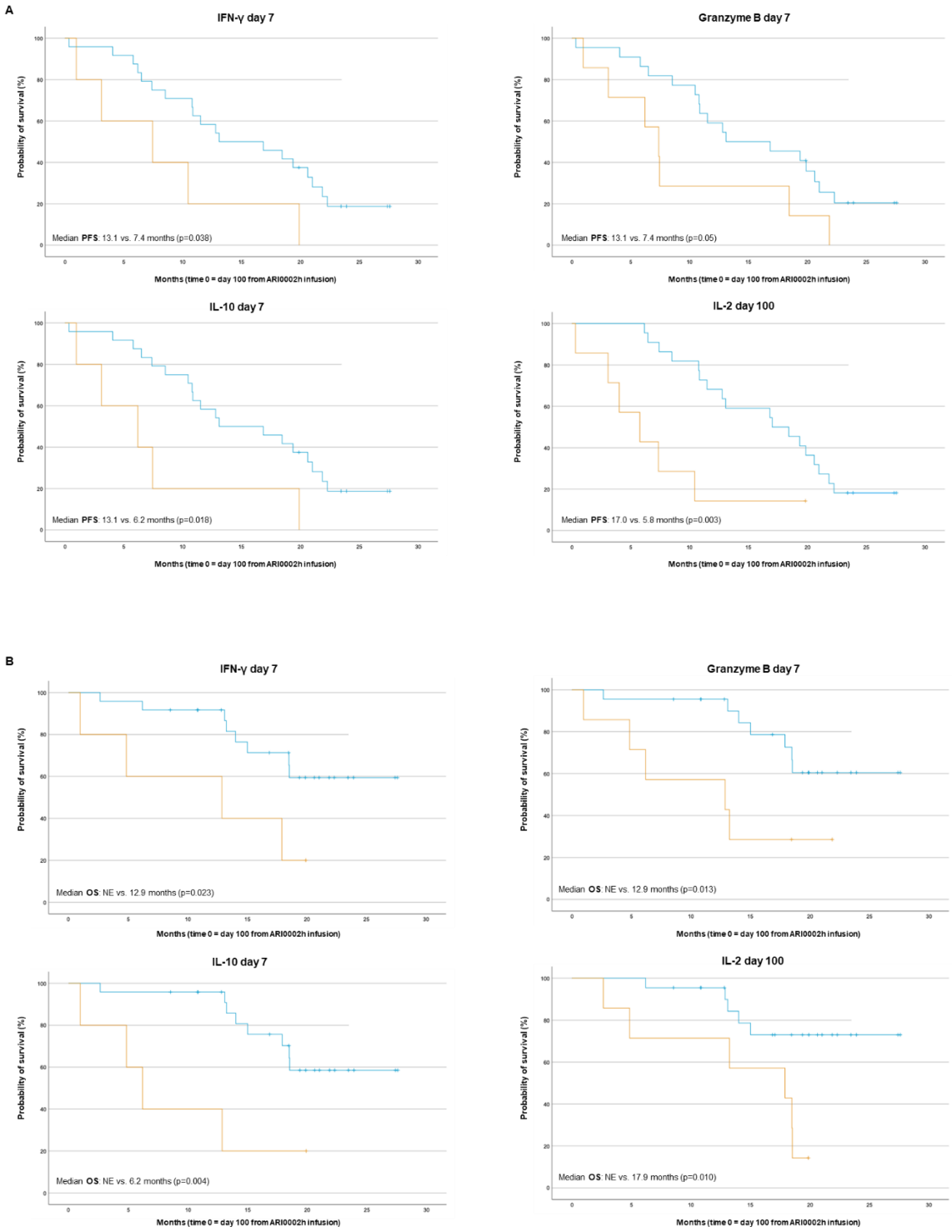

Table 5. Detection of CART antibodies at different timepoints.

	Day 28	Day 100	Month 6	Month 12	End of treatment
Patients with CART antibodies (n)	5	4	13	13	7
Samples available (n)	57	55	48	26	28
Patients with CART antibodies (%)	8.8	7.3	27.1	50	25

Table 6. Patients with positive antibodies against CART in the end-of-treatment (EOT) sample. In patient 1, several samples were positive before relapse; in patients 2 to 7 the positivity was in the EOT sample or very close to it. Note that one withdrawal was not due to relapse.

	Samples with positive CART antibodies	Time to withdrawal (months)	Reason for withdrawal
Patient 1	Months 12, 18, 24 and EOT.	24.0	Relapse
Patient 2	Month 12 and EOT.	12.3	AlloSCT
Patient 3	Only EOT.	13.8	Relapse
Patient 4	Only EOT.	11.5	Relapse
Patient 5	Only EOT.	10.2	Relapse
Patient 6	Only EOT.	8.8	Relapse
Patient 7	Only EOT.	4.6	Relapse

Table 7. Patients with positive antibodies against CART at some point that remain in response after a long follow-up (cohort 1).

	Samples with positive CART antibodies	Follow-up (months)
Patient 8	Day 28.	30.6
Patient 9	Months 6, 12, 18 and 24.	30.4
Patient 10	Month 18.	26.9
Patient 11	Month 18 and 24.	26.5
Patient 12	Month 12.	22.9

Table 8. Measurable residual disease results at different timepoints.

		Day 28	Day 100	Month 6	Month 12	Month 18	Month 24	End of treatment
Measurable residual disease general results								
Negative		40	49	41	21	8	6	3
Positive		1	2	4	4	5	3	16
Not evaluable		13	5	3	0	0	0	0
No sample		3	1	2	1	1	0	15
Out of study		3	3	8	17	25	30	-
Not reached		0	0	2	17	21	21	26
Total		60	60	60	60	60	60	60
Measurable residual disease negative results								
Negative (total n)		40	49	41	21	8	6	3
Sensitivity	**10^{-4}**	4	5	2	2	1	0	1
	10^{-5}	17	17	15	3	2	1	2
	10^{-6}	19	27	24	16	5	5	0
% negative at 10^{-6}		47.5	55.1	58.5	76.2	62.5	83.3	0
% negative at 10^{-5} and 10^{-6}		90	89.8	95.1	90.5	87.5	100	66.7

Figure 11. Progression-free survival according to MRD-negative sensitivity on day 28. Sensitivity of 10^{-6} = green, 10^{-5} = yellow, 10^{-4} = orange, hemodiluted = gray.

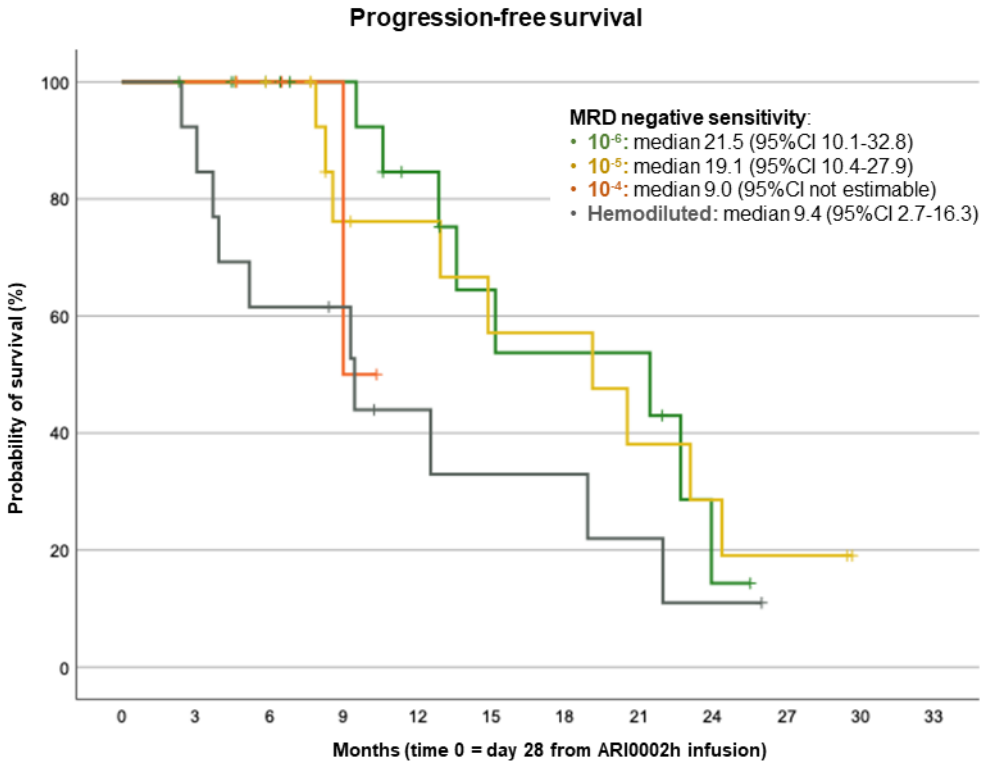

Figure 12. Time (months) from measurable residual disease positivity to overt relapse according to sensitivity of detection.

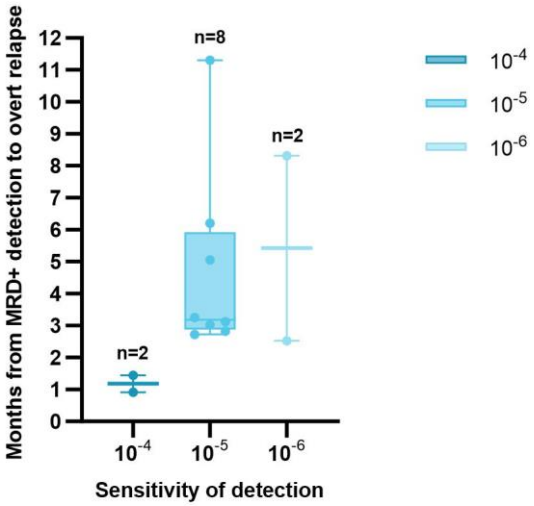

Figure 13. Progression-free survival according to **(A)** triple-refractoriness, **(B)** high-risk cytogenetics and the presence of extramedullary disease (**(C)** all plasmacytomas and **(D)** only those of extramedullary location). ISS was a better predictor when calculated at baseline **(E)**, rather than **(F)** at MM diagnosis.

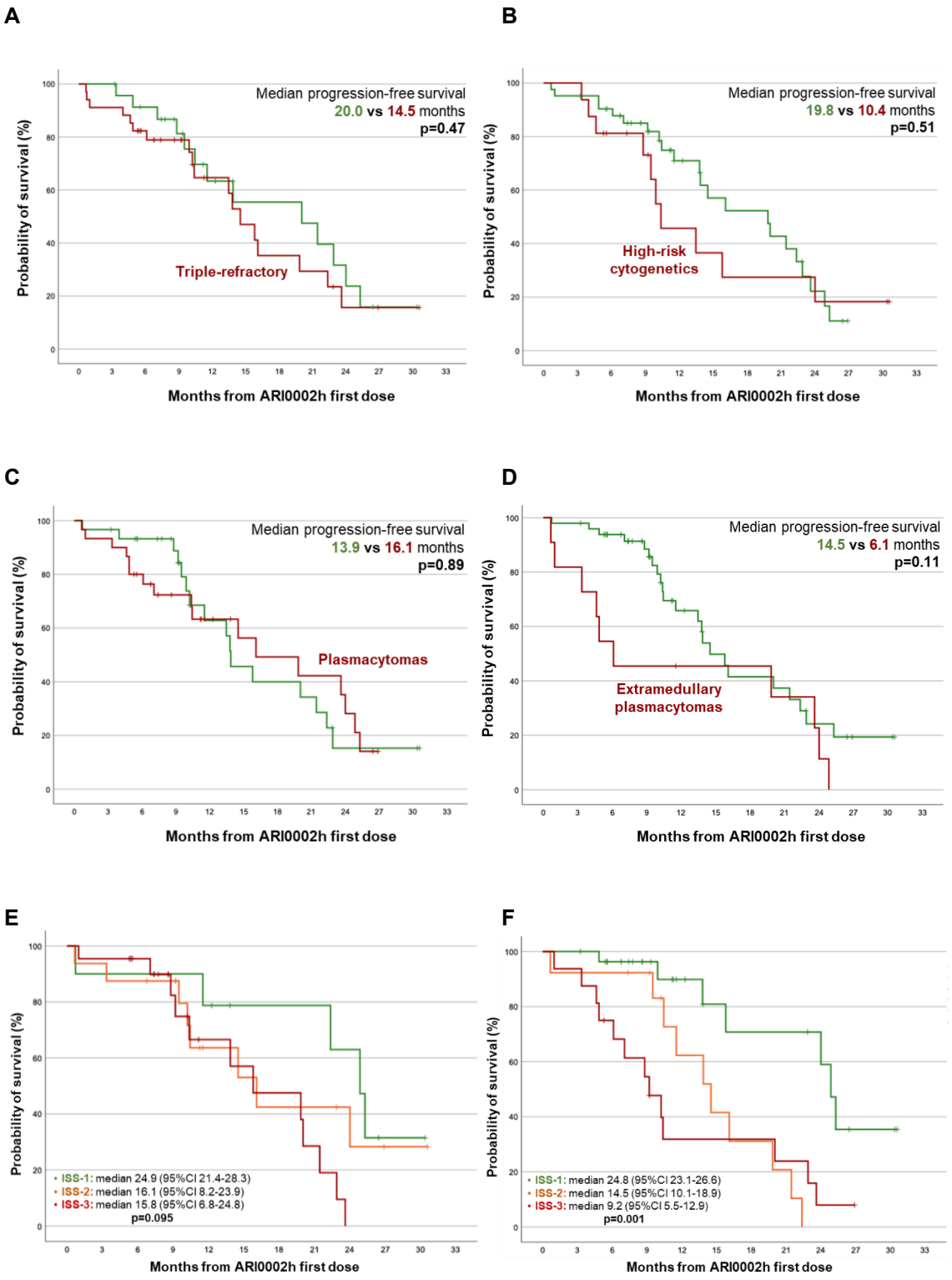

Table 9. Multivariate analysis of the impact in progression-free survival of clinical variables at baseline.

Variables at baseline	B	Exp(B) (95%CI)	p value
M-protein (g/L)	0.018	1.018 (1.001- 1.035)	0.039
Soluble BCMA (pg/mL)	0.0000012	1.0000012 (1.00000034-1.0000021)	0.006
ISS 2	1.427	4.165 (1.414-12.266)	0.010
ISS 3	1.276	3.582 (1.345-9.539)	0.011

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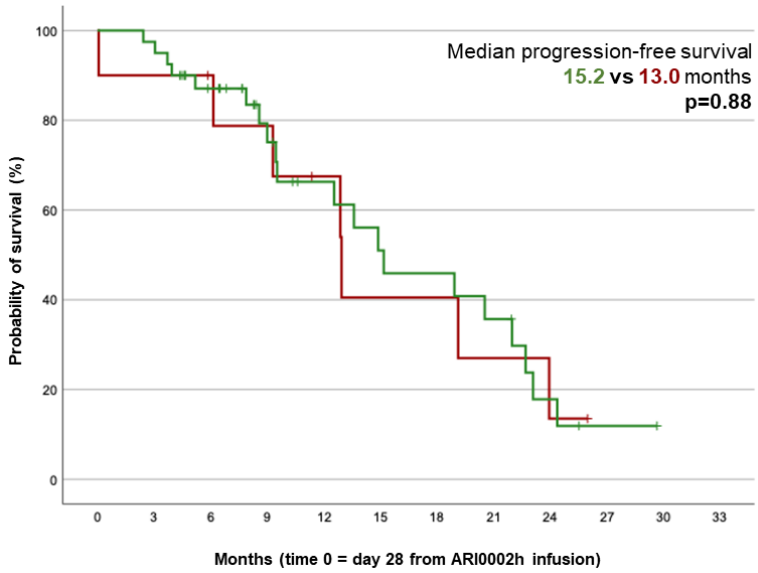
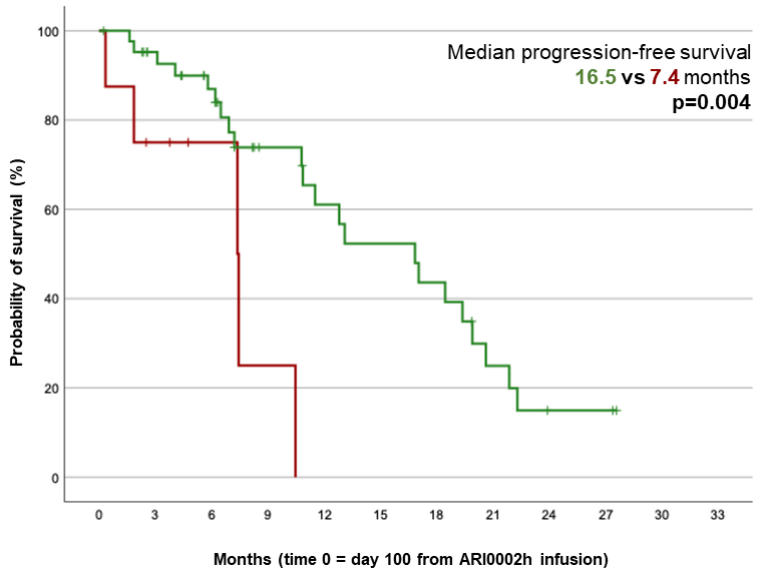
Figure 14. Progression-free survival according to involved serum free-light chain levels below the lower limit of normality (green) on **(A)** days 28 and **(B)** 100.

A



B



Article 3

TITLE: Different approaches to block TIGIT as a single target in the academic BCMA-CART ARI0002h fail to achieve a significant improvement in a model of multiple myeloma

Running title: TIGIT-blockade in the BCMA-CART ARI0002h

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TIGIT, immune checkpoint, CAR-T cell therapy, multiple myeloma, BCMA, CART, ARI0002h

DECLARATIONS

Ethics approval and consent to participate

The study protocol was approved by the Ethical Committee of Clinical Research (Hospital Clínic, Barcelona) and was carried out in compliance with the Declaration of Helsinki. For *in vivo* assays, ethical approval was obtained from the Ethical Committee of Animal Research (University of Barcelona, Spain). T-cells were obtained from patients of the Amyloidosis and Myeloma Unit at Hospital Clinic of Barcelona, after patient consent.

Consent for publication

Consent for publication was obtained.

Availability of data and material

Data and material described in the manuscript will be made available upon reasonable request.

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Authors' contributions

Conceptualization: AOC, AMB, JMP, LPA, MB, MC, HC, EL, MJ, BMA and CFL. Data curation: AOC, JMP, JC, SVS, MB and OC. Investigation: AOC, AMB, JMP, DFM, LGRL, EL and LR. Formal analysis: AOC and BMA. Funding acquisition: AOC, EL, BMA and CFL. Manuscript writing: AOC, BMA, CFL. All authors have reviewed and approved the final version of this manuscript.

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LIST OF ABBREVIATIONS

Ab: antibody

BCMA: B-cell maturation antigen

BM: bone marrow

B-NDG: NOD.CB17-*Prkdc*^{scid} *IL2rg*^{tm1}/BcgenHsd

B7H3: B7 homolog 3 protein

CAR: chimeric antigen receptor

CR: complete response

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

ffLuc: firefly liciferase

GFP: green fluorescent protein

gRNA: guide RNAs

IFN: interferon

IL: interleukin

IV: intravenous

KO: knock-out

LAG-3: lymphocyte-activation gene 3

MM: multiple myeloma

MOI: multiplicity of infection

MSLN: mesothelin

NSCLC: non-small-cell lung cancer

NSG: NOD-SCIDIL2gc-/-

NK: natural killer

N2: nectin-2

OR: overall response

OS: overall survival

PBMC: peripheral blood mononuclear cell

PCR: polymerase chain reaction

PD-1: programmed death 1

PFS: progression-free survival

PVR: poliovirus receptor

scFv: single-chain variable fragment

TGFβR: transforming growth factor β receptor

TIGIT: T cell immunoglobulin and ITIM domain

TIM-3: T-cell immunoglobulin and mucin-domain containing-3

TME: tumour microenvironment

TNF: tumour necrosis factor

UT: untransduced

WT: wild type

ABSTRACT

Background

CAR-T cell therapy has revolutionized the treatment of hematologic malignancies. Different BCMA-directed CAR-T therapies have shown promising results in multiple myeloma (MM), including ARI0002h, an academic CAR-T developed at our institution and administered to patients in a pivotal multicenter clinical trial. Despite optimal outcomes obtained with BCMA-directed CAR-T cell therapy in MM, patients continue to relapse. T-cell exhaustion plays a crucial role as a resistance mechanism to BCMA-CART. Indeed, an increased TIGIT and PD-1 expression was confirmed in patient samples included in the ARI0002h trial. In the past years, TIGIT has emerged as a potential immunotherapy target, with several clinical trials ongoing. In this study, we aimed to analyse the impact of blocking TIGIT in the efficacy of ARI0002h cells in a model of MM.

Methods

We used three different strategies to block TIGIT: *(1)* Addition of an external anti-TIGIT-antibody (Ab), *(2)* Modify ARI0002h into a 4th generation CAR-T, named ARITIGIT, capable of secreting a blocking scFv and *(3)* TIGIT knock-out in ARI0002h using CRISPR/Cas9. Each strategy was evaluated *in vitro* (cytotoxicity assays and cytokine production) and *in vivo* in NSG mice.

Results

Adding a TIGIT-blocking Ab to ARI0002h improved *in vitro* cytotoxicity, but failed to enhance mice survival. The new 4th generation CAR-T, ARITIGIT, was also unable to achieve better survival outcomes. In both cases, despite favoring the *in vivo* model by using a myeloma cell line with a high expression of the TIGIT ligand PVR and T-cells of a patient with high TIGIT expression, survival remained equal besides TIGIT-blockade.

Interestingly, when mice were challenged with a second infusion of tumor cells, mimicking a relapse model, ARITIGIT improved the survival, although differences did not reach statistical significance (p=0.11). Finally, TIGIT-knock-out on ARI0002h (TIGIT KO-ARI0002h) using CRISPR/Cas9 showed a similar *in vitro* activity compared to ARI0002h. In an *in vivo* stress model where ARI0002h did not improve survival significantly, TIGIT KO-ARI0002h improved it (p=0.02). However, this improvement was not significant compared to ARI0002h.

Conclusion

This study failed to demonstrate a siginificant benefit of TIGIT-blockade on ARI0002h cells despite using three different approaches, suggesting that targeting TIGIT as a single immune checkpoint may be insufficient.

KEY MESSAGES

What is already known on this topic –

Despite impressive outcomes of BCMA-directed CAR-T cell therapy in multiple myeloma, patients continue to relapse. Mechanisms of resistance to BCMA-CART mediated by immune checkpoint receptors (PD-1, TIGIT, TIM-3, LAG-3) seem crucial. In the past years, TIGIT has emerged as a novel immunotherapy target, with extensive clinical research ongoing. So far, only one published study has analyzed the efficacy of a single-TIGIT-blockade on CAR-T cells.

What this study adds –

In this study, we blocked TIGIT in ARI0002h, an academic BCMA-directed CART, administered to patients with relapsed-refractory multiple myeloma in a pivotal clinical trial. Despite using three different strategies to block TIGIT, a significant benefit of a TIGIT-blockade in ARI0002h could not be proven.

How this study might affect research, practice or policy -

Although further studies are needed to determine the potential value of TIGIT as an immunotherapy target, our results add insight to the idea that a single TIGIT-targeting strategy may not be sufficient to improve outcomes for immunotherapy in myeloma.

BACKGROUND

Chimeric antigen receptor (CAR)-T cell therapy has revolutionized the treatment of hematologic malignancies. Despite excellent outcomes obtained with the approved B-cell maturation antigen (BCMA)-directed CARTs for multiple myeloma (MM), idecabtagene vicleucel[1,2] and ciltacabtagene autoleucel[3,4], most patients still relapse. Our institution developed ARI0002h, an academic second-generation 4-1BB-based CAR, obtained by lentiviral transduction on autologous T cells, that has demonstrated promising efficacy and safety in a multicentre pivotal clinical trial for relapsed/refractory MM in Spain[5]. Overall/complete response (OR/CR) rates were 95/58%. After a median follow-up of 23.1 months, the estimated median progression-free survival (PFS) was 15.8 months, with overall survival (OS) rates at 12 and 18 months of 81% and 69%. Correlative studies performed on patients' samples revealed a decrease in BCMA molecules between inclusion and relapse, but none of the patients became BCMA negative. Interestingly, a marked increase of the exhaustion markers programmed death-1 (PD-1) and T cell immunoglobulin and ITIM domain (TIGIT) was observed between the final product and bone marrow samples (BM) obtained on days 28 and 100 after ARI0002h infusion; TIGIT expression in the BM on day 28 inversely correlated with CR rates on day 28, day 100 and as best response. Moreover, ARI0002h was detectable in 9 of 28 (32.1%) patients with an available sample at relapse, with no co-existing B-cell aplasia in 5 of 9, the expected *on-target off-tumor* effect. This suggests a dysfunctional status of these CAR T-cells, highlighting the role that exhaustion may play in relapse[6].

TIGIT, an inhibitory receptor expressed on T-cells and natural killer (NK)-cells, interacts with its main ligand, poliovirus receptor (PVR) or CD155, and nectin-2 (N2) or CD112, located on antigen-presenting cells and tumor cells. The TIGIT/CD226 pathway is constituted by different inhibitory (TIGIT, CD96, and CD112R) and activating (CD226) receptors that bind to their ligands (PVR, N2 and nectin-3) with different affinities[7,8]; TIGIT binds PVR with the highest affinity, which is lower for N2 and nectin-3. While

nectin-3 is restricted to non-hematopoietic tissues, PVR and N2 are expressed in hematopoietic cells and over-expressed in many human malignancies[9,10], including MM[11]. Previous preclinical studies have suggested that TIGIT blockade successfully reinvigorates T-cell function against MM[12,13]. The ClinicalTrials.gov database reports 65 trials using an anti-TIGIT Ab to treat solid and hematologic malignancies[14], with three active trials for MM. Furthermore, TIGIT has been described as a central player in mantle cell lymphoma relapse after CD19-CART therapy[15] and some studies, including ours, have reported a higher TIGIT expression on T cells and NK cells after relapse to BCMA-CART therapy for MM, compared to baseline[6,16,17]. Consequently, we decided to investigate the potential benefit of TIGIT blockade in ARI0002h efficacy.

MATERIALS AND METHODS

Study Design

TIGIT blockade on ARI0002h was performed using three different strategies: *(1)* The addition of an anti-TIGIT-antibody(Ab) to ARI0002h therapy. *(2)* We modified ARI0002h into a 4th generation CART, termed ARITIGIT, capable of secreting a TIGIT-blocking scFv, and *(3)* knocking-out (KO) TIGIT on ARI0002h cells using CRISPR/Cas9 (Figure 1A). Each strategy was evaluated *in vitro* by performing cytotoxicity assays, cytokine measurements (interferon (IFN)-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-α), and *in vivo* using NOD-SCIDIL2gc-/- (NSG) mice. To test the anti-TIGIT-Ab strategy, the BMS-986207 TIGIT-Ab was used (Bristol-Myers Squibb Company, New York, US).

Development of the ARITIGIT 4th generation CART

ARI0002h is a second-generation 4-1BB-based BCMA-directed CART. To transform it into a 4th generation CART capable of secreting a TIGIT-blocking single-chain variable fragment (scFv) we added the following structure to the ARI0002h sequence: P2A +

the signal peptide IgK VIII[18] + the scFv (variable heavy chain + linker + variable light chain) generated from the DNA sequence of the TIGIT antibody tiragolumab, Genentech® (publicly available[19]) + stop codon (Figure 1B). scFv secretion was confirmed by liquid chromatography-mass spectrometry of ARITIGIT culture supernatants using nanoAcquity liquid chromatographer (Waters, Massachusetts, US) and LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Massachusetts, US). Further information is provided in the Supplementary Material, page 2.

Cell lines and cell culture

The ARP-1 myeloma cell line was kindly provided by the Multiple Myeloma Research Center (Arkansas, US), and the U266 were obtained from the American Type Culture Collection (Maryland, US). HEK293T cells were obtained from Takara Bio. ARP-1 and U266 cells were modified to express green fluorescent protein (GFP) and firefly luciferase (ffLuc). BCMA expression was confirmed in both cell lines (Supplementary Figure 1). RPMI-1640 media containing 10% or 15% fetal bovine serum (FBS) was used for ARP-1 and U266 culture, respectively. DMEM + 10% FBS was used for HEK293T cells. T-cells were obtained from healthy donors provided by the blood and tissue bank (Banc de Sang i Teixits, Catalunya, Spain) and patients of the Amyloidosis and Myeloma Unit at Hospital Clinic of Barcelona, after patient consent. T-cells were cultured using T-cell media consisting of 47.5% Click's media, 47.5% RPMI-1640 and 5% human serum. All culture media were supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were cultured at 37ºC with 5% CO2.

CART production

Peripheral blood mononuclear cells (PBMCs) were purified from buffy coats or patients' peripheral blood after density-gradient separation using Histopaque-1077 (Sigma-Aldrich, San Luis, MO) and isolated by magnetic depletion with Pan T cell isolation kit and AutoMACS® (Miltenyi Biotec, Germany). Isolated T-cells were activated with

Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific, San Diego, CA). 100 UI/mL IL-2 (Miltenyi Biotec) or 10 ng/ml IL-15 (Miltenyi Biotec) were added according to the protocol. ARI0002h and ARITIGIT lentiviral particles were produced and titrated using HEK293T cells, as described elsewhere[20]. T-cells were transduced after 24-48h of activation, adding the appropriate amount of lentivirus at a multiplicity of infection (MOI) of 5-10 in the presence of polybrene (Merck Millipore, Burlington, MA) for 6-24h after spinoculation (2000rpm, 32ºC, 60 min). Untransduced (UT) T-cells were exposed to the same conditions to use as the control. A cell expansion period of 6-9 days was required before starting experiments. To assess CART transduction, cells were incubated with recombinant BCMA-Fc (Enzo Life Sciences, Farmingdale, NY) and stained with a BV421/BV510 anti-Fc antibody (Biolegend, San Diego, CA).

CART cells in the third strategy were genetically disrupted using CRISPR/Cas9. Three TIGIT-targeting guide RNAs (gRNA) were purchased (Synthego, Redwood City, CA): T45 UGUGCCUGUCAUCAUUCCUA, T46 UUGUGCCUGUCAUCAUUCCU and T52 UCUUCCCUAGGAAUGAUGAC. Using the Neon Transfection System (Thermo Fisher, Waltham, MA), ribonucleoprotein complexes formed by sgRNA plus Cas9 were inserted in T-cells by electroporation according to the manufacturer protocol. Control cells underwent the same steps without adding the sgRNAs, but the carrier buffer. DNA was extracted from TIGIT KO CAR-T cells and wild type (WT) CAR-T cells to evaluate KO efficiency using a DNeasy kit (Qiagen). After polymerase chain reaction (PCR) amplification, purified products were sequenced, and results were analyzed using the Interference of CRISPR Edits Analysis Tool (Synthego), using WT-ARI0002h as control.

Cytotoxicity assays

The killing capacity of each strategy was compared after co-culture of MM cell lines (ARP-1/U266/ARP-1-N2) with T cells (UT/ ARI0002h/ ARITIGIT/ TIGIT KO-ARI0002h) and the TIGIT-Ab if applicable (10μg/ml)[12], at different effector:target ratios (1:2 or

1:4) and timepoints (12-36h). Results were assessed using flow cytometry (proportion of alive GFP+ tumor cells) or bioluminescence (after the addition of luciferin). Since the killing ability of the groups of interest (ARI0002h vs. TIGIT-blocking strategy in ARI0002h) was very high, cytotoxicity results were considered valid if the proportion of killing for all CART groups compared to the UT group remained between 10-90%.

Cytokine profiling

To determine cytokine production, supernatants of MM cell lines and CART co-cultures were collected after 24h. IFN-γ, TNF-α and IL-2 were quantified by enzyme-linked immunosorbent assay (ELISA) using the ELISA MAX^{TM} Deluxe Set (Biolegend), following the manufacturer protocol.

***In vivo* assays**

10-20 week-old mice were irradiated (2Gy) 24-48h before intravenous (IV) inoculation of GFP-ffLuc positive MM cells. After 1-2 weeks, mice were randomized according to tumor burden measured by bioluminescence imaging using IVIS® (PerkinElmer, Waltham, MA) into the required treatment groups, and CART or UT cells were IV injected. MM and CART dosage and timings varied among experiments. Further details are provided for each experiment in the *Results* section. All experiments were performed using a subtherapeutic dose of CARTs (stress model) to avoid long-term remission of all mice, which would allow us to see differences between the control (ARI0002h) and the TIGIT-blockade groups. Disease progression was followed weekly by bioluminescence imaging using IVIS®. Based on previous reports[12] the starting BMS-986207 dose consisted of an intraperitoneal 200μg injection of TIGIT-Ab 2 times/week; however, frequency of dosage varied upon experiments. Mice were sacrificed based on the following prespecified criteria: tumor burden (average radiance $2x10^8$ p/sec/cm2/sr), weight loss > 20% or signs of welfare loss. In specific

experiments, mice tissues (BM and spleen) were collected, processed, and filtered (100µm) to analyze tumor and T-cell populations by flow cytometry.

Flow cytometry

T-cell subsets and TIGIT expression were analyzed by flow cytometry using fluorochrome-conjugated Ab: CD4-PerCP (cat.560650, BD Biosciences, Franklin Lakes, NJ), CD8-PE-Cy7 (cat.557746, BD Biosciences) and TIGIT-APC (cat.17-9500-42, ThermoFisher, Waltham, MA). In mice-processed tissues, cells were pre-treated with an anti-mouse Ab FC shield CD16/CD32 (Tonbo Biosciences, San Diego, CA). The BD FACSCanto™ (BD Biosciences) and Omnicyt™ CE-IVD (Cytognos, Salamanca, Spain) flow cytometers were used to obtain data. Analysis was performed using FlowJo software v.10 (Treestar, Ashland, OR).

Statistical analysis

Students' t-test and the Wilcoxon test were used to analyze the continuous variables according to normality adherence. Kaplan-Meier and the log-rank test were used to analyze survival. A two-sided p-value of <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism v10.2.0 (GraphPad Software, San Diego, CA) and IBM SPSS Statistics (v29.0).

Data visualization

Figures and data plotting were performed using Biorender (Toronto, Canada), GraphPad Prism v10.2.0 (GraphPad Software, San Diego, CA) and FlowJo v.10 (Treestar, Ashland, OR).

RESULTS

Characterization of TIGIT expression on T cells used in this study

We characterized the T-cell subsets and TIGIT expression of T-cells from each donor/patient before assay performance (days 7-10 from T-cell activation) (Supplementary Table 1). No differences in the CD4 and CD8 populations or TIGIT expression were observed between UT and CAR T-cells. We observed a heterogeneous TIGIT expression (range in CD4-UT: 4.1-36.7; range in CD4-CAR: 3.1-30.6; range in CD8-UT: 2.2-68.8; range in CD8-CAR: 4.0-41.8). TIGIT expression was similar between CD4 and CD8. T-cells obtained from healthy donors (n=12) and patients (n=4) did not differ in terms of TIGIT expression (Supplementary Figure 2).

The addition of a TIGIT blocking Ab to ARI0002h cells only improves their efficacy *in vitro*

Initially, we co-cultured tumor cells in the absence (control) and presence (test) of a TIGIT-blocking antibody (anti-TIGIT-Ab) to verify that neither the drug or its carrier were toxic for the tumor cells (data not shown). Then, co-culture of MM cell lines ARP-1 and U266 with ARI0002h in the presence of anti-TIGIT-Ab showed improved cytotoxicity, which was more evident against the U266 cell line. Moreover, anti-TIGIT-Ab induced a limited cytotoxic activity on UT T-cells (Figure 2A). No differences were observed in the pro-inflammatory cytokine production (IFN-γ or TNF-α), although a significant increase of IL-2 was observed in the U266 co-culture in the presence of anti-TIGIT-Ab (Figure 2B).

To test this strategy *in vivo*, NSG mice received 200µg of the anti-TIGIT-Ab twice a week for four weeks after MM (ARP-1) and ARI0002h or UT T-cells injection. Surprisingly, no survival differences were observed compared to the control groups (ARI0002h or UT without anti-TIGIT-Ab) (Figure 2C). As *in vitro* results did not replicate *in vivo*, we decided to favor the *in vivo* model towards the anti-TIGIT-Ab by modifying three features: i) using the U266 cell line instead, which had shown better outcomes *in vitro*, ii) manufacturing ARI0002h with patient T-cells expressing a considerable amount

of TIGIT (ARI0002h cells before injection had in 29.7% of CD4+TIGIT+ and 23.6% of CD8+TIGIT+) and iii) increasing the Ab dose to 200µg three times a week, indefinitely. Despite these changes, we observed no differences in mice disease progression or survival (Figure 2D).

ARITIGIT showed a limited improvement in efficacy in a relapse MM model

A 4th generation ARI0002h CAR, ARITIGIT, was designed. This construct was able to secrete a TIGIT-blocking scFv in the tumor microenvironment.

In vitro expansion of ARI0002h and ARITIGIT was equivalent (Supplementary Figure 3). When we compared ARITIGIT to ARI0002h, we observed a higher cytotoxic activity only against the U266 cell line with both healthy donor- and patient-derived CAR-T cells, which did not translate into a higher cytokine production (Figure 3A-B). When testing this strategy *in vivo*, with ARP-1 and patient-derived T-cells expressing TIGIT (17.9% of CD4+TIGIT+ and 20.3% of CD8+TIGIT+ cells) no differences in survival were observed (Figure 3C) even though ARITIGIT was able to block TIGIT *in vivo* in CAR-T cells (Supplementary Figure 4). Interestingly, when re-challenging the model with a second injection of tumor cells, mimicking a relapse of the disease, ARITIGIT showed a slight improvement in survival (Figure 3D). Since ARITIGIT showed augmented cytotoxicity in U266, we tested this strategy *in vivo* using the U266 cell line instead, with two different CAR-T cell doses (0.75 vs 1.5 x10^6 CAR-T+ cells), but, again, mice survival remained statistically comparable between ARI0002h and ARITIGIT (Figure 3E).

A high nectin-2 expression in MM cells did not increase the benefit of a TIGIT blockade

Two different TIGIT-blocking strategies using the anti-TIGIT-Ab and the 4th generation ARITIGIT CART demonstrated a better performance *in vitro* with the U266 cell line. In concordance, we observed a higher PVR expression in U266 compared to ARP-1.

However, N2 expression was similar in both cell lines (Figure 4A). We hypothesized that the higher amount of the ligand PVR expressed by the U266 cell line could explain the better results observed. Therefore, we decided to generate a high-expressing N2 ARP-1 cell line, named ARP-1-N2 (Figure 4B) to analyze the impact in our model with the anti-TIGIT-Ab and ARITIGIT. We observed no differences in cytotoxicity or cytokine production according to N2 expression on ARP-1 cells for the different treatments (Figure 4C-D).

TIGIT knockout on ARI0002h cells improved slightly ARI0002h efficacy in a stress MM model

In a third attempt to improve the CAR-T cell activity, TIGIT was removed from ARI0002h using CRISPR/Cas9. Three different gRNA were used for the KO: T45, T46, and T52. *In vitro* expansion was equivalent between the WT-ARI0002h and all 3 TIGIT KO-ARI0002h (Supplementary Figure 5A). KO efficiency was better with the gRNAs T45 and T52, which were selected for assay performance. Interestingly, despite DNA KO efficiencies around 40%, protein KO (reduction in protein expression measured by flow cytometry) was higher in CD4 and CD8, with all gRNAs (Supplementary Figure 5B-D).

In vitro comparison of ARI00002h *vs* KO-ARI0002h with T45 and T52 demonstrated similar cytotoxic efficacy (Figure 5A). Cytokine production was comparable for all groups, except for a lower IL-2 and TNF-α levels in the co-culture with ARP-1 only when the KO was performed using the gRNA T52 (Figure 5B).

In vivo comparison of the different treatments demonstrated that in a stress model where ARI0002h did not improve mice survival significantly, TIGIT KO-ARI0002h experienced prolonged survival compared to UT mice. Of note, this prolonged survival was not significant compared to the WT-ARI0002h ($p=0.07$), suggesting only a modest improvement achieved with the TIGIT KO (Figure 5C). Persistence of the TIGIT KO *in*

vivo was confirmed by analyzing TIGIT expression in T-cells of mice tissues eight days after CART injection (Supplementary Figure 6).

DISCUSSION

In the past years, TIGIT has emerged as an immunotherapy target, and several clinical trials are ongoing to evaluate the efficacy of TIGIT-blocking antibodies in cancer, including MM[14]. Besides the existing evidence of the immunosuppressive effect that TIGIT exerts in the tumor microenvironment[9,12,21,22], data show a detrimental impact of TIGIT also in CAR T cell therapy, where exhaustion plays a crucial role [6,16,23–25]. Overcoming this immunosuppressive microenvironment may be critical to maintaining durable responses. Therefore, combined therapies with TIGIT blockade and CART cells represent an area of expanding research. In this matter, we aimed to analyze a combined therapy based on TIGIT blockade and ARI0002h cells in a model of MM. We demonstrate that blocking TIGIT on BCMA CAR-T cells with three different approaches only provided a slight improvement in ARI0002h performance in a relapse model and in a stress model, using in both cases the strategies that involve genetic modification of ARI0002h, either secretion of a TIGIT-blocking scFv or KO of TIGIT. In agreement, other studies have also demonstrated the superiority of a mesothelin (MSLN)-CART secreting a TIGIT-blocking scFv compared to the addition of a TIGIT-blocking Ab in solid tumors[26]; and in neuroblastoma, where TIGIT-KO CAR-NK improved *in vitro* tumor control in long-term tumor microenvironment (TME) co-cultures[27]. Aside from these two studies, no additional publications have reported a benefit from a single TIGIT-blockade on CAR-T/-NK cells.

This study demonstrates only a modest advantage with single TIGIT-blockade on ARI0002h cells. In this regard, *in vivo* models in our study were performed on NSG mice, which are immunocompromised strains. This could undervalue the benefits of a TIGIT blockade due to the lack of a TME with the presence of other immune cells, such

as NK cells [27] or macrophages, that also benefit from TIGIT blockade [27,28]. Still, in the MSLN-CART study [26], assays were performed on B-NDG mice, a very similar immunocompromised model.

Moreover, our previous results in patients receiving ARI0002h cells demonstrated that the overexpression of TIGIT and PD-1 was associated with poor outcomes[6]. Conversely, there is growing evidence of a potential synergistic effect between PD-1 and TIGIT. Indeed, TIGIT is typically co-expressed with PD-1 and up-regulated after PD-1 blockade[29], only the inhibition of both allows a normal function of the activating receptor CD226[30], and dual blockade of TIGIT and PD-1 has yielded favorable results in preclinical studies[29,31,32]. These findings led to evaluating the combined inhibition of TIGIT/PD-1 in the clinical setting.

In detail, phase I clinical trials with anti-TIGIT Abs, alone or combined with PD-1 and radiation reported clinical benefit in advanced solid tumors[33–35]. However, advanced-phase clinical trials are still required. Tiragolumab (anti-TIGIT) combined with atezolizumab (anti-PD-L1) showed higher response rates and progression-free survival compared to atezolizumab alone in the phase II CITYSCAPE trial for advanced PD-L1-high non-small-cell lung cancer (NSCLC)[36]. These results motivated the development of the SKYCRAPER phase III clinical trials evaluating Tiragolumab combined with anti-PD-1 and chemotherapy for both NSCLC and small-cell lung cancer, but neither met the primary endpoints[37–39], raising concerns about the value of TIGIT. The most recent update of the phase Ib/II MORPHEUS-liver study comparing tiragolumab, atezolizumab and bevacizumab (anti-VEGF) vs. only atezolizumab and bevacizumab for hepatocellular carcinoma reported longer PFS in the treatment arm, although the OR rate in the control arm was lower than expected[40,41]. In hematologic malignancies, although numerous clinical trials are evaluating anti-TIGIT Abs, no definitive results have been reported yet.

Preliminary reports suggest a potential benefit of the combination of CAR T cells with PD-1 blockade in diffuse large B-cell lymphoma[42] and neuroblastoma[43], with

several clinical trials ongoing[44]. Moreover, simultaneous depletion of TIGIT with other immune check-points by small hairpin RNAs demonstrated that KO of PD-1, TIGIT and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) with KO of TGFβR, IL-10 receptor and IL-6 receptor, in epidermal growth factor receptor (EGFR)- and B7 homolog 3 protein (B7H3)-directed CARTs for cholangiocarcinoma showed a higher inhibitory effect on tumor growth, prolonging mice survival[45]. Of interest, another study examining shRNA KO combinations of PD-1/TIM-3, PD-1/lymphocyte-activation gene 3 (LAG-3), PD-1/CTLA-4 and PD-1/TIGIT on CD19-CAR T cells observed that only the PD-1/TIGIT downregulation improved antitumor effects compared to PD-1 KO alone [46]. Functional analyses suggested that downregulation of PD-1 enhances short-term effector function, whereas downregulation of TIGIT maintains a less differentiated/exhausted state, providing a potential mechanism for the observed synergy. These data led to the development of a clinical trial for R/R large B cell lymphoma (NCT04836507), with preliminary results of 34 patients reporting ORR of 85% and CR on day 28 of 74%[47].

On the other hand, in our study, TIGIT blockade was more effective *in vitro* in the U266 cell line. Since assays were equivalent, this could be related to its higher PVR expression compared to the ARP-1 cell line. However, the introduction of a high N2 expression cell line, ARP-1-N2, did not yield better results after TIGIT blockade. Thus, the role of PVR may be more relevant than that of N2, which is consistent with the already-known interactions between receptors and ligands of the TIGIT/CD226 pathway[41]. Interestingly, PVR is a biomarker of poor prognosis in several tumors[10,48–50].

In summary, although the preclinical evidence and also our previous results in patients receiving ARI0002h [6] supported a potential success of TIGIT blockade in cancer immunotherapy, our results in MM inhibiting TIGIT as a single target did not provide enough advantage to enhance CAR-T cell efficacy. Clinical results suggest that a combined TIGIT-PD-1 blockade could provide an advantage. However, single TIGIT

targeting and even in combination with anti-PD-1/PD-L1 agents in advanced-phase clinical trials for solid tumors, have provided limited results. Although clinical results of single TIGIT or dual TIGIT/PD-1 blockade in MM are still missing, the existing literature suggests that dual TIGIT and PD-1 blockade could be an interesting strategy. Whereas there is less evidence regarding TIGIT blockade applied to CAR-T/-NK therapy, particularly in hematologic malignancies, we suggest that identifying the best indications, patient populations, and the most appropriate multiple-targeting represents an area of improvement.

FIGURE LEGENDS

Figure 1. (A) TIGIT-blockade approaches tested in this study. **(B)** ARITIGIT 4th generation CART sequence scheme.

Figure 2. (A) Cytotoxicity results after co-culture of the MM cell lines ARP-1 and U266 with either ARI0002h or untransduced (UT) T-cells obtained from healthy donors, adding or not an anti-TIGIT-Ab (the Ab carrier was added to the control groups). **(B)** Pro-inflammatory cytokine production of IFN-γ, IL-2 and TNF-α after 24 hours of co-culture. **(C)** In vivo results of this strategy: mice were administered 1.5x10^6 MM ARP-1 cells on day 0, 24h after radiation with 2Gy. When disease luminescence was detectable, mice were injected with 0.5x10^6 of a healthy donor-derived ARI0002h CAR+ cells or UT T-cells. After that, the anti-TIGIT-Ab was administered intraperitoneally twice a week for 4 weeks in the respective groups. Mice were followed weekly using bioluminescence and sacrifice was performed when prespecified criteria were met. **(D)** In vivo results of the favoured model: mice were administered 1.5x10^6 MM U266 cells on day 0, 24h after radiation with 2Gy. When disease luminescence was detectable, mice were injected with 1.5x10^6 of patient-derived ARI0002h CAR+ cells or UT T-cells. After that, the anti-TIGIT-Ab was administered intraperitoneally three times a week, indefinitely, in the respective groups. Mice were followed weekly using bioluminescence and sacrifice was performed when prespecified criteria were met.

Figure 3. (A) Cytotoxicity results after co-culture of the MM cell lines ARP-1 and U266 with either ARI0002h or ARITIGIT manufactured from healthy donor or patient T-cells. **(B)** Pro-inflammatory cytokine production of IFN-γ, IL-2 and TNF-α after 24 hours of co-culture. **(C)** In vivo results of the unchallenged group: mice were administered 1.5x10^6 MM ARP-1 cells on day 0, 24h after radiation with 2Gy. When disease luminescence was detectable, mice were injected with 1.5x10^6 of patient-derived untransduced (UT)

vs. ARI0002h vs. ARITIGIT T-cells. Mice were followed weekly using bioluminescence and sacrifice was performed when prespecified criteria were met. **(D)** In vivo results of the re-challenged group: mice were administered $1.5x10^6$ MM ARP-1 cells on day 0, 24h after radiation with 2Gy. When disease luminescence was detectable, mice were injected with $3.5x10^6$ of patient-derived untransduced (UT) vs. ARI0002h vs. ARITIGIT T-cells. When clearance of the disease was observed in the CART groups, mice were readministered $1.5x10^6$ MM ARP-1 cells. Mice were followed weekly using bioluminescence and sacrifice was performed when prespecified criteria were met. **(E)** In vivo results of the favoured model: mice were administered $1.5x10^6$ MM U266 cells on day 0, 24h after radiation with 2Gy. When disease luminescence was detectable, mice were injected with either 0.75 or $1.5x10^6$ of patient-derived untransduced (UT) vs. ARI0002h vs. ARITIGIT T-cells. Mice were followed weekly using bioluminescence and sacrifice was performed when prespecified criteria were met.

Figure 4. (A) Flow cytometry staining of PVR and nectin-2 (N2) in U266 and ARP-1 cells. Gray = Isotype; Orange = PVR-expressing cells; Red = nectin-2-expressing cells. **(B)** The ARP-1 cell line was lentivirally transduced with a viral vector containing the N2 gene. Positive N2 cells were sorted by flow cytometry to obtain a 100% N2-positive ARP-1 population Gray = unstained cells; Red = nectin-2-expressing cells. **(C)** Cytotoxicity results after co-culture of the MM cell lines ARP-1 and ARP-1-N2 with ARI0002h vs. ARI0002h + TIGIT-AB vs. ARITIGIT. **(D)** Pro-inflammatory cytokine production of IFN-γ, IL-2 and TNF-α after 24 hours of co-culture.

Figure 5. (A) Cytotoxicity results after co-culture of the MM cell lines ARP-1 and U266 with either WT-ARI0002h, T52 gRNA-ARI0002h or T45 gRNA-ARI0002h. **(B)** Pro-inflammatory cytokine production of IFN-γ, IL-2 and TNF-α after 24 hours of co-culture. **(C)** In vivo results: mice were administered $1.5x10^6$ MM ARP-1 cells on day 0, 24h after radiation with 2Gy. When disease luminescence was detectable, mice were injected

with $1.5x10^6$ of untransduced (UT) vs. WT-ARI0002h vs. TIGIT KO-ARI0002h. Mice were followed weekly using bioluminescence and sacrifice was performed when prespecified criteria were met.

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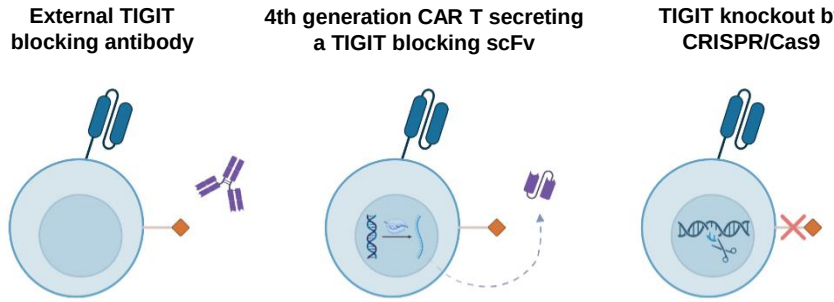
Figure 1

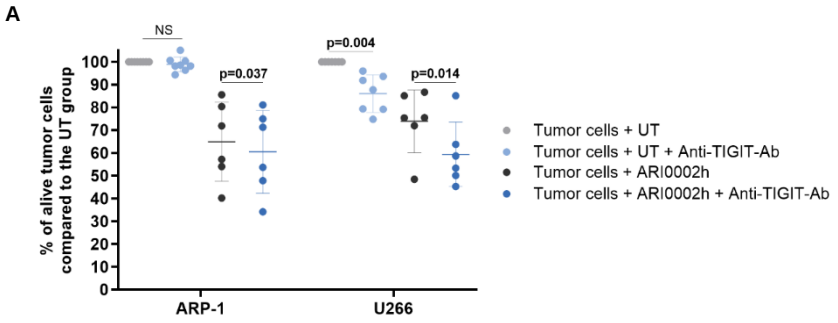
A

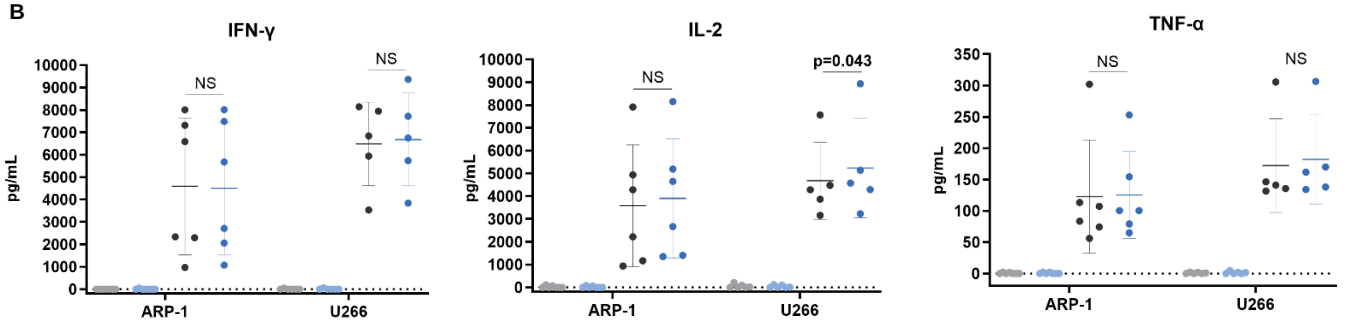

External TIGIT blocking antibody
4th generation CAR T secreting a TIGIT blocking scFv
TIGIT knockout by CRISPR/Cas9

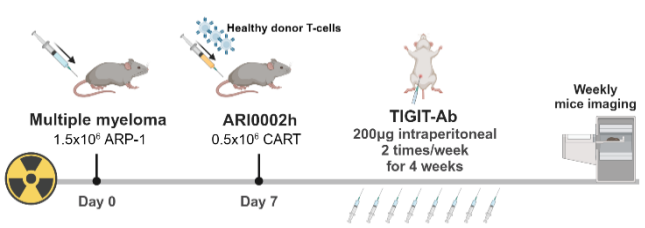
B

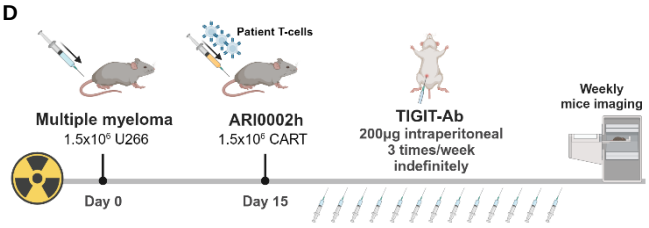

5’
3’
ARI0002h
P2A
Signal peptide – IgK VIII
TIGIT VH
linker
TIGIT VL
STOP codon

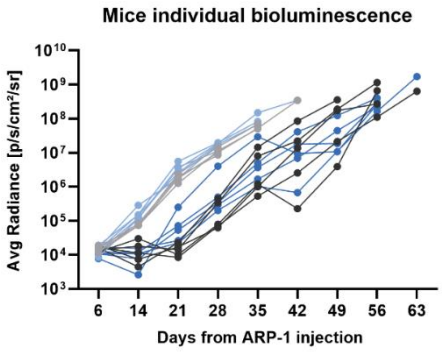
Figure 2

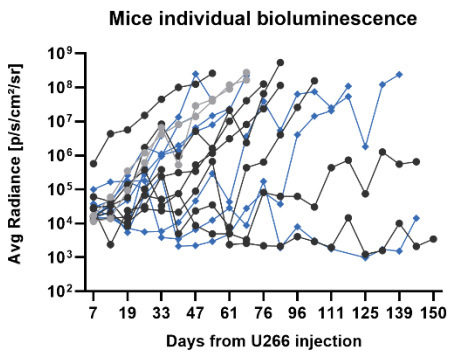

A
NS
p=0.037
p=0.004
p=0.014
% of alive tumor cells compared to the UT group
ARP-1
U266
Tumor cells + UT
Tumor cells + UT + Anti-TIGIT-Ab
Tumor cells + ARI0002h
Tumor cells + ARI0002h + Anti-TIGIT-Ab

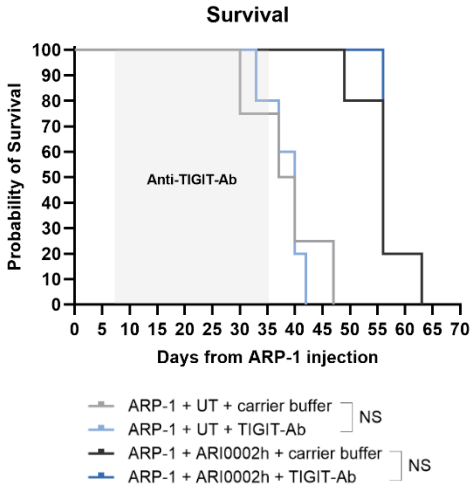

B
IFN-γ
IL-2
TNF-α
pg/mL
NS
p=0.043
ARP-1
U266


C
Healthy donor T-cells
Multiple myeloma
1.5x10⁶ ARP-1
ARI0002h
0.5x10⁶ CART
TIGIT-Ab
200µg intraperitoneal
2 times/week
for 4 weeks
Weekly
mice imaging
Day 0
Day 7


D
Patient T-cells
Multiple myeloma
1.5x10⁶ U266
ARI0002h
1.5x10⁶ CART
TIGIT-Ab
200µg intraperitoneal
3 times/week
indefinitely
Weekly
mice imaging
Day 0
Day 15


Mice individual bioluminescence
Avg Radiance [p/s/cm²/sr]
Days from ARP-1 injection


Mice individual bioluminescence
Avg Radiance [p/s/cm²/sr]
Days from U266 injection


Survival
Probability of Survival
Anti-TIGIT-Ab
Days from ARP-1 injection
ARP-1 + UT + carrier buffer
ARP-1 + UT + TIGIT-Ab
ARP-1 + ARI0002h + carrier buffer
ARP-1 + ARI0002h + TIGIT-Ab
NS
NS

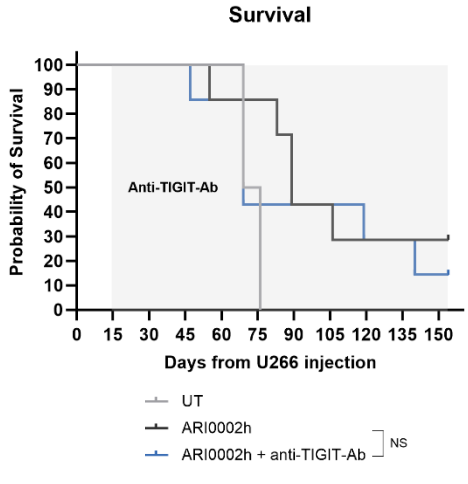

Survival
Probability of Survival
Anti-TIGIT-Ab
Days from U266 injection
UT
ARI0002h
ARI0002h + anti-TIGIT-Ab
NS

Figure 3

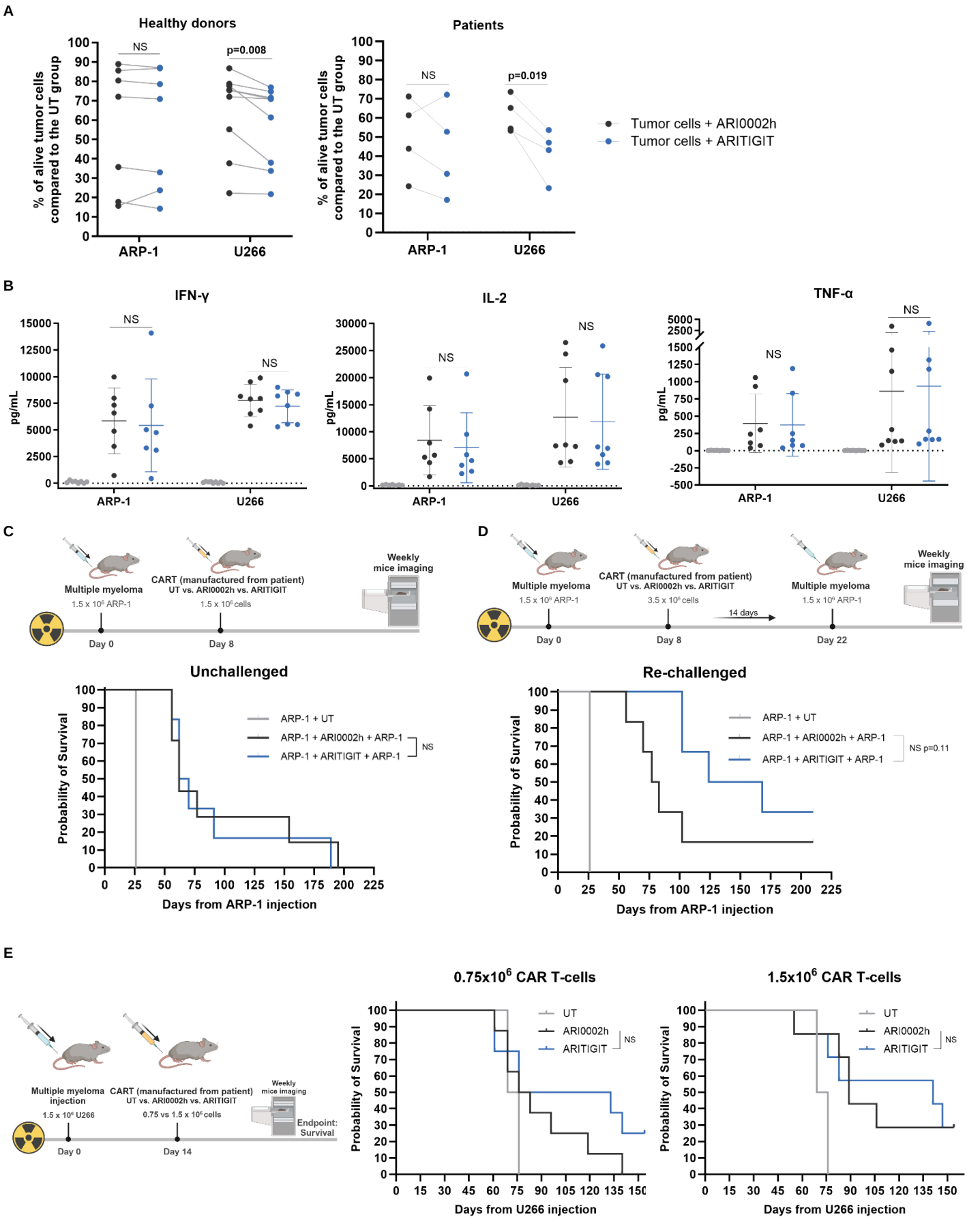

A
Healthy donors
Patients
% of alive tumor cells compared to the UT group
NS
p=0.008
p=0.019
ARP-1
U266
Tumor cells + ARI0002h
Tumor cells + ARITIGIT
B
IFN-γ
IL-2
TNF-α
pg/mL
NS
C
Multiple myeloma
1.5 x 10⁶ ARP-1
CART (manufactured from patient)
UT vs. ARI0002h vs. ARITIGIT
1.5 x 10⁶ cells
Weekly mice imaging
Day 0
Day 8
Unchallenged
Probability of Survival
ARP-1 + UT
ARP-1 + ARI0002h + ARP-1
ARP-1 + ARITIGIT + ARP-1
Days from ARP-1 injection
D
3.5 x 10⁶ cells
14 days
Day 22
Re-challenged
NS p=0.11
E
Multiple myeloma injection
1.5 x 10⁶ U266
0.75 vs 1.5 x 10⁶ cells
Endpoint: Survival
0.75x10⁶ CAR T-cells
1.5x10⁶ CAR T-cells
UT
ARI0002h
ARITIGIT
Days from U266 injection

Figure 4

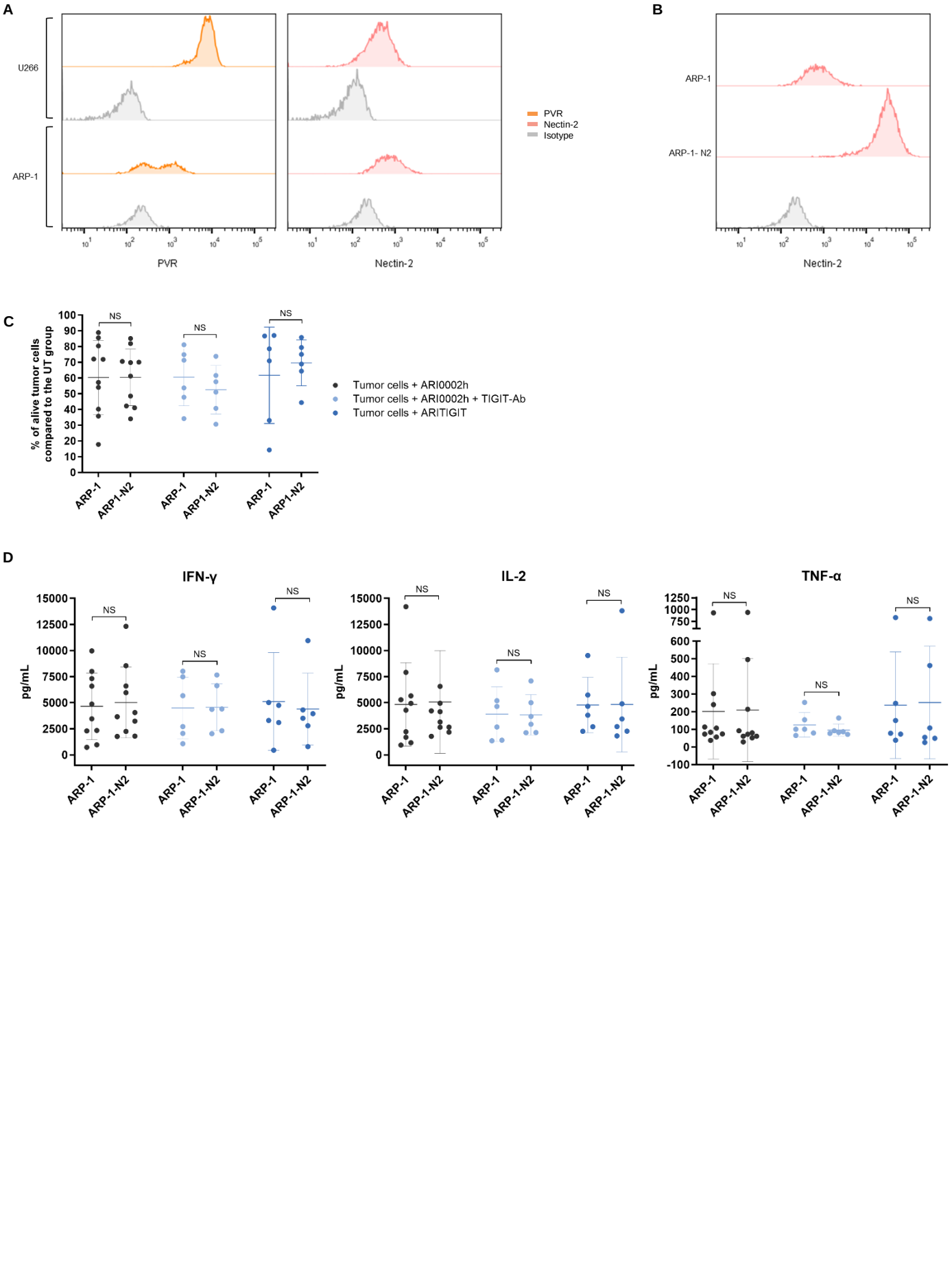
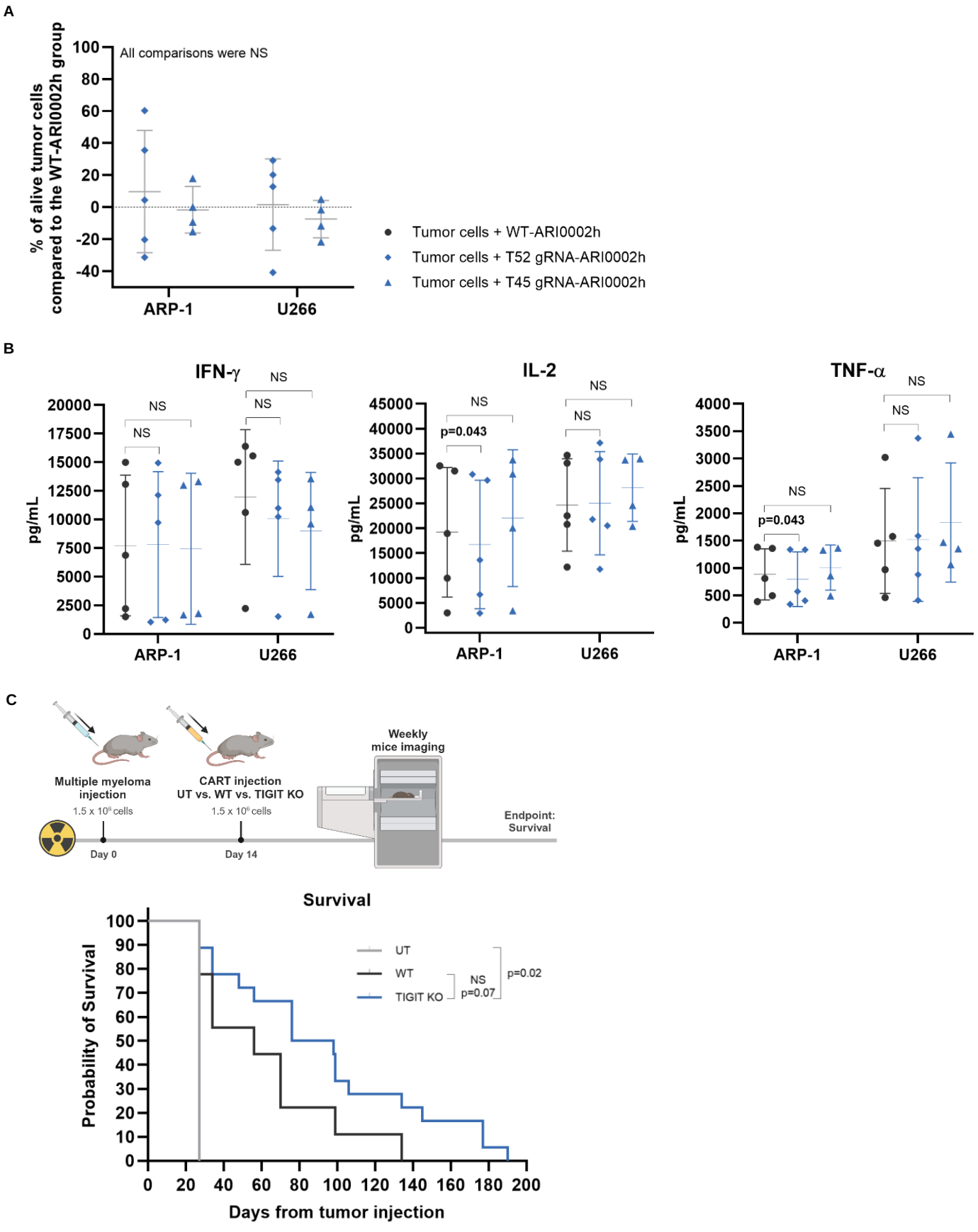

A
U266
ARP-1
PVR
Nectin-2
Isotype
PVR
Nectin-2
B
ARP-1
ARP-1- N2
Nectin-2
C
% of alive tumor cells compared to the UT group
NS
ARP-1
ARP1-N2
Tumor cells + ARI0002h
Tumor cells + ARI0002h + TIGIT-Ab
Tumor cells + ARITIGIT
D
IFN-γ
IL-2
TNF-α
pg/mL
NS
ARP-1
ARP-1-N2

Figure 5


A
All comparisons were NS
% of alive tumor cells compared to the WT-ARI0002h group
100
80
60
40
20
0
-20
-40
ARP-1
U266
Tumor cells + WT-ARI0002h
Tumor cells + T52 gRNA-ARI0002h
Tumor cells + T45 gRNA-ARI0002h
B
IFN-γ
IL-2
TNF-α
pg/mL
NS
p=0.043
ARP-1
U266
C
Multiple myeloma injection
1.5 x 10⁶ cells
Day 0
CART injection
UT vs. WT vs. TIGIT KO
1.5 x 10⁶ cells
Day 14
Weekly mice imaging
Endpoint: Survival
Survival
Probability of Survival
Days from tumor injection
UT
WT
TIGIT KO
NS
p=0.07
p=0.02

Supplementary Material Table of Contents

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Mass spectrometry – Protocol and results

1. Sample preparation. Protein Digestion.

Samples were centrifuged at 16.000xg to keep out debris. After that were centrifuged using an Amicon Ultra 50kDa (Millipore) at 3270xg using a SX4750A centrifuge. A filtrate liquid was concentrated against Amicon Ultra 10kDa (Millipore) again.

Concentrated samples were precipitated using 15% TCA (final concentration) at 4ºC and centrifuged at 14.000xg. Precipitated proteins were cleaned using acetone.

Direct digestion was performed as follow: samples were resuspended with 3 uL in 8M urea/50 mM ABC and proteins were reduced (DTT 21 mM; 1h, 32o C) and alkylated (iodoacetamide 25 mM; at room temperature for 30 min, in the dark) Afterwards, the samples were diluted down to 1 M urea with digestion buffer and proteins were digested for 2 hours with trypsin (Sequence grade modified porcine Trypsin, Promega; pH 8, 32o C) and were then digested overnight with the same enzyme. The resulting peptide mixtures were cleaned-up with a C18 tip (ZipTip) as per manufacturer´s protocol. Finally, the cleaned-up peptide solutions were dried-down and stored at -20 oC until the LCMS analyses 2.

2. Analytical procedure and equipment used. LC-MSMS.

The dried-down peptide mixture was analyzed in a nanoAcquity liquid chromatographer (Waters) coupled to a LTQ-Orbitrap Velos (Thermo Scientific) mass spectrometer. The tryptic digests were resuspended in 1% FA solution and an aliquot per sample was injected for chromatographic separation. Peptides were trapped on a Symmetry C18TM trap column (5µm 180µm x 20mm; Waters) and were separated using a C18 reverse phase capillary column (ACQUITY UPLC BEH column; 130Å, 1.7µm, 75 µm x250 mm, Waters). The gradient used for the elution of the peptides was 1 to 40 % B in 60 minutes, followed by a gradient from 40% to 60% in 10 min. (A: 0.1% FA; B: 100% ACN, 0.1%FA; flow rate: 250 nL/min). Column temperature: 40ºC.

Eluted peptides were subjected to electrospray ionization in an emitter needle (Thermo) with an applied voltage of 2000V. Peptide masses (m/z 300-1600) were analyzed in data dependent mode where a full Scan MS was acquired in the Orbitrap with a resolution of 60,000 FWHM at 400m/z. Up to the 15th most abundant peptides (minimum intensity of 500 counts) were selected from each MS scan and then fragmented in the linear ion trap using CID (38% normalized collision energy) with helium as the collision gas.The scan time settings were: Full MS: 250 ms (1 microscan) and MSn: 120 ms. Generated. raw data files were collected with ThermoXcalibur (v.2.2).

3. Data analysis: Database search and protein ID

The raw data files obtained in the mass spectrometry analyses were used to search against a modified version of the public database SwissProt containing selected TIGIT-blocking-scFv protein entry (from customer), with a small database containing laboratory contaminants. Database search was performed with SEQUEST search engine using Thermo Proteome Discover (v.1.4.1.14).

The following search parameters were applied:

Database/Taxonomy: SPH_230307_cont. fasta (with TIGIT-blocking-scFv)
Enzyme: Trypsin Missed cleavage: 2
Fixed modifications: Carbamidomethyl of cysteine
Variable modifications: Oxidation of methionine, deamidation of asparagine, deamidation of glutamine
Peptide tolerance: 10 ppm and 0.6 Da (respectively for MS and MS/MS spectra)
Percolator Target FDR (Strict): 0.01.
Target FDR (Relaxed): 0.05
Validation based on q-Value.

To improve the sensitivity of the database search, Percolator (semi-supervised learning machine) was used in order to discriminate correct from incorrect peptide spectrum matches. Percolator assigns a q-value to each spectrum, which is defined as the minimal FDR at which the identification is deemed correct. These q-values are estimated using the distribution of scores from the decoy database search.

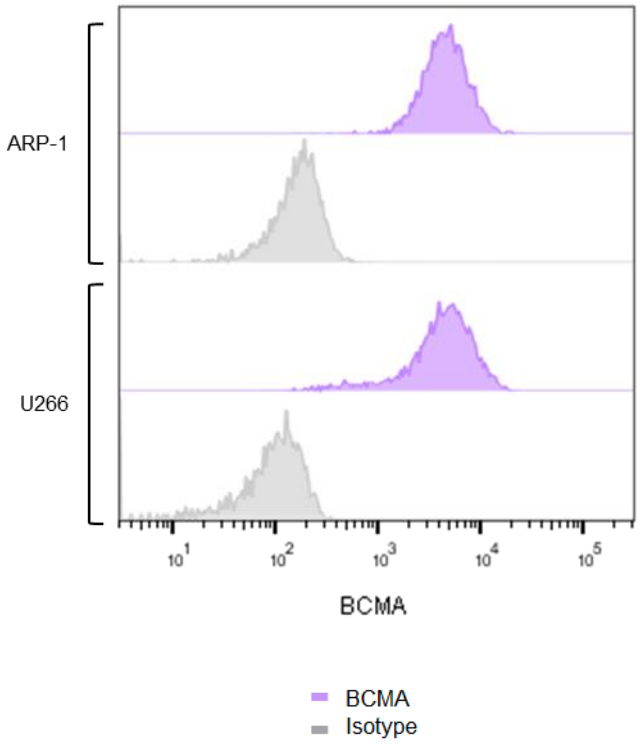
Results

The search results obtained with the SEQUEST search were visualized in Proteome Discoverer (v.1.4.1.14) and exported to Excel a list of identified proteins. The results have been filtered so only proteins identified with at least 2 peptides (FDR ≤ 5%) are included in the lists.

TIGIT-blocking-scFv protein was found in ARITIGIT and not in ARI0002h supernatants.

Supplementary Figure 1

B-cell maturation antigen (BCMA) expression on tumor cell lines. BCMA expression was confirmed in multiple myeloma cell lines used to perform assays: ARP-1 and U266.



Supplementary Table 1

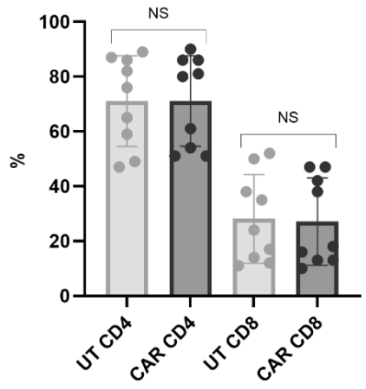
TIGIT expression on T-cells of subjects included in this study (HD: healthy donors and P: patients). Analyses were performed up to 24h before assays were started. Results refer to the expression of CD4, CD8, and TIGIT in untransduced (UT) T-cells and in CAR T-cells, if available.

	T-cell group	CD4/CD8 ratio	% CD4+TIGIT+	% CD8+TIGIT+
HD1	UT	69/27	36.7	56.8
HD2	UT	38/58	5.7	2.2
HD3	UT	39/60	9.65	6.98
HD4	UT	92/7	13.5	27.9
HD5	UT	62/31	11.9	39.5
HD6	UT	52/41	30.6	68.8
HD7	UT	78/19	7.0	16.4
HD8	UT	49/50	11.6	12.7
	ARI0002h	51/47	5.6	7.3
HD9	UT	86/14	5.8	12.2
	ARI0002h	86/13	7.3	11.1
HD10	UT	76/24	4.3	10.9
	ARI0002h	81/18	3.1	5.9
HD11	UT	47/52	4.1	3.4
	ARI0002h	51/47	4.7	4
HD12	UT	87/12	19.7	52.7
	ARI0002h	80/16	16.9	41.8
P1	UT	82/17	11.2	40.0
	ARI0002h	86/13	9.6	30.3
P2	UT	65/35	16.4	8.18
	ARI0002h	61/38	11.9	4.8
P3	UT	89/11	10.9	17.5
	ARI0002h	90/10	17.9	27.8
P4	UT	59/38	25.4	17.9
	ARI0002h	54/42	30.6	25.0

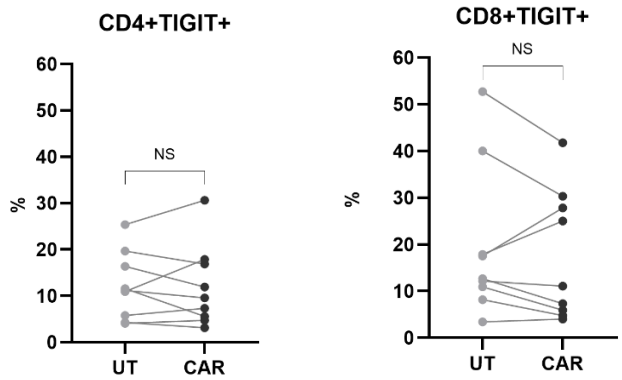
Supplementary Figure 2

Characterization of T-cells in the subjects included in the study in days 7-10 after T-cell activation, before assay performance. (A) CD4 and CD8 subsets in untransduced (UT) T cells and CAR-T cells. **(B)** Percentage of TIGIT expression comparing UT *vs* CAR-T cells for each sample. **(C)** Percentage of TIGIT expression in the different T cell subsets. **(D)** Percentage of TIGIT expression comparing healthy donors *vs* patients.

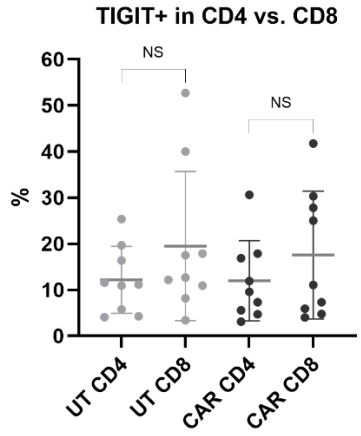
A



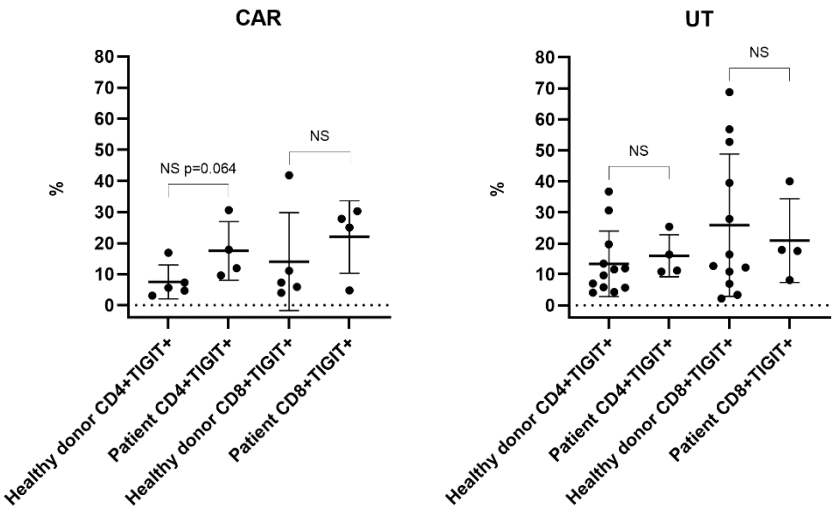
B



C

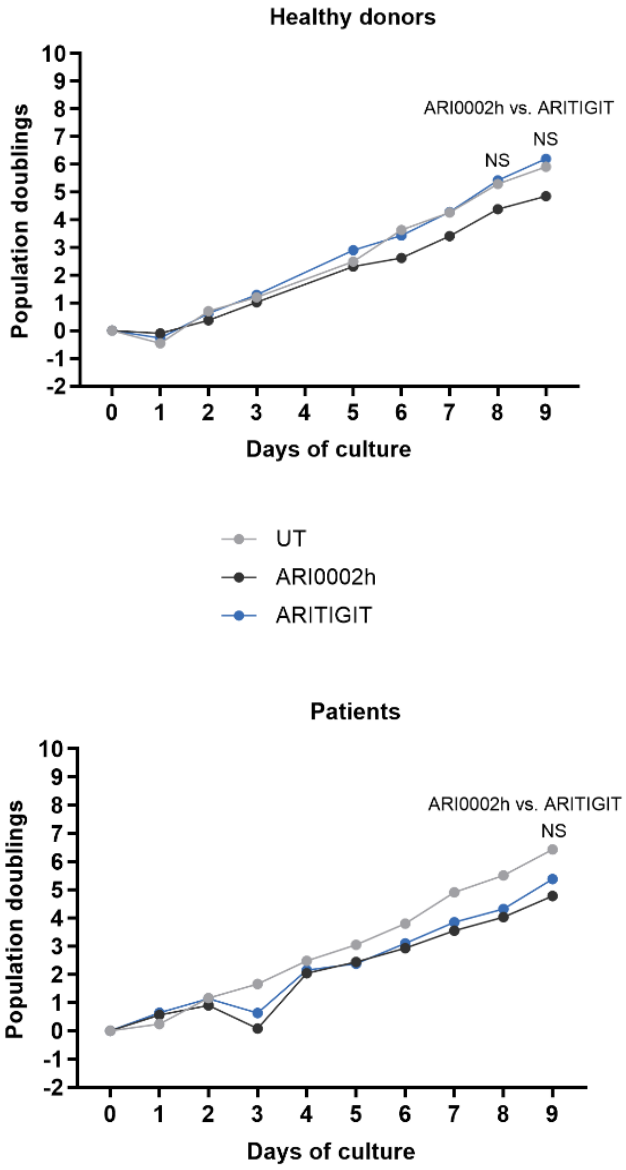


D


CAR
UT
%
NS p=0.064
NS
NS
NS
Healthy donor CD4+TIGIT+
Patient CD4+TIGIT+
Healthy donor CD8+TIGIT+
Patient CD8+TIGIT+

Supplementary Figure 3

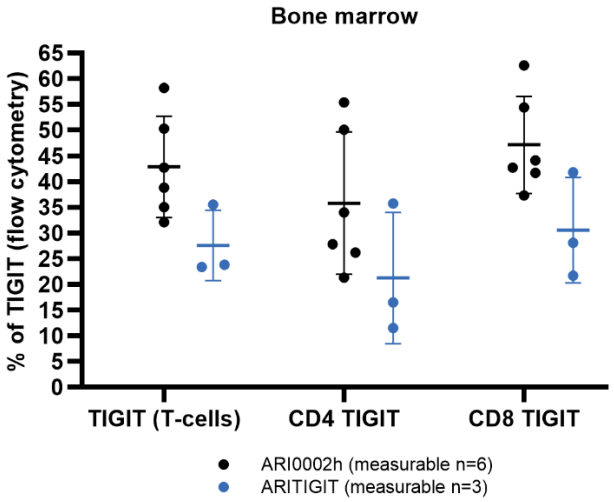
***In vitro* expansion of ARI0002h and ARITIGIT in healthy donors and patients..**



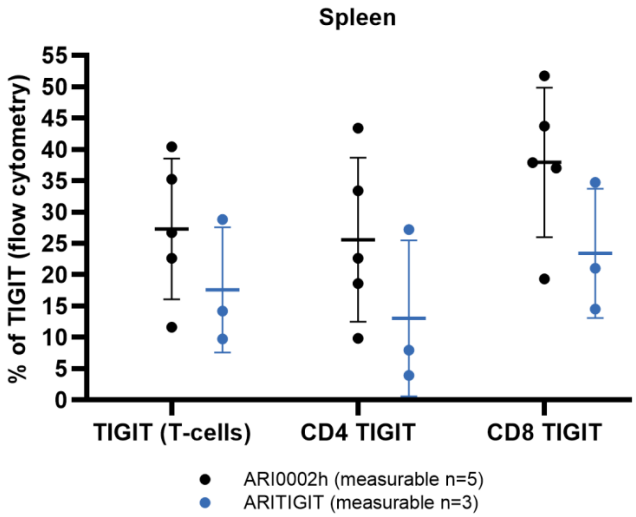
Supplementary Figure 4

Effective TIGIT blockade in mice experiments was confirmed for ARITIGIT. Mice were sacrificed 8 days after CART injection and tissues (bone marrow and spleen) were processed and analyzed by flow cytometry. TIGIT expression on T-cells (CD4 and CD8) of the bone marrow (**A**) and spleen (**B**) of ARI0002h-treated-mice *vs*. ARITIGIT-treated-mice.

A

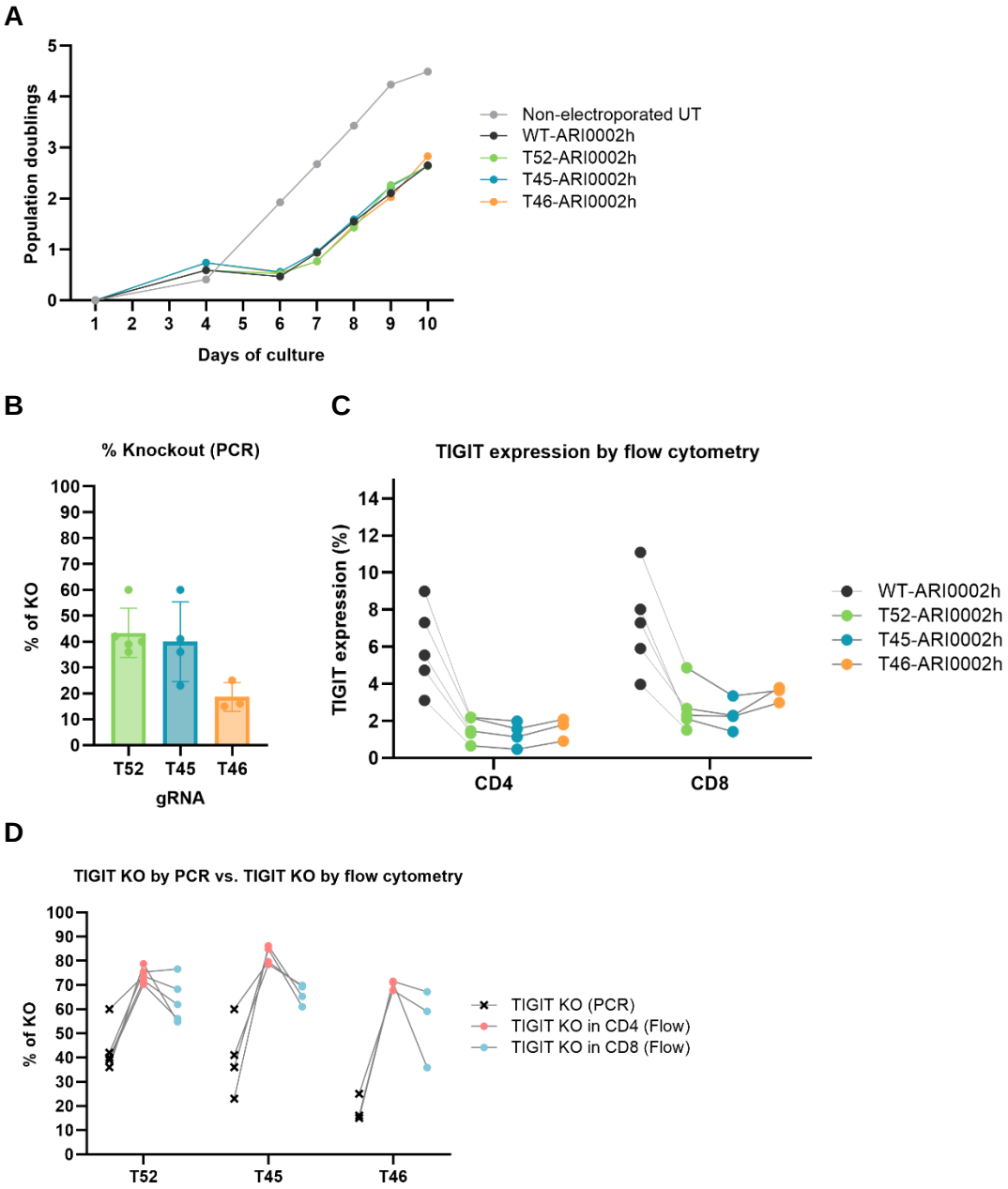


B



Supplementary Figure 5

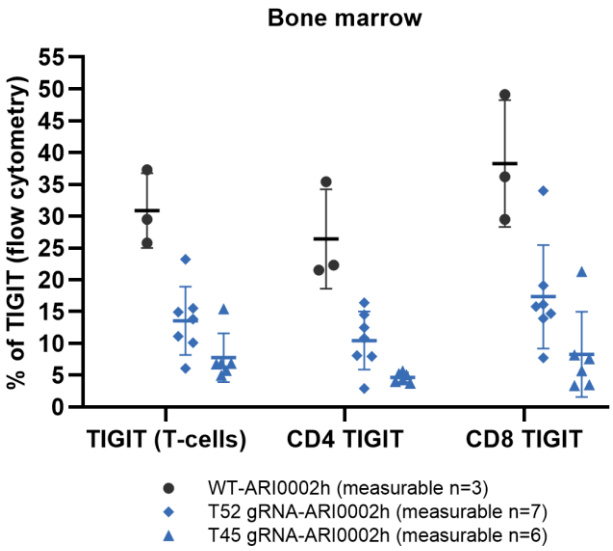
Three different guide RNAs (gRNA), T45, T46 and T52, were tested to remove TIGIT from ARI0002h cells. (A) *In vitro* expansion between wild type (WT)-ARI0002h and all 3 TIGIT-KO-ARI0002h. Electroporation was performed on day 4. **(B)** KO efficiency of TIGIT measured by PCR and sequencing. **(C)** TIGIT protein expression in CD4 and CD8 cells using flow cytometry. **(D)** Comparison of TIGIT KO values evaluated by PCR and flow cytometry in CD4 and CD8 in all groups.



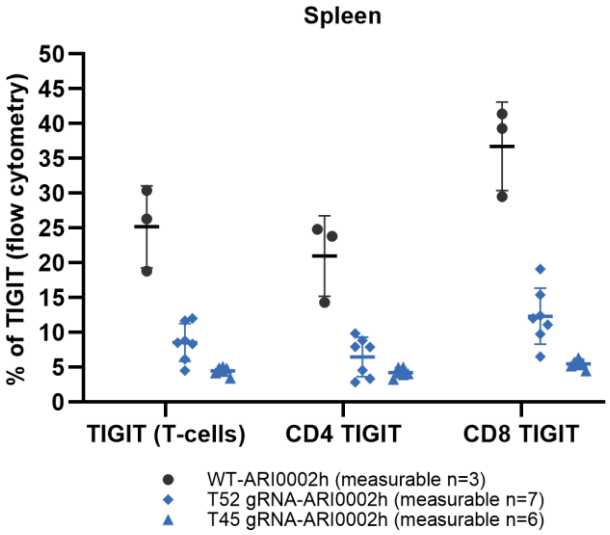
Supplementary Figure 6

Effective TIGIT blockade in mice experiments was confirmed for the TIGIT KO CART. Mice were sacrificed 8 days after CART injection and tissues (bone marrow and spleen) were processed and analyzed by flow cytometry. TIGIT expression on T-cells (CD4 and CD8) of the bone marrow (**A**) and spleen (**B**) in the different groups of treated mice.

A



B



DISCUSSION

Discussion

In the past decade, a new paradigm for cancer treatment has arisen in the form of cellular immunotherapies. CAR-T cell therapy has emerged as a transformative approach for hematologic malignancies, achieving impressive results in heavily pretreated B-ALL, NHL and MM patients, in which limited or no therapeutic options were available. The success observed in hematologic malignancies has not yet been reproduced for solid tumors. Tumor heterogeneity and the lack of specific and selective surface antigens in certain cancer subtypes, hinder finding a suitable target antigen. Proper tumor trafficking and infiltration, and the hostile tumor microenvironment, are other challenging features that CAR-T for solid tumors need to overcome(167,168). Still, efforts to improve CAR-T efficacy are being investigated in the preclinical setting and preliminary results of GD2- and HER2- directed CAR-T cells are encouraging(169–173), although validation on a larger clinical scale is needed. On the other hand, impressive results have been observed in non-malignant diseases after CD19-CART therapy in small cohorts of patients with autoimmune diseases, including lupus erythematosus, idiopathic inflammatory myositis, myasthenia gravis and systemic sclerosis(174,175).

The impressive results of CAR-T cell therapy in R/R patients with hematologic malignancies have encouraged its application into earlier lines of treatment. Two CD19-CART, axi-cel and lisocabtagene-maraleucel (liso-cel) have already been approved as second line of therapy for NHL refractory patients or those presenting a relapse within 12 months after the first line(176,177). In MM, several clinical trials are investigating the potential benefit of introducing the already approved BCMA-CART, cilta-cel and ide-cel, to the first line of treatment. Options that are being evaluated include the use of a BCMA-CART as a substitution of ASCT (NCT05257083; CARTITUDE-6), as maintenance treatment compared to RD in patients not intended for transplant (NCT04923893; CARTITUDE-5), and combined with lenalidomide maintenance, compared to lenalidomide alone, in patients who have a suboptimal response after ASCT (NCT06045806; KarMMa-9).

Our work proves the feasibility of ARI0002h manufacturing in two different academic sites, showing promising efficacy with a tolerable toxicity profile. Despite excellent outcomes observed in patients with MM after CAR-T therapy,

the disease remains incurable. Identifying reliable biomarkers of response to CAR-T therapy is still a critical area of investigation. Also, improving our knowledge on CAR-T resistance mechanisms and developing strategies to overcome them, is essential to improve CAR-T outcomes. We analyzed potential resistance mechanisms to CAR-T treatment that may be addressed. Based on the previous findings, we performed structural changes in ARI0002h to try to improve its efficacy, which were tested in the preclinical setting.

The CARTBCMA-HCB-01 pivotal study evaluated ARI0002h, an autologous BCMA-directed CAR-T, developed in an academic institution, for the treatment of patients with R/R MM. ARI0002h was successfully manufactured with a point-of-care approach in two academic facilities: Hospital Clínic de Barcelona and Clínica Universidad de Navarra. This clinical trial included some features not previously explored in the immunotherapy field of multiple myeloma: a fractionated administration of the first dose and a booster dose, together with a humanized scFV to potentially limit the immunogenicity.

Based on the knowledge acquired with the ARI-0001 clinical trial, CART19-BE-01(164,178), a decision was made to include a fractionated initial dose in the CARTBCMA-HCB-01 trial design, to reduce the severity of immune-related CAR-T side effects. The development of CRS with ARI0002h was similar to that of the approved BCMA-CAR T-cell therapies, showing a lower grade of severity, and only very few cases of grade I ICANS were observed. The onset of CRS was quite similar to the one observed with cilta-cel (around seven days, instead of the earlier one with ide-cel)(141,145). Importantly, no other neurotoxic events were reported after ARI0002h treatment. This differs from data reported from the CARTITUDE-1 study evaluating cilta-cel, in which 13% of patients developed neurotoxicity beside ICANS(146). A slightly delayed profile in CART kinetics and cytokine expression, compared to other BCMA-CART, may be explained by the fractionated infusion. This behavior might have had a different effect on immunologic parameters and could explain the lower neurologic events and grade ≥3 CRS observed after ARI0002h treatment. Still, these observations could only be confirmed with a randomized study. Although the fractionated administration may have favored an acceptable safety profile, other factors, such

as the antigen-binding domain affinity, the 41BB co-stimulatory domain and a weight-based calculated dose, might have also intervened in the lower severity of immune side effects observed(179,180).

Establishing the real incidence of HLH-like symptoms, recently termed IEC-HS, in CAR-T recipients is challenging since classic-HLH criteria rely on cytopenia, which can be confounding in patients who may have disease or lymphodepletion-related myelosuppression, but also due to overlapping symptoms with CRS(126). In a retrospective study which investigated the development of IEC-HS in BCMA-CART recipients based on ferritin, fibrinogen, LDH, triglycerides, soluble-IL-2 receptor, and NK cell activity, up to 21.8% of patients met IEC-HS criteria. The proportion of captured patients was higher compared to using HLH 2004 or CART-related MAS/HLH criteria(125). IEC-HS-related deaths have been reported after cilta-cel and ide-cel administration(143,145). One IEC-HS-related death after ARI0002h treatment set the focus on this adverse event. All cases that met any of the available criteria, even if only laboratory criteria were found, were carefully reported as IEC-HS. Currently, there is increasing recognition that IEC-HS may not be as superimposed or as directly associated with CRS severity as was initially described(126). In this regard, all ARI0002h patients who developed IEC-HS had prior CRS (4 grades 1 and 2 grades 2), with a delayed onset of IEC-HS in 5 of 6 and, in the remaining patients, the onset was concurrent, but IEC-HS lasted longer.

Other CAR-T-related side effects, such as cytopenias and infections, were common, with a similar profile to that reported in other BCMA-CART trials(141,144). Recently, concerns emerged regarding secondary neoplasias after CAR-T, prompted by the reporting of 22 cases of T-cell cancers in CART recipients; 14 with available data occurred within 2 years of CART infusion. In three cases for which genetic sequencing had been performed, the CAR transgene was detected in the malignant clone, which indicated that the CAR-T product was most likely involved in the development of the T-cell cancer. The small number of cases and variability of product use precluded conclusions of an association with a specific product. However, the overall rate of this adverse event compared to the large number of CAR-T infusions

appears to be low(181). In the ARI0002h trial, none of the secondary neoplasias were hematological, likely due to the small patient cohort included and the low incidence rates of this complication. Moving forward, especially if CAR-T treatment spreads to non-oncologic indications, new strategies involving targeting insertion of the CAR construct to specific loci might help reduce the risk of cancers due to integration of the CAR construct at oncogenic loci within the genome.

Regarding efficacy, ARI0002h provided deep and sustained responses in heavily pretreated patients, and only one patient (1.6%) was primarily refractory. Due to the fractionated design of the first dose, in some patients who developed early signs of CRS, a decision was made to suspend the third fraction, and so received a lower dose of CAR-T. Outcomes of patients who received only two fractions vs. the full first dose were comparable. The development of an early CRS in these patients who received only two fractions of the first dose, was probably reflecting a stronger *in vivo* expansion of ARI0002h, which might explain the analogous outcomes in terms of efficacy.

MRD negativity in the bone marrow, measured by next-generation flow cytometry (NGF) was achieved by almost all patients in the first 100 days after infusion, which made comparisons between MRD status and PFS or OS unfeasible. Of note, the proportion of MRD-negative patients was higher than those with CR, due to the serum M protein and free light chain evaluation requirement included in the classic IMWG criteria(9). The PETHEMA/GEM group analyzed MRD significance in R/R MM patients receiving cellular immunotherapies. Interestingly, after stratifying patients into MRD-/CR, MRD-/No CR, MRD+/CR, and MRD+/No CR, both the achievement of CR and MRD-negative CAR-T recipients had a prognostic impact(182), in contrast to newly diagnosed MM treated with conventional therapies, in which MRD negative patients have similar outcomes, regardless of the CR status(183). Interestingly, an involved serum FLC below the limit of normality in day 100 was predictive of longer PFS. In MRD negative patients or in those with hemodiluted samples, which is a significant issue in the first bone marrow evaluations after CAR-T infusion, FLC measurement could be

a predictive tool, although further prospective studies are required to confirm this finding.

The booster dose was introduced in the clinical trial design with the aim of deepening and lengthening responses. The rationale of this decision was that a new infusion of CAR-T cells not previously exposed to the target antigen would be active and non-exhausted, and could reinvigorate CAR-T function. Importantly, the booster dose was safe, and patients could be rapidly discharged, making an outpatient or at-home management an option. Both expansion and an improvement in responses were observed after booster dose administration. However, the contribution of the booster dose to the improvement of ARI0002h efficacy is unknown due to the non-randomized nature of this feature in the clinical trial and the great proportion of patients already in sCR before receiving the booster dose. The fact that a deepening of responses over time has been reported in other single-dose BCMA-CART trials also contributes to this uncertainty, but the median time to best response reported in the CARTITUDE-1 study was 2.6 months, suggesting that most patients achieved the deepest response within the first 3 months of infusion(144). A randomized study exploring this feature could be helpful to clarify the specific efficacy of this approach. Nevertheless, the feasibility in terms of manufacturing, the absence of related side-effects, the streamlined logistics for its administration and the potential benefit in efficacy warrant further investigation of the booster dose. The requirement of a second LD regimen in patients who no longer had ARI0002h persistence in the peripheral blood remains unclear. Expansion after booster dose was similar in both groups, regardless of a second LD administration, which suggests that it may not be mandatory. Yet, since LD was re-administered according to CART persistence, these observations are biased as groups are not balanced.

Significant limitations restrain the success of CAR-T therapy. Elevated costs, which are unaffordable for public health systems, complex logistics, and manufacturing limitations, such as slot-obtention delays and the required manufacturing periods, allow access to only a few patients to this therapy(155,184). The complexity is growing, with an escalating demand for

CAR-T, due to its exceptional outcomes and expanding indications in various diseases. Moreover, the presumable introduction of BCMA-CART into earlier lines of treatment in MM, as has already happened in NHL(185), will probably aggravate the situation.

ARI0002h can be manufactured at up to one-fourth/one-fifth of the cost of commercial CAR-T cells. In the CARTBCMA-HCB-01 clinical trial, a point-of-care approach proved to be feasible, facilitating accelerated final product release in specific cases if clinically required. An authorized compassionate use program has enabled the treatment of 65 additional patients with R/R MM in Spain since the completion of recruitment in the clinical trial. Without this program, these patients would not have had access to a BCMA-CART therapy, due to a lack of reimbursement agreement in our country. This fact is not unique; many other countries face similar challenges with very few exceptions. Beyond Europe, US and China, access to CAR-T cell therapies for MM is scarce(157). A different BCMA-directed academic CART, HBI0101, was also developed in Israel. Hematologic and organ responses were observed in 4 amyloidosis patients, including 3 with IIIa cardiac involvement(186). Also, a phase 1 dose-escalation study in 20 R/R MM patients reported promising efficacy with tolerable safety. Importantly, establishing a GMP infrastructure in this academic setting did not compromise regulatory requirements or product quality(187). Therefore, academic CAR-T manufacturing using a point-of-care approach could enhance access to this treatment. This collaborative effort between academic institutions and pharmaceutical companies could supplement the existing commercial slots. Overall, efforts to improve affordability, expand infrastructure, and advocate for equitable distribution are imperative to ensure that all patients have access to CAR-T treatments(188).

While the potential for academic CAR-T therapies to enhance global access and alleviate financial constraints appears evident, the CARTBCMA-HCB-01 clinical trial revealed that manufacturing capacity, as measured by median turnaround time, did not significantly differ from data reported in pivotal trials for approved BCMA-CART. In situations where urgent patient treatment is required and it is suspected that bridging therapies may not adequately control the disease until

the product becomes available, consideration should be given to off-the-shelf strategies such as bispecific T cell engagers. Alternatively, the emergence of allogeneic CART offers a viable option. A study investigating the first allogeneic BCMA-CART (ALLO-715) reported an ORR of 71%, with 25% achieving a CR, along with a short median time from enrolment to lymphodepletion (5 days, range 0-20)(189). However, a longer follow-up period is required to determine the feasibility of this strategy.

Despite promising results after BCMA-CART in MM, patients continue to relapse. Several determinants of response and mechanisms of resistance to CAR-T therapy have been proposed, and strategies to overcome these are an area of intensive research. Identifying determinants of response is essential for effectively guiding therapeutic decisions. Certain baseline characteristics, such as tumor burden, stage, prior lines/refractoriness, cytogenetics, or extramedullary disease, have been associated with poorer outcomes in patients with MM receiving CAR-T. Furthermore, numerous uncertainties have arisen concerning therapy-related aspects, such as the significance of the target antigen density on tumor cells, the potential sink-effect of sBCMA, and the importance of T cell population distribution in the apheresis and the FP. Following infusion, CAR-T kinetics, target-antigen loss, T cell exhaustion, and the potential immunogenicity of the non-human parts of the construct may also compromise efficacy(140,190,191).

A higher tumor burden has been correlated with worse outcomes in CAR-T recipients in terms of both efficacy and safety(192,193). In the BCMACART-HCB-01 trial, baseline serum M protein levels, but not the proportion of bone marrow plasma cells, correlated with shorter PFS after ARI0002h administration. Interestingly, the ISS was a better predictor of outcomes after CAR-T when calculated at CAR-T inclusion rather than at MM diagnosis. Extramedullary soft-tissue involvement is usual in relapsed MM patients. It is correlated with worse outcomes and entails an unmet need due to the lack of efficacious treatments(194). BCMA-CART constitute a useful tool to treat this subgroup with extramedullary plasmacytomas, since deep responses are observed in most patients. Still, a trend to shorter survival is observed compared to patients without

plasmacytomas or with paraskeletal plasmacytomas. In high-risk cytogenetic patients, poorer survival outcomes were reported with some constructs(143,145), but no differences were observed in ARI0002h-treated high-risk patients.

Correlative studies performed on patient samples highlight different features that might be responsible for poorer outcomes after ARI0002h treatment. Initially, there was a belief that sBCMA could reduce efficacy by acting as a sink for CAR-T cells, with some preclinical studies suggesting a potential impairment in cytotoxic activity after the addition of high doses of sBCMA. Results from different clinical trials reported no correlation of sBCMA with clinical outcomes. In the ARI0002h trial, higher baseline sBCMA correlated with shorter DOR, PFS and OS. Interestingly, a correlation was also observed with CRS severity. Since the binding of ARI0002h and sBCMA cannot be translated into a cytotoxic effect, the correlation between sBCMA with shorter survival and increased severity of immune events may obey to sBCMA as a surrogate marker for tumor burden.

The impact of BCMA density in CAR-T efficacy remains unclear. Although most studies have reported similar responses and survival regardless of surface BCMA expression(145), a clinical trial using a γ-secretase inhibitor (GSI), a molecule that prevents BCMA from shedding and increases target antigen density, showed impressive results in heavily pretreated MM patients, including a subset with prior BCMA-targeted therapy(195,196). While none of the patients in the ARI0002h trial became BCMA negative, a decrease in BCMA expression was observed between baseline and relapse and a resistance mechanism associated with this low-BCMA reservoir cannot be discarded. In this subset of cells, GSI could play a role in lengthening responses. Although BCMA antigen loss seems uncommon, genetic disruption of the gene has been reported in some cases(197,198). When suspected, assessing BCMA expression may be of special importance in patients in whom a subsequent BCMA-directed therapy is considered. In such cases, evaluating sBCMA could be contemplated, since genetic alterations would also impair sBCMA levels, if present. sBCMA analysis could prevent from performing more invasive procedures, although its measurement remains limited in availability across all healthcare centers(199–201).

To overcome antigen loss, several target antigens other than BCMA are under clinical evaluation. GPRC5D is the second most developed immunotherapeutic target in CART for MM, showing already promising results in bispecific TCE(138,150). Preliminary results of a phase 1 dose-escalation study with GPRC5D-directed CAR-T cells, including patients with prior BCMA treatment, reported responses in patients receiving the maximum tolerated dose identified at $150x10^6$ CAR+ cells. At the $450x10^6$ CAR+ cell dose, 1 patient had grade 4 cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome (ICANS), and 2 patients had a grade 3 cerebellar disorder of unclear cause. No cerebellar disorder, ICANS of any grade, or cytokine release syndrome of grade 3 or higher occurred at the lower doses(202). A report from the phase 1 POLARIS study with OriCAR-017 GPRC5D-CART showed ORR 100% (40% sCR) in 9 patients with R/R MM. No neurotoxicity or grade $\geq$3 CRS were reported(203). A phase 2 trial with 33 patients treated with a GPRC5D-CART reported ORR of 91% (sCR 33% and 30% CR). In 9 patients with prior exposure to BCMA-CART at least a partial response (PR) was observed in all of them. In terms of safety, CRS was reported in 76% (all grades 1-2), and neurotoxicities in 2 patients, including a grade 3 ICANS, and a grade 3 headache(204). However, the most effective strategy will probably be dual strategies targeting two antigens. Preclinical studies combining different target antigens have shown promising results(205,206). In the clinical setting, BCMA and CD19 dual targeting reported high response rates in several clinical trials, with an acceptable safety profile(207,208). A BCMA-CD38 approach showed also promising activity with acceptable toxicity(209). A major limitation is the single-arm design of these dual-targeting trials, which prevents from assessing the true added value that the dual approach offers.

T cell subset distribution in the apheresis and the final product is relevant in terms of CAR-T outcomes (192,210–213). A higher CD4/CD8 ratio has been associated with better responses after CAR-T(214–216). Moreover, the presence of long-lived self-renewing early memory phenotypes, such as naïve, central memory and stem cell memory T cells, seems to be essential to maintain profound and sustained responses besides initial CART expansion(137,217). Regulatory T cells (Tregs) are crucial to maintain immune tolerance, but in the setting of CAR-

T overabundance of Tregs could hinder CAR-T activity. A previous report suggested that the expression of the activation marker HLA-DR correlates with better outcomes, an observation also found in the ARI0002h trial(218).

Endogenous T cells used to produce autologous CAR-T in MM patients are affected by the immunosuppressive nature of an advanced disease. In addition, cumulative exposure to different anti-myeloma drugs may have reduced the number of T cells and increased T-cell defects. Also, the treatment administered prior to leukapheresis may alter the quality of the harvested T cells(219,220). Therefore, advancing CAR-T to earlier lines of therapy may allow for the obtention of better-quality products. Another strategy would consist of harvesting and cryopreserving T cells early after disease diagnosis, to be used later, if necessary(221). Since an ASCT is performed in the first line in transplant-eligible patients, harvesting additional cells, when possible, or employing unused cells could be an option to improve CAR-T fitness. In this sense, some preclinical studies have shown that mobilization agents such as G-CSF is not deleterious for BCMA-CART manufacturing, with equivalent CAR-T phenotype and activity(222).

The manufacturing process can also be modified to improve the composition of the T cell subsets in the final product towards a less differentiated stage. This can be achieved by reducing the length of the T cell expansion phase to minimize the progressive differentiation(223), use cytokine cocktails containing IL-7 or IL-15 over IL-2, and promoting a lower metabolic activity during the process by blocking pathways such as PI3K/AKT/mTOR using drugs such as idelalisib or rapamycin in the manufacturing process(224–226). T cell selection has also been investigated, but its benefit remains unclear and randomized trials are lacking(227).

The scFv of several CAR-T constructs derives from non-human species, potentially inducing immunogenicity and leading to the development of neutralizing CAR-T antibodies. This has raised concerns regarding reduced CAR-T persistence and efficacy. Most studies have detected CAR-T antibodies in BCMA-CART recipients, with only one study reporting shorter time to progression

in these patients(228,229). To mitigate this risk, ARI0002h was designed using a humanized scFv. However, 60% of patients in the CARTBCMA-HCB-01 trial still developed CART-antibodies. Interestingly, these were transient in most patients and their presence did not correlate with poorer expansion following booster dose administration or with worse survival outcomes. Nonetheless, their neutralizing ability and the potential deleterious impact on ARI0002h efficacy remain uncertain.

The importance of CAR-T kinetics in MM is controversial. A preliminary assumption is that prolonged persistence correlates with improved outcomes. However, it is imperative to differentiate between detection and functionality of CAR-T cells. Merely detecting CAR-T cells does not ensure efficacy, as they may be present but functionally exhausted, rendering them ineffective. In the context of CD19- and BCMA-targeted CAR-T therapies, the sustainment of B-cell aplasia could indicate that CAR-T cells are present and functional. However, loss of B-cell aplasia occurred soon before relapse in the majority of patients after infusion of ARI0002h. Moreover, the assessment of persistence is primarily performed in peripheral blood samples, which is not the disease compartment in MM, and may not be an accurate location for its measurement. Consequently, persistence may be underestimated. Additionally, during disease remission, owing to a lack of target-antigen stimulation, CAR-T cells might be present, but at undetectable levels using current molecular and/or flow cytometry techniques.

The brief persistence reported in BCMA-CART contrasts with prolonged DOR, PFS and OS(141,145). Therefore, persistence and loss of B cell aplasia seem unrelated to the probability of progression. In contrast, one-third of patients included in the ARI0002h trial had detectable CAR-T at relapse, which was not coexistent with B-cell aplasia in some patients. This can only be explained by the fact that these CAR-T were unable to maintain disease control. Furthermore, an increased expression of the exhaustion markers PD-1 and TIGIT was observed in bone marrow CAR-T as soon as on day 28 after ARI0002h infusion, which highlights the role that exhaustion may play in ARI0002h relapse. Mechanisms to overcome exhaustion, besides improving T-cell fitness of the FP, could include the combination of immune checkpoint blockade(190,191). Checkpoint blockade

can be achieved by concomitant systemic drug administration, or using localized approaches such as 4th generation CAR-T or gene-editing techniques to disrupt checkpoint receptor synthesis. Also, an earlier administration of the booster dose could be considered to provide unexhausted CAR-T when those administered in the first infusion already show signs of exhaustion.

In the past years, TIGIT has emerged as a promising immunotherapy target, with several clinical trials assessing the efficacy of TIGIT-blocking antibodies in cancer(84,85). In hematologic malignancies, no definitive results have been reported yet. Biological studies have highlighted the detrimental influence of TIGIT in the TME of MM, and raising evidence also suggests its adverse impact in CAR-T therapy, where T cell exhaustion constitutes a significant resistance mechanism(89,190,230). Overcoming exhaustion may be crucial to maintain durable responses following CAR-T therapy.

The combination of TIGIT blockade and CART through three different approaches - systemic addition of a blocking antibody, and two localized strategies involving a 4th generation CART secreting a blocking scFv and genetic disruption of TIGIT using CRISPR/Cas9- yielded humble benefits in our study. Localized strategies showed a modest enhancement of ARI0002h performance in a relapse and a stress model when tested *in vivo*. Findings of two studies testing (MSLN)-targeting CAR-T cells for solid tumors and TIGIT-KO CAR-NK in neuroblastoma align with the superiority of localized strategies over a systemic approach with a TIGIT blocking antibody(231,232). Interestingly, no additional publications have reported a benefit from a single TIGIT-blockade on CAR-T/-NK cells.

The use of immunocompromised strains for the *in vivo* models of this study may have underestimated the advantages of TIGIT blockade due to the absence of a TME with the presence of other immune cells(232,233). However, in the MSLN-CART study, assays were performed on B-NDG mice, a very similar immunocompromised model (231).

The observed increase of TIGIT and PD-1 in ARI0002h cells, coupled with emerging evidence insinuating a potential synergistic effect between PD-1 and

TIGIT, suggests that dual TIGIT and PD-1 blockade could represent an interesting therapeutic strategy(233–236). Since the use of PD-1 blocking antibodies is widely spread in the treatment of advanced solid tumors, a systemic dual TIGIT and PD-1 blockade has already been evaluated. Early phase clinical trials in non-small-cell lung cancer reported a benefit of a dual TIGIT and PD-1 blockade compared to PD-1 alone, but phase 3 trials failed to meet the primary endpoints(237–239). The most recent update of the phase Ib/II MORPHEUS-liver study comparing tiragolumab (anti-TIGIT), atezolizumab (anti-PD-1) and bevacizumab (anti-vascular endothelial growth factor (VEGF)) vs. only atezolizumab and bevacizumab for hepatocellular carcinoma reported longer PFS in the treatment arm, although the OR rate in the control arm was lower than expected(240,241). Trials testing the dual blockade combination in hematologic malignancies are lacking.

Several studies have analyzed the application of immune checkpoint blockade to CAR-T(242,243). Most studies were performed in small patient populations or were early-stage clinical trials with a limited follow-up. A meta-analysis that analyzed 9 available studies concluded that the combination of single PD-1 blocking agents with CD19-CART in B-ALL and DLBCL appeared to be safe, but efficacy results were heterogeneous. While the combination could be a promising option in B-ALL, with modest data, in DLBCL, although hopeful responses were observed, the combination did not appear better than CAR-T cell monotherapy(244). Additional prospective clinical trials are ongoing. The dual TIGIT and PD-1 blockade approach applied to CAR-T has yielded encouraging results in preclinical studies(245,246). In the clinical setting, results of 34 R/R DLBCL patients treated with a CD19-CART with a dual TIGIT and PD1 KO (NCT04836507), showed an ORR of 85% and CR on day 28 of 74%(247). No further clinical data are available.

Although biological evidence in MM patients and TIGIT upregulation observed in ARI0002h recipients supports a potential success of TIGIT blockade in BCMA-CART, our results blocking TIGIT as a single target in ARI0002h did not provide a significant benefit. However, there were no deleterious effects, and some results showed a trend towards a better outcome. The growing evidence

regarding a synergistic effect between TIGIT and PD-1 and results observed in a few preclinical and clinical studies, suggest that a dual blockade combination could improve CAR-T outcomes. Localized blocking approaches seem more efficacious than systemic combinations with blocking antibodies. However, available evidence is limited, and further studies are needed to confirm these hypotheses.

Looking ahead, the field of CAR-T cell therapy for MM continues to evolve, presenting both opportunities and challenges. Key areas for future research include enhancing CAR-T cell persistence and efficacy, optimizing treatment protocols and prophylaxis, refining biomarker-based patient selection and elucidating resistance mechanisms. Moreover, advancing regulatory frameworks, fostering international collaborations, and addressing logistical hurdles are essential to maximize the impact of CAR-T cell therapy on patient care. By addressing these challenges and harnessing the full potential of CAR-T cell therapy, a new era of precision medicine can start for patients with MM and other hematologic malignancies.

CONCLUSIONS

Conclusions

1. ARI0002h, an academic autologous CAR-T against BCMA, has demonstrated clinical activity, with an acceptable safety profile in patients with relapsed/refractory multiple myeloma enrolled in the clinical trial CARTBCMA-HCB-01.

2. The manufacturing of ARI0002h in two different academic centers was feasible in all the patients included, demonstrating the success of an academic point-of-care manufacturing model to increase access to CAR-T therapy.

3. A fractionated approach of the CAR-T administration may contribute to a reduced incidence of high-grade immune-related adverse events and neurotoxicity.

4. The administration of a booster dose of ARI0002h in patients with a previous response was found to be safe and could potentially enhance and/or prolong treatment responses.

5. Patients with myeloma and extramedullary plasmacytomas can benefit from BCMA-CART therapy, as responses are observed in this subgroup; however, progression-free survival may be inferior compared to patients without soft-tissue involvement or with paraskeletal plasmacytomas.

6. A higher tumor burden, measured by serum monoclonal protein and the amount of soluble BCMA, correlated with poorer outcomes in the ARI0002h trial, while a higher expression of the activation marker HLA-DR in the apheresis was predictive of longer progression-free survival.

7. Target antigen loss represents a rare mechanism of resistance to BCMA-CART therapy after ARI0002h, while impaired CAR-T cell function, partly driven by T cell exhaustion, with a marked increased of TIGIT and PD-1 expression in the patients enrolled in the trial, may be a pivotal factor in disease relapse.

8. Targeting the immune checkpoint receptor TIGIT in monotherapy in the CAR-T setting has yielded only modest improvements in a murine model, even when using different approaches, including the administration of a monoclonal antibody against TIGIT, the production of a soluble immune inhibitor by the same CAR-T cell, or the deletion of the TIGIT gene.

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