

ORIGINAL ARTICLE

Chimeric HLA antibody receptor T cells for targeted therapy of antibody-mediated rejection in transplantation

Sergi Betriu¹  | Jordi Rovira^{2,3}  | Carolt Arana^{2,4} |
 Ainhoa García-Busquets² | Marina Matilla-Martinez²  |
 Maria J. Ramirez-Bajo^{2,3}  | Elisenda Bañon-Maneus^{2,3} |
 Marta Lazo-Rodriguez² | Ariadna Bartoló-Ibars¹ | Frans H. J. Claas⁵ |
 Arend Mulder⁵ | Sebastiaan Heidt⁵  | Manel Juan¹ | Beatriu Bayés-Genís^{2,4} |
 Josep M. Campistol^{2,4} | Eduard Palou¹ | Fritz Diekmann^{2,3,4}

¹Department of Immunology, Clinic Barcelona, Barcelona, Spain

²Laboratori Experimental de Nefrologia i Trasplantament (LENIT), Fundació de Recerca Clinic Barcelona-Institut d'Investigacions Biomèdiques August Pi i Sunyer (FRCB-IDIBAPS), Barcelona, Spain

³Red de Investigación Renal (REDINREN), Instituto de Salud Carlos III, Madrid, Spain

⁴Department of Nephrology and Kidney Transplantation, Institut Clínic de Nefrologia i Urologia (ICNU), Clínic Barcelona, Barcelona, Spain

⁵Department of Immunology, Leiden University Medical Center, Leiden, The Netherlands

Correspondence

Eduard Palou, Department of Immunology, Clinic Barcelona, Barcelona, Spain.

Email: epalou@clinic.cat

Fritz Diekmann, Laboratori Experimental de Nefrologia i Trasplantament (LENIT), Fundació de Recerca Clinic Barcelona-Institut d'Investigacions Biomèdiques August Pi i Sunyer (FRCB-IDIBAPS), Barcelona, Spain.

Email: fdiekman@clinic.cat

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The presence of donor-specific antibodies (DSA), mainly against HLA, increases the risk of allograft rejection. Moreover, antibody-mediated rejection (ABMR) remains an important barrier to optimal long-term outcomes after solid organ transplantation. The development of chimeric autoantibody receptor T lymphocytes has been postulated for targeted therapy of autoimmune diseases. We aimed to develop a targeted therapy for DSA desensitization and ABMR, generating T cells with a chimeric HLA antibody receptor (CHAR) that specifically eliminates DSA-producing B cells. We have genetically engineered an HLA-A2-specific CHAR (A2-CHAR) and transduced it into human T cells. Then, we have performed in vitro experiments such as cytokine measurement, effector cell activation, and cytotoxicity against anti-HLA-A2 antibody-expressing target cells. In addition, we have performed A2-CHAR-Tc cytotoxic assays in an immunodeficient mouse model. A2-CHAR expressing T cells could selectively eliminate HLA-A2 antibody-producing B cells in vitro. The cytotoxic capacity of A2-CHAR expressing T cells mainly depended on Granzyme B release. In the NSG mouse model, A2-CHAR-T cells could identify and eradicate HLA-A2 antibody-producing B cells even when those cells are localized in the bone marrow. This ability is effector:target ratio dependent. CHAR

Sergi Betriu and Jordi Rovira contributed equally to this study.

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technology generates potent and functional human cytotoxic T cells to target alloreactive HLA class I antibody-producing B cells. Thus, we consider that CHAR technology may be used as a selective desensitization protocol or an ABMR therapy in transplantation.

KEYWORDS

ABMR therapy, antibody-producing B cells, chimeric HLA antibody receptor T cells (CHAR-Tc), desensitization protocol, donor-specific antibodies, HLA-sensitized patients

1 | INTRODUCTION

Solid organ transplantation (SOT) is the best replacement therapy for chronic organ dysfunction.^{1–4} HLA-A is a commonly mismatched antigen in transplantation and is associated with poor outcomes after hematopoietic stem cell transplantation^{5–7} and SOT.^{8,9} Specifically, *HLA-A*02* constitutes a common HLA allele group, with reports of 21.1%–27.7% of renal transplant recipients in Europe and the United States receiving an *HLA-A*02*-mismatched renal transplant.^{10–12}

HLA-sensitized patients, those with pre-existing donor-specific antibodies (DSAs), are limited regarding the access to a suitable donor due to the high rejection risk associated with pre-existing DSA. For highly sensitized patients there are only very few or no donors express acceptable HLA antigens. In addition, long periods on the waiting list increase the risk of death or unfavorable outcomes for these patients.^{13,14} Although current therapies can almost block cellular rejection, many recipients develop antibody-mediated rejection (ABMR), especially those highly sensitized.¹⁵ ABMR relates to HLA-incompatibilities between donor and recipient and pre-existing or de novo DSA.¹⁶

Thus, desensitization therapies, either pre- or post-transplant, constitute transplant options for these patients despite DSA. Currently, there are several desensitization protocols, which can include the elimination of DSA through plasma exchange or immunoadsorption and subsequent immunoglobulin replacement.¹⁷ In some centers, the elimination of B cells using anti-CD20 antibodies, or proteasome inhibitors (bortezomib or carfilzomib) to eliminate plasma cells has been assessed to eliminate the antibody-producing cells.¹⁸ These therapies have also been used to treat ABMR¹⁹ with variable success rates; in some patients, a remission of the rejection episode can be achieved, but in others, an acute or chronic deterioration of graft function with subsequent graft loss persists. The treatment of ABMR and its complications are critical aspects and are still an unmet need.^{20,21}

Donor-specific antibody removal techniques such as plasma exchange or immunoadsorption and IgG

replacement is a therapy that eliminates the DSA or modulate their effects for a specific time interval, but after some weeks, these HLA antibodies appear again.²² The use of anti-CD20 antibodies has been debated because they are not directed against plasma cells, the main alloantibody-producing cells, and are associated to adverse effects such as bacterial and especially viral infections, thus increasing the risk of mortality.^{23–25} On the other hand, using proteasome inhibitors eliminates all types of plasma cells; however, discordant results have been observed if used as a desensitization therapy and these drugs are associated with some severe side effects.²⁶

Over the last two decades, the development of chimeric antigen receptor (CAR) T cell therapies has opened the opportunity to target specific antigens with autologous cytotoxic T cells with remarkable clinical success in the treatment of hematological malignancies.^{27–29} At Hospital Clinic de Barcelona, CAR-19 therapies have been developed from Academia reaching the clinical phase in several oncological diseases.^{30,31}

In the field of transplantation, the development of alloantigen-specific T regulatory cells (Tregs) using a CAR targeting HLA-A2 has demonstrated efficacy in reducing graft versus host disease (GvHD)³² and prolonging skin allograft survival in a murine model.³³ This strategy has been tested in heterotopic heart transplantation in mice with good results.³⁴ Recently, Schreeb et al. described the design of the STEADFAST study, a first-in-human, phase I/IIa, multicenter, open-label, single-ascending dose, dose-ranging study to assess an autologous cell therapy (TX200-TR101) where an HLA-A2-CAR is introduced into autologous naive Tregs in living-donor renal transplant recipients.³⁵ These strategies generate a tolerogenic environment around the alloantigen, however, the generation of antibodies could be reactivated at any time.

The development of chimeric autoantibody receptor (CAAR) T lymphocytes has been postulated for targeted therapy of autoimmune diseases to eliminate the antibody-producing cells. Specifically, Ellebrecht et al. demonstrated that the engineered T cells, expressing the pemphigus vulgaris autoantigen desmoglein, could selectively eliminate anti-desmoglein target cells.³⁶ Based on

this idea, we considered the development of T cells that express a chimeric HLA antibody receptor (CHAR), which allows it to eliminate B cells that produce donor-specific HLA antibodies. In particular, we engineered HLA-A2-specific CHAR (A2-CHAR) T cells. This strategy could be a therapeutic approach for personalized desensitization of HLA-sensitized recipients and even for ABMR in SOT.

2 | METHODS

2.1 | A2-CHAR generation

Exons 2, 3 and 4 of the HLA-A gene, which codify for the extracellular domains $\alpha 1$, $\alpha 2$, and $\alpha 3$, were cloned from an *HLA-A*02:01* healthy volunteer. RNA was extracted from PBMCs using mRNA Isolation Kit (#11741985001; Roche), retrotranscribed to cDNA using the *Transcriptor First Strand cDNA Synthesis Kit* (#04379012001; Roche), and cDNA was used as a template to amplify extracellular domain sequence of *HLA-A*02:01*. The entire A2-CHAR sequence was generated by the fusion of extracellular *HLA-A*02:01* domains with the transmembrane and intracellular domain of ARI-001 CAR19.³⁰ The complete A2-CHAR sequence (including a signal peptide, *HLA-A*02:01* domains, CD8 hinge, and transmembrane regions 4-1-BB and CD3z) was cloned into the third-generation lentiviral vector pCCL (kindly provided by Dr. Luigi Naldini; San Raffaele Hospital, Milan) under the control of EF1 α promoter. Third-generation lentiviral vectors³⁷ were produced by HEK 293T cell transfection with our transfer vector (pCCL-EF1 α -A2-CHAR) together with packaging plasmids pMDLg-pRRE (Addgene, 12251), pRSV-Rev (Addgene, 12253), and envelope plasmid pMD2.G (Addgene, 12259), using linear PEI molecular weight (MW) 25,000 (Polysciences, 23966-1). Briefly, 24 h before a transformation, HEK 293 T cells were plated at a concentration of 10^6 cells per Petri 150 mm plate with DMEM supplemented with 10% of FBS and penicillin–streptomycin (100 UI/mL–100 μ g/mL). A plasmid-PEI mix was prepared and added slowly to each plate on the day of transfection. After 4–6 h of incubation, media was exchanged for new supplemented DMEM. The supernatant was harvested at 48 and 72 h and concentrated by LentiX–Concentrator (Takara Bio, 631232). The titers of concentrated virus were determined by limiting dilution on Jurkat cells.

2.2 | T cells isolation, transduction, and expansion

Human CD3+ T cells, isolated by negative selection using RosetteSep Human T Cell Enrichment Cocktail Kit (STEMCELL Technologies) from healthy *HLA-A*02:01*

negative donors buffy coats, were thawed, cultured with RPMI1640 supplemented with 10% of FBS, penicillin–streptomycin (100 UI/mL–100 μ g/mL), and IL-2 (50 IU/mL; R&D), and activated with anti-CD3 and anti-CD28 dynabeads (ThermoFisher) at 1:1 bead: cell ratio 24 h prior transduction. Cells were transduced the day after with the lentiviral particles at MOI (Multiplicity of Infection) of 5 lentiviral particles/cell and cultured for 10 days before conducting experiments.

2.3 | Flow cytometry

The immunophenotype of transduced A2-CHAR T cells and the evaluation of cytotoxicity were performed by flow cytometry. All monoclonal antibodies against human proteins and viability markers used were from BD bioscience: CD3-BV421 (#563798), CD4-PE (#565999), CD8-PerCP-Cy5.5 (#560662), CD19-PE (#561741), HLA-A2-APC (#561341), and 7-AAD (#559925). Briefly, cells were collected into a 5 mL Round Bottom Polystyrene Test Tube (Falcon #352008) and washed with FACS Buffer for 5 min at 300 g. Then, cells were stained for 15 min at room temperature, protected from light, and washed again with FACS Buffer for 5 min at 300 g.

Samples were run through the BD FACSCanto II (BD Biosciences) cytometer, and data were analyzed using the FlowJo 10.8.1.

2.4 | Cytotoxic activity in vitro

To analyze the cytotoxic properties of A2-CHAR T cells, target anti-*HLA-A*02* antibody-producing B cells (hybridomas SN230G6 and ROU2D3) were required and gently provided by Dr Claas group (currently directed by Dr Heidt at Leiden University Medical Center).³⁸ Briefly, SN230G6 cells produce anti-*HLA-A*02* IgG1 isotype, whereas ROU2D3 cells release IgM antibodies. Moreover, SN230G6 and ROU2D3 cells have low and high expression of anti-HLA-A2 antibody at surface, respectively. Antibody-producing B cell hybridomas were adjusted to 10×10^6 live cells/mL in supplemented RPMI medium. A total 250 μ L of cell mixture per well were added in a 48-well plate for a total of 2×10^5 live cells/well. Subsequently, A2-CHAR-T or UT-T cells (the starting point was a concentration of 8×10^5 live cells/mL) were cocultured at the indicated ratios.

The exposure to immunosuppressive drugs such as tacrolimus (TAC) at 10 ng/mL, prednisolone (PDN) at 400 μ g/mL, mycophenolate mofetil (MMF) at 10 μ M, and rapamycin (RAPA) at 10 ng/mL were analyzed to simulate in vivo serum concentrations of patients with kidney transplants.³⁹ Two different triple therapies (TAC + MMF + PDN and TAC + RAPA + PDN) were also tested.

In addition, the cytotoxicity activity was evaluated when the effector cells were not in contact with the target cells, and their effects were assessed after exposure to anti-*HLA-A*02* antibody-containing serum. For this purpose, transwells (TW) with 3 μm diameter pores (Falcon® Cell Culture Inserts #353096) were used. In these experiments, 24-well plates were used, 5×10^5 live SN230G6 target cells were plated with supplemented RPMI, then TW was placed on top, and A2-CHAR-T cells were introduced into the TW at 1:1 ratio. The cytotoxic activity was also evaluated in the presence of anti-*HLA-A*02* antibodies through the addition of 100 μL of supernatant (SN) from target cell cultures into the transwell.

After 16 h at 37°C, cells were collected from the well and marked for 15 min with CD45-PE, and then stained with 7-ADD and CountBright beads. Subsequently, cells were analyzed by flow cytometry, as described above.

2.5 | Activation of A2-CHAR-T cells by anti-*HLA-A*02* antibodies

The following experiment was designed to determine whether the presence of anti-*HLA-A*02* antibodies can activate A2-CHAR T cells without contact with the target cells. The A2-CHAR-T and UT-T cells were plated at 1.5×10^5 cells/well in a 96-well plate with supplemented RPMI in a volume of 150 μL , then 50 μL of donor serum containing antibodies were added. Sera containing anti-*HLA A*02* and Anti-*HLA-B*44* (non-specific antibody) antibodies were tested. The positive control was carried out with lectin phytohemagglutinin (PHA). Cells were incubated for 16 h and were subsequently analyzed for activation markers (CD137) by flow cytometry and granzyme B release by Luminex®.

In order to analyze the cytotoxic properties of A2-CHAR T cells, stable GFP/Luciferase-expressing target antibody-producing B cells (hybridomas SN230G6 and ROU2D3) were generated by genetic engineering. B cells were transduced with 3rd generation lentiviral vector with pLV_MSCV_Luc-T2A-GFP. GFP+ cells were sorted with FACS Aria cell sorting to obtain a homogeneous B cell population expressing GFP (Supplementary Figure S4).

2.6 | Interleukins and cytokines analysis after cytotoxic in vitro assays

After 16 h at 37°C incubation, 450 μL of supernatant was collected, and cytokine concentration levels were determined enzymatically using high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits IFN γ (88-7316-77 Invitrogen) and IL-2 (88-7025-77 Invitrogen). IFN γ and IL-2 concentrations were calculated using

standard curves. The data points are averages of three independent experiments performed in duplicate.

Subsequently, using 25 μL of supernatants, cytokine levels (IFN γ , IL-10, Granzyme B IL-2, IL-6, sFasL, TNF α , perforin) were analyzed using MILLIPLEX® xMAP KIT (Millipore®, Darmstadt, Germany) and Luminex® magnetic beads (Luminex, Austin, TX).

2.7 | Mice

Male NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (NSG, from Jackson Laboratories), 9–11 weeks of age, were provided by Dr. Guedan group and bred and housed at the Facultat de Medicina vivarium from Universitat de Barcelona on a 12-h light cycle with access to food and water ad libitum.

2.8 | Cytotoxic activity in vivo

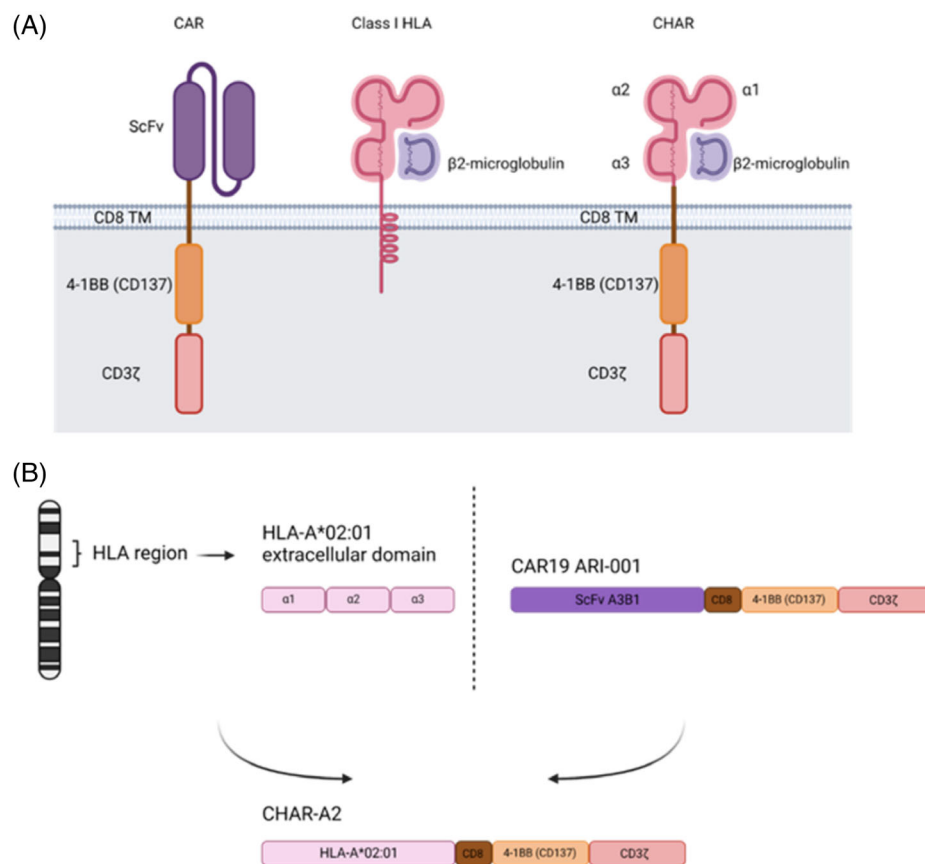
NSG mice were irradiated with 2Gy 1 day before injecting the target cells. The specificity conferred by A2-CHAR to T cells was assessed in the first set of experiments. The expansion of 5×10^5 SN230G6 cells on NSG irradiated mice that received 10^6 untransduced T cells or 10^6 A2-CHAR human T cells at day +1 was compared. In the second set of experiments, ROU2D3 or SN230G6 cells were administered by tail intravenous injection (10^7 cells) on fully irradiated NSG mice. At day +1, several mice received 10^7 A2-CHAR-T cells. Furthermore, other NSG mice with ROU2D3 or SN230G6 cells received 10^7 A2-CHAR-T cells at day +7. In the third set of in vivo experiments, repeated doses were dispensed, at D + 3 and D + 6, of 10^7 or 2×10^7 A2-CHAR-T cells on NSG irradiated mice that received 10^6 SN230G6 cells at D0.

The in vivo cytotoxicity was determined by bioluminescence assay, and mice were followed up weekly using in vivo imaging systems; Hamamatsu (Hamamatsu Photonics KK, Hamamatsu, Japan) or IVIS (Lumina III; Perkin-Elmer, Waltham, MA). Luciferin was administered to mice by intraperitoneal injection, and after 10 min of incubation, an image was taken. Bioluminescence images were analyzed and quantified with Hamamatsu or IVIS software.

2.9 | Statistics

All data are presented as mean $\pm$ SEM. Comparisons of quantitative variables between groups in the experiments of *HLA-A*02* expression in T cells and Cytotoxic activity, using *t*-student for parametric samples and Mann-Witney U test or Kruskal-Wallis test for non-parametric samples. Survival curves were estimated by the Kaplan–Meier

FIGURE 1 Engineering a chimeric HLA antibody receptor (CHAR). (A) Schematic representation of CAR19 molecule, Class I HLA molecule and engineered A2-CHAR. (B) Cloning strategy to develop A2-CHAR. *HLA-A*02:01* extracellular domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$) were cloned from healthy volunteers and then were coupled to a second-generation CAR19 instead of ScFv domain. The transmembrane portion was derived from CD8 molecule, and intracellular portion contained a costimulatory domain from 4-1BB molecule and a stimulatory domain from CD3 ζ molecule. Created with [BioRender.com](https://www.biorender.com).



method and compared with the log-rank test. A p -value less than 0.05 was considered statistically significant. Unless otherwise indicated, asterisks in graphs represent as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. The statistical analysis and graphical work were performed with GraphPad Prism version 8.0.

2.10 | Study approval

The study was reviewed and approved by the Ethics Committee of the Hospital Clinic de Barcelona (HCB/2017/0645). Animal experiments were approved by and conducted according to the guidelines of the Local Animal Ethics Committee (Comitè Ètic d'Experimentació Animal, CEEA, Decret 214/97, Catalonia, Spain).

3 | RESULTS

3.1 | Construction and validation of an A2-CHAR

We aimed to generate a new CHAR containing the extracellular domain of the *HLA-A*02* molecule, as this is the most prevalent HLA class I allele, present

at high frequencies in all ethnic populations. A2-CHAR was engineered by cloning and sequencing the *HLA-A*02:01* extracellular domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$) and fusing them to the transmembrane segment of CD8 and the intracellular signal transducing portions of 4-1BB, and CD3 ζ in a second-generation CAR structure (Figures 1 and S1).

3.2 | Generation of A2-CHAR-T cells

First, A2-CHAR was cloned into the pCCL and then transduced to HEK293 in combination with SuperLenti Packaging Mix. HEK293 cells produced high-titer packaged lentiviral particles (Figure S2A). To verify the A2-CHAR expression, we performed the lentiviral infection of Chinese Hamster Ovary cells (CHO) and JURKAT cells (Figure S2B,C). A2-CHAR human T cells were generated by purifying T cells from the peripheral blood of healthy *HLA-A*02* negative volunteers and then transducing them with A2-CHAR lentiviral particles. The expression of A2-CHAR increased from 50%–60% to 85% on A2-CHAR human T cells according to the multiplicity of infection (MOI) applied, with MOI of 5 or 10, respectively (Figure 2B). Considering the analysis of population doubling (Figure 2C) and the A2-CHAR expression in

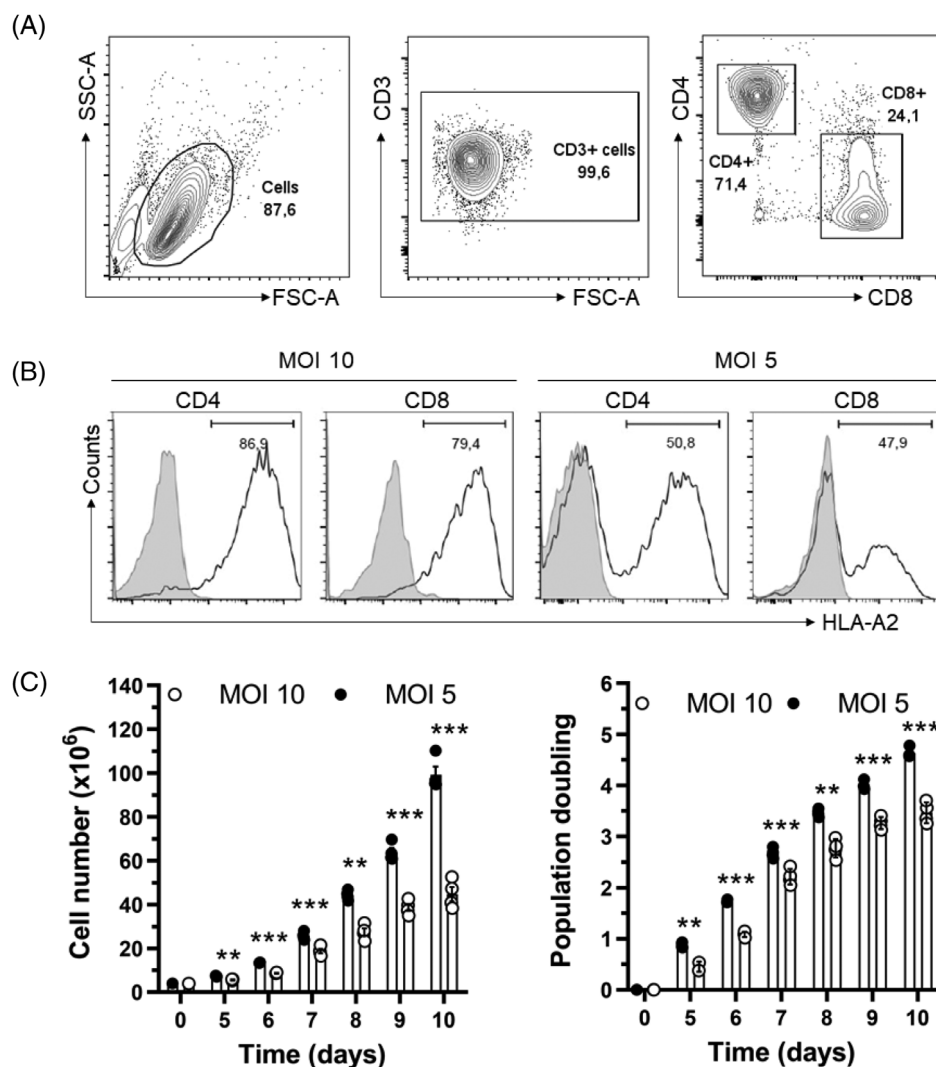


FIGURE 2 Lentiviral infection with A2-CHAR construct into primary T cells from healthy volunteers. (A) Flow cytometry approach to differentiate CD4 and CD8 T cells. (B) Expression of HLA-A2 in CD4 and CD8 T cells after lentiviral infection with A2-CHAR using MOI10 and MOI5. (C) Population doubling and cell number of T cells transduced with the lentiviral A2-CHAR construct at MOI10 and MOI5. MOI, multiplicity of infection. MOI 5, 5 million virions are added to 1 million cells. MOI10, 10 million virions are added to 1 million cells. Statistical differences between MOI10 and MOI5 ** $p < 0.01$; *** $p < 0.001$.

human T cells, the following experiments were conducted with an MOI of 5.

3.3 | Cytotoxic activity of A2-CHAR-T cells in vitro

To test the cytotoxic activity, we used two hybridomas as target cells, SN230G6 and ROU2D3, with low and high expression of the anti-HLA-A2 antibody at the surface, respectively.³⁸

A2-CHAR human T cells could eliminate both types of hybridomas in a dose dependent-manner reaching 80%–85% at 1:1 ratio. In contrast, untransduced T cells showed a background death rate of target cells up to 20% at 1:1 ratio (Figure 3A – left and middle plots). In addition, the specificity of A2-CHAR human T cells was tested in experiments that include Nalm-6 B cells, as cells do not express anti-HLA-A2 antibodies at the surface. In this setting, A2-CHAR

human T cells, like untransduced T (UT-T) cells, did not exhibit cytotoxic activity (Figure 3A – right plot). The analysis of interleukin/cytokine production in the supernatant revealed that A2-CHAR human T cells released IFN γ and IL-2 into the supernatant during cytotoxic experiments in a dose-dependent manner (Figure 3B, C, respectively).

3.4 | Impact of the immunosuppressive drugs on A2-CHAR-T cytotoxic capabilities

Several immunosuppressive drugs (TAC, MMF, RAPA, and PDN) and two different triple therapies (TAC + MMF + PDN and TAC + RAPA + PDN) were tested to analyze the cytotoxic properties of A2-CHAR-T cells against SN230G6 target cells. None of these immunosuppressants alone at therapeutic doses impacted the cytotoxic capacity of A2-CHAR-Tc (Figure 4A). Only the triple therapies TAC + MMF + PDN and TAC

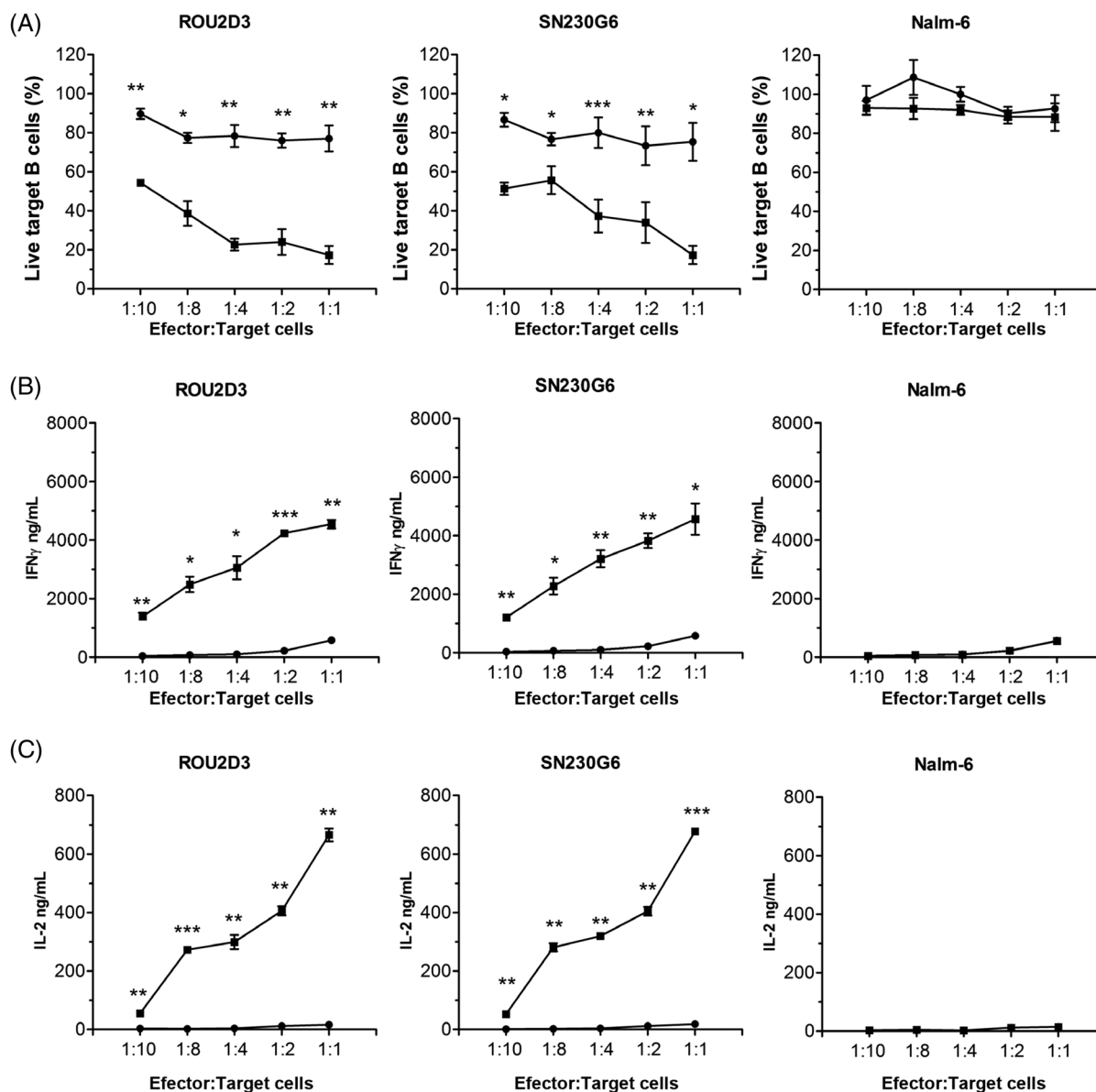


FIGURE 3 Cytotoxic activity of A2-CHAR human T cells in vitro. (A) Viability of target B cells after the co-incubation with untransduced T cells (UT-T, circle) or A2-CHAR-T cells (square). Target B cells were ROU2D3 (left), SN230G6 (middle), and NALM-6 (right). (B) IFN γ release into the supernatant after the cytotoxic assay. (C) IL-2 release into the supernatant after the cytotoxic assay. Statistical differences compared to UT-T cells release, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

+ RAPA + PDN statistically reduced the cytotoxic activity of A2-CHAR-Tc at E:T ratio of 1:2 and 1:1. The analysis of cytokines and interleukins into the supernatant of the cytotoxic assays (Figures 4B and S3) revealed that granzyme B was the only cytotoxic molecule that remained unaffected by the immunosuppressants even when A2-CHAR-T cells were cultured with the triple therapies. All treatments that include Tacrolimus (TAC alone, TAC + MMF + PDN, and TAC + RAPA + PDN) reduced the release of IFN γ , IL-2, TNF α , and IL-10 on the A2-CHAR-T cell assays to the same level of UT-T cells. MMF treatment reduced the release of IFN γ , IL-2,

and sFasL. RAPA did not modify the release of any cytokines or interleukins analyzed on the A2-CHAR-T cell assays, whereas PDN only reduced the release of IL-10.

3.5 | Assessment of non-contact cytotoxicity activity of A2-CHAR-T cells

The use of permeable cell culture inserts (TW) in the in vitro cytotoxic assays, avoiding cell-to-cell contact, significantly reduced the cytotoxic activity of A2-CHAR-T

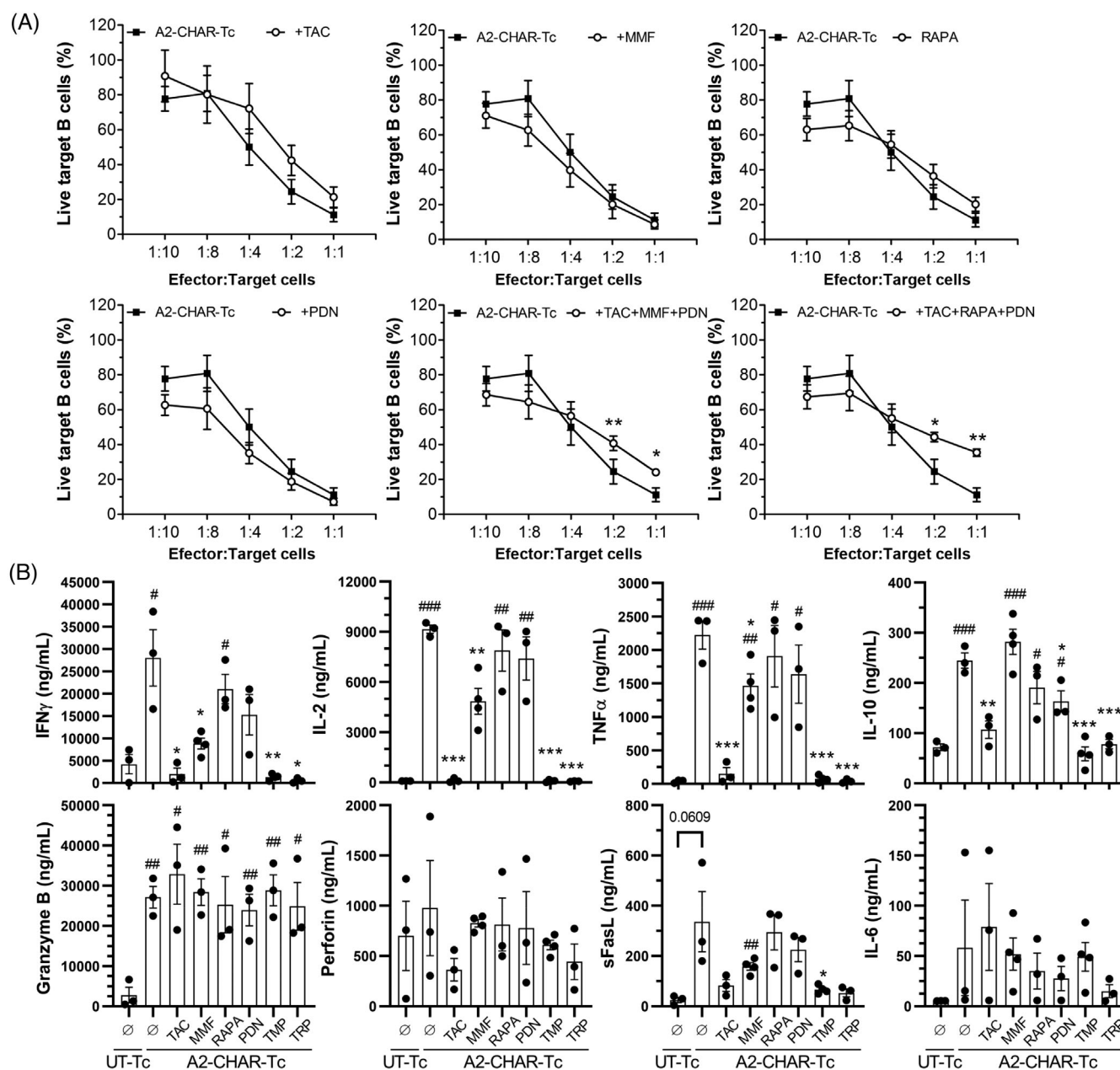


FIGURE 4 Impact of immunosuppressive drugs on cytotoxic activity of A2-CHAR human T cells in vitro. (A) Percentage of live target B cells after the cytotoxic assay in the presence of calcineurin inhibitor (tacrolimus, TAC), mycophenolate mofetil (MMF), mTOR inhibitor (rapamycin, RAPA), corticosteroids (prednisolone, PDN), the triple therapies; TAC + MMF + PDN (TMP) or TAC + RAPA + PDN (TRP). Statistical differences compare to A2-CHAR-T cells without immunosuppressant, * $p < 0.05$; ** $p < 0.01$. (B) Quantification of cytokine/interleukin into the supernatant after the cytotoxic assay at 1:1 effector:target B cell ratio. Statistical differences compared to UT-T cells, # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$. Statistical differences compared to A2-CHAR-T cells without any immunosuppressant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

cells. The addition of supernatant from the hybridomas cell culture, containing anti-*HLA-A*02* antibodies, increased the cytotoxic activity of A2-CHAR-T cells. UT-T cells revealed a background death rate of target cells of up to 10%–20%, which was not affected by the use of TW nor the addition of anti-*HLA-A*02* antibodies (Figure 5A).

3.6 | Activation of A2-CHAR-T cells with anti-*HLA-A*02* antibodies

After exposing A2-CHAR-T cells to anti-*HLA-A*02* containing serum, a significant increase in cell activation (measured by CD137 expression) was observed compared to the negative control

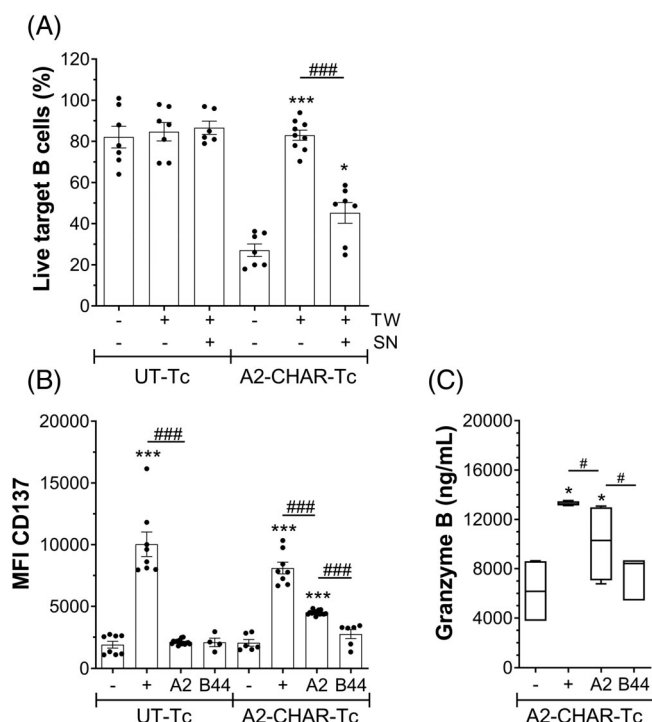


FIGURE 5 Impact of cell-to-cell contact and circulating antibodies on cytotoxic properties of A2-CHAR-T cells. (A) Cytotoxic assay using transwell (TW) to avoid cell-to-cell communication between UT-T or A2-CHAR-T cells with SN230G6 target cells at 1:1 effector:target ratio. In addition, the impact of supernatant (SN) of target cells cultures (containing anti-*HLA-A*02* antibodies) has been tested. Statistical differences compared to the experimental group without TW group, * $p < 0.05$; *** $p < 0.001$. Statistical differences compared to experimental groups with TW, ### $p < 0.001$. (B and C) Activation assay using serum samples containing anti-HLA antibodies. Control (–), A2-CHAR-T cells without any stimuli; Control (+), A2-CHAR-T cell stimulated with phytohemagglutinin; A2, A2-CHAR-T cells exposed to serum with anti-*HLA-A*02* antibodies; B44, A2-CHAR-T cells exposed to serum with anti-*HLA-A*B44* antibodies. (B) CD137 staining is represented by mean fluorescence intensity (MFI). Statistical differences compared to the negative control, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Statistical differences compared to experimental groups, ### $p < 0.001$. (C) Granzyme B was released in the supernatant after the activation assay. Statistical differences compared to the negative control, * $p < 0.05$. Statistical differences compared to experimental groups, # $p < 0.05$.

(Figure 5B), whereas UT-T cells were not activated by anti-*HLA-A*02* containing serum. However, no difference was observed when UT-T or A2-CHAR-T cells were exposed to serum with non-specific anti-HLA antibodies (anti-*HLA-B*44*). Furthermore, Granzyme B release increased when A2-CHAR-T cells were exposed to a serum with anti-*HLA-A*02* antibodies but not with anti-*HLA-B*44* antibodies (Figure 5C).

3.7 | Cytotoxic activity of A2-CHAR-T cells in vivo

To track target cells in mice, we introduced GFP and luciferase genes using a lentiviral vector into ROU2D3 and SN230G6 cells. Tracked target cells were transduced and then sorted by GFP. Then, cytotoxic assays were repeated to corroborate the ability of A2-CHAR-Tc to eliminate both modified target cells by bioluminescence (Figure S4).

We performed in vivo experiments to demonstrate that only cells incorporating A2-CHAR could localize and eliminate anti-HLA-A2 antibody-producing B cells. Immunodeficient NSG mice were fully irradiated (2Gy) the day before target cell infusion. Then, we infused 5×10^5 SN230G6 cells into NSG irradiated mice at D0, and the day after, 10^6 untransduced (UT) T cells, 10^6 A2-CHAR-T cells, or vehicle were administered (Figure 6). At D + 7, untreated mice and mice treated with UT-T cells showed bioluminescence localized mainly in the femur, sternum, and humerus, whereas mice treated with A2-CHAR-T cells showed no signs of bioluminescence. The follow-up revealed that SN230G6 cells achieved full expansion at D + 21 in untreated mice and in mice treated with UT-T cells, whereas only 2 out of 6 mice treated with A2-CHAR-T cells showed signs of bioluminescence at D + 21. This signal remained localized into the femur until D + 35 with a photons/sec inferior to the bioluminescence at D + 7 observed in the other two groups.

Next, at D0, 10^6 traceable target cells, either ROU2D3 (Figures 7B–D and S5B) or SN230G6 (Figures 7E–G and S5C), were infused by intravenous injection into NSG irradiated mice. At D + 1 or D + 7, A2-CHAR-T cells were administered by intravenous infusion. Both target cells revealed exponential growth, although SN230G6 cell expansion was even faster than ROU2D3, as they completely expanded in D + 14 versus D + 21. The early administration of A2-CHAR T cells at Day + 1 reduced the expansion of both traceable target cells. ROU2D3 cells were eliminated by A2-CHAR-T cells, and all mice survived until D + 35 without signs of bioluminescence. However, SN230G6 cells were eliminated partially by A2-CHAR-T cells, as the bioluminescence assay revealed that target cells started the expansion from D + 5. All mice survived until D + 28 (doubling the life expectancy of mice infused with SN230G6), then they were sacrificed at different time points due to ethical concerns.

The late administration of A2-CHAR-T cells at D + 7 showed discordant data on the target cell expansion into mice. On the one hand, ROU2D3 cells were partially eliminated by A2-CHAR-T cells, showing an extension of

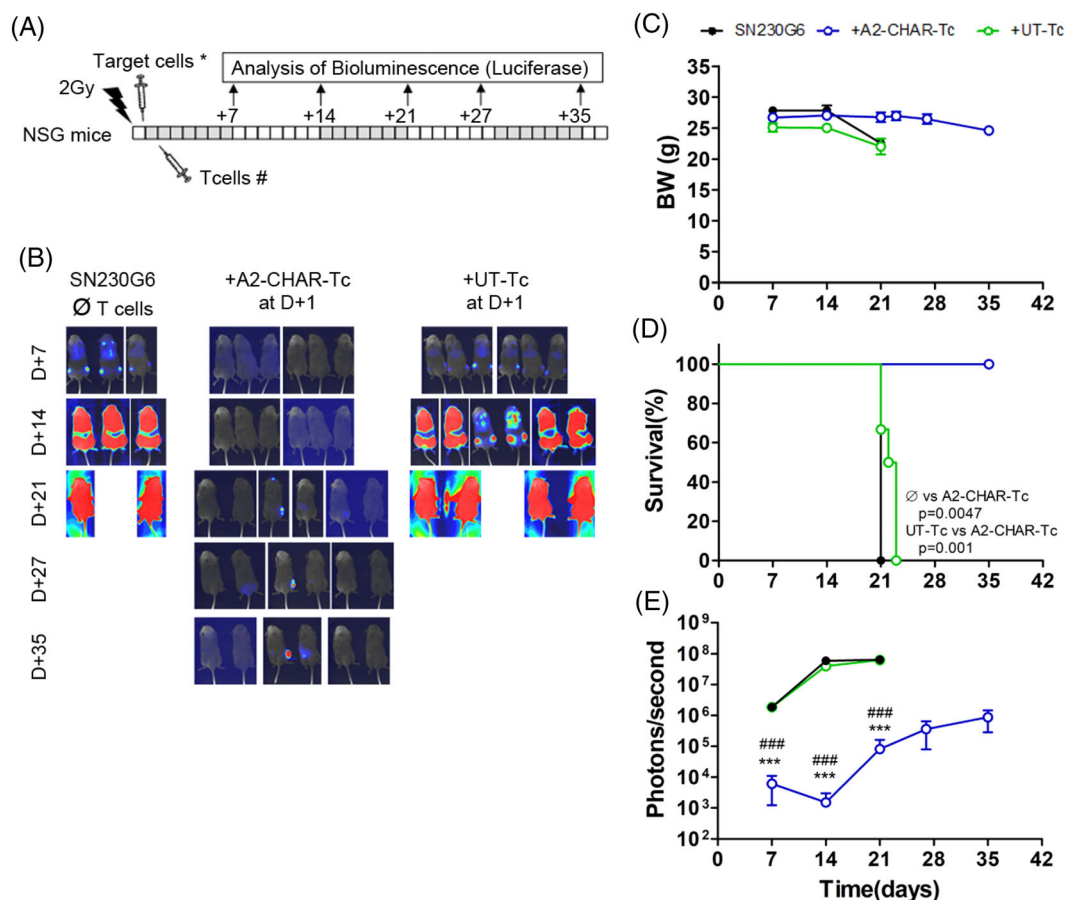


FIGURE 6 Cytotoxic activity of A2-CHAR human T cells in the specificity assay in vivo. (A) Scheme of the in vivo cytotoxic assay. NSG mice were irradiated at D-1, then modified GFP/Luciferase-SN230G6 cells (*) were infused at D0. At D + 1, three therapies (#) were applied; without T cells (Ø, physiologic serum was administered), Untransduced T cells (UT-T), and A2-CHAR-T cells. (B) Bioluminescence pictures were obtained with the Hamamatsu device. (C) Body weight (BW) follow-up. (D) Survival analysis. (E) Quantification of SN230G6 target cell expansion by bioluminescence due to Hamamatsu device. Statistical differences compared to NSG mice without T cells, *** $p < 0.001$. Statistical differences compared to NSG mice treated with UT-T cells, ### $p < 0.001$.

survival of NSG mice (Figure 7B–D), whereas NSG mice infused with SN230G6 cells and treated with A2-CHAR-T cells at D + 7 were sacrificed for ethical concerns before the control group mice (Figure 7E–G).

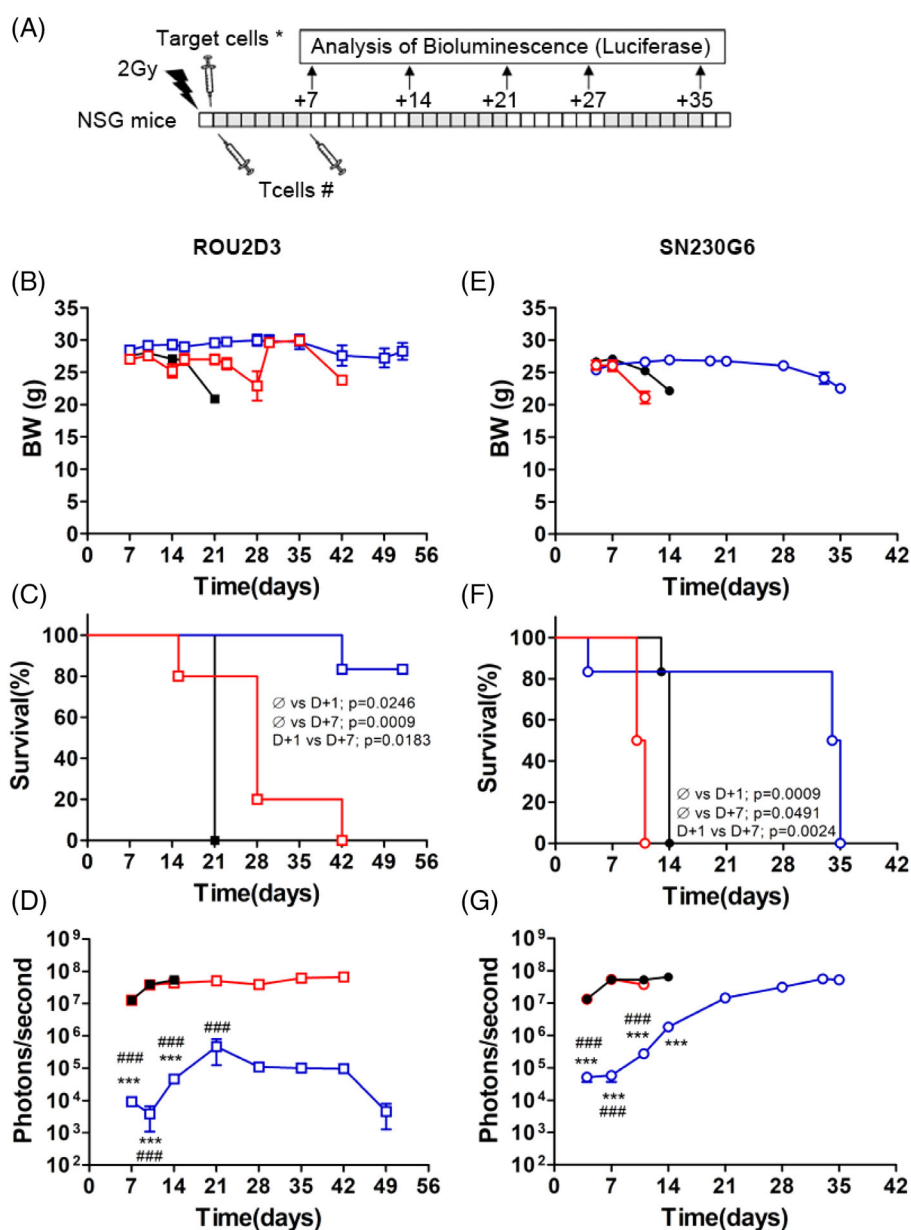
The last experiment, performed in a high-sensitivity device (Lumina IVIS), revealed that SN230G6 target cells localized to the spleen and bone marrow on day 3 post-infusion (Figure 8). In the non-treated NSG mice, SN230G6 cells were expanded over NSG mice until the full expansion at D + 24 to +27. Repeated doses of A2-CHAR-T cells (at 2×10^7 cells) prolonged the NSG mice survival due to the limitation of the SN230G6 cell expansion. However, the E:T ratio determined the ability of A2-CHAR-Tc to limit the expansion of the target cells. The dose of 10^7 A2-CHAR-Tc was insufficient to prevent the proliferation of the target cells, showing an expansion similar to that observed in non-treated mice.

4 | DISCUSSION

CAR-HLA modified Tregs have been proposed previously in the transplant field to inhibit alloimmune responses.^{32,35} However, the ability to specifically eliminate anti-HLA antibody-producing cells by the use of CAR-modified cytotoxic T cells has yet to be reported. On the other hand, CAAR T cell therapy has been proven effective and specific for eliminating autoantigen antibody-producing B cells in the pemphigus vulgaris mouse model.³⁶

This study is a proof-of-concept study in which we have shown for the first time that CAR-modified cytotoxic T cells could eliminate anti-HLA antibodies producing B cells which play a prominent role in transplant rejection. The use of the extracellular part of an HLA-A*02:01 molecule in a CAR-like construct allows a specific cytotoxic effect only against B cells that

FIGURE 7 Cytotoxic activity of A2-CHAR human T cells at different time points in vivo. (A) Scheme of the in vivo cytotoxic assay. NSG mice were irradiated at D-1, then modified GFP/luciferase-ROU2D3 cells or SN230G6 were infused at D0. The NSG mice groups were analyzed; without T cells (Ø, physiologic serum was administered, Black line); Blue-line, mice treated with A2-CHAR-T cells at Day + 1; Red-line, mice treated with A2-CHAR-T cells at Day + 7. The cytotoxic activity of A2-CHAR-Tc against ROU2D3 is shown in B–D plots, whereas cytotoxic activity against SN230G6 is in E–F plots. (B, E) Body weight (BW) follow-up. (C, F) Survival analysis. (D, G) Quantification of target cell expansion by bioluminescence due to the Hamamatsu device. Statistical differences compared to NSG mice without T cells, *** $p < 0.001$. Statistical differences compared to NSG mice treated with A2-CHAR-T cells at D + 7, ### $p < 0.001$.



express specific antibodies against *HLA-A*02:01* molecule on their plasma membrane. Unlike drugs that eliminate all B cells, such as rituximab,⁴⁰ our approach should spare the majority of B cells, only removing the allospecific cells. The so called A2-CHAR-T cells eliminated specifically anti-*HLA-A2* antibody-producing B cells under in vitro and in vivo conditions. Interestingly, A2-CHAR-T cells could be activated by anti-*HLA-A*02* antibodies, either IgG1 or IgM isotypes, produced by SN230G6 and ROU2D3, respectively.

Our cell product contains about 50% of A2-CHAR-T cells, which have been shown to completely eliminate target cells in vitro at an effector:target (E:T) ratio of 1:2. So, in fact, the ratio should be considered as 1:4. This difference between the real E:T ratio used and the

theoretical E:T ratio could have been a handicap when the in vivo study was carried out, where the target cells could not be completely eliminated. We have demonstrated that the E:T ratio is critical for defining the success of the therapy, being crucial in the in vivo assays where the target cells are distributed throughout the mouse body, mainly in the bone marrow (femur, humerus, and sternum) but also in the spleen and lymph nodes.

We considered it necessary to investigate the impact of immunosuppression (IS) on the cytotoxic properties of A2-CHAR-T cells in ABMR treatment because our kidney transplant recipients receive immunosuppressive therapies. Importantly, the cytotoxic properties were not affected by IS at the therapeutic range, although several

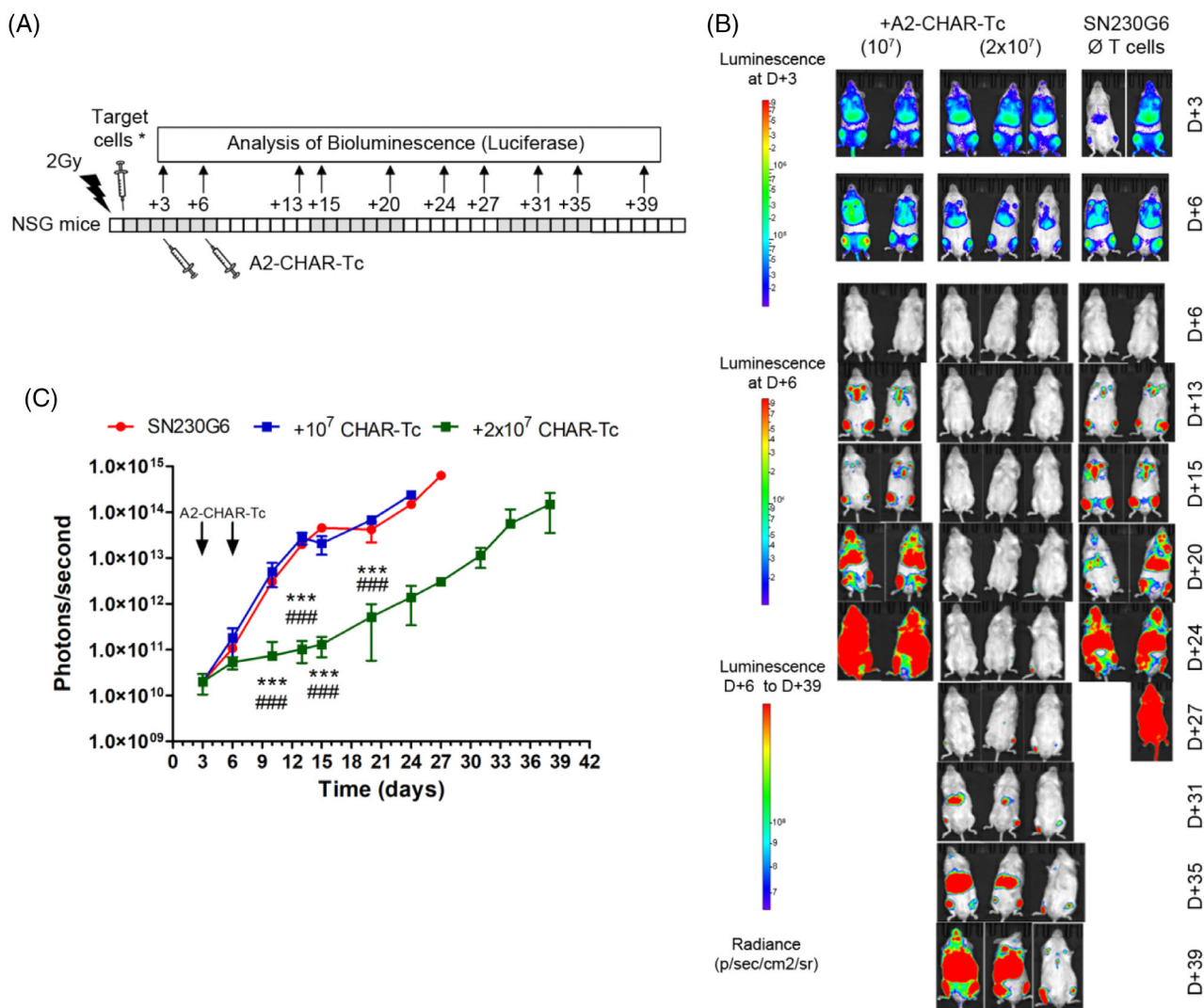


FIGURE 8 Cytotoxic activity of A2-CHAR human T cells in a multiple-dose assay in vivo. (A) Scheme of the in vivo cytotoxic assay. NSG mice were irradiated at D-1, then SN230G6 cells were infused at D0. T-cell therapy was administered twice, at D + 3 and D + 6. Three treatments were analyzed; without T cells (Ø, physiologic serum was administered), A2-CHAR-T cells 10⁷ cells per infusion, or 2 × 10⁷ cells per infusion. (B) Bioluminescence pictures obtained with the IVIS-Lumina device. (C) Quantification of SN230G6 target cell expansion by bioluminescence due to IVIS-Lumina device. Arrows indicate the time points when A2-CHAR-T cells were administered. Statistical differences compared to NSG mice without T cells, ****p* < 0.001. Statistical differences compared to NSG mice treated with 10⁷ A2-CHAR-T cells, ###*p* < 0.001.

interleukins and cytokines were completely down-regulated, specifically IL-2 and IFN γ . Both cytokines are under the control of the nuclear factor of activated T-cells (NFAT) transcription factor, which is completely repressed by the calcineurin inhibitor TAC.^{41,42} Steroids block T-cell cytokine expression, inhibit transcription of cytokine genes and, subsequently, decrease serum levels of IL-1, IL-2, IL-3, IL-6, TNF α , and IFN γ .⁴³ These observations open the possibility of exploring the use of other cell populations which could be less affected by the calcineurin inhibitors, such as NK cells.^{44,45}

The presence of circulating anti-HLA-A2 DSAs in sensitized patients could activate A2-CHAR-T cells

without any cell-to-cell interaction with the antibody-producing B cells, which would reduce the efficacy of the therapy or be potentially harmful. However, several well-known techniques could help us to solve this problem. Currently, plasma exchange and immunoadsorption are used in SOT to eliminate DSA either in desensitization protocols or during ABMR episodes.^{17,46} We consider that plasma exchange therapy should be performed before the application of A2-CHAR-T cell therapy to avoid unwanted side effects in the desensitization setting.

The NSG mice model allowed us to demonstrate that A2-CHAR-T cells could find target cells distributed in different mouse compartments and activate the cytotoxic

machinery to eliminate them. However, the *in vivo* assays do not reflect the clinical scenarios because the experiments were performed in immunodeficient mice, where mature T, B, and NK cells are absent. In NSG mice, expansion of target cells is easily achieved, and on the other hand, the cytotoxic properties of A2-CHAR-T cells are not altered by immune cells. Moreover, these experiments do not pretend to simulate the situation that could occur in a potential infusion in a human recipient. The number of target cells and the timing of their appearance will be completely different. Therefore, the doses of A2-CHAR-T cells and the infusion time necessary to eliminate the target cells in this animal model cannot be extrapolated to a future clinical trial. Interestingly, the number of target cells used in our approach was similar to those used in oncology.⁴⁷ However, in SOT, the number of antibody-producing B cells will be much lower, and the proliferation rate of these cells is also very likely to be lower than that of tumor cells. Additional experimental *in vivo* models, including immunocompetent or humanized mice, would be necessary to study whether A2-CHAR-Tc therapy is effective in a functional immune system.

Even though the specificity of A2-CHAR-T cells has been proved in this paper, since these cells cannot kill B cells that do not express specific anti-HLA antibodies, it is not known how A2-CHAR-T cells would behave in a human recipient. In addition, we cannot exclude potential off-target effects as the HLA Class I molecule expressed in their cell membrane could be the natural ligand of some other molecules, different from the specific anti-HLA antibodies. For instance, LILRB molecules have been described as such ligands, and their binding to the HLA class I molecules could lead to unexpected and unwanted cell killing.⁴⁸ On the other hand, A2-CHAR-T cells could also recognize alloreactive T cells that express a specific TCR able to bind the *HLA-A*02* molecule through a direct presentation. If this were the case, A2-CHAR-T cells could eliminate anti-HLA antibody-producing B cells and impair the T-cell alloresponse.

Patients with broad HLA sensitization have poor access to donor organs, high mortality while waiting for a solid organ transplant, and inferior graft survival after receiving an organ through regular allocation. Although current desensitization strategies permit the reduction of the impact of DSA, the B cell–response axis from germinal center activation to plasma cell differentiation remains intact. The selective elimination of alloreactive B cells that produce DSA could be a new personalized HLA desensitization strategy without increasing infectious adverse events. In addition, the appearance of *de novo* DSA after transplantation and the development of ABMR could be another indication for our A2-CHAR-T cell

therapy. Selectively eliminating alloreactive DSA B cells could improve graft survival without side effects, such as viral infections that compromise patient survival. Nevertheless, the low levels of expression of BCR in plasma cells raise doubt about the effectiveness of this therapy in eliminating these cells.

To our knowledge, this is the first study elucidating the cytotoxic effects of CHAR-T cells on antibody-producing cells, thus directly reducing the capacity of anti-HLA antibody synthesis. Further studies have to be performed to develop CHAR-T cells against alloreactive B cells that produce class II HLA antibodies, which are associated with long-term unfavorable outcomes in the solid organ transplant setting.⁴⁹

In conclusion, these findings demonstrate the efficacy of A2-CHAR-T cells against alloreactive B cells. The development of a chimeric HLA-antibody receptor library may represent an innovative therapeutic strategy to reach a personalized medicine capable of desensitizing patients with broad HLA sensitization and even treat ABMR episodes. In addition, this approach could avoid the risks of general IS.

AUTHOR CONTRIBUTIONS

Sergi Betriu and Jordi Rovira designed and performed experiments and analyzed data. Caroli Arana, Ainhoa García-Busquets, Marina Matilla-Martinez, Ariadna Bartoló-Ibars, Maria J. Ramirez-Bajo, Elisenda Bañon-Maneus, and Marta Lazo-Rodriguez performed experiments and analyzed data. Jordi Rovira and Sergi Betriu wrote the manuscript. Elisenda Bañon-Maneus, Marta Lazo-Rodriguez, Manel Juan, Beatriu Bayés-Genís, Josep M. Campistol, Eduard Palou, and Fritz Diekmann critically reviewed it. Frans H. J. Claas, Arend Mulder, and Sebastiaan Heidt contributed by providing SN230G6 and ROU2D3 hybridomas; both target cells have been essential for the study's development and contributed to the critical review of the manuscript. Beatriu Bayés-Genís, Josep M. Campistol, Eduard Palou, and Fritz Diekmann secured funding. Eduard Palou and Fritz Diekmann conceived of and directed the research and had overall oversight over the manuscript. The first authors have been determined alphabetically.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data are included in the manuscript and/or supporting materials.

ORCID

Sergi Betriu  <https://orcid.org/0000-0002-4199-1168>

Jordi Rovira  <https://orcid.org/0000-0001-8737-5005>

Marina Matilla-Martinez  <https://orcid.org/0000-0002-6970-0158>

Maria J. Ramirez-Bajo  <https://orcid.org/0000-0003-0119-1331>

Sebastiaan Heidt  <https://orcid.org/0000-0002-6700-188X>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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