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Chimeric HLA antibody receptor T cells to target HLA-specific B cells in solid organ transplantation

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HLA-sensitized patients on the transplant waiting list harbor antibodies and memory B cells directed against allogeneic HLA molecules, which decreases the chance to receive a compatible donor organ. Current desensitization strategies non-specifically target circulating antibodies and B cells, warranting the development of therapies that specifically affect HLA-directed humoral immune responses. We developed Chimeric HLA Antibody Receptor (CHAR) constructs comprising the extracellular part of HLA-A2 or HLA-A3 coupled to CD28-CD3ζ domains. CHAR-transduced cells expressing reporter constructs encoding T-cell activation markers, and CHAR-transduced $CD8^+$ T cells from healthy donors were stimulated with HLA-specific monoclonal antibody-coated microbeads, and HLA-specific B cell hybridomas. CHAR T cell activation was measured by upregulation of T cell activation markers and IFNγ secretion, whereas CHAR T cell killing of B cell hybridomas was assessed in chromium release assays and by IgG ELISpot. HLA-A2- and HLA-A3-CHAR expressing cells were specifically activated by HLA-A2- and HLA-A3-specific monoclonal antibodies, either soluble or coated on microbeads, as shown by CHAR-induced transcription factors. HLA-A2 and HLA-A3 CHAR T cells efficiently produced IFNγ with exquisite specificity and were capable of specifically lysing hybridoma cells expressing HLA-A2- or HLA-A3-specific B-cell receptors, respectively. Finally, we mutated the α3 domain of the CHAR molecules to minimize any alloreactive T-cell reactivity against CHAR T cells, while retaining CHAR activity. These data show proof of principle for CHAR T cells to serve as precision immunotherapy to specifically desensitize (highly)

Abbreviations: ABMR, antibody-mediated rejection; AM, acceptable mismatch; BAR, B-cell antibody receptor; BCR, B-cell receptor; CAAR, chimeric autoantibody receptor; CAR, Chimeric Antigen Receptor; CHAR, Chimeric HLA Antibody Receptor; DSA, donor-specific antibodies; Dsg, desmoglein; FVIII, Factor VIII; Ides, imlifidase; Ig, immunoglobulin; IRES, internal ribosome entry site; IVIg, intravenous immunoglobulin; KO, knock-out; mAbs, monoclonal antibodies; NGFR, nerve growth factor receptor; PBMC, peripheral blood mononuclear cell; PV, Pemphigus Vulgaris; scFv, single-chain variable fragment; SLE, systemic lupus erythematosus; TCR, T-cell receptor; TPR, Triple Parameter Reporter; Tregs, regulatory T cells; β2m, beta-2 microglobulin.

Mirjam H.M. Heemskerk and Sebastiaan Heidt shared senior authorship.

sensitized solid organ transplant candidates and to treat antibody-mediated rejection after solid organ transplantation.

KEYWORDS
cellular therapy, CHAR T cells, sensitization, solid organ transplantation

1 | INTRODUCTION

Transplantation is the treatment of choice for patients with end-stage organ failure. Unfortunately, the transplant waiting list includes an increasing number of patients that are sensitized against HLA. These patients harbor antibodies and memory B cells directed against allogeneic HLA due to immunizing events such as previous organ transplantation, blood transfusions or pregnancy.[1,2] HLA sensitization diminishes the chance of receiving a compatible organ offer,[3] with highly sensitized patients being disadvantaged the most.[4]

Several special allocation programs aim to increase the chance for highly sensitized patients to receive a compatible organ transplant, such as acceptable mismatch (AM) programs. These programs are very successful in transplanting highly sensitized patients with excellent long-term graft survival.[5–7] Nevertheless, a subset of highly sensitized patients can still not be transplanted within a reasonable time frame.[5] For these patients, desensitization strategies may be an option to facilitate a crossmatch negative transplant. Current desensitization strategies typically consist of intravenous immunoglobulin (IVIg) or low-dose IVIg in combination with plasmapheresis, or anti-CD20 monoclonal antibody (mAb) Rituximab. Recently, imlifidase (IdeS), was approved by the European Medicines Agency (EMA) for the desensitization of highly sensitized kidney transplant patients with a positive crossmatch against an available deceased donor. Imlifidase non-specifically cleaves all circulating IgG by enzymatic activity.[8] Thus far, all desensitization methods lack specificity as all circulating antibodies and/or B cells are affected, regardless of their target.

In line with this, Chimeric Antigen Receptor (CAR) T cells directed towards CD19 have proven to be highly effective in depleting all circulating B cells for the treatment of hematological malignancies.[9–11] CAR T cells recognize antigens via a single-chain variable fragment (scFv) domain, which is a fusion protein of the variable regions of the heavy and light chain of a specific immunoglobulin (Ig). The extracellular CAR domain is coupled to a costimulatory domain, such as CD28 or 4-1BB, and the intracellular signaling domain CD3ζ, allowing for T-cell activation. CD19 CAR T-cell products were the first CAR T-cell therapies to be authorized by the FDA for the treatment of B-cell acute lymphoblastic leukemia and B-cell lymphoma.[9,11] Since then, tumor-specific CAR T-cell therapies have been developed for targeting various tumor types.[12] In hematological malignancies, as well as autoimmune diseases CAR T cell therapy showed to be more effective than treatment with mAbs.

In the transplantation setting CAR regulatory T cells (Tregs) have been developed with an HLA-A2 scFv domain that binds HLA-A2 expressed on allogeneic cells, thereby directing the Tregs to the allograft. MacDonalds and colleagues were the first to successfully redirect Tregs towards HLA-A2 using CARs in a transplantation setting.[13] HLA-A2 CAR Tregs demonstrated specific migration towards allografts and suppression of alloreactive immune cells to ultimately reduce transplant rejection.[13–19] Since then, the first phase I/IIa multicenter study with HLA-A2 CAR Tregs started with the recruitment of kidney transplant recipients (NCT04817774).

CAR T cells have also been developed aiming to treat autoimmune diseases. Ellebrecht and colleagues repurposed CAR T cells for the treatment of the autoimmune disease Pemphigus Vulgaris (PV).[20] By engineering a chimeric autoantibody receptor (CAAR), B cells expressing anti-desmoglein (Dsg) 3 B-cell receptors (BCRs), responsible for the autoimmune response, could be eliminated. The CAAR consisted of the PV autoantigen, Dsg3 coupled to CD137-CD3ζ signaling domains, thus expressing the autoimmune target antigen instead of the classical scFv domain. A similar approach has been investigated for the treatment of Hemophilia A by eliminating Factor VIII (FVIII)-specific B cells.[21] These B-cell antibody receptor (BAR) T cells were able to eradicate FVIII-reactive B-cell hybridomas in vitro and in vivo.

Due to the pre-existing HLA-directed alloimmunity, it is unlikely that CAR Tregs will allow for HLA-incompatible transplants to be performed.[16] Therefore, analogous to CAAR T cells generated in the setting of autoimmune diseases, we developed Chimeric HLA Antibody Receptor (CHAR) T cells to specifically target and eliminate HLA-directed B cells. We hypothesize that CHAR T cells could serve as personalized precision immunotherapy to desensitize (highly) sensitized kidney transplant candidates and to treat humoral rejection after transplantation.

2 | METHODS

2.1 | Construct design and retroviral production

Retroviral vectors encoding CHAR molecules comprised the sequence of the extracellular part (the α1, α2, and α3 domain) of an HLA-A*02:01:01:02 or HLA-A*03:01:01:01 molecule coupled to the CD28 transmembrane and intracellular region, and CD3ζ intracellular signaling domains, coupled to an internal ribosome entry site (IRES) allowing for co-expression of nerve growth factor receptor (NGFR). As a control, a vector containing NGFR only was used. Phoenix-AMPHO (ATCC, CRL-3213) or Phoenix-GALV[22] were transiently transfected with different constructs using Fugene HD Transfection Reagent (Promega) and after 48 h retroviral supernatants were harvested and stored at −80°C.

2.2 | Generation of CHAR-expressing cells

For retroviral transduction, 24-well flat-bottom culture plates (Greiner Bio-One) were coated with 30 μg/mL retronectin (Takara) and blocked with 2% HSA (Sanquin). Retroviral supernatants were added and centrifuged at 3000*g* for 20 min at 4°C. After removal of the retroviral supernatant, cells were transferred to the virus-coated wells. After overnight incubation, cells were transferred to 24-well flat-bottom plates (Costar). Seven days after stimulation with autologous feeders irradiated at 35 Gy, supplemented with phytohaemagglutinin (PHA, Oxoid Microbiology Products, Thermo Fisher Scientific), transduced cells were MACS enriched for the gene marker NGFR, using an NGFR-APC antibody (Sanbio, clone ME20.4) and anti-APC MicroBeads (Milteny, clone BW135/80). The allo-HLA-reactive TCR derived from the previously described HSS12 T-cell clone recognizing the epitope USP11-FTW (RefSeq ID: NM_004651) presented in HLA-A*02:01,[23] was transduced in a similar manner.

2.3 | Human CD8 T-cell isolation and cell culture

HLA-A2 and HLA-A3 negative healthy donors were selected from the biobank of the Leiden University Medical Center Department of Hematology (HEM 008/SH/sh). PBMCs were isolated by standard Ficoll Isopaque separation and cryopreserved. HLA typing data is described in Supplementary Table 1.

PBMCs were thawed and enriched for $CD8^+$ T cells by positive selection using CD8 Microbeads (Miltenyi) according to the manufacturer's instructions. $CD8^+$ T cells were stimulated using autologous feeders irradiated at 35 Gy, supplemented with phytohaemagglutinin (PHA, Oxoid Microbiology Products, Thermo Fisher Scientific). T cells were cultured in IMDM (Lonza) containing 5% heat-inactivated FBS (Lonza), 5% heat-inactivated human serum (ABOS, Sanquin), 100 U/mL penicillin, 100 μg/mL streptavidin, 2.7 mM L-glutamine (Lonza), and 100 IU/mL IL-2 (Chiron).

2.4 | Cell lines and cell culture

K-562 (ATCC, CCL-243™), Jurkat clone E6-1 (ATCC, TIB-152™) and Jurkat E6-1 Triple Parameter Reporter (TPR) cells were cultured in IMDM containing 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptavidin, 2.7 mM L-glutamine (all from Lonza).

Human HLA-specific B cell hetero-hybridomas derived from pregnancy-immunized individuals were sorted for IgG expression using rat-α-human IgG-FITC (Dako, clone F0315) and used as targets for CHAR-expressing cells. Hybridoma SN607D8 secretes and expresses anti-HLA-A2/HLA-A28 mAbs, recognizing eplet 144TKH, and BRO11F6 HLA-A11/HLA-A3-specific mAbs, recognizing eplet 144TKR.[24] Hybridomas were cultured in IMDM containing 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptavidin, and 2.7 mM L-glutamine (all from Lonza). K-562 cells were knocked out for β2m using CRISPR-Cas9 techniques to investigate the role of β2m in CHAR cell surface expression. Knockout was performed using CRISPR-Cas9 similarly as previously described.[25] CHAR molecules were mutated in the α3 domain (D227K/T228A) to abrogate binding to the CD8 co-receptor.[26] Mutations were made to the original HLA-A2 CHAR constructs using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs [NEB]). All cell lines were tested for mycoplasma contamination on a regular basis.

2.5 | Generation of antibody-coated beads

Dynabeads M-450 Epoxy (Invitrogen) were washed in 0.1% BSA in PBS with 2 mM EDTA, followed by incubation with 200 ug/ml purified mAb overnight on a roller bench at room temperature. Human HLA-specific mAbs generated from human B cell hybridomas SN607D8, SN203G6, BRO11F6, and MUL2C6[27,28] were used for coating. Beads were washed thrice and stored in 0.02% Natriumazide at

4°C. Before use, beads were washed twice in 1% HSA/PBS and diluted accordingly. Successful mAb coating was confirmed by flow cytometry with HLA-specific PE-labeled pMHC-tetramers and/or APC-coupled IgG antibody (Jackson ImmunoResearch, clone 109-136-170).

2.6 | Flow cytometry

Cells were washed in PBS-GPO and stained with fluorochrome-conjugated antibodies for 20–30 min at 4°C. The purity of CD8 isolated fractions was checked with PE-labeled CD8β (Beckman Coulter, clone 2ST8.5H7) and FITC-coupled CD4 (BD/Pharmingen, clone RPA-T4). Cell surface expression of CHAR molecules was measured by FITC-coupled HLA-A2 (BD/Pharmingen, clone BB7.2) and PE-labeled HLA-A3 (BD, clone GAP.A3) specific antibodies. APC-labeled NGFR (Sanbio, clone ME20.4) was used to determine the transduction efficiency and purity of transduced cells. Cells were washed and fixed with 1% paraformaldehyde before acquiring them using LSRII or Fortessa flow cytometer instruments (BD) and were analyzed using FlowJo software (Tree star). Appropriate controls were included to verify antibody specificity. K-562 CHAR cells were gated on NGFR+ for the HLA-specific staining. CHAR-transduced Jurkat TPR cells were gated on NGFR-negative for cells transduced with TCRs or NGFR-positive fractions for cells transduced with CHARs.

2.7 | IFNγ ELISA

IFNγ secretion was quantified using an ELISA (Diaclone). Supernatants were collected after overnight cocultures of CHAR T cells and hybridoma cells, beads coated with mAbs, or soluble mAbs and diluted 1:5 and 1:125, to quantify IFNy production. In short, high-binding plates were coated with capture antibody overnight. Plates were blocked using 10%BSA for 2 h at room temperature. Supernatant and biotinylated detection antibodies were added for 2 h at room temperature. Next, streptavidin labeled HRP was added for 30 min at room temperature. Finally, a substrate consisting of 6 mg/mL Tetramethylbenzidine (TMB), 3% H_2O_2 was added and 2 M H_2SO_4 was added. The absorbance was measured at 450 nm using a microplate reader (Thermo Electron).

2.8 | Chromium release assay

Cytotoxicity was determined by 51-chromium (^{51}Cr)-release assays. Hybridoma target cells were labeled with 100 μCi ^{51}Cr for 1 h at 37°C. Cells were washed and cocultured with HLA-A2 CHAR, HLA-A3 CHAR or mock transduced T cells at different E: T ratios (9:1–0.3:1) in triplicate. Spontaneous ^{51}Cr release of the target cells was determined in culture medium alone, and maximum ^{51}Cr release was determined by adding 1% Triton-X100 (Sigma-Aldrich). Supernatants were harvested after 6 h and transferred to Lumaplates (Perkin Elmer). The amount of released ^{51}Cr was measured on a Microbeta counter (Perkin Elmer). The percentage of specific lysis was calculated as ((experimental ^{51}Cr release − average spontaneous ^{51}Cr release)/(average maximal ^{51}Cr release − average spontaneous ^{51}Cr release)) × 100.

2.9 | IgG ELISpot

ELISpot plates (Merck) were coated with 5 ug/mL goat-α-human IgG (Jackson ImmunoResearch, clone 109-005-098) overnight at 4°C. Plates were blocked using culture medium for 1 h at 37°C. HLA-A2, HLA-A3 CHAR or mock T cells were cocultured with hybridoma cell lines for 16–24 h at various E: T ratios. Cells were washed to remove secreted IgG and transferred to the coated ELISpot plates and incubated overnight at 37°C. Plates were extensively washed in 0.05% Tween/PBS (Braun) between each incubation step. Goat-α-human IgG-biotin (Invitrogen, clone A224480) in 1%BSA/0.025%Tween/PBS was added for 2 h at room temperature. Strept-avidine ALP (Sigma-Aldrich, E2636.2ML) in 0.1%Tween20/PBS was added for 1 h at room temperature. Finally, filtered BCIP/NBT Plus (Mabtech) was added for 5 min at room temperature and the reaction was stopped by the addition of tap water. The amount of IgG spots were quantified using a BioSys reader. Spots >70 μm were measured and the number of IgG spots per well was determined as a percentage of the number of spots when incubated with NGFR-mock T cells.

2.10 | Statistical analysis

Statistical analysis was performed using GraphPad Prism software (V.9.3.1). The statistical test used for each experiment is indicated in each figure legend, and $p < 0.05$ was considered significant. The Mann–Whitney test was used to determine the significance of unpaired data collected from different experiments and/or different donor T cells. The Wilcoxon test was used to determine the significance of paired data. Significance levels are indicated

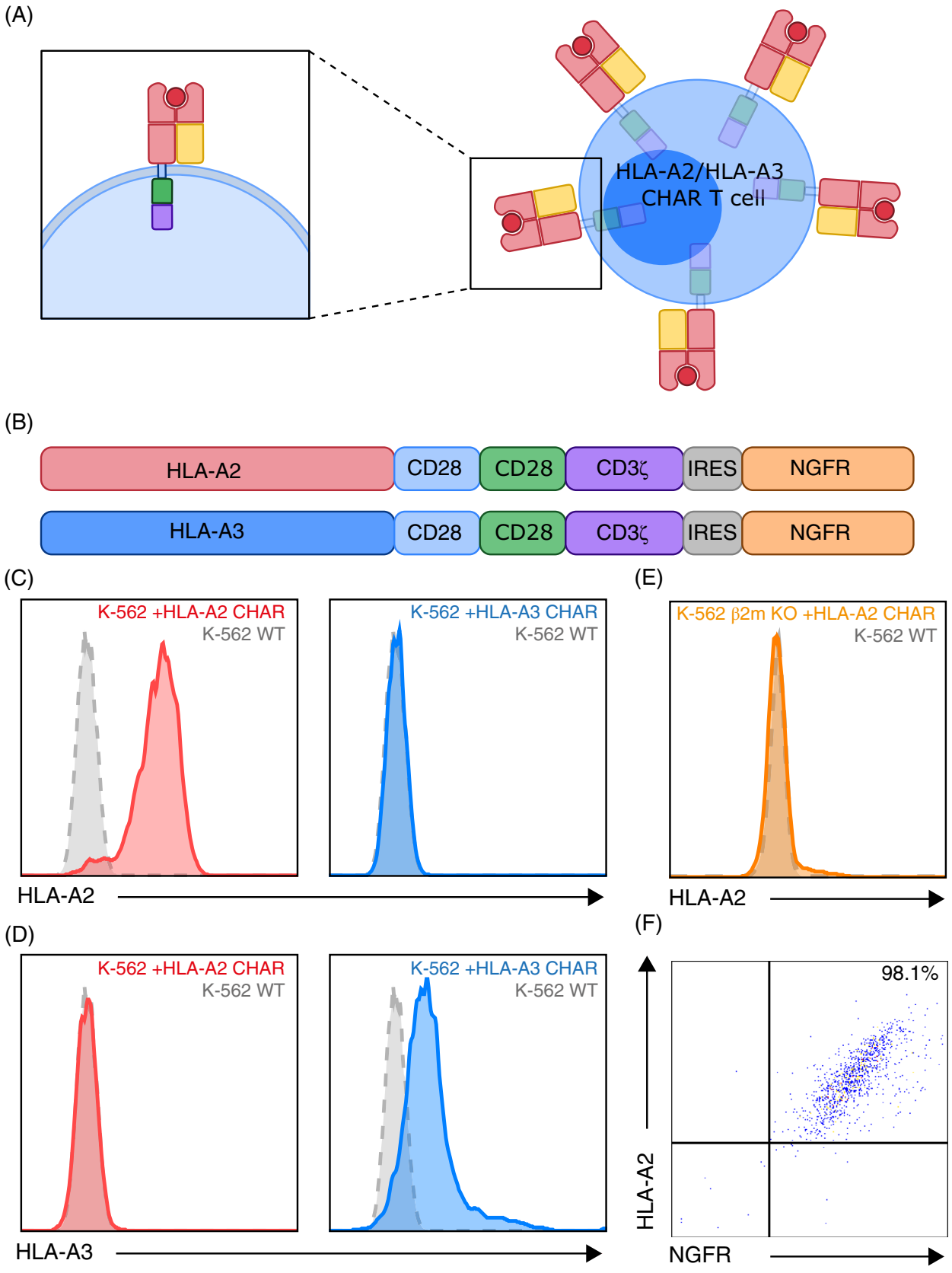

FIGURE 1 Legend on next page.

as $*p < 0.05$ and $**p < 0.01$. Data represent mean ± SD, or minimum to maximum, showing all data points.

2.11 | Study approval

Samples of healthy donors were used from the LUMC Biobank for Hematological Diseases, after approval by the Institutional Review Board of the LUMC, Leiden, the Netherlands (approval number 3.4205/010/FB/jr) and the METC-LDD (approval number HEM 008/SH/sh). Informed consent was obtained in adherence to the Declaration of Helsinki.

3 | RESULTS

3.1 | Generation of HLA-A2 and HLA-A3 CHAR T cells

We constructed CHAR molecules of two common HLA alleles, namely HLA-A*02:01 and HLA-A*03:01, that could additionally serve as specificity controls for each other (Figure 1A). Retroviral vectors encoding CHAR molecules were constructed comprising the sequence of the extracellular part of HLA-A*02:01 or HLA-A*03:01 coupled to CD28 costimulatory and CD3ζ intracellular signaling domains, and an IRES allowing for the co-

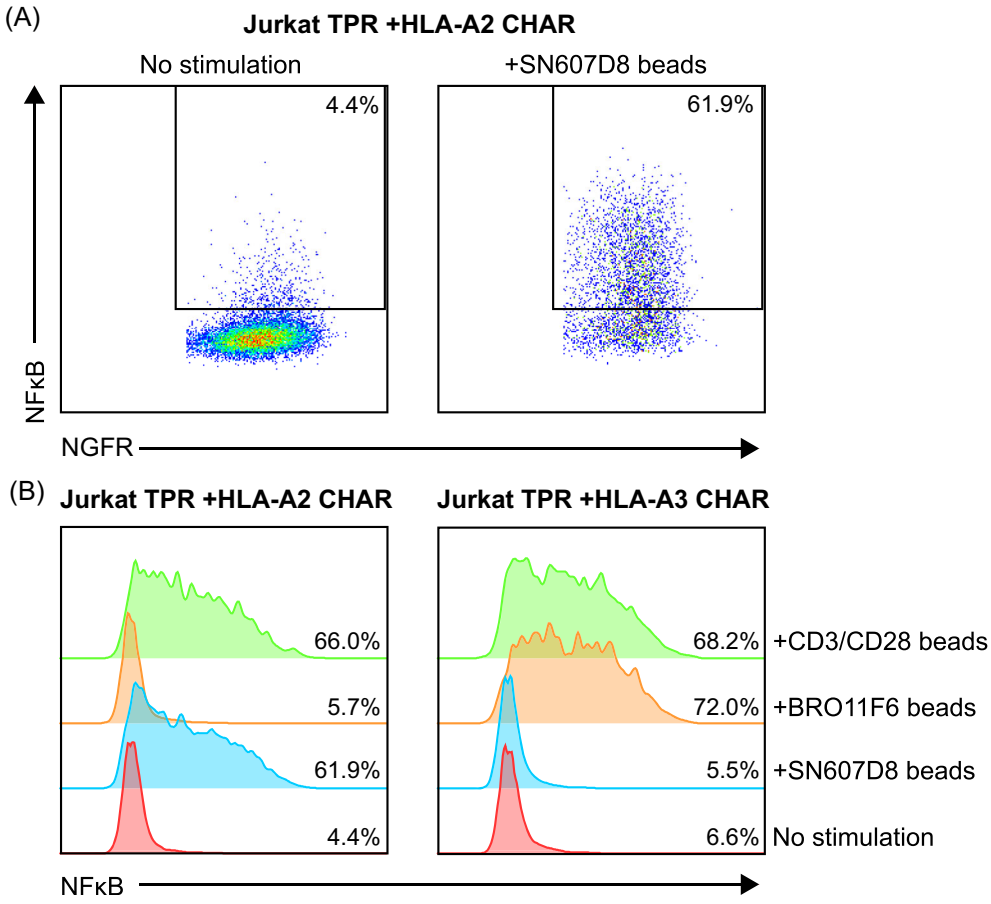

FIGURE 2 Signal transduction upon specific ligation of CHAR molecules. (A) Stimulation with αHLA-A2 SN607D8 mAb-coated beads results in NFκB expression in Jurkat TPR cells transduced with an HLA-A2 CHAR, at 1:1 effector: target ratio. (B) Representative figure of the effect of beads coated with HLA-A2 antibodies SN607D8 (blue), HLA-A3 antibodies BRO11F6 (orange), or αCD3/CD28 beads (green) on NFκB upregulation in Jurkat TPR cells transduced with HLA-A2 CHAR (left), or HLA-A3 CHAR (right). The negative control without stimulation is depicted in red.

FIGURE 1 CHAR molecules are successfully expressed on the cell surface. (A) Graphical representation of a T cell expressing an HLA-A2 or HLA-A3 CHAR. (B) Schematic overview of the HLA-A2 and HLA-A3 CHAR constructs, consisting of sequences of the extracellular part (α1, α2 and α3 domain) of an HLA-A*02 or HLA-A*03 molecule coupled to the CD28 costimulatory transmembrane and intracellular region, and CD3ζ intracellular signaling domains, coupled to IRES allowing for co-expression of NGFR. (C) CHAR cell surface expression of K-562 WT (gray, dashed line), K-562 + HLA-A2 CHAR (red, solid line, left panel) and K-562 + HLA-A3 CHAR (blue, solid line, right panel) measured by flow cytometry with an HLA-A2-specific antibody, and (D) HLA-A3-specific antibody. (E) Absence of CHAR cell surface expression of K-562 WT (gray, dashed line) and K-562 β2m KO + HLA-A2 CHAR (orange, solid line) shown using a fluorescently labeled HLA-A2 antibody. (F) Co-expression of the HLA-A2 CHAR and NGFR on HLA-A2-transduced Jurkat TPR cells. IRES: internal ribosome entry site; NGFR: nerve growth factor receptor.

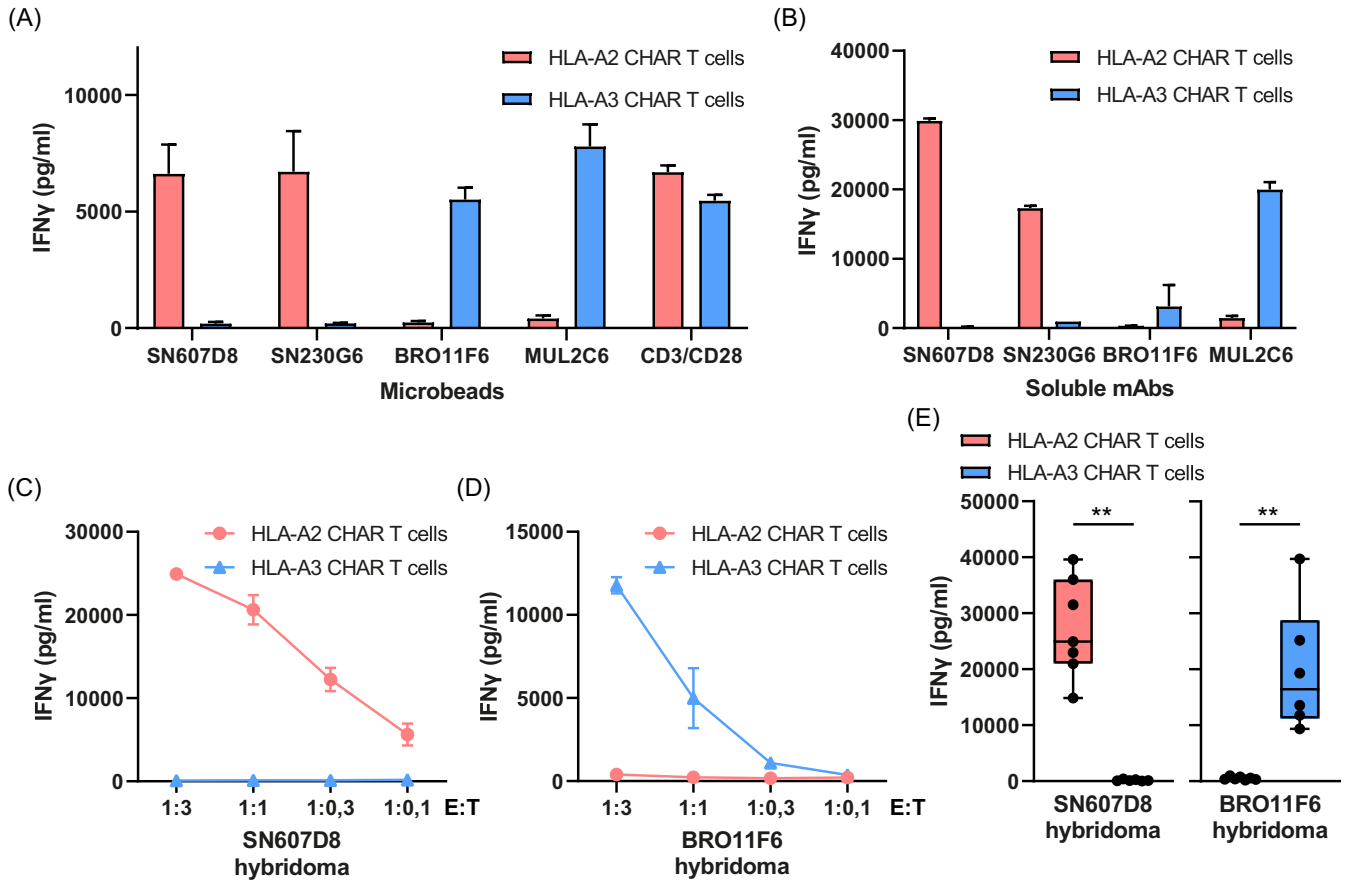

FIGURE 3 Primary CD8$^+$ CHAR T cells produce IFNγ upon HLA-specific stimulation. (A) IFNγ release by HLA-A2 CHAR (pink) or HLA-A3 CHAR (blue) T cells when incubated with beads coated with different antibodies at a 1:1 effector: target ratio, or (B) soluble antibodies. (C) IFNγ release by HLA-A2 CHAR (pink dots) or HLA-A3 CHAR (blue triangles) T cells when incubated with HLA-A2 specific hybridoma SN607D8, or (D) HLA-A3 specific hybridoma BRO11F6 at different E: T ratios. Repeated measurements show reproducibility, as the effect is consistently observed in multiple donors. (E) Overview of IFNγ release by T cells from different donors (represented by each dot) transduced with an HLA-A2 CHAR (pink), or HLA-A3 CHAR (blue) after co-culture with SN607D8 (left panel), and BRO11F6 (right panel) hybridomas. Significance was calculated using the Mann–Whitney test, $p = 0.001$ for both graphs (HLA-A2 CHAR: $n = 7$, HLA-A3 CHAR: $n = 6$).

expression of NGFR as a gene marker (Figure 1B,F). K-562 cells were retrovirally transduced with either HLA-A2 or HLA-A3 CHAR-containing vectors after which staining with fluorescently labeled HLA-A2 or HLA-A3 mAbs showed successful HLA-A2 and HLA-A3-CHAR expression, respectively (Figure 1C,D). Endogenous beta-2 microglobulin (β2m) is required for CHAR cell surface expression, as shown by the absence of CHAR expression in K-562 β2m knock-out (KO) cells (Figure 1E).

3.2 | CHARs transduce signals into the cell after HLA-specific stimulation

Jurkat Triple Parameter Reporter (TPR) cells allow simultaneous detection of intracellular T-cell activation markers. Response elements for NFκB, NFAT and AP1 drive the expression of the fluorescent proteins CFP, eGFP and mCherry, respectively, and were measured using flow cytometry. HLA-A2 CHAR-transduced Jurkat TPR cells showed NFκB, NFAT and AP1 expression specifically upon stimulation with microbeads coated with HLA-A2-specific SN607D8 mAbs (Figure 2A,B, Supplemental Figure 1A,B). In contrast, HLA-A3 CHARs only induced T-cell activation transcription factors when exposed to αHLA-A3 BRO11F6 beads, and not to αHLA-A2 SN607D8 beads (Figure 2B, Supplemental Figure 1A,B).

3.3 | Primary CD8$^+$ CHAR T cells produce IFNγ upon specific stimulation

Primary CD8$^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) obtained from HLA-

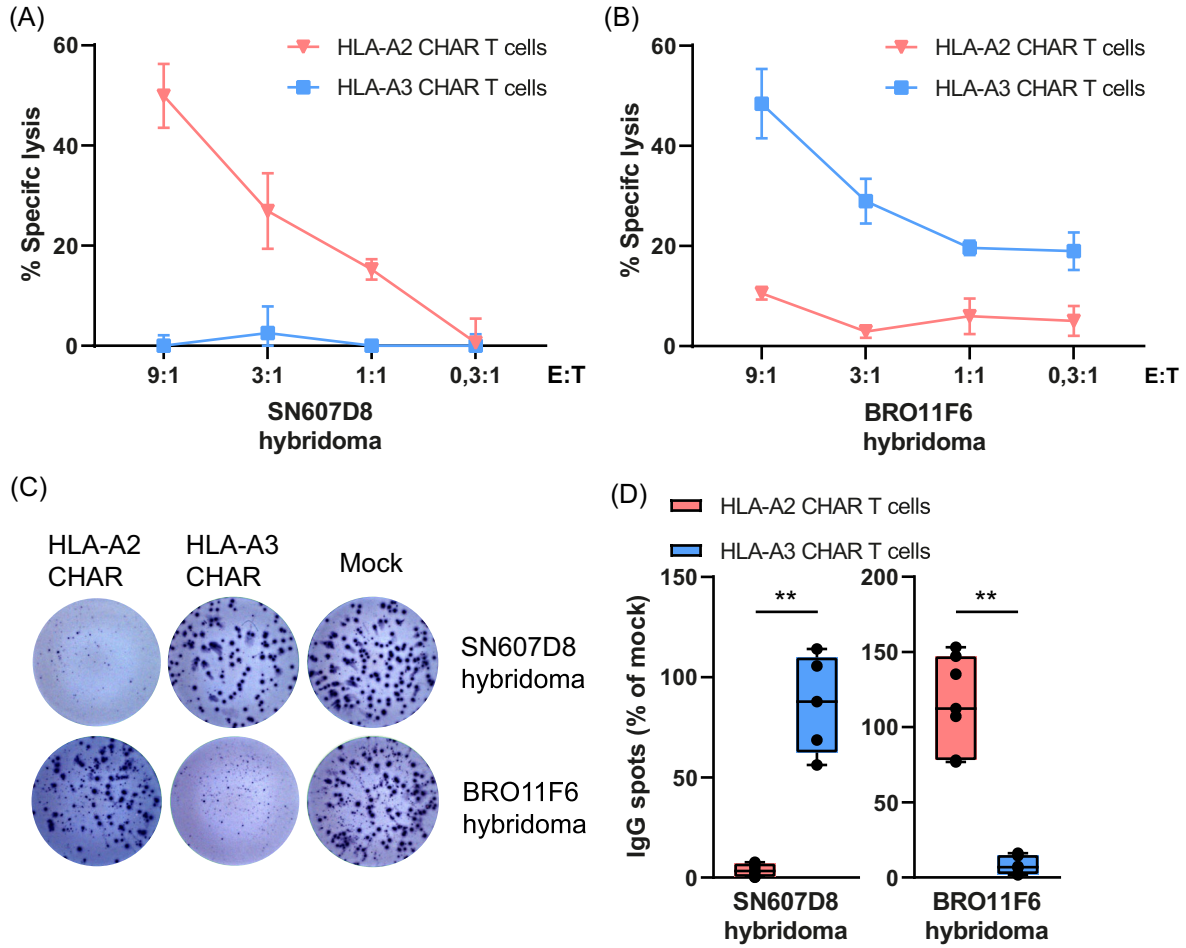

FIGURE 4 Specific lysis of HLA-specific hybridoma cells after incubation with CHAR T cells. (A) Representative figure ($n = 4$) of the percentage of specific lysis by HLA-A2 CHAR T cells (pink triangles) or HLA-A3 CHAR T cells (blue squares) of HLA-A2-specific hybridoma SN607D8, and (B) HLA-A3 specific hybridoma BRO11F6 at different effector: target (E: T) ratios. (C) Representative wells of spots produced by SN607D8 (top row) or BRO11F6 (bottom row) hybridomas when incubated with HLA-A2, HLA-A3 CHAR or mock-NGFR T cells. Spots >70um were measured. (D) Overview of the number of spots produced by SN607D8 hybridomas (left panel) or BRO11F6 hybridomas (right panel) after incubation with T cells derived from different donors (represented by each dot) expressing either HLA-A2 (pink) or HLA-A3 CHARs (blue) at E: T ratio 10:1. Data was corrected for technical differences and donor-specific effects by calculating the number of spots per well as a percentage of the number of spots produced after incubation with the corresponding mock-NGFR T cells. Significance was calculated using the Mann–Whitney test, SN607D8 hybridoma: $p = 0.003$, (HLA-A2 CHAR: $n = 7$, HLA-A3 CHAR: $n = 5$) and BRO11F7 hybridoma: $p = 0.001$ (HLA-A2 CHAR: $n = 7$, HLA-A3 CHAR: $n = 6$).

A2- and HLA-A3-negative healthy donors. These T cells were retrovirally transduced with the HLA-A2 CHAR, HLA-A3 CHAR, or mock-NGFR viral supernatant. Transduced cells were enriched for the gene marker NGFR resulting in >90% purity (Figure 1F). To assess whether CHAR T cells secrete IFNγ upon antigen-specific stimulation, $CD8^+$ CHAR T cells were incubated with microbeads coated with various HLA-specific mAbs. Two HLA-A2 wand two HLA-A3 mAbs, recognizing different epitopes on the same HLA molecule respectively, were tested for the induction of IFNγ release by CHAR T cells. HLA-A2 CHAR T cells secreted IFNγ upon incubation with either αHLA-A2 beads SN230G6 and SN607D8, and not with the αHLA-A3 beads BRO11F6 and MUL2C6 (Figure 3A). In contrast, HLA-A3 CHAR T cells produced IFNγ upon exposure to either αHLA-A3 beads, but not to αHLA-A2 beads (Figure 3A). In addition, soluble mAbs were added to CHAR T cells after which IFNγ secretion was determined. SN607D8 and SN230G6 mAbs induced IFNγ secretion by HLA-A2 CHAR T cells, whereas HLA-A3 CHAR T cells produced IFNγ upon HLA-A3-specific stimulation with BRO11F6 and MUL2C6 mAbs (Figure 3B).

Next, the parental hybridoma cell lines of SN607D8 and BRO11F6 were FACS sorted based on IgG surface expression and used as stimulator cells as a surrogate for HLA-specific B cells. HLA-A2 CHAR T cells specifically produced IFNγ when cocultured with the SN607D8

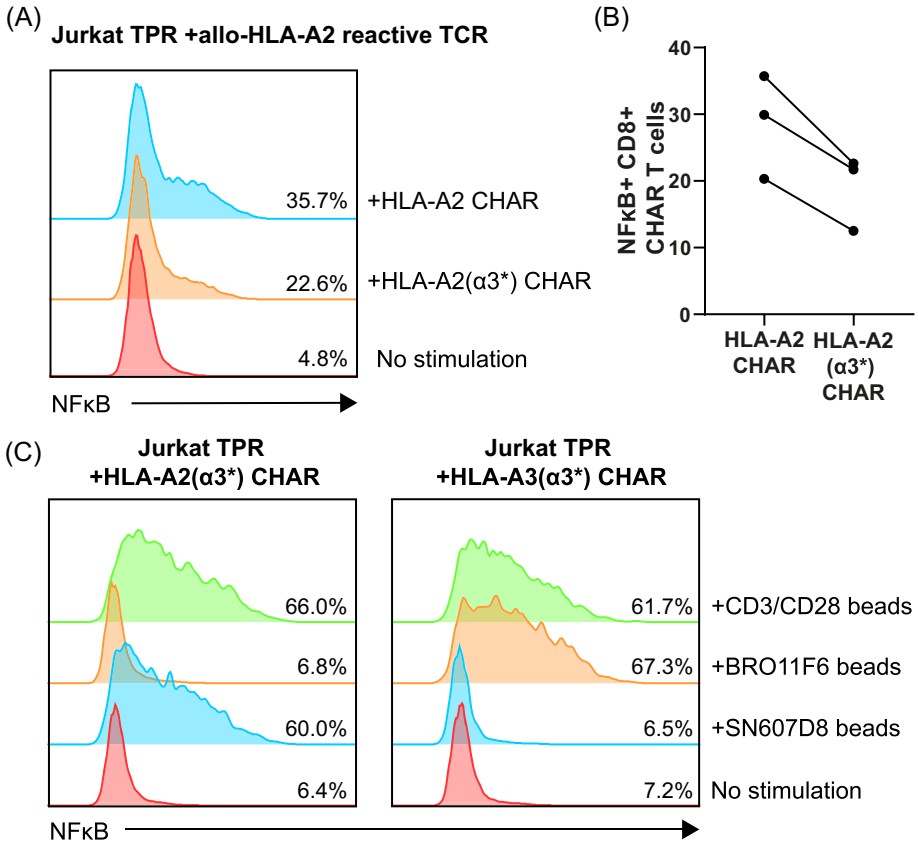

FIGURE 5 α3 mutated CHARs lead to decreased T-cell activation. (A) Representative ($n = 3$) NFκB upregulation by Jurkat TPR cells transduced with an alloreactive HLA-A2 TCR cocultured with Jurkat transduced with HLA-A2 CHAR (blue) or HLA-A2 CHAR with the α3 mutation (orange). (B) Overview of NFκB upregulation in Jurkat TPR cells transduced with an alloreactive HLA-A2 TCR after incubation with Jurkat cells transduced with HLA-A2 CHAR or the HLA-A2 (α3*) CHAR. (C) Representative figure of the effect of no stimulation (red), beads coated with HLA-A2 antibodies SN607D8 (blue), HLA-A3 antibodies BRO11F6 (orange), or αCD3/CD28 beads (green) on NFκB upregulation in Jurkat TPR cells transduced with HLA-A2(α3*) CHAR (left), or HLA-A3(α3*) CHAR (right).

hybridoma in a dose-dependent manner (Figure 3C). Similar effects were observed when incubating HLA-A3 CHAR T cells with the HLA-A3 specific hybridoma BRO11F6 (Figure 3D). The allotype-specific IFNγ release was significantly increased and highly reproducible in primary $CD8^+$ CHAR T cells derived from several donors (Figure 3E, $p = 0.001$).

3.4 | Primary $CD8^+$ CHAR T-cell cytotoxic capacity

To examine whether CHAR T cells have cytolytic activity towards HLA-specific BCR-expressing cells, chromium release assays were performed with HLA-A2 and HLA-A3 CHAR T cells and SN607D8 or BRO11F6 hybridoma cells at several effector: target (E: T) ratios. HLA-A2 and HLA-A3 CHAR T cells specifically lysed SN607D8 and BRO11F6 hybridomas, respectively, in a dose-dependent manner without any cytotoxic effect on the control hybridomas (Figure 4A,B). These results were corroborated by using ELISpot assays to quantify the number of IgG-producing hybridoma cells after incubation with CHAR T cells. The number of IgG spots was significantly decreased when HLA-A2 CHAR T cells were added to SN607D8 hybridomas, with no decrease when HLA-A3 CHAR T cells were added (Figure 4C,D). Similarly, HLA-A3 CHAR T cells reduced the number of IgG spots when cocultured with BRO11F6 hybridomas, whereas this was not observed for HLA-A2 CHAR T cells (Figure 4C,D). These data were highly reproducible as shown by the specific lysis of HLA-specific B cell hybridomas by primary $CD8^+$ CHAR T cells derived from different healthy donors (Figure 4D, SN607D8 hybridoma: $p = 0.003$, BRO11F7 hybridoma: $p = 0.001$).

3.5 | Allo-HLA-reactive T cell reactivity against CHAR T cells can be diminished

In vivo, alloreactive cytotoxic T cells of the recipient could potentially recognize and kill infused CHAR T cells, thereby reducing the potency of CHAR T-cell therapy. To investigate the effect of alloreactive T cells recognizing the HLA-A2 domain of the CHAR, we transduced Jurkat TPR cells with an HLA-A2 alloreactive T-cell receptor (TCR) specific for the household peptide USP11 presented in HLA-A*02:01.[23,29] TCR-transduced Jurkat TPR, upon stimulation with HLA-A2 CHAR Jurkat cells, upregulated NFκB, NFAT and AP1, similarly to stimulation with an HLA-A2 positive cell line (Figure 5A). In contrast, HLA-A2 CHAR T cells were not activated after co-culture with cells expressing the allo-HLA-A2-reactive TCR (Supplemental Figure 2B).

To reduce potential alloreactive T-cell reactivity against CHAR T cells we mutated the α3 domain of the extracellular part of the HLA-A2 CHAR molecule to abrogate CD8 binding, thereby reducing the affinity of the TCR complex with HLA class I.[30] Two mutations in the α3 domain (D227K/T228A) decreased the responsiveness of the allo-HLA-A2-reactive TCR to HLA-A2 CHAR T cells by 34.2% (Figure 5A,B). Importantly, the specificity of α3 mutated CHARs was retained and the CHAR T cells remained effective (Figure 5C).

4 | DISCUSSION

Sensitization against HLA poses a major barrier to organ transplantation. Around 30% of patients on the kidney transplant waiting list harbor antibodies against non-self HLA, with approximately 5% being highly sensitized.[4] Current desensitization strategies lack specificity, warranting the development of new strategies for targeting the HLA-specific humoral immune response. Here we introduce the concept of CHAR T cells as a novel approach to target HLA sensitization for (highly) sensitized patients on the transplant waiting list.

We set out to thoroughly characterize the CHAR molecules, their specificity and their functional capacity. As proof of concept, CHAR constructs were designed to contain the α1, α2 and α3 domains of HLA-A2 or HLA-A3, allowing for endogenous ß2m to form an HLA molecule in its native conformation. Indeed, by using ß2m KO cells, we could show the absolute requirement for ß2m to form a functional molecule. Our data on the HLA-A2 alloreactive TCR-transduced Jurkat cells further substantiate the formation of an intact HLA molecule with the correct conformation and containing a peptide, as this particular TCR only becomes activated by the complex of HLA-A2 containing the USP11 household peptide.

Since signal transduction from the CHAR molecules into the T cells is essential for their functionality, we made use of the Jurkat TPR system to formally address whether CHAR molecules could induce proper signaling. We observed a very strong NFκB signal upon specific ligation, with a somewhat weaker NFAT and AP1 response, which is in line with previously published data on Jurkat TPR PRAME-specific TCR transgenic cells incubated with PRAME-loaded JY-LCL cells.[31] Further functionality was shown by IFNγ secretion and cell lysis upon contact with BCR-expressing cells with exquisite specificity. We deemed it essential to use the HLA-A2 and HLA-A3 CHARs as their respective negative controls, as specificity is pivotal when infusing genetically modified cytotoxic T cells in a clinical setting.

It is conceivable that the binding of soluble HLA-specific antibodies to CHAR molecules could lead to the susceptibility of CHAR T cells to NK-mediated or complement-mediated killing. Indeed, we showed that soluble HLA-specific antibodies specifically resulted in IFNγ production by CHAR T cells, indicating the binding of soluble antibodies to the CHAR molecules. Additionally, the binding of soluble antibodies to CHAR molecules could potentially lead to CHAR T-cell exhaustion due to chronic stimulation.[32–34] Nonetheless, Ellebrecht and colleagues demonstrated that the binding of Dsg-CAAR molecules by soluble Dsg-specific antibodies actually promoted CAAR T-cell efficacy and persistence in vivo due to CD137-mediated costimulatory signals.[20] This would possibly allow for the administration of lower cell doses in a clinical setting. Whether CHAR T cells respond in a similar fashion as CAAR T cells remain to be explored. In case soluble HLA-specific antibodies negatively affect CHAR T-cell activity, co-treatment with plasmapheresis or immunoadsorption may facilitate optimal CHAR T-cell efficiency. Of note, bone marrow-residing plasma cells are the major source of circulating antibodies. These cells are challenging to target with CHAR T cells due to their lack of surface immunoglobulin expression. Nonetheless, Mackensen et al. showed clinical remission in patients with refractory systemic lupus erythematosus (SLE) using anti-CD19 CAR T cell therapy, even though they primarily targeted memory B cells and plasmablasts without significant effects on plasma cells.[35]

Alloreactive T cells are likely to be common in all individuals due to heterologous immunity of virus-specific T cells.[36,37] Since patients who have been exposed to foreign HLA will have elevated numbers of alloreactive T cells, we deemed it important to determine whether alloreactive T cells would be targeted by CHAR T cells, or vice versa. We demonstrated for a single TCR that alloreactive T cells can become activated after the ligation of CHARs, by using TCR transfer from an HLA-A2 alloreactive T-cell clone into the Jurkat TPR system. In contrast, CHAR T cells did not become activated upon ligation with cells expressing the allo-HLA-reactive TCR. This disparity may be strongly influenced by the affinity of the TCR with the CHAR molecule and should be investigated in more detail.

Nevertheless, since in the transplant setting activation of alloreactive T cells by CHAR T cells could potentially result in exacerbation of the alloimmune response, we set out to minimize the binding to CHAR molecules by allo-HLA-reactive TCR-bearing T cells. Using a previously described mutation in the α3 domain of the extracellular part of the CHAR we aimed to abrogate CD8 binding thereby decreasing activation of the T cell.[26] While a

decrease in T-cell activation with retained CHAR function was achieved by these mutations, complete abrogation of T-cell reactivity was not yet established. Therefore, other regions where CD8 binds to the HLA molecule will be explored for further mutations of the CHAR construct.

In this study, we have shown two specificities of CHAR T cells that can potentially be used for patients to increase the chance of receiving a compatible organ offer. Specificity is key in such cellular therapies, and we have shown by using both HLA-A2 and HLA-A3 CHAR molecules that exquisite specificity is feasible. HLA-A2 is the most common HLA antigen in populations worldwide and therefore is most often mismatched in the transplantation setting.[38] CHAR T cells with other specificities against different HLA-recognizing B cells can be easily designed to build a CHAR T-cell library to treat as many patients as possible. It would be interesting to explore whether different CHAR T cells can be combined to increase the chance for HLA-sensitized patients to become eligible for transplantation. Besides HLA class I CHARs, it will be of major interest to develop class II CHAR T cells to target HLA class II-specific B cells, as HLA class II-specific antibodies are associated with poor graft outcomes.[39] Especially HLA-DQ donor-specific antibodies (DSA) have been associated with rejection, and allograft loss.[40,41] In a post-transplantation setting where HLA-DQ antibody development is most prevalent, it would be of great interest to develop HLA-DQ CHAR T cells to counteract antibody-mediated rejection (ABMR).

The findings described herein are the first proof of concept of CHAR T cells to target HLA-specific B cells in a transplantation setting. With CHAR T cells having the potential to increase the chance of highly sensitized patients getting transplanted, they could also serve to prevent or treat the development of de novo DSA as well as ABMR. In the case of ABMR treatment, it needs to be investigated how CHAR T cells function under immunosuppressive agents. The two specificities of CHAR T cells described herein show great potential for CHAR T-cell therapy. We hypothesize CHAR T cells may become a potential personalized precision immunotherapy to target HLA-specific immune memory.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the research studies, Ilse Gille, PMWM and RSH conducted experiments, Ilse Gille performed data analysis, and Ilse Gille, Mirjam H.M. Heemskerk and Sebastiaan Heidt participated in manuscript writing and editing. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available upon reasonable request from the corresponding author, S. Heidt.

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