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Universal Cloaking of Allogeneic T Cell Therapies Against Natural Killer Cells Via CD300a Agonism

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Abstract:
Immunogenicity limits the persistence of off-the-shelf, allogeneic cell therapies and transplants. While ablation of human leukocyte antigen (HLA) removes most T cell and humoral alloreactivity, no solution has enabled universal protection against the resulting natural killer (NK) cell response. Here, we engineered Trans Antigen Signaling Receptors (TASR) as a new class of NK inhibitory ligands and discovered CD300a, a previously inaccessible receptor, as a functional target. CD300a TASR outperformed leading alternative strategies in focused screens, including CD47 and HLA-E, and was solely capable of universally protecting allogeneic T cells against a large human cohort (45/45 donors), spanning diverse demographics and NK cell phenotypes. A model allogeneic T cell therapy co-expressing an anti-CD19 Chimeric Antigen Receptor (CAR) and CD300a TASR, produced using multiplexed non-viral integration, exhibited enhanced B cell killing potency under allogeneic immune pressure. CD300 TASR represents a universal solution to NK alloreactivity, broadening the population that could be effectively treated by next-generation allogeneic cell therapies.

Conflict of interest: COI declared - see note

COI notes: All authors are current or former employees of Clade Therapeutics. C.C is a founder of Clade Therapeutics. A patent application has been filed by Clade Therapeutics for the technology described here.

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Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: All data supporting the findings of this study are within the main paper, Supplemental figures, and Supplemental tables. A list of antibody and protein reagents used are in Supplemental Table 1. A list of HDR templates and sgRNA sequences are in Supplemental Table 2 and 3. A list of mRNA-encoding DNA template sequences is in Supplemental Table 4. All other data may be requested by emailing the corresponding author.

Clinical trial registration information (if any):

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Universal protection of allogeneic T cell therapies from natural killer cells via CD300a agonism

Short Title: **Immuno-evasive allogeneic cells via CD300a agonism**

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Data Sharing Statement

All data supporting the findings of this study are within the main paper, Supplemental figures, and Supplemental tables. A list of antibody and protein reagents used are in Supplemental Table 1. A list of HDR templates and sgRNA sequences are in Supplemental Table 2 and 3. A list of mRNA-encoding DNA template sequences is in Supplemental Table 4. All other data may be requested by emailing the corresponding author.

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Key Points

- An engineered CD300a agonist ligand (CD300a TASR) universally protects HLA-deficient allogeneic T cells from NK-mediated rejection.
- CD300a TASR is more protective in CMV seropositive hosts than HLA-E ligand and enhances CAR-T efficacy under allogeneic immune pressure.

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Abstract

Immunogenicity limits the persistence of off-the-shelf, allogeneic cell therapies and transplants. While ablation of human leukocyte antigen (HLA) removes most T cell and humoral alloreactivity, no solution has enabled universal protection against the resulting natural killer (NK) cell response. Here, we engineered Trans Antigen Signaling Receptors (TASR) as a new class of NK inhibitory ligands and discovered CD300a, a previously inaccessible receptor, as a functional target. CD300a TASR outperformed leading alternative strategies in focused screens, including CD47 and HLA-E, and was solely capable of universally protecting allogeneic T cells against a large human cohort (45/45 donors), spanning diverse demographics and NK cell phenotypes. A model allogeneic T cell therapy co-expressing an anti-CD19 Chimeric Antigen Receptor (CAR) and CD300a TASR, produced using multiplexed non-viral integration, exhibited enhanced B cell killing potency under allogeneic immune pressure. CD300 TASR represents a universal solution to NK alloreactivity, broadening the population that could be effectively treated by next-generation allogeneic cell therapies.

Introduction

Off-the-shelf T and CAR-T cell therapies promise to build on the success of its autologous counterparts while providing patients a well-defined, scalable, and cost-efficient drug product that can be administered on-demand[1]. Host immune rejection, however, is a major obstacle that limits their persistence, efficacy, and redosing potential[2]. While elimination of donor HLA class I (HLA-I) is a common strategy to evade T cell alloreactivity and HLA-specific anti-donor antibodies (ADAs), this strategy also unleashes NK alloreactivity owing to loss of inhibitory signaling from Killer cell immunoglobulin-like receptors (KIR) binding to HLA-I that normally restrains the NK cell response[3]. Current approaches to reinstate NK inhibition rely on the expression of natural cloaking ligands such as HLA-E[4–6], HLA-A[7], HLA-C[8], and CD47[9] to agonize NKG2A, KIR, and SIRPα inhibitory receptors. These targets, however, are only expressed on limited subsets of NK cells or upon sustained cytokine stimulation, rendering the ligands ineffective against hosts with low frequencies of the corresponding NK

subset[5,10]. In this study, we discover and assess a universal solution against NK alloreactivity to enhance the persistence and efficacy allogeneic T cell therapies against all hosts.

Methods

T Cell Engineering

Human primary T cell targets are engineered for knock-out (KO) by CRISPR/Cas9 and knock-in (KI) by non-viral homology-directed repair (HDR) in accordance with previous protocols[11]. HLA-A2 allo-T cell clones are generated by orthotopic TCRαβ replacement as previously described[12]. For mRNA-based screening, 1-2 million activated T cells with Beta-2-Microglobulin (B2M) KO were electroporated (EP) in 20 µL P3 buffer containing 1.5 ug of mRNA using code CM-137 and rested overnight. Transgene expression was verified by staining with relevant antibody and/or recombinant protein ligand and acquired on flow cytometry. See supplemental methods for detailed T cell engineering, culture, and staining protocols.

Allo-T, NK Cell Challenge Assay

Cryopreserved allo-T cells are thawed, stimulated with Immunocult CD2/CD3/CD28 activator (StemCell 10990), and cultured for 6-7 days in Rh10pc. Cryopreserved NK cells are thawed and cultured in Rh10p (RPMI, 10% v/v human serum, 1x penicillin/streptomycin) + 10 ng/mL IL-15 for 3-5 days prior to start of co-culture. For co-cultures, 30,000 target T cells are plated with NK and/or allo-T cells at various E:T ratios in a 96 well round-bottom plate for 20 hours in R10p (RPMI, 10% v/v fetal bovine serum, 1x penicillin/streptomycin) + 10 ng/mL IL-15 and then subjected to Fluorescent Barcoding Flow Cytometry[13].

Amine-reactive dyes AF647 (Thermofisher A20006), IF700 (AATBioquest 71514), and IR800CW (Licor 929-70021) are resuspended in dimethyl sulfoxide (DMSO) and mixed in 8 combinations to make 100X working stocks of 0.2 mg/ml, 1 mg/ml, and 1.5 mg/ml, respectively, and stored in -80°C. The co-culture plate was resuspended to 50 µL phosphate buffer saline (PBS) by centrifugation at 500g for 2 minutes to decant supernatant. Fluorescent barcode working stocks were thawed, diluted 1:50 with PBS, and 50 µL of the appropriate barcode combination was immediately transferred to the corresponding wells of the co-culture plate using a multichannel pipette and incubated in the dark for 15 minutes room temperature. 100 µL of R10p was added to each well and incubated for 10 minutes to quench the reaction, followed by two washes in FACS buffer (PBS, 5 mM EDTA, 2% v/v fetal bovine serum) by centrifugation at 1200g for 2 minutes. Wells are mixed column-wise and transferred into a new 96-well round-bottom plate, spun 1200g 2 minutes, and then resuspended in the following antibody staining mix in FACS buffer: BV421 CD56, BV510 CD3, BV605 HLA-I, 7-AAD, PE Qbend10.

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The plate is washed twice in FACS buffer by centrifugation at 1200g for 2 minutes, and then acquired at equal volumes on flow cytometry.

PBMC Challenge Assay

Cryopreserved peripheral blood mononuclear cells (PBMCs) are thawed, assessed for phenotype by flow cytometry, and rested overnight in Rh10p + 40 ng/mL IL-2. 30,000 HDR edited T cells are plated with PBMCs at various E:T ratios in a 96 well flat-bottom plate for 3 days in R10p + 40 ng/mL IL-2. Cells are transferred to a 96 well round-bottom plate, and T cell survival is determined by fluorescent barcoding flow cytometry using the protocol outlined above using the following antibody staining mix: PE Qbend10, BV605 HLA-I, BV650 CD56, BV785 CD3, 7-AAD.

Allo-T + NK Cell Competition Assay

HDR edited T cells are pooled together at 1:1 ratio, seeded at 200,000 per well, and co-cultured with Allo-T and NK cells at various E:T ratios in a 96 well flat-bottom plate for 20 hours in R10p + 10 ng/mL IL-15. 10 µL of counting beads (Biolegend 424902) are added to each well and transferred to new 96 well round-bottom plate. The plate is spun 500g 3 min and resuspended into 50 µL of antibody staining mix, consisting of the following reagents in FACS buffer: BV421 Qbend10, BV510 CD3, BV605 HLA-I, BV785 CD56, PE 218 Linker, 7-AAD, PE-Cy7 HLA-E, AF647 G4S Linker, and APC-Cy7 CD47. After staining, cells washed twice with FACS buffer and acquired on Flow cytometry at equal volumes per well.

B Cell Depletion Assay

Cryopreserved PBMCs are thawed and cultured in Rh10p + 40 ng/mL IL-2 for 3 days. PBMCs are seeded at 250,000 per well and co-cultured with CAR-T cells at various CAR-T:PBMC ratios in a 96 well flat-bottom plate for 3 days in R10p + 40 ng/mL IL-2. Cells are transferred to a 96 well round-bottom plate spun by centrifugation at 500g for 3 minutes into following antibody staining mix in 30µL of FACS buffer: BV421 CD56, BV510 CD3, PE CD19, AF647 G4S Linker, 7-AAD are first added, and then 20µL of an antibody-based fluorescent barcoding mix are added. Barcode staining mix consists of a combination of anti-CD45 antibodies on the BV711, BV785, PE-Cy7, and APC-Cy7 Channels in FACS buffer that are unique to each row. The plate is washed twice in FACS buffer by

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centrifugation at 500g for 3 minutes. Wells are mixed column-wise and transferred into a 96-well deep well plate and then acquired at equal volumes on flow cytometry.

All primary cells are obtained from commercial sources from de-identified donors in adherence with the relevant institutional review board and with written informed consent.

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Results

Modeling Cellular Rejection of Allogeneic T Cells

We first developed an *ex-vivo* model of cellular rejection by measuring the survival of human allogeneic T cell targets co-cultured with human primary cell effectors using a modified fluorescently barcoded flow cytometry readout **(Supplemental Fig. 1a)**[13]. This method enabled sensitive and high-throughput measurement of target T cell survival by direct counting across multiple E:T ratios with high reproducibility in IC50 value **(Supplemental Fig. 1b,c)**. Analogous to dose-response curves, we define the IC50 value as the E:T ratio corresponding to 50% of target T cell survival, with higher values indicating enhanced T cell survival ability. To confirm that disruption of HLA-I eliminates T cell alloreactivity, we co-cultured target T cells from HLA-A2+ serotyped donors with HLA-A2- effector alloreactive T cells engineered to express a known HLA-A2 reactive T cell receptor (TCR) **(Fig. 1a)**. Target T cells were rejected in a dose-dependent manner but were rescued by disruption of HLA-I expression via CRISPR/Cas9-mediated KO of the B2M gene **(Fig. 1a,b)**. However, B2M KO sensitized target T cells to NK allorecognition, and neither HLA-I+ nor B2M KO target T cells survived when both NK and alloreactive T cells were added as effectors **(Fig. 1b)**. Live cell counts of allo-T and NK cell effectors at assay endpoint were similar when added alone or together across all E:T ratios against both B2M KO and HLA-I+ targets, demonstrating that cytotoxicity was restricted to target cells and negligible between effector cells **(Supplemental Fig. 2a)**. B2M KO-mediated NK cell alloreactivity was consistent across multiple donor replicates, confirming missing-self mechanism of NK recognition in this context (**Supplemental Fig. 2b)**[3,5]**.**

Screening and Discovery of NKG2A and CD300a TASR

We applied this experimental system to rapidly screen previously published “cloaking” strategies known to inhibit NK cell cytotoxicity **(Fig. 1c, Supplemental Fig. 3a)**. One set of strategies involves the removal of ligands that can agonize activating or adhesion receptors on NK cells [14–17]. A second set involves expression of ligands that agonize inhibitory receptors on NK cells or reduce the effect of cytotoxic granules[5,18–21]. We challenged B2M

KO T cells engineered with each strategy against human primary NK cells from a single donor at various E:T ratios and measured the fold-change in T cell survival relative to non-cloaked negative control using the IC50 value. Genetic ablation of CD48, CD54, CD58, and CD155 via CRISPR/Cas9 did not protect against NK cell challenge. This result was reproducible across two T cell lines per target, each engineered with a separate guide RNA with confirmed knockdown of surface expression. While all natural NK inhibitory ligands were successfully and uniformly expressed via mRNA electroporation, only the well-studied HLA-E enhanced the survival of B2M KO T cells against NK challenge, in line with prior works[5,6].

Constrained by the limited targeting repertoire of natural ligand-based approaches, we hypothesized that surface expression of single-chain antigen binding fragments (scFV) on an inert scaffold, herein termed Trans Antigen Signaling Receptors (TASRs), can agonize a wider repertoire of NK inhibitory receptors than is currently possible (**Fig. 1d**). We constructed TASRs using an initial CD8 hinge and transmembrane scaffold using scFV constructs of well-studied antibody clones derived from literature and patents to target inhibitory receptors known to be expressed on NK cells. We screened TASRs via mRNA electroporation of B2M KO primary T cells for expression and functional potency against human primary NK cells derived from one donor (**Supplemental Fig. 3a**). From this screen, we identified a NKG2A and a CD300a binding TASR that functioned in a dose-dependent manner **(Fig. 1c, Supplemental Fig. 3a-c)**. NKG2A TASR agonizes the same target as HLA-E, while CD300a is an unexplored target for this application.

Optimization of CD300a TASR

We set out to optimize the expression and function of CD300a TASR version 1 by testing several mutant constructs using our mRNA screening platform. We discovered a mouse B7-1 transmembrane (TM) and cytoplasmic (Cyt) domain that enhanced expression and an inverse correlation between TASR hinge length and the function of CD300a TASR but not NKG2A TASR **(Fig. 1e-g, Supplemental Fig. 4,5)**[22]. An optimized version 2, containing no hinge but stabilized by the mouse B7-1 cytoplasmic tail, and hereafter termed CD300a TASR, exhibited significantly enhanced function while maintaining a fully human extracellular domain **(Fig. 1h-j)**.

Validation of CD300a TASR

To model cloaking ligands under constitutive and physiologically-relevant levels of expression, we inserted transgenes via CRISPR/Cas9 mediated non-viral homology-directed repair (HDR) of human T cells. Transgenes were integrated into either the B2M locus using the endogenous B2M promoter concomitant with

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B2M KO, or AAVS1 locus using the exogeneous EF1α promoter **(Fig. 2a, Supplemental Fig. 6a)**. CD300a TASR expression was confirmed in both integration approaches by staining with either cognate CD300a protein ligand or an antibody specific to scFV linker sequence **(Fig. 2b, Supplemental Fig. 6b)**. CD300a TASR enhanced T cell survival in our standard 20-hour challenge assay with IL-15 treated NK cell effectors from multiple donors relative to non-cloaked B2M KO T cells **(Fig. 2c)**. This result was reproducible with non-cytokine treated NK cells and in long term 7-day co-cultures, with protection on par with HLA-I+ positive control. CD300a TASR also reduced the secretion of Interferon gamma (IFNγ) and Tumor Necrosis Factor alpha (TNFα) release after NK challenge relative to negative control B2M KO T cells, and to levels on par with HLA-I+ T cell positive control **(Supplemental Fig. 6c,d)**.

To confirm utilization of the CD300a pathway, we performed NK challenge in the presence of blocking antibodies. The addition of anti-CD300a blocking antibody but not isotype control or anti-CD300c, a close paralog of CD300a, completely abrogated the protective effect of CD300a TASR to non-cloaked levels (Fig. 2d). Anti-CD300a did not affect the survival of non-cloaked T cells. Thus, CD300a mediated inhibition is not functionally utilized during endogenous T cell:NK cell interactions and the protective effect of CD300a TASR relies on the CD300a signaling axis.

CD300a TASR outperforms alternative strategies

CD300a TASR outperformed the current best-in-class ligands HLA-E and CD47, as well as the recently published SIRPα engager in NK protection[5,6,9,10,23]. To provide head-to-head comparisons, we inserted each ligand into the B2M locus of human T cells concomitant with B2M KO and confirmed surface expression **(Fig. 3a)**. Challenged with NK cells, CD300a TASR and HLA-E outperformed CD47 and SIRPα engager, while CD300a TASR was the only protective ligand against a donor with high frequencies of an $NKG2A^{-}NKG2C^{+}$ NK cell phenotype, hereby termed $2C^{dom}$ (**Fig. 3b**)[5]. The dominance of CD300a TASR over CD47 was observed under all conditions tested, including when overexpressed via mRNA, inserted into the AAVS1 locus via HDR under EF1α promoter, and challenged with NK cells cultured in high-dose IL-2, which upregulates SIRPα (**Supplemental Fig. 7**)[10,23]. Using mRNA EP, we further determined that CD300a TASR outcompeted TIM3 engager against 4/4 NK donors under comparable levels of expression (**Supplemental Fig. 8**).

As an orthogonal readout, we pooled HDR-edited T cells from the previous experiment along with an HLA-I+ control and challenged with allo-T and NK cells in a competition assay. T cells expressing the indicated ligands were pooled in equal ratios and challenged with NK cells and HLA-A2 alloreactive T cells **(Fig 3a,c)**. The identity and frequency of each T cell member can be discerned based on antibody staining of the cloaking ligand

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and measured by conventional multicolor flow cytometry **(Supplemental Fig. 9a-e)**. CD300a TASR emerged as the dominant survivor when challenged with both NK and allo-T cells **(Fig. 3c)**. This result was reproducible across a $2A^{dom}$ and $2C^{dom}$ NK cell donor and two alloreactive T cell clones specific to HA-2 or EMC7 antigen, as well as any combination thereof. In this mixed T cell model, active T cell alloreactivity can be observed with both Allo-T cell effectors as the fraction of HLA-I+ T cells remain significantly lower relative to conditions without Allo-T cells. Likewise, active rejection of non-cloaked B2M KO T cells can be observed with both NK cell donors in this model relative to conditions without NK cells. Competition with additional NK donors in the absence of HLA-I+ control cells further corroborated dominance of CD300a TASR in $2C^{dom}$ NK donors **(Supplemental Fig. 9f)**.

CD300a TASR is a pan-NK inhibitor

We next assessed CD300a TASR and HLA-E, the most potent alternative, against a large human cohort to assess the heterogeneity of responses across donors and to identify correlates of protection. To model the physiological context of intravenously injected T cell therapies, we measured the survival of cloaked T cells challenged with PBMCs **(Fig. 4a, Supplemental Fig. 10)**. 45 PBMC donors were selected for diversity in ethnicity, age, gender, and CMV seropositivity due to their known effects on NK cell function and phenotype **(Fig. 4a)**[3,24–26]. While large variations in CD57, KIR, NKG2A, and NKG2C NK marker expression was observed, CD300a was uniformly expressed on NK cells in all donors **(Fig. 4b, Supplemental Fig. 11)**. CD300a TASR and HLA-E were expressed at comparable levels from the AAVS1 locus based on RQR8 expression **(Fig. 4c)**[27]. Within this cohort, CD300a TASR protected B2M KO T cells against all PBMC donors tested (45/45), indicating universal protection against NK cell reactivity **(Fig. 4d, Supplemental Fig. 12)**. CMV serostatus emerged as the most significant demographic determinant of reduced protection by HLA-E and correlated with the frequency of the well-known $NKG2C^{+}NKG2A^{-}CD57^{+}CD16^{+}CD56^{hi}$ adaptive NK cell subset (**Fig. 4e,f**)[28]. While HLA-E lost protection with increasing frequencies of adaptive NK cells, CD300a TASR maintained protection (**Fig. 4g**).

CD300a TASR enhances anti-CD19 CAR T cell killing under allogeneic conditions

Lastly, we integrated recent innovations to produce a model allogeneic CAR-T cell therapy co-expressing a clinically relevant anti-CD19 CAR into the TRAC locus and cloaking ligand into the B2M locus via multiplex non-viral HDR **(Fig. 5a, Supplemental Fig. 13a,b)**[5,11,29]. Expression of CD300a TASR, HLA-E, and RQR8 control cloaking ligand in CAR-T cells was confirmed (**Fig. 5b)**. To model CAR-T function under a physiological allogeneic environment, we dosed our engineered CAR-T cells into PBMCs and measured killing of CD19-expressing B cells

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under NK alloreactivity **(Fig. 5c, Supplemental Fig. 13c-g)**. CD300a TASR enhanced B cell killing potency in all PBMC donors (5/5) and outcompeted HLA-E in all donors with $2C^{dom}$ NK phenotype (**Fig. 5c**). This result was not due to intrinsic differences, as all CAR-T cells had similar potency against CD19-expressing Raji cells in the absence of allogeneic pressure (**Fig. 5d)**. In addition, TASR-mediated protection against NK cells was maintained in the presence of CAR (**Fig. 5e)**.

Discussion

Immunological rejection is a major barrier to the persistence and efficacy of allogeneic cell therapies and transplants[30]. In this study, we describe the discovery, engineering, and assessment of TASRs as a new modality to inhibit the alloreactive NK cell response. We demonstrate that an optimized CD300a TASR in combination with HLA-I ablation protected against both T and NK cell alloreactivity, exhibited superior function relative to alternative strategies, and possessed broad NK inhibitory potential against a large human cohort. Lastly, we show that CD300a TASR can be integrated into a next-generation allogeneic CAR-T product via multiplexed, non-viral targeted integration, and that the resulting product enhanced B cell killing potency under allogeneic immune pressure. We expect the B cell lysis model to be relevant for allogeneic CAR-T therapy against systemic lupus erythematosus (SLE), where B cell depletion is the main mechanism of action for dampening the autoreactive antibody response[31].

CD300a is a new and unexplored target for NK cloaking. CD300a is a classical inhibitory receptor expressed on all NK cells, and signals through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) upon binding its endogenous ligands phosphatidylserine (PS) and phosphatidylethanolamine (PE) on the surface of apoptotic cells[32–34]. Antibody cross-linking studies have also demonstrated SHP-1 and SHP-2 recruitment to ITIMs to be the primary mechanism of inhibition by CD300a[35–38]. Mechanistically, CD300a TASR mirrors the inhibitory effect of anti-CD300a antibody in redirected killing assays with NK effectors against P815 target cells[35]. CD300a TASR leaves the host immune system intact and functional, as non-cloaked T cells were still depleted in the presence of CD300a TASR expressing T cells in the competition assay. Thus, CD300a TASR could offer an enhanced safety profile over CAR or antibody-mediated approaches that ablate the host immune system and also leave the endogenous T and NK anti-tumor response intact[39].

The functionality of TASR is determined by both the antigen binding fragment and the scaffold structure. Despite attempts to reinstate KIR agonism after HLA-I ablation, none of the KIR-targeting TASR clones in our screen demonstrated efficacy. It is possible that our anti-KIR scFVs lack intrinsic agonistic activity or cross reacts with both inhibitory and activating KIRs, which can be difficult to distinguish with TASRs due to high sequence homology between KIR members[40]. The beneficial effect of hinge removal for CD300a TASR contrasts with CAR

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designs, where hinges can enhance CAR-T function and signaling[41,42]. Given that CD300a TASR is a mimic to the small phospholipids PS and PE, we suspect that hinge removal enhances CD300a agonism by reducing the TASR:CD300a interaction distance to be comparable to that of the endogenous PS/PE:CD300a interaction.

We expect the use of CD300a TASR in allogeneic cell therapies to significantly expand the addressable patient population. By comparing CD300a TASR and HLA-E against a large human cohort, we highlight how NK alloreactivity can exhibit large donor-to-donor variations that should be considered. While prior work has reported HLA-E's lack of efficacy against the $2C^{dom}$ NK phenotype, our study provides the first comprehensive link between cloaking ligand functional efficacy, NK phenotype, and patient demographics[5]. With this data, we were able to robustly show that HLA-E has diminished protection against NK cells isolated from CMV+ individuals while CD300a TASR retains function in all hosts independent of demographic determinants. 50% (12/24) of CMV+ individuals in our cohort harbored substantial adaptive NK cell frequency above 5% of total NK cells, which is in line with past estimates and corresponds to the point where HLA-E begins to lose potency in the PBMC challenge assay[28]. Given global CMV seroprevalence rates of 43-96% by country, we expect CD300a TASR to expand the addressable patient population by 22-48% over HLA-E and have the greatest impact in the elderly over 60 years of age, which have 80%+ CMV seropositivity rate, as is the case with CAR-T therapy for most cancer malignancies[43,44].

We note several limitations to our study design and scope. First, it remains to be seen if NKG2A and CD300a TASR can maintain NK protection across a broader range of therapeutic cell types beyond T cells. Second, while CD300a TASR enhanced the persistence and function of an allogeneic CAR-T cell produced by multiplexed non-viral integration, additional testing will be required to assess the feasibility of this drug product with respect to genomic integrity, cell phenotype, long-term function, and comparison to other production methods. Lastly, additional studies are warranted to investigate the efficacy of TASRs in-vivo.

In summary, we have demonstrated that CD300a TASR acts as a universal ligand against NK cell alloreactivity. Its combination with HLA and TCRαβ ablation to prevent T cell alloreactivity, ADAs, and GvHD paves the way for fully immuno-evasive CAR-T cells that are effective for anyone in need of treatment, from cancer to autoimmunity and beyond.

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Authorship Contributions

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S-Q.Z. conceived and developed the TASR modality. S-Q.Z. and C.C. conceived and designed the study. S-Q.Z, F.T., and J.F. designed, performed, and analyzed data for all experiments. S-Q.Z., K.A., C.C., and G.G.W. supervised the study. S-Q.Z. and G.G.W. wrote the manuscript with feedback from all authors.

Conflict of Interest Disclosure

All authors are former employees of Clade Therapeutics. C.C is a founder of Clade Therapeutics. A patent application has been filed by Clade Therapeutics for the technology described here.

References

1. Depil S, Duchateau P, Grupp SA, Mufti G, Poirot L. 'Off-the-shelf' allogeneic CAR T cells: development and challenges. *Nat. Rev. Drug Discov.* 2020;19(3):185–199.

2. Turtle CJ, Hanafi L-A, Berger C, et al. CD19 CAR–T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. *J Clin Invest*. 2016;126(6):2123–2138.

3. Anfossi N, André P, Guia S, et al. Human NK Cell Education by Inhibitory Receptors for MHC Class I. *Immunity*. 2006;25(2):331–342.

4. Degagné É, Donohoue PD, Roy S, et al. High-specificity CRISPR-mediated genome engineering in anti-BCMA allogeneic CAR T cells suppresses allograft rejection in preclinical models. *Cancer Immunol. Res.* 2024;

5. Jo S, Das S, Williams A, et al. Endowing universal CAR T-cell with immune-evasive properties using TALEN-gene editing. *Nat. Commun.* 2022;13(1):3453.

6. Gornalusse GG, Hirata RK, Funk S, et al. HLA-E-expressing pluripotent stem cells escape allogeneic responses and lysis by NK cells. *Nat Biotechnol*. 2017;35(8):765–772.

7. Furukawa Y, Ishii M, Ando J, et al. iPSC-derived hypoimmunogenic tissue resident memory T cells mediate robust anti-tumor activity against cervical cancer. *Cell Rep. Med.* 2023;4(12):101327.

8. Xu H, Wang B, Ono M, et al. Targeted Disruption of HLA Genes via CRISPR-Cas9 Generates iPSCs with Enhanced Immune Compatibility. *Cell Stem Cell*. 2019;24(4):566-578.e7.

9. Hu X, Manner K, DeJesus R, et al. Hypoimmune anti-CD19 chimeric antigen receptor T cells provide lasting tumor control in fully immunocompetent allogeneic humanized mice. *Nat. Commun.* 2023;14(1):2020.

10. Deuse T, Hu X, Agbor-Enoh S, et al. The SIRPα–CD47 immune checkpoint in NK cells. *J. Exp. Med.* 2021;218(3):e20200839.

11. Shy BR, Vykunta VS, Ha A, et al. High-yield genome engineering in primary cells using a hybrid ssDNA repair template and small-molecule cocktails. *Nat Biotechnol*. 2022;1–11.

12. Schober K, Müller TR, Gökmen F, et al. Orthotopic replacement of T-cell receptor α- and β-chains with preservation of near-physiological T-cell function. *Nat. Biomed. Eng.* 2019;3(12):974–984.

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13. Krutzik PO, Clutter MR, Trejo A, Nolan GP. Fluorescent Cell Barcoding for Multiplex Flow Cytometry. *Curr. Protoc. Cytom.* 2011;55(1):6.31.1-6.31.15.

14. Nakajima H, Cella M, Langen H, Friedlein A, Colonna M. Activating interactions in human NK cell recognition: the role of 2B4-CD48. *Eur. J. Immunol.* 1999;29(5):1676–1683.

15. Rölle A, Halenius A, Ewen E, et al. CD2–CD58 interactions are pivotal for the activation and function of adaptive natural killer cells in human cytomegalovirus infection. *Eur. J. Immunol.* 2016;46(10):2420–2425.

16. Differential Expression of Cell Adhesion Molecules CD54/CD11a and CD58/CD2 by Human Melanoma Cells and Functional Role in Their Interaction with Cytotoxic Cells1 | Cancer Research | American Association for Cancer Research.

17. Wang B, Iriguchi S, Waseda M, et al. Generation of hypoimmunogenic T cells from genetically engineered allogeneic human induced pluripotent stem cells. *Nat Biomed Eng*. 2021;5(5):429–440.

18. Wei Y, Ren X, GalboJr. PM, et al. KIR3DL3-HHLA2 is a human immunosuppressive pathway and a therapeutic target. *Sci. Immunol.* 2021;6(61):.

19. Zhang P, Lu X, Tao K, et al. Siglec-10 is associated with survival and natural killer cell dysfunction in hepatocellular carcinoma. *J. Surg. Res.* 2015;194(1):107–113.

20. Cohnen A, Chiang SC, Stojanovic A, et al. Surface CD107a/LAMP-1 protects natural killer cells from degranulation-associated damage. *Blood*. 2013;122(8):1411–1418.

21. Bhatt RS, Berjis A, Konge JC, et al. KIR3DL3 Is an Inhibitory Receptor for HHLA2 that Mediates an Alternative Immunoinhibitory Pathway to PD1. *Cancer Immunol. Res.* 2021;9(2):156–169.

22. Lin Y-C, Chen B-M, Lu W-C, et al. The B7-1 Cytoplasmic Tail Enhances Intracellular Transport and Mammalian Cell Surface Display of Chimeric Proteins in the Absence of a Linear ER Export Motif. *PLoS ONE*. 2013;8(9):e75084.

23. Gravina A, Tediashvili G, Zheng Y, et al. Synthetic immune checkpoint engagers protect HLA-deficient iPSCs and derivatives from innate immune cell cytotoxicity. *Cell Stem Cell*. 2023;30(11):1538-1548.e4.

24. Gumá M, Angulo A, Vilches C, et al. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood*. 2004;104(12):3664–3671.

25. Garff‐Tavernier ML, Béziat V, Decocq J, et al. Human NK cells display major phenotypic and functional changes over the life span. *Aging Cell*. 2010;9(4):527–535.

26. Cheng MI, Li JH, Riggan L, et al. The X-linked epigenetic regulator UTX controls NK cell-intrinsic sex differences. *Nat. Immunol.* 2023;24(5):780–791.

27. Philip B, Kokalaki E, Mekkaoui L, et al. A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy. *Blood*. 2014;124(8):1277–1287.

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28. Lopez-Vergès S, Milush JM, Schwartz BS, et al. Expansion of a unique CD57+NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc. Natl. Acad. Sci.* 2011;108(36):14725–14732.

29. Zhang J, Hu Y, Yang J, et al. Non-viral, specifically targeted CAR-T cells achieve high safety and efficacy in B-NHL. *Nature*. 2022;609(7926):369–374.

30. Wagner DL, Fritsche E, Pulsipher MA, et al. Immunogenicity of CAR T cells in cancer therapy. *Nat. Rev. Clin. Oncol.* 2021;18(6):379–393.

31. Mackensen A, Müller F, Mougiakakos D, et al. Anti-CD19 CAR T cell therapy for refractory systemic lupus erythematosus. *Nat Med*. 2022;28(10):2124–2132.

32. Zenarruzabeitia O, Vitallé J, Eguizabal C, Simhadri VR, Borrego F. The Biology and Disease Relevance of CD300a, an Inhibitory Receptor for Phosphatidylserine and Phosphatidylethanolamine. *J Immunol*. 2015;194(11):5053–5060.

33. Li S, Wang T, Xiao X, et al. Blockade of CD300A enhances the ability of human NK cells to lyse hematologic malignancies. *Cancer Biol. Med.* 2024;21(4):331–346.

34. Lankry D, Rovis TL, Jonjic S, Mandelboim O. The interaction between CD300a and phosphatidylserine inhibits tumor cell killing by NK cells. *Eur. J. Immunol.* 2013;43(8):2151–2161.

35. Cantoni C, Bottino C, Augugliaro R, et al. Molecular and functional characterization of IRp60, a member of the immunoglobulin superfamily that functions as an inhibitory receptor in human NK cells. *Eur. J. Immunol.* 1999;29(10):3148–3159.

36. Bachelet I, Munitz A, Moretta A, Moretta L, Levi-Schaffer F. The Inhibitory Receptor IRp60 (CD300a) Is Expressed and Functional on Human Mast Cells. *J. Immunol.* 2005;175(12):7989–7995.

37. Kim E, Lee S, Suk K, Lee W. CD300a and CD300f differentially regulate the MyD88 and TRIF-mediated TLR signalling pathways through activation of SHP-1 and/or SHP-2 in human monocytic cell lines. *Immunology*. 2012;135(3):226–235.

38. Vitallé J, Terrén I, Orrantia A, et al. CD300a inhibits CD16-mediated NK cell effector functions in HIV-1-infected patients. *Cell. Mol. Immunol.* 2019;16(12):940–942.

39. Qasim W. Genome-edited allogeneic donor “universal” chimeric antigen receptor T cells. *Blood*. 2023;141(8):835–845.

40. Czaja K, Borer A-S, Schmied L, et al. A comprehensive analysis of the binding of anti-KIR antibodies to activating KIRs. *Genes Immun.* 2014;15(1):33–37.

41. Fujiwara K, Tsunei A, Kusabuka H, et al. Hinge and Transmembrane Domains of Chimeric Antigen Receptor Regulate Receptor Expression and Signaling Threshold. *Cells*. 2020;9(5):1182.

42. Qin L, Lai Y, Zhao R, et al. Incorporation of a hinge domain improves the expansion of chimeric antigen receptor T cells. *J. Hematol. Oncol.* 2017;10(1):68.

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43. Zuhair M, Smit GSA, Wallis G, et al. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. *Rev. Méd. Virol.* 2019;29(3):e2034.

44. Staras SAS, Dollard SC, Radford KW, et al. Seroprevalence of Cytomegalovirus Infection in the United States, 1988–1994. *Clin. Infect. Dis.* 2006;43(9):1143–1151.

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Figure 1: Discovery and Optimization of CD300a TASR

a, Overview of allo-T + NK challenge assay. Survival of HLA-I+ and B2M KO target T cells from an HLA-A2+ donor challenged with NK and allo-T cell effectors from HLA-A2- donors. Allo-T cells express the AHIII.2 TCRαβ reactive to EMC7 peptide, ALWGFFPVL, presented by HLA-A2. To discern from allo-T cells, B2M KO target T cells were engineered to express RQR8 and HLA-I+ target T cells were engineered to express GFP. **b**, HLA-I expression by flow cytometry of HLA-I+ and B2M KO target T cells from (a). **c,** NK challenge assay to screen cloaking strategies on B2M KO T cells. Ligands are expressed by mRNA EP, and KO by CRISPR/Cas9. Y-axis shows ratio of IC50 value of the indicated cloaked T cell over uncloaked negative control. IC50 represents E:T ratio corresponding to 50% target T cell survival, derived from one 6–7-point curve of T cell survival at various E:T ratios challenged with one NK cell donor, with higher values indicating enhanced persistence. N = 1 technical replicate per condition. **d**, Annotation of TASR structure. (e-g), effect of hinge length on function of CD300a TASR. **e**, design of CD300a TASR variants with different hinge domains with the indicated amino acid lengths and their expression level after mRNA EP into B2M KO T cells. **f**, NK challenge assay expressing the indicated CD300a TASR variant from (e). N = 2 technical replicate curves per condition. **g**, correlation of NK protection from (f) as defined by IC50 value with hinge length from (e). Error bar indicates 95% confidence interval of IC50 value. (h-j)**,** Final optimization of CD300a TASR. **h**, CD300a TASR V1 and two optimized variants are tested for expression via mRNA EP of B2M KO T cells. **i**, NK challenge assay against the indicated CD300a TASR variant from (h), N = 1 technical replicate curve per condition. **j**, optimized CD300a TASR design and mechanism of action.

Figure 2: Constitutive expression of CD300a TASR robustly protects B2M KO T cells against NK alloreactivity via CD300a agonism

a, Experimental overview, NK challenge assay with T cells engineered to express cloaking transgene from the B2M locus via non-viral HDR concomitant with B2M KO. **b**, TASR and HLA-I Expression by flow cytometry of target T cells expressing CD300a TASR or RQR8 negative control transgene from the B2M locus, as well as HLA-I+

positive control T cells expressing GFP from the AAVS1 locus. CD300a TASR expression evaluated by antibody (left panel) and ligand-based (middle panel) staining. Dashed line indicate negative control T cells stained with the same marker. **c**, NK challenge assay with three NK cell donors against target T cells from (a,b) co-cultured for 1 and 7 days. NK cells were cultured in 10 ng/mL Il-15, while resting NK cells are thawed, rested overnight, and co-cultured in absence of exogeneous cytokines. N = 1-2 technical replicate curves per condition. **d**, 1-day NK challenge assay against target T cells from (a,b) in the presence of 5 μg/mL of anti-CD300a (clone MEM-260), anti-CD300c (clone TX45), or mIgG1 isotype control antibody. N = average of 2 technical replicate curves per condition.

Figure 3: B2M KO + CD300a TASR outcompetes HLA-E, CD47, and SIRPa engager cloaking ligands and protects against T and NK cells

a, Phenotype by flow cytometry of engineered T cells expressing the indicated transgenes at the target locus by non-viral HDR, gated single cell lymphocytes. grey dotted histograms indicate negative control T cells stained with the same markers. CD47 is endogenously expressed on T cells, and so the RQR8 epitope tag was co-expressed using 2A self-cleaving peptide to facilitate purification transgene-expressing cells. **b**, NK challenge assay with B2M KO T cells expressing cloaking transgene from the B2M locus from (a), challenged with three NK cell donors. Inset shows the NK cell phenotype by flow cytometry, gated CD3-CD56+. N= 2 technical replicate curves per condition. **c**, Competition assay of pooled T cell targets from a) co-cultured with the indicated allo-T cell clone and/or NK cell effector donor. Allo-T cells express TCRαβ specific for either minor histocompatibility antigen 2 (HA-2) or EMC7 peptide presented on HLA-A2. Target T cells are serotyped HLA-A2^{+}, and effector cells are HLA-A2^{-}. Y-axis shows frequency of the indicated T cell member after challenge with indicated effector cell. N = 3 technical replicates per condition.

Figure 4: CD300a TASR universally protects against NK cell alloreactivity

a, Study design and demographic overview of the PBMC donors used. T cells are engineered to co-express cloaking transgene and RQR8 via 2A self-cleaving peptide under control of EF1α promoter by non-viral HDR. **b**, Percentage of NK cells expressing the indicated markers by flow cytometry from the 45 PBMC donors in (a). **c**, Phenotype by flow cytometry of the three engineered T cell targets, gated on live single lymphocytes. Label indicates cloaking transgene. grey dotted histograms indicate negative control T cells stained with the same markers. **d**, Aggregate results as in (c) against 45 PBMC donors. Each datapoint represents IC50 value of the indicated cloaking ligand against PBMCs from one donor. Limit of quantitation in IC50 set to be twice the highest E:T ratio used. Wilcoxon matched pairs signed rank test. **e**, Association of PBMC donor demographics from (a)

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with functional data from (d), Kruskal-Wallis for Ethnicity, Mann-Whitney for other. Y-axis represents ratio of IC50 between CD300a TASR and HLA-E cloaking ligand, with one indicating equal protection. **f**, Adaptive NK cell frequency by CMV serostatus of PBMC donors from (a). Mann Whitney U Test. **g**, Relationship between the adaptive NK cell frequency of the PBMC donor from (f) and functional potency of CD300a TASR (left) and HLA-E (right), normalized to non-cloaked control from (d). Dotted line represents linear fit of log-log transformed data. N = 45 PBMC donors.

Figure 5: CD300a TASR enhances CAR-T functional potency under allogeneic immune pressure

a, Non-viral multiplexed targeted integration of human T cells to co-express cloaking and anti-CD19 CAR transgenes while ablating HLA-I and TCRαβ expression. **b**, phenotype of three engineered CAR-T cells by flow cytometry expressing the indicated cloaking transgene at B2M locus, gated live single cells. The same anti-CD19 CAR is used for all CAR-T cells. **c** (bottom row) B cell lysis assay for engineered CAR-T cell therapy containing the indicated cloaking transgene against the indicated PBMC donors. N = 2 technical replicate curves per condition. (top row) phenotype of NK cells from the respective PBMC donor. **d**, Cytotoxicity of indicated cloaked anti-CD19 CAR-T cell against CD19-expressing Raji cells. Grey no CAR control T cells contains CD300a TASR integrated into the B2M loci without anti-CD19 CAR. N = 3 technical replicate curves per condition. **e**, NK challenge of cloaked anti-CD19 CAR-T cells containing the indicated cloaking transgene with a $2A^{dom}$ and $2C^{dom}$ NK donor. N = 3 technical replicates curves per condition.

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Figure 1

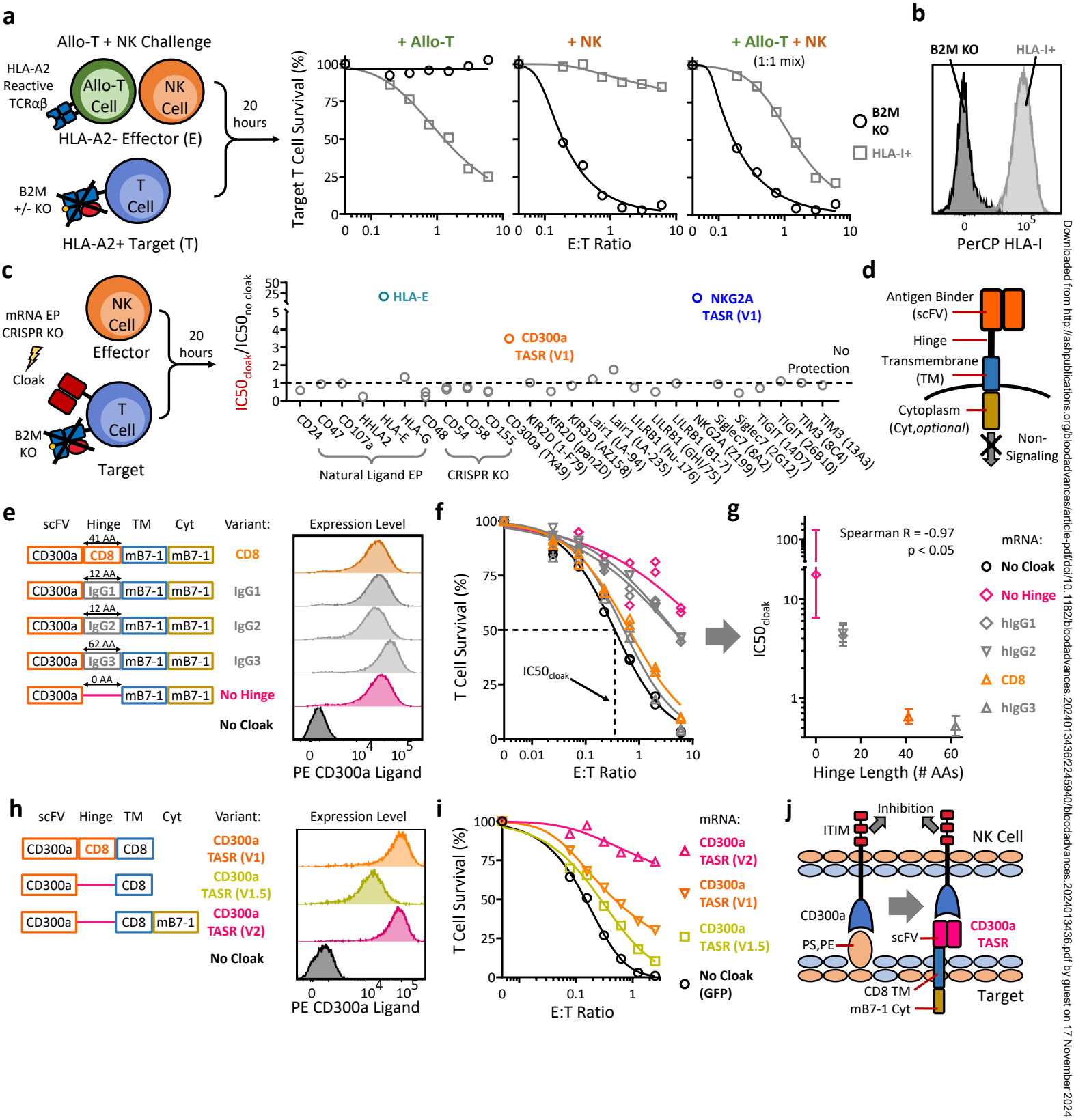

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Figure 2

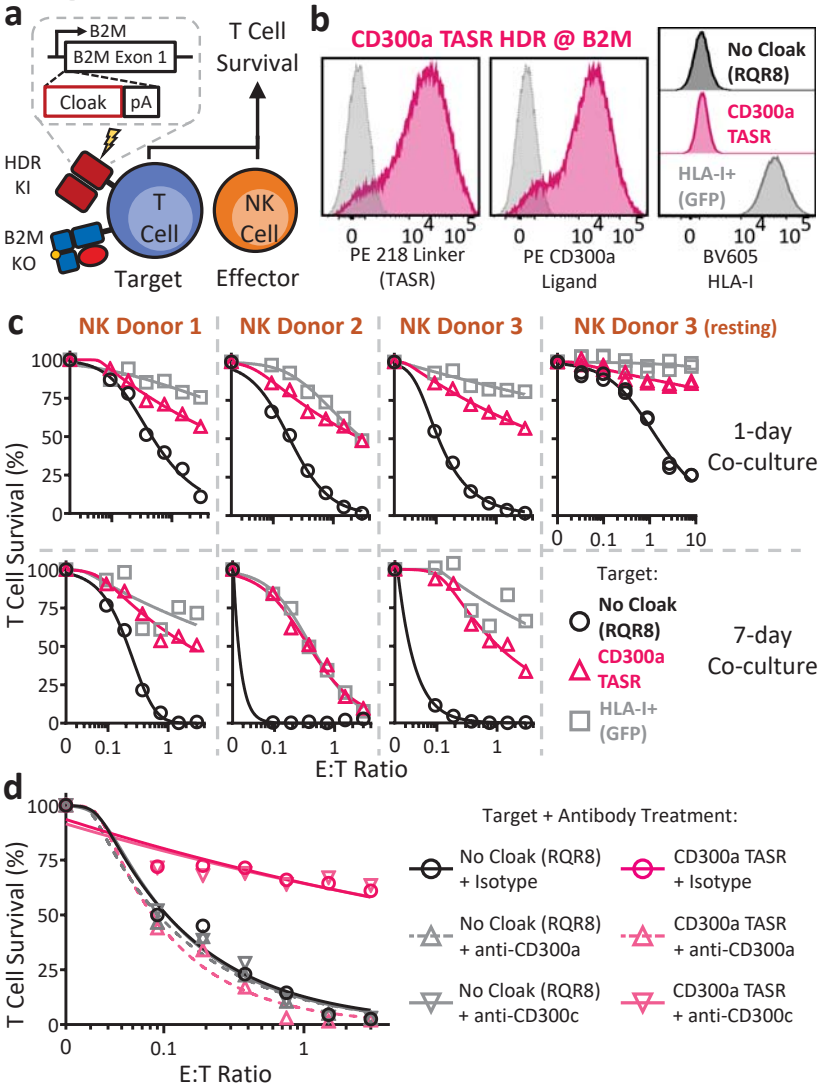

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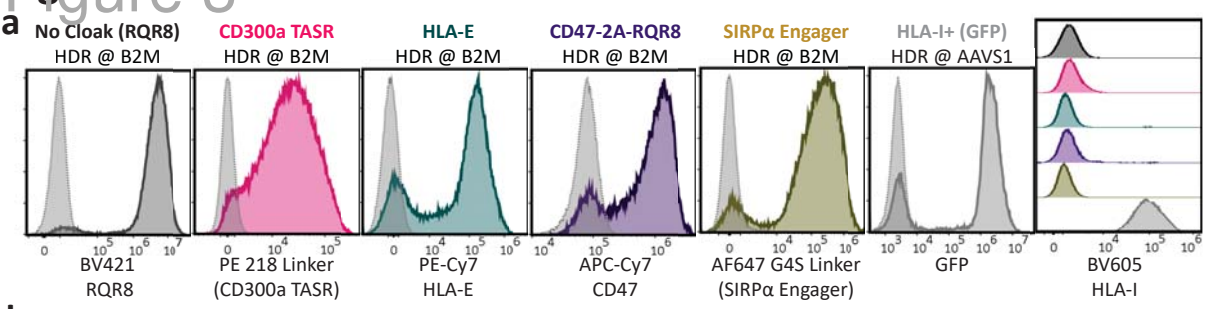
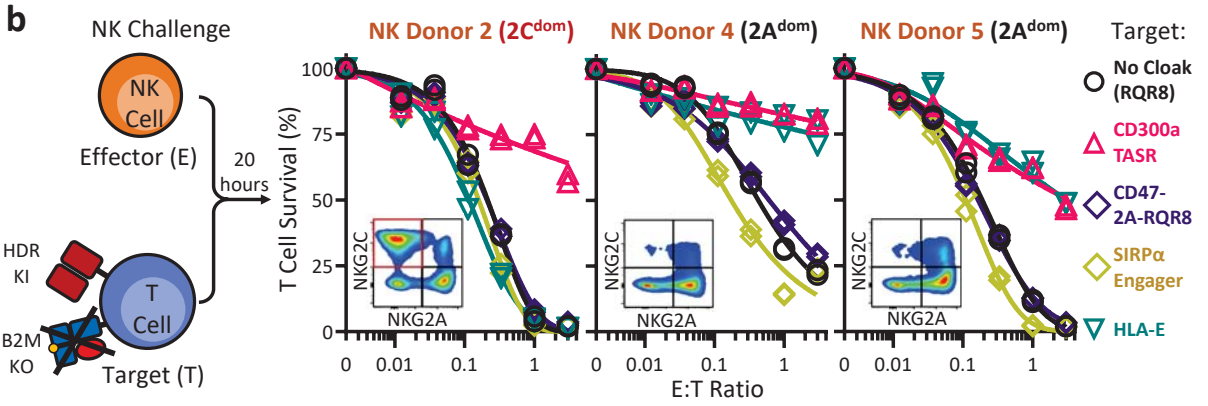
Figure 3





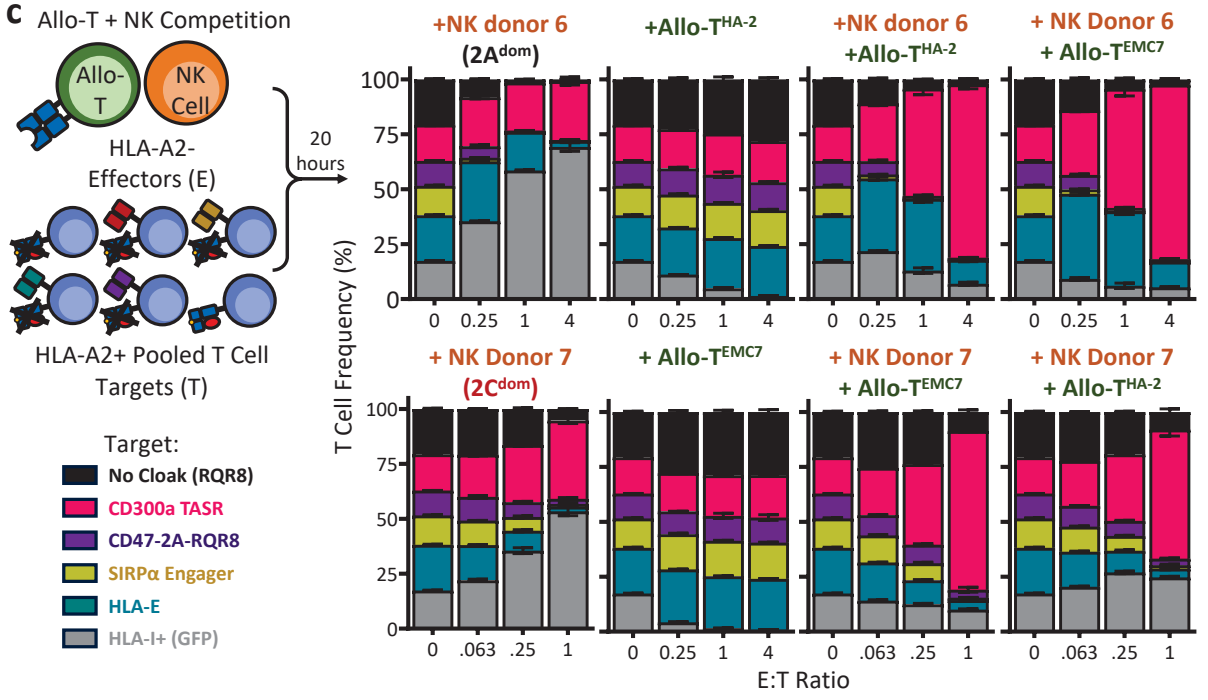


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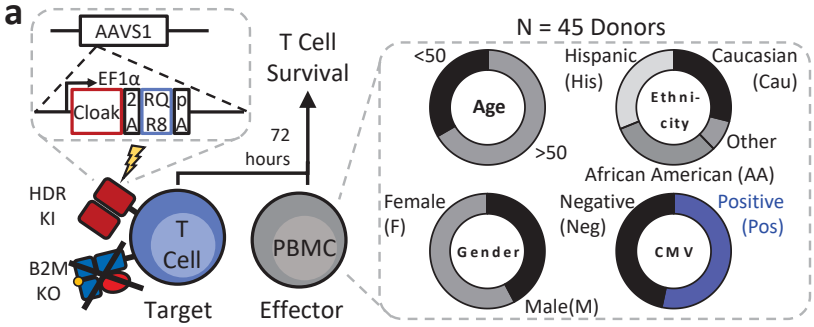
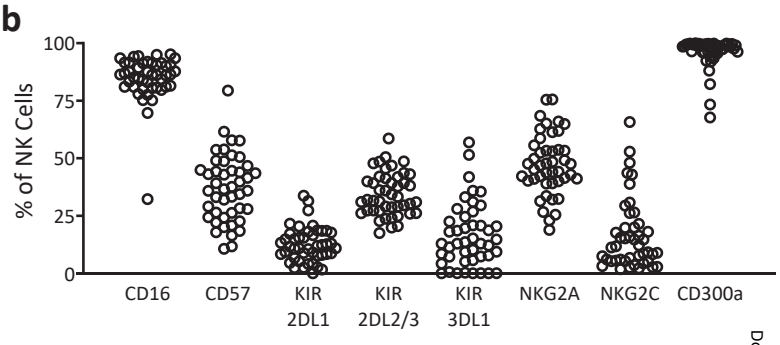
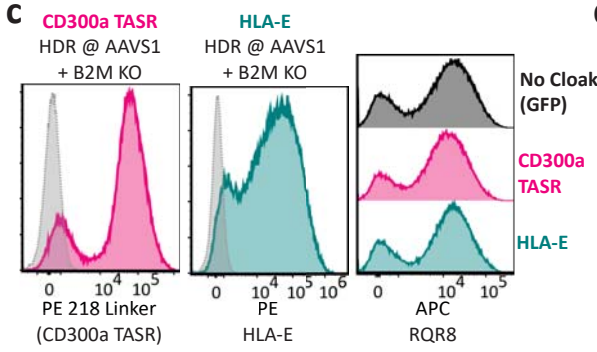
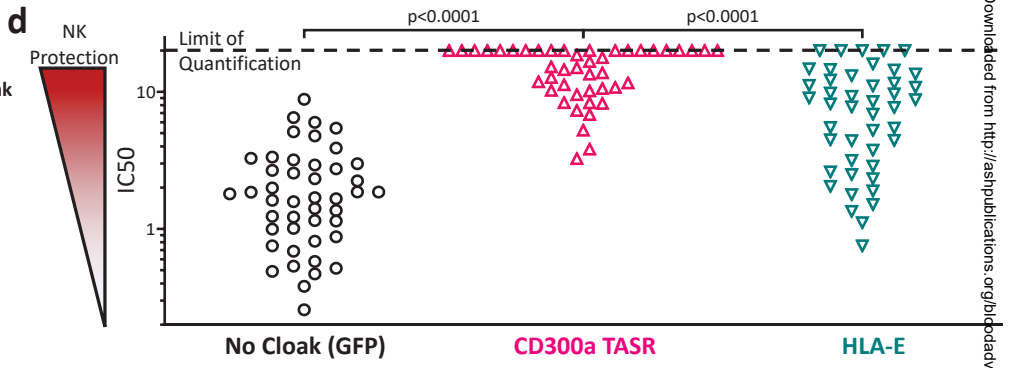
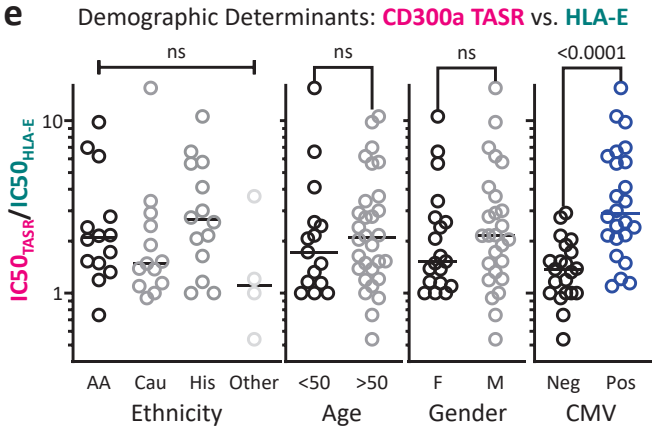
Figure 4

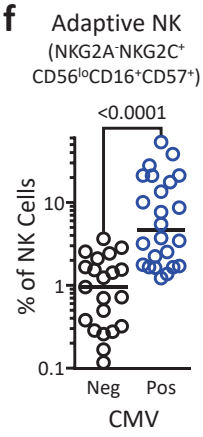












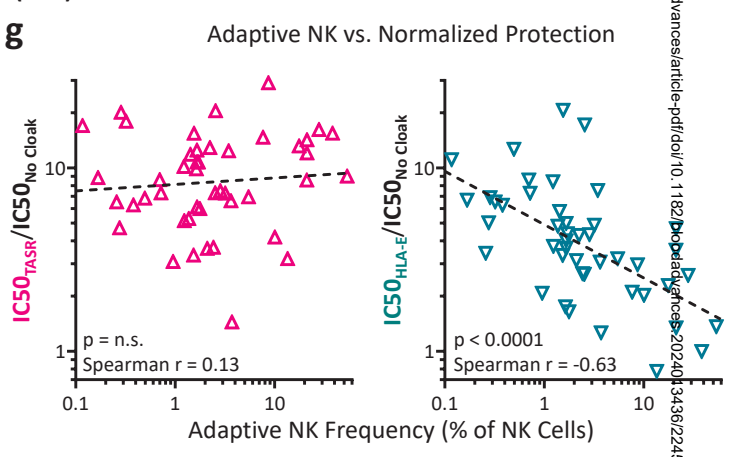


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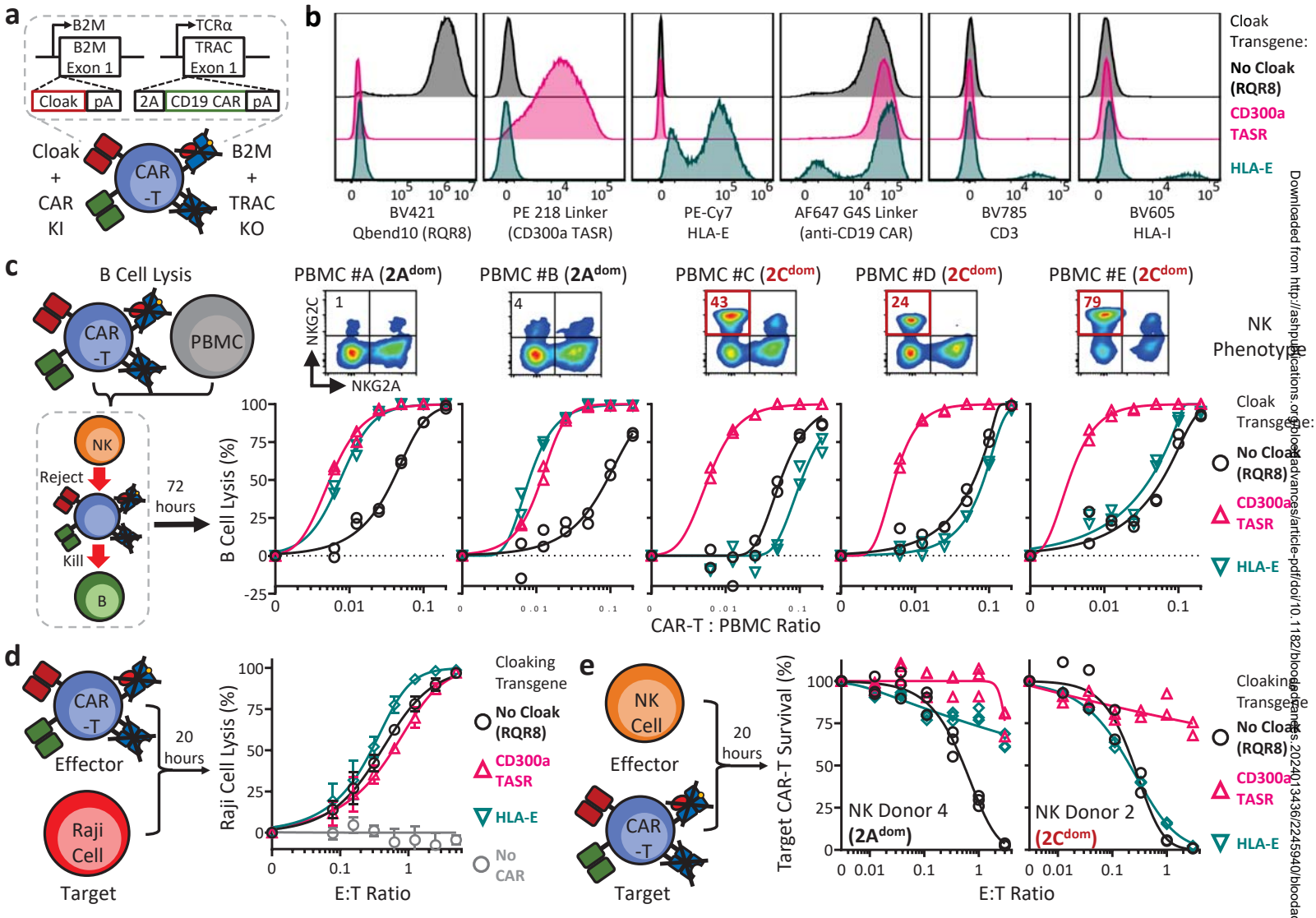
Figure 5



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