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Brief communication

Endowing human CD8 T cells with a veto-like recognition capacity via the electroporation of MHC-I/CD3ζ mRNA

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ABSTRACT

Graft-versus-host disease (GVHD) and transplant rejection as a result of host-versus-graft (HVG) response have remained two major complications of allogeneic hematopoietic stem cell transplantation (allo-HSCT). When donors are partially HLA-mismatched unrelated or haploidentical related, their severity correlates with the degree of HLA disparity. Specific elimination of alloreactive donor or recipient T cells targeting the mismatched HLA products could markedly alleviate both complications while only minimally affecting graft-versus-tumor (GVT) response or engraftment.

To redirect human CD8 T cells against alloreactive CD8 T cells we electroporate these cells with in-vitro-transcribed mRNA encoding MHC-I heavy chains fused with the signaling portion of CD3ζ. Here we show that peripheral blood human CD8 T cells expressing H-2K^b/CD3ζ or H-2K^d/CD3ζ respond to anti-MHC-I stimuli in a strictly specific manner. This study paves the way for further advancing this approach as a means to dampen GVHD and HVG that are caused by HLA disparity in allo-HSCT.

1. Introduction

Allo-HSCT is the treatment of choice for hematologic malignancies and non-malignant disorders and optionally for solid tumors [1–7]. Clinical HSC samples are prepared from donor bone marrow, peripheral blood or umbilical cord blood and usually contain significant numbers of T cells and other fully differentiated lymphocytes [2,3,8,9]. Both the GVT response and GVHD are largely attributed to these T cells and key to these immune reactions is the HLA makeup of both donor and recipient. Apart from GVHD, another potential complication in allo-HSCT is graft rejection due to an HVG response elicited, in part, by alloreactive anti-graft recipient T cells that endure the preconditioning protocol [2,3].

In the clinical setting of HLA-matched related donors the T cell-mediated GVT response is directed at HLA-bound peptides derived from polymorphic tissue-associated antigens referred to as minor histocompatibility antigens, or miHAs [4,5]. Distinct miHAs have been identified that are selectively expressed by hematopoietic cells and commonly serve as T cell targets in graft-versus-leukemia (GVL) reactivity. [4,5]. Yet, T cells reactive against miHAs that are expressed in non-hematopoietic tissues can elicit potent GVH responses [10,11]. Indeed, T cell depletion from bone marrow transplants of HLA-identical siblings resulted in a marked reduction in the level of acute and chronic GVHD, but also in a concomitant increase in leukemia relapse [12]. A retrospective comparison of HLA-matched unrelated donors with matched related donors revealed clear association of the former setting with lower relapse and superior progression-free survival that were not accompanied by increased severity of GVHD [13].

In reality, matched related or unrelated donors will not be available to a substantial proportion of patients requiring HSCT [14,15]. The use of partially HLA-mismatched unrelated donors or haploidentical related donors, which are readily available for most patients [16], are two alternative treatment options that are routinely implemented in the clinic. While the aforementioned miHAs-directed GVT and GVH reactivities across partial HLA disparity still influence the clinical outcome of HSCT, immune reactions are likely dominated by alloreactive responses directed against the products of mismatched HLA alleles. Clearly, the degree of HLA disparity correlates with GVHD and it has been repeatedly documented that a single HLA mismatch increases the risk of GVHD [17–20].

The marked effect of disparity in HLA-I alleles on both GVHD and allograft rejection points to a central role for alloreactive CD8 T cells in these complications. Undoubtedly, a major challenge in HSCT is how to blunt GVHD while only minimally affect GVT. In the case of HLA-I disparity, a highly selective approach for counteracting only alloreactive donor or recipient T cells which react against the mismatched

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HLA-I product(s) could reduce the incidence of both GVHD and graft rejection, respectively. At the same time, the beneficial GVT activity that is mediated by miHA-specific CD8 T cells which are restricted by the shared HLA-I alleles is expected to remain unaffected.

We previously converted MHC-I molecules into T cell activation receptors via a chimeric gene encoding the monomorphic MHC-I light chain β_2 microglobulin (β_2m), linked via a peptide bridge to the signaling domain of the T cell receptor (TCR) CD3ζ chain [21]. The resulting β_2m-ζ polypeptide was expressed in T cells as an integral membrane protein, paired with endogenous MHC-I heavy chains and triggered T cell activation upon heavy chain cross-linking. In principle, the expression of such a construct could endow T cells with a veto-like activity [22,23], defined as 'the ability of a cell to specifically suppress only T cells directed against its antigens' [23]. Yet, while potentially effective at eliminating alloreactive CD8 T cells, the expression of β_2m-ζ by recipient CD8 T cells can, in principle, also target donor CD8 T cells executing a beneficial GVT effect. The use of chimeric MHC-I-ζ receptors where the MHC-I component is the heavy chain of a mismatched allele can offer a more selective strategy for reducing the alloreactive response with only limited effect on the magnitude of the GVT activity.

The use of mRNA electroporation for delivery of in-vitro-transcribed mRNA into human immune cells, including T cells, routinely allows high yield and an exceptionally high level of uniform gene expression which is transient and safe for many therapeutic purposes (see, for example, our recently published work [24–26] and Levin et al. submitted and Weinstein-Marom et al., manuscript in preparation). Here we demonstrate, for the first time, that it is possible to redirect human CD8 T cells against MHC-I allele-specific targeting agents in a highly selective manner by virtue of chimeric MHC-I-ζ expression achieved via mRNA electroporation.

2. Materials and methods

2.1. Assembly of DNA constructs

DNA templates for mRNA encoding full-length H-2K^b and H-2K^d heavy chains have been cloned by reverse-transcriptase-PCR (RT-PCR) from mRNA of the mouse cell lines RMA and SP2/0, respectively. These DNA clones served as templates for cloning of the chimeric H-2K^b/CD3ζ (K^b-ζ) and H-2K^d/CD3ζ (K^d-ζ) genes, taking advantage of a *Pst*I restriction site at the α3-encoding sequence of both the H-2K^b and H-2K^d genes. The segment encoding the remaining ectodomain of K^b/ζ, including the N-terminal part of the bridge (Fig. 1) was PCR-amplified (Reddy Mix Extensor PCR Mix 1, Thermo Fisher Scientific, Waltham, MA) with the forward primer 5′ CGC CTG CAG GGG ATG GAA CCT TCC AGA AG 3′ and the reverse primer 5′ GCG CTC GAG GGC TCC CAT CTC AGG GTG AGG GG 3′, harboring an *Xho*I restriction site. The corresponding portion of K^d-ζ was similarly cloned with the forward primer: 5′ CGC CTG CAG GGG ATG GAA CCT TCC AG 3′ and the reverse primer 5′ GCG GCG GCC GCG GTG AAA TCC CCT GGC TG 3′. The DNA stretch encoding the C-terminal part of the bridge and human CD3ζ transmembrane and cytoplasmic domain was clones with the forward primer 5′ GCG CTC GAG CCA GCC CAC CAT CCC CAT CCT CTG CTA CCT GCT GGA TGG 3′ (with an *Xho*I site) and the reverse primer 5′ GCG GCG GCC GCG GTG AAA TCC CCT GGC TG 3′ containing a *Not*I site. The *Xba*I site at the 5′ end of the K^b- and K^d-encoding segments, together with the *Pst*I, *Xho*I and NotI sites were utilized for single-step cloning of the final K^b-ζ and K^d-ζ transcription units and inserted into the multiple cloning site of the pGEM4Z/EGFP/A64 vector (kindly provided by Dr. Eli Gilboa, University of Miami) (Fig. 1).

2.2. Cells

Jurkat cells were grown in complete RPMI 1640 medium containing 50 μM 2-mercaptoethanol, 1% non-essential amino acids, 2 mM L-glutamine, 10 mM HEPES (pH 7.4), 10% fetal-calf serum and combined antibiotics. Peripheral blood lymphocytes (PBLs) from a healthy individual were kindly provided by Dr. Yishai Ofran, Rambam Health Care Campus, Haifa, Israel (under Institutional Review Board approval RMB0141–10) and were grown in complete RPMI. CD8 T cells were sorted by positive selection using magnetic beads (BD Biosciences, San Jose, CA). Purified CD8 T cells were grown for 72 h in the presence of plate-bound anti-CD3 (OKT3) and 0.5 μg/ml soluble anti-CD28 mAbs and 100 U/ml recombinant IL-2 and then moved fresh medium with no antibodies or IL-2 for another 24 h.

2.3. mRNA preparation and electroporation

Plasmid DNA was linearized with *Spe*I. Transcription and capping reactions were performed with the T7 mScript Standard mRNA Production System (CellScript, Madison, WI) according to the manufacturer's instructions. Quality of the mRNA product was assessed by agarose gel electrophoresis and concentration was determined by spectrophotometric analysis. Purified mRNA was stored at −80 °C in small aliquots. Electroporation was performed with Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA) in cold 2 mm cuvettes at a concentration of 9×10^6 cells/100 μl, applying a square wave pulse of 400 V, 0.5 msec for Jurkat cells or at a concentration of 3×10^6 cells/100 μl and a square wave pulse of 500 V, 1 msec for human CD8 T cells. Following electroporation cells were transferred into tissue culture wells for 18 h and subjected to flow cytometry analysis.

2.4. Flow cytometry analysis

Cells were washed twice in 3 ml cold FACS buffer (PBS supplemented with 0.1% BSA and 0/1% sodium azide) and incubated on ice with primary antibody for 1 h. Cells were then washed twice with FACS buffer and incubated on ice for 20 min with secondary donkey anti-mouse IgG FITC or DyLight-conjugated antibodies (Jackson Laboratory, Bar-Harbor, ME). Following additional two washes cells were re-suspended in 300 μl FACS buffer and analyzed with FACSCalibur (Becton Dickinson, San Jose, CA). Data were analyzed by FCSexpress (DeNovo Software, Los Angeles, CA).

2.5. T cell activation assays

Jurkat cells: Nonspecific activation of Jurkat cells was performed in the presence of 10 ng/ml phorbol-12 myristate 13-acetate ester (PMA) and 0.5 μg/ml ionomycin. Transfected Jurkat cells or non-transfected controls were incubated with 5 μg/ml immobilized OKT3 (anti-human CD3), Y3 (anti-H-2K^b, the two antibodies were purified from B cell hybridomas) or SF1–1.1 (anti-H-2K^d, BD Pharmingen, Thermo Fisher Scientific) and 10 ng/ml PMA for 3.5 h at 37 °C. T cell activation was monitored by semi-quantitative RT-PCR (sqRT-PCR) of cellular mRNA using oligonucleotide primers specific for human IL-2.

Human CD8 T cell activation: Transfected and non-transfected purified CD8 T cells were incubated overnight at 37 °C with plate-bound OKT3, anti-H-2K^b or anti-H-2K^d antibodies (each of the latter two also serving as a negative control for the other). Growth medium was then collected and subjected to IFN-γ ELISA (R&D Systems Minneapolis, MN).

3. Results

3.1. Construct design and assembly

Four DNA constructs were cloned in the pGEM4Z/EGFP/A64 vector as templates for in-vitro transcription of mRNA: 1–2) K^b-ζ and K^d-ζ, encoding mouse H-2K^b and H-2K^d MHC-I heavy chains in which the transmembrane and cytoplasmic portions have been replaced with those of human CD3-ζ. 3–4) H-2K^b and H-2K^d, encoding the intact,

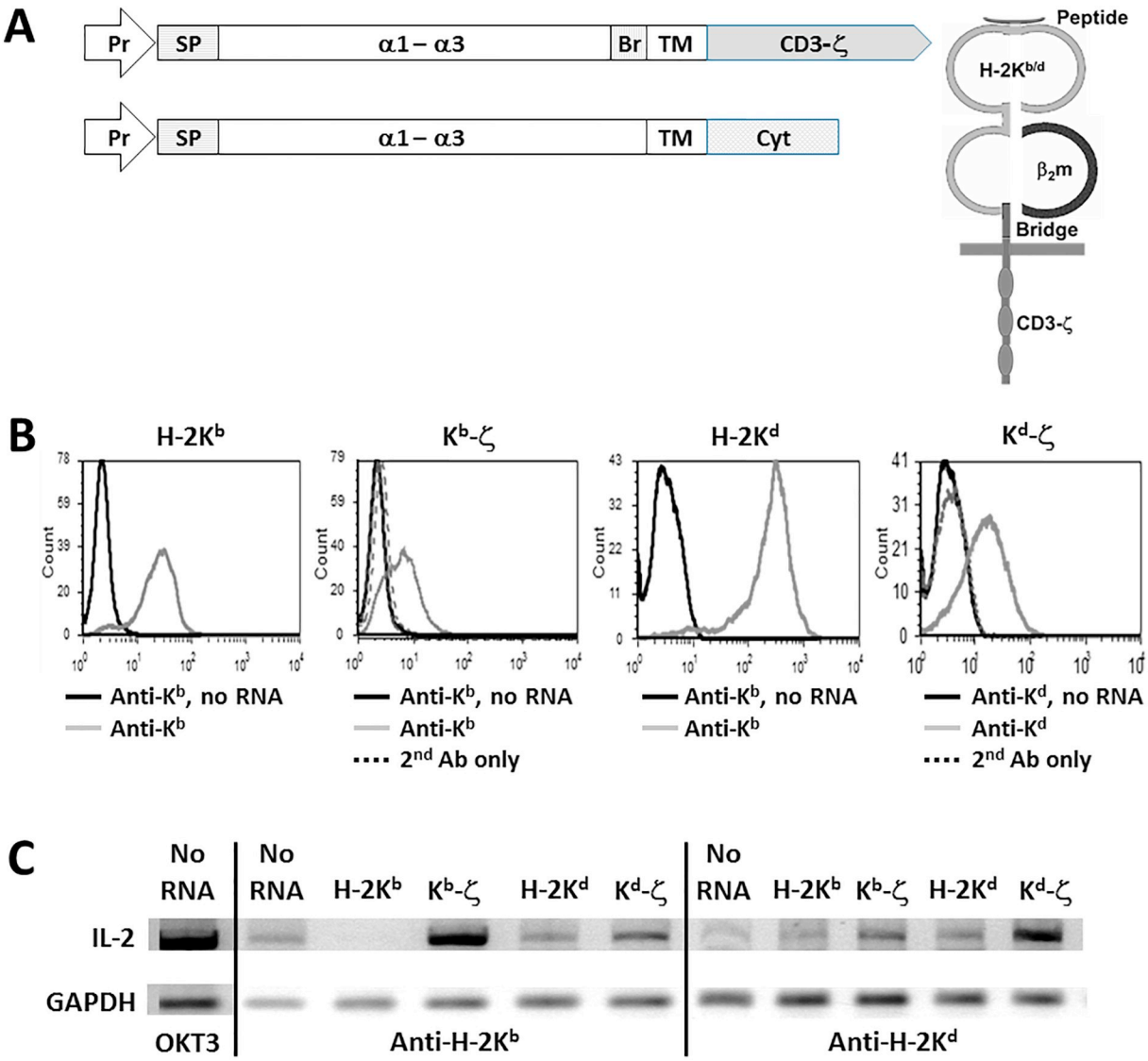

Figure 1

Fig. 1. Assembly, expression and function of the chimeric MHC-I-ζ receptors. A. Scheme of the genetic constructs and the anticipated protein products. SP, signal peptide; TM. Transmembrane; Cyt, cytoplasmic. B. Cell surface expression in Jurkat cells. Twenty four hours post-electroporation of Jurkat cells with all four mRNAs (as indicated above the panels), transfectants or non-transfected controls were stained with anti-H-2K^b or anti-H-2K^d mAbs followed by fluorophore-conjugated anti-mouse Abs or by anti-mouse Abs alone and subjected to flow cytometry analysis. C. Functional assessment of the constructs in Jurkat cells. Transfected cells were incubated overnight in the presence of the indicated mAbs. Cells were then harvested and their RNA was subjected to a semi-quantitative RT-PCR analysis using oligonucleotide primers specific for IL-2 or the housekeeping gene GAPDH, followed by agarose gel electrophoresis.

native H-2K^b and H-2K^d heavy chains, which served as positive controls for expression and negative controls for T cell activation (Fig. 1A).

3.2. Expression and function in Jurkat cells

To initially test surface expression of the chimeric constructs and evaluate their functionality in human T cells we chose the human Jurkat CD4 T cell line. Flow cytometry analysis revealed that both K^b-ζ and K^d-ζ were properly expressed at the cell surface of mRNA-transfected cells twenty four hours post-transfection, with H-2K^b > K^b-ζ and, similarly, H-2K^d > K^d-ζ (Fig. 1B). Of note, in both K^b-ζ and K^d-ζ the entire transmembrane domain of the heavy chain has been replaced with that of the ζ chain. There is evidence that the ER transmembrane chaperon calnexin which plays a role in the assembly of the MHC-I peptide loading complex in the ER may interact with the transmembrane domains of mouse MHC-I heavy chains [27,28]. Replacing this domain with that of the ζ chain may have reduced the efficacy of this process and, consequently, the level of surface expression. Furthermore, the ζ chain transmembrane cysteine residue which naturally forms CD3 ζ chain homodimers can potentially also mediate the formation of chimeric MHC-I-ζ homodimers, affecting the rate of complex assembly and surface expression.

To assess the functional potency of these constructs we employed a sqRT-PCR analysis for the level of cellular IL-2 mRNA following cell activation in the presence of immobilized anti-H-2K^b and anti-H-2K^d mAbs, where the anti-human CD3 mAb OKT3 serving as a positive control (Fig. 1C). Indeed, a reciprocal pattern of T cell activation could be observed, where only K^b-ζ transfectants could be activated by the

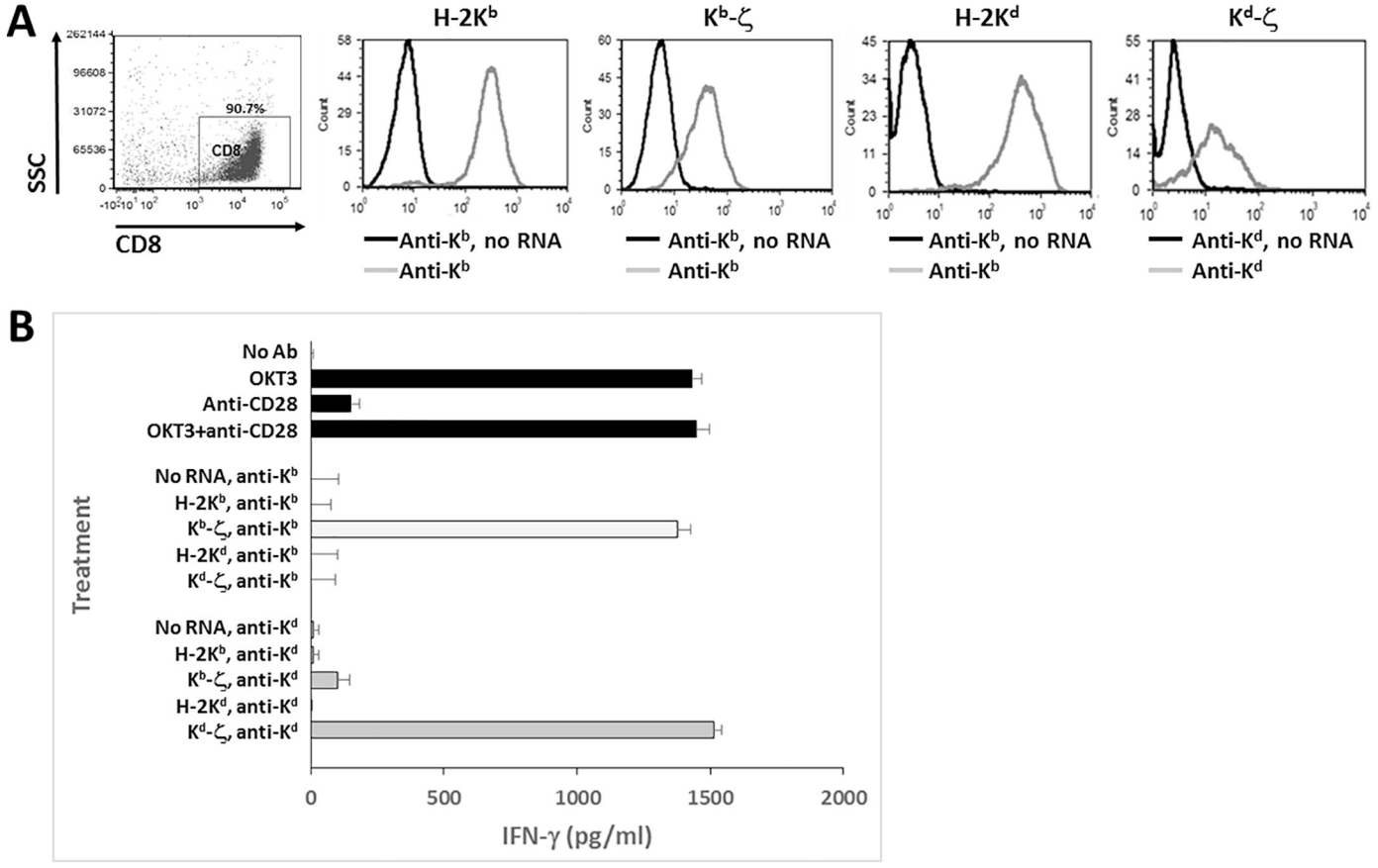

Fig. 2. Expression and function in PBL-derived human CD8 T cells. A. Cell surface expression of the four gene products was analyzed 24 h post-electroporation essentially as described above for Jurkat cells (Fig. 1B). B. T cell response to mAb-mediated cross-linking was monitored by a commercial IFN-γ ELISA kit, following overnight incubation of transfectants with immobilized anti-MHC-I mAbs in triplicate in 96 well plates. Results are presented as IFN-γ concentration in the growth medium.

anti-H-2K^b mAb while anti-H-2K^d could only activate K^d-ζ transfectants. Unlike, the two control mRNAs encoding native H-2K^b and H-2K^d did not confer any reactivity in response to stimulation with either of the two mAbs.

3.3. Expression and function in human CD8 T cells

Having established gene expression and reactivity of the products in the Jurkat human CD4 T cell line we went on to confirm expression and function in human CD8 T cells purified from PBLs of a health donor (Fig. 2A). Cell surface expression was analyzed 24 h post-electroporation and revealed >90% transfection efficacy and an expression pattern similar to that observed for Jurkat cells, namely, H-2K^b > K^b-ζ and H-2K^d > K^d-ζ (Fig. 2A).

We now examined the ability of the four constructs to activate transfectants in response to stimulation by the respective anti-MHC-I mAb. For this purpose we employed a similar experimental setting for T cell stimulation as described above for Jurkat cells, with the anti-human CD3 mAb OKT3 as a positive control, monitoring the secretion of IFN-γ to the growth medium (Fig. 2B). Indeed, as expected, only the right combinations of mAbs and MHC-I/ζ constructs triggered significant IFN-γ secretion.

4. Discussion

Allogeneic HSCT is a common treatment for various hematologic diseases, including different types of cancer. A desired GVT response is almost invariably accompanied by GVHD while an HVG reaction often prevents proper engraftment. The severity of these two complications increases in clinical procedures which involve partial HLA-I mismatch owing to a strong, two-way alloreactive response directed against the products of the mismatched allele(s). This work describes a gene-based strategy for the specific targeting of such alloreactive CD8 T cells.

Two related attempts in this direction have been described. In one, Nguyen et al. [29] assembled a series of chimeric receptors based on the mouse H-2K^b heavy chain (K^b), including K^b-ζ, K^b-CD28-ζ, K^b-CD28 and K^b-CD28-ζ-lck. These authors used retroviral transduction of mouse CD8 T cells to show that loading a synthetic antigenic-peptide selectively redirected these cells against antigen-specific T cells. In the other, McFarland et al. [30] generated transgenic mice expressing a chimeric H-2D^d construct under the control of the CD2 promoter (for assuring T cell expression) to assess whether it could affect H-2D^d allo-specific responses in-vitro and in-vivo. Surprisingly, these cells lacked TCR and displayed veto responses to H-2D^d in-vitro.

The current study provides the first demonstration that peripheral blood-derived human CD8 T cells can be endowed with an MHC-I-specific veto-like recognition capacity through the expression of chimeric MHC-I/CD3ζ receptors. Furthermore, these receptors can be expressed via the electroporation of in-vitro-transcribed mRNA, a safe and highly efficient gene delivery method which is increasingly practiced clinically in CAR-T cell therapy and other therapeutic applications [31]. Of note, repeated injections of mRNA-transfected cells can compensate, at least partially, for the transient nature of the introduced mRNA [32].

To dampen GVHD in the clinical setting, gene-modified recipient CD8 T cells will be co-cultured ex-vivo with the allograft and co-administered to the patient to maximize removal of alloreactive donor CD8 T cells. To eliminate alloreactive recipient CD8 T cells and confer allograft protection, similarly engineered donor CD8 T cells will be co-

transferred to the patient along with non-modified allograft cells. In this scenario, negative thymic selection will then act to reduce the emergence of donor-derived anti-recipient CD8 T cells and, consequently, GVHD.

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