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Selective immunotargeting of diabetogenic CD4 T cells by genetically redirected T cells

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Summary

The key role played by islet-reactive CD8 and CD4 T cells in type 1 diabetes calls for new immunotherapies that target pathogenic T cells in a selective manner. We previously demonstrated that genetically linking the signalling portion of CD3-ζ onto the C-terminus of β_2-microglobulin and an autoantigenic peptide to its N-terminus converts MHC-I complexes into functional T-cell receptor-specific receptors. CD8 T cells expressing such receptors specifically killed diabetogenic CD8 T cells, blocked T-cell-induced diabetes in immunodeficient NOD.SCID mice and suppressed disease in wild-type NOD mice. Here we describe the immunotargeting of CD4 T cells by chimeric MHC-II receptors. To this end we chose the diabetogenic NOD CD4 T-cell clone BDC2.5, which recognizes the I-A^{g7}-bound 1040-31 mimotope. We assembled several constructs encoding I-A^{g7} α- and β-chains, the latter carrying mim or hen egg lysozyme peptide as control, each supplemented with CD3-ζ intracellular portion, either with or without its transmembrane domain. Following mRNA co-transfection of reporter B3Z T cells and mouse CD8 and CD4 T cells, these constructs triggered robust activation upon I-A^{g7} cross-linking. A BDC2.5 T-cell hybridoma activated B3Z transfectants expressing the mimotope, but not the control peptide, in both configurations. Potent two-way activation was also evident with transgenic BDC2.5 CD4 T cells, but peptide-specific activation required the CD3-ζ transmembrane domain. Chimeric MHC-II/CD3-ζ complexes therefore allow the selective immunotargeting of islet-reactive CD4 T cells, which take part in the pathogenesis of type 1 diabetes.

Keywords: adoptive T-cell immunotherapy; chimeric receptors; mRNA transfection; T-cell activation; type 1 diabetes

Introduction

Type 1 diabetes (T1D) is an autoimmune disease that results from selective destruction of insulin-producing β cells in pancreatic islets by autoreactive CD8 and CD4 T cells. Cytotoxic CD8 T cells target β cells directly through MHC-I-bound peptides derived from endogenous, islet-specific proteins. MHC-II expression by β cells of T1D patients and non-obese diabetic (NOD) mice has been demonstrated, but it is not yet clear whether this expression is a trigger of disease or a response to inflammatory cytokines already present in the islets.[1] The current understanding is that T helper type 1 (Th1) CD4 T cells: (i) provide necessary help for autoreactive CD8 T cells both in the priming stage and in the effector phase; (ii) secrete pro-inflammatory cytokines, particularly tumour necrosis factor-α, interferon-γ (IFN-γ) and interleukin-1β (IL-1β), which exert a direct lytic effect on β cells; (iii) produce cytokines and chemokines that attract innate immune cells and activate them at the inflamed islets.[2–8] A diabetogenic role has also been attributed in recent years to IL-17-producing CD4 T cells (Th17)[9–12] but several studies maintain that the inflammatory conditions in the islets induce these cells to acquire an IFN-γ-secreting Th1 phenotype.[11,13–15]

The ongoing attempts to elucidate the contribution of Th1 cells to the initiation and progression of T1D have produced a growing list of MHC-II-restricted epitopes recognized by these cells. These have been identified both in humans, many of which restricted, by HLA-DR4 and

DQ8 and in the NOD mouse, binding I-A^{g7}.[16–18] These peptides derive from insulin (or pre-proinsulin), islet-specific glucose-6-phosphatase catalytic subunit-related protein, glutamic acid decarboxylases 65 and 67, heat-shock proteins 60 and 70), insulinoma-associated protein 2, zinc transporter ZnT8, islet amyloid polypeptide, chromogranin A and other self antigens. The discovery of these auto-antigenic peptides is not only instructive for understanding the immunopathological cascade in T1D, but also provides a valuable platform for the design of antigen-specific immunotherapies.[18–23]

Polyclonal T cells can be genetically redirected against an antigen of choice, using either an exogenous T-cell receptor (TCR) to target a conventional MHC-restricted epitope or chimeric antigen receptors (CARs), for recognizing cell surface antigens in an MHC-independent manner.[24–27] This approach is widely explored today, particularly in the field of cancer immunotherapy. We, and others, have realized that CARs can be used for targeting pathogenic T cells of known specificity. This is accomplished by incorporating the MHC ligand of the pathogenic TCR as the antigen-binding domain, so that pathogenic T cells are targeted by genetically modified T cells via CAR-TCR engagement. We recently employed this strategy for the selective immunotargeting of diabetogenic CD8 T cells. The underlying genetic design uses the invariant MHC-I light chain β_2-microglobulin (β_2m) as a scaffold. We first showed that the genetic engraftment of the intracellular domain of the CD3-ζ-chain onto the C-terminus of β_2m converts MHC-I molecules into activating TCR-like receptors when expressed in T cells.[28] Moreover, in the same work we showed that linking a selected MHC-I binding peptide to the N-terminus of β_2m redirects gene-modified T cells against CD8 T cells recognizing the particular MHC-I/peptide complex, offering a powerful tool for targeting autoreactive CD8 T cells displaying the corresponding specificity. For specific immunotargeting of diabetogenic CD8 T cells we chose the InsB15-23 peptide[29,30] and generated transgenic (Tg) NOD mice as a source for T cells expressing an InsB15-23/β_2m/CD3-ζ construct.[31] We recently reported that Tg CD8 T cells killed InsB15-23-reactive target CD8 T cells *ex vivo* and migrated to pancreatic lymph nodes of NOD mice *in vivo*. In adoptive transfer experiments, Tg CD8 T cells protected NOD.SCID mice from diabetes when co-transferred with the pathogenic T cells and significantly reduced the incidence of diabetes in wild-type NOD mice.[32]

In a series of reports, Geiger and his colleagues described an analogous genetic approach for immunotargeting autoreactive CD4 T cells. They exploited chimeric MHC-II/CD3-ζ polypeptides incorporating an autoantigenic peptide from myelin basic protein and the cytoplasmic (cyt) portion of CD3-ζ to target mouse or human encephalitogenic CD4 T cells.[33–37] This group demonstrated that the adoptive transfer of such redirected T cells to recipient mice eliminated antigen-specific CD4 T cells and suppressed experimental allergic encephalomyelitis.

For similarly targeting diabetogenic CD4 T cells we chose the I-A^{g7}-binding mimotope peptide (mim) recognized by the diabetogenic NOD CD4 T-cell clone BDC2.5.[38–40] We created several genetic constructs encoding I-$A^{d}\alpha$/CD3-ζ and mim/I-$A^{g7}\beta$/CD3-ζ or HEL/I-$A^{g7}\beta$/CD3-ζ (encoding the control hen egg lysozyme peptide), evaluating CD3-ζ either with the full transmembrane (tm) domain, including the cysteine residue, which allows ζ-ζ dimer formation or the cyt portion alone. For gene delivery into T cells we used *in vitro*-transcribed mRNA as a safe, effective and versatile methodology that can also be applied in the clinical setting.

Materials and methods

Cells and mice

B3Z is an $OVA_{257–264}$-specific, H-$2K^{b}$-restricted T-cell hybridoma harbouring the NFAT-lacZ reporter gene for T-cell activation,[41] a kind gift from Dr N. Shastri, University of California, Berkeley, CA.**2.5/BW1-31.3** (31.3) is a T-cell hybrid expressing the BDC2.5 TCR and was a kind gift from Dr K. Haskins, Colorado University, Denver, CO. Both cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids and combined antibiotics. NXA is a fusion between the A20 B-cell lymphoma and NOD splenocytes, which naturally express I-A^{g7}.

BALB/c mice were purchased from Harlan Laboratories (Rehovot, Israel). Transgenic BDC2.5 NOD mice carrying the rearranged TCR genes from the diabetogenic BDC2.5 T-cell clone were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in microisolators or individually ventilated cages in scantainers in a specific pathogen-free barrier facility. All procedures were performed in accordance with the protocols approved by the UK Home Office.

CD8 and CD4 T cells from homogenized spleens of female BALB/c mice were isolated with Anti-Mouse CD8 and Anti-Mouse CD4 Magnetic Particles (Becton Dickinson, San Jose, CA). Cells were washed and grown in six-well plates at 5×10^6 cells/well for 24 hr in the presence of IL-2 and IL-7. Cells were than washed and diluted to 1×10^6 cells/well and grown in the presence of IL-2 and IL-7 for another 24 hr before mRNA transfection.

Cloning and gene assembly

I-$A^{g7}\alpha$ (I-$A^{d}\alpha$) and β-chains were cloned by RT-PCR using mRNA template prepared from NOD splenocytes. All amplified fragments were cloned in the pGEM-T vector (Promega, Madison, WI) and their DNA sequence was

confirmed. Native I-$A^d\alpha$ was amplified with the forward primer 1221 and the reverse primer 1222 and I-$A^{g7}\beta$ with 1224 and 1225. The antigenic peptides were linked to the N-terminus of the mature β-chain through a short linker peptide, downstream to the leader peptide of mouse β_2m. As forward primer for both peptides we used 5′Xba lead; the reverse primer for mim was 1226 and for HEL 1227. As template we used a mouse β_2m clone. The extracellular domains of I-$A^{g7}\beta$ were cloned with primers 1228 and 1229 for the Pep/I-$A^{g7}\beta$/tmζ configuration and with primers 1228 and 1250 for Pep/I-$A^{g7}\beta$/cytζ. I-$A^d\alpha$/tmζ was amplified with primers 1221 and 1223 and I-$A^d\alpha$/cytζ with 1221 and 1248. The construction of the transmembrane and cytoplasmic portion of the mouse CD3-ζ-chain (tmζ) was described[28]. For the shorter, cytoplasmic-only portion of CD3-ζ (cytζ) we used primers 27246 and 1249. All final constructs were assembled in one step using the restriction sites incorporated into the respective primers and inserted into the pGEM4Z/GFP/A64 vector,[42] a kind gift from Dr E. Gilboa, University of Miami, FL, following the removal of the GFP insert.

PCR primers used for cloning (from 5′ to 3′):

1221: CGC TCT AGA CCA GAG ACC AGG ATG CCG TGC

1222: CGC GCG GCC GCC CTT CCT TTC CAG GGT GTG AC

1223: GCG GCT CGA GGG AAT CTC AGG TTC CCA GTG

1224: CCT CTA GAG CCT TAG AGA TGG CTC TGC AG

1225: CGC GCG GCC GCA CTG AGT CAA AAC ACT CTG AG

1226: GGG GGA TCC GCC CCC TCC GCT AGC CTC CAT CCT CAC CCA CAG AGG CCT CAC GTA AGC ATA CAA GCC GGT CAG

1227: GGG GGA TCC GCC CCC TCC GCT AGC CCT GTA GTT GTC CAG GCC GTG CCT TTT CAT AGC ATA CAA GCC GGT CAG

1228: GCG GGA TCC GGC GGA GGG GGA GAC TCC GAA AGG CAT TTC

1229: GGG CTC GAG GCC CTC CAC TCC ACA GTG ATG GG

5′Xbalead: GCG TCT AGA GCT TCA GTC GTC AGC ATG GCT CGC

1248: GCG CCC GGG TGA TCG CAG GCC TTG AAT GAT G

1249: GGC CCG GGA GAG CAA AAT TCA GCA GG

1250: GCG CCC GGG TTT CTG ACT CCT GTG ACG GAT G

27246: GGC CCG GGA GAG CAA AAT TCA GCA GG

mRNA synthesis and transfection

For *in vitro* transcription of mRNA, template DNA was prepared with the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA), linearized with *Spe*I and subjected to mRNA synthesis with the T7 mMessage mMachine Kit (Ambion, Austin, TX). For mRNA transfection, 3×10^6 B3Z cells in 100 μl were mixed in a 4-mm sterile electroporation cuvette with 10 μg of each mRNA. Transfection was performed with Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA) using a square wave pulse at 600 V, 1 ms.

Antibodies, peptides, reagents

The monoclonal hamster anti-mouse CD3 ε-chain 145-2C11 was purified from hybridoma supernatant. DyLight-conjugated donkey anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-mouse I-A^k monoclonal antibody (clone 10-3.6) binds I-$A^k\beta$ and cross-reacts with I-$A^{g7}\beta$ and was purchased from BioLegend (San Diego, CA). The FITC-conjugated goat anti-mouse IgG (Fab-specific) was from Sigma (St Louis, MO).

Mimotope 1040-31 (YVRPLWVRME) and HEL (MKRHGLDNYR) peptides were synthesized by GenScript Corp (Piscataway, NJ). For peptide loading, cells were washed twice with serum-free medium, incubated with peptide at 20 μg/ml for 2 hr at 37° in serum-free medium and then washed three times with PBS.

The IFN-γ ELISA was performed with a commercial kit from BioLegend or PeproTech (Rocky Hill, NJ).

Flow cytometry

Cells were harvested and washed twice with 2 ml FACS buffer (5% FCS, 0·1% sodium azide in PBS). Cells were stained with FITC-labelled antibody or unlabelled primary antibody at 1 μg/ml for 1 hr at 4°. Cells were washed twice with FACS buffer and incubated with 1 μg/ml of FITC-labelled secondary antibody for 1 hr at 4°. Cells were washed twice with FACS buffer, resuspended in 1 ml cold PBS and analysed by FACScalibur (Becton Dickinson). FACS results were analysed by FCS Express (De Novo Software, Los Angeles, CA).

B3Z activation assay

B3Z cells (5×10^4/well) were incubated at 37° overnight in triplicates in 96-well plates, either in wells precoated with activating monoclonal antibody (2C11 or anti-I-A^{g7}) or in the presence of activating cells at different ratios. Growth medium was removed and 100 μl of lysis buffer [9 mM $MgCl_2$, 0·125% Nonidet P-40, 0·3 mM CPRG (chlorophenol red-β-D-galactopyranoside) in PBS] was added to each well. 1–4 hr post-lysis optical density was monitored using a **Sunrise microplate reader (Tecan, Salzburg, Austria)** at 570 nm with 630 nm as reference.

Results

Construct assembly and expression

The BDC2.5 clone was isolated from a diabetic NOD mouse and first reported in 1990.[38,40,43,44] These cells are highly diabetogenic in young, asymptomatic NOD mice and are capable of transferring disease to NOD.SCID recipients. For more than two decades the identity of the islet autoantigen that activates these cells had remained elusive. Screening of BDC2.5 cells against a combinatorial decapeptide library[39] revealed several potently activating mimotopes, including 1040-31 (mim), which has since served in many studies on the pathogenic role of the BDC2.5 specificity. In 2010, a peptide with unusual binding to I-A^{g7} from chromogranin A, a protein found in the secretory granules of pancreatic β cells and additional neuroendocrine tissues, was identified as the natural BDC2.5 antigen.[40]

Two peptides were used in this study: mim and HEL. To evaluate our immunotargeting device, we assembled genes encoding four different heterodimeric configurations of I-A$^{d}\alpha$ and I-A$^{g7}\beta$ to produce the constructs shown in Fig. 1: (i) the native I-A^{g7} molecule; (ii) Pep/I-A^{g7}, comprising native I-A$^{d}\alpha$ and either mim or HEL linked to the N-terminus of the native I-A$^{g7}\beta$, yielding mim/I-A^{g7} and HEL/I-A^{g7}, respectively; (iii) Pep/I-A^{g7}/tm $\zeta\zeta$: mim/tm $\zeta\zeta$ and HEL/tm $\zeta\zeta$; (iv) Pep/I-A^{g7}/cyt $\zeta\zeta$ harboring the same peptides but lacking the CD3- ζ tm domain: mim/cyt $\zeta\zeta$ and HEL/cyt $\zeta\zeta$. Chimeric I-A$^{g7}\beta$ was provided with the leader peptide of mouse β_2m. The synthetic linker tethering the antigenic peptide to the C-terminus of the β-chain comprises ASGGGGSGGG[45]. For joining either α- or β-chain to the CD3-ζ moieties convenient restriction sites were incorporated that did not affect the amino acid composition. Following verification of the DNA sequences, all genetic constructs were cloned in the pGEM4Z/A64 vector to generate templates for *in vitro* transcription of mRNA.

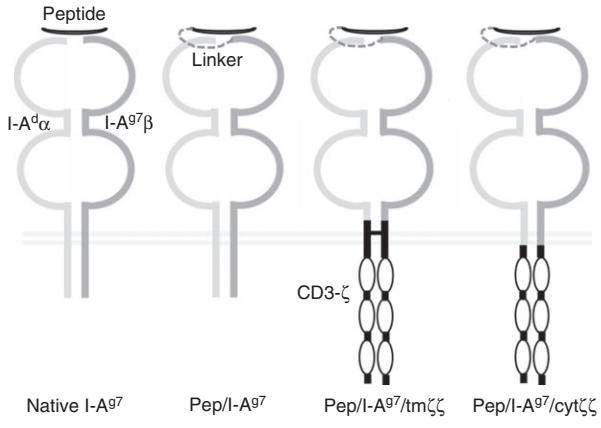

Figure 1. Scheme of native I-A^{g7} and the immunotargeting receptors at the cell surface. The four pairs of I-A$^{g7}\alpha$ and I-A$^{g7}\beta$ constructed and expressed in this work are shown.

Expression of the four chimeric I-A^{g7} T-cell activation receptors in the B3Z T-cell hybridoma following mRNA co-transfection of the separate α- and β-chain genes of each was confirmed by flow cytometry (Fig. 2). Although staining was moderate, it clearly revealed surface expression of all four, with HEL yielding a stronger signal than mim.

Functional assessment

Cross-linking of I-A^{g7} at the surface of T cells expressing the chimeric receptors is expected to trigger their activation similarly to TCR ligation. To test this assumption, we first incubated mRNA-transfected and non-transfected B3Z cells with immobilized anti-I-A^{g7} or anti-CD3 monoclonal antibodies and used a LacZ reporter assay to evaluate cell activation (Fig. 3a). Indeed, cross-linking of the two peptide/I-A^{g7}/tm$\zeta\zeta$ receptors, but not native I-A^{g7} resulted in robust T-cell activation, which was similar, or even stronger, in magnitude compared with that generated by TCR-mediated cross-linking. Marked activation was also observed with splenic CD8 and CD4 T cells from BALB/c mice co-transfected with mRNAs encoding two peptide/I-A^{g7}/tm $\zeta\zeta$-chains (CD8 > CD4) but not with mRNAs for the corresponding native peptide/I-A^{g7} configuration (Fig. 3b). Assessment of functional expression in B3Z cells at three time-points (Fig. 3c) revealed that the ability of all four receptors to transmit T-cell activation signal was higher than (for HEL), or comparable to (for mim) the endogenous TCR at least 24 hr post-transfection.

To assess peptide-specific activation of gene-modified B3Z cells by antigen-specific T cells we first used the T-cell hybrid of the BDC2.5 clone **designated 2.5/BW1-31.3** (31.3). The results of this experiment are presented in Fig. 4 and reveal that while transfectants expressing both mim- and HEL-bearing receptors responded robustly and in comparable magnitude to antibody-mediated cross-linking of I-A^{g7}, only transfectants expressing both configurations of mim, but not HEL, were activated by the 31.3 hybrid in a dose-dependent manner.

We expect that the engagement of gene-modified T cells and their target autoreactive T cells would lead to the activation of both cell populations. To confirm that this is indeed the case, we used as target cells CD4 T cells purified from BDC2.5 Tg mice. We took advantage of the fact that B3Z and BDC2.5 Tg T cells allow the simultaneous performance of two activation assays that fully discriminate between the two cell types: the enzymatic CPRG assay would only detect LacZ activity in activated B3Z cells whereas only the Tg T cells, but not B3Z, secrete IFN-γ in response to TCR cross-linking (as confirmed in

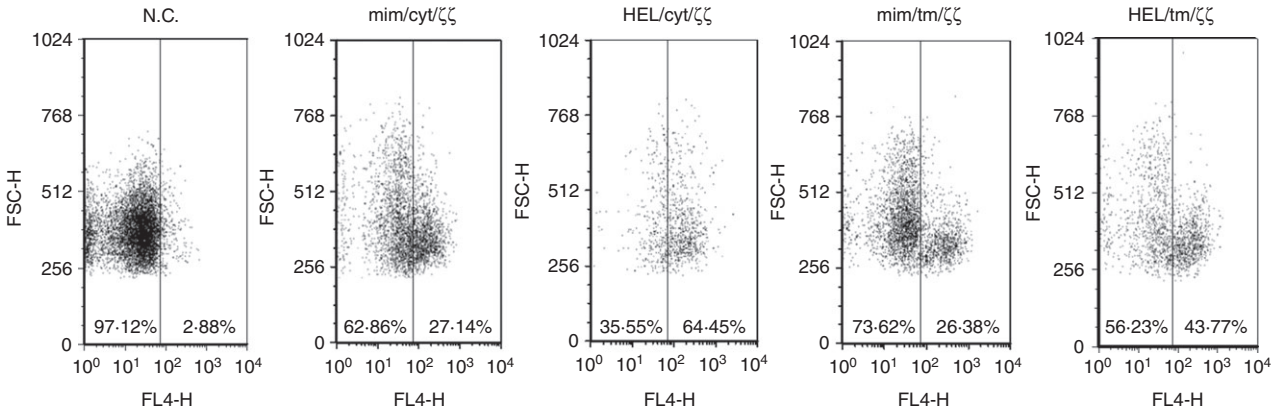

Figure 2. Flow cytometry analysis of B3Z cells for the expression of the chimeric I-A^{g7} activation receptors. Following electroporation of B3Z cells with the indicated mRNA or none (N.C.), cells were incubated overnight in triplicate in a 96-well plate in the presence of immobilized anti-I-A^{g7} monoclonal antibody. Cells were then detached from the plate by pipetting and stained for I-A^{g7} using DyLight-conjugated anti-mouse IgG antibodies.

a preparatory experiment and see Fig. 5a). The two assays (Fig. 5) reveal that both B3Z transfectants and BDC2.5 T cells were activated in a peptide-specific manner only by the tm ζζ. Unexpectedly, the cytζζ configuration triggered a considerable T-cell response not only from mim construct but also from HEL. Interestingly, this was neither the case for B3Z cells co-incubated with the 31.3 hybrid, which bears the same BDC2.5 TCR (Fig. 4), nor for the BDC2.5 CD4 T cells co-incubated with HEL-loaded NXA cells (Fig. 5a).

Discussion

An ultimate challenge in the immunotherapy of T1D is to suppress, or eliminate pathogenic T cells in a selective and effective manner. The gene-based device for immunotargeting islet-reactive CD4 T cells presented here is complementary to our genetic approach for targeting diabetogenic CD8 T cells[31,32] (and Fishman, S. *et al.*, manuscript in preparation) and is designed to meet these requirements.

Peptide selectivity was clearly evident for the tmζζ configuration as B3Z transfectants were activated by both the BDC2.5 31.3 hybrid and the Tg BDC2.5 T cells when presenting mim, but not HEL (Figs 4 and 5). Unlike the tmζζ configuration, the cytζζ constructs exhibited this selectivity only upon interacting with the hybrid, but not with the Tg T cells. However, in the same experiment these Tg T cells fully discriminated between the two peptides when these were presented by the native I-A^{g7} molecule on NXA cells (Fig. 5a). A partial explanation for this peculiarity may relate to the fact that the cytζζ configuration, which was also used by Geiger *et al.*,[33,37] is devoid of the transmembrane domain of the ζ-chain. This domain includes the cysteine residue responsible for ζ-chain dimerization and the aspartic acid, which is critical for the formation of the full TCR–CD3 complex.[46] The full ζ-chain transmembrane domain is also incorporated in the β_2m/CD3-ζ constructs that we have been using in our parallel study. Indeed, the chimeric polypeptide product of this construct was efficiently co-immunoprecipitated from T-cell detergent lysate with the endogenous CD3 ε-chain (Cafri, G, M.Sc. dissertation, unpublished observations), suggesting that tmζζ may also interact with the TCR–CD3 complex. Such interactions may, for example, subject tmζζ, but not cytζζ, receptors to the same regulation of TCR signalling thresholds allegedly executed by the associated transmembrane tyrosine phosphatase CD45.[47,48] Exclusion of cytζζ receptors from CD45 regulation may result in diminished receptor selectivity, which can be manifested in ζ-chain signalling following lower affinity interactions. The different outcome of engagement with the hybrid's TCR and the basis for the reciprocal effect observed for the activation of the Tg BDC2.5 T cells remain unexplained. Regardless of the explanation for these observations, the tmζζ composition displays the anticipated selectivity and is our configuration of choice for further investigation.

Although optimizing in-cell stability of the introduced mRNA and maximizing level and duration of expression were beyond the scope of this study, activation signals transmitted by the encoded receptors in B3Z cells and mouse splenic T cells were comparable in magnitude, and even superior, to those transmitted by the endogenous TCR (Figs 3–5). These results demonstrate the potency of mRNA electroporation and suggest that the moderate transfection yield and expression level that were detected by the flow cytometry analysis (Fig. 2) may reflect steric hindrance at the T-cell surface, which prevented antibody binding, rather than true paucity of functional MHC-II receptors.

The use of viral vectors for immunotherapeutic purposes entails the random integration of vector sequences

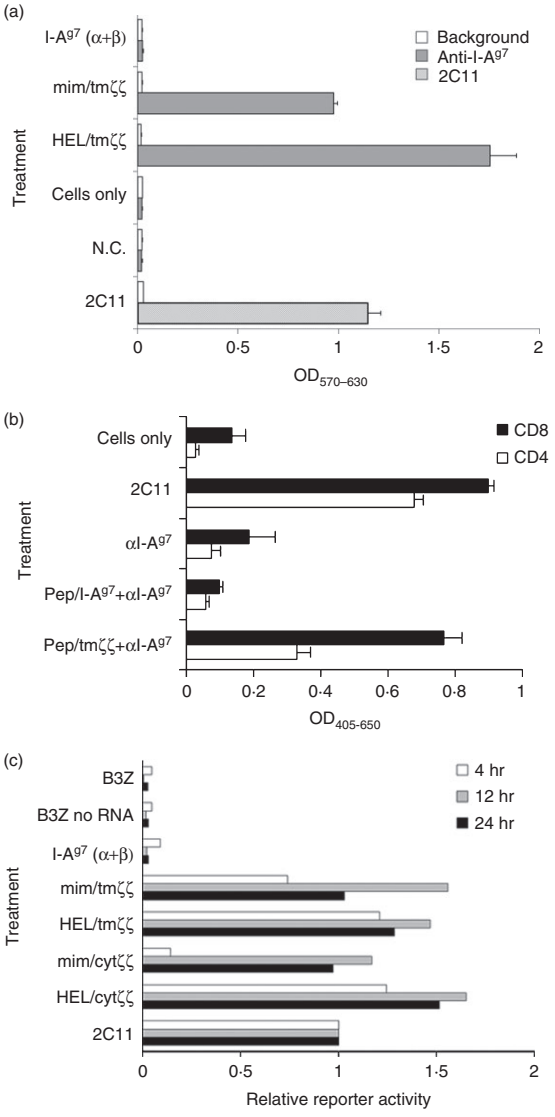

Figure 3. Activation of mRNA-transfected T cells with the anti-I-A^{g7} antibody. (a) B3Z cells were transfected with mRNAs encoding the two peptide/I-A^{g7}/tmζζ receptors and were incubated with the immobilized anti-I-A^{g7} antibody. Non-transfected cells and the anti-CD3 ε-chain monoclonal antibody 2C11 served as negative and positive controls, respectively. (b) Activation of BALB/c CD8 and CD8 T cells by peptide/I-A^{g7}/tmζζ. CD8 and CD4 T cells were co-transfected with the mRNAs pairs encoding either HEL/I-A^{g7}/tmζζ or HEL/I-A^{g7} (native). Transfected and non-transfected cells were incubated with the anti-I-A^{g7} antibody or 2C11 as control and 24 hr later the level of interferon-γ in the growth medium was monitored by ELISA. (c). Cell activation triggered by the four receptors relative to T-cell receptor-mediated activation monitored at the indicated time-points post-mRNA transfection. B3Z transfectants cells were incubated for 18 hr in triplicate in 96-well plates, coated with anti-I-A^{g7} antibody or non-coated wells as background. Non-transfected B3Z cells were similarly activated by immobilized 2C11 monoclonal antibody as a reference. Cells were then lysed and subjected to the β-Gal enzymatic assay using the colorimetric CPRG substrate. Non-transfected B3Z cells or cells electroporated with no RNA were used as negative controls.

into the host cell genome and is associated with the risk of insertional mutagenesis and cellular transformation. This risk is alleviated by the use of mRNA for gene delivery. Electroporation of mRNA is also fast, simple and efficient, usually drives high and uniform expression and requires only mild conditions, thereby preserving cell viability. Importantly, this methodology allows the co-introduction of several genes as pre-defined mixtures, which is often a limiting factor with viral vectors. Here we report the successful co-transfection of mRNA for delivering the two MHC-II-based immunotargeting genes into the same effector T cells. In a parallel study on peptide/MHC-I/CD3-ζ constructs (Fishman, S. *et al.*, manuscript in preparation) we present data on the efficient co-introduction of two mRNAs into primary mouse CD8 T cells.

What are the prospects for clinical implementation of such an approach? Exploiting mRNA for genetically redirecting primary human T cells against tumour antigens has gained considerable momentum in recent years (e.g. refs 49–52) and is currently evaluated in the clinic (ClinicalTrials.gov identifiers NCT01355965 and NCT01897415). In parallel, new methods are constantly developed for large-scale, clinical grade production and administration of mRNA.[53,54] Notably, essential steps toward the generation of off-the-shelf, universal human T cells for therapeutic use have been reported. Such cells are genetically edited to lack both TCR to prevent graft-versus-host reaction[55] and HLA molecules to avoid rejection.[56] When such a feat is clinically approved the need to derive and propagate autologous T cells will be obviated, rendering adoptive T-cell transfer a more acceptable treatment option not only for cancer but also for autoimmune diseases such as T1D.

Can the targeting of T cells reactive against a single self-peptide/MHC complex suffice to elicit a significant beneficial effect? In our previous *in vivo* studies evaluating the immunotargeting of CD8 T cells that recognize the InsB15-23/H-2K^{d} complex[32] 11/13 of NOD mice that received activated CD8 T cells from control NOD mice developed spontaneous diabetes compared with only 3/9 mice that received activated InsCD3-ζ transgenic CD8 T cells ($P = 0{\cdot}019$). These results are in line with previous studies in the NOD mouse indicating that CD8 T-cell clones specific to the InsB15-23/H-2K^{d} complex are the predominant population in the earliest CD8 T-cell infiltrate.[30,57] Yet, human T1D is complex and even if distinct T-cell specificities dominate the early infiltrate, epitope spreading is likely to obscure such putative hierarchy at later stages,[18,58] rendering the immunotargeting of a single specificity less feasible. One way to tackle this problem is to simultaneously target a number of specificities documented to prevail in the peripheral blood of T1D patients. Another intriguing approach is to deliver mRNA encoding peptide/MHC-I/CD3-ζ and peptide/MHC-II/CD3-ζ receptors not only to CD8 T cells, but

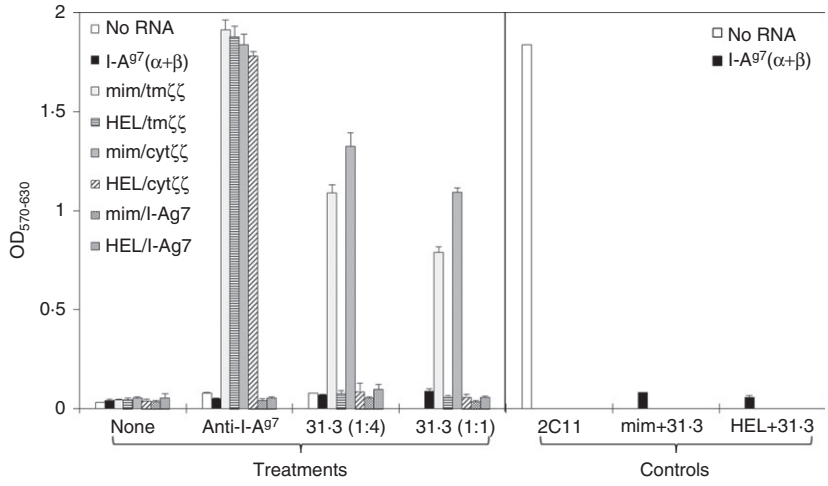

Figure 4. Activation of mRNA-transfected B3Z cells by a T-cell hybrid. B3Z cells were transfected with the mRNAs specified in the legend and incubated with either monoclonal antibodies, 31.3 cells at two 31.3:B3Z ratios (1 : 4 and 1 : 1) or at 1 : 1 ratio following loading with the synthetic peptides. Incubation with anti-CD3 (2C11) was performed only for non-treated B3Z or 31.3 cells. mim/I-A^{g7} HEL/I-A^{g7} mRNAs encode the corresponding peptide linked to native I-A^{g7} (Pep/I-A^{g7}, see Fig. 1). T-cell activation was monitored with CPRG. Representative results of two independent experiments are shown.

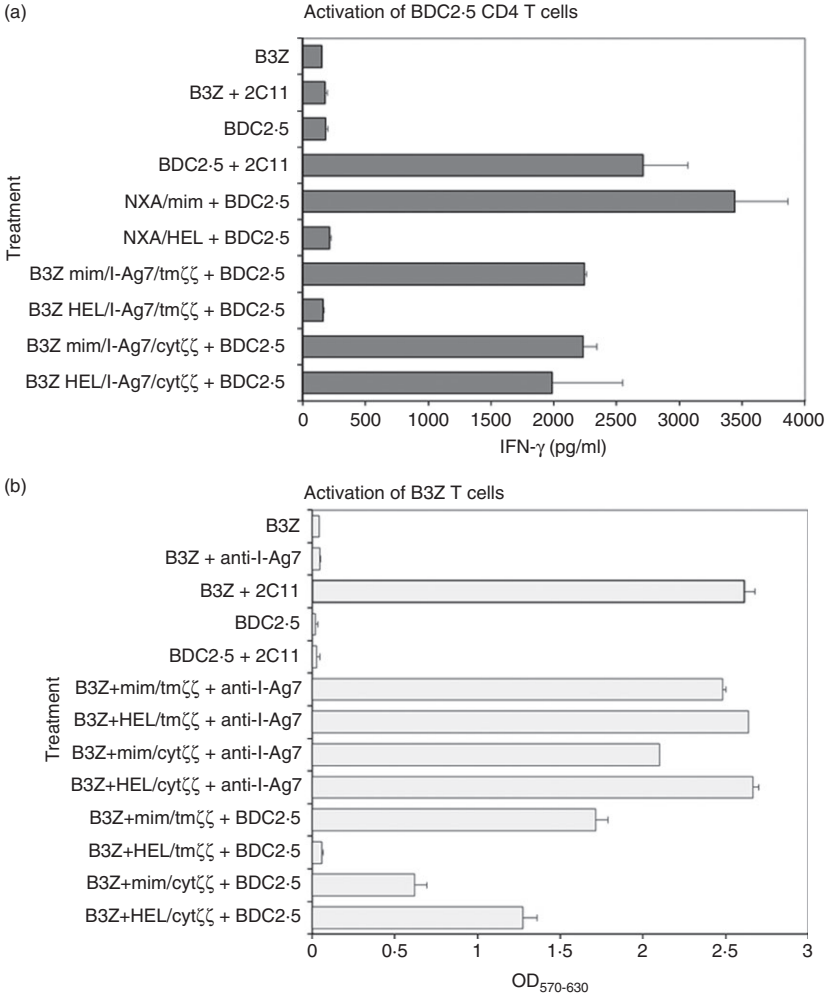

Figure 5. Messenger RNA encoding mim/tmζζ triggers peptide-specific, two-way T-cell activation. B3Z cells were transfected with the indicated mRNAs and co-incubated at 1 : 1 ratio for 24 hr with transgenic BDC2.5 CD4 T cells. (a) Secretion of interferon-γ (IFN-γ) to the assay growth medium was evaluated by ELISA (expressed as OD_{450}). NXA (NOD × A20) cells naturally expressing I-A^{g7} were loaded with each of the synthetic peptides to serve as activation reference. (b) B3Z activation was recorded with the CPRG assay. Incubation of non-transfected B3Z cells with the anti-CD3 (2C11) and anti-I-A^{g7} monoclonal antibodies served as positive and negative controls, respectively.

also to CD4 regulatory T (Tregs) cells, either natural or induced. In spite of the transient nature of mRNA expression, the immunotargeting of islet-reactive CD8 and CD4 cells by transfected Treg cells can potentially exert a durable tolerizing effect through the induction of infectious tolerance. While infectious tolerance was originally reported in a transplant acceptance setting, several studies have demonstrated that it can operate in autoimmune diseases, including diabetes[59–62] and see ref. 62 for a recent review. Importantly, redirected Treg cells from peptide/MHC-II/CD3-ζ Tg mice generated in the experimental allergic encephalomyelitis model prevented disease

and could also cure recipient mice even when diversification of the autoreactive T-cell repertoire could already be manifested.[36] Evidently, the adoptively transferred redirected Treg cells suppressed disease by converting autoreactive CD4 T cells into adaptive Treg cells that functioned independently of their inducer cells, which is the hallmark of infectious tolerance. In an attempt to harness reprogrammed Treg cells and their ability to initiate infectious tolerance for the treatment of T1D we are currently evaluating CD4 Treg cell reprogramming via mRNA in NOD mice.

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Disclosures

The authors have no conflicts of interest.

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