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Adoptive Transfer of mRNA-Transfected T Cells Redirected against Diabetogenic CD8 T Cells Can Prevent Diabetes

Sigal Fishman,[1,2,5] Mark D. Lewis,[3,5] L. Khai Siew,[3,5] Evy De Leenheer,[3,5] Dimitri Kakabadse,[3] Joanne Davies,[3] Doron Ziv,[1,4] Alon Margalit,[1,4] Nathan Karin,[2] Gideon Gross,[1,4,6] and F. Susan Wong[3,6]

[1]Laboratory of Immunology, MIGAL – Galilee Research Institute, Kiryat Shmona 11016, Israel; [2]Department of Immunology, Rappaport Family Institute for Research in the Medical Sciences, Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Haifa 3525433, Israel; [3]Diabetes Research Group, Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff University, Cardiff CF14 4XN, UK; [4]Department of Biotechnology, Tel-Hai College, Upper Galilee 12210, Israel

Chimeric major histocompatibility complex (MHC) molecules supplemented with T cell receptor (TCR) signaling motifs function as activation receptors and can redirect gene-modified T cells against pathogenic CD8 T cells. We have shown that β_2 microglobulin (β_2m) operates as a universal signaling component of MHC-I molecules when fused with the CD3-ζ chain. Linking the H-2K^d-binding insulin B chain peptide insulin B chain, amino acids 15–23 (InsB$_{15–23}$) to the N terminus of β_2m/CD3-ζ, redirected polyclonal CD8 T cells against pathogenic CD8 T cells in a peptide-specific manner in the non-obese diabetic (NOD) mouse. Here, we describe mRNA electroporation for delivering peptide/β_2m/CD3-ζ genes to a reporter T cell line and purified primary mouse CD8 T cells. The peptide/β_2m/CD3-ζ products paired with endogenous MHC-I chains and transmitted strong activation signals upon MHC-I cross-linking. The reporter T cell line transfected with InsB$_{15–23}$/β_2m/CD3-ζ mRNA was activated by an InsB$_{15–23}$-H-2K^d-specific CD8 T cell hybrid only when the transfected T cells expressed H-2K^d. Primary NOD CD8 T cells expressing either InsB$_{15–23}$/β_2m/CD3-ζ or islet-specific glucose-6-phosphatase catalytic subunit-related protein, amino acids 206–214 (IGRP$_{206–214}$)/β_2m/CD3-ζ killed their respective autoreactive CD8 T cell targets in vitro. Furthermore, transfer of primary CD8 T cells transfected with InsB$_{15–23}$/β_2m/CD3-ζ mRNA significantly reduced insulitis and protected NOD mice from diabetes. Our results demonstrate that mRNA encoding chimeric MHC-I receptors can redirect effector CD8 against diabetogenic CD8 T cells, offering a new approach for the treatment of type 1 diabetes.

INTRODUCTION

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease in which both CD4 and CD8 T cells (CTLs) target insulin-producing islet β cells. In human T1D, islet-specific CTLs have been identified and histology shows CTLs in the islets, whereas in the non-obese diabetic (NOD) mouse, CTLs are implicated in the initial stages as well as in progression of disease.[1–6] Selective immunotargeting of diabetogenic CTLs is therefore a promising avenue for immunotherapy of T1D.

The CD3-ζ chain is an essential signaling component of the T cell receptor (TCR) complex. T cells genetically redirected through major histocompatibility complex (MHC)-I heavy (α) chains fused with CD3-ζ and supplemented with a peptide of choice can target peptide-specific CD8 T cells, initially achieved through the expression of MHC-Iα/CD3-ζ fusion proteins. For example, T cells expressing chimeric H-2K^b/CD3-ζ and pulsed with a distinct peptide exhibited efficient cytolysis of antigen-specific cytotoxic CTL precursors.[7] Furthermore, transgenic T cells of a unique memory phenotype expressing an H-2D^d/CD3-ζ construct potently vetoed responses to H-2D^d in vitro.[8] The addition of a cognate H-2D^d peptide endowed these transgenic cells with cytolytic activity against an antigen-specific T cell hybridoma. The polymorphic MHC-I heavy chain is non-covalently associated with an invariant, non-MHC-encoded β_2 microglobulin (β_2m) light chain, not anchored to the plasma membrane. We have shown that β_2m can serve as a versatile molecular scaffold for chimeric MHC-I/CD3-ζ T cell activation receptors.[9] A single β_2m/CD3-ζ-based expression cassette enables covalent linking of any pre-selected peptide to the N terminus of β_2m, so as to redirect T cells at autoreactive CD8 T cells of a given specificity.

A number of cloned diabetogenic CTLs from the NOD mouse target identified antigens. Proinsulin is a major target antigen for diabetogenic CTLs, both in the NOD mouse[10] and in humans.[11–17] G9C8 is a highly pathogenic CTL clone that recognizes insulin B chain, amino acids 15–23 (InsB$_{15–23}$) in the context of H-2K^d in the

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[5]These authors contributed equally to this work.

[6]These authors contributed equally to this work.

Correspondence: Gideon Gross, Laboratory of Immunology, MIGAL – Galilee Research Institute, Kiryat Shmona 11016, Israel.
E-mail: gidi@migal.org.il

NOD mouse,[10,18] and the cells are a predominant population in the early CD8 T cell infiltrate detected as early as 4 weeks of age.[10,19] Later, CD8 T cells reactive against an H-2K^d-binding peptide from islet-specific glucose-6-phosphatase catalytic subunit-related protein, amino acids 206–214 (IGRP$_{206–214}$)[20–23] become dominant. A third islet-reactive, pathogenic NOD CTL, although initially thought to be specific to a dystrophia myotonica kinase, amino acids 138–146 (DMK$_{138–146}$) peptide, is actually reactive to insulin.[23–25] Interestingly, the relative distribution in the infiltrate of T cells varies considerably among individual mice, defining a unique immunological signature.[20–23] CD8 T cells reactive to glutamic acid decarboxylase (GAD65)—especially GAD65, amino acids 546–554 (GAD65$_{546–554}$)—have also been identified in the NOD mouse.[26,27]

Immune responses to proinsulin are necessary for IGRP-reactive CTLs to expand[28,29] and to cause diabetes. Therefore, early immunological intervention selectively targeting dominant CTL clones may arrest β cell destruction and inhibit, or entirely prevent, the onset of disease. As a proof of concept, we previously generated NOD mice expressing an InsB$_{15–23}$/β$_2$m/CD3-ζ construct in CD8 T cells.[30] CTLs from these mice killed InsB$_{15–23}$-reactive target CD8 T cells and protected NOD SCID (severe combined immunodeficiency) mice from diabetes when co-transferred with the pathogenic T cells and significantly reduced spontaneous diabetes in wild-type NOD mice.[31]

Transfection of mRNA to modify primary human and mouse T cells has drawn considerable interest. Electroporation of mRNA is fast, simple, and exceptionally efficient and drives high and uniform expression under mild conditions, thereby preserving cell viability. Although transient, mRNA transfection can drive functional expression of the introduced genes up to 5–7 days and more.[32–36] The use of mRNA entirely obviates the risk of cellular transformation and allows the co-introduction of several genes as pre-defined mixtures, which is often limited with other gene delivery vehicles.

Here we show that CD8 T cells can be reprogrammed to recognize diabetogenic T cells following the electroporation of mRNA encoding peptide/β$_2$m/CD3-ζ and this can target autoreactive CTLs in vivo to reduce insulitis and prevent autoimmune diabetes in the NOD mouse.

RESULTS

Assembly and Expression of Peptide/β$_2$m/CD3-ζ Constructs in Reporter T Cells

We first assembled four DNA templates for in vitro transcription of mRNA encoding peptide/β$_2$m/CD3-ζ, incorporating InsB$_{15–23}$, IGRP$_{206–214}$, DMK$_{138–146}$, or GAD65$_{546–554}$. Figures 1A and 1B, respectively, illustrate the genetic design and the anticipated MHC-I complexes comprising the chimeric polypeptide chains. Human β$_2$m (hβ$_2$m), which was used throughout this study, has been shown to associate efficiently with mouse MHC-I heavy chains[37–39] and is useful for detecting expression of the resulting MHC-I complexes. Flow cytometric analysis confirmed the expression of hβ$_2$m at the cell surface of CD8 T cells purified from BALB/c and NOD mice and the reporter B3Z T cell hybridoma following mRNA transfection (Figure 1C). This expression was reproducible over many experiments. We then evaluated the effect of the amount of InsB$_{15–23}$/β$_2$m/CD3-ζ mRNA used for electroporation and level of expression in B3Z cells. Indeed, both flow cytometry and western blot analyses for hβ$_2$m (Figures 1D and 1E) revealed dose-dependent expression, which was not accompanied by a reduction in cell viability at the higher mRNA concentrations (not shown). Western blot analysis, which was performed under reduced conditions, revealed a band of approximately 33 kDa, in accordance with the calculated molecular weight of the InsB$_{15–23}$/β$_2$m/CD3-ζ polypeptide product.

Function of Peptide/β$_2$m/CD3-ζ Constructs in Reporter T Cells

We tested whether the new MHC-I complexes expressed at the cell surface can indeed function as T cell activation receptors. To this end, we transfected B3Z reporter cells with mRNA encoding each of four peptide/β$_2$m/CD3-ζ polypeptides, with or without mRNA encoding the full-length H-2K^d heavy chain. The B3Z cells endogenously express the MHC-I H-2D^b, but not the H-2K^d necessary for antigen presentation of some of the transfected peptides in the NOD mouse, necessitating the transfection of the appropriate MHC-I complex in addition to the peptide/β$_2$m/CD3-ζ. The four peptides were derived from previously identified autoantigens in diabetes in the NOD mouse, which express the MHC-I H-2K^d and H-2D^b. These were insulin B chain, amino acids 15–23 (InsB$_{15–23}$), islet-specific glucose-6-phosphatase catalytic subunit-related protein, amino acids 206–214 (IGRP$_{206–214}$), and glutamic acid decarboxylase, amino acids 546–554 (GAD65$_{546–554}$), which are presented by MHC-I H-2K^d. Dystrophia myotonica kinase, amino acids 138–146 (DMK$_{138–146}$), binds H-2D^b, which is naturally expressed by B3Z cells. Transfected cells were then incubated in the presence or absence of immobilized antibodies against the respective MHC-I heavy chain and T cell activation was monitored by a reporter assay (Figure 2A). Clearly, this experiment indicates that the polypeptide products functionally paired with exogenous (H-2K^d) or endogenous (H-2D^b) heavy chains at the plasma membrane and, when triggered by the anti-MHC-I antibody binding and hence cross-linking MHC-I, potent activation signals were transmitted. These signals were comparable in magnitude to those produced by similarly cross-linking the endogenous TCR expressed by B3Z cells as shown by the response seen with anti-CD3 antibody 2C11. We then assessed the ability of InsB$_{15–23}$β$_2$m/CD3-ζ expressed by B3Z to mediate peptide-specific T cell activation in target T cells expressing an anti-InsB$_{15–23}$-H-2K^d TCR. To this end, we used the CHIB2 T cell hybridoma, which expresses the αβTCR of the diabetogenic G9C8 CD8 T cell clone that recognizes the insulin B chain peptide, amino acids 15–23. We observed construct-mediated recognition upon co-culture of the two cells (Figure 2B) only when B3Z expressed both InsB$_{15–23}$/β$_2$m/CD3-ζ and H-2K^d. As both cells express the same nuclear factor of activated T cells (NFAT)-lacZ

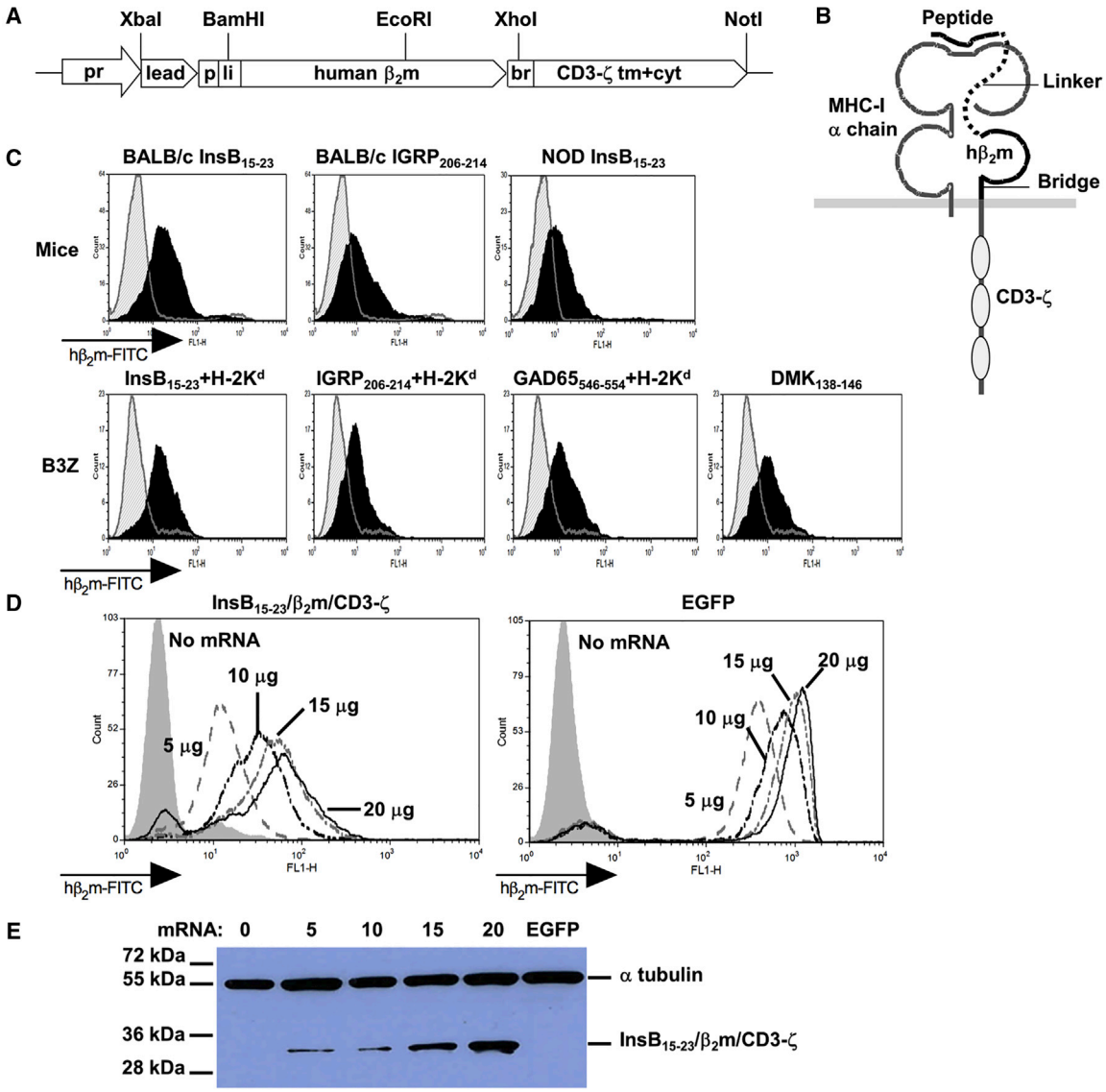

Figure 1. Gene, Product and Expression of Peptide/β_2m/CD3-ζ

(A) Genetic design. Selected restriction sites are shown. br, bridge; lead, leader peptide; li, linker; p, peptide; pr, promoter; tm+cyt, transmembrane and cytoplasmic domain. (B) Scheme of an intact, surface MHC-I complex comprising the chimeric peptide/β_2m/CD3-ζ chain. (C) Flow cytometric analysis for surface expression. Splenic CD8 T cells of BALB/c and NOD mice (upper panel) and B3Z cells (lower panel) were transfected with 10 μg mRNA encoding the indicated constructs. mRNA encoding H-2K^d was added to enable presentation of the respective peptides. Cells were stained with anti-human β_2m and detected with FITC-conjugated donkey anti-mouse IgG. Staining of non-transfected cells is presented by the gray histograms. (D) Flow cytometric analysis of B3Z cells electroporated with different amounts of mRNA (in micrograms), no mRNA, or EGFP mRNA and analyzed using anti-human β_2m mAb purified from the supernatant of hybridoma HB149. (E) Western blot analysis performed under reduced conditions with cell lysates of B3Z cells transfected with the same mRNA as in (D), with detection using rabbit anti-human β_2m polyclonal antibodies (Abs).

reporter gene, this assay does not discriminate between the two. However, it coincides with our previous work[31] showing two-way T cell activation mediated by the G9C8 TCR and the InsB$_{15-23}$/β_2m/CD3-ζ construct, as the G9TCR recognizes the construct and the triggering of the construct activates the transfected cell. Here we recapitulate this notion also for exogenous mRNA-driven expression.

Functional Expression of Peptide/β_2m/CD3-ζ in NOD CD8 T Cells

We went on to test whether polyclonal NOD CD8 T cells can be endowed with the ability to kill diabetogenic T cells following electroporation with peptide/β_2m/CD3-ζ mRNA. In the experiment presented in Figure 3A, we employed CHIB2 T cell hybridomas as target cells. Polyclonal CD8 T cells transfected to express InsB$_{15-23}$/β_2m/CD3-ζ

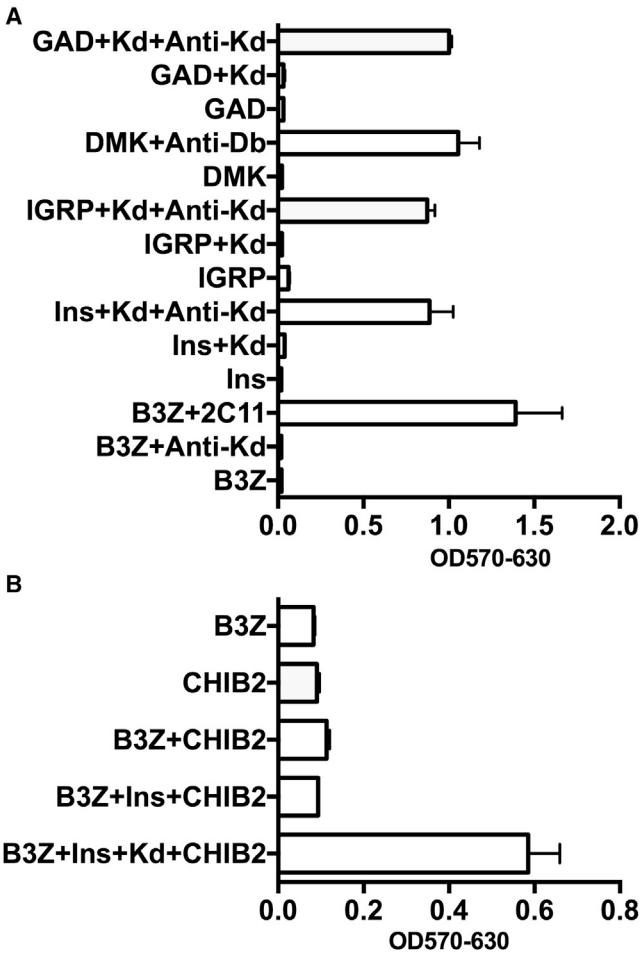

Figure 2. CD8 T Cell-Targeting Genes Are Functionally Expressed in a Reporter T Cell Hybridoma following mRNA Transfection

(A) B3Z hybridoma cells, endogenously expressing MHC H-2D^b, were used as reporter T cell hybridomas expressing the immunotherapeutic targeting constructs that encompassed peptides from four autoantigens important in diabetes: GAD, DMK, IGRP, and insulin. GAD, IGRP, and insulin peptides require presentation by MHC H-2K^d, which is not endogenously expressed by B3Z cells. Thus, the B3Z cells were transfected with 10 μg of each of the indicated mRNAs with or without H-2K^d mRNA. The peptide from DMK is presented by H-2D^b, endogenously expressed by the B3Z. The peptide/β_2m/CD3-ζ polypeptides pair with exogenous H-2K^d (GAD, IGRP, and insulin) or endogenous H-2D^b (DMK) heavy chains and transmit T cell activation signals upon MHC-I cross-linking by plate-bound anti-K^d or anti-D^b. The assay was performed by incubating the transfected cells overnight in 96-well plates coated with anti-K^d or anti-D^b. Cell lysates were prepared and subjected to a CPRG colorimetric assay. When these indicator T cells expressing the targeting construct were triggered, either by binding to an anti-K^d or anti-D^b antibody, a color change was seen in the lysates. The negative controls were cells with transfected construct, but no stimulation with anti-K^d or anti-D^b antibody, or in the case of GAD, IGRP, and insulin peptides, no exogenous MHC construct. The positive control is shown as B3Z stimulated by anti-CD3 antibody, 2C11, indicating that when the B3Z cells are triggered through their own endogenous TCR, they are able to effect the color change, which is a positive control for the cell activation. (B) The B3Z cells were transfected with the InsB$_{15–23}$ peptide construct with or without H-2K^d. The cells were co-incubated overnight in triplicate in a 96-well plate, the cells were lysed, and the lysate was analyzed by the CPRG colorimetric assay. The B3Z cells transfected with the InsB$_{15–23}$ peptide construct together with H-2K^d can be recognized by the CHIB2 cells and this recognition stimulates activation of the B3Z cells, indicated by the color change. The error bars represent SD of the mean from triplicate experiments.

(effectors) were incubated with CHIB2 (targets) at three different effector-to-target (E:T) ratios and dose-dependent killing of the target CHIB2 cells was observed. We previously showed that expression of the peptide/β_2m/anchor configuration resulted in exceptionally high occupancy of the restricting MHC-I product by the linked peptide.[9,40] To supplement the endogenous H-2K^d in the polyclonal NOD CD8 T cells, we tested the addition of mRNA encoding H-2K^d heavy chains. Figure 3A illustrates that the transfected polyclonal CD8 T cells have acquired the ability to kill the target CHIB2 cells and overexpression of H-2K^d had no discernible effect on the killing activity. Example flow cytometric plots are shown in Figure S1. Figure 3B presents the results of a similar cell cytotoxicity assay, showing that NOD CD8 T cells transfected with InsB$_{15–23}$/β_2m/CD3-ζ or IGRP$_{206–214}$/β_2m/CD3-ζ mRNA, respectively, kill target T cells expressing insulin-reactive G9C8 or IGRP-reactive NY8.3 TCRs purified from the corresponding TCR transgenic NOD mice. Example flow cytometric plots from Figure 3 are shown in Figure S1.

CD8 T Cells Redirected to Kill Insulin-Reactive CD8 T Cells Reduce Diabetes

Having shown that the redirected CD8 T cells were able to kill insulin-reactive or IRGP-reactive T cells in vitro, we then tested the effect of the redirected CD8 T cells on the incidence of diabetes in vivo. CD8 T cells transfected with either InsB$_{15–23}$/β_2m/CD3-ζ or IGRP$_{206–214}$/β_2m/CD3-ζ mRNA were adoptively transferred into 5- to 6-week-old female NOD mice in a single transfer. These mice were tested weekly for glycosuria and diabetes was confirmed by a blood glucose reading of >13.9 mmol/L. The mice were euthanized either when they became diabetic or at 35 weeks if they were non-diabetic. We observed a significant reduction in the incidence of diabetes when the cells were transfected with the InsB$_{15–23}$/β_2m/CD3-ζ mRNA construct but not with the IGRP$_{206–214}$/β_2m/CD3-ζ mRNA construct, as shown in Figure 4A. Furthermore, there was a clear reduction in insulitis, the infiltration of lymphocytes, in the non-diabetic mice, compared with the insulitis in diabetic mice, with significant differences in composition of the infiltration of CD4 and CD8 T cells as well as B cells (Figures 4B and 4C).

DISCUSSION

In this study, we have shown that mRNA constructs encoding autoantigenic peptide/β_2m/CD3-ζ can be used to transfect both T cell hybrids as well as polyclonal T cells and redirect these T cells against autoreactive pathogenic CTLs. This means of selectively targeting antigen-specific cells is a modification of the successful chimeric antigen receptor (CAR) strategy adopted for tumor adoptive cell therapy[41] and could be used in adoptive cell therapy in type 1 diabetes. There is good expression of the constructs and we have shown that functionally, the redirected targeting cells are recognized specifically

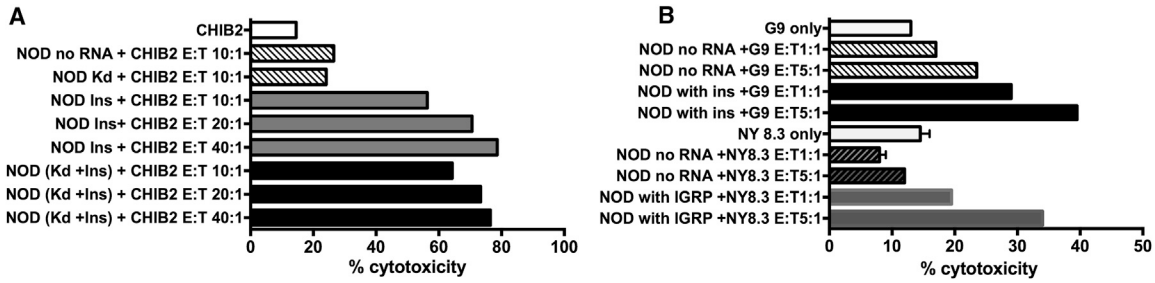

Figure 3. Peptide/β_2m/CD3-ζ mRNA Redirects NOD CD8 T Cells to Kill Insulin Reactive CHIB2 T-Hybridoma Cells and Insulin Peptide-Specific CD8 T Cells
(A) Effector cells transfected with $InsB_{15–23}/\beta_2$m/CD3-ζ mRNA (NOD Ins; shown in gray filled bars) or $InsB_{15–23}/\beta_2$m/CD3-ζ mRNA together with additional H-2K^d [NOD (K^d+Ins)]; shown in the black filled bars) were co-incubated for 24 hr with PKH26-labeled target CHIB2 cells at effector target ratios of 10:1, 20:1, and 40:1. TO-PRO-3 was added to the cells and the percentage of lysed target CHIB2 cells was determined by flow cytometric analysis. As controls (shown in the top three bars), target CHIB2 cells alone (white open bars), effector cells electroporated in the absence of mRNA (NOD no RNA; light hatched bars), or effector cells electroporated with mRNA encoding only the MHC (NOD K^d) but not the peptide (dark hatched bars) were each incubated with target cells. (B) Effector cells transfected with $InsB_{15–23}/\beta_2$m/CD3-ζ mRNA (NOD with ins; shown in filled black bars) or IGRP/β_2m/CD3-ζ mRNA (NOD with IGRP; shown in filled gray bars) were co-incubated for 24 hr with PKH26-labeled target $InsB_{15–23}$-reactive G9 or $IGRP_{206–214}$-reactive NY8.3 CD8 T cells, respectively, at effector-to-target (E:T) ratios of 1:1 or 5:1, in duplicate. As controls, the same target cells were incubated with cells electroporated in the absence of mRNA (NOD no RNA), as shown in light hatched bars or dark hatched bars, respectively. TO-PRO-3 was added to the cells and the percentage of lysed cells was determined by flow cytometric analysis. Controls showing target G9 T cells or NY8.3 T cells are shown in the open bars. The graph shown is representative of nine similar experiments. The error bars represent SD of the mean.

by autoreactive CD8 T cells. The autoreactive CD8 T cells are selectively removed, as illustrated by the in vitro assays. We have also shown that peptide/β_2m/CD3-ζ can target specific autoreactive CD8 T cells in vivo and reduce the incidence of autoimmune diabetes.

The simultaneous immunotargeting of a number of dominant autoreactive CD8 T cell clones by the peptide/β_2m/CD3-ζ constructs can be attained either by the co-transfection of the same polyclonal CD8 T cells with a combination of mRNAs encoding the respective peptide/β_2m/CD3-ζ polypeptides or the co-administration of a mixture of T cell transfectants, each of which expresses a different peptide/β_2m/CD3-ζ mRNA. In the current study, we have demonstrated the functional expression of the different mRNA species, each co-transfected (when needed) with mRNA encoding the H-2K^d heavy chain (Figure 2A). In this experiment, the activation of transfectants by MHC-I cross-linking required the functional expression and pairing of the polypeptide products of both mRNA encoding MHC-I as well as the peptide/β_2m/CD3-ζ. Nevertheless, it was almost as powerful as cellular activation triggered by TCR cross-linking under exactly the same conditions. In an accompanying study,[42] we recently showed that mRNA encoding peptide/MHC-II/CD3-ζ can be similarly used to efficiently redirect T cells against pathogenic CD4 T cells in a peptide-specific manner. Likewise, in that study, T cell activation through the chimeric MHC-II molecules, which also required co-expression and pairing of the two products, was comparable to TCR-mediated activation. The current achievement complements our arsenal of genetic immunotargeting devices against both CD4 and CD8 islet-reactive T cells in the NOD mouse.

The demonstration that redirected polyclonal NOD CD8 T cells kill autoreactive CD8 T cells (Figure 3) recapitulates our previous findings[31] in which the insulin-reactive CD8 T cells were killed by preactivated T cells that expressed $InsB_{15–23}/\beta_2$m/CD3-ζ as a transgene. However, while those transgenic experiments illustrated the principle that CD8 T cells could be effectively redirected to kill antigen-specific targets, this procedure would not be translatable for immunotherapy in humans. In the current study, the strategy brings the procedure a step nearer to translation, in that we have shown that primary cells can be readily transfected with the mRNA construct and can kill insulin-reactive targets in a similar manner.

When the $InsB_{15–23}/\beta_2$m/CD3-ζ or $IGRP_{206–214}/\beta_2$m/CD3-ζ mRNA-transfected cells were transferred to young NOD female mice, protection from diabetes was only seen when the cells targeted $InsB_{15–23}$-reactive T cells. The injection was done at a time (5–6 weeks of age) when insulin-reactive cells are detected in the pancreatic lymph nodes in a higher proportion than at later time points. In contrast, IGRP-reactive T cells tend to rise later on, so that injecting the cells at this young age may be more likely to target insulin-reactive T cells.[10,19] In principle, cell transfer aiming at immunotargeting T cells of both autoreactivities can be carried out at different time points, so that the earlier transfer targets insulin-reactive T cells and the later one targets IGRP-reactive cells. The current study demonstrates proof of principle and further studies will be carried out in the future to optimize timing of injections, including injections at later time points and whether more than one adoptive cell transfer may be required.

To our knowledge, this work is the first demonstration that mRNA-transfected T cells expressing chimeric MHC complexes can selectively immunotarget pathogenic T cells in vivo and inhibit or reduce the incidence of autoimmune diabetes. We are also evaluating the similar reprogramming of regulatory T cells in an ongoing study, hypothesizing that the targeting of a narrow population of autoreactive CD8 or CD4 T cell clones may suffice for eliciting a broad tolerizing effect.

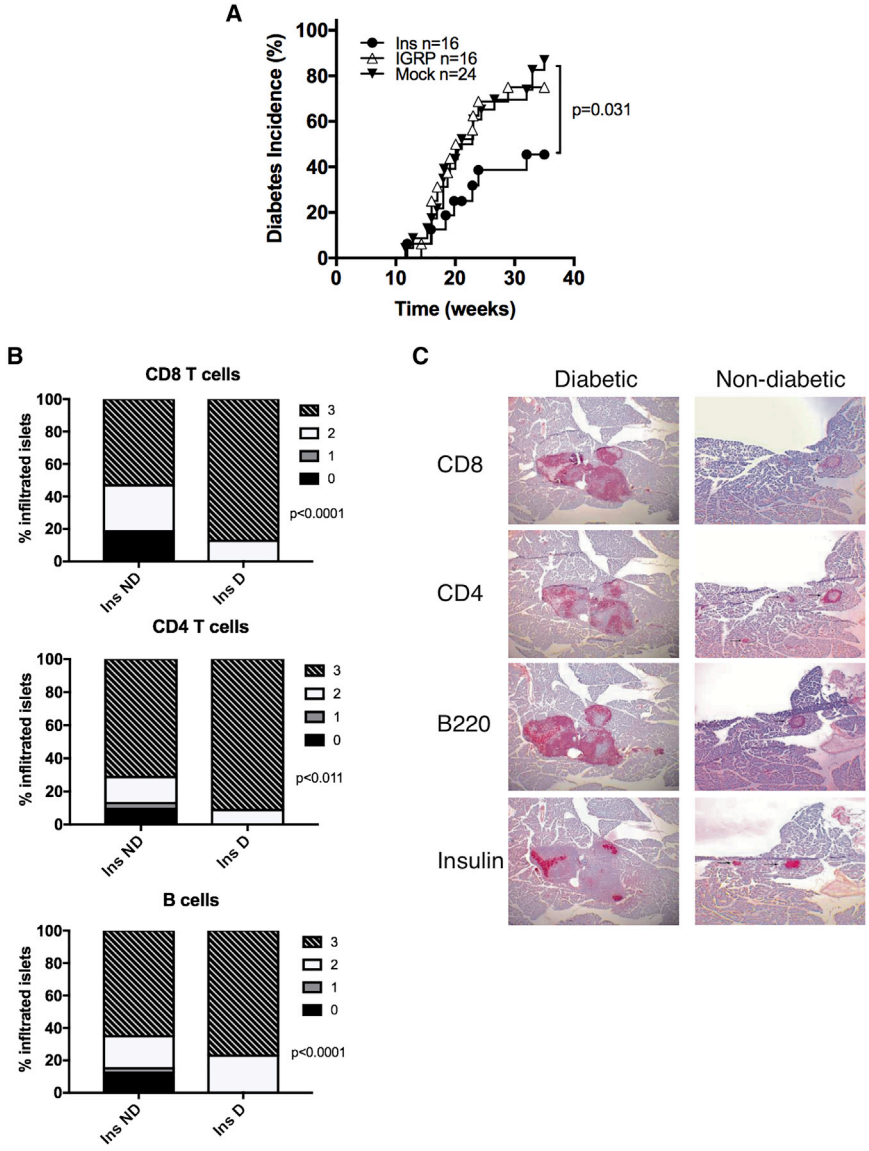

Figure 4. Insulitis and Diabetes in NOD Mice following Transfer of mRNA-Transfected Cells

Female NOD mice were transferred with 10^7 NOD CD8 T cells that had been transfected with $InsB_{15–23}/\beta_2m$/CD3-ζ mRNA (Ins; n = 16), $IGRP_{206–214}/\beta_2m$/CD3-ζ mRNA (IGRP; n = 16), or control cells electroporated but without mRNA (Mock; n = 24). The mice were screened weekly for glycosuria and following two consecutive positive readings, diabetes was confirmed by a blood glucose reading of >13.9 mmol/L. (A) Incidence of diabetes is shown with data that were analyzed by log-rank survival statistics, comparing the Ins group with the Mock group (p = 0.031). (B) Insulitis scores either when the mice developed diabetes or for the non-diabetic mice at 35 weeks of age are shown. We examined 80–115 islets from at least three mice in each group. The sections were stained with anti-CD8 (top), anti-CD4 (middle), and anti-B220 for B cells (bottom). Scoring for insulitis was as follows: 0, no insulitis; 1, peri-insulitis; 2, less than 50% infiltration; and 3, more than 50% infiltration. Data were analyzed by the χ^2 test. (C) Examples of the insulitis are shown, with separate staining for CD8 and CD4 T cells, B cells (B220), and insulin, all stained in red, in a diabetic mouse and a non-diabetic mouse. Magnification is 100×.

MATERIALS AND METHODS

DNA Vectors and Plasmids

The pGEMT vector for direct cloning of PCR products was from Promega. Template DNA for in vitro transcription of mRNA was cloned into the pGEM4Z/GFP/A64 vector,[43] a kind gift from Dr. E. Gilboa (University of Miami) following the removal of the GFP insert (pGEM4Z/A64).

Assembly of Genetic Constructs

All PCR products were sub-cloned and their DNA sequence was confirmed prior to insertion in expression vectors. For cloning of the four constructs encoding peptide/human β_2m/mouse CD3-ζ, we used essentially the same cloning strategy we described previously.[9] The gene segment encoding the full leader sequence of human β_2m, the antigenic peptide, and the N-terminal part of the linker was amplified from a hβ_2m cDNA clone (prepared from Jurkat cells) with the forward primer 5′-GGG TCT AGA GCC GAG ATG TCT CGC TCC GTG-3′ and one of the following reverse primers: 5′-CGC GGA TCC GCC ACC TCC CAC ACG CTC CCC ACA CAC CAG GTA GAG AGC CTC AAG GCC AGA AAG-3′ for the $InsB_{15–23}$ variant peptide G9V (LYLVCGERV[44]), 5′-CGC GGA TCC GCC ACC TCC AAG AAA AAC ATT AGT TTT AAG ATA AAC AGC ATA CAA GCC GGT CAG-3′ for $IGRP_{206–214}$ (VYLKTNVFL), 5′-CGC GGA TCC GCC ACC TCC AAC TTT ATC ACC AAG AGG TTG ATA AGA AGC ATA CAA GCC GGT CAG-3′ for $GAD65_{546–554}$ (SYQPLGDKV), and 5-CGC GGA TCC GCC ACC TCC AAG ATA AAG ATA ATT TTC ATC TTG AAA AGC ATA CAA GCC GGT CAG-3′ for $DMK_{138–146}$ (FQDENYLYL). The resulting XbaI/BamHI fragment and a BamHI/EcoRI fragment encoding the hβ_2m/CD3-ζ portion of the chimeric polypeptide[9] were inserted into pGEM4Z/A64.

Cell Lines

B3Z[45] is an ovalbumin $(OVA)_{257–264}$-specific, H-$2K^b$-restricted CTL hybridoma and was a kind gift from Dr. N. Shastri (University of California, Berkeley). CHIB2 is an $InsB_{15–23}$-specific hybrid of the G9C8 T cell clone with the BW5147 thymoma expressing CD8, H-$2K^d$-restricted T cell hybridoma.[10] Both B3Z and CHIB2 harbor the nuclear factor of activated T cells-lacZ inducible reporter gene.[46]

In Vitro Transcription and Electroporation of mRNA

Template DNA cloned in pGEM4Z/A64 was prepared with the Endo-Free Plasmid Maxi Kit (Promega) and linearized using the SpeI restriction site positioned at the 3′ end of the poly(A) tract of the vector. In vitro transcription was conducted in a 20-μL reaction mix at 37°C using the T7 mScript Standard mRNA Production System (CellScript) to generate 5′-capped in vitro-transcribed mRNA. Cells were washed twice with OptiMEM medium (Gibco) and re-suspended in 200 μL OptiMEM containing the required amount of in vitro-transcribed mRNA (10–20 μg in most experiments). Electroporation was performed with Gene Pulser Xcell (Bio-Rad Laboratories) in 2-mm cuvettes using a square wave pulse (1 millisecond, 300 V) for the experiments with cell lines, or with BTX Harvard Apparatus ECM830 (1 millisecond, 300 V) for the primary cells.

Mice

NOD mice, originally from the NOD/CaJ colony at Yale University, and G9 TCR transgenic mice[47] have been bred at Cardiff University for 5 years. NY8.3 TCR transgenic mice[48] were purchased from Jackson Laboratory. These mice were housed in microisolators at the specific pathogen-free facility at Cardiff University. BALB/c mice were maintained at the animal facility of the Bar-Ilan University Faculty of Medicine. All procedures were performed in accordance with protocols approved by the UK Home Office and the Israeli National Committee Institutional Animal Care and Use Committee.

Antibodies and Reagents

Anti-H-2K^d monoclonal antibody (mAb) (clone SF1-1.1) was from Pharmingen and anti-H-2D^b mAb was purified from the supernatant of the 28-14-8 hybridoma. 2C11 is a hamster mAb specific to mouse CD3ε. mAb against hβ_2m (clone BM-63) was from Sigma. Rabbit polyclonal antibodies against hβ_2m were from Dako. Hamster mAb to mouse CD3ζ (clone H146-968) was from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated goat antibodies against rabbit IgG were from Jackson Laboratory. Rabbit antibodies against hamster IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and FITC-conjugated donkey anti-mouse IgG were from Sigma and Jackson Laboratory, respectively. HRP-conjugated rabbit antibodies against hamster IgG were from Sigma. Biotinylated rat anti-mouse CD4 (clone GK1.5) was purchased from Biolegend. Biotinylated rat anti-mouse CD8a (clone 53-6.7) and rat anti-mouse B220 (clone RA3-6B2) were from BD Pharmingen and biotinylated mouse anti-insulin antibody (ICBTACLS) was from eBioscience.

Flow Cytometry

One million cultured cells or primary cells harvested from the spleen of mice were used. Single cell suspensions were either stained with the respective monoclonal antibodies in a direct staining procedure or, for indirect staining, the cells were incubated with the primary antibody for 1 hr and then washed before further incubation with fluorophore-labeled secondary antibody at 4°C. Cells were washed twice, re-suspended in 1 mL cold PBS, and analyzed by a FACSCalibur or FACSCanto system (Becton Dickinson). Data were analyzed by FCS Express (version 4; De Novo Software) or FlowJo (version 7.6.5; Tree Star) software.

Chlorophenol Red β-D-Galactopyranoside Assay

B3Z transfected cells were incubated in plates coated with immobilized MHC-I antibody or with CHIB2 hybridoma cells overnight. The culture medium was removed and 100 μL lysis buffer (9 mM $MgCl_2$, 0.125% NP-40, and 0.3 mM chlorophenol red β-D-galactopyranoside [CPRG] in PBS) was added to each well. One to 24 hr post-lysis, the optical density (OD) of each well was measured using an ELISA reader (at 570 nm, with 630 nm as reference).

Immunoblot Analysis

Protein samples were boiled for 3 minutes, separated on a 10% non-reducing SDS polyacrylamide gel at 50 mA, and transferred onto a nitrocellulose membrane. The membrane was blocked with milk buffer (0.3 g Na_2HPO_4, 2.19 g NaCl, 25 mL water, and 225 mL 1% low-fat milk) overnight at 4°C and 1 hr at room temperature, washed twice with PBS, and incubated for 2 hr with the primary antibody. The membrane was then washed six times for 6 min with PBS, incubated for 1 hr with the secondary peroxidase-conjugated antibody, washed six times for 6 min with TPBS (PBS with 0.05% Tween 20), washed six times for 6 min with PBS, and then developed using an ECL (enhanced chemiluminescence) kit (Pierce-Thermo Fisher Scientific) and X-ray film 100NIF (Fuji).

Cytotoxicity Assay

CD8 T cells were isolated from the spleen of 5- to 6-week-old NOD mice and separated by negative selection (MACS; Miltenyi Biotec). The purified CD8 T cells were then activated and expanded by stimulation using plate-bound anti-CD3 and anti-CD28 antibodies, interleukin (IL)-2 and IL-7 as previously described.[49] The activated CD8 T cells were transfected with mRNA encoding InsB_{15-23}/β_2m/CD3-ζ or IGR$P_{206-214}$/β_2m/CD3-ζ. These cells were the effector cells of the assay. G9 and NY8.3 TCR transgenic CD8$^+$ T cells were purified by negative selection (MACS; Miltenyi Biotec) from the spleens of G9 and NY8.3 transgenic mice, respectively. Activated mRNA-transfected T cells (effectors) were incubated with purified G9 or NY8.3 T cells (targets), labeled with PKH-26 (Sigma), at an effector-to-target ratio of 1:1 and 5:1 for 16 hr at 37°C. To detect cell death, TO-PRO-3 iodide (Thermo Fisher Scientific) was added immediately prior to flow cytometric analysis. Single PKH-26$^+$TO-PRO-3$^+$ cells were gated, and cytotoxicity was expressed as the percentage of dead cells/total targets.[31] Activated CD8 T cells with no transfected mRNA served as controls for the effector T cells.

Adoptive Transfer

NOD CD8 T cells were isolated, activated, expanded, and transfected with mRNA as described for the cytotoxicity assay. As a control, CD8+ T cells were subjected to the electroporation procedure but with no added mRNA (mock transfection). Transfected cells (peptide/β_2m/CD3-ζ construct and mock-transfected) were adoptively transferred into 6-week-old NOD mice recipients by injection into the tail vein (6–10 × 10^6 cells per mouse).

Diagnosis of Diabetes

Mice were screened weekly for glycosuria (Diastix; Bayer) and, following two consecutive positive readings, diabetes was confirmed by a blood glucose reading of >13.9 mmol/L.

Histology

The pancreas was fixed in paraformaldehyde lysine periodate buffer overnight and then infused with 10% sucrose followed by 20% sucrose, as previously described.[31] The pancreas was then embedded in OCT and snap frozen for immunohistochemistry. Frozen sections of 10-μm thickness were cut and stained with rat anti-mouse CD4, CD8, B220, and anti-insulin antibodies and detected with streptavidin-alkaline phosphatase and a Vector Red AP substrate kit (Vector Laboratories). The sections were counter-stained with hematoxylin. Insulitis was assessed from at least three mice per group and 80–115 islets were scored. Scoring for insulitis is shown in the legend for Figure 4.

Statistical Analysis

Log-rank analysis was carried out for the adoptive transfer experiments. The χ^2 test was performed for the analysis of insulitis. For all tests, $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at http://dx.doi.org/10.1016/j.ymthe.2016.12.007.

AUTHOR CONTRIBUTIONS

G.G. and F.S.W. conceived and designed the study. S.F., M.D.L., L.K.S., E.D.L., D.K., J.D., and D.Z. performed experiments. A.M., N.K., G.G., and F.S.W. supervised the study. S.F., G.G., and F.S.W. wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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YMTHE, Volume *25*

Supplemental Information

Adoptive Transfer of mRNA-Transfected T Cells Redirected against Diabetogenic CD8 T Cells Can Prevent Diabetes

Sigal Fishman, Mark D. Lewis, L. Khai Siew, Evy De Leenheer, Dimitri Kakabadse, Joanne Davies, Doron Ziv, Alon Margalit, Nathan Karin, Gideon Gross, and F. Susan Wong

Legend for supplementary figure S1.

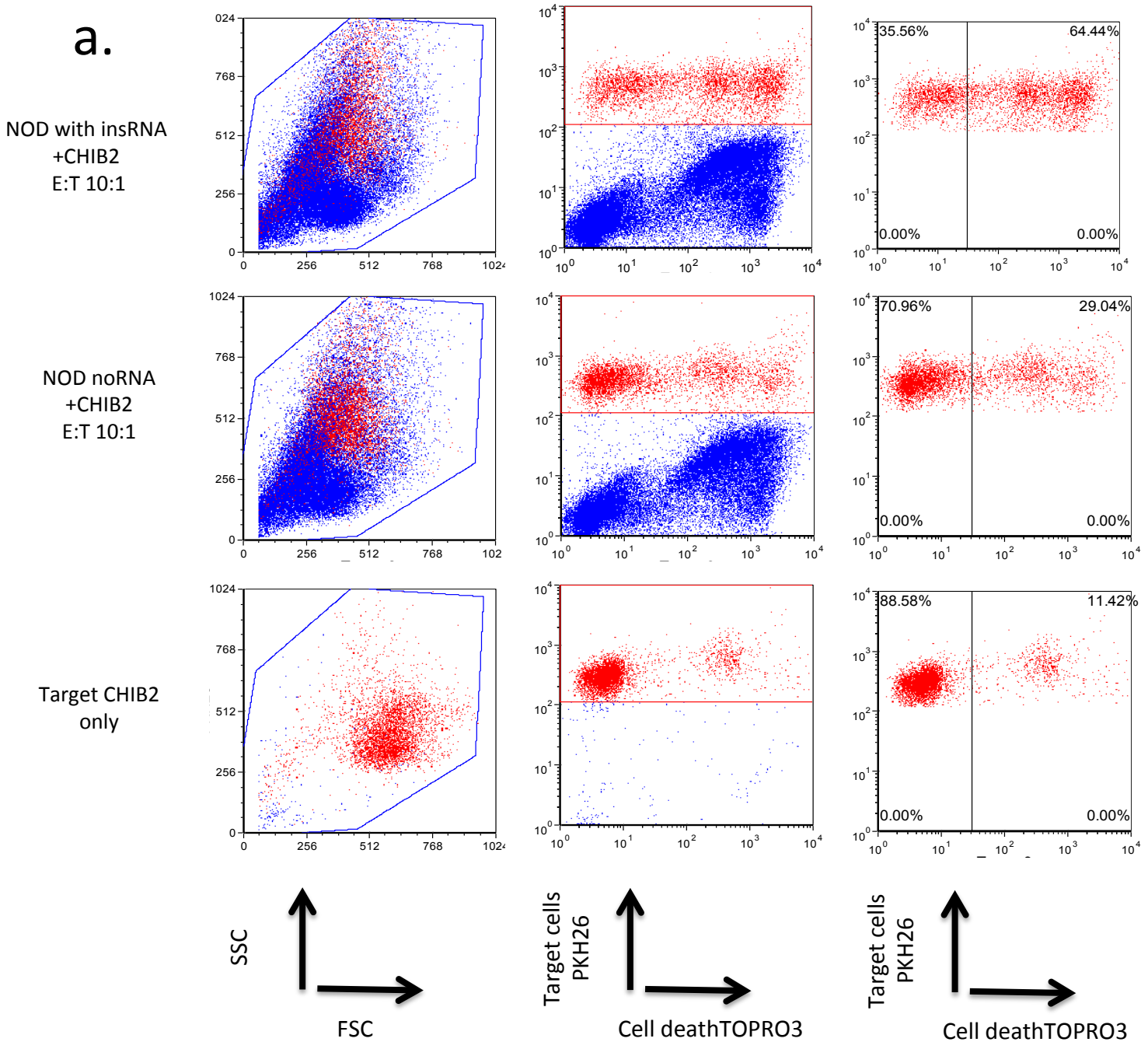
S1 Gating strategy for cytotoxicity assay

a. NOD T cells were electorporated with $InsB_{15\text{-}23}/\beta_2m$/CD3-ζ mRNA (top row) or electroporated without RNA (second row) and were incubated with PKH26-labeled target CHIB2 hybridoma cells, at an effector:target E:T ratio of 10:1 for 24 hours. PKH26-labeled target cells alone are shown in the bottom row. The analysis was performed by flow cytometry, detecting cells damaged by cytotoxicity, with TOPRO-3 added just prior to analysis. The gating strategy is shown. Total cells were gated and then after gating for single cells, the populations that were labeled with PKH26 and those stained by TOPRO-3 are shown in the middle panel. The % killed cells are shown in the right panels with cells stained with TOPRO3 calculated as a percentage of total PKH26-labeled target cells.

b. NOD T cells were electorporated with $InsB_{15\text{-}23}/\beta_2m$/CD3-ζ mRNA (top row) or electroporated without RNA (second row) and were incubated with PKH26-labeled target G9 insulin-reactive CD8 T cells at an effector:target E:T ratio of 5:1 for 24 hours. PKH26-labeled target cells alone are shown in the bottom row. The analysis was performed by flow cytometry, detecting cells damaged by cytotoxicity, with TOPRO-3 added just prior to analysis. The gating strategy is shown. Total cells were gated and then, after gating for single cells, the populations that were labeled with PKH26 and those stained by TOPRO-3 are shown in the middle panel. The % killed cells are shown in the right panels with cells stained with TOPRO3 calculated as a percentage of total PKH26-labeled target cells.

S1

a.


NOD with insRNA
+CHIB2
E:T 10:1
NOD noRNA
+CHIB2
E:T 10:1
Target CHIB2
only
35.56%
64.44%
0.00%
0.00%
70.96%
29.04%
0.00%
0.00%
88.58%
11.42%
0.00%
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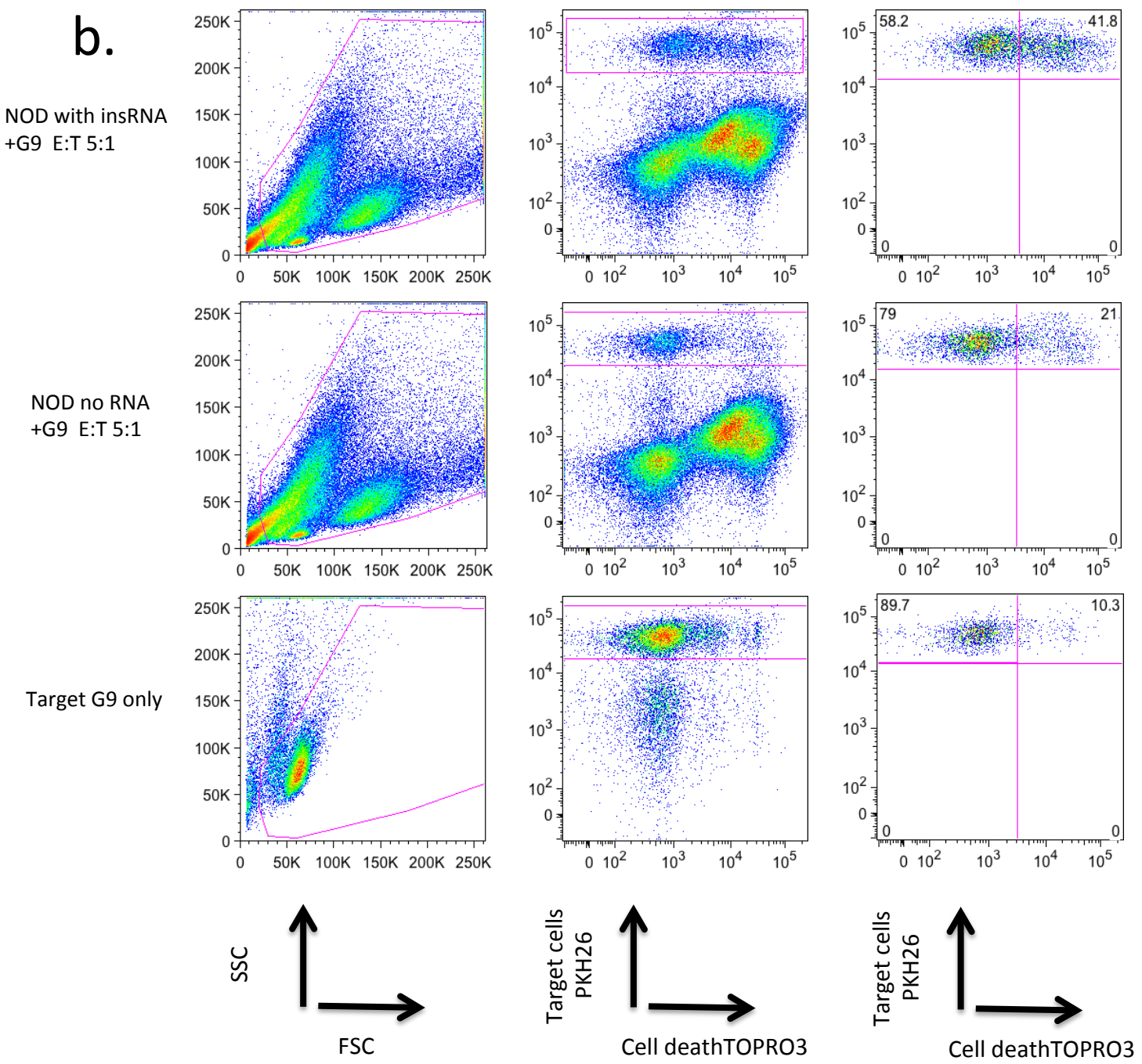
SSC
FSC
Target cells
PKH26
Cell deathTOPRO3
Target cells
PKH26
Cell deathTOPRO3

S1


b.
NOD with insRNA
+G9 E:T 5:1
NOD no RNA
+G9 E:T 5:1
Target G9 only
58.2
41.8
79
21
89.7
10.3
SSC
FSC
Target cells PKH26
Cell deathTOPRO3
Target cells PKH26
Cell deathTOPRO3