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Co-delivery of antigen-encoding mRNA and signal 2/3 mRNAs to PBMCs by Cell Squeeze® Technology generates SQZ® eAPCs that prime CD8+ T cells in a humanized mouse model

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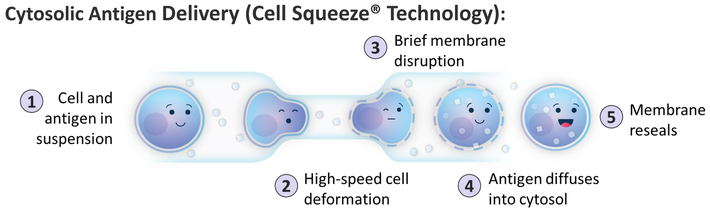
Background

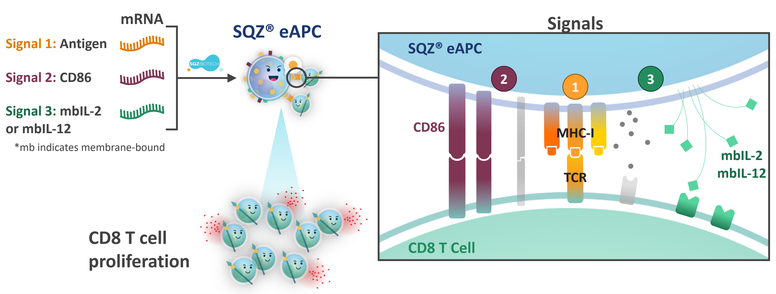
Antigen-specific CD8+ T cells are critical for mounting an effective immune response against tumors. Generation of antigen-specific T cells require interactions with multiple signals produced by antigen presenting cells (APCs). These signals are comprised of three components: (signal 1) the peptide-MHC complex binding to the T cell receptor, (signal 2) costimulatory molecules on the surface of APCs, and (signal 3) inflammatory cytokines binding to cognate receptors on T cells. To engineer all major cell subsets of human peripheral blood mononuclear cells (PBMCs) to become enhanced APCs (eAPCs), we used Cell Squeeze® technology to deliver multiple mRNA encoding for non-self-antigens (signal 1), CD86 (signal 2), and/or membrane-bound cytokines (signal 3). The signal 3 molecules, membrane-bound IL-12 (mbIL-12) and membrane-bound IL-2 (mbIL-2), are chimeric proteins designed to increase the localized concentration of the cytokines and limit off-target effects. Flow cytometry confirmed translation of delivered signal 2/3 mRNA by all major subsets within PBMCs: T cells, B cells, NK cells, and monocytes. The potency of these eAPCs was assessed in vitro by culturing the APCs with antigen-specific T cells for multiple days before measuring the functionality of antigen-specific T cells via intracellular cytokine staining or ELISA. Using this approach, we demonstrate that Cell Squeeze® technology co-delivery of antigen mRNA and signal 2/3 mRNA significantly enhances CD8+ T cell responses to a variety of antigens, including CMV pp65, Influenza M1, HPV16 E6, and HPV16 E7. Furthermore, we demonstrate that SQZ® eAPCs drive significant expansion of antigen-specific CD8+ T cells in a humanized mouse model. Thus, we demonstrate that the Cell Squeeze® technology can deliver mRNA encoding signals 1, 2, and 3 to human PBMCs and has the potential to generate eAPCs that drive strong CD8+ T cell responses against multiple antigens. The versatility of this approach has the potential to enable rapid exchange of mRNA to code for other antigens or T cell activation signals.

SQZ® Technology and Enhanced APC Platform

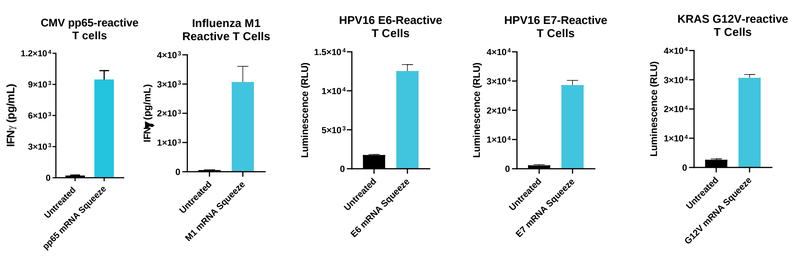
Cytosolic Antigen Delivery (Cell Squeeze® Technology):



Generation of Enhanced APCs through Antigen and Signal 2/3 mRNA Delivery via Cell Squeeze® Technology (SQZ® eAPCs):

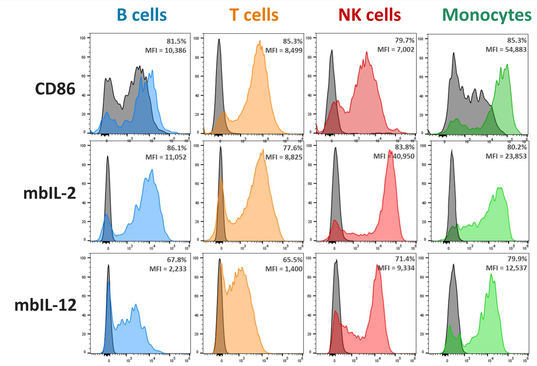


PBMCs Squeezed with Antigen-Encoding mRNA Leads to Robust MHC-I Presentation

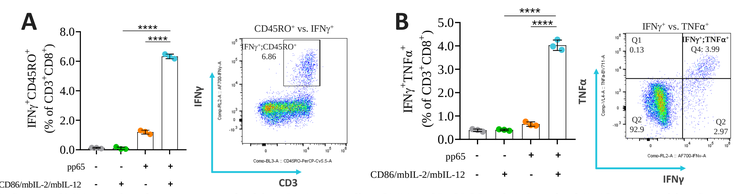


Human PBMCs squeezed with antigen-encoding mRNA activate antigen-specific T cells in vitro. Isolated human PBMCs were squeezed in the presence of CMV pp65, Influenza M1, HPV16 E6, HPV16 E7, or KRAS G12V mRNA. Untreated or squeezed PBMCs were then incubated with responder cells to measure MHC-I presentation of specific epitopes. For CMV pp65 and Influenza M1, primary CD8+ T cells from HLA-A*02+ donors were expanded against CMV $pp65_{495-503}$ or Influenza $M1_{58-66}$ epitopes and used as responders. IFNγ production after overnight culture was measured in the supernatant by ELISA. For HPV16 E6, HPV16 E7, and KRAS G12V reactivity, Jurkat NFAT reporter cells were transduced with TCRs specific for $E6_{29-38}$, $E7_{11-19}$, or KRAS $G12V_{8-16}$ and used as responders for untreated PBMCs or PBMCs squeezed with the corresponding antigen-encoding mRNA. Luciferase production in the supernatant was measured by spectrophotometer.

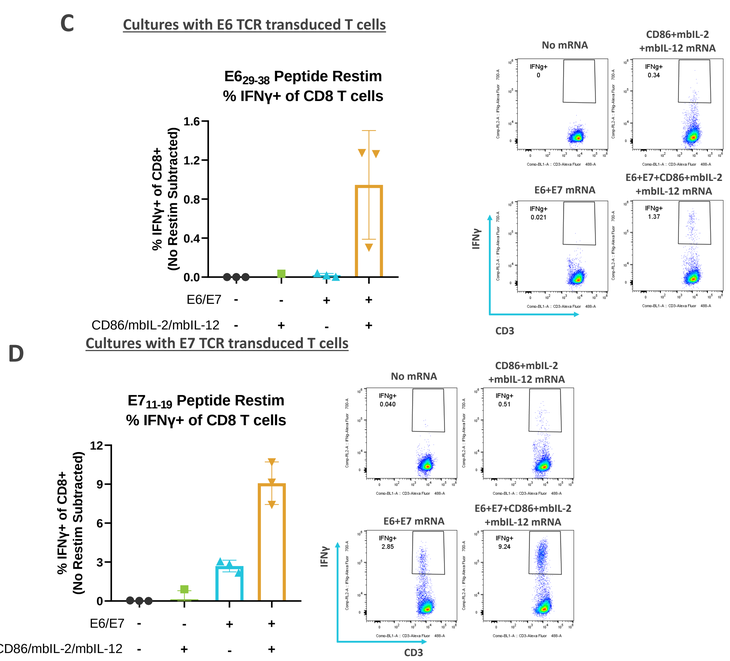
Co-squeezing Signal 2/3 mRNAs Specifically Enhance Antigen-Specific CD8+ T Cell Responses



Membrane-bound IL-2 and IL-12 mRNA delivery via the Cell Squeeze® system leads to surface expression of the cytokines in four human PBMC subsets and yields in functional IL-2 and IL-12 signaling. Human PBMCs were squeezed without cargo (black histograms) or squeezed in the presence of either CD86, mbIL-2, or mbIL-12 mRNA. **A)** FACS analysis was performed to confirm expression of CD86, mbIL-2, and mbIL-12 on four human PBMC subsets at 4 hours post squeeze.

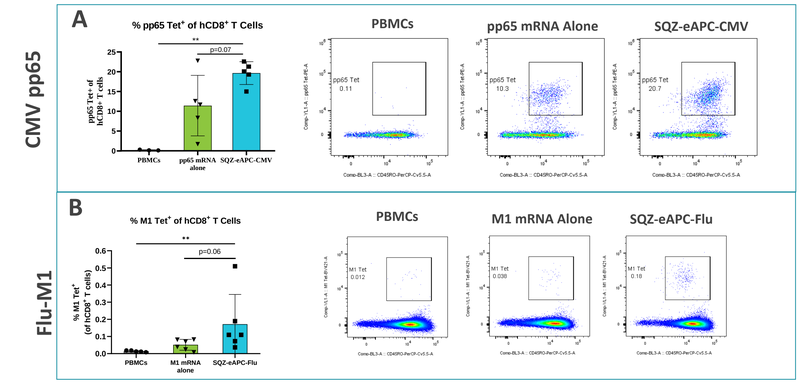


PBMCs from a CMV reactive donor squeezed with mRNAs encoding CMV pp65, CD86, mbIL-2 and mbIL-12 are capable of expanding autologous CD8+ T cells. Cell Squeeze® processed PBMCs were co-cultured with donor matched unprocessed PBMCs that served as a source of CMV reactive CD8+ T cells. Following a 5-day co-culture, cells were stimulated with a mixture of overlapping 15-mer peptides spanning across the whole sequence of CMV pp65 (CMV pp65 PepMix) and evaluated via intracellular cytokine staining for IFNγ **(A)** and TNFα **(B)**. Squeezing all 4 mRNAs into PBMCs resulted in a significant increase in IFNγ and TNFα levels compared CMV pp65 mRNA alone or CD86+mbIL-2+mbIL-12 mRNAs alone.

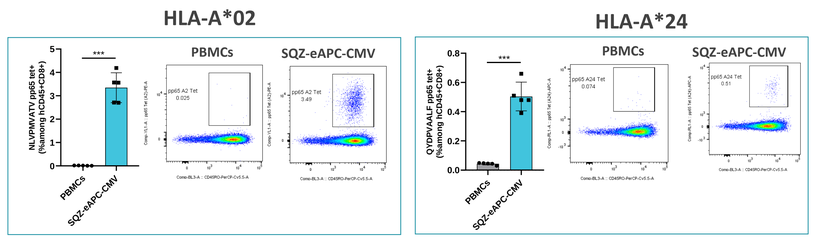


PBMCs squeezed with mRNAs encoding E6, E7, CD86, mbIL-2 and mbIL-12 are capable of expanding CD8+ T cells transduced with HPV16-specific TCRs. Isolated CD8+ T cells from an HLA-A*02 donor were transduced with HPV16 E6 TCR or E7 TCR lentiviruses. The transduced CD8+ T cells were cultured with autologous PBMCs that were squeezed in the presence of E6+E7 mRNA and/or CD86+mbIL-2+mbIL-12 mRNA. To allow for expansion of antigen-specific CD8+ T cells, squeezed PBMCs were cultured with the E6 TCR or E7 TCR transduced T cells for 6 days. Cells were then restimulated with the E6 or E7 minimal epitopes. Intracellular cytokine staining for IFNγ was done to measure the percentage of E6 TCR T cells **(C)** or E7 TCR T cells **(D)**. Squeezing all 5 mRNA into PBMCs resulted in a significant increase in the percentage of the cognate HPV16-specific CD8+ T cells compared to E6+E7 mRNA alone or CD86+mbIL-2+mbIL-12 mRNA alone.

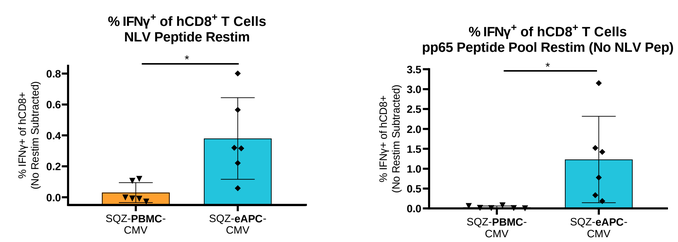
SQZ® eAPCs Drive Expansion of Antigen-Specific CD8+ T Cells in a Humanized Mouse Model



Humanized mice immunized with SQZ® eAPCs generate CD8+ T cell responses that trend higher than mice immunized with PBMCs squeezed with antigen alone. (**A**) NSG MHC-I/II DKO mice were engrafted on day 0 with $5x10^6$ PBMCs. On day 0 and day 7, mice were injected with $5x10^6$ unprocessed PBMCs, $5x10^6$ PBMCs squeezed with pp65 mRNA alone, or $5x10^6$ SQZ-eAPC-CMV cells. On Day 14, mice were sacrificed and CMV pp65-specific cells in the spleen were measured by tetramer staining. (**B**) NSG MHC-I/II DKO mice were engrafted on day 0 with $9x10^6$ unprocessed PBMCs. On day 0 and 14, mice were immunized with $1x10^6$ unprocessed PBMCs, $1x10^6$ PBMCs squeezed with M1 mRNA alone, or $1x10^6$ SQZ-eAPC-Flu cells. On Day 21, mice were sacrificed and Flu M1-specific cells in the spleen were measured by tetramer staining. Representative plots are shown for **A** and **B**.



SQZ® eAPCs elicit responses across multiple HLA types in humanized mice. NSG MHC-I/II DKO mice were engrafted on day 0 with $9x10^6$ unprocessed PBMCs. On days 0 and 14, mice were immunized with $1x10^6$ unprocessed PBMCs or $1x10^6$ SQZ-eAPC-CMV cells. On day 21, mice were sacrificed and pp65-specific responses in the spleen were measured using an HLA-A*02-restricted tetramer (left) and HLA-A*24-restricted tetramer (right).



SQZ® eAPCs elicit CD8+ T cell responses in humanized mice that are higher than those to SQZ-PBMCs across multiple HLA types. NSG MHC-I/II DKO mice were engrafted on day 0 with $9x10^6$ unprocessed PBMCs. On days 0 and 14, mice were immunized with $1x10^6$ SQZ-PBMC-CMV cells or $1x10^6$ SQZ-eAPC-CMV cells. SQZ-PBMC-CMV cells were PBMCs squeezed with a $pp65_{485-519}$ synthetic long peptide containing the HLA-A*02 restricted epitope, NLVPMVATV, and matured for four hours with the TLR9 agonist, CpG. SQZ-eAPC-CMV cells were PBMCs squeezed with full-length pp65 mRNA, as well as CD86, mbIL-2, and mbIL-12 mRNA. On day 21, mice were sacrificed and spleens were restimulated with the HLA-A*02 restricted epitope, NLVPMVATV, or a pool of seven pp65 minimal epitopes predicted to bind HLA-A*01, HLA-A*11, HLA-A*24, HLA-B*07, or HLA-B*35. Responses were measured by intracellular cytokine production of IFNγ.

Summary

- In vitro, squeezing of human PBMCs with antigen and CD86, mbIL-2, and mbIL-12 mRNAs augments antigen-specific CD8+ T cell responses compared to PBMCs squeezed with antigen mRNA alone
- In humanized mouse models, squeezing of human PBMCs with antigen and CD86, mbIL-2, and mbIL-12 mRNAs enhances antigen-specific CD8+ T cell responses and generates responses across multiple HLA types