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TECHNOLOGY EXPLAINED

Cell Squeeze: driving more effective CD8 T-cell activation through cytosolic antigen delivery

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Cell Squeeze is a novel technology that relies on temporarily disrupting the cell membrane to deliver cargo directly into the cytosol. This approach is applicable to a broad range of cell types (peripheral blood mononuclear cells, red blood cells, hematopoietic stem cells, etc.) and cargos (peptides, proteins, small molecules, nucleic acids, and gene-editing complexes) while minimally disrupting normal cell function. By enabling direct cytosolic delivery, one can use this technology to dramatically enhance major histocompatibility complex (MHC) class I presentation of antigens (Ags) for CD8+ T-cell activation—a longstanding challenge for the therapeutic cancer vaccine field that has generally relied on cross-presentation of endocytosed Ags. In addition, by coupling improved MHC class I presentation with coexpression of additional stimulatory factors or systemic immune modulators, one can further enhance the potential impact of an antitumor CD8 response. Pursuing a more direct cellular engineering strategy, which is independent of viral transduction, genetic manipulation, and expansion steps, enables <24 h manufacturing of autologous cell therapies. Through generation of more sophisticated, multifunctional, cell-based vaccines, clinical testing of this technology will elucidate its potential for impact across multiple tumor types.

Key words: Cell Squeeze, therapeutic cancer vaccine, cell therapy, MHC class I antigen presentation

BACKGROUND

As immuno-oncology is better understood, it is evident that CD8+ T-cell activation and infiltration into the tumor microenvironment (TME) correlate with the response across tumor types; however, it has been historically challenging to elicit this CD8+ T-cell response.[1] To date, therapies aimed at priming the CD8+ T-cell response have had limited success.

Approved chimeric antigen receptor T-cell (CAR-T cell) therapy, an autologous CD4+ and CD8+ T-cell product genetically modified to express CD19, has had success in certain hematological malignancies; however, this success has not translated into effective treatment against solid tumors as CAR-T cells may not be able to infiltrate the TME[2] or can drive fatal toxicity.[3,4] In addition, delivery of the transgene into the T cell to manufacture CAR-T cells for clinical use is complicated. Established methods of delivering cargo into the cell, such as viral vectors or electroporation, could be used to deliver immunogenic cargo, but these techniques present different challenges (Table 1). Viral integration of the transgene has oncogenic and mutagenic potential, limits the size of the transgene, and necessitates more cumbersome release testing to control for these risks. Electroporation can be used as an alternative to viral vectors; however, it tends to be less efficient for integration and has been shown to disrupt normal cell function.[5] Harsh preconditioning regimens, high manufacturing costs, and lengthy turnaround times further contribute to the difficulties of broadly implementing current cell therapies.

While immune checkpoint inhibitors (ICIs) have aimed to rescue exhausted CD8+ T cells by inhibiting negative regulators of T-cell function, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death-ligand 1 (PD-L1), only a minority of patients respond to treatment, suggesting that ICI therapy itself is not sufficient. Failure to ICI therapy is not fully understood, but impaired formation of antitumor T cells [including lack of response to tumor-specific antigens (Ags) and inability to infiltrate the TME] is thought to contribute.[6]

Therapeutic cancer vaccines are a potentially promising approach to improve Ag-specific immune responses against cancer cells. With a long list of identified neoantigens and cancer-associated viral Ags, the as yet unrealized benefits of

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Table 1. Comparison of different approaches to intracellular delivery

	Viral	Electroporation	Cell Squeeze
Minimal cell perturbation	Tendency to trigger innate antiviral responses. Risk of integration of viral components into the genome	Disruption of normal gene expression that can interfere with important cell functions such as differentiation, expansion, cytokine secretion, etc.	Minimal perturbation of gene expression or normal cell functions
Scalability	Production of good manufacturing practice virus with sufficient batch-to-batch consistency can be a challenge	Flow-based systems are scalable; cuvette-based systems have challenges	Scalable through channel parallelization
Universal across cell types	Only applicable to certain cell types and often require different viral constructs for different cell types	Only applicable to certain cell types	Applicable to all mammalian cell types attempted to date
Material independent	No, can only deliver nucleic acids compatible with viral packaging	No, complex delivery mechanism favors highly charged materials such as nucleic acids	Diffusion-mediated delivery is mostly cargo agnostic. Relatively large (>100 nm) nanoparticles can become a challenge
Compatible with intracellular targeting	No, viral delivery mechanism provides little flexibility	Some flexibility to accommodate various targeting domains to direct cargo to different intracellular locations	Yes
Dosage control	Stochasticity of viral transduction can lead to high variability	Some control; however, nonlinear optimization space of electroporation parameters poses a challenge	Predictable diffusion-mediated mechanism ensures a fairly linear control by adjusting material concentration in the buffer
Cost	High: production of viral material is complex and expensive	Low	Low

Many intracellular delivery techniques have been developed. These are covered in detail in Stewart et al.[24]; however, the most commonly used approaches in current cell therapy manufacturing are viral transduction or electroporation.

a therapeutic cancer vaccine could be substantial.[7] Many clinical trials to demonstrate cancer vaccine proof-of-concept are currently being conducted in patients with human papillomavirus (HPV)-associated cancers. Given the viral nature of HPV-driven tumors, which constitutively express oncogenic E6 and E7 proteins that are not present in noncancerous cells, these cancers are ideal for evaluating a therapeutic cancer vaccine.

The prospect of a safe and effective therapeutic vaccine could be transformative; however, cancer vaccines have had limited success, in part due to the lack of CD8+ T-cell response elicited by vaccination.[7] Many vaccine modalities have had success in the generation of antibody and CD4 responses,[8-10] but despite some promising preclinical data,[11,12] there has been limited CD8 infiltration and subsequent clinical benefit. A significant consideration is that most vaccine strategies primarily rely on cross-presentation of Ag that is endocytosed by a resident dendritic cell for cytotoxic or effector T-cell activation. Endocytosis is the process by which cells engulf material, resulting in the material being segregated from the cytosol in an endosome. This results primarily in major histocompatibility complex (MHC) class II presentation and subsequent CD4+ helper T-cell activation. Through a process referred to as cross-presentation, a small amount of endosomal material can be presented on MHC class I to yield some CD8+ T-cell activation.[13] However, this process is inefficient, resulting in suboptimal priming of $CD8^+$ T-cell responses with antitumor or antiviral activity.

Therefore to achieve effective CD8 responses one must likely provide significant Ag load to the patient with these technologies. While this has been possible in murine systems, scaling to humans has been infeasible (Table 2). In some cases, the human dose is the same or very similar to a murine dose on a total mass basis, therefore is dramatically lower on a surface area or mass scaling basis.[11-14]

By solving some of the underlying problems related to the enablement of MHC class I presentation to CD8 T cells, the Cell Squeeze technology may provide a path to achieving a more potent therapeutic cancer vaccine. In preclinical models, Cell Squeeze has demonstrated the ability to induce robust MHC class I presentation across multiple target Ags, subsequently creating strong CD8+ T-cell responses capable of tumor infiltration.[15] This review explores the potential impact of this approach.

Table 2. Comparison of preclinical versus clinical dosing in different HPV16 cancer vaccines

	SQZ APCs (SQZ-eAPC-HPV)	BioNTech mRNA (HPV16 RNA-LPX)	ISA (ISA101)
Mouse dose[a]	40×10^6 cells/kg[18]	1600 μg/kg[11]	6000 μg/kg[12]
Human high dose[b]	10×10^6 cells/kg[c]	1 μg/kg[25,26]	4.3 μg/kg[14]
Fold difference	4×	1600×	1400×

A comparison of preclinical and clinical doses in different HPV16 cancer vaccines. The preclinical doses of BioNTech and ISA vaccines were 1000× greater than the high doses used in human trials; scaling preclinical doses to humans has been infeasible.
HPV, human papilloma virus; mRNA, messenger RNA.
[a] Assumes a 25 g mouse.
[b] Assumes a 70 kg human.
[c] Intended high dose, not yet tested in clinic.

CELL SQUEEZE TECHNOLOGY

Using the Cell Squeeze microfluidics technology, cargo can be delivered intracellularly. Cells are flowed in a solution of cargo at high speeds through a microfluidic constriction that is smaller than the diameter of the cell, which results in the temporary disruption of the cell membrane (Figure 1).[16] This enables the target cargo to enter directly into the cytosol of the cells. Cell Squeeze can be used to deliver many types of molecules, including peptides, proteins, small molecules, nucleic acids, and gene-editing complexes, to a diversity of cell types.[17] Many of these material classes, such as proteins, peptides, and nucleic acids, have been difficult to deliver with existing delivery techniques. Further, multiple materials can be delivered into the cells simultaneously, thus enabling the ability to multiplex and engineer several cellular functions in a single step.

In the context of dendritic cells, for example, direct cytosolic delivery of protein Ag was shown to be ~1000 times more effective than cross-presentation after endocytic uptake of protein Ag.[15] These engineered cells also showed potent abilities to stimulate previously activated CD8+ T cells, both *in vitro* and *in vivo* (Figure 2). We also demonstrate that microfluidic squeezing can enable Ag presentation by human cells at a manufacturing scale for potential clinical application. Finally, we show that immune responses elicited by squeezed cells, in combination with an adjuvant, are capable of driving antitumor effects that correlate with an influx of tumor-specific CD8+ T cells. By overcoming the fundamental barrier to effective MHC class I presentation of Ag, squeeze-engineered cells could potentially be used as the basis for a potent, rapid turnaround, cell-based vaccine that is applicable across tumor types.[15]

When assessed *in vivo*, mouse splenocytes squeezed with <100 ng of Ag elicit an antitumor response that is better than subcutaneous injection of 150 µg of the same Ag, a difference of three orders of magnitude.[15] The first clinical candidate, SQZ-PBMC-HPV, is in a phase I/II study in patients with HPV16+ recurrent, locally advanced, or metastatic solid tumors as a monotherapy and in combination with ICIs. The preliminary findings are discussed later in this review. The product is based on *ex vivo* engineering of autologous peripheral blood mononuclear cells (PBMCs) to create antigen-presenting cells (APCs) that are then transferred back to the patient.

A significant challenge for existing cell therapies is the time, cost, and complexity of manufacturing (Table 3). By potentially enabling rapid, cost-effective manufacturing of

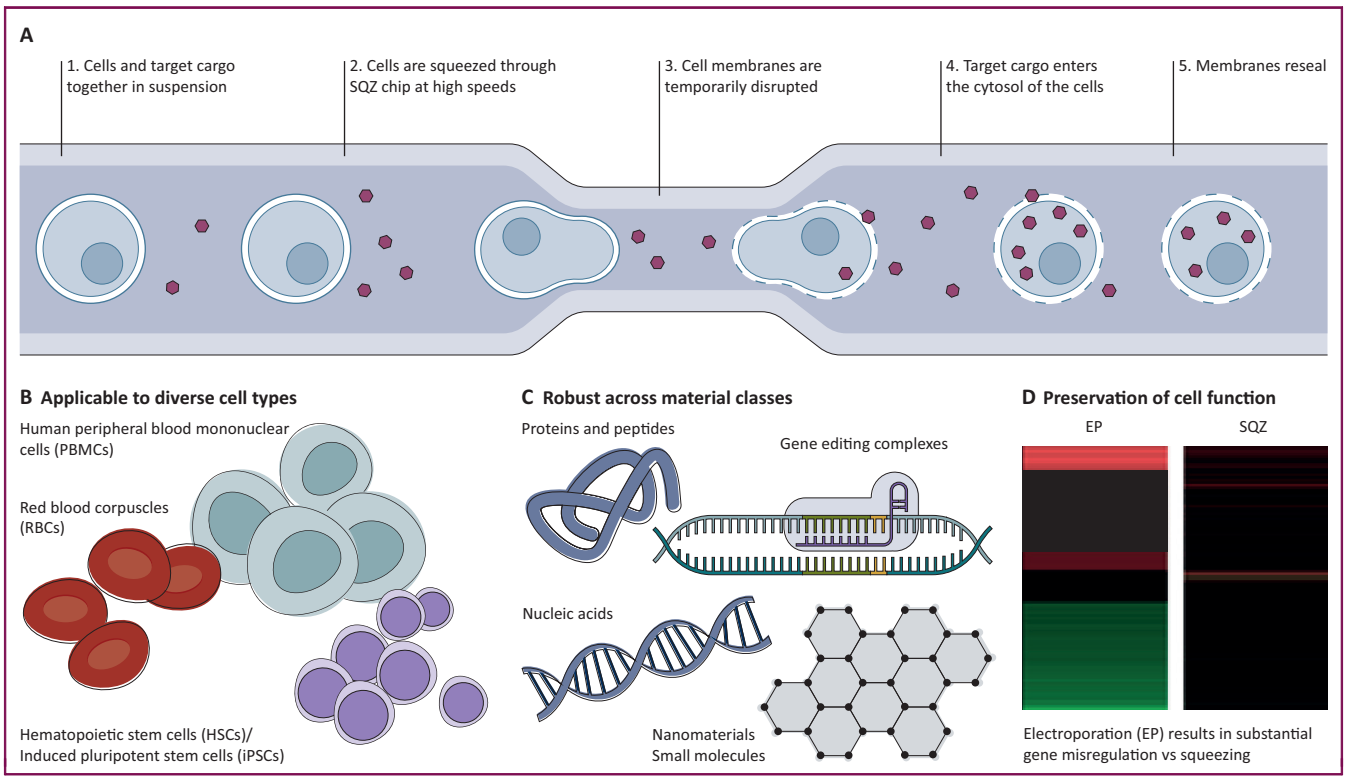

Figure 1. Overview of the Cell Squeeze technology. There are two categories of approaches one can pursue with the Cell Squeeze technology to engineer an anticancer vaccine: (1) Leveraging the direct cytosolic delivery capabilities of the technology to by-pass cross-presentation and facilitate direct MHC class I expression of target antigens. (2) Using the ability to deliver material to red blood cells to transport material to the professional DCs that are most adept at antigen presentation *in vivo*. This technology is currently in clinic; however, because the mechanism of action is significantly different, it is not covered in this paper. (A) The Cell Squeeze technology passes cells through a restriction point, temporarily disrupting the cell membrane, allowing for delivery of cargo. (B) The Cell Squeeze technology is applicable to a broad array of cell types. Currently, RBC, PBMCs, and HSC/iPSCs are currently being evaluated as clinical candidates. (C) A variety of cargos may be introduced into the cell, including proteins, peptides, nanomaterials, nucleic acids, and small molecules. (D) Heat map showing gene misregulation in human T cells 6 h after delivery of fluorescently labeled dextran via either electroporation or Cell Squeeze.[16]
DC, dendritic cell; HSC, hematopoietic stem cell; iPSC, induced pluripotent stem cell; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; RBC, red blood cell.

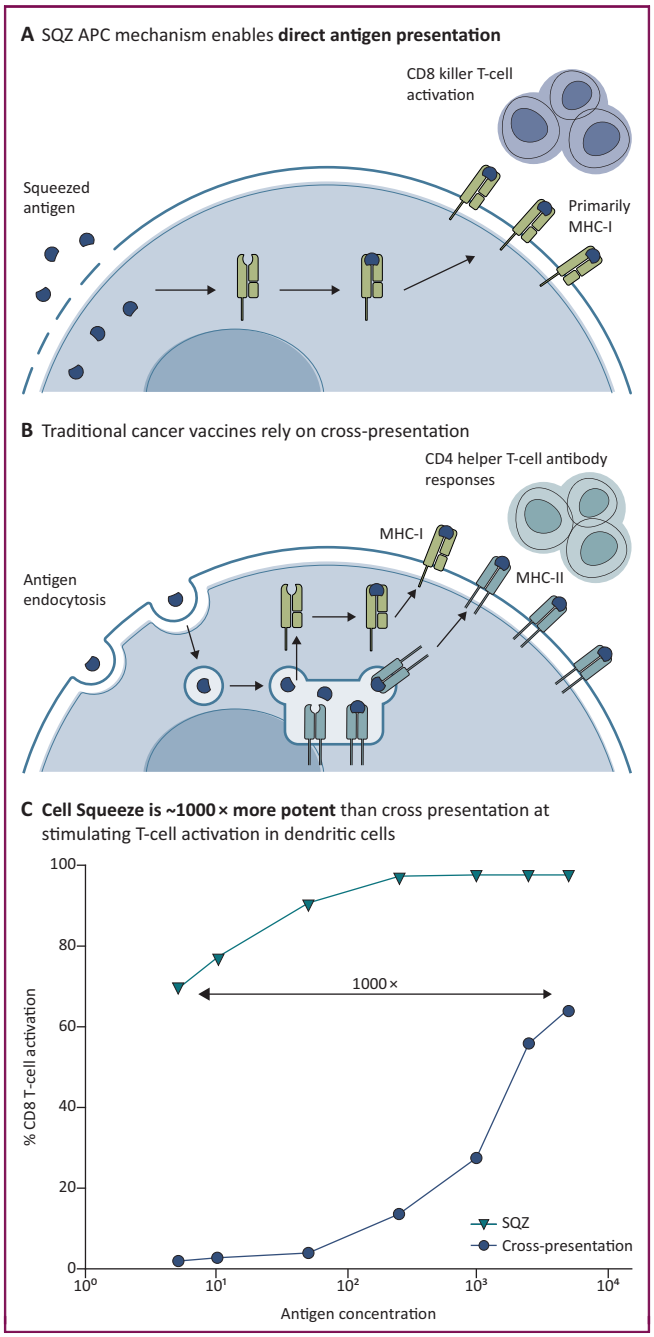

Figure 2. Traditional cancer vaccine endogenous cross-presentation on MHC class II versus Cell Squeeze Cancer vaccine direct presentation on MHC class I. (A) Cell Squeeze delivers cargo directly into the cytosol of the cell, resulting in primarily MHC class I presentation. (B) Traditional cancer vaccines have relied on endocytosis to process antigen, which results primarily in MHC class II presentation. (C) Control murine bone marrow-derived dendritic cells (BMDCs) and BMDCs Cell Squeezed with fluorescently labeled OVA were cultured with CD8+ T cells for 24 h, then assessed by flow cytometry.[15] Cell Squeeze is ~1000× more potent than cross-presentation at stimulating T-cell activation.[23] MHC, major histocompatibility complex.

cell therapies, the Cell Squeeze technology could potentially improve the feasibility and accessibility of cell therapies for many indications. The current SQZ process for producing clinical material is a fully closed system that maintains aseptic process conditions. The system utilizes a disposable kit that includes the microfluidic chips and incorporates integrity tests on the kit prior to the initiation of the manufacturing process. The microfluidic chip has hundreds of parallel constriction channels. The manufacturing time for the current sterile product candidate is under 24 h, with a vein-to-vein time of ~1 week. Sipuleucel-T (Provenge, Dendreon Pharmaceuticals, Seal Beach, CA) has a shorter vein-to-vein time (~3 days), but the drug product is released at risk; final sterility information is not available until after the patient has received drug, at least 7 days after the start of the manufacturing process.[18] Current sterile cell therapies typically can have vein-to-vein times of 4-6 weeks.

The automation of the entire manufacturing process is underway to create a point-of-care system to further reduce the manufacturing time and cost. By integrating upstream and downstream manufacturing operations with the Cell Squeeze system, a fully closed, automated system suitable for on-site cell therapy production without a clean room is potentially enabled. This decentralized manufacturing approach is designed to enable rapid access to a cell therapy intervention at community sites or field clinics, reduce treatment time from days to hours and improve patient access.

Currently, the Cell Squeeze technology has been used to create drug products and is being tested in two ongoing clinical trials with the APC platform (NCT04084951) and with the Activating Antigen Carrier (AAC) platform (NCT04892043).

The development plan of a therapeutic vaccine using the Cell Squeeze technology aims to demonstrate proof-of-concept as monotherapy and also enhanced efficacy in combination with ICIs that are presently part of the therapeutic arsenal for HPV-driven tumors.

ICIs, in particular those acting on the PD-1—PD-L1 axis, have been effective in treating multiple tumors, including HPV-positive tumors, by allowing the exhausted T cells to once again recognize the malignant cells. However, only a subset of patients treated with ICIs responds and most eventually experience disease progression. The combination of a therapeutic vaccine with anti-PD-1 checkpoint inhibitors has been evaluated in HPV-induced cancers with promising results.[19] Already, combination treatment with ICI and interleukin-2 (IL-2) has demonstrated preliminary efficacy in patients refractory to ICI monotherapy,[20] suggesting that patients may be able to derive additional benefit by enhancing the tumor-specific T-cell response in combination with ICI treatment. The vaccine approach is expected to induce Ag-specific antitumor immune response that would work synergistically with the checkpoint inhibitors in removing the brakes of the CD8+ T cells and restoring the ability to attack the tumor. In preclinical studies, recombinant anti-PD-1 IL-2v fusion protein (PD1-IL2v) and M-SQZ-PBMC-HPV resulted in dramatic infiltration of E7-specific CD8+ T cells into the TME and subsequent cures in treated animals (Figures 3 and 4).[21] The mechanism of action is thought to be targeted delivery of IL-2 to PD-1-expressing cells and these results highlight the potential of multiple immune modulating modalities, in this case, a cytokine and checkpoint inhibitor, to behave synergistically with an effective vaccine.

Table 3. Overview of cancer vaccine manufacturing

Cell type	Drug product	Cell yield	Manufacturing time	Estimated vein-to-vein	Drug product release characterization	Other parameters
DCs	Sipuleucel-T[18]	50-100 M cells	3-4 days	~3 days[a]	At risk	3 leukaphereses
PBMCs	SQZ-PBMC-HPV	5-10 B cells	<24 h	~1 week	Sterile	1 leukapheresis
RBCs	SQZ-AAC-HPV	TBD	<24 h	~1 week	Sterile	1 whole blood collection
CAR-T	Tisagenlecleucel[27,28]	60-600 M	~23 days	~3-8 weeks	Sterile	1 leukapheresis
TIL	TIL[29]	>1 B	~20 days	~30 days	At risk	Isolated from resected metastatic lesion

A summary of cancer vaccines using different cell types. TIL and CAR-T vaccines have a lengthy manufacturing due to the *ex vivo* expansion of cells, leading to longer vein-to-vein times for patients and increased cost. TIL and DC vaccines are released before all quality control testing is complete, that is, at risk. TIL and DC vaccines also have more involved cell harvesting processes than other vaccines. Cancer vaccines developed using the Cell Squeeze technology are manufactured using only one blood collection (either whole blood or leukapheresis), have a rapid manufacturing time, and are confirmed sterile at the time of release.
CAR-T, chimeric antigen receptor T; DC, dendritic cell; PBMC, peripheral blood mononuclear cell; RBC, red blood cell; TIL, tumor-infiltrating lymphocyte.
[a] Sipuleucel-T has an estimated vein-to-vein time of ~3 days. This rapid vein-to-vein time is due to the product being released before microbial and sterility testing is resulted i.e. at risk. Vein-to-vein time would be at least 7 days (sterility test incubation time) otherwise.

The current clinical APC platform drug product, SQZ-PBMC-HPV, has completed the monotherapy dose-escalation portion of the study. SQZ-PBMC-HPV monotherapy was found to be safe and well-tolerated; no dose-limiting toxicities were observed.[22] Encouraging antitumor activity was observed in patients treated with SQZ-PBMC-HPV monotherapy. Currently, the study is enrolling into the combination phase of the study, in which participants will be treated with SQZ-PBMC-HPV and an ICI.

While SQZ-PBMC-HPV has shown promising results in the clinic, one can leverage the flexibility of the Cell Squeeze technology to engineer additional stimulatory domains in cell therapy. SQZ-eAPC-HPV, a next-generation product, improves upon the first by engineering costimulatory and cytokine expression simultaneously with Ag presentation (Figure 5). This is accomplished by squeezing messenger RNA transcripts for E6, E7, CD86, membrane-bound IL-2, and membrane-bound IL-12 into PBMCs, resulting in a multifunctional cell therapy capable of more powerful T-cell modulation.

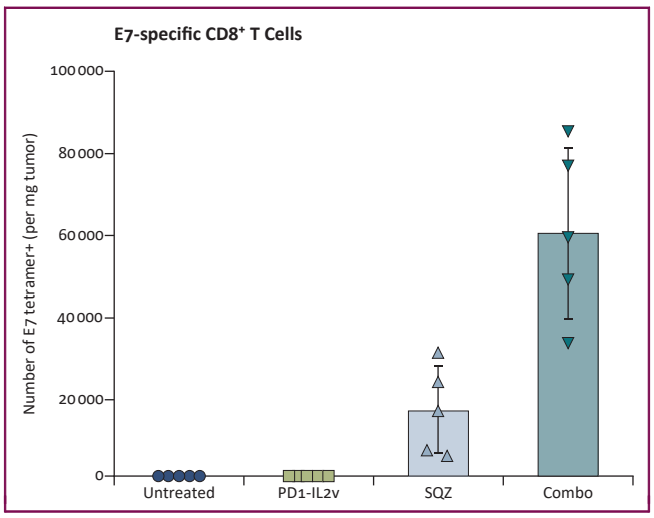

Figure 3. CD8+ infiltration into the TME. SQZ and combo mice were treated with 1 × 10^6 M-SQZ-PBMC-HPV 14 days after TC-1 inoculation. PD1-IL2v and Combo mice were dosed with 0.5 mg/kg PD1-IL2v on day 21. Tumors were harvested on day 24 and TILs analyzed by flow cytometry.[21]
TIL, tumor-infiltrating lymphocyte; TME, tumor microenvironment.

If successful in these initial studies, the Cell Squeeze cancer vaccine strategies could be rapidly adapted for implementation across applications beyond HPV-driven cancers. The technology's flexibility around Ag cargo enables the creation of therapeutic vaccines for immuno-oncologic targets such as Epstein—Barr virus, mutant KRAS, mutant TP53, and patient-specific neoantigens.[7] Moreover, the clinical safety results to date are supportive of the potential implementation of these therapeutic concepts in early-line settings, including combination approaches with currently approved ICIs. This provides the opportunity to complement or displace standard-of-care regimens with cancer vaccines that have the potential for durable patient impact.

The ability to more directly engineer MHC class I presentation marks a potentially transformative advancement

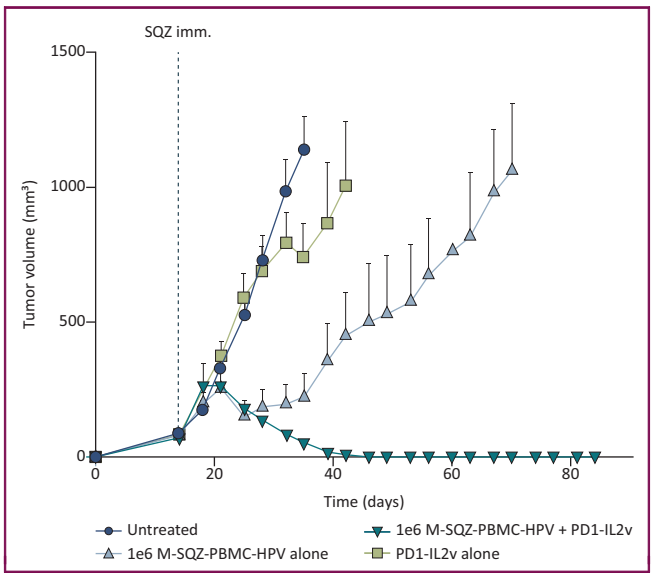

Figure 4. M-SQZ-PBMC-HPV vaccination has synergistic antitumor activity when combined with ICI. Mice were implanted with TC-1 cells. For mice treated with M-SQZ-PBMC-HPV, vaccination occurred 14 days later. Mice receiving PD1-IL2v were administered drug intravenously 21, 28, and 35 days after implantation. Greater tumor reduction was observed in mice treated with M-SQZ-PBMC-HPV and PD1-IL2v than in mice treated with M-SQZ-PBMC-HPV alone or PD1-IL2v, suggesting there is a synergistic drug effect.[21]
ICI, immune checkpoint inhibitor.

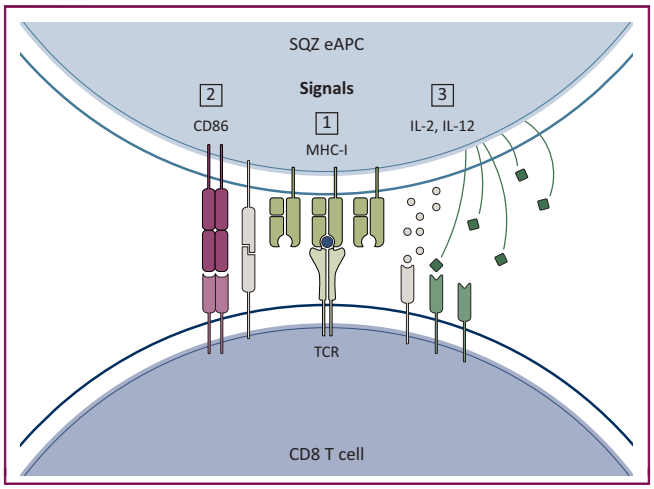

Figure 5. Anticipated clinical translation of multifunctional eAPCs. As SQZ-eAPC-HPV is manufactured by using Cell Squeeze to insert mRNA that will translate full-length E6, full-length E7, membrane-bound CD86, membrane-bound interleukin-2, and membrane-bound interleukin-12, SQZ-eAPC-HPV is expected to have enhanced CD8+ activation and broader antigen repertoire than SQZ-PBMC-HPV, which is manufactured using only the immunodominant synthetic long peptides for E6 and E7. SQZ-eAPC-HPV is also expected to have enhanced signal 1 (due to E6 and E7), signal 2 (due to CD86), and signal 3 (due to interleukin-2 and interleukin-12).
IL, interleukin; mRNA, messenger RNA; TCR, T-cell receptor.

for the cancer vaccine field. As ongoing studies continue to explore this potential, careful consideration of biomarkers and trial design factors can help improve our understanding of the impact of this technological advance and how best to harness it for improved patient outcomes. Continued investment in future generations of multifunctional cell therapies, such as the eAPCs, can further enhance the ultimate probabilities of success while the development of more streamlined manufacturing can broaden the accessibility of these cell therapies. Ultimately, this latest generation of Cell Squeeze-enabled cancer vaccines may finally achieve the patient impact this field has long aspired to.

DISCLOSURE

HB, SL, RZ, JJ, and AS are employees of SQZ Biotech. VN, CK, LCD, PU, and CT are employees of Roche. JCP has declared no conflicts of interest.

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