Targeted Cell-to-Cell Delivery of Protein Payloads via the Granzyme-Perforin Pathway

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There is great potential for engineering cellular therapeutics by repurposing biological systems. Here, we report utilization of the granzyme-perforin pathway of cytotoxic lymphocytes as a cell-to-cell protein delivery module. We designed and constructed granzyme B-derived chaperone molecules fused to a fluorescent protein payload and expressed these constructs in natural killer (NK) cells. Using confocal microscopy and flow cytometry, we investigated the co-localization of the chaperones with lytic granules and the chaperone-mediated transfer of the fluorescent protein payload from NK to target cells in co-culture experiments. A synthetic chaperone consisting of the granzyme B ER signal peptide and a domain encompassing putative N-linked glycosylation sites in granzyme B is insufficient for payload transfer to target cells, whereas full-length granzyme B is sufficient for payload delivery. Combining our functional data with an analysis of the crystal structure of granzyme B suggests that the necessary motifs for granzyme B loading into lytic granules are dispersed throughout the primary amino acid sequence and are only functional when contiguous in the tertiary structure. These results illustrate that using granzyme B as a molecular chaperone the granzyme-perforin pathway can be exploited as a programmable molecular delivery system for cell-based therapies.

INTRODUCTION

With their ability to sense and integrate a wide range of signals, actively move to specific tissue compartments, and actuate context-dependent responses, engineered cell-based therapeutics are emerging as the next major class of medical intervention. Chimeric antigen receptor T cells (CAR Ts) are highly effective in treating hematological malignancies and many mesenchymal stem cell therapies are at various stages of development for use in cardiac, neurological, and malignant disease. These advances are a result of recombining the diverse functionality of biological systems to generate new functional biological molecules and pathways.

For any useful cellular device, three primary components are required: input modules, control logic, and output modules. Significant progress has been made with the former two, including a variety of transcriptional control circuits as well as highly specific and configurable chimeric antigen receptors and synthetic notch receptors. Output modules for cellular devices have been less developed and investigated. Most rely on passive, nonspecific secretion, activated by a combination of sensory input modules, modulated by control circuits. This approach has potential for a variety of metabolic derangements, but also has a significant limitation in that transcriptionally activated secretion creates a temporal delay between activation and actuation. For many applications, an ideal output module would have the deliverable payload pre-positioned for rapid actuation and delivery. Furthermore, secretion only achieves regional specificity at best. In many cases cell-specific delivery would be extremely valuable, both as a research tool, as well as in therapeutic contexts.

To date the most investigated and best understood cell-to-cell delivery systems are bacterial in origin, specifically bacterial type III and type IV secretion systems. Other synthetic biology approaches have also been developed using, for example, invasin triggered by environmental signals or quorum sensing. These designs achieve cell-specific delivery, yet they retain two major weaknesses. First, they are for the most part transcriptionally controlled, resulting in a lag between activation and delivery, and second, they are bacterial in origin, likely raising prohibitive immunological obstacles to human therapeutic applications.

We set out to develop a cell-to-cell delivery module in which payload delivery could be rapidly initiated by surface receptors, with this activation resulting in the transfer of the payload from the delivery cell to a target cell in a cell-specific manner. Further, we desired that delivery originate from a pre-positioned source, such that the time between initiation of target recognition and completion of payload delivery is a short, tightly coupled process. The granzyme-perforin pathway, in the context of a cytotoxic lymphocyte chassis, is a unique and potentially broadly applicable cell-to-cell delivery system that fulfilled these criteria. This pathway is one of the main ways in which cytotoxic lymphocytes kill target cells. Its main constituent components are the serine protease granzymes and the pore forming protein perforin, both of which are stored in membrane-bound secretory lysosomes, or

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forin from the lytic granules into the synapse between the two cells. Upon target cell recognition, the cytotoxic lymphocyte forms a tight apposition with the target cell, forming the immunological synapse. Surface receptor signaling results in the endocytic release of granzymes and perforin, which targets the protein to the Golgi network once synthesis is complete. In the Golgi, the N-glycan is further phosphorylated. This phosphosugar moiety on granzyme B then binds to the mannose-6-phosphate receptor, which targets the protein to lytic granules, where it is sequestered until target cell recognition, resulting in granzyme B release into the immune synapse. Importantly, recent work has shown that following release into the immune synapse, the trafficking of granzyme B to the target cell membrane and entry into the target cell via perforin pores is likely a result of passive diffusion only.

RESULTS

Design of Granzyme B-Derived Molecular Chaperones

Granzyme B is synthesized as a pre-pro-protein, with an 18 amino acid N-terminal endoplasmic reticulum (ER) signal peptide, followed by an inhibitory dipeptide, followed by the rest of the protein. Upon initiation of translation, the ER signal peptide directs the nascent protein to the ER, where it is co-translationally inserted into the ER. As the protein is synthesized in the ER, an N-glycan is added, which targets the protein to the Golgi network once synthesis is complete. In the Golgi, the N-glycan is further phosphorylated. This phosphosugar moiety on granzyme B then binds to the mannose-6-phosphate receptor, which targets the protein to lytic granules, where it is sequestered until target cell recognition, resulting in granzyme B release into the immune synapse. Importantly, recent work has shown that following release into the immune synapse, the trafficking of granzyme B to the target cell membrane and entry into the target cell via perforin pores is likely a result of passive diffusion only.

We used this information to guide our design of chaperones for granzyme-perforin-mediated delivery. Since the steps in this process that are downstream of lytic granule exocytosis appear to be passive, we hypothesized that a chaperone that successfully directed a payload to be loaded into lytic granules would also be sufficient for payload delivery to the target cell. In designing such a chaperone, we adopted two strategies: rational and empirical (schemas for all constructs used in this paper are found in Figure 1).

For our rational design, we sought to develop a set of minimal granzyme B domains that would shuttle a protein payload to lytic granules. We took this set to be an ER localization domain and a lytic granule localization domain. For the former, we used the granzyme B domains that would shuttle a protein payload to lytic granules. For the latter, we used a 53 amino acid motif surrounding two computationally predicted N-linked glycosylation sites (GZBSM). Our rationale for this choice was that if there were unknown domains within granzyme B other than the region surrounding the N-linked glycosylation sites that were necessary for lytic granule loading or if the necessary domains are adjacent to the N-linked glycosylation sites only in the tertiary structure of granzyme B, then they

This combination of the granzyme-perforin pathway and cytotoxic lymphocyte chassis fulfill all of the requirements for a cellular device output module listed above: native and engineered targeting modules (T cell receptors or chimeric antigen receptors) that naturally interface with a signaling cascade that initiates release of pre-positioned molecules, that are delivered in a cell-specific manner, as defined by the original surface receptor recognition of the target cell.

Granzyme B (GZB) is a well-studied effector molecule that transits the granzyme-perforin pathway. Here, we engineer granzyme B-derived chaperones and trace chaperone mediated trafficking of a functional fluorescent protein payload through this pathway from effector to target cells. This demonstrates the implementation of a programmable cell-to-cell transfer module that can potentially be used to deliver different ectopic protein payloads to targeted tissues or cells.

Figure 1. Design of Payload Delivery Module Chaperones

(A) Granzyme B as a model protein that transits the granzyme-perforin pathway. The full-length coding sequence is shown in green, with the ER signal peptide in light green (GZBSS). The two putative N-linked glycosylation motifs are shown in blue (NLG), with the encompassing putative sort motif (GZBSM) in yellow. The boundaries of the first and second halves of granzyme B used in this study (GZB1 and GZB2) are shown above the full-length coding sequence. (B) Schematic of the constructs used in this study. crmCherry (MCH, red), an RFP protein was used as a model payload, and a flexible glycine serine linker (GSL, purple) was used to join GZB, GZB1, and GZB2 to MCH.
would be captured in the full-length protein. As a first step toward delineating the location of these critical motifs within full-length granzyme B, we also investigated the first and second halves of granzyme B (GZB1 and GZB2, respectively) as independent chaperones. If either of these halves were functional as a chaperone, not only would it indicate that a minimal motif was present in them, from a practical standpoint this would be useful as it would yield a smaller chaperone, potentially much smaller if the process were repeated and the minimal motif further constrained. We placed the GZBSS ER signal peptide at the N terminus of both GZB1 and GZB2. To keep these granzyme B domains in as native a form as possible, we fused the MCH payload to the C terminus of GZB, with the two proteins connected by a flexible glycine-serine linker.

As controls, we also generated two additional constructs: MCH alone and GZBSS followed by MCH.

**Screening Chaperone Designs by Assessing Lytic Granule Co-localization Using Confocal Microscopy**

Since our hypothesis was that lytic granule loading of a payload would be sufficient for payload delivery, we first investigated the subcellular localization of the chaperones using confocal microscopy. Using electroporation, we expressed the candidates in the natural killer cell line YT-Indy, which retains a functional granzyme-perforin pathway and has well-characterized target cell lines. Following enrichment for mCherry⁺ cells via cell sorting, we stained the cells for the lysosomal and lytic granule marker Lamp1 and then acquired images of the cells using confocal microscopy. As expected, the Lamp1 distribution was punctate in nature, but the MCH distribution was highly variable (Figure 2).

Due to the range of phenotypes observed in the images, we sought to evaluate the degree of payload (MCH) co-localization with Lamp1 in an unbiased manner. To do this, we developed a semi-automated image filtration and analysis pipeline. The algorithm is illustrated in Figure 3 and seeks to eliminate both local background and bleed as well as pixel noise. This is achieved using both local and global image information to filter each pixel. This filtering is critical to enable quantification of co-localization, as it eliminates the background noise from the regions of the image in which there are no cells, as well as regions that are adjacent to granules that have moderate signal intensity, both of which could give a spurious contribution to any quantitative metric of co-localization. The efficacy of this method can be observed by examining the progression of the columns from top to bottom of Figure 3: note that pixel intensities of the punctate structures remain relatively intact, whereas the binarized images (showing the extent of the background signal) change from containing large homogeneous structures to puncta that closely resemble those in the pixel intensity images. To our knowledge, this combination of local background subtraction with global pixel noise filtration has not been reported previously. We feel that it is particularly useful for studies involving small, subcellular structures, and in the hopes that it will be of use to the community, we have included the source code that implements the algorithm in the Supplemental Information.

Using this approach, we quantified the co-localization between MCH and Lamp1 in these filtered images using the Manders M1 coefficient and Pearson correlation coefficient (PCC) (Figure 4). Both metrics indicated that MCH alone had a low degree of co-localization with lytic granules, which would be expected as the lytic granules are small dense granules, and unfused MCH is distributed throughout the cytosol. These quantitative metrics were less useful for differentiating between the remaining fusion proteins. This motivated us to qualitatively assess the morphology of the distributions of MCH and Lamp1 and use this as a guide to selecting candidate chaperones. GZBSS-MCH had a perinuclear and membrane distribution, consistent with it entering the secretory pathway. GZB-MCH displayed a punctate cytosolic distribution consistent with granule loading. GZBSS-MCH-GZBSM exhibits a heterogeneous distribution that is partially punctate and granular (similar to GZB-MCH) and partially of moderate intensity, diffuse, and cytosolic (similar to unfused MCH). GZB1-MCH has an interesting membranous phenotype, which we hypothesize might be a result of this fusion protein entering the secretory pathway. GZB2-MCH has a highly punctuate distribution, but one that is considerably divergent from that of the Lamp1 distribution. The marked difference in the morphology of the subcellular localization of the two halves of granzyme B is interesting, and we discuss this further below. In summary, our assessment of the subcellular distribution of the various chaperone mCherry fusion proteins led us to conclude that GZB-MCH and GZBSS-MCH-GZBSM had the most potential as candidate chaperones.

**Transfer of Fusion Proteins from Effector to Target Cells**

We next characterized the capacity of two candidate chaperones (GZBSS-MCH and GZBSS-MCH-GZBSM) to facilitate transfer of the payload through the granzyme perforin pathway to target cells. To do this, we conducted a series of co-culture experiments, again using mCherry as a fluorescent reporter payload that was easily traceable. Effectors expressing a variety of mCherry fusion proteins were co-cultured with fluorescently labeled target cells, and then analyzed for evidence of mCherry in the target cell populations. We used the B cell lymphoblastoid cell line 721.221 (hereafter 721) as target cells, as they are a well-known YT-Indy target.

We started by testing GZB-MCH, GZBSS-MCH, and MCH alone. GZB-MCH was the chaperone our microscopy images had suggested was most likely to load payloads into lytic granules, while MCH alone was clearly not loaded into lytic granules and thus a good control. GZBSS-MCH was included to confirm that it was not being loaded into lytic granules and hence would not be transferred to target cells. YT-Indy cells were transfected with plasmids coding for these chaperones and then FACS enriched for RFP⁺ cells. The various effector cell types were separately co-cultured with 721 target cells that had been labeled with a fluorescent dye, to distinguish between the effector and target cells. This mixed cell population was then analyzed via flow cytometry (Figure 5). Target 721 cells that were co-cultured with YT-Indys expressing GZB-MCH show an increase in mCherry signal, as compared to 721 s alone, 721 s co-cultured with unmodified YT-Indys, and 721 s co-cultured with YT-Indys expressing either
Figure 2. Subcellular Distribution of Candidate Chaperone-mCherry Fusion Proteins
YT-Indys expressing the candidate fusion proteins (labeled at left) were stained for the lytic granule marker Lamp1 and imaged using confocal microscopy. Shown are merged red (mCherry) and green (Lamp1) channels for three representative cells for each sample. White scale bar, 5 μm.
MCH or GZBSS-MCH. Notably, this increase is most prominent in the dead cell fraction (DAPI+) of the target cells. Since the majority of these cells are dying due to YT-Indy attack, DAPI positivity can be viewed as a proxy for YT-Indy targeting. Therefore, the increase in MCH signal in dead (DAPI+) targets co-cultured with YT-Indys expressing GZB-MCH, but not MCH or GZBSS-MCH, suggests that GZB-MCH is transferred to target cells specifically via chaperone-mediated trafficking through the granzyme-perforin pathway.

We then investigated if the rationally designed chaperone (GZBSS-MCH-GZBSM) would perform similarly to GZB-MCH. We conducted the same type of experiment as above, comparing YT-Indys expressing GZBSS-MCH, GZBSS-MCH-GZBSM, and GZB-MCH. We selected GZBSS as the comparator so that all constructs would have the same N-terminal ER signal peptide and potential for secretion. This would allow us to differentiate between non-specific and mCherry signal in target cells and mCherry signal in target cells resulting from chaperone-mediated transfer. The data (Figure 6) are consistent with our initial co-culture experiments and indicate that GZB transfers MCH to target cells, but GZBSS or GZBSS in combination with GZBSM does not. The fact that GZBSS-MCH-GZBSM did not transfer MCH to target cells is interesting. Given the mixed phenotype observed in YT-Indys expressing this construct in our microscopy images, we thought there was a possibility that it might also traffic to target cells. The fact that it does not provides useful information concerning the nature and location of the granzyme B motifs responsible for its trafficking through the granzyme-perforin pathway, which we discuss below.

Finally, to confirm at the protein level that granzyme B transfers the MCH payload to target cells, we repeated the above experiments, with the additional, post-co-culture, step of collecting live and dead (DAPI− and DAPI+, respectively) 721 target cells from each co-culture via fluorescence-activated cell sorting (FACS). Data from this sort is shown in Figure 7A, and it is consistent with the flow cytometry data from our other experiments. Whole-cell lysates from the sorted target cell populations were then size-separated by gel electrophoresis and probed for mCherry via western blot (Figure 7B). A prominent 60-kDa band consistent with GZB-MCH is observed in lysates of 721s co-cultured with YT-IndyS expressing GZB-MCH. There is also a background band of approximately 30 kDa, consistent with unfused mCherry in the lysate of 721s co-cultured with YT-Indys expressing GZBSS-MCH. This is not unexpected, as the ER signal peptide in GZBSS-MCH directs mCherry to the secretory pathway, resulting in extracellular mCherry, some of which is likely taken up by
the target 721s. However, if non-specific uptake were the main mechanism of MCH transfer from effector to target cell for all samples, then we would not expect to see any difference between 721s co-cultured with YT-Indys expressing GZB-MCH compared to 721s co-cultured with YT-Indys expressing GZBSS-MCH. Instead, of the DAPI+ 721 samples, only the sample from targets that were co-cultured with YT-Indys expressing GZB-MCH has detectable amounts of mCherry protein, and this band is detected at approximately 60 kDa, the expected size of GZB-MCH. Conversely, there is no detectable analogous 30-kDa band corresponding to MCH in the lysates from DAPI+ targets co-cultured with YT-Indys expressing GZBSS-MCH. That the putative GZB-MCH band in the DAPI+ 721 sample co-cultured with YT-Indys expressing GZB-MCH is even detectable is noteworthy given the actual amount of protein loaded is quite small, as demonstrated by the lack of a vinculin-loading control band. This is despite equal cell numbers for all lanes being sorted and lysed and is because the DAPI+ dead cells are apoptotic and rapidly degrading, which results in a loss of protein.

Taken together, these results and analysis suggest that while there is some background, non-specific 721 uptake of MCH from the co-culture media, YT-Indys expressing GZB-MCH specifically transfer the fusion protein to targeted 721s, while YT-Indys expressing GZBSS-MCH and GZBSS-MCH-GZBSM do not specifically transfer MCH to targeted 721s. Thus, granzyme B appears to be a suitable chaperone protein for delivery of protein payloads via the granzyme-perforin pathway.

DISCUSSION
Cellular therapeutics that repurpose and recombine biological function in a cellular chassis are transforming medicine. These efforts will rely heavily on the development of modules and systems that perform specific sensory, computational, and effector functions. We report here our efforts to develop a cell-to-cell delivery module for cellular therapeutics by repurposing the granzyme-perforin pathway of cytotoxic lymphocytes. Our results support the use of granzyme B as a molecular chaperone for inserting protein payloads into this pathway and facilitating payload delivery to target cells.

We hypothesized that lytic granule loading of a payload would be sufficient for transfer to a target cell, and that loading could be achieved by fusing a chaperone to the payload. We designed two candidate chaperones derived from granzyme B, fused them to mCherry, and investigated their subcellular localization in the natural killer cell line YT-Indy.

All constructs containing an N-terminal ER signal peptide exhibited a high degree of co-localization with Lamp1, as opposed to MCH alone.
This result is best understood by considering the biological distribution of Lamp1 and the cellular compartments in which the co-localization occurs. The primary route of newly synthesized Lamp1 follows the secretory pathway to exosomes at the cell membrane and is then recaptured in early endosomes and eventually fuses with nascent lysosomes. In the case of GZBSS-MCH, the ER signal peptide would direct the protein to the secretory pathway, so GZBSS-MCH is expected to be found co-localized with Lamp1 in a perinuclear distribution in the ER and Golgi and in punctate granules at the cell membrane, but not in cytoplasmic lytic granules. This is what we observe in cells expressing GZBSS-MCH, in contrast to those expressing GZB-MCH, in which the observed co-localization is primarily in cytoplasmic puncta, consistent with lytic granules. These observations are supported by our co-culture experiments, which indicated that GZB-MCH was transferred from effector to target cell, but not GZBSS-MCH. This interpretation is predicated on the assumption that entry into the target cell via perforin pores is passive, which, while historically controversial, is supported by most recent experimental and theoretical data.

We postulated that combining an ER signal peptide with a putative N-linked glycosylation motif would be sufficient for payload delivery, but our co-culture results clearly refuted this. That GZBSS-MCH-GZBSM did not transfer to target cells has several interesting implications surrounding the intracellular trafficking of granzyme B. The first is that the putative N-linked glycosylation sites we computationally identified and their flanking amino acids are insufficient for granule loading. While we cannot rigorously exclude the possibility, we do not believe that these results are simply due to faulty glycosylation of GZBSM, since this process occurs co-translationally and only depends on local sequence. This suggests then that GZBSM is not being phosphorylated, likely because the binding domain for the GlcNAc-1-phosphotransferase, which adds a phosphate group to the mannose of the N-linked glycan in the Golgi, is not faithfully recapitulated in GZBSS-MCH-GZBSM. This could either be due to a lack of actual amino acids that are present elsewhere in the full-length granzyme B protein or that the phosphotransferase-binding domain is conformation dependent, as has been suggested elsewhere in the literature, or both. Much work has been invested into characterizing this domain, but its exact nature remains elusive. Our results suggest that whatever the exact composition, its constituent residues are likely distributed throughout the primary amino acid sequence and hence were not captured in GZBSM, which is why it failed to facilitate transfer of the MCH payload to target cells. This conclusion is consistent with the location of the asparagine residues within the context of GZBSM and the full-length granzyme B protein. As shown in Figure 8, both of the N-linked glycosylation sites we computationally identified are located immediately adjacent to residues that lie external to GZBSM. In particular, N104 is located at a junction in which residues on one side of N104 are located within GZBSM, while those on the other side are located in the other half of the granzyme B protein (Figures 8A and 8C). Also of note is that the other N-linked glycosylation site (N71) is surrounded by a triangular pattern of lysines (Figure 8D), a pattern which some experimental data suggests is the phosphotransferase binding site.

The conclusion that the domains necessary for lytic granule (LG) loading lie throughout the granzyme protein is further supported by our microscopy data, which showed that neither half of granzyme B co-localized with Lamp1 in a similar fashion to full-length granzyme B. This data is also consistent with the arginine residue structural analysis presented above. Beyond corroboration, the
The successful transfer of GZB-MCH highlights two unique and highly desirable features of the granzyme-perforin pathway: modularity and prepositioning. The first is important in that all that is required to deliver a payload is to fuse it to the chaperone. In principle, no further modifications are required, regardless of the payload. This modularity suggests that the system might be widely applicable as a means of cellular delivery, either in cytotoxic lymphocytes, or in the long term, in other, orthogonal, highly engineered cellular chassis. The second advantage is that, as opposed to producing a payload in response to target cell recognition using transcriptional control, a pre-synthesized payload loaded into a lytic granule can be released...
on the timescale during which the immune synapse remains intact, and hence cell-to-cell specificity is maintained.

A critical consideration in using this system is the design and construction of functional fusion proteins that traffic the pathway and remain functional in the target cell. Several issues bear discussion in this regard. Future payloads may not be stable in the harsh environment of the lytic granule, which is acidic and contains many proteases. This might limit the range of applications such a system could be used for, although it is possible that the payload could be engineered to increase its ability to survive the lytic granule, for example by removing a protease cleavage site. The size of the payload is also important, since the internal lumen of the perforin pore has been observed to be 10–20 nm, leaving an appreciable window for a variety of payloads. The diffusivity of the fusion protein is important to consider, as this dictates the propensity for the granzyme fusion protein to both spread throughout the synapse, translocate through the pore, and escape the synapse. While empirical measurement of the fusion protein diffusivity would be a substantial undertaking, we can at least make estimates regarding the change in diffusivity using the Stokes-Einstein relationship, in which the diffusivity \( D \) is proportional to the inverse of the radius \( r \) of the molecule. (The exact relationship is \( D = \frac{K_B T}{6 \pi \eta r} \), where \( K_B T \) is the thermal energy and \( \eta \) is the solvent viscosity. Note that aside from \( r \), all other terms are constants between different proteins). The radius of granzyme B and mCherry have both been experimentally determined to be approximately 2.5 nm. Thus, the radius of the fusion protein could be expected to be approximately the average of its long and short dimensions (that is 2.5 and 5 nm), or 3.25 nm. Thus, the ratio of the radius of the fusion protein to wild-type granzyme is approximately 1.3. Therefore, using the Stokes-Einstein relationship, we would predict that the diffusivity of the fusion protein would be approximately 77% that of the wild-type granzyme. Of course, this analysis only considers the size of the protein and ignores any residue-specific protein-protein interactions, intraprotein effects, effects on solubility, and many other factors. We have conducted a computational study of some of these factors and have found that the rate of perforin pore formation is by far the most important factor in determining the efficacy of granzyme B internalization. Finally, the effect of each fusion partner on the other’s function is an important consideration. We have attempted to mitigate this by joining the two by a flexible glycine-serine linker, this has proven successful in maintaining function of granzyme B internalization.33 Finally, the effect of a variety of payloads fused to granzyme B (data not shown). However, in the absence of reliable computational prediction of protein structure and function, the function of both granzyme and the payload of each new fusion would have to be empirically validated, although we note that as more fusions are generated, design rules may become apparent.

As currently implemented, this approach relies upon using a cytotoxic lymphocyte chassis as the delivery cell. Therefore, any eventual application would have to consider the native cytotoxic effector mechanisms of the lymphocyte chassis. Unmodified cytotoxic lymphocytes are appropriate vehicles to deliver payloads to target cells with the intent of killing them, as would be the case with tumor cells. However, for other applications—for example delivery of pro-survival factors in degenerative diseases, deficient enzymes in metabolic diseases, reprogramming cells, or delivery of gene-editing tools in research contexts—the granzyme-perforin delivery functionality...
would have to be decoupled from the delivery cell cytotoxicity. This may be possible either through attenuation or knockout of the native effector mechanisms in a cytotoxic lymphocyte or by reconstituting the pathway in an independent, non-cytolytic cell chassis. While both of these suggestions would be challenging undertakings, there have been some remarkable successes in similar endeavors, most notably the reconstitution of basic T cell receptor signaling. The granzyme chaperone itself should be readily catalytically inactivated, as has been previously reported. Alternatively, other classes of chaperone could be considered. Promising candidates include other lytic granule constituents, such as other granzymes, and the cathepsins, as well as derivatives thereof. However, we feel strongly that even if all of these approaches were found to be intractable, this system warrants further investigation as a potential therapeutic modality in the context of CAR T cell therapy for cancer.

We have repurposed the granzyme-perforin pathway as a cell-to-cell delivery module for cellular therapeutics. By facilitating targeted transfer of arbitrary payloads with single-cell precision, this system is an important addition to the part set of synthetic immunology.

**Figure 8. Spatial Context of Putative N-linked Glycosylation Sites in Granzyme B**

(A) Schematic of primary amino acid structure of granzyme B. The coloring corresponds to the crystal structures below. Numbers are amino acid residues. (B and C) Three-dimensional crystal structure of granzyme B, colored as in (A), highlighting the potential importance of surface-exposed residues that are immediately adjacent to the N-linked glycosylation sites (blue), but are not contained within the GZBSM (yellow). Note that these regions are quite far from the GZBSM in primary amino acid space, as shown in (A), and in (B) and (C) by the labeling of representative amino acids in these regions. (D) Lysines have been colored in white, to show their inverted triangular pattern surrounding the N71 putative glycosylation site. (E) Location of putative N-linked glycosylation sites throughout the protein. The residues have been colored from red through white, to cyan, according to their position in the primary amino acid sequence.

**MATERIALS AND METHODS**

**Computational Identification of N-Linked Glycosylation Motifs**

The granzyme B coding sequence was downloaded from NCBI RefSeq gene (NCBI: NG_028340.1). We then used NetNGlyc 1.0 to predict putative N-linked glycosylation sites, of which there were two, 33 residues apart. Since the NX(S/T) consensus sequence is necessary but insufficient for glycosylation and the glycosylation occurs co-translationally, it follows that local sequence context surrounding the consensus site is critical. Therefore, we extracted a 53-amino acid domain from granzyme B, extending from 10 amino acids N-terminal of the first putative glycosylation site, to 10 amino acids C-terminal of the second site. Intriguingly, this domain was also present in human granzyme H.

**Plasmids**

A custom mammalian expression vector was used in this work (Figure S1). This pdL vector was constructed in house, based on a pcDNA3.1(+) (Thermo Fisher Scientific) backbone. Specifically, the mammalian and bacterial selectable markers and all origins of replication are derived from pcDNA3.1(+), corresponding to bases 1670 (CGATTTCGGCCTATTGGTTA) to 5396 (...TAAACAAATAGGGTTCCGC). A custom expression cassette was cloned into this backbone. This cassette consisted of eukaryotic and prokaryotic promoters and ribosomal binding sequences, followed by the open reading frame, followed by eukaryotic and prokaryotic transcriptional termination sites. For the mammalian promoter, we used the CAG
promoter for its ability to drive high levels of expression in a variety of tissues. The sequence was amplified from pEMS1157. This was followed by a hybrid T7 prokaryotic promoter, taken from pCMV
cTN (Promega). This was followed by consensus Shine-Dalgarno and Ko-

The full plasmid sequence for the base pdL vector is in the Supplemental Information, along with the full coding sequence for all plasmids used. All plasmids were constructed through a combination of PCR, synthesis, and restriction/ligation cloning. All PCR amplicons and coding sequences were sequence verified.

Cell Culture
YT-Indy and 721.221 cells were a gift from Judy Lieberman (Harvard University). YT-Indy cells were cultured in RPMI 1640 media, supplemented with 20% heat inactivated fetal bovine serum, 1× GlutaMAX, 1 mM sodium pyruvate, 10 mM HEPES, 0.1 mM beta-mercaptoethanol. 721 cells were cultured in DMEM, sup-

Transfection
YT-Indy cells were electroporated using the Neon system (Thermo Fisher Scientific), using the 100 μL tip. 6 × 10⁶ cells were washed once in PBS, and resuspended in Buffer R along with 20 μg plasmid DNA, in a final volume of 110 μL. The extra volume ensures no bubbles are generated in aspirating the cell mixture into the electro-

Flow Cytometry
Cells were harvested and resuspended in PBS supplemented with 10% complete media and 1 μg/mL of DAPI (Sigma) as a viability stain. If cells were to be sorted, they were passed through a 35-μm nylon filter (BD Falcon). Cells were kept on ice and then analyzed on a BD Fortessa II or sorted on either a BD Aria III or Fusion. For sorting, cells were sorted into complete media. In all flow cytometry experiments, two initial gating steps were used. Debris was excluded by excluding cells at the bottom left corner of a propidium iodide (PI) versus FSC-A (forward scatter area) gate. Doublets were excluded using a hierarchical gating scheme: all cells with a wider pulse width signal were excluded first in FSC-W versus FSC-H (forward scatter width versus height) and then SSC-W versus SSC-H (side scatter width versus height). All flow cytometry data was analyzed in FlowJo.

Microscopy
Transfected cells were first FACS sorted for moderate intensity RFP⁺ cells. 2.5 × 10⁶ cells resuspended in 50 μL complete media (RPMI-1640 supplemented with 10% fetal calf serum, 2 mM glutami-

Image Analysis
Image filtering was done using a custom script written in MATLAB. The green channel (Lamp1) was filtered as follows: The localized background of the image was calculated for each pixel as the median intensity of a 25 × 25 pixel square centered on that pixel. This background pixel intensity was subtracted from the original pixel intensity. Any pixels with negative intensity after this step were set to zero. This step aids in distinguishing small punctate structures from one another. Next, a pixel noise threshold was calculated as follows. First the median absolute deviation (MAD) of all nonzero pixels from the raw image was calculated. From this, the standard deviation of the pixel intensity was approximated as 1.4 times the MAD, which is a reasonable estimate of the pixel noise. Finally, the noise threshold was taken as 6 times this value (that is 6 × 14 × MAD). Any pixels in the background subtracted image whose intensity were below this value were set to zero. The red channel (mCherry) was filtered in the same way, except localized background was not subtracted. The MATLAB script implementing this algorithm is in the Supplemental Information.
Colocalization analysis was also conducted in MATLAB. For paired red and green channel images, with pixel intensities \( R_i \) and \( G_i \) respectively, PCC was calculated as

\[
PCC = \frac{\sum_i \sum_j (R_i - \overline{R})(G_i - \overline{G})}{\sqrt{\sum_i \sum_j (R_i - \overline{R})^2 \sum_i \sum_j (G_i - \overline{G})^2}}
\]  
(Equation 1)

where \( \overline{R} \) and \( \overline{G} \) are the mean pixel intensities. The Manders M1 coefficient was calculated as

\[
M_1 = \frac{\sum_i c_{ij} R_i}{\sum_i R_i}, \quad c_{ij} = \begin{cases} 
1, & G_i > 0 \\
0, & G_i = 0
\end{cases}
\]  
(Equation 2)

These co-localization scores were calculated separately for each sample of each image and then plotted using RStudio.

Cell Labeling
Cells were fluorescently labeled with carboxyfluorescein succinimidyl ester (CFSE; eBioscience) following the manufacturer’s protocol, except that only one PBS wash prior to labeling was done and only one media wash after labeling was done.

Co-culture Experiments
YT-Indy cells were transfected with mCherry fusion proteins, and 48 hr later FACS were sorted for viable RFP+ cells. The following day \( 4 \times 10^5 \) YT-Indy effector cells were combined with \( 1 \times 10^5 \) CFSE-labeled target 721 cells at a 4:1 effector:target (E:T) ratio in a final volume of 500 µL YT-Indy media in 5 mL polystyrene round-bottom tubes (BD Falcon). The cell suspension was gently pelleted by spinning it at 200 g for 15 s. The tubes were then incubated at 37°C for 90 min, and then prepared for flow cytometry or FACS sorting as above.

Statistical Analysis
mCherry median fluorescent intensity was tabulated for each target cell population using flow cytometry data from above. For each target cell population, a single factor analysis of variance was conducted to determine if the MCH median fluorescent intensity (MFI) means were the same for all effector cell populations using the model MFI ~ EffectorPopulation. We then used these results as input for a Tukey’s honest significant difference (HSD) test of the difference between sample means within each target cell population. Statistical tests were conducted in R, using the aov and TukeyHSD commands respectively.

Western Blotting
\( 3 \times 10^4 \) cells were sorted into PBS in microcentrifuge tubes. Cells were kept on ice thereafter. Cells were then pelleted, resuspended in 10 µL PBS, and lysed directly by adding 10 µL 2× Laemmli sample buffer. Samples were incubated at 95°C for 10 min and then stored at −20°C.

For blotting, samples were boiled again at 95°C for 10 min and then loaded onto pre-cast 4%-12% Bis-Tris polyacrylamide gels (Thermo Fisher Scientific). Proteins were size separated by gel electrophoresis by running the gel at 150 V for 75 min. Proteins were transferred to a nitrocellulose membrane using a standard wet transfer at 300 mA for 2 hr.

The blot was cut horizontally at 100 kDa and then was blocked in tris-buffered saline with Tween 20 (TBS-T) with 5% skim milk powder at room temperature for 1 hr and then incubated with primary antibody in sealed pouches at 4°C overnight. The primary antibodies used were rabbit anti-mCherry (Biovision cat. #5993-100) and rabbit anti-vinculin (Abcam cat. #EPR8185) as a loading control. The dilutions were 1:500 (mCherry) with 5% skim milk powder, 1:10,000 (vinculin) with 2% skim milk powder, both in TBS-T. Blots were then washed with TBS-T and incubated with horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology cat. #sc-2004) for 1 hr. The dilution was 1:5,000 in TBS-T, with 5% skim milk powder (anti-mCherry) and 2% skim milk powder (anti-Vinculin). Finally, the blots were washed with TBS-T and then developed using Bio-Rad Clarity Western ECL (enhanced chemiluminescence) substrate reagent, following the manufacturer’s protocol. Blots were imaged using a Bio-Rad Chemidoc MP Imaging System, with exposure times ranging from 1 to 100 s.

Crystal Structure Analysis
To visualize the location of the various motifs of granzyme B in the three dimensional protein, we downloaded the granzyme B crystal structure from the Protein Data Bank (PDB: 1FQ3) and rendered the base crystal structure and custom annotations using PyMOL (Schrödinger). Surface-exposed residues adjacent to the N-linked glycosylation sites were determined by first selecting all residues that were within 15Å of the glycosylated residue. We then selected the subset of these residues that were surface exposed, using a custom PyMOL script written by Jason Vertrees.

SUPPLEMENTAL INFORMATION
Supplemental Information includes one figure, a base pdL plasmid sequence, coding sequence inserts, and MATLAB source code for image filtering algorithm and can be found with this article online at https://doi.org/10.1016/j.omtm.2017.10.003.

AUTHOR CONTRIBUTIONS
D.J.W. and R.A.H. designed the research and the chaperones. D.J.W. and L.D. generated the plasmids and performed the western blots. D.J.W. and L.A. acquired the microscopy images. D.J.W. conducted the co-culture experiments, developed the image-analysis methods, and conducted statistical and crystal structure analyses. D.J.W. and R.A.H. analyzed the data and wrote the paper.

CONFLICTS OF INTEREST
R.A.H. and D.J.W. are listed as inventors on a patent application (WO2015157864A1) that covers some of the work presented in this manuscript.
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REFERENCES

1. Fischbach, M.A., Bluestone, J.A., and Lim, W.A. (2013). Cell-based therapeutics: the next pillar of medicine. Sci. Transl. Med. 5, 179ps7.
2. Maude, S.L., Frey, N., Shaw, P.A., Apenc, R., Barrett, D.M., Bunin, N.J., Chew, A., Gonzalez, V.E., Zheng, Z., Lacey, S.F., et al. (2014). Chimeric antigen receptor T cells for sustained remissions in leukemia. N. Engl. J. Med. 371, 1507–1517.
3. Davila, M.L., Riviere, L., Wang, X., Barido, S., Park, J., Curran, K., Chng, S.S., Stefanski, J., Borquez-Ojeda, O., Obizewski, M., et al. (2014). Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. Sci. Transl. Med. 6, 224ra25.
4. Lee, D.W., Kochenderfer, J.N., Stetler-Stevenson, M., Cui, Y.K., Delbrook, C., Feldman, S.A., Fry, T.J., Orentas, R., Sabatino, B., Nah, N.N., et al. (2015). T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet 385, 517–528.
5. D’Souza, N., Rossignoli, F., Golinielli, G., Grisendi, G., Spano, C., Candini, O., Osturu, S., Catani, F., Paolucci, F., Horwitz, E.M., and Dominici, M. (2015). Mesenchymal stem/stromal cells as a delivery platform in cell and gene therapies. BMC Med. 13, 186.
6. Suresh, S.C., Selvaraju, V., Thirunavukkarasu, M., Goldman, J.W., Husain, A., Alexander, P., Fauser, C., Brook, D., Feldman, S.A., Fry, T.J., Orentas, R., Sabatino, B., Nah, N.N., et al. (2015). T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukemia in children and young adults: a phase 1 dose-escalation trial. Lancet 385, 517–528.
7. Cheng, Z., Ou, I., Zhou, X., Li, F., Jia, X., Zhang, Y., Liu, X., Li, Y., Ward, C.A., Melo, L.G., and Kong, D. (2008). Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infected myocardium improves cardiac performance. Mol. Ther. 16, 571–579.
8. van Velthoven, C.T., Braccioli, L., Willemen, H.L., Velaars, A., and Heijnen, C.J. (2014). Therapeutic potential of genetically modified mesenchymal stem cells after neonatal hypoxic-ischemic brain damage. Mol. Ther. 22, 645–654.
9. Sage, E.K., Kolluri, K.K., McNulty, K., Da Silva Lourenco, S., Kalber, T.L., Ordonez, K.L., Davies, D., Gao, X., Li, X., Guan, Y., and Janes, S.M. (2014). Systemic but not topical TRAIL-expressing mesenchymal stem cells reduce tumor growth in malignant mesothelioma. Thorax 69, 638–647.
10. Niess, H., Bao, Q., Conard, C., Zischek, C., Notohabimiprodjo, M., Schwaab, F., Schwarz, B., Huss, R., Jauch, K.W., Nelson, P.J., and Bruns, C.J. (2011). Selective targeting of genetically engineered mesenchymal stem cells to tumor stroma microenvironments using tissue-specific suicide gene expression suppresses growth of hepatocellular carcinoma. Ann. Surg. 254, 767–774, discussion 774–775.
11. Sage, E.K., Thakrar, R.M., and Janes, S.M. (2016). Genetically modified mesenchymal stem cells in cancer therapy. Cytotherapy 18, 1435–1445.
12. Lim, W.A. (2010). Designing customized cell signalling circuits. Nat. Rev. Mol. Cell Biol. 11, 393–403.
13. Woodworth, D.J., and Holt, R.A. (2017). Cell-based therapeutics: making a faustian pact with biology. Trends Mol. Med. 23, 104–115.
14. Stanton, B.C., Siciliano, V., Ghodasara, A., Wroblewska, L., Clancy, K., Trefzer, A.C., Chesnut, J.D., Weiss, R., and Voigt, C.A. (2014). Systematic transfer of prokaryotic sensors and circuits to mammalian cells. ACS Synth. Biol. 3, 880–891.
15. Sadelain, M., Brentjens, R., and Riviere, I. (2013). The basic principles of chimeric antigen receptor design. Cancer Discov. 3, 388–398.
16. Roybal, K.T., Rupp, L.J., Morsut, L., Walker, W.L., McNally, K.A., Park, I.S., and Lim, W.A. (2016). Precision tumor recognition by T cells with combinatorial antigen-sensing circuits. Cell 164, 770–779.
17. Morsut, L., Royal, K.T., Xiong, X., Gordley, R.M., Coyle, S.M., Thomas, M., and Lim, W.A. (2016). Engineering customized cell sensing and response behaviors using synthetic notch receptors. Cell 164, 780–791.
18. Kemmer, C., Gitzinger, M., Daoud-El Baba, M., Djonov, V., Stelling, J., and Fussenegger, M. (2010). Self-sufficient control of urine homeostasis in mice by a synthetic circuit. Nat. Biotechnol. 28, 355–360.
19. Ye, L., Chang, Y.H., Xiong, Q., Zhang, P., Zhang, L., Somasundaram, P., Lepley, M., Swingle, C., Su, L., Wendel, J.S., et al. (2014). Cardiac repair in a porcine model of acute myocardial infarction with human induced pluripotent stem cell-derived cardiovascular cells. Cell Stem Cell 15, 750–761.
20. Green, E.R., and Mecas, J. (2016). Bacterial secretion systems: an overview. Microbiol. Spectr. 4, 10.1128/microbiolspec.VMBF-0012-2015.
21. Costa, T.R.D., Felisberto-Rodrigues, C., Meir, A., Prevost, M.S., Redzej, A., Troke, M., and Waksman, G. (2015). Secretion systems in Gram-negative bacteria: structural and mechanistic insights. Nat. Rev. Microbiol. 13, 343–359.
22. Reeves, A.Z., Spears, W.E., Du, J., Tan, K.Y., Wagers, A.J., and Lesser, C.F. (2015). Engineering Escherichia coli into a protein delivery system for mammalian cells. ACS Synth. Biol. 4, 644–654.
23. Anderson, J.C., Clarke, E.J., Arkin, A.P., and Voigt, C.A. (2006). Environmentally controlled invasion of cancer cells by engineered bacteria. J. Mol. Biol. 355, 619–627.
24. Anderson, J.C., Voigt, C.A., and Arkin, A.P. (2007). Environmental signal integration by a modular AND gate. Mol. Syst. Biol. 3, 133.
25. Barry, M., and Bleackley, R.C. (2002). Cytotoxic T lymphocytes: all roads lead to rat. Nat. Rev. Immunol. 2, 401–409.
26. Montel, A.H., Morse, P.A., and Brahmi, Z. (1995). Upregulation of B7 molecules by activated T cells for sustained remissions in leukemia. N. Engl. J. Med. 333, 528.
27. Ye, L., Chang, Y.H., Xiong, Q., Zhang, P., Zhang, L., Somasundaram, P., Lepley, M., Swingle, C., Su, L., Wendel, J.S., et al. (2014). Cardiac repair in a porcine model of acute myocardial infarction with human induced pluripotent stem cell-derived cardiovascular cells. Cell Stem Cell 15, 750–761.
28. Ye, L., Chang, Y.H., Xiong, Q., Zhang, P., Zhang, L., Somasundaram, P., Lepley, M., Swingle, C., Su, L., Wendel, J.S., et al. (2014). Cardiac repair in a porcine model of acute myocardial infarction with human induced pluripotent stem cell-derived cardiovascular cells. Cell Stem Cell 15, 750–761.
37. Shimizu, Y., and DeMars, R. (1989). Production of human cells expressing individual transferred HLA-A, -B, -C genes using an HLA-A, -B, -C null human cell line. J. Immunol. 142, 3320–3328.

38. Braulke, T., and Bonifacino, J.S. (2009). Sorting of lysosomal proteins. Biochim. Biophys. Acta 1793, 605–614.

39. Saftig, P., and Klumperman, J. (2009). Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. Nat. Rev. Mol. Cell Biol. 10, 623–635.

40. Lopez, J.A., Jenkins, M.R., Rudd-Schmidt, J.A., Brennan, A.J., Danne, J.C., Mannering, S.I., Trapani, J.A., and Vosbeinok, I. (2013). Rapid and unidirectional perforin pore delivery at the cytotoxic immune synapse. J. Immunol. 191, 2328–2334.

41. Breitling, J., and Aebi, M. (2013). N-linked protein glycosylation in the endoplasmic reticulum. Cold Spring Harb. Perspect. Biol. 5, a013359.

42. van Meel, E., Lee, W.S., Liu, L., Qian, Y., Doray, B., and Kornfeld, S. (2016). Multiple domains of GlcNAc-1-phosphotransferase mediate recognition of lysosomal enzymes. J. Biol. Chem. 291, 8295–8307.

43. Warner, J.B., Thalhauser, C., Tao, K., and Sahagian, G.G. (2002). Role of N-linked oligosaccharide flexibility in mannose phosphorylation of lysosomal enzyme cathepsin L. J. Biol. Chem. 277, 41897–41905.

44. Qian, Y., Lee, I., Lee, W.S., Qian, M., Kudo, M., Cantfield, W.M., Lobel, P., and Kornfeld, S. (2010). Functions of the alpha, beta, and gamma subunits of UDP-GlcNAcylosomal enzyme N-acetylgalactosamine-1-phosphotransferase. J. Biol. Chem. 285, 3360–3370.

45. Waugh, S.M., Harris, J.L., Fletterick, R., and Craik, C.S. (2000). The structure of the pro-apoptotic protease granzyme B reveals the molecular determinants of its specificity. Nat. Struct. Biol. 7, 762–765.

46. James, J.R., and Vale, R.D. (2012). Biophysical mechanism of T-cell receptor triggering in a reconstituted system. Nature 487, 64–69.

47. Kurschus, F.C., Kleinschmidt, M., Fellows, E., Dormair, K., Rudolph, R., Lilie, H., and Jenne, D.E. (2004). Killing of target cells by redirected granzyme B in the absence of perforin. FEBS Lett. 562, 87–92.

48. Gupta, R., and Brunak, S. (2002). Prediction of glycosylation across the human proteome and the correlation to protein function. Pac. Symp. Biocomput. 2002, 310–322.

49. Blázquez, M., and Shennan, K.I.J. (2000). Basic mechanisms of secretion: sorting into the regulated secretory pathway. Biochem. Cell Biol. 78, 181–191.

50. Portales-Casamar, E., Swanson, D.J., Liu, L., de Leeuw, C.N., Banks, K.G., Ho Sui, S.J., Fulton, D.L., Ah, J., Amirabbasi, M., Arenillas, D.J., et al. (2010). A regulatory toolbox of MiniPromoters to drive selective expression in the brain. Proc. Natl. Acad. Sci. USA 107, 16589–16594.