Orthogonalized human protease control of secreted signals

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Abstract

Synthetic circuits that regulate protein secretion in human cells could support cell-based therapies by enabling control over local environments. While protein-level circuits enable such potential clinical applications, featuring orthogonality and compactness, their non-human origin poses a potential immunogenic risk. Here, we developed Humanized Drug Induced Regulation of Engineered CyTokines (hDIRECT) as a platform to control cytokine activity exclusively using human-derived proteins. We sourced a specific human protease and its FDA-approved inhibitor. We engineered cytokines whose activities can be activated and abrogated by proteolytic cleavage. We utilized species specificity and re-localization strategies to orthogonalize the cytokines and protease from the human context that they would be deployed in. hDIRECT should enable local cytokine activation to support a variety of cell-based therapies such as muscle regeneration and cancer immunotherapy. Our work offers a proof of concept for the emerging appreciation of humanization in synthetic biology for human health.

Intercellular communication, mediated by the secretion and display of soluble factors (cytokines and growth factors) and receptors, plays a crucial role in health and diseases. Cell therapies aim to treat complex diseases using human cells through the modulation of intercellular communication[1], the chimeric antigen receptor (CAR) T cell therapy being the most notable example[2]. Aside from treating cancer, another promising example is treating autoimmune diseases and support muscle regeneration using mesenchymal stem cells (MSCs)[3–6]. Despite their promise, many cellular therapeutics are susceptible to changes in the local microenvironment[7–9]. To address this hurdle, researchers have "armed" cell therapies to constitutively secrete cytokines to tune their local environments to promote proliferation and efficacy, such as driving CAR-T activation in solid tumors using IL-12[10], and improving the survival of transplanted MSCs using the anti-inflammatory IL-10[11]. While constant expression of these powerful signals improve efficacy, increased toxicity was also observed, indicating a need for further control[10].

The field of synthetic biology offers a plethora of tools and methods for localized, conditional control of cytokine activity. In particular, compared to the more conventional transcriptional control, protein-level controls hold advantages for potential clinical use such as fast operation, compact single-transcript delivery, and context-independent performance[12]. Notably, orthogonal viral proteases have emerged as useful post-translational tools due to their ability to control the activity, degradation, and localization of target proteins[13,14]. These orthogonal proteases are highly versatile and can be combined to implement robust sense-and-response behaviors in mammalian cells. They are uniquely positioned to serve as cargos on mRNA vectors that are safer and more scalable than conventional DNA vectors[15]. They have been successfully applied to the control of protein secretion and display[16,17].

However, despite the promise of proteases as control knobs, there remains a critical constraint: the non-human origin and potential immunogenicity of viral proteases may preclude their translational potential. In analogous scenarios, immune responses against other synthetic biology tools such as *Streptococcus pyogenes* Cas9 protein (SpCas9) and synthetic CAR-T receptors have been documented with compromised efficacy and duration, highlighting the need to reduce immunogenicity of synthetic tools[18,19]. Even more notably, we are inspired by the decades of efforts to "humanize" monoclonal antibodies[20,21], and we reason that, if synthetic biology is to deliver its promise in human health, it is imperative that we preempt similar challenges as we engineer the fundamental tools. In an emerging concensus to "humanize" synthetic biology, groups have developed tools derived largely from human-derived components such as zinc finger transcriptional regulators (synZiFTRs)[22], CRISPR-CAS-inspired RNA targeting systems (CIRTS)[23], and synthetic intramembrane proteolysis receptors (SNIPRs)[24]. While these papers have demonstrated humanized transcriptional and translational regulation, the humanization efforts have yet to reach the post-translational level.

Here, we sought to develop protease-based control of intercellular communication for potential application in cellular therapeutics. We developed our system with five features for potential clinical suitability, combining general humanization considerations[22] with the unique benefits of protease control: (i) use of human-derived proteins to minimize the risk of immunogenicity; (ii) use of orthogonalized components with high specificity and minimal crosstalk with native signaling pathways; (iii) ability to control exogenously using FDA-approved small molecules; (iv) compact, single-transcript design to facilitate delivery into therapeutic cells; (v) compatibility with mRNA delivery for potential *in vivo* gene delivery applications.

We identified a human protease, renin, as the control knob. Renin, in contrast to most human proteases, exhibits high substrate specificity[25]. Additionally, renin comes with a clinically-viable mode of exogenous control in the form of its FDA-approved small-molecule inhibitor, aliskiren[26]. In contrast to previously used cytosolic proteases, renin functions extracellularly and in the secretory pathway, offering an external interface to directly control intercellular communication.

In this paper, we introduce Humanized Drug Induced Regulation of Engineered CyTokines (hDIRECT), using renin to control intercellular communication. We engineered a variety of cytokines, whose activities are increased or decreased by renin-mediated proteolysis and can be tuned exogenously via aliskiren. We used structure-guided mutagenesis and re-localization strategies to create renin mutants with new and orthogonal substrate specificity from wildtype proteins. Finally, we showed that cytokine activity can be tuned with aliskiren in stably transduced mammalian cells. We envision that conditional control of cytokine activity has immediate use for supporting and controlling cell therapies. This study demonstrates a proof-of-principle for the orthogonalization of human proteases and serves as an example of making biomolecular controls more clinically compatible.

Results

Engineering caged cytokines.

hDIRECT consists of three components: a membrane-bound renin, renin's FDA-approved small-molecule inhibitor, aliskiren, and engineered cytokines modulated by renin cleavage (Fig. 1a, b). Renin is known to function extracellularly as it is natively secreted by juxtaglomerular cells in the kidney[25]. To localize renin to the plasma membrane to enable the autonomous control of engineered cells, the active form of renin was fused to the transmembrane domain of human CD28 (Fig. 1b). Here, constitutive expression of membrane-bound renin can control the activity of co-expressed secreted cytokines. We first evaluated a variety of strategies to place cytokines under the control of renin.

To engineer cytokines activated by renin cleavage, we drew inspiration from previous work on attenuated cytokines, specifically, receptor-masked interleukin-2 (IL-2).[27,28] Here, IL-2 activity was tied to proteolytic cleavage by fusing IL-2 with a receptor subunit via a matrix metalloproteinase (MMP)-cleavable linker. Cytokine activity is inhibited ("caged") in the absence of protease, and conditionally activated ("uncaged") by proteolytic cleavage. Using this general framework, we demonstrated the possibility to cage structurally and functionally distinct cytokines. Inspired by MSC and CAR T applications, we chose interleukin-10 (IL-10, an immuno-suppressive cytokine that plays a role in limiting host immune responses as well as promoting wound healing and muscle regeneration[29]), interleukin-6 (IL-6, generally associated with inflammation, but also implicated with the activation of satellite cells and promotion of myotube regeneration for muscle regeneration[30–32]), and IL-2 (a potent factor for the proliferation and activation of lymphocytes, and widely studied for activating effector T cells against tumors[33–35]).

We started by designing a caged IL-10. IL-10 is composed of a homodimer that binds to a tetrameric IL-10 receptor complex formed by two IL-10Ra and two IL-10Rb subunits.[36] Since effectively caging a dimeric protein may be difficult due to having multiple epitopes to interact with receptors, we also tested a previously engineered monomeric variant of IL-10 (R5A11M) with boosted affinity for IL-10Rb and potent immunomodulatory effects.[37] We created two caged IL-10 constructs. Both designs contain a renin cleavable linker (cut site and glycine-serine,

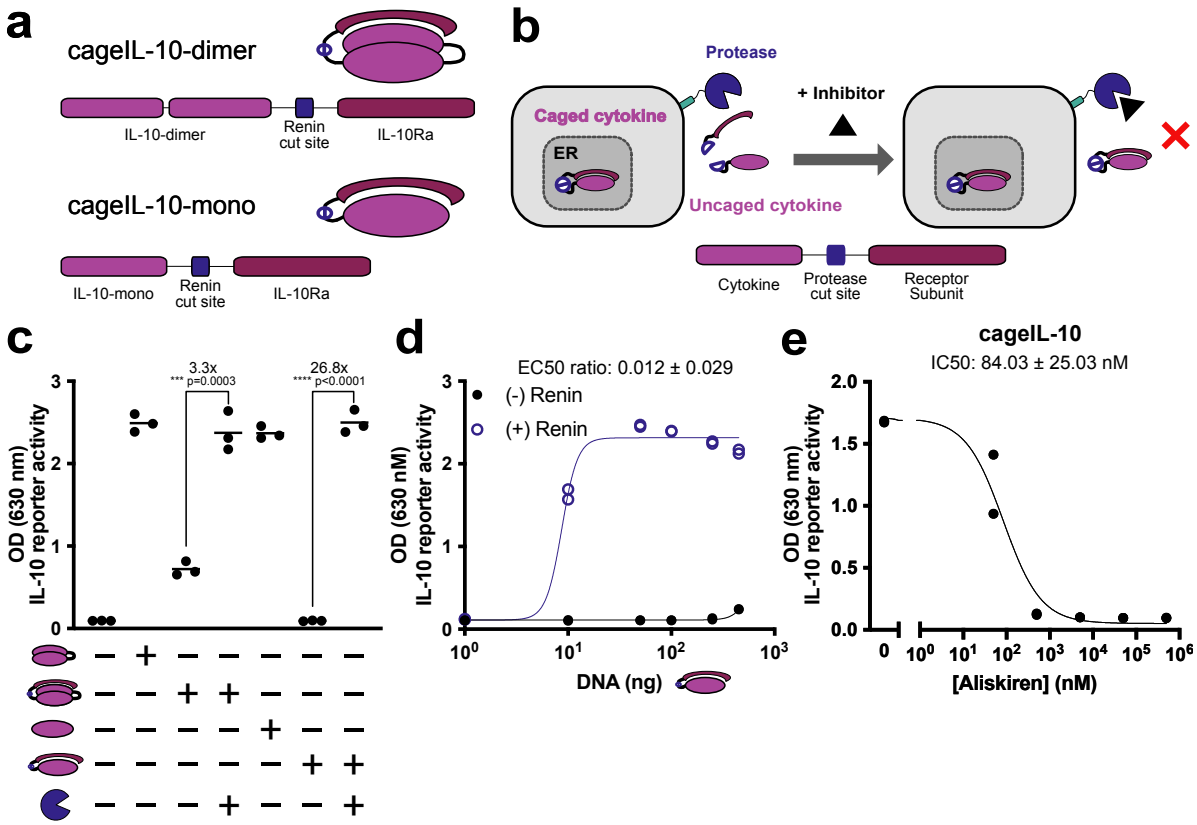

Fig. 1: Engineering caged IL-10. a, Caged cytokine design for dimeric and monomeric IL-10. Cytokines are fused with renin-cleavable linkers to cognate receptor subunits, inhibiting binding to cellular receptors. **b,** hDIRECT circuit using caged cytokines to control cytokine activity. Cells constitutively secrete attenuated caged cytokines and cytokine activity can be tuned down via small-molecule inhibitor for co-transfected protease, renin. **c,** Functional activity of caged IL-10 constructs with or without renin co-expression. **d,** Functional activity of cageIL-10 plasmid transiently transfected with increasing amounts, with or without renin co-expression. **e,** Functional activity of cageIL-10 co-expressed with renin at increasing aliskiren concentrations. (For **c-e**: Cytokine activity measured from supernatant using HEKBlue IL-10™ reporter cells. For **c**: mean of n = 3 biological replicates; Unpaired two-tailed Student's t-test. For **d-e**: n = 2 biological replicates; data represent mean ± s.e.m. *** $p \leq 0.001$, **** $p < 0.0001$.)

GS, linker) and the extracellular portion of specific IL-10R subunits for caging. The first construct was created by fusing two WT IL-10 monomers together with IL-10Ra (CageIL-10-dimer – Fig. 1a). The second construct used the monomeric R5A11M IL-10 and IL-10Rb (CageIL-10-mono – Fig. 1a). We hypothesized that aliskiren could be used to control renin activity and removal of the caged portion of hDIRECT to control cytokine activity (Fig. 1b).

Taking advantage of our fast design-build-test cycle, we transiently transfected human embryonic kidney (HEK) 293 cells using plasmids encoding the caged cytokines and renin. Supernatant was transferred to HEK-Blue™ IL-10 reporter cells to assess functional activity. Here, signal transduction from IL-10 receptor activation results in expression of secreted embryonic alkaline phosphatase (SEAP), which can be assayed via absorbance following

129 incubation with a colorimetric substrate. A schematic for this cytokine reporter assay can be
130 found in Supplementary Fig. 1. Unless specified otherwise, the same workflow was used
131 throughout this work. In the absence of renin, cageIL-10-mono was caged more efficiently than
132 the cageIL-10-dimer (Fig. 1c). CageIL-10-mono was also able to fully reconstitute activity with
133 protease present (Fig. 1c). From here on, we refer to this optimal variant as "cageIL-10". Next, to
134 explore the extent of IL-10 caging and linker cleavability, we titrated the amount of transfected
135 cageIL-10 plasmid DNA. We observed efficient caging even at high doses of cageIL-10 (Fig.
136 1d), and renin-activated IL-10 activity at low doses of cageIL-10 (Fig. 1d). We then validated
137 that aliskiren enables dose-dependent control of hDIRECT (Fig. 1e).
138 To expand the cytokine output of hDIRECT, we next developed caged designs for IL-6.
139 Structurally, IL-6 is a monomeric four-α helical bundle typical of most cytokines (distinct from
140 the dimeric IL-10). Canonically, IL-6 binds to its high affinity receptor, IL-6Ra, on cell surfaces,
141 followed by complexing on the membrane with IL-6Rb (gp130) which transduces signal
142 activation across the membrane[38]. However, IL-6 activity has also been implicated in an
143 alternative pro-inflammatory pathway wherein IL-6 binds to soluble IL-6Ra (sIL-6Ra) and
144 mediates transactivation on cells expressing IL-6Rb[39]. To avoid transactivation of our caged IL-6
145 complex and bias IL-6 signaling to a regenerative phenotype, we sought to cage IL-6 with an IL-
146 6Ra mutant (A228D/N230D/H280S/D281V) that has abrogated activation of IL-6Rb. We denote
147 this IL-6Ra mutant as IL6Ra_M4.
148 Inspection of the IL-6-receptor complex structures suggested that placing IL-6Ra at the
149 N-terminus of the fusion protein would allow IL-6 to bind IL-6Ra in its canonical conformation.
150 Consistent with this observation, our data shows improved caging when the receptor is fused to
151 the N-terminus, although fusion at either terminus exhibits substantial renin-independent
152 activation (Supplementary Fig. 2). We hypothesized that caging could be improved if we fused
153 an additional receptor subunit. Therefore, we created a multi-cageIL-6 by fusing IL-6Ra_M4 to
154 the N-terminus and IL-6Rb to the C-terminus of IL-6 with renin-cleavable linkers (Fig. 2a).
155 Indeed, multi-cageIL-6 demonstrated vastly improved caging over the initial design (Fig. 2b),
156 albeit with reduced activity in the presence of renin (Fig. 2b). However, we found that IL-6
157 activity in response to renin can be increased at higher doses of multi-cageIL-6 plasmid (Fig. 2c).
158 Finally, to show broad generalizability in cytokines and therapeutic applications, we
159 ported over previous caged IL-2 constructs to hDIRECT[27]. A mutant IL-2 (sumIL-2) was
160 recently developed with enhanced affinity for IL-2Rb (i.e., effector T cells) over IL-2Ra (i.e.,
161 regulatory T cells)[28]. We leveraged this insight to design a caged sumIL-2 with a renin-cleavable
162 linker and C-terminal IL-2Rb (Fig. 2d). We also investigated whether varying the linker length
163 on either side of the renin cut site would improve cleavability but did not find any significant
164 effect (Supplementary Fig. 3). CageIL-2 is efficiently caged at high doses of cytokine (Fig. 2e)
165 and exhibits renin-activation at low doses (Fig. 2e). IL-2 activity was tuned sensitively by
166 aliskiren upon drug titration (Fig. 2f), indicating that aliskiren control is also generalizable
167 between different cytokines. Altogether, we established three different caged cytokines
168 compatible with renin-aliskiren control.

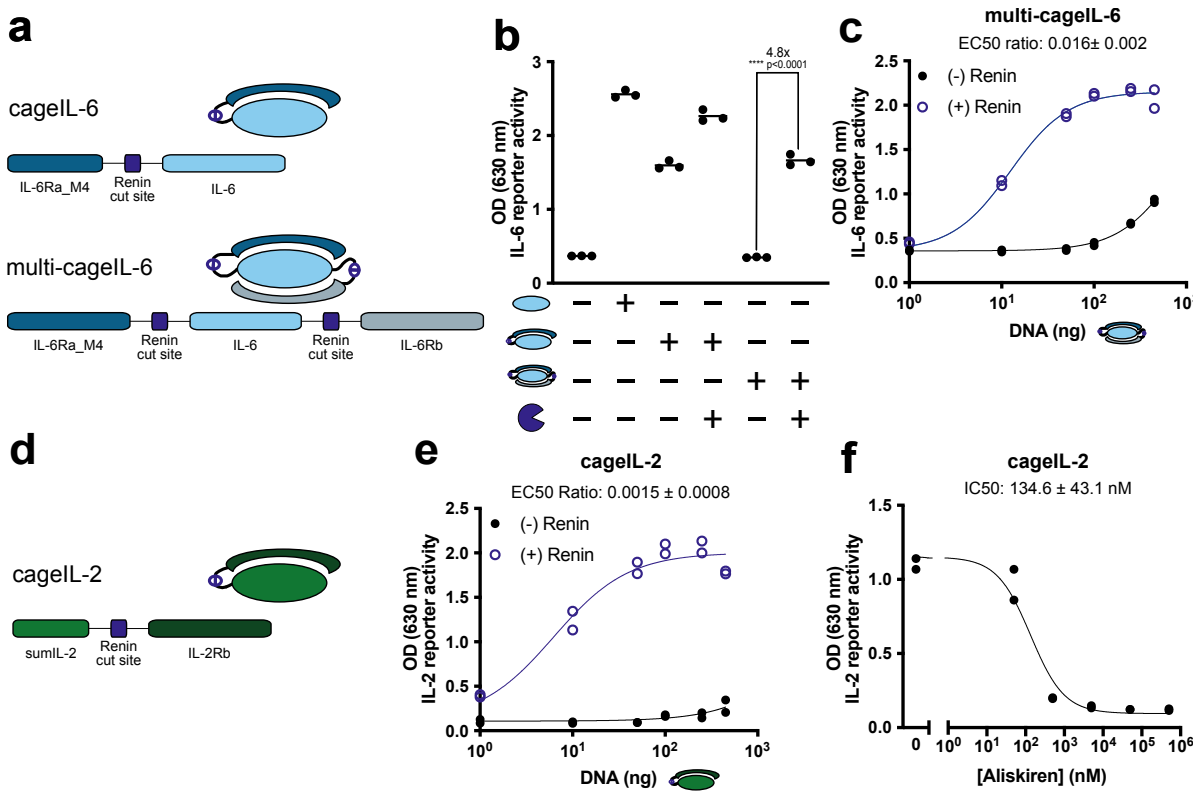

Fig. 2: Engineering caged IL-6 and IL-2. a, Caged cytokine design for single and multi-caged IL-6 constructs. **b,** Functional activity of caged IL-6 constructs with or without renin co-expression. **c,** Functional activity of cageIL-6 plasmid transiently transfected with increasing amounts, with or without renin co-expression. **d,** Caged cytokine design for caged IL-2. **e,** Functional activity of cageIL-2 plasmid transiently transfected with increasing amounts, with or without renin co-expression. **f,** Functional activity of cageIL-2 co-expressed with renin at increasing aliskiren concentrations. (Cytokine activity measured from supernatant using HEKBlue IL-6™ (**b-c**) and IL-2™ (**e-f**) reporter cells. For **b**: mean of n = 3 biological replicates; Unpaired two-tailed Student's t-test. For **c,e-f**: n = 2 biological replicates; data represent mean ± s.e.m. **** $p < 0.0001$.)

Engineering cleavable cytokines.

To expand the potential of protease regulation, we sought to design cytokines whose activities would be abrogated (rather than activated) by cleavage. Compared to the caged design, this modality would allow cytokine activity to be "turned on" or restored by a small-molecule input. It could be particularly useful in applications where temporary, rather than constitutive, cytokine activity is desired, such as transient IL-2 activation to support CAR-T cell proliferation and cytotoxicity while avoiding exhaustion and overstimulation[40].

We hypothesized that cytokines could be directly inactivated if they were engineered to include protease cut sites. We inserted protease cut sites within flexible loops of cytokines so as not to perturb receptor binding regions. Like the caged cytokines, we envision engineered cells constitutively expressing cleavable cytokines and membrane-bound renin. In this fashion, cytokine activity can be tuned up on demand upon drug input and protease inhibition (Fig. 3a).

182 We started designing cleavable cytokines using the previously mentioned mono-IL-10.
183 Researchers engineered this functional mono-IL-10 by inserting a 6 amino acid GGGSGG linker
184 between the D and E helices of human IL-10. We leveraged this insight to create a cleavable
185 mono-IL-10 by replacing this glycine linker with the 13 amino acid renin cleavage site.
186 CleaveIL-10 retained activity in the absence of protease and was abrogated upon co-transfection
187 with renin (Fig. 3b). To explore the range at which cleaveIL-10 is sensitive to renin cleavage, we
188 co-transfected cells with or without renin while titrating the amount of cleaveIL-10 DNA and
189 observed a substantial shift of the titration curves by renin (Fig. 3c).
190 Next, we sought to demonstrate the generalizability of this method to native cytokines
191 that lack synthetic linkers to substitute. Our general method was to find sites in human IL-6 and
192 IL-2 tolerable (retaining activity) to insertion of a protease cut site. We scanned various solvent
193 exposed (protease accessible) flexible loops between helices (Supplementary Fig. 4) and then
194 optimized linkers to improve their cleavability. Cleave IL-6 and IL-2 were designed through
195 direct insertion of the renin cut site with or without a five amino acid GS linker into flexible
196 loops at residues 127-140 in IL-6 and 74-81 in IL-2. Both cleaveIL-6 (Fig. 3d) and cleaveIL-2
197 (Fig. 3e) retained their functional activity in the absence of protease. Additionally, we observed
198 substantial shifts in response to renin in both cleaveIL-6 (Fig. 3d) and cleaveIL-2 (Fig. 3e).
199 To demonstrate that this cleavable method can be used to turn on cytokine activity by a
200 small-molecule input, we determined that cleaveIL-10 activity can be tuned via aliskiren (Fig.
201 3f). Taken together, these results demonstrate successful engineering of cleavable cytokine
202 constructs for three structurally distinct cytokines also compatible with renin-aliskiren control.

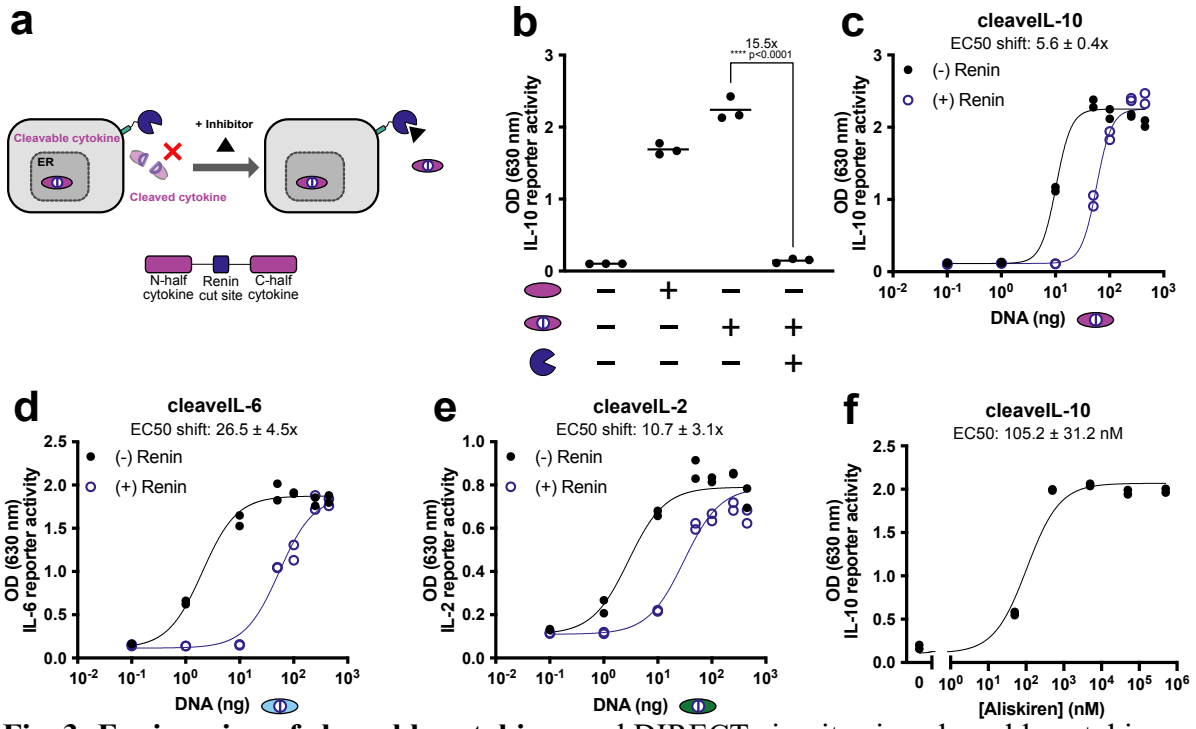

Fig. 3: Engineering of cleavable cytokines. a, hDIRECT circuit using cleavable cytokines to control cytokine activity. Cells constitutively secrete cleavable cytokines and cytokine activity can be tuned via small-molecule inhibitor for co-transfected protease, renin. **b,** Functional activity of cleaveIL-10 with or without renin co-expression. **c,** Functional activity of cleaveIL-10 plasmid transiently transfected with increasing amounts, with or without renin co-expression. **d,** Functional activity of cleaveIL-6 plasmid transiently transfected with increasing amounts, with or without renin co-expression. **e,** Functional activity of cleaveIL-2 plasmid transiently transfected with increasing amounts, with or without renin co-expression. **f,** Functional activity of cleaveIL-10 co-expressed with renin at increasing aliskiren concentrations. (Cytokine activity measured from supernatant using HEKBlue IL-10™ (**b-c,f**), IL-6™ (**d**), and IL-2™ (**e**) reporter cells. For **b**: mean of n = 3 biological replicates; Unpaired two-tailed Student's t-test. For **c-f**: n = 2 biological replicates; data represent mean ± s.e.m. **** $p < 0.0001$.)

203

204 **Orthogonalization of hDIRECT from its human counterparts.**

205 After establishing renin-regulated secreted cytokines, our next goal was to orthogonalize
206 the system from human hosts. In contrast to parts sourced from other organisms (e.g., viral
207 proteases), utilizing human parts bears the distinct and critical risk of off-target effects due to
208 crosstalk with other human proteins. In particular, renin is endogenously secreted into the blood
209 to regulate blood pressure via proteolytic cleavage of angiotensinogen[25]. Therefore, to avoid
210 crosstalk with the host, there are two issues to address: the activation of angiotensinogen by cell-
211 surface, exogenous renin and the cleavage of engineered cytokines by endogenous renin.

212 To address the first problem, we took inspiration from our previous work and
213 hypothesized that we could spatially separate renin from circulation by localizing it to the
214 endoplasmic reticulum (ER) of the engineered cell[16,41]. We fused renin to the CD4

transmembrane domain and a cytosolic ER-retention motif (RXR). Here, renin regulates engineered cytokines in cis as the cytokines move through the secretory pathway, while remaining sequestered from renin's endogenous substrate in the blood (Fig. 4a). Using cageIL-10 as a test case, we validated that ER-retained renin maintained the ability to activate secreted cageIL-10 (Fig. 4b). Next, to ensure that aliskiren could permeate engineered cells and inhibit ER-renin with a similar sensitivity (as it typically acts extracellularly), we performed aliskiren titration and validated that ER-renin retains aliskiren sensitivity (Fig. 4c).

To address the second problem of endogenous renin cleaving the engineered cytokines, we took inspiration from renin's diversity of specificities across species. In particular, human and mouse angiotensinogen are not recognized by the opposing renins[42]. We hypothesized that we could use the mouse cut site in engineered cytokines to avoid their cleavage by circulating human renin, and in parallel minimally engineer human renin to recognize the mouse site such that the protease is still unlikely to be recognized as non-human. To introduce mouse substrate recognition, we compared substrate bound renin structures across species. Compared to human renin, mouse renin has a larger binding pocket to accommodate bulkier substrate residues (L35 and Y36). We hypothesized that by mutating four substrate-binding residues in human renin to corresponding residues in mouse renin with smaller side chains (L147I, R148H, I203V, L290V) we could expand the binding pocket to accommodate the mouse substrate. We denote this mutant renin as human-to-mouse (H2M) renin. We then tested the orthogonality of the renin protease and found that the human and mouse renins only recognize their corresponding cut sites while the H2M renin's specificity is expanded to recognize both (Fig. 4d). We confirmed that H2M, ER-localized renin remains sensitive to aliskerin (Fig. 4e) and that ER-localization does not affect orthogonality (Fig. 4f).

In summary, human renin was successfully orthogonalized using structure-guided mutation and localization motifs. Importantly, these orthogonalization strategies did not compromise renin function or sensitivity to aliskiren. We expect to use ER-retained H2M renin and caged and cleavable cytokines harboring the mouse cut site, with minimal crosstalk with endogenous human renin and angiotensinogen.

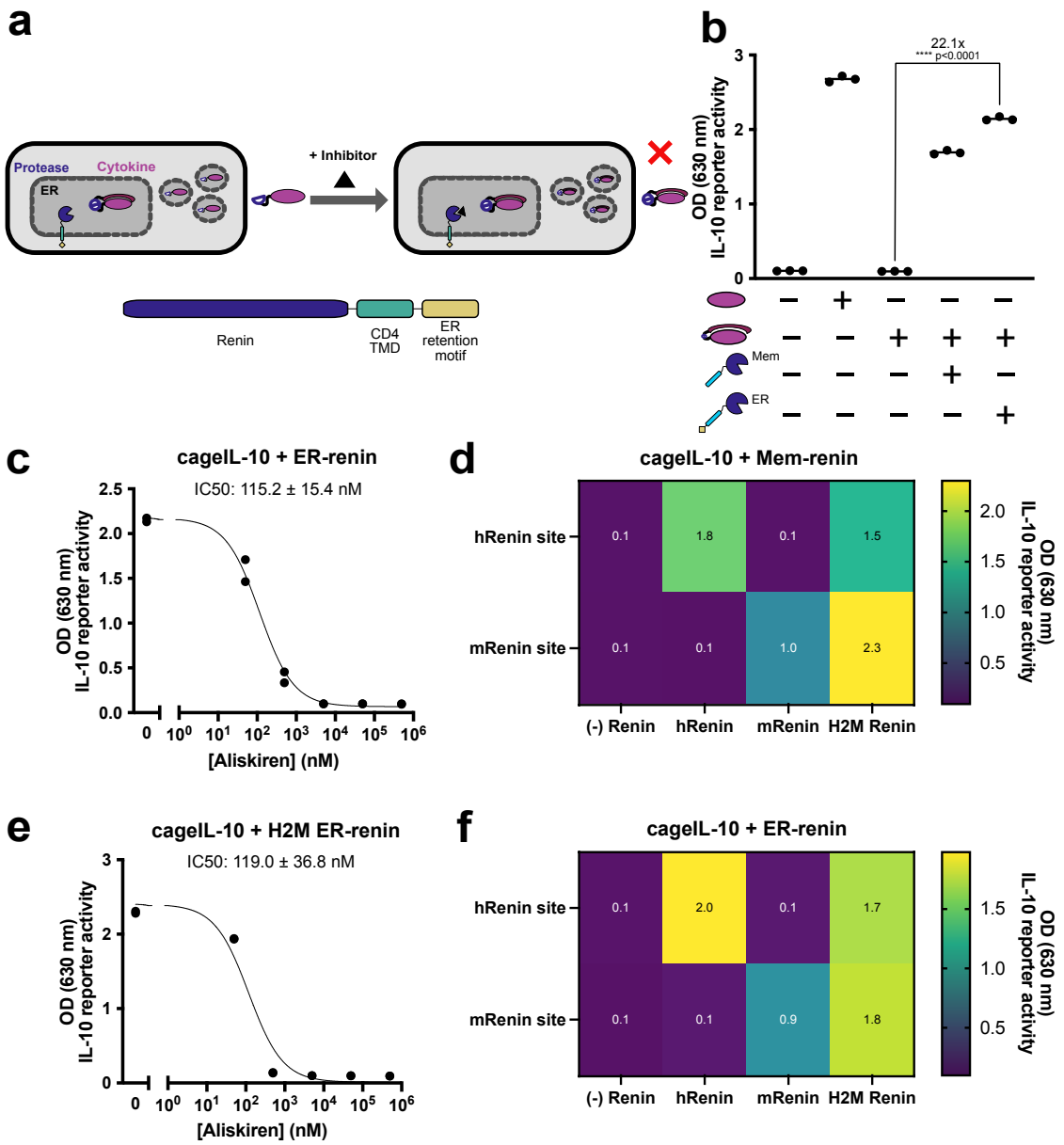

Fig. 4: Orthogonalization of renin. a, hDIRECT circuit using caged cytokines and ER-retained renin to control cytokine activity. Cells constitutively secrete caged cytokines and cytokine activity can be tuned via small-molecule inhibition of a co-expressed protease, renin. ER-renin is retained in ER via cytosolic motif and can cleave secreted cytokines. **b,** Activity of cageIL-10 transiently transfected with membrane or ER-retained renin. **c,** Activity of cageIL-10 co-expressed with ER-renin at increasing aliskiren concentrations. **d,** Orthogonal renin matrix. Functional activity of cageIL-10 constructs containing human (h) or mouse (m) cut sites transiently transfected with membrane-bound human, mouse, or engineered H2M renin. **e,** Functional activity of cageIL-10 with an mRenin cut-site co-expressed with H2M ER-renin at increasing aliskiren concentrations. **f,** Orthogonal renin matrix. Functional activity of cageIL-10 constructs containing human (h) or mouse (m) cut sites transiently transfected with ER-retained human, mouse, or engineered H2M renin. (Cytokine activity measured from supernatant using HEKBlue IL-10™ (**b-f**) reporter cells. For **b,d,f**: mean of n = 3 biological replicates; Individual replicate data for **d,f** can be found in Supplementary Fig. 5. Unpaired two-tailed Student's t-test. For **c,e**: n = 2 biological replicates; data represent mean ± s.e.m. **** $p < 0.0001$.)

Compact and stable transduction of hDIRECT in mammalian cells.

Clinically, *ex vivo* gene delivery to engineer therapeutic cells is bottlenecked by viral packaging limits of ~5 kb (adeno-associated virus) to ~10 kb (lentivirus)[43,44]. Compact genetic constructs are therefore a critical requirement for translational applications. To demonstrate that hDIRECT enables compact delivery, we created single-transcript constructs encoding engineered cytokines and renin separated by 2A "self-cleaving" peptides[45].

We created a polycistronic construct containing cageIL-10, membrane-bound renin, and a fluorescent co-transfection marker, mCherry (Fig. 5a). Transient transfection of this construct in HEK293 cells enables inhibition of IL-10 activity upon dosing with aliskiren (Fig. 5a). However, immune and regenerative responses are often mediated by the action of multiple cytokines with characteristic dosing and temporal profiles, rather than the activity of a single cytokine.[46,47] For example, muscle regeneration is mediated by an initial pro-inflammatory regime (i.e. IL-6), stimulating and recruiting the innate immune system, followed by an anti-inflammatory regime (i.e. IL-10) to indirectly promote the differentiation of muscle stem cells.[48] To demonstrate that hDIRECT can simultaneously control the activity of multiple cytokines, we created single-transcript constructs containing both cageIL-10 and cleaveIL-6 (Fig. 5b) to mimic a cytokine response for promoting muscle regeneration. In this manner, delivery with drug should allow an initial regime of IL-6 activity, followed by a switch to IL-10 activity when drug is removed. HEK293 cells transiently transfected with this single-transcript construct demonstrated bidirectional control of IL-10 and IL-6 activity with aliskiren (Fig. 5b).

Finally, we tested whether hDIRECT's performance is robust against different delivery methods or cellular contexts. First, we created lentiviral constructs using a weaker constitutive promoter, EF1-α, and the polycistronic cageIL-10 design (from Fig. 5a). We chose a weak promoter since stronger promoters during our pilot study exhibited leaky cytokine activity (data not shown). Stable transduction of HEK293 cells enabled regulation of IL-10 activity with aliskiren (Fig. 5c). Next, we sought to test hDIRECT in another cell line to validate its generalizability to different cell types. We chose Jurkat cells as an accessible proof of concept model for regulating IL-2 activity in the context of a CAR-T cell therapy. We designed lentiviral constructs using the EF1-α promoter and a polycistronic cageIL-2 design (Fig. 5d). Stably transduced Jurkats exhibited lower levels of secretion compared to HEK293s, but retained high sensitivity to aliskiren regulation of IL-2 activity (Fig. 5d). These results demonstrate that hDIRECT can be delivered as a stably expressed, single transcript in a commonly used viral vector, one step closer to the engineering of therapeutic cells.

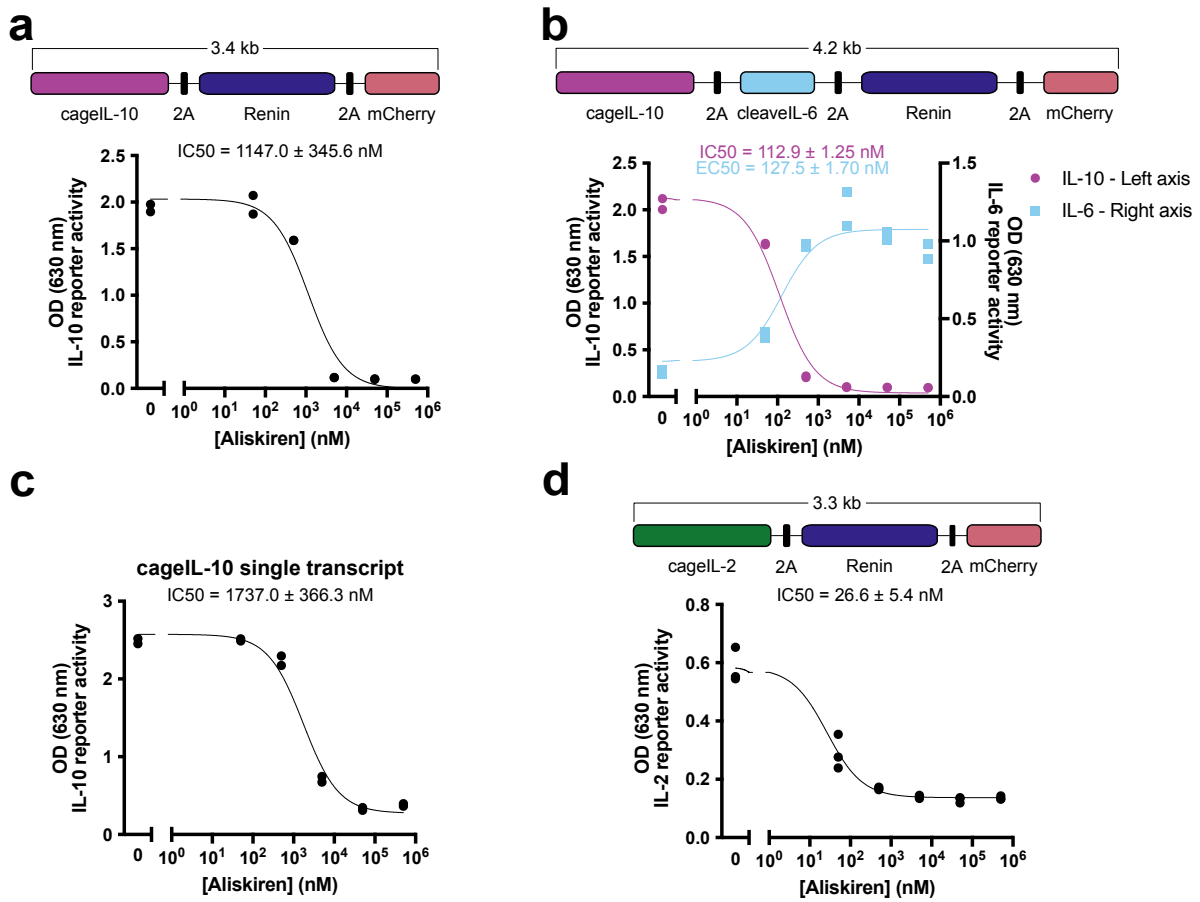

Fig. 5: Compact delivery of hDIRECT in mammalian cells. a, Polycistronic design of co-expressed cageIL-10 and renin (top cartoon). Aliskerin-regulated activity of cageIL-10 in HEK293 cells transiently transfected with cageIL-10 single-transcript design. **b,** Polycistronic design of cageIL-10, cleaveIL-6, and renin (top). Aliskiren-regulated activity of cageIL-10 (left-axis) and cleaveIL-6 (right-axis) in HEK293 cells transiently transfected with cageIL-10/cleaveIL-6 single-transcript design. **c,** Aliskiren-regulated activity of cageIL-10 in HEK293 cells stably transduced with cageIL-10 single-transcript design (from **a**). **d,** Polycistronic design of co-expressed cageIL-2 and renin (top cartoon). Aliskiren-regulated activity of cageIL-2 at increasing aliskiren concentrations from Jurkat cells stably transduced with cageIL-2 single-transcript design. (Cytokine activity measured from supernatant using HEKBlue IL-10™ (**a-c**), IL-6™ (**b**), and IL-2™ (**d**) reporter cells. For **a-d**: n = 2 biological replicates; data represent mean ± s.e.m.)

Discussion

Here, we have presented a post-translational, humanized platform, hDIRECT, for drug-mediated regulation of secreted cytokine activity in mammalian cells. Exclusively using human proteins and domains, hDIRECT confers protease control with opposing signs ("caged" and "cleavable") over cytokine activity. Co-expression of engineered cytokines renin allows the control of cytokine activity using renin's FDA-approved inhibitor, aliskiren. We used species specificity and re-localization strategies to orthogonalize both renin and engineered cytokines to avoid crosstalk with their human counterparts. Lastly, we showed that hDIRECT is compact and can regulate the activities of multiple cytokines encoded on a single transcript in different

mammalian cells. hDIRECT therefore represents a proof-of-concept towards humanized engineering for post-translational tools to control next generation cell therapies.

While we have shown that hDIRECT can regulate cytokines, we expect that the proteolytic control module can be extended to a variety of secreted and membrane proteins of interest. Growth factors and chemokines, which regulate the proliferation, differentiation, and migration of cells, could be similarly engineered for proteolytic regulation. In addition, we envision that receptors with cleavable pro-domains or competitive caging domains might offer small-molecule control of receptor availability which, for example, could tune the activity of CAR-T cells to limit exhaustion[40].

We envision that hDIRECT will find immediate applications in cellular therapeutics. hDIRECT could be used to temporarily supply CAR-T cells with IL-2 to improve proliferation and cytotoxicity. In applications where the temporal profile of cytokine activity is critical, such as MSC therapy for muscle regeneration, hDIRECT can be used to activate pro-inflammatory IL-6 followed by regenerative, anti-inflammatory IL-10. While this work demonstrates the control of two cytokines simultaneously, lentiviral packaging could allow for the delivery of a single transcript encoding up to five engineered cytokines. hDIRECT could also be used for research in basic science investigating the timing and dosage of various cytokine regimes by imparting temporal control over the activity of multiple cytokines.

While our system is built from native human proteins, the presence of junctions from fusion of domains and small number of mutations introduced for improving function and orthogonalization may still be recognized as foreign. Although immunogenicity can be estimated *in silico* using methods such as MHC binding algorithms, empirically validating these predictions will require clinical studies on human patients[49]. Nonetheless, we anticipate that human-derived engineering is a step towards immune-tolerized synthetic circuits.

While clinically suitable, hDIRECT still requires further work for potential use in humans. Notably, most experiments here were conducted in the model mammalian cell line HEK293. Future work in primary cell lines more aligned with an intended clinical application, such as CAR-T cells, could demonstrate the functional efficacy of this platform more. Subsequently, *in vivo* experiments in mice will help to determine the pharmacokinetics of small-molecule dosing and localized cytokine activity in a more complex environment. Furthermore, the use of aliskiren (approved as an antihypertensive) in a patient requiring a cellular therapeutic might have possible side effects. To minimize side effects, renin could be further engineered to be more sensitive to aliskiren so lower doses can be used.

In summary, hDIRECT is the first post-translational control platform using orthogonalized humanized components in mammalian cells. Using hDIRECT, we demonstrate small-molecule control of multiple secreted cytokines simultaneously. We envision that hDIRECT is generalizable towards a wide variety of extracellular factors and receptors to tune the. We expect our future engineering efforts to establish hDIRECT as a powerful tool routinely used in cell therapies.

References

1. Fischbach, M. A., Bluestone, J. A. & Lim, W. A. Cell-based therapeutics: the next pillar of medicine. *Sci. Transl. Med.* **5,** 179ps7 (2013).
2. Labanieh, L., Majzner, R. G. & Mackall, C. L. Programming CAR-T cells to kill cancer. *Nat. Biomed. Eng.* **2,** 377–391 (2018).
3. Ninagawa, N. T. *et al.* Transplantated mesenchymal stem cells derived from embryonic stem

336 cells promote muscle regeneration and accelerate functional recovery of injured skeletal
337 muscle. *Biores. Open Access* **2,** 295–306 (2013).
338 4. Winkler, T. *et al.* Dose-response relationship of mesenchymal stem cell transplantation and
339 functional regeneration after severe skeletal muscle injury in rats. *Tissue Eng. Part A* **15,**
340 487–492 (2009).
341 5. Munir, H. & McGettrick, H. M. Mesenchymal stem cell therapy for autoimmune disease:
342 risks and rewards. *Stem Cells Dev.* **24,** 2091–2100 (2015).
343 6. Squillaro, T., Peluso, G. & Galderisi, U. Clinical trials with mesenchymal stem cells: an
344 update. *Cell Transplant.* **25,** 829–848 (2016).
345 7. Marofi, F. *et al.* CAR T cells in solid tumors: challenges and opportunities. *Stem Cell Res*
346 *Ther* **12,** 81 (2021).
347 8. Preda, M. B. *et al.* Short lifespan of syngeneic transplanted MSC is a consequence of in vivo
348 apoptosis and immune cell recruitment in mice. *Cell Death Dis.* **12,** 566 (2021).
349 9. Kim, N. & Cho, S.-G. New strategies for overcoming limitations of mesenchymal stem cell-
350 based immune modulation. *Int J Stem Cells* **8,** 54–68 (2015).
351 10. Kerkar, S. P. *et al.* Tumor-specific CD8+ T cells expressing interleukin-12 eradicate
352 established cancers in lymphodepleted hosts. *Cancer Res.* **70,** 6725–6734 (2010).
353 11. Nitahara-Kasahara, Y. *et al.* Enhanced cell survival and therapeutic benefits of IL-10-
354 expressing multipotent mesenchymal stromal cells for muscular dystrophy. *Stem Cell Res*
355 *Ther* **12,** 105 (2021).
356 12. Chen, Z. & Elowitz, M. B. Programmable protein circuit design. *Cell* **184,** 2284–2301
357 (2021).
358 13. Gao, X. J., Chong, L. S., Kim, M. S. & Elowitz, M. B. Programmable protein circuits in
359 living cells. *Science* **361,** 1252–1258 (2018).
360 14. Fink, T. *et al.* Design of fast proteolysis-based signaling and logic circuits in mammalian
361 cells. *Nat. Chem. Biol.* **15,** 115–122 (2019).
362 15. Sahin, U., Karikó, K. & Türeci, Ö. mRNA-based therapeutics--developing a new class of
363 drugs. *Nat. Rev. Drug Discov.* **13,** 759–780 (2014).
364 16. Vlahos, A. E. *et al.* Protease-controlled secretion and display of intercellular signals. *Nat.*
365 *Commun.* **13,** 912 (2022).
366 17. Praznik, A. *et al.* Regulation of protein secretion through chemical regulation of
367 endoplasmic reticulum retention signal cleavage. *Nat. Commun.* **13,** 1323 (2022).
368 18. Ferdosi, S. R. *et al.* Multifunctional CRISPR-Cas9 with engineered immunosilenced human
369 T cell epitopes. *Nat. Commun.* **10,** 1842 (2019).
370 19. Lamers, C. H. J. *et al.* Immune responses to transgene and retroviral vector in patients
371 treated with ex vivo-engineered T cells. *Blood* **117,** 72–82 (2011).
372 20. Riechmann, L., Clark, M., Waldmann, H. & Winter, G. Reshaping human antibodies for
373 therapy. *Nature* **332,** 323–327 (1988).
374 21. Jones, P. T., Dear, P. H., Foote, J., Neuberger, M. S. & Winter, G. Replacing the
375 complementarity-determining regions in a human antibody with those from a mouse. *Nature*
376 **321,** 522–525 (1986).
377 22. Li, H.-S. *et al.* Multidimensional control of therapeutic human cell function with synthetic
378 gene circuits. *Science* **378,** 1227–1234 (2022).
379 23. Rauch, S. *et al.* Programmable RNA-Guided RNA Effector Proteins Built from Human
380 Parts. *Cell* **178,** 122–134.e12 (2019).
381 24. Zhu, I. *et al.* Design and modular assembly of synthetic intramembrane proteolysis receptors

382 for custom gene regulation in therapeutic cells. *BioRxiv* (2021).
383 doi:10.1101/2021.05.21.445218
384 25. Danser, A. H. J. & Deinum, J. Renin, prorenin and the putative (pro)renin receptor.
385 *Hypertension* **46,** 1069–1076 (2005).
386 26. Vaidyanathan, S., Jarugula, V., Dieterich, H. A., Howard, D. & Dole, W. P. Clinical
387 pharmacokinetics and pharmacodynamics of aliskiren. *Clin Pharmacokinet* **47,** 515–531
388 (2008).
389 27. Puskas, J. *et al.* Development of an attenuated interleukin-2 fusion protein that can be
390 activated by tumour-expressed proteases. *Immunology* **133,** 206–220 (2011).
391 28. Hsu, E. J. *et al.* A cytokine receptor-masked IL2 prodrug selectively activates tumor-
392 infiltrating lymphocytes for potent antitumor therapy. *Nat. Commun.* **12,** 2768 (2021).
393 29. Wang, X., Wong, K., Ouyang, W. & Rutz, S. Targeting IL-10 Family Cytokines for the
394 Treatment of Human Diseases. *Cold Spring Harb. Perspect. Biol.* **11,** (2019).
395 30. Sarvas, J. L., Khaper, N. & Lees, S. J. The IL-6 Paradox: Context Dependent Interplay of
396 SOCS3 and AMPK. *J. Diabetes Metab.* **Suppl 13,** (2013).
397 31. Serrano, A. L., Baeza-Raja, B., Perdiguero, E., Jardí, M. & Muñoz-Cánoves, P. Interleukin-
398 6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. *Cell Metab.*
399 **7,** 33–44 (2008).
400 32. Zhang, C. *et al.* Interleukin-6/signal transducer and activator of transcription 3 (STAT3)
401 pathway is essential for macrophage infiltration and myoblast proliferation during muscle
402 regeneration. *J. Biol. Chem.* **288,** 1489–1499 (2013).
403 33. Liao, W., Lin, J.-X. & Leonard, W. J. Interleukin-2 at the crossroads of effector responses,
404 tolerance, and immunotherapy. *Immunity* **38,** 13–25 (2013).
405 34. Rosenberg, S. A. Interleukin 2 for patients with renal cancer. *Nat. Clin. Pract. Oncol.* **4,** 497
406 (2007).
407 35. Hawkins, E. R., D'Souza, R. R. & Klampatsa, A. Armored CAR T-Cells: The Next Chapter
408 in T-Cell Cancer Immunotherapy. *Biologics* **15,** 95–105 (2021).
409 36. Logsdon, N. J., Jones, B. C., Josephson, K., Cook, J. & Walter, M. R. Comparison of
410 interleukin-22 and interleukin-10 soluble receptor complexes. *J. Interferon Cytokine Res.*
411 **22,** 1099–1112 (2002).
412 37. Gorby, C. *et al.* Engineered IL-10 variants elicit potent immunomodulatory effects at low
413 ligand doses. *Sci. Signal.* **13,** (2020).
414 38. Garbers, C. *et al.* Plasticity and cross-talk of interleukin 6-type cytokines. *Cytokine Growth*
415 *Factor Rev.* **23,** 85–97 (2012).
416 39. Wolf, J., Rose-John, S. & Garbers, C. Interleukin-6 and its receptors: a highly regulated and
417 dynamic system. *Cytokine* **70,** 11–20 (2014).
418 40. Kwon, B. The two faces of IL-2: a key driver of CD8+ T-cell exhaustion. *Cell Mol Immunol*
419 **18,** 1641–1643 (2021).
420 41. Shikano, S. & Li, M. Membrane receptor trafficking: evidence of proximal and distal zones
421 conferred by two independent endoplasmic reticulum localization signals. *Proc. Natl. Acad.*
422 *Sci. USA* **100,** 5783–5788 (2003).
423 42. Fukamizu, A. *et al.* Dependence of angiotensin production in transgenic mice carrying either
424 the human renin or human angiotensinogen genes on species-specific kinetics of the renin-
425 angiotensin system. *Arzneimittelforschung.* **43,** 222–225 (1993).
426 43. Wu, Z., Yang, H. & Colosi, P. Effect of genome size on AAV vector packaging. *Mol. Ther.*
427 **18,** 80–86 (2010).

44. Kalidasan, V. *et al.* A guide in lentiviral vector production for hard-to-transfect cells, using cardiac-derived c-kit expressing cells as a model system. *Sci. Rep.* **11,** 19265 (2021).
45. Tang, W. *et al.* Faithful expression of multiple proteins via 2A-peptide self-processing: a versatile and reliable method for manipulating brain circuits. *J. Neurosci.* **29,** 8621–8629 (2009).
46. Subramanian, N., Torabi-Parizi, P., Gottschalk, R. A., Germain, R. N. & Dutta, B. Network representations of immune system complexity. *Wiley Interdiscip Rev Syst Biol Med* **7,** 13–38 (2015).
47. Orzechowski, A. in *The plasticity of skeletal muscle* (ed. Sakuma, K.) 113–139 (Springer Singapore, 2017). doi:10.1007/978-981-10-3292-9_5
48. Yang, W. & Hu, P. Skeletal muscle regeneration is modulated by inflammation. *J. Orthop. Translat.* **13,** 25–32 (2018).
49. Calis, J. J. A. *et al.* Properties of MHC class I presented peptides that enhance immunogenicity. *PLoS Comput. Biol.* **9,** e1003266 (2013).

Methods

Plasmid generation. All plasmids were constructed using general practices. Backbones were linearized via restriction digestion, and inserts were generated using PCR, phosphorylated annealing, or purchased from Twist Biosciences. A complete list of plasmids used for each experiment (Supplementary Data 1) and the respective amounts used for transfection can be found in Supplementary Data 2. In addition, the DNA sequences of all the plasmids used in this study can be found in the source data, and all new plasmids with annotations will be available on Addgene.

Tissue culture. WT Human Embryonic Kidney (HEK) 293 cells (ATCC, catalog no. CRL-1573) and HEK293T-LentiX (Takara Biosciences, catalog no. 632180) were cultured in a humidity-controlled incubator under standard culture conditions (37 °C with 5% CO_2) in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Fisher Scientific, catalog no. FB12999102), 1 mM sodium pyruvate (EMD Millipore, catalog no. TMS-005-C), 1× penicillin–streptomycin (Genesee, catalog no. 25-512), 2 mM l-glutamine (Genesee, catalog no. 25-509) and 1× MEM non-essential amino acids (Genesee, catalog no. 25-536). HEKBlue™ IL-2, IL-6, and IL-10 cells were purchased from Invivogen (catalog no. hkb-il2, hkb-hil6, hkb-il10, respectively). Cells were cultured as per manufacturer's protocol. T-REx™ Jurkat cells (Invitrogen, catalog no. R72207) were cultured as per manufacturer's protocol.

Transient transfection. HEK293 cells were cultured in 96-well tissue culture-treated plates under standard culture conditions. When cells were 70-90% confluent, the cells were transiently transfected with plasmid constructs using the jetOPTIMUS DNA transfection reagent (Polyplus, catalog no. 117-15), as per manufacturer's instructions using 0.375 µL of reagent per 50 µL of jetOPTIMUS buffer for 500 ng total DNA transfections. Experiments requiring incubation with aliskiren (Sigma Aldrich, catalog no. SML2077) were treated with drug at time of transfection through media exchange.

Lentiviral transduction. LentiX cells were cultured in 6-well tissue culture-treated plates under standard culture conditions. When cells were 70-90% confluent, the cells were transiently transfected with plasmid constructs (600 ng PAX2, pMD2g, and 1,100 ng transfer target) using

the jetOPTIMUS DNA transfection reagent (Polyplus catalog no. 117-15), as per manufacturer's instructions using 1.5 μL of reagent per 200 μL of jetOPTIMUS buffer for 2,000 ng total DNA transfections. Cells were incubated for 24 h under standard culture conditions. Afterwards an additional 3 mL of DMEM media was added carefully to each well. Lentivirus was concentrated 24 hrs afterward using viral precipitation. For each lentiviral prep, media was filtered using a syringe and 0.45 μm filter into 15 mL conical tubes. 5x Lentivirus Precipitation Solution (Alstem catalog no. VC100) was mixed with each prep and incubated at 4 °C overnight. Then, virus was spun down at 1500xg for 30 min at 4 °C. Supernatant was aspirated, and virus was resuspended using 200 μL Dulbecco's PBS (Genesee catalog no. 25-508). Virus was added dropwise onto 24-well tissue culture plates containing HEK293 or Jurkat cells seeded at 250k cells/well. 72 h after incubation, the percentage of mCherry-positive cells were quantified using flow cytometry. The cells were selected using 1000 ng/mL of puromycin (ThermoFisher Scientific; catalog# J61278-MB) for one week until >90% of cells were mCherry-positive before being used for experiments.

Cytokine reporter cell assay. Supernatant (10 μL) from HEK293 cells transiently transfected 24 h prior were incubated with 5 x 10^4 HEKBlue™ cells (190 uL) in 96-well tissue culture-treated plates for 24 h under standard culture conditions. Afterwards, 20 μL HEKBlue™ cell supernatant was collected and incubated with 180 μL Quanti-Blue reagent (Invivogen) for 2 h at 37 C as per manufacturer protocol. The mixture was read at 630 nm using the Spark® Multimode microplate reader (TECAN). Cartoon schematic can be found in Supplementary Fig. 1.

For stable transductions, stable HEK293 cells were seeded at 1.5 x 10^4 cells in 96-well tissue culture-treated plates for 48 h under standard culture conditions. Media was changed either with or without aliskiren 24 h into the incubation. Supernatant transfer to HEKBlue™ cells and assay proceeded the same as prior. Jurkat cells were seeded at 5 x 10^4 cells in U-bottom 96-well plates for 48 h under standard culture conditions. Cells were spun down in a centrifuge at 300 xg for 7 min and media was replaced with or without aliskiren 24 h into the incubation. 48 h after seeding, cells were spun down in a centrifuge at 300 xg for 7 min again and supernatant was transferred to HEKBlue™ cells and assay proceeded the same as prior.

Statistical analysis. An unpaired *t*-test was used to determine the statistically significant difference between two groups. Single, double, triple, and quadruple asterisks indicate $P < 0.05$, $P < 0.005$, $P < 0.001$ and $P < 0.0001$. All statistical analyses were performed using GraphPad Prism 10.0.0.

Data availability.

All data reported in this paper are available from the corresponding author on request. Plasmid maps for all plasmids used in this study will be available as GenBank files. The plasmids used in this study will be deposited on Addgene, with complete and annotated GenBank files on their website. All figures and supplemental figures have associated raw data. There are no restrictions on data availability.

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Author contributions

C.A. and X.J.G. conceived and directed the study. C.A. performed all the experiments. A.E.V. created the transmembrane domain and ER-retention motifs for renin constructs. J.P. and Q.C. provided recommended mutations for the H2M renin. C.A. analyzed the data for the manuscript. C.A. and X.J.G. wrote the manuscript. All authors provided feedback on the manuscript.

Competing interests

The board of trustees of the Leland Stanford Junior University have filed a patent on behalf of the inventors (C.A.A. and X.J.G.) of the small-molecule control of membrane and secreted proteins using human proteases platform described (US provisional Application No. 63/458833). An additional provisional patent application has also been filed on behalf of the inventors (C.A.A., X.J.G., J.P, and Q.C.) for the orthogonalization of human renin. X.J.G. is a co-founder and serves on the scientific advisory board of Radar Tx.