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1 **Title:** Differential effects of voclosporin and tacrolimus on insulin secretion from human islets

2

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19

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27 **Abstract**

28 ***Context.*** The incidence of new onset diabetes after transplant (NODAT) has increased over the
29 past decade, likely due to calcineurin inhibitor-based immunosuppressants, including tacrolimus
30 (TAC) and cyclosporin (CsA). Voclosporin (VCS), a next generation calcineurin inhibitor is
31 reported to cause fewer incidences of NODAT but the reason is unclear.
32
33 ***Objective.*** Whilst calcineurin signaling plays important roles in pancreatic β-cell survival,
34 proliferation, and function, its effects on human β-cells remain understudied. In particular, we do
35 not understand why some calcineurin inhibitors have more profound effects on the incidence of
36 NODAT.
37
38 ***Methods.*** We compared the effects of TAC and VCS on the dynamics of insulin secretory
39 function, programmed cell death rate, and the transcriptomic profile of human islets. We studied
40 two clinically relevant doses of TAC (10 ng/ml, 30 ng/ml) and VCS (20 ng/ml, 60 ng/ml), meant to
41 approximate the clinical trough and peak concentrations.
42
43 ***Results*****.** TAC, but not VCS, caused a significant impairment of 15 mM glucose-stimulated and
44 30 mM KCl-stimulated insulin secretion. This points to molecular defects in the distal stages of
45 exocytosis after voltage-gated Ca^{2+} entry. No significant effects on islet cell survival or total insulin
46 content were identified. RNA sequencing showed that TAC significantly decreased the expression
47 of 17 genes, including direct and indirect regulators of exocytosis (*SYT16*, *TBC1D30*, *PCK1*,
48 *SMOC1*, *SYT5, PDK4*, and *CREM*), whereas VCS has less broad and milder effects on gene
49 expression.
50
51 ***Conclusions****.* Clinically relevant doses of TAC, but not VCS, directly inhibit insulin secretion from
52 human islets, likely via transcriptional control of exocytosis machinery.
53
54

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55 **Introduction**

56 New onset diabetes after transplant (NODAT) is a clinical problem that has increased in
57 incidence over the past decade. It is widely believed that NODAT is caused by exposure to
58 steroids or high dose calcineurin inhibitors, including tacrolimus (TAC; also known as FK506) and
59 cyclosporin (CsA) (1,2). These drugs are shown to damage cell types critical for the maintenance
60 of glucose homeostasis, particularly pancreatic β-cells (3). TAC is a mainstay for prevention of
61 transplant rejection, but effective immunosuppression targeting a concentration range of 4-11
62 ng/mL still results in NODAT in > 20% of patients (1). There is an unmet clinical need for
63 calcineurin inhibitors that do not cause diabetes in this already vulnerable patient population.

64 Voclosporin (VCS) is a next-generation calcineurin inhibitor that is structurally related to
65 cyclosporine A (CsA) with the addition of a carbon molecule at amino acid-1 of CsA. This
66 modification results in enhanced binding of the VCS-cyclophilin complex to calcineurin and shifts
67 metabolism, resulting in increased potency and a consistently better pharmacokinetic-
68 pharmacodynamic profile as compared to CsA. VCS was previously evaluated in a Phase IIb,
69 multi-center, open-label, concentration-controlled study in patients undergoing *de novo* renal
70 transplantation, evaluating the efficacy and safety of three doses of VCS (0.4, 0.6, and 0.8 mg/kg
71 BID) as compared to TAC (4). In that study, the low-dose VCS group had similar efficacy to
72 tacrolimus in controlling acute rejection with a significantly decreased incidence of NODAT (1.6%
73 vs. 16.4%, respectively, p=0.031) (4). In the AURA study for lupus nephritis, 1.1% of patients
74 receiving VCS (23.7 mg) and 1.1% of patients in the placebo group reported diabetes (5),
75 contrasting with NODAT in 25.7% of patients while taking a standard dose of TAC for 1 year (6).
76 Thus, there is an unexplained difference between the clinical diabetes seen after treatment with
77 VCS, and currently prescribed immunosuppressants, TAC and CsA.

78 While the etiology of NODAT is not well understood, calcineurin and nuclear factor of activated
79 T-cells (NFAT) are involved in a number of cellular processes outside of immunosuppression,
80 including direct roles in normal pancreatic β-cell development and physiology (7-9). For example,

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81 Heit et al. reported that β-cell selective knockout of calcineurin in mice reduced functional β-cell
82 mass and caused diabetes that could be rescued by over-expressing active NFATc1 (8). The
83 effects of acute calcineurin inhibition on human and mouse islet function was previously
84 investigated in a study by our group comparing clinically relevant concentrations of TAC, CsA,
85 and rapamycin (7). *In vitro* experiments showed a direct decrease in β-cell function after only 24
86 hours of exposure to TAC (7). *In vivo* studies in mice from our group and others have shown that
87 inhibition of the calcineurin pathway by TAC impairs the function of transplanted human islet grafts
88 (7,10). Thus, while previous data from animal models and human islets studies clearly
89 demonstrates that TAC has multiple pathological effects on pancreatic islets, the mechanisms by
90 which TAC impairs insulin secretion from human islets remained unresolved. Most importantly,
91 there are no published studies directly comparing the direct effects of TAC versus VCS on human
92 islets.

93 Here, we directly tested the hypothesis that VCS has lower human β-cell toxicity compared
94 with TAC, and we conducted studies designed to investigate the molecular mechanisms involved
95 (Fig 1A). We found that human islets exposed to TAC exhibited significantly reduced distal steps
96 of glucose-stimulated insulin secretion, while VCS showed no statistically significant inhibition at
97 a dose that elicits sufficient immunosuppression. RNA sequencing showed that TAC, and to a
98 lesser extent VCS, decreased the expression of genes that specifically regulate the distal steps
99 of insulin secretion. In support of the clinical observations, our data suggest that VCS is less toxic
100 to human pancreatic β-cells at clinically relevant doses.

101

102 **Methods**

103 *Human islet culture*

104 High-quality research specific human islets from cadaveric donors were obtained from
105 IsletCore Laboratory in Edmonton Alberta (11), and used under ethical approval from the UBC

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106 Clinical Research Ethics Board (H13-01865). Islets were shipped in CMRL media (Thermo Fisher
107 Scientific) overnight from Edmonton to the University of British Columbia. Upon arrival, islets were
108 immediately purified by handpicking under a stereomicroscope and suspended in RPMI 1640
109 medium (Thermo Fisher Scientific) supplemented with 5.5 mmol glucose, 10% FBS, and 100
110 units/ml penicillin/streptomycin. Groups of 100 islets were placed in non-treated 100-mm
111 polystyrene petri dishes (Fisher Scientific) followed by 48-hr incubation at 37°C and 5% CO_2 (Fig.
112 1A). We chose 48-hr as an incubation time to balance our efforts to model long-term (years)
113 exposure to these drugs in the clinical setting with our experience that human islet function
114 degrades after 4-5 days in culture. Donor and isolation characteristics can be found in Figure 1B.
115
116 *Immunosuppression-related assays*
117 The Calcineurin inhibitor assay and the T-cell proliferation assay are described in more detail
118 elsewhere (12,13). Briefly, peripheral blood mononuclear cell (PBMC) lysates were used in the
119 calcineurin assay after 25 min stimulation with PMA-A23187. After lysis with a calcineurin assay
120 buffer (no detergents) and 3 freeze-thaw cycles, the soluble fraction was probed by Western blot
121 with 1:200 anti-NFAT2 antibody (Santa Cruz Biotechnology Cat# sc-13033, RRID:AB_2152501).
122 Western blot analysis of NFAT accumulation after treatment with indicated doses of VCS and
123 TAC was then conducted to identify the relative concentrations that elicited a similar response.
124
125 *Dynamic analysis of insulin secretion from human islets*
126 To define the dynamic effects of VCS and TAC on insulin secretion from human islets, we used
127 human islet perifusion studies (7). We assessed the effects of TAC (10 ng/ml, 30 ng/ml) and VCS
128 (20 ng/ml, 60 ng/ml) on the dynamics of insulin secretion in response to two stimuli that are
129 diagnostic of changes in metabolism/signalling or exocytosis. Our standard approach (7,14-19)
130 compared the response to 15 mM glucose stimulation and direct depolarization with 30 mM KCl.
131 More specifically, 65 islets per column were perifused (0.4 ml/min) with 3 mM glucose KRB

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132 solution as described previously (7) for 60 min to equilibrate the islets to the KRB and flow rate,
133 and then with the indicated condition (as described in corresponding figure legends). First phase
134 insulin release was defined as the amount of insulin secreted during the first 20 min of 15 mM
135 glucose stimulation, while the remaining 40 of stimulation were defined as second phase. DMSO,
136 VCS and TAC were present at the indicated concentrations during the entirety of the perifusion
137 experiment. We repeated this experiment on islets from 17 donors to take into account the
138 significant variability between human islet preparations (11). Samples were stored at −20 °C and
139 insulin secretion was quantified using human insulin radioimmunoassay kits (Cedarlane).
140
141 *Cell death assays*
142 Pancreatic islets from human cadaveric donors were dispersed and seeded into 384-well
143 plates and cultured in similar manner as previously described (20-23). Cells were stained with 50
144 ng/mL Hoechst 33342 (Invitrogen) and 0.5 μg/mL propidium iodide (PI) (Sigma-Aldrich) in RPMI
145 1640 medium (Invitrogen) with 5 mM glucose (Sigma-Aldrich), 100 U/mL penicillin, 100 μg/mL
146 streptomycin (Invitrogen), 10% vol/vol fetal bovine serum (FBS) (Invitrogen) with or without a
147 cytokine cocktail (25 ng/mL TNF-α, 10 ng/mL IL-1β, and 10 ng/mL IFN-γ; R&D Systems). We
148 have previously shown that neither of these chemicals affect islet cell viability on their own at
149 these concentrations (23). After 2 hours of staining, cells were incubated with different
150 concentrations of VCS or TAC (1, 10, 20, 30, 60, 120 ng/ml) or vehicle (DMSO) and with a
151 cytokine cocktail (25 ng/mL TNF-α, 10 ng/mL IL-1β, and 10 ng/mL IFN-γ; R&D Systems).
152 Immediately after the addition of the test drugs, the cells were imaged at 37°C and 5% CO_2.
153 Images were captured every second hour for 48 hours using a robotic microscope
154 (ImageXpress^{MICRO} XLS, Molecular Devices) and analyzed using MetaXpress multi-wave cell
155 score Software (Molecular Devices). To adjust for the difference in basal cell death for each
156 individual run, each timepoint was normalized to the first timepoint then further normalized to the
157 cytokine control.

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158 *RNA-sequencing and pathway analysis*

159 Groups of 200 islets from 7 independent donors were treated for 48 hours with vehicle (DMSO),
160 TAC (30 ng/ml) or VCS (60 ng/ml) then immediately flash-frozen in liquid nitrogen and stored at -
161 80°C. RNA isolation, library preparation, RNA sequencing and bioinformatics support were
162 provided by the UBC Biomedical Research Centre Core Facility. Briefly, sample quality control
163 was performed using the Agilent 2100 Bioanalyzer. Qualifying samples were then prepped
164 following the standard protocol for the NEBnext Ultra ii Stranded mRNA (New England Biolabs).
165 Sequencing was performed on the Illumina NextSeq 500 with Paired End 42bp × 42bp reads. De-
166 multiplexed read sequences were then aligned to the reference sequence using STAR aligners
167 (STAR, RRID:SCR_015899) Assembly were estimated using Cufflinks (Cufflinks,
168 RRID:SCR_014597) through bioinformatics apps available on Illumina Sequence Hub. Raw
169 counts were analyzed by NetworkAnalyst 3.0 (www.networkanalyst.ca) (24). DeSeq package was
170 selected for differential expression of the genes. Differentially expressed genes were identified as
171 those with an adjusted p value < 0.05 using the comparison of TAC vs DMSO or VCS vs DMSO.

172 To determine the relationship between the differentially expressed genes and the calcineurin-
173 NFAT pathway, we extracted all 49 genes in the Gene Ontology Term "calcineurin-NFAT
174 pathway" (accessed July 30, 2020 from
175 http://www.informatics.jax.org/vocab/gene_ontology/GO:0033173). We removed genes from the
176 calcineurin-NFAT list without human homologues and that were not expressed in our human islet
177 RNAseq. We then combined the expressed calcineurin-NFAT list with the genes that were
178 differentially expressed between DMSO and TAC and used the protein-protein network interaction
179 analysis tools at string-db.org with the following setting (active interaction sources – all; medium
180 confidence 0.4; max interactors 0).

181 *Statistics and data analysis*

182 Data were analyzed using a one-way multiple comparisons ANOVA followed by a *post hoc* t-
183 test using the Tukey HSD method, unless otherwise indicated. Data are expressed as means ±

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184 SEM, and $p < 0.05$ was considered significant. n values represent the number of unique islet
185 donors studied.
186
187 **Results**
188 *Effects of TAC and VCS on NFAT activity*
189 Both TAC and VCS exert their immunosuppressive effects through inhibition of calcineurin
190 phosphatase activity, preventing the dephosphorylation and activation of NFATc (25,26). Each of
191 these two drugs is distinct with regard to its molecular mechanism of action. TAC interacts with
192 the immunophilin FKBP12 prior to binding calcineurin, whereas VCS interacts with cyclophilins.
193 The *in vitro* activity of TAC, VCS (and the structurally related inhibitor CsA) were compared in a
194 calcineurin inhibition assay and for their downstream ability to inhibit the division of activated T
195 cells. The IC_{50} of TAC against calcineurin was found to be approximately 4-fold lower than VCS,
196 in accordance with clinical dosing where the therapeutic range of TAC is 5-30 ng/mL and VCS
197 target trough concentrations are around 20-60 ng/mL (Fig 2A). Surprisingly, the potency
198 difference between TAC and VCS widened to 50-fold, in favor of TAC, when looking at inhibition
199 of T-cell proliferation (Fig 2A), leading to additional experiments comparing the impact of each
200 drug on their common pathway effector, NFAT. Maximal activation of NFAT was observed at 56
201 ng/mL of VCS and only 1.4 ng/mL of TAC, a potency difference of 40-fold (Fig. 2B,C,D). These
202 data demonstrate that, at therapeutic concentrations, TAC has a far more profound impact on
203 NFAT than VCS (Fig. 2E).
204
205 *Effects of TAC or VCS on basal and glucose stimulated insulin secretion*
206 Insulin is released from pancreatic islets, *in vivo* and *in vitro*, in a biphasic pattern that includes
207 both a rapid 1st phase of pre-docked insulin granules and a 2nd sustained phase involving granules
208 that are recruited to the plasma membrane (27). There is significant inter-individual variability in
209 the rate of insulin secretion from human islets (11) and the effects of immunosuppressants (7),

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210 so we examined insulin secretion from high quality human islets isolated from 17 donors
211 exclusively for research purposes. We employed the dynamic perifusion system with frequent
212 sampling to evaluate basal insulin secretion at 3 mM glucose, both phases of insulin secretion
213 during 15 mM glucose stimulation, and insulin secretion in response to direct depolarization with
214 30 mM KCl. TAC at 10 ng/ml (clinical trough) and 20 ng/ml (clinical peak) significantly inhibited
215 total insulin secretion at 15 mM glucose, whereas VCS did not (Fig. 3A,B). TAC exhibited a
216 specific effect on the 2nd phase but not the 1st phase of glucose-stimulated insulin secretion (Fig.
217 3C,D). The insulin secretory response to 30 mM KCl was also significantly inhibited by both
218 concentrations of TAC (Fig. 3E). In contrast, VCS concentrations approximately 2 times higher
219 than TAC had milder effects on glucose-stimulated insulin secretion. Specifically, the clinical
220 trough concentration of 20 ng/ml VCS was ~50% less inhibitory to the total and 2nd phase of insulin
221 secretion and did not result in statistically significant inhibition (Fig. 3A,B,D). No significant
222 differences in basal insulin secretion were observed at 3 mM glucose for either drug at either of
223 the concentrations tested (Fig. 3F). Similar results were observed when the perifusion data were
224 normalized to basal insulin secretion (Fig. 3G,H). We were unable to detect significant differences
225 in acid-ethanol extractable insulin protein content in human islets treated with either drug, at either
226 concentration, for 48 hr (Fig. 4). These results indicate that the specific significant inhibitory effects
227 of TAC on total and 2nd phase glucose-stimulated insulin secretion, as well as insulin secretion
228 stimulated by direct depolarization, are likely to be direct effects on the exocytosis machinery
229 rather than a result of reduced insulin production. Collectively, these data also demonstrate that
230 VCS has milder effects on human islet secretory function when compared with TAC in this
231 sensitive *in vitro* assay system.
232
233 *Effects of TAC or VCS on human islet cell survival*
234 We next assessed the effects of TAC and VCS on the survival of dispersed human islet cells
235 using our long-term high-content imaging protocols that measure both apoptotic and non-

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236 apoptotic cell death over multiple days of culture in the presence of proinflammatory cytokines
237 (20-23) (Fig. 5A,B). We studied both TAC and VCS over a 5 point-dose response curve. We
238 observed no significant differences in human islet cell survival following either drug treatment (Fig.
239 5C). This is consistent with our data showing no changes in insulin content following 48 hrs of
240 drug treatment, further pointing to the primacy of the direct effect of TAC on insulin secretion.
241

242 *Effects of TAC or VCS on human islet gene expression*

243 In order to provide clues as to the underlying molecular mechanisms by which TAC, but not
244 VCS, significantly inhibits insulin secretion we performed RNAseq and network analysis on human
245 islets from 7 donors (Fig 1B). Despite significant donor to donor variation in gene expression, we
246 were able to identify multiple significantly differentially expressed genes in human islets treated
247 with these immunosuppressant drugs, compared to DMSO (Fig. 6A). Specifically, TAC
248 significantly decreased the expression of synaptotagmin 16 (*SYT16*), TBC1 domain family
249 member 30 (*TBC1D30*), phosphoenolpyruvate carboxykinase 1 (*PCK1*), SPARC related modular
250 calcium binding 1 (*SMOC1*), synaptotagmin 5 (*SYT5*), muscular LMNA interacting protein (*MLIP*),
251 coiled-coil domain containing 184 (*CCDC184*), ankyrin repeat and SOCS box containing 4
252 (*ASB4*), pyruvate dehydrogenase kinase 4 (*PDK4*), long intergenic non-protein coding RNA 473
253 (*LINC00473*), long intergenic non-protein coding RNA 602 (*LINC00602*), SLC8A1 antisense RNA
254 1 (*SLC8A1-AS1*), leucine rich repeat LGI family member 2 (*LGI2*), islet amyloid polypeptide
255 (*IAPP*), cAMP responsive element modulator (*CREM*), B9 domain containing 1 (*B9D1*), and
256 scavenger receptor class B member 2 (*SCARB2*). VCS only significantly decreased the
257 expression of *SYT16*, *TBC1D30*, and *PCK1*, and to a qualitatively lesser degree.

258 Next, we examined the relationship between the calcineurin-NFAT gene network, as defined
259 by its Gene Ontology term, and the TAC-modulated genes in human islets using single String
260 protein-protein interaction network modelling (Fig. 6B). These analyses showed that CREM was
261 the TAC-target mostly highly connected to calcineurin-NFAT network, with highest confidence-

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262 level interactions with NFATC1, NFATC2, and NFATC3, and a medium confidence link to GSK3B.
263 MILP showed high confidence interactions with a module including Cardiomyopathy Associated
264 5 (CMYA5) and Myozenin 2 (MYOZ2). PCK1 showed a medium confidence interaction with
265 GSK3B. PDK4 showed a high confidence interaction with MTOR, and also with PCK1.
266 We found medium confidence interactions between SMOC1 and Leucine Rich Repeat LGI
267 Family Member 2 (LGI2), between B9D1 and Mitogen-Activated Protein Kinase 7 (MAPK7), and
268 between IAPP and Prion Protein (PRNP). Together, these analyses show how VCS and TAC
269 treatment affect the expression of specific genes via calcineurin signalling and lead us to our
270 current working model (Fig. 7C).
271

272 **Discussion**

273 The effects of calcineurin inhibitors on human β-cells remain understudied. A deeper
274 understanding of how immunosuppressive agents affect human islets is necessary to elucidate
275 the pathogenesis of NODAT and to identify immunosuppressants with improved safety profiles.
276 Thus, the objective of the present study was to determine why the next-generation calcineurin
277 inhibitor, VCS, has lower human β-cell toxicity compared with TAC. To examine this, we compared
278 the effects of TAC and VCS on the dynamics of insulin secretory function, the programmed cell
279 death, and the transcriptomic profile of isolated human islets. We found that TAC, but not VCS,
280 caused a significant impairment of both 15 mM glucose and 30 mM KCl-stimulated insulin
281 secretion at clinically relevant doses. We saw no effect on total insulin content or human islet cell
282 survival with either drug. Gene pathway enrichment analysis of RNAseq data showed that TAC
283 decreased the expression of genes that regulate exocytosis and genes that regulate transport
284 from the ER to the Golgi apparatus. VCS treatment also induced these changes; however, it did
285 so to a lesser extent. Thus, this greater inhibition of insulin secretion could provide an explanation
286 of why the risk of NODAT is higher in patients taking TAC than the next generation calcineurin

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287 inhibitor VCS. The equivalently immunosuppressive dose ranges of VCS and TAC we used have
288 dramatically different potencies on NFAT, suggesting that NFAT-mediated gene expression
289 mediates the observed effects on exocytosis regulating gene expression.

290 Previous studies have found that calcineurin inhibitors impair glucose-stimulated insulin
291 secretion in rodent islets and rodent cell lines (8,28-39), although through mechanisms that we
292 show here are not validated in human islets. For example, we do not see evidence for a robust
293 effect of TAC or VCS on insulin production or gene expression. Moreover, the majority of rodent
294 studies (8,28-39) and previous studies using human islets (7,10,40-47) used concentrations of
295 calcineurin inhibitors that are higher than seen clinically. Our study extends these previous
296 findings by using clinically relevant doses of TAC and VCS, rigorously examining the kinetics of
297 insulin release in human islets and by implicating a direct effect on insulin granule trafficking. TAC
298 at 10 ng/ml (clinical trough) and 20 ng/ml (clinical peak) significantly inhibited total insulin
299 secretion in response to 15 mM glucose and this inhibition was directly due to a specific effect on
300 the 2nd phase of insulin release. Furthermore, KCl-stimulated insulin secretion was decreased to
301 a similar degree, implicating secretory pathways distal to Ca^{2+} entry via voltage-gated Ca^{2+}
302 channels. Together, these data suggest that the readily releasable pool of insulin granules is not
303 robustly affected by TAC-mediated calcineurin inhibition; instead, TAC most likely impairs
304 pathways associated with insulin granule trafficking to the plasma membrane. While we did not
305 directly look at exocytotic events in this study, the results of our RNAseq analysis are consistent
306 with a TAC-mediated effect on insulin granule traffic and release. TAC has previously been
307 suggested to impair glucose-stimulated insulin secretion in rat islets via a mechanism involving
308 insulin granule exocytosis (39). Future studies using electrophysiology and imaging to examine
309 insulin granule docking, priming and release will provide a more detailed biophysical explanation
310 of TAC effects in human β-cells.

311 Our RNAseq data provided novel insights into the molecular mechanisms controlling insulin
312 secretion from human β-cells, the effects of these drugs, and impetus for follow-up studies on

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313 several gene products. A prime candidate is SYT16, which has not previously been directly
314 implicated in glucose-stimulated insulin secretion but that is known to be glucose responsive in
315 human islets (48) and under circadian control in mouse islets (49). The causal role of SYT5 in
316 insulin secretion remains unclear in primary human β-cells (50), but should also be investigated
317 further. TAC likely also has direct effects on insulin granule trafficking given that TBC1D30 is an
318 activating GTPase of RAB3 (51). MLIP activates AKT, which can promote insulin secretion (52),
319 and is regulated by CREB/CREM transcription factors. Indeed, TAC significantly inhibited CREM,
320 a member of the CREB family of transcription factors known as master regulators of β-cell function
321 (53). Previous studies in a β-cell line have shown that TAC inhibits CREB transcriptional activity
322 (54). *B9D1* contributes to the biogenesis of the primary cilium, which is required for normal insulin
323 secretion (55). *PCK1* and *PDK4* regulate cellular metabolism, but require additional studies in
324 human islets. TAC reduced *IAPP* expression but the functional consequence of this is unclear.
325 Thus, the majority of significantly downregulated genes point to the distal stages of insulin
326 trafficking and exocytosis as the primary sites of TAC-induced β-cell dysfunction. We used
327 protein-protein interaction mapping to predict the most likely link(s) between calcineurin signaling
328 and the TAC-downregulated genes. Indeed, this network analysis suggested direct and robust
329 links to CREM in TAC-treated human islets. Collectively, these data further solidify the role of
330 calcineurin signaling and NFAT-mediated gene transcription in human β-cell function and provide
331 insight into the molecular mechanisms controlling 2nd phase insulin exocytosis.

332 Calcineurin activity has been ascribed a number of functions in pancreatic β-cells (8-10,41,56-
333 59). For example, cultured insulinoma cells treated with the calcineurin inhibitor
334 cypermethrin exhibited decreased insulin exocytosis in response to both glucose and KCl
335 stimulation (60). In contrast to this, some studies suggest that calcineurin has a negative role in
336 insulin exocytosis. However, these were indirect findings obtained by examining the inhibition of
337 β-cell exocytosis via neurotransmitter-mediated activation of calcineurin (61,62), as opposed to

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338 direct effects observed following inhibition of calcineurin. Nevertheless, our study now shows that
339 VCS is gentler on human islets because it does not cause the same level of inhibition on insulin
340 exocytosis compared to TAC.

341 In our previous work, we examined the different effects of immunosuppressive drugs (TAC,
342 cyclosporine, and rapamycin) on induced endoplasmic reticulum (ER) stress and caspase-3-
343 dependent apoptosis in human islets (7). In that study, we found that TAC impaired glucose-
344 stimulated insulin release from human islets, but did not significantly elevate the protein levels of
345 CHOP or cleaved caspase-3, indicating that it's effects were mostly on β-cell function rather than
346 fate (7). In our current study we measured human islet cell survival over a longer period by using
347 our high-content imaging protocol. We found that neither TAC nor VCS had significant deleterious
348 effect on islet cell survival or insulin content over 48 hours, under the current testing conditions.
349 Similarly, our post RNA-seq pathway analysis did not identify significant changes in genes
350 involved in β-cell apoptosis and/or survival. While an increase in β-cell ER-stress and apoptosis
351 is a plausible pathophysiological mechanism for the diabetic effects of immunosuppressants in
352 the post-transplant clinical setting, it is unlikely that this is the mechanism explaining why the risk
353 of NODAT is higher in patients taking TAC when compared with VCS.

354 Limitations of our study include the following. Although we had the benefit of directly examining
355 human islets, our *in vitro* experiments may be impacted by islet isolation and culture.
356 Nevertheless, the fundamental *in vivo* mechanisms appear to be well conserved in high quality
357 isolated islets such as those used in our study (11). Mechanistically, our analysis of insulin
358 secretion in response to high glucose and high KCl, together with the transcriptomic profiling,
359 points to direct effects on the distal processes of insulin exocytosis. Future studies could directly
360 measure exocytosis with electrophysiological capacitance measurements. Our data point to
361 differences in NFAT activation between VCS and TAC than can explain the distinct impacts on
362 insulin secretion, but our work employed non-β-cells for the dose-finding experiment and ideally

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363 future studies will be repeated in human β-cells or human β-cell lines. We chose the
364 concentrations for our *in vitro* studies based on clinical efficacy. The observation that lower
365 concentrations of TAC were more toxic than VCS favours this next generation
366 immunosuppressant. Given the clinical importance of calcineurin inhibitor mediated β-cell
367 dysfunction, we hope that our work stimulates more experimentation in human islets and other
368 key tissue governing glucose homeostasis.

369 In summary, we have demonstrated that TAC, but not VCS, significantly inhibits the total
370 response of glucose-stimulated insulin secretion from human islets. This decrease in secretion is
371 not mediated by effects on islet cell health or insulin content and is most likely a direct
372 consequence of decreased insulin granule exocytosis. VCS, a next generation calcineurin
373 inhibitor, does not cause the same degree of inhibition of insulin secretion. Thus, this is a plausible
374 physiological mechanism explaining the lower incidence of NODAT in patients taking VCS.

375

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384 **Author Contributions:**

385 JK designed, conducted and analyzed experiments, and co-wrote the manuscript.

386 LB conducted and analyzed experiments and edited the manuscript.

387 PO designed, conducted and analyzed experiments, and edited the manuscript.

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388 HHC designed and analyzed experiments and edited the manuscript.

389 EP designed, conducted and analyzed experiments, and edited the manuscript.

390 DRU designed, conducted and analyzed experiments, and edited the manuscript.

391 JLC designed experiments and edited the manuscript.

392 RBH designed experiments and edited the manuscript.

393 JDJ designed and analyzed experiments and co-wrote the manuscript.

394

395 **Data Availability:**

396 All data generated or analyzed during this study are included in this published article or in the

397 data repositories listed in References.

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639 **Figure Legends**

640

641 **Figure 1| Experimental design, and human islet donor and isolation characteristics (A)**
642 Experimental design for study of VCS and TAC effects on high quality primary human islets
643 isolated from cadaveric donors. Details of each isolation are available from IsletCore.ca (the
644 example image shown from the R297 isolation). **(B)** High-quality, research-specific human islets
645 from consenting donors were obtained from IsletCore Laboratory in Edmonton, Alberta. Islet
646 donors and isolation characteristics are listed here. Stimulation index is the average of 3 technical
647 replicates of the 16.7 mM to 2.8 mM glucose response as provided by the IsletCore Laboratory
648 quality control database.

649

650 **Figure 2| Differences between TAC, VCS and CsA on calcineurin activity, lymphocyte**
651 **proliferation assay, and NFAT accumulation. (A-D)** Sister aliquots of PBMC were treated in
652 the presence of drug or vehicle control for analysis of lymphocyte cell division (Div), calcineurin
653 activity (CN), and cytosolic NFAT. **(A)** Lymphocyte division was determined by CFSE analysis
654 following 3-day stimulation with CD3 antibody. Calcineurin activity was determined by radioactive
655 32P-RII peptide assay following 30-min stimulation with PMA+A23187. **(B)** Cytosolic NFAT
656 relative to CypA (loading control protein) was determined by Western blotting and densitometric
657 quantitation following 30-min stimulation with PMA+A23187. **(C)** K562 cells stably transfected with
658 an NFAT-luciferase reporter were stimulated for 22 hr with PMA + A23187 in the presence of drug
659 or vehicle control. Cells were separated into separate aliquots for measurement of calcineurin
660 activity and luciferase activity for NFAT promoter activation. **(D)** Percent inhibition was determined
661 by normalizing values to vehicle control (100% activity) and to 2 μg/ml VCS (100% inhibition).
662 These observations were replicated 5 times. **(E)** Calculated IC50 values in PBMC and K562-
663 NFAT-Luc. n = 8-12 experiments for PBMC and 4-7 experiments for K562-NFAT-Luc.

664

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665 **Figure 3| Basal, glucose-stimulated and KCl-stimulated insulin secretion from human**
666 **islets treated with peak and trough concentrations of VCS and TAC. (A)** Averaged traces of
667 dynamic insulin secretion measurements in the context of 3 mM glucose, 15 mM glucose, or 30
668 mM KCl (as indicated). **(B)** Total area under the curve (AUC) of the 15 mM glucose response. (**C,**
669 **D, E)** AUCs of 1st phase and 2nd phase 15 mM glucose responses, as well as the KCl response.
670 **(F)** Baseline insulin secretion. **(G, H)** Insulin secretion normalized to baseline, including AUC.
671 n=17
672
673 **Figure 4| Insulin content from isolated human islets treated with VCS and TAC.** Insulin
674 protein measured by radioimmunoassay from 20 islets treated for 48 hours with VCS or TAC
675 (concentrations as indicate) after acid-ethanol extraction of insulin.
676
677 **Figure 5| Islet cell survival from dispersed human islets treated with VCS and TAC. (A,B)**
678 Cell death was assessed every 30 minutes over 48 hours using Propidium Iodide (PI) and Hoechst
679 staining, with a significant increase seen in the presence of a β-cell toxic cytokine cocktail. **(C)**
680 Shown is the maximum cell death normalized measurement (see methods) for each condition
681 TAC and VCS dose, normalized to the DMSO control cultures. Results shown are quantified from
682 5 cultures per drug condition, over two separate runs.
683
684 **Figure 6| RNA sequencing analysis and protein-protein interaction networks of human**
685 **islets treated with TAC and VCS at peak doses. (A)** mRNAs that were nominally differentially
686 expressed (adjusted $p < 0.05$) are shown **(B)** Protein-protein interaction networks highlight
687 connections between calcineurin/NFAT pathway and deferentially expression genes in **(A)** VCS
688 or **(B)** TAC treatment. Up or down regulated genes are shown in red or green, respectively.
689

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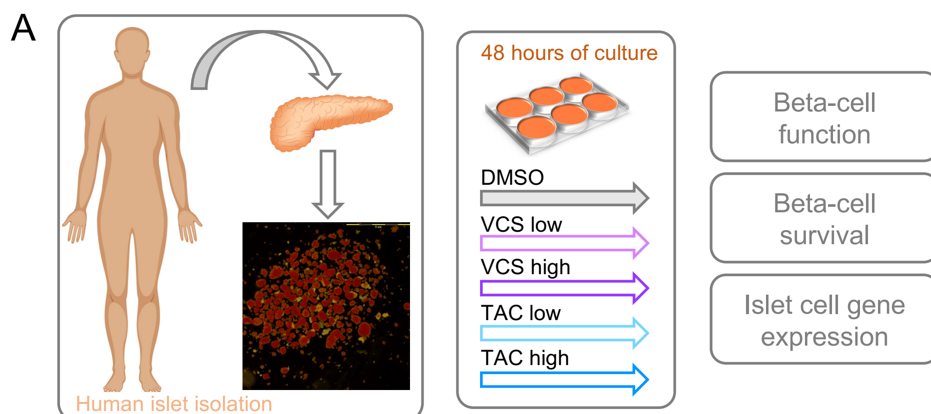
690 **Figure 7| Working model of differential regulation of insulin exocytosis by VCS and TAC**.
691 In our model, TAC has more potent inhibitory effects on NFAT, and therefore results in the
692 stronger suppression of key genes. TAC-sensitive gene products with known functions related to
693 insulin secretion are shown in orange.

Figure 1

Overall study design and human islet donor characteristics

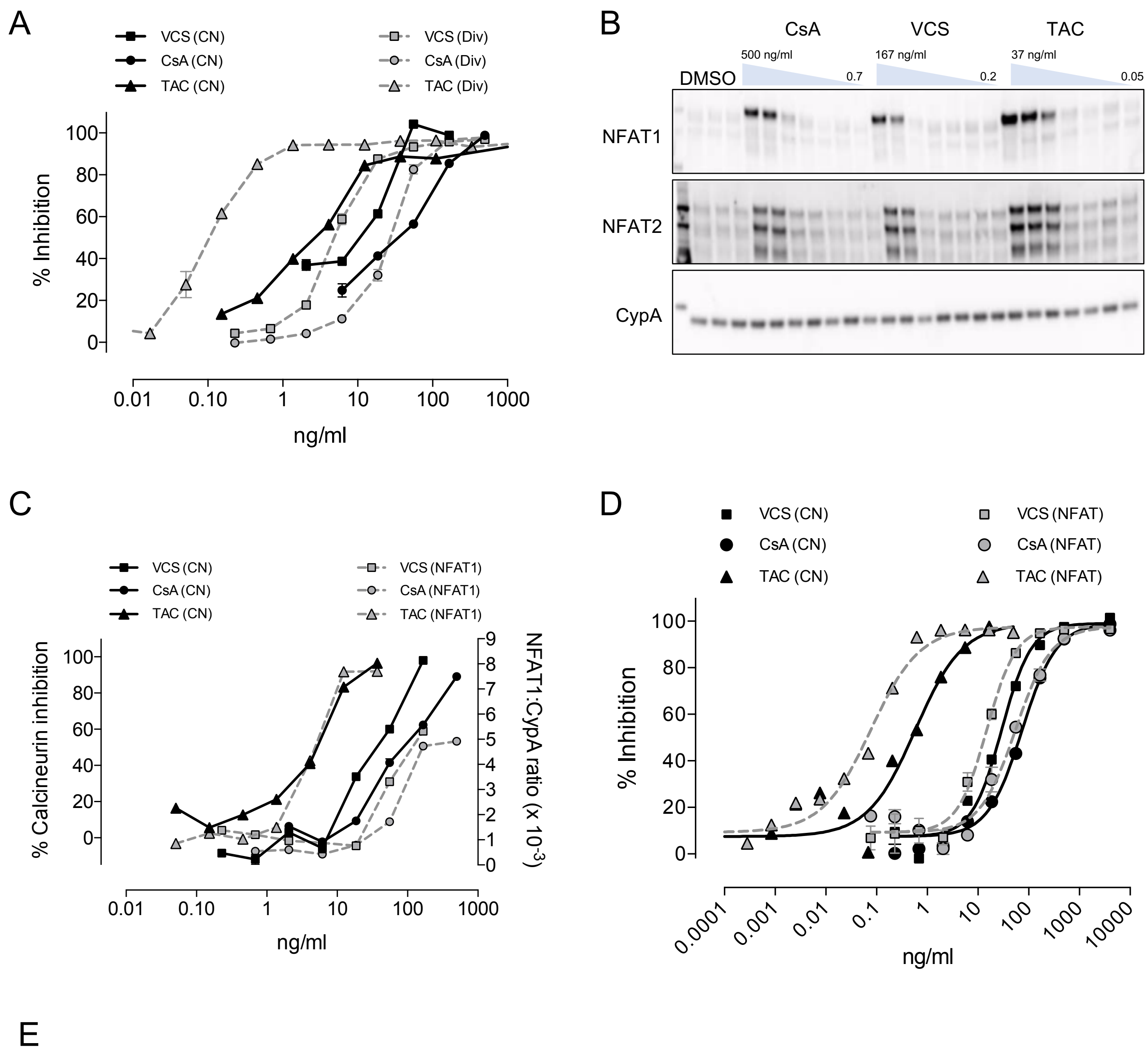
A



B

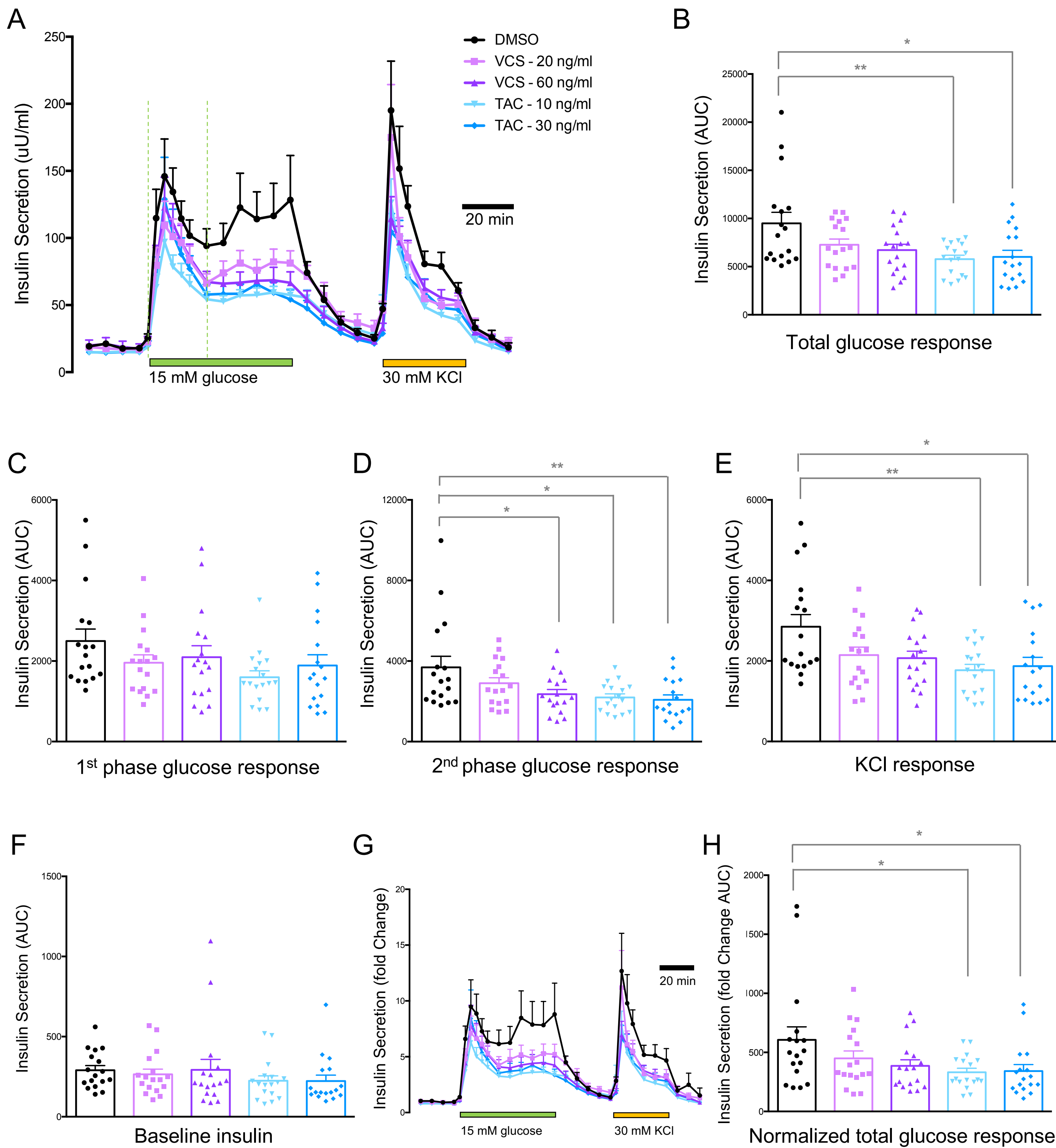
Donor ID	Age (years)	Sex	HbA1c (%)	BMI	Islet Purity	Stimulation Index	Experiments
R297	69	Male	No data	27.2	90%	5.05	Perifusion
R300	30	Female	No data	25.3	75%	2.05	Perifusion
R301	18	Male	5.0	19.0	75%	9.21	Perifusion
R302	62	Female	6.2	32.3	40%	3.57	RNAseq
R303	56	Female	No data	24.1	85%	2.64	Perifusion
R305	60	Male	5.6	21.0	80%	1.29	Perifusion, RNAseq
R306	22	Female	5.3	21.1	80%	19.13	Perifusion
R308	20	Male	5.5	19.8	80%	5.90	Perifusion
R309	47	Female	5.5	27.4	80%	9.08	Perifusion, RNAseq
R310	25	Male	5.4	26.4	90%	3.81	Perifusion, RNAseq
R314	31	Female	5.0	30.3	80%	6.53	Perifusion, RNAseq
R316	52	Male	5.7	26.0	50%	6.93	Perifusion, RNAseq
R317	54	Male	5.1	26.4	90%	10.78	RNAseq
R318	54	Male	5.0	20.5	90%	2.76	Perifusion
R319	68	Male	5.0	27.8	90%	5.04	Perifusion
R322	44	Female	4.9	23.2	90%	3.09	Perifusion
R325	50	Male	No data	30.3	75%	2.86	Perifusion
R326	26	Male	5.5	27.0	90%	5.34	Perifusion
R327	57	Male	5.8	33.9	95%	3.59	Perifusion
R335	25	Male	4.9	24.6	80%	11.92	Cell death
R338	30	Male	5.3	25.5	90%	6.73	Cell death

Figure 2



	PBMC Calcineurin Activity		PBMC Cell Division		K562-NFAT-Luc Calcineurin Activity		K562-NFAT-Luc NFAT Activity	
	IC50 (±SE) ng/ml	Relative Potency	IC50 (±SE) ng/ml	Relative Potency	IC50 (±SE) ng/ml	Relative Potency	IC50 (±SE) ng/ml	Relative Potency
CsA	83.7 (±18.7)	1	50.5 (±7.8)	1	201.6 (±52.7)	1	53.4 (±10.2)	1
VCS	26.3 (±4.1)	3.2	13.8 (±2.1)	3.7	65.2 (±15.9)	3.1	11.0 (±2.4)	4.9
TAC	2.6 (±0.7)	32	0.3 (±0.2)	168	1.1 (±0.3)	183	0.054 (±0.02)	989

Figure 3



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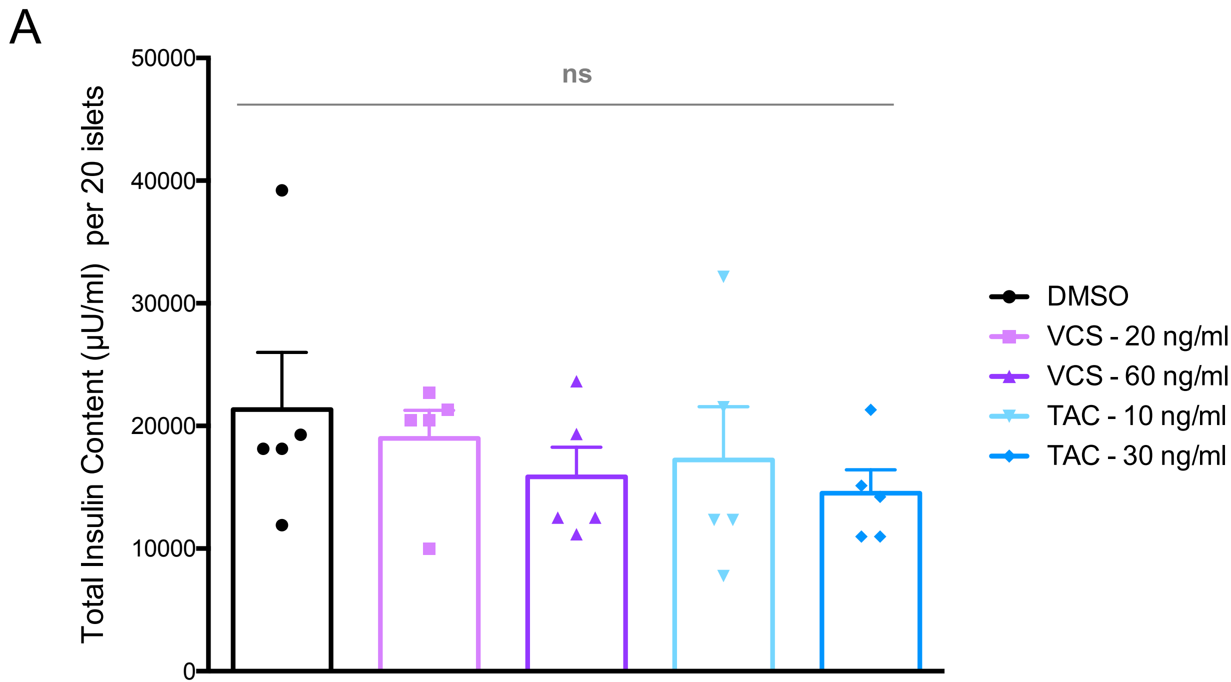

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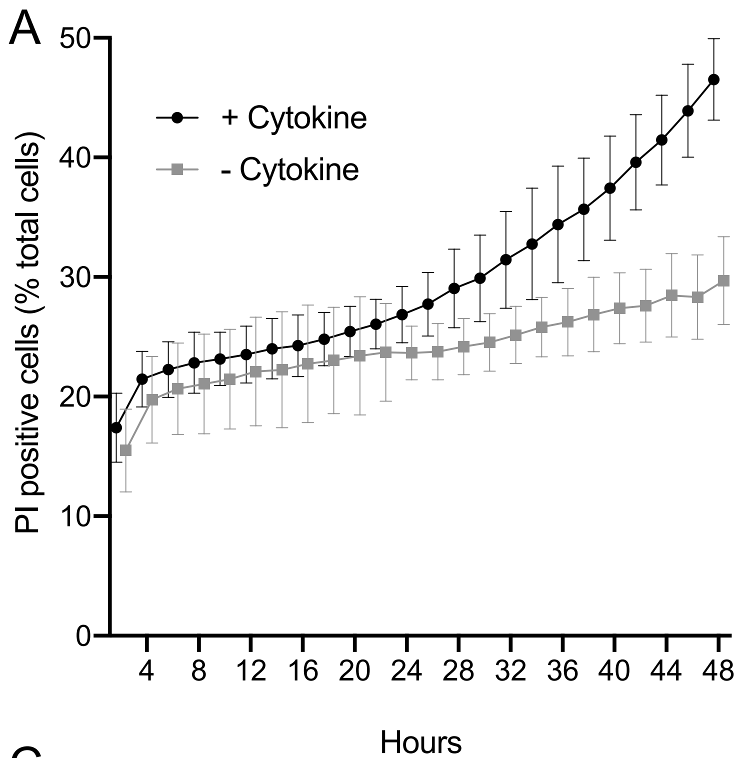

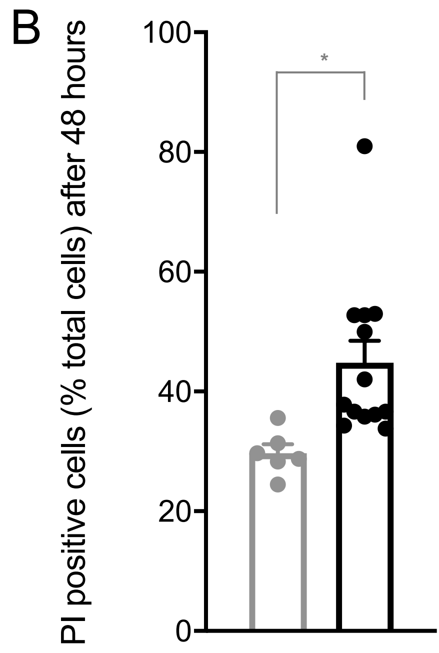

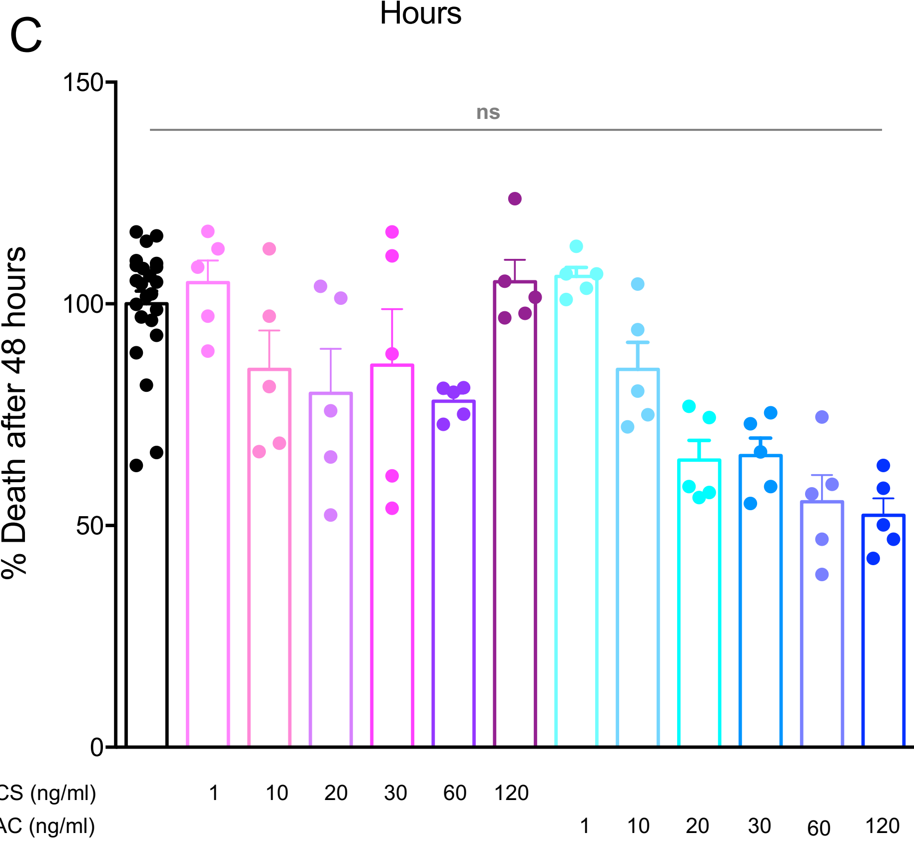

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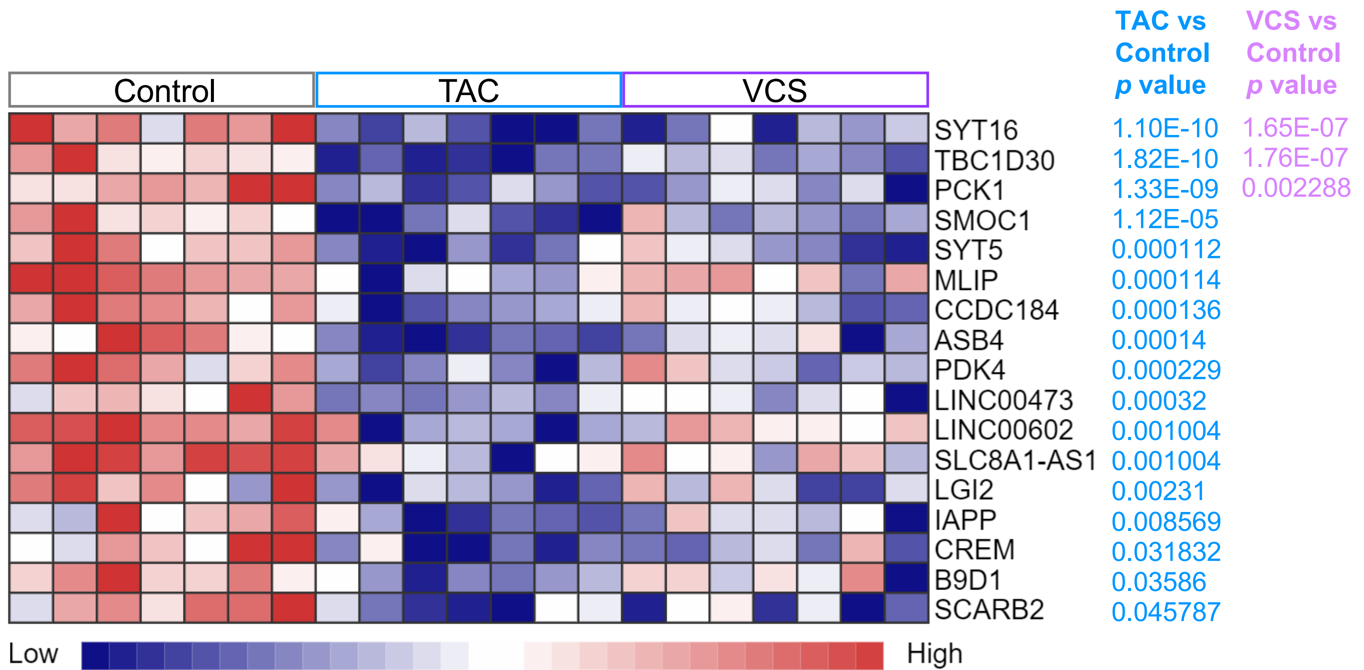

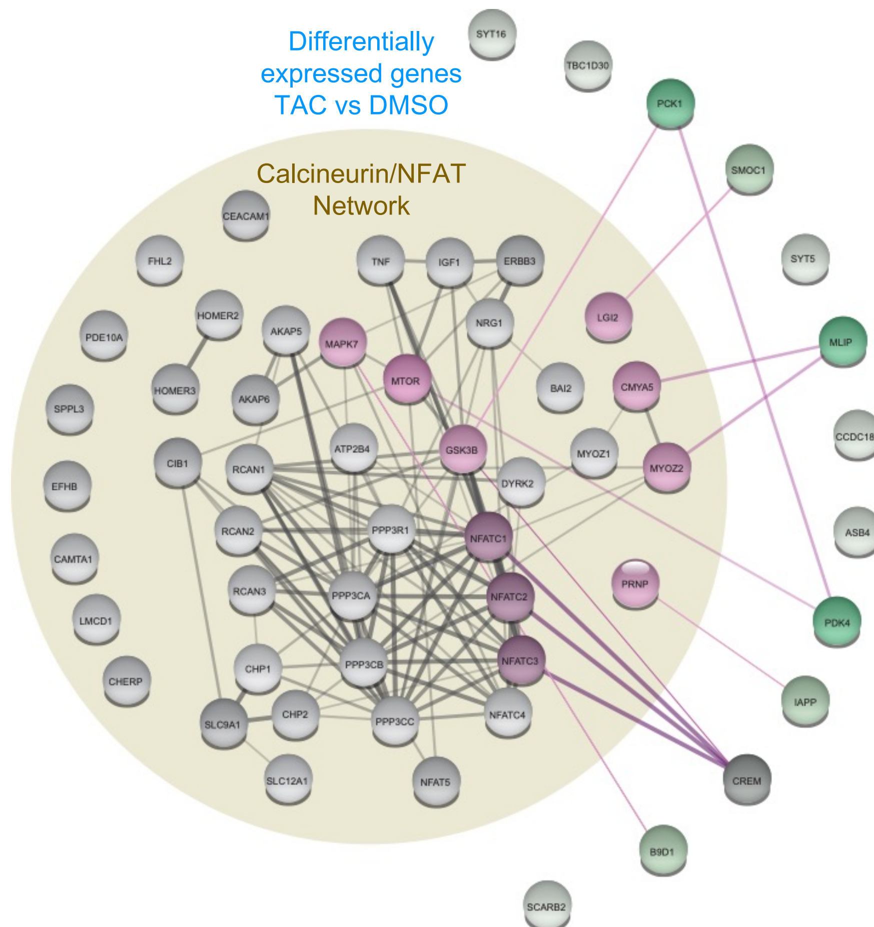

Figure 7