Similarities Between Bacterial GAD and Human GAD65: Implications in Gut Mediated Autoimmune Type 1 Diabetes

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Abstract

A variety of islet autoantibodies (AAbs) can predict and possibly dictate eventual type 1 diabetes (T1D) diagnosis. Upwards of 75% of those with T1D are positive for AAbs against glutamic acid decarboxylase (GAD65), a producer of gamma-aminobutyric acid (GABA) in human pancreatic beta cells. Interestingly, bacterial populations within the human gut also express GAD65 and produce GABA. Evidence suggests that dysbiosis of the microbiome may correlate with T1D pathogenesis and physiology. Therefore, autoimmune linkages between the gut microbiome and islets susceptible to autoimmune attack need to be further elucidated. Utilizing *silico* analyses, we show here that 25 GAD sequences from different human gut bacterial sources show sequence and motif similarities to human beta cell GAD65. Our motif analyses determined that a majority of gut GAD sequences contain the pyroxical dependent decarboxylase domain of human GAD65 which is important for its enzymatic activity. Additionally, we showed overlap with known human GAD65 T-cell receptor epitopes which may implicate the immune destruction of beta cells. Thus, we propose a physiological hypothesis in which changes in the gut microbiome in those with T1D result in a release of bacterial GAD, thus causing miseducation of the host immune system. Due to the notable similarities, we found between humans and bacterial GAD, these deputized immune cells may then go on to target human beta cells leading to the development of T1D.

38 **Introduction**

39 Glutamic acid decarboxylase (GAD65) is a prominent autoantibody in type 1 diabetes (T1D). For
40 those diagnosed as a teen, in addition to having an HLA DR3/DR2 signature, it is likely to be the
41 first autoantibody clinically detected in the blood. GAD65 antibodies are prevalent in the majority
42 of T1D cases, occurring in over 70% of patients [1]. Autoantibodies directed against GAD65
43 recognize linear and conformational epitopes throughout the protein. The functional region of
44 GAD65 contains a catalytic group involving several residues that are distantly located in the
45 primary amino acid sequence of the protein. The enzymatic function of GAD65 and subsequent
46 regulation of GABA production is dependent on a key residue within the catalytic loop, Lys396
47 [2]. This residue covalently binds pyridoxal 5'-phosphate (PLP), a co-factor that is essential for the
48 production of GABA by GAD65. Thus, T1D-related immune recognition of GAD65 is highly
49 dependent on the exposure of these regions to autoreactive antigen-presenting cells (APCs). These
50 activated APCs can then go on to attack GAD65-expressing pancreatic beta cells leading to their
51 destruction and eventual T1D diagnosis.

52 In addition to beta cells, a multitude of bacteria within the gut express GAD (bacterial
53 nomenclature for human GAD65). While there is great diversity within the gut microbiota, more
54 than 95% of gut bacterial species fall into four major microbial phyla: Firmicutes, Bacteroidetes,
55 Actinobacteria, and Protecteobacteria [3]. Bifidobacterium adolescentis, in particular, is an
56 important gut bacterial GABA producer and has the highest prevalence of gad genes in their
57 genomes [4]. These species provide a homeostatic environment that fosters and facilitates gut
58 epithelial integrity and immunity, along with providing energy. Varied literature supports both a
59 decrease in microbial diversity and an increase in a “leaky gut” phenotype throughout T1D [5].

60 GABA producing Bifidobacterium bacterial counts drop as the Firmicutes/Bacteroides (F/B) ratios
61 decrease in T1D [6]. There is also a reduction in the abundance of Clostridium clusters IV and
62 XIVa and mucin-degrading bacteria such as Prevotella and Akkermansia. An important metabolite
63 produced by these symbiotic bacterial populations, butyrate, supports gut integrity and has beta
64 cell-specific anti-inflammatory effects [7, 8, 9, 10, 11]. The reduction of these microbes and their
65 byproducts may be a contributing factor to why children at risk for T1D have higher intestinal
66 permeability and T1D-associated gut dysbiosis.
67
68 One hypothesis for the interrelationship between dysbiosis and T1D is that as these bacterial
69 populations die, they release GAD65 mimetics that trigger the immune system. Presumably, as
70 GABA-producing bacteria like B. adolescentis die, either from antibiotic misuse or other
71 pathology, immune cells located in Peyer's patches and deep in the enterocyte border detect the
72 inappropriate presence of GAD. Thus, APCs can act to further inform CD8+ T cells in nearby
73 lymph nodes. The lymphatic pathways between the intestinal submucosa and pancreatic lymph
74 nodes may provide a route for these “miseducated” CD8+ T cells to gain access to GAD65+ beta
75 cells. GAD65 may also originate from beta cells leading to an increase in epitope spreading and a
76 misguided immune attack on these insulin-producing cells.
77
78 To initiate the exploration of this hypothesis, we utilized in silico methodologies to compare gut
79 microbial species that decline at the onset of T1D and express GAD, with GAD65 sequences in
80 pancreatic beta cells. We show that there is notable sequence similarity, including relevant motif
81 and T-cell epitope overlaps between several bacterial species and beta cells particularly in the
82 functionally important pyroxical dependent decarboxylase domain of GAD65. This in silico

83 exercise suggests that further investigation is warranted of the relationship between microbial
84 disappearance and appearance of GAD65 autoantibodies during the prodrome and presentation of
85 T1D.
86
87 **Materials and Methods**
88
89 **GAD Sequence and T cell receptor epitope curation**
90 Twenty-five bacterial GAD proteins from species of interest, alongside those of mouse,
91 chimpanzee, and human GAD65 sequences were curated from the NCBI genes database (Table
92 1). The bacterial species were chosen by considering their association with the gut microbiome,
93 their significance in the gut microbiome alterations seen in T1D patients, human-related pathogen
94 status, and non-human-related environmental species. GAD65 T cell receptor epitopes were
95 curated from a literature review.
96
97 All GAD sequences in bacteria and the three mammalian species under consideration were
98 obtained by performing a BLASTP search, with human GAD65 as the query sequence.
99 Furthermore, only those sequences with significant e and p values were well annotated on the
100 NCBI database and/or RefSeq and were identified as GAD proteins were considered. All other
101 hits, including unnamed protein products, were ignored.
102
103 **Multiple Sequence Alignment and Motif Identification**
104 Multiple sequence alignment was performed using ClustalW (Ver. 1.2.2) with a gap opening
105 penalty of 10 and a gap extension penalty of 0.2 [12]. The resulting alignment is visualized and

106 annotated with CLC Sequence Viewer (Ver. 8.0) to highlight conservation and amino acid
107 properties. Identification of ungapped motifs was done using the MEME tool (Ver. 5.4.1) under
108 the MEME suite tools for motif discovery and enrichment [13]. The output consisted of 10 motifs,
109 shared by various (not necessarily all) organisms into consideration. Subsequently, each motif was
110 run against protein families, using a Pfam (Ver 33.1) search. The Motifs identified by MEME were
111 mapped against the query sequences using MAST(Ver. 5.4.1), to obtain the coordinates of motifs,
112 in each gad sequence [13].
113
114 **Logo plot & T Cell Receptor Epitopes**
115 The multiple sequence alignment obtained was converted into a logo plot, using Weblogo (Ver
116 2.8.2) [14]. Here, each logo consists of a stack of symbols, and each symbol's height is indicative
117 of the amino acid conservation, at a particular location. All motifs and T-cell receptor epitope
118 coordinates, obtained in the previous steps, were marked on the logo plot. The plot was then
119 trimmed, with a special emphasis on regions with high overlap between motifs and T-cell receptor
120 epitopes.
121
122 **Results**
123
124 **Multiple sequence alignment highlights several conserved residues between human and**
125 **bacterial GAD**
126 A total of 25 GAD protein sequences, including 3 mammalian GAD65 (human, chimpanzee, and
127 mouse) and 22 bacterial GAD, were aligned (Table 1 & Fig. 1). The 3 mammalian GAD65
128 exhibited high conservation with each other, while 20 out of the 22 bacteria GAD exhibited high

129 similarity to one another. Two bacteria, *Bdellobibrio sp. Qaytius* and *Sediminibacterium spp.* did
130 not exhibit any protein conservation. This may be due to a misannotation within the RefSeq
131 genome. We also found interesting similarities and differences between the mammalian and
132 bacterial GAD sequences. There were two regions of interest: AA389-397 and AA418-425.
133 AA389-397 contained several charged residues interspaced with hydrophobic residues, potentially
134 a conserved alpha-helix structure. AA418-AA425 contains conserved HK residues. These two
135 residues represent a known pyridoxal phosphate-binding site in *E.coli* GAD (H275 and K276),
136 that is likely conserved in mammalian GAD65. The length of mammalian GAD65 was ~120 amino
137 acids longer than bacterial GAD. Based on our alignment, this difference is mostly centered around
138 the N-terminus, which suggests adaptations and inclusions of signal peptides required for complex
139 cellular trafficking in mammalian cells. Also, we identified a few small gaps within the core
140 regions of GAD between species, these gaps are unlikely of any biological significance.

141 **Table 1. GABA producing bacterial species and their respective GAD gene**

Category	Species	Gene ID
Human	Human	2572
Primate	Chimpanzee	466026
Mouse	Mouse	14417
T1D	Streptococcus pneumoniae	Z49109.1
T1D	Bifidobacterium moukalabense	61040350
T1D	Bifidobacterium adolescentis	56675515
T1D	Bacteroides fragilis	61595025
T1D	Ruminococcus	Genome not found
T1D	Veillonella	Genome not found
T1D	Blautia	Genome not found
T1D	Lachnospira	Genome not found
T1D	Alistipes obesi	Genome not found
T1D	Clostridium spp.	29570578
T1D	Prevotella	Genome not found
T1D	Parabacteroides distasonis	CP040468
Gut	Escherichia coli	946058
Gut	Listeria monocytogenes	985123
Gut	Salmonella enterica	13913147
Gut	Streptomyces sp. NRRL B-24085	6211654
Gut	Carnobacterium maltaromaticum	56848084
Gut	Nocardia brasiliensis	13733698
Gut	Lactobacillaceae	56991876
Pathogen	Gammaproteobacteria	QNEL01000191.1
Pathogen	Klebsiella pneumoniae IS22	12545741
Environment	Planctomycetes bacterium	KAA3606119.1
Environment	Sediminibacterium sp.	JADMJG010000006.1
Environment	Moorea producens	CP017599.1
Environment	Magnetococcales bacterium	JADGBX010000054.1
Environment	Scytonema hofmannii	ANNX02000047.1
Environment	Candidatus Aminicenantes bacterium	WVZL01000014.1
Environment	Bdellovibrio sp. qaytius	CP025734.1
Environment	Verrucomicrobia	NZEQ01000004.1

142 **Motif analysis uncovers regional similarities of human and bacterial GAD enzymatic regions**

143 Our motif analysis found 10 ungapped motifs with each motif present in at least 10 of the
144 sequences under consideration (Fig. 2). Even though multiple common motifs between human
145 GAD65 and bacterial GAD can be observed, it is noteworthy that two motif blocks are conserved
146 all across the organisms considered, which is indicative of the sequence similarity observed in the
147 multiple sequence alignment. In addition, except for *Streptococcus pneumoniae,* one motif block
148 was consistently present in all sequences. Furthermore, these motifs were queried on the Pfam and
149 the CDD databases, and all 10 motifs were identified as matches to the pyridoxal-dependent
150 decarboxylase (PDP) conserved domain, which is instrumental in the biosynthesis of GABA, from
151 glutamate. This indicates that human GAD65 and bacterial GAD share sequence similarities in a
152 functional region of GAD.

153

154 **Overlap exists between T cell receptor epitopes of GAD65 and bacterial GAD**

155 To compare the overlap between T cell receptor epitopes, human GAD65, and bacterial GAD, a
156 logo plot of the multiple sequence alignment was made using three representative bacterial GAD
157 and three animal GAD65 proteins. Motif positions for each sequence were manually annotated
158 using the motif coordinates obtained in motif discovery and enrichment analysis. Thereafter, the
159 logo plot was annotated with T cell receptor epitopes and the overlapping regions were observed.
160 The fine lines indicate the motif coordinates, while the red blocks indicate the CD8+ epitopes and
161 blue blocks indicate the CD4+ epitopes. Three regions overlapped with a motif, which as stated in
162 the motif analysis result description, hit the PDP domain (Fig. 3). This was also confirmed by a
163 CDD search. Of the amino acids in which both CD4 and CD8 receptor epitopes overlap, there is
164 at least one amino acid residue that is conserved across bacterial GAD and GAD65 (Fig. 4). Such

165 overlaps emphasize a possible explanation for how T cells could recognize both bacterial and
166 human GAD65.
167
168 **Discussion**
169 In this study, we explored the sequence similarities between GAD in twenty GABA-producing
170 bacteria and GAD65 in humans. We found that human and bacterial GAD have similar motifs and
171 conserved residues. We identified notable conservation centered around the pyridoxal dependent
172 decarboxylase domain of GAD65 found in pancreatic beta cells, particularly around two key
173 substrate-binding residues. Moreover, some conserved sequences in bacterial GAD overlap with
174 known human GAD65 T cell receptor epitopes. As one example, GAD65-specific HLA-DR4
175 (DRB1*0401)–restricted murine T-cell hybridoma line T33.1 recognizes the GAD65 274-286
176 epitope, and this epitope was conserved across bacteria and human samples examined here (Figure
177 1). This *in silico* proof-of-concept suggests a possible relationship between disappearance of GAD
178 containing GABA-producing bacteria in the human gut microbiome and the appearance of GAD65
179 autoantibodies during the onset of T1D that needs to be further elucidated.
180
181 It is important to note a few limitations within our analyses. Many gut microbial genomes and
182 genome annotations were incomplete, particularly for some GABA-producing bacterial species of
183 importance that are significantly changed in T1D. We did not include several species due to
184 missing GAD genome annotations. Together, only 20 sequences were analyzed for their
185 homology, with many belonging to environmental organisms. A larger cohort would further
186 strengthen our sequence analyses and hypotheses. Furthermore, we hypothesized that the change
187 in anti-GAD65 is partly due to the changes in the gut microbiome. Additional studies are needed

188 to determine what insult(s) would cause such a disturbance and what cellular mechanisms are
189 involved in the miseducation of immune cells due to the release of GAD in the gut. The window
190 for the disappearance of the *gad* containing bacterial species should be identified as to whether it
191 precedes or follows the appearance of autoantibodies to GAD65 in humans. Lastly, the analysis
192 relied on known and curated T cell epitopes of GAD65 from the literature review, in which we
193 demonstrated similarity to bacterial GAD. Whether or not the bacterial peptides bind to human T
194 cell receptors needs to be further verified *in vitro.*
195
196 We postulate that bacterial GAD found in GABA producing bacteria may act as an antigen to
197 activate submucosal T cells as a result of gut microbiome bacterial destruction, either viral or
198 antibiotic induced. Further, we posit these primed bacterial GAD T cells bind GAD65 in the islet
199 environment due to the GAD and GAD65 epitope similarity. That leaves us to explore how
200 GAD65, an intracellular enzyme found in alpha and beta islet cells, might get recognized by
201 bacterial derived anti-GAD T cells.
202
203 In beta cells, GAD65 is intracellular and interacts with the cytosolic leaflet of Golgi membranes
204 and secretory vesicles due to its post-translational palmitoylation (lipidation) at cysteines 30 and
205 45 [15]. GAD65's shuttling to the Golgi for palmitoylation requires a signal located between
206 residues 1-27 of the protein [16, 17]. GAD65 may transiently interact with the cell's cytosolic
207 leaflet upon exocytosis of beta cell granules [18]. Pre-clinical studies demonstrated that GAD65
208 can associate with the plasma membrane and enter the extracellular space [19].
209

210 GAD65 is certainly present in alpha and beta cells and may interact with a GAD T cell from the
211 gut at the plasma membrane of both islet cell types, but there may be another route of contact. A
212 recent review highlights how beta cells can become "deputized" and act as antigen-presenting cells
213 (APCs) under certain conditions [20] . In this scenario, "deputized" beta cells that present GAD65
214 might have an opportunity to interact with a GAD sensitized T cell. Endothelial cells in the highly
215 vascularized islet environment have been named as additional "deputized" APCs. These
216 endothelial cells may be exposed to transient plasma membrane resident GAD65, or GAD65
217 containing EVs from stressed pancreatic beta cells [21, 22] and act to present them to anti-GAD
218 gut derived T cells.
219
220 A "two-hit" scenario is one where bacterial GAD primed T cells recognize alpha and beta cell
221 derived GAD65 to impact both intra islet cell interaction as well as simultaneously disrupting local
222 immune response. In islet α-cells, GABA induces membrane hyperpolarization and suppresses
223 glucagon secretion, while in islet β-cells GABA induces membrane depolarization and increases
224 insulin secretion [23]. One consequence of a decrease of GAD65 in the islet environment is the
225 resultant decrease in GABA production resulting in hyperglycemia which is toxic to beta cells.
226 GABA is also an immune cell modulator, shown to increase Tregs, decrease Th1 and CTLs
227 fostering a tolerant immune environment [23]. A GAD65 depletion leading to a GABA decrease
228 in the islet could pack a double punch of generating dysfunctional alpha and beta cells operating
229 in a more highly reactive immune micro-environment.
230
231 One can imagine a scenario where GAD primed T cells from the gut, become initially alerted to
232 the islet environment as GAD65 is presented via deputized alpha, beta, endothelial cells or some

233 combination of the three. Once alerted, GAD primed T cells destroy beta or alpha cells that display
234 GAD65 at the plasma membrane, reducing the overall GABA levels in the islets with resulting
235 consequences of hyperglycemia which destabilizes beta cells, in addition to altered immune
236 phenotype that favors attack of the islets.
237
238 Taken together, our *in silico* analyses of genome sequences and motifs from bacterial, human, and
239 T cell epitopes identified several homologies that may inform further experimentation and inquiry
240 into the relationship between T1D pathogenesis and microbiome dysbiosis.

241 **References**

242

243 [1] Petersen JS, Hejnaes KR, Moody A, et al. Detection of GAD65 antibodies in diabetes and other
244 autoimmune diseases using a simple radioligand assay. Diabetes. 1994;43(3):459-467.
245 doi:10.2337/diab.43.3.459

246 [2] Fenalti G, Law RH, Buckle AM, et al. GABA production by glutamic acid decarboxylase is
247 regulated by a dynamic catalytic loop. Nat Struct Mol Biol. 2007;14(4):280-286.
248 doi:10.1038/nsmb1228

249 [3] Sommer F, Bäckhed F. The gut microbiota--masters of host development and physiology. Nat
250 Rev Microbiol. 2013;11(4):227-238. doi:10.1038/nrmicro2974

251 [4] Duranti S, Ruiz L, Lugli GA, et al. Bifidobacterium adolescentis as a key member of the human
252 gut microbiota in the production of GABA. Sci Rep. 2020;10(1):14112. Published 2020 Aug 24.
253 doi:10.1038/s41598-020-70986-z

254 [5] Zhou H, Sun L, Zhang S, Zhao X, Gang X, Wang G. Evaluating the Causal Role of Gut
255 Microbiota in Type 1 Diabetes and Its Possible Pathogenic Mechanisms. Front Endocrinol
256 (Lausanne). 2020;11:125. Published 2020 Mar 24. doi:10.3389/fendo.2020.00125

257 [6] Murri M, Leiva I, Gomez-Zumaquero JM, et al. Gut microbiota in children with type 1 diabetes
258 differs from that in healthy children: a case-control study. BMC Med. 2013;11:46. Published 2013
259 Feb 21. doi:10.1186/1741-7015-11-46

260 [7] de Goffau MC, Fuentes S, van den Bogert B, et al. Aberrant gut microbiota composition at the
261 onset of type 1 diabetes in young children. Diabetologia. 2014;57(8):1569-1577.
262 doi:10.1007/s00125-014-3274-0

263 [8] Prause M, Pedersen SS, Tsonkova V, Qiao M, Billestrup N. Butyrate Protects Pancreatic Beta
264 Cells from Cytokine-Induced Dysfunction. Int J Mol Sci. 2021;22(19):10427. Published 2021 Sep
265 27. doi:10.3390/ijms221910427
266 [9] Leiva-Gea I, Sánchez-Alcoholado L, Martín-Tejedor B, et al. Gut Microbiota Differs in
267 Composition and Functionality Between Children With Type 1 Diabetes and MODY2 and Healthy
268 Control Subjects: A Case-Control Study. Diabetes Care. 2018;41(11):2385-2395.
269 doi:10.2337/dc18-0253
270 [10] Harbison JE, Roth-Schulze AJ, Giles LC, et al. Gut microbiome dysbiosis and increased
271 intestinal permeability in children with islet autoimmunity and type 1 diabetes: A prospective
272 cohort study. Pediatr Diabetes. 2019;20(5):574-583. doi:10.1111/pedi.12865
273 [11] Soyucen E, Gulcan A, Aktuglu-Zeybek AC, Onal H, Kiykim E, Aydin A. Differences in the
274 gut microbiota of healthy children and those with type 1 diabetes. Pediatr Int. 2014;56(3):336-343.
275 doi:10.1111/ped.12243
276 [12] Thompson, J D et al. “CLUSTAL W: improving the sensitivity of progressive multiple
277 sequence alignment through sequence weighting, position-specific gap penalties and weight
278 matrix choice.” Nucleic acids research vol. 22,22 (1994): 4673-80. doi:10.1093/nar/22.22.4673
279 [13] Timothy L. Bailey, James Johnson, Charles E. Grant, William S. Noble, "The MEME Suite",
280 Nucleic Acids Research, 43(W1):W39-W49, 2015.
281 [14] Crooks, Gavin E et al. “WebLogo: a sequence logo generator.” Genome research vol. 14,6
282 (2004): 1188-90. doi:10.1101/gr.849004
283 [15] Kanaani J, Diacovo MJ, El-Husseini Ael-D, Bredt DS, Baekkeskov S. Palmitoylation controls
284 trafficking of GAD65 from Golgi membranes to axon-specific endosomes and a Rab5a-dependent
285 pathway to presynaptic clusters. J Cell Sci. 2004;117(Pt 10):2001-2013. doi:10.1242/jcs.01030

286 [16] Solimena M, Dirkx R Jr, Radzynski M, Mundigl O, De Camilli P. A signal located within
287 amino acids 1-27 of GAD65 is required for its targeting to the Golgi complex region. J Cell Biol.
288 1994;126(2):331-341. doi:10.1083/jcb.126.2.331
289 [17] Christgau S, Aanstoot HJ, Schierbeck H, et al. Membrane anchoring of the autoantigen
290 GAD65 to microvesicles in pancreatic beta-cells by palmitoylation in the NH2-terminal domain.
291 J Cell Biol. 1992;118(2):309-320. doi:10.1083/jcb.118.2.309
292 [18] Kanaani J, el-Husseini Ael-D, Aguilera-Moreno A, Diacovo JM, Bredt DS, Baekkeskov S. A
293 combination of three distinct trafficking signals mediates axonal targeting and presynaptic
294 clustering of GAD65. J Cell Biol. 2002;158(7):1229-1238. doi:10.1083/jcb.200205053
295 [19] Jin H, Wu H, Osterhaus G, et al. Demonstration of functional coupling between gamma -
296 aminobutyric acid (GABA) synthesis and vesicular GABA transport into synaptic vesicles. Proc
297 Natl Acad Sci U S A. 2003;100(7):4293-4298. doi:10.1073/pnas.0730698100
298 [20] Li Y, Sun F, Yue TT, et al. Revisiting the Antigen-Presenting Function of β Cells in T1D
299 Pathogenesis. Front Immunol. 2021;12:690783. Published 2021 Jul 14.
300 doi:10.3389/fimmu.2021.690783
301 [21] Cianciaruso C, Phelps EA, Pasquier M, et al. Primary Human and Rat β-Cells Release the
302 Intracellular Autoantigens GAD65, IA-2, and Proinsulin in Exosomes Together With Cytokine-
303 Induced Enhancers of Immunity. Diabetes. 2017;66(2):460-473. doi:10.2337/db16-0671
304 [22] Greening JE, Tree TI, Kotowicz KT, et al. Processing and presentation of the islet autoantigen
305 GAD by vascular endothelial cells promotes transmigration of autoreactive T-cells. Diabetes.
306 2003;52(3):717-725. doi:10.2337/diabetes.52.3.717

[23] Wan Y, Wang Q, Prud'homme GJ. GABAergic system in the endocrine pancreas: a new target for diabetes treatment. Diabetes Metab Syndr Obes. 2015;8:79-87. Published 2015 Feb 3. doi:10.2147/DMSO.S50642

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Author Contributions

M.W. devised the original experimentation and acquired funding; M.W., S.B., and B.J. contributed to the conceptualization; S.B. and B.J. performed the investigation and formal analysis; M.W., F.S.L.B, S.B., B.J., and T.M.R. interpreted the results; M.W., T.M.R., B.J., S.B., and H.S. wrote the original draft; all authors reviewed and edited the manuscript.

328 **Figure 1. Multiple sequence alignment of twenty bacterial GAD protein and three animal**
329 **GAD65 protein highlights conserved residues.** Across a diverse number of bacterial species
330 and 3 animal species, 12 amino acids were found to be 100% conserved (shaded in blue). Amino
331 acids 1-110 are likely signal peptides required for eukaryotic protein translation and cellular
332 transport not needed in bacteria. H275 and K276 are key residues required for PDP binding and
333 are conserved in human GAD65 and bacterial GAD.
334

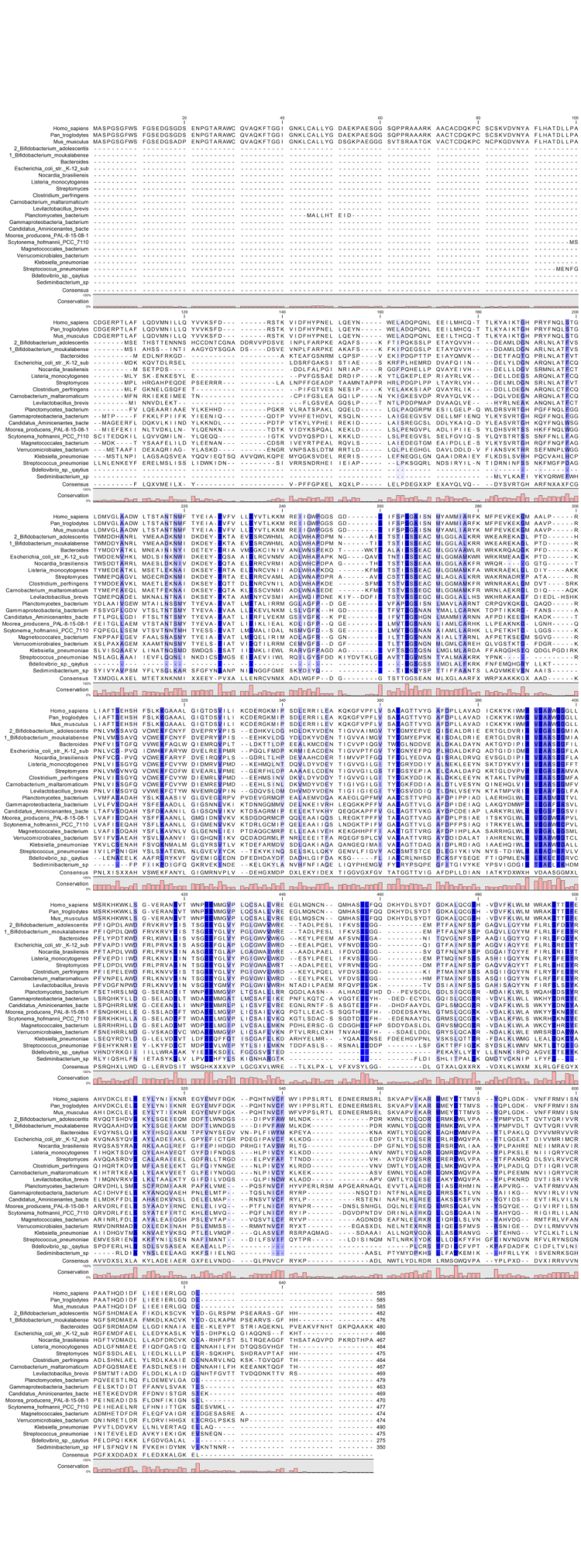

Homo_sapiens
Pan_troglodytes
Mus_musculus
2_Bifidobacterium_adolescentis
1_Bifidobacterium_moukalabense
Bacteroides
Escherichia_coli_str._K-12_sub
Nocardia_brasiliensis
Listeria_monocytogenes
Streptomyces
Clostridium_perfringens
Carnobacterium_maltaromaticum
Leviactobacillus_brevis
Planctomycetes_bacterium
Gammaproteobacteria_bacterium
Candidatus_Aminicenantes_bacte
Moorea_producens_PAL-8-15-08-1
Scytonema_hofmannii_PCC_7110
Magnetococcales_bacterium
Verrucomicrobiales_bacterium
Klebsiella_pneumoniae
Streptococcus_pneumoniae
Bdellovibrio_sp._qaytius
Sediminibacterium_sp
Consensus
Conservation

Figure 2. Motif Discovery Analysis of twenty bacterial GAD protein and three animal GAD65 proteins to identify motifs conserved across various species and their functional relevance with GAD protein. Across all 23 GAD sequences under consideration, 10 motifs were each found in at least 10 of the sequences used for motif discovery. Each motif appears in a separate color below with the individual sequences described as well. In addition, 2 of the 10 motifs were found in all of the sequences. Each motif was then queried for, using Pfam and CDD searches and it was found that all 10 of the motifs, hit the PDP domain in the GAD65 protein. All motifs match the Pyridoxal-dependent decarboxylase (PDP) conserved domain

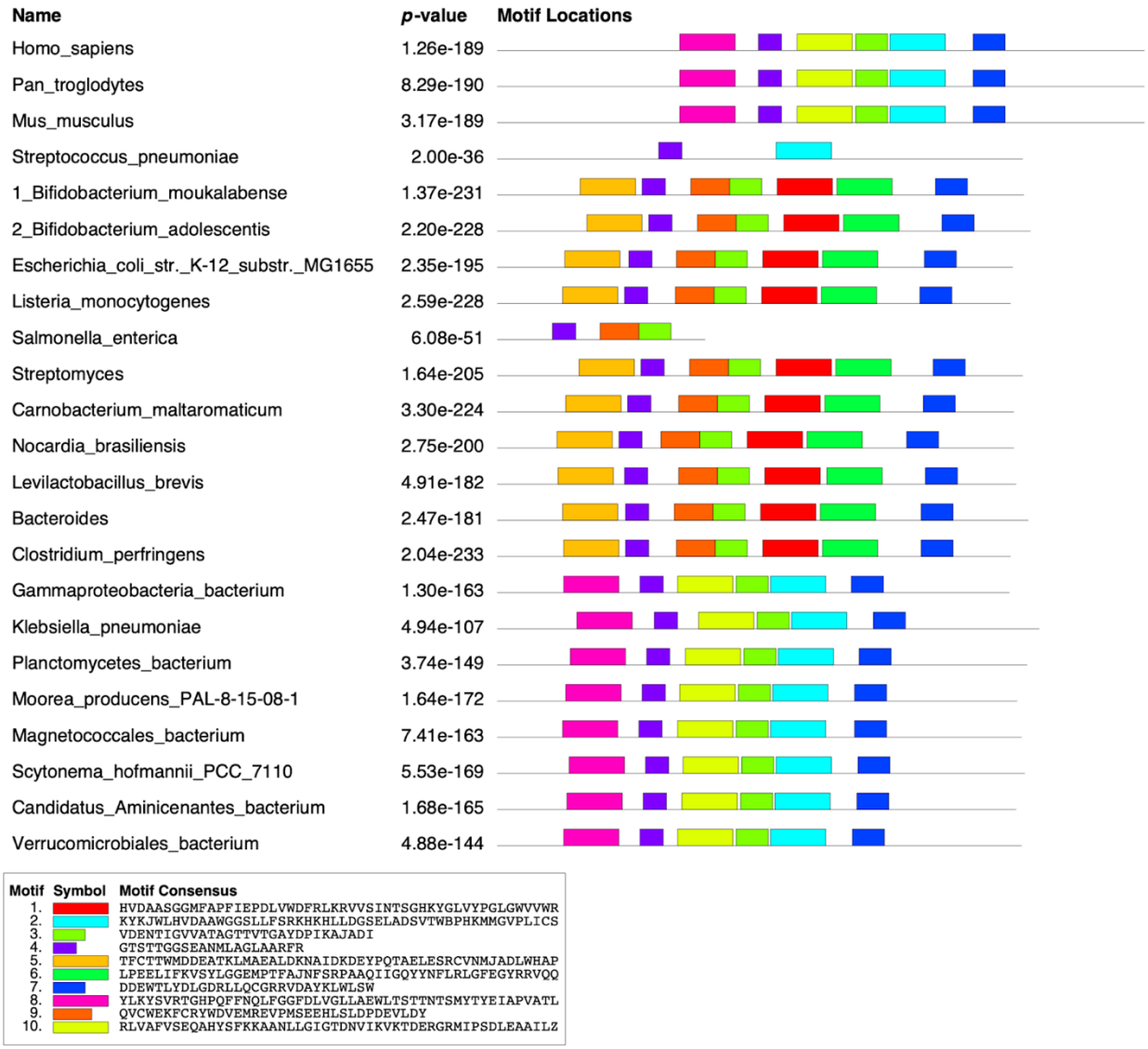
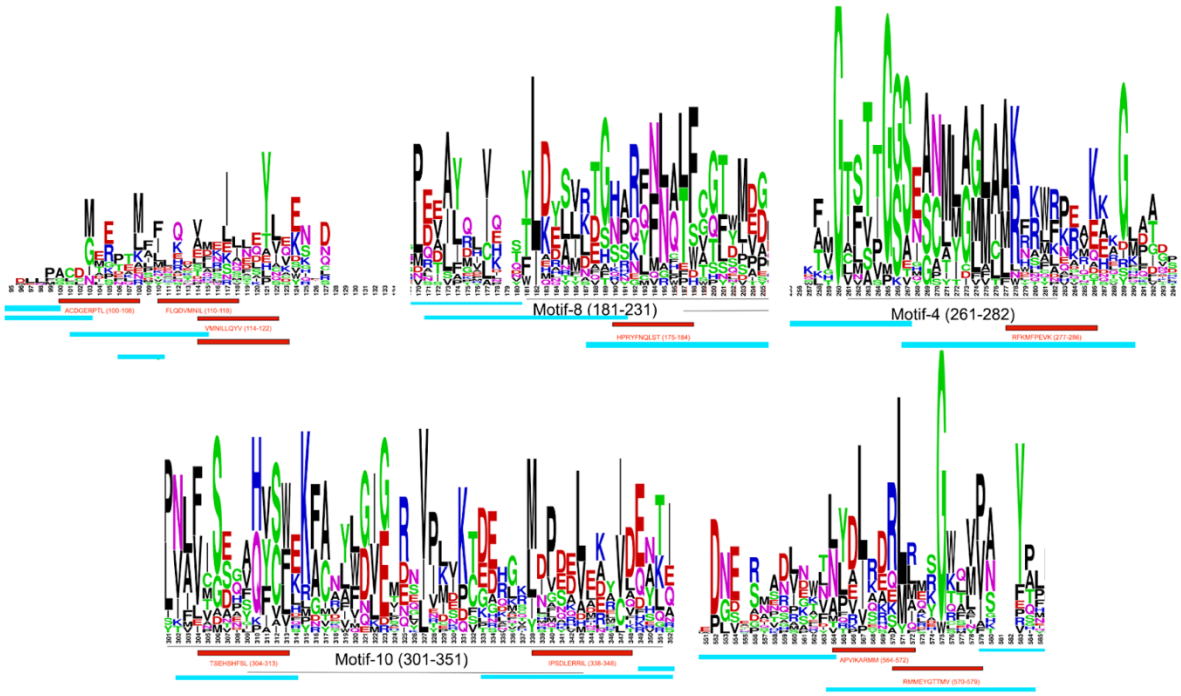
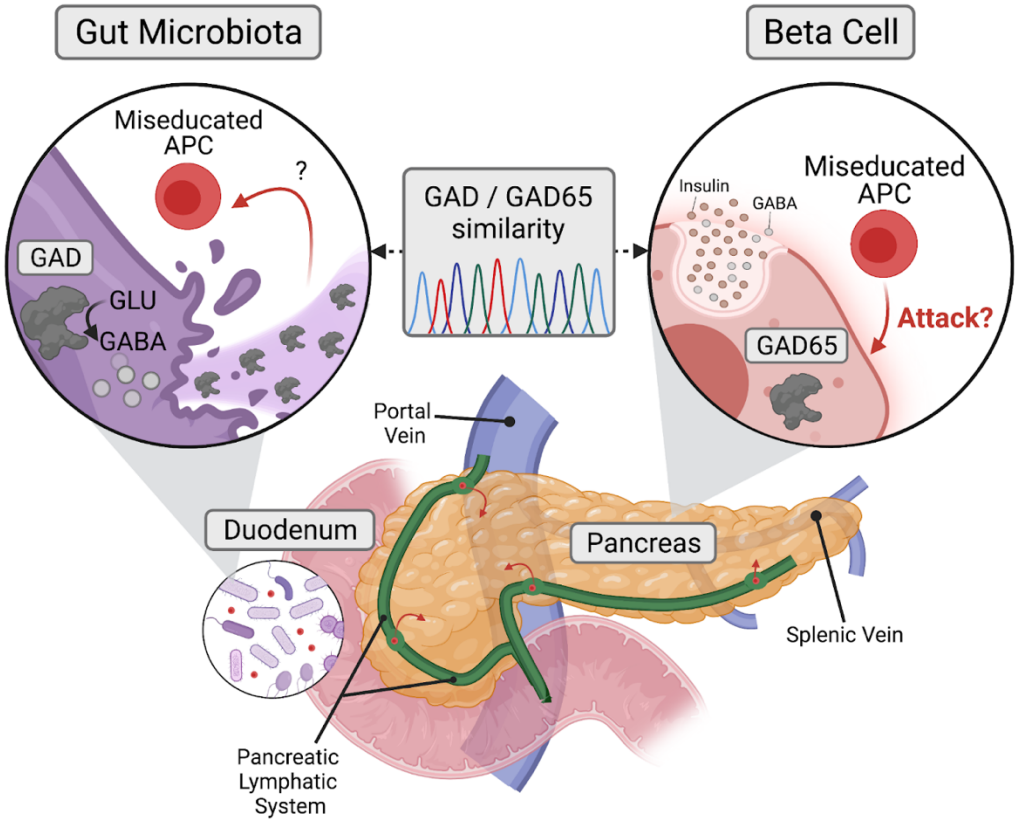


Figure 3. Conserved regions of GAD share common CD8+ and CD4+ T cell receptor epitopes. A total of five regions of interest were identified by overlapping T cell receptor epitopes to the sequence logo of three representative bacterial GAD and three animal GAD65 proteins. Three regions contained a motif match to Group II pyridoxal-dependent decarboxylases. Of the amino acids in which both CD4+ and CD8+ receptor epitopes overlap, there is at least 1 amino acid residue that is conserved across bacterial GAD and GAD65. Red is CD8+ epitopes and Blue is CD4+ epitopes.



352 **Figure 4. Miseducation of antigen-presenting cells (APCs) by bacterial GAD may**
353 **contribute to pancreatic beta cell immune-mediated destruction.** The death of GABA-
354 producing bacteria at the onset of T1D may release bacterial GAD65 which could lead to the
355 miseducation of APCs. Therefore, this miseducation could provoke the immune system to attack
356 and produce antibodies targeting human GAD65 expressing beta cells because of the similarity
357 and conservation between bacterial GAD and human GAD65. The pancreatic lymphatic system
358 could provide a pathway for these miseducated APCs to traverse from the duodenum into
359 pancreatic islets.