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1 **Activation of the Nlrp3 inflammasome by islet amyloid polypeptide**
2 **provides a mechanism for enhanced IL-1 β in type 2 diabetes**

3

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20

21

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23

24 **Abstract**

25

26 IL-1 β is an important inflammatory mediator of type 2 diabetes (T2D). Here we
27 show that oligomers of islet amyloid polypeptide (IAPP), a protein that forms
28 amyloid deposits in the pancreas during T2D, trigger the Nlrp3 inflammasome and
29 generate mature interleukin (IL)-1 β . A T2D therapy, glyburide, suppresses IAPP-
30 mediated IL-1 β production *in vitro*. Processing of IL-1 β initiated by IAPP first
31 requires priming, a process that involves glucose metabolism and can be facilitated
32 by minimally oxidized low density lipoprotein. Finally, mice transgenic for human
33 IAPP have increased IL-1 β in pancreatic islets, which colocalizes with amyloid and
34 macrophages. Our findings reveal novel mechanisms in the pathogenesis of T2D and
35 treatment of pathology caused by IAPP.

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39

40 **Introduction**

41

42 Type 2 diabetes (T2D) is characterized by insulin resistance and islet beta cell
43 dysfunction, with the progressive loss of insulin release being responsible for ever
44 increasing glucose concentrations in the absence of treatment ¹. Obesity is
45 frequently the basis for insulin resistance in T2D, and is associated with increased
46 concentrations of oxidized low density lipoprotein (LDL) ², free fatty acids (FFAs),
47 and pro-inflammatory cytokines ¹. One cytokine in particular, interleukin-1 beta (IL-
48 1 β), has been found to have profound effects on the function of pancreatic beta cells,
49 inducing them to undergo apoptosis ³. Abundant literature describes the
50 involvement of IL-1 β in type 1 diabetes (T1D), however IL-1 β is also a risk factor for
51 T2D ⁴, and a recent clinical trial of IL-1 blockade in T2D has been very encouraging ⁵.
52 These data clearly suggest a role for IL-1 β in the pathogenesis of T2D, however the
53 events leading to increased concentrations of active, secreted IL-1 β in this disease
54 remain unclear.

55

56 Recently a large number of publications have elucidated mechanisms by which
57 biologically active IL-1 β is made in the cell. This usually involves activation of a
58 protein complex termed the inflammasome, which is formed by a nucleating sensor
59 protein (typically a member of the nucleotide-binding domain and leucine-rich
60 repeat containing, NLR, family) that oligomerises the adaptor protein ASC to

61 dimerise and activate caspase-1 and thus cleave pro-IL-1 β into the processed,
62 secreted form. Before this can happen, certain cell types require priming to induce
63 suitable amounts of pro-IL-1 β and NLR proteins, this is sometimes referred to as
64 'signal 1'. The 'signal 2' factors that can activate the nucleating receptor of the
65 complex are still being determined. These include microbial DNA, RNA, cell wall
66 components and toxins. With reference to inflammatory diseases such as gout and
67 fibrosing disorders, endogenous factors and environmental contaminants have also
68 been discovered, such as uric acid crystals ⁶ and asbestos or silica respectively ⁷.
69 Most of these molecules activate the inflammasome complex nucleated by Nlrp3
70 (also known as Nalp3 or cryopyrin), however there is no structural similarity to
71 indicate that they would bind to it directly. Instead Nlrp3 is probably a sensor of
72 some homeostatic intracellular process that, if perturbed, will activate the
73 inflammasome. Certainly a large number of the Nlrp3 activating agents seem to
74 perturb the lysosomal vacuole, some due to their size through a process that has
75 been termed "frustrated phagocytosis", and others that are not degraded normally
76 once phagocytosed ⁸. This prompted us to ask if there might also be an endogenous
77 factor in T2D that could activate the Nlrp3 inflammasome in this way.

78

79 A hallmark feature of T2D is the deposition of amyloid in the pancreas of most
80 patients, with this morphological change being associated with the loss of insulin-
81 producing beta cells ⁹. The unique polypeptide constituent of amyloid found in
82 pancreatic islets is islet amyloid polypeptide (IAPP, also known as amylin) ^{10,11}, a 37
83 amino acid peptide co-secreted by the beta cell with insulin. There are a number of

84 lines of evidence that strongly implicate this deposition in disease progression. First
85 there is species specificity whereby IAPP in certain animals, such as rodents, is
86 unable to form amyloid due to the alteration of certain critical residues, however
87 when a human IAPP transgene is expressed in mice or rats, amyloid is deposited
88 within pancreatic islets, leading to loss of beta cell mass ¹²⁻¹⁴. Second, experiments
89 have been performed using non-human primates in which the concentration of
90 amyloid in the pancreas clearly correlates with the severity of disease and beta cell
91 function ¹⁵. Third, in humans a rare polymorphism in IAPP, S20G, is associated with
92 increased incidence or severity of T2D in some populations ¹⁶, and this version of
93 the protein has a higher propensity to form amyloid ¹⁷. IAPP oligomerization might
94 also have a role in preventing successful islet transplantation, which is a potential
95 treatment for T1D. Amyloid deposition is observed when human IAPP transgenic
96 mouse islets, or human islets are transplanted into mice ^{18,19}, and this could also be
97 associated with the failure of islet transplantation in humans ²⁰.

98

99 Initially it was established that IAPP is cytotoxic ²¹, and this could be due to effects
100 at the cell membrane ²². Mechanistically, this might also involve reactive oxygen
101 species (ROS) because ROS inhibition prevents IAPP-mediated beta cell apoptosis *in*
102 *vitro* ²³. The process of IAPP incorporation into amyloid is thought to proceed via the
103 aggregation of prefibrillar oligomers. These soluble oligomers, rather than mature
104 fibrils, could be the cytotoxic component ²⁴. Amyloid builds up extracellularly but
105 phagocytosed IAPP can be detected within the lysosomal compartment of human
106 pancreatic macrophages *in vivo*, where due to its amyloid structure, it is not

107 degraded normally ^{25, 26}. There are also reports showing induction of IL-1 β by IAPP
108 ^{27, 28}, however these studies were performed before the identification of the
109 inflammasome, and did not examine islets or primary macrophages and dendritic
110 cells (DC), the latter two of which could be the main source of pathogenic IL-1 β in
111 the pancreas. This observation prompted us to evaluate the potential for IAPP to
112 activate the Nlrp3 inflammasome through the mechanism of phagosomal
113 destabilization ⁸. We show that amyloidogenic human IAPP, but not the non-
114 amyloidogenic rat form of IAPP can trigger inflammasome activation and IL-1 β
115 production in lipopolysaccharide- (LPS) primed macrophages or DC, and that this
116 was dependent on Nlrp3. This then allowed us to investigate the effect of glyburide,
117 a common treatment in T2D, which is known to inhibit the inflammasome ²⁹. We
118 also observe that minimally oxidized LDL could prime for IL-1 β processing, and that
119 priming (signal 1) was dependent on glucose metabolism. Finally, we employed a
120 mouse model where the human IAPP gene is expressed in islet beta cells, and found
121 increased IL-1 β expression that broadly co-localizes with amyloid and also
122 macrophages. Our results therefore provide a possible mechanism for IL-1 β
123 activation in T2D.
124

125 **Results**

126

127 **IAPP activates the inflammasome to produce mature IL-1 β**

128 Bone marrow-derived dendritic cells (BMDC) and bone marrow-derived
129 macrophages (BMDM) are commonly used to investigate the inflammasome because
130 of their high expression of pro-IL-1 β following LPS priming. We compared the
131 ability of IAPP to stimulate production of mature IL-1 β from LPS primed BMDC with
132 known inflammasome-activating agents like particulate adjuvants³⁰. Overnight
133 stimulation with human IAPP was able to generate comparable amounts of IL-1 β to
134 these adjuvants, but rat IAPP which is unable to form amyloid did not stimulate IL-
135 1 β release (**Fig. 1a**). Human IAPP also stimulated the release of IL-1 α which, like IL-
136 1 β , has been shown to rely on the activity of caspase-1³¹, but could also represent
137 paracrine induction of IL-1 α by IL-1 β . IL-1 α in cell lysates was not decreased,
138 suggesting that full length IL-1 α is not released by cell lysis (**Supplementary Figure**
139 **1a**). TNF and IL-6 production (**Fig. 1a**) were undetectable without LPS stimulation,
140 indicating that the IAPP preparations were not contaminated with LPS. The
141 concentrations of TNF and IL-6 were not altered by the presence of IAPP, indicating
142 that there was a specific effect on IL-1 β production and not a general increase in
143 pro-inflammatory cytokine production, and that there was no obvious cell death
144 before these cytokines were produced.

145

146 Immunoblotting confirmed that the detected IL-1 β was the cleaved 17.5 kDa
147 fragment. Cleaved IL-1 β was increased in the presence of IAPP and the

148 inflammasome-activating agents uric acid crystals (MSU) and ATP (**Fig. 1b**).
149 Measurement of lactate dehydrogenase (LDH) in the supernatants was carried out
150 along with the measurement of IL-1 α and IL-1 β ; this confirmed that there was no
151 obvious cytotoxicity under these conditions (**Supplementary Figures 1a-d**). Both
152 DCs and macrophages are found in pancreatic islets, and these cells have been
153 observed to phagocytose IAPP in T2D ²⁶. Like BMDC, BMDM also produced a
154 significant amount of IL-1 β in response to IAPP. In comparison, the production of IL-
155 1 β by purified mouse pancreatic islets *ex vivo* and the rat beta cell line Rin-5F was
156 very low, even when these cells were cultured under hyperglycemic conditions (**Fig.**
157 **1c**). These results show that amyloidogenic human IAPP can trigger processing of
158 IL-1 β from BMDM and BMDC.

159

160 **IAPP activates caspase-1 and ASC speck formation**

161 Caspase-1 is the protease activated in the inflammasome complex to cleave IL-1 β
162 and we measured active caspase-1 with a fluorescent cell-permeable probe that
163 binds activated caspase-1 (FAM-YVAD-fmk). Flow cytometry indicated a large
164 increase in the percentage of BMDC that contain active caspase-1 after being
165 stimulated with IAPP for 1 hour (**Fig. 1d**). The mechanism by which caspase-1 is
166 activated to produce mature IL-1 β requires the apoptosis-associated speck-like
167 protein containing a CARD (ASC), and its ability to form the multimeric
168 inflammasome complex. Normally ASC is evenly distributed throughout the cell
169 cytoplasm, but when the inflammasome complex becomes activated, ASC aggregates
170 to a single foci within the cell, known as a speck. We used immortalized BMDM that

171 stably express a fluorescent ASC protein to measure speck formation. LPS-treated
172 cells that were activated with IAPP induced formation of an intense, single
173 fluorescent speck in the cell, indicative of inflammasome activation (**Fig. 1e**,
174 **Supplementary Figure 1e**). In addition to IL-1 β processing, IAPP therefore also
175 activates caspase-1 and the ASC inflammasome complex.

176

177 **IAPP oligomers activate the Nlrp3 inflammasome**

178 Having shown that an inflammasome complex containing caspase-1 and ASC is
179 activated by IAPP to cleave IL-1 β , we next examined which NLR protein nucleates
180 this inflammasome complex. We elected to study Nlrp3, which is required for
181 inflammasome activation by particles, rather than IPAF, Nlrp1, Nod1 or Nod2 which
182 are not. Accordingly, for Nlrp3-deficient BMDC, mature IL-1 β production was
183 completely abrogated in response to IAPP (**Fig. 2a**). These cells still produced TNF
184 and IL-6, attesting to the specificity of Nlrp3 deletion, and IL-1 β mRNA expression
185 was not decreased (**Supplementary Figure 1f**). To determine whether oligomers or
186 IAPP fibrils activate the Nlrp3 inflammasome, we employed the organic solvent
187 HFIP to dissolve amyloid fibrils. The HFIP was then removed using nitrogen gas and
188 the IAPP reconstituted in H₂O or phosphate buffered saline (PBS) and incubated at
189 room temperature. Thioflavin T fluorescence was used to indicate fibrillar content.
190 IAPP fibrils were formed over 24 h and this was accelerated when reconstituted in
191 PBS compared to H₂O (**Fig. 2b**, top panel). This increase in fibrillar content
192 coincided with a decrease in IL-1 β production from BMDC (**Fig. 2b**, lower panel). To
193 further elucidate the active species, preparations of IAPP were subjected to size

194 fractionation, generating samples with predominantly fibrillar (>100 kDa) or
195 oligomeric (<100 kDa) species. We found that the IL-1 β activating potential was
196 predominantly retained in the oligomeric fraction <100 kDa from freshly
197 reconstituted IAPP (**Fig. 2c**). Collectively this data suggests that the Nlrp3-activating
198 constituent is a soluble oligomer of IAPP, rather than a highly fibrillar species of
199 high molecular weight.

200

201 **Inflammasome activation involves the phagolysosome**

202 To confirm the role of caspase-1 and support the use of inhibitors to investigate
203 mechanisms of inflammasome activation, a caspase-1 inhibitor was added to BMDC
204 before IAPP activation. Caspase-1 inhibition reduced IL-1 β production in a dose-
205 dependent manner (**Fig. 2d**). IAPP is thought to signal through a complex of
206 receptor activity-modifying proteins with the calcitonin receptor, and the biological
207 effects of IAPP can be blocked at this point using a specific peptide inhibitor
208 (AC187)³². To determine if recognition of IAPP by the receptor complex is required
209 for inflammasome activation a titration of up to 40 μ M AC187 (a 4 fold molar
210 excess) was used, however this had no effect on IL-1 β (**Fig. 2d**). Alternatively IAPP
211 could be phagocytosed by BMDM and thereby activate the inflammasome in a
212 manner similar to other species of amyloid³³. Inhibition of IL-1 β production
213 following the addition of cytochalasin D confirmed that inflammasome activation
214 was dependent on phagocytosis (**Fig. 2d**). Furthermore, inhibition of the vacuolar
215 H⁺ ATPase by bafilomycin A restricted the effect of IAPP on IL-1 β secretion (**Fig. 2d**).

216 Taken together this suggests that phagolysosomal processes downstream of
217 acidification are altered to trigger Nlrp3 activation.

218

219

220 **IAPP inflammasome activation is prevented by glyburide**

221 Two mechanisms have been proposed to account for perturbations in phagocytic
222 processes that activate the inflammasome. Previously cathepsin B was implicated in
223 this process for amyloid beta ³³ while IAPP ²³ and MSU ⁶, have been found to induce
224 ROS. We used the ROS inhibitor (2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylic acid
225 (APDC) and a cathepsin B inhibitor (CA-074 Me) to confirm their role in IAPP
226 activation of the inflammasome (**Fig. 2e**). Glyburide is a second generation
227 sulfonylurea, an agonist of the potassium channel composed of SUR1 and KIR6.2
228 that signals for increased insulin secretion. Additionally, this small molecule agonist
229 inhibits the inflammasome and IL-1 β production, but this is independent of its
230 interaction with the SUR1, KIR6.2 complex ^{29,34}. We found that activation of the
231 Nlrp3 inflammasome by IAPP was also inhibited by glyburide (**Fig. 2e**).

232

233 **Txnip is not involved in IAPP activation of IL-1 β**

234 The thioredoxin interacting protein Txnip was recently identified as a redox
235 sensitive ligand of Nlrp3 ³⁵. This study suggested that an increased concentration of
236 glucose alone could induce pancreatic beta cell Txnip to activate the inflammasome,
237 and that glyburide could prevent this. However it was also shown that expression of
238 Txnip in macrophages is constitutively high, so it was unlikely that this would be the

239 mechanism of inflammasome inhibition by glyburide in macrophages. Nevertheless,
240 we formally examined Txnip mRNA levels both before and after LPS priming in
241 BMDM, and found no difference due to glyburide in either setting (**Supplementary**
242 **Figure 2a**). Furthermore, LPS decreased expression of Txnip arguing against this
243 protein as the instigator of Nlrp3 activation in BMDM. We then tested Txnip-
244 deficient macrophages to see if this Nlrp3 ligand was required for IL-1 β production
245 in response to IAPP (**Supplementary Figure 2b**). We could find no difference in IL-
246 1 β secretion in response to IAPP, or indeed other inflammasome activators such as
247 MSU or ATP in BMDM *ex vivo*. In addition we directly compared BMDM from mice
248 lacking Txnip or Nlrp3 for IL-1 β secretion and caspase-1 activation and found that
249 these parameters were only decreased in the *Nlrp3*^{-/-} mice (**Supplementary Figure**
250 **2c-f**). Therefore our studies to date do not find a role for Txnip regulating the effect
251 of glyburide on the inflammasome, or IL-1 β production by the inflammasome.

252

253 **Glucose metabolism is required for inflammasome priming**

254 Elevated concentrations of D-glucose, similar to those encountered in T2D, can
255 induce pro-IL-1 β ^{36,37} although this is debated ³⁸. Growing BMDM in different
256 concentrations of glucose influenced cell proliferation, to which BMDC were more
257 resistant. We therefore tested BMDC in hyperglycemic conditions (25 mM D-
258 glucose) for up to 24 h either before or after priming with LPS. We saw no
259 significant difference in IL-1 β production after IAPP stimulation (**Fig. 3a**). We also
260 tested D-glucose analogs; one that is not metabolized by the cell and provides a
261 similar osmotic environment to D-glucose (L-glucose), and another that actually

262 inhibits the processing of D-glucose and subsequent metabolic processes in the cell
263 (2-deoxy-D-glucose, 2DG). L-glucose had no effect (**Fig. 3a**), however we found that
264 low concentrations of 2DG prevented IL-1 β release due to IAPP in a dose dependent
265 manner (**Fig. 3b**). 2DG had no effect when added after the addition of LPS, but only
266 before the addition of IAPP (**Fig. 3b**), and we found that 2DG prevented IL-1 β mRNA
267 induction during LPS priming of the inflammasome (**Fig. 3c**). There was also a small
268 decrease in the production of IL-6 at the highest concentration of 2DG (**Fig. 3b**),
269 however the production of TNF and the viability of cells in general were not
270 perturbed (**Fig. 3b and Supplementary Fig. 3a**). Sufficient glucose metabolism is
271 therefore required *in vitro* during priming (signal 1) of the inflammasome.

272

273 **mmLDL primes the inflammasome activated by IAPP**

274 Our studies to this point showed that glucose metabolism was required for LPS to
275 prime the inflammasome after which IAPP can activate Nlrp3, caspase-1 and ASC,
276 leading to IL-1 β processing. The question then arose as to what the endogenous
277 priming agent of the Nlrp3 inflammasome might be i.e. a potential host factor as
278 opposed to LPS. It has been reported that a particular form of LDL, minimally
279 oxidized LDL (minimally modified LDL or mmLDL), can engage Toll-like receptor 4
280 (TLR4) in a manner similar to LPS, while extensively oxidized LDL does not ³⁹.
281 Specifically, mmLDL can be elevated in individuals with T2D ^{40, 41}, and can cause
282 apoptosis and a loss of insulin secretion by islets in culture ⁴². We therefore tested
283 preparations of LDL that had been oxidized to different extents. Although the level
284 of IL-1 β produced after IAPP activation of the inflammasome was low when first

285 primed with mmLDL, it was clearly higher than priming with more extensively
286 oxidized species of LDL, or LDL that was not oxidized (**Fig. 4a**). mmLDL recognition
287 depended on TLR4, because C3H/HeJ mice which have a non-functional TLR4, were
288 unable to prime the inflammasome via LPS or mmLDL. mmLDL also resulted in the
289 dose-dependent production of TNF and IL-6 (**Fig. 4b**). Although LPS was not
290 detected in mmLDL preparations, an LPS inhibitor, polymyxin B, inactivated mmLDL
291 (**Supplementary Figure 3b**). While trace amounts of LPS might be specifically
292 presented to TLR4 by mmLDL, it is also possible that polymyxin B neutralizes
293 mmLDL in the same way as LPS. There are thought to be two prerequisites for
294 inflammasome priming, the induction of pro-IL-1 β , and Nlrp3⁴³, however we
295 observed that mmLDL only induced IL-1 β mRNA, in a time-dependent manner (**Fig.**
296 **4c**). This lack of Nlrp3 upregulation might help explain the relatively low level of IL-
297 1 β activation when compared to LPS priming. Nevertheless these data suggest that
298 mmLDL can promote IL-1 β by providing signal 1 (priming) for the inflammasome,
299 which may be particularly relevant in T2D.

300

301 **IAPP induces IL-1 β *in vivo***

302 As mouse IAPP is not amyloidogenic, mice that are transgenic for the expression of
303 human IAPP have been generated and extensively characterized as a rodent model
304 of islet amyloid formation⁴⁴. In particular, when these mice are fed a high fat diet,
305 amyloid deposition within islets of the pancreas occurs and is associated with the
306 impaired function of insulin-producing cells⁴⁵. We compared wild-type mice
307 (control) and IAPP transgenic mice, both fed on a high fat diet for one year (the

308 length of time required in this model), and then determined whether insulin and IL-
309 1 β were present in pancreatic islets by immunofluorescence (**Fig. 5a**). Although
310 variable, IAPP transgenic mice had marked regions within the islet that did not stain
311 for insulin, and instead these regions displayed evidence of IL-1 β immunoreactivity.
312 However the IL-1 β staining co-localized to regions where amyloid was deposited, as
313 determined by staining with thioflavin S (**Fig. 5b**). To identify a cell type in the islet
314 that produces this IL-1 β we stained with an antibody that is particularly useful for
315 detecting peripheral tissue macrophages, MoMa2 (**Fig. 5c**). Most of the
316 macrophages in the islet were positive for IL-1 β , but there was still a large area
317 staining for IL-1 β outside these cells. When quantified, a statistically significant
318 decrease in the area of insulin-producing cells was observed (**Fig. 5d**), with a
319 concurrent increase in the amount of amyloid (**Fig 5e**), and the area of IL-1 β
320 expression (**Fig. 5f**) within pancreatic islets of IAPP transgenic mice. There was no
321 significant difference in the area of MoMa2 reactivity within the islet (**Fig. 5g**). This
322 mouse model highlights that the presence of IAPP amyloid promotes IL-1 β *in vivo*.

323

324 **Discussion**

325

326 This work describes the mechanism whereby an endogenous molecule that is
327 deposited in the pancreas during T2D can potentiate the processing of IL-1 β , a
328 pathogenic cytokine that causes beta cell death. We also saw release of IL-1 α due to
329 IAPP, which could augment sterile inflammation in pancreatic islets. Our
330 observation that IAPP can activate the Nlrp3 inflammasome is consistent with
331 similar activation from a different amyloidogenic peptide, amyloid beta ³³, and
332 literature showing that the disease-causing species of IAPP might be oligomers ⁴⁶.
333 Macrophages and DC are professional phagocytic cells that take up IAPP in
334 pancreatic islets, and we found that these cell types produce significant amounts of
335 IL-1 β in response to IAPP oligomers. Using multiple inhibitors we showed that
336 perturbation of the phagolysosomal pathway seems to be involved in Nlrp3
337 activation by IAPP, however the role of ROS may be complex. Recently a number of
338 papers have shown that patients deficient for ROS production still activate the
339 inflammasome, to an even higher degree than healthy controls with normal ROS
340 production ⁴⁷. This suggests that the effect of the ROS inhibitor that we used might
341 be non-specific, and indeed this is a caveat raised against the use of inhibitors in
342 general. Although the effects of the inhibitors we used probably point towards a
343 common mechanism of inflammasome activation during the phagocytosis of IAPP,
344 their precise target in this pathway remains to be elucidated.

345

346 We found no evidence that the signaling cascade leading to Nlrp3 activation
347 requires Txnip. Particularly with regard to T2D where Txnip is known to be a ROS-
348 sensitive molecule implicated in disease, we had hypothesized that IAPP-induced
349 changes would utilize this pathway. While it is possible that our *in vitro* experiments
350 have not fully recapitulated *in vivo* mechanisms of disease, they clearly show that
351 Nlrp3 is a critical mediator of IL-1 β release while Txnip is not. Certainly cell type
352 differences might account for our observation since Txnip mRNA levels are not
353 altered by glyburide in macrophages, while others report that glyburide might
354 prevent Txnip induction in beta cells ³⁵. Because IAPP can induce significant IL-1 β
355 production from macrophages and this is inhibited by glyburide while Txnip mRNA
356 levels remain unchanged, a separate mechanism for this inhibition must exist, and is
357 sure to attract interest in the future. Although we found that glyburide can inhibit
358 IAPP-induced IL-1 β *in vitro* at concentrations above 5 μ g/ml, it is unlikely that the
359 concentration of glyburide currently used clinically would reach this level. New
360 small molecules that can target this inflammatory pathway *in vivo* will likely be
361 attractive therapeutic candidates.

362

363 Identification of IAPP as a possible trigger for inflammasome activation in T2D still
364 leaves open the question as to which endogenous factors prime the inflammasome
365 in this disease. High glucose alone was not able to replace LPS as the priming agent,
366 or influence IL-1 β mRNA levels. We found that mmLDL could prime the
367 inflammasome through TLR4, however extensively oxidized LDL, which does not
368 engage this receptor, could not. Potentially this is a mechanism by which mmLDL

369 might be associated with T2D, and other IL-1 β -dependent manifestations such as
370 atherosclerosis⁴⁸. Recently, a further involvement of LDL in inflammasome
371 activation was also described, where it facilitates cholesterol crystal deposition in
372 the vessel wall⁴⁸. While the role of mmLDL in T2D has not been extensively
373 characterized, FFAs are commonly thought of as potentially pathogenic in T2D.
374 Based on the literature, we would expect that FFAs could also prime the
375 inflammasome by interaction with TLRs⁴⁹.

376

377 Although increasing glucose concentration had no effect on IL-1 β activation in our
378 *in vitro* assays, we found that glucose metabolism is absolutely required for LPS to
379 induce the expression of pro-IL-1 β in macrophages. To inhibit glucose metabolism
380 we used 2DG, which has also been found to reduce IL-1 β *in vivo*, as for
381 atherosclerosis-prone, insulin-resistant rats⁵⁰. Clearly the *in vivo* effects of IL-1 β
382 with regard to fever and inflammation are highly energy-dependent processes. Thus
383 it would be appropriate for cells to prevent IL-1 β production unless there is
384 sufficient glucose that can be metabolized to mediate these biological responses. A
385 small decrease in IL-6 production was observed, however it is likely that the factors
386 controlling IL-1 β synthesis that are inhibited by 2DG are also shared in some part by
387 other pro-inflammatory cytokines. Because we have found glucose metabolism to be
388 a limiting step in inflammasome priming, this could be a physiologically relevant
389 mechanism in the pathogenesis of T2D where persistent hyperglycemia might
390 abolish any such limits. Indeed the beta cell loss in high fat fed IAPP transgenic mice
391 is greatly increased when they develop amyloid deposition⁴⁵, and we now report

392 that this is associated with a significant increase in IL-1 β immunostaining. The IL-1 β
393 observed in islets of IAPP transgenic mice co-localized with macrophages, and also
394 with amyloid. We could find no clear evidence of beta cells producing IL-1 β , and if
395 they do it is likely to be at a low level, or a rare event. While IL-1 β staining in
396 macrophages might represent intracellular pro-IL-1 β , staining outside these cells
397 that is coincident with amyloid could represent the secreted, processed form, as
398 there are few live cells in these regions. Our analysis is somewhat limited in this
399 respect, as the antibody we used to detect IL-1 β would recognize both the processed
400 and pro-IL-1 β species.

401

402 Our data is unambiguous as to the ability of IAPP to activate the Nlrp3
403 inflammasome and generate processed IL-1 β . This might be a key mechanism for IL-
404 1 β production in T2D. It could be that amyloid deposition in the pancreas only
405 happens later in the disease process, and some have argued that this would be as a
406 result, and not as a cause of disease. However it is also possible that even if this
407 buildup is secondary to the initiation of disease, that it nevertheless aids and abets
408 in the progressive loss of beta cell function that is a key component of T2D. Future
409 studies on patients should identify whether targeting IL-1 has most benefit only
410 later in the disease progression or for those with more severe disease pathology. It
411 is also becoming apparent that IAPP could contribute to the death of transplanted
412 islets being used as therapy in T1D ¹⁸⁻²⁰. The current Edmonton protocol of
413 immunosuppression used during this transplant procedure could conceivably be
414 improved with anti-inflammatory therapy targeting IL-1 β activated by IAPP. To

415 conclude, our findings highlight new roles for well-known entities in the
416 pathogenesis and treatment of diabetes and will hopefully spur new research
417 targeted at the mechanisms of this debilitating, chronic disease.
418

419

420

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422

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432

433

434 **Author contributions**

435

436 S.L.M. designed and performed experiments, analyzed data and wrote the paper;
437 L.A.J.O. and E.C.L. conceived ideas and oversaw research; A.D., S.L.S., R.L.H., G.M.T.,
438 F.A.S., C.B., L.F., E.Y., Z.C., N.M., L.A.M., J.H. and R.C.C., performed experiments;
439 K.H.G.M, K.H.M., P.N., G.N., J.Y., and S.E.K. provided advice and reagents.

440

441

442 The authors declare no competing financial interests.

443

444

445 **Figure legends**

446

447 **Figure 1. Inflammasome activation and IL-1 β production induced by human**

448 **IAPP. (a)** IAPP from different species or polystyrene beads (adjuvant) were

449 incubated overnight with BMDC with and without 3 hours priming using LPS.

450 Supernatants were then analyzed for IL-1 β , IL-1 α , IL-6 and TNF by ELISA. **(b)**

451 Immunoblotting for IL-1 β was performed using supernatants from BMDC primed

452 with LPS, then activated with IAPP, MSU or Alum for 6 hours. **(c)** IL-1 β secretion

453 from BMDM and BMDC compared to purified mouse pancreatic islets or Rin-5F cells

454 (a pancreatic beta cell line), stimulated with LPS and IAPP. Rin-5F cells were also

455 cultured for different times with elevated glucose (25 mM D-glucose). **(d)** Flow

456 cytometric analysis of BMDC stimulated with IAPP for 1 hour then treated with

457 FAM-YVAD-fmk which covalently binds to active caspase-1 and fluoresces. Control is

458 shaded under the curve, IAPP stimulated is unshaded. **(e)** Immortalized BMDM

459 expressing YFP-ASC were primed with LPS, then activated with IAPP overnight and

460 imaged with a fluorescent microscope (40x objective). Arrows indicates speck

461 formation. Means \pm SD, * $p < 0.01$, ** $p < 0.001$. All data representative of three

462 independent experiments.

463

464 **Figure 2. IAPP oligomers activate the Nlrp3 inflammasome which is prevented**

465 **by glyburide, and inhibitors targeting phagocytosis, ROS and Cathepsin B. (a)**

466 WT or Nlrp3 deficient (*Nlrp3*^{-/-}) BMDM were primed with LPS and then activated

467 with uric acid crystals (MSU), Alum or IAPP overnight after which IL-1 β , TNF and IL-
468 6 production was measured. **(b)** Thioflavin T fluorescence was used to indicate IAPP
469 fibrillar content after reconstitution in H₂O or PBS for different times at room
470 temperature. These different preparations of IAPP were then used to activate BMDC
471 as in **(a)**, and IL-1 β was measured. **(c)** IAPP reconstituted in H₂O for different times
472 was separated based on size into fractions >100 kDa and <100 kDa, then used to
473 activate BMDC as in **(a)**, and IL-1 β was measured. **(d)** BMDC were treated as in **(a)**
474 for IAPP, with the addition of various inhibitors 1 hour before inflammasome
475 activation; 1 μ g/ml caspase-1 inhibitor, 5 μ M cytochalasin D, 250 μ M bafilomycin A
476 or 40 μ M IAPP receptor inhibitor peptide (AC187). Starting at the highest
477 concentration indicated, two 10-fold dilutions of the inhibitor were also tested, or in
478 the case of AC187, two 4 fold dilutions. **(e)** BMDC were treated as in **(a)** for IAPP,
479 with the addition of various inhibitors 1 hour before inflammasome activation; 1
480 μ g/ml ROS inhibitor (APDC), 10 μ M cathepsin B inhibitor (CA-074 Me) and 50
481 μ M glyburide. Starting at the highest concentration indicated, two 10 fold dilutions
482 of the inhibitor were also tested, or in the case of glyburide, two 4 fold dilutions.
483 Means \pm SD, * p<0.05, ** p<0.01, *** p<0.001. All data representative of three
484 independent experiments.

485

486 **Figure 3. Priming the inflammasome requires glucose metabolism.** **(a)** BMDC
487 were treated with 25 mM D- or L-glucose for different periods of time prior to LPS
488 priming and inflammasome activation with IAPP, after which IL-1 β production was
489 measured. **(b)** BMDC were treated with increased amounts of 2-deoxy-D-glucose

490 (2DG) either 3 hours before or 3 hours after priming with LPS. Cells were then
491 activated with IAPP and the cytokines IL-1 β , IL-6 and TNF were measured. (c) IL-1 β
492 mRNA levels were determined after 3 hours LPS stimulation of BMDC in the
493 presence of increased amounts of D-glucose and its analogues for a total of 6 hours.
494 Means \pm SD, * p<0.001. All data representative of three independent experiments.

495

496 **Figure 4. Minimally oxidized LDL can prime for inflammasome activation by**
497 **IAPP.** (a) IAPP activation of the inflammasome after 3 hrs priming with LPS, human
498 plasma LDL, or LDL oxidized to different extents. LDL oxidation is quantified by
499 relative electrophoretic mobility (REM). BMDM from C3H/HeN mice were
500 compared to those from C3H/HeJ mice that do not have functional TLR4. (b) Dose
501 response for minimally oxidized LDL (mmLDL) priming of IAPP inflammasome
502 activation and IL-1 β production. IL-6 and TNF are also produced in a dose-
503 dependent fashion. (c) IL-1 β and Nlrp3 mRNA levels were measured after priming
504 with mmLDL for different times. Means \pm SD, * p<0.05, ** p<0.01, *** p<0.001. All
505 data representative of three independent experiments.

506

507 **Figure 5. Increased IL-1 β expression in islets of mice transgenic for human**
508 **IAPP.** (a) Immunofluorescent analysis (20x objective) of sections from the pancreas
509 of wild-type (WT) and IAPP transgenic (IAPP-TG) mice, both fed a high fat diet for
510 one year. Sections were stained for insulin (red) and IL-1 β (green). (b) Pancreatic
511 sections from IAPP-TG mice stained for amyloid (red) and IL-1 β (green). (c)
512 Pancreatic sections from WT and IAPP-TG mice stained for macrophages (red) and

513 IL-1 β (green). All images representative of three individual mice. **(d)** The area
514 within the islet expressing insulin was quantified for multiple islets of three
515 individual mice. **(e)** The area of amyloid deposition in the islet, measured as for
516 insulin. **(f)** The islet area expressing IL-1 β , measured as for insulin. **(g)** The islet area
517 stained for macrophages, measured as for insulin. Means \pm SD, * p=0.0427, **
518 p=0.0261, n=3.
519
520

521

522 **Materials and Methods**

523

524 **Cell culture**

525 Bone marrow from C57BL/6 mice was differentiated for 10 days in GM-CSF (4%
526 J588 myeloma cell supernatant) or 7 days in M-CSF (20% L929 cell supernatant) in
527 typical media preparations to make bone marrow derived dendritic cells (BMDC)
528 and macrophages (BMDM) respectively *Nlrp3* KO mice were from Jurg Tschopp
529 (University of Lausanne) or Millennium Pharmaceuticals. Pancreatic islet cells were
530 obtained from collagenase digested mouse pancreatic islets and cultured overnight
531 ⁵¹. The Rin-5F cell line was from ECACC and cultured according to their protocol.

532

533 **Materials**

534 Unless stated, cells were primed for 3 hrs with 100 ng/ml LPS (Alexis) or 10 µg/ml
535 human plasma LDL oxidized with copper(II) sulphate for different times (Kalen
536 biomedical), then activated with 10 µM human IAPP (Sigma), 10 µM rat IAPP
537 (Bachem), 20 µg/ml MSU (Opsona), 100 µg/ml Alum (Brenntag biosector) or 0.6
538 mg/ml 430 nm polystyrene beads (Corpuscular) for 16 hours. The following
539 inhibitors or glucose analogs were added 2 hours after LPS priming, 1 hour before
540 inflammasome activation at the highest concentration indicated, unless stated; 1
541 µg/ml Caspase-1 inhibitor VI (Calbiochem), 5 µM cytochalasin D, 250 µM
542 bafilomycin A, 10 µM cathepsin B inhibitor (CA-074 Me), 1 µg/ml (2R,4R)-4-
543 Aminopyrrolidine-2,4-dicarboxylic acid (APDC), 50 µM N-acetyl-cysteine (NAC), 50

544 μ M glyburide, 25 mM D-glucose and its analogs (all from Sigma), and 40 μ M IAPP
545 receptor inhibitor peptide AC187 (Tocris biosciences). 100 μ g/ml Polymyxin B
546 (Sigma) was also added to 1 μ g/ml LPS or 100 μ g/ml mmLDL overnight at 4° C
547 before use in some experiments with a 1/10 dilution.

548

549 **Measurement of caspase-1 activity**

550 BMDM were stimulated with IAPP for 1 hour before the addition of FAM-YVAD-fmk
551 according to manufacturer's instructions (Immunochemistry Technologies).

552 Fluorescent cells were measured by flow cytometry.

553

554 **Analysis of ASC speck formation**

555 Immortalized BMDM expressing YFP-ASC were generated as described previously ⁸.

556 These cells were primed with LPS then activated with IAPP overnight and imaged
557 using a fluorescent microscope with a 40x objective lens.

558

559 **Immunoblotting**

560 To blot for IL-1 β in supernatants, BMDM grown in 24 well plates in 0.5 ml media
561 containing 1% FCS were stimulated, supernatants collected and precipitated with 1
562 volume methanol, 1/4 volume chloroform, then the precipitate was washed in 1
563 volume methanol and finally resuspended in 60 μ l SDS loading buffer for running on
564 15% Tris-glycine polyacrylamide gels. Proteins were transferred to PVDF
565 membranes, blocked in 5% skimmed milk then probed with goat anti-mIL-1 β (RnD
566 systems, AF-401) and detected with HRP-donkey anti-goat antibody (Upstate).

567 Alternatively, cell lysates were prepared by lysis in RIPA buffer, followed by
568 electrophoresis and blotting as above, then probed with goat anti-IL-1 α (RnD
569 systems, AF-400), rabbit anti-ASC (Alexis), mouse anti-Txnip (MBL international) or
570 mouse anti-caspase-1 (developed in-house) followed by a suitable detection
571 antibody. Immunoblots were quantified using ImageJ software.

572

573 **Cytokine measurements**

574 For cytokine measurements, BMDM or BMDC were cultured in 96 well plates,
575 stimulated in triplicate, supernatants were then collected and ELISA performed for
576 mL-1 β , mL-1 α , mL-6 and mTNF (RnD systems).

577

578 **Q-PCR**

579 BMDM were cultured in 24 well plates, stimulated in triplicate, RNA prepared using
580 the RNeasy kit (Qiagen) and then Q-PCR performed using Taqman probes specific
581 for IL-1 β (Mm01336189_m1), Nlrp3 (Mm00840904_m1) or Txnip
582 (Mm00452393_m1).

583

584 **Cytotoxicity assay**

585 Cytotoxicity was determined by an LDH assay (Promega) according to
586 manufacturer's instructions.

587

588 **Thioflavin T fluorescence and size fractionation**

589 IAPP was dissolved in HFIP (Sigma) which was subsequently removed using
590 nitrogen gas. Aliquots were then resuspended at 500 μ M in H₂O or PBS for different
591 times. This was diluted to 10 μ M in RPMI and thioflavin T added to a concentration
592 of 20 μ M after which fluorescence measurements were recorded according to
593 protocol ⁵² using a FP6200 fluorescence spectrometer (JASCO). To separate
594 fractions based on size, resuspended IAPP was diluted to 20 μ M in RPMI and quickly
595 filtered through a 100 kDa cut-off membrane (Amicon). The concentrated fraction
596 greater than 100 kDa was supplemented to the original volume with RPMI and both
597 fractions were diluted 1/2 onto BMDC for a final IAPP concentration of 10 μ M.

598

599 **Immunofluorescent analysis of sections from IAPP transgenic mice**

600 IAPP transgenic mice were fed a high fat diet for one year then the pancreas was
601 sectioned as described ⁴⁵. Immunofluorescent staining was performed using
602 thioflavin S (Sigma), mouse anti-insulin (Sigma, I2018), rabbit anti-IL-1 β (Santa
603 Cruz Biotechnology, sc-7884) and specific fluorescently-conjugated secondary
604 antibodies (Molecular Probes), or Alexa647 conjugated anti-MoMa2 (AdbSerotech).
605 Analysis of images performed using ImageJ software.

606

607 **Statistical analysis**

608 Data is presented as mean \pm SD. Significance was determined by two-tailed unpaired
609 t-test with the exception of insulin staining in pancreatic islets, for which a one-
610 tailed unpaired t-test was used as the direction of the outcome can be anticipated
611 based on similar experiments we have published previously.

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