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IL-1 Blockade Attenuates Islet Amyloid Polypeptide-Induced Proinflammatory Cytokine Release and Pancreatic Islet Graft Dysfunction

Clara Westwell-Roper,* Derek L. Dai,* Galina Soukhatcheva,* Kathryn J. Potter,* Nico van Rooijen,† Jan A. Ehses,‡ and C. Bruce Verchere*,‡

Islets from patients with type 2 diabetes exhibit β cell dysfunction, amyloid deposition, macrophage infiltration, and increased expression of proinflammatory cytokines and chemokines. We sought to determine whether human islet amyloid polypeptide (hIAPP), the main component of islet amyloid, might contribute to islet inflammation by recruiting and activating macrophages. Early aggregates of hIAPP, but not nonamyloidogenic rodent islet amyloid polypeptide, caused release of CCL2 and CXCL1 by islets and induced secretion of TNF-α, IL-1α, IL-1β, CCL2, CCL3, CXCL1, CXCL2, and CXCL10 by C57BL/6 bone marrow-derived macrophages. hIAPP-induced TNF-α secretion was markedly diminished in MyD88−, but not TLR2- or TLR4-deficient macrophages, and in cells treated with the IL-1R antagonist (IL-1Ra) anakinra. To determine the significance of IL-1 signaling in hIAPP-induced pancreatic islet dysfunction, islets from wild-type or hIAPP-expressing transgenic mice were transplanted into diabetic NOD/SCID recipients implanted with mini-osmotic pumps containing IL-1Ra (50 mg/kg/d) or saline. IL-1Ra significantly improved the impairment in glucose tolerance observed in recipients of transgenic grafts 8 wk following transplantation. Islet grafts expressing hIAPP contained amyloid deposits in close association with F4/80-expressing macrophages. Transgenic grafts contained 50% more macrophages than wild-type grafts, an effect that was inhibited by IL-1Ra. Our results suggest that hIAPP-induced islet chemokine secretion promotes macrophage recruitment and that IL-1R/MyD88, but not TLR2 or TLR4 signaling is required for maximal macrophage responsiveness to prefibrillar hIAPP. These data raise the possibility that islet amyloid-induced inflammation contributes to β cell dysfunction in type 2 diabetes and islet transplantation. The Journal of Immunology, 2011, 187: 000–000.

Patients with type 2 diabetes mellitus are unable to secrete sufficient insulin to compensate for increased peripheral insulin resistance (1). Their pancreatic islets exhibit progressive β cell loss (2), likely due in part to increased expression of proinflammatory cytokines (3, 4), macrophage infiltration (5, 6), and islet amyloid deposition (7). Similarly, immune cell infiltration and proinflammatory cytokine release impair β cell function (8). Islet amyloid deposits form by aggregation of human islet amyloid polypeptide (hIAPP), a 37-aa peptide cosecreted with insulin by β cells (18). Fibrils comprised of aggregated hIAPP are phagocytosed but ineffectively degraded by islet macrophages (19). The increased diabetes susceptibility observed in transgenic mice with β cell expression of hIAPP suggests that hIAPP aggregation is a cause and not merely a consequence of β cell dysfunction (20–22). Furthermore, prefibrillar aggregates are directly toxic to β cells in vitro (23, 24). hIAPP aggregation is also associated with β cell death in cultured human islets (10, 25) and with recurrence of hyperglycemia following islet transplantation (9, 12). Rodent islet amyloid polypeptide (rIAPP) does not share this toxicity because three proline substitutions between aa 20 and 29 disrupt the β-sheet formation required for aggregation (26).

Aggregates of hIAPP share a common cross β-sheet structure with those of other amyloidogenic peptides known to induce a potent proinflammatory response. Peptides such as enterobacterial CsgA and mammalian amyloid-β (Aβ), which forms amyloid plaques in Alzheimer’s disease, induce TLR signaling in monocytes (27), providing one potential pathway for amyloid-induced pro–IL-1β synthesis (28, 29). Aβ also promotes pro–IL-1β maturation (the second signal required for secretion of mature IL-1β) via activation of the NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome (30). We therefore hypothesized that hIAPP may contribute to islet inflammation by

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Abbreviations used in this article: Aβ, amyloid-β; BMMDC, bone marrow-derived macrophage; hIAPP, human islet amyloid polypeptide; IAPP, islet amyloid polypeptide; IL-1Ra, IL-1R antagonist; LDH, lactate dehydrogenase; NLRP3, NACHT, LRR, and PYD domain-containing protein 3; qRT-PCR, quantitative RT-PCR; rIAPP, rodent IAPP.

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activating the same innate immune pathways. To address this hypothesis, we first characterized the effects of hIAPP aggregation on cytokine release by bone marrow-derived macrophages (BMDMs) and chemokine release by islets. We next assessed the effects of IL-1R and TLR signaling on the macrophage response to hIAPP. Finally, we evaluated the effects of IL-1Ra on hIAPP-induced islet dysfunction and macrophage recruitment in a rodent model of islet transplantation.

Materials and Methods

Animals

C57BL/6J, B6.129-Tlr2tm1Kir/J (Tlr2-/-), C57BL/10J, C57BL/10ScNJ (Tlr4-/-), FBV/N-Tg(Ins2-IAPP)/RHFSoi3l (hIAPP transgenic), and NOD.CB17-Prkdcscid/LtJ (NOD/SCID) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), C57BL/6 Myd88-/- mice were purchased from OrientalBioScience (Kyoto, Japan). Heterozygotes were bred in-house to produce littermate controls. All animals were maintained in compliance with Canadian Council on Animal Care guidelines, and studies were approved by the University of British Columbia Committee on Animal Care.

Cell culture

All incubations were performed at 37°C with 5% CO2. THP-1 monocytes (ATCC TIB-202) were maintained in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 ml Glutamax, 10 µM HEPES, 1 mM sodium pyruvate, and 10% FBS (all from Invitrogen, Burlington, ON, Canada). To generate BMDMs, bone marrow was flushed out of mouse femurs and plated in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM Glutamax, 1 mM sodium pyruvate, 10% FBS, and 15% L929-conditioned media (AbLab, Biomedical Research Centre, University of British Columbia). After 24 h, nonadherent cells were transferred to tissue culture-treated plates and incubated for 7 d, with addition of fresh media on days 3 and 6. Adherent BMDMs were detached by gentle scraping in PBS and plated at a density of 100,000 cells/well of a 96-well plate. After 24 h, cells were starved of L929 media for 10–12 h prior to each experiment.

Peptides and inhibitors

hIAPP and rIAPP (Bachem, Torrance, CA) were dissolved in hexafluoro-2-propanol for 3 h, lyophilized, and stored at −20°C until use. Immediately prior to each experiment, islet amyloid polypeptide (IAPP) was dissolved in 0.1 M acetic acid, which was diluted in culture media to a final maximum concentration of 395 nM acetic acid. Inhibitors were added to BMDMs as indicated, including polymyxin B (Sigma-Aldrich, Oakville, ON, Canada), clodronate, polymyxin B (Sigma-Aldrich, Oakville, ON), caspase-1 inhibitor Z-YVAD-FMK (Sigma-Aldrich), and the actin polymerization inhibitor cytochalasin D (Sigma-Aldrich), the cathepsin B inhibitor CA-074-Me (EMD Chemicals, Gibbstown, NJ), the NLRP3 inhibitors glibenclamide and Bay 11-8072 (Sigma-Aldrich), and thapsigargin B inhibitor CA-074-Me (EMD Chemicals, Gibbstown, NJ), and treated with varying concentrations of hIAPP or rIAPP for the indicated length of time. Supernatants were collected and stored at −80°C until analysis. Thawed supernatants were diluted 1:1 with RPMI 1640 containing 0.1% BSA, and 70 µl of the suspension was added to the upper chamber of the Transwell plate. After 4 h, the media in the bottom wells were collected and the number of cells was determined by analyzing each sample on a flow cytometer for 15 s and interpolating from a standard curve. The chemotactic index was the ratio of the concentration of migrated cells in the test sample to that in the media control, because in our preliminary experiments IAPP alone did not induce monocyte chemotaxis.

Iset culture

Islet macrophage depletion

Prior to evaluation of hIAPP-induced islet CCL2 release, isolated islets were allowed to recover overnight and incubated for 24 h with 1 mg/ml clodronate delivered in liposomes to deplete islets of resident macrophages. Liposomes were prepared, as described previously (33), using phosphatidylcholine (LIPOID E PC; Lipoid, Ludwigshafen, Germany) and cholesterol (Sigma-Aldrich). Clodronate (or clodronate) was a gift of Roche Diagnostics (Mannheim, Germany). Islets were washed prior to subsequent experiments, and depletion of macrophages was verified by quantitative RT-PCR (qRT-PCR).

Global gene expression

C57BL/6 BMDMs were treated with hIAPP or rIAPP for 12 h. RNA was isolated using an RNeasy mini kit (Qiagen, Mississauga, ON, Canada), and quality was assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA). All samples had RNA integrity number scores >9.2. Global gene expression was evaluated with an Illumina MouseRef-8 v2 Expression Bead Chip with an Illumina Beadstation 500GX BeadArray Reader (Illumina, San Diego, CA). Data were analyzed with Bead Studio software using rank-invariant normalization. Only transcripts with detection and differential expression values <0.05 were included in the analysis. Differentially expressed genes (absolute fold difference >1.5) were uploaded to the Database for Annotation, Visualization, and Integrated Discovery functional clustering tool (34). Functional annotation analysis was used to evaluate overrepresentation of biological pathways based on Gene Ontology terms. The significance of overrepresented terms was determined by a Benjamini p value to correct for multiple comparisons. Array data were deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus database as accession number GSE23534 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23534).

qRT-PCR

cDNA was synthesized using 100 ng RNA and a qScript cDNA synthesis kit, according to the manufacturer’s instructions (Quanta Biosciences, Gaithersburg, MD). qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, U.K.), and reactions were run on an ABI 7500 Fast Real-Time PCR System. Primer sequences were as follows (all mouse specific): Tnf (PrimerBank ID 7305585a1) forward 5′-CCCTCACACTAGATCCTTCT-3′, reverse 5′-GATACGACCTGATGGCTACAG-3′; Il1b (PrimerBank ID 6680415a1) forward 5′-GCACTGTTCGCCAATTCACT-3′, reverse 5′-ATCATTTTGGGTCCTGGCAACT-3′; Il1a (PrimerBank ID 6679937a1) forward 5′-CAGGCCGAGAGGGAGTCAAC-3′, reverse 5′-CAGGAACTTGGCTCATTGAT-3′; Emr1 (PrimerBank ID 2078512a1) forward 5′-GTCCTGAACATCAGGCCTAC-3′, reverse 5′-ATCATTTTGGGTCCTGGCAACT-3′; Il4 (PrimerBank ID 13262688b1) forward 5′-CATGATCCCTCAAGGAAGTGC-3′, reverse 5′-ACGCCTTGCTGCTGATGTC-3′; Ccl2 (PrimerBank ID 6755430a1) forward 5′-TCAATGGGCTTGAACACACAAA-3′, reverse 5′-TCCTGAGGTATTTAGATGGCTG-3′; Gsdm1 (PrimerBank ID 6679937a1) forward 5′-AGTGGCTGGTTGAGCGATTTT-3′, reverse 5′-TGATAGCATCGTATGGACGTCAC-3′; and Rplp0 (PrimerBank ID 6671569a1)
forward 5'-AGATTCCGGATATGCTGTTGGC-3', reverse 5'-TCGGGTCTTAGACCGTGTTC-3'. Differential expression was determined by the 2−ΔΔCt method (37) using GAPDH (macrophages) or Rppl0 (islets) as the internal control.

**Islet transplantation**

Islets were isolated from 12- to 16-wk-old FVB/N-Tg(Ins2-IAPP)RHFSoel/J mice or wild-type littermate controls and allowed to recover overnight prior to transplantation. Recipient 11-wk-old NOD/SCID mice were rendered hyperglycemic by a single i.p. injection of 200 mg/kg streptozotocin (Sigma-Aldrich) in citrate buffer. One day prior to transplantation, recipient mice were implanted with an ALZET mini-osmotic pump (Durect, Cupertino, CA) containing PBS or IL-1ra. Pumps were replaced every 2 wk for the duration of the experiment. Recipient mice were transplanted with 150 islets into the left renal subcapsular space 4 d after streptozotocin injection, when blood glucose levels were >20 mmol/l. Animals were monitored twice weekly for nonfasting tail vein blood glucose levels using a glucometer (OneTouch, Burnaby, BC, Canada). After 8 wk, glucose tolerance was evaluated by measurement of glucose at 0, 15, 30, 60, and 120 min following i.p. injection of 1.5 g dextrose per kg body weight. Recipient mice were implanted with an ALZET mini-osmotic pump (Durect, Cupertino, CA) containing PBS or IL-1ra. Pumps were replaced every 2 wk for the duration of the experiment. Recipient mice were transplanted with 150 islets into the left renal subcapsular space 4 d after streptozotocin injection, when blood glucose levels were >20 mmol/l. Animals were monitored twice weekly for nonfasting tail vein blood glucose levels using a glucometer (OneTouch, Burnaby, BC, Canada). After 8 wk, glucose tolerance was evaluated by measurement of glucose at 0, 15, 30, 60, and 120 min following i.p. injection of 1.5 g dextrose per kg body weight. Removal of the islet graft-bearing kidney was performed to rule out effects of residual pancreatic islet function, and all mice included in our analyses returned to hyperglycemia within 24 h. Grafts were fixed in 10% formalin and processed for histology.

**Thioflavin assays**

hIAPP aggregation was monitored by Thioflavin T fluorescence, as described previously (38). In brief, peptide was dissolved in phenol red-free DMEM containing 10 μM Thioflavin T. Fluorescence emission was evaluated on a Fluoroskan Ascent reader (excitation 444 nm; emission 485 nm). Cell viability was assessed using the colorimetric MTT assay. Cytotoxicity was assessed using the luminometric LDH release assay (39). All image analyses, including deconvolution of z-stacks, were performed using Image-Pro software (MediaCybernetics, Bethesda, MD).

**Immunohistochemistry**

Paraffin-embedded sections (5 μm) from different regions of each islet graft were deparaffinized and rehydrated. Ag retrieval was performed by incubation in Target Retrieval Solution (Dako, Carpinteria, CA) in a steamer for 20 min. Sections were blocked with 2% normal goat serum (Vector Laboratories, Burlingame, CA) and visualized on a BX61 microscope (Olympus, Center Valley, PA). All image analyses, including deconvolution of z-stacks, were performed using Image-Pro software (MediaCybernetics, Bethesda, MD).

**Cytokine and chemokine analyses**

Supernatants were stored at −80°C prior to analysis. An array of 12 cytokines and chemokines (CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL2, CXCL10, IL-1α, IL-1β, IL-6, TNF-α, IL-12p70) was assessed by multiplex assay (Millipore, Bedford, MA) and analyzed on a Luminex-100 system, according to the manufacturer's instructions (Luminex, Austin, TX). For inhibitor studies, TNF-α, IL-1β, and CCL2 levels were determined by ELISA (eBioscience, San Diego, CA, or BioLegend, San Diego, CA).

**Statistical analyses**

Data are expressed as mean ± SD of the indicated number of replicates. All cell culture experiments were performed with three to four replicate wells per treatment. Data were analyzed using a nonlinear regression analysis program (GraphPad Prism, La Jolla, CA). Differences between two groups were evaluated with a two-tailed t test. Differences among three or more groups were evaluated with a one-way ANOVA and Bonferroni posttests to compare all replicate means or Dunnett posttests to compare all means to a single control. Differences among three or more treatments from multiple groups were evaluated with a two-way ANOVA and Bonferroni posttests. For figures in which representative experiments are shown, data from other experiments showed the same statistically significant differences, but with different absolute values due to variation in IAPP activity among lots of peptide. A p value <0.05 was considered significant.

### Results

**Prosurvival effects of IAPP in BMDMs**

To evaluate the possible effects of hIAPP on macrophage viability, BMDMs were treated with hIAPP or rIAPP (15 μM) in the absence of M-CSF for 12 h. Aggregated peptide was observed by Thioflavin S staining of hIAPP-treated cells, often in association with cell membranes (Fig. 1A). Interestingly, both hIAPP and rIAPP inhibited LDH release associated with M-CSF deprivation, with a reduction to 83 ± 7% of the untreated control for rIAPP and 62 ± 3% for hIAPP after 12 h (Fig. 1B). Similarly, IAPP protected against macrophage loss, with an average cell number per field of 175 ± 26 for hIAPP and 80 ± 14 for rIAPP compared with 57 ± 2 in the untreated control after 12 h (Fig. 1C). These data suggest that IAPP can provide a prosurvival signal to macrophages even in the absence of aggregation; however, the more dramatic effect of hIAPP compared with rIAPP suggests that peptide aggregation provides an additional survival stimulus, similar to proinflammatory agents such as LPS.

**Proinflammatory cytokine release by macrophages in response to hIAPP**

To determine whether hIAPP induces expression of proinflammatory genes, we performed global gene expression analysis to compare expression profiles between hIAPP- and rIAPP-treated BMDMs after 12 h. Functional annotation analysis revealed significant enrichment of Gene Ontology terms among genes that were differentially expressed in response to hIAPP (Supplemental Table I). Overrepresented pathways among the upregulated genes included those associated with actin polymerization; angiogenesis and wounding; and regulation of the immune response, including cytokine and chemokine expression (Table I). Downregulated groups included genes associated with nucleosomes, membrane-enclosed lumens, regulation of cell death, and protein serine/threonine kinase activity.

To explore further the cytokine profile of hIAPP-treated cells, we analyzed supernatants from BMDM cultures treated with IAPP for 12 h (Fig. 2A). hIAPP, but not rIAPP, induced significant levels of IL-1α (11.7 ± 2.3 versus <2.9 pg/ml), IL-1β (27.2 ± 2.7 versus 2.6 ± 1.4 pg/ml), TNF-α (134 ± 23 versus 5.9 ± 0.4 pg/ml), CCL2 (169 ± 8 versus 18 ± 2 pg/ml), CXCL1 (522 ± 110 versus 91 ± 23 pg/ml), CCL3 (6.96 ± 2.1 versus 0.10 ± 0.01 ng/ml), CCL4 (4.97 ± 1.4 versus 0.08 ± 0.01 mg/ml), CCL5 (7.6 ± 5.9 versus <3.0 pg/ml), and CXCL5 (2.95 ± 0.64 versus 1.92 ± 0.25 ng/ml). A trend toward increased IL-6 was observed but did not reach statistical significance (31.0 ± 19.1 versus 14.4 ± 10.1 pg/ml). Induction of mRNA encoding IL-1α, IL-1β, and TNF-α was also confirmed by qRT-PCR (Fig. 2B).

Because the combination of TNF-α and IL-1β has well-characterized inhibitory effects on insulin secretion and at high concentrations promotes β cell apoptosis (39), we chose these cytokines for further mechanistic studies. TNF-α induction in BMDMs after 24 h was hIAPP concentration dependent (Fig. 3A) and associated with increased cell viability relative to rIAPP-treated cells (181 ± 5 versus 142 ± 5% of the untreated control, respectively; Fig. 3B). No apoptotic death of hIAPP-treated BMDMs was observed at concentrations up to 15 μM after 24 h (Fig. 3C). The LPS inhibitor polymyxin-B had no effect on hIAPP-induced TNF-α release (Fig. 3D), suggesting that these effects were not due to endotoxin contamination.
Effect of hIAPP aggregation state on TNF-α release

To determine which state of hIAPP fibrillogenesis is responsible for TNF-α and IL-1β induction, IAPP was dissolved in media and aggregation was monitored by Thioflavin T fluorescence (Fig. 4A). Thioflavins undergo a red shift in their emission spectra upon binding to β-sheet motifs present in fibrillar hIAPP aggregates (40). BMDMs were treated with freshly dissolved peptide or peptide that had been allowed to aggregate prior to the experiment. Maximal release of TNF-α (Fig. 4B), IL-1α (Fig. 4C), and IL-1β (Fig. 4D) was observed with freshly dissolved peptide presumably containing primarily prefibrillar aggregates, whereas hIAPP that had been allowed to aggregate for 6 h prior to addition to BMDMs induced much smaller increases in cytokine levels (TNF-α, 260 ± 43 versus 968 ± 235 pg/ml; IL-1α, 3.2 pg/ml versus 199 ± 68; IL-1β, 160 ± 22 versus 451 ± 127 pg/ml). Following 7 d of aggregation, induction of both IL-1α and IL-1β by hIAPP fibrils was even further reduced (Fig. 4C, 4D). Consistent with these observations, TNF-α secretion induced by freshly dissolved hIAPP did not follow the kinetics of LPS-induced release (Fig. 4E), sug-

Table I. Functional annotation analysis of hIAPP-induced gene expression in BMDMs

<table>
<thead>
<tr>
<th>Pathway</th>
<th>FE</th>
<th>p Value</th>
<th>Differentially-Expressed Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin filament-based process (GO:0030029)</td>
<td>3.2</td>
<td>0.011</td>
<td>MYL6, MTSS1, LIMA1, PDGFB, PDLIM7, PDGFA, GM8034, SRF, PFN1, GM5915, EZR, LOC100044675, BCL2, LOC100044177, GM3787, PLCG1, FMR1, ACTN4, ACTN1, TMSB10, GM10080, GM8984, ELM01, LOC100048142, NAPR, APBP2, GM9844, LCPI1</td>
</tr>
<tr>
<td>Vascular development (GO:0001944)</td>
<td>2.9</td>
<td>0.003</td>
<td>GNA13, TNFRSF12A, PDGFA, EDN1, ANPEP, SRF, CITED2, ARHAGAP22, LOC100048867, HMOX1, ITGA5, TGM2, IL1B, RHOB, LOX, PPA2B, RAPGEF1, COL18A1, PDPN, PHK1, MMP14, JUNB, ANXA2, VEGFC, JMD16, HBE2GF</td>
</tr>
<tr>
<td>Response to wounding (GO:0009611)</td>
<td>2.7</td>
<td>0.002</td>
<td>GNA13, CXCL1, CCL3, GM8762, TNF, CCL2, NFKBI, TOLLIP, PPARC, CXCL2, SGMS1, CCL4, CCL7, MIF, CD44, BCL2, MTPN, IL1B, GM16379, IL1A, KLF6, GM5365, F10, PLEK, PIK3CB, GM6097, GM10169, MAP2K3, TLR7, SPH1K1, ANX5, NLRP3, CD180, PLAUR, HBE2GF, LOC100044948, CD14, LCPI1</td>
</tr>
<tr>
<td>Immune response (GO:0006955)</td>
<td>2.3</td>
<td>0.005</td>
<td>CXCL1, CCL3, GM8762, TNF, CCL2, TOLLIP, CXCL2, RSAD2, IL7R, CCL4, CCL7, MIF, KHL6, CLEC4E, SQSTM1, GP49A, LOC641240, OASL2, IL1B, GM16379, IL1A, RCMS2, GM6097, H2-M2, GM10169, TLR13, H2AB1, MAL1, TNFSP9, NLRP3, VAV1, CD180, LOC100046079, LAT2, FCGR2B, GADD45G, LILRB4, OAS1A, LOC100044948, OAS1G, CLEC5A, CD14, LCPI1, TNFAIP1, CD300LD</td>
</tr>
</tbody>
</table>

Cells were treated with hIAPP or rIAPP for 12 h (n = 4 per treatment). Global gene expression was analyzed by mouse 8-chip Illumina array. Pathway overrepresentation analysis was performed on genes upregulated by at least 1.5-fold using the Database for Annotation, Visualization, and Integrated Discovery functional annotation tool. Enriched Gene Ontology terms with a Benjamini p value < 0.01 were considered highly significant. FE, fold enrichment.
gesting a mechanism distinct from immediate cell surface TLR activation and a delay in response until the proinflammatory species was formed. Furthermore, hIAPP-induced TNF-α release was almost completely inhibited by Congo red (Fig. 4F), an inhibitor of IAPP fibril formation (41). Taken together, these data suggest that exposure of macrophages to intermediate hIAPP species, rather than preformed fibrils, is required for maximal cytokine release.

**FIGURE 3.** hIAPP-induced TNF-α release is dose dependent and not due to cell death or endotoxin contamination. A, TNF-α production in response to treatment with 5–20 μM IAPP for 24 h was determined by ELISA. B, Viability was assessed by Alamar Blue fluorescence. C, Cells were fixed after 24 h, and cell death was evaluated by ArrayScan VTI HCS analysis of TUNEL staining. D, hIAPP-induced TNF-α release was evaluated in the presence of the cationic peptide polymyxin-B. Data are representative of five independent experiments with three replicates per treatment. ***p < 0.001.

**FIGURE 2.** hIAPP induces proinflammatory cytokine release by BMDM. A, Cells were incubated with hIAPP or rIAPP (15 μM) for 12 h. Supernatants were assessed for proinflammatory cytokines and chemokines with a multiplex assay. B, Gene expression was evaluated by qRT-PCR. Data are representative of three independent experiments with four replicates per treatment. **p < 0.01, ***p < 0.001.
Role of MyD88 and IL-1R signaling in hIAPP-induced TNF-α release

To determine whether TLR pathways are involved in the macrophage response to hIAPP, as described for other amyloidogenic peptides, we evaluated TNF-α induction in BMDMs from mice lacking TLR signaling pathway components. hIAPP-induced TNF-α release was markedly attenuated in BMDMs lacking MyD88 (0.13 ± 0.01 versus 5.27 ± 0.69 ng/ml; Fig. 5A), but not TLR2 (Fig. 5C) or TLR4 (Fig. 5D). A specific peptide inhibitor of MyD88 homodimerization also reduced hIAPP-elicited TNF-α release in wild-type BMDMs from 3.66 ± 0.28 to 1.68 ± 0.53 ng/ml (Fig. 5B). Thus, the MyD88 dependency of this cytokine cannot be attributed to nonredundant TLR2 or TLR4 signaling.

Because the IL-1R also uses the adaptor protein MyD88, and IL-1 is known to induce TNF-α (42), we evaluated the effect of IL-1 signaling inhibition on hIAPP-induced TNF-α release. Inhibition of pro-IL-1β cleavage with a caspase-1 inhibitor and blockade of IL-1 signaling with IL-1Ra significantly attenuated hIAPP-induced TNF-α release (Fig. 5E). To determine whether NLRP3 inflammasome signaling might be involved in caspase-1 activation leading to IL-1β secretion (a process that requires both synthesis of pro-IL-1β and cleavage of the propeptide to its mature form), we evaluated the effects of NLRP3 inflammasome inhibitors on hIAPP-induced IL-1β and TNF-α secretion (Fig. 5F, 5G). Cytokine induction was significantly reduced by Bay 11-8072, an inhibitor of both NF-κB and the NLRP3 inflammasome (43), and by glibenclamide, an inhibitor of ATP-sensitive potassium channels that also blocks NLRP3 activation (44). Cytokine release was dependent on actin polymerization, because it was blocked by cytochalasin D. The potential requirement for phagocytosis is further emphasized by the significant overrepresentation of genes related to actin filament-based processes in our microarray study (Table I). As described for other inflammasome activators, cytokine release was blocked by CA-074-Me, an inhibitor of the lysosomal enzyme cathepsin B. Thus, hIAPP can provide both signals required for IL-1β secretion, and autocrine or paracrine signaling by IL-1α or IL-1β appears to be at least partially responsible for amplification of TNF-α release.

Effect of hIAPP on islet chemokine release and monocyte recruitment ex vivo

To determine whether hIAPP aggregation causes chemokine release from islets, we measured CCL2 and CXCL1 release by whole islets treated with rIAPP or hIAPP for 24 h. We found that both chemokines were markedly upregulated in response to hIAPP, but not rIAPP (Fig. 6A). This effect was preserved following macrophage depletion of islets with clodronate-liposomes, verified by decreased expression of genes encoding F4/80 and CD11b with no significant effect on Ins2 or Ccl2 expression (Supplemental Fig. 1). To assess the effect of endogenously produced hIAPP on CCL2 and CXCL1 release, we cultured islets from transgenic mice expressing hIAPP under the control of a β cell-specific promoter. Male homozygous FVB/N-Tg(Ins2-IAPP)RHFSoel/J mice spontaneously develop diabetes by 8 wk of age (20), whereas the hemizygous animals are phenotypically normal and do not...
develop detectable amyloid deposits (45). However, isolated hemizygous islets form extracellular amyloid deposits after 8 d of culture with high glucose (46). In our experiments, a small amount of Thioflavin S-positive material was also observed in islets cultured with 11 mM glucose for 7 d, but not in freshly isolated islets (Supplemental Fig. 2). After 96 h in culture, we observed higher levels of CCL2 (288 ng/ml) and CXCL1 (1294 ng/ml) for 12 h prior to addition of IAPP (15 μM) or LPS (10 ng/ml). B, Wild-type BMDMs were pretreated with MyD88 homodimerization inhibitory peptide or control peptide (both 100 μM) for 12 h prior to addition of IAPP (15 μM) or LPS (10 ng/ml) for 24 h. TNF-α levels were determined by ELISA. C, The caspase-1 inhibitor Z-YVAD-FMK (40 μM) and IL-1Ra (4 μg/ml) were added to BMDM cultures 2 h prior to treatment with hIAPP for 24 h. TNF-α levels were determined by ELISA. D, TNF-α (F) and IL-1β (G) levels were determined by multiplex assay following 24-h culture with hIAPP in the presence of the NLRP3 inhibitors Bay 11-8072 (Bay, 5 μM), glibenclamide (GLB, 200 μM), cytochalasin D (CD, 5 μM), or CA-074-Me (CA-074, 80 μM). Statistical significance is shown relative to the hIAPP-treated control. Data are representative of two (C, D, F, G), three (B, E), or six (A) independent experiments. **p < 0.01, ***p < 0.001. UT, untreated.

These results are consistent with the hypothesis that chemokines produced by resident islet cells attract immune cells to sites of hIAPP aggregation.

**Effect of IL-1Ra on islet function and macrophage recruitment in vivo**

To determine whether hIAPP expression is associated with impaired islet function in vivo, we transplanted islets from hIAPP transgenic and wild-type littermate control mice into diabetic NOD/SCID recipients treated with 50 mg/kg/d IL-1Ra or PBS. Recipients of transgenic islet grafts displayed impaired glucose tolerance 8 wk following transplantation compared with recipients of wild-type grafts (area under the curve = 1940 ± 80 versus 1260 ± 60, p < 0.001). Administration of IL-1Ra reduced nonfasting hyperglycemia associated with islet hIAPP expression (Supplemental Fig. 3) and significantly improved glucose tolerance in recipients of transgenic grafts (area under the curve = 1470 ± 160 versus 1940 ± 80, p < 0.05), but not wild-type grafts (Fig. 7A, 7B). No differences in body weight were observed among transplant recipients (Supplemental Fig. 3). Quantification of intragraft staining for the macrophage glycoprotein F4/80 demonstrated that hIAPP-expressing grafts contained 50% more macrophages than wild-type grafts, an effect that was significantly inhibited by IL-1Ra (Fig. 7C). Furthermore, a 5-fold reduction in amyloid area was observed in transgenic grafts from IL-1Ra–treated recipients (Fig. 7D).
Interestingly, most Thioflavin S-positive amyloid deposits in transgenic grafts were associated with F4/80-expressing macrophages, both extra- and intracellularly (Fig. 7E).}

**Discussion**

hIAPP fibrils are present within the lysosomes of pancreatic macrophages in type 2 diabetic humans, monkeys, and hIAPP-expressing transgenic mice (47). Autopsy studies have also demonstrated more islet macrophages together with increased IL-1β expression in type 2 diabetic islets compared with nondiabetic controls (3, 5, 6, 17). Our data suggest a possible link between hIAPP aggregation and islet inflammation, with four novel findings, as follows: 1) prefibrillar hIAPP species induce proinflammatory cytokine and chemokine release by BMDMs, an effect that is blocked by inhibition of fibrillogenesis; 2) hIAPP-induced TNF-α release is almost entirely dependent on MyD88, an effect that is at least partially explained by autocrine/paracrine effects of IL-1α and/or IL-1β; 3) hIAPP expression is associated with chemokine release by islets ex vivo and increased islet macrophage content in vivo; and 4) inhibition of IL-1R signaling improves hIAPP-induced pancreatic islet graft dysfunction.  

Our observation that hIAPP aggregation induces release of multiple proinflammatory cytokines and chemokines by BMDMs is consistent with previous studies in neurons and glia. Like Aβ, hIAPP induced IL-6 and IL-8 secretion by human astrocytoma cells (48) and release of IL-1β, TNF-α, IL-6, IL-8, CCL3, and CCL4 by LPS-activated THP-1 monocytes, a model for human microglia (29). Our data suggest that prefibrillar hIAPP induces proinflammatory cytokine release by both macrophages and islets in the absence of priming by a distinct TLR ligand, consistent with previously reported increases in monocyte IL-1β and TNF-α mRNA in response to hIAPP alone (29) and in response to Aβ (30). Unlike monocytes, macrophages do not express constitutively active caspase-1 (49), and we found that hIAPP mediates IL-1β secretion in a caspase-1–dependent manner. hIAPP-induced NLRP3 activation in dendritic cells was recently described by Masters et al. (50), although the lack of significant TNF-α induction reported by these authors may be explained by peptide handling affecting the aggregation state of the peptide or by cell type-specific effects.

TLR4 signaling is required for efficient microglial clearance of Aβ deposits in the mouse brain (51) and is also involved in Aβ-induced neuronal cell death (52). Both CD36-mediated TLR4–TLR6 heterodimer formation (53) and TLR2 activation (27, 54) have also been implicated in the response to Aβ. We found hIAPP-induced TNF-α release from macrophages to be largely MyD88 dependent. TNF-α levels in MyD88-deficient BMDMs were nevertheless significantly increased compared with untreated BMDMs.
controls and reached ∼3% of the levels observed in wild-type cells, which may implicate other, as yet undefined mechanisms. In any case, because TLR2 and TLR4 deficiency had no detectable effect on hIAPP-induced TNF-α release, these pathways do not appear to represent necessary mechanisms of innate immune activation by all amyloidogenic peptides.

That amplification of hIAPP-induced TNF-α release was inhibited not only by MyD88 deficiency, but also by blockade of caspase-1 and IL-1R suggests a role for IL-1α or IL-1β release in the autocrine or paracrine amplification of other cytokines. Recent data suggest that hIAPP-induced NLRP3 activation, like that of Aβ, is associated with lysosomal damage in dendritic cells (50), and hIAPP fibrils condense within macrophage lysosomes to form proteolysis-resistant protofilaments (19). Consistent with these reports, we have shown that hIAPP-induced IL-1β secretion in macrophages is blocked by the cathepsin B inhibitor CA-074-Me. Because CA-074-Me and the inhibitor of actin polymerization cytochalasin D also attenuated TNF-α release, lysosomal damage may be a major mechanism mediating the proinflammatory activity of hIAPP. However, additional studies are required to identify the initial stimulus for pro–IL-1β gene transcription, which presumably may also contribute to Tnf transcriptional activation. Possible mechanisms include events downstream of lysosomal dysfunction, endosomal TLR signaling, and initial activation of the IL-1R by hIAPP-induced IL-1α release.

The toxicity of synthetic hIAPP toward β cells depends on the peptide’s aggregation state and on the association of growing fibrils with the plasma membrane (23). Our data support the hypothesis that prefibrillar hIAPP aggregates also act as the principal proinflammatory stimulus, because the ability of hIAPP to induce both TNF-α and IL-1β release declined when the peptide was added to macrophages in a more aggregated form. This finding is consistent with a previous report of maximal TNF-α induction by prefibrillar Aβ species (55). Although TNF-α induction by hIAPP was significantly reduced in the presence of the dye Congo red, an inhibitor of amyloid formation (41), this dye could also act by inhibiting interactions between hIAPP and the cell membrane. Nevertheless, inhibitors of hIAPP aggregation, which protect cultured human islets from hIAPP-induced cell death (10), may achieve this effect in part by limiting IL-1β secretion.

Hemizygous expression of the hIAPP transgene in islet β cells induces islet amyloid formation under conditions that increase β cell secretory demand and dysfunction, including high fat feeding (21), ex vivo culture (46), and islet transplantation (12). In this study, we have shown that both synthetic and endogenously produced hIAPP potentiate islet chemokine release ex vivo and are
associated with increased numbers of islet macrophages following transplantation. Migration of monocytes toward media conditioned by hIAPP-treated human islets also suggests that the chemokine release observed in transgenic islets has functional implications. One previous study reported no difference in macrophage density between amyloid-containing and amyloid-free human and monkey islets (47); however, immune cell recruitment may occur prior to formation of extensive fibrillar deposits, which most likely accompany resolution of the inflammatory response. This hypothesis is consistent with our finding of maximal proinflammatory activity of prefibrillar hIAPP in BMDM cultures and with chemokine release by transgenic islets in the absence of widespread amyloid deposition.

Recruitment of macrophages to islet grafts may result directly from hIAPP-induced islet chemokine release, or could be an indirect consequence of sterile inflammation caused by hIAPP-induced cell death. We found no significant differences in the number of TUNEL-positive β cells among the treatment groups 8 wk following transplantation (data not shown), suggesting that extensive hIAPP-induced cell death is not a requirement for graft inflammation. Furthermore, we have demonstrated hIAPP-induced chemokine release from transgenic mouse islets ex vivo in the absence of significant cell death, as determined by LDH release and Alamar Blue reduction. Although our in vitro studies suggest that interaction of hIAPP with macrophages induces release of cytokines that at high local concentrations are known to cause β cell dysfunction (4), we cannot rule out the possibility that macrophages play an important role in regulating islet homeostasis. Indeed, our microarray data suggest hIAPP-induced upregulation of pathways involved in angiogenesis and wound healing, consistent with the proangiogenic role of islet macrophages described in a mouse model of chronic pancreatitis (56). Nevertheless, improved glucose tolerance was associated with a reduction in the number of F4/80-positive cells within transgenic grafts from recipients treated with IL-1Ra. Further phenotypic and functional characterization of these cells will help to elucidate their potential effects on islet function.

The improved glucose tolerance in IL-1Ra–treated recipients of hIAPP transgenic islets suggests a common mechanism of hIAPP-induced β cell dysfunction in type 2 diabetic and transplanted islets. Inhibition of hIAPP-mediated IL-1 signaling may partially explain the improvement in β cell secretory function in type 2 diabetic patients receiving anakinra (15), and our data suggest that blockade of IL-1 signaling may also promote long-term islet graft function. Although systemic IL-1Ra may also increase peripheral insulin sensitivity, we have observed no differences in insulin resistance between hemizygous hIAPP transgenic mice and wild-type littermate controls (C. Westwell-Roper, unpublished data). Furthermore, there was no significant effect of IL-1Ra on glucose tolerance in recipients of wild-type grafts in our NOD/SCID transplant model, suggesting a specific effect on hIAPP-associated islet pathology. Interestingly, we observed decreased amyloid deposition in hIAPP-expressing grafts in recipients of IL-1Ra, suggesting that IL-1–mediated islet inflammation may not only be a consequence, but also a cause of hIAPP aggregation.

Finally, a number of potential macrophage-independent mechanisms for hIAPP-induced inflammation must be considered in future studies, some of which may involve fibrils rather than prefibrillar species. For example, fibrillar IAPP induces complement activation and may contribute to β cell death via anaphylotoxin release or membrane attack complex formation (57). In addition to islet macrophages, endocrine cells, endothelial cells, and dendritic cells may modulate hIAPP-induced effects in vivo. Our data demonstrate that hIAPP aggregation causes release of chemokines such as CCL2 by whole islets, including β cells, and that this effect is preserved upon macrophage depletion. Consistent with hIAPP-induced monocyte recruitment to the islet, we observed more macrophages within hIAPP transgenic grafts, and these cells were present in association with amyloid plaques. Whether IL-1Ra–mediated improvements in islet function are due to inhibition of β cell chemokine release, attenuation of macrophage cytokine secretion, blockade of IL-1–induced β cell dysfunction, or a combination of these effects remains to be addressed.

In conclusion, this study suggests that prefibrillar hIAPP aggregates promote islet dysfunction not only via direct toxicity to β cells, but also by triggering a localized inflammatory response mediated by IL-1. Thus, strategies aimed at reducing hIAPP expression and aggregation may not only protect β cells from apoptosis, but also ameliorate deficits in insulin secretion associated with proinflammatory cytokine release. Because IL-1R signaling contributes to the amplification of hIAPP-induced cytokine and chemokine release by islets and macrophages, these data suggest a possible mechanism by which blockade of IL-1 signaling or hIAPP aggregation may significantly alter the inflammatory milieu of the pancreatic islet to improve β cell function in both type 2 diabetes and islet transplantation.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Fig. S1. Macrophage depletion does not affect hIAPP-induced Ccl2 expression. Islets were isolated from 12-wk-old C57BL/6 mice and incubated for 24 h with liposomes containing PBS or clodronate (1 mg/ml; PBS-lip or CLO-lip, respectively). (A) Expression of the macrophage markers Emr1 (F4/80) and Itgam (CD11b) was assessed by qRT-PCR to verify clodronate-mediated macrophage depletion. (B) Ins2 expression was assessed by qRT-PCR. (C) Islets were washed and treated with hIAPP or LPS for 24 h. Ccl2 expression was evaluated by qRT-PCR. *p < 0.05; **p < 0.01. Data show means ± SD of islets from 3 mice per group and are representative of 3 independent experiments.
Fig. S2. hIAPP transgenic islets develop Thioflavin S-positive deposits ex vivo. Islets were isolated from 12-wk-old transgenic hIAPP mice and wild-type littermate controls and cultured for 7 d in RPMI containing 11 mM glucose. Paraffin-embedded sections were stained for insulin (red), amyloid (Thioflavin S, green), and DAPI (blue). Scale bar: 25 μm.
Fig. S3. IL-1Ra ameliorates hyperglycemia in recipients of hAPP transgenic islet grafts. 150 islets from 12- to 16-wk-old wild-type or hAPP transgenic mice were transplanted into NOD/SCID recipients implanted with mini-osmotic pumps releasing PBS or IL-1Ra (50 mg/kg/d) for the duration of the experiment. (A) Morning non-fasting glucose levels in tail vein blood and (B) body weight were assessed 8 wks following transplantation. *, p < 0.05; **, p < 0.01. Data represent mean±SD.
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1Cells were treated with hIAPP or rIAPP for 12 h ($n=4$ per treatment). Global gene expression was analyzed by Illumina microarray. Pathway over-representation analysis was performed on genes up- and down-regulated by at least 1.5-fold using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional annotation tool. Enriched Gene Ontology (GO) terms with a Benjamini $p$-value $< 0.05$ are shown. FE: fold enrichment.