The intermediate proteasome is constitutively expressed in pancreatic beta cells and upregulated by stimulatory, non-toxic concentrations of interleukin 1 β

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Short title: The intermediate proteasome expression in pancreatic beta cells.

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Abbreviations

i-proteasome:	Immunoproteasome
int-proteasome:	Intermediate proteasome
s-proteasome:	Standard proteasome
$\beta 1$:	beta subunit 1
$\beta 2$:	beta subunit 2
$\beta 5$:	beta subunit 5
$\beta 1i$:	inducible beta subunit 1
$\beta 2i$:	inducible beta subunit 2
$\beta 5i$:	inducible beta subunit 5
PSMB8:	Proteasome subunit beta type-8 = $\beta 5i$
PSMB9:	Proteasome subunit beta type-9 = $\beta 1i$
PSMB10:	Proteasome subunit beta type-10 = $\beta 2i$
HBSS:	Hank's balanced salt solution
IL-1 β :	Interleukin 1 β
INF:	Interferon
MHC:	Major histocompatibility complex

48 **Abstract**

49 A central and still open question regarding the pathogenesis of autoimmune diseases, such as type 1
50 diabetes, concerns the processes that underlie the generation of MHC-presented autoantigenic
51 epitopes that become targets of autoimmune attack. Proteasomal degradation is a key step in
52 processing of proteins for MHC class I presentation. Different types of proteasomes can be expressed
53 in cells dictating the repertoire of peptides presented by the MHC class I complex. Of particular
54 interest for type 1 diabetes is the proteasomal configuration of pancreatic β cells, as this might
55 facilitate autoantigen presentation by β cells and thereby their T-cell mediated destruction. Here we
56 investigated whether so-called inducible subunits of the proteasome are constitutively expressed in β
57 cells, regulated by inflammatory signals and participate in the formation of active intermediate or
58 immuno-proteasomes.

59 We show that inducible proteasomal subunits are constitutively expressed in human and rodent islets
60 and an insulin-secreting cell-line. Moreover, the $\beta 5i$ subunit is incorporated into active intermediate
61 proteasomes that are bound to 19S or 11S regulatory particles. Finally, inducible subunit expression
62 along with increase in total proteasome activities are further upregulated by non-toxic concentrations
63 of IL-1 β stimulating proinsulin biosynthesis. These findings suggest that the β cell proteasomal
64 repertoire is more diverse than assumed previously and may be highly responsive to a local
65 inflammatory islet environment.

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71 **Introduction**

72 The proteasome is a multi-subunit complex essential for the proteolytic degradation of cellular
 73 proteins and in the generation of specific sets of bioactive peptides (1) influencing a variety of cellular
 74 processes e.g. transcriptional regulation, signaling and the regulation of the cell cycle progression (2-
 75 5).

76 Proteasomal activity is executed by the proteolytic core, known as the 20S proteasome. It consists of
 77 a stack of four heptameric rings: two outer α and two inner β rings (1). The β rings are composed of
 78 catalytically active subunits (β 1, β 2 and β 5) that cleave peptide bonds at the C-terminal side of
 79 proteins (6) with caspase-, trypsin- and chymotrypsin-like activities, respectively (7, 8). The standard
 80 26S proteasome contains a 19S regulatory cap that binds the polyubiquitin chain, denatures the
 81 protein, and feeds it into the proteolytic core of the proteasome (9).

82 Standard proteasomes (s-proteasome) assembled with β 1, β 2 and β 5 subunits are ubiquitously
 83 expressed, but specialized proteasomes also exist and are constitutively expressed by e.g. immune
 84 cells (9), where they represent the dominant form. Formation of the proteolytic core of these
 85 specialized proteasomes involves substitution of the constitutively expressed catalytic β 1, β 2 and β 5
 86 subunits with the interferon (IFN)- γ -inducible β 1i, β 2i and β 5i subunits (alternatively termed
 87 Psmb9/LMP2, Psmb10/MECL-1/LMP10 and Psmb8/LMP7, respectively) (6, 10, 11). The immune-
 88 proteasome (i-proteasome) has an alternative 20S catalytic core where all β -subunits are replaced by
 89 IFN- γ inducible β -subunits and where the 20S-associated 19S can be replaced by the 11S (also termed
 90 PA28 $\alpha\beta$) proteasome regulator (9, 12, 13).

91 When standard and inducible subunits are present in cells, the latter are preferentially incorporated
 92 into newly produced 20S proteasomes (14, 15). Interestingly, co-expression of standard and inducible
 93 β subunits enables cells to assemble a variety of distinct 20S complexes, collectively referred to as

intermediate proteasomes (int-proteasomes) (9). The two most common int-proteasomes are composed of two inner rings containing either $\beta 1/\beta 2/\beta 5i$ or $\beta 1i/\beta 2/\beta 5i$. These int-proteasomes are not exclusive, as other combinations have been observed, including 20S proteasome with one constitutive ($\beta 1/\beta 2/\beta 5$) and one immune ($\beta 1i/\beta 2i/\beta 5i$) inner ring (also called asymmetric proteasomes, (16-18)).

Immune cells permanently and many other cells under conditions of oxidative stress, inflammation, cytokine stimulation, or viral and bacterial infection express and assemble i- and int-proteasomes (9, 19). Recently, induction of expression of such proteasomes upon exposure of human pancreatic islets and rat and mouse insulinoma cells to $INF\gamma$ and β but not toxic concentrations of IL-1 β , was reported (18, 20). Furthermore, int-proteasomes (but not i-proteasomes) are constitutively expressed in various cells, including liver, heart, kidney, lung or colon (16, 21-24). They constitute between 1 % (heart) to 50 % (liver) of the total proteasome pool (16, 21, 23, 24).

The proteasomal composition in cells has broad implications, as proteasomes exhibit diverse substrate specificities. This affects the peptide repertoire generated for presentation on major histocompatibility complex (MHC) class I molecules (13, 19, 25), signal transduction via e.g. nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) (26) and protein degradation e.g. of proinsulin (27).

The s-proteasome is known to improve glucose-stimulated insulin secretion (28), regulate intracellular proinsulin levels (27) or protect against lipotoxic endoplasmic reticulum stress (29). However, the functions of i- and int-proteasomes are poorly defined. Importantly, *constitutive* expression of inducible proteasome subunits in pancreatic β cells has not been described, but their induction upon $INF\gamma$ and β treatment has been suggested to play a protective role against cytokine-induced apoptosis (20) and during antiviral responses (18).

Of special interest to type 1 and 2 diabetes pathogenesis is the constitutive profile of the β cell proteasomes and their regulation. Type 1 diabetes (T1D) is an autoimmune disease, in which

117 tolerance to β cells is broken, with proinsulin serving as a major autoantigen. T1D is histologically
 118 characterized by pancreatic islet inflammation with increased levels of cytokines i.e. IL-1 β , INF- γ / β
 119 and TNF- α , in the islet microenvironment (30). Type 2 diabetes (T2D) arises when insulin secretion
 120 fails to meet demands mainly due to impaired insulin sensitivity, with β -cell oxidative and
 121 endoplasmic reticulum stress, lipotoxicity and glucotoxicity as consequences causing progressive loss
 122 of β cell functional mass (31). All these cellular stresses induce an inflammatory response or are
 123 exacerbated by or associated with low-grade systemic inflammation via production of interleukin 1 β
 124 (IL-1 β) and IL-6 and recruitment and activation of innate immune cells (32, 33). As i- and int-
 125 proteasomes can modify e.g. signal transduction and MHC I peptide presentation, their constitutive
 126 and/or induced expression in β cells by inflammatory cytokines is of high interest and therapeutic
 127 potential.

128 Here, we hypothesized that β cells constitutively express active non-standard proteasomes and that
 129 the expression is upregulated by innate inflammatory signals at non-toxic levels. We therefore set out
 130 to analyze the composition of proteasomes in human and mouse islets as well as in the commonly
 131 used β -cell model INS-1E cell line in non-stimulated or cytokine-stimulated conditions. We report
 132 constitutive transcription and translation of inducible proteasome subunits (β 1i/ β 2i/ β 5i) in β -cells,
 133 albeit with lower expression levels compared to immune cell-lines. Of the inducible subunits, β 5i is
 134 incorporated into active proteasomes in non-stimulated INS-1E cells, forming intermediate
 135 proteasomes that constitute 14 % of total proteasomes in these cells. Furthermore, mRNA and protein
 136 expression of inducible subunits is upregulated by *non-toxic* concentrations of IL-1 β . β 5i and β 1i
 137 subunits were induced in all tested cellular models while β 2i was induced in mouse (but not human)
 138 islets and INS-1E cells. Consequently the composition and both constitutive and stimulated activity
 139 of proteasomes in β cells has to be considered when investigating degradation mechanisms and
 140 antigen presentation on MHC I molecules of proinsulin and other β -cell proteins.

141 **Materials and methods**

142 **Cell culture**

143 The rat insulinoma INS-1E cell line, a gift from Claes Wollheim and Pierre Maechler, University
 144 Medical Center, Geneva, Switzerland, was maintained as previously described (11). The mouse
 145 insulinoma MIN6 cell line, was cultured in DMEM (Life Technologies, Nærum, Denmark) with 25
 146 mM glucose, supplemented with 10 % FBS, 0.1 % Penicillin/Streptomycin (P/S), 50 uM β -
 147 mercaptoethanol and 2 mM L-glutamine. The mouse lymphocyte cell line A20, donated by Prof.
 148 Søren Buus, Department of Immunology and Microbiology, University of Copenhagen, Denmark,
 149 was cultured in RPMI-1640 (Life Technologies, Nærum, Denmark), containing 10 % FBS, 1 % P/S,
 150 10 mM HEPES, 50 uM β -mercaptoethanol and 4.5 g/L D-glucose. The human T lymphocyte cell line
 151 Jurkat, also from Prof. Buus, was cultured in RPMI-1640 with 10 % FBS and 1 % P/S. All cells were
 152 maintained at 37° C with 5 % CO₂. All cell-lines were *Mycoplasma* negative.

153 **Animal Care**

154 B6 C57BL/6NRJ mice were housed, handled and sacrificed according to Danish legislation for
 155 animal experimentation and with prior approval from the local animal ethics committee, issued by
 156 the Department of Experimental Medicine, University of Copenhagen. Animal handling and
 157 procedures were conducted by researchers with FELASA certification and supervised by
 158 veterinarians.

159 **Islet isolation and culture**

160 Mouse islets were isolated by injection of LiberaseTM TL (Roche®, Hvidovre, Denmark) through
 161 the common bile duct to digest exocrine tissue. Islets were handpicked and either lysed immediately

162 or cultured for 3-5 days in RPMI-1640 supplemented with 10 % FBS and 1 % P/S, at 37° C and 5 %
163 CO₂.

164 Human islets were isolated from healthy, heart-beating donors by the European Consortium for Islet
165 Transplantation (ECIT) in Milan, Italy, with local ethical approval. The obtained islets were ~ 90%
166 pure and no apparent difference in their quality was observed. Details on islet donors are included in
167 the Table 1. Islets were cultured as previously described in (11).

168 **Table 1. Human islets donors information and islet preparations used during the investigation.**

Islet donors information				
Donor	1	2	3	4
Age	63	62	58	20
Gender (M/F)	F	F	M	M
BMI	19.5	29.3	27.8	21.8
blood group	A+	A+	O+	B+
HLA (A:B)	2,11 : 18,57	26,29 : 7,18	2,26 : 35,55	11,24 : 18,51
HLA (DR)	11,17	4,15	14,16	1,11
Cold ischemia time (h)	9	5	8.5	8
Islets culture duration (h)*	16	14	20	42
Cause of death	Cerebral bleeding	Cerebral bleeding	Cerebral bleeding	Anoxia
Source of islets	ECIT	ECIT	ECIT	ECIT
Estimated viability (%)	95	95	95	95
Estimated purity (%)	90	90	90	90
Any additional note	EBV positive			EBV positive
*time from islets isolation to shipment				

169 Cytokine exposure

170 INS-1E cells were exposed to 10 ng/mL rat IFN- γ (R&D, Minnesota, USA) or 15 or 150 pg/mL rat
 171 IL-1 β (BD Bioscience, Lyngby, Denmark) or control medium for 24h. Human islets were exposed to
 172 10 ng/ml human IFN- γ (BD Bioscience, New Jersey, USA) or 30 or 300 pg/ml rat IL-1 β , while
 173 mouse islets were exposed to either 10 ng/mL rat IFN- γ or 50 or 300 pg/mL rat IL-1 β or control
 174 medium, both for 24 hours prior to experiments.

175 Western Blotting

176 Prior to experiments cells or islets were lysed in lysis buffer, consisting of 100 mM Tris (pH 8.0), 30
 177 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 2 % NP-40, 20 mM iodoacetamide and protease inhibitor
 178 cocktail (Life Technologies, Nærum, Denmark). Protein concentrations were measured using Bio-
 179 Rad Protein Assay Dye Reagent (Bio-Rad, Copenhagen, Denmark). Indicated amounts of proteins
 180 were loaded on Nu-Page 4-12 % bis-tris gels (Thermo Fisher Scientific, Hvidovre, Denmark), and
 181 proteins were separated by SDS-PAGE. Gels were transferred to PVDF membranes using the
 182 iBLOT2 system (Thermo Fisher Scientific, Hvidovre, Denmark). Membranes were cut prior to
 183 incubation with primary antibodies (Table 2) overnight. Primary antibodies were diluted in 2 % BSA
 184 in TBST (50 mM Tris pH 8, 150 mM NaCl, 0.1 % Tween). Membranes were blotted with appropriate
 185 secondary antibodies for 1 hour. Blots were developed using chemiluminescence and captured using
 186 the Azure®Saphire Biomolecular Imager. Western blots were quantified using ImageJ software (v.
 187 1.52a, (34)).

188 **Table 2. Primary and secondary antibodies used during the investigation.**

Antibody target	Company	Cat#	Dilution
β li	Abcam	ab243556	1:10.000

β5i	Abcam	ab3329	1:5.000
β2i	Abcam	ab183506	1:1.000
Tubulin	Sigma	T6074	1:10.000
Actin	Thermofisher Scientific	MA5-11869	1:15.000
Insulin	Cell signaling	8138S	1:5.000
Anti-mouse secondary	Cell signaling	7076S	1:10.000
Anti-rabbit secondary	Cell signaling	7074S	1:10.000

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190 **Proteasome activity**

191 INS-1E, A-20 and Jurkat cells, human and mouse islets were plated in duplicates or triplicates in 96-
192 well plates and treated with 50 nM ONX-0914, a selective inhibitor of the β5i subunit activity (Selleck
193 Chemicals, Rungsted, Denmark, IC50: ~10 nM for β5i, (35)) or 2 μM MG132, a broad proteasome
194 inhibitor (Sigma-Aldrich, Søborg, Denmark) or control medium for 2 hours prior to experiments.
195 Chymotrypsin-, trypsin- and caspase-like activity was measured through luminescent assay using
196 commercially available Proteasome-Glo™ Assay (Promega, Nacka, Sweden) according to the
197 manufacturer's protocol. Depicted data are averages of either technical duplicates or triplicates as
198 indicated. The added trypsin-like, chymotrypsin-like and caspase-like activity is referred to as total
199 proteasome activity.

200 **RNA Sequencing**

201 Five hundred mouse islets were plated and exposed to IL-1β (50 pg/mL) for 10 days. Total mRNA
202 was extracted from the islets by employing RNeasy ® Micro Kit (Qiagen, Vedbæk, Denmark).
203 Single-stranded, single-end sequencing libraries were generated using 35 ng of extracted RNA by

means of TruSeq® Stranded mRNA Library Prep (Illumina®, Copenhagen, Denmark), and library sequencing was done with the HiSeq 4000 System (Illumina®, Copenhagen, Denmark). Sequence files were drawn to the UCSC mouse genome NCB137/mm9. Further technical and analysis details in (36) and RNA-seq raw data are accessible here: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110691>.

Single-Cell RNA Sequencing of Pancreatic Islets

Islet cell subpopulations were analyzed for *PSMB8*, *PSMB9* and *PSMB10* genes expression using published human islet single-cell sequencing data (37). FastQ files were downloaded from ArrayExpress (accession: E-MTAB-5061). Data was analyzed with bcbio-nextgen (<https://github.com/chapmanb/bcbio-nextgen>), using the hisat2 algorithm (38) to align reads to human genome version hg38 and the Salmon algorithm (39) for quantitation of gene counts. Only cells that passed the quality control in the original study (37) were maintained for further analysis, and the cell type classification from the original study was also maintained.

Mass spectrometry for proteasome composition analysis

INS-1E cells were grown to 90 % confluence in T175 flasks. The cells were washed with HBSS before incubation with pre-warmed culture media complemented with 0.1 % formaldehyde for cross-linking for 15 minutes. Next, 125 mM glycine was added for 10 minutes at 37° C to quench the formaldehyde. The cells were then washed three times with HBSS and centrifuged, and pellets were stored at -80°C for later proteasome composition analysis. Immuno-purification of the proteasomes from the *in-vivo* cross-linked lysates, was performed as previously described (40). Briefly, proteasomes were purified by incubating the lysates with CNBr sepharose beads (GE Healthcare) covalently bound to the antibody specific for the $\alpha 2$ subunit of the proteasome (MCP21) (100 mg of

beads for 0.8 mg antibody), using 150 million cells per 50 mg of grafted beads. The supernatant was collected, and the beads were washed three times with 40 bead volumes of washing buffer (20 mM Tris-HCl pH 7.6, 1 mM EDTA, 10% glycerol, 150 mM NaCl, 0.1% NP-40, 2 mM ATP and 5 mM MgCl₂). Finally, proteins were eluted with 0.5 ml of elution buffer (20 mM Tris-HCl pH 7.6, 1 mM EDTA, 10% glycerol, 3 M NaCl, 2 mM ATP and 5 mM MgCl₂). Two additional cycles of purification were conducted, reincubating the collected supernatant with antibody-grafted beads. All fractions were pooled. LC-MS/MS analysis was performed as previously described (21, 41). Briefly, immunopurified proteasome samples were precipitated with 20% trichloroacetic acid (TCA), washed with cold acetone and then denatured by boiling at 95°C for 30 min in the Laemmli buffer, also reversing the cross-links (40). Proteins were alkylated and concentrated on 12% acrylamide SDS-PAGE gel as a single band, which was cut and washed. Trypsin digestion was then performed overnight at 37°C and the peptides were extracted from the gel. The digestion mixture was then dried in a Speed-Vac and resuspended with 2% acetonitrile, 0.05% trifluoroacetic acid. The peptide mixture was then analyzed by nano-LC-MS/MS using an UltiMate3000 system (Dionex) coupled to Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Proteins identification, validation and relative quantification were performed as previously published (40).

Statistical analysis

Differences between two groups were assessed by a two-tailed Student's t-test. All statistical analyses were done using GraphPad Prism (v. 6, La Jolla, CA). Data is represented as means ± SD or SEM. P-values of ≤0.05 were considered significant.

250 **Results**

251 **Inducible proteasome subunits are constitutively expressed in** 252 **pancreatic islets and β -cell lines**

253 To investigate whether proteasome inducible β subunits are expressed in non-stimulated β cells we
254 re-analyzed RNA-sequencing data of single-cells dispersed from pancreatic islets from healthy
255 individuals (37) and found that between 3.5 to 40 % of β , α and δ cells express constitutively mRNA
256 of all inducible subunits (Fig. 1A-B).

257 Next, we lysed human and mouse islets, INS-1E (β -cell insulinoma), A20 (B cell lymphoma) and
258 Jurkat (T cell leukemia) cells and analyzed their protein contents by SDS-PAGE and Western
259 blotting. As expected, immune cell lines (A20 and Jurkat) showed high expression of the inducible
260 subunits (Fig. 1C-E). Interestingly, we detected relatively low but consistent expression of all three
261 inducible subunits (β 1i, β 2i and β 5i) in human and mouse islets and INS-1E cells (Fig. 1C-E).

262

263 **Figure 1. Constitutive expression of proteasome inducible subunits in islets and cell lines. (A-B)**

264 Single cell RNA sequencing analysis of β 1i, β 2i and β 5i gene expression in human pancreatic islet
265 alpha, beta and delta cells from healthy individuals (n=6). The data is shown as means with SEM.
266 Table presents the percentage of cells with detectable levels of inducible subunit mRNA. **(C-D)** SDS-
267 PAGE and Western blot analysis of basal expression of proteasome inducible subunits in immune
268 cell lines A20 and Jurkat, in insulinoma beta cell line INS-1E, and human islets (H-islets) and mouse
269 islets (M-islets). Values on top of the Western blots show the amount of protein loaded. C and D are
270 representative blots of n=3. **(E)** Quantification of relative expression levels of inducible proteasome

271 subunits normalized to tubulin (C) or actin (D) in tested cell lines/islets (n=3). The data is shown as
272 means with SD.

273

274 **INS-1E cells contain two major types of proteasomes**

275 We next investigated the composition of proteasomes in INS-1E cells through immunoprecipitation
276 (IP) of the 20S $\alpha 2$ proteasome subunit that is an obligatory member of all types of assembled
277 proteasomes (42).

278 *In vivo* gently cross-linked INS-1E cells proteasomes were immunoprecipitated and samples analyzed
279 by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). About 86 % of the
280 total proteasomes were found to be s-proteasomes with enzymatically active rings composed of $\beta 1$ -
281 $\beta 2$ - $\beta 5$ subunits (Fig. 2). However, almost 14 % of the precipitated proteasomes contained $\beta 5i$
282 replacing the standard $\beta 5$ subunit, forming an intermediate proteasome $\beta 1$ - $\beta 2$ - $\beta 5i$. We found neither
283 $\beta 1i$ nor $\beta 2i$ in protein complexes precipitated with $\alpha 2$ subunit.

284 Finally, our MS data indicated the presence of proteasomal regulatory particles 19S and 11S
285 (PA28 $\alpha\gamma$) within $\alpha 2$ proteasome complexes at the rate of 55.8 % and 5.1 %, respectively (Fig. 2).
286 However, with our experimental approach we cannot assign proteasome types to the detected specific
287 regulatory particles. The remaining 39.1 % of 20S corresponds to free (unactivated) proteasome (21,
288 40).

289

290 **Figure 2. Identification of intermediate proteasomes in INS-1E cell line.** Four $\times 10^8$ cells were
291 cross-linked, lysed, their proteasomes immunoprecipitated with mAb MCP21 and analyzed by LC-
292 MS/MS. The absolute quantities of each of the six catalytic subunits measured by the LC-MS/MS

method were computed to calculate the stoichiometry of 20S proteasome subtypes and the fractions of regulatory particles associated with the 20S core particle, as detailed in Experimental Procedures. INS-1E cells were cultured at standard conditions and four biological replicates were analyzed.

The β 5i-selective small-molecule inhibitor ONX-0914 reduces chymotrypsin-like activity of the β cell proteasome

We next examined the profile of proteasome proteolytic activities: chymotrypsin-, trypsin- and caspase-like. The proteolytic activities were tested in unstimulated live cells by addition of specific substrates to the medium (Cell-Based Proteasome-Glo™ Assay). As shown in Fig. 3 A-C intact human and mouse islets, INS-1E, MIN6 and Jurkat cells exhibited strikingly similar profiles. Under basal conditions, chymotrypsin-like activity constituted 50-60 % of total proteasome activity, with the remaining 40-50 % of activity almost equally divided between trypsin- and caspase-like activities. In A20 cells, trypsin- and caspase-like activities constituted a larger part of the proteasome activity than did chymotrypsin-like activity, despite the fact that they expressed the highest amounts of β 5i and β 1i (both subunits have chymotrypsin-like activities).

We next probed what portion of the observed proteasome chymotrypsin-like activity can be attributed to the inducible subunit β 5i. We took an advantage of a selective and potent β 5i subunit inhibitor, ONX-0914. Pretreatment of human and mouse islets and INS-1E cells with 50 nM ONX-0914 for 4 hours reduced chymotrypsin-like activity by 40 %, indicating that β 5i is enzymatically active in those islets and cells (Fig. 3D-F). At the same time, trypsin- and caspase-like activities were not affected by ONX-0914. As expected two μ M of MG-132 treatment (broad proteasome inhibitor) almost completely blocked all three types of enzymatic activities in all tested cells and islets.

315

316 **Figure 3. Total and proteolytic-specific enzymatic activities of proteasomes.** (A) Proteasome
317 activity in human (n=3) and mouse islets (n=4), (B) beta cell lines: INS-1E (n=6) and MIN6 (n=3)
318 and (C) immune cells: A20 and Jurkat (n=3). Proteolytic-specific activities exhibited by proteasomes
319 subunits, treated with $\beta 5i$ subunit specific inhibitor ONX-0914 (2 h, 50 nM) or non-specific
320 proteasome inhibitor MG132 (2 h, 2 μ M), in (D) human islets (n=3), (E) mouse islets (n=4) and (F)
321 INS-1E cells (n=4). Proteasome activity was evaluated in cultured cells/islets using Promega
322 Proteasome Glo assay. The data is shown as luminescence per islet/cell. Statistical analysis was
323 performed by paired t-test of treatments versus control. The data is presented as means with SD.

324

325 **Non-toxic concentrations of IL-1 β upregulate $\beta 1i$, $\beta 2i$ and $\beta 5i$ subunit**
326 **expression in β cells**

327 Previous work has shown that the $\beta 1i$, $\beta 2i$ and $\beta 5i$ proteasome subunits are expressed in response to
328 IFN- γ/β in cells other than that of hematopoietic origin including β -cells (20, 21, 40) but their
329 expression was not regulated by the toxic concentrations of IL-1 β treatment (20). Here we asked, if a
330 similar to IFN- γ/β expression upregulation can be achieved by mimicking low-grade inflammation
331 with the application of a non-toxic stimulatory concentration of IL-1 β (36).

332 Mouse islets exposed to 50 pg/ml of IL-1 β for 10 days exhibited a significantly higher mRNA
333 expression of $\beta 5i$ (*Psmb8*), $\beta 2i$ (*Psmb9*) and $\beta 1i$ (*Psmb10*) compared to the untreated islets (Table 3).
334 The mRNA levels for $\beta 5$ (*Psmb5*), $\beta 2$ (*Psmb6*) and $\beta 1$ (*Psmb7*) genes that encode standard subunits
335 remained unchanged after the same exposure.

336 **Table 3. Non-toxic concentrations of IL-1 β induce β subunit mRNA expression in mouse islets.**

Gene	Untreated	IL-1 β treatment	P value
β 5i (PSMB8)	12.6386	34.7718	0.00005
β 1i (PSMB9)	8.77503	19.8912	0.00005
β 2i (PSMB10)	15.6204	45.1648	0.00005
β 5 (PSMB5)	51.4583	52.8289	0.77065
β 1 (PSMB6)	134.512	132.595	0.84375
β 2 (PSMB7)	110.75	106.398	0.5821

337

338 **Table 3.** Upregulation of inducible proteasome subunits upon prolonged, low-dose exposure to IL1-
339 β . Five hundred mouse islets were cultured and exposed to IL-1 β (50 pg/mL) for 10 days. Total
340 mRNA was extracted, sequenced and genes identified using the UCSC mouse genome
341 NCB137/mm9. mRNA levels of *PSMB8*, *PSMB9* and *PSMB10* for inducible subunits β 5i, β 1i and
342 β 2i, respectively, were significantly ($p < 0.0005$ each) upregulated by IL-1 β exposure while *PSMB5*,
343 *PSMB6* and *PSMB7* for corresponding standard proteasome subunits β 5, β 1 and β 2 remained
344 unchanged. Results were analyzed by Student's unpaired t-test, $n=3$.

345

346 Furthermore, exposure of human and mouse islets, as well as INS-1E cells, to non-toxic concentration
347 of IL-1 β for 24 h (15 pg/ml for INS-1E, 30 pg/ml for human islets and 50 pg/ml for mouse islets)
348 induced expression of β 1i, β 2i and β 5i (Fig. 4A-C) with the exception of β 2i in human islets. The
349 toxic concentration of IL-1 β (150 pg/ml for INS-1E and 300 pg/ml for human/mouse islets) further
350 induced β 1i and β 5i expression (but not β 2i) in INS-1E cells, but failed to induce upregulation in the
351 subunit expression in human and mouse islets (Fig. 4A-B). As expected, non-toxic IFN- γ treatment

for 24 h induced expression of all inducible subunits (Fig. 4 A-C). Concentrations of IL-1 β in the non-toxic range are known to increase insulin biosynthesis (43, 44). Interestingly, induction of inducible proteasome expression by non-toxic concentrations of IL-1 β or IFN- γ was associated with increased proinsulin expression levels in INS-1E cells while toxic concentrations of IL-1 β diminished proinsulin expression (S1 Fig.).

357

Figure 4. Cytokines induce upregulation of inducible proteasome subunits in islets/cells. Human (A, n=4) and mouse islets (B, n=3) and INS-1E cells (C, n=4) were exposed for 24 h to IL-1 β at low (50 pg/ml for mouse islets and 15 pg/ml for INS-1E) or high dose (300 pg/ml for human/mouse islets and 150 pg/ml for INS-1E) or IFN- γ (10 ng/ml). Islets/cells were lysed and protein content analyzed by SDS-PAGE and Western blotting. Representative blots of four independent experiments are shown (left,) and quantification of inducible subunit bands relative to the tubulin (A and C) or actin (B) is presented (right). Statistical analysis was performed by paired t-tests of treatments versus control. Experiments done on individual islet donors (A and B) or biological cell replicates (C) are connected by lines.

Non-toxic concentrations of IL-1 β increase proteasome activity in β cells

INS-1E cells exposed to either non-toxic concentrations of IL-1 β or IFN- γ showed significant increase in all three proteasome catalytic activities. Furthermore, toxic concentration of IL-1 β increased chymotrypsin-like and trypsin-like activity compared to controls, although the increase was less significant than that observed for low concentration exposure (Fig. 5A). Mouse islets showed a similar pattern of increasing chymotrypsin-, trypsin- and caspase-like activity, when exposed to non-toxic concentrations of IL-1 β or IFN- γ (Fig. 5B). A toxic concentration of IL-1 β did not have a

375 significant effect on any of the proteasome-based catalytic activities in mouse islets. Human islets
376 from two donors were tested (Fig. 5C), and showed similar changes or lack thereof in proteasome
377 activities as in mouse islets and INS-1E cells.

378

379 **Figure 5. Basal and cytokine induced activity of proteasome subunits in (A) INS-1E, (B) mouse**
380 **islets (both n=3) and (C) human islets (n=2).** Cells and islets were exposed to low IL-1 β dose (30
381 pg/ml for human islets, 50 pg/ml for mouse islets and 15 pg/ml for INS-1E for 24 hours), high IL-1 β
382 dose (300 pg/ml for human islets, 300 pg/ml for mouse islets and 150 pg/ml for INS-1E for 24 hours),
383 INF- γ (10 ng/ml for human islets, 10 ng/ml for mouse islets and INS-1E for 24 hours) or control
384 media. Statistical analysis was performed by paired t-tests of treatments versus control. Experiments
385 done on individual islet donors (B and C) or biological cell replicates (A) are connected by lines.

386

387 Discussion

388 The present work shows that 1) inducible proteasome subunits are constitutively expressed in human
389 and rodent islets and a β -cell line, 2) β 5i is incorporated into an active proteasome, forming int-
390 proteasomes and 3) inducible subunit expression is upregulated by non-toxic IL-1 β concentrations.
391 The cellular composition of proteasomes and their expressional regulation is of particular interest,
392 because different types of proteasomes degrade proteins and peptides with different efficiency and
393 specificity (45) influencing a variety of cellular processes including antigen presentation and thereby
394 maintenance of peripheral tolerance or induction of autoimmunity (8, 13).

395 The presence of int-proteasomes as normal constituents in different tissues has been established
396 before as they have been reported to constitute up to 50 % of the total proteasome pool, depending
397 on the tissue (16, 24), but a comprehensive investigation of proteasome composition in primary β

cells or β cell models in non-stimulated conditions has not been performed. The Human Protein Atlas RNA-seq data indicated constitutive expression of the inducible $\beta 1i$, $\beta 2i$ and $\beta 5i$ subunits in human pancreas, with 10 % of the RNA sequencing reads originating from the islets of Langerhans and 75 % coming from exocrine glandular cells (46). Immuno-staining of the islets for specific inducible subunits detected $\beta 5i$ protein by one of two employed antibodies, while $\beta 1i$ and $\beta 2i$ proteins were not detected (46). To clarify this issue, we first investigated the expression of inducible proteasome subunits in unstimulated human dispersed islet cells. Re-analysis of the previously published data set of single-cell RNA sequencing (37) uncovered substantial subpopulations of α -, β - and δ -cells that constitutively express all inducible subunits (Fig. 1A-B) placing human islets on par with other tissues that express those subunits constitutively (9, 16). When testing β cell models, as well as human and mouse islets, we found that all three inducible β -subunits were detectable at the protein level without the need for cytokine stimulation (Fig. 1C-E), although the expression levels of each subunit varied substantially between tested groups. β cells can therefore potentially assemble i- and int-proteasomes containing one, two or three inducible subunits without immune-stimulation. It is however important to stress that the number of $\beta 1i$ -positive β cells is low in human islets (Fig. 1B) thus substantially limiting the possibility to assemble i-proteasome. This observation should be taken into account while investigating proteasome function and composition in pancreatic β cells.

The similar profile of expression of inducible β subunits in β , α and δ cells indicates that those subunits play parallel roles in degradation of the hormones abundantly handled by each cell type ER: insulin, glucagon and somatostatin, respectively. The cellular localization of the i- and int-proteasomes may also play a role in that process, as $\beta 5i$ and $\beta 1i$ subunits are found in close proximity to the ER while the s-proteasomes are homogenously distributed in both nucleus and cytoplasm (47).

420 Next, we used mass spectrometry to identify the proteasome subtypes in β cells. By
 421 immunoprecipitating the $\alpha 2$ subunit from INS-1E cell lysates, we purified active proteasomes.
 422 Eighty-six % of total proteasomes contained standard β subunits forming s-proteasomes (Fig. 2). The
 423 remaining 14 % contained only $\beta 5i$ subunit, whereas $\beta 1i$ and $\beta 2i$ could not be detected. Therefore,
 424 INS-1E cells constitutively express two types of proteasomes, the s-proteasome and an int-
 425 proteasome, where at least one β - ring contains a $\beta 5i$ subunit. We neither detected i-proteasomes nor
 426 int-proteasomes with incorporation of inducible subunits other than $\beta 5i$, despite their expression in
 427 unstimulated INS-1E cells. According to the rules of cooperative assembly, $\beta 1i$ cannot be
 428 incorporated without $\beta 5i$ but the opposite is feasible (48) and thus it is theoretically possible but
 429 biologically less plausible that $\beta 1i$ and $\beta 2i$ are expressed but do not participate in the formation of a
 430 pool of active proteasomes. Alternatively, sensitivity of the antibody used for the detection of $\beta 1i$
 431 may be high relative to the sensitivity of the other antibodies used for subunits detection, distorting
 432 the evaluation of the intracellular stoichiometry of the inducible subunits. The MS data that failed to
 433 detect $\beta 1i$ and $\beta 2i$ incorporation into proteasomes may thus be a more valid measure of the actual
 434 subunit stoichiometry in INS-1E cells. Furthermore, according to the rules of proteasome assembly,
 435 the lack of incorporation of $\beta 1i$ would prevent the incorporation of $\beta 2i$ subunit (48). However, we
 436 cannot rule out that $\beta 1i$ and $\beta 2i$ are incorporated but constitute the minor portion of active
 437 proteasomes, below the detection limit of our MS method.

438 The enzymatically active proteasomes are generally capped on one or both ends of the central 20S
 439 proteasomal core by regulatory particles 19S or 11S, but the method employed in our study does not
 440 distinguish which type of proteasome is associated with a given regulatory particle. We have found
 441 that 56 % of proteasomes in INS-1E cells contained the 19S particle known to associate with all types
 442 of proteasomes (21, 42, 49, 50), while 5.1 % proteasomes contained 11S that preferentially associates
 443 with int- and i-proteasomes (12, 21, 42, 51). This would indicate that about one third of all INS-1E

int-proteasomes are bound to 11S particles while the other two third is associated with 19S particle, (hybrid int-proteasome) or not associated with any regulatory particle and thus presumably not active. Regulatory particles dictate substrate availability and specificity with 19S recognizing client proteins marked by polyubiquitin chains and 11S being involved in the degradation of short and non-ubiquitinated peptides and antigen processing for MHC I presentation (52). As a result, their presence within assembled and active proteasomes demonstrates that unstimulated β cells contain specialized and mixed populations of proteasomes, possibly reflecting functional specificity.

When profiles of proteasome substrate-specific activities were analyzed, we found that the islets and cell lines (with the exception of A20) all showed similar proteolytic profiles. Chymotrypsin-like activity constituted between 50 and 60 % of the total proteasome activity, while trypsin- and caspase-like activities were responsible for the remaining 40-50 % (Fig. 3A-C). Immune cell lines have generally been reported to express a higher basal level of inducible subunits, and the i-proteasome constitutes a dominant form of their proteasomes (8). The fact that INS-1E cells and islets share a similar proteasomal catalytic activity profile indicates that inducible subunits codetermine the activity profiles not only in immune cells. We thus next pretreated islets and cells with a $\beta 5i$ selective small-molecule inhibitor, ONX-0194, and found a 30 to 50 % reduction in chymotrypsin-like activity, further indicating that the $\beta 5i$ subunit is proteolytically active in β cells (Fig. 3D-F).

Interpretation of the pathophysiological consequences of the proteasomes diversity in β cells requires better understanding of factors influencing its expression and composition. The human genes coding for $\beta 5i$ and $\beta 1i$ map to chromosome 6 precisely between the DNA sequences coding for human leucocyte antigen (HLA)-DQ, HLA-DM and Transporter 1 ATP Binding Cassette Subfamily B Member (TAP) 1 and 2 (S2 Fig), genes known to be major determinants of antigen presentation and predisposing to autoimmune diseases, including type 1 diabetes (53). The promoter region of $\beta 5i$ contains binding sites for the NF κ B transcription factor (S2 Fig.), but toxic concentrations of IL-1 β ,

a strong inducer of NF κ B (54), do not increase β 5i expression (20). Accordingly, studies in neurons have shown that toxic concentration of IL-1 β induces Early growth response 1 protein (Egr1) that strongly inhibits transcriptional activity at the β 5i promoter (55). At the same time, Freudenburg et al. speculated that since viral infections induce IL-1 β synthesis, iNOS expression and nitric oxide production impeding on mitochondrial function, the resulting reduction in ATP levels would trigger i-proteasome activation and generation of altered peptides that may be immunogenic and enable killing of infected target cells as an appropriate host antiviral response (18). How can these apparently disparate IL-1 β functions be reconciled? We suggest that cytokine concentration and/or duration of exposure are a key determinants of cell fate. It has been reported that low IL-1 β concentrations (0.01–0.1 ng/ml) are stimulating and protective for β cells e.g. they improve insulin biosynthesis and secretion and increase β cell proliferation, while higher concentrations (5–20 ng/ml) can induce cell apoptosis and necrosis through e.g. induction of endoplasmic reticulum and mitochondrial stress (43, 44). These two outcomes employ different cellular pathways, the stimulatory pathway depending on PKC and phospholipases and the toxic pathway on NF κ B signaling. We therefore used 10-100 fold lower IL-1 β concentrations in our experiments compared to previous publications (20). Indeed, we found that treatment of human and mouse islets and INS-1E cells with non-toxic concentrations of IL-1 β increased the mRNA (Table 3) and protein expression of all inducible proteasome β subunits (except of β 2i in human islets, Fig. 4), while it had no impact on mRNA levels of standard subunits (Table 3) and significantly increased all substrate-specific proteolytic activities in mouse islets and INS-1E cells (Fig. 5). Human islets showed similar trends, with the caveat that only two islet donors were tested. Our results indicate that cytokine concentration is critical when evaluating the regulatory role of cytokines in proteasome expression and activity.

Proteasomes process proteins of both endogenous and exogenous origin and produce peptides that are complexed with MHC I. The shift in composition of proteasomes towards i-proteasomes, changes

the peptide repertoire from non-immunogenic to immunogenic (18, 56) and can contribute to the progression towards autoimmune diabetes (18, 20, 57, 58). Importantly, the observed differences in cytokine action may reflect changing conditions in the islet microenvironment during inflammatory or metabolic stress. IL-1 β is a central promoter of low-grade inflammation and protection against certain viral infections, including influenza (59). One of the possible host protective mechanisms engaged by this cytokine could involve expression and assembly of int- and i-proteasomes that would result in an increased presentation of viral antigens and/or modified self-antigens, thereby enabling T effector-cell dependent eradication of infected cells. Similarly, low grade inflammation and local IL-1 β production in the islet microenvironment, could facilitate neoepitope presentation by β cells through preferential incorporation of inducible subunits to form int- or i-proteasomes. Interestingly, cells deficient in β 5i show lower MHC I expression and peptide presentation, and β 5i pharmacological inhibition slows disease progression in mouse models of inflammatory diseases such as arthritis and lupus (35, 60). Furthermore, β 5i has also been implicated in type 1 diabetes, and its inhibition has been shown to have a protective effect (35). This could reflect the fact that the MHC I peptides repertoire is at least in part dependent upon the activity of β 5i subunit in β cells expressing int- or i-proteasomes.

The role of immune- and, especially, intermediate proteasomes in β cell pathophysiology remains to be uncovered in detail. The perspective that differential proteasome subunit expression dictates the repertoire of β -cell neoepitopes presented by MHC I deserves future investigation. Discoveries in this field could lead to targeted proteasome inhibition as treatment options in diseases with an autoimmune component. In this study, we lay the groundwork for such future investigations. For the first time, we show that int-proteasomes are constitutively expressed and active in β cells and that inducible proteasome subunits can be upregulated in β cells in response to stimulatory non-toxic concentrations of IL-1 β along with increases in total proteasome activities.

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531 **Conflict of Interest**

532 The authors declare that there are no conflicts of interests.

534 **Author Contributions**

535 MSK, DV, SEB, THB, CP, KK developed the protocols for the experiments, conducted experiments,
 536 performed the statistical analysis, constructed figures and tables and wrote and reviewed the

537 manuscript. TD performed human islet experiments and participated in writing the manuscript.
 538 Bioinformatics analysis of single-cell RNA-sequencing data was conducted by BT. MCBP and NB
 539 generated and shared the data for Table 3. M-PBD and DZ performed and analyzed mass spectrometry
 540 experiment and wrote the manuscript. TMP developed the protocols for the experiments, provided
 541 experimental tools, participated in critical data analysis and writing the manuscript. MTM is the study
 542 guarantor and was its initiator and leading supervisor; developed the protocols for the experiments,
 543 conducted experiments, performed the statistical analysis and constructed figures and tables as well
 544 as wrote the manuscript.

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Supporting information

S1 Fig. Cytokine induced increase in proinsulin levels in INS-1E cells. Lysates of cells exposed to IL-1 β at low (15 pg/ml) and high concentration (150 pg/ml), IFN- γ (10ng/ml) and control medium, were run on SDS-PAGE and subjected to Western blotting in **A**) and proinsulin band intensity normalized to tubulin in **B**). Statistical analysis was performed by paired t-tests of treatments versus control. The data is shown as means with SD.

731

732 **S2 Fig. Genetic localization of $\beta 5i$ and $\beta 1i$ genes.** Genes for $\beta 5i$ (*PSMB8*) and $\beta 1i$ (*PSMB9*) reside
733 in the MHC-II region on human chromosome 6. Presented transcription factors were included in
734 UCSC genome browser genome GRCh37 and visualized with integrated regulation from ENCODE-
735 track option. For clarity and in relevance to the current publication, only some transcription factors
736 are presented.

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Figure 1

Figure 1

Figure 2

Mass spectrometry analysis of active proteasomes in INS-1E cells

Figure 2

Figure 3

Basal proteasome activity profile

Inhibition of proteasome proteolytic activities by β 5i-specific ONX-0914 inhibitor and pan-proteasome MG132 inhibitor

Figure 3

Figure 4

Cytokine induced upregulation of inducible proteasome subunits in islets/cells

Figure 4

Figure 5

Basal and cytokines induced proteasome activity in islets/INS-1E

Figure 5

Suppl. Figure 1

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Suppl. Figure 2