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***Chlamydia pneumoniae* Promotes Dysfunction of Pancreatic Beta Cells**

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Abstract

The human pathogen *Chlamydia pneumoniae* has been implicated in chronic inflammatory diseases including type 2 diabetes. Therefore, we designed a study to evaluate pancreatic beta cells and mast cells during chlamydial infection. Our study revealed that *C. pneumoniae* infected mast cells significantly ($p < 0.005$) decreased beta cell ATP and insulin production, in contrast to uninfected mast cells co-cultured with beta cells. Infected mast cells exhibited pyknotic nuclei and active caspase-3 and caspase-1 expression. Additionally, *ex vivo* analyses of tissues collected from *C. pneumoniae* infected mice showed increased interleukin-1 β production in splenocytes and pancreatic tissues as was observed with *in vitro* mast cell-beta cell co-cultures during *C. pneumoniae* infection. Notably, infected mast cells promoted beta cell destruction. Our findings reveal the negative effect of *C. pneumoniae* on mast cells, and the consequential impact on pancreatic beta cell function and viability.

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Keywords

Type 2 diabetes; mast cells; obesity; insulin; beta cells

1. INTRODUCTION

Greater than 35% of Hispanic Americans and 40% of African Americans are categorized as obese and experience a high incidence of type 2 diabetes [1–4]. Type 2 diabetes is associated with the induction of inflammatory cytokines including IL-1 β , IL-6 and interferon-gamma (IFN γ) [5]. Importantly, active caspase-1 cleaves pro-IL-1 β to promote IL-1 β secretion during cellular stress [6]. The transition to an inflammatory state is a result of a lack of homeostatic balance between inflammatory (e.g., IL-6 and IL-1 β) and anti-inflammatory (e.g., IL-4 and IL-10) components. To this end, IL-4 has been shown to up-regulate GLUT-1 expression [7], and insulin up-regulates GLUT-4 [8] thereby promoting glucose uptake.

Recently, our laboratory demonstrated that mast cell/IL-4 enhances macrophage ATP production and phagosome acidification to promote bacterial killing [9]. Interestingly, addition of IFN- γ to infected mast cells increases production of IL-4, demonstrating a mast cell mechanism to maintain homeostatic balance. Genes that impact type 2 diabetes include those that regulate phosphorylation and the ATP sensitive potassium channel, which are critical for pancreatic beta cell insulin secretion [10, 11], suggesting a plausible link between immune cells, specifically mast cells, and pancreatic beta cell function during stress and infection.

Mast cells shape the immune system via release of several proteases [12] and other products [13, 14] including leukotrienes, prostaglandins, TNF- α , IL-6, and IL-4 [15]. Additionally, our laboratory [16, 17] and others have shown that mast cells phagocytose and kill bacteria [18, 19]. Mast cells and macrophages are early responders during innate immune responses; however, these cell types also are noted in several pathologies such as heart disease and type 2 diabetes [20, 21]. We have previously shown that mast cells are critical responders in control of *Francisella tularensis* pulmonary infection. However, the current study reveals the detrimental effects of *Chlamydia pneumoniae* on mast cells and subsequent alteration of beta cell function and viability. *C. pneumoniae* causes acute and chronic infection, particularly of young and aged individuals [22, 23], and is associated with inflammation and heart disease.

Our results showed a significant influence of *C. pneumoniae*-infected mast cells on beta cell ATP production and insulin secretion. We analyzed beta cell function and insulin production during *C. pneumoniae* infection using the TC6 beta cell-line [24], primary beta cells, as well as a diabetic obese mouse model (db/db) and littermate controls (db/+) [25, 26] to evaluate tissues and pancreatic cell populations. Additionally, we evaluated immune cells in co-culture with pancreatic beta cells. Flow cytometry analyses revealed altered immune cell populations in the spleen, pancreas and liver in diabetic (db/db) compared to normal (db/+) mice. Beta cells co-cultured with mast cells and high glucose resulted in increased ATP production, while *C. pneumoniae* infected mast cells co-cultured with beta cells and high

glucose showed a significant decrease in both beta cell ATP and insulin production ($p<0.005$) at 1 h, and an increase in insulin production at 15 h. Additionally, a significant increase ($p<0.005$) in IL-1 β production was noted, compared to infected co-cultures without glucose, at 15 h. These results provide additional insights into how mast cells may contribute to altered pancreatic beta cell function and exacerbate type 2 diabetes.

2. MATERIALS AND METHODS

2.1. Mice

Specific pathogen-free 8–10 week old mice were utilized for all procedures. C57BL/6 and Lpr (leptin receptor) db/+ (C57BLKS-Lpr^{db}) mice [27] were purchased from the Jackson Laboratory. All experimental procedures and animal care were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

Mice were fed regular chow or a high fat plus sucrose diet (Harlan) *ad libitum* for 3 weeks and then infected with *C. pneumoniae* (1×10^5 IFU) intranasally or mock (PBS) infected. Mice were sacrificed by CO₂ asphyxiation and cervical dislocation; tissues were collected and placed in 10% RPMI plus gentamicin, penicillin and streptomycin, washed 1 $\times$, and processed (at 37°C) with collagenase $\times$ (1 mg/mL, Sigma). The cells were then analyzed by flow cytometry or cultured for 48h with antibiotics to evaluate intracellular *C. pneumoniae* tissue burdens.

2.2. Bacteria

Chlamydia pneumoniae (from Anand Ramasubramanian, UTSA) was grown in Hep2 cells (Hep G2, ATCC[®] HB-8065[™]) as previously described [28] and stored at –80°C in sucrose-phosphate-glutamine buffer.

2.3. Generation of primary cells and in vitro infection

Femurs were removed from sacrificed mice and flushed with RPMI 1640 (supplemented with 10% FBS and penicillin-streptomycin). Collected cells were washed and seeded into T75 culture flasks, and after 24 h, non-adherent cells were used for differentiation of mast cells. Generation of primary mast cells was performed by resuspending collected cells in recombinant IL-3 (5 ng/mL; PeproTech) and stem cell factor (5 ng/mL; PeproTech). The mast cells were harvested at 4 weeks for all experiments. The purity of mast cells was confirmed by flow cytometry using FITC (fluorescein isothiocyanate) conjugated CD117 (c-Kit) and anti-Fc ϵ RI (PE clone: Mar-1; e-Bioscience) and was found to be at least 95% pure, in agreement with our previous study [16]. Mast cells were infected with *C. pneumoniae* (1 MOI) *in vitro* for 2h in 6-well plates (or uninfected for controls), followed by 1h gentamicin treatment, washed 1 $\times$ and resuspended in 10% RPMI plus gentamicin or in the same medium plus glucose (65mg/dL- total glucose versus 20mg/dL- total glucose in the control system), and added to beta cells (Beta-TC-6, ATCC[®] CRL11506[™]) at a 1:2 ratio (mast cells: 2.5×10^5 /well and beta cells: 5×10^5 /well). In separate experiments, mast cells were infected with *Francisella tularensis* LVS (10 MOI) or *Klebsiella pneumoniae* (10 MOI) for 2 h, followed by 1h gentamicin treatment, washed and resuspended in 10% RPMI prior to addition to beta cell cultures at a 1:2 ratio (mast cells:beta cells). The samples and cultures

with the added glucose (65 mg/dl total glucose, a physiologically relevant glucose concentration in diabetic mice) are designated as “plus (or added) glucose” throughout this article; the samples and cultures without added glucose (20mg/dl total glucose) are designated as “without added glucose.” The beta cells used in all experiments were the Beta-TC-6 (beta) cell-line, unless otherwise noted. *C. pneumoniae* infected cultures were evaluated by immunofluorescent staining and flow cytometry as previously described [29, 30]. Beta cells alone were resistant to *C. pneumoniae* (1 or 5 MOI) infection. However, mast cells and Hep2 cells were susceptible at 1 MOI. Infected and mock (PBS) cells were treated similarly for flow cytometry. Supernatants from the tissue culture cells were collected at intervals, filtered and stored at -80°C for cytokine (IL- 1β , TNF- α or IL-6) analysis using BD OptEIA kits (BD Biosciences) as described by the manufacturer. In separate experiments, cellular lysates were prepared by rinsing the cells $1\times$ in PBS and treating with 0.1% Triton X100 plus protease inhibitor for 20 min with gentle rocking. The lysates were placed in 1.5 ml tubes and centrifuged to remove debris and stored at -20°C for subsequent ATP assays using the Sigma ATP Bioluminescent Assay Kit (FLAA Sigma) and Bio-Rad protein assay for equilibration.

2.4. Flow cytometry

Cells were collected and washed (1% FBS in $1\times$ PBS with 0.09% sodium azide) at 3, 12 or 24 h. Samples were subsequently blocked with anti-mouse CD16/CD32 (BD Biosciences) at 4°C followed by addition of fluorescent conjugated antibodies. Fluorescent antibodies included Fc ϵ RI (PE), c-Kit FITC (Clone 2B8, eBioscience), MHC Class II APC (clone, M5/114, BD Biosciences) or appropriate isotype controls. Samples were incubated at 4°C for 30 min and washed with 1% PBS. Intracellular staining was performed using the BD mAb Apoptosis Kit (BD Biosciences) as described by the manufacturer. Briefly, after surface staining, the cells were fixed in Cytfix/CytopermTM for 20 min, washed with 1% PBS, and permeabilized with $1\times$ PermWashTM. Antibody or isotype control was added to appropriate samples. Samples were incubated for 45 min, washed in $1\times$ PermWashTM, and resuspended in 2% paraformaldehyde for analysis by flow cytometry (FACSCalibur, BD Biosciences). Analyses included side scatter versus c-Kit or Fc ϵ RI gates for primary mast cells or F4/80 for macrophages. Analyses were performed using CellQuest Pro software (BD Biosciences).

2.5. Confocal microscopy

C. pneumoniae elementary bodies (EB) were labeled with Lucifer yellow (Sigma) at 37°C , washed and used for *in vitro* infection as described in the previous section. Mast cells and/or beta cells were cultured on coverslips in 24-well plates for confocal analysis. Cells were stained with fluorescent conjugated antibodies, c-Kit FITC, mitochondrial stain or acidification probe for beta cells along with DAPI for nuclear staining, and placed on slides. For active caspase-1, cells were blocked and incubated overnight with antibody to active caspase -1 (Santa Cruz), washed 3 times, stained with secondary antibody (donkey anti-goat alexa488) for 1h and washed 2 times. Samples were examined using a confocal microscope (Zeiss 710 Meta, Carl Zeiss Microimaging Inc.) and analyzed with Imaris software (Bitplane, Inc.).

2.6. Electron microscopy imaging

Scanning electron microscopy (SEM) imaging was carried out with an ultra-high resolution field emission scanning electron microscope (UHR FE-SEM), HITACHI In-Lens S-5500. The cells were fixed with PBS-4% formaldehyde/1% glutaraldehyde, pH 7.2 for 1h at room temperature, and then post-fixed with 1% osmium tetroxide (OsO₄, Electron Microscopy Sciences) in PBS for 1h. After dehydration through a graded series of ethanol concentrations (25, 50, 75, 95 and 100 %) for 15 min each, samples were processed with a critical point dryer (Leica EM CPD300) and finally sputter coated with gold (Pelco SC-7 Auto Sputter Coater). SEM imaging was performed with an accelerating voltage of 5 or 10 kV, and images processed with Quartz PCI.

2.7. Statistics

Analyses were evaluated by Student's t-test using the statistical software program SigmaStat (Chicago, IL). A *p* value of 0.05 or less was considered statistically significant. Data are representative of experiments repeated two to three times.

3. RESULTS

3.1. Mast cells and macrophages are mobilized and are increased in number in the spleen and pancreas of obese mice

Given that mast cells and other immune cell-types have been implicated in type 2 diabetes [20, 21, 31], we evaluated selected immune cell populations in the pancreas, liver, and spleen of obese mice (db/db), compared to littermate control (db/+) mice, by flow cytometry. Adult db/+ mice had an average mass of 25.1g (+/-4.7) while age matched db/db mice had an average 2-fold greater mass of 52.0g (+/- 3.6) [Fig. 1A]. Both the spleen and pancreatic tissues of obese (db/db) mice had increased levels of mast cells (CD117⁺), as shown in Figure 1B, whereas mast cells were not detected in the livers of either mouse type. Obese mice additionally exhibited increased CD4⁺ T cells in the pancreas, but with decreased numbers in the liver (Fig. 1C). Total macrophages (F4/80⁺) were markedly increased in the pancreas of obese mice, but like CD4⁺ T cells were reduced in the livers of obese mice (Fig. 1D). Although the total macrophage population was not altered within the spleen, MHCII expression was increased (11.2%) in the spleens of obese db/db compared to db/+ mice (5.9%, Fig. 1E). These results further support a role for obesity in altering immune cell populations.

3.2. Mast cells are susceptible to *C. pneumoniae* infection

C. pneumoniae has been associated with inflammation and type 2 diabetes [32, 33]. However, the mechanisms and role of *C. pneumoniae* during progression to type 2 diabetes are not well understood, and the contribution of mast cells during this infection has not been examined. Therefore, we first evaluated *C. pneumoniae* infection in mast cells and beta cells. Beta cells were resistant to *C. pneumoniae* infection (Fig. 2A). However, bone marrow derived mast cells (MC) infected with 1 MOI of *C. pneumoniae* showed positive immunofluorescent staining (*C. pneumoniae* LPS antibody) at 24h. At 48h, mast cells exhibited lysis (data not shown). *C. pneumoniae* infected approximately 10% of MC at 24h,

as revealed by confocal microscopy evaluation. Hep2 cells exhibited approximately 50% *C. pneumoniae* positive cells, whereas beta cells were resistant (<1% positive) to infection (Fig. 2A). We then evaluated infected MC by SEM at 20 and 24 h (Fig. 2B–D). SEM imaging revealed prominent protrusions (red arrows, Fig. 2C) extending from infected MC at 20h in contrast to uninfected mast cells. Additionally, at 24h the MC appeared to have altered cellular morphologies and damage (yellow arrows, Fig. 2D). Therefore, we next analyzed *C. pneumoniae* infected MC for active caspase-3 expression at 24h by confocal microscopy and flow cytometry (Fig. 2 E, F). Active caspase-3 and pyknotic nuclei were readily apparent in confocal images of infected MC grown with added glucose (Fig. 2E). Flow cytometry analyses demonstrated significantly ($p < 0.05$) increased active caspase-3 levels with addition of glucose at 24h, while propidium iodide staining was slightly decreased (Fig. 2F). These data demonstrate that MC are susceptible to *C. pneumoniae* infection and that glucose alters infection and cell death dynamics.

3.3. *C. pneumoniae* infected mast cells alter beta cell ATP and insulin production

Given that increased MC were identified in association with the pancreatic tissue of obese mice, we next evaluated beta cells and MC in co-culture to assess mast cell influence on beta cell function. ATP and insulin production were evaluated in MC and beta cell co-cultures during *C. pneumoniae* infection, with or without added glucose. To this end, beta cell ATP production has been correlated with insulin production [10, 11, 34–38]. Our results showed that uninfected beta cells cultured with high (added) glucose displayed a significant increase ($p < 0.0005$) in ATP production (Fig. 3A) and a significant increase ($p < 0.05$) of insulin production (Fig. 3B) by 1h, as previously reported [34]. Additionally, beta cells co-cultured with uninfected MC and high glucose showed an even greater increase in ATP production and insulin secretion ($p < 0.0005$ and $p < 0.005$, respectively, Fig. 3) by 1h. In contrast, *C. pneumoniae* infected MC plus glucose showed a significant decrease ($p < 0.005$) in cellular ATP compared to uninfected MC plus glucose or *C. pneumoniae* infected MC without added glucose. Additionally, both ATP and insulin production in the absence of added glucose were significantly ($p < 0.0005$ and $p < 0.005$, respectively) decreased in beta cells co-cultured with *C. pneumoniae* infected MC (Fig. 3A and B). Interestingly, insulin production was significantly ($p < 0.005$) increased in beta cells cultured with *C. pneumoniae* infected MC plus glucose at 15h (Fig. 3B). These results support a role for mast cells in altering beta cell function.

3.4. *C. pneumoniae* infection promotes IL-1 β production and beta cell destruction

Uninfected and *C. pneumoniae* infected MC were cultured with beta cells, with or without added glucose, and examined by confocal microscopy at 24h. The cells were fixed, permeabilized, and anti-*C. pneumoniae* and insulin antibodies were added, followed by secondary staining and addition of DAPI to visualize nuclei. The uninfected co-cultures showed numerous dense beta cell clusters (blue) with insulin (red) staining, in contrast with *C. pneumoniae* infected co-cultures which revealed sparse beta cell populations (blue) with *C. pneumoniae* (green) and insulin (red) staining (Fig. 4A). Quantitative analyses showed a decrease of approximately 50% of the beta cells at 24h in co-culture with infected MC, compared to uninfected MC (Fig. 4B). These data further suggest that *C. pneumoniae* infected mast cells promote beta cell destruction.

IL-1 β has been shown to be critical for defense during *C. pneumoniae* lung infection [39]. However, IL-1 β production also is implicated in inflammation, type 2 diabetes and pancreatic beta cell destruction [40]. Active caspase-1 cleaves pro-IL-1 β to promote secretion of the cytokine [41]. Therefore, we evaluated active caspase-1 and IL-1 β production. Active caspase-1 was located primarily at the cellular periphery in uninfected MC as revealed by confocal microscopy (Fig. 4C). However, during *C. pneumoniae* infection, active caspase-1 was clustered within the cell. Flow cytometry analysis also showed an increase in active caspase-1 expression by MC with infection (Fig. 4C). Additional analyses revealed that uninfected MC beta cell co-cultures and *C. pneumoniae* infected co-cultures without added glucose produced minimal IL-1 β (Fig. 4D). In contrast, *C. pneumoniae* infected mast cells in co-culture with beta cells plus added glucose produced significantly ($p<0.005$) greater levels of IL-1 β . These data show that added glucose impacts *C. pneumoniae* infected mast cell function and IL-1 β production.

We next used live cell imaging to assess mast cell-beta cell interactions. Live imaging (Fig. 5, live imaging frame) revealed that MC exhibited acidification (blue) and mitochondrial (red) staining throughout the cells, while *C. pneumoniae* infected MC exhibited reduced acidification regions, but intense mitochondrial staining regions indicative of increased mitochondrial activity following infection. Importantly, the acidification regions did not localize with Lucifer-yellow labeled *C. pneumoniae* (green). In contrast, we have previously shown that MC acidified phagolysosomes encompass *Francisella* bacteria for efficient killing [9].

Given that primary MC and beta cells showed increased IL-1 β production with added glucose, we next collected pancreatic beta cells from *C. pneumoniae* infected obese (db/db) and control (db/+) mice fed regular chow or a high fat plus sucrose diet and analyzed by flow cytometry (Fig. 6A and B). Pancreatic tissues from *C. pneumoniae* infected and uninfected obese mice on regular chow had comparable levels of MC to uninfected or infected control mice. In contrast, *C. pneumoniae* infected obese mice fed a high fat plus sucrose diet had significantly decreased levels of MC compared to infected control mice. Additionally, beta cells collected from infected obese mice on the high fat plus sucrose diet produced increased levels of IL-1 β compared to infected control mice on the same diet (Fig. 6B). Together, these results support a role for *C. pneumoniae* infection contributing to inflammation and beta cell dysfunction.

4. DISCUSSION

In this study, we have defined a previously unexplored role for mast cells and *C. pneumoniae* infection in pancreatic beta cell dysfunction and type 2 diabetes. The detrimental effect of MC was dependent on combinatorial events, including the presence of high glucose, *C. pneumoniae* infection, and MC products. Additionally, MC were present at increased numbers in the spleen and pancreatic regions of obese mice. Importantly, *C. pneumoniae* infected MC contributed to altered pancreatic beta cell ATP production, and ultimately insulin production.

Many diabetics experience complications during pulmonary infection [32, 33]. Chlamydial species have been shown to alter glucose metabolism and ATP production [42, 43] via stimulation of glucose transport in HeLa cells [42]. In our study, as shown in the model in Figure 7, *C. pneumoniae* infection of bone marrow derived MCs led to increased active caspase-1 expression and significantly decreased cellular ATP in the presence of high glucose. Also, IL-1 β was elevated in *C. pneumoniae* infected obese mice, which may be a consequence of elevated active caspase-1. Importantly, the observed MC destruction by *C. pneumoniae* infection also may result in release of pre-stored granules containing inflammatory mediators and active caspase-1 which has been predicted to cleave numerous cellular products and contributed to cellular destruction and pathogenesis.

Uninfected MC co-cultured with beta cells showed a significant increase in ATP and insulin production with addition of glucose (Fig. 3A and Fig.7). Additionally, *C. pneumoniae* infected MC plus glucose showed a significant decrease ($p<0.005$) in cellular ATP compared to uninfected MC plus glucose or *C. pneumoniae* infected MC without added glucose. It is important to note that mast cells infected with *Francisella tularensis* LVS or *Klebsiella pneumoniae* also resulted in a decrease of ATP production in beta cell-mast cell co-cultures (Supplemental Figure 1). However, infection of MC with *C. pneumoniae* plus added glucose resulted in approximately a 20-fold decrease in ATP production, while infection with the pulmonary bacterial pathogens *Francisella tularensis* LVS or *Klebsiella pneumoniae* plus glucose resulted in a 2-fold decrease in ATP production suggesting *C. pneumoniae* infection may have more detrimental impact on beta cells. These findings highlight a relevant role for mast cells, Gram-negative bacterial pathogens and their interaction with beta cells. We also observed that high caloric intake and obesity leads to increased immune cell (MC, macrophage, and CD4+ T cell) migration to the pancreas. Importantly, increased ATP and insulin production has several downstream signaling and cellular effects including increased cell survival, proliferation and chemotaxis [44]. Therefore, MC enhanced ATP and insulin production may ultimately lead to inflammation and pathological conditions related to obesity. In addition, the reduction of MC produced by *C. pneumoniae* infection may suggest a relevant association with the increased health complications noted in diabetic individuals, given the prominent role of mast cells in control of bacterial infection, wound healing and angiogenesis [45]. It is critical to note that pancreatic beta cell destruction and innate immune aspects also are associated with type 1 diabetes. Park and associates have shown that toll-like receptor 2 (TLR2) polymorphisms are linked to type 1-diabetes susceptibility [46]. In a separate study conducted by Ururahy and associates, TLR2 expression also was increased in patients with type 1 diabetes [47]. Both mast cells and monocytes express high levels of TLR2. Importantly, *C. pneumoniae* heat shock protein 60 has a critical role in TLR2 signaling initiation [48, 49]. Therefore, given the high rate of *C. pneumoniae* infection in the young, this pulmonary pathogen also may provide a critical link in the development of type 1 diabetes.

In this study as shown in the model (Fig. 7), *C. pneumoniae* infection of mast cells altered the function of beta cells in co-culture and caused a significant decrease in beta cell ATP levels and insulin production, and finally resulted in beta cell destruction. The decrease in ATP levels was followed by an early decrease in insulin. However, a spike in insulin

production at 15 h was observed in infected co-cultures with added glucose. The late spike in insulin production may be related to chlamydial replication during this interval, release of EBs, and/or cellular destruction. These cultures also exhibited increased IL-1 β production compared to cultures without added glucose. Moreover, pancreatic cells from *C. pneumoniae* infected mice (db/db and db/+) exhibited IL-1 β production, with greater IL-1 β production in cells collected from the obese mice.

Interleukin-1 β has been suggested to be important for control of *C. pneumoniae* pulmonary infection in mice in a caspase-1 dependent manner [39]. Additionally, IL-1 β is a primary cytokine during the acute phase of infection [50, 51] and requires stringent regulation to avoid inflammation related pathogenesis, as may be the case with *C. pneumoniae* infected mast cells in this study. However, obesity and inflammation may promote production of additional inflammatory cytokines, including TNF- α , and IL-6 [2, 5]. Also, additional cell types, such as macrophages, may contribute to IL-1 β production [41, 50, 51]. In our study, uninfected MC increased beta cell ATP production in co-culture, and exogenous ATP is often added to macrophage cultures to induce IL-1 β production. Hence, MC may act on macrophages or other immune cells through upregulation of ATP production to induce IL-1 β production and influence beta cell dysfunction. In summary, our data provides additional insight into mast cells and obesity during *C. pneumoniae* infection, and the deleterious impact of infected mast cells on pancreatic beta cell function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Mast cells are susceptible to *Chlamydia pneumoniae* infection.
- Obese mice have increased splenic and pancreatic mast cells.
- *C. pneumoniae* infected mast cells alter beta cell ATP and insulin production.
- Infected mast cells promote pancreatic beta cell destruction.

Figure 1. Increased numbers of mast cells and macrophages are present in the spleen and pancreas of obese mice

(A). Adult db/db mice fed regular chow diet were markedly obese compared to db/+ littermates. The spleen, pancreas and liver were collected from obese (db/db) and normal (db/+) littermate controls and assessed by flow cytometry. (B) The spleen and pancreas showed increased mast cells (CD117). (C) CD4+ T cells were increased in the pancreas, but decreased in the liver. (D) Splenic macrophage (F4/80) levels were similar; however, the pancreas showed increased numbers of macrophages, while the liver showed decreased macrophages in obese db/db mice. (E) Dot plots of spleen total macrophage populations. Macrophage F4/80+MHCII+ expression was increased in obese (db/db) [$11\% \pm 2.2$] compared to normal db/+ mice ($5.9\% \pm 1.9$). * $p < 0.05$, ** $p < 0.005$; $n = 3$, (two independent experiments). ND=Not detected; Error bars represent the mean $\pm$ SD.

Figure 2. *Chlamydia pneumoniae* infects mast cells and promotes cell death

(A) Confocal microscopy representative images of resistant beta cells (Beta), compared to susceptible mast cells (MC) and Hep2 cells infected with *C. pneumoniae* at 24h; *C. pneumoniae* (green), DAPI (blue); Quantitative analysis showing differences in susceptibility of the cell types to infection. (B-D) Scanning electron microscopy. (B) Uninfected MC at 20h. (C) *C. pneumoniae* infected MC at 20h with protrusions (red arrows). (D) *C. pneumoniae* infected MC at 24h with cellular alterations (yellow arrow). (E) Confocal microscopy, *C. pneumoniae* infected MC plus glucose showing DAPI stained nuclear protrusions and cellular active caspase-3 staining (DAPI-blue, active caspase-3 red) 630 $\times$. (F) Active caspase-3 expression is significantly elevated in *C. pneumoniae* infected MC (24h) with added glucose, propidium iodide staining is not significantly altered at this time by addition of glucose; flow cytometry analyses, percentage of positive cells; ($p < 0.05$); three independent experiments; Error bars represent the mean $\pm$ SD.

Figure 3. *C. pneumoniae* infected mast cells alter beta cell ATP and insulin production

A) Cellular ATP was decreased in *C. pneumoniae* infected MC with glucose (1h); cellular lysate ATP assay. (B) Insulin production (ELISA analysis) by beta cells was increased by added glucose alone and in co-culture with uninfected MC at 1h. Insulin production was decreased in co-cultures (MC + Beta) during *C. pneumoniae* infection at 1h, but was increased in *C. pneumoniae* infected co-cultures (MC + Beta) with added glucose at 15h; three independent experiments; Error bars represent the mean $\pm$ SD.

Figure 4. *C. pneumoniae* promotes beta cell destruction

(A) Confocal images: Beta cells plus uninfected MC and added glucose at 24h, Insulin (red), DAPI (blue); Beta cells plus *C. pneumoniae* infected MC and added glucose at 24h, Insulin (red), *C. pneumoniae* (green), DAPI (blue); 400 $\times$. (B) Beta cell recovery and enumeration. (C) Active caspase-1 expression: Representative images of uninfected and *C. pneumoniae* infected MC; Active caspase-1 (green) DAPI (Blue); Flow data showing increased active caspase-1 following infection. (D) IL-1 β production in *C. pneumoniae* infected MC +beta cell cocultures plus glucose; culture supernatants analyzed at 15h by ELISA; three independent experiments, Error bars represent the mean $\pm$ SD.

Figure 5. *C. pneumoniae* evades mast cell acidification and killing

(A) Beta cells plus uninfected MC; acidification label (blue), mitochondrial dye (red). (B) Beta cells plus infected MC (Lucifer labeled *C. pneumoniae*, green), acidification label (blue) does not encompass the *C. pneumoniae*, with increased mitochondrial activity (mitochondrial dye, red); 630 $\times$.

Figure 6. *C. pneumoniae* infection of mice alters pancreatic beta cell function

(A) Pancreatic tissue MC (c-Kit⁺) populations in obese (db/db) and control (db/+) mice at day 7 post challenge. (B) Pancreatic cells from *C. pneumoniae* infected control and obese mice at day 7 post challenge were collected, cultured, and analyzed for IL-1β production by ELISA, showing increased IL-1β production in obese compared to control pancreatic cells following infection; n=3, Error bars represent the mean ± SD

Figure 7. Model of *C. pneumoniae* infection of mast cells promoting beta cell dysfunction and death

Glucose uptake and ATP production by beta cells increase in a high glucose environment; ATP sensitive potassium channels modulate insulin secretion [42]. Uninfected mast cells may further promote beta cell ATP production and insulin exocytosis. In contrast, *C. pneumoniae* infection in a high glucose environment decreases MC ATP, and leads to increased active caspase-1 (and caspase-3, not shown), IL-1 β expression, and pyknotic MC. Pancreatic beta cell ATP and insulin production decrease, and ultimately infected MC promote pancreatic beta cell destruction.