Original Article

MOLECULAR METABOLISM

C3aR1 on β cells enhances β cell function and survival to maintain glucose homeostasis

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ABSTRACT

Objective: Pancreatic β cell dysfunction is critical to the development of type 2 diabetes (T2D). Our previous studies suggested that C3aR1 on β cells promotes insulin secretion and cell survival. However, as C3aR1 is expressed on many other cell types including within the islets, whole-body C3aR1 knockout models confound the analyses of direct impacts on β cells.

Methods: To clarify the role of C3aR1 in β cells under T2D conditions, we generated β cell-specific C3aR1 knockout mice. We assessed glucose homeostasis, focusing on β cell function and mass under metabolic stress conditions, to interrogate the effects of C3aR1 on β cells in a mouse model of T2D. We performed proteomic analyses on islets from control and β cell-specific C3aR1 knockout mice. To determine potential translational relevance, *C3AR1* was assessed alongside glucose-stimulated insulin secretion in human islets.

Results: We show that the complement receptor C3aR1 on β cells plays an essential role in maintaining β cell homeostasis, especially under the metabolic duress of obesity and T2D. Male mice with β cell specific deletion of *C3ar1* (β-C3aR1 KO) exhibit worse glucose tolerance and lower insulin levels when fed regular or high fat diet. Under high fat diet, β-C3aR1 KO also have diminished β cell mass. Islets from β-C3aR1 KO mice demonstrate impaired insulin secretion. β cells lacking C3aR1 display increased susceptibility to lipotoxicity-mediated cell death. Markers of β cell identity are decreased in β-C3aR1 KO mice while stress markers are elevated. Disruption of *C3ar1* on β cells ablates the insulin secretory response to C3a, establishing a signaling axis between C3a and β cell-derived C3aR1. Islet proteomic analyses highlight the MAPK pathway and mitochondrial dysfunction with C3aR1 loss in β cells. Finally, we show that *C3AR1* is positively correlated with insulin secretion in human islets.

Conclusions: These findings indicate that C3aR1 expression on β cells is necessary to maintain optimal β cell function and preserve β cell mass in T2D.

Keywords Beta cell; Type 2 diabetes; Complement; Insulin secretion; Beta cell failure

1. INTRODUCTION

Type 2 diabetes (T2D) is a multi-organ disease with profound impacts on cancer, cardiovascular, kidney and retinal diseases [1,2]. Diabetes mellitus has become a pandemic afflicting over 38 million people in the USA with over 90 % having T2D [3]. In the US alone, the economic costs of diabetes in 2022 accounts for $306 billion in direct medical costs [4]. β cell dysfunction is key to developing T2D with insufficient insulin secretion to compensate for the insulin resistance [5,6]. A hallmark of chronic T2D is pancreatic β cell failure, resulting in insulinopenia and severe hyperglycemia that requires insulin therapy [7]. There are two major antihyperglycemic pharmacological therapies that target β cells in current clinical use for T2D: sulfonylureas and incretin mimetics or dipeptidyl peptidase-4 (DPP-4) inhibitors that act on the same pathway [2]. Sulfonylureas have been associated with premature β cell failure [8]. Incretin mimetics also target appetite suppression in the brain with GLP-1R and GIP-R co-agonists and tri-agonists with GCG-R [9,10].

β cell defects in T2D can be attributed to β cell dysfunction and/or loss of β cell mass. Both of which can act alone or in combination to result in insufficient insulin secretion. β cell attrition from the metabolic stress of T2D can be due to cell death and trans- or de-dedifferentiation [11,12]. It is unknown if β cells that have adopted a different cellular identity can regain their β cell phenotype to secrete insulin. Strategies to augment β cell mass in T2D have yet to make it into the clinic and include inducing β cell proliferation and trans-plantation. Current therapies include medical therapies that protect functional β cell mass by reducing β cell workload [13].

Complement proteins are expressed within the pancreatic islets and in β cells and are capable of regulating insulin secretion [14]. We had previously shown that the adipokine adipsin/complement factor D (cfd) was part of an adipose-pancreas organ cross talk that boosted glucose-stimulated insulin secretion through the C3a peptide that is generated downstream of adipsin action [15,16]. Furthermore, adipsin prevented the development of β cell failure in genetically obese *db/db* mouse model of severe T2D [17]. In humans, patients with T2D or

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worse β cell functional parameters such as HOMA-β have lower circulating adipsin levels compared to controls at earlier stage of T2D or without disease [18,19]. Higher circulating adipsin levels associate with freedom from incident T2D in middle-aged adults [17]. Adipsin may also play a protective role in protection against β cell failure in T2D in humans as patients with T2D and β cell failure have lower adipsin levels than those with T2D without β cell failure [16].

Our previous work suggested that adipsin catalyzes the formation of the C3 convertase which then generates C3a to act on the G-protein coupled receptor (GPCR) C3aR1 on the β cell to augment insulin secretion and protect against cell death and dedifferentiation [15,20]. A pharmacological agonist of C3aR1 also potentiates glucose-stimulated insulin secretion and protects against lipotoxicity in human and mouse islets in vitro [21]. Together these studies suggest that C3a directly acts on C3aR1 on the β cell. However, C3aR1 is expressed on many different cell types in the islet including macrophages [14,22]. Whole body C3aR1 knockout mice are protected from diet-induced obesity and insulin resistance, confounding studies on islets and β cell function [23]. To determine the role of C3aR1 on β cells in T2D, we generated β cell-specific C3aR1 knockout mice and assessed glucose homeostasis in T2D conditions, focusing on β cell function and mass. Here we show that C3aR1 on the β cell plays a critical role in maintaining β cell function and numbers especially under metabolic stress conditions of T2D.

2. RESULTS

2.1. C3aR1 on β cells regulates glucose homeostasis and insulin secretion

We previously found that the adipokine adipsin augments glucose stimulated insulin secretion through generation of the complement component C3a to act on islet cells [16]. To directly interrogate the role of *C3ar1* on β cells in glucose homeostasis and insulin secretion, we generated β cell-specific C3aR1 knockout (β-C3aR1 KO) mice by crossing *C3ar1* floxed mice with *Ins1-Cre* transgenic mice. Successful deletion of *C3ar1* in pancreatic β cells from the β-C3aR1 KO mouse was confirmed by >99 % decrease in *C3ar1* mRNA expression compared to *Ins1-Cre* heterozygous controls in sorted β cells (Figure 1A). We confirmed by Western blotting that C3aR1 protein was almost completely absent in isolated islets from constitutive and β-cell-specific C3aR1 knockout mice (Figure 1B). 15- and 30-weeks old control and β-C3aR1 KO mice were challenged with a glucose tolerance test. Under the non-diabetogenic conditions of a regular diet for 15 weeks, male and female β-C3aR1 KO mice displayed similar glucose tolerance compared to controls (Supplementary Figs. 1A–D). However, 30 weeks old β-C3aR1 KO male mice had moderately impaired glucose tolerance (Figure 1C,D) and insulin secretion (Figure 1E) compared to control mice. Female β-C3aR1 KO mice showed similar glucose tolerance but exhibited a 34 % and 18 %

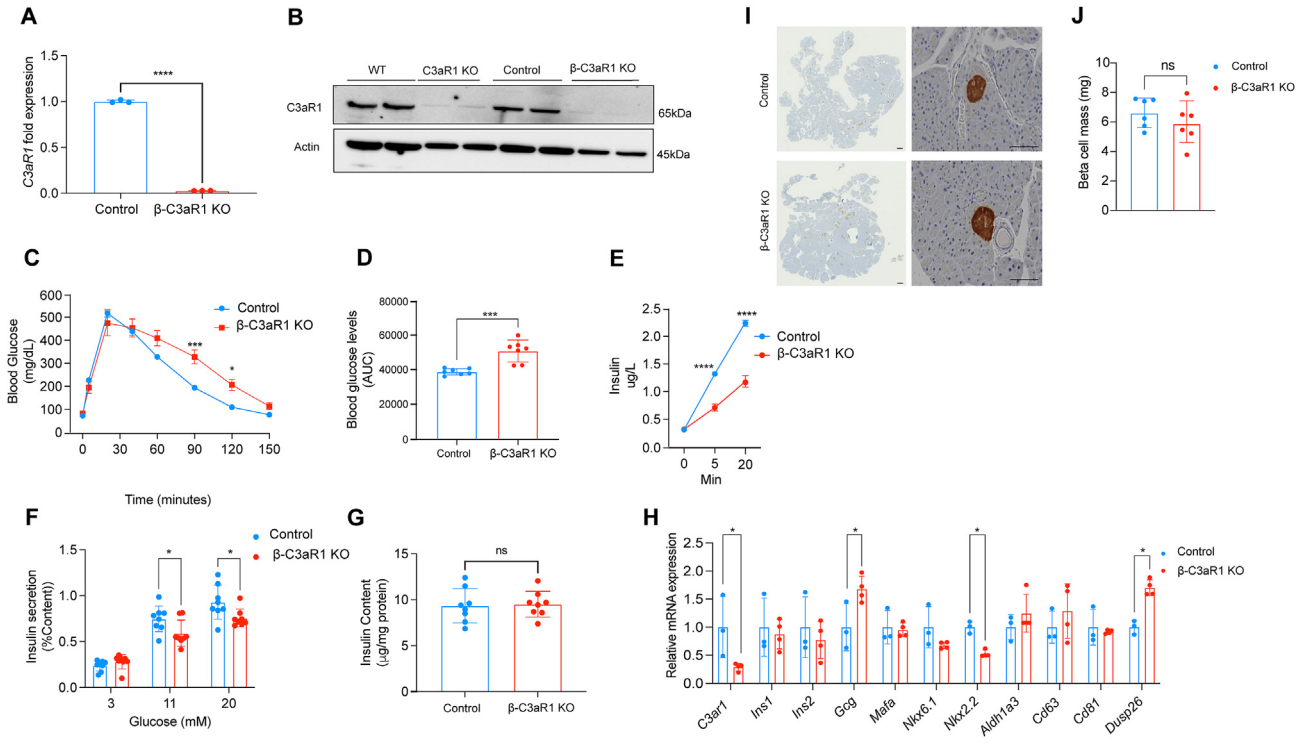

Figure 1: C3aR1 on β cells regulates glucose homeostasis and insulin secretion.
A. Relative mRNA levels of *C3ar1* from sorted β cells in control and β-C3aR1 KO islets in male (n = 3/group). **B.** Western blot for C3aR1 from islets of wild-type, C3AR1 constitutive knockout, and β-C3aR1 KO mice. Western blot was performed at least three times in independent cohorts with similar results. **C**. Glucose tolerance test (GTT) was performed on 30-weeks-old control and β-C3aR1 KO male mice (n = 7/group). **D.** Glucose area under curve (AUC) during GTT in male mice (**C**) (n = 7/group). **E.** Plasma insulin levels assayed at the indicated time points after i.p. glucose challenge in male mice (n = 7/group). **F.** Glucose-stimulated insulin secretion assay on islets from 30-weeks-old control and β-C3aR1 KO male mice (n = 8/group). **G.** Insulin content of control and β-C3aR1 KO male mice normalized by intracellular protein (n = 8/group). **H.** Relative gene expression of key transcription factors essential for β cell development and immature β cell in islets from male mice (n = 3/group). **I.** Representative IHC staining for insulin (brown) in pancreases of control and β-C3aR1 KO male mice. Scale bars 100 µm. IHC was performed at least twice, independently, with similar results. **J.** Quantification of β cell mass in control and β-C3aR1 KO male mice (n = 6/group). Data are presented as mean ± SEM. Unpaired 2-tailed t test is used for figures A, D, F, G, H and J and ANOVA two-way followed by Šídák's test for multiple comparisons test is used for figures C and E. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs β-C3aR1 KO mice.

reduction in insulin secretion at 5 and 20 min after glucose challenge, respectively, compared to controls (Supplementary Figs. 2A and B).

To directly interrogate whether loss of C3aR1 in β cells impaired β cell function, we isolated islets from control and β-C3aR1 KO mice. Islets from β-C3aR1 KO mice exhibited approximately 20 % and 18 % decrease in insulin secretion following stimulation with 11 and 20 mM glucose, respectively, for both male (Figure 1F,G) and female mice (Supplementary Figs. 2C and D), recapitulating the in vivo phenotype. In addition, islets from β-C3aR1 KO mice exhibited a marked reduction in the expression of *Nkx2.2*, a critical transcription factor essential for the development and maintenance of β cells, and elevations in *Gcg* and *Dusp26* (Figure 1H). We previously showed that C3a negatively regulates *Dusp26* expression in β cells, while palmitate enhances its expression, suggesting that C3a plays a protective role in mitigating β cell dysfunction under lipotoxicity by inhibiting *Dusp26* [17]. To assess for a potential quantitative defect in β cells, we performed insulin immunohistochemistry and found no significant differences in islet morphology and β cell mass between control and β-C3aR1 KO mice (Figure 1 I,J). Together these results reveal a role for C3aR1 on β cells in maintaining glucose homeostasis and insulin secretion under normal physiological conditions.

2.2. β cell-derived C3aR1 is important in maintaining glucose homeostasis and insulin secretion under the metabolic stress of obesity

At 4 weeks of age, we subjected control and β-C3aR1 KO mice to high fat diet (HFD) feeding to explore the role of C3aR1 on β cells in regulating whole-body energy and glucose homeostasis under metabolic stress. No significant differences in body weights were found between control and β-C3aR1 KO male and female mice fed a HFD (Figure 2A; Supplementary Fig. 3A). After 25 weeks of HFD, male β-C3aR1 KO mice exhibited a moderate impairment in glucose tolerance compared to controls (Figure 2 B,C), with similar results after 15 weeks HFD (Supplementary Figs. 3D—E). In female mice, C3aR1-deficiency in β cells did not have an impact on glucose homeostasis at 15 and 25 weeks HFD. (Supplementary Figs. 3B—C; Supplementary Fig. 4 A-B). Control and β-C3aR1 KO mice on a HFD demonstrated similar insulin tolerance tests, suggesting that changes in insulin sensitivity were not responsible for the differences in glucose tolerance (Figure 2 D,E).

To evaluate the role of C3aR1 on β cells in regulating insulin secretion, we measured insulin levels in control and β-C3aR1 KO mice subjected to a HFD. Male and female β-C3aR1 KO mice on a longer term HFD exhibited lower insulin levels following a glucose challenge compared

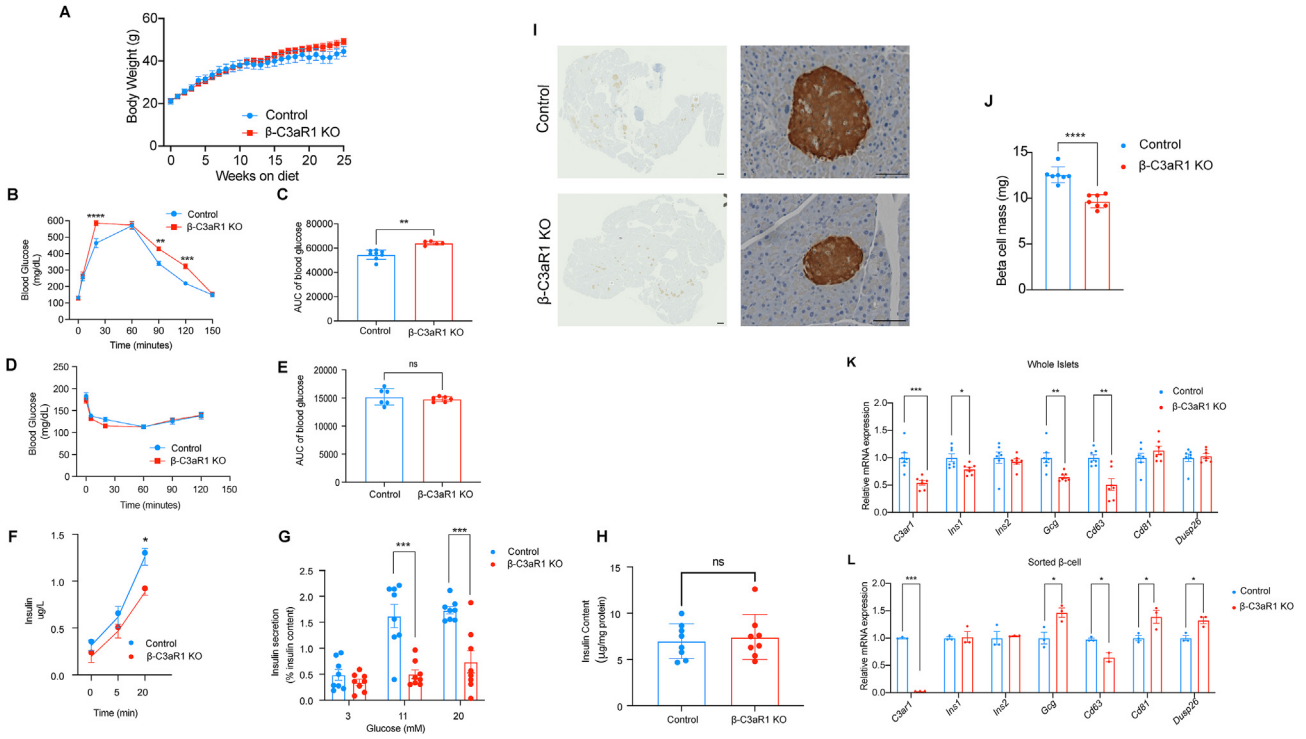

Figure 2: β cell-derived C3aR1 is important in maintaining glucose homeostasis and insulin secretion under the metabolic stress of obesity.
A. Body weights of control and β-C3aR1 KO male mice fed a high fat diet (HFD) for 25 weeks starting at 4 weeks (n = 8/group). **B.** Glucose tolerance test (GTT) was performed on control and β-C3aR1 KO mice (n = 5—8/group). **C.** Glucose area under curve (AUC) during GTT in (**B**) (n = 5—8/group). **D**. Insulin tolerance test (ITT) was performed on control and β-C3aR1 KO mice fed a HFD for 25 weeks with measurement of blood glucose concentrations (n = 6/group). **E**. Glucose area under the curve (AUC) during ITT in **D** (n = 6/group). **F**. Plasma insulin levels from control and β-C3aR1 KO male mice on HFD after i.p. glucose challenge at the indicated time points (n = 5—8/group). **G.** Glucose-stimulated insulin secretion assay on islets from control and β-C3aR1 KO male mice after 25 weeks on HFD (n = 8/group). **H.** Insulin content of control and β-C3aR1 KO male mice normalized by intracellular protein (n = 8/group). **I.** Representative IHC staining for insulin (brown) in pancreases of control and β-C3aR1 KO male mice fed a HFD for 25 weeks. Scale bars 100 μm. IHC was performed at least twice, independently, with similar results. **J.** Quantification of β cell mass in control and β-C3aR1 KO mice (n = 7/group). **K,L**. Relative gene expression of **(K)** whole islets (n = 7/group) and **(L)** sorted β cells (n = 3/group) from control and β-C3aR1 KO male mice on HFD for 25 weeks. Data are presented as mean ± SEM. Unpaired 2-tailed t test is used for figures C, E, G, H, J, K and L or ANOVA two-way followed by Šídák's test for multiple comparisons test is used for figures A, B, D and F. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

to controls, suggesting an important role for C3aR1 on β cells to induce insulin secretion (Figure 2F and Supplementary Fig. 4C). These findings implicated β cell insufficiency as an explanation for the exacerbated hyperglycemia in β-C3aR1 KO mice. This may result from decreased β cell mass and/or β cell dysfunction. To directly interrogate whether loss of C3aR1 in β cells impaired β cell function, we performed glucose stimulated insulin secretion (GSIS) on isolated islets. Islets from male β-C3aR1 KO mice displayed a dramatic reduction in insulin secretion in response to glucose compared to controls (Figure 2G—H). There was a similar phenotype in females with a moderate reduction in glucose-stimulated insulin secretion (Supplementary Fig. 4. D-E). To assess β cell mass, we performed insulin immunohistochemistry on pancreatic sections and observed that C3aR1 knockout in β cells led to an approximately 23 % reduction in β cell mass in male mice (Figure 2 I,J). Collectively, these results indicate that C3aR1 is important for both the maintenance of insulin secretion and the preservation of pancreatic β cells.

To further interrogate the role of C3aR1 in β cell maintenance, we analyzed genes involved in β cell identity and function. In islets from male mice, we observed that loss of C3aR1 on β cells resulted in a mild-moderate decrease in *Ins1* and halving of *Cd63*, a marker of a subset of β cells with enhanced insulin secretion (Figure 2K) [24]. In sorted β cells from male mice, we found a moderate reduction in *Cd63* alongside increases in *Gcg*, *Dusp26*, and *Cd81*, the last of which is a marker of β cell stress (Figure 2L) [24,25]. In islets from female mice, *Gcg* was similarly increased in the β-C3aR1 KO group compared to controls (Supplementary Fig. 4F). Collectively, these results indicate that C3aR1 on β cells is required to maintain euglycemia and preserve β cell mass in the context of obesity and T2D.

2.3. C3aR1 on β cells is important for maintenance of β cell identity

To determine if C3aR1 on β cells plays a role in β cell identity, we analyzed the expression of key transcription factors essential for β cell identity. In whole islets, we observed that loss of C3aR1 on β cells resulted in >50 % reduction in *Mafa* in β-C3aR1 KO mice compared to controls (Figure 3A). *Mafa* was decreased by ∼70% and *Nkx6.*1 by ∼25 % in the β-C3aR1 KO group in sorted β cells (Figure 3B). *Aldh1a3* was elevated with C3aR1 deficiency in both islets and sorted

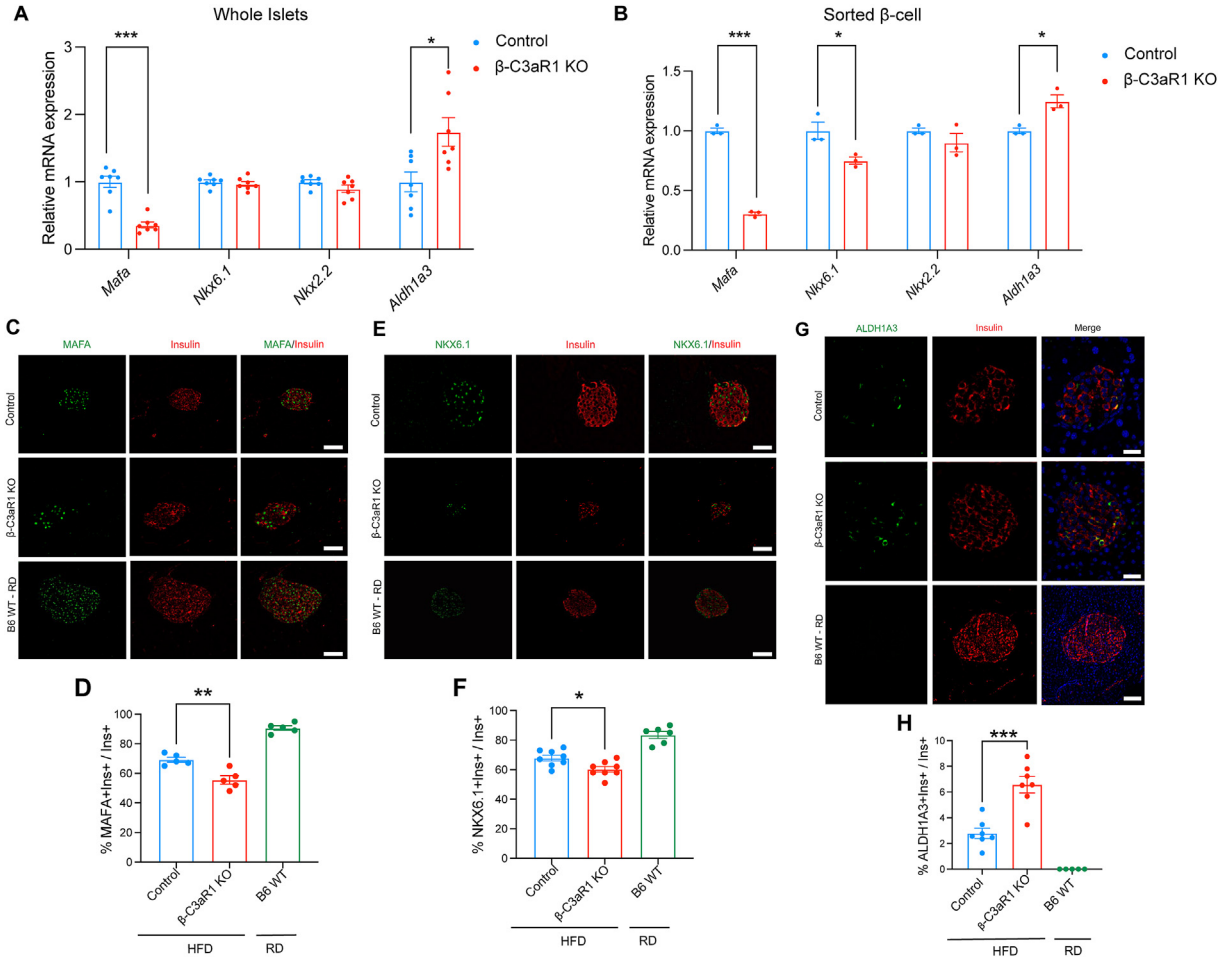

Figure 3: C3aR1 on β cells is important for maintenance of β cell identity.
A,B. Gene expression of transcription factors essential for β cell identity in whole islets **(A)** (n = 7/group) and sorted β cells **(B)** (n = 3/group) from control and β-C3aR1 KO male mice fed a high fat diet (HFD) for 25 weeks. **C,D.** Representative images of MAFA and insulin immunofluorescence (IF) in B6 WT mice on regular diet (RD) and control and β-C3aR1 KO mice on HFD with quantification of MAFA+ β cells **(D)** (n = 5/group). **E,F.** Representative images of NKX6.1 and insulin IF in B6 WT mice on RD and control and β-C3aR1 KO mice on HFD with quantification of NKX6.1+ β cells **(F)** (n = 6—8/group). **G,H.** Representative images of ALDH1A3 and insulin IF in B6 WT mice on RD and control and β-C3aR1 KO mice on HFD with quantification of ALDH1A3^{hi} β cells **(H)** (n = 5—7/group). Scale bars 100 μm. Data are presented as mean ± SEM. Unpaired 2-tailed t test is used for comparison between two groups. *P < 0.05, **P < 0.01, ***P < 0.001 vs. β-C3aR1 KO mice.

β cells (Figure 3A,B). Islets from female mice also demonstrated marked reductions in islet *Mafa* and *Nxk6.1* in the β-C3aR1 KO group compared to controls (Supplementary Fig. 4F).

To investigate whether β cell C3aR1 influences the maintenance of β cell transcriptional identity at the single cell level, we performed immunostaining for MAFA, NKX6.1, and NKX2.2. Deletion of C3aR1 on β cells led to a 20 % decrease in β cells expressing MAFA, and ∼1 0 % decrease in NKX6.1 without significant changes in NKX2.2 (Figure 3C—F and Supplementary Figs. 5A and B). We also performed immunostaining for glucagon and aldehyde dehydrogenase 1a3 (ALDH1A3), which is molecular marker of failing or dedifferentiated β cells. β cell specific loss of C3aR1 doubled the number of ALDH1A3^{hi} β cells, but had no significant impact on the number of glucagon positive cells on islets (Figure 3G,H and Supplementary Figs. 5C and D). Our data suggest that C3aR1 protects against β cell failure by maintaining β cell transcriptional identity.

2.4. β cell C3aR1 in C3a-mediated insulin secretion, cell death, and Human Islet Function

β cell death can also contribute to decreased β cell mass and failure and the pathogenesis of T2D. We assessed β cell death by TUNEL staining in the mice fed a HFD. Loss of C3aR1 on β cells resulted in a dramatic 4-fold increase in β cell death by TUNEL (Figure 4A,B). We had previously shown that C3a can protect against palmitate-induced cell death of islet cells [17]. It was not definitively known if C3aR1 expression on β cells is a prosurvival factor. We found that ablation of C3aR1 on β cells resulted in a 30 % increase in palmitate-induced β cell death after 48 h of exposure (Figure 4C,D). Together these results highlight the crucial role of C3aR1 on β cells in survival from the metabolic stress of diabetes.

We had previously shown that C3a enhances GSIS on islets [16]. It was unclear if this occurs through a direct interaction with C3aR1 on β cells or if another islet cell type expressing C3aR1 may be involved. To

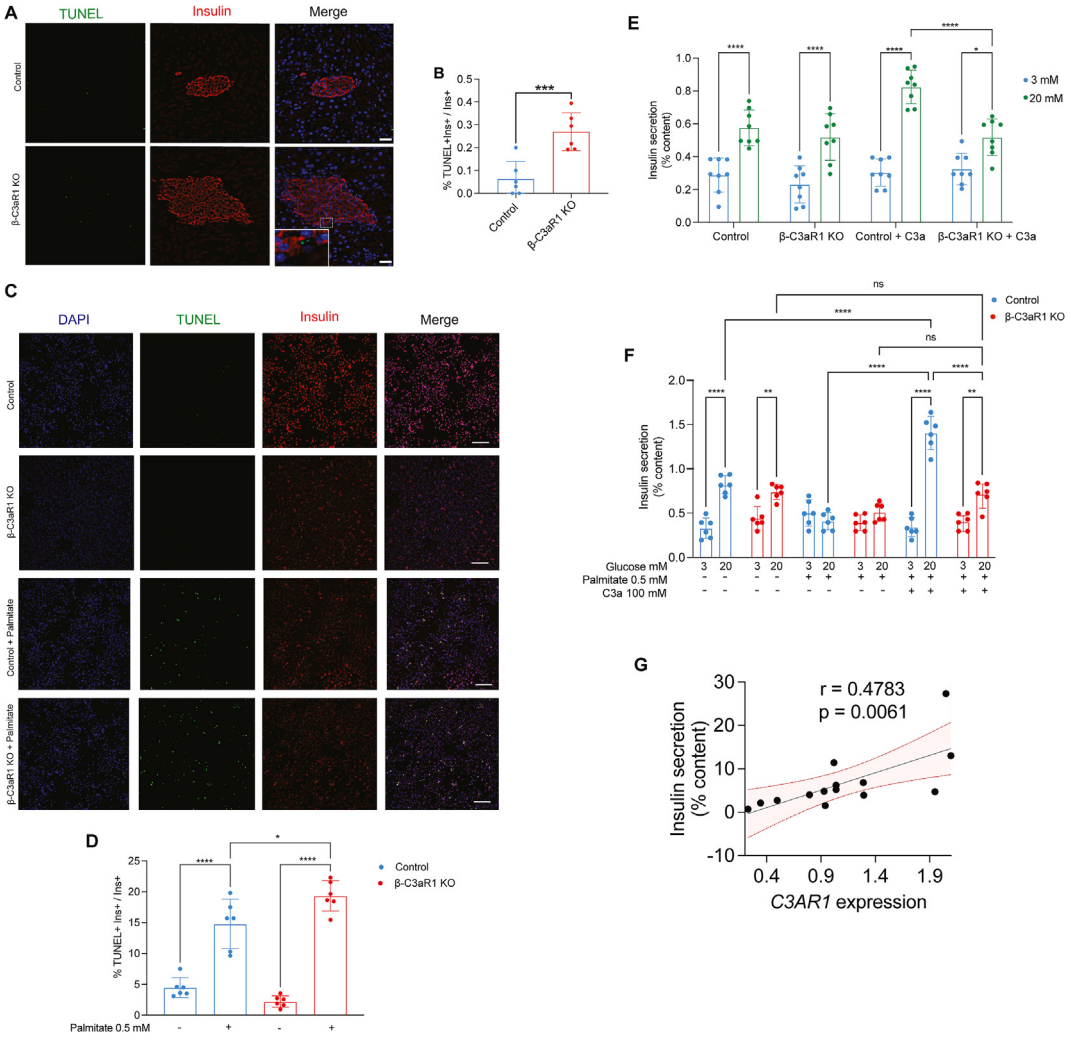

Figure 4: β Cell C3aR1 in C3a-mediated Insulin Secretion, Cell Death, and Human Islet Function.
Representative images of immunofluorescence (IF) staining for insulin and TUNEL assay in pancreases of control and β-C3aR1 KO male mice fed a high fat diet (HFD) for 25 weeks. White dashed box indicates region magnified in white panel. **B.** Quantification of TUNEL + beta cells as determined by IF (n = 6 group). Scale bars. **C.** Representative images of IF staining for insulin and TUNEL assay in dispersed islets. Images are representative of two independent experiments. **D.** Quantification of TUNEL+ β cells as determined by IF in (**C**) (n = 6/group). Scale bars, 100 μm. **E.** Islets from control and β-C3aR1 KO mice on a regular diet were subjected to a glucose-stimulated insulin secretion (GSIS) assay with or without recombinant C3a (100 nM) at the indicated concentrations of glucose (n = 8/group). **F.** Islets were treated with vehicle (Veh) or palmitate (Pa) (0.5 mM) followed by a GSIS assay with control or recombinant C3a (100 nM) at the indicated concentrations of glucose (n = 6/group). **G.** Correlation between human islet *C3AR1* expression and insulin secretion to 16.7 mM glucose (n = 14 donors). Data are presented as mean ± SEM. Unpaired 2-tailed t test is used for figure B or ANOVA one-way for multiple comparations is used for figures D, E. and F. *P < 0.05, ***P < 0.001, ****P < 0.0001 vs. β-C3aR1 KO mice. Linear regression with Pearson correlation coefficient analysis (bars indicate the 95 % confidence intervals).

directly interrogate the requirement of β cell C3aR1 on the effect of C3a on GSIS, islets from control and β-C3aR1 KO mice fed a RD was subjected to a GSIS assay in the presence of C3a. Acute administration of C3a increased insulin secretion by 40 % under high, but not low glucose conditions in control islets (Figure 4E). However, in islets from β-C3aR1 KO mice, C3a did not further enhance insulin secretion, suggesting that C3aR1 on the β cell is essential for C3a to augment glucose-stimulated insulin secretion (Figure 4E). Next, we assessed the β cell response to acute lipotoxicity and its response to C3a for insulin secretion. Islets from control and β-C3aR1 KO mice on a RD were exposed to palmitate for 18 h. While control islets robustly responded to C3a even after palmitate treatment, islets from the β-C3aR1 KO group failed to respond (Figure 4F). These results suggest that C3aR1 on the β cell is required for C3a augmentation of GSIS under basal conditions and after exposure to lipotoxicity.

We assessed the potential translational relevance of C3aR1 on β cells in human diabetes and β cell function. We analyzed the relationship between *C3AR1* expression and insulin release in human pancreatic islets. Our results revealed that *C3AR1* positively correlates with insulin secretion in islets from non-diabetic human donors, suggesting that C3aR1 may play a role in modulating insulin secretion in human islets (Figure 4G). These findings provide new insights into the function of C3aR1 on β cells and the role C3aR1 plays in the complex regulation of pancreatic β cell function under normal physiologic and pathological conditions of T2D.

2.5. Proteome controlled by C3aR1 on β cells

To gain further mechanistic insight into how ablation of C3aR1 drives impairments in insulin secretion, glucose homeostasis, and beta cell survival in β-C3aR1 KO islets, we performed proteomic analysis of pancreatic islets isolated from 12-week-old male control and β-C3aR1 KO mice fed with regular diet. As expected, β-C3aR1 knockout led to distinct alterations in protein abundance, with this KO group clustering when compared to controls (Figure 5A—C: 314 differentially abundant proteins).

Proteins that were increased in abundance in the β-C3aR1 KO were functionally associated with mitochondrial protein degradation, MAPK6/MAPK4 signaling, membrane organization, and RNA metabolism (Figure 5D). Consistent with these findings, independent confirmation of protein by Western blot and mRNA analyses by qPCR revealed a significant reduction in mitochondrial activity markers in the β-C3aR1 KO group, further supporting mitochondrial dysfunction in this condition (Figure 5E—H). Additionally, we observed increased phosphorylation of MAPK6/ERK3, along with increases in CDK1 and MAPKAPK2 protein levels (consistent with data in Figure 5I) in the KO group, reinforcing the role of C3aR1 in regulating MAPK signaling pathways. (Figure 5I—K). In contrast, proteins that were decreased in abundance in the β C3aR1 KO were linked to GTP hydrolysis, the 60 S ribosomal subunit, translation initiation complex formation, cellular responses to stimuli, mRNA processing, and regulation of intracellular transport (Supplementary Fig. 6 A-D). These results suggest and are consistent with the role of mitochondrial dysfunction, which plays a central role in the impairment of type 2 diabetes (T2D) and the survival of β cells.

3. DISCUSSION

Our study demonstrates that C3aR1 on β cells plays an important non-redundant role in insulin secretion and preservation of β cell mass

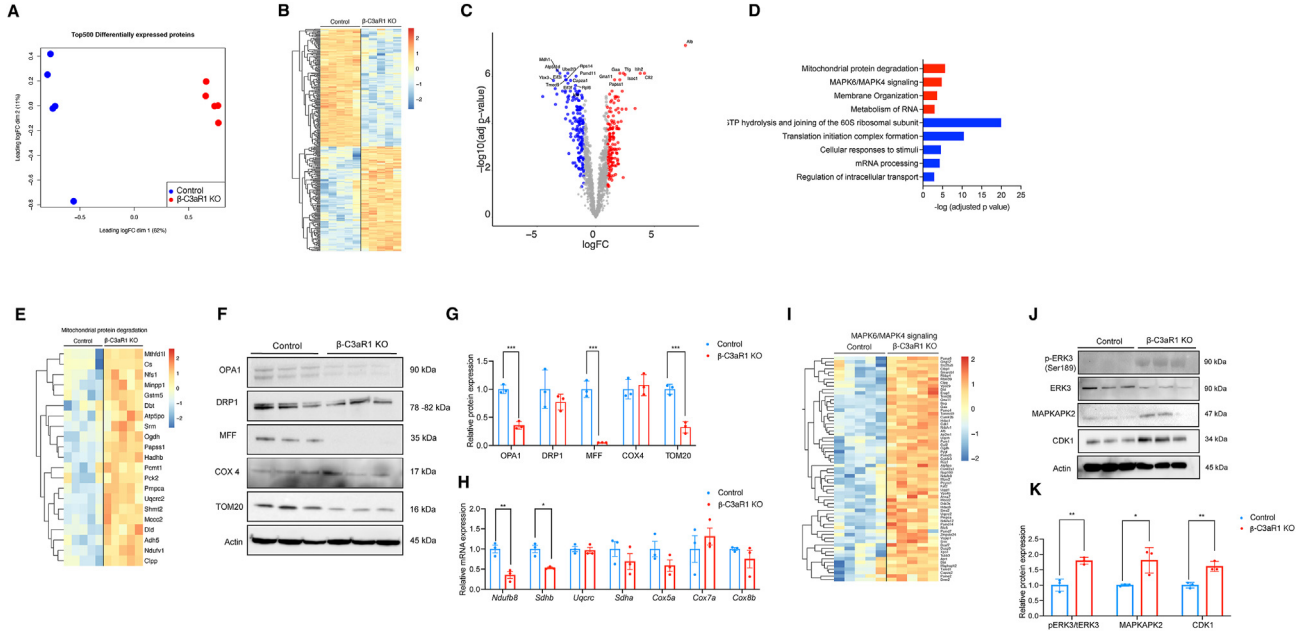

Figure 5: Proteome controlled by C3aR1 on β cells.

A. Multidimensional scaling (MDS) plot for islets from control and β-C3aR1 KO male mice fed with RD. **B.** Protein abundance heatmap between control and β-C3aR1 KO islets (cutoff values logFC ≥1 or logFC ≤1 and adjusted p ≤ 0.05). **C.** Volcano plot for top 20 protein abundance between control and βC3aR1 KO islets (cutoff values logFC ≥1 or logFC ≤1 and adjusted p ≤ 0.05). **D.** Functional enrichment analysis of protein abundance comparing control and β-C3aR1 KO islets. Red indicates upregulated pathways, while blue indicates downregulated pathways. The analysis was performed using Metascape. **E.** Heatmap of mitochondrial degradation proteins from control and β-C3aR1 KO islets (cutoff values logFC ≥1 or logFC ≤1 and adjusted p ≤ 0.05). **F-G.** Western blot analysis and quantification (**G**) of mitochondrial markers in whole islets from control and β-C3aR1 KO (n = 3). **H.** Gene expression of mitochondrial genes in whole islets from control and β-C3aR1 KO (n = 3). **I.** Heatmap of MAPK6/MAPK4 signaling proteins from islets of control and β-C3aR1 KO male mice (cutoff values logFC ≥1 or logFC ≤1 and adjusted p ≤ 0.05). **J-K.** Western blot analysis and quantification (K) of MAPK6/4 pathway proteins in whole islets from control and β-C3aR1 KO (n = 3). Data are presented as mean ± SEM. Unpaired 2-tailed t test is used for comparison between the two groups. *P < 0.05, **P < 0.01, ***P < 0.001 vs. β-C3aR1 KO mice.

(Supplemental Table 3). The effects with loss of C3aR1 seen in this study occur with older age or longer exposure to diet-induced obesity. Overall, a similar and milder phenotype is present in female mice. Our group recently reported sex-dependent regulation of thermogenic adipose tissue mediated by adipocyte-derived C3aR1 [26]. However, in β cells, our findings do not suggest significant sex differences. C3aR1 on the β cell protects against cell death. We have shown this in the context of lipotoxicity with palmitate, which is a commonly used trigger in experimental models. Mice with C3aR1 deficiency specifically in β cells also exhibit a loss of key β cell transcription factors essential for maintaining β cell identity, such as MAFA and NKX6.1, which are responsible for β cell maturation, insulin gene transcription, and GSIS, all of which are crucial for blood glucose homeostasis [27]. Furthermore, our study showed that mRNA levels of *Cd63*, a marker of β cells with higher metabolic activity and insulin secretion [24], are downregulated in β-C3aR1 KO mice. Likewise, the loss of C3aR1 specifically in β cells leads to increased levels of stress markers such as *Cd81*, *Aldh1a3*, and *Dusp26* and misexpression of *Gcg* in sorted β cells, highlighting the importance of C3aR1 in protecting β cells from failure and maintenance of identity. This effect is particularly significant as it underscores the role of C3aR1 specifically in β cells, rather than in the entire islet, in maintaining their transcriptional identity and function. The downstream C3aR1 signaling pathways in β cells that lead to the prosurvival and enhanced insulin secretion phenotype remain to be determined [17]. One major candidate that arose was DUSP26, a dual specificity phosphatase that was decreased by adipsin and C3a [17]. It is not clear how much DUSP26 contributes to the protection from cell death and dedifferentiation conferred by C3aR1. Future studies elucidating the pathophysiological role of DUSP26 in β cells should address this.

We establish critical roles for C3a on β cell action in this and prior studies [16,17]. Atanas et al. had demonstrated that pharmacological activation of C3aR potentiates glucose-stimulated insulin secretion and protects against fatty acid-induced apoptosis in both human and mouse islets in vitro [21]. Pharmacological agents may have off target effects that depend on dosing and can also affect multiple cell types, including other islet cell types such as macrophages expressing C3aR1. While their findings relied on pharmacological activation over hours to days, we provide new insights into the endogenous role of C3aR1 specifically on β cell pathophysiology in a mouse model of T2D spanning months [21]. Our data suggest that C3aR1 not only contributes to insulin secretion under physiological conditions but also plays a critical non-redundant role in maintaining β cell viability under metabolic stress in vivo. This positions C3aR1 as a key player in the crosstalk between innate immune responses and pancreatic β cell function, with potential implications for the development of therapeutic strategies aimed at preserving β cell mass and function in T2D. What still remains unknown is the source of C3a. Does C3a derive locally or from the circulation from C3? Since C3a acts as an anaphylatoxin and can be deactivated to C3a-desArg by carboxypeptidases, systemic levels of C3a are generally not abundant. β cells can also make C3 though they generally are thought to lack the machinery to process it to C3a [28]. Rather intracellular C3 in β cells have been shown to protect against stress and death from cytokines [28,29]. It is possible that another islet cell type such as the macrophage may locally secrete adipsin. There may be more than one beneficial signal from C3 and its products to β cell health and protection against T2D. An open question remains as to whether and to what extent TLQP-21, a peptide derived from VGF through proteolytic processing and known to modulate energy metabolism, differentiation, and cellular stress responses, can activate C3aR1 on β cells and provide protection against T2D [30,31].

This study shows a positive correlation with *C3AR1* in human islets with glucose-stimulated insulin secretion. It is tempting to speculate that lower C3aR1 on human β cells would lead to decreased insulin secretion that would eventually cause β cell dysfunction and T2D. We do not formally know if C3aR1 declines in β cells in humans with T2D. Or that loss of the receptor or abrogated C3aR1 signaling in human β cells is part of the pathophysiology of T2D in humans or potentially in a subset of patients [32]. A previous study showed that serum complement C3 is independently associated with β cell function in T2D, with higher baseline C3 levels correlating with better β cell performance in humans [33]. In contrast, a recent study reported that C3 protein might promote β cell dedifferentiation in T2D by activating the Wnt/β-catenin signaling pathway [34]. Additionally, higher circulating levels of C3 have been associated with metabolic syndrome [35] and an increased risk of developing diabetes [36]. Despite these differing findings, targeting C3aR1 in β cells could still represent a promising therapeutic approach for T2D. However, a potential challenge in targeting C3aR1 is that its ligand, C3a, is an anaphylatoxin and is rapidly inactivated in the serum by carboxypeptidases. Nevertheless, with biased GPCR agonists it might be possible to separate out the anaphylatoxin properties from the protective β cell effects. Another future possibility is to direct the ligand to the β cell without off-cell effects like the mast cell. Other avenues include assessing what is downstream of the C3aR1 in β cells. For example, inhibition of the phosphatase DUSP26 could be a viable therapeutic option without the untoward side effects of systemic C3aR1 agonism.

The proteomic studies conducted here suggest that C3aR1 ablation on β cells could enhance mitochondrial degradation and activate stress-related MAPK signaling cascades. This disruption could further exacerbate β cell dysfunction and apoptosis. Mitochondrial dysfunction has been shown to impair insulin secretion and increase oxidative stress in β cells, which can lead to cellular apoptosis and β cell mass reduction. These processes are critical in the pathogenesis of T2D, as they contribute to the progressive loss of β cell function and insulin production. Additionally, perturbations in mitochondrial dynamics, including alterations in mitophagy and mitochondrial biogenesis, have been implicated in β cell failure, further linking mitochondrial health to long-term β cell survival. Our study confirmed the downregulation of key mitochondrial regulators, including TOM20, OPA1, and MFF, which play essential roles in maintaining mitochondrial integrity, dynamics, and function [37]. The reduction of these proteins suggests impaired mitochondrial fusion and protein import, processes critical for β cell survival and insulin secretion. This disruption could make β cells more susceptible to metabolic stress and apoptosis, further exacerbating β cell dysfunction in T2D. Despite these changes, the mitochondrial mass may be unaltered, as the levels of COX4 and DRP1 were not significantly affected. This finding suggests that alterations in mitochondrial quality, rather than quantity, may drive β cell impairment in this context. Moreover, another key mechanism involved in β cell apoptosis is the activation of the MAPK signaling pathways. Similar to other MAPKs, JNKs are activated through a cascade that involves the sequential activation of dual-specificity MAPK kinases, including MKK4 and MKK7. While MKK7 is a specific activator of JNKs, MKK4 can also phosphorylate the Thr-Gly-Tyr motif of p38 MAPK, contributing to stress responses. Activation of both JNK and p38 MAPK is well-known to induce β cell apoptosis, thereby exacerbating β-cell dysfunction in the context of metabolic stress and T2D [38,39]. Furthermore, our study confirmed the increased phosphorylation of MAPK6, as well as the upregulation of CDK1 and MAPKAPK2 expression. MAPKAPK2, a downstream target of the p38 MAPK pathway, plays a critical role in regulating β

cell function and insulin secretion, influencing glucose homeostasis and potentially contributing to the development of diabetes [40]. These findings suggest a broader activation of stress-related MAPK pathways, which contribute to β cell apoptosis and dysfunction in response to C3aR1 ablation. Thus, the interplay between mitochondrial dysfunction and MAPK pathway activation creates a detrimental environment for β cells, driving both impaired insulin secretion and increased apoptosis, which are hallmarks of β cell failure in T2D. Notably, our findings suggest that the loss of C3aR1 signaling in β cells triggers these molecular pathways. As such, C3aR1 may represent a crucial regulator of these processes, positioning it as a potential therapeutic target. Future studies should investigate how C3aR1 influences these molecular pathways, including mitochondrial function and MAPK signaling, to better understand its role in β cell survival and its therapeutic potential in the context of T2D.

There are some limitations to our study. While we show an essential role for C3aR1 on β cells in glucose-stimulated insulin secretion, this does not formally rule out the possibility that other cell types expressing C3aR1 in the islets or elsewhere could have an indirect impact on the β cells. The direct and potential indirect effects of C3a and C3aR1 are not mutually exclusive. As stated earlier, the human *C3AR1* islet data with insulin secretion is a correlation. Studies to knockout/knockdown *C3AR1* in human β cells would be needed to show causality and is beyond the scope of this paper. Our studies were performed in the B6/J strain of mice; the generalizability to other strains of mice is not known. This could be important as immune responses and obesity varies depending on the genetic background.

4. MATERIAL AND METHODS

4.1. Animals

C3ar1 flox/flox mice were on the C57BL/6 J background as described [26]. *Ins1-Cr*e transgenic mice were purchased from The Jackson Laboratory (strain 26801). Mice were bred from few different strategies including *Ins1-Cre C3ar1* fl/+ X *C3ar1* fl/fl, *Ins1-Cre* X *C3ar1* +/+, and *Ins1-Cre C3ar*1 fl/fl X *C3ar1* fl/fl mice. Mice were age and sex-matched for the studies, including proteomics. *Ins1-Cre* heterozygous mice were used in the experiments as controls. All mice were maintained in plastic cages under a 12-hour light/12-hour dark cycle at constant temperature (22 °C) with free access to water and food. Mice were fed a regular chow diet (RD; 5053, LabDiet) or a 60 % high fat diet (HFD; D12492i, Research Diets) after weaning.

4.2. Blood chemistry and serum insulin analysis

Mice were fasted for 16 h prior to the glucose tolerance tests and injected intraperitoneally with syringe-filtered D-glucose solution (2 g/kg). For insulin tolerance test, mice were fasted for 6 h and injected of insulin (0.5 units/kg body weight). Blood glucose levels were assayed by commercial glucometer (OneTouch) by tail vein blood samples. Tail vein blood was collected into tubes, centrifuged at 2000×*g* at 4 °C, and plasma insulin levels were determined by ELISA using a standard curve (Mercodia).

4.3. Pancreatic islet isolation and culture

Mouse pancreatic islets were isolated by perfusion of the pancreases with ClZyme (VitaCyte) through the common hepatic duct as previously described [17]. Islets were then hand-picked and processed for different applications. For treatment with palmitate and/or recombinant C3a, an equal number of whole handpicked islets were cultured in 3 mM glucose and 0.5 mM palmitate or vehicle and then harvested. For flow cytometry and FACS sorted islet cells, dissociation was performed with 0.05 % trypsin.

For static incubation assays, islets were handpicked and transferred into basal Krebs buffer containing 3 mM glucose. After 1 h of starvation, islets were transferred into Krebs solution containing 11 or 20 mM for 1 h at 37 °C. Supernatants were centrifuged and collected. The pellet was used to determine intracellular insulin content. Insulin secretion was measured with Insulin ELISA kit (Mercodia).

4.4. Flow cytometry

β cells were gated and purified based on granularity and FAD/FMN (FITC) autofluorescence. FAD and NAD(P)H levels were assessed by measuring autofluorescence at and excitation wavelength of 488 and 350 nm respectively using a Sony MA-900 sorter as described [24,41].

4.5. TUNEL assay

For determination of apoptosis in dispersed islets, a TUNEL assay (Promega) was performed according to the manufacturer's instructions. TUNEL + nuclei in insulin-positive staining was determined with Fiji/ImageJ (NIH). For determination of cell viability, 50,000 cells per well were seeded in a 24-well plate and subjected to palmitate treatment and C3a (100 mM) for 18 h.

4.6. RNA extraction and qPCR analysis

RNA isolation was performed using the RNeasy Micro and Mini kits (Qiagen). cDNA was synthesized through reverse transcription using a cDNA synthesis kit (Thermo). cDNA was analyzed by real-time PCR using specific gene primers and a SYBR Green Master Mix (Quanta). Primer sequences are shown in Supplementary Table 1.

4.7. Western blot

Isolated islets were homogenized in RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail. Clarified protein extracts were obtained through multiple centrifugation steps at 13,400 g for 10 min at 4 °C. Protein extracts were separated on a NuPAGE Bis-Tris gel (Thermo Fisher Scientific) and transferred onto a PVDF membrane. Membranes were incubated overnight at 4 °C with the appropriate primary antibodies. Anti-TOM20 (42406), anti-OPAl (80471), anti-DRP1 (8570), anti-MFF (84580), anti-MAPKAPK-2 (3042), anti-CDC2/CDK1 (77055), anti-ERK3 (4067) and anti-β-actin (4970) were from Cell Signaling, anti-pMAPK6/Erk3 (ab74032) and anti-COX4 (ab14744) were from Abcam, anti C3aR1 (sc-133172) was from Santa Cruz. Detection of proteins was carried out by incubations with HRP-conjugated secondary antibodies described above followed by enhanced chemiluminescence detection reagents. Band density was quantified using ImageLab (Bio-Rad).

4.8. IHC and IF analyses

For histological studies of islets, pancreases were dissected, laid flat for paraffin embedding and fixed in 10 % neutral-buffered formalin (VWR) overnight at 4 °C. Tissues were then transferred to 70 % ethanol and subsequently embedded in paraffin and sectioned at 5 μm thickness. Sections were dewaxed, and antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) at boiling temperature for 14 min. For insulin IHC to determine beta cell mass, anti-insulin (Dako, IR002) antibody was incubated overnight at 4 °C, followed by incubation with corresponding biotinylated secondary antibodies. Signals were developed using 3,3′-diaminobenzidine (DAB). Sections were then counterstained with hematoxylin. For IF staining, appropriate primary antibodies were incubated overnight at 4 °C, followed by incubation with Alexa-Fluor conjugated secondary

antibodies (Thermo). For MAFA (Bethyl, IHC-00352), NKX6.1 (DSHB, F55A12), NKX2.2 (Atlas Antibodies, HPA003468), ALDH1A3 (Novus, NBP2-15339) and Glucagon (Phoenix, H-028-05) stainings, Tyramide Signal Amplification (TSA; Thermo) was used. To calculate beta cell mass, whole sections stained with insulin and developed with DAB were scanned using a Zeiss Axioscan7 whole-slide scanner. The fraction of insulin-positive areas compared to total pancreatic tissue area (hematoxylin) was determined with Fiji/ImageJ (NIH). Beta cell mass and IF analyses were determined as previously described [17]. To assess apoptosis in paraffin-embedded pancreatic sections, a TUNEL assay (Promega) was performed according to the manufacturer's instructions. TUNEL + nuclei surrounded by insulin-positive staining were analyzed using Fiji/ImageJ (NIH).

4.9. Human samples

Frozen human pancreatic islets were obtained through the Integrated Islet Distribution Program (IIDP) at City of Hope (Duarte, CA) (https://iidp.coh.org) (Supplementary Fig. Table 2). Insulin secretion assays were conducted using an in vitro perifusion system, which integrates both insulin and glucagon secretion from islets with high temporal resolution. These experiments were performed, and results were provided by the IIDP. Additionally, *C3AR1* mRNA expression was assessed by qPCR. The use of human islets was approved by the IIDP as previously described [24].

4.10. Proteomics for pancreatic islets

Pancreatic Islets were pooled and lysed in RIPA buffer and sonicated for 5 min. DTT (100 mM) was added to lysates, followed by incubation at 60 °C for 30 min. Iodoacetamide (200 mM) was added and incubated for 30 min at room temperature in the dark. Samples were transferred to a KingFisher 96 deep-well plate, where SpeedBead magnetic carboxylate beads (12 μL) and MeCN (54 μL) were added. The mixture was gently agitated for 15 min. Beads were then washed using the KingFisher Flex 96 magnetic system with 80 % EtOH MeCN. After several wash cycles, beads were transferred to a plate containing trypsin (0.2 μg/well in 50 mM NH4HCO3) for digestion. Digestion involved multiple bead washes in trypsin solution.

Tryptic peptides were desalted using an Oasis HLB 96-well plate, conditioned with MeCN +0.1 % formic acid and H2O + 0.1 % formic acid. Peptides were eluted twice using H2O + 0.1% formic acid, followed by a final elution with 80 % MeCN +0.1 % formic acid. Volatiles were removed by vacuum concentration, and samples were reconstituted in 40 μL of 2 % MeCN +0.1 % formic acid. Samples were analyzed on a Bruker nanoElute 2/timsTOF Pro 2 system using a two-column nano-UHPLC separation method with a data-independent analysis (DIA) MS method. Peptides were trapped on a C18 column and eluted with a 2–35 % gradient of MeCN +0.1 % FA. The MS analysis was conducted with a DIA method using HyStar v. 6.2 software.

Data were processed using DIA-NN 2.0 with a Mouse FASTA database (UniProt). Parameters included 1 % precursor FDR, trypsin digestion, and carbamidomethylation as a fixed modification. The 'smart profiling' library generation strategy was employed for data analysis. DIA-NN output files were processed in R using the MSnbase package. Data were normalized, imputed, and differential expression was analyzed using linear models with Benjamini-Hochberg FDR adjustment. Volcano plots were generated to compare log2 fold change and -log10 (p-value) for protein expression.

4.11. Statistical analysis

Data are presented as mean ± s.e.m. Data are derived from multiple experiments unless stated otherwise. If not mentioned otherwise in the figure legend, statistical significance is indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. Statistical analysis was carried out using unpaired, two-tailed t-test or two-way ANOVA followed by Šídák's multiple comparisons test. GraphPad Prism 10 was used for statistical analysis. Pearson analysis was used for correlations with bars indicating the 95 % confidence intervals.

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CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Renan Pereira de Lima: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Ang Li:** Methodology, Investigation. **Ankit Gilani:** Methodology, Investigation. **Alfonso Rubio-Navarro:** Methodology, Investigation. **Charles D. Warren:** Methodology, Formal analysis. **Isabella Y. Kong:** Formal analysis. **Jacob B. Geri:** Methodology, Formal analysis. **James C. Lo:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY

Western blot original images are available at doi: 10.17632/t8nmk94n92.1. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD062327.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2025.102134.

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