LUDC-IRC Postdoctoral Program 2020 – Project 3

Role of intracellular C3 in homeostatic autophagy and survival in pancreatic β-cells

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Purpose and aims

To investigate the function of intracellular C3 within pancreatic β-cells, in cytoprotective autophagy.

- Firstly, to investigate blood glucose homeostasis in a new novel mouse model, a β-cell specific C3 knockout
- Secondly, to determine the mechanisms of C3 upregulation in response to diabetogenic stress
- Thirdly, to investigate interactions between C3 and autophagy machinery, in particular ATG16L1, within cells, largely using confocal microscopy and fluorescently labeled proteins.

State-of-the-art/background

C3 is a highly abundant acute phase serum protein involved in innate immunity, becoming activated in the presence of danger- or pathogen-associated molecular patterns. On activation, a small pro-inflammatory peptide, C3a, is cleaved off, and can signal to nearby surrounding cells via a cell surface G-protein coupled receptor (C3aR). The remaining fragment of C3, C3b, undergoes a conformational change, revealing a reactive thioester group, which covalently reacts with amine and carboxyl groups, therefore irreversibly depositing C3 onto the surface of pathogens. C3b is then recognized by activatory receptors on phagocytes, resulting in clearance and killing. C3 is therefore well understood as a multifunctional extracellular protein involved in innate immunity.

Surprisingly, we have recently discovered that C3 is also highly expressed within human pancreatic islets[1]. Islet C3 expression correlated with type 2 diabetes (T2D) status, as well as with BMI, HbA1c, and inflammatory status of the islets. C3 expression was also upregulated in isolated islets of several rodent models of diabetes. We have discovered that C3 is not only secreted from islet cells, but is also present within the cytosol, produced from an alternative translational start site[1] downstream of the secretory signal peptide. Running protein interaction microarrays, we discovered that C3 interacts with ATG16L1, a key component of autophagy machinery. CRISPR/Cas9 mediated C3 KO INS-1 cells suffered dysfunctional autophagy, and underwent more rapid apoptosis when faced with challenges usually alleviated by autophagy (for example, lipotoxicity).

The C3/ATG16L1 interaction has also been verified by others in the context of targeted killing of cyto-invasive pathogens by xenophagy[2], and others have shown intracellular C3 to be cytoprotective in immune cells[3], lung epithelial cells[4], and beta-cells. However, we are the only group working in the context of intracellular C3 and autophagy in pancreatic islet function. Autophagy is known to be cytoprotective for β-cells, and we therefore hypothesise that C3 upregulation is required for efficient autophagy enhancement and preservation of β -cell function[5].

Significance and scientific novelty

The truly cytosolic presence of C3 has been very controversial in the field of complement research in the last few years, but we have been first to demonstrate the mechanism of an alternative translational start site by which it reaches this cellular compartment, by the use of an alternative translational start site. The continued verification of functions of cytosolic C3 will therefore demonstrate the physiological roles of this protein, for which numerous targeting reagents have been developed in the treatment of inflammatory disease. Understanding that C3 is present intracellularly therefore opens possibilities not only for its pharmacological intervention, but also raises awareness that targeting of extracellular C3 could have unforeseen side effects. The field of intracellular C3 is therefore a hot and rapidly developing topic of investigation.

Likewise, within the field of diabetes research, better understanding of mechanisms controlling and alleviating β-cell stress would aid in the development of novel strategies for extending β-cell function and

survival. Endoplasmatic reticulum (ER) stress is a contributing factor to β-cell failure and apoptosis, leading to loss of β-cell mass and subsequent insulin deficiency. Autophagy becomes dysfunctional in β-cells in T2D, and autophagy induction has been shown to alleviate ER stress and prevent loss of β-cells in diabetic models[6,7]. Understanding mechanisms by which this occurs can therefore lead to targeted treatments aimed at sustaining β-cell function.

C3 is part of innate immunity, and inappropriate complement activation contributes to autoinflammatory disease. We have seen that increased C3 secretion from isolated diabetic islets also leads to auto-activation of C3 secreted into the supernatant, with cleavage of additional complement proteins. This should lead to production of C3a, which has proinflammatory cyto- and chemokine-like properties. This could conceivably lead to increased inflammation in the pancreatic islet, and influx of inflammatory cells, just as C3a has been linked to inflammation and subsequent insulin resistance in obese adipose tissue[8]. Understanding this dual role of C3 in pancreatic islets is therefore of utmost importance; on the one hand, a cyto-protective intracellular role, but a proinflammatory role in the extracellular environment[5]. We are developing methods to specifically and separately target either intra- or extra-cellular C3, in order to assess each function separately. This is particularly important given the current development and clinical introduction of C3-targeting pharmacological and biologic therapies.

Preliminary and previous results

We previously found that C3 was highly expressed in isolated human pancreatic islets, and that this expression was significantly higher in diabetic compared to healthy donors, a finding that was replicated in several mouse models of diabetes[1] (figure 1). In addition, human islet C3 expression correlated significantly with donor BMI and HbA1c. We found that C3 was present within the cytosol of human cells, interacted with ATG16L1, and that C3-knockout INS-1 cell clones had dysfunctional autophagy, and underwent increased levels of apoptosis under challenge with free fatty acids or islet amyloid polypeptide. We therefore hypothesise that C3 is upregulated in response to diabetogenic stress, in order to facilitate increased levels of cytoprotective autophagy.

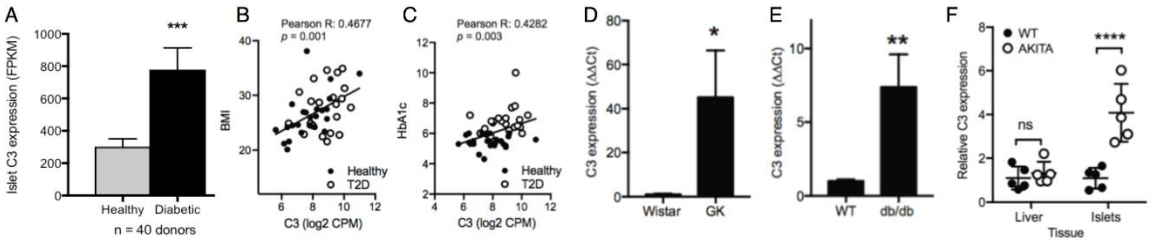


Figure 1: A) C3 expression is higher in freshly isolated human islets from diabetic compared to healthy donors, and B) correlates with donor BMI and C) HbA1c. D) Similar results were seen in isolated islets from diabetic GK rats, E) db/db mice, and F) diabetic Akita mice. C3 expression was not increased in the liver, showing islet specificity.

Since publication, we have investigated the mechanisms by which C3 enters the cytosol of cells, demonstrating that a non-canonical translational start site is used, downstream of the signal peptide that normally feeds the nascent polypeptide into the secretory pathway. This alternative isoform therefore bypasses ER, the site of post-translational modification such as glycosylation and processing of pro-C3 to separate alpha and beta chains. We have constructed gene-edited cell lines lacking the canonical C3 translational start site, therefore expressing only intracellular C3, and we are using these to specifically study the roles of intracellular rather than secreted C3. We have demonstrated that the intracellular pro-C3 found in the cytosol is unglycosylated, as expected due to ER bypass, and have raised specific antibodies recognizing unglycosylated, but not glycosylated C3, therefore enabling specific detection of the cytosolic isoform. These will be a vital tool in investigating and visualizing the functions of intracellular C3.

In C3 knockout cell lines, autophagy becomes dysfunctional, and we have been able to demonstrate rescue of autophagy by assessing C3-ΔATG1 gene-edited clones, which lack the conventional C3 translational start site, and therefore express only cytosolic, but not secreted C3. This rescues the phenotype seen in KO cells, demonstrating that it is the non-canonical cytosolic C3 isoform, and not canonical secreted C3, that is involved in autophagy (figure 2).

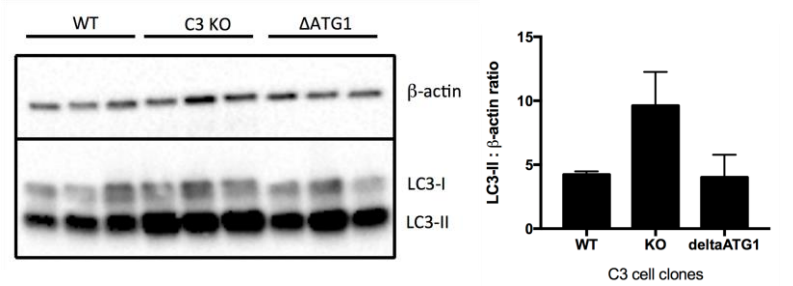


Figure 2: comparison of autophagic turnover, as measured by LC3-II levels, in WT, C3-KO and gene edited ΔATG1 clones, which have only intracellular but not canonical secreted C3. A reversal of the dysfunctional autophagic phenotype in ΔATG1 clones shows a role for cytosolic C3.

Research plan

We already have prepared a panel of cDNA constructs of C3 mutants, including with fluorescent tags, to be used to rescue autophagy in C3-knockout INS-1 cells, with readout of LC3I/II and P62 western blotting, as well as fluorescent confocal microscopy to visualize LC3-positive autophagosomes. We also have ATG16L1 and LC3 fluorescent constructs for studying protein colocalisation and recruitment to autophagosomes by confocal microscopy. We will also use deglycosylated C3-specific antibodies in combination with a large panel of C3-specific antibodies to study the processing of C3 by intracellular cleavage, and how this is used to regulate C3 function in autophagy at different stages, as hypothesized previously[5]. The C3 knockout INS-1 cells also provide a system by which we can test whether various C3 mutants can rescue this phenotype, allowing us to investigate which features of C3 are required for this function, for example a putative ATG16L1-interaction motif[9] that is also present within C3, or the reactive C3 thiolester group and cleavage of C3a peptide. Several such mutants have been already constructed and confirmed by Sanger sequencing.

These C3 constructs include ΔATG1 mutants lacking the canonical ATG start site, therefore preventing expression and secretion of canonical C3, but allowing non-canonical C3 to be expressed from a downstream translational start site in the cytosol. We hypothesize that cytosolic C3 is translated from non-canonical start codons within the canonical mRNA, dependent on conserved mRNA secondary structure, which we will test by introducing destabilising synonymous mutations. Use of non-canonical start codons can be dependent on the action of non-canonical translation initiation factors. We will investigate the function of these factors, their regulation by diabetogenic conditions e.g. glucolipotoxicity, and their involvement in production of cytosolic C3.

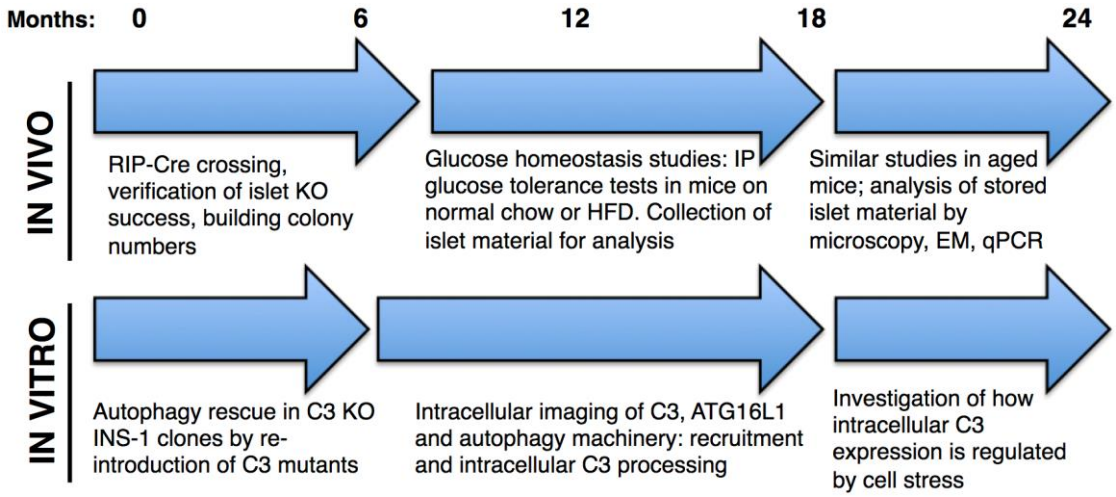
Finally we will cross the floxed C3 mice with RIP-Cre mice and then assess the resulting β-cell specific C3 knockouts in terms of blood glucose homeostasis (oral and peritoneal glucose tolerance tests), in young and aged animals, on normal and high-fat diets as well as after low dose streptozotocin injections to aggravate the development of diabetes. Isolated islets will also be assessed by western blotting for autophagy markers, as well as by electron microscopy to assess insulin content and for the appearance of autophagosomes, identifiable by their distinctive double membranes. Relative β-cell mass will be assessed by insulin staining. We expect that loss of C3, as a cytoprotective factor, will lead to diminished β-cell mass and a resultant decrease in blood glucose homeostasis, in particular in older mice on high-fat diets. This phenotype in the presence of normal blood levels of C3 will also strengthen the evidence that intracellular, and not canonical secreted C3, is the relevant isoform for this cytoprotective function. Since wt C3 is co-expressed with fluorescent dTomato reporter, we will be able to detect C3 expression in various cell types in pancreas using co-staining for other cell-type specific proteins like insulin, glucagon and somatostatin.

We will therefore use a combination of molecular and cell biology approaches, using gene-edited INS-1 β-cell clones, confocal microscopy, protein-protein interaction studies and functional/phenotypic readouts, in combination with a novel whole-animal β-cell specific C3 KO model. With this combination of approaches we hope to establish both the molecular interactions responsible for the function of intracellular C3, as well as the physiological outcomes at the whole-organism level.

The project is currently funded by a 5-year 18 million kronor grant from the Knut and Alice Wallenberg foundation to Professor Anna Blom (Wallenberg Scholar from 2020). Anna is the PI for this project and it is also being overseen by senior researcher Dr Ben King, who established the CRISPR/Cas9 gene edited and knockout INS-1 cell clones, and who also has relevant training for working with animals. A PhD student, Klaudia Kulak, is currently working specifically on this project, and is expected to finish her PhD in spring 2021 after which we plan to recruit new student. An experienced biomedical analyst Frida Mohlin is also aiding the experimental work for the project. The larger research group also contains further technicians and masters students who will be assisting in the laboratory work, in cell culture, protein analyses and western blotting.

Ethical permission for animal work and in particular running of diabetes models is held by Professor Erik Renström who is the crucial collaborator for the project due to his extensive competence in β-cell pathophysiology. All the techniques have previously been carried out within the groups of the lead PIs, as proven by publications.

As we already have established cell lines, methods, and all required cDNA constructs, we expect the in vitro work to proceed rapidly. Animal work will take a longer time due to breeding times, but within 2 years we expect to be able to gather in vivo data that will complement our in vitro findings. A 2-year timeplan for the project is outlined below:



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