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LUND
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Abstract
The Complement system is a main effector mechanism of the innate immune system, acting to enhance clearance of pathogens, but also aids removal of biological debris from the body, including immunocomplexes, apoptotic/necrotic cells and protein aggregates. Complement regulators serve to prevent excessive inflammation and their interaction with the same materials targeted by the complement system results in 'silent' cleaning of wastes. Type 2 diabetes (T2D) is characterized by insulin resistance in peripheral tissues resulting in an initial compensatory upregulation of insulin production but ultimately leading to failure of blood glucose homeostasis and death of insulin-secreting pancreatic β-cells. T2D is now understood to have several components, which drives pancreatic islet dysfunction: high glucose concentration, proinflammatory cytokines, long chain free fatty acids, increased insulin synthesis demand and increased exposure to islet amyloid polypeptide (IAPP). IAPP a hormone co-secreted with insulin from pancreatic β-cells is capable to form amyloid and intermediate species; oligomers that are highly cytotoxic for β-cells. IAPP oligomers have been also shown to activate the NOD-like receptor pyrin domain containing-3 (NLRP3) inflammasome leading to production of the pro-inflammatory cytokine IL-1β, which in high concretions is a driver of β-cell pathology. Previously we described binding of complement regulator C4-binding protein (C4BP) to IAPP amyloid that affected transition of IAPP monomers and oligomers to mature IAPP fibrils. Therefore, we hypothesized that C4BP might inhibit IAPP oligomer-induced death of β-cells, and limit inflammasome activation and IL-1β secretion secondary to β-cell failure. Presence of C4BP with IAPP monomers, which tend to assemble into oligomers and amyloid, resulted in better survival of cultured rat insulinoma INS-1 β-cells compared to cells treated with IAPP alone. Similarly, addition of C4BP with IAPP to macrophages limited IAPP-dependent inflammasome activation and IL-1β release, ensuring protection of β-cells against IL-1β-driven toxicity.

Dysregulated autophagy in β-cells coincides with failure of β-cells as well. Autophagy, a housekeeping activity, necessary for elimination and recycling of unwanted cellular components, supports β-cell health under metabolic challenges. A hub of the complement protein cascade, complement component 3 (C3) has been found to be highly expressed in human pancreatic islets with increased expression after exposure to β-cell specific stressors: IL-1β, palmitic acid (PA) and IAPP. We found that C3 regulates the process of autophagy and improves viability of INS-1 cells under IAPP and PA treatments. Furthermore, we found C3 to be cytoprotective against IL-1β induced death of β-cells. IL-1β driven upregulation of proapoptotic signalling does not seem to be dependent on canonical autophagy, but surprisingly intracellular/cytosolic C3 conferred protection to β-cells exposed to IL-1β.

Key words: Complement system, Diabetes, NLRP 3 inflammasome, IAPP, C4BP, C3, IL-1β, ATG16L1

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Coverphoto represents different components of type 2 diabetes: obesity, increased blood glucose and lipid levels, inflammation, risk of cardiovascular complications and genetic susceptibility (DNA, as a minor component).

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MADE IN SWEDEN

‘’First principle: never to let one’s self be beaten down by people or by events’’

(Maria Sklodowska Curie)

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***** *These authors contributed equally*

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Outside in : Roles of complement in autophagy. *(Review).* British Journal of Pharmacology. 2020 Jul. doi: 10.1111/bph.15192

Abbreviations

Aβ	Amyloid β
AD	Acidic transactivation domain
ASC	Apoptosis-associated speck like protein containing a CARD
ATG16L1	Autophagy Related 16 Like 1
BIR	Baculoviral inhibition of apoptosis protein repeat domain
CARD	Caspase activation and recruitment domain
CCP	Complement control protein
C3	Complement component 3
C4BP	C4b-binding protein
DM	Diabetes mellitus
DAMPs	Damage-associated molecular patterns
ER	Endoplasmic reticulum
FFA	Free fatty acids
IAPP	Islet amyloid polypeptide
IL-1β	Interleukin-1β
IL-1R	IL-1 receptor
LRR	Leucine rich repeats
LAMPs	Lifestyle-associated molecular patterns
MASP	Small MBL associated protein
MBL	Mannose-binding lectin
NLRs	NOD-like receptors
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NOD	Nucleotide-binding and oligomerization domain
PA	Palmitic acid
PAMPs	Pathogen-associated molecular patterns
PI3P	Phosphatidylinositol 3-phosphate
PRRs	Pattern recognizing receptors
PS	Protein S
PYD	Pyrin domain
ROS	Reactive oxygen species
TIR	Toll/interleukin-1 receptor
TLRs	Toll-like receptors
T1D	Type 1 diabetes
T2D	Type 2 diabetes
MAC	Membrane attack complex
mTOR	Mammalian target of rapamycin

Introduction: from the beginning to type 2 diabetes

The enormous diversity of multicellular life that we can observe today arose from single-celled organism, roughly 4 billions year ago. Through evolution, to adapt to new environments or changes, cells discovered that there is strength in numbers, aggregated into a riot of shapes and developed into a complex organisms with multiple organs. In order to function in multicellularity, cells took over specialized roles like for example coordination of movement, nutrient transport, elimination of wastes. Instructions necessary to define cell roles are contained in a strand of DNA, genetic information for making proteins. Every cell in a body contains the same DNA, inherited from parents, but, the utilization of different parts of DNA in different extents produces unique pools of proteins giving rise to structural and functional diversity of cells. Further, in order to transit from self-sufficient cell to well coordinated cellular team, cells developed communication systems. Cells use their surface as a point for information exchange, and the circulatory system as a carrier of cellular messages over long distance. Thus, a cell is the smallest building block of all living things and modifications at cellular level drive biological diversification, however, alterations that occur at the level of individual cells may lead to disease development as well.

All diseases start as disturbances in health at the cellular level. This might involve changes in the strand of DNA, inherited from parents, spontaneous mutations as a results of errors randomly occurring during formation of new cells, and induced mutations caused by agents in the environment that affects DNA structure. This might lead to changes in protein expression pattern and/or production of malfunctioning protein. Nonetheless, a lot of diseases that increased substantially in prevalence over the last 30 years seems to be determined overwhelmingly by non-genetic factors like diet and lifestyle choices (1). Those are, for example, cardiovascular diseases, diabetes, chronic lung diseases. Evolutionarily conserved biological systems that promote survival are designed to respond to environmental threats. The good example of that is fever that is an evolved defence, occurring as a consequence of inflammation caused by viruses or bacteria evasion and represents a beneficial response. Fever and inflammation behind it, promote action of immune system to fight the infection, but if excessive, might induce damage. Therefore, persistent exposure of cells to stress factors might lead to hyperactivation of

physiological survival pathways within the cells, initiating stress, injuries and even organ dysfunction. Type 2 diabetes (T2D) is strongly associated with environmental influences. Genetic predisposition to T2D development exists; however, clinical data frequently show that diet and other lifestyle choices might reverse the disease (2). In addition, the observed epidemic growth of T2D indicates genetic predisposition as a minor component. In T2D nutritional surplus, mainly high intake of fats and obesity behind it, are strong risk factors for disease development. Constant exposure to metabolic overload can yield hyper-reactivity of physiological pathways and these altered responses contribute to the disease phenotype. The epidemic prevalence of T2D and other chronic diseases implies that evolution has had no time to cope with rapidly changing environment and lifestyle of the modern world. The aim of this thesis was therefore to investigate how one particular pathway, the ancient complement system, may be involved in type 2 diabetes and how this may contribute to this modern disease.

Starting point of the study

The structural and functional features of the human body have been shaped over millions of years. There is no doubt that the evolutionary arms race of pathogens that invade our bodies has sharpened our evolution as well. This applies mainly to our immune system. The complement system, which will be described more in details in the next section, is an important part of innate immunity. Primitive complement proteins have been found in cnidaria suggesting that the origin of the system was established over 500 million years ago (3). For a long time the complement system has been viewed as a biochemical cascade of proteins, operating outside of the cells to detect and to destroy invading pathogens, as well as disposing of products of inflammatory injury like cellular debris or apoptotic cells, to ensure protection and homeostasis. Therefore, considering the well-known role of complement as a danger sensor (4), the interaction of complement components with varied environmental factors is plausible. Furthermore, taking into account the ancient origin of complement and the general evolutionary track, from cell to complex organism, implication in intracellular pathways should not come as a surprise. Indeed, close crosstalk between complement and metabolic disorders (5), tissue homeostasis (6) and intracellular actions of complement (7) have been identified. In addition, the complement system is an important part of innate immunity, which in recent years has emerged as a key mediator of both insulin resistance and defective insulin secretion during T2D development (8). All of these resulted in our exploration of complement gene expression in human pancreatic islets (9). In this study, evidences for significant expression of several complement genes in human pancreatic islets opened up new lines of investigation into the implication of complement components in (patho)physiological processes in β-cells (10).

The immune system

The immune system is traditionally divided into two parts: innate and adaptive, which work closely together to protect against the universe of pathogenic microbes. The innate branch is the first line of defence that acts in a very fast way. It is always present at the site of infection and ready to fight with inducers responding to activation by common mechanisms, recognizing molecules that are broadly shared by microbes. This is why the innate response is also called non-specific. The innate immune response consists of physical barriers like skin and mucous membranes, phagocytes like monocytes, macrophages, neutrophils and dendritic cells, and plasma proteins like cytokines and complement. Unlike innate immunity, the adaptive immune system manifests exquisite specificity, but develops over days or even weeks. The adaptive branch is primarily based on the development of antigen-specific receptors by T- and B-lymphocytes. The constituents of innate immunity, however, play a critical role in the initiation and subsequent direction of the adaptive immune system, as well as participation in the removal of pathogens that have been targeted by adaptive responses (11). Furthermore, innate immunity not only participates in response to infection but also plays an essential role in tissue-repair reactions. The table below shortly summarizes differences between innate and adaptive immunity.

Table 1	Components and main actions of innate and adaptive immunity			
	Innate	**Function**	**Adaptive**	**Function**
Cellular barriers	Skin Mucosal epithelium	Prevention of microbial entry	Intraepithelial lymphocytes	Cytokine release and killing of infected target cells, damage prevention of the epithelium
Blood effectors	Complement	Inflammation, leukocyte recruitment and activation, opsonization, microbial killing,	Antibodies	Recognition of antigens of foreign objects, activation of complement and effector cells via Fc receptors
	Cytokines	Systemic inflammatory responses, leukocyte recruitment and activation, modulation of inflammation	Cytokines	Lymphocytes are source of cytokine as wells, which function to mediate inflammation, and cell and inflammation modulation too
Cells	Phagocytes	Phagocytosis and killing, cytokine production, antigen presentation	B-lymphocytes	Antibodies production, antigen presentation
	Natural killer cells	Elimination of intracellular infection – direct killing of infected cell	T-lymphocytes	Helper T-cells produce cytokines to orchestrate response of other cell types, cytotoxic T-cells eliminate intracellular infection by direct killing of selected infected cells. Self-reactivity might lead to autoimmunity
Time course				
	Hours		**Days, weeks**	

Recognition of patterns of pathogen- or damage-associated molecules by innate immune cells

Upon a microbial infection, the body is alerted to the presence of the potential harmful pathogen thorough specialized receptors that are predominantly expressed by the cells of epithelial barrier and sentinel cells patrolling all the tissues, macrophages, neutrophils and dendritic cells. Those receptors, known as pattern recognizing receptors (PRRs) recognize conserved microbial signatures referred as pathogen-associated molecular patterns (PAMPs). Those motifs are specific to microorganisms like bacteria, viruses, parasites and fungi and are often a key element to ensure functionality or viability of the microbe, thus less subjected to changes, for example microbial nucleic acids, lipopolysaccharide (LPS) or peptidoglycan that is the major component of the cell wall of Gram-negative or Gram-positive bacteria respectively. In addition, PRRs sense endogenous, host-derived biomolecules known as danger-associated molecular patterns (DAMPs). DAMPs are for instance materials released upon cellular stress or from damaged or dying cells. Binding of PAMPs and DAMPs to PRRs prompts downstream

signalling cascades triggering inflammation resulting in production of cytokines/chemokines, induction of antimicrobial peptides and recruitment of phagocytic cells. Of note, DAMP-induced inflammation, which has a crucial contribution in inflammatory diseases, is termed sterile inflammation when it occurs in the absence of any infection (12). Interestingly, sterile inflammation might also be initiated by lifestyle-associated molecular patterns (LAMPs). This term has been proposed recently (13) and unlike DAMPs, which in the classical model of sterile inflammation are triggered by physical, chemical or metabolic noxious stimuli damaging the cell, the LAMPs typify for lifestyle factors such as high-fat diet-induced cholesterol or uric acid crystals, or asbestos/silica particles. While eradication strategies targeting PAMPs and DAMPs evolved over millions of years, and is followed by a return to homeostasis, efficient elimination strategies targeting LAMPs invoke rather improvised mechanisms that do not deal effectively or appropriately with modern and persistent lifestyle-associated immunostimulatory influences (13).

The main PRRs of the innate immune system are divided into four major classes (12) - Toll-like receptors (TLR) and NOD-like receptors (NLRs) are among them and are of particular relevance in this thesis, as they seem to be highly significant in inflammatory disorders, including type 2 diabetes.

TLRs and NLRs

TLRs are a family of the best-characterized receptors among the signalling PRRs, present at the plasma membrane and membranes of intracellular endosomes, ensuring detection of extracellular and intracellular PAMPs and DAMPs. NLRs, in turn, constitute a family of cytosolic PRRs, thus the primary role of these is recognition of cytoplasmic threats. The common feature between TLRs and NLRs is the presence of leucine-rich repeats (LRR) that are believed to sense cognate structural motifs of PAMPs and DAMPs. The second essential, conserved part in TLRs and NLRs is an intracellular signalling domain(s). This domain (TIR for TLRs and CARD (caspase-recruitment domain), PYD (pyrin domain), BIR (Baculoviral inhibition of apoptosis protein repeat domain), AD (acidic transactivation domain) for NLRs) recruits adaptor proteins, mediating signal transduction events, which ultimately leads to upregulation or suppression of genes and orchestration of inflammatory responses. The third canonical domain, present in NLRs but absent in TLRs, includes the central NOD domain (nucleotide-binding oligomerization domain) important for self-oligomerization of NLRs. Self-oligomerization through NOD-NOD interaction results in the exposure of the signalling domain promoting the recruitment and activation of downstream, effector molecules (12, 14).

The distinct spatial localization of TLRs and NLRs, divergent interactions of TLRs and NLRs paralogs with their ligands, and finally their conjugation with different adaptor molecules provides good protection of the whole cell against potential

insults. Further, to mount an effective immune response, substantial crosstalk between TLRs and certain NLRs and their signal transduction pathways occur. To date, 10 genes encoding functional TLRs (TLR1-10) and 22 NLRs have been identified in humans. NLRs are grouped into several subfamilies, based on the intracellular signalling domain.

The NLRP3 inflammasome

Signalling pathways downstream of TLRs and NLRs cumulates into the activation of transcription factors, which regulate the expression of proinflammatory cytokines favouring inflammation as a defensive response. Cytokines usually contain a signal peptide, directing proteins for secretion into the extracellular space (15). Nonetheless, transcription of proinflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL18) genes, which play a critical role in host defence, produces precursor forms of these proteins that need to be proteolytically cleaved for activation and secretion. IL-1β has been recognized as an important player initiating and sustaining inflammation in both type 1 diabetes (T1D) and T2D (16). Processing of proIL-1β and proIL-18 is mediated by caspase-1, which is also stored in an inactive state. Caspase-1 is, in turn, activated within a multi-protein complex called the inflammasome, that exists as inactive, monomeric cytoplasmic proteins, until stimulation causes them to oligomerise (15, 17). Considering the high potency of inflammasome activation products, these layers of controls are needed to prevent aberrant inflammasome activation. While adequate levels of inflammation are absolutely necessary for defence against pathogens (18), over-activation and self-sustained inflammation might cause pathological damage.

Inflammasomes are assembled by certain NLRs, upon recognition of specific ligands. One of these is the protein NLRP3 (NLR family pyrin domain (PYD) containing 3) senses a broad array of PAMPs and DAMPs resulting in the activation and formation of the NLRP3 inflammasome. The NLRP3 inflammasome is the most extensively studied of the identified inflammasomes. Due to the high diversity of NLRP3 inflammasome activators related to metabolic deregulations that are involved in induction of sterile inflammation, potential mechanisms underpinning NLRP3 inflammasome modulation have garnered a lot of attention in recent years (19).

The NLRP3 inflammasome responds to a disparate assortment of antagonists probably by sensing common cellular abnormalities triggered by these antagonists. The main abnormalities are potassium efflux, lysosomal disruption and mitochondrial dysfunction (17). The complete activation of the NLPR3 inflammasome occurs via 2 steps: priming and activation. The first or priming step involves the transcriptional upregulation of NLRP3 and IL-1β genes, and posttranslational modification such as deubiquitination (20) and phosphorylation

(21) of NLRP3 to allow for inflammasome oligomerization. PAMPs and DAMPs signalling via TLR pathways, and some cytokines stimulate the nuclear translocation of transcription factor NF-κß promoting expression of NLRP3 and pro-IL-1β. After the priming step, if the target ligand for NLRP3 is present, the NLRP3 inflammasome assembles (fig.1). Upon sensing of specific ligands through the LRR domain, NLRP3 undergoes a conformational change and thereby exposes a PYD domain allowing for interaction with the PYD domain of ASC (apoptosis-associated speck-like protein containing a CARD). The CARD domain of ASC then interacts with the CARD domain of pro-caspase-1, enabling self-cleavage of pro-caspase-1 to its enzymatically active form, which subsequently cleaves its targets, including pro-IL-1β (fig. 1) (17).

In addition to inflammatory cytokine production, NLRP3 inflammasome activation triggers pyroptosis, which is a lytic and highly pro-inflammatory form of cell death. NLRP3 inflammasome-activated caspase-1 cleaves gasdermin D that oligomerizes into the membrane, and forms pores initializing membrane rapture, release of cytokines and DAMPs and further perpetuating the inflammatory cascade (22) (fig.1). Therefore, two-step activation of the NLRP3 inflammasome represents an import regulatory checkpoint to avoid excessive production of potent inflammasome products that might harm the host. Although, in some scenarios, the inflammasome might be activated without priming (non-canonical inflammasome pathway) or activating steps (alternative inflammasome pathway (23)), the lack of either of these in the canonical pathway of NLRP3 inflammasome activation will result rather in a nominal magnitude response (17, 24).

The spectrum of severity of diseases caused by mutations affecting regulation of NLRP3 inflammasome ranges from relatively mild manifestations like familial cold autoinflammatory syndrome (FCAS), a disease triggered by exposure to cold characterized by episodic and recurrent rash, joints and muscle pain, headache and other symptoms of systemic inflammation, to the seriously disabling complications like neonatal-onset multisystem inflammatory disease (NOMID). NOMID is present at and within days of birth and involves inflammation through most of the body that can sustain permanent damages and lead to skeletal deformity and central nervous system abnormalities (25), demonstrating the potentially severe outcomes of unregulated inflammasome activation.

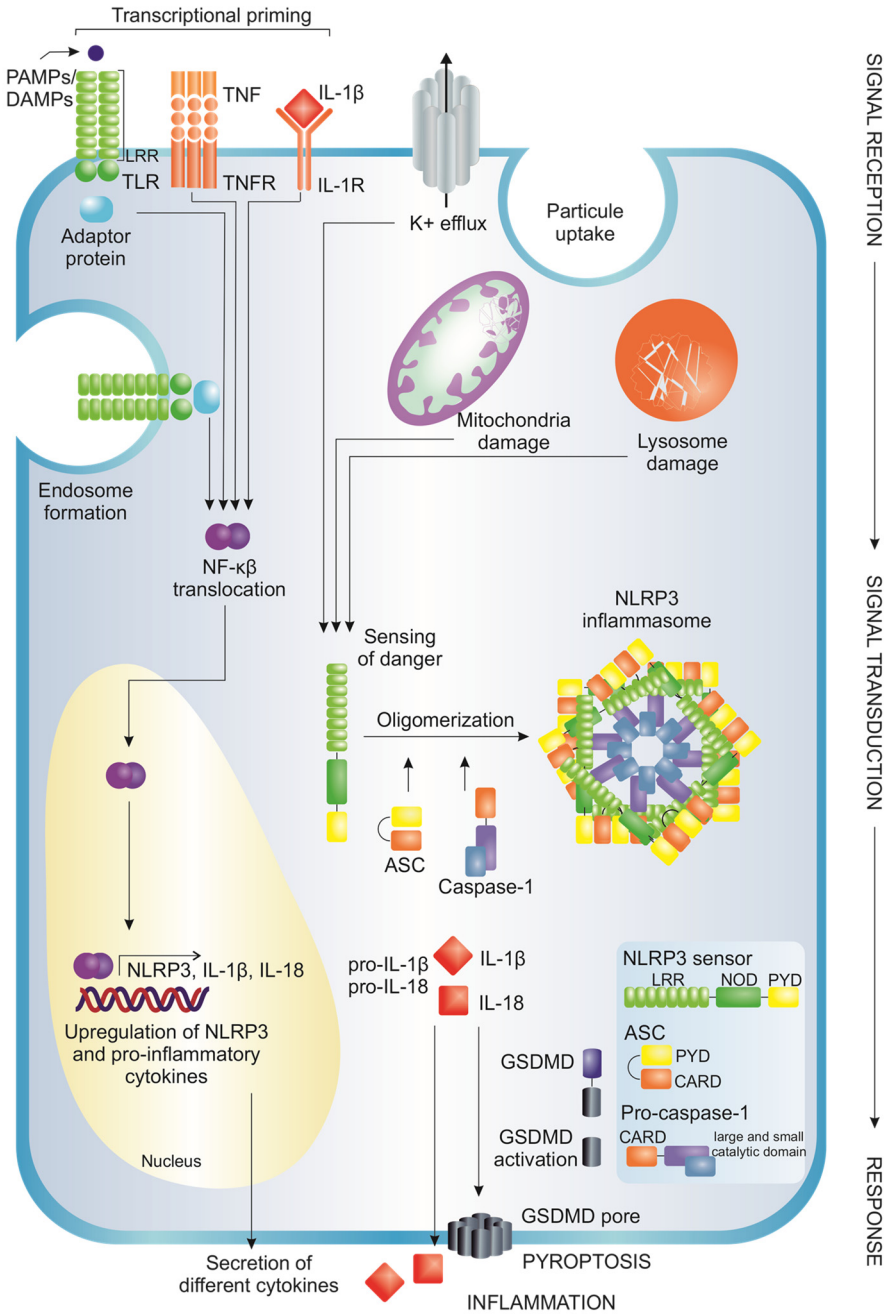
Transcriptional priming
PAMPs/
DAMPs
LRR
TLR
TNF
TNFR
IL-1β
IL-1R
K+ efflux
Particule
uptake
Adaptor
protein
Endosome
formation
NF-κβ
translocation
Mitochondria
damage
Lysosome
damage
NLRP3
inflammasome
Sensing
of danger
Oligomerization
ASC
Caspase-1
NLRP3, IL-1β, IL-18
Upregulation of NLRP3
and pro-inflammatory
cytokines
Nucleus
pro-IL-1β
pro-IL-18
IL-1β
IL-18
NLRP3 sensor
LRR
NOD
PYD
ASC
PYD
CARD
Pro-caspase-1
CARD
large and small
catalytic domain
GSDMD
GSDMD
activation
GSDMD pore
PYROPTOSIS
Secretion of
different cytokines
INFLAMMATION
SIGNAL RECEPTION
SIGNAL TRANSDUCTION
RESPONSE

Figure 1. Simplified model of action of TLRs and NLRs with focus on NLRP3 inflammasome assembly. LRR of TLRs located outside of the cell ensures detection of extracellular insults, while intracellular localization of LRR of TLRs (endosomes) and NLRs secure cell interior. Upon sensing of cognate ligand, PRRs undergo conformational changes exposing binding side of the intracellular biding domain allowing them to interact with adaptor proteins. Adaptor proteins transduce received signals to the cytoplasmic signalling pathways and govern further signalling and specificity. Initiated signal transduction cascades finalize at the step of transcription regulation. Translocation of NF-κβ into nucleus triggers upregulation of NLRP3 and pro-inflammatory cytokines expression resulting in secretion of cytokines, except for pro IL-1β and pro-IL-18 that need to be cleaved in the inflammasome to achieve activity. Trascription upregulation might also be triggered by TNF-alpha and IL-1β cytokines in autocrine loop. These signal-transducing events serve as a priming step for NLRP3 inflammasome activation as well. Primed NLRP3 inflammasome is ready to oligomerize in the presence of NLRP3 ligand, creating a platform for pro-caspase-1 self-cleavage. Therefore, NLRP3 inflammasome is composed of the sensor (NLRP3), adaptor (ASC) and an effector (caspase-1) that are bind to each other through homotypic interactions – PYD – PYD interaction between NLRP3 sensor and ASC adaptor and CARD – CARD interaction between ASC adaptor and caspase-1 effector. Assembly of the NLRP3 inflammasome triggers caspase-1 dependent cleavage of pro-IL-1β, pro-IL-18 and Gasdermin D, subsequently promoting potent pro-inflammation responses and pyroptotic cell death.

Recognition of patterns of pathogen- or damage-associated molecules by the complement system

In contrast to TLRs and NLRs that remain associated with cells, a number of molecules function in the bloodstream in soluble forms (26), scavenging for danger motifs that might have exogenous or endogenous origin. Every pathogen breaching the epithelial barrier encounters circulating, abundantly presented PRRs (26) and natural or specific antibodies existing freely in the bloodstream (27). Natural antibodies that are presented in pathogen-free conditions recognize evolutionary fixed antigens on foreign surfaces and specific antibodies are released upon recognizing an antigen that the body has previously encountered or produced after a certain time upon identifying a new infectious agent. Soluble PRRs (28), as well as antibodies (29) are also involved in elimination of the organism's own apoptotic cells, contributing to homeostatic maintenance of proper number of cells within tissues. Excessive and dysregulated responses against the body's self-material by PRRs and antibodies, however, might lead to immune tolerance breakdown and development of autoinflammatory or autoimmune diseases. Nevertheless, binding of soluble PRRs or antibodies to their corresponding PAMPs or DAMPs is able to activate the complement system.

The complement system is composed of around 50 proteins that are found in plasma as inactive precursors or embedded in cell surfaces functioning there as receptors or regulators of complement activation. Cooperation of complement proteins provides a powerful machinery for host defence against pathogens. Recognition of pathogenic and immunogenic surfaces by soluble complement PRRs and antibodies respectively, is a critical step in complement activation. The complement system is activated through three different pathways: classical, lectin and alternative. Natural or specific antibodies that form immune complexes with target surfaces primarily initiate the classical pathway through interactions with complement danger sensor C1q, which then undergoes conformational changes sequentially activating

associated serine proteases C1r and C1s. Circulating PRRs like mannose-binding lectins (MBLs) and ficolins, in turn, activate the lectin pathway. Once MBLs and ficolins recognize their ligands, which are predominately sugar groups found on the surface of pathogens, activation of MBL-associated serine proteases (MASPs) initiate the lectin route of complement cascade. The alternative pathway, in contrast to classical and lectin pathways, functions in a less specific manner, described below. It is constitutively active in the bloodstream but is also involved in the classical and lectin pathways where it acts as a powerful enhancer, amplifying complement system signals (fig 2).

The proteases activated upon binding of the complement danger sensors of the classical and lectin pathways, C1r/C1s and MASPs, cleave subsequent components of complement, C4 and C2, into C4a, C4b and C2a and C2b, producing the C3 convertase, composed of C4b and C2b fragments. The C4b2b complex forms a convertase that cleaves a hub protein of the complement cascade, that is complement component 3 (C3). Of note, cleavage fragments of complement proteins are distinguished from the parent molecule by suffix letter, designed accordingly to their relative size, with “a” fragments being smaller than “b” fragments (30).

C3 reaches concentrations between 1 to 1.5 mg/ml in serum and is the one of the most abundant proteins in circulation. One characteristic of C3 is the presence of a reactive thioester moiety that reacts with hydroxyl (-OH) and amine ($-NH_2$) groups present for example in the carbohydrates and proteins, respectively, allowing it to bind covalently to the cell surface of cells. Due to its high reactivity, the thioester is tightly hidden in native C3, and only revealed once C3 is activated. Although C3 circulates predominantly as an idle protein, a small portion of it is constantly activated by spontaneous hydrolysis of the thioester, which is referred to as the tick-over mechanism of the alternative pathway. Generation of hydrolyzed C3 – C3(H2O) – which has a different conformation to C3, increases its reactivity allowing for the binding of protease factor B. The bound factor B can be then proteolytically cleaved by serum protein factor D into Ba and Bb, leaving the C3(H2O)Bb complex that is the C3 convertase of the alternative pathway (31). The alternative pathway C3 convertase similarly to the classical and lectin pathway convertase, cleaves further copies of C3, producing smaller fragments carrying specific functions. Therefore, all complement pathways result in the generation of C3 convertases, leading to C3 cleavage (fig. 2).

Native C3 is a relatively inert protein but the reaction catalyzed by C3 convertases forms powerful and multifunctional molecules. The potency of this protein lies in its high transformability. Indeed, C3 is the most versatile and flexible immune mediator with many known binding partners (32) and with additional ligands still waiting to be verified (33) and maybe discovered. C3 is comprised of α- and β-chains connected by a disulfide bond. The first cleavage by C3 convertases occurs in the alpha chain, releasing a small sub-fragment of C3 – C3a with potent

inflammation-modulatory network activities (34). The remaining larger part, C3b, undergoes conformational changes, exposing the thioester group that attaches C3b to either of the two chemical groups widely present on the cellular surface of our cells or microbes: amino or hydroxyl groups (35). C3b opsonization is one of the key functions of the complement system, aiding recognition of pathogens by professional phagocytes, thus accelerating removal of unwanted material (fig. 2). To prevent deposition of C3b on healthy host tissues, all cells in our body employ complement regulators and an additional arsenal of efficient regulators circulate as soluble proteins. Complement regulators are designed to prevent the spontaneously activated complement cascade in physiological conditions and counteract host cell damage in the presence of infections (36). However, limited complement activation that takes place on the surface of host apoptotic and necrotic cells, inaugurated by complement initiating molecules: antibodies (37, 38), MBLs (39, 40), ficolins (41) or by direct binding of C1q sensor (42, 43) is a desired mechanism to maintain tissue homeostasis and induce clearance of apoptotic material and therefore limit the presence and immunogenicity of auto-antigens, and prevent autoimmunity. For example, C1q deficiency is associated with limited clearance, production of auto-antibodies, and systemic lupus erythematosus (SLE) a disease that involves widespread inflammation and tissue damage (44).

Along with complement regulators functioning to limit complement activation on healthy surfaces, the high reactivity of the C3 thioester with H_2O itself restricts C3b deposition to be a local event and prevents it from occurring far from the site of complement activation. Importantly, generation of C3b enables the binding of factor B that upon transformation by factor D forms the alternative C3 convertase (C3bBb). C3bBb cleaves more C3 into C3b, creating an amplification loop of the alternative pathway. Therefore complement regulators and short-lived thioester are important breakpoints of the complement cascade, preventing over-activation of complement system that might harm the host. Nonetheless, an additional effect of newly formed C3b, apart from opsonization, is generation of the C5 convertase.

High local concentrations of C3b on the target surface pressures binding of newly formed C3b to existing C3 convertases, converting C3 convertase specificity from C3 to C5, allowing the cleavage of C5 into C5a and C5b. C5a, similarly to C3a is an anaphylotoxin, however, with much stronger capacity to induce inflammation, including anaphylactic responses. In addition to the induction of inflammation, both anaphylatoxins are chemoattractants, thereby guiding the recruitment of various leukocytes expressing their receptors (neutrophils, eosinophils, and basophils, monocytes/macrophages, and mast cells) to inflammation sites during infection and tissue injury. Further, C5b as opposed to C3b doesn't have the ability to attach to surfaces due to lack of a thioester group, but it is anchored into lipid bilayers of cell membrane through the interaction with further proteins in the complement cascade, C6 and C7. The C5b-7 complex undergoes a conformational change exposing a hydrophobic site on C7 that allows the C7 to insert the complex into the hydrophobic

portion of lipid bilayer of targeted surfaces. Subsequent binding of the complex to C8 enables incorporation of multiple copies of C9 creating a ring structure through the membrane. The assembled C5b-C9 complex (also called the membrane attack complex (MAC)) forms pores, disrupting cell membrane integrity and inducing cellular lysis of susceptible pathogens (45), achieving the terminal event of the complement pathway (fig. 2). Given the strong inflammatory properties of C5a and MAC-induced disruption of cell membrane and thus release of potential DAMPs that might further perpetuate inflammation, inhibition of the complement cascade from the C5 level of the terminal pathway activity is reasonable when considering therapeutic inhibition of complement in chronic and severe acute diseases and to avoid interference with beneficial, homeostatic effects of complement e.g. C3b/iC3b opsonizing stimulation of immune complexes and apoptotic removal via phagocytes. Indeed, blocking of the terminal pathway with C5 inhibitor (eculizumab) and its use to treat some complement-mediated diseases has boosted the clinical development of new therapeutic strategies that target the complement system, however targeting C3 still might be advantageous in some contexts, and is under development (46).

Accordingly, the common functions of the complement can be shortly summarized:

- Anaphylatoxic events - C3a, C4a, C5a as anaphylatoxins, C4a of which is the weaker and C5a the strongest, trigger release of inflammatory mediators (like histamines and vasoactive effectors) eliciting, among other things, vascular permeability that results in tissue edema and leukocytic infiltration to engage pathogens in the infected tissues,
- Chemotactic effect – C3a and C5a are know chemoattractants, receptor-mediated movement guides immune cells to site of C3a and C5a release,
- Opsonising effect – deposition of C3b on pathogens and opsonization of apoptotic and necrotic cells by complement initiating molecules tags them for removal by phagocytes, significantly contributing to removal of unwanted materials,
- Immunomodulatory effects – the effects of complement that has not been mentioned above, but regulate how the rest of the immune system reacts to threat. C3a and C5a through their receptors act as an adjuvants of TLRs response resulting in augmentation of pro-inflammatory reactions (47-49). Furthermore, complement strongly mediates adaptive immunity reactions driving B-cell and T-cell responses in direct or indirect ways (50).

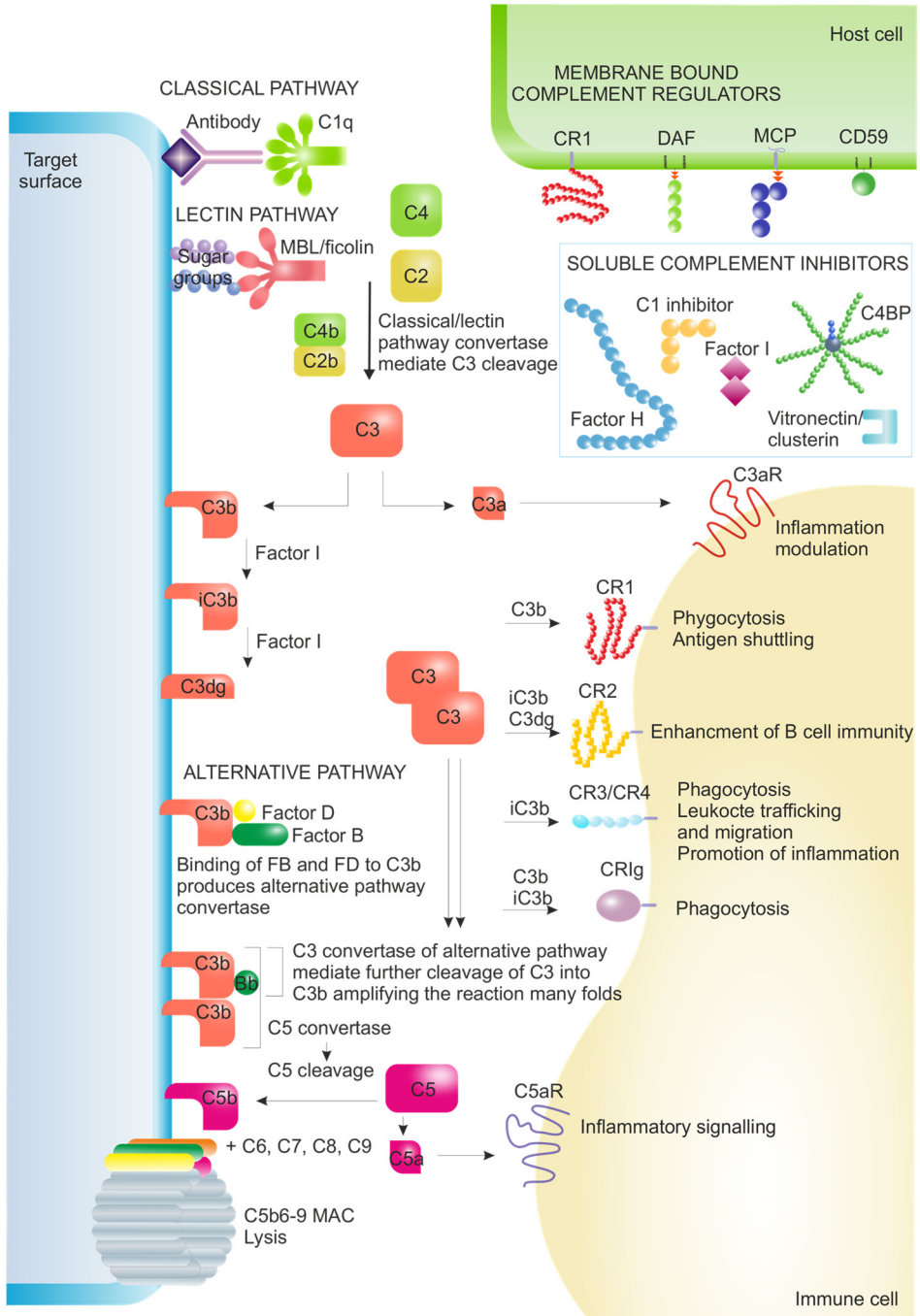
Host cell
MEMBRANE BOUND
COMPLEMENT REGULATORS
CR1
DAF
MCP
CD59
CLASSICAL PATHWAY
Antibody
C1q
Target
surface
LECTIN PATHWAY
Sugar
groups
MBL/ficolin
C4
C2
C4b
C2b
Classical/lectin
pathway convertase
mediate C3 cleavage
SOLUBLE COMPLEMENT INHIBITORS
C1 inhibitor
C4BP
Factor I
Factor H
Vitronectin/
clusterin
C3
C3b
C3a
C3aR
Inflammation
modulation
Factor I
iC3b
C3b
CR1
Phygocytosis
Antigen shuttling
Factor I
C3dg
C3
C3
iC3b
C3dg
CR2
Enhancment of B cell immunity
ALTERNATIVE PATHWAY
C3b
Factor D
Factor B
Binding of FB and FD to C3b
produces alternative pathway
convertase
iC3b
CR3/CR4
Phagocytosis
Leukocte trafficking
and migration
Promotion of inflammation
C3b
iC3b
CRIg
Phagocytosis
C3b
Bb
C3 convertase of alternative pathway
mediate further cleavage of C3 into
C3b amplifying the reaction many folds
C3b
C5 convertase
C5 cleavage
C5b
C5
C5aR
Inflammatory signalling
+ C6, C7, C8, C9
C5a
C5b6-9 MAC
Lysis
Immune cell

Figure 2. Schematic representation of complement system activations and regulations, fate of C3 and its cleavage products. Complement cascade can be initiated via one of the three canonical routs: classical, lectin and alternative. While classical and lectin pathways trigger the cascade more specifically by their corresponding pattern recognizing molecules: C1q and MBLs/ficolins/collectins, respectively, the alternative rout probes indiscriminately every surface and complement regulators occupying host cells, or in soluble forms counteract self-damages. Activation any of complement pathway merges at the step of proteolitical cleavage of C3 into C3a anaphylotoxin and C3b opsonin via bimolecular convertases. C3b is then incorporated in close proximity of the initiating surface. If it covalently attaches to pathogen surface lacking regulatory proteins, C3 convertases can form and persist to produce more C3 active products. Alternative pathway greatly contribute to production of C3b that constantly reinitiates the pathway by forming C3bBb convertase of alternative pathway, thereby amplify initial trigger many-fold. If C3b reaches healthy host surfaces complement-regulatory proteins either block formation of C3 convertases or promote their rapid dissociation. C3 convertase formation might be prevented by cleaving C3b into iC3b and C3dg in the presence of Factor I and complement regulators with cofactor activity. The cleavage fragment of C3 exerts function via binding to their cognate receptors. The next step in the cascade is generation of C5 convertase, formed by association of C3b with bimolecular C3 convertases. C5 captured with C5 convertase is then processed into C5a and C5b. C5b initiates assembly of the further components of complement system, their insertion into the pathogenic/immunogenic surfaces and formation of pores (MAC) that disrupt the membrane of targeted cells. Cascading arrangement and action of complement components mediates vigorous performance of the system effector functions.

A hub of complement cascade – C3 within the complement cascade

Complement C3 is characterized by a high versatility of spatial arrangement that controls its interactions and provides distinct and dynamic functions of the protein (51). Release of C3a from C3 after cleavage by the C3 convertase is just a first series of conformational changes leading to exposition of the shielded thioester and multiple cryptic binding sides in C3b. C3a exerts biological activities through ligation of the cognate receptor C3aR. Activity of C3a is controlled by serum carboxypeptidase N which rapidly cleaves off a C-terminal arginine residue, converting C3a to its des-Arg derivative which lacks pro-inflammatory activity due to loss of binding to C3aR. C3b, which propagates the complement cascade, is further inactivated (to iC3b) from functioning in the C3 and C5 convertases in a reaction mediated by factor I (in association with membrane-bound or soluble regulatory cofactors). Although iC3b cannot any more participate in the amplification loop of the complement cascade, it remains bound to the surface and largely enhances complement-mediated phagocytosis by gaining extra affinity to more phagocytic receptors (CR3 and CR4) in comparison to C3b (phagocytosis through CRIg) (fig. 2). Furthermore, conversion into iC3b exposes an additional binding site for the CR2 receptor that is present on B-cells and signals to stimulate B-cell responses and antibody production. The small C3f peptide cleaved out from C3b, has been demonstrated to increase vascular permeability and to act as a weak spasmogen (52). The third cleavage site in iC3b produces C3c and C3dg. The thioester bond within C3dg and C3d, which is a further final proteolytic product of C3dg, enables both these protein fragments to stay covalently attached to the targeted surface. C3dg loses affinity to CRIg but is able to signal through CR2. C3d acts as a strong molecular adjuvant increasing immunogenic reactions (53) (fig.2). All of these conversions of C3 into its smaller fragments occur by sequential cleavage by factor I. To sum it up, the sequential cleavage of C3b is not only important in regulating the chain reaction of complement activation, but drives a dynamic process by which the subsequently generated products lose binding to

different molecules to gain affinity to others. Nonetheless, the role of C3 does not seem to be restricted only to the complement cascade regulating innate and adaptive immunity but is much wider than just described.

A hub of complement cascade – C3 beyond the complement cascade

Over the last 2 decades the continued progress in the field of complement research led to dramatic changes in perception of the system. The complement system, discovered over 100 years ago for a long time has been perceived as solely an anti-microbial system. In the late nineteenth century, George Nuttall, Paul Ehrlich, and Jules Bordet were focusing on a thermolabile factor being present in serum and exhibiting bactericidal activity without involvement of immune cells. However the activity of serum was also dependent on the thermostable factor that turn out to be antibodies while thermolabile factors has been termed 'complement', as it aided the antibacterial activity of antibodies (54). Complement components are mainly produced by hepatocytes, from which they are distributed intravascularly (55). Due to the high concentration of complement proteins in the blood and the complementary activity with antibodies, it was obvious to hypothesize that complement mainly acts to offer protection against threats. Today, however, it is clear that complement proteins are synthesized by different cell types and the physiological spectrum of complement components by far exceeds host antimicrobial protection, and that the complement system, or its components on its own are highly intertwined with a wide array of bodily and even intracellular systems. This applies to C3 as well. C3 itself and C3 effectors have been implicated in regulation of a variety of systems in different cell types, deviating C3 from its traditional proinflammatory profile.

Lipid and glucose metabolism

Among complement researches, C3a des-Arg is rather considered as a non-operating byproduct of C3a degradation as it does not seem to have functional activity in the complement pathway (56). However, C3a des-Arg has been described in numerous studies in 90's and in the early 2000's from the Cianflone's laboratory (reviewed in (57) and (58)) to possess metabolic hormone properties that increases lipogenesis and glucose uptake by adipocytes and skin fibroblasts (59, 60). This has been linked to the to the pathogenesis of obesity via enhancement of lipid and glucose storage in adipocytes (61). Insulin secretion from β cells has also been shown to be augmented after C3a des-Arg treatment (62). Nonetheless, due to use of supraphysiologic doses of C3 des-Arg in the studies, and erroneous identification of the C5L2 receptor through which C3a des-Arg could exert theses effects (63-65), (other scientists have ruled out binding of C3a des-Arg to this receptor (66)), the presented data are controversial, but does not fully exclude implication of C3a des-Arg in lipid and glucose metabolism. It is worth noticing, C5L2 $^{-/-}$ mice displayed

reduced adipose tissue triglyceride synthesis (65), similarly to C3 $^{-/-}$ mice, which are unable to generate C3a des-Arg (67). However, some of the observed differences in metabolism could not be independently replicated in similar knockouts on a different genetic background (68), and it has been noted elsewhere that exact genetic matching and use of littermate controls is of vital importance when comparing metabolics in mice, due to significant differences even between different B6 substrains (69).

In 2014 a group of independent researches, identified C3a as a potent insulin secretion stimulator, used in relatively low doses, signalling through C3aR being expressed in the islets (70) and pointed to complement factor D (also known as adipsin) as an important mediator of this effect catalysing C3a. Calcium ions stimulate insulin release (71) and activation of C3aR leads to calcium influx in different cell types (72-74) which has also been shown to be the case for C3a-treated insulin producing β-cells, providing a potential mechanism by which C3a treatment of β-cells augments insulin secretion (70). In a follow-up study, it has been presented that chronic replenishment of factor D in diabetic *db*/*db* mice, used as a model that exhibits phenotype of human T2D including obesity and β-cell failure, ameliorates hyperglycemia and limits β-cell death. Further, factor D replenishment and C3a treatment downregulated expression of Dusp26, overexpression of which had deleterious effect on β cells, and its inhibition improved β-cell function in diabetic mice and human islets (75). Together with the reported diminished level of circulating factor D found in obese and diabetic patients and animal models, this suggests a model whereby C3a production, dependent on adipocyte-derived factor D, is required for optimal insulin secretion and metabolic control.

Bone remodelling

Several lines of evidence point to involvement of C3 in the bone remodeling process. Osteoclasts and osteoblasts are two main cell types participating in this process. While osteoclasts are responsible for aged bone degradation, osteoblasts manage new bone formation. It has been reported that activated form of vitamin D3 (1 alpha,25-dihydroxyvitamin D3) stimulate bone marrow cells to locally produce C3 that further lead to osteoclast differentiation (76) by modulating local IL-6 cytokine production (77). Moreover, C3a des-Arg and C3a have been shown to promote engraftment of hematopoietic stem and progenitor cells to bone marrow spaces. Hematopoietic stem and progenitor cells form all types of blood cells and engraftment of them takes place after transplantation, when the transplanted stem cells find their way to the bone marrow niches where they can survive and proliferate. This has been shown to happen in a C3aR-independant manner (78), enhancing responsiveness of the cells to stromal cell-derived factor 1, or directly through the C3a-C3aR axis by augmenting C3aR mediated secretion of matrix metalloprotease-9 and cell adhesion (79). Recruitment of bone morrow-derived mesenchymal stem cells to distant tissue, thus the opposite direction, has also been

reported (80). Mesenchymal stem cells are important in skeletal tissue repair due to their ability to differentiate into bone, cartilage, muscle and fat cells and their migration also involved C3a-C3aR signalling (80).

Development of central nervous system

C3a signalling plays an important role in central nervous system development as well as regulating neurogenesis (81-83) and neuronal migration (83-86). The effect of C3a-stimulated migration of neuronal progenitor cells was promoted by low dosages of stromal cell-derived factor 1 (83). Further, C3b/iC3b takes a part in remodelling of synaptic connections. C3b/iC3b mediated signalling promoted engulfment of improper synaptic connections through recognition by C1q (87, 88), regulating brain development as well as memory formation and retainment.

Liver regeneration

Other studies demonstrated a crucial role of C3 fragments in liver regeneration (89-91). C3a promoted hepatocyte proliferation while C3b/iC3b facilitated removal of damaged cells after induction of liver lesion with toxic carbon tetrachloride (89). Similarly, C3a was required for normal liver regeneration upon liver resection (90). In the other study (91), $C3^{-/-}$ mice suffered increased injury following 70% hepatectomy, in agreement with previous data (90), and in addition, increased steatosis, a pathologic condition where excess fat accumulates within hepatocytes due to a failure in lipid metabolism. In view of this finding and previously reported involvement of C3a des-Arg in lipid metabolism, administration of recombinant C3a des-Arg to $C3^{-/-}$ mice resulted in reduced steatosis and hepatic injury, and restoration of the proliferative response and survival of hepatocytes (91). Surprisingly, mice deficient in C5L2, a controversial proposed C3a des-Arg receptor, displayed similar phenotype as C3 deficient mice i.e. increased hepatic injury, mortality and steatosis and reduced regeneration following partial hepatectomy (91).

Skeletal muscle regeneration and differentiation

Activating components of complement: C3a and C4a have been shown to be upregulated in serum of healthy subjects after prolonged exercise, implying that complement activation might be an important part of a clearance mechanism of tissue debris released during physical work by metabolic tissue alteration (92). This conclusion seems to be confirmed by recently published findings, proving a crucial role of the alternative pathway activation in production of C3a and C3aR signalling in macrophage recruitment and macrophage-dependent muscle regeneration following cardiotoxin-induced injury (93). In addition, some indications exist pointing toward the involvement of C3 in production of muscle tissues. Proteomic and transcriptomic analyses revealed the complement C3 as an important factor secreted by muscle-resident preadipocytes that is internalized into myogenic cells

and promotes their differentiation (94). Additional proof of implication of C3 in myogenic differentiation comes from research on the regenerative biology involving amphibians (see below) (95).

Limb, lens and retina regeneration in non-mammalian species

Among vertebrates, amphibians are regarded as champions of regeneration. As such, they can fully regenerate their limbs (96), tail and spinal cord (97, 98), jaws (99), lens (100) and retina (101) and heart upon cardiac injury induction (102). It is realized by orchestrating complex morphogenetic and developmental strategies including rearrangement of pre-existing tissue, the use of tissue stem cells and dedifferentiation and/or transdifferentiation programs (103). It has been shown that C3 is specifically expressed in the regenerating limb during the cell reprograming and dedifferentiation processes and its expression in pluripotent blastema cells transforming toward the myotube lineage suggest involvement of C3 in muscle differentiation (95). In conjugation with C3 involvement in limb regeneration, C3 in cooperation with C5 has been found to promote lens regeneration and as previously, it was mainly synthetized in blastema cells (104). Further, C3a also induced repair and regeneration of the embryonic chick retina (105).

Intracellular sensing and action of C3

In recent years, a perception of C3 as an entirely intravascular and extracellular mediator has changed. It has been shown that intracellular sensing of C3, carried on the surface of pathogens provides signals restricting pathogen infections. It has been demonstrated that C3 directs autophagy against pathogens extracellularly opsonized with C3 that enter into the cytosol and hence, limits their intracellular growth. This is achieved via an interaction of C3 with Autophagy Related 16 Like 1 (ATG16L1), an intracellular protein involved in autophagosome biogenesis,. (106). Autophagy is a natural process allowing for utilization of the cell's own damaged organelles and/or subcellular debris for energy supply and for renewing cellular structures. Autophagic phenomenon that specifically captures intracellular microbes is called xenophagy. Analogous to this finding, a previous study reported that internalized C3 covalently attached to pathogens in the extracellular space triggers responses in non-immune cells via intracellular receptors (107). This has been shown to be achieved by activation of MAVS (mitochondrial antiviral-signalling protein) resulting in signalling and proteasome mediated degradation of viral particles (107). While C3 is brought into intracellular space from the outside of cells, reports exist suggesting the existence of intracellular complement proteins and intracellular mechanisms of activation that play homeostatic functions (108). C3aR engagement has been shown to provide a survival signal for effector T-cells (109) that has later been connected to mTOR activity (110). Intriguingly, it has been shown that resting T-cells contain lysosomal and endosomal C3 stores and intracellular generation of C3a mediated by cathepsin L cleavage and activation of it cognate receptor present on lysosomes contribute to these effects (7). Upon T-cell receptor activation, the

intracellular activation system shuttles to the cells surface, where binding of C3a and C3b to their corresponding receptors, C3aR and CD46 in an autocrine fashion, upregulates INF-γ production and induce T helper type 1 (Th1) differentiation (7). In addition, intracellular C3a presence is not T-cell restricted but occurred in other tested cell types, including immune cells other than T-cells and non-immune cells, suggesting that intracellular C3 activation might be a broad phenomena (7). One very interesting observation of the study was that C3 mRNA generates intracellular C3 being actively processed to its active forms C3a and C3b in T-cells of patients with primary serum C3-deficiency (7). Although this article was the first reporting intracellular processing of C3 in a C3-convertase independent manner, the cellular process of control of C3 activation or deactivation by self-contained proteases has already been outlined. Mast cells are long-lived tissue-resident cells with readily available pre-formed granules filled with inflammatory mediators making them perfectly poised to mediate inflammatory responses after infection (111). Both tryptase (112) and chymase (113, 114), one of the major proteases of mast cell secretory granules have been shown to cleave C3 giving rise to C3a (112, 114). These indicate that cellular control mechanisms independent from the conventional complement pathways exist in mast cells and point that cell-dependent arsenal of C3 cleavage meditators might exist. Interestingly some subset of mast cells express C3 (114, 115), pointing to possible self-sufficient regulation of C3a-driven inflammation.

Furthermore, intracellular C3 has been described to be important to maintain homeostatic autophagy flux in β-cells via interaction with ATG16L1, with C3 deficiency resulting in defective autophagy ((33)-paper III). Autophagy is constantly active within cells, but under adverse conditions might accelerate and then usually represents a pro-survival mechanism. Accordingly, β-cells deficient for C3, exposed to their typical damage-causing agents that usually induce protective autophagy, underwent higher apoptosis comparing to wild type counterparts ((33)-paper III). The paper also revealed that a mutation of the canonical start codon within the C3 sequence results in translation of a non-secreted, cytosolic C3 from a non-canonical in-frame start site, providing a mechanism by which downstream start sites might yield context-dependent C3. Indeed, cytosolic expression of C3 has been found to be crucial for β-cell protection against IL-1β-induced cytotoxicity (paper IV). Interesting, extracellular addition of C3a conferred partial protection as well as, although not statistically significant (paper IV). Further, it has been shown that C3(H2O) can be internalized from the extracellular space by endocytosis in diverse types of cells that can be cleaved and produce C3a fragments (116). The exogenous cytoprotective nature of C3 has been demonstrated in airway epithelial cells, which despite possessing their own intracellular C3, also utilize internalized exogenous C3 to mitigate oxidative stress-induced apoptosis (117). These studies imply a possibility of cooperation between exogenous and endogenous C3 pools to ensure homeostasis, but it might be cell and context dependent. On the other hand, the strong potential of complement to trigger inflammation can quickly turn the

system, with C3 being in the centre of the cascade, from homeostatic effector to a driver of immune and inflammatory diseases (44). Therefore a potential functional difference and dissociation between liver-derived and intracellular C3 might exist and it is of great interest to understand the character of dynamics in the conversions and interaction of these networks in order to rationalize therapeutic approaches. Furthermore, the discovery from T-cells of an intracellular complement activation system that translocates to the cells surface and its autocrine stimulation providing different outcomes on T-cell physiology, indicates that complement activities are strongly dictated by the location of both complement activation and complement receptors (7). Similarly, distinct localization of C3 within the cells; within the lumen of vesicles such as endosomes and lysosomes (7, 116) vs. cytosolic ((33)-paper III, paper IV, (106, 107) might drive different activities of complement and govern its signalling. To increase the degree of intricacy of C3 linked to its localization, conformational versatility and activation adaptability it is noted that C3 internalized from the surrounding milieu might modulate gene expression in B cells, but whether this occurs in different cell types, has not been addressed (118).

Regulators of complement cascade

Given that the complement system is liable to provoke damages, and considering the way in which it is serially activated and rapidly amplified, it must be subjected to tight control to protect host cells. One of the safeguards in fast inactivation of the strongest effectors, C3a and C5a, by arginine removal is mediated by the action of carboxypeptidases, and C3b by rapid hydrolysis of the externalized thioester, if it does not bind nearby surfaces. The second safeguard is provided by an arsenal of complement regulators that are either anchored within the membranes, or circulate in blood. Complement inhibitors cover major checkpoints of complement activation and combine to prevent complement activation from proceeding.

Membrane bound inhibitors

The externalized thioester bond in C3b has no mechanisms to distinguish between an acceptor amino or hydroxyl groups on the host cell from those on the surface of a pathogen. Although, there are two other activating routes of complement cascade (classical and lectin), the alternative pathway might contribute up to 80% of the overall response (119). Because, most of complement activities take place on the surfaces, the membrane bound inhibitors play an important role to protect host cells from uncontrolled attack. Most of them interact with C3b and either:

- Prevent the convertase from forming – complement receptor 1 (CR1) that sequesters C3b and C4b from participating in pathway activation, and

membrane cofactor protein (MCP/CD46), a cofactor for Factor I catalysing cleavage of C3b into inactive derivative iC3b,

- Promote rapid dissociation of C3b and C4b from convertase complexes - decay accelerating protein (DAF/CD55) binds C3b and C4b and dissociates these subunits of C3-convertases.

An additional main regulator is CD59 that inhibits the closing phase of complement pathway – MAC formation.

Soluble inhibitors

Fluid phase inhibitors are particularly important to limit overproduction of C3b by circulating C3 convertases. Among them are inhibitors of initiation pathway: C1 inhibitor, inactivating C1r, C1s and MASP-2, and sMBL (small MBL associated protein) and MBL-1, which bind to MBL and regulate the lectin pathway. Other soluble complement inhibitors target C3 convertases by displacing Bb from the alternative pathway convertase – this is mediated by Factor H, or by displacing C4b from classical/lecting C3-convertase what is achieved by action of C4b binding protein (C4BP). Factor I, in turn inactivates C4b and C3b from functioning in convertases when they are complexed to C4BP and Factor H respectively. Although the alternative pathway is consider to be mainly controlled by Factor H, C4BP has also been found to be cofactor for Factor I mediated cleavage of C3b (120). Vitronectin and clusterin prevent MAC formation. The importance of some of these inhibitors are nicely illustrated by deficiencies. For example, C1 inhibitor deficiency cause hereditary angioedema characterized by recurrent attacks of severe swelling (angioedema) of the skin and mucus membranę, and factor H deficiency is associated with kidney diseases such as: atypical hemolytic uremic syndrome (HUS), C3 glomerulopathies, and IgA nephropathy (44).

In the class of soluble regulators, C4BP is a principal interest of this thesis.

C4BP

C4BP mainly regulates complement activation in the fluid-phase. However, C4BP also engages host-specific surface pattern such as glycosoaminoglycans (121), thereby also regulating complement on many self-surfaces. Non-synonymous polymorphisms at C4BP locus are associated with spontaneous pregnancy loss (122), but complete C4BP deficiency has not been reported, indicating a high importance of the protein. C4BP is an oligomeric protein with the major circulating form being organized in a octopus-like structure, comprised of 7 identical α-chains assembled from 8 CCP domains and one β-chain composed of 3 CCP domains connected through a central core (fig. 3). CCP stands for **c**omplement **c**ontrol

protein modules and are typical for complement regulators. CCP 1-3 (123) and CCP 1-4 (120) of each α-chain act as cofactors to factor I in the cleavage of C4b and C3b respectively, and are thus involved in regulating convertases of all three complement pathways. The β-chain binds and controls activity of anti-coagulation protein S (PS) contributing to regulation of the coagulation pathway (124). PS is also known to bind to negatively charges phosphatidylserine, exposure of which on the surface of cells represent one of the earliest stage of apoptosis, and facilitates prompt removal of cells undergoing apoptosis by phagocytosis (125). PS accompanied with C4BP binding to the surface of apoptotic cells (126) potentially counteracts inflammation arising from complement activation on these cells. C4BP binding to DNA exposed during induced cell death also resulted in complement activation attenuation and decreased production of TNF-alpha (127). Apoptotic cells downregulate expression of their own cell-surface complement inhibitors, and C4BP and factor H also limit complement activation and complement mediated lysis (128) that could prompt release of DAMPs and lead to unnecessary high inflammation. C4BP has been also shown to bind to islet amyloid polypeptide (IAPP) (129) and amyloid β (Aβ) (130) as well as amyloidogenic human prion protein (131). Amyloids are proteinaceous deposits located extracellulary that accumulate in tissues and organs, are lacking biological functions and strongly predispose to degenerative diseases: IAPP, which is a hormone co-secreted with insulin and thus overproduced with the increased secretory demand for insulin during insulin resistance that is associated with T2D, (IAPP will be described more in the next section) predisposes for T2D, Aβ for Alzheimer disease (132) while human prion proteins also lead to neurodegeneration (133). All types of amyloids bind C1q allowing for activation of the classical complement cascade, but a lack of MAC deposition (129, 131, 134) suggest an inhibitory effect of C4BP on later activation stages. The observed presence of C3 cleavage products allows for removal by phagocytosis aided by complement receptors. Apart from inhibiting complement activation, C4BP, might also suppress inflammatory responses by other

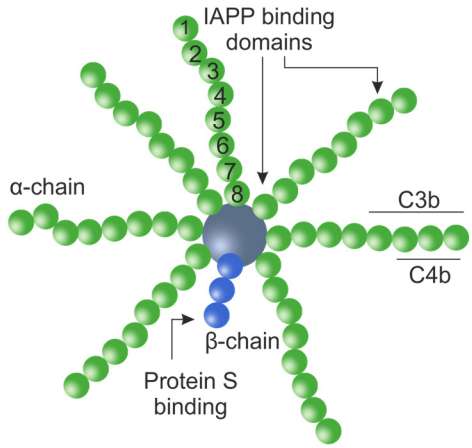

Figure 3. Structure of C4BP. C4BP has an octopus-like structure with major form being composed of seven, elongated and flexible alpha-chains and one β-chain that are linked to central oligomerization domain. C4BP form lacking β-chain is known as an acute phase variant, produced during infections. C4BP is dominated by CCPs, with 8 and 3 CCP domains in alpha-chains and β-chain, respectively. CCP1 of the β-chain contains high affinity to Protein S, while complement regulatory activities are located on alpha chains. CCP 1-3 and 1-4 binds C4b and C3b, respectively and identified binding sides for IAPP are on 2 and 8 domains.

mechanisms. We showed that C4BP affects the transition of IAPP monomers and oligomers to IAPP fibrils (129, 135), oligomers of which are considered as a strong diabetogenic factor that produces cytotoxicity (136). IAPP oligomers exert cytotoxic effects on β-cell directly through interaction and disruption of the cellular membrane, or indirectly through their engulfment by infiltrating macrophages, which triggers phagolysosomal membrane destabilization and inflammasome-dependent IL-1β production. Consequently, shortening the half-life of pro-inflammatory IAPP oligomers mediated by C4BP, translated into better survival and functionality of β cells limiting membrane damage ((137) – paper I) and by preventing phagolysosomal instability and resultant inflammasome-activated production of IL-1β in macrophages ((135) – paper II). It is noteworthy that in addition to liver as the main source, significant expression of C4BP is evident in lungs and pancreas (http://www.gtexportal.org), which might indicate a high importance of tissue-specific C4BP gene function. Indeed, C4BP is expressed in pancreatic islets with upregulation after exposure to prolonged high glucose concentration ((137) – paper I) and inflammatory stimulation with IL-1β ((135) – paper II). C4BP, therefore, seems to be an important endogenous, cytoprotective player that in the pancreatic environment allows for down-regulation of inflammation by limiting β-cell death ((137) – paper I) and by allowing silent phagocytosis of apoptotic/necrotic cells (127) and IAPP deposits ((135) – paper II). To further support an immunomodulatory action independent of complement regulation, the acute form of C4BP, without β-chain, has been shown to regulate TLR4-induced responses in dendritic cells and promote differentiation of dendritic cells toward a tolerogenic, anti-inflammatory phenotype with retained high endocytotic activity (138). Furthermore, a non-canonical immunosuppressant role for C4BP has been found in a mice model of antibody-driven lupus nephritis, a disease leading to kidney damage (139). Interestingly, an intracellulsar action of C4BP has been described in a recent study (140), where it interacts with cytosolic RelA, one of the subunits of the NF-κβ complex, providing a possibility of regulation of processes involving NF-κβ action.

Involvement of complement in diseases

Given the role of complement in danger sensing and its primary function as a surveillance system, it not surprising that small changes in this network can tip the balance of the system and provoke disease development. First of all, deficiencies, polymorphisms and dysfunction of complement components are risk factors for deregulation of the system and to inducing serious clinical manifestations including autoimmune, inflammatory, degenerative, hematological, and ischemic disorders. The common plot of implication of complement system in disease involves sensing of potential insults, an amplified inflammatory reaction due to excessive activation and/or inadequate control, and activation of downstream inflammatory responses that might fuel vicious cycles from overloading with damage. Exaggerated

complement activation in the presence of intact complement factors takes place as well. This usually occurs when the host is confronted with an overwhelming amount of PAMPs, DAMPs or persistent presence of undesired factors through the constant exposition of the body to noxious stimuli and/or inefficient elimination (reviewed in (44, 141).

In recent years, innate immunity and complement have been linked to both insulin resistance and defective insulin secretion (reviewed in (5, 8)). Given no supporting evidence that complement gene variations result in higher susceptibility to develop diabetes mellitus, it seems that involvement of complement factors in diabetes, is rather incidental due to the presence of triggers that can lead to destabilization of the complement system.

Diabetes mellitus

Nowadays, diabetes is a highly common, significant and costly lifelong health burden with increasing worldwide incidence. According to the International Diabetes Federation diabetes affects almost 9% of the world population and just in 2019 year alone diabetes contributed to 4.2 millions of deaths (https://idf.org). Diabetes falls into two main categories: type 1 and type 2 diabetes, but the most prevalent is T2D, accounting for about 85-90% of all diagnosed cases of diabetes (https://www.idf.org). T1D and T2D are metabolic disorders characterized by increased blood glucose levels over a prolonged period of time resulting from defects in insulin secretion, insulin action or both, respectively. Insulin is a hormone produced in regions of the pancreas the islets of Langerhans, by endocrine β-cells. The most important stimulus for insulin production includes the rise of postprandial blood glucose concentration. Increased production of insulin and its influence on target tissues, primarily adipose tissue, skeletal muscle and liver, which act as blood glucose buffer systems, improves the level of blood glucose. First, insulin promotes glucose uptake to insulin-sensitive tissues that convert glucose into energy straight away, or store it for later use. Second, insulin supresses hepatic glucose and triglyceride production, and inhibits adipose tissue lipolysis. Therefore optimal insulin synthesis and insulin metabolism is important to keep physiological concentrations of sugar level in the bloodstream and prevent complications that might occur if the insulin signalling network fails. The elevated blood glucose level and poor blood flow and mechanisms of injury behind it, might lead to development of micro- and macrovasular diseases (142). Macrovascular complications, affecting large blood vessels, are associated with development of atherosclerotic coronary heart disease, peripheral vascular disease and strokes. Microvascular disease (small vessel disease), in turn might induce damage in the retina (retinopathy) leading to blindness, kidney damage and failure (nephropathy), and peripheral impairment of neurons (neuropathy) that in combination with peripheral vessels disease are leading causes of nontraumatic amputations. Diabetes is a progressive degenerative disorder consuming around 10% of the total healthcare expenditure worldwide (143).

Pancreatic inflammation is the one characteristic that occurs in both types of diabetes, although development of the diseases involves different factors. While T1D involves destruction of β-cells driven by overt autoimmune attack, T2D is a result of a combination of insulin resistance and impaired insulin secretion that is associated with autoinflammation (16). Despite being of different background of

development and thus, treatment approaches (144, 145), both are characterized by hyperglycaemia and insulitis. Constantly present cellular stress from sustained hyperglycaemic environment might generate building ups of different kinds of intracellular DAMPs (like DAMPs released from damaged cells, accumulation of unfolded/misfolded proteins) and extracellular DAMPs (for example advanced glycated proteins formed after high sugar exposure). The physiological role of immunity is to eliminate DAMPs to maintain homeostasis, however this seems to be deregulated in diabetes in favour of detrimental effects over the beneficial ones. The table below summarizes general hyperglycaemic effects on innate immunity that are present in both types of diabetes. Information in the table was largely inspired from (146).

Table 2	Systemic dysregulation of innate immunity action in hyperglycaemia					
	Innate immune cells	**Results**	**Ref.**	**Complement**	**Results**	**Ref.**
Underactivation	Phagocytosis	Reduced movement of innate immune cells and reduced bacteria killing diminish host resistance to infections and wound healing	(147-150)	Inhibitor CD59	High and prolonged blood sugar level leads to glycation of CD59 inactivating the protein from effective prevention of MAC deposition. Increased MAC deposition is observed in target organs of diabetes complications.	(143)
	Chemotaxis					
	Bactericidal activity					
Overactivation	TLR 2/4, RAGE	Increased TLR 2/4, RAGE (PRRs of advanced glycated proteins) expressions and higher production of pro-inflammatory cytokines contribute to tissue damage and diabetes complications. Increased expression of adhesion molecules enhances adhesion of leukocytes to the endothelium	(151)	Autoantibodies	Autoantibodies primrily present in T1D might be present in T2D as well, and initiate complement activation	(152, 153)
	Inflammatory cytokines		(16)	MBL	MBL increases in both types of diabetes and might activate complement cascade by binding to neoepitopes induced by hyperglycemia	(154, 155)
	Adhesion molecules		(156)	C3	Elevated plasma level of C3 is observed in both T1D and T2D	(157, 158)

Type 2 diabetes

T2D is associated with obesity and aging and low-grade inflammation is a feature of the disease. The course of disease involves insulin resistance of peripheral tissues followed by increased burden on β-cells for overproduction of insulin in order to compensate for insulin insensitivity, which eventually can lead to hypersecretion-induced β-cell dysfunction. Obesity is estimated to cause 65-80% of new diabetes

cases, while physical inactivity alone accounts for around 7% of new cases (https://www.euro.who.int/en). In healthy individuals postprandial hyperglycaemia stimulates macrophages to produce IL-1β that potentiates glucose-stimulating insulin secretion via the IL-1 receptor (IL-1R) that is more abundantly expressed on β cells than in any other cell types. In turn, insulin reinforces macrophage glucose uptake and IL-1β production, generating a feed-forward loop between IL-1β and insulin for glucose disposal (159). Over-nutrition thus might lead to unresolved inflammation, activating the innate immunity network to mount pro-inflammatory reactions contributing to insulin resistance, β-cell damage and diabetic complications. Indeed, decreased insulin sensitivity and β-cell failure is accompanied by an increased number of pro-inflammatory cells that infiltrate adipose and muscle tissues, and the pancreas in obese individuals. At very low concentrations, IL-1β initially stimulates insulin secretion (159, 160), as well as β-cell proliferation (161, 162), however, it can turn against β-cells and switch to trigger β-cell death. Long-term exposure to IL-1β contributes to β-cell exhaustion and triggers proapoptotic signalling. In turn, increased glucose and IL-1β autostimulation of inflammasome priming establish a vicious circle. In further support of IL-1β as a diabetogenic cytokine, reports from human clinical trials provide evidence of improved β-cell function after blocking IL-1β signalling using IL-1R antagonist anakinra (163, 164), or blocking IL-1β activity by the specific antibody canakinumab (164-166). IL-1β interfering agents resulted in reduction of levels of glycated hemoglobin (HbA1c), which reflects blood glucose levels over a 6- to 12- week period, and also decreased risk of cardiovascular complications. Despite the achieved benefits of the treatment, for the median 4 years duration of the study, IL-1β blockade failed to reduce the incidence of diabetes (166).

Several cellular stresses that can originate from nutritional surplus have been implicated to mediate both insulin resistance and β-cell failure and those include: oxidative stress, endoplasmic reticulum (ER) stress, lipotoxicity, glucotoxicity and dysregulated autophagy. IAPP amyloid deposition is an exception and its damaging potential seems to be confined mainly to the pancreatic environment. Each of these cellular burdens are associated with inflammatory reactions, either induce or can be exacerbated by inflammation and are strongly linked together (8, 167). However, before a short description of those factors and their contribution to inflammation and *vice versa*, it is noteworthy to recall that IL-1β is produced in a 2-step mechanism of inflammasome activation typically by immune cells, but of note, β-cells are also a source of low doses of IL-1β.

Generation of the active, mature form of IL-1β requires two steps: while the first step is necessary to initiate pro-IL-1β expression and inflammasome components, the second signal is required for inflammasome oligomerization to cleave IL-1β and appears to be triggered by cellular abnormalities like potassium efflux, lysosomal disruption, mitochondrial dysfunction/reactive oxygen species (ROS) overproduction and ER stress. In T2D, free fatty acids (FFA), and glucose may serve

as a priming step. These endogenous factors are elevated in obese and T2D individuals and are able to prime the process of inflammasome activation signaling via TLR2 and TLR4 that results in NF-κβ translocation and upregulation of expression of the NLRP3 inflammasome components (168, 169). Considering that a higher concentration of FFA and glucose is closely associated with western lifestyle and diet, while elevated concentration of it after a meal is likely easily resolved in healthy individuals with adequate lifestyle choices, FFA and glucose might qualify as the recently proposed term of LAMPs (13). No priming step results in nominal or no IL-1β generation. Indeed, diet and exercise seem to have a great power in prevention of T2D (2). The available data to date points that genetics plays a role in development of diabetes, but it seems that environmental and lifestyle risk factors play a first violin in the majority of cases. Epigenetic analysis, detecting changes in functioning of genes that do not have an altered DNA sequence, could provide better understanding of the disease (170), but due to technical limitations are not currently widely available. Nonetheless, high incidence and cost of care of diabetes and diabetic complications necessitate the understanding of the pathological mechanisms that drive DM progress. Given the complexity of the disease, insulin resistance and β-cell death are likely a result of an interplay of multiple factors.

Glucotoxicity and lipotoxicity

Over time, chronic elevated levels of glucose and FFA that result in glucolipotoxicity contribute to the progressive deterioration of β-cell function and induce β-cell apoptosis, effecting gene transcription in β-cells and triggering metabolic alterations such as oxidative stress, ER stress and autophagy impairment (171). Among dietary fats, saturated FFAs, among them palmitic acid seems to exert the most detrimental effect on β-cell survival (171). Higher glucose and FFAs also induce and amplify macrophage-meditated inflammation (168, 169).

Oxidative stress

High glucose concentration contributes to formation of reactive oxygen species. β-cells are particularly susceptible to oxidative stress due to a high ROS production after increasing metabolic activity for insulin demand, and at the same time, they express low levels of antioxidative enzymes (172). Oxidative stress and ROS contribute to suppression of the insulin response and also contribute to development of insulin resistance (173).

ER stress

Proteins destined for different compartments of the endomembrane system, or to secretion, initially fold, mature and undergo quality control checks within the ER. The human insulin gene encodes preproinsulin. In the ER, preprosinsulin is converted to proinsulin upon cleavage of the signal peptide and then folded into the correct conformation. Further maturation to insulin is achieved in the Golgi complex, where insulin is packed with processing enzymes into secretory granules. The high demand of insulin synthesis and chronic insulin overproduction might lead to accumulation of unfolded protein molecules and tilt the balance toward ER stress (174) and the unfolded protein response (UPR). ER stress is also thought to be implicated in insulin resistance (175).

IAPP

Islet amyloid polypeptide (IAPP), or amylin is a hormone expressed in β-cells and co-secreted with insulin. IAPP contributes to maintenance of glucose levels namely by controlling gastric emptying and satiety, and by suppression of glucagon secretion (176). Human IAPP is a highly amyloidogenic polypeptide and hyperamylinemia in the pancreas is strongly associated with β-cell death. IAPP amyloid deposits are one of the pathological hallmarks of the pancreas in T2D patients (177). Additionally, the severity of T2D is closely correlated with the amount of amyloid deposition (178).

Expression of IAPP is regulated by glucose metabolism and calcium concentrations, but is activated by inflammatory mediators and fatty acids as well as (179). On glucose stimulation, increased secretion of insulin is accompanied by augmented concentrations of IAPP (180). IAPP is synthetized as a preproprotein, the signal peptide is cleaved off in the ER to form pro-IAPP, which is further converted to the mature protein in the Golgi apparatus, by packing with insulin and processing enzymes into insulin granules. Once produced, it is co-released with insulin upon stimulation. However, increases in IAPP expression together with an overwhelming of ER processing machinery and non-efficient conversion of pro-IAPP into IAPP favour accumulation of intermediates of IAPP, and excess of pro-IAPP (136). Human IAPP that normally has an alpha-helical structure adopts a non-native β-sheet conformation, a critical state of the protein in the early stage of amyloid aggregation. Pro-IAPP is fibrillogenic (181) and thus might result in assembly of toxic aggregates and serve as a template to promote polymerization into amyloid through a seeding-nucleation model (182). The process of oligomerization of IAPP in the extracellular space involves interactions of IAPP with lipid membranes that favour IAPP β-sheet formation and assembly to amyloid. Self assembly of the IAPP β-sheet monomers into higher structures produces oligomers, which enlarge into protofibrils that elongate and assemble the mature amyloid fibrils. The current

consensus is that amyloid monomers and extended fibrils are not the main cause of toxicity, but it is mostly ascribed to oligomers and protofibrils (136). Insertion of oligomeric IAPP species into membranes forms ion-leaking pores and reduces cell viability (183). Oligomers that enter the cytosol might perforate membranes of other organelles, such as permeabilized mitochondrial membranes seen in human IAPP transgenic mice (183), adding to the mechanisms of cytotoxicity. Of note, mouse and rat IAPP are not amyloidogenic (136), although human-IAPP transgenic mice develop spontaneous T2D symptoms in accordance with pancreatic amyloid deposition. Oligomers of IAPP are also a strong inducer of the NLRP3 inflammasome activation, contributing to IL-1β production (184) in T2D.

Given the high potential of IAPP to induce β-cell damage through either a direct mechanism of membrane permabilization, or indirectly via activation of NLRP3 inflammasome and IL-1β release, development of IAPP amyloid modulating drugs represents a considerable interest for treatment of T2D. Although, a numerous developed compounds that affect IAPP amyloid formation have shown a great success in limiting IAPP-mediated cytotoxicity (136, 179), clinical studies have not yet been reported.

Autophagy

Autophagy is a housekeeping activity, necessary to recycle intracellular material and remove material with potential cytotoxic properties. It is an evolutionarily conserved process occurring in all eukaryotic cells and being constantly active at a basal level, ensures breakdown of surplus or redundant proteins and damaged organelles for reuse and recycling, or for energy production. Autophagy is a general term of the process of degradation of the cellular components by delivering them to lysosomes. Among the 3 types of autophagy: macroautophagy, microautophagy and chaperon-mediated autophagy, the most studied is macroautophagy, which is mediated by generation of organelles termed the autophagosomes. Macroautophagy (referred to as autophagy hereafter) is a dynamic process that is assembled via several sequential steps: induction/recognition of the cargo, nucleation of the isolation membrane/phagophore, formation of the enclosed autophagosome and completion by fusion with a lysosome and formation of the autolysome. I will briefly summarize the mechanism of autophagy to increase the understanding of its role in β-cells.

A brief description of autophagy mechanisms

Induction of selective autophagy, that targets unneeded or harmful material, starts with recognition of cargo with a cellular signal that brings the labelled content through either direct or indirect interaction with Atg (autophagy-related genes) proteins to the nucleation site of the pre-autophagosomal structure (185). Bulk or non-selective autophagy that is induced upon nutrient deprivation involves

inhibition of mechanistic target of rapamycin complex 1 (mTORC1) and random uptake of regions of the cytoplasm into a precursor of the autophagosome called the phagophore. Even though they are initiated by two different mechanisms, selective and bulk autophagy seem to follow one path to create the autophagosome (186).

In humans, nucleation of the autophagosomal membrane appears to originate through extension of membranes that might derive from ER, mitochondria, ER-mitochondria junctions, ER-Golgi intermediate compartments, Golgi-endosomal membranes and plasma membranes (187), and requires activation of ULK complex that tethers into the site of phagophore nucleation (188). Further, the nucleation stage involves activation of the class III PI3K complex (consisting of PI3K, Beclin-1, VSP34 and ATG14L) through ULK-dependent phosphorylation of the key components. The complex then, promotes local production of phosphatidylinositol 3-phosphate (PI3P), leading to the recruitment of PI3P-binding partners (such as WIPI1/2) and the subsequent recruitment of proteins involved in expansion of the isolation membrane, resulting in the formation of the double-membraned autophagosome. The elongation and closure of the isolation membrane is regulated by two conjugation systems. The first, mediates the formation of the ATG12-ATG5 complex, which then binds to ATG16L1, which in turn might be recruited to the isolation membrane through interaction of ATG16L1 with WIPI2. The ATG12-ATG5-ATG16L1 complex stimulates lipidation of LC3-I, produced through a second conjugation system. The LC3 conjugation system functions to cleave cytoplasmic LC3 into LC3-I and adds phosphatidylethanolamine (PE, a major component of various biological membranes), to LC3-I to produce the membrane associated form called LC3-II. LC3-II is specifically and stably associated with the phagophore structures and mature autophagosomes, thus its abundance correlates with autophagosome numbers. Lipidation of LC3 facilitates autophagosome maturation. Finally, after successful initiation and elongation, mature autophagosomes reach the lysosome, either directly forming an autolysosome, or indirectly by fusing with endosomes, creating the intermediate amphisome. Fusion of the autophagosome with endosomes or lysosomes is mediated by a set of SNARE proteins (185) (189).

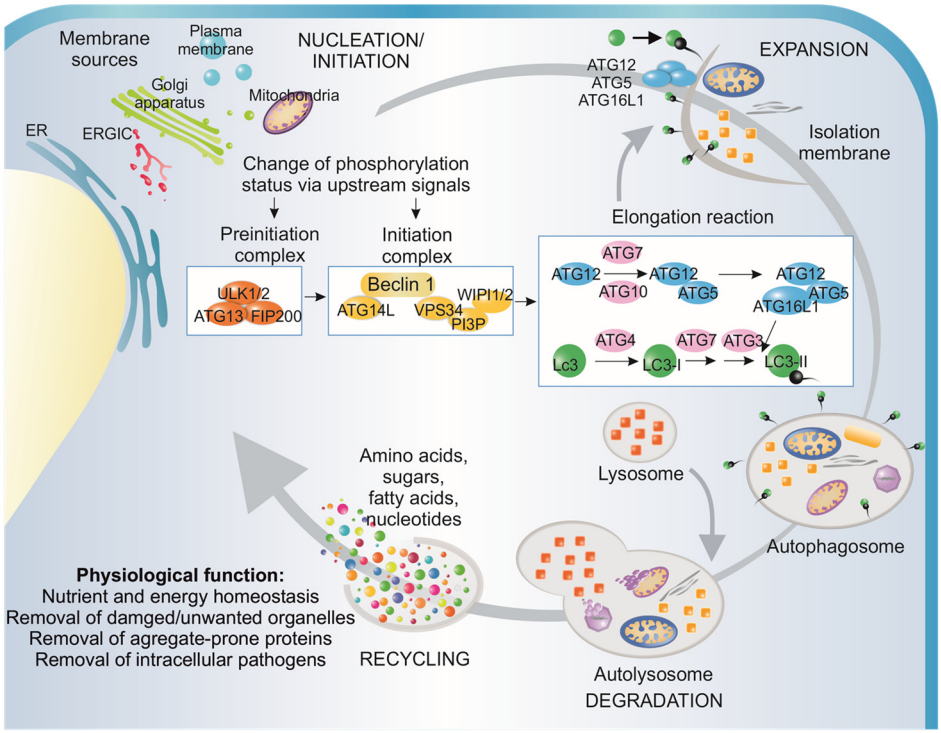

Figure 4. General autophagy pathway. Autophagy is a complex series of events and process is still poorly understood. The endoplasmic reticulum (ER), ER-Golgi intermediate compartment (ERGIC), mitochondria, Golgi apparatus and plasma membrane are suggested to supply lipids to the growing isolation membrane. Signals involved in autophagy induction change the phosphorylation status of ULK1/2, which then activated the 'initiation' complex (also called PI3K complex). 'Initiation' complex might likely be activated by other mechanisms as well. Vsp34 within the complex specifically phosphorylate phosphatidylinositol (PI) to produce phosphatidylinositol (PI) 3-phosphate (PI3P) at the side of nucleation of the isolation membrane, This leads to binding PI3P binding partners, including WIPI1/2 that participate in subsequent recruitment of proteins involved in elongation step. Two ubiquitin-like protein conjugation systems are involved in autophagy expansion: the first mediate the conjugation of ATG12 with ATG5 (via ATG7 and ATG10). ATG12-ATG5 conjugate reacts with ATG16L1 generating complex, which functions as part of the second ubiquitin-like conjugation system. LC3 is cleaved via ATG4 forming cytosolic LC3-I, which is then activated by ATG7 and transferred to ATG3 and linked to membrane phosphatidylethanolamine (PE) to generated membrane bound form of LC3-I called LC3-II (or LC3-PE). The transfer reaction of LC3 from ATG3 to PE is mediated by ATG12-ATG5-ATG16L1 complex, which also specifies the side of LC3 lipidation. LC3 is the only autophagy protein stably associated with mature autophagosomes and is widely used as an autophagy marker. Further steps include fusion of completed autophasome with lysosome, degradation and reuse of degraded material. Examples of autophagy physiological activities are listed in the figure. The figure is an adaptation of an existing one (189).

Autophagy in β-cells

A growing body of research indicates that compromised autophagy in β-cells is one of the components of diabetes, and enhanced autophagy induced by insulin resistance triggered by obesity and/or physical inactivity serves as a protective mechanism against β-cell dysfunction (reviewed in (190, 191)). A significant contribution to understanding a role of autophagy in β-cells derived from studies employing mice with β-specific knockouts of ATG7, which is involved in autophagosome formation, taking part in both conjugation systems facilitating

conjugation of ATG5 to ATG12 and LC3 lipidation (192) (fig.4). Lack of ATG7 resulted in a loss of β-cell architecture, mass and function (193) and high fat diet in these mice (194) or β-cell loss of ATG7 in obese ob/ob mice (195) led to induction of diabetes, due to impaired glucose tolerance and decreased levels of insulin, and accumulation of protein aggregates within β-cells, strongly supporting protective roles of basal and inducible autophagy against β-cell damage. Metabolic challenge of β-cells with FFA like palmitate (lipotoxicity) or human IAPP oligomers initially triggers autophagy acceleration, and autophagy-insufficent or deficient β-cell are more susceptible to these stresses. Long-term exposure is associated with impairment in autophagy (reviewed in (190, 191)) consistent with reports of dysfunctional autophagy in β-cells of T2D donors (196, 197) and indicating that autophagy insufficiency leads to an impairment in adaptive response to cellular stress. Owing to an intense research in recent years, there is rather consensus regarding the homeostatic function of autophagy in β-cells and the autophagy-mediated preservation of function of the organelles such as mitochondria and ER. In line with this, autophagy enhancements by therapeutic agents is a rapidly emerging research area to treat or prevent diabetes (190, 191).

Type 1 diabetes

T1D, unlike T2D is characterized as an autoimmune disorder, resulting from failing to distinguish between 'self' and 'non-self' structures by adaptive immunity, and subsequent destruction of the insulin-producing β-cells. T1D affects mostly children (below 15 years of age) but may develop in adults as well. The pathogenesis of the disease is thought to involve cytotoxic T-cells, which via the T-cell receptor recognize β-cell antigens presented by antigen presenting cells. In addition, exposure of B-cells to β-cell specific autoantigens induces production of islet-targeting autoantibodies. A specific trigger prompting β-cell-targeted autoimmunity is unknown, but association with genetic factors such as HLA (human leukocyte antigen) high-risk alleles suggest autoantigen presentation, and polymorphisms within PTPN22, CTLA4 and IL2RA contribute to higher activation of T-cells. Environmental factors that affect immune regulation, such as virus infections, and gestational events, have also been implicated (198, 199). During the autoimmune assault, pro-inflammatory cytokines, including IL-1β, are released and signal to β-cells resulting in their loss (198). Long-term innate immunity activation might contribute to metabolic interferences through inflammation, glucotoxicity and lipotoxicity, oxidiative stress and ER stress. Nonetheless, IL-1β blockage in patients with new-onset T1D has been shown to be ineffective to meet the primary outcome of protection of β-cells but the approach is still attractive with combination of adaptive immune modulators, although this requires new studies (16).

Hypothesis and major findings of the papers

Paper I: C4b-binding Protein Protects β-Cells from Islet Amyloid Polypeptide-induced Cytotoxicity

Hypothesis: Previously, we found that C4BP binds to and alters IAPP fibril formation; therefore we hypothesized that it might limit deleterious effects of toxic IAPP oligomers and protect β-cells against IAPP oligomer-induced cell death.

Major findings: Monomeric IAPP forms de novo aggregates and intermediate IAPP oligomers are particularly toxic for β-cells. In line with this, addition of monomeric IAPP resulted in lysis of erythrocytes, in contrast to membrane-inert IAPP fibrils. Erythrocyte lysis induced by IAPP oligomers was prevented by presence of C4BP. Accordingly, C4BP co-presence with IAPP monomers resulted in reduction of cytotoxicity and preserved the ability of rat insulinoma INS-1 cells to secrete insulin, and also preserved insulin secretion from primary rat islets compared to IAPP treated islets alone. Co-treatment of C4BP with IAPP invoked overexpression of genes involved in cholesterol synthesis compared to IAPP alone. Cytoprotective effects of C4BP were completely abolished upon acute membrane cholesterol depletion, indicating a process for cholesterol-dependent regulation of protective C4BP action.

Paper II: The human serum protein C4b-binding protein inhibits pancreatic IAPP-induced inflammasome activation

Hypothesis: IAPP oligomers have been recognized as a proinflammatory trigger for NLRP3 inflammasome activation, after being taken up by macrophages and causing phagolysosomal membrane integrity loss. C4BP, by shortening the half-life of pro-inflammatory IAPP oligomers, may inhibit IAPP-induced IL-1β secretion from macrophages.

Major findings: Using plasma-purified human C4BP, THP-1 monocytes differentiated into macrophages and primary human peripheral blood monocyte derived macrophages, we showed that C4BP significantly limits IL-1β production triggered by IAPP in a dose dependent fashion using physiological concentrations. C4BP did not affect IL-1β production induced by inflammasome activators that do not cause lysosomal destabilization, whereas it was also able to inhibit NLRP3 activation in response to other lysosomal damage-dependent inflammasome triggers. IAPP and C4BP/IAPP complexes were actively taken up into macrophages' phagolysosomes, and presence of C4BP prevented IAPP-induced loss of phagolysosomal membrane integrity. C4BP-mediated decrease of IL-1β concentration translated into better survival and functionality of INS-1 β-cells. We have found that human pancreatic islets themselves secrete C4BP protein, with upregulation in response to IL-1β stimulation. Altogether, C4BP has been identified as an inhibitor of IAPP-mediated NLRP3 inflammasome activation and resultant pancreatic islet inflammation in T2D.

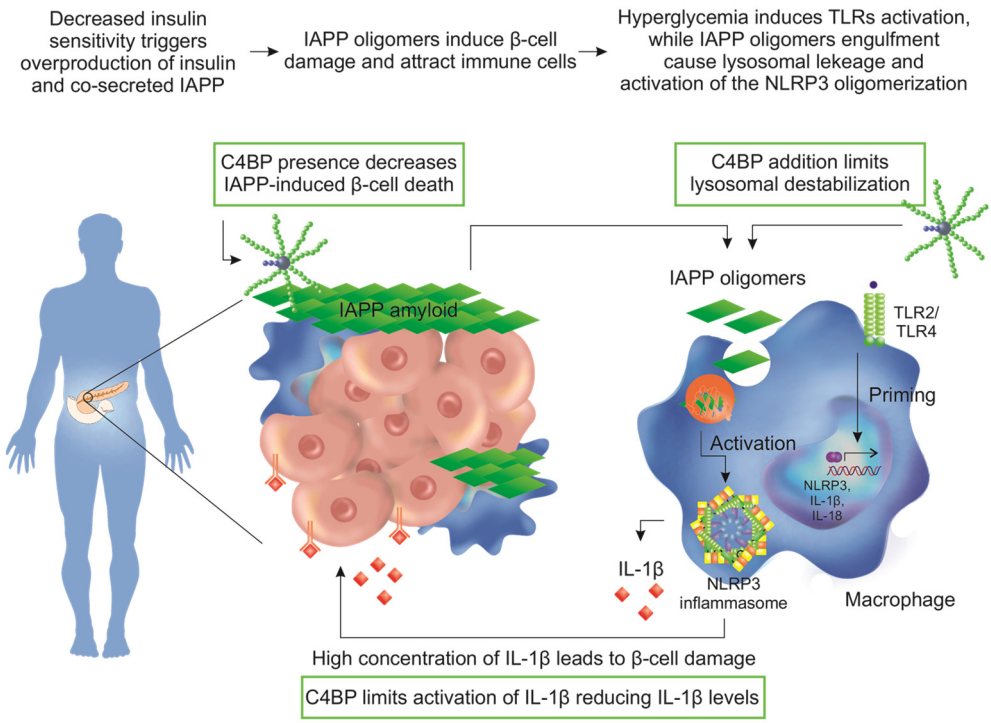

Figure 5. C4BP counteracts IAPP-induced damage of β-cells and IAPP-mediated NLRP3 inflammasome activation, and improves β-cells survival and function

Paper III: Complement component C3 is highly expressed in human pancreatic islets and prevents β cell death via ATG16L1 interaction and autophagy regulation

Hypothesis: A level of expression of C3 in human pancreatic islets higher than ubiquitously expressed complement inhibitors CD46 and CD55 (9) might be indicative of an important function of C3 in β-cells. Using protoarray and revealing ATG16L1, an important component of macroautophagy, as a potential C3 binding partner, we aimed to test the hypothesis if C3 might regulate macroautophagy in β-cells.

Major findings: Firstly, we found high expression of C3 in human pancreatic islets and positive correlation with T2D-related attributes like body mass index and HbA1c, and inflammatory markers. Following protoarray results and confirming C3 interaction with ATG16L1, C3 has been found to be important to maintain the rate of autophagy flux as evidenced by accumulation of LC3-II and p62 in CRISPR/Cas9 C3 knockout INS-1 cells, as well as isolated pancreatic islets from C3 deficient mice compared to wild type counterparts. β-cell specific autophagy inducers (IAPP and palmitate) led to increased cell death of C3 deficient INS-1 cells, which were characterized by autophagy insufficiency. In addition, using cDNA construct with a mutated canonical start site within the C3 sequence, we demonstrated that a non-canonical start site might be utilized and produce protein directly to cytosol, where it could interact with ATG16L1 and exert pro-autophagic and pro-survival functions.

Paper IV: Intracellular C3 protects β-cells from IL-1β-driven cytotoxicity

Hypothesis: In our previous investigation we found significant positive correlation of C3 expression with pro-inflammatory cytokines profile, including IL-1β, which is particularly toxic for β-cells. We aimed to investigate the influence of IL-1β on viability of CRISPR/Cas9 INS-1 clones and test the possibility of cytosolic C3 involvement by generating INS-1 clones producing a non-secreted, cytosolic isoform of C3 from a non-canonical start site.

Major findings: Using gene-edited clonal β-cells expressing C3, with no expression of C3, or expressing non-secreted cytosolic C3 from an alternative start site, we demonstrated considerable effect of intracellular C3 in prevention of IL-1β induced cell death. Addition of whole serum, or purified C3 could not rescue

increased cell death of C3 knockout clones, whereas treatment with recombinant C3a partially reserved the effect of higher cell death, although did not reach statistical significance it might point that C3a might be a cytoprotective C3 cleavage fragment and the possibility of cooperation of both autocrine and intracrine C3 sources to support β-cell survival. Gene expression studies revealed altered expression of genes involved in β-cell identity and response to cytokines, like IL-1R, CXCL10, JUN, DUSP26 in C3 deficient cells, that were restored in cells expressing cytosolic C3. Our data provide evidence that intracellular C3 confers protection against IL-1β-induced cytotoxicity, and may maintain β-cell function.

Summary and future perspectives

Given the multifaceted role of the complement system in health and disease, the continued studies of molecular mechanisms involved in complement-mediated effects is essential. This is further supported by the availability of FDA-approved drugs targeting complement for treatment of some diseases, and prevalence of new therapeutics in clinical trials (46).

We have described a safeguarding function of C4BP against IAPP-induced toxicity in β-cells and IAPP-driven NLRP3 inflammasome activation in macrophages. However, C4BP has also been found to limit NLRP3 inflammasome activation initiated by monosodium urate (MSU) and silica particles (SiO_2), which similarly to IAPP activate the phagolysosomal NLRP3 pathway. Currently we are evaluating the potential of C4BP to inhibit inflammasome activation caused by MSU and SiO_2. In addition, C4BP has been found to bind Aβ (130) and prion protein (131), both of which contribute to neurodegeneration, and activate the NLRP3 inflammasome through lysosome destabilization. Considering the high expression of C4BP in circulation (200 ug/ml) (200) and in locations relevant to diseases: lungs, pancreas (http://www.gtexportal.org), and its higher local presence in Alzheimer's disease brain (130) and synovial fluid of patients with rheumatoid arthritis (201) that frequently display MSU crystals deposits (202), C4BP might be a broad NLRP3 inflammasome inhibitor with potential to be a therapeutic modulator of amyloid- and particulate-induced inflammasome activation-driven disorders. Apart from confirming those assumptions using *in vitro* model, there is necessity to advance it using an *in vivo* model. To confirm and asses the potential of C4BP in inhibition of NLRP3 activation induced by lysosomal destabilization, C4BP knockout mice injected with mentioned NLRP3 inflammasome inducers, or even C4BP knockout mice crossed with mouse models mimicking human IAPP and Aβ-driven pathologies (like human IAPP transgenic mice and transgenic mice models of Alzheimer disease) will serve as a good tool for developing predictions in whole organisms. Reversing this, mouse models of IAPP/Aβ driven toxicity, might be injected with C4BP to evaluate effectiveness of C4BP in limiting disease onset. Furthermore, C4BP-mediated inhibition of NLRP3 inflammasome activation does not fully rely on its ability to modulate fibrillation (like in the case of IAPP), since MSU and SiO_2 are non-fibrillating agents. If C4BP simply covers those particles limiting their toxicity, or whether the molecular basis of inflammasome inhibition depends on some intracellular events, will be further addressed.

The second part of the thesis describes a protective effect of cytosolic C3 against β-cell stressors present during T2D (IAPP and palmitate), and both T2D and T1D (IL-1β). While the involvement of C3 in decreasing the vulnerability of cells to IAPP and PA might be explained by a requirement of C3 for effective autophagy, the mechanism implicating IL-1β driven toxicity in C3 knockout cells remains to be elucidated. Fyn-related kinase (FRK known also as a GTK/RAK/BSK/IYK), recognized on the protoarray as a potential binding partner of C3, is an attractive candidate that can participate in regulation of C3-IL-1β reactions, as it has been shown to be implicated in survival and response of β cells to cytokines (203, 204). The precise mechanism of C3 involvement in autophagy should also be further evaluated (205). Both studies, however, revealed cytosol C3-driven events. While fascinating, a lot of questions surround the origin and structure of cytosolic C3. Utilization of alternative translational start sites ((33)-paper III, paper IV) within the C3 sequence might give rise to different isoforms of C3 with presumably different binding partners. There is a need to further characterize C3 isoforms and accumulate more evidences for their existence and function. Furthermore, if cytosolic C3 can originate from the Golgi network and what structure it assembles should be approached as well.

IL-1β has also been found as a strong stimulator of C3 expression and secretion from human pancreatic β-cells and INS-1 cells ((33) paper III and paper IV). Elevated plasma levels of C3 are found in both T2D and T1D (158) and serum C3 has been described as a strong marker of insulin resistance (206). Adipose tissue is a source of components of the alternative pathway, including C3, FB and FD, FH, FI and properdin, indicating that complement components and activation products might have substantial effect on adipose tissue biology. Indeed, FD has been found to mediate pro-adipogenic effects via C3aR signalling (207). Nonetheless, C3aR and C5aR antagonists administrated to obese rats inhibit diet-induced obesity, metabolic dysfunction and adipocyte and macrophage pro-inflammatory signalling (208). Similarly, C3aR knockout mice display lower macrophage infiltration during high-fat diet and decreased insulin resistance (209) indicating complement activation-mediated recruitment of inflammatory cells and elevation of immune responses under unresolved inflammation, amplified by high fat diet (210). In a similar fashion, complement might contribute to pancreatitis. In fact, C3 upregulated in pancreatic islets of T2D donors and a positive correlation with inflammatory traits makes it a good islet marker for diabetes ((33) paper III). Although, we have discovered protective, intracellular functions for C3 in β-cells under acute metabolic challenges (IAPP, PA, IL-1β), a persistent onslaught on the β-cell originating from metabolic challenge and a likely continuous secretion of C3 might promote unsettled local inflammation and switch the signalling to favour detrimental effects over the protective ones under chronic conditions. This might be analogous to IL-1β, which after a meal stimulates insulin secretion to handle

increased amount of circulating substrates, but during chronic islet inflammation significantly contributes to β-cell death (16). To determine the effect of pancreatic-derived C3 under acute and chronic conditions, β-cell specific C3 knockout mice will be used. Furthermore, relating to therapeutics, there is further need of investigation and diversification between extracellular and intracellular functions of C3 in order to optimally design therapeutics, taking into account cell-membrane permeability of developing drugs. Our studies show that cytosolic C3 prevents IAPP-, PA- and IL-1β induced cytotoxicity in β-cells; nonetheless, hyperactive intracellular C3 activation has been observed in T-cells from patients suffering with autoimmune arthritis, that resulted in exaggerated Th1 responses and could be normalized *in vitro* by cathepsin inhibitors (7). Cathepsin inhibition also resulted in prevention of intestinal injury, where over-activation of C3 within the intestinal epithelial cells has been observed (211). This implies that intracellular C3 can take up on aberrant character and can be a target of therapeutics as well.

Popular science summary

Diabetes means that glucose (or sugar) from food, accumulates in your blood instead of entering into cells, with help of insulin. Insulin guides the movement of glucose from blood into your cells to give them energy. In type 1 diabetes (T1D), adaptive immunity, which tailors the defence to strategically thwart pathogens by production, among others, of specific antibodies, mistakenly attacks the body's own insulin producing β-cells. With type 2 diabetes (T2D), which affects 85-95% of all diabetic patients, your body's tissues are less responsive to insulin, which is followed by an increased burden of β-cells for overproduction of insulin in order to compensate for insulin insensitivity, and a subsequent exhaustion or dysfunction of β-cells. In contrast to T1D, T2D pathology seems to mainly involve on-going widespread low-level activation of innate immunity. Innate immunity is comprised of the sentinel immune cells patrolling the tissues like macrophages, which are a source of cytokines, small messaging proteins acting to alert cells to put up defences against potential danger. A second component of innate immunity is the complement system, that is considered as a system made up from proteins circulating in the blood, acting in concert to facilitate a search for and removal of a threat, and assists or 'complements' the other aspects of immunity. Newest studies, however, suggest that the complement system might support defence and other processes important for cell health, from inside of the cells. Although adaptive and innate immunity are fine-tuned systems with a capacity to distinguish between danger and non-danger signals, a persistent presence of undesired threats might lead to an unwarranted activation and following over-inflammation that can damage your tissues.

The development of T2D is closely linked to the growing problem of obesity, since accumulation of fats makes our innate immunity system more active, which is a time of progressing insulin insensitivity of tissues. β-cells then overproduce insulin, to meet the increased demand, which comes along with another protein called islet amyloid polypeptide, or IAPP for short. IAPP adds a 'stop eating' signal targeting the brain stem, and slows down digestion of food in the stomach, thereby preventing too much high sugar levels rising after a meal. Although IAPP participates in controlling blood sugar levels, the diabetes research community has realized that IAPP can be thought of as a 'bad' IAPP. Lots of it buds into build-ups, called amyloid that is lacking 'good' functions and instead causes damage to β-cells. We have found that a protein that belongs to complement system and is physiologically present in our bodies, binds to bad IAPP and limits its harmful effect on β-cells in two different ways. Firstly, however, it is important to grasp what bad IAPP exactly is. Formations of IAPP amyloids start with excessive production of IAPP that

becomes unstable and gathers together. Then it becomes larger, forming intermediate conformations termed oligomers that in turn are building blocks of fibrillar IAPP, which is the final stage of IAPP amyloid formation. This is significant, as oligomers are particularly toxic and it is these that are considered as serious elements of crime against the wellness of β-cells during the course of T2D, whereas pre- or post-oligomer states are relatively inert for cells.

The protein referred to above that has a potential to limit cytotoxicity of IAPP oligomers is termed C4BP. C4BP binds to IAPP and 'arrests' IAPP in a pre-oligomer, non-toxic state, but also has a potential to accelerate formation into IAPP fibrils, limiting the duration of action of toxic oligomers as well. Therefore we asked if C4BP could prevent the toxic effect of IAPP oligomers. IAPP oligomers make pores within the walls of cells and the presence of C4BP, resulted in improved β-cell health and activity. Nonetheless, IAPP cytotoxicity is not only limited to its direct effect on β-cells. IAPP amyloid is tagged by our immune system serving as a message for our immune cells to fight with it. Macrophages attracted by a high concentration of tagging signals, starts to 'eat' or phagocytose the IAPP amyloid. As it is with β-cells, engulfed IAPP oligomers makes pores, but from inside of the macrophages. This provokes a strong immune response and releases a potent cytokine from macrophages, deadly for β-cells. In the presence of C4BP the amount of the destructive cytokine production from macrophages decreases, and increases the number of viable β-cells, able to secrete insulin. Together, this leads us to suggest that our own endogenous protein C4BP is a forceful β-cell cytoprotective player, and increasing its local concentration might be beneficial for T2D patients.

Another important insight into β-cell physiology was the discovery of a new functional feature of a complement protein being associated with type 2 diabetes, but the role of this protein in T2D was not clear. The protein, called C3, has been found to regulate a process termed autophagy, literally meaning self-cannibalism. Despite appearances, self-cannibalism at the level of the cell is a required process by which cells digest and recycle their constituent parts and turn them into building blocks or food for other parts of the cell. Autophagy prevents piling up of damaged proteins or other debris and allows the cell to function well. When autophagy goes wrong, it can lead to many human diseases, including type 2 diabetes. C3 has been found to be necessary for well functioning autophagy. Moreover, C3 has been found to support β-cell health when exposed to detrimental cytokines, present in excessive amounts in both T2D and T1D. Understanding the mechanisms of these phenomena gives the possibility to intervene in precise ways and could help humans live healthier; C3 protein may serve as a new therapeutic target to achieve it.

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References

1. Sly PD, Carpenter DO, Van den Berg M, Stein RT, Landrigan PJ, Brune-Drisse MN, et al. Health Consequences of Environmental Exposures: Causal Thinking in Global Environmental Epidemiology. Ann Glob Health. 2016;82(1):3-9.
2. Magkos F, Hjorth MF, Astrup A. Diet and exercise in the prevention and treatment of type 2 diabetes mellitus. Nat Rev Endocrinol. 2020;16(10):545-55.
3. Nonaka M. Evolution of the complement system. Subcell Biochem. 2014;80:31-43.
4. Kohl J. The role of complement in danger sensing and transmission. Immunol Res. 2006;34(2):157-76.
5. Phieler J, Garcia-Martin R, Lambris JD, Chavakis T. The role of the complement system in metabolic organs and metabolic diseases. Semin Immunol. 2013;25(1):47-53.
6. Hajishengallis G, Reis ES, Mastellos DC, Ricklin D, Lambris JD. Novel mechanisms and functions of complement. Nat Immunol. 2017;18(12):1288-98.
7. Liszewski MK, Kolev M, Le Friec G, Leung M, Bertram PG, Fara AF, et al. Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation. Immunity. 2013;39(6):1143-57.
8. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nat Rev Immunol. 2011;11(2):98-107.
9. Krus U, King BC, Nagaraj V, Gandasi NR, Sjolander J, Buda P, et al. The complement inhibitor CD59 regulates insulin secretion by modulating exocytotic events. Cell Metab. 2014;19(5):883-90.
10. King BC, Blom AM. Non-traditional roles of complement in type 2 diabetes: Metabolism, insulin secretion and homeostasis. Mol Immunol. 2017;84:34-42.
11. Dempsey PW, Vaidya SA, Cheng G. The art of war: Innate and adaptive immune responses. Cell Mol Life Sci. 2003;60(12):2604-21.
12. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140(6):805-20.
13. Zindel J, Kubes P. DAMPs, PAMPs, and LAMPs in Immunity and Sterile Inflammation. Annu Rev Pathol. 2020;15:493-518.
14. Schroder K, Tschopp J. The Inflammasomes. Cell. 2010;140(6):821-32.
15. Kwak A, Lee Y, Kim H, Kim S. Intracellular interleukin (IL)-1 family cytokine processing enzyme. Arch Pharm Res. 2016;39(11):1556-64.
16. Donath MY, Dinarello CA, Mandrup-Poulsen T. Targeting innate immune mediators in type 1 and type 2 diabetes. Nat Rev Immunol. 2019;19(12):734-46.

17. Kelley N, Jeltema D, Duan Y, He Y. The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. Int J Mol Sci. 2019;20(13).
18. Bauernfeind F, Hornung V. Of inflammasomes and pathogens--sensing of microbes by the inflammasome. EMBO Mol Med. 2013;5(6):814-26.
19. Swanson KV, Deng M, Ting JP. The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nat Rev Immunol. 2019;19(8):477-89.
20. Juliana C, Fernandes-Alnemri T, Kang S, Farias A, Qin F, Alnemri ES. Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. J Biol Chem. 2012;287(43):36617-22.
21. Song N, Liu ZS, Xue W, Bai ZF, Wang QY, Dai J, et al. NLRP3 Phosphorylation Is an Essential Priming Event for Inflammasome Activation. Mol Cell. 2017;68(1):185-97 e6.
22. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature. 2015;526(7575):660-5.
23. Gaidt MM, Ebert TS, Chauhan D, Schmidt T, Schmid-Burgk JL, Rapino F, et al. Human Monocytes Engage an Alternative Inflammasome Pathway. Immunity. 2016;44(4):833-46.
24. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. Nat Rev Immunol. 2013;13(6):397-411.
25. Wilson SP, Cassel SL. Inflammasome-mediated autoinflammatory disorders. Postgrad Med. 2010;122(5):125-33.
26. Smole U, Kratzer B, Pickl WF. Soluble pattern recognition molecules: Guardians and regulators of homeostasis at airway mucosal surfaces. Eur J Immunol. 2020;50(5):624-42.
27. Holodick NE, Rodriguez-Zhurbenko N, Hernandez AM. Defining Natural Antibodies. Front Immunol. 2017;8:872.
28. Devitt A, Marshall LJ. The innate immune system and the clearance of apoptotic cells. J Leukoc Biol. 2011;90(3):447-57.
29. Gronwall C, Vas J, Silverman GJ. Protective Roles of Natural IgM Antibodies. Front Immunol. 2012;3:66.
30. Bohlson SS, Garred P, Kemper C, Tenner AJ. Complement Nomenclature-Deconvoluted. Front Immunol. 2019;10:1308.
31. Forneris F, Ricklin D, Wu J, Tzekou A, Wallace RS, Lambris JD, et al. Structures of C3b in complex with factors B and D give insight into complement convertase formation. Science. 2010;330(6012):1816-20.
32. Sahu A, Lambris JD. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. Immunol Rev. 2001;180:35-48.
33. King BC, Kulak K, Krus U, Rosberg R, Golec E, Wozniak K, et al. Complement Component C3 Is Highly Expressed in Human Pancreatic Islets and Prevents beta Cell Death via ATG16L1 Interaction and Autophagy Regulation. Cell Metab. 2019;29(1):202-10 e6.

34. Coulthard LG, Woodruff TM. Is the complement activation product C3a a proinflammatory molecule? Re-evaluating the evidence and the myth. J Immunol. 2015;194(8):3542-8.
35. Janssen BJ, Christodoulidou A, McCarthy A, Lambris JD, Gros P. Structure of C3b reveals conformational changes that underlie complement activity. Nature. 2006;444(7116):213-6.
36. Schmidt CQ, Lambris JD, Ricklin D. Protection of host cells by complement regulators. Immunol Rev. 2016;274(1):152-71.
37. Ogden CA, Kowalewski R, Peng Y, Montenegro V, Elkon KB. IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo. Autoimmunity. 2005;38(4):259-64.
38. Quartier P, Potter PK, Ehrenstein MR, Walport MJ, Botto M. Predominant role of IgM-dependent activation of the classical pathway in the clearance of dying cells by murine bone marrow-derived macrophages in vitro. Eur J Immunol. 2005;35(1):252-60.
39. Ogden CA, deCathelineau A, Hoffmann PR, Bratton D, Ghebrehiwet B, Fadok VA, et al. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J Exp Med. 2001;194(6):781-95.
40. Nauta AJ, Raaschou-Jensen N, Roos A, Daha MR, Madsen HO, Borrias-Essers MC, et al. Mannose-binding lectin engagement with late apoptotic and necrotic cells. Eur J Immunol. 2003;33(10):2853-63.
41. Kuraya M, Ming Z, Liu X, Matsushita M, Fujita T. Specific binding of L-ficolin and H-ficolin to apoptotic cells leads to complement activation. Immunobiology. 2005;209(9):689-97.
42. Korb LC, Ahearn JM. C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J Immunol. 1997;158(10):4525-8.
43. Nauta AJ, Trouw LA, Daha MR, Tijsma O, Nieuwland R, Schwaeble WJ, et al. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. Eur J Immunol. 2002;32(6):1726-36.
44. Ricklin D, Reis ES, Lambris JD. Complement in disease: a defence system turning offensive. Nat Rev Nephrol. 2016;12(7):383-401.
45. Sonnen AF, Henneke P. Structural biology of the membrane attack complex. Subcell Biochem. 2014;80:83-116.
46. Ricklin D, Mastellos DC, Reis ES, Lambris JD. The renaissance of complement therapeutics. Nat Rev Nephrol. 2018;14(1):26-47.
47. Zhang X, Kimura Y, Fang C, Zhou L, Sfyroera G, Lambris JD, et al. Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. Blood. 2007;110(1):228-36.
48. Hajishengallis G, Lambris JD. Crosstalk pathways between Toll-like receptors and the complement system. Trends Immunol. 2010;31(4):154-63.

49. Takabayashi T, Vannier E, Clark BD, Margolis NH, Dinarello CA, Burke JF, et al. A new biologic role for C3a and C3a desArg: regulation of TNF-alpha and IL-1 beta synthesis. J Immunol. 1996;156(9):3455-60.
50. Bennett KM, Rooijakkers SH, Gorham RD, Jr. Let's Tie the Knot: Marriage of Complement and Adaptive Immunity in Pathogen Evasion, for Better or Worse. Front Microbiol. 2017;8:89.
51. Janssen BJ, Huizinga EG, Raaijmakers HC, Roos A, Daha MR, Nilsson-Ekdahl K, et al. Structures of complement component C3 provide insights into the function and evolution of immunity. Nature. 2005;437(7058):505-11.
52. Ganu VS, Muller-Eberhard HJ, Hugli TE. Factor C3f is a spasmogenic fragment released from C3b by factors I and H: the heptadeca-peptide C3f was synthesized and characterized. Mol Immunol. 1989;26(10):939-48.
53. Dempsey PW, Allison ME, Akkaraju S, Goodnow CC, Fearon DT. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science. 1996;271(5247):348-50.
54. Chaplin H, Jr. Review: the burgeoning history of the complement system 1888-2005. Immunohematology. 2005;21(3):85-93.
55. Morgan BP, Gasque P. Extrahepatic complement biosynthesis: where, when and why? Clin Exp Immunol. 1997;107(1):1-7.
56. Muller-Eberhard HJ, Schreiber RD. Molecular biology and chemistry of the alternative pathway of complement. Adv Immunol. 1980;29:1-53.
57. Cianflone K, Maslowska M, Sniderman AD. Acylation stimulating protein (ASP), an adipocyte autocrine: new directions. Semin Cell Dev Biol. 1999;10(1):31-41.
58. Cianflone K, Xia Z, Chen LY. Critical review of acylation-stimulating protein physiology in humans and rodents. Biochim Biophys Acta. 2003;1609(2):127-43.
59. Cianflone K, Roncari DA, Maslowska M, Baldo A, Forden J, Sniderman AD. Adipsin/acylation stimulating protein system in human adipocytes: regulation of triacylglycerol synthesis. Biochemistry. 1994;33(32):9489-95.
60. Baldo A, Sniderman AD, St-Luce S, Avramoglu RK, Maslowska M, Hoang B, et al. The adipsin-acylation stimulating protein system and regulation of intracellular triglyceride synthesis. J Clin Invest. 1993;92(3):1543-7.
61. Cianflone K, Kalant D, Marliss EB, Gougeon R, Sniderman AD. Response of plasma ASP to a prolonged fast. Int J Obes Relat Metab Disord. 1995;19(9):604-9.
62. Ahren B, Havel PJ, Pacini G, Cianflone K. Acylation stimulating protein stimulates insulin secretion. Int J Obes Relat Metab Disord. 2003;27(9):1037-43.
63. Kalant D, Cain SA, Maslowska M, Sniderman AD, Cianflone K, Monk PN. The chemoattractant receptor-like protein C5L2 binds the C3a des-Arg77/acylation-stimulating protein. J Biol Chem. 2003;278(13):11123-9.
64. Kalant D, MacLaren R, Cui W, Samanta R, Monk PN, Laporte SA, et al. C5L2 is a functional receptor for acylation-stimulating protein. J Biol Chem. 2005;280(25):23936-44.

65. Paglialunga S, Schrauwen P, Roy C, Moonen-Kornips E, Lu H, Hesselink MK, et al. Reduced adipose tissue triglyceride synthesis and increased muscle fatty acid oxidation in C5L2 knockout mice. J Endocrinol. 2007;194(2):293-304.
66. Johswich K, Martin M, Thalmann J, Rheinheimer C, Monk PN, Klos A. Ligand specificity of the anaphylatoxin C5L2 receptor and its regulation on myeloid and epithelial cell lines. J Biol Chem. 2006;281(51):39088-95.
67. Murray I, Sniderman AD, Cianflone K. Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance. J Lipid Res. 1999;40(9):1671-6.
68. Wetsel RA, Kildsgaard J, Zsigmond E, Liao W, Chan L. Genetic deficiency of acylation stimulating protein (ASP(C3ades-Arg)) does not cause hyperapobetalipoproteinemia in mice. J Biol Chem. 1999;274(27):19429-33.
69. Fontaine DA, Davis DB. Attention to Background Strain Is Essential for Metabolic Research: C57BL/6 and the International Knockout Mouse Consortium. Diabetes. 2016;65(1):25-33.
70. Lo JC, Ljubicic S, Leibiger B, Kern M, Leibiger IB, Moede T, et al. Adipsin is an adipokine that improves beta cell function in diabetes. Cell. 2014;158(1):41-53.
71. Draznin B. Intracellular calcium, insulin secretion, and action. Am J Med. 1988;85(5A):44-58.
72. Norgauer J, Dobos G, Kownatzki E, Dahinden C, Burger R, Kupper R, et al. Complement fragment C3a stimulates Ca2+ influx in neutrophils via a pertussis-toxin-sensitive G protein. Eur J Biochem. 1993;217(1):289-94.
73. Werfel T, Kirchhoff K, Wittmann M, Begemann G, Kapp A, Heidenreich F, et al. Activated human T lymphocytes express a functional C3a receptor. J Immunol. 2000;165(11):6599-605.
74. Ischenko A, Sayah S, Patte C, Andreev S, Gasque P, Schouft MT, et al. Expression of a functional anaphylatoxin C3a receptor by astrocytes. J Neurochem. 1998;71(6):2487-96.
75. Gomez-Banoy N, Guseh JS, Li G, Rubio-Navarro A, Chen T, Poirier B, et al. Adipsin preserves beta cells in diabetic mice and associates with protection from type 2 diabetes in humans. Nat Med. 2019;25(11):1739-47.
76. Sato T, Abe E, Jin CH, Hong MH, Katagiri T, Kinoshita T, et al. The biological roles of the third component of complement in osteoclast formation. Endocrinology. 1993;133(1):397-404.
77. Tu Z, Bu H, Dennis JE, Lin F. Efficient osteoclast differentiation requires local complement activation. Blood. 2010;116(22):4456-63.
78. Ratajczak MZ, Reca R, Wysoczynski M, Kucia M, Baran JT, Allendorf DJ, et al. Transplantation studies in C3-deficient animals reveal a novel role of the third complement component (C3) in engraftment of bone marrow cells. Leukemia. 2004;18(9):1482-90.
79. Wysoczynski M, Reca R, Lee H, Wu W, Ratajczak J, Ratajczak MZ. Defective engraftment of C3aR-/- hematopoietic stem progenitor cells shows a novel role of the C3a-C3aR axis in bone marrow homing. Leukemia. 2009;23(8):1455-61.

80. Schraufstatter IU, Discipio RG, Zhao M, Khaldoyanidi SK. C3a and C5a are chemotactic factors for human mesenchymal stem cells, which cause prolonged ERK1/2 phosphorylation. J Immunol. 2009;182(6):3827-36.
81. Coulthard LG, Hawksworth OA, Conroy J, Lee JD, Woodruff TM. Complement C3a receptor modulates embryonic neural progenitor cell proliferation and cognitive performance. Mol Immunol. 2018;101:176-81.
82. Rahpeymai Y, Hietala MA, Wilhelmsson U, Fotheringham A, Davies I, Nilsson AK, et al. Complement: a novel factor in basal and ischemia-induced neurogenesis. EMBO J. 2006;25(6):1364-74.
83. Shinjyo N, Stahlberg A, Dragunow M, Pekny M, Pekna M. Complement-derived anaphylatoxin C3a regulates in vitro differentiation and migration of neural progenitor cells. Stem Cells. 2009;27(11):2824-32.
84. Benard M, Raoult E, Vaudry D, Leprince J, Falluel-Morel A, Gonzalez BJ, et al. Role of complement anaphylatoxin receptors (C3aR, C5aR) in the development of the rat cerebellum. Mol Immunol. 2008;45(14):3767-74.
85. Gorelik A, Sapir T, Haffner-Krausz R, Olender T, Woodruff TM, Reiner O. Developmental activities of the complement pathway in migrating neurons. Nat Commun. 2017;8:15096.
86. Carmona-Fontaine C, Theveneau E, Tzekou A, Tada M, Woods M, Page KM, et al. Complement fragment C3a controls mutual cell attraction during collective cell migration. Dev Cell. 2011;21(6):1026-37.
87. Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, et al. The classical complement cascade mediates CNS synapse elimination. Cell. 2007;131(6):1164-78.
88. Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, et al. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron. 2012;74(4):691-705.
89. Markiewski MM, Mastellos D, Tudoran R, DeAngelis RA, Strey CW, Franchini S, et al. C3a and C3b activation products of the third component of complement (C3) are critical for normal liver recovery after toxic injury. J Immunol. 2004;173(2):747-54.
90. Strey CW, Markiewski M, Mastellos D, Tudoran R, Spruce LA, Greenbaum LE, et al. The proinflammatory mediators C3a and C5a are essential for liver regeneration. J Exp Med. 2003;198(6):913-23.
91. He S, Atkinson C, Qiao F, Cianflone K, Chen X, Tomlinson S. A complement-dependent balance between hepatic ischemia/reperfusion injury and liver regeneration in mice. J Clin Invest. 2009;119(8):2304-16.
92. Dufaux B, Order U. Complement activation after prolonged exercise. Clin Chim Acta. 1989;179(1):45-9.
93. Zhang C, Wang C, Li Y, Miwa T, Liu C, Cui W, et al. Complement C3a signaling facilitates skeletal muscle regeneration by regulating monocyte function and trafficking. Nat Commun. 2017;8(1):2078.
94. Rouaud T, Siami N, Dupas T, Gervier P, Gardahaut MF, Auda-Boucher G, et al. Complement C3 of the innate immune system secreted by muscle adipogenic cells promotes myogenic differentiation. Sci Rep. 2017;7(1):171.

95. Del Rio-Tsonis K, Tsonis PA, Zarkadis IK, Tsagas AG, Lambris JD. Expression of the third component of complement, C3, in regenerating limb blastema cells of urodeles. J Immunol. 1998;161(12):6819-24.
96. Dinsmore CE. Urodele limb and tail regeneration in early biological thought: an essay on scientific controversy and social change. Int J Dev Biol. 1996;40(4):621-7.
97. Holtzer S. The inductive activity of the spinal cord in urodele tail regeneration [Thesis]. n.p.,: University of Chicago.
98. Diaz Quiroz JF, Echeverri K. Spinal cord regeneration: where fish, frogs and salamanders lead the way, can we follow? Biochem J. 2013;451(3):353-64.
99. Goss RJ, Stagg MW. Regeneration in lower jaws of newts after excision of the intermandibular regions. J Exp Zool. 1958;137(1):1-11.
100. Tsonis PA, Madhavan M, Tancous EE, Del Rio-Tsonis K. A newt's eye view of lens regeneration. Int J Dev Biol. 2004;48(8-9):975-80.
101. Mitashov VI. Mechanisms of retina regeneration in urodeles. Int J Dev Biol. 1996;40(4):833-44.
102. Neff AW, Dent AE, Armstrong JB. Heart development and regeneration in urodeles. Int J Dev Biol. 1996;40(4):719-25.
103. Sanchez Alvarado A, Tsonis PA. Bridging the regeneration gap: genetic insights from diverse animal models. Nat Rev Genet. 2006;7(11):873-84.
104. Kimura Y, Madhavan M, Call MK, Santiago W, Tsonis PA, Lambris JD, et al. Expression of complement 3 and complement 5 in newt limb and lens regeneration. J Immunol. 2003;170(5):2331-9.
105. Haynes T, Luz-Madrigal A, Reis ES, Echeverri Ruiz NP, Grajales-Esquivel E, Tzekou A, et al. Complement anaphylatoxin C3a is a potent inducer of embryonic chick retina regeneration. Nat Commun. 2013;4:2312.
106. Sorbara MT, Foerster EG, Tsalikis J, Abdel-Nour M, Mangiapane J, Sirluck-Schroeder I, et al. Complement C3 Drives Autophagy-Dependent Restriction of Cyto-invasive Bacteria. Cell Host Microbe. 2018;23(5):644-52 e5.
107. Tam JC, Bidgood SR, McEwan WA, James LC. Intracellular sensing of complement C3 activates cell autonomous immunity. Science. 2014;345(6201):1256070.
108. Liszewski MK, Elvington M, Kulkarni HS, Atkinson JP. Complement's hidden arsenal: New insights and novel functions inside the cell. Mol Immunol. 2017;84:2-9.
109. Strainic MG, Liu J, Huang D, An F, Lalli PN, Muqim N, et al. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. Immunity. 2008;28(3):425-35.
110. Strainic MG, Shevach EM, An F, Lin F, Medof ME. Absence of signaling into CD4(+) cells via C3aR and C5aR enables autoinductive TGF-beta1 signaling and induction of Foxp3(+) regulatory T cells. Nat Immunol. 2013;14(2):162-71.
111. Krystel-Whittemore M, Dileepan KN, Wood JG. Mast Cell: A Multi-Functional Master Cell. Front Immunol. 2015;6:620.
112. Schwartz LB, Kawahara MS, Hugli TE, Vik D, Fearon DT, Austen KF. Generation of C3a anaphylatoxin from human C3 by human mast cell tryptase. J Immunol. 1983;130(4):1891-5.

113. Kajita T, Hugli TE. Evidence for in vivo degradation of C3a anaphylatoxin by mast cell chymase. I. Nonspecific activation of rat peritoneal mast cells by C3ades Arg. Am J Pathol. 1991;138(6):1359-69.
114. Lipitsa T, Naukkarinen A, Laitala J, Harvima IT. Complement C3 is expressed by mast cells in cutaneous vasculitis and is degraded by chymase. Arch Dermatol Res. 2016;308(8):575-84.
115. Fukuoka Y, Hite MR, Dellinger AL, Schwartz LB. Human skin mast cells express complement factors C3 and C5. J Immunol. 2013;191(4):1827-34.
116. Elvington M, Liszewski MK, Bertram P, Kulkarni HS, Atkinson JP. A C3(H20) recycling pathway is a component of the intracellular complement system. J Clin Invest. 2017;127(3):970-81.
117. Kulkarni HS, Elvington ML, Perng YC, Liszewski MK, Byers DE, Farkouh C, et al. Intracellular C3 Protects Human Airway Epithelial Cells from Stress-associated Cell Death. Am J Respir Cell Mol Biol. 2019;60(2):144-57.
118. Kremlitzka M, Nowacka AA, Mohlin FC, Bompada P, De Marinis Y, Blom AM. Interaction of Serum-Derived and Internalized C3 With DNA in Human B Cells-A Potential Involvement in Regulation of Gene Transcription. Front Immunol. 2019;10:493.
119. Harboe M, Ulvund G, Vien L, Fung M, Mollnes TE. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. Clin Exp Immunol. 2004;138(3):439-46.
120. Blom AM, Kask L, Dahlback B. CCP1-4 of the C4b-binding protein alpha-chain are required for factor I mediated cleavage of complement factor C3b. Mol Immunol. 2003;39(10):547-56.
121. Hessing M, Vlooswijk RA, Hackeng TM, Kanters D, Bouma BN. The localization of heparin-binding fragments on human C4b-binding protein. J Immunol. 1990;144(1):204-8.
122. Mohlin FC, Mercier E, Fremeaux-Bacchi V, Liszewski MK, Atkinson JP, Gris JC, et al. Analysis of genes coding for CD46, CD55, and C4b-binding protein in patients with idiopathic, recurrent, spontaneous pregnancy loss. Eur J Immunol. 2013;43(6):1617-29.
123. Ziccardi RJ, Dahlback B, Muller-Eberhard HJ. Characterization of the interaction of human C4b-binding protein with physiological ligands. J Biol Chem. 1984;259(22):13674-9.
124. Dahlback B, Smith CA, Muller-Eberhard HJ. Visualization of human C4b-binding protein and its complexes with vitamin K-dependent protein S and complement protein C4b. Proc Natl Acad Sci U S A. 1983;80(11):3461-5.
125. Anderson HA, Maylock CA, Williams JA, Paweletz CP, Shu H, Shacter E. Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. Nat Immunol. 2003;4(1):87-91.
126. Webb JH, Blom AM, Dahlback B. Vitamin K-dependent protein S localizing complement regulator C4b-binding protein to the surface of apoptotic cells. J Immunol. 2002;169(5):2580-6.

127. Trouw LA, Nilsson SC, Goncalves I, Landberg G, Blom AM. C4b-binding protein binds to necrotic cells and DNA, limiting DNA release and inhibiting complement activation. J Exp Med. 2005;201(12):1937-48.
128. Trouw LA, Bengtsson AA, Gelderman KA, Dahlback B, Sturfelt G, Blom AM. C4b-binding protein and factor H compensate for the loss of membrane-bound complement inhibitors to protect apoptotic cells against excessive complement attack. J Biol Chem. 2007;282(39):28540-8.
129. Sjolander J, Westermark GT, Renstrom E, Blom AM. Islet amyloid polypeptide triggers limited complement activation and binds complement inhibitor C4b-binding protein, which enhances fibril formation. J Biol Chem. 2012;287(14):10824-33.
130. Trouw LA, Nielsen HM, Minthon L, Londos E, Landberg G, Veerhuis R, et al. C4b-binding protein in Alzheimer's disease: binding to Abeta1-42 and to dead cells. Mol Immunol. 2008;45(13):3649-60.
131. Sjoberg AP, Nystrom S, Hammarstrom P, Blom AM. Native, amyloid fibrils and beta-oligomers of the C-terminal domain of human prion protein display differential activation of complement and bind C1q, factor H and C4b-binding protein directly. Mol Immunol. 2008;45(11):3213-21.
132. Rambaran RN, Serpell LC. Amyloid fibrils: abnormal protein assembly. Prion. 2008;2(3):112-7.
133. Wathne GJ, Mabbott NA. The diverse roles of mononuclear phagocytes in prion disease pathogenesis. Prion. 2012;6(2):124-33.
134. Veerhuis R, van der Valk P, Janssen I, Zhan SS, Van Nostrand WE, Eikelenboom P. Complement activation in amyloid plaques in Alzheimer's disease brains does not proceed further than C3. Virchows Arch. 1995;426(6):603-10.
135. Kulak K, Westermark GT, Papac-Milicevic N, Renstrom E, Blom AM, King BC. The human serum protein C4b-binding protein inhibits pancreatic IAPP-induced inflammasome activation. Diabetologia. 2017;60(8):1522-33.
136. Raleigh D, Zhang X, Hastoy B, Clark A. The beta-cell assassin: IAPP cytotoxicity. J Mol Endocrinol. 2017;59(3):R121-R40.
137. Sjolander J, Byman E, Kulak K, Nilsson SC, Zhang E, Krus U, et al. C4b-binding Protein Protects beta-Cells from Islet Amyloid Polypeptide-induced Cytotoxicity. J Biol Chem. 2016;291(41):21644-55.
138. Olivar R, Luque A, Naranjo-Gomez M, Quer J, Garcia de Frutos P, Borras FE, et al. The alpha7beta0 isoform of the complement regulator C4b-binding protein induces a semimature, anti-inflammatory state in dendritic cells. J Immunol. 2013;190(6):2857-72.
139. Luque A, Serrano I, Ripoll E, Malta C, Goma M, Blom AM, et al. Noncanonical immunomodulatory activity of complement regulator C4BP(beta-) limits the development of lupus nephritis. Kidney Int. 2020;97(3):551-66.
140. Olcina MM, Kim RK, Balanis NG, Li CG, von Eyben R, Graeber TG, et al. Intracellular C4BPA Levels Regulate NF-kappaB-Dependent Apoptosis. iScience. 2020;23(10):101594.
141. Ricklin D, Lambris JD. Complement in immune and inflammatory disorders: pathophysiological mechanisms. J Immunol. 2013;190(8):3831-8.

142. Rask-Madsen C, King GL. Vascular complications of diabetes: mechanisms of injury and protective factors. Cell Metab. 2013;17(1):20-33.
143. Ghosh P, Sahoo R, Vaidya A, Chorev M, Halperin JA. Role of complement and complement regulatory proteins in the complications of diabetes. Endocr Rev. 2015;36(3):272-88.
144. Pathak V, Pathak NM, O'Neill CL, Guduric-Fuchs J, Medina RJ. Therapies for Type 1 Diabetes: Current Scenario and Future Perspectives. Clin Med Insights Endocrinol Diabetes. 2019;12:1179551419844521.
145. Marin-Penalver JJ, Martin-Timon I, Sevillano-Collantes C, Del Canizo-Gomez FJ. Update on the treatment of type 2 diabetes mellitus. World J Diabetes. 2016;7(17):354-95.
146. Xu H, Chen M. Diabetic retinopathy and dysregulated innate immunity. Vision Res. 2017;139:39-46.
147. Cutler CW, Eke P, Arnold RR, Van Dyke TE. Defective neutrophil function in an insulin-dependent diabetes mellitus patients. A case report. J Periodontol. 1991;62(6):394-401.
148. Krause S, Brachmann P, Losche W, Hoffmann T, Gangler P. Defective function of blood granulocytes in patients with diabetes mellitus-associated marginal periodontitis. Folia Biol (Praha). 1992;38(6):358-63.
149. Lecube A, Pachon G, Petriz J, Hernandez C, Simo R. Phagocytic activity is impaired in type 2 diabetes mellitus and increases after metabolic improvement. PLoS One. 2011;6(8):e23366.
150. Alba-Loureiro TC, Munhoz CD, Martins JO, Cerchiaro GA, Scavone C, Curi R, et al. Neutrophil function and metabolism in individuals with diabetes mellitus. Braz J Med Biol Res. 2007;40(8):1037-44.
151. Yehualashet AS. Toll-like Receptors as a Potential Drug Target for Diabetes Mellitus and Diabetes-associated Complications. Diabetes Metab Syndr Obes. 2020;13:4763-77.
152. Pihoker C, Gilliam LK, Hampe CS, Lernmark A. Autoantibodies in diabetes. Diabetes. 2005;54 Suppl 2:S52-61.
153. Itariu BK, Stulnig TM. Autoimmune aspects of type 2 diabetes mellitus - a mini-review. Gerontology. 2014;60(3):189-96.
154. Axelgaard E, Ostergaard JA, Thiel S, Hansen TK. Diabetes Is Associated with Increased Autoreactivity of Mannan-Binding Lectin. J Diabetes Res. 2017;2017:6368780.
155. Flyvbjerg A. The role of the complement system in diabetic nephropathy. Nat Rev Nephrol. 2017;13(5):311-8.
156. Kolluru GK, Bir SC, Kevil CG. Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodeling, and wound healing. Int J Vasc Med. 2012;2012:918267.
157. Hess K, Alzahrani SH, Mathai M, Schroeder V, Carter AM, Howell G, et al. A novel mechanism for hypofibrinolysis in diabetes: the role of complement C3. Diabetologia. 2012;55(4):1103-13.

158. Hertle E, Stehouwer CD, van Greevenbroek MM. The complement system in human cardiometabolic disease. Mol Immunol. 2014;61(2):135-48.
159. Dror E, Dalmas E, Meier DT, Wueest S, Thevenet J, Thienel C, et al. Postprandial macrophage-derived IL-1beta stimulates insulin, and both synergistically promote glucose disposal and inflammation. Nat Immunol. 2017;18(3):283-92.
160. Spinas GA, Palmer JP, Mandrup-Poulsen T, Andersen H, Nielsen JH, Nerup J. The bimodal effect of interleukin 1 on rat pancreatic beta-cells--stimulation followed by inhibition--depends upon dose, duration of exposure, and ambient glucose concentration. Acta Endocrinol (Copenh). 1988;119(2):307-11.
161. Maedler K, Fontana A, Ris F, Sergeev P, Toso C, Oberholzer J, et al. FLIP switches Fas-mediated glucose signaling in human pancreatic beta cells from apoptosis to cell replication. Proc Natl Acad Sci U S A. 2002;99(12):8236-41.
162. Boni-Schnetzler M, Hauselmann SP, Dalmas E, Meier DT, Thienel C, Traub S, et al. beta Cell-Specific Deletion of the IL-1 Receptor Antagonist Impairs beta Cell Proliferation and Insulin Secretion. Cell Rep. 2018;22(7):1774-86.
163. Larsen CM, Faulenbach M, Vaag A, Volund A, Ehses JA, Seifert B, et al. Interleukin-1-receptor antagonist in type 2 diabetes mellitus. N Engl J Med. 2007;356(15):1517-26.
164. Kataria Y, Ellervik C, Mandrup-Poulsen T. Treatment of type 2 diabetes by targeting interleukin-1: a meta-analysis of 2921 patients. Semin Immunopathol. 2019;41(4):413-25.
165. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. N Engl J Med. 2017;377(12):1119-31.
166. Everett BM, Donath MY, Pradhan AD, Thuren T, Pais P, Nicolau JC, et al. Anti-Inflammatory Therapy With Canakinumab for the Prevention and Management of Diabetes. J Am Coll Cardiol. 2018;71(21):2392-401.
167. Biasizzo M, Kopitar-Jerala N. Interplay Between NLRP3 Inflammasome and Autophagy. Front Immunol. 2020;11:591803.
168. Yin J, Peng Y, Wu J, Wang Y, Yao L. Toll-like receptor 2/4 links to free fatty acid-induced inflammation and beta-cell dysfunction. J Leukoc Biol. 2014;95(1):47-52.
169. Dasu MR, Devaraj S, Zhao L, Hwang DH, Jialal I. High glucose induces toll-like receptor expression in human monocytes: mechanism of activation. Diabetes. 2008;57(11):3090-8.
170. Kido Y. Gene-environment interaction in type 2 diabetes. Diabetol Int. 2017;8(1):7-13.
171. Lytrivi M, Castell AL, Poitout V, Cnop M. Recent Insights Into Mechanisms of beta-Cell Lipo- and Glucolipotoxicity in Type 2 Diabetes. J Mol Biol. 2020;432(5):1514-34.
172. Wang J, Wang H. Oxidative Stress in Pancreatic Beta Cell Regeneration. Oxid Med Cell Longev. 2017;2017:1930261.
173. Tiganis T. Reactive oxygen species and insulin resistance: the good, the bad and the ugly. Trends Pharmacol Sci. 2011;32(2):82-9.

174. Ghosh R, Colon-Negron K, Papa FR. Endoplasmic reticulum stress, degeneration of pancreatic islet beta-cells, and therapeutic modulation of the unfolded protein response in diabetes. Mol Metab. 2019;27S:S60-S8.
175. Salvado L, Palomer X, Barroso E, Vazquez-Carrera M. Targeting endoplasmic reticulum stress in insulin resistance. Trends Endocrinol Metab. 2015;26(8):438-48.
176. Lutz TA. Control of energy homeostasis by amylin. Cell Mol Life Sci. 2012;69(12):1947-65.
177. Westermark P, Grimelius L. The pancreatic islet cells in insular amyloidosis in human diabetic and non-diabetic adults. Acta Pathol Microbiol Scand A. 1973;81(3):291-300.
178. Guardado-Mendoza R, Davalli AM, Chavez AO, Hubbard GB, Dick EJ, Majluf-Cruz A, et al. Pancreatic islet amyloidosis, beta-cell apoptosis, and alpha-cell proliferation are determinants of islet remodeling in type-2 diabetic baboons. Proc Natl Acad Sci U S A. 2009;106(33):13992-7.
179. Asthana S, Mallick B, Alexandrescu AT, Jha S. IAPP in type II diabetes: Basic research on structure, molecular interactions, and disease mechanisms suggests potential intervention strategies. Biochim Biophys Acta Biomembr. 2018;1860(9):1765-82.
180. Mulder H, Ahren B, Sundler F. Islet amyloid polypeptide and insulin gene expression are regulated in parallel by glucose in vivo in rats. Am J Physiol. 1996;271(6 Pt 1):E1008-14.
181. Paulsson JF, Westermark GT. Aberrant processing of human proislet amyloid polypeptide results in increased amyloid formation. Diabetes. 2005;54(7):2117-25.
182. Mukherjee A, Morales-Scheihing D, Salvadores N, Moreno-Gonzalez I, Gonzalez C, Taylor-Presse K, et al. Induction of IAPP amyloid deposition and associated diabetic abnormalities by a prion-like mechanism. J Exp Med. 2017;214(9):2591-610.
183. Gurlo T, Ryazantsev S, Huang CJ, Yeh MW, Reber HA, Hines OJ, et al. Evidence for proteotoxicity in beta cells in type 2 diabetes: toxic islet amyloid polypeptide oligomers form intracellularly in the secretory pathway. Am J Pathol. 2010;176(2):861-9.
184. Masters SL, Dunne A, Subramanian SL, Hull RL, Tannahill GM, Sharp FA, et al. Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. Nat Immunol. 2010;11(10):897-904.
185. Anding AL, Baehrecke EH. Cleaning House: Selective Autophagy of Organelles. Dev Cell. 2017;41(1):10-22.
186. Hurley JH, Young LN. Mechanisms of Autophagy Initiation. Annu Rev Biochem. 2017;86:225-44.
187. Hamasaki M, Shibutani ST, Yoshimori T. Up-to-date membrane biogenesis in the autophagosome formation. Curr Opin Cell Biol. 2013;25(4):455-60.
188. Carlsson SR, Simonsen A. Membrane dynamics in autophagosome biogenesis. J Cell Sci. 2015;128(2):193-205.

189. Green DR, Levine B. To be or not to be? How selective autophagy and cell death govern cell fate. Cell. 2014;157(1):65-75.
190. Lee YH, Kim J, Park K, Lee MS. beta-cell autophagy: Mechanism and role in beta-cell dysfunction. Mol Metab. 2019;27S:S92-S103.
191. Vivot K, Pasquier A, Goginashvili A, Ricci R. Breaking Bad and Breaking Good: beta-Cell Autophagy Pathways in Diabetes. J Mol Biol. 2020;432(5):1494-513.
192. Ohsumi Y, Mizushima N. Two ubiquitin-like conjugation systems essential for autophagy. Semin Cell Dev Biol. 2004;15(2):231-6.
193. Jung HS, Chung KW, Won Kim J, Kim J, Komatsu M, Tanaka K, et al. Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. Cell Metab. 2008;8(4):318-24.
194. Ebato C, Uchida T, Arakawa M, Komatsu M, Ueno T, Komiya K, et al. Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. Cell Metab. 2008;8(4):325-32.
195. Quan W, Hur KY, Lim Y, Oh SH, Lee JC, Kim KH, et al. Autophagy deficiency in beta cells leads to compromised unfolded protein response and progression from obesity to diabetes in mice. Diabetologia. 2012;55(2):392-403.
196. Masini M, Bugliani M, Lupi R, del Guerra S, Boggi U, Filipponi F, et al. Autophagy in human type 2 diabetes pancreatic beta cells. Diabetologia. 2009;52(6):1083-6.
197. Mizukami H, Takahashi K, Inaba W, Tsuboi K, Osonoi S, Yoshida T, et al. Involvement of oxidative stress-induced DNA damage, endoplasmic reticulum stress, and autophagy deficits in the decline of beta-cell mass in Japanese type 2 diabetic patients. Diabetes care. 2014;37(7):1966-74.
198. Katsarou A, Gudbjornsdottir S, Rawshani A, Dabelea D, Bonifacio E, Anderson BJ, et al. Type 1 diabetes mellitus. Nat Rev Dis Primers. 2017;3:17016.
199. Roep BO, Thomaidou S, van Tienhoven R, Zaldumbide A. Type 1 diabetes mellitus as a disease of the beta-cell (do not blame the immune system?). Nat Rev Endocrinol. 2020.
200. Marcovina SM, Zoppo A, Vigano-D'Angelo S, Di Cola G, D'Angelo A. Determination of serum levels of complement component C4b-binding protein: influence of age and inflammation. Int J Clin Lab Res. 1991;21(2):171-5.
201. Sanchez-Pernaute O, Esparza-Gordillo J, Largo R, Calvo E, Alvarez-Soria MA, Marcos ME, et al. Expression of the peptide C4b-binding protein beta in the arthritic joint. Ann Rheum Dis. 2006;65(10):1279-85.
202. Petsch C, Araujo EG, Englbrecht M, Bayat S, Cavallaro A, Hueber AJ, et al. Prevalence of monosodium urate deposits in a population of rheumatoid arthritis patients with hyperuricemia. Semin Arthritis Rheum. 2016;45(6):663-8.
203. Anneren C. Dual role of the tyrosine kinase GTK and the adaptor protein SHB in beta-cell growth: enhanced beta-cell replication after 60% pancreatectomy and increased sensitivity to streptozotocin. J Endocrinol. 2002;172(1):145-53.
204. Welsh M, Welsh C, Ekman M, Dixelius J, Hagerkvist R, Anneren C, et al. The tyrosine kinase FRK/RAK participates in cytokine-induced islet cell cytotoxicity. Biochem J. 2004;382(Pt 1):261-8.

205. King BC, Kulak K, Colineau L, Blom AM. Outside in: Roles of complement in autophagy. Br J Pharmacol. 2020.
206. Muscari A, Antonelli S, Bianchi G, Cavrini G, Dapporto S, Ligabue A, et al. Serum C3 is a stronger inflammatory marker of insulin resistance than C-reactive protein, leukocyte count, and erythrocyte sedimentation rate: comparison study in an elderly population. Diabetes care. 2007;30(9):2362-8.
207. Song NJ, Kim S, Jang BH, Chang SH, Yun UJ, Park KM, et al. Small Molecule-Induced Complement Factor D (Adipsin) Promotes Lipid Accumulation and Adipocyte Differentiation. PLoS One. 2016;11(9):e0162228.
208. Lim J, Iyer A, Suen JY, Seow V, Reid RC, Brown L, et al. C5aR and C3aR antagonists each inhibit diet-induced obesity, metabolic dysfunction, and adipocyte and macrophage signaling. FASEB J. 2013;27(2):822-31.
209. Mamane Y, Chung Chan C, Lavallee G, Morin N, Xu LJ, Huang J, et al. The C3a anaphylatoxin receptor is a key mediator of insulin resistance and functions by modulating adipose tissue macrophage infiltration and activation. Diabetes. 2009;58(9):2006-17.
210. de Vries MA, Klop B, Janssen HW, Njo TL, Westerman EM, Castro Cabezas M. Postprandial inflammation: targeting glucose and lipids. Adv Exp Med Biol. 2014;824:161-70.
211. Satyam A, Kannan L, Matsumoto N, Geha M, Lapchak PH, Bosse R, et al. Intracellular Activation of Complement 3 Is Responsible for Intestinal Tissue Damage during Mesenteric Ischemia. J Immunol. 2017;198(2):788-97.

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Innate immunity in diabetes mellitus

Type 2 diabetes continues to increase in prevalence and is a significant cause of human suffering. Despite enormous investment in research and clinical care there is no apparent reduction of its incidence. In fact, around 80% of cases of type 2 diabetes are related with bad life style choices: poor diet and limited physical activity. If you have been diagnosed for type 2 diabetes and your disease arose as a result of an unhealthy lifestyle, then you are lucky, the future is in your hands!

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