Physiological synergy between IL-1β and insulin on glucose disposal and macrophage activity

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ABSTRACT

Type 2 diabetes (T2D) is an inflammatory disease associated with infiltration of immune cells into various tissues and increased levels of inflammatory factors including Interleukin-1 β (IL-1β). In pancreatic islets elevated glucose levels stimulate IL-1β production resulting in impaired function and survival of β cells. Blockade of IL-1β improves T2D, pointing on a major role for IL-1β in the development and T2D. While the deleterious role of chronic activation of the IL-1 system in T2D is well documented, little is known about its potential physiological role(s).

The aim of the present study is to reveal the physiological role of inflammation and specifically of IL-1β in metabolism.

In vivo acutely administered IL-1β dose dependently induced insulin secretion even at IL-1β concentrations in the circulation that are below the detection limit. Together with glucose IL-1β promotes insulin secretion via parasympathetic nerve stimulation. In vitro IL-1β had a dual effect on glucose stimulated insulin secretion: it was beneficial at low doses and deleterious at high doses. The endotoxin lipopolysaccharide (LPS) strongly induced IL-1β, stimulated insulin secretion and improved glucose tolerance. Interestingly, the improvement of glucose tolerance was not only a consequence of increased insulin levels but was also due to direct IL-1β mediated glucose uptake into various tissues. One of the compartments responding to IL-1β with elevated glucose uptake was the cells of the immune system, mainly macrophages. We further show that macrophages significantly contribute to IL-1β mediated glucose disposal from the circulation. In addition, this work provides evidence for a role of insulin in mounting an immune response. Indeed, insulin increased the secretion of IL-1β via the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome preferentially in inflammatory M1 macrophages but not from alternatively activated M2 macrophages. In line with this response, M1 macrophages expressed higher levels of insulin receptors than naïve or M2. Further, insulin had an overall pro-inflammatory effect in naïve and inflammatory macrophages, which could be attributed to increased glucose uptake via the GLUT1 glucose transporter.

This study uncovered that insulin and IL-1β stimulate each other. In a physiological context IL-1β together with insulin may activate innate immunity in order to respond to post-meal intruding gut microbiota or to cope with infections. Indeed, acute phase inflammatory responses following an infection are associated with insulin resistance increasing the demand for insulin. Elevated insulin levels together with IL-1β may supply glucose to the immune compartment to provide the energy required for its activation. Hence elevated insulin levels in obesity may contribute to the development of insulin resistance and worsening of T2D by chronically activating the immune system.

List of abbreviations

AC	Adenylyl cyclase
ACh	Acetylcholine
AMP	Adenosine monophosphate
ASC	Apoptosis-associated specklike protein
ATP	Adenosine triphosphate
C2TA	MHC class 2 transcription activator
cAMP	Cyclic AMP
CARD	Caspase activation and recruitment domains
CNS	Central nervous system
CXCL1	Chemokine (C-X-C motif) ligand 1
DAG	Diacylglycerol
DAMP	Danger associated molecules
DIO	Diet induced obese
ER	Endoplasmic reticulum
FFA	Free fatty acid
G protein	Guanine nucleotide-binding protein
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon like peptide 1
GLUT	Glucouse transporter
GPCR	G protein coupled receptor
GSIS	Glucose stimulated insulin secretion
GTT	Glucose tolerance test
HET-E	Incompatibility locus protein from Podospora anserina
IFN	Interferon
IKK	I kappa B kinase
IL	Interleukin
IL-1R1	IL-1 receptor type 1
IL-1Ra	IL-1 receptor antagonist
IL-1RAcP	IL-1 receptor associated protein
INSR	Insulin receptor
IP3	Inositol 1,4,5-triphosphate

IRAK	IL-1 receptor-associated kinases
IRS	Insulin receptor substrate
jnk	c-Jun N-terminal kinases
LPS	Lipopolysaccharide
LRR	Leucin rich regions
MAP2K	Mitogen-activated protein kinase kinase
MKP-1	MAPK phosphatase 1
MyD88	Myeloid differentiation primary response gene (88)
NACHT	NAIP, C2TA , HET-E and TP1
NAIP	Neuronal apoptosis inhibitor protein
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NLR	NOD–like receptor
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NOD	Nucleotide-binding oligomerization domain
NTPase	Nucleoside triphosphatases
P38MAPK	P38 mitogen-activated protein kinases
PAMP	Pathogen-associated molecular patterns
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase c
PLC	Phospholipase c
PRR	Pattern recognition receptors
PYD	Pyrin domain
RAG	Recombination-activating genes
ROS	Reactive oxygen spieces
T2D	Type 2 diabetes
TAB1	Mitogen-activated protein kinase kinase kinase 7-interacting protein 1
TAK1	TGF beta Activated Kinase 1
TLR	Toll like receptor
TNF	Tumor necrosis factor
TOLIPP	Toll interacting protein
TP1	Telomerase-associated protein

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1 INTRODUCTION

1.1. Overview

The majority of cases of diabetes (80%) are attributable to the increasing incidence of obesity. Nutrient excess and adiposity activate pathways implicated in the development of insulin resistance and induce inflammation[1]. Chronic inflammatory conditions have a major impact on the development of metabolic diseases including diabetes. However, the physiological role of inflammation in metabolism and short-term metabolic stress is still unclear.

The deleterious role of IL-1β in T2D is well-described[2]. IL-1β is one of the major cytokines governing inflammation, but it also has a role in the pathology of insulin secretion[3]. Whereas, *In vitro*, chronic or high doses of IL-1β directly impair insulin secretion, acute exposure or low doses improve it[4]. The focus of this work is to understand the *in vivo* consequences on the effect of systemic increase in IL-1β on insulin secretion and action, and its consequence on glucose metabolism.

1.2 Alpha (α) and beta (β) cells of the pancreas and their hormones

Insulin and glucagon, the two counteracting hormones of the endocrine pancreas, are responsible for maintaining blood glucose levels in a narrow range. Insulin is produced by pancreatic islet β-cells and is secreted in response to rising blood glucose concentrations. By mediating glucose uptake in the periphery and enhancing glycogen synthesis in the liver, insulin promotes energy storage in the fed state and thus maintains blood glucose levels in the normal range. In contrast, glucagon is secreted by pancreatic α-cells when blood glucose levels drop. Glucagon stimulates glycogenolysis and gluconeogenesis in the liver thereby preventing hypoglycaemia. Therefore glucagon is essential for maintaining glucose homeostasis in the fasting state.

1.3 Insulin secretion

Insulin, the key hormone in regulating glucose metabolism, is secreted from pancreatic β-cells. The process of its secretion is a highly dynamic and regulated

by multiple factors. Nutrient status, hormonal factors, and neuronal factors[5, 6, 7], regulate insulin secretion at multiple levels: at the level of single β-cells, the pancreatic islet, the whole pancreas, and the intact organism. Hence, *in vivo*, the dynamics of insulin secretion is the consequence of an integration of all of these systems.

Stimulus-secretion coupling is an essential biologic event in glucose regulated insulin secretion in pancreatic β-cells. Ca^{2+}, ATP, cAMP, and phospholipid-derived signals such as diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) are the major intracellular signals during insulin secretion[8, 9].

Glucose-stimulated insulin secretion (GSIS) is the principal mechanism of insulin secretion. Glucose is transported into the β-cell mainly by GLUT2 glucose transporters, and then metabolized, leading to an increase of the ATP/ADP ratio, closure of ATP-sensitive K^+ (K_{ATP}) channels, depolarization of the β-cell membrane, and opening of the voltage-dependent Ca^{2+} channels (VDCCs), resulting in Ca^{2+} influx[10]. The resulting rise in intracellular Ca^{2+} concentration in the β-cell triggers insulin secretion. In addition, there is also a K_{ATP} channel–independent pathway that amplifies the effects of Ca^{2+} on exocytosis and does not require an additional increase in intracellular Ca^{2+} concentration[11].

1.3.1 Islet G coupled receptors mediated insulin secretion

Various signals regulate glucose stimulated insulin secretion, including hormones (glucagon-like peptide-1; GLP-1, and glucose-dependent insulinotropic polypeptide; GIP), neurotransmitters (e.g. acetylcholine; ACh) and nutrients (e.g. free fatty acids; FFAs). These factors signal through guanine nucleotide-binding protein (G protein)-coupled receptors (GPCR) to enhance GSIS. The islet GPCR mainly couple to adenylate cyclase and to phospholipase C (PLC).

1.3.1.1 Acetylcholine-potentiated GSIS

ACh, the major neurotransmitter of the peripheral parasympathetic nervous system, is known to facilitate the release of insulin in a glucose-dependent fashion. ACh is released from intra-pancreatic parasympathetic (vagal) nerve

endings during the pre-absorptive and, most likely, also during the absorptive phase of feeding. Of note, vagal nerves release at least five different neurotransmitters. Muscarinic ACh receptor subtypes (M1–M5) are widely expressed in a complex and overlapping pattern in many peripheral tissues and most regions of the brain. In β-cells the M3 muscarinic ACh receptor is the major muscarinic receptor expressed. β-cell specific M3 muscarinic receptor ko mice have impaired glucose tolerance and impaired insulin secretion and islets isolated from these mice have impaired GSIS[12]. In β-cells ACh activates several signal transduction pathways. One of the major pathways targeted by ACh is PLC, which mainly generates IP_3 and DAG. They are potent PKC activators, which lead to an increased efficiency of free cytosolic Ca^{2+} in the stimulation of exocytosis of insulin granules. ACh also depolarizes the plasma membrane of β-cells by Na^+- or nonspecific cationic-dependent mechanisms. This depolarization is small and reaches the threshold for the activation of voltage-dependent Ca^{2+} channels only if the plasma membrane is already depolarized by secretagogues such as glucose. The glucose dependence of this depolarization largely contributes to the glucose-dependence of ACh stimulated insulin secretion. The insulinotropic effect of ACh results from two mechanisms: one involves a rise in intracellular calcium concentration and the other involves a marked, PKC-mediated increase in the efficiency of Ca^{2+} to stimulate exocytosis of insulin vesicles.

1.3.1.2 Incretin-potentiated GSIS

Incretin hormones are polypeptides released from endocrine cells in response to nutrient ingestion, which stimulate insulin secretion in a glucose dependent manner. Both GLP-1 and GIP are incretin hormones.

GLP-1 is produced mainly in the distal jejunum, ileum and colon by L cells. GIP is produced in the proximal small intestine (duodenum and jejunum) by the intestinal K cells. The most studied incretin effect is the stimulation of insulin secretion by GLP-1. It induces insulin secretion and biosynthesis via GLP-1 receptor activation, followed by activation of adenylate cyclase (AC) and increased cAMP production. In addition, β-cell apoptosis is inhibited and ER stress is reduced through activation of AC whereas β-cell proliferation and neogenesis are

mediated via phosphatidylinositol-3 kinase (PI3K) activated pathways. Furthermore, GLP-1 inhibits glucagon secretion from the α-cells in vivo. This inhibitory effect may involve direct binding of GLP-1 to its receptor on α-cells, alternatively GLP-1 may inhibit glucagon secretion indirectly by stimulating insulin and somatostatin secretion.

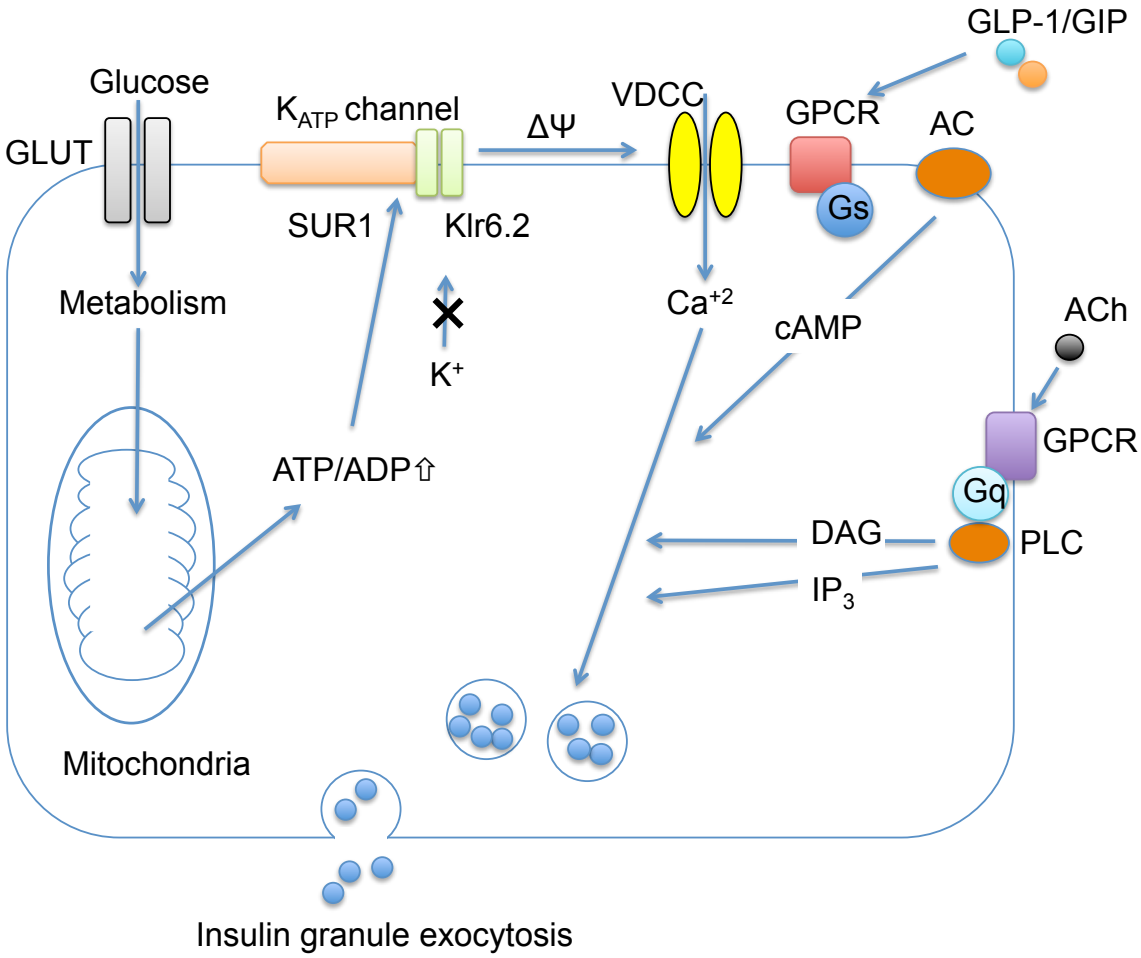


Figure 1. Model of insulin secretion. Glucose-induced insulin secretion and its potentiation constitute the principal mechanism of insulin release. Glucose is transported by the glucose transporter (GLUT) into the pancreatic β-cell. Metabolism of glucose increases ATP production (and the ATP-to-ADP ratio), closing the K_{ATP} channels, resulting in membrane depolarization (ΔΨ), opening of the voltage-dependent Ca^{2+} channels (VDCCs), and Ca^{2+} influx, which triggers insulin granule exocytosis. Insulin granule exocytosis is also regulated by hormones (GLP-1/GIP) and neurotransmitters (ACh), which generate intracellular signals such as cAMP, diacylglycerol (DAG), and inositol triphosphate (IP_3). Image adapted from[13]

1.4 Type 2 diabetes

1.4.1 Overview

In individuals suffering from T2D the pancreatic β-cells fail to produce sufficient insulin to compensate for peripheral insulin resistance, resulting in hyperglycemia. Obesity is associated with insulin resistance and is thus a risk factor for developing T2D. The lack of responsiveness to insulin eventually increases the demand for insulin and in order to adapt, the functional β cell mass in the endocrine pancreas increases. Nevertheless, this adaption can fail, eventually leading to diabetes.

The pathophysiology of T2D is characterized by impaired β-cell secretory function and decreased β-cell mass due to increased apoptosis[14]. In addition, T2D is accompanied by morphological alterations of pancreatic α-cells, increased α-cell mass and dysregulation of glucagon secretion. Recently, innate immunity and inflammatory processes were shown to contribute to the development of T2D.

1.4.2 T2D as an auto-inflammatory disease

Auto-inflammatory diseases are characterized by sterile inflammation predominantly mediated by the cells and molecules of the innate immune system[15]. Some auto-inflammatory diseases are attributed to a dysfunctional monocyte caspase 1 activity which is responsible for the conversion of cytoplasmic pro-IL-1β to its active, secreted form; Blocking IL-1β results in a rapid and sustained reduction in the severity of most auto-inflammatory diseases including T2D[16].

Pancreatic islet inflammation plays a role in the development and progression of T2D. Islets of T2D patients display clear signs of inflammatory processes, such as cytokines, apoptosis markers, immune cell infiltration and fibrosis[17, 18, 19]. In addition, free fatty acids and glucose induce endoplasmatic reticulum (ER) stress and a pro-inflammatory pattern that results in dysfunctional pancreatic islet[20, 21, 22, 23]. Thus the combination of dyslipidemia, hyperglycemia and islet amyloidosis[24, 25] in T2D is probably the driving force behind this inflammatory response.

IL-1β not only has a deleterious effect on insulin secretion but also contributes to the development of insulin resistance. Interestingly, IL-1β induced insulin resistance in adipocytes[26] and the NLRP3 inflammasome which regulates IL-1β processing and secretion contributes to obesity induced insulin resistance[27]. In addition, NLRP3-derived IL-1β induced by FFAs prevented normal insulin signaling in insulin target tissues leading to insulin resistance[28]

All together these data support the concept that T2D is an inflammatory disease.

1.5 Innate immunity and inflammatory processes

1.5.1 Overview

The innate immune system serves as the first line of defense to protect the body from infection or irritation in a non-specific manner. This means that the cells of the innate system recognize and respond to pathogens in a generic way[29]. Inflammation is one of the first reactions of the innate immune system and is stimulated by various factors released by injured cells. Inflammation serves to protect against stressors, and to restore tissue and organism homeostasis. The process of acute inflammation is initiated by cells, mainly macrophages that are present in all tissues. These cells present receptors on the cell surface or within the cell, known as pattern recognition receptors (PRRs). These receptors recognize molecules that are shared by different pathogens, collectively termed as PAMPs (pathogen-associated molecular patterns)[30]. At the onset of stress, these cells undergo activation e.g. Toll like receptors (TLR) bind LPS, and release inflammatory mediators responsible for the clinical signs of inflammation[31]. This process is largely driven by IL-1β, one of the first cytokines discovered [4]. Prolonged activation of the immune system may eventually promote the development of a chronic inflammatory disease. Interestingly, over-nutrition and obesity lead to such a chronic activation of the innate immune system, eventually leading to metabolic diseases[32, 33, 34, 35, 36]. Indeed, acute-phase proteins, cytokine and chemokine levels as well as immune cell types and numbers are altered in the circulation and in various tissues of patients with metabolic diseases[19, 37]. A crucial role in the initiation of an innate immune response and of auto inflammatory diseases including T2D is attributed to macrophages, the main

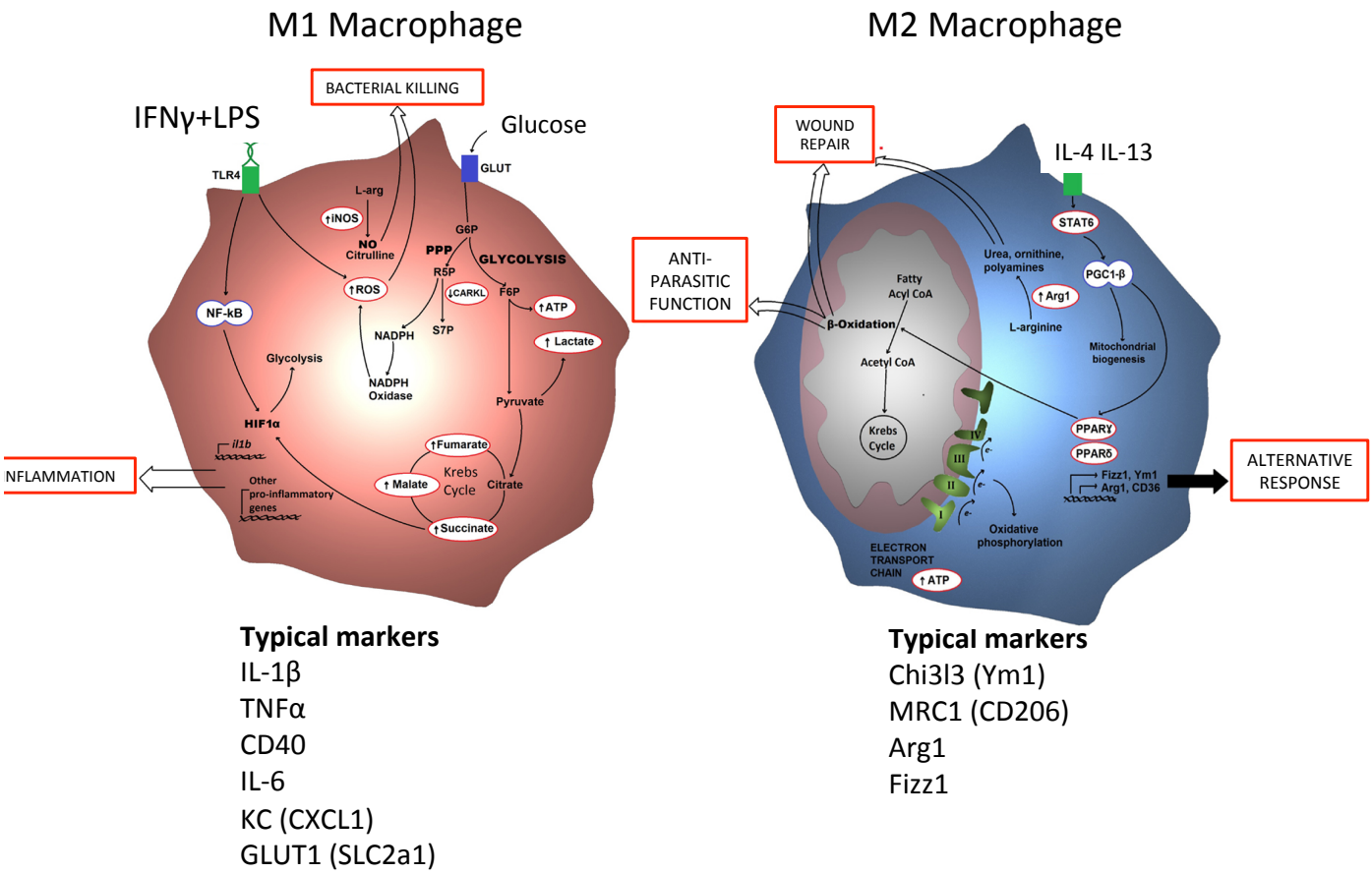
source of IL-1β in the body.

1.5.2 Macrophages

Macrophages are distributed throughout the body. In some tissues, they represent 10 – 20% of all cells and become highly specialized cells such as Kupffer cells in the liver, osteoclasts in the bone or microglia in the brain. Macrophages are versatile cells and can adopt specialized functions at particular tissue locations. Macrophages are derived from circulating precursors, the monocytes. Circulating monocytes are released from the bone marrow as non-differentiated cells, circulate in the blood for a few days in a process called rolling and are poised to rapidly extravate to inflamed tissues. Upon local inflammation, circulating monocytes undergo extravasation, which involves adhesion and transmigration into a target tissue. Upon infiltration into the inflamed tissue, monocytes readily differentiate into mature macrophages. Stimulation of macrophages with cytokines such as IFNγ alone or together with other cytokines (e.g. TNFα) or bacteria derived stimuli (e.g. LPS) promotes the maturation of "classically" activated macrophages termed M1. These cells are characterized by the production of of high levels of toxic intermediates (e.g. reactive oxygen species, nitric oxides), a high capacity to present antigens and high secretion levels of inflammatory cytokines. M1 macrophages are the main source for IL-1β in the body. In contrast, various signals (e.g. IL-4, IL-13) induce “alternative” maturation termed M2 macrophages. These cells are able to down-tune inflammatory responses, scavenge debris, and promote angiogenesis and tissue remodeling.

Recently, many studies focused on molecules that could play a role in macrophage polarization, such as cytokines, transcription factors or enzymes. Interestingly, as macrophages switch from a quiescent (naïve) to an activated state, their metabolic activity is modified. Recent evidence suggests that immune cells adopt specific metabolic signatures[38, 39]. Metabolic changes in cells that participate in inflammation, such as activated macrophages (and T-helper 17 cells), include a shift towards enhanced glucose uptake, glycolysis and increased

activity of the pentose phosphate pathway. In contrast, anti-inflammatory cells, such as M2 macrophages (and regulatory T cells and quiescent memory T cells), have lower glycolytic rates and higher levels of oxidative metabolism. This is presented in the following figure. An important question in the field of "immunometabolism" is whether metabolic pathways themselves can alter immune cell differentiation and thus have direct effector function. Glucose is a critical component in the pro-inflammatory response of macrophages. In pro-inflammatory polarized M1 macrophages, GLUT1 (SLC2A1) is the primary rate limiting glucose transporter and metabolic reprogramming via increased glucose availability modulates macrophage inflammatory response[40]. This suggests that T2D hyperglycemic milieu may act as a driving force of M1 macrophage polarization.



Typical markers
IL-1β
TNFα
CD40
IL-6
KC (CXCL1)
GLUT1 (SLC2a1)

Typical markers
Chi3l3 (Ym1)
MRC1 (CD206)
Arg1
Fizz1

Figure 2 Metabolic profile and typical markers of polarized macrophages: Classically activated macrophages (M1) induce an aerobic glycolytic (will be reflected in extracellular acidification rate; ECAR) program that results in lactate production and increased levels of intermediates of the Krebs cycle. The hypoxia-inducible factor (HIF) 1α transcription factor also becomes activated and can drive production of pro-inflammatory cytokines. Alternatively activated macrophages (M2) trigger a metabolic program including the electron transport chain as well as fatty acid β-oxidation (that will be reflected in oxygen consumption rate; OCR). Taken from[41].

1.6 Interleukin-1

1.6.1 Overview

The IL-1 family of cytokines consists of 11 known members that are encoded by distinct genes and includes IL-1α, IL-1β, and the IL-1 Receptor antagonist (IL-1Ra). The major role of IL-1 type cytokines is to control pro-inflammatory reactions in response to tissue injury[42] - either due to recognition of PAMPs (e.g. the bacterial wall product LPS) or of danger associated molecular patterns (DAMPs; e.g. uric acid) by a target cell. IL-1α is a membrane-anchored protein that upon its release, signals through autocrine or juxtracrine mechanisms. IL-1β is processed from its zymogen form (pro-IL-1β) by caspase-1 (IL-1β converting enzyme) and upon secretion acts in a paracrine or systemic manner. Of note, IL-1β is predominantly produced by macrophages and monocytes, but to a much lesser extent also by other cell types such as epithelial cells, fibroblasts, endothelial cells and pancreatic islets including the insulin expressing beta cells[21, 43]. Secretion of IL-1β is a highly regulated process that requires the formation of a protein complex called the inflammasome[44]. There are several variations of this multi-protein complex, The NLRP3 inflammasome is the most fully characterized form. The NLRP3 inflammasome contains the adaptor protein apoptosis-associated specklike protein (ASC), the proinflammatory caspase, caspase-1 and NLRP3. NLRP3 belongs to the nucleotide-binding oligomerization domain (NOD)–like receptor (NLR) family of PRRs. Upon activation, NLRP3 is thought to oligomerize via homotypic interactions between NACHT (NTPase) domains and by presenting clustered pyrin (PYD) domains enabling an interaction with the PYD domain of ASC. ASC assembly, in turn, presents clustered caspase activation and recruitment domains (CARDs) for interaction with the CARD of procaspase-1. Procaspase-1 clustering enables auto-cleavage and activation; activated caspase-1 can cleave other cytosolic targets, including IL-1β. Thus unlike IL-1α the expression of IL-1β mRNA is necessary but not sufficient to activate IL-1 signaling[16].

1.6.2 Interleukin-1 signaling

IL-1 has two isoforms, IL-1α and IL-1β. Both bind to the same cellular receptor, the Type 1 IL- 1 receptor (IL-1R1) to induce signaling. Upon receptor engagement, IL-1R1 forms a heterodimer with its co-receptor IL-1 receptor accessory protein (IL-1RAcP). IL-1RAcP cannot bind directly to IL-1 but is essential for IL-1-mediated signaling. Binding of IL-1 to this receptor complex leads to the activation of the transcription factor NF-κB through different signaling mechanisms. IL-1 receptor-associated kinases, IRAK-1, IRAK-2 and IRAK4 have been implicated in the activation of NF-κB. IRAKs function as adapter proteins and protein kinases to transmit downstream signals. This leads to the recruitment of TNF receptor associated factor 6 (TRAF6) to the IL-1 receptor complex via an interaction with IL-1RAcP. Oligomerization of TRAF6 and subsequent formation of TAK1 and MEKK3 signaling complexes relays the signal leading to NF-kB activation (see figure below).

In addition, activation of mitogen activated protein kinases, including JNKs and p38 MAPK through various MAP2Ks also plays an important role in mediating IL-1 responses by enhancing transcription through the activator protein-1 (AP-1) transcription factor.

These signaling events co-operatively induce the expression of IL-1 target genes such as IL-8 (KC/CXCL1 in mice) and IL-6.

IL-1 signaling is regulated by various mechanisms. The IL-1 family member IL-1Ra binds with a similar affinity to the IL1-R1 receptor as the agonistic ligands IL-1α and β, but it is incapable of activating the signaling response. The type 2 IL-1 receptor is a decoy receptor that binds to IL-1α and β but lacks signaling capacity. The naturally occurring 'shed' domains of the extracellular IL-1 receptor chains (soluble IL-1R1, IL-1R2 and IL- 1RAcP) also act as inhibitors of IL-1 signaling. In the cell, IL-1R binds to toll- interacting protein (TOLLIP), which results in the inhibition of IRAK1 and subsequent targeting of the internalized IL-1R to endosomes leading to efficient degradation. Other mechanisms such as p38MAPK mediated phosphorylation of TAB1 which results in the inactivation of TAK1, and expression of genes including MAPK phosphatase 1 (MKP-1) and

Inhibitor of kappa B alpha (NFKBIA) that inhibit IL-1 signaling components also serve as negative regulators of IL-1 signaling as shown in the following figure.

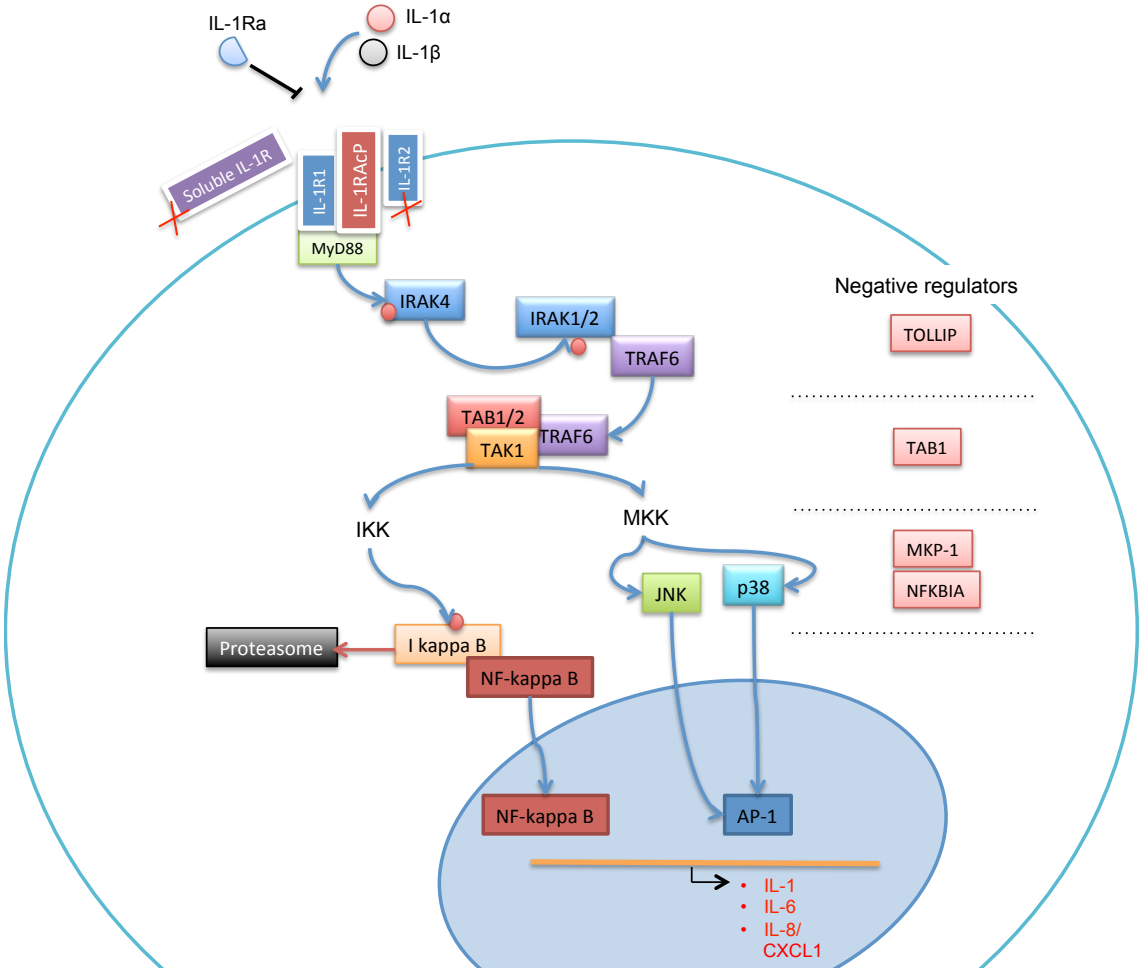


Figure 3. Scheme for IL-1 signaling. Upon binding of IL-1β or IL-1α to IL-1R1 and IL-1RAcP, MyD88 is recruited, IRAKs are being phosphorylated leading to formation of protein complex that activate NF- kappa B or AP-1 via degradation of I kappa B or activation of JNK and p38 respectively. Resulting in cytokine expression. Negative regulation is done in multiple levels, from receptor antagonist (IL-1Ra) and decoy receptors (IL-1R2 or soluble receptors) to inhibition of IRAK by TOLLIP, TAK1 by TAB1, MKK by MKP-1 and NF-kappa B by NFKBIA in addition IL-1R1 signaling results in receptor internalization mediated by TOLLIP.

1.6.3 Interleukin-1β in T2D

To describe the role of IL-1β in T2D, this chapter is divided to 2 parts, one part focuses on IL-1β in obesity and insulin resistance and the other one on the role of IL-1β in islet dysfunction.

The discovery of protective effects of tumor necrosis factor (TNF) neutralization on glucose uptake in obese rats was one of the first observations linking inflammation to the development of insulin resistance[33]. Cytokines such as IL-1β, TNF and IL-6 induce the expression of suppressors of cytokine signaling and limit inflammatory signals. These suppressors interfere with tyrosine kinases including the one of the insulin receptor[45]. Furthermore, inflammatory signals, including IL-1β, result in the ubiquination and breakdown of insulin substrates, IRS1 and IRS2[46]. In vivo, the importance of IL-1β in metabolic disorders was demonstrated by studies showing the crucial role of NLRP3 inflammasome in the development of obesity and insulin resistance. Mice deficient for NLRP3 or the pro-IL-1β converting enzyme, caspase-1, are protected from diet induced obesity[47], have improved insulin sensitivity[48] and reduced IL-18, interferon gamma (IFNγ) and effector T cell numbers in adipose tissue. In obese humans with T2D the improved insulin sensitivity upon weight loss is associated with reduced NLRP3 expression[27]. In addition, FFAs activate the NLRP3 inflammasome[28] by increasing reactive oxygen species (ROS) which leads to more cytokine production and enhanced insulin resistance. Of note, insulin resistance does not result in an overall reduced glucose consumption as other glucose transporter (GLUT1) are upregulated in adipocytes treated with IL-1β[26], i.e. it is the insulin regulated glucose uptake via GLUT4 that is impaired while basal glucose consumption is increased, leading to increased ROS production.

Besides the described effects on insulin sensitivity, inflammation has a role in the impairment of insulin secretion. High IL-1R1 expression in insulin producing beta cells[20] renders them sensitive to the deleterious effects of IL-1β. These include the formation of nitric oxide[49] followed by induction of the death receptor Fas[50] and necrosis[51]. Islets treated with high concentrations of IL-1β have impaired glucose stimulated insulin secretion and increased beta cell death. Interestingly, high concentrations of glucose also induced apoptosis in cultured islets[52]. Further

investigation revealed that glucose induces IL-1β in islets and that the deleterious effects of glucose on islet function and survival were diminished when islets were treated with IL-1Ra[21]. These findings were confirmed and extended by the discovery showing that high glucose concentrations activate the NLRP3 inflammasome in islets[53]. Later, other metabolites and proteins typically altered in T2D state have been described to act as activators of the NLRP3 inflammasome[24, 28].

All of the above explains why out of the 11 members of the IL-1 family, IL-1β had emerged as the primary therapeutic target in auto inflammatory diseases[54, 55].

However, the observation of high expression of IL-1R1 in β cells also suggests a physiological function of IL-1β in pancratic beta cells that has been neglected in the past years. Indeed old publications demonstrate a dual role of IL-1β on insulin release of pancreatic islets[4]. Furthermore, low dose treatment with IL-1β induced beta cell proliferation and secretion in human islets[52] also hinting to more complex roles of IL-1β in islets. This work focuses on the physiological role of IL-1β in insulin secretion and its consequences.

1.7 Aim of study

Numerous studies associated IL-1β with pathological outcomes such as impaired β-cell function and survival, however, little is known about its acute, physiological role *in vivo* in metabolism and in the development of diabetes or other auto inflammatory diseases.

The aim of this study was to investigate the acute effects of exogenous and endogenously produced IL-1β on glucose metabolism in mice and on related parameters such as insulin secretion and insulin resistance. Inflammatory processes and activation of the immune response require major changes in the metabolic profile of immune cells e.g. increased glycolytic capacity. Since both, glucose, as a main fuel for an immediate immune response, and insulin, the main regulator of glucose homeostasis, are elevated in the metabolic syndrome, we speculated that there might be a link between insulin, glucose metabolism and the activation of the immune system. The aim of the present work is to study this

possible link, specifically between IL-1β and insulin in glucose metabolism and initiation of inflammation.

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Physiological synergy between IL-1β and insulin on glucose disposal and macrophage activity

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The deleterious role of chronic activation of the IL-1β system in type 2 diabetes and other metabolic diseases is well documented. However, little attention has been paid to a possible physiological role of IL-1β in the regulation of glucose metabolism. Here we show that acute injections of IL-1β strongly induced insulin secretion and improved glucose disposal in normal, obese and diabetic mice. This insulin secretagogue effect was potentiated by glucose and involved neuronal stimulation. LPS induced similar effects but not in the presence of the IL-1Ra or in mice deficient for the NLRP3 inflammasome. IL-1β alone and together with insulin increased glucose uptake into muscle, adipose tissue and in the immune compartment, mainly in macrophages. In polarized M1 macrophages, insulin reinforced a pro-inflammatory pattern via its upregulated receptors, elevated AKT phosphorylation, increased glycolytic activity and increased inflammasome-mediated IL-1β secretion. Our findings identify a physiological role for IL-1β and insulin in the regulation of both metabolism and immunity.

INTRODUCTION

Activation of the innate immune system is an initial response of the body to infections and injuries. The resulting inflammation aims at protecting against stressors, and at restoring tissue and organism homeostasis. This process is largely driven by interleukin-1β (IL-1β), one of the first described cytokines[4]. However, prolonged activation of the immune system may eventually promote the development of a chronic inflammatory disease. Increasing evidence shows that over-nutrition and obesity lead to such a chronic activation of the innate immune system and subsequent metabolic diseases[32, 33, 34, 35, 36]. Indeed, acute-phase proteins, cytokine and chemokine levels as well as immune cell types and numbers are altered in the circulation and in various tissues of patients with metabolic diseases[19, 37]. A critical sensor of nutrient overload is the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome, which promotes the maturation of IL-1β in various metabolic disorders. This is the case for uric

acid crystals which activate the NLRP3 inflammasome in gout[56], for cholesterol crystals which induce it in atherogenesis[57], and for glucose, fatty acids, and islet amyloid which contribute to impaired insulin secretion and action via NLRP3 in type 2 diabetes[21, 24, 27, 28, 48]. Importantly, a causal link between IL-1β-induced inflammation and these metabolic diseases has been demonstrated by various genetic and pharmacological approaches in animal models[47, 53, 58, 59, 60, 61] and therapeutic interventions in humans[54, 62, 63, 64, 65, 66]. While these studies shed light on the pathological aspects of IL-1β and chronic inflammation in metabolism, the expected (acute) physiological role of IL-1β in metabolic control remains largely unexplored.

In response to an infection, a massive expansion and activation of the immune system occurs. To cope with the energy requirement of this process profound changes in the metabolism of immune cells take place[40, 67, 68, 69]. Beyond supplying energy, nutrients can also act as cell signaling molecules in the activation of the immune system. Indeed, elevated glucose levels and metabolites such as succinate may drive production of IL-1β in macrophages[21, 53, 70].

The main immediate source of energy in the body is glucose, which is primarily regulated by insulin. Interestingly, the insulin-producing β-cells express the highest level of IL-1 receptor compared to other tissues and are highly sensitive to IL-1β[20, 71, 72]. While a large body of *in vivo* and *in vitro* data demonstrate a deleterious role of high dose and long-term IL-1β exposure on islet function and mass, a few mainly *in vitro* observations hint to a possible beneficial role of IL-1β in the function and survival of β-cells[4, 52, 73]. Therefore, we hypothesized that an acute increase in IL-1β has a physiological role in whole body glucose homeostasis that is to recruit the energy needed to activate the innate immune system. We provide evidence that *in vivo* an acute increase in IL-1β shifts glucose disposal from the circulation to classical insulin sensitive tissues and to the immune compartment partly via potent neuronal stimulation of insulin secretion. In macrophages, IL-1β and insulin promote glucose uptake via the glucose transporter GLUT1, and increase glucose metabolism and cytokine production. Interestingly, insulin stimulates IL-1β production in polarized macrophages via glucose uptake and the NLRP3 inflammasome and therefore directly modulates the immune system.

RESULTS

IL-1β is a potent insulin secretagogue and improves glucose disposal in normal, obese and diabetic mice.

In order to test the acute *in vivo* effects of IL-1β we first evaluated the pharmacokinetic of increasing concentrations of intraperitoneal (ip) IL-1β injections in normal mice. Basal circulating IL-1β levels were 2.8 ± 2.5 pg/ml and peaked after 15 minutes at 9.0 ± 2.0, 12.7 ± 7.7, 46.1 ± 12.1 and 178.5 ± 28.7 pg/ml after injection of 0.1, 0.25, 0.5 and 1 µg/kg IL-1β, respectively (Fig. 1a, Supplementary Fig. 1a). Based on these data we injected IL-1β 18 minutes before an ip glucose tolerance test (ipGTT) in subsequent studies. In a dose dependent manner, IL-1β strongly induced insulin secretion along with a profound improvement of glucose clearance (peak insulin concentration in plasma 2.0 ± 0.2, 2.1 ± 0.3 and 3.6 ± 0.3 ng/ml following 0.25, 0.5 and 1 µg/kg IL-1β respectively; Fig. 1b,c). Of note, although the lowest injected dose of 0.1 µg/kg IL-1β only marginally increased the concentration of circulating IL-1β, it was sufficient to significantly elevate insulin levels, demonstrating the potency of IL-1β (Supplementary Fig. 1b). Since insulin resistance is associated with systemic inflammation[33], we assessed insulin sensitivity with an ip insulin tolerance test (ITT) and during hyper-insulinemic-euglycemic clamp studies. Following an acute ip injection of 1 µg/kg IL-1β, insulin sensitivity and hepatic glucose production remained comparable to the control group (Fig. 1d-f,

Supplementary Fig. 1c,d). Because diet induced obese (DIO) mice have an impaired glucose tolerance, we tested whether acute IL-1β is still beneficial in this context. Mice fed with a high fat diet for 24 weeks were injected with 1 µg/kg IL-1β prior to an ipGTT. Treated mice had improved glucose tolerance along with a strong stimulation of insulin secretion (Fig. 1g,h). As a second model of impaired glucose metabolism, 16 week old db/db mice were injected ip with 1 µg/kg IL-1β prior to an ipGTT. Even in these severely insulin resistant animals, IL-1β improved glucose tolerance and strongly increased insulin secretion (Fig. 1i,j). To demonstrate that the observed effects of IL-1β are mediated by the IL-1 receptor signal transduction pathways, we used IL-1 receptor-associated kinase-4 (IRAK-4) deficient mice. In contrast to wild-type mice (not shown), IRAK-4 deficient mice injected with IL-1β before an ipGTT showed no change in blood glycaemia and no improvement in insulin secretion (Fig. 1k,l). Next we assessed the insulin secretagogue capacity of IL-1β or glucose alone compared to the combination of both. Injection of IL-1β or glucose alone led to a small induction of insulin secretion and a mild decrease in blood glucose concentration. In contrast, IL-1β combined with glucose strongly potentiated the effect of either glucose or IL-1β alone. This effect of the combination was more than additive (Fig. 1m-o). To assess the direct effect of IL-1β on insulin secretion, we first identified the IL-1 receptor type 1 and detected it in a subpopulation of β-cells (Fig. 1p) at a much higher expression level than in islet resident immune cells (Fig. 1q). *In vitro* in isolated mouse pancreatic islets, the presence of IL-1β for 30 minutes at 2.8 mM glucose (priming) had a biphasic effect on subsequent glucose stimulated insulin secretion (16.7 mM): low concentrations of IL-1β up to 0.5 ng/ml increased, whereas 2 ng/ml IL-1β blunted glucose stimulated insulin secretion (Fig. 1r). IL-1β priming also improved glucose stimulated insulin secretion of human islets at 0.5 ng/ml (Fig. 1s) and at 1 ng/ml in the human β-cell line ENDOC (Fig. 1t). Therefore, IL-1β priming also potentiates glucose stimulated insulin secretion *in vitro*. Of note, the IL-1β secretagogue effect was much stronger *in vivo* than *in vitro*, pointing to additional indirect effects of IL-1β on insulin secretion and tissue glucose uptake.

Endogenous IL-1β plays a role in insulin secretion

Next we tested whether the effects of exogenous IL-1β on insulin secretion and glucose disposal are physiological or merely pharmacological by inducing endogenous IL-1β production with LPS. We first measured the kinetics of LPS-induced IL-1β secretion. Injection of 1 mg/kg of LPS increased circulating IL-1β levels and reached a peak after 3 hours (Fig. 2a), which was paralleled by a drop in blood glucose levels (Fig. 2b) and an increase in insulin concentration (Fig. 2c). In contrast, LPS injection in IL-1β deficient mice resulted in blunted insulin secretion compared to wild type mice (Fig. 2d). To substantiate the role of endogenous IL-1β, we then injected LPS in chow and high fat diet fed mice and performed an ipGTT. Similar to IL-1β, LPS strongly improved glucose tolerance and stimulated insulin secretion (Fig. 2e,f). In the presence of the IL-1 receptor antagonist (IL-1Ra; Fig. 2g) or in mice deficient for the NLRP3 inflammasome (Fig. 2h), the LPS-induced insulin secretory effect was almost completely blocked. Finally, since obesity is associated with elevated IL-1β[27], we investigated in obese mice the effect of endogenous IL-1β and evaluated its effect on islet function by acute IL-1 antagonism. Acute injections of IL-1Ra resulted in lower fasting insulin levels in high fat fed mice (Fig. 2i) without changing insulin sensitivity or hepatic glucose production (Fig. 2j, Supplementary Fig. 1e,f). Similarly, acute IL-1Ra reduced basal insulin levels in db/db mice (Fig 2k). Altogether, this suggests a physiological role of endogenously produced IL-1β in the regulation of insulin secretion under various conditions.

***In vivo*, only prolonged administration of IL-1β impairs glucose metabolism**

The role of IL-1β in islet failure and insulin resistance in patients with type 2 diabetes is well established[36]. Due to the unexpected above described beneficial effects of acute IL-1β injections, we investigated under which conditions IL-1β becomes deleterious *in vivo*. First we tested the response to a higher dose of IL-1β (10 µg/kg), given either acutely as described above or for 5 consecutive days. Both treatments improved glucose tolerance during ipGTT to a similar level (Fig. 3a), although the mice injected for 5 days displayed a tendency to less pronounced improvement of insulin secretion compared to the single injection (Fig 3b). Of note, the discrepancy between the similar glucose lowering effects at different circulating insulin levels pointed to insulin independent IL-1β effects on glucose disposal, as depicted below. As a next step we increased the dose of IL-1β to 35 µg/kg for 3 days and still observed improved glycaemia induced by IL-1β, however, in the absence of stimulated insulin secretion (Fig. 3c-d). In order to perform prolonged IL-1β applications, we then implanted mini-osmotic pumps continuously releasing IL-1β (0.1 µg/kg/day) into mice for 31 days. At day 17, continuous subcutaneous release of IL-1β slightly improved glucose tolerance without effect on insulin secretion (Fig. 3e,f) and after 31 days of treatment, glycaemia and insulin secretion, started to deteriorate (Fig. 3g,h). Finally, we injected mice daily with 1 µg/kg of IL-1β for 2 weeks and observed impaired glucose tolerance (Fig. 3i,j). To verify that IL-1β reached the islets, we isolated them and observed increased inflammatory parameters (Fig. 3k). Thus, only prolonged exposure to IL-1β for several weeks impairs glucose metabolism.

Improvement of glycaemia by IL-1β is partly mediated via neuronal stimulation of insulin secretion and is independent of the incretin system

To better understand the beneficial role of IL-1β we performed oral GTT (oGTT), the physiological way of glucose ingestion. Mice were acutely injected with IL-1β followed by oral gavage of glucose. Similar to the ipGTT (Fig. 1b), 1 µg/kg of IL-1β strongly improved glucose clearance (Fig. 4a). Surprisingly, insulin levels were similar to the control group (Fig 4b). Since acute IL-1β did not affect insulin sensitivity according to insulin tolerance tests and hyper-insulinemic euglycemic clamps (Fig 1d-f), we first considered an incretin effect to explain the difference between ip and oGTT. Therefore we injected mice with the GLP-1 receptor blocker exendin 9-39 and repeated the ipGTT. As expected, exendin 9-39 alone impaired insulin secretion and glucose tolerance, however, it failed to block the effect of IL-1β (Fig. 4c,d). Furthermore, circulating active GLP-1 was not increased upon acute IL-1β (Fig. 4e). To definitively rule out an incretin effect, we injected GIP/GLP-1 double receptor KO mice with IL-1β and observed the same robust improvement in glycaemia and increase in insulin secretion than with wild type control mice during an ipGTT (Fig. 4f,g). We then hypothesized that IL-1β may stimulate insulin secretion via neuronal transmission. Therefore we repeated the ipGTT after administration of the muscarinic acetylcholine antagonist, atropine, and observed an attenuation of the IL-1β effect on insulin secretion by 80% without affecting blood glucose levels (Fig. 3h-j). Of note, also under this condition insulin secretion and changes in blood glucose were disconnected, further pointing to additional IL-1β effects on glucose disposal. In contrast, darifenacin, a specific muscarinic 3 receptor (the isoform expressed in β cells[74, 75]) antagonist that does not cross the blood brain barrier, did not prevent the IL-1β effect on glucose tolerance and insulin secretion (Fig. 4k,l), suggesting a central neuronal effect. Furthermore, in isolated human but not mouse islets, atropine treatment blocked IL-1β promoted insulin release (Fig. 4m,n), confirming acetylcholine detection in human

islets[76]. This points to a paracrine effect involving IL-1β and acetylcholine in insulin secretion in human but not in mouse islets. Interestingly, IL-1β induced mRNA expression of vesicular acetylcholine transporter (vAChT) in cultured mouse and human islets treated with IL-1β *in vitro* (Fig. 4o,p), and in islets isolated from mice chronically injected with the cytokine (Fig 4q). In addition, as a model for low-grade chronic inflammation, islets isolated from high fat fed mice had higher vAChT expression than islets from chow fed mice (Fig. 4r). The neuronal role of IL-1β mediated insulin secretion was also apparent in mice fed a high fat diet, where atropine strongly inhibited the effect of IL-1β on glucose tolerance (Fig. 4s,t). To confirm the neuronal involvement we used GFRα2 deficient mice that lack the parasympathetic innervation in the islets[77]. GFRα2 deficient mice injected with IL-1β displayed a weaker improvement in glycaemia and insulin secretion as compared to wild type littermates (Fig. 4u,v). However, this effect is independent of neurons expressing the Glut 2 transporter (Supplementary Fig 1g). Overall, these data show that improvement of glycaemia by IL-1β is partially mediated via neuronal stimulation of insulin secretion and is independent of the incretin system.

IL-1β promotes insulin independent glucose uptake in muscle, fat and immune cells but not in the liver.

To understand the IL-1β mediated and insulin independent decrease in blood glucose (Fig. 4a,b), we performed glucose bio-distribution assays, using trace amounts of radiolabeled 2-deoxy glucose following an injection of 1 μg/kg IL-1β. In the absence of metabolizable glucose, IL-1β did not stimulate insulin secretion (Fig. 5a). IL-1β increased glucose uptake of muscle and adipose tissue but not of the liver (Fig. 5b-e). In addition, glucose uptake was increased in circulating white blood cells and in the spleen (Fig. 5f,g). We followed up by injections of 35 μg/kg of IL-1β in mice for 3 days. This led to elevated numbers of peritoneal cells and lymphocytes, and splenomegaly along with increased glucose uptake in lymphocytes and macrophages (Fig. 5h-l). In order to confirm our *ex vivo* results, we exposed isolated T-cells and macrophages to IL-1β and observed an increased glucose uptake (Fig. 5m,n). Furthermore, blocking endogenously produced IL-1 with IL-1Ra resulted in decreased glucose uptake in macrophages (Fig. 5o). To further investigate the contribution of immune cells to the glucose disposal, we first used T and B cell deficient (RAG2 KO) mice. These mice showed similar glucose disposal in an oGTT upon IL-1β as their littermate controls (Fig. 5p). When we then additionally ablated the macrophages in RAG2 KO mice with clodronate liposomes (Fig. 5q), the IL-1β effect on glucose disposal was impaired, suggesting that immune cells contribute to IL-1β-induced glucose uptake (Fig. 5r). Thus IL-1β promotes glucose uptake selectively in fat, muscle and immune cells, but not into the liver and this happens independently of insulin.

Insulin regulates macrophage metabolism and stimulates IL-1β via the NLRP3 inflammasome.

Because IL-1β enhanced glucose stimulated insulin secretion (Fig. 1), we hypothesized that insulin may contribute to the regulation of the immune system. Therefore, we first tested the effect of insulin on glucose uptake in naïve peritoneal macrophages. Insulin alone induced glucose uptake and this effect was additive to IL-1β (Fig 6a). Next we investigated the expression of the insulin receptor and found it upregulated in the pro-inflammatory M1 macrophages whereas it was downregulated in the anti-inflammatory M2 compared to naïve macrophages (M0; Fig. 6b; as a control for the polarization see mRNA expression of CD40, IL-1β and Chi3l3 in the Supplementary Fig. 1h). Furthermore, insulin induced AKT phosphorylation in naïve M0, to a greater extent in M1 but not in M2 macrophages (Fig. 6c). In line with this pattern, as shown by extra cellular acidification rate (ECAR),

insulin treatment increased the glycolytic activity selectively in M1 macrophages, while it was not stimulated in naïve or M2 macrophages (Fig. 6d). Furthermore, insulin increased basal oxygen consumption rate (OCR) in M0, but had no clear effects on this parameter in M1 and M2 macrophages (Fig. 6e). Similar to the pattern of insulin receptor expression and activation, at mRNA level, insulin promoted an overall pro-inflammatory state in M0 and M1 macrophages and an anti-inflammatory in M2 macrophages (Fig. 6f). At protein level, insulin induced secretion of mature IL-1β preferentially in M1, to a much lesser extent in M0 and not in M2 macrophages (Fig. 6g). In parallel, upon stimulation with IL-1β, IL-1Ra was strongly induced in M0, less in M1 and remained unchanged in M2 macrophages (Fig. 6h). This insulin effect on IL-1β secretion was lost in the presence of the glucose transporter GLUT1 (also known as Slc2a1) inhibitor fasentin (Fig. 6i). Finally, the insulin stimulated effect on IL-1β secretion and cytokine mRNA expression was lost in macrophages from NLRP3 deficient mice (Fig. 6j,k). Interestingly insulin also stimulated the expression of GLUT1 and hexokinase II (HKII), the rate-limiting enzyme of glycolysis. In addition we detected increased NLRP3 expression upon insulin treatment (Fig. 6j,k). Overall, these data show that insulin directly regulates macrophage energy metabolism and function, and induces inflammasome mediated IL-1β secretion. These effects are dependent on the activity status of macrophages.

DISCUSSION

The deleterious effects of IL-1β on islet function and survival are well described and IL-1β was frequently used as a tool to provoke β-cell demise[49, 71, 72]. Paradoxically, at low concentrations or upon short exposure, IL-1β modestly stimulates insulin secretion in isolated islets indicating that IL-1β may not only be detrimental for β-cells but may have more complex biological functions[4, 52]. A possible physiological role of IL-1β in islets is also suggested by our previous observation of high levels of IL-1 receptor mRNA expression in whole islets[20] and in the present study by its prominent expression in β-cells. Furthermore, injections of IL-1β shortly prior to a glucose bolus massively improved glucose disposal, supporting previous findings with pharmacological doses of IL-1β[73, 78]. We show that this was due to increased insulin secretion via activation of neurons involved in the release of insulin and due to direct IL-1β-mediated glucose uptake. Of note, IL-1β increased glucose uptake in muscle, fat and immune tissues but not in the liver and without changes in insulin sensitivity. The physiological relevance of these findings was demonstrated by LPS-induced endogenous IL-1β secretion, which was equally effective as the injection of exogenous IL-1β.

The observed beneficial effects of IL-1β on glucose homeostasis is in apparent contrast to the glucose lowering effects of IL-1 antagonism in patients with type 2 diabetes[54]. The most obvious explanation is the difference between acute and chronic effects. Indeed, prolonged administration of IL-1β impaired glucose metabolism in mice. However, the positive effect of acute IL-1β on insulin secretion was maintained in animal models of diabetes despite chronically increased endogenous IL-1β levels. Similarly, fasting insulin levels of db/db mice and of diet induced obese mice were decreased by IL-1Ra treatment without changes in insulin sensitivity. A possible explanation to reconcile these findings is the concept of β-cell “rest”. Indeed, potassium channel openers, which decrease insulin secretion, improve insulin secretion in patients with type 2 diabetes[79]. Possibly, the benefit of IL-1 antagonism in patients with type 2 diabetes is also due to β-cell rest and not only due to the postulated direct toxic effects of IL-1β. An alternative or additional

explanation for the contrasting effects of acute versus chronic IL-1β exposure is based on the local production of IL-1β by islets in response to a metabolic stress[21, 53]. This islet-derived IL-1β may drive local production of chemokines followed by recruitment of detrimental immune cells. In contrast, systemic IL-1β will not create a chemokine gradient around the islets and subsequent insulitis will not occur.

Based on our and previous studies, we propose that the physiological role of IL-1β in metabolism is to adapt glucose homeostasis to the needs of an innate immune response (Fig. 7). Indeed, IL-1β increases body temperature during infections[4]. This requires glucose uptake into muscle, which is directly promoted by IL-1β and indirectly via IL-1β stimulated insulin secretion. Furthermore, an infection often leads to insulin resistance in liver and adipose tissues, increasing the availability of glucose for immune cells. Importantly, our current work showed that IL-1β also supplies glucose to immune cells. Interestingly, insulin proved to be an enhancer of this IL-1β effect. In macrophages the role of insulin appeared to be most explicit in activated M1 macrophages, which displayed higher levels of insulin receptor, allowing AKT to be phosphorylated, glycolytic activity to be increased and an overall pro-inflammatory pattern to be established. Finally, we showed that insulin is a previously unknown activator of the NLRP3 inflammasome, inducing macrophage-derived IL-1β by enhanced glucose uptake through the glucose transporter GLUT1. Hence insulin, which is increased in early stages of type 2 diabetes, may sustain the inflammatory state and may therefore directly contribute to the chronic low grade inflammation associated with metabolic diseases. Conversely, IL-1β may stimulate insulin secretion to compensate for the increased demand of insulin during obesity.

Altogether, our findings show that both IL-1β and insulin have potent effects on glucose homeostasis and on the immune system further supporting the emerging concept of immunometabolism. Understanding the physiological synergy between IL-1β and insulin on glucose disposal and macrophage activity may have important implications for the development and use of drugs modulating IL-1β and insulin actions in pathological conditions.

METHODS

Human pancreatic islets

Human islets were isolated in the islet transplantation centres of Lille and Geneva from pancreata of cadaver organ donors in accordance with the local Institutional Ethical Committee. They were obtained via the “islet for research distribution program” through the European Consortium for Islet Transplantation, under the supervision of the Juvenile Diabetes Research Foundation (31-2008-416). Islets were cultured in CMRL-1066 medium containing 5 mmol/l glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM Glutamax and 10 % FCS (Invitrogen) on extracellular matrix-coated 24-well plates (Novamed Ltd.) in humid environment containing 5 % CO_2. Some islets were treated for 0.5-2h with 0.1, 0.5 and 1 ng/ml recombinant human IL-1β (R&D), 20 µM atropine (Sigma), or 10 µM carbachol (Sigma).

Mouse pancreatic islets

To isolate mouse islets, pancreata were perfused through the sphincter of oddi with a collagenase solution (Worthington) and digested in the same solution at 37°C, followed by sequential filtration through 500 µm and 70 µm cell strainers (BD). Islets were handpicked and cultured on extracellular matrix-coated 24-well plates (Novamed Ltd.) in RPMI-1640 (GIBCO) containing 11.1 mM glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamax, 50 µg/ml gentamycin, 10 µg/ml Fungison and 10 % FCS. Islets were either collected directly for RNA extraction or cultured for 36 hours on extracellular matrix-coated

24-well plates and treated for 24 hours with or without 1ng/ml recombinant mouse IL-1β for subsequent RNA extraction as well as protein measurements in the supernatant or they were used for glucose-stimulated insulin secretion experiments.

Animal experiments

All animal experiments were performed in mice on a C57BL/6 background unless otherwise specified. WT mice were obtained from Charles River. For the diet induced obesity (DIO) experiments, 4 week old mice were fed a high fat diet (D12331, Research Diets; containing 58, 26 and 16 % calories from fat, carbohydrate and protein, respectively) for 20-25 weeks. Leptin receptor deficient (db/db) mice were obtained from Jackson laboratories at the age of 4 weeks, and housed until the age of 16 weeks. IRAK 4 KO mice[80] on a Balb/c background were kindly provided by Amgen. IL-1β KO mice were produced by gene targeting as previously described[81]. GLP1R GIPR KO mice were generated as described[82]. GFRα2-KO mice were obtained and genotyped as described earlier[77]. NLRP3 KO mice were generated as described[56]. RAG2 KO mice were bred in house. Crossing of Glut2 floxed mice[83] with nestin-Cre transgenic mice was performed as previously described[84] to generate mice deficient in Glut2 in nestin expressing cells.

All animal experiments were conducted according to the Swiss Veterinary Law and Institutional Guidelines and were approved by the Swiss Authorities. All animals were housed in a temperature-controlled room with a 12 h light – 12 h dark cycle and had free access to food and water. All metabolic experiments using transgenic mice were performed using wild type littermates as controls. The mice were between 12 and 29 weeks of age. Mice that did not gain weight in diet induced obesity experiments were excluded. All experiments were performed at least twice with weight-matched mice and with at least 4 animals per group. For drug applications, each cage included mice receiving all treatments in order to avoid cage dependent differences.

Glucose tolerance tests (GTTs)

For glucose tolerance testing, mice were fasted for 6 hours starting in the morning and injected ip or by gavage with 2 g glucose per kg body weight. LPS (1 mg/kg body weight) was applied 3 hours, atropine (Sigma; 5 mg/kg body weight) or darifenacin (Santa Cruz; 5 mg/kg body weight) or exendin 9-39 (Bachem; 1 µg/kg body weight) 36 minutes and, recombinant mouse IL-1β (0.1-1 µg/kg) 18 minutes before the start of the GTT. IL-1Ra (10 mg/kg body weight) was ip injected twice, 3 hours and 36 minutes prior to the glucose injection. Blood glucose was measured using a glucometer (Freestyle; Abbott Diabetes Care Inc.). For detection of active GLP-1, mice were ip injected with 25 µg/kg sitagliptin (Sigma) 30 minutes prior to the GTT.

ENDOC cells

The human β-cell line (ENDOC) was kindly provided by R. Sharfmann and cultured and subjected to glucose-stimulated insulin secretions as described earlier[85]. Human IL-1β (R&D) was added at the indicated concentrations for 30 minutes at 2.8 mM glucose concentration prior to the incubation with 16.7 mM glucose.

Glucose-stimulated insulin secretion assay in islets

For glucose-stimulated insulin secretion experiments, islets or ENDOC cells were cultured for 2 days and pre-incubated for 30 minutes in modified Krebs-Ringer bicarbonate buffer (KRB; 115 mM NaCl, 4.7 mM KCl, 2.6 mM $CaCl_2$ $2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ $2H_2O$, 10 mM HEPES, 0.5 % bovine serum albumin, pH 7.4) containing 2.8 mM glucose. KRB was then replaced by KRB with 2.8 mM glucose and collected after 1 hour to determine the basal insulin release. IL-1β was added at the indicated concentrations for the last 30 minutes of the 1-hour period of the basal insulin

release (priming). This was followed by 1 hour in KRB with 16.7 mM glucose to determine the stimulated insulin release. The stimulatory index was defined as the ratio of insulin secretion at 16.7 mM to 2.8 mM glucose/hour and expressed as percent of untreated control.

Primary immune cell isolation and cultivation

Mouse macrophages, splenocytes and T-cells were isolated from male C57BL/6 mice after euthanization in a CO_2 chamber. To obtain circulating white blood cells (WBC), the hearth was punctured and the collected blood was incubated briefly with red blood cells lysis buffer (154mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA). To isolate macrophages, the peritoneum was infused with a PBS 1% FCS solution and the lavage was filtered through 70 µm cell strainer (BD). To isolate splenocytes, the spleen was minced and cells were passed through a 70 µm cell strainer and incubated with red blood cells lysis buffer. Cells were centrifuged at 350 x g for 5 minutes at 4^{o}C. Macrophages and splenocytes were resuspended in culture media (same media used for mouse pancreatic islets). For T-cell isolation splenocytes were re-suspended according to the T-cell isolation kit protocol (Dynabeads untouched mouse T-cells, Invitrogen). Cells were cultured in 48 or 96 well plates (TPP). Splenocytes and T-cells were kept in suspension for glucose uptake measurements. Macrophages were allowed to adhere for at least 4 hours, naïve macrophages were used for glucose uptake assays or were polarized to M1 or M2 phenotypes as follows: 2 hours (serum free) or 16 hours treatment with LPS (100 ng/ml) + IFNγ (10 ng/ml) for M1 and IL-13 (10 ng/ml) + IL-4 (10 ng/ml) for M2 polarization, followed by 2 or 24 hours, with or without 1 µg/ml insulin in the presence or absence of fasentin (50 µM; Sigma). Supernatants were collected and stored at -80 ^{o}C and cells were harvested for RNA extraction (see RNA extraction and qPCR).

RNA extraction and qPCR

Total RNA was extracted using the Nucleo Spin RNA II Kit (Machery Nagel). cDNA was prepared with random hexamers (microsynth) and Superscript II (Invitrogen) according to the instructions of the supplier. RNA expression was determined with TaqMan assays and the real time PCR system 7500 (Applied Biosystems). The following TaqMan assays were used:

Human: SLC18a3 (Vesicular acetylcholine gene,VAChT) Hs00268179_s1, 18s Hs99999901_s1. Mouse: GAPDH: Mm99999915_g1, TNFa: Mm00443258_m1, YM1, chitinase 3-like 3 (chi3l3): Mm00657889_m1, mannose receptor 1 (MRC1): Mm00485148_m1; IL-1Ra: Mm00446185_m1, SLC2a1 (GLUT1): Mm00441480_m1. IL-1β: Mm0043228_m1, insulin receptor: Mm01211875_m1, KC (Cxcl1): Mm04207460_m1, CD40: Mm00441891_m1, Hexokinase 2 (HKII): Mm00443395_m1, IL-1α: Mm00439621_m1, NLRP3: Mm00840904_m1, IL-1 Receptor type 1 (IL-1R1): Mm00434237_m1, IL-6: Mm0046190_m1, F4/80 (Emr1): Mm00802529_m1.

Data were normalised with 18s for islets mRNA measurements or geometrical mean of GAPDH, 18s, actin for macrophage RNA and quantified using the comparative $2^{-\Delta\Delta CT}$ method.

Protein measurement assays

Insulin concentrations were determined using human insulin ultrasensitive ELISAs (Mercodia) or mouse/rat insulin kits (Mesoscale Discovery). Mouse IL-1β and active GLP-1 concentrations were assayed using the appropriate assays (Mesoscale Discovery). IL-1Ra was determined using ELISA assays (R&D).

Glucose bio-distribution assay

Male C57BL/6 mice were fasted for 3 hours in the morning, ip injected with IL-1β (1 µg/kg body weight) or saline and 18 minutes later with ^{3}H labeled 2-deoxy glucose (10 µCi per mouse, Perkin Elmer). After 30 minutes mice were sacrificed, liver, quadriceps muscle,

visceral adipose tissue, and spleen were weighed, washed immediately in ice cold PBS and incubated with lysis buffer (10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 62.5mM Tris pH6.8, 6M urea) followed by sonication. WBC were isolated as described above, washed twice with ice cold PBS, counted and lysed with 0.1% SDS. Triplicate samples were then measured in a beta counter. Data are presented as percentage beta counts per minute per mg tissue, in organs or beta counts per cell in WBC.

***In vitro* glucose uptake assay**

For *in vitro* treatment, macrophage, and T-cells were incubated for 2 hours (Macrophages) or 40 minutes (T-cells) with 1 mM glucose KRB (as described in GSIS section) with or without the indicated treatment. To determine glucose uptake, macrophages, T-cells, and splenocytes were then incubated for 30 minutes with 0.4 nCi ^{3}H labeled 2-deoxy glucose (Perkin Elmer), washed twice with ice cold PBS, lysed with 0.1 % SDS and transferred into scintillation fluid. ^{3}H labeled 2-deoxy glucose uptake was measured in a beta counter.

Glucose clamp studies

Glucose clamp studies were performed in freely moving mice as previously described[86]. Steady state glucose infusion rate was calculated once glucose infusion reached a constant rate with blood glucose levels at 5 mmol/l (70-80 minutes after the start of insulin infusion). Thereafter, blood glucose concentration was kept constant at 5 mmol/l for 15-20 minutes and glucose infusion rate was calculated. Glucose disposal rate, endogenous glucose production and insulin-stimulated glucose disposal rate were calculated as described[87].

Implantation of osmotic pumps releasing IL-1β

C57BL/6, male mice, were subcutaneously implanted with osmotic mini pumps (Alzet 2006) releasing either recombinant mouse IL-1β (R&D; 0.1 µg/kg/day) or saline for 31 days. Surgery was done in an SPF environment; animals were anaesthetised with Ketalar (65 mg/kg) and Xylasol (13 mg/kg) by ip injections. After falling asleep eye creme (Floxal) was applied to avoid dry eyes and the neck was shaved. As preparation to the pump implantation, an air pocket was created under the skin. The sterile pumps were inserted in the pocket and the wound was closed with two wound clips. Mice were single caged and kept under a warming lamp. After the first signs of waking, painkiller (Temgesic; 0.05 mg/kg) was injected subcutaneously. After 24 hours and eventually 48 hours (if the mice were scratching themselves), another shot of painkiller was given. Healing of the wound and the health state of every mouse was observed and recorded on a score sheet every day for one week. All animals were housed single caged. On days 17 and 31 mice underwent glucose tolerance testing.

***In vivo* daily injections of IL-1β**

Low dose: male C57BL/6 mice were injected daily at 16:00 with the same dose used for acute IL-1β (1 µg per kg body weight) or saline for either 6 or 13 days. Intermediate dose: male C57BL/6 mice were injected daily at 8:00 with saline or 10 µg/kg body weight for 5 days or once on the day of GTT. High dose: male C57BL/6 mice were injected daily at 16:00 with saline or 35 µg/kg IL-1β for 3 days.

Western blotting.

We separated proteins (8-12 µg) in 4-12 % NuPAGE gels (Invitrogen), blotted them onto nitrocellulose membranes (Bio-Rad) and incubated them with antibodies against total AKT (pan AKT; #4691), pAKT (s473; #9271) (Cell Signaling). Blots were analyzed using image lab 4.1 software (Bio-Rad).

Cellular respiration and extracellular acidification measurements.

An XF96e Extracellular Flux analyser (Seahorse Biosciences) was used to determine the bioenergetic

profile of macrophages. Peritoneal cells were plated at a density of 300'000 cells per well in XF96 plate, incubated for 4 hours, and washed vigorously before being stimulated with LPS (100 ng/ml) + IFNγ (10 ng/ml) or IL-4\IL-13 (10 ng/ml) overnight. The next day insulin was added to the media (1 µg/ml end concentration) for 2 hours. Prior to the assay, cells were incubated in unbuffered RPMI (Seahorse Biosciences) containing 11.1mM glucose for 1 hour. Then oxygen consumption rate and extracellular acidification rate were assessed during 2 minutes. Four basal measurements were followed by 5 measurements upon injection of the following agents: Glucose (26.8 mM), oligomycin (1 uM), carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP) (2 µM) and rotenone (1 uM). Oligomycin, FCCP and rotenone were purchased from Sigma.

Macrophage ablation

Clodronate or PBS liposomes (ClodronateLiposomes.org) were injected on two consecutive days, the first day ip and the second day intravenously (50ul per 10 grams bodyweight each injection). Mice were sacrificed at the end of the procedure and in order to control macrophage depletion, splenocytes and peritoneal cells were isolated as described above and analysed by FACS analysis as described below. Macrophages were defined as $CD11b^+$ $F4/80^+$ double positive cells.

Flow cytometry

To obtain single cells, islets were dispersed with trypsin (Invitrogen) for 6 minutes at 37°C, washed with PBS, pelleted at 300 x g, 5 minutes, 4°C and re suspended in FACS buffer (PBS with 0.5% BSA and 2 mM EDTA). After 15 minutes incubation with an Fc blocker (Anti-mouse CD16/CD32; eBioscience 14-0161) splenocytes or single islet cells were stained with the appropriate antibody for 30 minutes at 4°C in the dark. To control the effect of the clodronate depletion splenocytes from RAG2 KO mice were stained with anti F4/80-PE (Clone BM8; 12-4801), anti CD11b-eFluor660 (Clone M1/70; 50-0112) and CD45-FITC (Clone 30-F11; 11-0451). Single islet cells were stained with CD45-PE-cy7 (Clone 30-F11; 25-0451) for immune cells (all antibodies were purchased from eBioscience). Stained cells were washed twice with FACS buffer prior to FACS. Splenocytes from chlodronate treated mice were analyzed on an Accuri C6 flow cytometer (BD Bioscience). Dispersed islet cells were analyzed and sorted on a FACS ARIA III cell sorter (BD Biosciences) using FACS Diva software (BD Biosciences). All samples were stained with appropriate isotype control antibodies; viability staining was done using 7-AAD (Sigma) or DAPI (for the sort). Data were analysed using Flow Jo 9.4 software (Tree Star).

Immunofluorescence staining

Pancreata were fixed overnight in 4% paraformaldehyde at 4°C, followed by paraffin embedding. Sections were deparaffinized, re-hydrated, and incubated 1 hour at room temperature with guinea pig anti-insulin antibody (Dako; A0564), followed by detection with a fluorescein-conjugated donkey anti-guinea pig antibody (Dako). Subsequently, the sections were labeled for IL-1R1 with goat anti IL-1-R1 antibody (R&D; AF771), followed by detection with a fluorescein-conjugated donkey anti-goat antibody (Invitrogen).

Statistics

Appropriate statistical tests were performed where required. Two-sided unpaired Student's *t*-tests were performed for all statistical analyses unless otherwise specified using GraphPad Prism 5 (GraphPad Software). Data are expressed as means ± s.e.m. and statistical significance is denoted as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and, $****P < 0.0001$. n numbers indicate biological replicates for *in vitro* experiments or number of mice for *in vivo* experiments.

ACKNOWLEDGMENTS

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FIGURES AND FIGURE LEGENDS

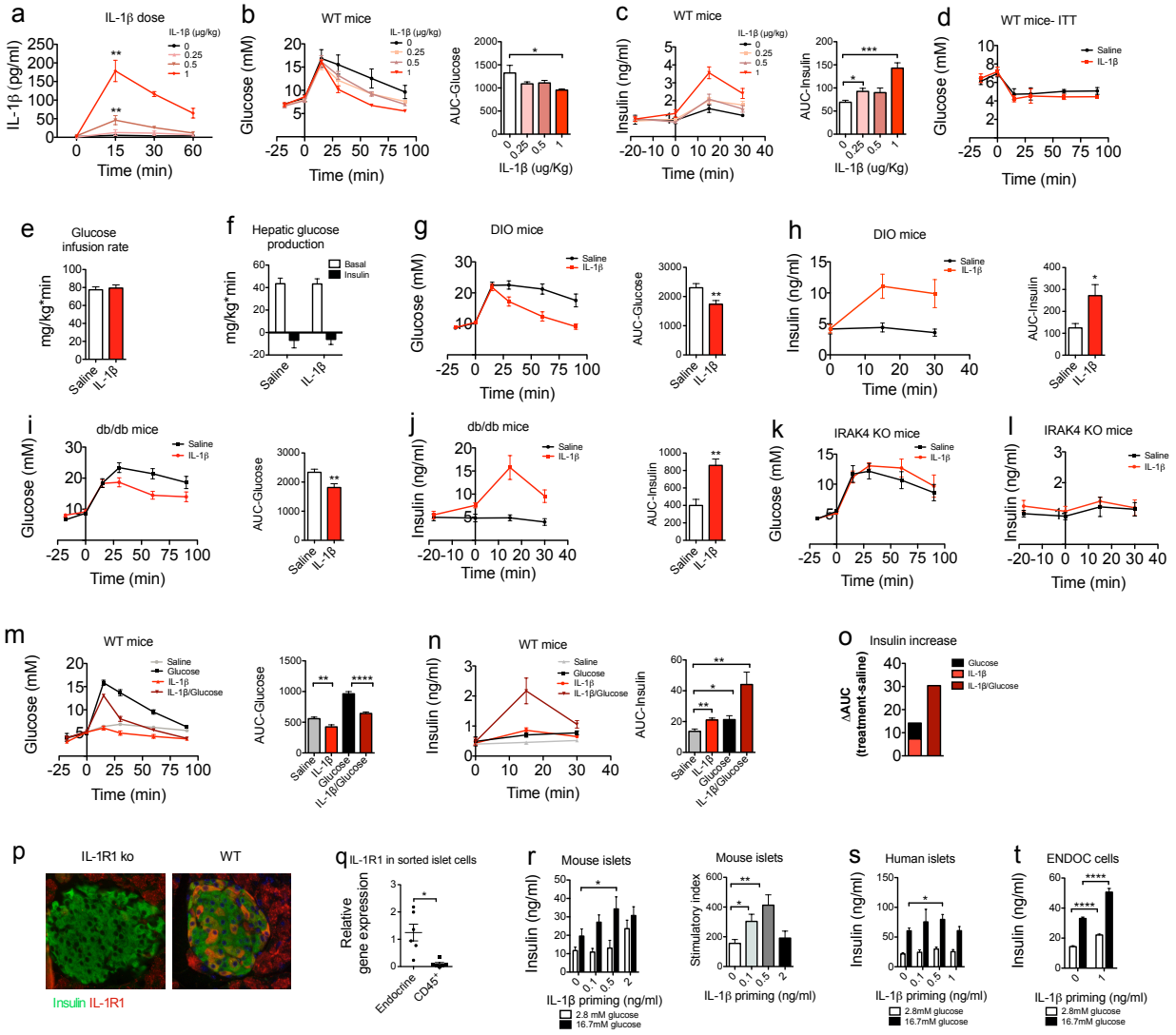


Figure 1. IL-1β acutely is a potent insulin secretagogue and improves glucose disposal in normal, obese and diabetic mice. (a) Plasma concentrations of IL-1β following an intraperitoneal (ip) injection of IL-1β into wild type (WT) mice. Mice were injected with saline (n=4) or 0.25 (n=6), 0.5 (n=5), and 1 (n=9) μg/kg IL-1β at time 0. (b) Glucose and (c) insulin levels during an ip glucose tolerance test (ipGTT) in mice 18 minutes after a single injection with saline (n=5) or 0.25 (n=7), 0.5 (n=8), 1 (n=6) μg/kg IL-1β. (d) Insulin tolerance test (ITT) 18 minutes after an injection of 1 μg/kg IL-1β. Hyper-insulinemic euglycemic clamp in mice pre-treated with 1 μg/kg IL-1β (n=5): (e) glucose infusion rate, (f) hepatic glucose production. Glucose (g, i, k) and insulin (h, j, l) levels during an ipGTT with diet induce obese (DIO) (g, h), db/db (i, j) and IRAK4 knockout (KO) (k, l) mice, pre-treated with 1 μg/kg IL-1β (n=12, 12 and 6 per group, respectively). (m) Glucose and (n) insulin levels in mice injected with 1 μg/kg IL-1β or saline alone or in combination with glucose (n=8). (o) Delta AUC insulin of IL-1β-saline and glucose-saline alone or in combination. (p) Double immunostaining of IL-1 receptor type 1 (IL-1R1) and insulin in pancreatic tissue sections of WT and IL-1R1 KO mice. (q) IL-1R1 mRNA expression in FACS sorted islet cells (n=6). (r-t) Insulin concentrations in cultured media of islets isolated from mice (r; left to right: n=9,13,9,12; 3 experiment) and humans (s; left to right: n=44,9,34,15; 8 experiment) and of ENDOC cells (t; n=9; 3 experiment) pre-incubated with IL-1β followed by incubation at low (2.8 mM) and high (16.7 mM) glucose. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001. Statistical significance (*P*) was determined by Student's t test. All error bars denote s.e.m.

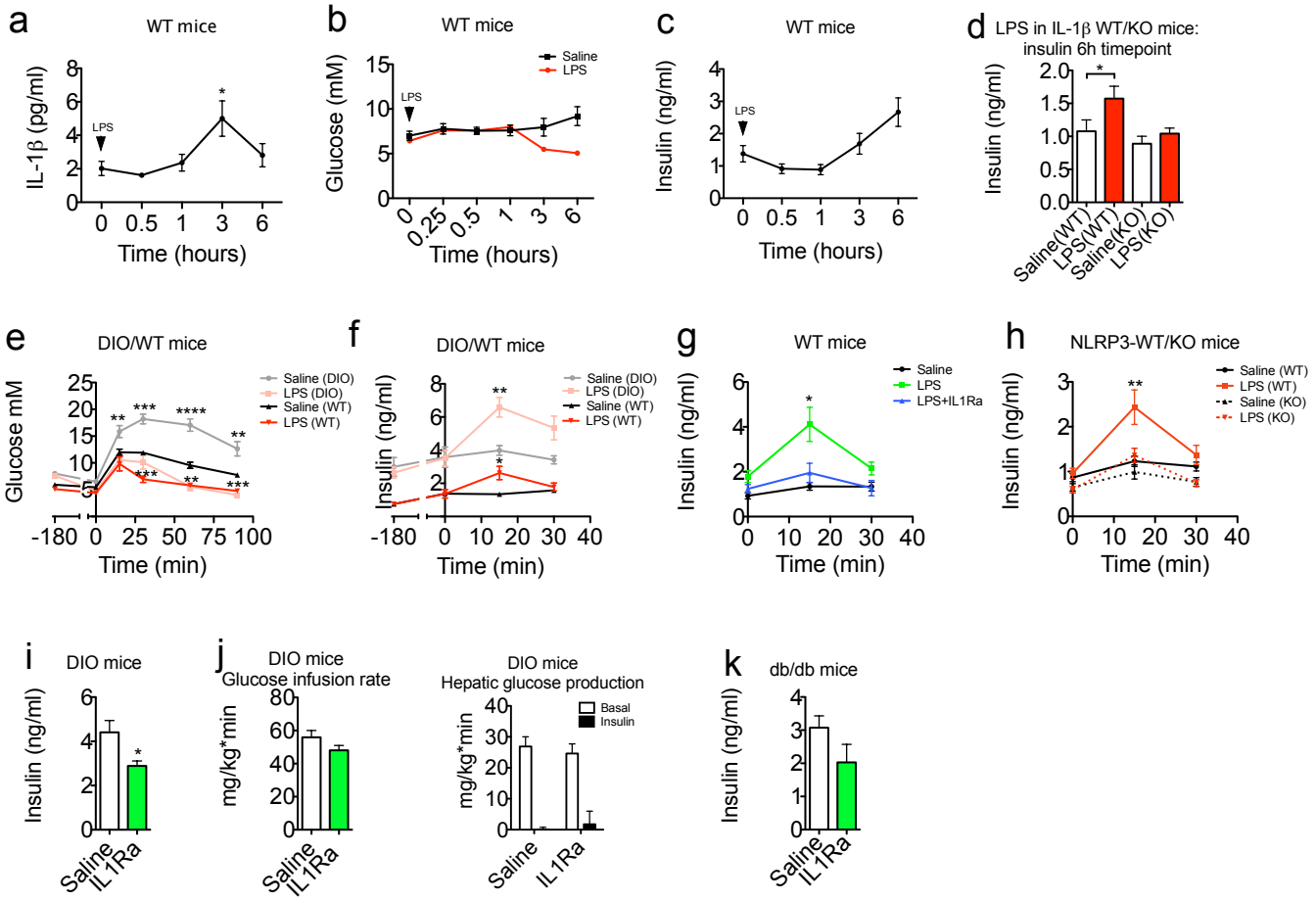


Figure 2. Endogenous IL-1β plays a role in insulin secretion. (a-c) Plasma concentrations of IL-1β (a) blood glucose (b) and insulin (c) after an intraperitoneal (ip) injection of 1 mg/kg LPS in mice (n=8). (d) Insulin levels of wild type (WT) and IL-1β knockout (KO) mice injected with LPS (n=7; ANOVA). (e) Glucose and (f) insulin levels during an ip glucose tolerance test (ipGTT) in normal or diet induced obese (DIO) mice pre-injected with LPS 3 hours before (n=5; significance level indicating differences between saline and LPS treated WT or DIO mice). (g) Circulating insulin levels during an ipGTT from mice pre-injected with LPS in combination with or without IL-1Ra (n=5; significance level indicating differences between LPS and LPS+IL-1Ra treated mice). (h) Circulating insulin levels during an ipGTT from WT or NLRP3 KO mice pre-injected with LPS (n=8). (i) Basal insulin levels following acute injections of 10 mg/kg IL-1Ra in DIO mice. (j) Hyper-insulinemic euglycemic clamp in DIO mice pre-injected with saline or IL-1Ra (n=4,5 respectively). (k) Basal insulin levels after acute saline or IL-1Ra injections in db/db mice (n=6,5 respectively). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Statistical significance (P) was determined by Student's t test and in (d) by ANOVA. All error bars denote s.e.m.

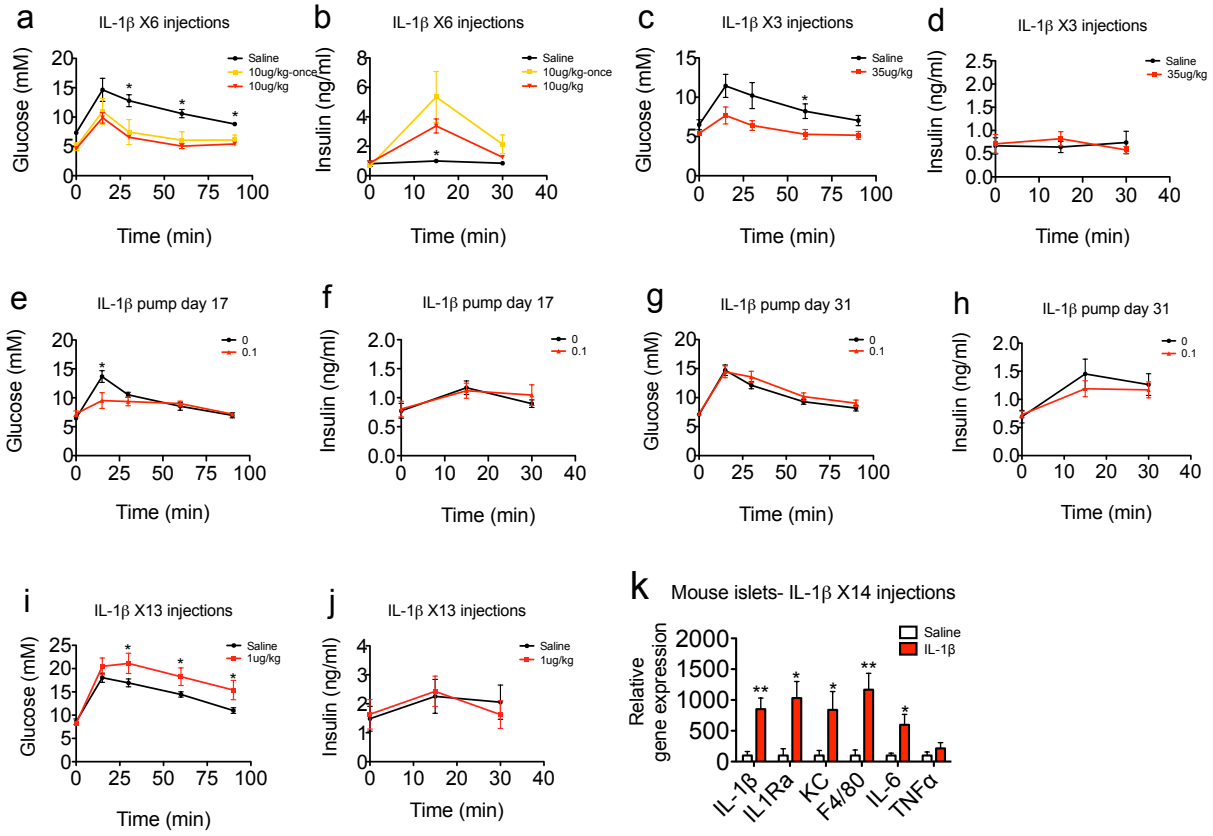


Figure 3. Long term IL-1β administration impairs glucose metabolism. Blood glucose (a, c, e, g, i) and insulin (b, d, f, h, j) levels during an intraperitoneal glucose tolerance test (ipGTT) in (a, b) mice following a single (n=6), or daily for 6 days injection of saline or 10 μg/kg IL-1β (n=5,7 respectively), or (c,d) after daily injections for 3 days with 35 μg/kg IL-1β (n=5), or after (e, f) 17 and (g, h) 31 days after implantation of pumps delivering IL-1β (n=6), or (I, j) following daily injections of 1 μg/kg IL-1β for 13 days (n=10). (k) mRNA expression of islets isolated from mice injected daily with saline or 1 μg/kg IL-1β for 14 days (n=7,9 respectively). **P* < 0.05, ***P* < 0.01. Statistical significance (*P*) was determined by Student's t test. All error bars denote s.e.m.

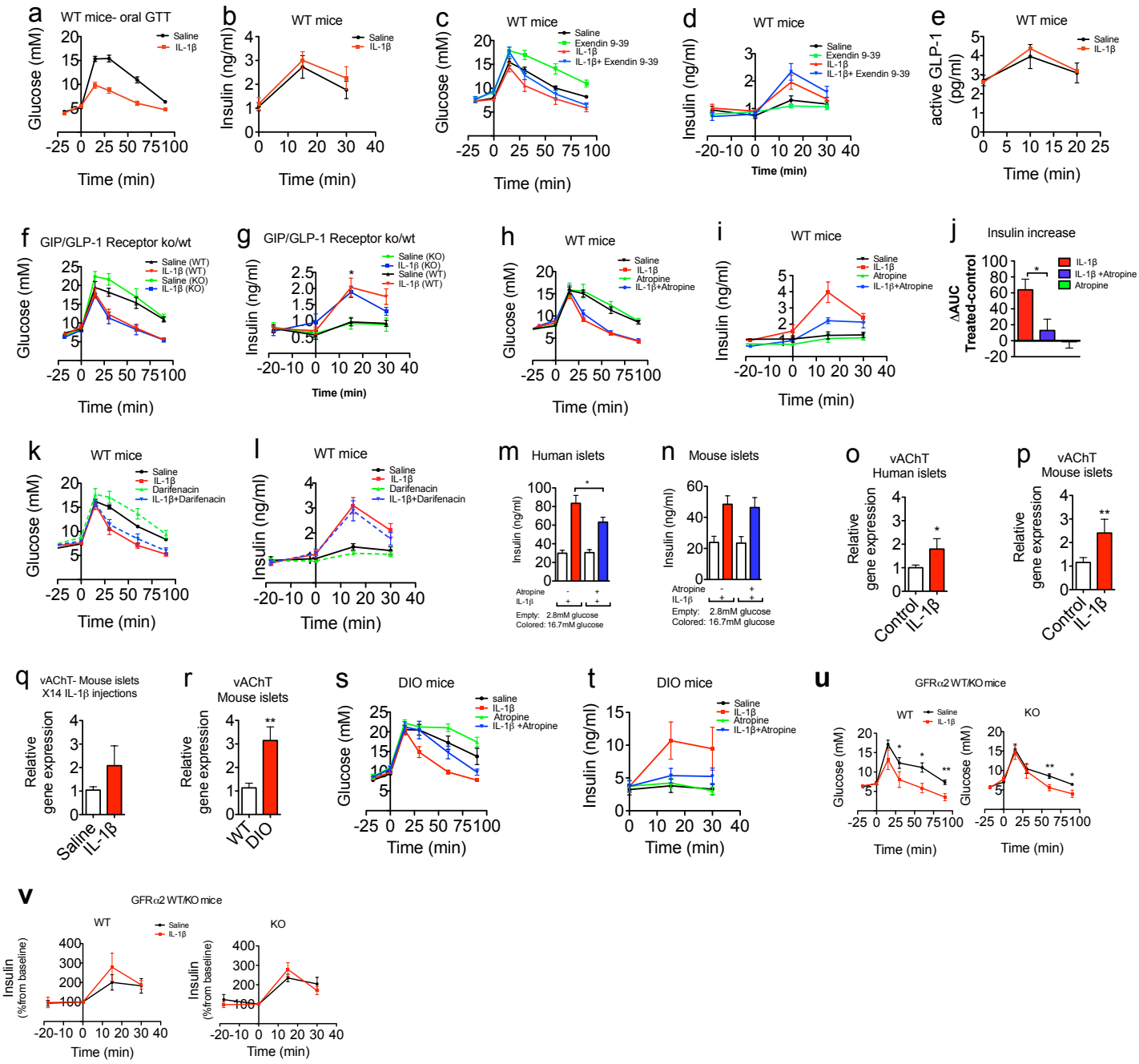


Figure 4. IL-1β induced insulin secretion is partly mediated by neuronal stimulation and is independent of the incretin system. (a) Blood glucose (n=8) and (b) insulin (n=16) levels during an oral glucose tolerance test (oGTT) in mice 18 minutes after a single injection of 1 μg/kg IL-1β. (c) Blood glucose (n=7) (d) insulin (n=7) and (e) active GLP-1 (saline; n=13, IL-1β; n=14) during an intraperitoneal glucose tolerance test (ipGTT) in mice 18 minutes after a single injection of 1 μg/kg IL-1β with or without exendin 9-39. (f) Blood glucose (g) and insulin during an ipGTT in GIP and GLP-1 receptor double knockout (KO; n=7) or wild type (WT; n=8) littermate control mice 18 minutes after a single injection of saline or 1 μg/kg IL-1β. (h, k) Blood glucose and (i, j, l) insulin from mice after a single injection of 1 μg/kg IL-1β with or without 5 mg/kg atropine (n=7) or 5 mg/kg darifenacin (n=12). (m-n) Insulin concentrations of culture media supernatants of islets isolated from human (m; n=34) or mouse (n; n=24) pre-incubated with 0.5 ng/ml IL-1β with or without 20 μM atropine followed by incubation at 2.8 mM and 16.7 mM glucose. (o-r) Vesicular acetylcholine transporter (vAChT) mRNA expression in: human (o; n=33) and mouse (p; n=16) islets treated for 24h with IL-1β (1ng/ml) and *ex vivo* in islets isolated from mice treated for 14 days with saline or IL-1β (q; n=6,9 respectively), or from WT mice and diet induced obese (DIO) mice (r; n=29,28 mice, respectively). (s) Blood glucose and (t) insulin levels during an ipGTT in DIO mice 18 minutes after a single injection of saline 1 μg/kg IL-1β with or without 5 mg/kg atropine (n=7,8,7,7, respectively). (u) Blood glucose and (v) insulin levels during an ipGTT in WT and GFRα2 KO littermate mice 18 minutes after a single injection of 1 μg/kg IL-1β (n=5). **P* < 0.05, ***P* < 0.01. Statistical significance (*P*) was determined by Student's t test. All error bars denote s.e.m.

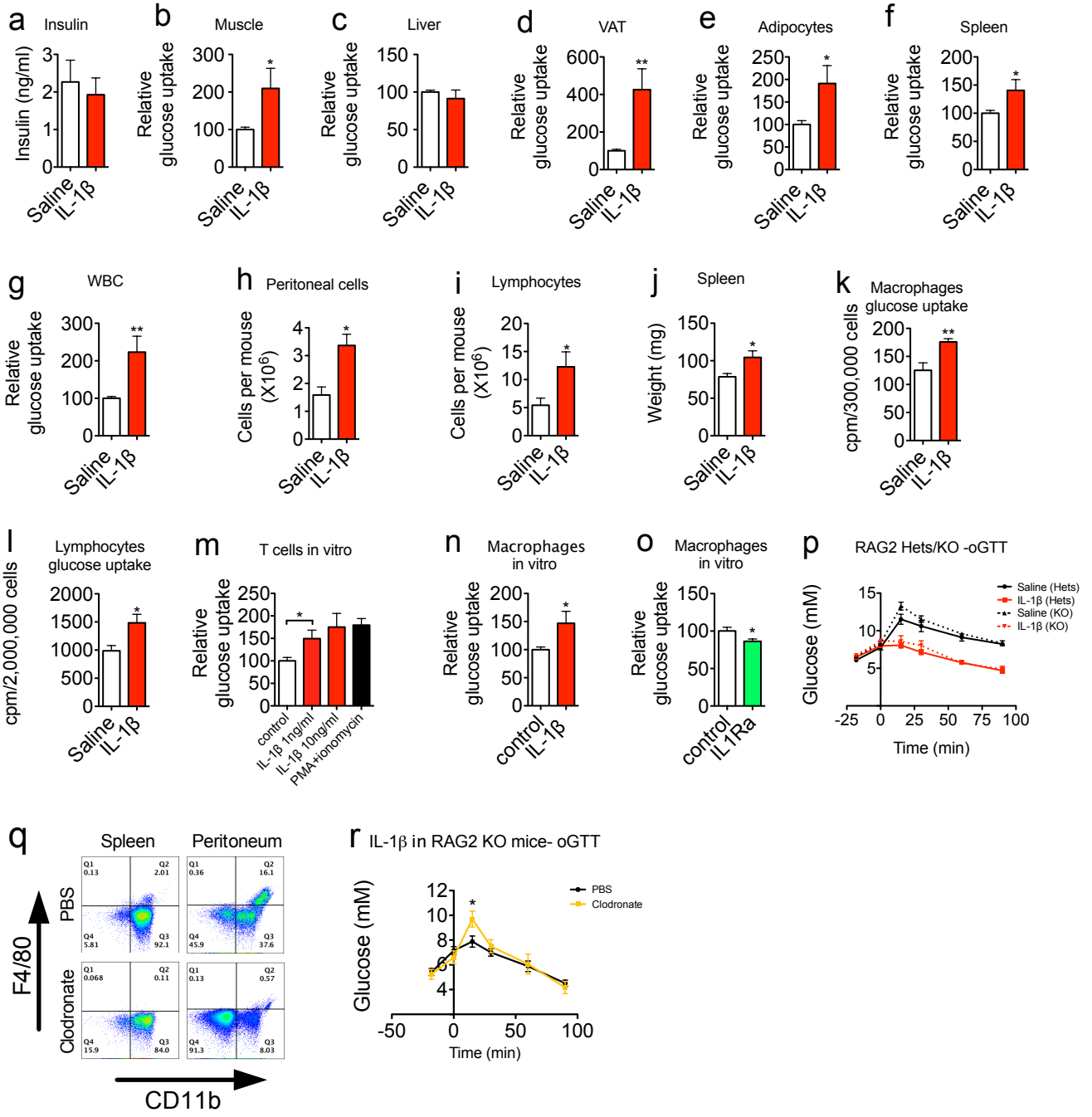


Figure 5. IL-1β promotes insulin independent glucose uptake in muscle, fat and immune cells but not in the liver. (a-g) Mice were injected intraperitoneally (ip) with 1 μg/kg IL-1β and 10 μCi ^{3}H labeled 2-deoxy glucose 18 minutes prior to sacrifice and assessment of circulating insulin (a; n=4); glucose uptake in muscle (b; n=8), liver (c; n=8), visceral adipose tissue (VAT; d; n=8), adipocytes isolated from epidydymal fat pads (e; n=4), spleen (f; n=4), and circulating white blood cells (WBC; g; n=8). (h-l; n=5) Mice were injected once a day for 3 days with 35 μg/kg IL-1β: number of peritoneal cells (h) and of lymph node cells (i), spleen weight (j), and glucose uptake in macrophages (k) and lymph node cells (l). (m-o) *In vitro* glucose uptake in: T cells incubated with IL-1β for 30 minutes (m; n=32, 3 experiments) and, macrophages incubated with IL-1β for 2 hours (n; n=15, 11, respectively, 3 experiments) and, in macrophages incubated with saline or IL-1Ra for 3 hours (o; n=14,9 respectively; 3 experiments). (p) Blood glucose levels during an oGTT 18 minutes after a single injection of saline or 1 μg/kg IL-1β in RAG2 deficient (n=6) and littermate heterozygous (Hets; n=7) mice. (q,r) macrophage (CD11b^{+}, F4/80^{+} double positive cells) ablation in RAG2 ko mice: (q) Representative flow cytometer analysis of macrophage depletion in spleen and peritoneal cells from RAG2 deficient mice, using 2 injections of 10 ml/kg clodronate or PBS liposomes, and (r) blood glucose levels during an oGTT after treatment with 1 μg/kg IL-1β (PBS; n=13, clodronate; n=9). *P < 0.05, **P < 0.01,. Statistical significance (P) was determined by Student's t test. All error bars denote s.e.m.

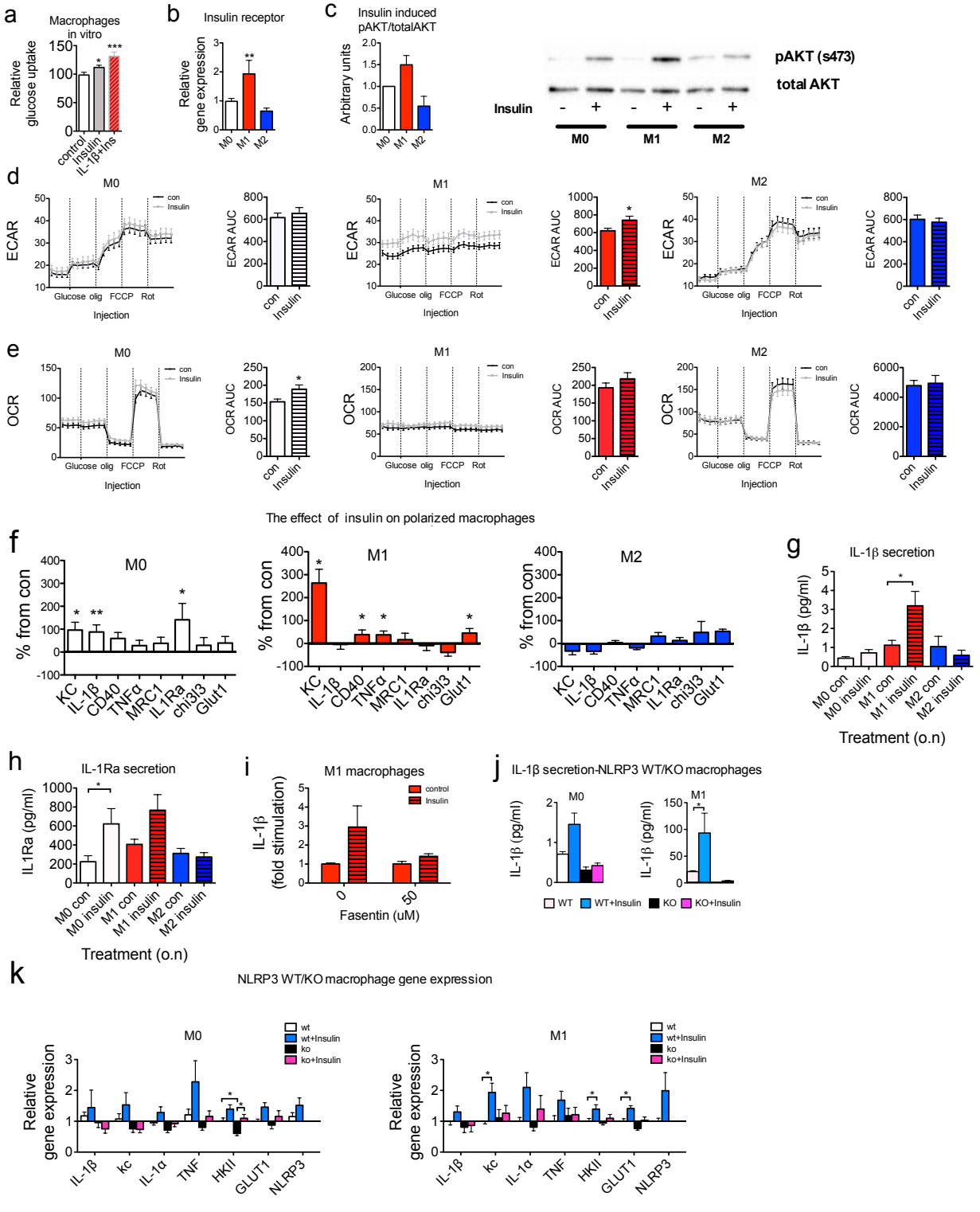


Figure 6. Insulin regulates macrophage metabolism and stimulates IL-1β via the NLRP3 inflammasome. (a) Glucose uptake in naïve macrophages (control; n=21; 3 experiments) or incubated for 3 hours with 1 μg/ml insulin alone (n=28; 3 experiments) or in combination with 1 ng/ml IL-1β (n=15; 3 experiments). (b) Insulin receptor mRNA expression in naïve (M0; n=11; 3 experiments), pro-inflammatory M1 (LPS+IFNγ; n=11; 3 experiments) and alternative M2 (IL-4+IL-13; n=12; 3 experiments) polarized macrophages (ANOVA). (c) Insulin induced (s473) phospho-AKT in polarized macrophages, data presented as ratio of insulin to non-insulin treated cells after normalization to total AKT (n=3 experiments) and a representative Western blot. Seahorse measurements and AUC of (d) extracellular acidification rate (ECAR; mpH/min) and of (e) oxygen consumption rate (OCR; pmol/min) from polarized macrophages incubated for 2 hours in the presence or absence of insulin. (n=12,15 respectively; 3 experiments). (f) Gene expression from naïve and polarized macrophages with or without 1 μg/ml insulin: data are expressed as percentage change from untreated controls (n=12, 3 experiments). (g) IL-1β and (h) IL-1Ra protein secretion from polarized macrophages with or without 1 μg/ml insulin (n=12). (i) 2 hour IL-1β protein secretion from M1 macrophages with or without 50 μM fasentin and 1 μg/ml insulin: data are presented as fold stimulation from non-insulin treated (n=14, 3 experiments). (j) 2 hour IL-1β protein secretion from polarized macrophages isolated from wild type (WT) or NLRP3 knockout (KO) mice with or without 1 μg/ml insulin and (k) corresponding gene expression (n=9, 3 experiments). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Statistical significance (*P*) was determined by Student's t test and in (b) by ANOVA. All error bars denote s.e.m.

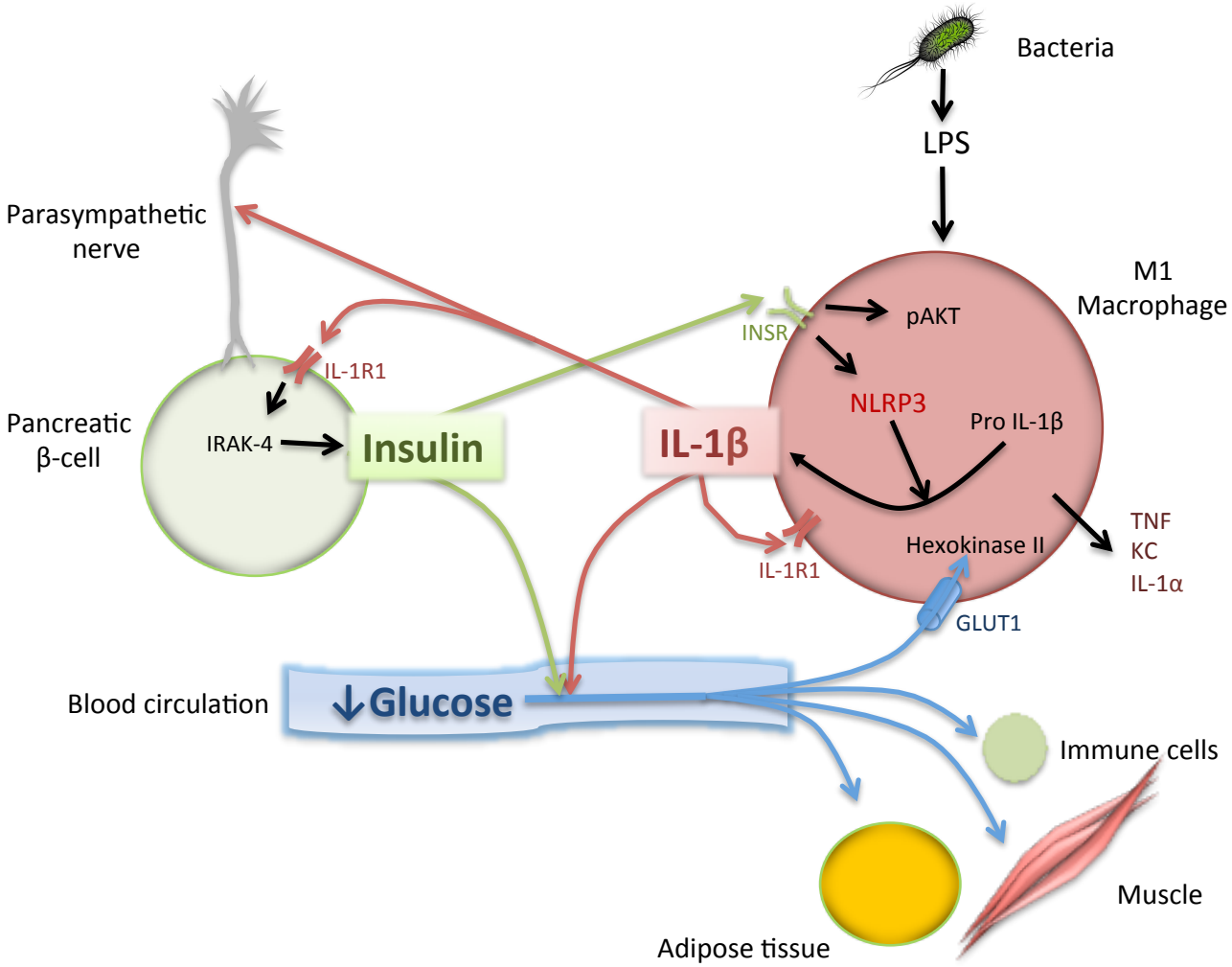
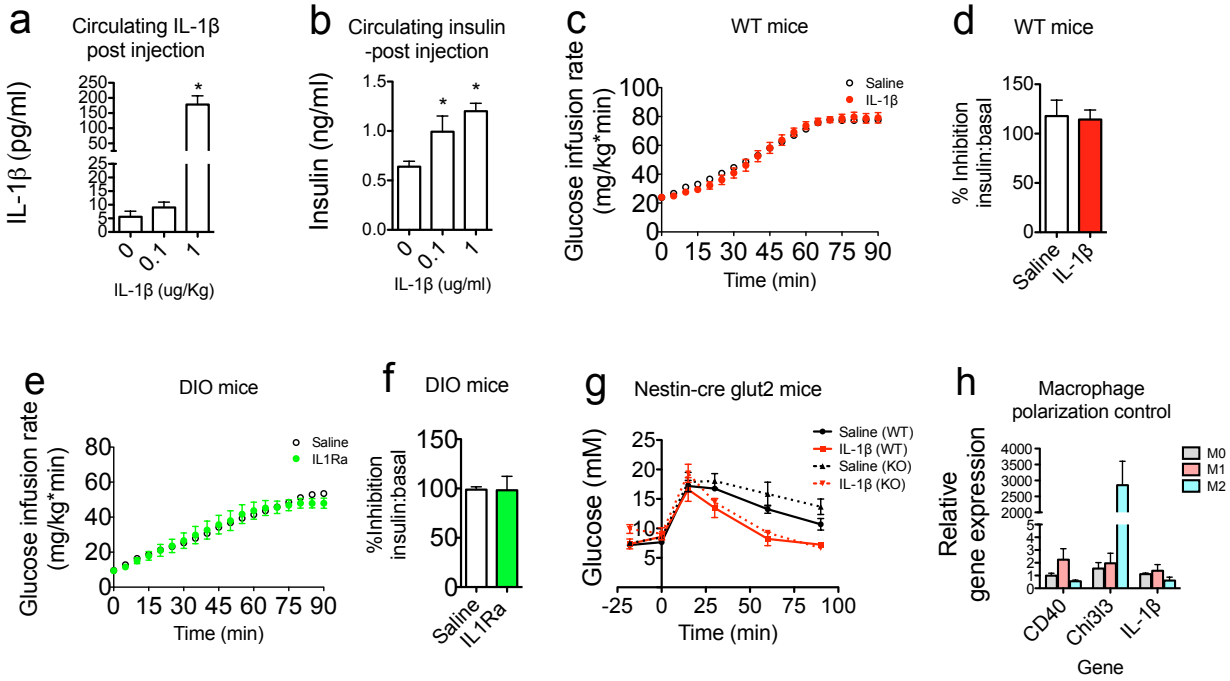


Figure 7 Proposed model describing the physiological role of IL-1β and insulin in the regulation of glucose metabolism during acute activation of the innate immune system. Bacterial cell wall products such as lipopolysaccharide (LPS) induce IL-1β secretion from macrophages. IL-1β then stimulates insulin secretion directly from pancreatic β-cells via the highly expressed IL-1 receptor 1 (IL-1R1) and the IL-1 receptor-associated kinase-4 (IRAK-4), and indirectly via parasympathetic nerves. The secreted insulin binds to its receptor (INSR), which is up-regulated in activated M1 macrophages, leading to enhanced AKT phosphorylation, glycolytic activity and cytokine expression such as TNF, KC and IL-1α. Insulin signaling also enhances macrophage-derived IL-1β by increasing glucose uptake through the glucose transporter GLUT1 and activation of the NLRP3 inflammasome. Increased levels of IL-1β alone and in combination with insulin enhance glucose uptake into muscle, adipose tissue, and in macrophages as well as other immune cells, and consequently decrease glycaemia.



Supplementary Figure Plasma concentrations of (a) IL-1β and (b) insulin 15 minutes after an intraperitoneal (ip) injection of saline or 0.1 or 1 μg/kg IL-1β. (c, e) Glucose infusion rate and (d, f) percent inhibition of hepatic glucose production by insulin in (c, d) wild type (WT) mice primed with 1 μg /kg IL-1β, and in (e, f) diet induced obese (DIO) mice injected with 10 mg/kg IL-1Ra. (g) Blood glucose during an ipGTT in nestin-cre-glut2 KO and littermate controls mice primed with saline 1 μg/kg IL-1β, 18 minutes before glucose injections (n=4,5 respectively). (h) IL-1β, CD40 (pro-inflammatory, M1 marker) and chitinase-like 3 (Chi3l3; alternative, M2 marker) gene expression from naïve (M0), M1 and, M2 polarized macrophages (n=12).

3 Unpublished results

3.1 Glucose did not induce IL-1β in an oGTT

Since LPS derived or exogenously administered IL-1β induced insulin secretion in vivo (Fig. 1, Fig. 2), the involvment of IL-1β in insulin secretion after oral ingestion of glucose was evaluated. Since glucose is the main fuel for IL-1β producing macrophages and FFAs activates NLRP3 inflammasome, WT mice received glucose or corn oil orally; blood was collected for evaluation of circulating IL-1β levels. Control mice received water. As seen in Fig. 4 (unpublished), no significant differences between the groups were detected.

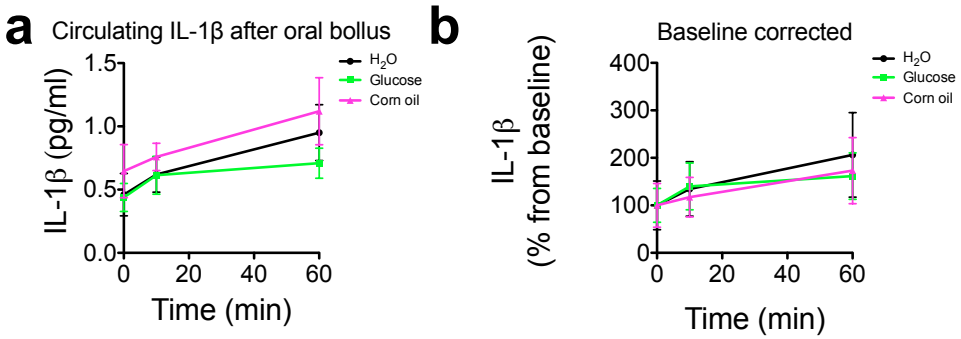


Figure 4. Circulating IL-1β levels post glucose ingestion. (a) IL-1β levels percentage in the circulation or (b) percentage change from baseline, from mice after oral administration (5 times bodyweight) of water, glucose, or corn oil (8 calories/kg, equivalent to 2gr/kg glucose; n=14,16,10, respectively,).

3.2 Potential role of IL-1β in the inhibition of GLP-1 secretion.

A disconnection between insulin levels and blood glucose levels in IL-1β injected mice is presented in the above manuscript. A further example for this disconnection is shown in Fig. 4a,b. Oral administration of glucose resulted in improved glycaemia in mice treated with IL-1β compared to saline treated mice, however, in contrast to intraperitoneal glucose administration, no difference in circulating insulin levels were detected. Since the major cause for the difference between oral and intraperitoneal administration of glucose is the incretin effect, the effect of IL-1β on incretin secretion was evaluated. Mice were injected with sitagliptin (as described in methods) followed by injection of 1ug/kg IL-1β, and 18 minutes later an oral glucose tolerance test was performed. Interestingly, IL-1β

treated mice had lower levels of circulating active GLP-1 than the control group (Saline; unpublished, Fig. 5).

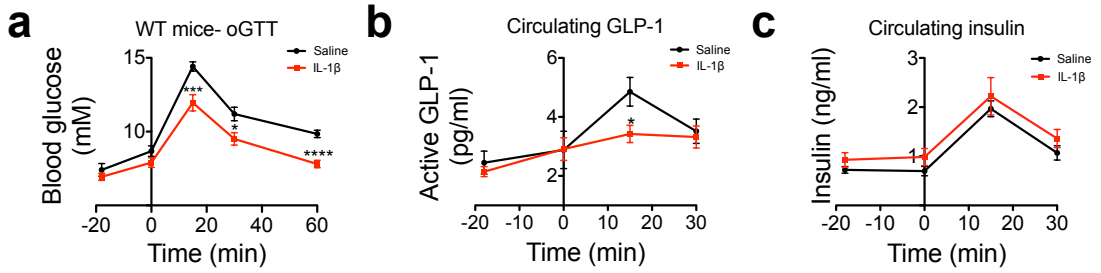


Figure 5. Lowered circulating active GLP-1 in mice injected with IL-1β. Data from oral glucose tolerance test (oGTT) performed in wild type (WT) mice 18 minutes after injection of saline or 1 µg/kg IL-1β (n=13,12, respectively). (a) Blood glucose levels. (b) Circulating GLP-1. (c) Circulating insulin.

3.3 Diet induce obese mice have higher IL-1R1 expression

To further investigate the physiological role of IL-1β in insulin assessment of IL-1R1 expression in DIO compared to WT mice was performed (unpublished, Fig. 6). Immunohistochemistry of pancreata of DIO mice displayed 2.5 fold more IL-1R1 expressing β-cells than WT mice.

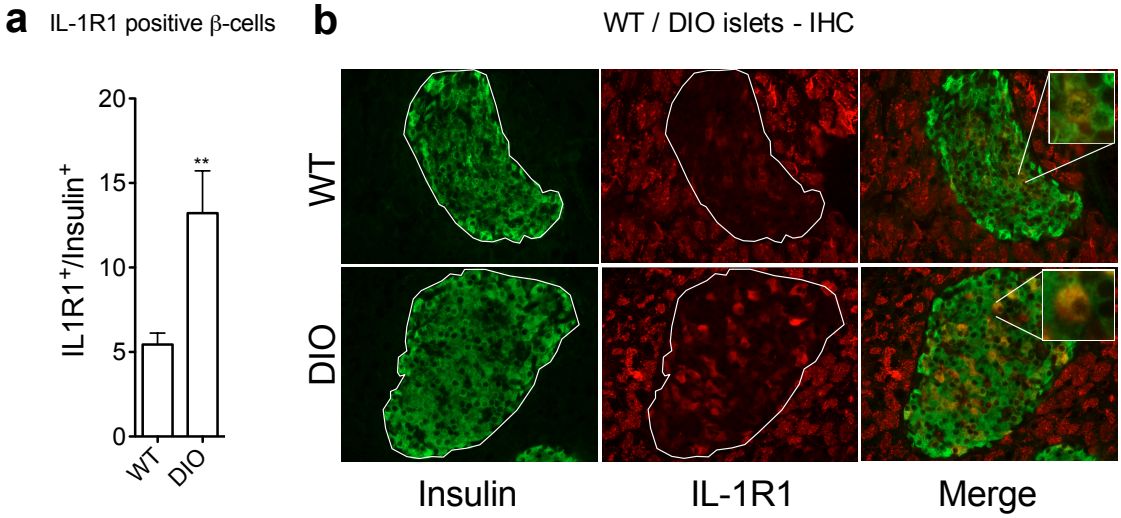


Figure 6. IL-1R1 expression in islets from WT and DIO mice. (a) Analysis of β-cells expressing IL-1R1, data are presented as ratio between IL-1R1 positive and insulin positive cells (n=24, 6 islets from 4 mice per group). (b) Representative insulin (green), IL-1R1 (red) staining of pancreatic islet (surrounded in white) section (X40 magnification). For IL-1R1 staining control see Fig. 1 in submitted manuscript. All pictures were acquired using the same settings.

4 Discussion

4.1 IL-1β is a potent insulin secretagogue in normal, obese and, diabetic mice

The deleterious effects of IL-1β on islet function and survival are well described and IL-1β was frequently used as a tool to provoke β-cell demise[49, 71, 72]. Surprisingly, at low concentrations or upon short exposure, IL-1β modestly stimulates insulin secretion in isolated islets indicating that IL-1β may not only be detrimental for β-cells but may have more complex biological functions[4, 52]. The insulin secretagogue effect of IL-1β is potentiated by glucose and conserved in human and mouse models. A possible physiological role of IL-1β in islets is also suggested by the previous observation of high levels of IL-1 receptor mRNA expression in whole islets[20] and in the present study by its prominent expression in β-cells. Interestingly, not all β-cells were positive for IL-1R1 (Fig. 1). This means that IL-1R1 is a novel marker for a β-cell sub population. Of note, IL-1R1 expression in DIO mice was higher compared to WT mice (unpublished data, Fig. 3), further pointing to a physiological role of IL-1β in insulin secretion and adaptation to the increased demand of insulin in insulin resistance. Indeed, acute IL-1Ra injections in DIO mice attenuated basal insulin secretion. Injections of IL-1β shortly prior to a glucose bolus massively improved glucose disposal in WT, DIO and, db/db mice supporting previous findings with pharmacological doses of IL-1β[73, 78]. Of note, these mouse models of insulin resistance secrete large amounts of insulin to compensate for the resistance and hardly develop β-cell failure. The present study shows that as long as functioning β-cells are present, acutely administrated IL-1β will result in increased insulin secretion. This occurs via direct action on islet β cells and via activation of neurons involved in the release of insulin. Furthermore, the glucose lowering effect of IL-1β is also due to direct IL-1β-mediated glucose uptake into various tissues as will be further discussed.

4.2 Endogenous IL-1β plays a role in insulin secretion

The physiological relevance of the present findings was demonstrated by LPS-induced endogenous IL-1β secretion which was equally effective as the injections of exogenous IL-1β. Furthermore, a role of endogenous IL-1β in the stimulation of insulin secretion was also demonstrated by blocking IL-1 signaling with IL-1Ra in DIO mice.

The bacterial wall component LPS is an inducer of the innate immune system by promoting inflammation via activation of the toll like receptor and by the subsequent secretion of various cytokines including IL-1β. Although LPS injected mice reached a peak concentration in the circulation of only 5 pg/ml IL-1β, insulin was strongly induced, but not in the presence of IL-1Ra or in NLRP3 deficient mice, indicating the potency of LPS derived IL-1β as an insulin secretagogue. However, it is likely that the levels of IL-1β in the circulation do not reflect its concentration in target cells and organs.

Numerous studies link high energy and fat rich diet with activation of the immune system and endotoxemia[53, 88], a state where LPS levels are elevated in the circulating. In addition, recent reports provide evidence that free fatty acids are activators of TLRs and inducers of the NLRP3 inflammasome[28]. The present study demonstrates the importance of elevated IL-1β levels after LPS administration for insulin secretion. Elevation of FFAs after a high fat meal as well as elevation of LPS levels, that can occur due to intestine permeability allowing gut microbiota to penetrate into the circulation, may lead to IL-1β induced insulin secretion. In turn, IL-1β reduces glycaemia and activates the immune system to fight intruding microbiota. However as shown in unpublished results (fig. 1), circulating IL-1β levels were not elevated after high fat or high glucose ingestion. This may be the case for three reasons, first, circulating IL-1β levels do not reflect its local production, second, the settings of the experiment does not reflect physiological food intake and a mixed meal rather than glucose or oil gavage may be the proper way to perform this assay. Third, IL-1β is an extremely potent cytokine acting at levels bellow the detection of commercially available assays. Hence in order to evaluate the role of endogenously produced IL-1β we blocked its activity with IL1Ra. Indeed, DIO and db/db mice acutely injected with IL-1Ra

had basally lower circulating insulin levels which were not due to altered insulin sensitivity as demonstrated in clamp studies. Altogether, this provides evidence that IL-1β has a physiological role in insulin secretion and the adaptation to the increased demand of insulin in states of obesity and insulin resistance.

4.3 Improvement of glycaemia by IL-1β is partly mediated via neuronal stimulation of insulin secretion and is independent of the incretin system.

In this study, the effect of IL-1β was much stronger when applied together with a glucose bolus. This suggests that IL-1β potentiates glucose stimulated insulin secretion resembling the effect of G-coupled receptors as described above (Introduction; 1.3.1). Hence, the involvement of major G coupled receptor agonists involved in insulin secretion (incretins and acetylcholine) were investigated. According to the results presented in Fig. 4 c,d,f,g, incretins do not contribute to IL-1β stimulated insulin secretion. Both WT mice treated with GLP-1 receptor antagonist (exendin 9-39) and incretin receptor ko mice responded as their untreated or littermate controls, respectively. In addition, GLP-1 levels were not different in IL-1β injected mice compared to saline injected controls. However upon oral glucose administration, the physiological way of glucose ingestion, IL-1β treated mice had lower GLP-1 levels in the circulation compared to saline injected controls while insulin levels were equal (unpublished data Fig 2). A possible explanation for this observation is that upon oral ingestion IL-1β inhibits the incretin secretion but at the same time directly induces insulin. However, once applied intraperitoneally, glucose does not stimulate the secretion of incretins therefore the effect of IL-1β on insulin secretion is detectable. Interestingly, even though circulating insulin levels were similar and GLP-1 levels were lower, IL-1β treated mice had improved glucose tolerance pointing on a role of IL-1β in glucose clearance as discussed below.

The present study results indicate that besides its direct secretagogue effect in islets, IL-1β mediates insulin secretion via neuronal stimulation. Atropine partially blocked the effect of IL-1β in WT and DIO mice demonstrating the contribution of acetylcholine acting via muscarinic receptor in IL-1β mediated insulin secretion

(Fig. 4 h-j, s-v). In addition, darifenacin, an M_3 muscarinic receptor blocker, that does not cross the blood brain barrier, failed to block the effect of IL-1β indicating that the central nervous system (CNS) participates in the observed effect.

The role of IL-1β in the CNS is well described[89] and the role of the CNS in immunity is emerging[90]. One may speculate that IL-1β signals to the brain that an inflammatory process is taking place, thus temperature homeostasis is disturbed and this in turn initiates shivering in order to increase the body temperature which in turn increases the glucose demand. At the same time, nerves stimulate insulin that enhances glucose uptake to the deprived compartments. As shown before nerves may signal to the immune system further activating or attenuating inflammatory processes[91, 92] as a positive or a negative feedback.

Moreover, in vitro, IL-1β stimulated insulin secretion was blocked by atropine confirming the observation of the presence of ACh in human islets[76]. This inhibitory effect of atropine appeared only in human islets. Interestingly, RNA levels of vesicular ACh transporter (vAChT) were elevated upon treatment with IL-1β in both murine and human islets or when isolated from DIO mice. These findings indicate a possible role of chronically elevated IL-1β in the induction of ACh as a paracrine inducer of insulin. Of note, it has been previously demonstrated that treatment with cholinergic agonist normalized glucose tolerance. Further, insulin secretion is more pronounced in DIO mice than in WT mice[93]. This may be partially attributed to IL-1β as it also increased the levels of M_3 muscarinic receptor in mouse islets (preliminary- not shown).

4.4 IL-1β promotes insulin independent glucose uptake in muscle, fat and immune cells but not in the liver

In the present study we observed several times that the link between insulin and reduced glycaemia was disconnected. In some experimental settings blood glucose levels were different between treatments while insulin levels were unchanged (Fig. 3, Fig 4 a,b). Since IL-1β had no effect on insulin sensitivity (Fig. 1 d-f, sup Fig. 1), the direct effect of IL-1β on glucose disposal was investigated. Three approaches were used to adress this. In the first approach (Fig. 5 a-g), IL-1β was injected in the absence of a glucose bolus. Glucose uptake was evaluated

using trace amounts of radiolabeled, non-metabolizable glucose and therefore insulin levels were not changed. Various tissues had increased glucose uptake upon IL-1β treatment . This suggests that the observed increase in muscle, adipose tissue, adipocytes, spleen and, circulating white blood cells (WBC) is due to a direct effect of IL-1β on glucose uptake. Thus, the role of IL-1β as a pyrogen during infections[94] may be attributed to a direct increase in glucose uptake (and indirectly via IL-1β stimulated insulin secretion) in muscle. Additionally, this increase in glucose uptake in various tissues may contributes to insulin resistance as seen previously in adipocytes[26]. In the second approach (Fig 5 h-o), IL-1β was administered or blocked at ambient glucose levels *in vivo* or *in vitro*, and the focus was on immune cells glucose uptake. IL-1β increased immune cell glucose consumption. This was associated with an elevation of the glycolytic capacity which may promote the activation of the immune system and of inflammatory processed. Moreover, endogenously produced IL-1β acts in an autocrine manner elevating glucose uptake (Fig. 5 o). In the third approach (Fig 5 q,r), the contribution of macrophages to changes in circulating glucose levels was evaluated. IL-1β was injected into macrophage ablated and T and B cell deficient mice (RAG2 KO) during an oral glucose bolus. In these mice, the glucose lowering effect of IL-1β was dampened, demonstrating the potency of immune cells, mainly macrophages, to consume glucose and contribute to glucose disposal.

Furthermore and in line with these results, an infection often leads to insulin resistance in liver and adipose tissues, increasing the availability of glucose for immune cells.

Altogether, these results provide a firm proof that the immune system not only influences whole body glucose metabolism via changes in insulin sensitivity (as it is well established[28, 47, 48]) but also via glucose consumption and thereby contributes to changes in glycaemia. The physiological role of IL-1β in metabolism is to adapt glucose homeostasis to the needs of an innate immune response.

4.5 Insulin regulates macrophage metabolism and stimulates IL-1β via the NLRP3 inflammasome

The current work shows two main functions of IL-1β. First, IL-1β is a potent insulin secretagogue and second, IL-1β directly enhances the glucose supply into immune cells. Interestingly, insulin proved to be an enhancer of this IL-1β effect. In macrophages the role of insulin appeared to be most explicit in activated M1 macrophages, which express increased insulin receptor density, leading to enhanced AKT phosphorylation, glycolytic activity and cytokine expression such as TNF, KC and IL-1α. Finally, insulin is a previously unknown activator of the NLRP3 inflammasome, inducing macrophage-derived IL-1β by enhanced glucose uptake through the glucose transporter GLUT1. Hence insulin, which is increased in early stages of type 2 diabetes, may sustain the inflammatory state and may therefore directly contribute to the chronic low-grade inflammation associated with metabolic diseases. Conversely, IL-1β may stimulate insulin secretion to compensate for the increased demand of insulin during obesity, and this is via its up-regulated receptors (IL-1R1) on a β-cell subpopulation.

4.6 The logic behind IL-1 receptor antagonism as treatment for T2D

The observed beneficial effect of IL-1β on glucose homeostasis is in apparent contrast to the glucose lowering effects of IL-1 antagonism in patients with type 2 diabetes[54]. The most obvious explanation is the difference between acute and chronic effects. Indeed, prolonged administration of IL-1β impaired glucose metabolism in mice (Fig. 3). However, the positive effect of acute IL-1β on insulin secretion was maintained in animal models of diabetes despite chronically increased endogenous IL-1β levels. Similarly, fasting insulin levels of db/db mice and of diet induced obese mice were decreased by IL-1Ra treatment without changes in insulin sensitivity. A possible explanation to reconcile these findings is the concept of β-cell “rest”. Indeed, potassium channel openers, which decrease insulin secretion, improve insulin secretion in patients with type 2 diabetes[79]. Possibly, the benefit of IL-1 antagonism in patients with type 2 diabetes is also due to β-cell rest and not only due to the postulated direct toxic effects of IL-1β. An alternative or additional explanation for the contrasting effects of acute versus

chronic IL-1β exposure is based on the local production of IL-1β by islets in response to a metabolic stress[21, 53]. This islet-derived IL-1β may drive local production of chemokines followed by recruitment of detrimental immune cells. In contrast, systemic IL-1β will not create a chemokine gradient around the islets and subsequent insulitis will not occur.

5 Conclusion and outlook

Overall, the findings from this work show that both IL-1β and insulin have potent effects on glucose homeostasis and on the immune system further supporting the emerging concept of immunometabolism. Understanding the physiological synergy between IL-1β and insulin on glucose disposal and macrophage activity may have important implications for the development and use of drugs modulating IL-1β and insulin actions in pathological conditions.

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