Targeting GPCR-Signaling in Macrophages to Combat Obesity and Type 2 Diabetes

Introduction:

Type 2 diabetes (T2D), a growing global health concern affecting over 462 million people worldwide [1].T2D is still a focus in the field of metabolic research because it is primarily characterized by obesity-related insulin resistance. With its complex immunological etiology, T2D raises several challenges in terms of treatment. Adipose tissue has emerged as a critical immunological organ, especially in the state of obesity. Notably, obese adipose tissue contains approximately 2-4 million stromal vascular cells per gram, and immune cells contributes to 60-70% of the total stromal vascular cell population. [2].

Although specialised public health strategies and treatment initiatives have been devised to combat the obesity pandemic, the prevalence of obesity has increased at an alarming rate[3], potentially leading to increased numbers of patients affected by complications of obesity, and associated metabolic disorders including T2D[4]. Chronic hyperglycemia is a hallmark of T2DM, often accompanied by comorbidities including cardiovascular disease, obesity, microangiopathy, and renal failure[5]. Current therapies either improve insulin sensitivity or introduce exogenous insulin to treat diabetic hyperglycemia, which is caused by a reduction in insulin sensitivity and excessive insulin production. However, these treatments do not achieve long-term glycemic control nor do they halt the disease's progression[6].

The International Diabetes Federation estimates that by 2045, 700 million people would have diabetes, with annual health costs of $850 billion[7-9]. Adipose tissue, skeletal muscle (SKM), and the liver all acquire insulin resistance as a result of excessive dietary intake. Pancreatic beta cells' compensatory insulin release causes hyperinsulinemia, which promotes liver steatosis, hyperlipidemia, and the expansion of adipose tissue[10]. These mechanisms further encourage the malfunctioning of different metabolic organs, which results in hyperglycemia and ultimately the development of T2D. A specific signaling pathway can be amplified or suppressed in different cells or tissues as a very successful pharmacological therapy strategy. G protein-coupled receptors (GPCRs) emerged as the most extensively researched therapeutic targets in contemporary medicine. GPCRs are seven transmembrane receptors that are present on almost all cell types and control a wide range of physiological processes[11]. GPCRs primarily transmit their signals through the alpha subunits of heterotrimeric G proteins after activation[12]. Based on the functional roles of their alpha subunits, G proteins are divided into four main families: Gαs, Gαi/o, Gαq/11, and Gα12/13. While stimulation of Gαi/o suppresses adenylate cyclase activity and controls the function of different ion channels, activation of Gαs promotes adenylate cyclase and raises levels of cyclic adenosine monophosphate (cAMP)[12]. By activating phospholipase Cβ, the Gαq/11 protein stimulates intracellular calcium levels, while the

Gα12/13 protein stimulates the activity of several Rho and MAP kinases[13]. GPCRs control a wide range of metabolic processes, including glucose synthesis, glucose uptake, insulin action, insulin secretion, and cell type differentiation and proliferation. The fact that GPCRs have become a top therapeutic target for the treatment of metabolic illnesses including obesity and T2D[14-16]. While the roles of Gs-coupled receptors in regulating macrophage function and overall glucose homeostasis have been extensively studied, there is limited knowledge about the physiological and pathological roles of Gi-coupled GPCRs in macrophages. Similar to most GPCRs, Gi-linked GPCRs in macrophages, LTB4, and its receptor Ltb4r1 play a critical role in obesity-induced insulin resistance by promoting inflammation and disrupting insulin signaling in major insulin target tissues[17]. Inhibiting Ltb4r1 provides significant improvement against systemic insulin resistance and hepatic steatosis, highlighting its potential as a therapeutic target for metabolic diseases[17]. Therefore, assessing the in vivo metabolic roles of Gi-coupled receptors in macrophages cannot be achieved simply by observing the effects of receptor subtype-specific agonists or antagonists in vivo[17].

The hM4Di receptor belongs to a novel class of designer GPCRs called DREADDs (Designer Receptors Exclusively Activated by a Designer Drug)[18, 19]. These DREADDs are typically mutant muscarinic receptors that exhibit minimal to no activity in response to acetylcholine, the natural muscarinic receptor agonist[18, 19].

Rosa-LSL-hM4Di mice, referred to as Rosa-LSL-GiD mice, have been used to express the hM4Di (GiD) designer receptor in various metabolically important cell types, including mouse pancreatic α-cells[20], hepatocytes[21], and adipocytes[22]. Treating these mutant mouse strains with Deschlorochlozapine (DCZ)selectively stimulates Gi signaling in specific cell types in vivo. This selective targeting cannot be achieved with receptor subtype-selective GPCR agonists targeting native GPCRs, as most GPCRs are expressed across various tissues.

In this study, we employed a chemogenetic strategy to create a mouse model with a Gi-coupled designer GPCR (Gi DREADD; also referred to as hM4Di or GiD) selectively expressed in macrophage (lyzM-GiD mice). Administering deschlorochlozapine (DCZ) to these lyzM-GiD mice, a synthetic compound that specifically activates GiD and similar DREADDs while remaining pharmacologically inert at the correct doses led to targeted stimulation of Gi signaling in macrophages.

Objectives:

1. To investigate the effects of Gi signaling activation in macrophages in the context of diet-induced obesity and T2D.
2. The molecular mechanism(s) and signaling pathways by which Gi DREADD regulates macrophage function.

Materials and methods:

Generation of Mutant Mice Expressing GiD in monocytes/macrophages:

To obtain transgenic mice selectively expressing GiD (DREADDs that stimulate Gi signaling upon activation) in monocytes/macrophages, LysM-cre mice were bred with R26-LSL-Gi-DREADD mice. LysM-cre mice, which express Cre recombinase under the control of the mouse lysozyme 2 promoter, were active specifically in the myeloid cell lineage (monocytes and macrophages; JAX Stock No. 004781). R26-LSL-Gi-DREADD mice were purchased from Jackson Laboratories (Stock No. 026219). The expression of Cre recombinase in monocytes/macrophages excised the floxed-STOP cassette, enabling the selective expression of DREADDs in macrophages.

Acute Activation of Macrophage Gi Signaling in Mice Maintained on Standard Chow and High-fat diet:

Given that different macrophage, G protein signaling pathways play critical roles in various signaling processes, it was anticipated that acute activation of Gi signaling in macrophages would lead to significant metabolic phenotypes. Therefore, it is of great interest to examine how the acute activation of these pathways in the DREADD mice affect whole-body glucose and lipid metabolism, as well as energy homeostasis.

We performed the following metabolic tests to assess the role of macrophage Gi signaling on metabolism:

In Vivo Metabolic Tests:

Mice maintained on a standard diet (15% kcal fat, energy density 3.1 kcal/g), and high-fat diet (60 kcal/g) at least for 8-12 weeks old, were used for these experiments. Mice without Cre recombinase but carrying the DREADD gene were used as control group.

Impairments in glucose homeostasis and insulin action are known major contributors of T2D. To investigate whether activation of Gi signaling in macrophages affected glucose metabolism, we conducted a glucose tolerance test (GTT). GiD and control mice were fasted overnight and injected with glucose and DCZ, with blood glucose levels measured periodically through the tail vein. To determine the effect of macrophage GiD signaling activation on insulin sensitivity, we carried out an insulin tolerance test (ITT). Both groups of mice received insulin and DCZ, with blood glucose levels measured periodically after DCZ treatment. We also performed a pyruvate tolerance test (PTT) to elucidate the effect of macrophage GiD activation on liver gluconeogenesis.

To study glucose-stimulated insulin secretion (GSIS), mice were fasted overnight and then injected intraperitoneally (i.p.) with 1 or 2 g/kg of glucose, as specified. Blood samples were collected at specific time points post-injection, and plasma insulin levels were measured using a mouse insulin ELISA kit (Crystal Chem Inc.), following the manufacturer's instructions.

Plasma glycerol, triglyceride, and free fatty acid (FFA) levels were determined using commercially available kits from Sigma-Aldrich and Fujifilm Wako Diagnostics. Plasma cytokine levels was measured using procartaplex cytokine/chemokine panel from Invitrogen.

Western-blot studies:

Bone marrow-derived macrophages from lyzM-GiD and control mice were cultured in 6-well plate. Cells were treated with 100nM DCZ and lysed using radioimmunoprecipitation assay (RIPA) buffer supplemented with cOmplete EDTA-free protease inhibitor cocktail (Sigma-Aldrich). Lysate were centrifuged at 18000 X g for 15mins at 4ºC. Supernatant were collected into fresh 1.5ml tubes and protein concentration was determined using BCA protein assay kit (Pierce). 2mg/ml protein samples were prepared with 4x Laemmli sample buffer. Samples were denatured at 95ºC and ran on different percentages of gel dependent (4–12% SDS–polyacrylamide gel electrophoresis) on the protein of interest. Proteins were transferred to PVDF membranes and incubated for blocking in 5% BSA at room temperature and overnight at 4°C with the primary antibody. The following day, the membranes were thoroughly washed and incubated with HRP-conjugated anti-rabbit secondary antibodies. Protein bands were visualized using ECL Substrate (Biorad) on the azure600 Imaging System (Azure Biosystems). Immunoreactive bands were quantified using ImageJ Software.

Measurement of Mouse Plasma Cytokine Levels:

Blood was collected from the mouse tail vein into K2-EDTA tubes and quickly centrifuged at 4°C to obtain plasma. Plasma cytokine levels were measured using the procartaplex cytokine/chemokine panel from Invitrogen according to the manufacturer's instructions. The concentrations of cytokines were determined using the bio-plex MAGPIX multiplex reader as specified by the manufacturer.

Real-time qRT-PCR Gene Expression Analysis:

Mouse tissues and BMDM cells isolated from lyzM-GiD and Control mice were collected, and total mRNA was extracted and purified using the RNeasy mini kit with the RNase-free DNase set from Qiagen, following the manufacturer's protocol. cDNA synthesis was performed with PrimeScript™ 1st strand cDNA Synthesis Kit. Real-time qPCR was conducted using SYBR Green (Takara), and primer sets for real-time PCR were designed using Harvard primer bank and purchased from Eurofins. RNA expression data were normalized to the expression of β-actin or 18S rRNA.

Immunofluorescence: BMDM cells from lyzM-GiD and control mice were cultured in 96 well plates. Cells were starved with basal media for 1hour and DCZ treatment was given at different time intervals (1hour,2hour). Media was aspirated and cells were washed with PBS. It is then fixed with 4% PFA was 10mins and washed with PBS thrice, followed by membrane permeabilization with 1XPBST + 0.01%Triton X100 for 10mins. Cells were washed with PBS three times and blocked with 3%BSA in PBST. The primary antibody was incubated overnight at 4º in 1:100 dilution. Cells were then washed

thrice with PBS and Alexa fluro-488 secondary antibody (1:1000) + DAPI (1:1000) in PBS was incubated for 2 hours at room temperature. Fluorescent intensity was measured using high-content microscopy.

Statistical analysis: Statistical analysis was performed with data presented as means ± standard error of the mean (s.e.m.). For significance testing, a two or one-tailed unpaired Student's t-test or one-way ANOVA was applied using GraphPad. A p-value of less than 0.05 was considered statistically significant.

Results:

1. Gi-DREADD activation in macrophages stimulates the release of pro-inflammatory cytokines in mouse plasma.

We expressed Gi-DREADD in macrophages using LyzM-Cre and Gi-DREADD flox mice, resulting in two groups: one with GiD expression in macrophages (experimental group) and a control group with no GiD expression. We confirmed Gi-DREADD expression in different tissues and bone marrow-derived macrophages (Fig. 1a-b). To validate its functionality, we performed a cAMP-HTRF assay, observing a significant decrease in cAMP levels as expected (Fig. 1c). No significant differences were observed when treated with PTX (a Gnai inhibitor) Fig 1d. We conducted a multiplex cytokine profile using plasma from LyzM-GiD and control mice collected at baseline and 30 minutes post-DCZ treatment. Notably, we found increased levels of IL-1β and CXCL1 in LyzM-GiD mice (Fig. 1e-k), indicating a pro-inflammatory state upon Gi activation.

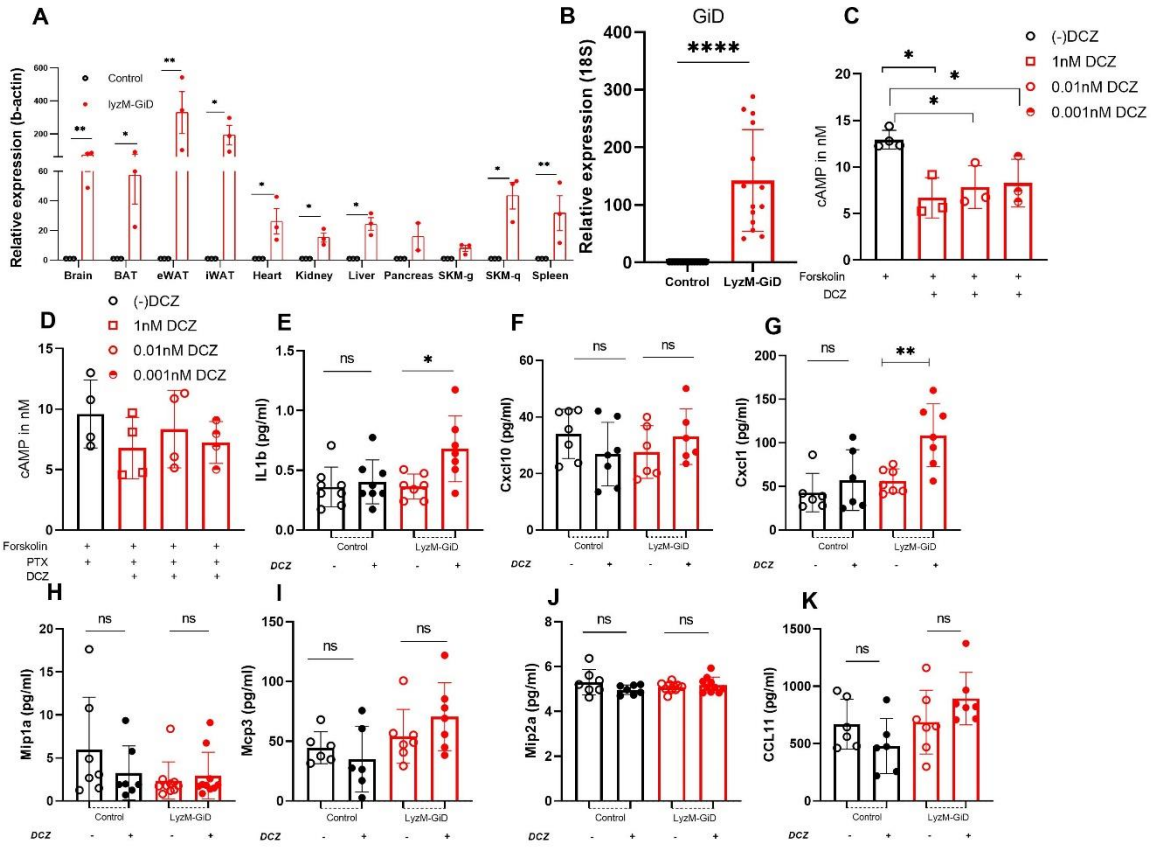


Figure 1: a-b: Confirmation of Gi-DREADD expression in tissues and bone marrow-derived macrophages. b: cAMP-HTRF assay results show a significant decrease in cAMP levels in Gi-DREADD-expressing macrophages. d: Effects of PTX treatment on cAMP levels in Gi-DREADD-expressing macrophages. No significant differences were observed. e-k: Multiplex cytokine profile showing increased levels of IL-1β and CXCL1 in Gi-DREADD mice post-DCZ treatment, indicating a pro-inflammatory state

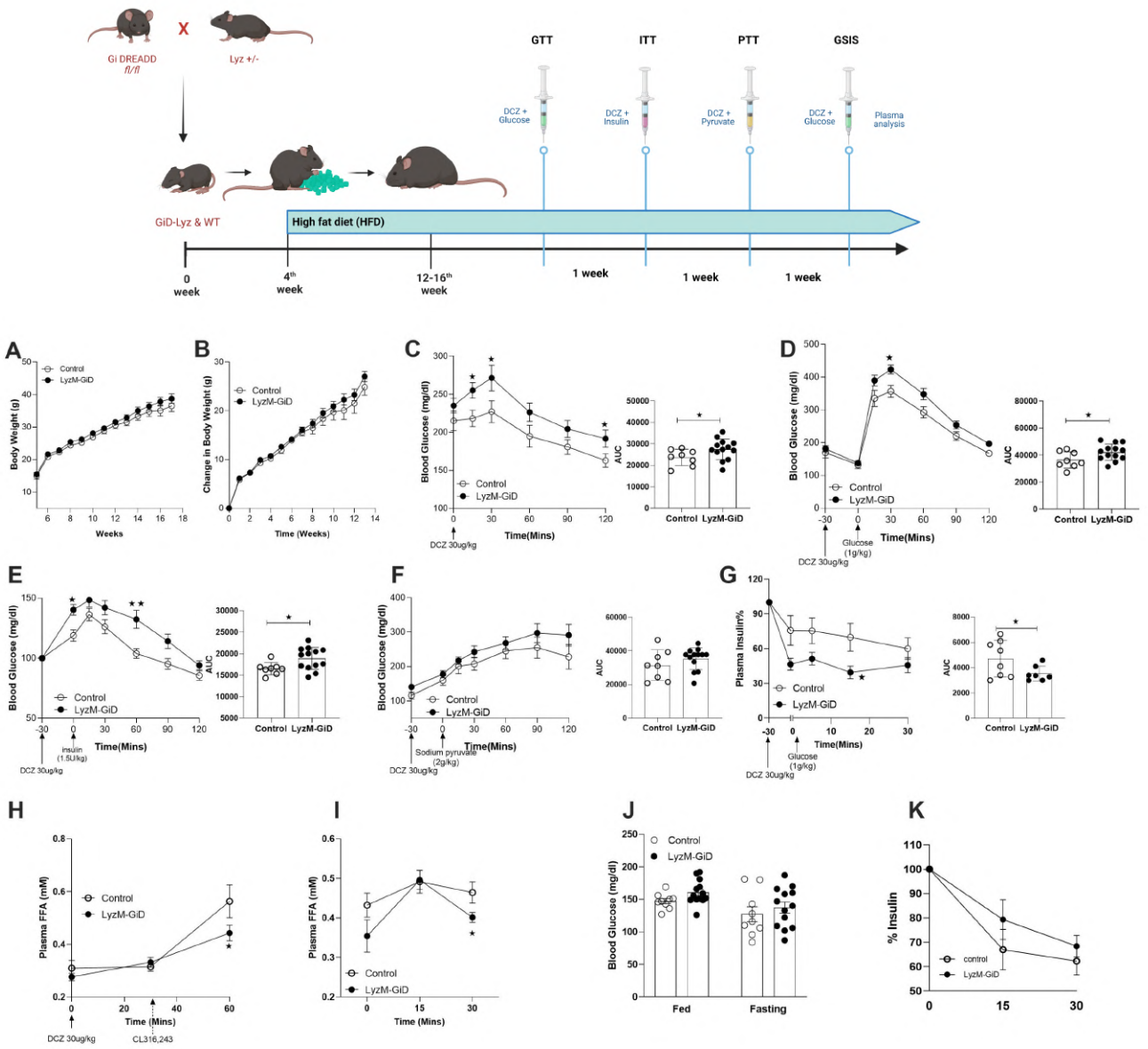
2. Gi- DREADD mice showed impaired glucose metabolism in high-fat diet conditions.

We maintained a cohort of lyzM-GiD and control mice on a high-fat diet (HFD) for 12 weeks before commencing metabolic tests. No significant differences in body weight were observed (Figs. 2a-b).

Upon performing the DCZ challenge test to evaluate the impact of Gi signaling in macrophages on glucose homeostasis, we found an increase in blood glucose levels in lyzM-GiD mice (Fig. 2c). Unexpectedly, the GTT revealed an impaired glucose clearance in the HFD group, indicating reduced glucose uptake by skeletal muscle and adipose tissue (Fig. 2d). Additionally, impaired insulin sensitivity was observed in lyzM-GiD mice (Fig. 2e), though no differences were seen in the pyruvate tolerance test (PTT) (Fig. 2f).

A significant decrease in insulin secretion by beta cells in response to glucose was observed during the glucose-stimulated insulin secretion (GSIS) test (Fig. 2g). Lipolysis was inhibited when CL216,243 was administered 30 minutes after DCZ (Fig. 2h), and plasma-free fatty acid levels were decreased with DCZ alone (Fig. 2i). However, there were no differences in fed or fasting blood glucose and insulin levels with DCZ alone (Figs. 2j-k).

In conclusion, in mice fed a HFD, the activation of Gi signaling in macrophages results in elevated blood glucose levels, reduced glucose clearance, and decreased insulin sensitivity. This is accompanied by a significant decrease in insulin secretion and lipolysis, indicating an impaired metabolic response.



significant differences in body weight were observed between the two groups. c: Blood glucose levels following DCZ challenge test (DCT) in Gi-DREADD and control male mice on a high-fat diet. An increase in blood glucose levels was observed in LyzM-GiD mice. d: Glucose tolerance test (GTT) results in male mice on a high-fat diet. Impaired glucose clearance was observed in the experimental group, indicating reduced glucose uptake. e: Insulin tolerance test (ITT) results in male mice on a high-fat diet. Impaired insulin sensitivity was observed in LyzM-GiD mice. f: Pyruvate tolerance test (PTT) results in male mice on a high-fat diet. No significant differences were observed between the experimental and control groups. g: Glucose-stimulated insulin secretion (GSIS) test results in male mice on a high-fat diet. A significant decrease in insulin secretion was observed in LyzM-GiD mice. h: Lipolysis inhibition test results in male mice on a high-fat diet following Cl216243 and DCZ administration. Inhibition of lipolysis was observed in LyzM-GiD mice. i: Plasma-free fatty acid levels in male mice on a high-fat diet following DCZ administration. Decreased levels were observed in LyzM-GiD mice. j-k: Fed and fasting blood glucose and insulin levels in male mice on a high-fat diet. No significant differences were observed between the experimental and control groups.

3. Activation of macrophage Gi signaling increases the expression of proinflammatory genes.

Next to understand the molecular events that led to impaired metabolism, we conducted bulk RNA sequencing using BMDM from GiD and control mice. Our analysis revealed numerous differentially expressed genes in LyzM-GiD macrophages (Fig. 3a). Our transcriptome differential expression analysis confirmed increase in expression of proinflammatory genes. Gene ontology analysis showed an increase in the endosomal transport and a decrease in the negative regulation of NFκB transcription factor activity. These findings suggest heightened inflammatory activity in the macrophages (Fig. 3b-c). Consequently, we performed qPCR for genes associated with pro-inflammatory responses and observed increased expression of CXCL1, MCP1, CXCL2, IL-6, IL-1α, NFκB, and SREBP (Fig. 3D).

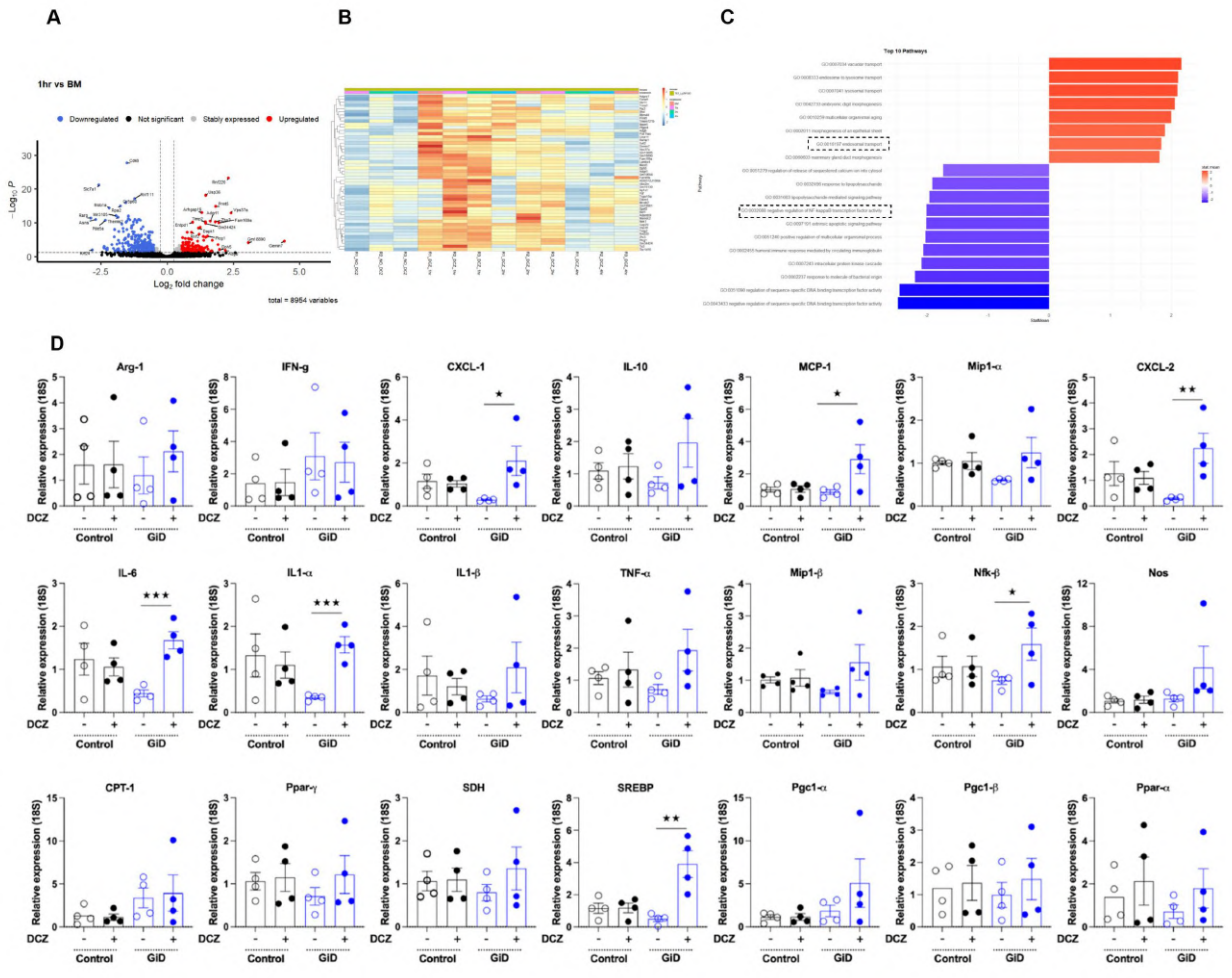


Figure 3: (A) Volcano plot for differentially expressed genes consisting of both upregulated and downregulated genes (B) Heatmap illustrating differential gene expression profiles across different groups. The intensity of the colors represents the level of expression, with red indicating upregulation and blue indicating downregulation. Columns represent individual samples, and rows represent genes. (C) GO enrichment analysis highlighting significantly upregulated and downregulated biological processes in the experimental groups. Blue bars denote highly downregulated GO terms. (D) Quantitative real-time PCR analysis of mRNA expression levels of inflammatory cytokines, lipid metabolism-related genes, and transcription factors across different treatment groups (Control, DCZ, GID, and GID+DCZ). Data are presented as mean ± SEM. Statistical significance was determined using t-test. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the Control group.

4. Macrophage Gi signaling regulates expression of interleukins via AKT/mTOR-NFκB axis.

Upon activation of Gi-DREADD, we observed an increase in s6kinase, NFκB, Akt and Jnk. The expression of IL-1β is higher in the LyzM-GiD group compared to the control, particularly at the 30-

minute time point. The bar graphs quantitate the activation (phosphorylation) of proteins (NF-kB, AKT) and cytokine (IL-1β) release. There is a significant increase in these signaling molecules in the LyzM-GiD group compared to the control group,. The figure 4d illustrates the proposed signaling pathway, indicating that the LyzM-GiD group shows increased cytokine release (IL-1α, IL-1β, IL-6) due to heightened activation of the AKT/mTORC1 and NF-kB pathways, possibly leading to increased inflammatory responses. Figure 5a shows an increase in IL6 protein in lyzM-GiD upon Gi-Dreadd activation using a high-content microscope.

The LyzM-GiD group exhibits enhanced activation of the AKT and NF-kB signaling pathways, leading to increased expression of inflammatory cytokines such as IL-1β. This suggests a potential role for these pathways in mediating inflammatory responses in the LyzM-GiD condition.

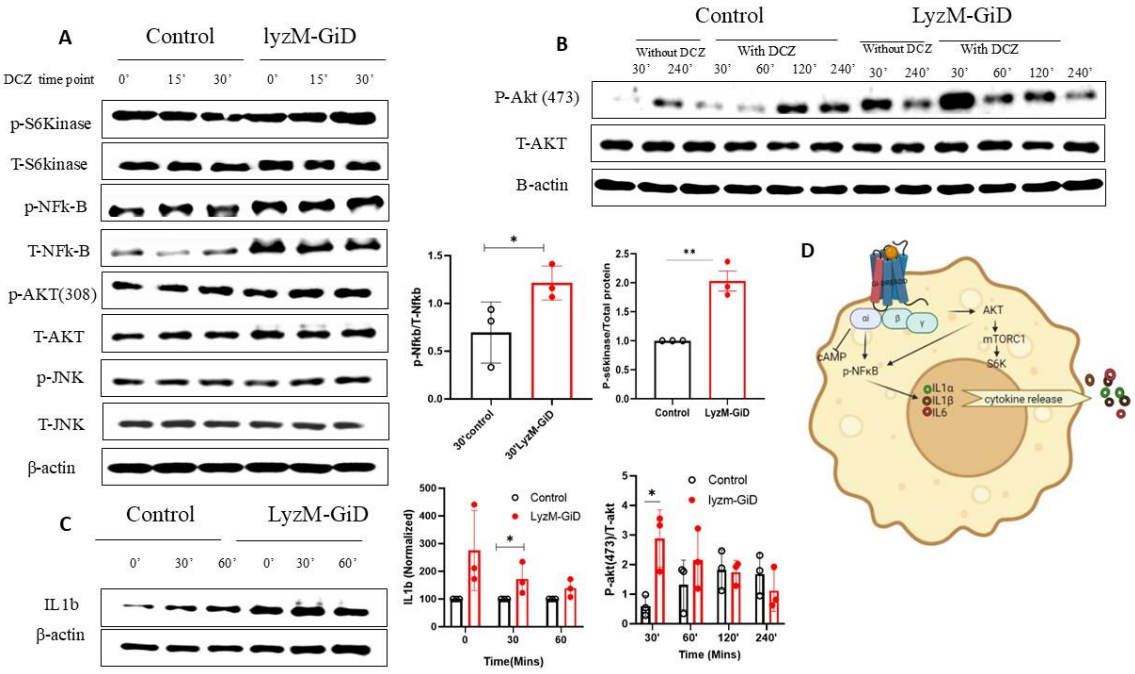
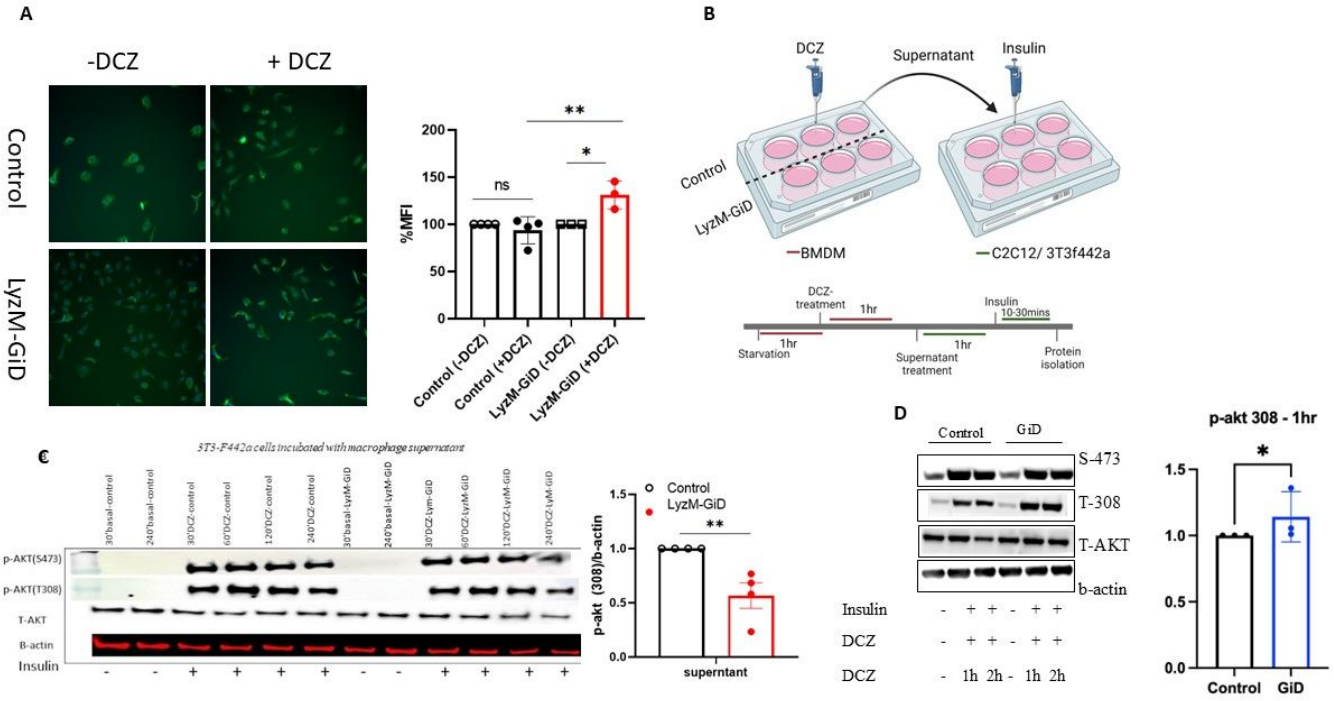


Figure 4: Activation of inflammatory signaling pathways in LyzM-GiD cells. **(A)** Representative Western blot images showing the phosphorylation (activation) and total levels of key signaling proteins (S6Kinase, NF-kB, AKT, and JNK) in control and LyzM-GiD groups. Cells were treated with DCZ, and samples were collected at indicated time points (0, 15, 30 minutes). β-actin is used as a loading control. **(B)** Western blot analysis of AKT phosphorylation at Ser473 (p-AKT (473)) and total AKT (T-AKT) in control and LyzM-GiD groups, with or without DCZ treatment. Samples were collected at various time points (30, 60, 120, 240 minutes). The bar graph on the right quantifies p-AKT (473) levels normalized to total AKT. *p < 0.05 compared to control. **(C)** Western blot showing the expression of IL-1β in control and LyzM-GiD groups after treatment with DCZ at different time points (0, 30, 60 minutes). β-actin is used as a loading control. The bar graph below quantifies IL-1β levels normalized to β-actin. *p < 0.05 compared to control. **(D)** Schematic representation of the proposed signaling pathway. LyzM-GiD induces the activation of AKT and NF-kB signaling, leading to the activation of mTORC1 and increased expression of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6), which may contribute to cytokine release and inflammation.

5. Activation of Macrophge Gi signaling regulates insulin signaling in adipocyte and skeletal muscle cells.

BMDM cells were cultured and Gi-Dreadd activated at different time point. The collected supernatant was transferred to 3t3F442a and C2C12 cells which are adipocyte and myocyte cell lines respectively.

Insulin signaling was studied to understand the effect of macrophage's cytokine on insulin sensitivity.



high-content microscopy showed an increase in IL6 in LyzM-GiD macrophages. B schematic representation of supernatant treatment to 3t3f442a and c2c12. C. macrophage supernatant treatment to 3t3f442a, decreased p-akt (308). D. macrophage supernatant treatment to c2c12 showing an increase in insulin signaling.

Discussion

We report that expression and activation of Gi- signaling in macrophages leads to strong pro-inflammatory responses characterized by elevated levels of cytokines including CXCL1 and IL-1β. These cytokines are well-known mediators of inflammation, hence their increased occurrence in the plasma of LyzM-GiD mice means that systemic inflammation is promoted via the activation of Gi signaling. Moreover, our metabolic analysis indicated that glucose homeostasis is severely impaired by macrophage Gi signaling. Specifically, HFD-fed LyzM-GiD animals showed reduced insulin sensitivity, diminished insulin production as well as poor glucose tolerance. These metabolic abnormalities are indicative of impaired insulin signaling in peripheral organs which is a characteristic feature of type 2 diabetes mellitus. We showed that lipolysis is inhibited and the levels of free fatty acids in the plasma decreases significantly after DCZ treatment, which is quite interesting because it implies that macrophages play a important role in disruption of normal lipid homeostasis in obese and T2D individuals. Macrophages derived from bone marrow using transcriptome analysis in LyzM-GiD mice provided additional insights into molecular mechanisms underlying these phenotypic changes. An intricate interplay between metabolic and inflammatory pathways exists in macrophages as evidenced

by enhanced NF-kappaB transcription factor activity concurrent with overexpression of inflammatory pathway genes and TCA cycle. This was confirmed through qPCR data where we observed up-regulation of pro-inflammatory genes including CXCL1, MCP1 and IL-6 among others suggesting that they are responsible for systemic inflammation found in LyzM-GiD animals. Studies used before were mainly focused on how Gs/Gq-coupled receptors control macrophage activity and glucose homeostasis. Yet our results establish that Gi-linked receptors play a distinct but critical role in inciting pro-inflammatory responses and metabolic malfunctioning. Hence, the therapeutic potential for treating obesity-related metabolic disorders is present through targeting of Gi signaling in macrophages. The exact mechanisms by which β-cell function and insulin secretion are regulated by Gi signaling in macrophages have not been fully elucidated. Although our research has shown the impact of Gi signaling on fat and glucose metabolism, it needs to be further explored whether these effects are directly mediated by macrophages or indirectly by other metabolic tissues such as liver, skeletal muscle or adipose tissue.

In conclusion, we focused on the pharmacodynamics of Gi-signaling resulting in inflammation and impaired glucose and lipid homeostasis. As a result, insulin resistance as well as type 2 diabetes is developed. Therefore, these findings suggest that Gi signaling in macrophages may represent a new therapeutic target for the regulation of metabolism in obesity-related metabolic diseases. Henceforth, more research should be done to define specific signaling pathways involved and evaluate whether cutting-edge T2D therapies can be fostered by Gi-coupled receptor antagonists.

Impact of Research and Benefit to Mankind:

1. This study's findings have a huge impact on ways of improving human health with regard to combating the metabolic diseases that are now widespread throughout the globe. Understanding Gi signaling in macrophages opens up new avenues for therapeutic intervention in these metabolic diseases that affect millions of people worldwide.
2. Progression Prevention of Diseases: From the molecular understanding revealed in this study, early interventions could halt the progression of obesity-induced effects such as liver disease, cardiovascular disorder and other comorbidities associated with type 2 diabetes. It is possible that healthcare providers can prevent or reverse the metabolic disorders' progression by intervening earlier in its natural course, directed at macrophage-mediated inflammation specific pathways.
3. Decreasing Healthcare Costs: There is an overwhelming global burden of obesity and type 2 diabetes upon health systems. This research may lead to new therapeutic targets for better drugs relieving the severity and frequency of these diseases. Consequently, the reduction in financial implications for managing chronic metabolic illnesses would be beneficial to both individuals and societies.

4. Better Quality of Life: Wellness in their quality of life would result if people who have T2D and are obese get focused medications to better control their diseases. Fewer complications due to decreased inflammation along with better glucose homeostasis such as neuropathy, renal damage, and cardiovascular problems can make patients live a healthier life by being more active.

We investigated immune system regulation and metabolic processes in mice, thus contributing to immediate clinical applications. There is great potential for the field of Gi signaling in macrophages to revolutionize the treatment of metabolic diseases. There is hope that our study will lead to better therapies that could improve the lives of millions and therefore address the root causes behind inflammation and disrupted metabolism for all humanity.

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