Chapter 8

Isoliquiritigenin: A Unique Component That Attenuates Adipose Tissue Inflammation and Fibrosis by Targeting the Innate Immune Sensors

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Abstract

Recent studies have suggested that pattern recognition receptors, including inflammasomes and TLRs, in the innate immune system recognize various kinds of endogenous ligands and have critical roles in initiating or promoting obesity-associated chronic inflammation. These findings have provided new therapeutic strategies based on regulation of the innate immune system. With the rapid advancement of novel technologies and the increased research on natural products, many new plant-derived extracts and active compounds have been identified to exhibit anti-inflammatory effects. Isoliquiritigenin (ILG) is a flavonoid derived from *Glycyrrhiza uralensis* with a chalcone structure. We have reported that ILG inhibits NLRP3 inflammasome activation resulting in the improvement of diet-induced adipose tissue inflammation and insulin resistance. Furthermore, we have also demonstrated that ILG improves diet-induced fibrosis in adipose tissue by inhibiting TLR4- and Mincle-induced expression of fibrosis-related genes in obese adipose tissue and macrophages. Thus, ILG can suppress two important dysfunctions of obesity, adipose tissue inflammation and fibrosis by targeting innate immune sensors. Here we overview ILG as a potential therapeutic agent for the treatment of obesity-associated diseases. We also summarize anti-inflammatory actions of other constituents of licorice.

Keywords: chronic inflammation, diabetes, inflammasome, innate immunity, metabolic syndrome

1. Introduction

Obesity has become a worldwide health problem because it is strongly associated with metabolic syndromes including type 2 diabetes mellitus (T2DM), atherosclerosis and ischemic heart diseases [1, 2]. Accumulating evidence indicates that chronic low-grade inflammation has a

crucial role in the pathogenesis of obesity-associated metabolic dysfunction [3, 4]. The chronic inflammatory alternations are associated with dynamic changes in the composition and function of immune cells in various tissues such as adipose tissue, pancreatic islet, liver, muscle, and hypothalamus [5–7]. A large number of inflammatory immune cells infiltrate into adipose tissue during the course of obesity. M1-like macrophages, an inflammatory type of macrophage, accumulate in adipose tissue and are major sources of inflammatory mediators such as tumor necrosis factor (TNF)-α and IL-6 [8].

The connective fiber content of adipose tissue dramatically increases by the upregulation of collagen expression, which in turn elevates the overall rigidity of adipose tissue and finally leads to fibrosis. The deficiency of collagen 6, a key component of the extracellular matrix in adipose tissue, significantly improves the phenotypes of obese mice, including adipocyte death and adipose tissue inflammation [9]. This implies that alterations in the extracellular matrix in adipose tissue are linked to the development of inflammation. Thus, inflammation and fibrosis are important targets for the treatment of obesity.

An obese state results in an elevation of circulating levels of fatty acids (FAs) and, subsequently, an increase in inflammation of adipose tissue [10]. Adipose macrophages play critical roles in the immune responses through several FA-sensing mechanisms, such as pattern recognition receptors (PRRs). PRRs such as toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) quickly recognize pathogenic agents [11]. It is now becoming even more apparent that these PRRs are not only able to recognize microbial components but also mediate immune responses to endogenous molecules, including those arising in metabolic disorders, such as FAs. These endogenous molecules have been termed danger-associated molecular patterns (DAMPs) and have similar functions as microbial components to activate immune responses [12]. In the obese state, TLR4 may be activated by saturated free FAs, such as palmitic acid, derived from hypertrophied adipocytes as a DAMP and promote adipose tissue inflammation and insulin resistance [13–15]. Inflammasomes are multimeric protein complexes that are crucial for caspase-1, IL-1β, and IL-18 production [16]. The nucleotide-binding domain, leucine-rich repeats containing family, pyrin domain-containing-3 (NLRP3) inflammasome also senses obesity-associated FAs and contributes to obesity-induced inflammation and insulin resistance [17, 18]. Moreover, IL-1β inhibits insulin signaling in the insulin-target organs, including adipose tissue, liver, and skeletal muscle, and also induces dysfunction and cell death of insulin-producing pancreatic β cells [19]. Macrophage-inducible C-type lectin (Mincle) recognizes not only cord factor, a mycobacterial glycolipid, but also SAP130 released from dead cells [20, 21]. Furthermore, Mincle is highly expressed in M1 macrophages in adipose tissue and involved in the induction of adipose tissue fibrosis and insulin resistance during obesity [22, 23].

Plant-derived natural products and their derivatives or synthetic mimics make up a considerable portion of current drugs. These products have played an important role in treating T2DM, especially in Asian countries. We previously reported that glycyrrhizin (GL) and isoliquiritigenin (ILG), components of *Glycyrrhiza uralensis* (*G. uralensis*), inhibit TLR4 signaling at the receptor level on the cell surface, resulting in inhibition of NF-κB and mitogen-activated protein kinases (MAPKs) activation [24]. Furthermore, ILG potently inhibits NLRP3

inflammasome activation independent of its inhibitory action on TLR4 [25]. Our *in vivo* study revealed that ILG attenuated high-fat diet (HFD)-induced adipose tissue inflammation and insulin resistance by inhibiting NLRP3 inflammasome activation in adipose tissue [25]. In addition to inflammation, we recently reported that ILG impacts fibrogenesis in adipose tissue by targeting TLR4 and Mincle activation in macrophages. On the basis of these findings, natural products derived from *G. uralensis* may serve as lead compounds for the development of new anti-T2DM drugs.

This review article highlights the recent discoveries of the anti-inflammatory and anti-fibrotic effects and mechanisms of action of *G. uralensis* components that target PRRs. We will also overview our findings that ILG targets activation of TLR4, NLRP3 inflammasome, and Mincle. We will discuss the roles of innate immunity and potential mechanisms by which it participates in obesity-associated inflammation and fibrosis.

2. PRRs that link with adipose tissue inflammation and fibrosis

2.1. Toll-like receptors

TLRs are transmembrane proteins that recognize conserved structural moieties of microorganisms and for the subsequent induction of pro-inflammatory responses [26]. They directly bind to their ligands and activate the NF-κB and MAPK pathways to induce the production of pro-inflammatory cytokines that are important for evading pathogens. It is widely suggested that TLRs also sense non-microbial endogenous ligands, such as dietary FAs [12]. Activation of TLRs by the endogenous ligands similarly induces pro-inflammatory pathways as microbial ligands in various organs, such as adipose tissue and the liver.

TLR4 is the most important TLR for LPS-mediated inflammatory responses [27]. There is a body of evidence suggesting that TLR4 is an attractive candidate for linking innate immune responses to obesity-associated dysfunction. For example, TLR4 expression is increased in inflammatory macrophages derived from obese adipose tissue [13, 28]. TLR4 KO mice or mice with a loss-of-function mutation in the TLR4 gene are protected from obesity-associated insulin resistance [13, 29]. Furthermore, hematopoietic cell-specific deletion of TLR4 ameliorates HFD-induced hepatic insulin resistance [30]. Intriguingly, saturated FAs released by adipocyte lipolysis can be endogenous TLR4 ligands and activate the NF-κB pathway on macrophages [15]. Another paper reported that resistin derived from adipose tissue directly bound to TLR4 in the hypothalamus and leads to the activation of MAPKs signaling and promoting insulin resistance through MyD88 [31], suggesting that resistin is an endogenous TLR4 ligand, which links hypothalamic inflammation with insulin resistance.

We previously demonstrated that the development of obesity-related inflammation required radioprotective 105 (RP105) rather than TLR4 [32]. RP105 was identified as a first mammalian homologue of Drosophila toll that expressed on B cells [33] and suggested to be involved in LPS-induced B-cell responses. In fact, RP105-deficient mice show reduced LPS-dependent proliferation and CD86 upregulation in B cells, albeit to a lesser extent than TLR4-deficient

mice [34, 35]. Among B cell subsets, marginal zone (MZ) B cells express high density of RP105. We have showed that RP105 is indispensable for TLR4-dependent plasma cell differentiation and IgM production in MZ B cells [36]. Additionally, M1 macrophages of murine epididymal white adipose tissue (eWAT) highly express RP105 [32]. This expression is markedly increased by HFD supplementation [32]. Furthermore, HFD-induced obesity, adipose tissue inflammation, and insulin resistance are severely attenuated in RP105 KO mice compared with wild-type (WT) and TLR4 KO mice. In contrast to TLR4, RP105 is not activated by palmitic acid [32]. Our results suggest that ligands and signaling pathways involved in RP105-mediated adipose tissue inflammation do not completely overlap with those utilized by TLR4. Future investigations will determine an endogenous ligand and a signaling pathway of RP105 in adipose tissue.

2.2. NLRP3 inflammasome

Inflammasomes are cytoplasmic receptors and play an important role in the host defense against microbial infection. Activation of NLRP3 inflammasome is regulated by various sterile stimuli, including cholesterol crystals, β-amyloid, palmitic acid, and ceramides. It is generally accepted that two signals are required for NLRP3 inflammasome activation. One is an NF-κB-dependent priming step that induces the transcription of pro-IL-1β and NLRP3. Another is an activation step that induces the activation of caspase-1. Normal activation of NLRP3 inflammasome contributes to host defense, but several studies suggest that excessive activation leads to the development of obesity-associated inflammation.

Islet amyloid polypeptide (IAPP) is deposited in the pancreas and associated with the loss of β cell function in T2DM. The observation of NLRP3-dependent IL-1β production by macrophages in response to IAPP implied a potential role for NLRP3 in promoting IL-1β secretion in T2DM [37]. Interestingly, an anti-diabetic drug glyburide inhibits NLRP3 activation by macrophages in response to IAPP. Direct involvement of NLRP3 in obesity has been confirmed in studies that NLRP3 KO mice fed HFD display reduced caspase-1 activation and pro-IL-1β expression in adipose tissue compared with WT mice [38].

2.3. C-type lectin Mincle

C-type lectin receptors elicit inflammation and innate immune responses through activation of multiple signaling cascades. Mincle recognizes cord factor, a mycobacterial glycolipid, and transduces activation signals by associating with the Fc receptor common γ-chain, which contains immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domain. The phosphorylated ITAM recruits Syk (spleen tyrosine kinase), leading to activation of NF-κB and MAP kinases [39, 40].

The expression level of Mincle is increased by various cellular stresses and stimuli. Mincle expression is upregulated in patients with rheumatoid arthritis and is increased in microglia, neuron, and endothelial cells in the brain after ischemic stroke [41, 42]. Furthermore, Mincle is highly expressed in inflammatory M1 macrophages in adipose tissue and involved in the induction of adipose tissue fibrosis and insulin resistance [22, 23]. These results suggest that

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Mincle is involved in the pathogenesis of various inflammation and represents a potential target molecule for the treatment of inflammatory diseases, including rheumatoid arthritis, brain infarction, and T2DM.

3. Natural products that target innate immune system

Chinese herbal formulas with anti-diabetic effects are well developed such that a number of these formulas have commonly been used in diabetic patients since ancient times. The most frequently used 10 Chinese herbs in the period from 2004 to 2009, for the treatment of T2DM, include *Radix Astragali*, *Rhizome Dioscoreae*, *Radix Rehnabbiae*, *Radix Salviae Miltiorrhizae*, *Radix Puerariae*, *Rhizoma Coptidis*, *Fructys Lycii*, *Poria*, *Rhizoma Alismatis*, and *Fructus Corni* [43]. However, it is unclear whether these Chinese herbs have as potent anti-inflammatory effects as those of Western anti-diabetic drugs.

Glycyrrhiza plants (licorice) have been used as herbal medicine worldwide for over 4000 years [44]. Several studies have reported that Chinese and Japanese herbal medicines or their components regulate innate immunity. Among *Glycyrrhiza* plants, *G. uralensis* is one of the most used herbal medicines in Asian countries. Various components have been isolated from licorice, for example triterpene saponins, flavonoids, isoflavonoids, and chalcones. Therefore, we have focused on the anti-inflammatory effects of *G. uralensis* and its components on innate immune responses.

3.1. Glycyrrhizin

Glycyrrhizin (GL), a triterpene saponin, is considered to be the major biological active ingredient of *G. uralensis*. It has been reported that GL inhibits LPS-induced TLR4 internalization [45]. Our experiments confirmed that GL suppressed lipid A moiety of LPS-induced IL-6 production in mouse macrophages [24]. Furthermore, GL treatment significantly suppressed the production of inflammatory cytokines, including TNF-α and IL-6, in LPS-injected mice [24]. We further demonstrated that GL attenuated lipid A-mediated activation of NF-κB and MAPKs, including JNK, p38, and ERK. It was suggested that GL might be incorporated into lipid bilayers and suppress the plasma membrane integrity [45]. LPS binds to MD-2 and this triggers homodimerization of the TLR4/MD-2 complex, resulting in the induction of signal transduction [46]. Our data demonstrated that GL inhibited LPS binding to the complex in a dose-dependent manner. Accordingly, LPS-induced TLR4 homodimerization was suppressed by GL stimulation. Moreover, GL inhibited not only LPS- but also TLR9 ligand CpG-DNA-induced inflammatory responses (our unpublished data), as suggested previously [45]. Thus, GL inhibits the activation of multiple TLRs at the plasma membrane by altering membrane integrity.

GL also inhibits the activation of another PRR, NLRP3 inflammasome, induced by various stimuli, including adenosine triphosphate (ATP), monosodium urate (MSU) and nigericin [25]. LPS is a potent inducer of the priming of the NLRP3 inflammasome. However, the inhibitory

activity of GL on NLRP3 inflammasome is independent of its effect on TLR4 activation [25]. Intriguingly, activation of another inflammasome, absent in melanoma 2 (AIM2) inflammasome, was suppressed by GL stimulation [25], suggesting that GL inhibits multiple inflammasomes activation. However, a high concentration of GL is required for these inhibitory effects on inflammasomes. Therefore, GL may not be suggested to be a potential therapeutic agent for the treatment of obesity-associated inflammation.

3.2. Isoliquiritigenin

ILG, another component of *G. uralensis*, is a flavonoid with a chalcone structure. It has a wide variety of biological activities, including anti-allergic, anti-tumor growth, and anti-platelet aggregation activity. We recently demonstrated that ILG potently inhibited activation of both TLR4/MD-2 and NLRP3 inflammasome [24, 25]. ILG significantly suppressed LPS-induced production of inflammatory cytokines not only *in vitro* but also *in vivo* [24]. Whereas GL stimulation affects the binding of LPS to TLR4/MD-2, the amount of bound LPS was not decreased by ILG stimulation. However, LPS-induced homotypic interaction of TLR4 was potently inhibited by ILG stimulation in a dose-dependent manner. Additionally, ILG blocked lipid A-induced NF-κB and MAPKs activation [24]. This may be due to the inhibition of TLR4 homodimerization. Beside this, it has been reported that ILG interacts with IKK directly and inhibits its kinase activity [47]. Thus, ILG suppresses TLR4/MD-2-mediated immune responses in multiple steps, at the receptor level and the downstream signaling level (**Figure 1**).

3.2.1. Inhibitory effects of isoliquiritigenin on PRR-mediated adipose tissue inflammation

In addition to the inhibitory effects of ILG on the TLR4/MD-2 complex, we demonstrated that it was highly effective in inhibiting NLRP3 inflammasome activation by various stimuli including ATP, MSU, and nigericin [25]. These responses were suppressed by a low concentration of ILG (10~30 μM). The IC50 for the inhibitory effect of ILG is 0.4936 μM. Furthermore, ILG was more effective in inhibiting NLRP3 inflammasome activation than GL (IC50, 358.9 μM) and parthenolide, a known inhibitor of NLRP3 inflammasome. Contrary to GL, ILG did not inhibit poly(dA:dT)-induced activation of the AIM2 inflammasome. As ILG suppresses NLRP3- but not AIM2-induced formation of ASC pyroptosome, it is unlikely that ASC is a molecular target of ILG. IAPP is a unique polypeptide constituent of amyloid deposited in pancreatic islets [48, 49]. This deposition is associated with disease progression of T2DM and triggers NLRP3 inflammasome activation in islet macrophages [37]. The sulfonylurea drug glyburide inhibits IAPP-induced activation of NLRP3 inflammasome [37]. It is noteworthy that a low concentration of ILG is more effective in inhibiting IAPP-induced activation of NLRP3 inflammasome than that of glyburide [25] (**Figure 1**).

The above observations led us to investigate the inhibitory effects of ILG on TLR4- and NLRP3-associated inflammation in obesity. ILG supplementation remarkably improved obesity, hyperlipidemia, hepatic steatosis, and insulin resistance in HFD-fed wild-type mice [25].

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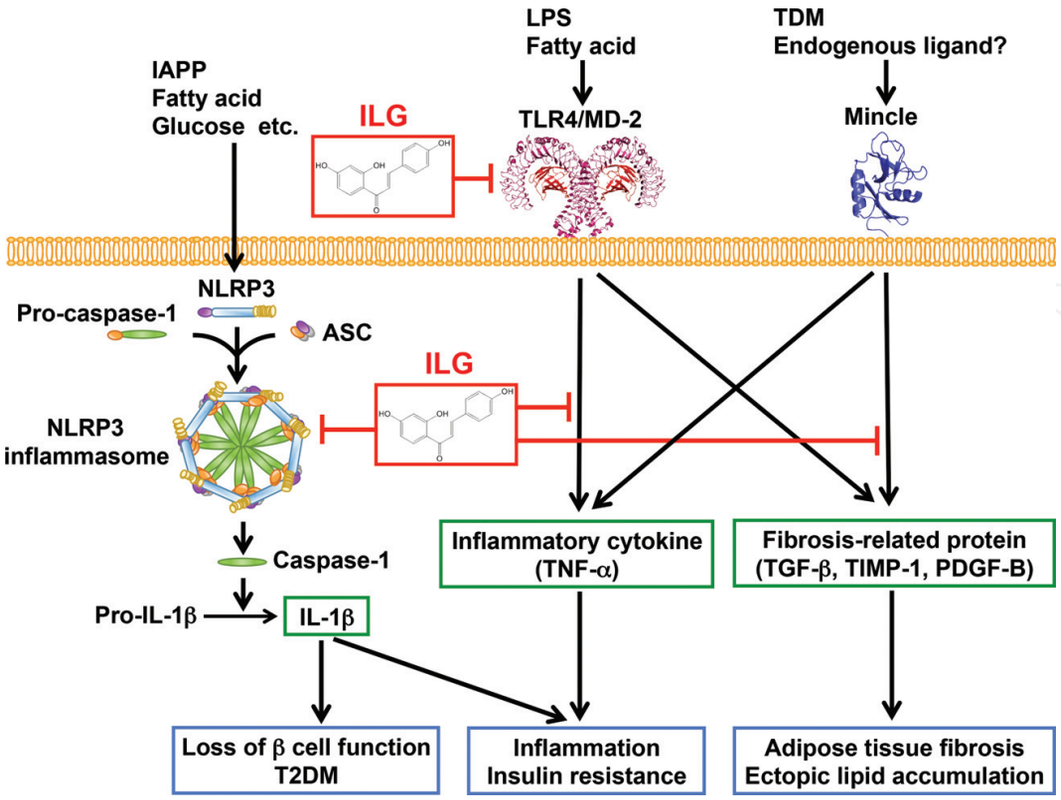

Figure 1. Schematic diagram of ILG-mediated suppression of PRR activation in macrophage. NLRP3 inflammasome is activated by various DAMPs, including IAPP and free FAs. The inflammasomes assemble into oligomeric complex with ASC and activate caspase-1. Activated caspase-1 processes pro-IL-1β into mature IL-1β. ILG potently inhibits IAPP-induced NLRP3 inflammasome activation. The LPS sensor TLR4/MD-2 also recognizes free FAs such as palmitic acid. ILG inhibits TLR4/MD-2-induced inflammation and fibrogenic responses in multiple steps, at the receptor level and the downstream signaling level. Mincle recognizes a mycobacterial glycolipid TDM, also named cord factor. TDM-stimulated inflammatory and fibrogenic responses are attenuated by ILG treatment. Because HFD-induced adipose tissue inflammation and fibrosis are greatly improved in Mincle-deficient mice compared with WT mice, it is suggested that Mincle may recognize an endogenous ligand derived from inflamed tissues, resulting in the induction of inflammation and fibrosis.

Furthermore, ILG supplementation inhibited IL-1β and caspase-1 production in eWAT from wild-type mice fed with HFD for 4 weeks. At this time point, TNF-α production was not increased in eWAT from HFD-fed mice compared with that from normal diet-fed mice. Thus, inflammasome activation occurs in eWAT at an early time point during obesity before TNF-α-associated inflammation.

Because ILG inhibits the priming and activation steps of the inflammasome via TLR4 and NLRP3, respectively, the therapeutic effects of ILG on HFD-induced adipose tissue inflammation and insulin resistance may be attributed to the inhibition of not only NLRP3 but also TLR4 pathways. Indeed, TLR4 is shown to have a critical role in the pathogenesis of obesity-induced inflammation using TLR4-deficient mice [13, 29]. Therefore, ILG is a potential therapeutic agent that targets NLRP3- and TLR4-associated adipose tissue inflammation in obesity.

3.2.2. Inhibitory effects of isoliquiritigenin on PRR-mediated adipose tissue fibrosis

In addition to inflammation, fibrosis may have an important role in adipose tissue dysfunction [9]. Because TLR4 signaling in immune cells has a key role in the development of obesity- and endotoxin-mediated adipose tissue fibrosis [50], we examined whether ILG attenuated TLR4-stimulated expression of fibrosis-related genes in peritoneal macrophages and stromal vascular fraction (SVF) of obese eWAT. Lipid A stimulation increased the expression of fibrosis-related genes, such as TGF-β and TIMP-1 (tissue inhibitor of metalloproteinase-1) in these cells. These increases were significantly attenuated by ILG stimulation (**Figure 1**).

Mincle stimulation is also crucial for fibrogenesis in SVF of obese adipose tissue [22]. The SVF from HFD-fed mice was stimulated with a Mincle ligand trehalose-6,6′-dimycolate (TDM), a mycobacterial cell wall glycolipid [22, 51]. TDM stimulation significantly increased TIMP-1 and PDGF-B mRNA expression in the SVF and these increases were significantly attenuated by ILG stimulation [51] (**Figure 1**).

Finally, we examined whether ILG improved HFD-induced adipose tissue fibrosis. Histological analysis revealed that HFD treatment induced extensive interstitial fibrosis in eWAT, which was markedly suppressed by ILG supplementation (0.5% w/w in HFD) [51]. HFD treatment increased collagen 1, TGF-β, TIMP-1, and PDGF-B mRNA expression in eWAT. These expressions were markedly decreased by ILG supplementation. These results highlight that ILG is a promising therapeutic candidate for HFD-induced adipose tissue fibrosis by suppressing the activation of innate immune sensors.

4. Concluding remarks

It has now become clear that *G. uralensis*-derived components including ILG have a major impact in innate immunity to prevent adipose tissue inflammation and fibrosis. On the other hand, ILG also acts on adipocytes, and consequently suppresses inflammatory changes elicited by macrophage-derived mediators such as TNF-α [51], suggesting that ILG targets multiple cells that constitute adipose tissue. Moreover, activation of various innate immune sensors is affected by ILG stimulation, consequently suppressing adipose tissue inflammation and fibrosis. A better understanding of these mechanisms will be addressed in the near future. With these new findings, we will be enabled to design better therapeutic strategies based on innate immunity through the usage of ILG to combat obesity-associated diseases.

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Conflict of interest statement

The authors declare no competing financial interests.

Abbreviations

AIM2	Absent in melanoma 2
ATP	Adenosine triphosphate
DAMP	Danger-associated molecular pattern
eWAT	Epididymal white adipose tissue
FA	Fatty acid
GL	Glycyrrhizin
G. uralensis	*Glycyrrhiza uralensis*
HFD	High-fat diet
IAPP	Islet amyloid polypeptide
ITAM	Immunoreceptor tyrosine-based activation motifs
ILG	Isoliquiritigenin
MAPK	Mitogen-activated protein kinases
Mincle	Macrophage-inducible C-type lectin
MSU	Monosodium urate
MZ	Marginal zone
NLR	NOD-like receptors
NLRP3	Nucleotide-binding domain, leucine-rich repeats containing family, pyrin domain-containing-3
NOD	Nucleotide oligomerization domain
PRR	Pattern recognition receptor
RP105	Radioprotective 105
SVF	Stromal vascular fraction
TDM	Trehalose-6,6′-dimycolate
TIMP-1	Tissue inhibitor of metalloproteinase-1
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T2DM	Type 2 diabetes mellitus
WT	Wild-type

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