Involvement of Cbl-b-mediated macrophage inactivation in insulin resistance

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Abstract

Aging and overnutrition cause obesity in rodents and humans. It is well-known that obesity causes various diseases by producing insulin resistance (IR). Macrophages infiltrate the adipose tissue (AT) of obese individuals and cause chronic low-level inflammation associated with IR. Macrophage infiltration is regulated by the chemokines that are released from hypertrophied adipocytes and the immune cells in AT. Saturated fatty acids are recognized by toll-like receptor 4 (TLR4) and induce inflammatory responses in AT macrophages (ATMs). The inflammatory cytokines that are released from activated ATMs promote IR in peripheral organs, such as the liver, skeletal muscle and AT. Therefore, ATM activation is a therapeutic target for IR in obesity. The ubiquitin ligase Casitas b-lineage lymphoma-b (Cbl-b) appears to potently suppress macrophage migration and activation. Cbl-b deficiency enhances ATM accumulation and IR in aging- and diet-induced obese mice. Cbl-b inhibits migration-related signals and SFA-induced TLR4 signaling in ATMs. Thus, targeting Cbl-b may be a potential therapeutic strategy to reduce the IR induced by ATM activation. In this review, we summarize the regulatory functions of Cbl-b in ATMs.

Key words: Casitas b-lineage lymphoma-b; Insulin resistance; Macrophage; Obesity; Toll-like receptor 4

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Core tip: Obesity leads to the development of chronic inflammation and insulin resistance (IR). Adipose tissue macrophages (ATMs) play a crucial role in the development of obesity-induced IR. Therefore, ATMs are attractive therapeutic targets for IR. Recently, we demonstrated that the ubiquitin ligase Casitas b-lineage lymphoma-b (Cbl-b) negatively regulates the migration and activation of ATMs.
Here, we review key aspects of Cbl-b function in the regulation of ATMs.

INTRODUCTION

In 2014, more than 1.9 billion adults were overweight, and of these, over 600 million were obese[4]. Obesity is a risk factor for the development of insulin resistance (IR), diabetes mellitus, hepatic steatosis and hypertension[5], resulting in escalating healthcare costs in several developed countries. Thus, it is important to elucidate the mechanism for obesity-associated IR and develop attractive therapeutic strategies for treating IR. A combination of various factors, such as diet, lifestyle, genetic background, psychological stress and aging, leads to obesity. In particular, aging and nutritional excess play critical roles in the development of obesity.

Aging causes decreases in physical activity, lean body mass and anti-oxidant defenses, thus increasing oxidative stress and the number of damaged cells[6]. These changes are associated with lipid accumulation in white adipose tissue (WAT) due to decreased energy expenditure. The oxidative stress induced by aging causes mitochondrial dysfunction and muscle atrophy. Sarcopenia, aging-induced skeletal muscle loss, decreases energy expenditures and causes obesity[7]. An excessive intake of carbohydrates and lipids causes the accumulation of triacylglycerols in adipocytes, which produces expansion of the adipocyte. Obesity causes inflammatory responses in WAT. It is well-known that in addition to its roles in fat storage, AT also plays key roles in endocrine system. AT secretes lipids, adipokines and chemokines to maintain homeostasis. The hypertrophy of the AT alters adipokine and chemokine secretion[2,5]. It is well-known that diverse immune cells reside in WAT of both lean and obese individuals, and these cells release inflammatory cytokines during obesity. Resident eosinophils and regulatory CD4+ helper T cells maintain homeostasis in the AT of lean subjects[6]. In contrast to CD4+ T cells, CD8+ T cells increase in number in the AT of obese subjects and promote the inflammatory responses mediated by macrophages[7]. AT macrophages (ATMs) also release various inflammatory mediators. Because ATMs play a key role in obesity-associated inflammatory action, the suppression of ATM activation is a potent therapeutic strategy for treating IR induced by obesity. Recently, several studies demonstrated that the ubiquitin ligase Casitas b-lineage lymphoma-b (Cbl-b) is a key regulator of macrophage activation[8-10]. Here, we review the key roles of Cbl-b in ATM activation and the pathogenesis of IR in obesity.

THE UBIQUITIN LIGASE CBL-B

In mammalian cells, there are three major intracellular protein degradation pathways. The calpain pathway, the autophagy-lysosome pathway, and the ubiquitin (Ub)-proteasome system play important roles in maintaining cellular homeostasis. In particular, the Ub-proteasome system is regulated by three types of enzymes: A Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2) and a Ub ligase (E3). In the initial step, the activation of Ub proteins by E1 enzymes is critically dependent on the presence of ATP. An E1 enzyme transfers a Ub protein to E2 enzyme. And then, the E2 enzymes shuttle a Ub protein to an E3 enzyme, which ubiquitinates the specific target protein. The proteins tagged with Ub are specifically degraded by the proteasome. Therefore, E3 enzymes are important for determining the specific target proteins that will be degraded by proteasome[11].

The Cbl proteins in mammalian (c-Cbl, Cbl-b and Cbl-c), which were originally identified as adaptor molecules, function as ubiquitin ligases (Figure 1). A number of studies show that Cbl proteins inhibit the signal transduction by receptor and non-receptor tyrosine kinases[12-14]. The protein tyrosine kinase-binding (TKB) and really interesting new gene (RING) finger (RF) domains are highly conserved in the N-terminal domains of all Cbl homologues. The TKB domain, which is a specific domain in Cbl proteins, binds to the phosphorylated tyrosines of the substrates through Src-homology (SH) 2 domains[15]. The RF catalytic domain has the E3 ubiquitin ligase activity because it binds to E2 enzymes[16]. Cbl-b is a substrate of tyrosine kinases, and the ubiquitin ligase activity is regulated by the phosphorylation of some tyrosine residues[14,17,18]. Increasing evidence indicates that Cbl-b is abundantly expressed in leukocytes and decreases the activation of various immune cells. Therefore, loss-of-function mutations of Cblb cause various autoimmune diseases[19-21]. Interestingly, a Cblb mutation was identified as factor associated with diabetes in a rat model of human type 1 diabetes[20,22]. Yokoi et al[22] reported that F328L is a loss-of-function mutation in T cells that was identified in Japanese subjects. These studies reveal that the function of Cbl-b is connected to diabetes.

INFLAMMATORY ACTIONS OF MACROPHAGES IN ADIPOSE TISSUE

Various immune cells, such as macrophages, T cells, mast cells, natural killer cells and eosinophils, reside in WAT along with adipocytes. The expansion of adipocytes alters these populations in WAT. ATMs increase the number of cells in the AT of obese mice[23]. ATMs play important roles in the AT of lean and obese humans and rodents. In the AT of lean subjects, resident M2-like or alternatively activated ATMs preferentially maintain homeostasis by secreting anti-inflammatory cytokines. In contrast, in obesity, the M1-like or classically activated ATMs in WAT induce inflammation mediated by the release of inflammatory cytokines and
Cbl-b proteins contain highly conserved tyrosine kinase-binding (TKB), linker (L), RING finger (RF) and proline-rich (PR) domains. 4H: Four-helix bundle; SH2: Src-homology 2; UBA: Ubiquitin-associated domain.

Figure 1  The primary structure and domain organization of human Casitas b-lineage lymphoma family proteins. Cbl-b proteins contain highly conserved tyrosine kinase-binding (TKB), linker (L), RING finger (RF) and proline-rich (PR) domains. 4H: Four-helix bundle; SH2: Src-homology 2; UBA: Ubiquitin-associated domain.

Cbl-b (982aa)

Cbl-c (474aa)

Cbl-b (906aa)

chemokines. ATMs are activated by saturated fatty acids (SFAs) through toll-like receptor 4 (TLR4). Although TLR4 was identified as the receptor for lipopolysaccharide (LPS), which is a component of the outer membrane of gram-negative bacteria, SFAs also activate TLR4 signaling in macrophages. The global mutation or the bone marrow-specific deficiency of TLR4 abrogated the systemic IR induced by the consumption of a high-fat diet (HFD). However, the molecular mechanism of TLR4 activation by SFAs is poorly understood. It is thought that SFAs fail to directly bind to TLR4. A recent study showed that SFAs activate the TLR4 signaling mediated by fetuin-A, a 64 kDa glycoprotein released from the liver in response to HFD consumption. Fetuin-A mediates SFA-induced activation of TLR4 by directly interacting with TLR4 in macrophages and adipocytes. Interestingly, treatment with the insulin sensitizer pioglitazone suppresses fetuin-A expression through peroxisome proliferator-activated receptor-γ activation in hepatoma cells. SFA treatments induce the activation of nuclear factor κB (NF-κB) and Jun N-terminal kinase (JNK), which are TLR4 signaling molecules in macrophages. In fact, the inhibition of NF-κB or JNK ameliorates IR by activating ATMs in obese rodents. Therefore, the regulation of ATM activation is a potent therapeutic target for obesity-associated IR.

CBL-B IN ATM RECRUITMENT

Aging and overnutrition cause the hypertrophy of AT, resulting in the accumulation of ATMs. The activated ATMs induce peripheral and systemic IR through the release of inflammatory cytokines. JNK is a TLR4 signaling molecule and mediates the expression of inflammatory cytokines in macrophages. Bone marrow-specific deficiency of JNK1 ameliorated diet-induced IR by suppressing AT inflammation in mice. We demonstrated that depletion of Cbl-b exacerbated obesity and IR induced by aging and HFD in mice. We also found that ATM activation was enhanced in Cbl-b knockout (Cbl-b-/-) mice. In 30-wk old Cbl-b-/- mice, we observed hypertrophy of AT, IR, hepatic steatosis and β cell dysfunction (Table 1). Interestingly, the ATM accumulation was dramatically increased in WAT. This event was caused by two factors in Cbl-b-/- mice. One factor was the high levels of monocyte chemotactic protein (MCP)-1/CC chemokine ligand 2 protein in circulation and WAT. MCP-1 is a member of CC chemokines, and causes the chemotaxis of leukocytes. Previous reports demonstrated that MCP-1 and CC chemokine receptor type 2 (CCR2), the receptor for MCP-1, are associated with obesity-induced IR, inflammation and ATM accumulation. In addition, CCR2 causes hepatic infiltration of macrophages and steatosis in mice. Taken together, the data indicate that the inhibition of CCR2 is a potent therapeutic strategy for treating obesity-induced inflammation and IR.

Furthermore, it is known that Cbl-b decreases the migration-related signaling in macrophages. Macrophage migration is regulated by activation of the guanine nucleotide exchange factor Vav1. Previous studies demonstrated that phosphorylation of Vav1 at Tyr267 mediated by spleen tyrosine kinase (Syk) is critical for Vav1 activation in leukocytes. Cbl-b directly binds to Vav1 in T cells. Although Vav1 phosphorylation is inhibited by Cbl-b, Cbl-b does not induce the degradation of Vav1. We also demonstrated that the depletion of Cbl-b promoted tyrosine phosphorylation in Vav1 in peritoneal macrophages from mice. These results indicated that the increased MCP-1 released from WAT and Vav1 phosphorylation cause ATM accumulation in Cbl-b-/- mice. Thus, Cbl-b may serve as a therapeutic target to reduce the IR mediated by the accumulation of ATMs.

CBL-B IN TLR4 SIGNALING

Several ubiquitin ligases have been identified as negative regulators of TLR4 signaling. Triad3A is a RF ubiquitin ligase and directly binds to TLR4, resulting in ubiquitination and proteolytic degradation. Recent reports indicate that TLR4 signaling is inhibited by Cbl-b in macrophages and neutrophils. Han et al. demonstrated that TLR4 signaling induced by LPS was suppressed in macrophages.
by Cbl-b-mediated ubiquitination and breakdown of toll/IL-1 receptor domain-containing adapter inducing interferon-β (TRIF) and MyD88, which are adaptor molecules for TLR4 signal transduction. This suppression by Cbl-b was dependent on the presence of integrin αM (CD11b). In neutrophils, Cbl-b also suppresses LPS signaling by preventing the formation of the TLR4-MyD88 complex. These reports suggest that Cbl-b is a critical regulator of the macrophage activation mediated by LPS-induced TLR4 signaling.

TLR4 activation by SFAs thought to play a pivotal role in ATM activation-induced IR. Diet-induced obesity increases the circulating levels of free FAs. SFAs directly induce IR in the liver, skeletal muscle and AT. Furthermore, SFAs cause chronic inflammation through ATM activation, which is mediated by TLR4 signal transduction. Recently, we demonstrated that the knockout of Cbl-b promoted and IR through ATM accumulation in HFD-fed mice. In addition to increased ATM accumulation, inflammatory cytokine secretion was increased in the AT of obese Cbl-b−/− mice. In addition to aging, the consumption of a HFD increases MCP-1 expression in WAT. We found that depletion of Cbl-b in murine peritoneal macrophages promotes SFA-induced TLR4 signal transduction (Figure 3). Palmitate-induced JNK phosphorylation and IL-6 expression were enhanced in Cbl-b-deficient peritoneal macrophages. We also showed that TLR4 is a substrate for Cbl-b in the presence of SFAs. Overexpression of Cbl-b increased the ubiquitination and breakdown of TLR4 after palmitate treatment. Consistent with this finding, the TLR4 protein expression levels on the surface of Cbl-b-deficient peritoneal macrophages were increased. It is well known that LPS treatment induces the phosphorylation of 2 tyrosine residues of human TLR4. The phosphorylation of TLR4 is required to activate signaling by promoting an interaction with Syk in macrophages. It remains unknown whether SFAs also induce the TLR4 tyrosine phosphorylation in macrophages. Although LPS induces the ubiquitination and degradation of MyD88 and TRIF, SFAs do not induce these pathways in macrophages. These differences between LPS and SFAs are not fully understood. Further investigations are needed to elucidate the mechanism of SFA-induced phosphorylation of TLR4.

Recently, Lu et al. reported that treatment with a TLR4 antagonist improves insulin sensitivity and macrophage accumulation in the atherosclerotic lesions of low-density lipoprotein receptor-deficient mice. We demonstrated the TLR4 signaling was strongly associated with the development of IR in obese Cbl-b−/− mice using eritoran, a TLR4 antagonist. The eritoran treatment reduced the insulin sensitivity and glucose tolerance in obese Cbl-b−/− mice. This phenomenon may be caused by a decrease in ATM accumulation. In fact, we found that an anti-TLR4 antibody inhibited SFA-induced TLR4 signal transduction in murine peritoneal macrophages. Our data suggest that TLR4 antagonists are potent therapeutic drugs that can be used to treat the IR mediated by ATM activation.

CONCLUSION

Obesity causes various diseases through the development of IR, which is a clinical feature of patients with type 2 diabetes. Prediabetes is defined as impaired fasting glucose, impaired glucose tolerance and/or high levels of plasma glycated hemoglobin and is a critical risk factor for cardiovascular diseases. AT inflammation is thought to be associated with the onset of prediabetes. Therefore, to prevent type 2 diabetes, the development of an effective therapeutic strategy for obesity-induced IR is urgently needed.

Aging- and diet-induced obesity causes the IR mediated by ATM activation. However, the mechanisms underlying ATM activation are poorly understood. We showed that Cbl-b reduces IR by suppressing macrophage migration and activation in mice. However, several questions remain about the biological implication of Cbl-b in human cells. The molecular mechanism underlying the effects of Cbl-b

### Table 1 Phenotypes of Cbl-b−/− mice

<table>
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<tr>
<th>Age and diet</th>
<th>Phenotypes</th>
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<tbody>
<tr>
<td>30-wk old, normal diet</td>
<td>Adipose tissue inflammation</td>
<td>[36]</td>
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<tr>
<td>30-wk old, normal diet</td>
<td>Adiposity</td>
<td>[35]</td>
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<td>30-wk old, normal diet</td>
<td>Fasting hyperinsulinemia</td>
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<tr>
<td>30-wk old, normal diet</td>
<td>Hepatic steatosis</td>
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<td>30-wk old, normal diet</td>
<td>Impaired glucose tolerance</td>
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<td>30-wk old, normal diet</td>
<td>Insulin resistance</td>
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<tr>
<td>30-wk old, high-fat diet</td>
<td>Adipose tissue inflammation</td>
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<tr>
<td>13-wk old, high-fat diet</td>
<td>Insulin resistance</td>
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Figure 2 Casitas b-lineage lymphoma-b suppresses macrophage migration. Monocyte chemoattractant protein (MCP)-1 causes macrophages to infiltrate adipose tissue via C-C chemokine receptor 2 (CCR2). Phosphorylation (P) of Vav1 mediates macrophage migration, and Cbl-b negatively regulates macrophage migration by suppressing Vav1 phosphorylation.
in macrophages is unknown. Further investigations are essential to identify new tyrosine kinases for Cbl-b. Recently, it was shown that macrophages infiltrate the fatty liver and AT in obesity. Cbl-b may suppress the macrophage activation in fatty liver. The side effects of Cbl-b activation remain unclear. We also showed that Cbl-b disturbed insulin-like growth factor signaling through ubiquitination and degradation of insulin receptor substrate-1 in skeletal muscle under unloading conditions\[61\]. Although we did not observe an enhancement of insulin signal transduction in lean Cbl-b\(-/-\) mice, tissue-specific Cbl-b activation may be important when using a drug delivery system, such as liposomes. A better understanding of Cbl-b-mediated ATM activation may provide the basis for developing novel therapeutic strategies that can be used to treat IR.

REFERENCES


22. Yokoi N, Hayashi C, Fujiiwara Y, Wang HY, Seino S. Genetic reconstitution of autoimmune type 1 diabetes with two major
susceptibility genes in the rat. *Diabetes* 2007; 56: 506-512 [PMID: 17259398 DOI: 10.2337/db06-1027]


Inflammatory Responses by Two Sequential and Distinct Mechanisms. 

*J Immunol* 2015; 195: 4415-4425 [PMID: 26392463 DOI: 10.4049/jimmunol.1500859]


60 **Festa A**, Hanley AJ, Tracy RP, D’Agostino R, Haffner SM. Inflammation in the prediabetic state is related to increased insulin resistance rather than decreased insulin secretion. *Circulation* 2003; 108: 1822-1830 [PMID: 14517163 DOI: 10.1161/01.CIR.0000091339.70120.53]


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