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Original Article

Soluble CLEC2 Extracellular Domain Improves Glucose and Lipid Homeostasis by Regulating Liver Kupffer Cell Polarization

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ABSTRACT

The polarization of tissue resident macrophages toward the alternatively activated, anti-inflammatory M2 phenotype is believed to positively impact obesity and insulin resistance. Here we show that the soluble form of the extracellular domain (ECD) of C-type lectin-like receptor 2, CLEC2, regulates Kupffer cell polarization in the liver and improves glucose and lipid parameters in diabetic animal models. Over-expression of Fc-CLEC2(ECD) in mice via in vivo gene delivery, or injection of recombinant Fc-CLEC2(ECD) protein, results in a reduction of blood glucose and liver triglyceride levels and improves glucose tolerance. Furthermore, Fc-CLEC2(ECD) treatment improves cytokine profiles and increases both the M2 macrophage population and the genes involved in the oxidation of lipid metabolism in the liver. These data reveal a previously unidentified role for CLEC2 as a regulator of macrophage polarity, and establish CLEC2 as a promising therapeutic target for treatment of diabetes and liver disease.

1. Introduction

Chronic inflammation is thought to contribute to the development of obesity and metabolic syndromes (Hotamisligil, 2006; Shoelson et al., 2006). Aberrant pro-inflammatory immune responses are found in many organs of diabetic individuals, including the pancreas, liver, adipose, heart, brain, and muscle (Lumeng and Saltiel, 2011). For example, many pro-inflammatory proteins, including TNF-α, interleukin 6 (IL-6) and inducible nitric oxide synthase, secreted from adipose tissue macrophages (ATM), are found at higher levels in adipose tissue from obese individuals compared to lean individuals (Harkins et al., 2004; Hotamisligil, 2006). Increased adiposity promotes macrophage infiltration and local inflammation, which in turn contributes to increasing insulin resistance (Weisberg et al., 2003; Xu et al., 2003). Inflammatory responses in the liver, another major metabolic organ, have also been implicated in obesity, type 2 diabetes and fatty liver diseases. Activation of the resident macrophages in the liver, Kupffer cells, induces hepatotoxicity in obese mice (Li and Diehl, 2003) and regulates hepatic glucose metabolism and insulin resistance (Huang et al., 2010; Lanthier et al., 2010).

Macrophages are derived from monocyte precursors and undergo specific differentiation and activation depending on the local tissue environment and cytokine milieu (Steinman and Idoyaga, 2010). Two distinct states of polarized activation for macrophages have been defined: the classically activated macrophage phenotype, M1, and the alternatively activated macrophage phenotype, M2 (Gordon and Taylor, 2005; Mantovani et al., 2002). M1 macrophages are effector cells in T_H1 cellular immune responses, whereas M2 macrophages appear to promote immune suppression and wound healing/tissue repair (Gordon and Taylor, 2005; Mantovani et al., 2002). Recent evidence demonstrates that in lean animals, higher numbers of macrophages are M2 polarized, possessing anti-inflammatory potential by producing IL-10, while obesity drives pro-inflammatory M1 polarization (Lumeng et al., 2007a,b; Mjosberg et al., 2011). Thus, the M1/M2 switch may occur within local tissues such as fat and liver (Kang et al., 2008; Odegaard et al., 2008), and the balance between M1 and M2 macrophages contribute to the onset of insulin resistance (Charo, 2007; Lumeng et al., 2007a,b). Locally produced T_H2-type cytokines, such as IL-4 and IL-13, and activation of peroxisome proliferator-activated receptor δ/β (PPARδ/β) or PPARγ, result in the activation of M2 macrophages. Disruption of either PPARδ/β or PPARγ in myeloid cells may impair the alternative activation of M2 macrophages in the adipose tissue and liver, resulting in impaired glucose tolerance and exacerbated insulin resistance under high fat diet conditions (Hevener et al., 2007; Kang et al., 2008; Odegaard et al., 2007, 2008).

C-type lectin-like receptor 2 (CLEC2) was initially identified through a computational approach searching for sequences similar to known

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C-type lectin-like receptors expressed on immune cells (Colonna et al., 2000). CLEC2, a member of the type II transmembrane C-type lectin-like receptor family, has a single YXXL/hemi-ITAM (immuno-receptor tyrosine-based activation motif) within its cytoplasmic domain. Expression of CLEC2 has been detected on the surface of platelets and a number of different immune cells, including dendritic cells, neutrophils, and Kupffer cells (Colonna et al., 2000; Mourao-Sa et al., 2011; Tang et al., 2010). The gene encoding CLEC2 is located in a genetic locus proximal to a distinct cluster of related receptors, including CLEC7A, LOX-1 and CLEC9A; most of which are expressed in myeloid populations (Sobanov et al., 2001).

The first identified ligand for CLEC2 was rhodocytin, a toxin from snake venom that induces platelet aggregation (Hooley et al., 2008; Suzuki-Inoue et al., 2006). More recently, podoplanin, a membrane glycoprotein, was proposed as an endogenous ligand for CLEC2 (Christou et al., 2008; Kato et al., 2008; Suzuki-Inoue et al., 2007). The interaction between CLEC2 and podoplanin is critical for the separation of blood and lymphatic vessels during embryonic development and during some pathophysiological conditions, such as tumor metastasis (Bertozzi et al., 2010). The interaction between the two proteins during embryogenesis is exemplified by the finding that mice deficient for CLEC2 display a similar phenotype as mice deficient for podoplanin, including bleeding and defects in vascular connections. However, in the normal adult state, while CLEC2 is predominantly expressed on cells located within blood vessels, podoplanin is expressed on cells lining lymphatic vessels (Suzuki-Inoue et al., 2007) and thus interaction between the two is unlikely. Therefore, it is possible that other, yet unidentified CLEC2 ligands may exist.

Besides playing a role in platelet aggregation (Chang et al., 2010; Kerrigan et al., 2009; Mourao-Sa et al., 2011), a function for CLEC2 on other immune cells has yet to be defined. Here we show that soluble CLEC2 regulates Kupffer cell polarization in the liver and improves glucose and lipid parameters in diabetic animals, thus revealing a novel physiological role for CLEC2 in both inflammation and metabolism. Our results demonstrate a previously unknown connection between CLEC2 and glucose and lipid metabolism, and support CLEC2 as a potential target for treating diabetes.

2. Materials and Methods

2.1. Fc-CLEC2(ECD)

To design a stable soluble CLEC2 protein that may block endogenous CLEC2 activity, the C-terminal extracellular domain of murine CLEC2 (51-229) was fused with a human Fc protein at its N-terminus connected by a flexible glycine linker (G4SG4), yielding Fc-CLEC2(ECD). DNA vectors for hydrodynamic injection, recombinant AAV preparation and recombinant protein expression all carry the same coding sequence for this Fc-CLEC2(ECD) protein. Recombinant Fc-CLEC2(ECD) was expressed through transient transfection of HEK293 cells and purified via Protein A affinity chromatography.

2.2. Animal Studies

All animal experiments were approved by the Institutional Animal Care and Use Committee of Amgen and cared for in accordance to the *Guide for the Care and Use of Laboratory Animals*, 8th Edition (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al., 2011). Mice were housed in an air-conditioned room at 22 ± 2 °C with a 12 hour light and 12 hour darkness cycle (0600–1800 h). Mice were randomized into control and treatment groups to achieve similar baseline levels of body weight, fasting glucose and insulin levels. No animals were excluded from the statistical analysis, and data collection was blinded in the studies.

The efficacy of recombinant Fc-CLEC2(ECD) protein was tested in 14-week-old, male B6D2F1/J mice (The Jackson Laboratory) fed for at least 8 weeks with a 60% fat diet, D12492 (Research Diet). The mice received intraperitoneal injection of Fc-CLEC2(ECD) protein diluted in 0.2 ml PBS. The control group received intraperitoneal injections of PBS. Injections were performed 1 to 2 h prior to the dark phase of the light cycle except on days when oral glucose tolerance test (oGTT) was performed. Proteins were then administered 2–3 h before fasting glucose measurement.

Hydrodynamic Tail Vein (HTV) studies were carried out in 12-week-old male B6D2F1/J mice (The Jackson Laboratory) fed with a 60% high fat diet for 6 weeks. Mice were randomized in respective groups based on body weight and both fasting (4 h) blood glucose and serum insulin levels, all of which measured the day before HTV injection. An endotoxin free DNA construct expressing Fc-CLEC2(ECD) was diluted in a saline solution to the concentration of 8 μg/ml. The injection volume was calculated based on body weight, approximately 100 ml/kg, but never exceed 2.5 ml per animal. The DNA solution was injected into the tail vein of mice within a 5–8 second timeframe. The construct carrying human Fc protein alone was used as a negative control.

Recombinant adeno-associated virus (rAAV) expressing Fc-CLEC2(ECD) was produced by transient transfection into 293T cells using the helper-free system, purified by gradient centrifugation, buffer exchanged. Mice were injected through the tail vein with 2–8 × 10^{12} virus particles per mouse of either Fc-CLEC2(ECD), or an empty vector (EV) as the negative control, in PBS.

An oGTT was performed after 4 h of fasting. Mice were injected with a bolus of glucose (10 ml/kg body weight of 20% glucose) into the stomach by a gavage needle (20 G × 1.5 in.) (Popper and Sons). Blood glucose levels were measured with a glucometer from tail tip blood collected at 0, 20, 40, 60, and 90 min after glucose dosing. Serum insulin levels were measured using a mouse insulin ELISA kit (ALPCO Diagnostics). The assay was performed as described by the manufacturer's protocol.

Hepatic triglyceride content was determined using homogenized liver extracts using chloroform/methanol (2:1 v/v) and lysed using a Qiagen tissue lyzer for 30 s to 1 min. Samples were first transferred to 12 × 75 mm glass test tubes and incubated at room temperature for 30–45 min. Samples were then washed with 50 mM NaCl, vortexed, centrifuged at 1500 *g* for 10 min and the organic phase was removed and placed into a new glass tube. The organic phase was washed twice with 0.36 M $CaCl_2$/methanol and centrifuged at 1500 *g* for 10 min. The triglyceride levels were measured using an Infinity triglyceride assay kit (Thermo Scientific).

Serum cytokine measurements were performed using the Bio-Plex Pro cytokine multiplex assay (BioRad) to measure the levels of cytokines in serum samples. Samples were diluted 2-fold in Bio-Plex sample diluent for the assay. The assay was performed according to the manufacturer's protocol. Plates were read on a Bio-Plex system and data was obtained using Bio-Plex Manager software.

2.3. H&E Staining and Immunohistochemistry

All collected tissues were fixed in 10% Neutral Buffered Formalin (NBF) for 24 h, processed to paraffin blocks, and cut into 4 micron sections. The sections were dried overnight in a 37 °C oven, followed by 1 hour incubation in a 60 °C oven prior to deparaffinization. Deparaffinization and H&E staining (Surgipath, Buffalo Grove, IL, USA) were performed on an automated multistainer (Leica ST 5020, Buffalo Grove, IL, USA). The F4/80 antibody, an IgG2b affinity purified rat monoclonal antibody, was purchased from AbD Serotec. The α smooth muscle actin antibody was purchased from Abcam. Morphometric analysis was performed using a Scan Scope XT (Aperio) and both Image Scope (Aperio) and Indica Lab (Indica Lab) software. For each animal, nine 3.6 × 10^5 μm^2 areas were evaluated for the percent F4/80 positive staining per area of tissue.