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C5aR and C5L2 act in concert to balance immunometabolism in adipose tissue

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ABSTRACT

Recent studies suggested that the immunometabolic receptors; C5aR and C5L2, constitutively self-associate into homo-/heterodimers and that acylation stimulating protein (ASP/C3adesArg) or C5a treatment of adipocytes increased their colocalization. The present study evaluates the C5aR contribution in adipocytes to the metabolic and immune responses elicited by ligand stimulation.

The effects of C5a, ASP, and insulin on cytokine production, triglyceride synthesis (TGS), and key signaling pathways were evaluated in isolated primary adipocytes and cultured 3T3-L1 differentiated adipocytes. In addition, mRNA expression of *IRS1* and *PGC1α* was compared in adipose tissue samples from WT vs. C5aRKO mice.

Both C5a and ASP directly increased MCP-1 (238 ± 4%; $P < 0.001$, and 377 ± 2% vs. basal 100%; $P < 0.001$, respectively) and KC (413 ± 11%; $P < 0.001$, and 529 ± 16%; $P < 0.001$ vs. basal 100%, respectively) secretion, TGS (131 ± 1%; $P < 0.001$, and 152 ± 6%; $P < 0.001$, vs. basal 100% respectively), and Akt/NFκB phosphorylation pathways in adipocytes. However, in C5aRKO adipocytes, C5a effects were disrupted, while stimulatory effects of ASP were mostly maintained. Addition of C5a completely blocked ASP signaling and activity in both C5aRKO and WT adipocytes as well as 3T3-L1 adipocytes. Furthermore, C5aRKO adipocytes revealed impaired insulin stimulation of cytokine production, with partial impairment of signaling and TGS stimulation, consistent with decreased *IRS1* and *PGC1α* mRNA expression in adipose tissue.

These observations indicate the importance of C5aR in adipose tissue metabolism and immunity, which may be regulated through heterodimerization with C5L2.

1. Introduction

It is now well accepted that adipocytes play a dynamic role in metabolic regulation, with active synthesis and secretion of cytokines, adipokines, complement proteins, and molecules associated with inflammation (MacLaren et al., 2008). Investigations have revealed major contributions of adipocytes to immunity, with adipocytes interacting and signaling with immune cells, including both resident and infiltrating macrophages, within the microenvironment of adipose tissue (Klos et al., 2009; Poursharifi et al., 2013; Tom et al., 2013). Recently, complement protein/receptor interactions with adipokines have attracted attention, with "immunometabolism" being the subject of a number of reviews (MacLaren et al., 2008; Nikolajczyk et al., 2012; Schâffler and Schôlmerich, 2010; Schipper et al., 2012).

Many inflammatory responses in the immune system are related to the complement cleavage fragments, C3a and C5a, also known as anaphylatoxins (Klos et al., 2009). These proteins mediate their signaling activities through several serpentine 7-transmembrane G protein-coupled receptors (GPCRs), which are expressed by various immune as well as non-immune cells (Klos et al., 2009). C5a is regarded as one of the most potent inflammatory factors known, and functions via its receptor C5aR, but also binds C5aR-like receptor 2 (C5L2) (Cui et al., 2009b; DeMartino

Abbreviations: ASP, acylation stimulating protein; C5L2, C5aR-like receptor 2; GPCR, G protein-coupled receptor; KC, keratinocyte chemoattractant; MCP-1, monocyte chemoattractant protein -1; TGS, triglyceride synthesis.

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et al., 1994; Ohno et al., 2000; Okinaga et al., 2003). Both C5L2 and C5aR are expressed on myeloid and non-myeloid cells such as adipocytes and preadipocytes, although *C5aR* mRNA levels are typically higher than *C5L2* (Bamberg et al., 2010; Chen et al., 2007; Ohno et al., 2000). As previously shown, phosphorylation of C5aR and C5L2 leads to association with β-arrestin proteins and internalization into clathrin-coated vesicles (Bamberg et al., 2010; Braun et al., 2003; Cui et al., 2009b). Notably, the complex array of C5a functional activities, including chemotaxis, enzyme/cytokine release, and the respiratory burst, are mostly attributed to its binding to C5aR (Okinaga et al., 2003), with stimulation of MAPK, ERK, diacylglycerol and Akt signaling pathways (Monk et al., 2007). While C5a binds C5L2 with the same high affinity as C5aR, the potential functions and signaling pathways mediated through C5L2 are still controversial (Cain and Monk, 2002; Okinaga et al., 2003).

C3a and acylation stimulating protein (ASP/C3adesArg) both stimulate triglyceride synthesis (TGS) and glucose transport in adipocytes (Cianflone et al., 1989; Kalant et al., 2005). Adipocytes produce C3, factor B, and adipsin, leading to production of ASP within the adipocyte microenvironment upon activation of the alternative complement pathway (Baldo et al., 1993). ASP induces Akt, MAPK, ERK1, and NFκb signaling pathways in 3T3-L1 adipocytes and/or preadipocytes (Maslowska et al., 2006; Poursharifi et al., 2013; Tom et al., 2013). Tom et al. demonstrated ASP stimulation of monocyte chemoattractant protein (MCP)-1 and keratinocyte chemoattractant (KC) production in adipocytes, an effect blocked with PI3-kinase and NFκB inhibitors (Tom et al., 2013). Further, complement C3 knockout mice, which are deficient in C3, the precursor of ASP, and therefore obligately deficient in ASP, demonstrated altered energy metabolism and fat storage (Paglialunga et al., 2008; Roy et al., 2008).

The two receptors, C5aR and C5L2, have been proposed to have closely linked physical and functional interactions (Bamberg et al., 2010; Poursharifi et al., 2013). Likewise, it has been demonstrated that C5aR and C5L2 are both capable of forming homo- and heterodimers (Poursharifi et al., 2013). Interestingly, both ASP and C5a have been found to stimulate internalization/colocalization of C5aR and C5L2 in J774 macrophages and 3T3-L1 adipocytes (Poursharifi et al., 2013). The consequences of homo- or heterodimerization are not yet clear; however, this could be linked to alternative signaling or regulatory cell- and ligand-dependent responses to severe inflammatory conditions or metabolic modulations. Further, the distinctive roles of C5aR and C5L2 in immunity and adipocyte metabolism are presently clouded by controversy.

The aim of the current study was to investigate (i) C5a–C5aR functions and signaling pathways and (ii) their possible regulatory effects on ASP/C5a signaling pathways in adipocytes using C5aR knockout (C5aRKO) mice and *in vitro* studies.

2. Methods

2.1. Materials

All tissue culture reagents, including Dulbeccos's modified Eagle's medium/F-12 (DMEM/F12), phosphate buffered saline (PBS), fetal bovine serum (FBS), trypsin and tissue culture supplies were from Gibco (Burlington, ON). Triglyceride (TG) mass was measured using an enzymatic colorimetric assay (Roche Diagnostic, Indianapolis, IN). Recombinant ASP (rASP) was prepared as previously described in detail (Cui et al., 2009a), and assessed for purity by mass spectrometry (Cui et al., 2009a; Murray et al., 1997), and was verified to be endotoxin-free. Recombinant C5a (rC5a) (purity ⩾95% by SDS–PAGE) was purchased from EMD Biosciences (Gibbstown, NJ). Physiological concentrations of C5a (20 nM) and ASP (100 nM) were used in all of the experiments.

2.2. Cell culture of 3T3-L1 preadipocytes and differentiation into mature adipocytes

3T3-L1 preadipocytes, obtained from the American Type Culture Collection (Manassas, VA), were maintained in DMEM/F12 supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in 5% CO_2. Differentiation was induced two days post confluence in medium containing 10 μg/ml insulin, 1.0 μM dexamethasone, and 500 μM isobutylmethylxanthine (IBMX). After three days, the differentiation cocktail was replaced with insulin supplementation for 2 more days, then changed to 10% FBS in DMEM/F12 only. Media was changed on fully differentiated 3T3-L1 adipocytes (⩾80% differentiated as determined by microscopic evaluation of multiple lipid droplets) every two days and were used for functional and signaling assays on days 9–10 after differentiation was initiated. Throughout all experiments, cells were transferred to serum free (SF) medium 2 h prior to the treatments.

2.3. Mice

BALB/c mice and C5aRKO mice on a BALB/c background were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed in a sterile barrier facility with a 12 h light: 12 h dark cycle. All protocols were pre-approved by the Laval University Animal Care Committee and were conducted in accordance with the Canadian Council of Animal Care (CACC) guidelines. Animals were fed a standard chow diet *ad libitum*.

2.4. Isolation and culture of primary mouse adipocytes

Mice were euthanized and gonadal fat pads from wild type (WT) and C5aRKO ($n = 9$–11 in each group) were collected in Krebs–Ringer buffer (KRB), pH 7.4. The tissue was minced and incubated in KRB buffer containing collagenase (collagenase type II, 0.1% (w/v)) at 37 °C for 45 min. The resulting suspension was filtered through a nylon mesh (250 μm) and separated into two parts (floating mature adipocytes and pelleting stromal cells) by low-speed centrifugation. The mature adipocyte fraction was rinsed three times with KRB buffer, and adipocytes were counted and aliquoted for the various experiments of the study.

2.5. Fluorescent fatty acid uptake into mature adipocytes

Uptake and incorporation into lipids of fluorescently-labeled fatty acid was measured using the QBT™ fatty acid uptake assay kit (Molecular Devices, Sunnyvale, CA) in 3T3-L1 adipocytes and primary adipocytes, according to the manufacturer's instructions. C5aR antagonist (3D53), which binds specifically to C5aR (Finch et al., 1999; Monk et al., 2007; Wong et al., 1998), was used to pre-treat 3T3-L1 adipocytes for 30 min prior to addition of the treatments where indicated.

2.6. Akt/NFκB/ERK phosphorylation

Total and phosphorylated Akt (Ser^{473}), ERK ($Thr^{202/204–185/187}$), and NFκB (Ser^{536}) were quantified directly in lysed cells using ELISA-based assays, as previously published (Poursharifi et al., 2013; Tom et al., 2013). This methodology allows direct evaluation of both phosphorylated and total forms of the proteins simultaneously, reducing the technical manipulations of protein extraction, homogenization, and gel separation used in traditional western blot analysis.

Akt and NFκB activation were measured in 3T3-L1 adipocytes by Fast Activated Cell-based ELISA kit (Active Motif, Carlsbad, CA) as described by the manufacturer. Briefly, cells seeded in 96-well plates were stimulated with ASP (200 nM) and/or C5a (20 nM)