Immunobiology 218 (2013) 127–133

Contents lists available at SciVerse ScienceDirect

Immunobiology

journal homepage: www.elsevier.de/imbio

C5L2 receptor disruption enhances the development of diet-induced insulin resistance in mice

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ARTICLE INFO

Article history:
Received 9 December 2011
Accepted 17 April 2012

Keywords:
Obesity
Diabetes
Inflammation
Complement
Adipokine

ABSTRACT

Introduction: Acylation stimulating protein (ASP) is a hormone secreted by the adipose tissue that has been shown to increase triglyceride storage and glucose transport in adipocytes. These effects are mediated by C5L2 receptor, which has also been associated with inflammatory effects. C5L2 deficient mice on a low-fat diet are hyperphagic yet lean due to increased energy expenditure. The present study assessed insulin sensitivity and metabolic and inflammatory changes in C5L2KO mice *vs* WT in diet-induced obesity.
Methods: We placed C5L2KO and WT mice on a diabetogenic diet for 12 weeks and examined *in vivo* and *ex vivo* metabolism.
Results: C5L2KO mice on a diabetogenic diet exhibit decreased insulin sensitivity. Whole body substrate partitioning is evidenced through increased glucose uptake by the liver and decreased uptake by adipose tissue and skeletal muscle. Lipid content of both liver and skeletal muscle was higher in C5L2KO mice *vs* WT. Furthermore, elevated levels of macrophage markers were found in adipose tissue, liver and skeletal muscle of C5L2KO mice *vs* WT. Several inflammatory cytokines such as IL-6, MIP-1α and KC were also elevated in plasma of C5L2KO mice *vs* WT.
Conclusions: Overall, we demonstrated that C5L2KO mice fed a diabetogenic diet develop more severe insulin resistance than WT mice through altered substrate partitioning, ectopic fat deposition and a pro-inflammatory phenotype.

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Introduction

In humans, excess food energy intake results in increased adipose tissue mass, which can lead to lipotoxicity in organs such as skeletal muscle and liver. Such ectopic fat deposition is a well-known cause for further metabolic dysfunctions such as type 2 diabetes and cardiovascular diseases. Strategies aimed at reducing the capacity of adipose tissue to store triglycerides (TGs) are faced with the question of whether this could result in excess lipid overflow into other tissues (Cianflone 2003).

The inflammatory component is another important factor in the development of obesity-related pathologies. Chronic low-grade inflammation has been associated with type 2 diabetes, cardiovascular diseases and obesity, and is evidenced in part through a cross-talk between immune cells and resident tissue cells (Hotamisligil 2006). Macrophages, infiltrated and activated in the adipose tissue, muscle and liver, release several factors such as TNF-α, IL-6, IL-10 or MCP-1 that, in addition to adipokines, alter the inflammatome profile in the organism.

Acylation stimulating protein (ASP) is an adipokine derived from the alternative complement branch of the innate immune system. Proteolytic cleavage of complement C3 by factor B and adipsin leads to the formation of C3a, which is rapidly desarginated in circulation by carboxypeptidase B to produce C3adesArg, also termed ASP (Cianflone et al. 2003). ASP has been shown to promote fatty acid uptake and glucose transport in adipocytes. These effects are directly mediated through increased diacylglycerol acyltransferase

Abbreviations: ACACA, acetyl-CoA carboxylase 1; ADRA1B, alpha-1B adrenergic receptor; AdT, adipose tissue; ASP, acylation stimulating protein; C3KO, complement C3 knockout; C5L2KO, C5L2 receptor knockout; FATP4, long-chain fatty acid transport protein 4; FBP1, fructose 1,6-bisphosphatase; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; GTT, glucose tolerance test; HADH, Hydroxyacyl-Coenzyme A dehydrogenase; HF/HS, high fat–high sucrose; ITT, insulin tolerance test; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemoattractant protein 1; MIP-1α, macrophage inflammatory protein 1α; NEFA, non-esterified fatty acid; RANTES, regulated upon activation, normal T-cell expressed and secreted; TG, triglyceride; TNF-α, tumor necrosis factor α; WT, wild type.

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0171-2985/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.imbio.2012.04.001

activity (Yasruel et al. 1991) and increased translocation of glucose transporters GLUT1 and GLUT4 to the cell surface (Maslowska et al. 1997; Tao et al. 1997). Inhibition of hormone-sensitive lipase (Van Hermelen et al. 1999) and facilitation of lipoprotein lipase activity (Faraj et al. 2004) by ASP result in increased uptake and intracellular accumulation of TG in adipocytes. In humans, ASP levels are increased in subjects with obesity, type 2 diabetes and/or cardiovascular diseases (Onat et al. 2011).

C5L2 has been identified as a functional receptor for ASP (Kalant et al. 2003, 2005) and is expressed in multiple tissues such as white and brown adipose tissue, brain, muscle, liver and several types of immune cells, suggesting potential direct effects of ASP on many types of tissues (Kalant et al. 2005). Furthermore, the effects of ASP stimulation have been extensively evaluated in adipocytes but not as thoroughly described in other cell types. In addition to binding ASP, C5L2 also binds the proinflammatory molecule C5a; however, this does not result in an immune response (Scola et al. 2009). Such a process suggests a potential C5a-sequestering role for C5L2, which could lead to anti-inflammatory effects (Fisette and Cianflone 2010). An association of ASP with chronic inflammation in ASP–C5L2 deficient models was indicated by reciprocal links between IL-6 and ASP in previous studies (Paglialunga et al. 2010; Wernstedt et al. 2006). Studies have previously questioned whether C5L2 binds and/or is activated by ASP and C3a (Johswich and Klos 2007; Scola et al. 2009). We and others have however demonstrated the role of C5L2 in the signaling of ASP/C3a (Chen et al. 2007; Cui et al. 2009; Kalant et al. 2003, 2005).

ASP deficient (C3KO) and C5L2 deficient (C5L2KO) models have been used to further study the role of ASP *in vivo*. Complement C3 knockout mice are obligatorily ASP deficient, as C3 is the direct precursor of ASP, while C5L2 knockout mice lack the ASP receptor. A delay in postprandial TG and non-esterified fatty acids (NEFA) clearance is observed in both models (Murray et al. 1999b; Paglialunga et al. 2007) and is normalized in ASP deficient mice by ASP injection (Murray et al. 1999a; Xia et al. 2004). The disruption of the ASP–C5L2 pathway in these knockout models has also been shown to confer an obesity-resistant phenotype. Both ASP deficient (C3KO) and C5L2KO resulted in similar phenotypes, characterized by decreased fat storage in spite of increased food intake, accompanied by increased energy expenditure (Paglialunga et al. 2007; Roy et al. 2008; Xia et al. 2002, 2004). The metabolic similarities exhibited by the ASP deficient C3KO mice and by the C5L2KO mice further suggest that C5L2 is a functional ASP receptor. Additionally, we have shown that short-term chronic injection of ASP and C5L2 antibodies induce the same phenotype in wild type mice (Cui et al. 2007).

This evidence suggests that inactivation of the ASP–C5L2 signaling pathway could be a potential therapy for tipping the balance from energy storage to energy usage (Cianflone 2003). The metabolic changes observed in ASP or C5L2 deficient mice point toward a healthier and leaner phenotype (Paglialunga et al. 2007; Roy et al. 2008; Xia et al. 2002, 2004). However, the delay in postprandial fat clearance and the increased food consumption exhibited by these animal models could also potentially lead to deleterious consequences. In spite of higher energy usage, a redistribution of lipids away from adipose tissue toward other tissues, such as the liver, could potentially lead to ectopic fat deposition. Indices of insulin resistance were found in C5L2 deficient mice fed a diabetogenic diet (Paglialunga et al. 2007). Additionally, ASP has been shown to increase glucose-stimulated insulin secretion *ex vivo*, an important component of insulin action and sensitivity (Ahren et al. 2003).

The aim of the present study was to assess the influence of the ASP–C5L2 pathway in the development of insulin resistance using C5L2KO mice. We hypothesized that, under a diabetogenic diet, C5L2 deficient mice would exhibit decreased insulin sensitivity compared to wild type mice due to higher ectopic fat deposition and inflammation. The metabolic and inflammatory states were physiologically evaluated and compared with parameters of glucose handling and insulin sensitivity.

Materials and methods

Animals

Whole-body-C5L2KO mice were obtained and genotyped as previously described (Paglialunga et al. 2007). All mice were on a C57Bl/6 background and had been backcrossed for at least eight generations. Heterozygous breeding in our internal colony generated C5L2KO and wild type (WT) littermates. All mice were individually housed in a sterile barrier facility with a 12 h light:12 h dark cycle. At 8 weeks, mice were placed on high fat–high sucrose (HF/HS) diet (58% kcal fat D12331; Research Diets Inc., New Brunswick, NJ, USA) for 12 weeks. At the end of the protocol, overnight-fasted mice received an intraperitoneal injection of glucose (2 g/kg body weight) supplemented with radioactive deoxyglucose (0.5 mCi [^{3}H]deoxyglucose/kg of body weight) and were sacrificed exactly 2 h later. Animals were euthanized using a ketamine–xylazine mix. Blood was collected through cardiac puncture and tissues (muscle, liver, abdominal (perigonadal) adipose tissue) were harvested and immediately frozen in liquid nitrogen. Tissues were subsequently transferred to −80 °C and stored for further analysis. All protocols were approved and were conducted in accordance with the CACC guidelines and approved by the Laval University Animal Care Committee.

Plasma analysis

Blood was collected through cardiac puncture. Plasma TG, NEFA and glucose were measured using colorimetric enzymatic kits as follows: plasma TG (Roche Diagnostics, Richmond, VA, USA), NEFA (Wako Chemicals, Richmond, VA, USA), and glucose (Sigma). Mouse IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17A, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α, ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1 and resistin were measured using suspension bead array immunoassay kits following manufacturer's specifications (Bio-Plex Pro Mouse Cytokine Assay 23-plex and Bio-Plex Pro Mouse Diabetes Assay 8-plex, Biorad, Mississauga, ON, Canada) on a Bio-Plex series 100 instrument (Biorad, Mississauga, ON, Canada).

Glucose and insulin tolerance tests

Glucose tolerance test (GTT) was performed on mice (at week 11 of their regimen) following an overnight fast. Blood samples were taken from saphenous vein bleeding at 0, 15, 30, 60, and 90 min after an intraperitoneal glucose injection (2 g/kg body weight). Mice were allowed 5 days to recover. An insulin tolerance test (ITT) was then performed following a 4-h fast. Blood samples were taken from saphenous vein bleeding at 0, 15, 30, 60 and 120 min after an intraperitoneal insulin injection (1 U/kg of body weight). Glucose was measured using colorimetric enzymatic kit (Sigma) while insulin was measured using RIA kit (Linco, St Charles, MO, USA).

Glucose uptake and oxidation

Glucose uptake in abdominal adipose tissue, muscle and liver was evaluated as the accumulation of radioactive [^{3}H] deoxyglucose in tissue lysates. Results were corrected for total protein content (muscle and liver) or weight (adipose tissue) and are expressed as relative deoxyglucose uptake. Glucose oxidation was measured *ex vivo* as previously described (Roy et al. 2008) with

the following modification: benzethonium hydroxide was added as the CO_2 trapping agent, rather than KOH. Results are expressed as pmole of oxidized glucose per gram (g) of protein content.

HADH activity

Hydroxyacyl-Coenzyme A dehydrogenase (HADH) maximal (V_{max}) enzyme activity was assayed as previously described (Marcus and Hosey 1980; Roy et al. 2008).

Lipid and glycogen measurements

Liver and muscle lipids (TG and fatty acids) were extracted from tissue pieces (40 mg) using 2 mL of heptane:isopropanol (3:2). The extract was transferred while the remaining tissue (liver and muscle) was air dried, dissolved in 0.3 N NaOH and assessed for protein content using the Bradford method (Bio-Rad, Mississauga, ON, Canada). Organic extracts were lyophilized and lipids were redissolved in 10% Triton X-100 aqueous solution. TG and fatty acids were measured using commercial colorimetric kits as described above. Results are expressed as mmol of TG or NEFA per kilogram (kg) of protein. Liver glycogen was measured as previously described (Paglialunga et al. 2010). Results are expressed as μmol of glucose per gram (g) of liver wet weight.

Real-time quantitative PCR

Tissue mRNA was extracted, purified and reverse transcribed into cDNA using RNeasy Mini kits or RNeasy Lipid Tissue Mini kit and Quantitect Reverse Transcription kits (Qiagen, Gaithersburg, MD, USA). mRNA for ACACA, ADRA1B, FATP4, FBP1, GAPDH, GLUT2 and GLUT4 genes were measured using QuantiTect Primer Assays (Qiagen, Gaithersburg, MD, USA). mRNA for F4-80, CD11c and CD163 was quantified using custom primers (F4-80 forward: CTTTGGCTATGGGCTTCCAGTC reverse: GCAAGGAGGACAGAGTTTATCGTG SEQUENCE, CD11c forward: CTGGATAGCCTTTCTTCTGCTG reverse: GCACACTGTGTCCGAACTC, CD163 forward: GGGTCATTCAGAGGCACACTG reverse: CTGGCTGTCCTGTCAAGGCT). Relative gene expression was calculated and corrected using GAPDH as the housekeeping gene. All procedures followed manufacturer's instructions and MIQE guidelines (Bustin et al. 2009).

Statistical analysis

Results are expressed as mean ± SEM. Groups were compared using two-way ANOVA with SNK *post hoc* test or *t*-test using Prism 5.0 software (GraphPad, CA, USA). Statistical significance was set as $p < 0.05$, where * <0.05, ** <0.01, and *** <0.001.

Results

C5L2KO mice on HF/HS show an exacerbated phenotype of insulin resistance

C5L2KO mice have fasting glucose similar to the WT controls. However, fasting insulin is almost two-fold higher in C5L2KO (Table 1). A glucose tolerance test showed that C5L2KO mice have similar glucose clearance than WT mice (Fig. 1A). However, this clearance rate was obtained with much higher insulin levels, as shown in Fig. 1B ($p < 0.001$). In addition, a state of pronounced insulin resistance was confirmed through an insulin tolerance test (Fig. 1C), where C5L2KO failed to respond as efficiently to insulin as WT controls ($p < 0.05$). Furthermore, glucagon levels following the GTT (Post-GTT) were significantly higher in C5L2KO mice (Table 1), confirming the diabetic state. Meanwhile, plasma leptin and PAI-1 concentrations, both strongly associated with insulin resistance (Kahn and Flier 2000; Ma et al. 2004), were significantly higher in C5L2KO mice *vs* WT controls (Table 1).

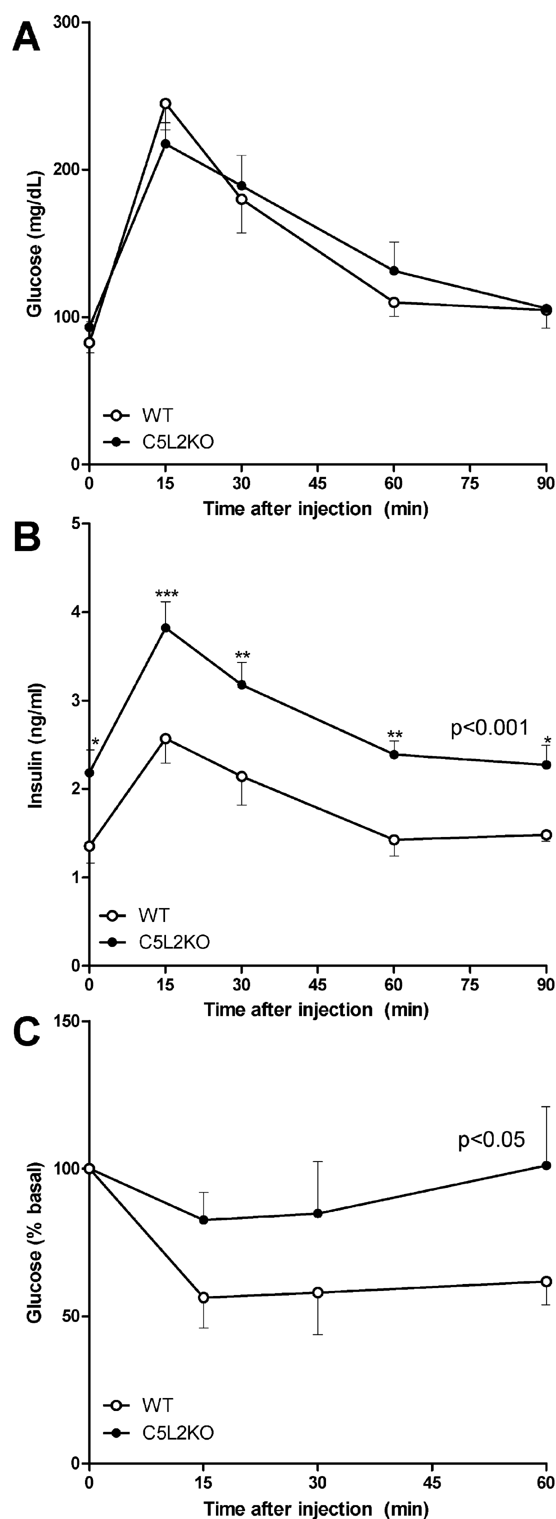

Fig. 1. Insulin resistance in C5L2KO mice. The results of a glucose tolerance test (panels A and B) and of an insulin tolerance test (panel C) are shown. Results are expressed as mg/dL of glucose (A), as ng/mL of insulin (B) or as % of basal glucose levels (C). Results are expressed as mean ± SEM where differences *vs* WT controls are expressed as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

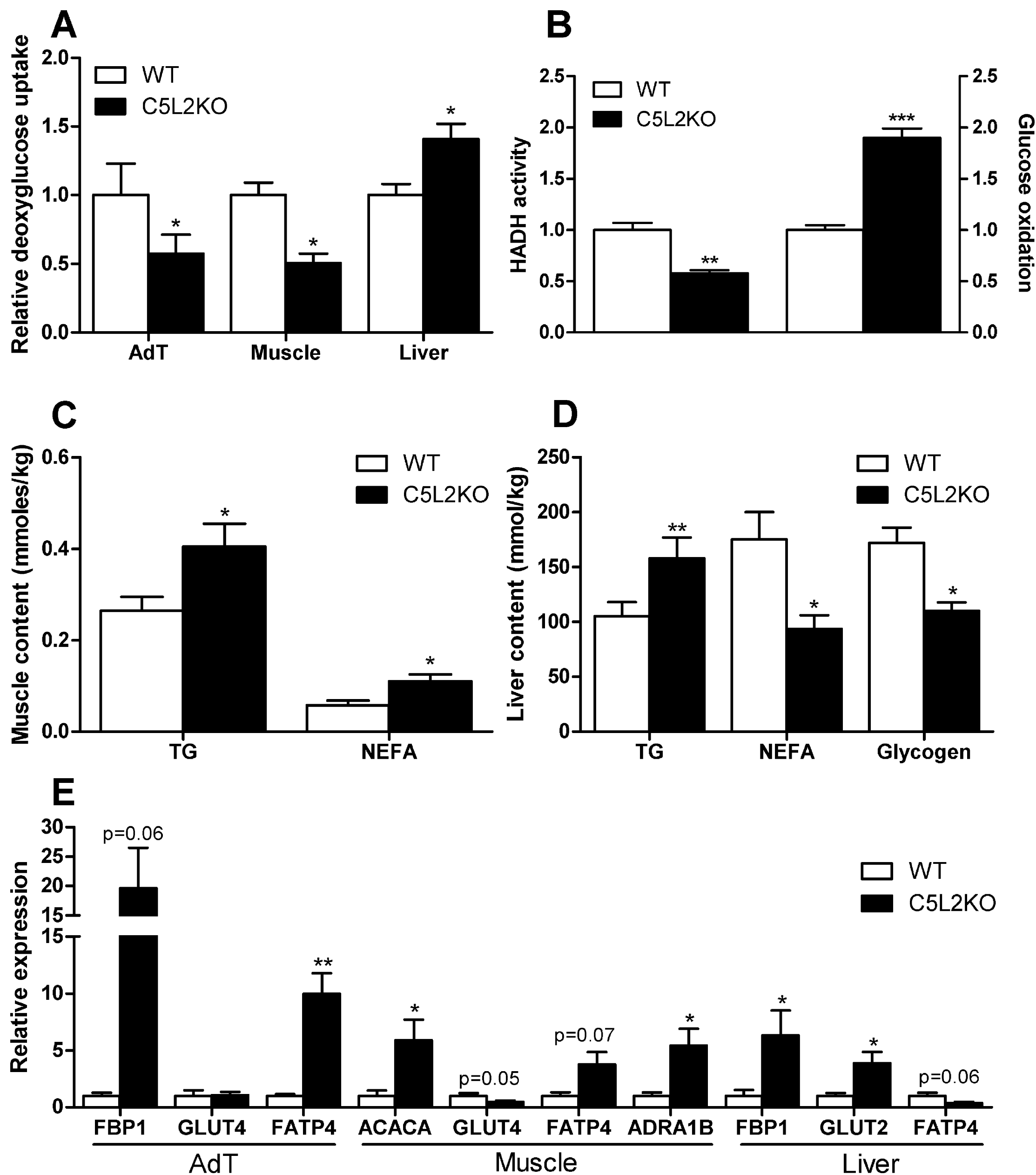

Fig. 2. Altered substrate absorption, storage and oxidation in C5L2KO mice. Relative deoxyglucose uptake (panel A), hepatic *ex vivo* substrate oxidation (panel B), muscle TG and NEFA content (panel C), liver TG, NEFA and glycogen content (panel D) and adipose tissue, muscle and liver genetic analysis (panel E) are shown. Results are expressed as relative deoxyglucose absorption (A), relative HADH activity or relative glucose oxidation capacities (B), mmol/kg (C and D) or relative gene expression (E). Results are expressed as mean ± SEM where differences *vs* WT controls are expressed as * $p<0.05$, ** $p<0.01$, and *** $p<0.001$. ACACA, acetyl-CoA carboxylase 1; ADRA1B, alpha-1B adrenergic receptor; AdT, adipose tissue; FATP4, long-chain fatty acid transport protein 4; FBP1, fructose 1,6-bisphosphatase; HADH, Hydroxyacyl-Coenzyme A dehydrogenase; NEFA, non-esterified fatty acids; TG, triglycerides.

C5L2 is critical for appropriate absorption, storage and oxidation of energetic substrates

Previous studies have shown that ASP and C5L2 deficient mice display altered substrate partitioning. In the present study, we examined glucose metabolism in the tissues of the "metabolic triad". Interestingly, deoxyglucose uptake was reduced in abdominal adipose tissue (−42%, $p<0.05$) and skeletal muscle (−49%, $p<0.05$) of C5L2KO mice and increased in the liver (+41%, $p<0.05$), all *vs* WT controls (Fig. 2A). Furthermore, *ex vivo* C5L2KO liver glucose oxidation was increased two-fold ($p<0.001$) while the rate-limiting enzyme of fatty acid oxidation (HADH) showed a significantly lower activity ($p<0.01$) (Fig. 2B). Adipose tissue GLUT4 levels did not change significantly in C5L2KO mice while muscle GLUT4 expression was reduced and liver GLUT2 expression increased, following the trends seen in deoxyglucose absorption. Meanwhile, skeletal muscle lipid content of C5L2KO mice was increased, as both TG ($p<0.05$) and fatty acids ($p<0.05$) were found in higher concentration (Fig. 2C). Liver TG content was also increased ($p<0.01$), but fatty acid concentrations were lower (Fig. 2D, $p<0.05$), suggesting less reliance on fat as an energy source. Gene expression mRNA analysis showed that both the adipose tissue ($p<0.01$) and muscle ($p=0.07$) of C5L2KO mice exhibit higher FATP4 mRNA than WT controls while there is a trend for reduction in the liver ($p=0.06$) (Fig. 2E). Finally, liver glycogen was significantly lower in C5L2KO mice ($p<0.05$) (Fig. 2D). FBP1 mRNA was increased in both adipose tissue ($p=0.06$) and the liver ($p<0.05$) of C5L2KO mice *vs* WT

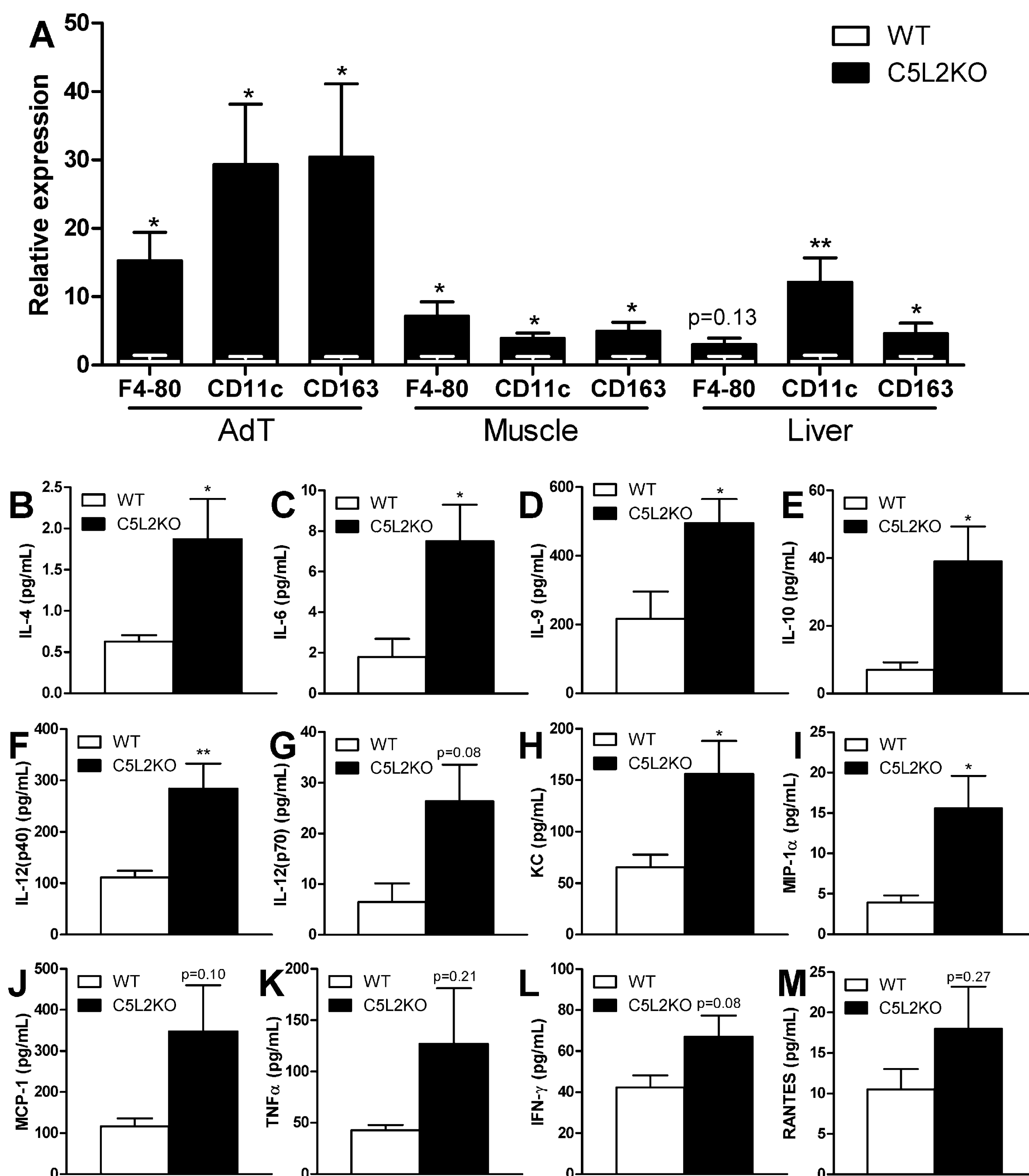

Fig. 3. Increased inflammation and macrophage infiltration in C5L2KO mice. Macrophage total (F4-80), M1 subtype (CD11c) and M2 subtype (CD163) (panel A), IL-4 (panel B), IL-6 (panel C), IL-9 (panel D), IL-10 (panel E), IL-12(p40) (panel F), IL-12(p70) (panel G), KC (panel H), MIP-1 (panel I), MCP-1 (panel J), TNF-α (panel K), IFN-γ (panel L) and RANTES (panel M) are shown. Results are expressed as relative gene expression (A) or pg/mL (B-M). Results are expressed as mean ± SEM where differences *vs* WT controls are expressed as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

controls. ACACA ($p < 0.05$) and ADRA1B ($p < 0.05$) mRNA were in turn higher the skeletal muscle of C5L2KO mice *vs* WT controls.

Disruption of C5L2 modulates the systemic inflammatory phenotype

We evaluated macrophage infiltration and polarization in abdominal adipose tissue, skeletal muscle and liver. While M1 macrophages are associated with the secretion of proinflammatory molecules, M2 macrophages mostly show an anti-inflammatory cytokine secretion profile. For all three tissues, markers of total macrophages (F4-80), M1 subtype macrophage (CD11c) and M2 subtype macrophage (CD163) increased at least threefold in C5L2KO mice *vs* WT counterparts, as seen in Fig. 3A. M1 and M2 macrophages markers increase in a similar ratio in the adipose tissue and skeletal muscle, but M1 marker increase ratio is higher in the liver. Plasma concentrations of several pro- and anti-inflammatory cytokines were evaluated: IL-4, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), KC, MIP-1α, MCP-1, TNF-α, IFN-γ and RANTES. Interestingly, all cytokine levels were elevated in C5L2KO mice (Fig. 3B–M).

Table 1
C5L2KO and WT mice basal parameters.

Genotype	WT ($n = 8$)	C5L2KO ($n = 10$)
Age (weeks)	21	21
Fasting glucose (mg/dL)	82.6 ± 6.7	93.1 ± 3.9
Fasting insulin (ng/mL)	1.35 ± 0.19	2.18 ± 0.26*
Post-GTT TG (mmol/L)	1.05 ± 0.18	1.29 ± 0.09
Post-GTT NEFA (mmol/L)	0.68 ± 0.05	0.61 ± 0.04
Post-GTT glucagon (pg/mL)	0.31 ± 0.06	2.49 ± 0.54*
Post-GTT PAI-1 (ng/mL)	0.83 ± 0.17	2.93 ± 0.35***
Post-GTT leptin (ng/mL)	14.1 ± 5.5	34.3 ± 4.6*

Basal characteristics of C5L2KO mice *vs* WT controls.
* Age, plasma TG, NEFA, fasting glucose, insulin, postprandial glucagon, PAI-1 and leptin levels were compared by *t*-tests where $p < 0.05$.
*** Age, plasma TG, NEFA, fasting glucose, insulin, postprandial glucagon, PAI-1 and leptin levels were compared by *t*-tests where $p < 0.001$.

Discussion

In the present study, we aimed at evaluating the effects of C5L2 disruption on the development of insulin resistance. This study is the first to demonstrate a deleterious metabolic effect of ASP–C5L2 pathway disruption. On standard chow low-fat diet or a high-fat only diet, the disruption of ASP–C5L2 pathway has been previously shown to induce beneficial effects (Cianflone 2003; Paglialunga et al. 2007; Roy et al. 2008; Xia et al. 2002, 2004). However, the joint presence of elevated fat content and sucrose in the diet pushes the balance toward a stronger negative adaptation in C5L2KO mice, which do not handle substrate overload properly under these circumstances when compared to wild type controls.

Here we show that C5L2 disruption exacerbates the development of insulin resistance in mice through two different pathways. First, C5L2KO mice demonstrate substrate partitioning typical of insulin resistant mice: reduced glucose uptake in the muscle and adipose tissue, increased glucose absorption by the liver, associated with the subsequent consequential changes in substrate oxidation. This partitioning is not deleterious *per se* in low-fat diet fed animals, but could worsen the condition of animals fed a calorie dense diet, as shown in this study. Redistribution of substrates in excess of tissue oxidation capacities – such as in the liver or skeletal muscle – can result in ectopic fat deposition and is known to lead to local and systemic insulin resistance (Kahn and Flier 2000). Such negative consequences of substrate partitioning have been shown in several knockout models (Goldberg et al. 2009; Kim et al. 2001; Kotani et al. 2004), while other examples exhibit a sufficient increase in energy expenditure to overcome the repartitioning and avoid ectopic fat deposition (Franckhauser et al. 2008; Smith et al. 2000). As ASP was demonstrated to be implicated in glucose-stimulated insulin release from pancreatic cells (Ahren et al. 2003), a dysfunctional ASP–C5L2 pathway could therefore unbalance this role and trigger a compensatory mechanism.

Further, the presence of a chronic state of low-grade inflammation has been established in diet-induced obesity and this can also contribute to the development of insulin resistance (Hotamisligil 2006). Molecules such as TNF-α and MCP-1, secreted by infiltrating macrophages and adipocytes, have been shown to induce insulin resistance through several mechanisms (Hotamisligil 2006). We show in this study that C5L2KO mice exhibit a more pronounced state of chronic inflammation that wild type controls, a phenotype that likely contributes to the more severe insulin resistance. The proinflammatory effects of C5L2 disruption could be due to several processes: the absence of ASP-mediated inflammatory effects in metabolic and immune cells; the increased oxidative stress due to higher substrate oxidation; the lack of C5a sequestration by C5L2, which could be an ASP-independent process; or the absence of potential C5a receptor and C5L2 heterodimerization (Fisette and Cianflone 2010). It is interesting to note that C5a have been shown to play a role in the polarization of macrophages (Langer et al. 2010).

In conclusion, targeting ASP–C5L2 interaction to induce substrate repartitioning and potentially reduce the incidence of obesity has been previously suggested (Cianflone 2003). Our study demonstrates that in the presence of a high-fat–high-sucrose diet, a dysfunctional C5L2 receptor worsens the state of insulin resistance in mice models through metabolic and proinflammatory changes.

Acknowledgments

This work was supported by a grant from NSERC (to KC) and from NIH (AI068730 to JDL). A. Fisette and M.N. Munkonda hold CIHR doctoral scholarships. K. Oikonomopoulou is a recipient of an NSERC postdoctoral fellowship. K. Cianflone holds a Tier 1 Canada Research Chair in Adipose Tissue. J.D. Lambris holds the Dr. Ralph and Sallie Weaver Professor of Research Medicine endowed chair. We would like to acknowledge the help of Jennifer Dumais, CRIUCPQ, for animal handling and Melanie Cianflone for manuscript preparation.

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