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

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Abstract

The link between immunity and metabolism in adipose tissue also extends to the complement system. Recent studies demonstrate complement receptor C5aR heterodimerization with C5L2, which we propose as a receptor for both complement C5a and acylation stimulating protein (ASP/C3adesArg), a lipogenic adipokine. The present study evaluates the C5aR contribution to regulation of adipocyte lipid metabolism, immune responses and signaling induced by C5a and/or ASP.

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

Both ASP and C5a directly increased MCP1 and KC secretion, TGS, and Akt/NFκB phosphorylation pathways in adipocytes. However C5a effects were absent in C5aRKO adipocytes, while ASP effects were mostly maintained. Addition of C5a completely blocked ASP signaling and activity in both C5aRKO and WT adipocytes. C5a also blocked effects of ASP in 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 are consistent with roles for C5aR in both metabolism and immunity in adipose tissue, which may be regulated through heterodimerization with C5L2.

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1. Introduction[1]

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 R 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 A et al. 2009, Poursharifi P et al. 2013, Tom FQ et al. 2013). Recently, complement protein/receptor interactions with adipokines have attracted attention, with "immunometabolism" being the subject of a number of reviews (MacLaren R et al. 2008, Nikolajczyk BS et al. 2012, Schâffler A and Schôlmerich J 2010, Schipper HS et al. 2012).

Many inflammatory responses in the immune system are related to the complement cleavage fragments, C3a and C5a, known as anaphylatoxins (Klos A 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 A 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 W et al. 2009b, DeMartino JA et al. 1994, Ohno M et al. 2000, Okinaga S 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 CE et al. 2010, Chen NJ et al. 2007, Ohno M et al. 2000). As previously shown, phosphorylation of C5aR and C5L2 leads to

[1] **Abbreviations:** ASP, acylation stimulating protein; C5L2, C5aR-like receptor 2; GPCR, G protein-coupled receptor; TGS, triglyceride synthesis

association with β-arrestin proteins and internalization into clathrin-coated vesicles (Bamberg CE et al. 2010, Braun L et al. 2003, Cui W 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 S et al. 2003), with stimulation of MAPK, ERK, diacylglycerol and Akt signaling pathways (Monk PN et al. 2007, Ward PA 2009). While C5a binds C5L2 with the same high affinity as C5aR, the potential functions and signaling pathways mediated through C5L2 are still controversial (Cain SA and Monk PN 2002, Okinaga S et al. 2003).

C3a and acylation stimulating protein (ASP/C3adesArg) both stimulate triglyceride synthesis (TGS) and glucose transport in adipocytes (Cianflone KM et al. 1989, Kalant D 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 A et al. 1993). ASP induces Akt, MAPK, ERK1, and NFκb signaling pathways in 3T3-L1 adipocytes and/or preadipocytes (Maslowska M et al. 2006, Poursharifi P et al. 2013, Tom FQ 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 FQ et al. 2013). Further, complement C3 knockout mice, which are deficient in C3, the precursor of ASP, and therefore obligately deficient in ASP, demonstrate altered energy metabolism and fat storage (Paglialunga S et al. 2008, Roy C et al. 2008).

The two receptors, C5aR and C5L2, have been proposed to have closely linked physical and functional interactions (Bamberg CE et al. 2010, Poursharifi P et al. 2013). Likewise, it has been demonstrated that C5aR and C5L2 are both capable of forming homo- and heterodimers (Poursharifi P 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 P 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 C5a-C5aR functions and signaling pathways and 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 W et al. 2009a), and assessed for purity by mass spectrometry (Cui W et al. 2009a, Murray I 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 AM et al. 1999, Monk PN et al. 2007, Wong AK et al. 1998), was used to pre-treat 3T3-L1 adipocytes for 30 min prior to addition of the treatments.

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 P et al. 2013, Tom FQ 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) for the indicated incubation times, and fixed with 4% formaldehyde. Following washing and blocking steps, cells were incubated overnight with anti-phospho or anti-total Akt and NFκB. Following incubation with anti-HRP conjugated IgG for 1 h at room temperature, a colorimetric assay was performed and absorbance at 450 nm was determined. The values were subsequently corrected for cell number using the absorbance values at 595 nm determined by crystal violet cell staining.

Akt, ERK, and NFκB phosphorylation in primary adipocytes were analyzed by InstantOne ELISA kits purchased from eBioscience (San Diego, CA), following the manufacturer's protocol for non-adherent cells. In summary, freshly isolated primary adipocytes were seeded in 96 well plates (20,000 cells/well), treated for stimulation, then lysed and incubated with antibodies against phospho- and total-Akt/ERK/NFκB overnight and following colorimetric processing, absorbance readings were obtained at 450 nm/650 nm.

2.7. Hormone Measurements

MCP-1 and KC concentrations were measured using mouse ELISA kits (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions. Differentiated 3T3-L1 adipocytes and freshly isolated primary adipocytes (100,000 cells/1 mL) were incubated overnight (24 h) in SF media with the indicated treatment concentrations, and the media was then collected. All samples were assayed in triplicate. Following colorimetric processing (according to the instruction), optical density was determined by a microplate reader set to 450 nm with the wavelength correction set to 540 nm.

2.8. RNA Extraction and qPCR Analysis

RNA was extracted from the whole gonadal fat pad of C5aRKO and WT mice (RNeasy Plus Universal Mini Kit) and cDNA was synthesized using RT^2 First Strand kit (Qiagen Inc., Mississauga, ON, Canada). mRNA expression level of each gene was quantified by $CFX96^{TM}$ Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON, Canada). Primers were designed using the Mouse qPrimerDepot resource site (mouseprimerdepot.nci.nih.gov) and were purchased from Alpha-DNA (Montreal, Canada). Results were analyzed by the ΔΔCt relative quantification method and normalized to *Eef2* As housekeeping gene. Primer sequences were as follows: mouse *IRS1*, forward 5'-CTATGCCAGCATCAGCTTCC-3' and reverse 5'-GGAGGATTTGCTGAGGTCAT-3'; mouse *PGC1α*, forward 5'-TGTAGCGACCAATCGGAAAT-3' and reverse 5'-TGAGGACCGCTAGCAAGTTT-3'; mouse *Eef2*, forward 5'- GCTTCCCTGTTCACCTCTGA-3' and reverse 5'-CGGATGTTGGCTTTCTTGTC-3'.

2.9. Statistical Analysis

All values are presented as mean ± standard error of the mean (SEM), with 5-9 mice per group, or multiple wells from cell experiments (as indicated). Groups were compared using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA) by two-way analysis of variance (ANOVA) followed by Bonferroni post-test, or by Student's t test, as indicated. Statistical significance was indicated as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

3. Results

3.1. C5aR is Required for C5a Metabolic, Immune and Signaling in Adipocytes

As C5a has been shown to interact with both C5aR and C5L2, receptors involved in immune and lipid storage functions, the effects of C5a on MCP-1 and KC production, triglyceride synthesis (TGS), as well as Akt, ERK and NFκB signaling pathways were evaluated. MCP-1 and KC secretion increased significantly in WT adipocytes treated with 20 nM C5a ($P < 0.001$) (Fig 1A and B). In adipocytes from C5aRKO mice, the majority of C5a stimulation was lost ($P < 0.001$, WT+C5a vs C5aRKO+C5a), although there still remained a small significant C5a effect as compared to basal level (Fig 1A and B). C5a also increased TG storage, an important adipocyte function, in WT adipocytes ($P < 0.001$) (Fig 1C). By contrast, there was no stimulatory effect of C5a on TGS in C5aRKO adipocytes (Fig 1C). As shown in Figure 1D, in adipocytes from WT, C5a induced rapid phosphorylation of Akt on Ser^{473} by 5 min, reaching a peak at 30 min. In the absence of C5aR, C5a failed to induce Akt phosphorylation (two-way ANOVA, WT vs C5aRKO, P=0.0052). There was no C5a activation of NFκB phosphorylation (Ser^{536}) in adipocytes from either WT or C5aRKO (Fig 1E). While there was a small stimulation of C5a on ERK phosphorylation ($Thr^{202/204\text{-}185/187}$) in WT adipocytes (Figure 1F), interestingly, the C5a impact on ERK phosphorylation increased in C5aRKO adipocytes (two-way ANOVA, WT vs C5aRKO, $P<0.0001$).

3.2. C5a Impaired ASP Activity in WT and C5aRKO Primary Adipocytes

ASP is known (i) to stimulate triglyceride synthesis through C5L2 interaction (Cui W et al. 2007, Kalant D et al. 2005), (ii) to increase MCP1 and KC secretion in adipocytes (Tom FQ et al. 2013), and also (iii) to enhance colocalization of C5aR with C5L2 in adipocytes and

macrophages (Poursharifi P et al. 2013). Based on this, the dual effect of ASP and C5a combined actions were evaluated together. Consistent with previous results, ASP alone strongly induced MCP-1 and KC production, as well as TGS in WT adipocytes (Fig 2A-C). In WT adipocytes, although addition of C5a had no effect on ASP stimulation of TGS, C5a reduced the ASP effect on MCP-1 and KC (although still remaining significant). By contrast, in C5arKO adipocytes, ASP effects on MCP-1 secretion and TGS were comparable, but were reduced for KC secretion although still remaining significant ($P<0.0001$). However addition of C5a completely blocked all functional effects of ASP in C5aRKO adipocytes, with responses decreased to basal levels (Fig 2A-C).

3.3. C5a Blocked ASP Signaling Pathways in C5aRKO and WT Primary Adipocytes

Based on the C5a interference with ASP functionality, signaling pathways were further assessed to determine the mechanism of negative C5a feedback. As shown in Figure 3A, while ASP stimulated time-dependent Akt (Ser^{473}) phosphorylation in WT adipocytes, addition of C5a partially blocked the ASP effect. Similarly, ASP stimulation of NFκB (Ser^{536}) phosphorylation in WT adipocytes was blocked by addition of C5a (Fig 3D). By contrast, in C5aRKO adipocytes, there was little effect of either ASP or ASP+C5a (Figs 3B and E), as with the lack of C5a response (Figure 3C and F). ERK activation followed a different profile: while there was no significant change in phospho-ERK relative to total ERK between ASP treated versus ASP+C5a stimulated adipocytes (Fig 3G and H), adipocytes from C5aRKO mice were more responsive than WT, regardless of the ligand stimulation (ASP, ASP+C5a or C5a, Figure 3I).

3.4. C5a Interferes with ASP Function and Signaling in 3T3-L1 Adipocytes

Similar experiments were also conducted in 3T3-L1 adipocytes to confirm the results obtained from primary adipocytes. As shown in Figure 4 A-D, C5a and ASP both independently increased MCP-1 and KC secretion as well as Akt and NFκB phosphorylation. However, treatment of 3T3-L1 adipocytes simultaneously with ASP and C5a significantly diminished the ASP effects on MCP-1 and KC secretion as well as on Akt and NFκB phosphorylation. Interestingly, treating the cells with the combination of ASP and 3D53, a specific C5aR antagonist that does not bind either C5L2 or C3aR (Lim et al., 2013), blocked the ASP effect, decreasing TGS to basal levels (Fig 4E and F).

3.5. C5aR Disruption Induced Partial Insulin Resistance in Primary Adipocytes

As demonstrated above the presence of C5aR influenced most adipocyte immune and metabolic responses; this suggested a potential involvement of C5aR receptors in insulin resistance. To address this possibility, adipocytes from WT and C5aRKO mice were assessed for insulin effects on cytokine production, TGS, signaling pathways, and gene expression. As shown in Figure 5, insulin stimulated MCP-1 and KC secretion (Fig 5A and B), TGS (5C), and Akt, NFκB, and ERK phosphorylation (Fig 5D-F) in WT adipocytes. Interestingly, C5aRKO adipocytes revealed impaired insulin stimulation of MCP-1 and KC production (Fig 5A, B) and NFκB phosphorylation on Ser^{536} (Fig 5E) with partial impairment of TGS (Fig 5C) and Akt phosphorylation (Ser^{473}) (Fig 5D). Of relevance, mRNA expression of *IRS1* and *PGC1α* was lower in adipose tissue from C5aRKO (Fig 5G). By contrast, as shown in Figure 5F, there was an increase of insulin-stimulated ERK phosphorylation ($Thr^{202/204\text{-}185/187}$) in C5aRKO mice in comparison to WT (two-way ANOVA, P=0.04).

4. Discussion

The present study extends previous findings for C5aR and C5L2 interaction, by proposing new insights into the C5a-C5aR role in adipocyte metabolism and immunity, and demonstrating regulatory impact on ASP and insulin-mediated functions. The close physical interaction of C5aR and C5L2 has been recently demonstrated in several studies, with heterodimerization of C5aR and C5L2 in transfected cells (Poursharifi P et al. 2013), colocalization of internalized C5aR with both C5L2 and β-arrestin in neutrophils (Bamberg CE et al. 2010), and internalization/colocalization of C5aR and C5L2 in C5a- or ASP-stimulated macrophages and adipocytes (Poursharifi P et al. 2013). Although there are only a few studies using C5aR and/or C5L2 antibodies/antagonists or knockout models, the results are consistent with coupled immune-metabolic aspects in adipocytes (Cui W et al. 2007, Lim J et al. 2013). However, the individual contributions of C5aR and C5L2 to adipose tissue functions and signaling are not yet clear. Lim *et al.* demonstrated C5a effects on lipid and glucose metabolism in adipocytes by using a C5aR-selective antagonist which interfered with C5a-mediated fatty acid uptake (Lim J et al. 2013). In the present study, where the potential cross-talk between C5aR and C5L2 in adipocytes was evaluated, the use of C5aRKO models allowed us to investigate (i) C5a-C5L2 function and signaling, (ii) C5a effects on ASP function and signaling, and (iii) the potential role of C5aR in insulin sensitivity in adipocytes.

C5a, a potent mediator of inflammation, has also been shown to be associated with adipocyte metabolism, by influencing lipogenesis, glucose uptake, and lipolysis (Lim J et al. 2013). These insulin-like effects in adipocytes have previously been well-established for ASP (Cianflone KM et al. 1989, Kalant D et al. 2005). Moreover, Akt and ERK have been shown to be involved in insulin- and ASP-mediated TGS lipid storage pathways in 3T3-L1 preadipocytes

(Maslowska M et al. 2006). Additionally, *in vitro* studies by Tom *et al.* suggested that ASP-mediated stimulation of the inflammatory factors, MCP-1 and KC, involved both NFκB and Akt mediated pathways (Tom FQ et al. 2013). In accordance with our findings, both C5a and ASP independently enhanced adipocyte function and signaling, however the disruption of C5aR reduced the C5a effects on inflammatory cytokine secretion, TGS, and Akt phosphorylation, whereas ASP effects mostly remained intact. Collectively, we speculate that the responses to C5a in primary adipocytes are primarily mediated through C5aR, while ASP effects, in certain cases, were manifested only in the presence of both C5aR and C5L2 receptors, possibly as heterodimers. Accordingly, *in vitro* assays with 3T3-L1 adipocytes pre-treated with a C5aR antagonist showed significantly reduced ASP effects on TGS. Of note, C5aR does not seem to play a role in C5a or ASP activation of ERK, since ERK phosphorylation remained comparable or greater in C5aRKO adipocytes, possibly pointing to a role for C5L2 in this process. Of relevance, neutrophils and macrophages obtained from C5L2KO mice showed absence of ERK phosphorylation following C3a stimulation, in contrast to the WT mice (Chen NJ et al. 2007).

This study has shown that C5a interfered with ASP functions and signaling in primary adipocytes and that the effects were more pronounced in C5aRKO adipocytes. In like manner, combination of C5a and ASP stimulation on 3T3-L1 adipocytes, negatively influenced ASP impact on cytokine secretion and signaling. Since ASP and C5a do not appear to compete for the same binding sites on C5L2 (Kalant D et al. 2003), then the C5a negative influence on ASP action could support the hypothesis that ASP does indeed bind to C5L2. This contrasts with the interpretation of other studies (Johswich K et al. 2006, Johswich K and Klos A 2007) reporting no such binding, but those studies were based solely on lack of competitive radioligand displacement with labelled C5a. If so, this would be consistent with C5a-C5aR/C5L2 exerting

regulatory effects via ASP-C5L2 pathway in adipocytes. This type of ligand-receptor blocking has been demonstrated elsewhere. For example, in an analogous fashion, a study by Pello *et al.* indicated that the simultaneous stimulation of CXCR4- and δ-opioid receptor (DOR)-expressing cells blocked the individual responses of the receptors. It was proposed that formation of CXCR4/DOR heterodimers, as a silent signaling complex, resulted following exposure to the combination of ligands (Pello OM et al. 2008).

Studies with C5L2KO mice have demonstrated a pathophysiological role for C5L2 in insulin resistance, lipid metabolism and sepsis (Fisette A et al. 2013, Gao H et al. 2005, Gerard NP et al. 2005, Paglialunga S et al. 2007). Further, it is suggested that disruption of an ASP-C5L2 dependent signaling pathway may contribute to altered energy metabolism (Cianflone K et al. 2003) and induce beneficial effects (Paglialunga S et al. 2007, Roy C et al. 2008). By contrast, the absence of C5L2 has been shown to accentuate insulin resistance induced by a high-fat-high-sucrose diet (Fisette A et al. 2013). Further, recent *in vivo* studies with C5aR and C3aR selective inhibitors revealed an improved inflammatory profile, suggesting specific roles for C5aR and C3aR in metabolic dysfunction such as obesity (Lim J et al. 2013). Based on our current results, in the absence of C5aR, adipocytes appear to lose the pro-inflammatory effects of insulin on MCP-1 and KC production, and NFκB phosphorylation, while maintaining ERK activation along with a trend towards lower expression of genes responsible for metabolic disorders, including insulin resistance (*IRS1* and *PGC1α*). Interestingly, survival in high-grade (100% lethality) sepsis required simultaneous blockade of C5aR and C5L2 receptors (Rittirsch D et al. 2008). Thus, synergic pro-inflammatory contributions of C5aR and C5L2 in sepsis might reflect an adaptive C5L2 mechanism in response to chronic levels of C5a.

5. Conclusion

In conclusion, these novel findings provide evidence that C5a may function as a negative regulator of ASP, which acts via both C5aR and C5L2. Together, these findings reveal that C5aR is associated with multiple inflammatory mechanisms in adipocytes, which could be regulated by heterodimerization with C5L2, in a ligand dependent manner. Preclinical/clinical trials have been undertaken to target C5a-C5aR interaction, following development of compounds acting on either C5 (to prevent its cleavage), or on C5aR (to prevent activation by C5a). The chronic antagonism of C5aR may adversely affect host defence and other beneficial complement functions, however this has recently been addressed by developing alternative tissue specific antibody therapies (Durigutto P et al. 2013). Given the potential therapeutic value of inhibiting C5a-C5aR function to prevent or slow progression of inflammation (Monk PN et al. 2007), further studies are essential to better understand how C5a-C5aR/C5L2 alternative pathways adjust to different pathophysiological environments, and might have unexpected influences on adipose tissue.

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7. Conflict of interest statement

Authors have no competing financial interests to declare in relation to the work described.

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10. Figure Legends

Figure 1. C5aR is Required for C5a Metabolic, Immune and Signaling in Adipocytes. Mouse adipose tissue from WT and C5aRKO mice was dissected, and primary adipocytes were prepared by collagenase digestion. *A* and *B:* Mature adipocytes were treated with C5a for 24 h, then MCP-1 and KC secretion were evaluated in cell culture medium. *C,* Primary adipocytes were treated with C5a for 1 h, then uptake and incorporation of fluorescently-labeled fatty acid into lipids was measured. *D to F:* Freshly isolated adipocytes were seeded in 96 well plates (20,000 cells/well) and were treated with C5a (T=0, 15, 30, 45 min) at 37°C, then Akt (*D*), NFκB (*E*), and ERK (*F*) phosphorylation and total protein were analyzed. Values are means ± SEM; white bars represent baseline with only PBS and striped bars represent C5a (20 nM). Significant differences were analyzed by t-test or two-way ANOVA: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. BSL.

Figure 2. C5a Impaired ASP Activity in WT and C5aRKO Primary Adipocytes. *A* and *B,* Mouse gonadal adipose tissue obtained from WT and C5aRKO mice was dissected, and primary adipocytes were prepared by collagenase digestion. Mature adipocytes were treated with ASP and ASP+C5a for 24 h, then MCP-1 and KC secretion were evaluated in cell culture medium. *C,* Primary adipocytes were treated with ASP and ASP+C5a for 1 h then uptake and incorporation of fluorescently-labeled fatty acid into lipids was measured. Values are means ± SEM, where white bars represent baseline with only PBS (BSL), striped bars represent ASP (200 nM), and checkered bars represent ASP (200 nM) + C5a (20 nM). Significant differences were analyzed by t-test: $**P < 0.01$, $***P < 0.001$ vs. BSL.

Figure 3. C5a Blocked ASP Signaling Pathways in C5aRKO and WT Primary Adipocytes. Freshly isolated primary adipocytes from WT and C5aRKO mice were seeded in 96 well plates (20,000 cells/well) and were treated with ASP (200 nM) or the combination of ASP (200 nM) and C5a (20 nM) for 15, 30, and 45 min at 37°C, then Akt (*A* and *B*), NFκB (*D* and *E*), and ERK (*G* and *H*) phosphorylation and total protein were analyzed. The ratio of phosphorylation/total (%) area under the curve (AUC) for Akt (*C*), NFκB (*F*), and ERK (*I*) in WT and C5aRKO adipocytes are presented. Treatments: C5a (white bars), ASP (striped bars), and ASP+C5a (checkered bars). Values are presented as means ± SEM. Significant differences were analyzed by t-test or two-way ANOVA: $*P < 0.05$, $**P < 0.01$ vs. BSL.

Figure 4. C5a Interferes with ASP Function and Signaling in 3T3-L1 Adipocytes. 3T3-L1 adipocytes were differentiated and then incubated in serum-free media 2 h prior to treatments. Treatments: PBS (BSL), insulin (INS; 100 nM), ASP (200 nM), and C5a (20 nM). *A* and *B*, 3T3-L1 adipocytes were treated with C5a (20 nM) and/or ASP (200 nM) for 24 h, then MCP-1 and KC secretion were evaluated in cell culture medium. 3T3-L1 adipocytes were treated with either ASP (200 nM, T=0, 15, 30, 45 min) and/or C5a (20 nM, T=0, 15, 30, 60, 90) at 37°C, then Akt (*C*) and NFκB (*D*) phosphorylation and total protein were measured. *E*, 3T3-L1 adipocytes were pre-treated with C5aR antagonist (3D53; 1 μM) for 30 min prior to addition of the treatments. Cells were incubated with the treatments for 1 h at 37 °C. Uptake and incorporation of fluorescently-labeled fatty acid into lipids was measured over 115 min. *F*, Results are presented for fatty acid uptake area-under-the-curve (AUC). Values are means ± SEM. Significant differences were analyzed by t-test or two-way ANOVA: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. BSL.

Figure 5. C5aR Disruption Induced Partial Insulin Resistance in Primary Adipocytes. *A* and *B,* Mouse gonadal adipose tissue from WT and C5aRKO mice was dissected, and primary adipocytes were prepared by collagenase digestion. Mature adipocytes were treated with insulin for 24 h, and then MCP-1 and KC secretion were evaluated in cell culture medium. White bars represent baseline with only PBS treatment and striped bars represent insulin (100 nM) treatment. Freshly isolated primary adipocytes were seeded in 96 well plates (20,000 cells/well) and were treated with insulin for 15, 30, and 45 min at 37°C, then analyzed for Akt (*D*), NFκB (*E*), and ERK (*F*) phosphorylation and total protein. *G*, *IRS1* (insulin receptor substrate 1) and *PGC1α* (peroxisome proliferator-activated receptor-γ co-activator 1-α) gene expression was evaluated in adipose tissue obtained from WT (striped bars) and C5aRKO (white bars) mice by RT-quantitative PCR, expressed relative to the housekeeping gene *Eef2* (eukaryotic elongation factor 2). Values are presented as means ± SEM; n = 5-9 per group. Significant differences were analyzed by t-test or two-way ANOVA: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. BSL.

HIGHLIGHTS:

- ASP and C5a increase MCP1, KC and TGS via Akt/NFκB pathways in adipocytes.
- C5a effects are absent in C5aRKO adipocytes, while ASP effects are mostly maintained.
- C5a blocks ASP signaling in C5aRKO and WT adipocytes, and 3T3-L1 adipocytes.
- C5aR is implicated in immunity and metabolism in adipose tissue.
- C5aR may mediate effects via C5L2 heterodimerization.

Figure 1

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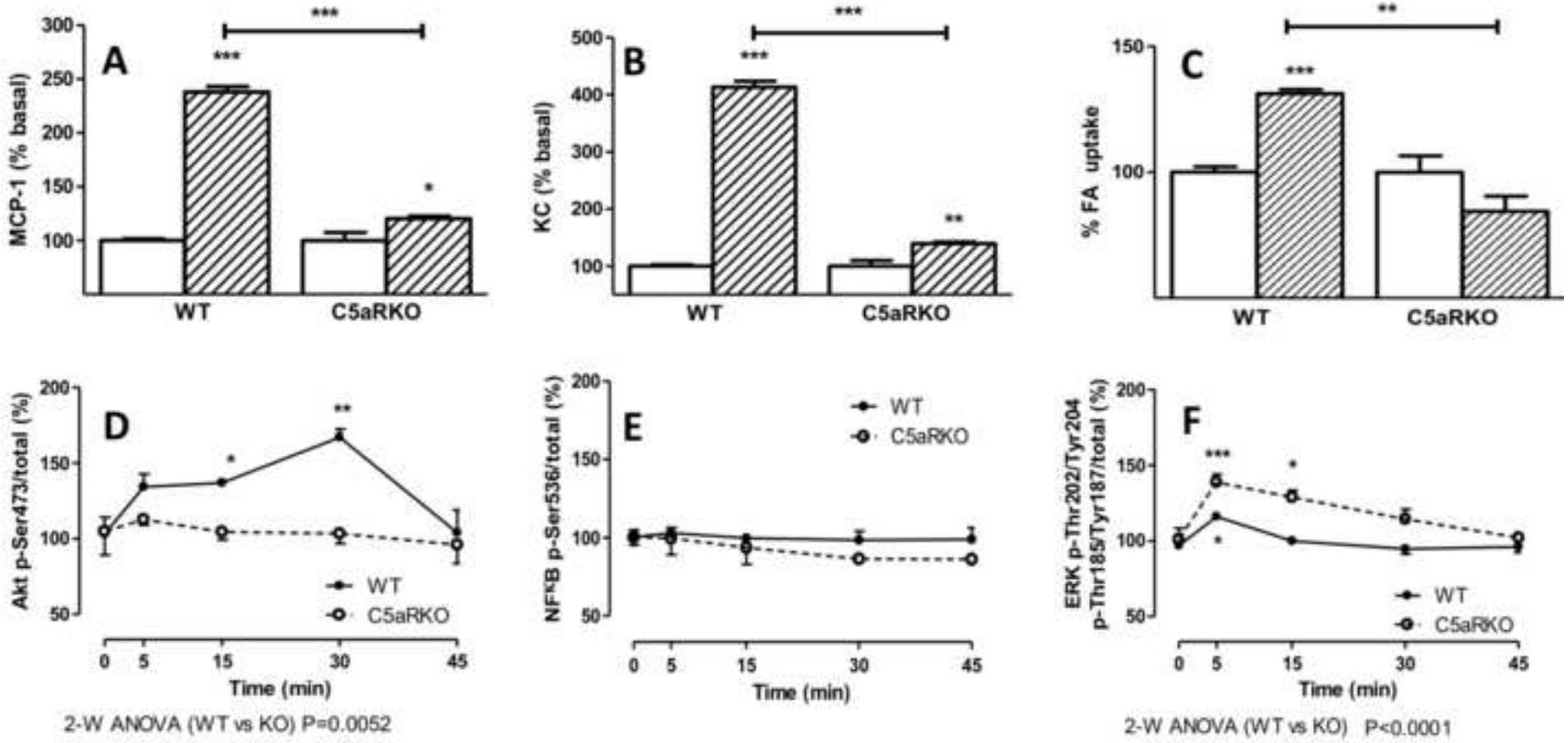

Figure 1

Figure 2

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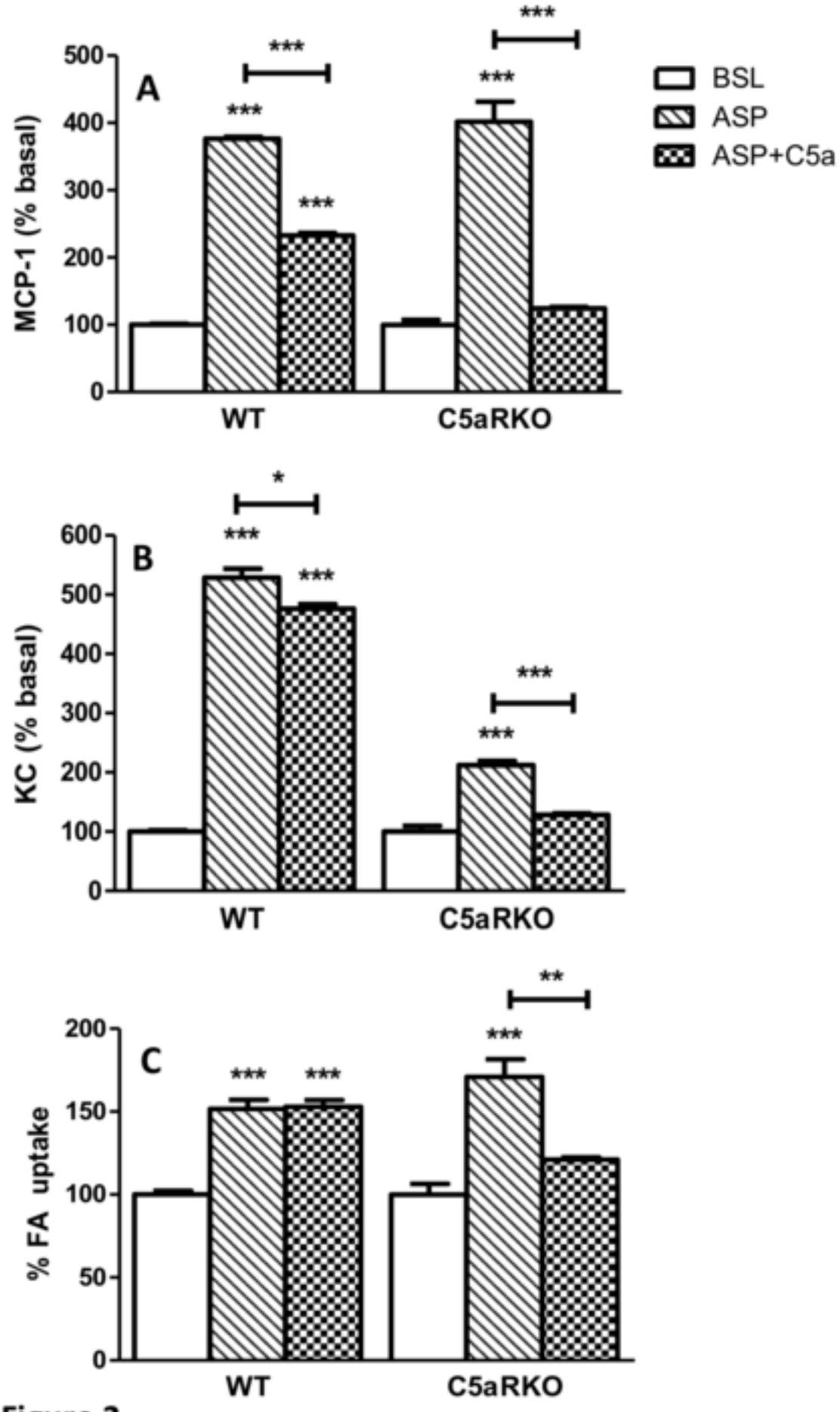

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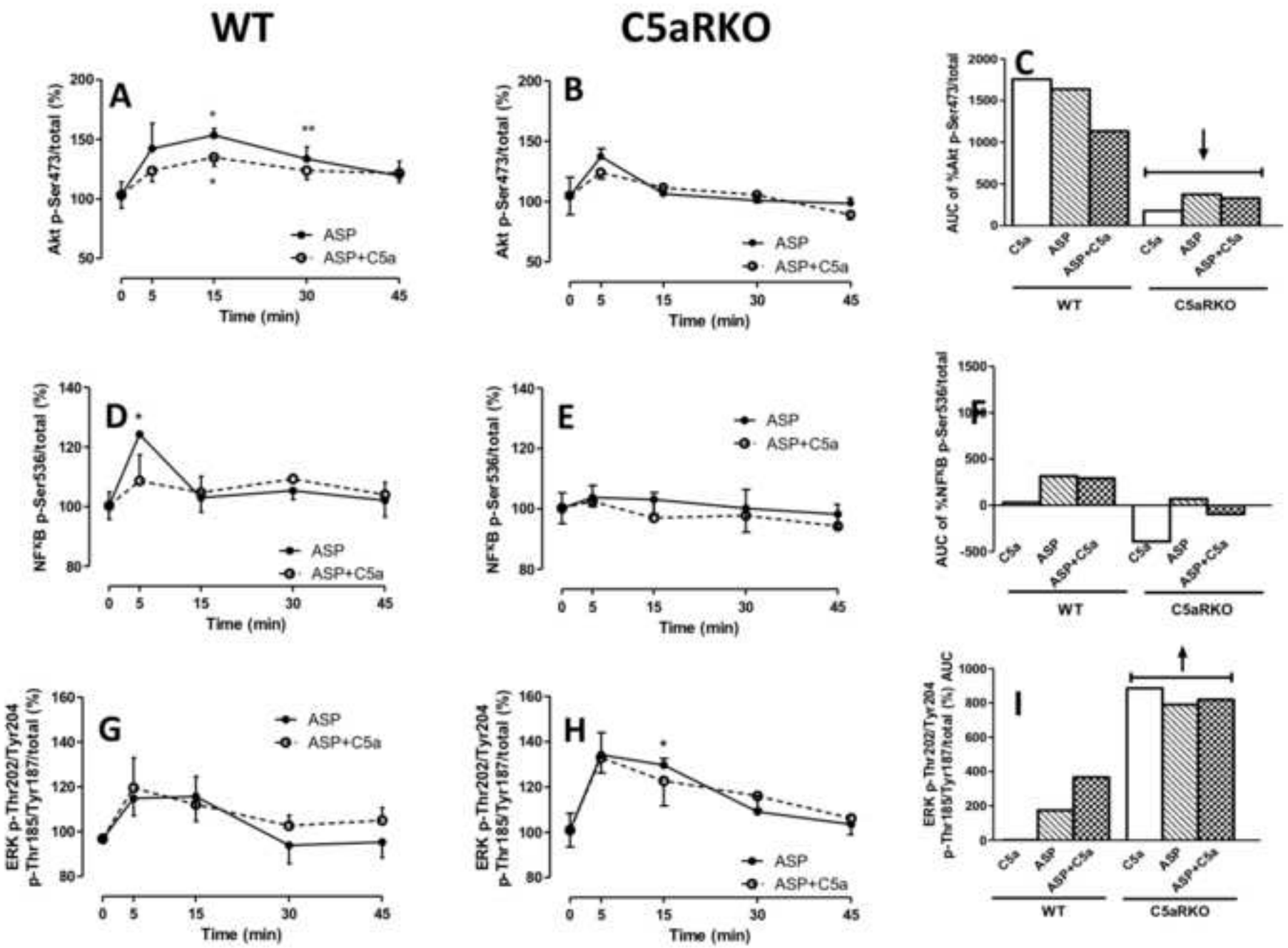

Figure 3

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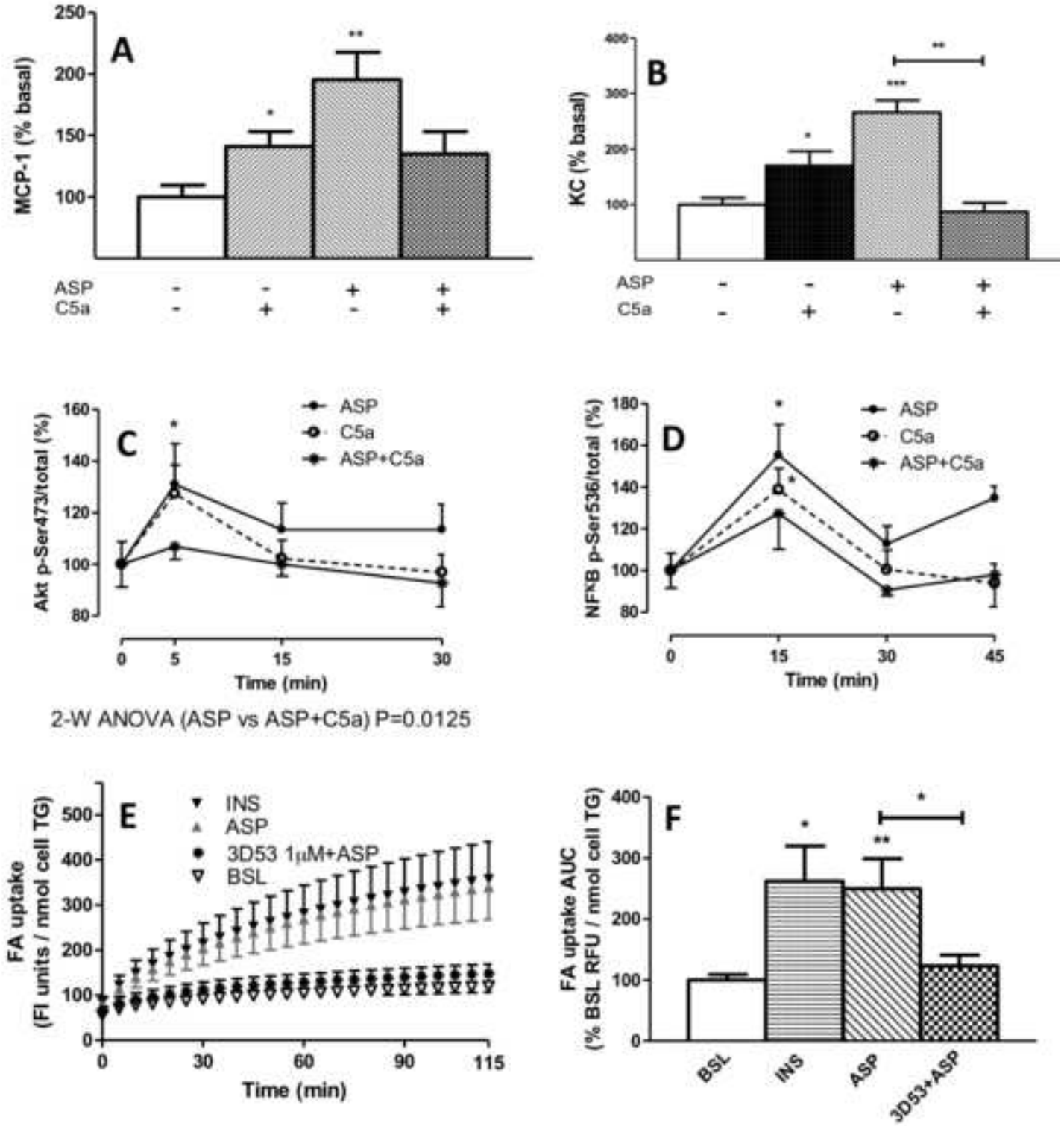

Figure 4

Figure 5

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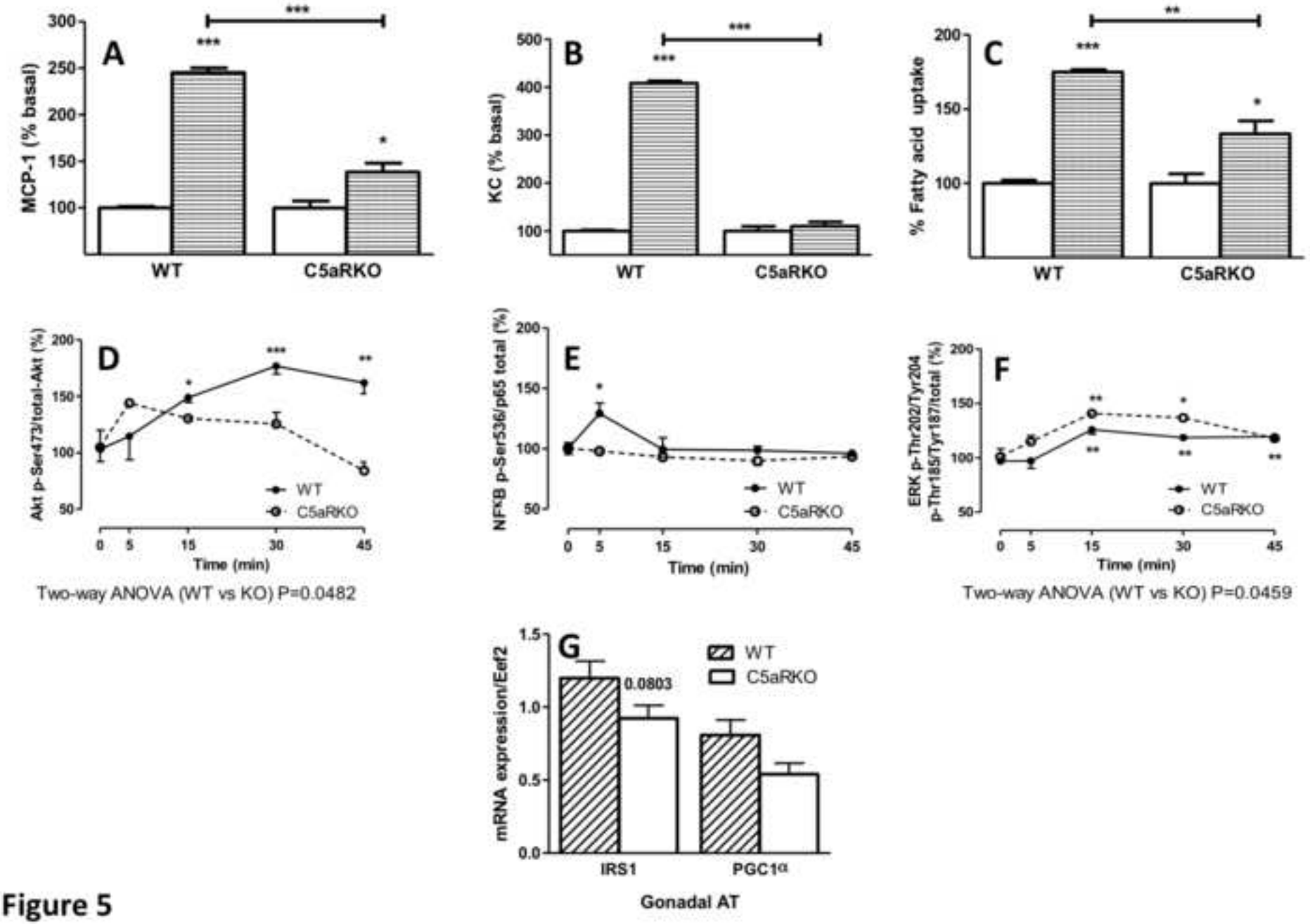

Figure 5