**UNIVERSIDADE FEDERAL DE MINAS GERAIS**
**Faculdade de Farmácia**
**Programa de Pós-Graduação em Ciências de Alimentos**

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# PARTICIPAÇÃO DAS CITOCINAS IL-18 E TNF, E DO RECEPTOR CCR5 EM ALTERAÇÕES METABÓLICAS E INFLAMATÓRIAS NA OBESIDADE INDUZIDA POR DIETA

Belo Horizonte
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Tese apresentada ao Programa de Pós-Graduação em Ciências de Alimentos da Faculdade de Farmácia da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do grau de Doutora em Ciências de Alimentos.

Orientadora: Profª. Drª. Adaliene Versiani Matos Ferreira

Coorientadora: Profª. Drª Marina Chaves de Oliveira

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# FOLHA DE APROVAÇÃO

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## JAQUELINE PEREIRA LANA

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIA DE ALIMENTOS, como requisito para obtenção do grau de Doutor em CIÊNCIA DE ALIMENTOS, área de concentração CIÊNCIA DE ALIMENTOS.

Aprovada em 03 de agosto de 2018, pela banca constituída pelos membros:

Prof(a). Adaliene Versiani Ferreira Matos - Orientador
UFMG

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Belo Horizonte, 3 de agosto de 2018.

# ATA DA DEFESA DE TESE DA ALUNA
# JAQUELINE PEREIRA LANA

Realizou-se, no dia 03 de agosto de 2018, às 14:00 horas, Sala 430, na Escola de Enfermagem da Universidade Federal de Minas Gerais, a defesa de tese, intitulada *PARTICIPAÇÃO DAS CITOCINAS IL-18 E TNF, E DO RECEPTOR CCR5 EM ALTERAÇÕES METABÓLICAS E INFLAMATÓRIAS NA OBESIDADE INDUZIDA POR DIETA*, apresentada por JAQUELINE PEREIRA LANA, número de registro 2014719815, graduada no curso de NUTRIÇÃO, como requisito parcial para a obtenção do grau de Doutor em CIÊNCIA DE ALIMENTOS, à seguinte Comissão Examinadora: Profa. Dra. Adaliene Versiani Ferreira Matos (Orientadora e presidente da Comissão Examinadora), Profa. Dra. Marina Chaves de Oliveira, Profa. Dra. Simone de Vasconcelos Generoso, Profa. Dra. Letícia Cordeiro, todas da Escola de Enfermagem da UFMG, Profa. Dra. Thereza Christina Barja Fidalgo, da UERJ, Profa. Dra. Albena Nunes da Silva, da UFOP.

A Comissão considerou a tese:

(X) Aprovada

( ) Reprovada

Finalizados os trabalhos, lavrei a presente ata que, lida e aprovada, vai assinada por mim e pelos membros da Comissão.

Belo Horizonte, 03 de agosto de 2018.

Profa. Dra. Adaliene Versiani Ferreira Matos (Orientadora e presidente da Comissão)

Profa. Dra. Marina Chaves de Oliveira

Profa. Dra. Simone de Vasconcelos Generoso

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# RESUMO

A obesidade caracteriza-se pela expansão exacerbada do tecido adiposo associada à disfunção metabólica e inflamatória, sendo classificada como um estado de inflamação crônica de baixo grau. Nesse contexto, vias e mediadores pró-inflamatórios têm demonstrado papel importante no desenvolvimento da doença metabólica. A resposta inflamatória associada à obesidade é desencadeada pela integração de diferentes vias do sistema imunológico, com marcada liberação de citocinas pró-inflamatórias no tecido adiposo. Diversos leucócitos e os próprios adipócitos contribuem com esse aumento de citocinas em tal tecido, sendo que essas, por si só, também estimulam vias de transcrição de outros mediadores pró-inflamatórios. Assim, o presente trabalho avaliou o papel das citocinas IL-18, TNF e também do receptor CCR5 no remodelamento do tecido adiposo, bem como no metabolismo intermediário. Para desenvolvimento do presente estudo foram utilizados camundongos IL-18$^{-/-}$, TNFR1$^{-/-}$ e CCR5$^{-/-}$, e animais selvagens (WT), alimentados com dieta controle ou rica em carboidratos refinados (HC). Mostrou-se que a ablação da citocina IL-18 ou do receptor 1 do TNF levou ao aumento na produção de citocinas no fígado e à piora do escore histopatológico nesse órgão, mesmo quando esses animais foram tratados com dieta controle (magros). No entanto, o tratamento com dieta HC não exacerbou essa inflamação nos mesmos animais. Com relação a expansão e inflamação no tecido adiposo, camundongos TNFR1$^{-/-}$ ganharam mais adiposidade, independentemente da composição da dieta (controle ou HC). Além disso esses camundongos apresentaram supressão na concentração das

citocinas TNF, IL-6 e IL-10 no tecido adiposo. Por outro lado, no contexto da dieta HC, a ablação do TNFR1$^{-/-}$ levou à melhora na sensibilidade à insulina. Com relação aos camundongos IL-18$^{-/-}$, esses também apresentaram maior adiposidade, sendo esse aumento associado à maior concentração de citocinas pró-inflamatórias no tecido adiposo, à diminuição da tolerância à glicose, mesmo quando alimentados com dieta controle. O tratamento com dieta HC não exacerbou essas respostas. Realizou-se também um modelo de quimeras para avaliar em quais células a ausência dessa citocina estaria desencadeando o fenótipo observado. Mostrou que a expansão do tecido adiposo observada nos animais IL-18$^{-/-}$ parece estar relacionada à deficiência de sua secreção em células não hematopoiéticas, enquanto que a menor tolerância à glicose parece estar relacionada à ablação da citocina em células hematopoiéticas. Já sobre os camundongos CCR5$^{-/-,}$ mostrou-se que a deficiência desse receptor de quimiocinas, apesar de não alterar o ganho de peso corporal e a adiposidade visceral, aumenta o compartimento inguinal, prejudicando o metabolismo de glicose e lipídios, além de contribuir com o acúmulo de gorduras no fígado. Quando alimentados com dieta HC os camundongos CCR5$^{-/-}$, apresentaram piora na resistência à insulina, bem como na esteatose hepática. Como conclusão do presente trabalho, destaca-se que além da participação de diferentes vias inflamatórias no desenvolvimento e progressão da obesidade, tais vias parecem também estar relacionadas à manutenção da homeostase metabólica

Palavras Chave: Obesidade; citocinas; inflamação; tecido adiposo; quimicionas, dieta; dieta HC; IL-18; TNF; CCR5.

## ABSTRACT

Obesity is characterized as an exacerbated expansion of adipose tissue associated with metabolic and inflammatory dysfunction, and with a low-grade chronic inflammation state. In this context, the role of proinflammatory pathways and mediators have been shown on the development of that metabolic disease. The inflammatory response observed is an integration between differents pathways of the immune system, and during besity, there is a marked increase in proinflammatory cytokines in the adipose tissue. Leukocytes and adipocytes contribute to the high level of cytokines, and that cytokines can also stimulate the transcription of other proinflammatory mediators. Thus, the present study evaluated the role of the cytokines IL-18 and TNF, and also the role of CCR5 receptor on the adipose tissue remodeling and on the metabolic complications of obesity. IL-18$^{-/-}$, TNFR1$^{-/-}$ , CCR5$^{-/-}$ and wild type mice, fed with chow or rich in refined carbohydrates diet (HC) were used. Lack of IL-18 or TNF receptor 1 have led to increased cytokine production in the liver and higher histopathological score, even in the lean animals (fed on a control diet). However, HC diet did not exacerbate hepatic inflammation. About adipose tissue expansion, TNFR1$^{-/-}$ mice gained more adiposity regardless of diet composition. Also, they showed decreased levels of TNF, IL-6 and IL-10 cytokines on the tissue. On the other hand, the ablation of TNFR1$^{-/-}$ mice improved insulin sensitivity during a challenge with HC diet. The IL-18$^{-/-}$ mice gained more weight and adiposity, and have shown increased proinflammatory cytokines in adipose tissue, associated to a decreased glucose tolerance even when fed with control diet. HC diet challenge

did not exacerbate those responses. We also studied a chimeric model, to evaluate which cells were involved in the IL-18 $^{-/-}$ mice phenotype. We have shown that the expansion of adipose tissue observed in IL-18$^{-/-}$ mice appears to be related to the deficiency of this cytokine in non-hematopoietic cells, whereas the decrease in glucose tolerance seems to be related to the cytokine ablation in hematopoietic cells. To evaluate the role of recruitment, we used CCR5$^{-/-}$ mice. We have shown that CCR5 absence did not alter body weight gain and visceral adiposity, but increased inguinal fat pad, impaired glucose and lipid metabolism and contributed to fat accumulation in the liver. When fed with HC diet, CCR5$^{-/-}$ mice showed impaired insulin sensitivity, as well as hepatic steatosis. We concluded that despite different inflammatory pathways were associated with development and progression of obesity, they also seem to be related to maintenance of metabolic homeostasis.

Key words: Obesity; cytokines; inflammation; adipose tissue; chemokines; diet; HC diet; IL-18; TNF; CCR5.

SUMÁRIO

# 1. INTRODUÇÃO

## 1.1. Obesidade

A obesidade é uma doença crônica e tornou-se um grave problema de saúde pública no mundo, apresentando, em 2014, prevalência estimada de 13% na população adulta mundial e de 17,9% no Brasil (ABESO, 2016; WHO, 2016). A projeção é que, em 2025, cerca de 2,3 bilhões de adultos tenham sobrepeso e mais de 700 milhões, sejam obesos (WHO, 2016). A obesidade também está intrinsecamente associada ao desenvolvimento de doenças, como diabetes mellitus tipo II, doenças cardiovasculares, problemas psicossociais, apneia do sono, desordens musculoesqueléticas e alguns tipos de câncer. As comorbidades associadas à obesidade foram responsáveis por 68% das mortes no mundo em 2012, sendo mais de 40% delas consideradas mortes prematuras (WHO, 2014).

De origem multifatorial, a obesidade pode ser desencadeada tanto por fatores endógenos como por fatores exógenos, culminando num conjunto de alterações que a tornam uma doença complexa e de difícil controle (DÂMASO, 2003). Dentre esses fatores, o ambiente predominante nos países ocidentais favorece o desenvolvimento de tal morbidade, uma vez que se caracteriza pela oferta de alimentos de baixo custo, palatáveis, práticos e de alta densidade energética. Nas últimas décadas, o incentivo para a ingestão de dietas com baixo teor de gorduras impulsionou o aumento do consumo de açúcar e outros carboidratos a fim de acentuar o sabor e o conteúdo calórico das refeições, contribuindo assim com o desenvolvimento da obesidade e comorbidades associadas. Estudos mostram que o consumo de dietas ricas em carboidratos refinados ou gorduras saturadas contribuem com o aumento de adiposidade em animais (BHERING MARTINS *et al.*, 2017; HARRIS; APOLZAN, 2012; MENEZES-GARCIA *et al.*, 2013; OLIVEIRA, MARINA C. *et al.*, 2013) e humanos (MALIK; WILLETT; HU, 2013). Aliado à isso, a diminuição da atividade física também agrava esse quadro (HILL; PETERS, 1998; KANNEGANTI; DIXIT,

2012). Assim, entende-se que o desequilíbrio crônico entre o gasto e a ingestão energética causa aumento de peso corporal, com expansão da massa adiposa para armazenar o excesso de nutrientes/calorias.

O tecido adiposo localiza-se perifericamente nas regiões subcutânea e visceral, e é composto por adipócitos, células do sistema imunológico, tecido conjuntivo, nervoso e vascular (FONSECA-ALANIZ; ALONSO-VALE; LIMA, 2006; JACENE *et al.*, 2011; OUELLET *et al.*, 2011). Os adipócitos são as únicas células especializadas no armazenamento de lipídios sob a forma de triacilglicerol (TAG) em seu citoplasma, sem que isso seja nocivo para sua integridade funcional. A capacidade de expansão do tecido adiposo representa uma adaptação crítica à exposição crônica ao excesso de calorias e/ou nutrientes. O remodelamento da massa adiposa decorrente de obesidade é desencadeado tanto pelo aumento do volume do adipócito (hipertrofia) quanto pela multiplicação do seu número (hiperplasia) (HAUSMAN *et al.*, 2001; SUGANAMI; OGAWA, 2010). Essa expansão, principalmente devido à hipertrofia dos adipócitos, pode desencadear um quadro de hipóxia tecidual, levando à morte celular, à superprodução de matriz extracelular, ao aumento da produção de adipocitocinas de perfil mais pró-inflamatório, ao aumento da expressão de moléculas de adesão em células do endotélio vascular, além do recrutamento de células imunológicas para o tecido (CURAT *et al.*, 2004).

O papel dos mediadores inflamatórios e adipocitocinas expressos no tecido adiposo no contexto da obesidade tem sido amplamente estudado. Sabe-se que na obesidade o tecido adiposo altera seu perfil de secreção de adipocitocinas, substâncias bioativas produzidas principalmente por esse tecido, com ação local ou sistêmica. Essa alteração na secreção de adipocitocinas pode levar à desordens metabólicas, imunológicas e neuroendócrinas (SPIEGELMAN, B M; FLIER, 2001). O aumento da adiposidade reduz a produção de adiponectina, adipocitocina de perfil anti-inflamatório, ligada à diminuição da gliconeogênese hepática e ao aumento da oxidação lipídica no músculo (ARITA *et al.*, 2012). Em contrapartida, promove também aumento da produção de leptina, que apesar de participar do controle da ingestão alimentar, apresenta-se

como uma adipocitocina de perfil principalmente pró-inflamatório (MARTIN; QASIM; REILLY, 2008). Em adição, o influxo de células imunológicas para o tecido adiposo, desencadeado pelo aumento exacerbado da massa adiposa, do próprio adipócito e consequente hipóxia tecidual, também participa da elevação na produção de mediadores pró-inflamatórios. Dentre esses mediadores destacam-se as citocinas Fator de necrose tumoral (TNF), Interleucina-6 (IL-6), Interleucina-1β (IL-1β), Interleucina-18 (IL-18) e também as quimiocinas (ESPOSITO, 2002; FANTUZZI, 2005; OLUSI; AL-AWADHI; ABRAHAM, 2003). Nesse contexto, o remodelamento do tecido adiposo associado ao desequilíbrio na secreção de diferentes moléculas caracteriza a obesidade como estado de inflamação crônica de baixo grau (FANTUZZI, 2005; TILG; MOSCHEN, 2008; WELLEN; HOTAMISLIGIL, 2003). Essa inflamação decorrente da obesidade pode ainda prejudicar a cascata de sinalização do receptor da insulina, culminando num quadro de resistência à ação desse hormônio. Assim, a menor sensibilidade à ação da insulina em células alvo pode gerar hiperglicemia e hiperinsulinemia. Essas alterações metabólicas estão associadas a menor captação de glicose por tecidos dependentes da insulina, como o músculo esquelético e adipócitos (JELLINGER, 2009).

Embora sejam inquestionáveis os efeitos deletérios do quadro de inflamação crônica associada à obesidade, ainda é necessário elucidar o papel de moléculas e vias inflamatórias no desenvolvimento das disfunções características dessa condição. Nosso grupo de pesquisa tem trabalhado com a hipótese de que a inflamação, para além de mediar as alterações metabólicas da obesidade, num primeiro momento, possa apresentar-se como um dos mecanismos regulatórios para manutenção do metabolismo basal e controle da expansão da massa adiposa. Rodrigues et al. (2014) mostrou que mesmo o consumo de ração equilibrada leva ao aumento da resposta inflamatória pós-prandial. Além disso, trabalho de Oliveira et al. (2013) mostrou que camundongos respondem de forma aguda (1-3 dias) à sobrecarga de carboidratos refinados, demonstrando alterações na resposta imunológica, aumento da massa adiposa, principalmente no compartimento visceral, além de

alterações no metabolismo glicídico e lipídico. De forma congruente, Menezes-Garcia et al. (2013) mostrou que a redução do *milieu* inflamatório desencadeado pela ablação do receptor do fator de ativação plaquetária (PAF) em camundongos, apesar de se mostrar protetora contra resistência à insulina induzida por dieta rica em carboidratos refinados, gera também expansão exacerbada da massa adiposa, evidenciando possível papel da inflamação no controle da homeostase metabólica. Em adição, Bhering Martins et al. (2017) mostrou em seu trabalho que apesar do enfoque patológico dado à citocina TNF no contexto da obesidade, essa molécula parece contribuir para limitar o aumento exacerbado da massa adiposa. Diante desse contexto, no qual inúmeros componentes inflamatórios integram a indução da doença metabólica, pode ser que exista também a participação dos mesmos componentes no controle da homeostase. Assim, com o objetivo de elucidar parte desse cenário, no presente trabalho, estudamos 3 vias inflamatórias importantes, as vias das citocinas IL-18 e TNF e a via de quimiotaxia do receptor CCR5 na modulação do metabolismo intermediário e expansão do tecido adiposo.

## 1.2. Participação da IL-18 na obesidade

### 1.2.1. A citocina IL-18

A citocina IL-18 é um membro da família IL-1 expressa por diversos tipos celulares, incluindo macrófagos, células endoteliais, células da musculatura vascular lisa, células dendríticas, células de Kupffer, pré-adipócitos e adipócitos (AKDIS *et al.*, 2011; SKURK *et al.*, 2005; WOOD *et al.*, 2005). Foi descrita primeiramente como fator indutor de Interferon γ (IFN-γ) após ser isolada do soro de camundongos que receberam injeção intraperitoneal de endotoxina. Após sua purificação a partir do fígado desses camundongos, em 1995, a proteína foi identificada como uma nova citocina e o nome alterado para IL-18 (OKAMURA *et al.*, 1995).

Essa citocina é sintetizada como precursor biologicamente inativo, que sem sinal peptídico, permanece como citocina intracelular. É preciso que esse

precursor seja clivado para torná-lo uma molécula biologicamente ativa, sendo essa clivagem dependente da ativação do inflamassoma (DINARELLO, C A, 1999; DINARELLO, CHARLES A *et al.*, 2013). O inflamassoma é um complexo multiproteico intracelular que atua na ativação de enzimas da família cisteína-aspartato proteases (CASPASES), sendo estrutura essencial para a regulação da imunidade em condições fisiológicas, assim como no reconhecimento de sinais de perigo desencadeados por diferentes componentes. O inflamassoma é composto pela proteína adaptadora intracelular ASC (apoptosis-associated speck-like protein containg a CARD), por um receptor NLR, e pela enzima caspase-1. A ativação de NLR leva ao recrutamento da ASC e da caspase-1, com formação do complexo pentamérico ou heptamérico. A formação desse complexo culmina na ativação da caspase-1, que atua então clivando e ativando citocinas, como IL-1β, IL-18 e IL-33 (AREND; PALMER; GABAY, 2008; KUFER; FRITZ; PHILPOTT, 2005; MEYLAN; TSCHOPP; KARIN, 2006; ZOU *et al.*, 1999). Apesar de essencial para as respostas imunológicas, a ativação persistente do inflamassoma tem sido descrita como fator preponderante para o desencadeamento de algumas doenças inflamatórias, como gota, aterosclerose e diabetes mellitus tipo II (AKDIS *et al.*, 2011).

Evolutivamente, os mecanismos de sensibilidade à patógenos e à nutrientes tem sido conservados entre as espécies fazendo com que os sistemas imunológico e metabólico funcionem de forma integrada (HOTAMISLIGIL, GÖKHAN S; ERBAY, 2008). Nesse contexto, tem sido proposto que receptores do sistema imune inato, como os receptores do tipo NOD e os receptores de membrana do tipo TOLL, reconheçam além de componentes microbianos e sinais de dano celular, também o excesso de nutrientes como sendo nocivo. Durante o desenvolvimento da obesidade, as concentrações elevadas de glicose e de ácidos graxos saturados de cadeia longa tem sido apontadas como ativadores do receptor NLRP3 do inflamassoma, por meio da produção de espécies reativas ao oxigênio (WEN *et al.*, 2011; ZHOU *et al.*, 2010). Adicionalmente, ceramidas, ácido úrico e cristais de colesterol também estão envolvidos na ativação do NLRP3 em doenças metabólicas. Em geral, a ativação

desses receptores induz uma resposta inflamatória devido à clivagem de citocinas pró-inflamatórias, podendo desencadear resistência à insulina e desequilíbrio energético, contribuindo para as complicações metabólicas associadas a obesidade, como diabetes e aterosclerose (GREGOR; HOTAMISLIGIL, 2011).

### 1.2.2. Transdução de sinal da citocina IL-18

Após clivagem, a IL-18 pode então ser liberada e ligar-se ao seu receptor, ativando sua cadeia de transdução de sinal. O receptor de IL-18 (IL-18R) consiste em um heterodímero composto por duas cadeias: a cadeia α (IL-18Rα), elemento de ligação ao ligante; e a cadeia β (IL18Rβ), elemento de sinalização. (DINARELLO, CHARLES A *et al.*, 2013).A ligação da citocina ao receptor resulta na degradação da proteína inibitória de NF-κB (IkB), com consequente liberação e ativação do fator de transcrição nuclear κB (NF-κB). Esse, quando ativado, pode migrar para o núcleo da célula, induzindo a produção de diversas proteínas envolvidas nas respostas inflamatórias e imunológicas (DINARELLO, CHARLES A *et al.*, 2013).

A IL-18 pode ainda aumentar a expressão de moléculas de adesão celular, a síntese de óxido nítrico e a produção de quimiocinas (DINARELLO et al., 2013). A indução de quimiocinas das famílias CC e CXC coloca a IL-18 em papel estratégico no desenvolvimento da resposta inflamatória. A IL-18 pode também atuar induzindo Fas ligante, resultando em apoptose celular (DINARELLO, CHARLES A, 2006).

### 1.2.3. A citocina IL-18 no contexto da obesidade

Trabalhos realizados nos últimos anos sugerem que a citocina IL-18 possa atuar como modulador fisiológico do consumo alimentar e do metabolismo energético. De forma similar às outras adipocitocinas, as concentrações séricas de IL-18 estão associadas ao estado metabólico, relacionando-se ao volume de massa adiposa, à perda de peso, à hiperglicemia e à ingestão de gorduras

(Esposito et al., 2002a; Esposito et al., 2002b; Esposito et al., 2003). Camundongos deficientes em IL-18 (IL-18$^{-/-}$) ou em componente do receptor IL-18R$^{-/-}$ apresentam hiperfagia tanto quando alimentados com dieta padrão (LINDEGAARD *et al.*, 2013; NETEA, MIHAI G *et al.*, 2006; ZORRILLA *et al.*, 2007), quanto com dieta rica em lipídeos (LINDEGAARD *et al.*, 2013), ou ainda com dieta com baixo teor desse nutriente (ZORRILLA *et al.*, 2007). Esses animais apresentam, alterações lipídicas, aumento de lesões ateroscleróticas, resistência à insulina e tornam-se obesos (LINDEGAARD *et al.*, 2013; NETEA, MIHAI G *et al.*, 2006), revelando que a sinalização da IL-18 pode modular o consumo alimentar, o metabolismo e a adiposidade. De fato, a deleção global de IL-18 desencadeia a obesidade e resistência à insulina ao passo que a administração exógena de IL-18 recombinante pode reverter esse fenótipo (NETEA, MIHAI G *et al.*, 2006; ZORRILLA *et al.*, 2007). O mecanismo responsável pelo aumento da ingestão alimentar em camundongos IL-18$^{-/-}$ tem sido atribuído à perda do controle de apetite pelo sistema nervoso central. Relata-se que camundongos IL-18$^{-/-}$ comem durante todo o dia, enquanto camundongos selvagens apresentam hábitos noturnos (DINARELLO, CHARLES A *et al.*, 2013). Em adição, (ERIC P. ZORRILLA, PH.D.1,2 AND BRUNO CONTI, 2015), mostraram que a deficiência de IL-18 promove balanço energético positivo, uma vez que animais IL-18$^{-/-}$ gastam menos energia do que os animais selvagens (WT) sob as mesmas condições.

Conforme descrito anteriormente, a clivagem da IL-18 está associada a ativação do inflamassoma, assim, a retirada de algum componente desse complexo multiprotéico por interferir na atividade dessa citocina. De forma interessante, trabalhos com camundongos deficientes em caspase-1 ou NLRP3 mostram que esses animais apresentam menor adiposidade quando alimentados com dieta HF (STIENSTRA, R; JOOSTEN; KOENEN, 2010; STIENSTRA, RINKE *et al.*, 2011; VANDANMAGSAR *et al.*, 2011), efeito esse não esperado.

Em estudos com humanos obesos a concentração de IL-18 circulante apresentou-se elevada (ESPOSITO, 2002; OLUSI; AL-AWADHI; ABRAHAM,

2003). Observou-se também aumento de RNA mensageiro desta citocina no tecido adiposo subcutâneo de indivíduos obesos (LEICK *et al.*, 2007), sendo que a perda de peso resultou em diminuição das concentrações séricas da mesma (VILARRASA et. al, 2007). O aumento de IL-18 também tem sido relacionado à resistência à insulina (LEICK *et al.*, 2007). De forma interessante, apesar de sua ausência em animais levar à resistência à insulina, em humanos, a alta concentração de IL-18 associa-se a obesidade e ao diabetes. Dessa forma, tem sido sugerido que indivíduos obesos com resistência à insulina apresentam altas concentrações de IL-18 circulante devido a menor resposta a essa citocina, analogamente aos casos de resistência à leptina (TACK *et al.*, 2012).

Apesar de trabalhos anteriores já demostrarem a participação da IL-18 no controle da ingestão alimentar e, consequentemente, no desenvolvimento da obesidade, ainda é incipiente o entendimento do papel da IL-18 na modulação da inflamação do tecido adiposo e consequentemente no remodelamento desse tecido. Em adição apesar das evidencias do papel da IL-18 no controle do metabolismo basal, não se sabe se essa participação se dá pela sua atuação em células hematopoiéticas ou em células não derivadas da medula óssea, como os adipócitos.

## 1.3. Papel do receptor CCR5 na obesidade

### 1.3.1. Quimiocinas e seus receptores

A migração direcional é fundamental para o influxo de células do sistema imunológico para os sítios de inflamação. Participam dessa migração de leucócitos as quimicionas, as moléculas de adesão, e outros. As quimicinas são pequenas proteínas de baixo peso molecular (7 a 15kDa) com aproximadamente 70 a 80 aminoácidos de comprimento (ALLEN; CROWN; HANDEL, 2007; MAÑES *et al.*, 2005). São conhecidas mais de 50 quimiocinas, identificadas e categorizadas de acordo com o número e espaçamento dos aminoácidos existentes nos dois primeiros resíduos de cisteína da extremidade N-terminal. Tais proteínas são classificadas em quatro subfamílias: CXC, CC, CX3C e C,

nas quais C representa cisteína e X ou X3 representa um ou três aminoácidos. A família CC é considerada a mais ampla e possui dois resíduos de cisteína adjacentes. As quimiocinas dessa família são caracterizadas pela atração de células mononucleares para sítios de inflamação crônica (ALLEN; CROWN; HANDEL, 2007; CHARO; RANSOHOFF, 2006; HORUK, 2007).

As quimiocinas exercem sua função quimiotática por meio da ligação a seus respectivos receptores, expressos de forma diferenciada por todos os leucócitos. Os receptores de quimiocinas são receptores acoplados à proteína G (GPCRs). Tais receptores apresentam sete domínios hidrofóbicos transmembranares em alfa-hélice, com o terminal amino no meio extracelular e o terminal carboxila no meio intracelular. A ligação da quimiocina ao receptor dissocia as subunidades GαI e Gβγ da proteína G resultando na ativação de efetores, como fosfatidilinositol 3-quinase (PI3K) e das vias de sinalização de GTPases Rho, entre outros (VIOLA; LUSTER, 2008). Os GPCRs podem reconhecer mais de uma quimiocina, porém estão praticamente restritos a uma única subfamília. Desta forma, sua nomenclatura baseia-se na especificidade do receptor para a subfamília de quimiocinas (MURPHY, P M *et al.*, 2000). Foram identificados 19 receptores de quimiocinas: CXCR1 a CXCR6 (ligação às quimiocinas CXC), CCR1 a CCR11 (ligação às quimiocinas CC), CX3 CR1 (liga-se à fractalcina) e XCR1 liga-se à linfotactina (CHARO; RANSOHOFF, 2006; MANTOVANI *et al.*, 2004; MURPHY, P M *et al.*, 2000; MURPHY, PHILIP M, 2002; PROUDFOOT, 2002).

Após se ligarem a seus receptores em leucócitos, as quimiocinas induzem a cascata de sinalização intracelular, que resulta em aumento da avidez de ligação da integrina, levando a adesão firme dos leucócitos ao endotélio e transmigração. Já nos tecidos, as quimiocinas coordenam a migração direcionada de leucócitos para áreas específicas (BUTCHER; PICKER, 1996; SPRINGER, 1994).

### 1.3.2. Importância do Receptor de quimiocinas CCR5 e seu papel na obesidade

O CCR5 é expresso, preferencialmente, em linfócitos Th1, macrófagos, em tecidos linfóides e não linfóides e em células dendríticas (OPPERMANN, 2004). Apresentam alta afinidade para o receptor CCR5 os ligantes CCL3 (MIP-1αS), CCL3LI (MIP-1αP), CCL4 (MIP-1β), CCL5 (RANTES), e CCL8 (MCP-2) (VIOLA; LUSTER, 2008). Já CCL2, CCL11, CCL13 e CCL14 são seus ligantes adicionais (OPPERMANN, 2004). O CCR5 é conhecido por ser um dos principais co-receptores do vírus HIV, controlando a susceptibilidade à infecção e à evolução da doença (MURPHY, P M *et al.*, 2000; OPPERMANN, 2004). A deleção em 32 pares de bases na estrutura genética do gene CCR5 resulta na perda de expressão funcional desse receptor. Indivíduos homozigóticos para esse gene exibem elevada resistência à infecção pelo HIV, enquanto que os heterozigóticos, quando infectados pelo vírus, progridem menos rapidamente para a imunodeficiência (DAVID; MORTARI, 2000; LUSSO, 2006; MURPHY, P M *et al.*, 2000; OPPERMANN, 2004). Estes dados sugerem a importância do CCR5 e seus ligantes em processos inflamatórios/infecciosos.

Receptores de quimiocinas também têm sido relacionados ao processo inflamatório decorrente da obesidade. Como a obesidade induz o acúmulo de macrófagos no tecido adiposo, o recrutamento de tais células contribui para a produção de moléculas pro-inflamatórias e consequentemente para desenvolvimento e manutenção da inflamação no referido tecido (CURAT *et al.*, 2004; WEISBERG, STUART P *et al.*, 2003; XU; BARNES; YANG, 2003). O receptor de quimiocinas CCR2 e o seu ligante CCL2 são considerados fundamentais para desenvolvimento de respostas inflamatórias, assim como para recrutamento de células do sistema imunológico para os locais de inflamação. Dessa forma, ambos são relacionados ao recrutamento de macrófagos para o tecido adiposo, e desenvolvimento de resistência à insulina. Camundongos com supressão nesses genes apresentaram diminuição do conteúdo de macrófagos no tecido adiposo, com consequente diminuição da

resposta inflamatória e possível efeito protetor contra resistência à insulina induzida por dieta rica em gordura (KANDA; TATEYA; TAMORI, 2006; WEISBERG, SP; HUNTER, 2006). Em adição, camundongos com superexpressão de CCL-2 em tecido adiposo apresentaram aumento do número de macrófagos na região, sendo esse aumento associado ao desenvolvimento de resistência à insulina (KAMEI *et al.*, 2006; KANDA; TATEYA; TAMORI, 2006). Portanto considera-se que o eixo CCL-2/CCR2 é de grande importância no recrutamento de macrófagos para o tecido adiposo, além de participar do desenvolvimento da resistência à insulina.

Nesse contexto outras quimiocinas e receptores também têm sido relacionados à infiltração de macrófagos no tecido adiposo de camundongos obesos (MANTOVANI *et al.*, 2004). Um dos possíveis receptores é o CCR5. Huber et al. (2008), mostraram que o CCR5 e os seus ligantes também estão aumentados em tecido adiposo de humanos obesos, quando comparados aos controles. Além disso, Kitade et al. (2012) mostraram que o CCR5 e seus ligantes CCL3/MIP-1a, CCL4/MIP-1b, CCL5/RANTES, e CCL8/MCP-2 estão desregulados no tecido adiposo epididimal de camundongos obesos. No mesmo trabalho, houve também maior expressão de CCR5 e seus ligantes no fígado dos animais em dieta indutora de obesidade. Wu et al (2007), também mostraram que o mRNA de RANTES e CCR5, estavam significativamente aumentados em tecido adiposo de camundongos obesos. Outros trabalhos também mostram expressão elevada de CCL2, CXCL14, CCL-3, CCL8, CCL7 e CCL5/RANTES no tecido adiposo de camundongos com modificação genética para receptores de quimiocinas ou em dieta indutora de obesidade (KANDA, 2006; WEISBERG, 2006).

Embora alguns trabalhos já tenham associado o CCR5 e seus ligantes à obesidade em animais, a real contribuição dessa molécula no desfecho inflamatório e metabólico ainda é controversa. Alguns trabalhos sugerem que o menor recrutamento de macrófagos para o sítio de inflamação desencadeado pela ablação do CCR5, poderia apresentar-se como protetor no contexto da obesidade (HUH *et al.*, 2018; KITADE *et al.*, 2012). Outro já demonstra que a

diminuição da resposta inflamatória pode prejudicar o metabolismo glicídico e a sensibilidade à insulina (KENNEDY *et al.*, 2013). Como o recrutamento de macrófagos é um evento importante para o desenvolvimento das alterações metabólicas vistas na obesidade, é necessário elucidar o papel desse receptor nos compartimentos adiposos (visceral e subcutâneo), bem como em órgãos metabólicos como o fígado. Em adição, ainda não havia sido avaliado o papel desse receptor em dietas com maior teor de carboidratos, refletindo de forma mais fidedigna o padrão de consumo alimentar ocidental.

### 1.4. Função do Fator de Necrose Tumoral (TNF) na obesidade

O TNF é uma proteína solúvel da classe das citocinas pró-inflamatórias e possui efeitos pleiotrópicos. As atividades biológicas do TNF são mediadas via sua interação com receptores específicos (TNFR), que são o TNFR1 constitutivamente expresso em todas as células, com exceção dos eritrócitos, e o TNFR2 que é geralmente induzido e preferencialmente expresso em células endoteliais e em células hematopoiéticas. A interação do TNF com o TNFR pode levar à ativação de NF-κB, que controla a expressão de genes de mediadores inflamatórios, ou à ativação de uma via de caspases, causando apoptose. Assim, a sinalização via TNFR1 pode levar à ativação celular ou à apoptose, enquanto a sinalização via TNFR2 não leva diretamente à apoptose, mas pode contribuir com o TNFR1 para induzi-la (WARZOCHA, BIENVENU, COIFFIER, & SALLES, 1995;TRACEY, KLARESKOG, SASSO, SALFELD, & TAK, 2008). A secreção do TNF pode ser induzida por vários estímulos, como produtos bacterianos, mediadores lipídicos, complexos imunológicos, entre outros, participando assim de vários processos patológicos (GWOZDZIEWICZOVÁ *et al.*, 2005). Diante disso, o TNF está relacionado à várias atividades biológicas que incluem a proliferação, a diferenciação e apoptose celular (BOUCHER *et al.*, 2005; PAUSOVA *et al.*, 2000), estímulo da produção de colagenases, aumento da expressão de moléculas de adesão, bem como ao desenvolvimento e a expressão fenotípica da obesidade (LYON; LAW; HSUEH, 2003).

Durante a obesidade a expressão do TNF no tecido adiposo está aumentada tanto em modelos experimentais (HOTAMISLIGIL, GS; SHARGILL; SPIEGELMAN, 1993), como em humanos (HOTAMISLIGIL, G S *et al.*, 1995; KERN, P A *et al.*, 1995). Esse aumento se dá tanto pela maior secreção dessa citocina pelos adipócitos hipertrofiados quanto pelos macrófagos infiltrados em resposta à hipóxia. No tecido adiposo o TNF inibe a esterificação de ácidos graxos aos estar associado com o aumento da lipólise e inibição da lipogênese, regulando assim os estoques de energia. Por outro lado, a elevação de ácidos graxos livres circulantes pode prejudicar a sinalização de insulina. Além disso, o TNF pode participar da fosforilação do resíduo de serina no substrato do receptor de insulina impedindo assim a continuidade da cascata de sinalização que tem por último estágio a translocação de GLUT 4 em adipócitos e células musculares, intensificando o quadro de resistência à insulina. TNF também está associado à diminuição da secreção de adiponectina e inibição do transporte de glicose para células do fígado. Ademais, sabe-se que ele estimula a produção de outras citocinas, como IL-6, e proteínas de fase aguda associadas ao processo inflamatório (LIU *et al.*, 1998). Dessa forma, tem sido proposto que o aumento da produção de TNF nos adipócitos possa ser um mecanismo para induzir a resistência insulínica local e assim limitar a expansão exacerbada da massa adiposa (ARGILÉS *et al.*, 1997; GULLER *et al.*, 1988; SPIEGELMAN, BRUCE M.; HOTAMISLIGIL, 1993).

Outros tecidos também estão expostos ao influxo de ácidos graxos livres decorrentes do aumento da lipólise no tecido adiposo, o que pode acarretar acúmulo ectópico de triglicérides em órgãos como fígado, músculo esquelético e pâncreas, além de oxidação reduzida de ácidos graxos (FOROUHI *et al.*, 1999; JACOB *et al.*, 1999; MAERSK *et al.*, 2012; OKAMURA *et al.*, 1995; PAN *et al.*, 1997). Esses fatores podem interferir na função celular e, consequentemente, na função do órgão, além de serem associados ao desenvolvimento de resistência à insulina. Embora o acúmulo de gordura nos hepatócitos possa ser mecanismo natural de armazenamento de energia, em situação de sobrecarga, pode levar a doença hepática gordurosa não alcoólica (DHGNA). Em modelos experimentais

de obesidade, a expressão gênica de citocinas pró-inflamatórias, como IL-6, TNF-α e IL-1β, encontram-se aumentadas no fígado e isso parece decorrer do acúmulo de gordura no órgão (CAI *et al.*, 2005). Como o TNF-α participa do desencadeamento de resistência à insulina, está intimamente relacionado ao desenvolvimento de doença hepática (XU; BARNES; YANG, 2003). Parece que além de atuar induzindo tanto a morte celular quanto a proliferação de hepatócitos, está também envolvido na patogênese da fibrose hepática (WULLAERT *et al.*, 2007). Assim, outros órgãos, como o fígado, também são comprometidos pelo desenvolvimento e progressão da obesidade.

Apesar da ampla associação do TNF ao desenvolvimento de obesidade e resistência à insulina, pouco se estudou sobre seu impacto no fígado, seja em situação de dieta normal ou em situação de sobrecarga de nutrientes específicos.

# 2. JUSTIFICATIVA

A resposta inflamatória decorrente da obesidade é caracterizada pela integração de diferentes vias. Um ponto importante no desenvolvimento da doença metabólica é a liberação de citocinas pró-inflamatórias no tecido adiposo, sendo que diversos leucócitos participam dessa resposta. Macrófagos polarizados para o fenótipo M1, células de resposta Th1 $CD4^+$ e células T efetoras $CD8^+$ contribuem de forma massiva para o aumento de citocinas inflamatórias no tecido adiposo (OSBORN; OLEFSKY, 2012). A clivagem da citocina pró-inflamatória IL-18, dependente da ativação do inflamassoma, converge para a ativação da via do fator de transcrição nuclear NF-KB, sendo esse fator responsável pelo aumento da transcrição de mediadores pró-inflamatórios, como as citocinas TNF, IL-6, IL-8 e IL-1β.

O TNF foi a primeira citocina a ser descrita nesse contexto, e sua associação com a obesidade e inflamação tem sido amplamente investigada. Sabe-se que a via do TNF desencadeia aumento da produção de outras citocinas pró-inflamatórias, como IL-6 e IL-1β, também pela ativação do fator NF-KB (MCARDLE *et al.*, 2013). Essa resposta leva ao aumento da lipólise (RYDÉN *et al.*, 2004), reduz da sensibilidade à insulina (STEPHENS; PEKALA, 1991) e inibe a adipogênese. Apesar dos efeitos deletérios da elevação crônica do TNF na obesidade, ainda não é claro o papel fisiológico, se existente, dessa resposta inflamatória no tecido adiposo. Além disso, pouco se sabe sobre seu impacto em órgãos metabólicos que também respondem à obesidade, como o fígado.

Vista a importância das citocinas na progressão da obesidade, é inquestionável a participação do recrutamento de leucócitos para o tecido adiposo nesse processo. A quimiotaxia de leucócitos é dependente da ligação das quimiocinas liberadas no sítio de inflamação aos seus respectivos receptores na membrana celular dos leucócitos circulantes. Os receptores de quimiocinas CCR2 e CCR5, assim como suas quimiocinas ligantes, estão aumentados no tecido adiposo de camundongos obesos e consequentemente

relacionados ao maior recrutamento de macrófagos e desenvolvimento de resistência à insulina.

Assim, cabe destaque às citocinas e quimiocina IL-18, TNF e o receptor de quimiocinas CCR5 no desenvolvimento da obesidade e alterações metabólicas. Entretanto, alguns pontos ainda permanecem por serem melhor compreendidos e envolvem: (i) o entendimento do papel fisiológico dessas citocinas no controle do metabolismo intermediário; (ii) a avaliação do papel desses mediadores em células hematopoiéticas ou em células não derivadas da medula óssea, e ainda (iii) a análise desses mediadores no contexto de diferentes composições dietéticas, incluindo a dieta rica em carboidratos refinados.

# 3. OBJETIVOS

## 3.1. Objetivo geral

Investigar o papel da IL-18, do TNF e do CCR5 no desenvolvimento e progressão da obesidade induzida por dieta rica em carboidratos refinados.

## 3.2. Objetivos específicos

(i) Verificar o papel da IL-18 e do TNF nas alterações hepáticas de camundongos alimentados com dieta rica em carboidratos refinados.

(ii) Verificar o papel do TNF nas alterações metabólicas inflamatórias no tecido adiposo de camundongos alimentados com dieta rica em carboidratos refinados.

(iii) Investigar o papel da IL-18 no remodelamento e inflamação do tecido adiposo de camundongos alimentados com dieta controle ou dieta rica em carboidratos refinados.

(iv) Avaliar se a ausência do receptor CCR5 altera as respostas metabólicas e inflamatórias desencadeadas por dieta rica em carboidratos refinados em camundongos.

# 4. CAPÍTULO 1

## TNF and IL-18 cytokines may regulate liver fat storage under homeostasis conditions

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# 5. CAPITULO 2

## Paradoxical role of Tumor Necrosis Factor on metabolic dysfunction and adipose tissue expansion in mice

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# 6. CAPITULO 3

## IL-18 controls adipose tissue expansion and inflammation in normal condition but not upon a high-carbohydrate diet challenge

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***Manuscrito em finalização***

**Abstract**

Obesity is characterized by adipose tissue expansion associated with metabolic and inflammatory dysfunction, described as a low-grade inflammation state. In this context, proinflammatory cytokines have been associated with the development of metabolic diseases. However, the role of cytokines in the obesity progress is not fully elucidated. Our aim was to evaluate the role of IL-18 cytokine in metabolic and inflammatory obesity-related alterations. Male C57BL/6 and interleukine-18 deficient (IL-18$^{-/-}$) mice were fed with chow or high refined carbohydrate-containing (HC) diet for 8 weeks. IL-18$^{-/-}$ mice showed increased body weight gain and adiposity, and hyperphagia. In relation to metabolic alterations, IL-18$^{-/-}$ mice presented higher glucose serum levels and glucose intolerance. IL-18$^{-/-}$ mice showed higher serum levels of leptin and lower adiponectin. Furthermore, these mice presented higher levels of TNF and IL-6 in epididymal adipose tissue when fed chow diet. However, upon a refined-carbohydrate diet challenge IL-18$^{-/-}$ mice did not intensify the obesity phenotype or metabolic dysfunction. To verify in which site the lack of IL-18 was important to trigger the alterations observed in mice fed chow diet, a chimeric model was performed. IL-18 appears to play a role in the control of adipose tissue expansion mainly due to IL-18 presence in other tissues than in bone marrow cells. However, IL-18 in bone marrow cells are important to maintain the adipose tissue inflammation and glucose metabolism. Interestingly, adipocytes from IL-18$^{-/-}$ mice secretes more TNF and IL-6. Therefore, IL-18 cytokine seems to be important to constrain adipose tissue expansion and inflammation by acting upon its secretion by bone marrow cells and possibly adipocytes.

Key words: obesity, IL-18, cytokine, glucose intolerance, adiposity, diet.

## Introduction

Obesity has been considered as a global epidemic and is defined as an abnormal accumulation of body fat that may become harmful to the health (CHAN; WOO, 2010). This disease is associated with pathophysiological changes in the adipose tissue by altering its profile of immune cells and, consequently, the pattern of cytokines released, leading to a chronic low grade inflammation (GREGOR; HOTAMISLIGIL, 2011; HOTAMISLIGIL, GS; SHARGILL; SPIEGELMAN, 1993; SUN; KUSMINSKI; SCHERER, 2011; WELLEN; HOTAMISLIGIL, 2003). It is also widely known that in obesity, pro-inflammatory cytokines, as tumor necrosis factor (TNF) or Interleukin-6 (IL-6), which are released by adipocytes, play a significant role in obesity-associated co-morbidities, contributing to the development of type 2 diabetes, cardiovascular diseases and some types of cancer (DE LUCA; OLEFSKY, 2008; GUTIERREZ; PUGLISI; HASTY, 2009; KERN, PHILIP A *et al.*, 2001).

Beyond classical cytokines, i.e. TNF and IL-6, others have been identified as important as during the development of obesity and its comorbidities. The cytokine interleukin-18 (IL-18) has received attention in this context since it is related to inflammasome activation, which is an important proinflammatory route. This cytokine, also called interferon (IFN)-γ-inducing factor, belongs to the IL-1 family and it is produced by Kupffer cells, activated macrophages, keratinocytes, intestinal epithelial cells, adipocytes and preadipocytes (AKDIS et al., 2011; DINARELLO, C A, 1999; SKURK et al., 2005; WOOD et al., 2005). Its role is best known on inflammation, inducing IFN-γ in T cells and natural killer cells, up-regulating Th1 cytokines, stimulating the proliferation of activated T cells (POINT; DINARELLO, 1999), and enhancing Fas ligand expression in Natural Killer cells

and cytotoxic T lymphocytes (DAO et al., 1996). Moreover, Il-18 can directly activate nuclear factor-κB (NF-κB) and induce the production of different cytokines, including TNF, IL-6, IL-8 and IL-1β (KASHIWAMURA; UEDA; OKAMURA, 2002; NAKANISHI et al., 2001; NETEA, MG; KULLBERG, 2000; PUREN et al., 1998).

In obese context, human serum levels of IL-18 were directly associated with body mass index (BMI), adiposity, insulin resistance, hypertriglyceridemia, and metabolic syndrome (ESPOSITO, 2002; OLUSI; AL-AWADHI; ABRAHAM, 2003). In contrast, the lack of IL-18 or IL-18 receptor in mice led to a state similar to metabolic syndrome (AKIRA, 2000; LINDEGAARD *et al.*, 2013; NETEA, MIHAI G *et al.*, 2006). This ablation also leads to hyperphagia and higher body fat mass (LINDEGAARD *et al.*, 2013; NETEA, MIHAI G *et al.*, 2006). Although it was previously demonstrated that IL-18 control food intake and consequently the development of obesity, is still incipient the role of IL-18 in modulating inflammation of adipose tissue and consequently in the remodeling of this tissue. In addition, despite some evidences pointing out to the role of IL-18 on the control of basal metabolism, it is not known whether hematopoietic cells or non-hematopoietic cells, as adipocytes are the main source of IL18 that determines the metabolic effects. In addition, is not yet clear the role of IL-18 on adipose tissue expansion after a dietary challenge rich in refined-carbohydrates, but isocaloric.

Herein, we showed that the ablation of IL-18 increases the body weight gain, the food intake and adiposity. We also found an altered profile of cytokines in white adipose tissue and lower glucose tolerance in mice deficiently in IL18 fed a chow diet. However, in general, the absence of IL-18 signaling did not impair the metabolic and inflammatory pathways in mice fed an obesogenic diet rich in refined carbohydrates. By using a chimeric model we also showed that the ablation of IL-18 in cells other than bone marrow derived cells, possible adipocytes, may contribute to the adipose tissue inflammation and insulin resistance.

## Materials and Methods

### Animal, diet, and tissue collection

Male C57bl/6 wild-type (WT) mice at 6-7 weeks of age were obtained from the animal care center of Universidade Federal de Minas Gerais (CEBIO-UFMG). Male mice lacking IL-18 (IL-$18^{-/-}$) at 6-7 weeks of age were kindly provided by Leda Quercia Vieira (Laboratory of Gnotobiology and Immunology, UFMG, Brazil). All animals were kept in an environmentally controlled room under a 14/10 h light-dark cycle and had free access to water and food. They were maintained according to ethical guidelines of our institution (protocol numbers 347/2013 and 367/2016).

The animals were fed standard laboratory chow (LABINA) or high refined carbohydrate-containing (HC) diet for eight weeks. The HC diet was composed of 45% condensed milk, 10% refined sugar, and 45% chow diet. The macronutrient composition of the chow diet (4.0 kcal/g) was 65.8% carbohydrate, 3.1% fat, and 31.1% protein; the HC diet (4.4 kcal/g) was 74.2% carbohydrate, 5.8% fat, and 20% protein. It is important to note that HC diet contains at least 30% refined sugars, mostly sucrose.

Mice were collectively housed and weighed once a week. Food intake was measured twice a week for eight weeks. At the end of the dietary treatment, animals were anesthetized with ketamine (130 mg/kg) and xylazine (0.3 mg/kg) and killed. Samples of blood, epididymal, retroperitoneal, mesenteric, and subcutaneous white adipose tissue were collected. The adiposity index was calculated as a percentage of body fat (the sum of epididymal, mesenteric, retroperitoneal and subcutaneous adipose tissue) divided by body weight.

### Oral Glucose Tolerance Test

For oral glucose tolerance test, D-glucose (2 mg/g body weight) was given orally to fasted mice (4 hours) at week seven after diet initiation. Glucose levels

were monitored from tail blood samples at 0, 15, 30, 60, 90 and 120 minutes after glucose overload using an Accu-Check glucometer (Roche Diagnostics, Indianapolis, IN).

**Determination of Serum Parameters**

Fasting glucose, total cholesterol and triglycerides were assayed using enzymatic kits (KATAL Belo Horizonte, MG, Brazil). By using ELISA test we measured fasting serum levels of adiponectin, resistin, and leptin, according to manufacturer instructions (all R&D systems Europe Ltd., Abington, UK).

**Histology**

Samples from epididymal adipose tissue were fixed in phosphate-buffered formaldehyde solution for 48 h and then incubated in 70% ethanol. Samples were then dehydrated and embedded in paraffin and sections of tissue were stained with hematoxylin-eosin. Images of six fields from each animal were captured using a digital camera coupled to a microscope (200X). In the analysis of epididymal adipose tissue, the area of 50 cells was measured in each animal using Image Pro-Plus software (Media Cybernetics, USA) and was used ImageJ (National Institutes of Health, Bethesda, Maryland, USA) to calculate the mean adipocyte area ($\mu m^2$).

**ELISA Assay**

IL-6, TNF-α, IL-10, IL-1β, INF-γ assays were performed by using DuoSet ELISA kits and according to the instructions provided by the manufacturer (R&D System, Inc., Minneapolis, USA).

**Adipocyte isolation and cytokines measurements**

Adipocytes were isolated from epididymal fat pads, as described by Rodbell (14). Briefly, digestion with collagenase (1 mg/ml) was carried out at 37ºC with constant shaking (140 cycles/min) for 40 min. Cells were filtered through

nylon mesh and washed three times with buffer plus 1% bovine fatty acid free-serum albumin. After that, adipocytes were separated from stromal vascular cells. Then, they were incubated in DMEM medium for 4 hours at 37°C, and at the end of the incubation period, infranatant was collected. IL-6, TNF and IL-10 were measured by using ELISA assay.

**Chimeric model**

Chimeras with IL-18 deficiency in hematopoietic or in non-hematopoietic derived cells were generated. The chimeras were obtained through BM transplantation between WT and IL-18 knockout mice, as previously described (Castor et al, 2010). Before receiving the hematopoietic cells from the donors, mice aging 8 weeks were subjected to a lethal dose of 4.5 gray of gamma irradiation (source CO60) for bone marrow ablation. Two hours later, the mice received intravenous injection of 1 x 107 BM cells from femur and tibia of the syngeneic WT or IL-$18^{-/-}$ donors. One day before and 15 days later BM transplantation, the mice were treated with ciprofloxacin (70 mg/l), by oral suspension in water. WT mice receiving WT BM cells (WT-BM→ WT) were the control of the experiment. The other groups were constituted of IL-$18^{-/-}$ mice receiving IL-$18^{-/-}$ cells (IL-$18^{-/-}$ -BM→ IL-$18^{-/-}$), WT mice receiving IL-$18^{-/-}$ cells (IL-$18^{-/-}$ BM→ WT) and IL-$18^{-/-}$ mice receiving WT hematopoietic cells (WT-BM→ IL-$18^{-/-}$).

**Statistical Analysis**

Results are expressed as means ± SEM and analyzed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). All data were analyzed for normality of distribution using Kolmogorov-Smirnov test and were found to be normal. Comparison between two groups was performed using Student's t test and multiple comparisons performed using one-way ANOVA with Student-Newman-Keuls post-hoc analysis. Statistical significance was set at $P < 0.05$.

**Results**

**Lack of IL-18 induces an increase in body weight gain and adiposity in mice fed with chow diet**

Wild-type mice fed with HC diet presented higher final body weight gain compared with the same strain fed with chow diet. Interestingly, IL-18$^{-/-}$ mice fed with chow diet or HC diet showed higher body weight gain compared with WT mice (Figure 1A). Although wild-type mice showed no differences in food intake when fed with chow or HC diet, IL-18$^{-/-}$ mice showed higher food intake apart the diet composition (Figure 1B).

Wild-type mice fed with HC diet showed higher adiposity and adipocyte area compared with WT-C group (Figure 1C-D-E). IL-18$^{-/-}$ mice fed chow diet also showed higher adiposity and adipocyte area when compared with WT mice fed the same diet. Interestingly, upon an HC diet challenge, IL-18$^{-/-}$ mice did not worst the adiposity index or adipocyte hypertrophy (Figure 1C-D-E).

Consistently with the increase in adiposity and adipocyte area, serum leptin levels were increased in wild-type mice fed with HC diet. Concomitantly, IL-18$^{-/-}$ mice fed with chow diet showed higher leptin levels compared with WT-C group. However, IL-18$^{-/-}$-HC group did not show any changes in leptin levels when compared either with WT-HC group or IL-18$^{-/-}$-C group (Figure 1E).

**Lack of IL-18 augments cytokine levels in white adipose tissue of mice fed a chow diet, but do not upon a HC diet challenge**

The levels of cytokines in epididymal adipose tissue is shown in Figure 2. It was observed an increase in proinflammatory cytokines TNF (Figure 2A) and IL-6 (Figure 2B) of wild-type mice fed with HC diet when compared with WT-C group. IL-18$^{-/-}$ mice fed chow diet also showed a higher content of TNF and IL-6 in the adipose tissue when compared with WT-C group. When mice deficient in IL-18 were challenged with a diet rich in refined carbohydrates they do not show any increment in the content of cytokines in the adipose tissue compared either WT-HC group or IL-18$^{-/-}$-C group (Figure 2A-B). In addition, there were no

differences in the levels of IL-10 anti-inflammatory cytokine between the experimental groups (Figure 2C).

There were no differences in IL-1β levels between WT or IL-18$^{-/-}$ mice fed with HC diet compared with their respective controls. However, IL-18$^{-/-}$ mice fed with chow diet presented higher IL-1β levels compared with wild-type mice fed with the same diet (Figure 2D). About IFN-Y levels, the consumption of HC diet did not alter the production of this cytokine in wild-type mice. Interestingly, IL-18$^{-/-}$ mice fed chow diet showed increasing levels of IFN when compared to the wild-type fed the same diet. However, IL-18$^{-/-}$ mice fed with HC diet showed lower levels of this cytokine compared to the littermate control (Figure 2E).

**Glucose metabolism is altered by lacking IL-18**

An oral glucose tolerance test was performed to assess glucose metabolism in mice lacking IL-18 either upon a control or HC diet. After a glucose overload, wild-type mice fed with HC diet showed a decrease in glucose tolerance when compared to the wild-type control mice. Interestingly, IL-18$^{-/-}$ mice fed chow diet already showed a decrease in glucose tolerance. There was no difference in glucose tolerance in IL-18$^{-/-}$ mice fed with chow or HC diet (Figure 3A and 3B)

It was not observed difference in fasting glucose comparing WT-HC mice and WT control mice. IL-18$^{-/-}$ fed with chow or HC diet presented higher values of fasting glucose in relation to their respective wild-type controls. No differences were observed between IL-18$^{-/-}$ mice fed with chow or HC diet (Figure 3D). Adiponectin levels were also measured. Wild-type mice fed with HC diet showed a decrease in this adipokine when compared to the respective control. IL-18$^{-/-}$ mice fed a chow diet showed a reduction in serum adiponectin levels when compared with WT-C group. We also observed that IL-18$^{-/-}$ mice fed HC diet

presented lower adiponectin levels when compared with IL-18$^{-/-}$-C mice (Figure 3C).

**Metabolic and inflammatory changes in mice lacking IL-18 seem to be related to the activity of this cytokine on bone marrow cells and outsider tissues**

Adiposity and adipocyte area did not change in WT mice receiving bone marrow cells from IL-18$^{-/-}$ mice. As expected, the whole body IL-18 deficiency mice showed a higher adiposity and bigger adipocytes. Interestingly, IL-18 deficiency mice that received bone marrow from WT mice showed a comparable adiposity seen in whole body IL-18 deficiency mice. It may indicate that hematopoietic cells producing IL-18 did not constrain the adipose tissue expansion (Figure 4 A and B) indicating that cells other than hematopoietic cells are the IL-18 source that mediate the control of the adipose tissue enlargement. Leptin serum levels were also higher in whole body IL-18 deficiency mice and in IL-18 mice receiving BMC from WT, but not alters (Figure 4C).

Oral glucose tolerance test was also performed in chimeric mice. WT mice receiving BMC from IL-18$^{-/-}$ mice showed decreased glucose tolerance. As expected, the whole body IL-18 deficiency mice showed worst glucose tolerance. It is possible that hematopoietic cells producing IL-18 did impact in glucose tolerance. In addition, the whole body IL-18 deficiency mice showed a decrease in glucose tolerance (Figure 5A and 5B). Nevertheless, serum glucose levels (Figure 5C) or adiponectin levels (Figure 5D) did not alter in WT mice receiving bone marrow cells from IL-18$^{-/-}$ mice. IL-18$^{-/-}$ to IL-18$^{-/-}$ group showed increased serum glucose (Figure 5C) and decreased adiponectin levels (Figure 5D), as expected.

Interestingly, IL-6 and TNF levels in the in epididymal adipose tissue were increased when WT mice received BMC transplant from IL-18$^{-/-}$ mice (Figure 6 A and B). As expected, the whole body IL-18 deficiency mice showed increased

levels of IL-6 and TNF in epididymal adipose tissue compared with WT to WT mice. In the IL-10 analysis, the transplant of BMC cells did not alter the cytokine content in the epididymal adipose tissue (Figure 6C).

To evaluate whether the IL-18 alters the cytokine secretion in adipocytes, fat cells were isolated from WT and IL-18$^{-/-}$ mice and incubated in normal media. Adipocytes from IL-18$^{-/-}$ mice secreted more IL-6 (Figure 6D) and TNF (Figure 6E) when compared to adipocytes harvested from WT. We found no changes concerning adipocytes secretion of IL-10 by IL-18$^{-/-}$ or WT mice (Figure 6F).

## Discussion

Obesity and metabolic dysfunction are linked by a state of low grade inflammation due to the expansion of the adipose tissue mass. In this context, many cytokines have been show be important in the development of metabolic disease. Herein, we have shown that IL-18 have a physiological role to constrain adipose expansion and protect mice from glucose intolerance. The major points of this study were: (i) the lack of IL-18 in mice fed with chow diet leads to the development of metabolic and inflammatory alterations, as hyperglycemia, increased body weight gain and augmentation of cytokine levels in adipose tissue; (ii) when IL-18$^{-/-}$ mice were challenged with an overload of nutrients containing refined carbohydrates, they did not present exacerbation of these parameters; and (iii) the major adipose tissue expansion observed in mice lacking IL-18 seems to be related to absence of IL-18 release from non-hematopoietic cells, while the glucose intolerance seems to be due to its absence in hematopoietic cells.

IL-18$^{-/-}$ mice showed hyperphagia, higher body weight gain and adiposity than WT mice, regardless of the diet composition. Indeed, these data corroborate with findings of other authors, which demonstrated that the lack of IL-18 leads to hyperphagia and obesity in animal models, irrespective of gender or diet

(LINDEGAARD *et al.*, 2013; NETEA, MIHAI G *et al.*, 2006; ZORRILLA *et al.*, 2007). Lindegaard et al. (2013) also showed that the intake of a high-fat diet, despite to promote an increase in adipose tissue in WT mice, did not exacerbate the fat enlargement in IL-18$^{-/-}$ mice. Thus, IL-18 ablation is sufficient to determine an increase in adipose tissue expansion but the consumption of a high carbohydrate diet or a high fat diet did not aggravate the phenotype.

It was also observed an increase in serum leptin levels in IL-18$^{-/-}$ mice fed with chow diet. This augmentation is related to the increase in adiposity in those mice, since the rate of leptin release is proportional to fat mass (CONSIDINE; SINHA, 1996; FREDERICH; HAMANN; ANDERSON, 1995), and it shows a high correlation with adipocyte size (ZHANG, YIYING; GUO; DIAZ, 2002). The role of leptin in food intake and energy expenditure is already widely known. Thus it may impact directly in weight gain (NETEA, MIHAI G *et al.*, 2006). Netea et al. (2006) also showed an increase in serum leptin levels of IL-18$^{-/-}$ mice fed with chow diet, and this augmentation was highly associated with major body weight. In the present study, IL-18$^{-/-}$ mice fed with HC diet did not present major leptin levels compared to WT mice fed the same diet. In agreement, Wang et al. (2013) showed that caspase-1 deficiency, which impairs IL-18 cleavage, did not exacerbate the hyperleptinemia in animals fed with an obesogenic diet. Moreover, Kimura et al.(2016) also assessed serum levels of leptin in high-fat feeding Caspase-1 deficient mice and found that they were significantly increased compared with high-fat feeding WT mice.

Insulin resistance is highly present in the obese state (REAVEN, 1995). Then, we also evaluated some parameters related to glucose metabolism. We showed that IL-18$^{-/-}$ mice showed glucose intolerance and higher glucose serum levels. Literature data show consistently that glucose clearance is impaired in IL-18$^{-/-}$ mice, accompanied to an increase in gluconeogenesis enzymes (LINDEGAARD *et al.*, 2013; NETEA, MIHAI G *et al.*, 2006). One of the pathways activated by IL-18 receptor is the signal transducer and activator of transcription 3 (STAT3). An increase in STAT3 phosphorylation leads to a reduction in the

expression of proteins related to gluconeogenesis (GROSS, D N; VAN DEN HEUVEL; BIRNBAUM, 2008; WANG, RUI-HONG *et al.*, 2011). Indeed, Netea et al. (2006) showed that STAT3 phosphorylation appears to be impaired in IL-18$^{-/-}$ mice resulting in higher glucose levels. Our study also showed that the levels of IL-6, TNF and IL-1β were increased in IL-18$^{-/-}$ mice. Such increase may also contribute to worsening glucose metabolism since those cytokines disrupt important insulin signaling pathways (BARBARROJA *et al.*, 2010; UYSAL *et al.*, 1997; XU; BARNES; YANG, 2003). Accordingly, IL-18$^{-/-}$ mice also showed a decrease in serum adiponectin levels. Adiponectin stimulates glucose uptake (MARCELL et al., 2005) and participates in the maintenance of metabolic homeostasis (LEE; SHAO, 2013). The lower levels of adiponectin may be related with a higher content of proinflammatory cytokines in the adipose tissue from IL-18 $^{-/-}$ mice. Indeed, adipose tissue inflammation during obesity may suppress the adiponectin release (OUCHI et al., 2003). Thus, the increase in proinflammatory cytokines levels in epididymal adipose tissue of IL-18$^{-/-}$ mice could modulate the secretion of adiponectin and consequently the glucose tolerance.

Deficiency in other inflammasome compounds can result in a decrease in IL-18 cleavage. Studies have shown that Caspase1 $^{-/-}$ mice gained significantly more body weight, visceral and subcutaneous fat contents than WT mice treated with an obesogenic diet (KIMURA *et al.*, 2016; KOTAS *et al.*, 2013; WANG, H *et al.*, 2013). As the caspase-1 activity culminates in both IL-18 and IL-1β cleavage, Kimura et al. (2016) also assessed the effect of high-fat diet on the development of obesity using IL-1β$^{-/-}$ mice. These mice significantly gained less body weight than WT mice. On the other hand, Stienstra et al. (2011) showed that caspase-1, ASC protein and Nlrp3 (NOD-like receptor family pyrin domain containing 3) deficiencies protect mice from diet induced obesity. Moreover, as the activation of inflammasome culminates in both IL-18 and IL-1β activation (AKDIS *et al.*, 2011), we evaluated IL-1β levels in adipose tissue of IL-18$^{-/-}$ mice. IL-1β is increased in epididymal adipose tissue of IL-18$^{-/-}$ mice fed with chow diet, but this was not observed in IL-18$^{-/-}$ mice fed with HC diet. Although IL-18 is known to

induce IFN-γ production (NAKANISHI *et al.*, 2001), we assessed IFN-γ in adipose tissue of IL-18$^{-/-}$ mice. IL-18$^{-/-}$ mice fed with chow diet showed an increase in IFN-γ levels in epydidimal adipose tissue, but it was not observed in IL-18$^{-/-}$ mice fed with HC diet. Kimura et al.(2016) also showed that levels of IFN-γ and IL-1β tend to be elevated in the Caspase 1$^{-/-}$ mice, but it did not reach statistical significance. These data demonstrate that some impairment in inflammasome activation can alters obesity outcomes. Our data support that the release of IL-18, a downstream cytokine, is important to the maintenance of basal metabolism and adipose tissue mass control.

To evaluate which cells play the major role in IL-18 secretion and consequently in mantainance of the homeostatic balance, we performed a chimeric model. We showed that transplantation of BMC of IL-18$^{-/-}$ to WT mice did not lead to the adipose tissue expansion. These data can indicate that IL18 secreted from non-hemapoietic cells may constrain the adipose tissue expansion. On the other hand, the transplantation of BMC of IL-18$^{-/-}$ to WT mice altered their glucose tolerance accompanied by major inflammation in the adipose tissue. The absence of IL-18 release in hematopoietic cells can impair glucose tolerance by promote an increase in proinflamatory cytokines release. Thus, we believe that the role of IL-18 in homeostasis and in intermediate metabolism was due to its release by both hematopoietic cells and by other cells, as adipocytes.

In summary, the present study showed that IL-18 plays an important role in body weight and food intake control. Also, its presence seems to be important for the regulation of metabolic and inflammatory responses in metabolic organs, including adipose tissue. The secretion of IL-18 by other cells than hematopoietic cells seems to regulate adipose tissue enlargement. On the other, hematopoietic cells, secreting IL-18 seems to control glucose homeostasis. Despite, the obese phenotype seen in mice lacking IL-18, the nutrient overload triggered by HC diet did not exacerbate the obesity or metabolic dysfunction. Then, the phenotype related to the IL-18 lack may highlight this cytokine as an important mediator in the control of body weight gain and adipose tissue expansion.

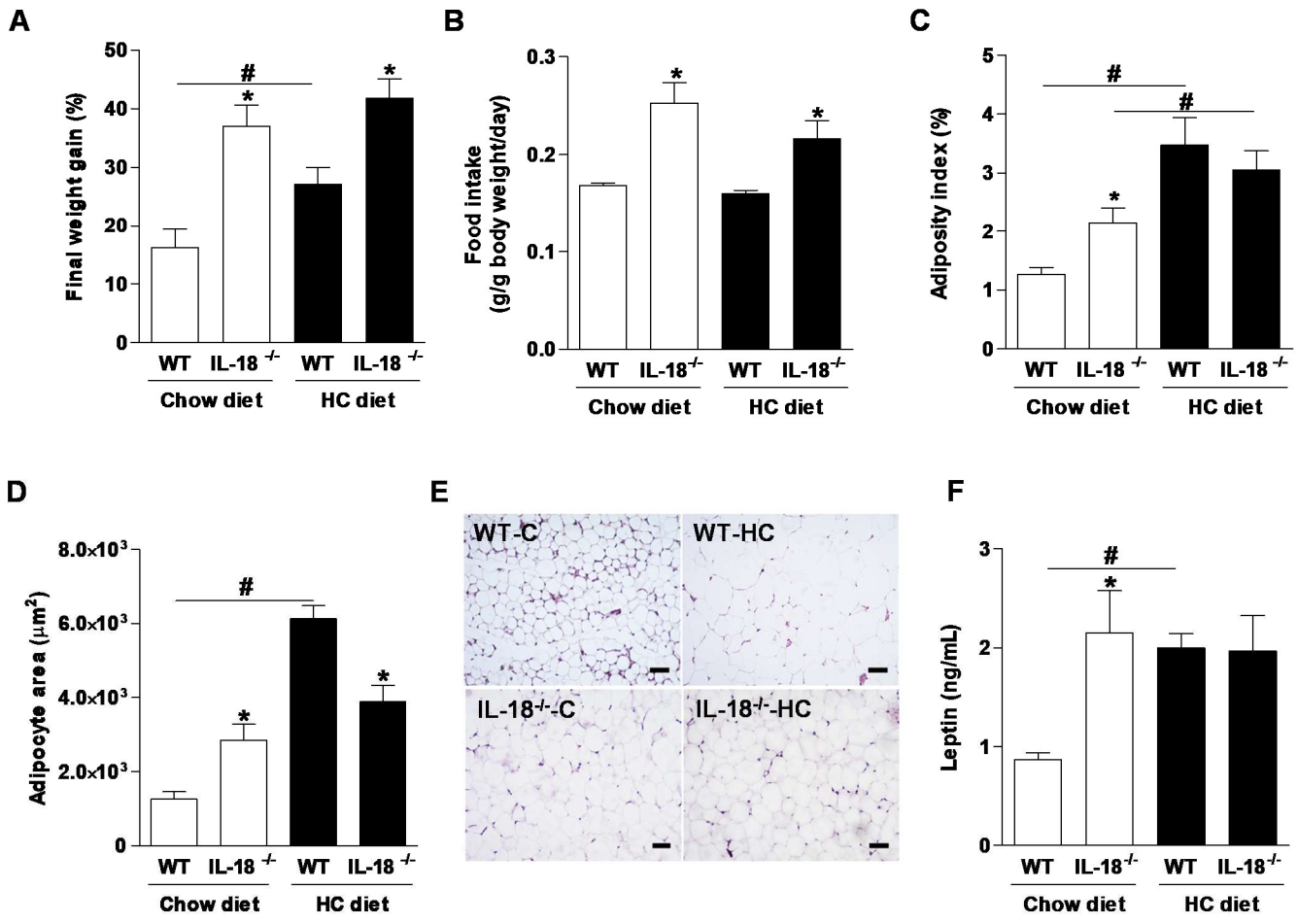


**Figure 1** - (A) Representation of final body weight gain in percentage; (B) daily food intake; (C) evaluation of the adiposity index (mass sum of epididymal, mesenteric, and retroperitoneal adipose tissues x 100 / body weight); (D)

determination of adipocyte area in epididymal adipose tissue; (E) histological analysis of epididymal adipose tissue sections (100x), bars represent 50μm; and (F) serum leptin levels of wild-type (WT) and knockout IL-18 (IL-18$^{-/-}$) mice fed with chow diet or high refined carbohydrate-containing (HC) diet during 8 weeks. The bars represent the mean ± standard error of the mean, n = 6-8. * $P < 0.05$ vs. WT; # $P < 0.05$ vs. chow diet.

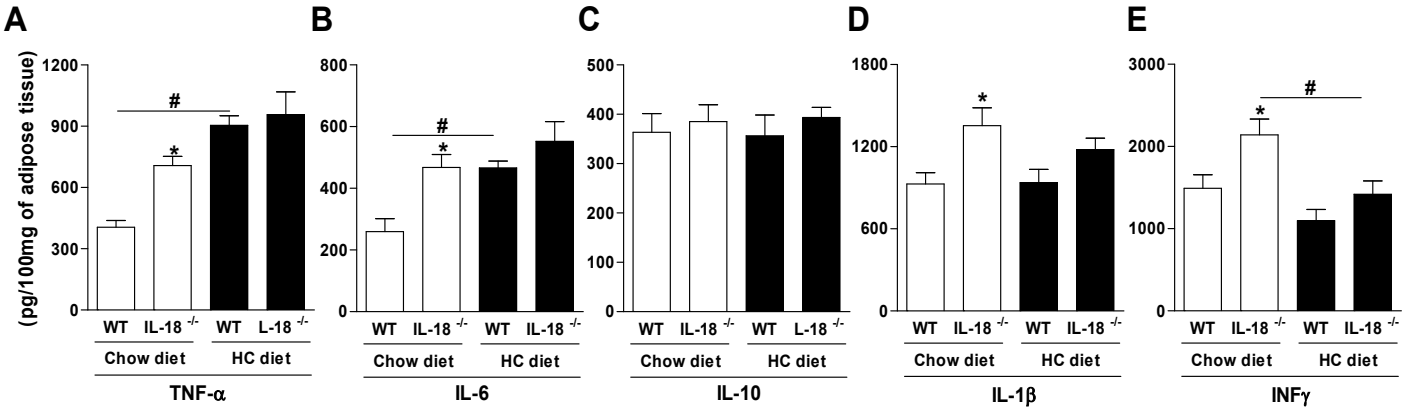


**Figure 2** - Levels of cytokines (A) TNF, (B) IL-6, (C) IL-10, (D) IL-1β, (E) IFN-γ in the epididymal adipose tissue of wild-type (WT) and knockout IL-18 (IL-18$^{-/-}$) mice fed with chow diet or high refined carbohydrate-containing (HC) diet during 8 weeks. The bars represent the mean ± standard error of the mean, n = 5-9. * $P < 0.05$ vs. WT; # $P < 0.05$ vs. chow diet.

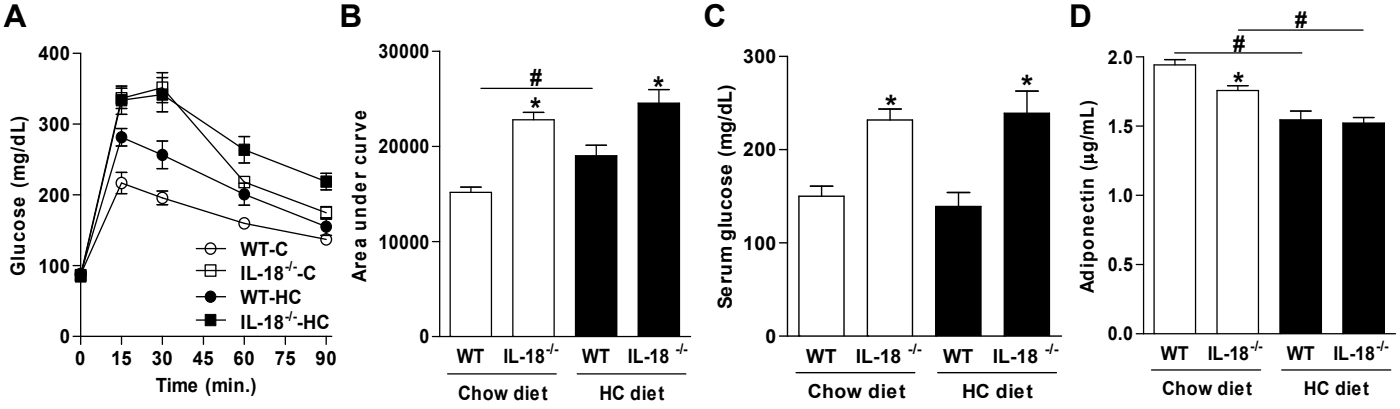


**Figure 3** - (A) Oral glucose tolerance test (OGTT), glucose curve in relation to baseline (time 0 - fasting) at 15, 30, 60 and 90 minutes after gavage of 2 mg / g body weight of glucose. (B) Representation of the area under the OGTT curve. Metabolic analyses in the serum of (C) glucose and (D) adiponectin of wild-type (WT) and knockout IL-18 (IL-18$^{-/-}$) mice fed with chow diet or high refined carbohydrate-containing (HC) diet during 8 weeks. The bars represent the mean ± standard error of the mean. n = 5-9. * $P < 0.05$ vs. WT; # $P < 0.05$ vs. chow diet.

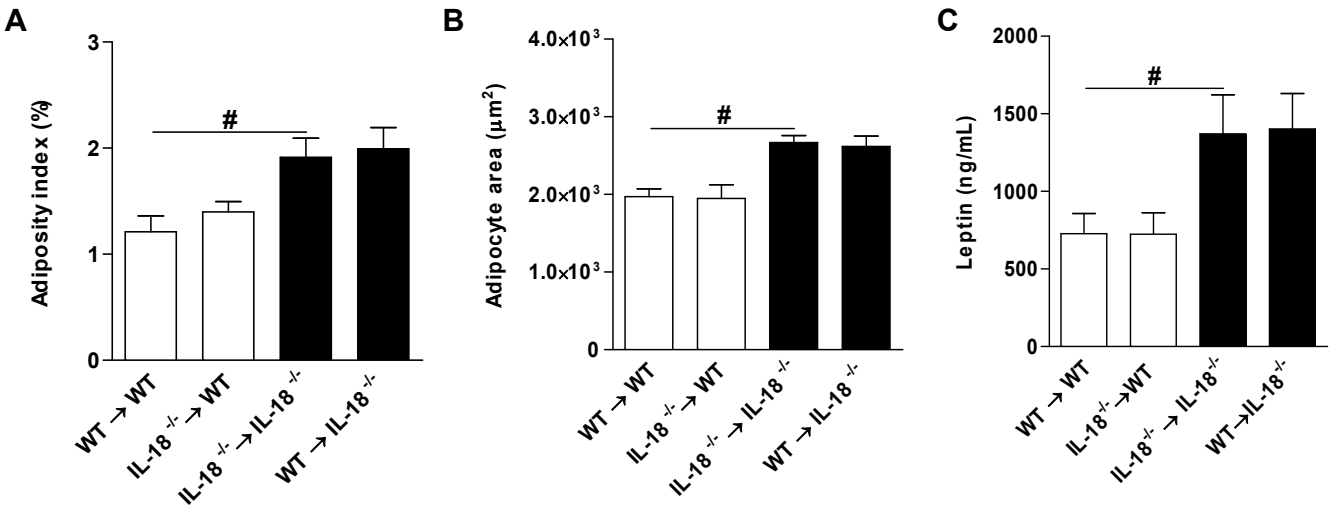


**Figure 4 -** (A) Evaluation of the adiposity index (mass sum of epididymal, mesenteric, and retroperitoneal adipose tissues x 100 / body weight); (B) determination of adipocyte area in epididymal adipose tissue; (C) serum leptin levels of chimeric mice fed with chow diet. The bars represent the mean ± standard error of the mean, n = 6-8. * *P* <0.05 vs. WT to WT or IL-18$^{-/-}$ to IL-18$^{-/-}$; # *P* <0.05 vs. inverse chimeric.

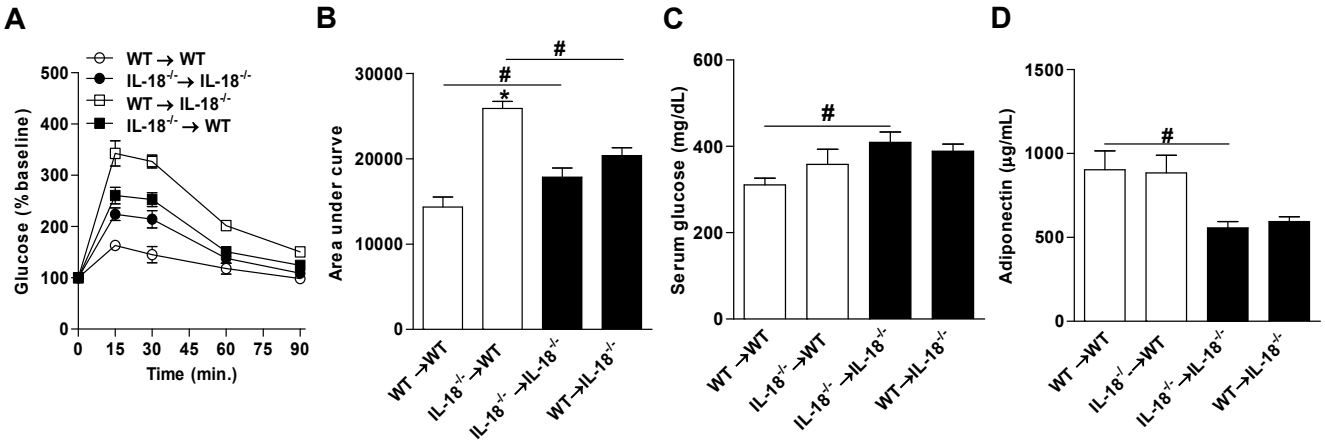


**Figure 5** - (A) Oral glucose tolerance test (OGTT), glucose curve in relation to baseline (time 0 - fasting) at 15, 30, 60 and 90 minutes after gavage of 2 mg / g body weight of glucose. (B) Representation of the area under the OGTT curve. Metabolic analyses in the serum of (C) glucose and (D) adiponectin of chimeric mice fed with chow diet. The bars represent the mean ± standard error of the mean, n = 6-8. * *P* <0.05 vs. WT to WT or IL-18$^{-/-}$ to IL-18$^{-/-}$, # *P* <0.05 vs. inverse chimeric.

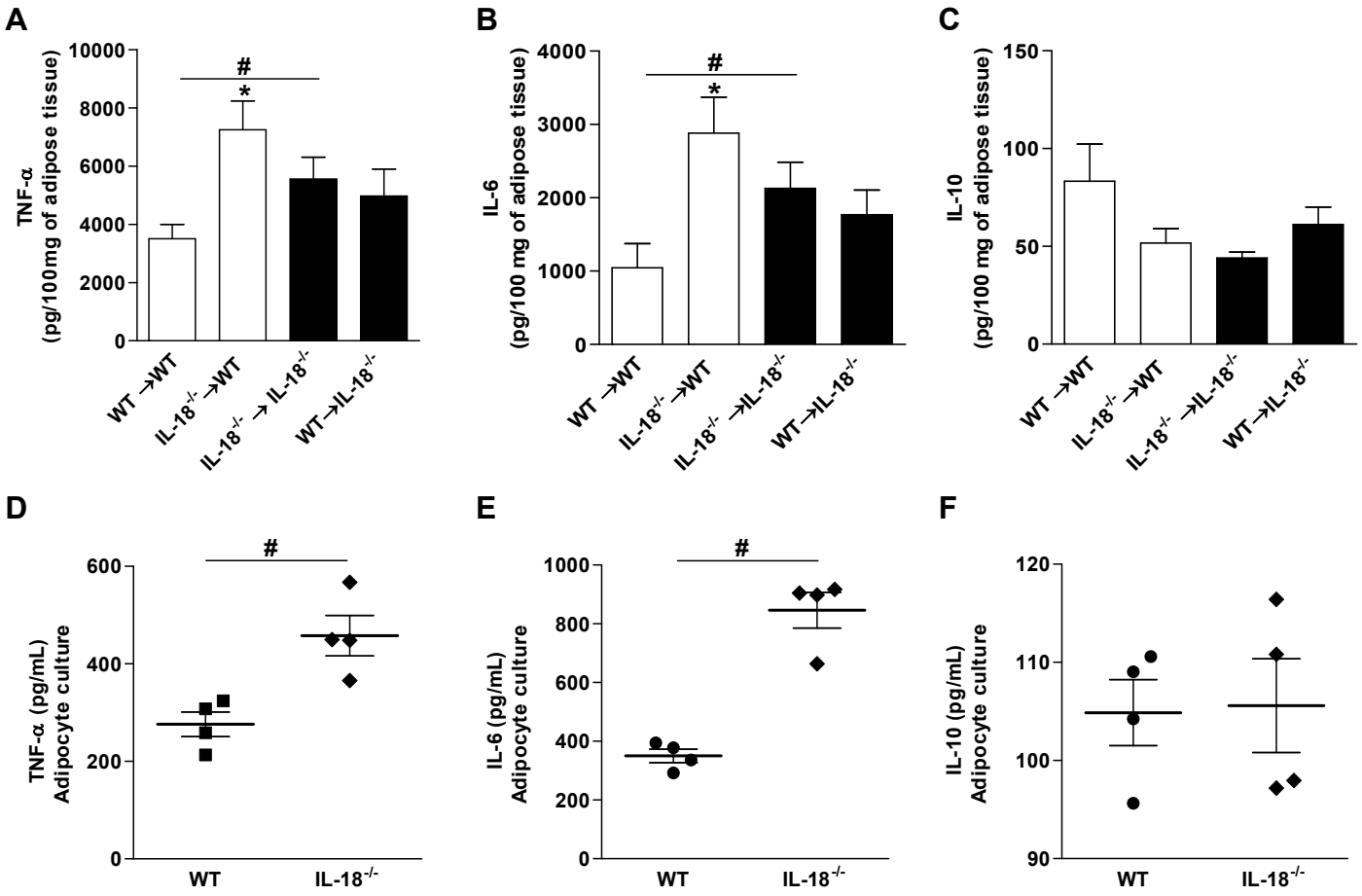


**Figure 6**- Levels of cytokines (A) TNF, (B) IL-6 and (C) IL-10 of chimeric mice fed with chow diet. The bars represent the mean ± standard error of the mean, n = 6-8. **P* <0.05 vs. WT to WT or IL-18$^{-/-}$ to IL-18$^{-/-}$; # *P* <0.05 vs. inverse chimeric. Levels of cytokines (A) TNF, (B) IL-6 and (C) IL-10 of infranatant of adipocyte culture from wild-type (WT) and knockout IL-18 (IL-18$^{-/-}$) mice. The bars represent the mean ± standard error of the mean, n = 4. **P* <0.05 vs. WT mice.

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# 7. CAPITULO 4

## Effect of CCR5 deficiency on metabolic and inflammatory changes resulting from intake of high-refined carbohydrate-containing diet

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***Manuscrito em preparação.***

**Abstract**

Obesity is related to a state of chronic low-grade inflammation characterized by the increased production of inflammatory mediators and presence of immune cells. The recruitment of proinflammatory macrophages and T cells to adipose tissue plays an essential role in the disease development. Although some receptors of chemokines are overexpressed in obesity context, it is controversial the role of the role of C-C motif chemokine receptor 5 (CCR5) in metabolic and inflammatory changes in adipose tissue and the liver upon overload of nutrients, specifically triggered by the consumption of a refined carbohydrate diet. Then, we aimed to evaluate whether CCR5 ablation alters metabolic and inflammatory responses related to obesity. Male C57BL/6 and CCR5 deficient ($CCR5^{-/-}$) mice were fed with chow diet or high refined carbohydrate-containing diet (HC) for eight weeks. We evaluated the effect of CCR5 ablation in adipose tissue and liver of those mice. We demonstrated that CCR5 deficiency in mice did not alter body weight gain and visceral adiposity but increases inguinal fat pad. Metabolic alterations were also observed by impaired glucose levels, insulin sensitivity and glucose tolerance, and altered systemic lipids reflected by increased triglycerides content in the liver. In general, the inflammation was reduced systemic and in the adipose tissue of $CCR5^{-/-}$ mice treated with HC diet. Therefore, the decreased inflammatory response caused by a deletion of CCR5 seems to contribute to metabolic dysfunction, indicating that the inflammatory response related to leukocytes may contribute to metabolic homeostasis.

Key words: obesity, CCR5, cytokine, chemokine, glucose intolerance, adiposity, diet.

## Introduction

Obesity is related with a state of chronic low-grade inflammation, characterized by increased concentrations of circulating proinflammatory cytokines and the activation of inflammatory pathways (FANTUZZI, 2005; GREGOR; HOTAMISLIGIL, 2011; HOTAMISLIGIL, GS; SHARGILL; SPIEGELMAN, 1993; WELLEN; HOTAMISLIGIL, 2003). Its development triggers the recruitment of proinflammatory macrophages and T cells to metabolically activated tissues, such as adipose tissue (CALDER *et al.*, 2011; OH *et al.*, 2012; OSBORN; OLEFSKY, 2012), liver and muscle (LI; SOLOSKI; DIEHL, 2005; OBSTFELD *et al.*, 2010; STANTON *et al.*, 2011). These cells release inflammatory mediators such as TNF, IL-6 and CCL2, which impair insulin signaling and lead to the development of insulin resistance, contributing to the progress of type 2 diabetes, metabolic syndrome and non-alcoholic fatty liver disease (FANTUZZI, 2005; KANDA; TATEYA; TAMORI, 2006; KITADE *et al.*, 2012; STRISSEL *et al.*, 2009; WEISBERG, SP; HUNTER, 2006; WINER *et al.*, 2009). Thus, studying the recruitment of immune cells arising from the state of obesity becomes important, since there is a diversity of inflammatory mediators that are not well explored in this disorder.

It is known the role of chemokines and their receptors in immune cell chemotaxis. Chemokines are small proinflammatory cytokines with chemoattractant properties through binding to specific G protein-coupled receptors (CHARO; RANSOHOFF, 2006; RANSOHOFF, 2009). C-C motif chemokine receptor 2 (CCR2) and its ligand monocyte chemoattractant protein-1 (CCL2) participate in the development of insulin resistance (Kanda et al., 2006; Weisberg & Hunter, 2006). Also, human studies have shown upregulation in the expression of not only MCP-1 and CCR2, but also other CC chemokines and their

receptors in the visceral fat of obese individuals (HUBER *et al.*, 2008). Although receptors of chemokines are expressed in adipose tissue, it is not yet clear the role of C-C motif chemokine receptor 5 (CCR5) in metabolic and inflammatory changes due to obesity development.

CCR5 is a receptor for RANTES, CCL5, MIP1-α, CCL3, MIP1-β, CCL4, MIP-1β, CCL-8. These ligands are important in the recruitment of monocytes, macrophages, activated T cells, natural killer and Th1 cells (MANTOVANI *et al.*, 2004). Kitade et al. (2012) showed that CCR5 and its ligands are upregulated in white adipose tissue of obese mice, particularly in the macrophage fraction. Although some studies have linked CCR5 and its ligands to obesity in experimental models, the role of this molecule in obesity outcomes is still controversial. Some studies suggest that the lower recruitment of macrophages to the site of inflammation triggered by CCR5 ablation could be protective in obesity (HUH et al., 2018; KITADE et al., 2012). Another demonstrated that the decreased inflammatory response might impair glucose metabolism and insulin sensitivity (KENNEDY et al., 2013). Despite macrophage recruitment is an important step for the development of metabolic alterations related to obesity, it is still necessary to elucidate the role of this receptor in the adipose (visceral and subcutaneous) compartments, as well as in metabolic organs such as the liver. Also, the function of the CCR5 upon a nutrient overload caused by diets with higher carbohydrate content had not been evaluated.

Then, we evaluated the effect of high-refined carbohydrate-containing (HC) diet on adipose tissue, liver and systemic alterations in mice lacking CCR5. Herein, we demonstrated that CCR5 deficiency does not alter body weight gain and visceral adiposity, but increases inguinal fat pad, impairs glucose and lipid metabolism contributing to fatty liver. In general, when these mice are fed with HC diet, the insulin resistance is aggravated as well as the liver steatosis without an increment at inflammation.

## Materials and Methods

### Animal, diet, and tissue collection

Male CCR5$^{-/-}$ mice and C57bl/6 mice at 6-7 weeks of age were obtained from the animal care center of Universidade Federal de Minas Gerais (CEBIO-UFMG) and kept in an environmentally controlled room under a 14/10 h light-dark cycle. Animals had free access to water and food and were maintained according to ethical guidelines of our institution (Protocol nº 295 / 2014). Animals were fed with a standard laboratory chow (LABINA) or high-refined carbohydrate-containing (HC) diet for 8 weeks.

The HC diet was composed of 45% condensed milk, 10% refined sugar, and 45% chow diet. The macronutrient composition of the chow diet (4.0 kcal/g) was 65.8% carbohydrate, 3.1% fat, and 31.1% protein; the HC diet (4.4 kcal/g) was 74.2% carbohydrate, 5.8% fat, and 20% protein. The HC diet contains at least 30% refined sugars, mostly sucrose (Oliveira et al, 2013).

Mice were collectively housed and weighed once a week. Food intake was measured twice a week for eight weeks. At the end of the experimental period, animals were anesthetized with ketamine (130 mg/kg) and xylazine (0.3 mg/kg) and killed. Samples of blood, epididymal, retroperitoneal, mesenteric, and subcutaneous adipose tissue and liver were collected. The adiposity index was calculated as a percentage of body fat (the sum of epididymal, mesenteric, retroperitoneal adipose tissue) divided by body weight.

**Oral glucose tolerance Test**

For oral glucose tolerance test, D-glucose (2 mg/g body weight) was given orally to mice that were fasted overnight. This test was performed at the week seven after initiation of diets. Glucose levels were monitored from tail blood samples at 0, 15, 30, 60, 90 and 120 minutes after glucose overload using an Accu-Check glucometer (Roche Diagnostics, Indianapolis, IN).

**Determination of serum parameters**

Fasting glucose, cholesterol, triglycerides (KATAL Belo Horizonte, MG, Brazil) and non-esterified fatty acids (NEFA) levels (Wako chemicals, USA) were assayed using enzymatic kits. ELISA analyses determined the fasting serum levels of insulin (Millipore, Missouri, MO, USA), adiponectin, resistin, and leptin (R&D systems Europe Ltd., Abington, UK). The homeostatic model assessment-insulin resistance (HOMA-IR) was calculated as follows: HOMA-IR = fasting glucose level (mmol/L) × fasting insulin level (μU/mL) ÷ 22.5.

**Histology**

Samples from epididymal adipose tissue, subcutaneous adipose tissue and liver were fixed in phosphate-buffered formaldehyde solution for 48h and then incubated in 70% ethanol. Samples were then dehydrated and embedded in paraffin and sections of the tissue were stained with hematoxylin-eosin. Images of six fields from adipose tissue of each animal were captured using a digital camera coupled to a microscope (100x). For analysis both epididymal adipose tissue and subcutaneous adipose tissue, area of 50 cells was measured in each animal using Image Pro-Plus software (Media Cybernetics, USA) and was used ImageJ (National Institutes of Health, Bethesda, Maryland, USA) to calculate mean adipocyte area ($\mu m^2$). For liver analysis was performed the histopathological score it was used an index measuring 10 random fields to determine the mean of each liver. The inflammatory infiltrate, blood vessel

inflammation sinusoidal cells and steatosis were evaluated using the method proposed by Kleiner et al. (2005).

**Isolation and purification of total lipids**

The extractions were performed as described by Folch et al. (1957), using the original extraction ratio of 20 parts 2:1 chloroform/ methanol to 1 part tissue. A weak salt solution (NaCl 0.9%) was then added to achieve a final ratio of 8:4:3 chloroform/methanol/water after including the water contained in the tissue. Total liver fat was determined by gravimetry of the evaporated solution. The extract previously obtained was mixed with 500 µL of isopropanol, and the triglyceride and total cholesterol levels were evaluated using enzymatic kits (KATAL Belo Horizonte, MG, Brazil).

**ELISA assay**

IL-6, TNF-α, IL-10 assays were performed in epididymal and subcutaneous adipose tissues using DuoSet ELISA kits and according to the instructions provided by the manufacturer (R&D System, Inc., Minneapolis, USA).

**Statistical analysis**

Results are expressed as means $^{+}_{-}$ SEM and analyzed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). All data were analyzed for normality of distribution using Kolmogorov-Smirnov test and were found to be normal. Comparison between two groups was performed using Student's t test and multiple comparisons performed using one-way ANOVA with Student-Newman-Keuls post-hoc analysis. Statistical significance was set at $P < 0.05$.

**Results**

**CCR5 deficiency does not alter body weight and visceral adiposity but increases inguinal fat mass**

There was no difference in body weight gain between WT-HC and WT-C groups or between CCR5$^{-/-}$-HC and CCR5$^{-/-}$-C groups (Figure 1A). Nevertheless, WT mice fed with HC diet presented an increase in visceral adiposity (Figure 1B) and adipocyte area in epididymal adipose tissue (Figure 1D and 1G) compared with the WT mice fed with chow diet, as well as observed in CCR5$^{-/-}$-HC group compared with the CCR5$^{-/-}$ mice fed with chow diet.

As observed in visceral adiposity, WT mice fed with HC diet have shown an increase in inguinal adipose tissue depots as well as in its adipocyte area compared with the WT-C group (Figure 1C, 1E and 1G). However, this increase was not observed between CCR5$^{-/-}$- HC and CCR5$^{-/-}$-C mice in relation to inguinal fat mass or its adipocyte area (Figure 1C, 1E and 1G). Interestingly, CCR5$^{-/-}$ mice fed with chow diet presented an increase in those parameters compared with WT-C mice. However, CCR5$^{-/-}$-HC mice did not demonstrate an increment in adipocyte area of inguinal adipose tissue compared with WT-HC group (Figure 1C, 1E and 1G).

Leptin is an adipokine related to appetite, but also to adipose tissue volume (FREDERICH; HAMANN; ANDERSON, 1995). Then, we analyzed it in the serum of all groups. Both WT and CCR5$^{-/-}$ mice fed with HC diet presented an increase in this parameter compared with the respective strain fed with chow diet. There was also an increase in leptin levels between CCR5$^{-/-}$ mice fed with chow diet compared with WT mice fed with the same diet (Figure 1F).

**Impact of CCR5 lack in the profile of cytokines in white adipose tissue**

TNF and IL-6 proinflammatory cytokines were assessed in adipose tissues of mice. In epididymal adipose tissue, both cytokines were increased in WT-HC group compared with WT-C mice. However, there were no differences between CCR5$^{-/-}$-HC compared with the CCR5$^{-/-}$-C mice, since CCR5$^{-/-}$-HC group presented lower levels in those cytokines compared with WT-HC mice (Figure 2A and 2B). On the other hand, in inguinal adipose tissue, WT-HC mice showed a

decrease in TNF-α compared with the WT-C mice. Already, CCR5$^{-/-}$-HC mice presented an increase in those parameters compared with CCR5$^{-/-}$-C group. Also, CCR5$^{-/-}$-C showed a decrease in TNF-α levels compared with WT-C mice (Figure 2C). There were no differences in IL-6 levels in inguinal depots between groups (Figure 2D).

**Lack of CCR5 induces insulin resistance**

We also evaluated whether glucose metabolism would be altered by lacking CCR5 in mice. Then, the oral glucose tolerance test was performed. Mice fed with HC diet, independent of the strain, presented glucose intolerance when compared with the mice fed with chow diet of the same strain. Moreover, this intolerance was also observed CCR5$^{-/-}$ groups in relation to their WT controls (Figure 3A and 3B).

Some parameters were also evaluated in the serum and HOMA-IR index was determined. In an analysis of serum glucose, WT-HC mice showed an increase in glucose compared with the WT-C group. There were no differences between CCR5$^{-/-}$-C and CCR5$^{-/-}$-HC groups. However, both CCR5$^{-/-}$-C and CCR5$^{-/-}$-HC presented an increase in serum glucose levels compared with their respective WT controls (Figure 3C).

There were no differences in insulin between WT-HC and WT-C groups. However, CCR5$^{-/-}$-HC mice presented an increase in insulin levels compared with the CCR5$^{-/-}$-C mice. In addition, both CCR5$^{-/-}$ groups, independent of diet, showed an increase in this parameter compared with the respective WT controls (Figure 3D). The same profile was also observed in relation to the HOMA-IR index (Figure 3E).

We also evaluated the serum levels of adipokines related to inflammation, but also to glucose metabolism, adiponectin and resistin. As expected, WT-HC mice presented a decrease in adiponectin levels compared with WT-C group. However, a reduction in this adipokine was also observed CCR5$^{-/-}$-C mice

compared with the WT-C mice and demonstrated similar levels to CCR5$^{-/-}$-HC (Figure 3F).

On the other hand, it was observed that resistin levels were increased in WT-HC mice compared with WT-C group. There were no differences between CCR5$^{-/-}$-HC and CCR5$^{-/-}$-C groups, however CCR5$^{-/-}$-HC showed lower resistin levels than WT-HC mice (Figure 3G).

The increased circulating levels of non-esterified fatty acids (NEFA) may be associated with insulin resistance. Then, we analyzed NEFA in the serum. It was observed an increase of it in WT-HC mice compared with WT-C group. There were no differences between CCR5$^{-/-}$-HC and CCR5$^{-/-}$-C groups. Nevertheless, CCR5$^{-/-}$-C mice showed an increase in NEFA levels compared with the WT-C mice (Figure 3H).

**Circulating leukocytes are not augmented in CCR5 deficient mice fed with HC diet**

Since CCR5 is a receptor for chemokines, we evaluated the circulating number of leukocytes. It is observed in the total leukocyte count, WT-HC mice presented an increase in circulating leukocytes compared with the WT-C mice. However, CCR5$^{-/-}$-HC mice showed a lower number of total leukocytes compared with WT-HC group (Figure 4A). The profile observed in the number of total leukocytes was similar to multinucleated cells (Figure 4B), but there were no differences between mononucleated cells (Figure 4C).

**CCR5 deficiency impairs lipid metabolism and leads to fatty liver**

We also analyzed the metabolism of lipids in the serum and liver alterations caused by the lack of CCR5. Both stains showed an increase in circulating triglycerides when consumed HC diet compared with their respective chow diet control. However, triglycerides levels were higher in both CCR5$^{-/-}$ groups compared with their respective WT (Figure 5A). In serum cholesterol

analysis, WT-HC mice showed an increase compared with WT-C mice, but it was not observed differences between the other groups (Figure 5B).

Associated to that, the lipid content in the liver was analyzed. In relation to total lipid content, both WT-HC and CCR5$^{-/-}$-HC presented an increase compared with their respective controls. The similar alteration was demonstrated when liver triglycerides were evaluated (Figure 5D). In addition, the CCR5$^{-/-}$-HC group presented an increase in total fatty compared with the WT-HC mice (Figure 5C). However, there were no differences between groups in relation to hepatic total cholesterol levels (Figure 5E).

In the histological liver score, mice fed with HC diet showed higher score compared with their respective controls. However, the lack of CCR5 appears to intensify the steatosis in the liver (Figure 5F).

**Discussion**

Chemokines are essential molecules related to the recruitment of leukocytes into different tissues in the body (LUSTER, 1998). In this study, we evaluated the effect of an overload of a diet rich of refined carbohydrates in mice lacking the receptor for CCR5 chemokine. We demonstrated that CCR5 deficiency: (i) leads to an increase in inguinal adipose tissue and its adipocyte area; (ii) impairs glucose metabolism that is even higher after consumption of HC diet; (iii) reduces adipose tissue and systemic inflammation; and (iv) impairs lipid metabolism and steatosis in the liver at basal levels and upon an HC diet feeding.

We evaluated the impact of CCR5 deficiency on obesity development. There were no differences in body weight gain between experimental groups, and CCR5 lack did not exacerbate adiposity-induced by HC diet. Although CCR5 deficiency did not alter visceral adiposity, interestingly, we observed an increase in inguinal fat depots and their adipocyte area in CCR5$^{-/-}$ mice fed with chow diet. Kennedy et al. (2013), using a high-fat diet, also showed that an overload of nutrients did not exacerbate body weight gain, total fat mass in CCR5$^{-/-}$ mice.

Kitade et al. (2012) also showed that CCR5$^{-/-}$ mice did not differ significantly from WT mice in body weight gain and epididymal adipose tissue weight maintained on either standard chow or high-fat diet. It was observed an increase in leptin levels in CCR5$^{-/-}$ mice fed with chow diet, but the HC diet consumption did not exacerbate this parameter in knockout mice. Despite there was no increase in visceral adipose tissue in CCR5$^{-/-}$ mice, the augments in inguinal fat mass and in its adipocyte size may be responsible for the increase in leptin release. According to some studies leptin mRNA levels were higher in subcutaneous than in visceral adipose tissues in humans (MASUZAKI *et al.*, 1995; MONTAGUE *et al.*, 1998; VAN HARMELEN *et al.*, 1998).

Insulin resistance is quite common in obesity (GREGOR; HOTAMISLIGIL, 2011). CCR5 deficiency also impairs glucose metabolism. These mice presented glucose intolerance, an increase in fasted glucose, serum insulin and HOMA-IR, regardless of the diet. However, these parameters were impaired when mice were challenge with HC diet. In agreement, Kennedy et al. (2013) showed that CCR5$^{-/-}$ mice on a high-fat diet had impairment in glucose tolerance compared with WT mice. In contrast, Kitade et al. (2012) showed that CCR5$^{-/-}$ mice on a standard chow diet had slight better glucose tolerance than WT mice, and that glucose intolerance and hyperinsulinemia on a high-fat diet were significantly improved in KO mice. However, consistent with worsening of glucose metabolism, we observed a reduction in adiponectin levels in the serum of CCR5$^{-/-}$ mice fed with chow or HC diet, since adiponectin stimulates glucose uptake and is involved in insulin sensitivity (MARCELL *et al.*, 2005). Moreover, the increased NEFA in the serum of CCR5$^{-/-}$ mice also impairs glucose metabolism. NEFA compete with glucose for utilization by insulin-sensitive peripheral tissues, such as skeletal muscle, leading to reduced glucose utilization. NEFA also stimulate hepatic glucose production, thereby decreasing glucose tolerance (FRAYN, 1998). Then, our data are consistent to believe that CCR5 deficiency contributes to insulin resistance, and this is aggravated after consumption of a diet rich in refined carbohydrate.

In obesity, inflammation into adipose tissue is increased and characterized by presence of pro-inflammatory mediators (ESPOSITO, 2002; FANTUZZI, 2005; OLUSI; AL-AWADHI; ABRAHAM, 2003) and infiltration of leukocytes, such as neutrophils, and monocytes that change into macrophages (KANDA; TATEYA; TAMORI, 2006; OLIVEIRA, MARINA C. *et al.*, 2013). There were no differences in epididymal adipose tissue inflammation between CCR5$^{-/-}$ and WT mice on chow diet. However, levels of TNF and IL-6 in that tissue were lower in CCR5$^{-/-}$-HC mice than WT mice fed with the same diet. Macrophages are one of the main cells attracted by chemokines dependent of CCR5 ligand (BAGGIOLINI, 1998) and they contribute more in the production of these pro-inflammatory cytokines into adipose tissue (OH *et al.*, 2012; OSBORN; OLEFSKY, 2012). In fact, Kitade et al. (2012) showed that the presence of macrophages in adipose tissue was reduced when CCR5$^{-/-}$ mice were fed with high-fat diet. We suggest that a lower inflammation in epididymal adipose tissue could be related to a reduced presence of leukocytes in it. Moreover, despite there were no differences between mononucleated circulating cells, we observed a reduction in multinucleated cells in CCR5$^{-/-}$-HC group compared with WT-HC group and similar to the inflammatory profile showed in epididymal adipose tissue. Multinucleated cells include neutrophils, which are critical for innate immunity and acute inflammation (KOBAYASHI *et al.*, 2005). In response to an inflammatory challenge, circulating neutrophils adhere to the vasculature and undergo diapedesis to the site of inflammation (LEY, 2002; VON ANDRIAN *et al.*, 1991). In addition, activated neutrophils may interact with and activate other inflammatory cells, such as macrophages and lymphocytes (MANTOVANI *et al.*, 2011). Therefore, lower circulating neutrophils also contribute to decreasing in an inflammatory response that could be reflected in epididymal adipose tissue inflammation.

In inguinal tissue, we observed a decrease of TNF levels in CCR5$^{-/-}$-C mice. Several activities differentiate visceral and subcutaneous adipose tissue, such as adipokine and cytokine release, adipogenic potential and the ability to store and mobilize lipids (FOSTER; PAGLIASSOTTI, 2012). For example, leptin

and adiponectin gene expression (FAIN *et al.*, 2004; MONTAGUE *et al.*, 1998; SAMARAS *et al.*, 2010) and release (BAGLIONI *et al.*, 2012; VAN HARMELEN *et al.*, 1998) appear to be higher in subcutaneous adipose tissue compared with visceral, while cytokine expression, such as IL-6, IL-8, MCP-1 and visfatin, seems to be greater in visceral fat compared with subcutaneous fat (MIRZA, 2011). Our data indicate that subcutaneous adipose tissue is more sensible to the lack of CCR5 than visceral adipose tissue, either for increasing mass or influencing inflammatory response in the tissue.

The liver is an important organ in the control of lipid metabolism (BECHMANN *et al.*, 2011). We showed that the lack of CCR5 induces hypertriglyceridemia, and $CCR5^{-/-}$ mice fed with HC diet presented higher fatty content in the liver. Conversely, Kitade et al. (2012) showed that CCR5 deletion is associated with protection from hepatic steatosis in mice fed with HF diet. $CCR5^{-/-}$ mice showed a decrease in inflammatory responses and presented more fatty content in the liver. Similarly, OLIVEIRA *et al.*, 2015 showed that $PAFR^{-/-}$ mice, which is known for diminished inflammatory milieu, when fed with HC diet also showed more steatosis, and higher transaminases levels associated with lower inflammation. As observed in $CCR5^{-/-}$ mice, the consumption of a HC diet worse metabolic response in the liver in $PAFR^{-/-}$ mice. Moreover, $CCR5^{-/-}$ showed hyperinsulinemia and hyperglycemia. In insulin-resistant states, insulin and glucose activate transcription factors involved in hepatic *de novo* lipogenesis, such as Sterol regulatory element-binding protein-1 (SREBP-1) and carbohydrate response element binding protein (ChREBP), contributing to excess of acetyl-CoA that can be used in FFA synthesis. These alterations in glucose and inflammatory response may lead to hepatic lipogenesis and the development of nonalcoholic fatty liver disease (GUILHERME *et al.*, 2008; HWANG *et al.*, 2007; KORENBLAT *et al.*, 2008).

In summary, the present study showed that CCR5 deficiency leads to an increase in inguinal fat mass and consequently an increase in serum leptin levels. In addition, CCR5 lack induces insulin resistance that is impaired upon refined

carbohydrate overload. The lower circulating immune cells in mice fed HC die may also contribute to decreased levels of proinflammatory cytokines in epididymal adipose tissue, demonstrating a generally reduced inflammation. Thus, lower inflammation caused by a deletion of a receptor for important chemokines may contribute to a worsening in glucose and lipid metabolism, indicating that the inflammatory response leaded by leukocytes may, at least in part, controls metabolic alterations, as observed in an obese state.

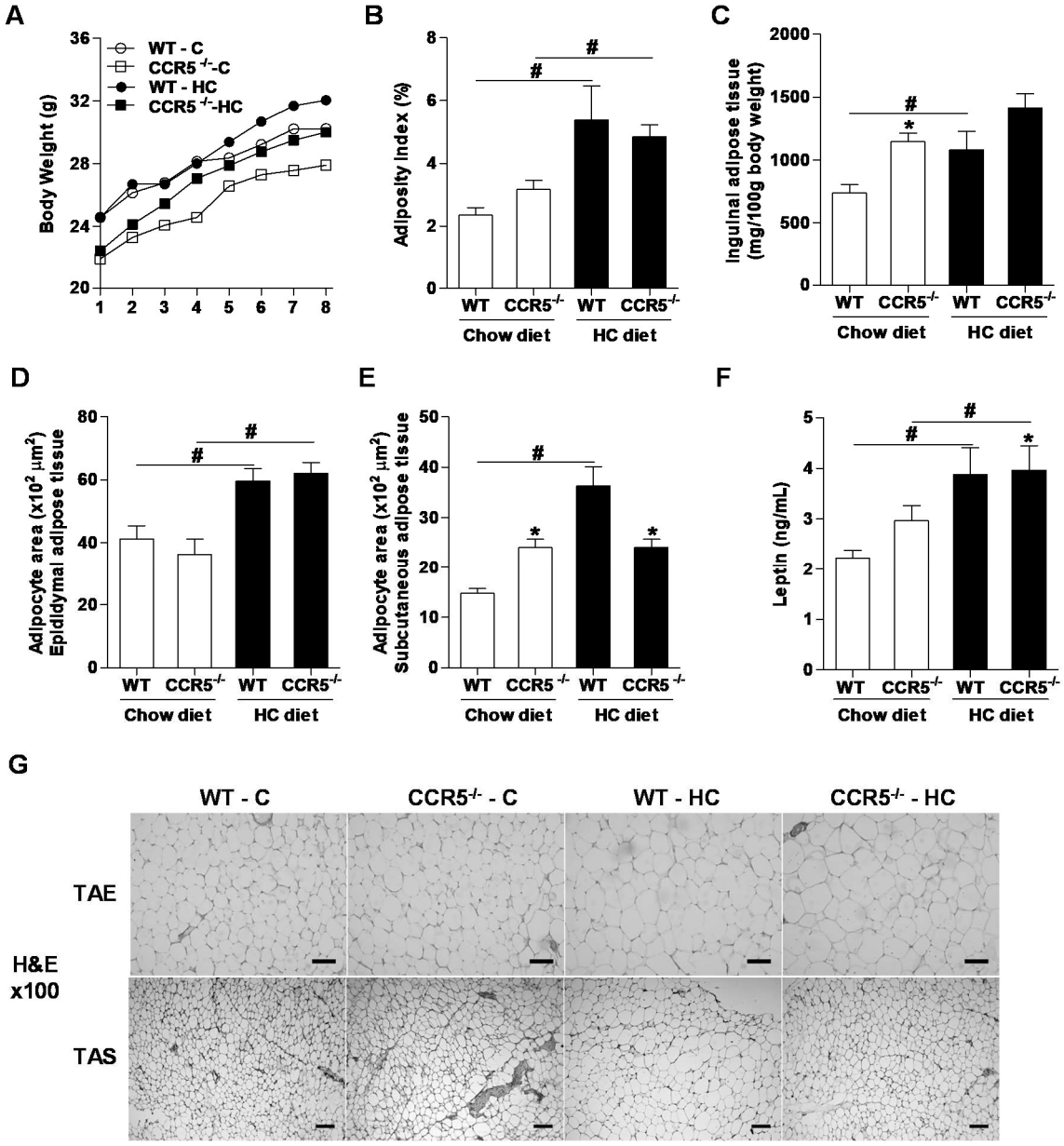


**Figure 1** - (A) Representation of body weight gain in percentage; (B) daily food intake; (C) evaluation of the adiposity index (mass sum of epididymal, mesenteric, and retroperitoneal adipose tissues x 100 / body weight); (D) inguinal adipose tissue mass; (E) adipocyte area of epididymal adipose tissue; (F) adipocyte area of inguinal adipose tissue; (G) histological pictures of epididymal and inguinal adipose tissue sections (100x), bars represent 50µm; (H) dosage of serum leptin of wild-type (WT) and CCR5 knockout mice (CCR5$^{-/-}$) fed with chow diet or high

refined carbohydrate-containing (HC) diet during 8 weeks. The bars represent the mean ± standard error of the mean, n = 6-8. * $P < 0.05$ vs. WT; # $P < 0.05$ vs. chow diet.

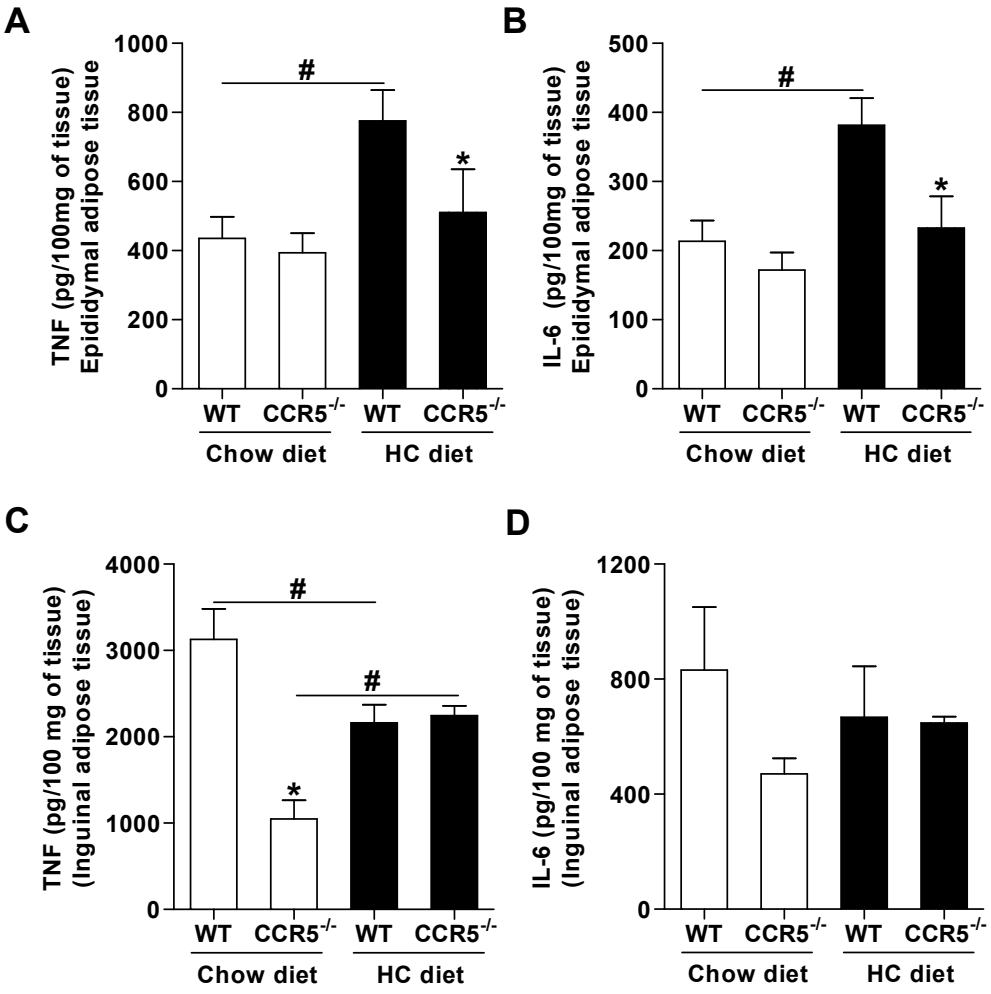


**Figure 2** - Levels of (A) TNF, (B) IL-6 in epididymal adipose tissue and levels of (D) TNF, (E) IL-6 and (F) IL-10 in inguinal adipose tissue of wild-type (WT) and CCR5 knockout mice ($CCR5^{-/-}$) fed with chow diet or high refined carbohydrate-containing (HC) diet during 8 weeks. The bars represent the mean ± standard error of the mean, n = 5-9. * $P < 0.05$ vs. WT; # $P < 0.05$ vs. chow diet.

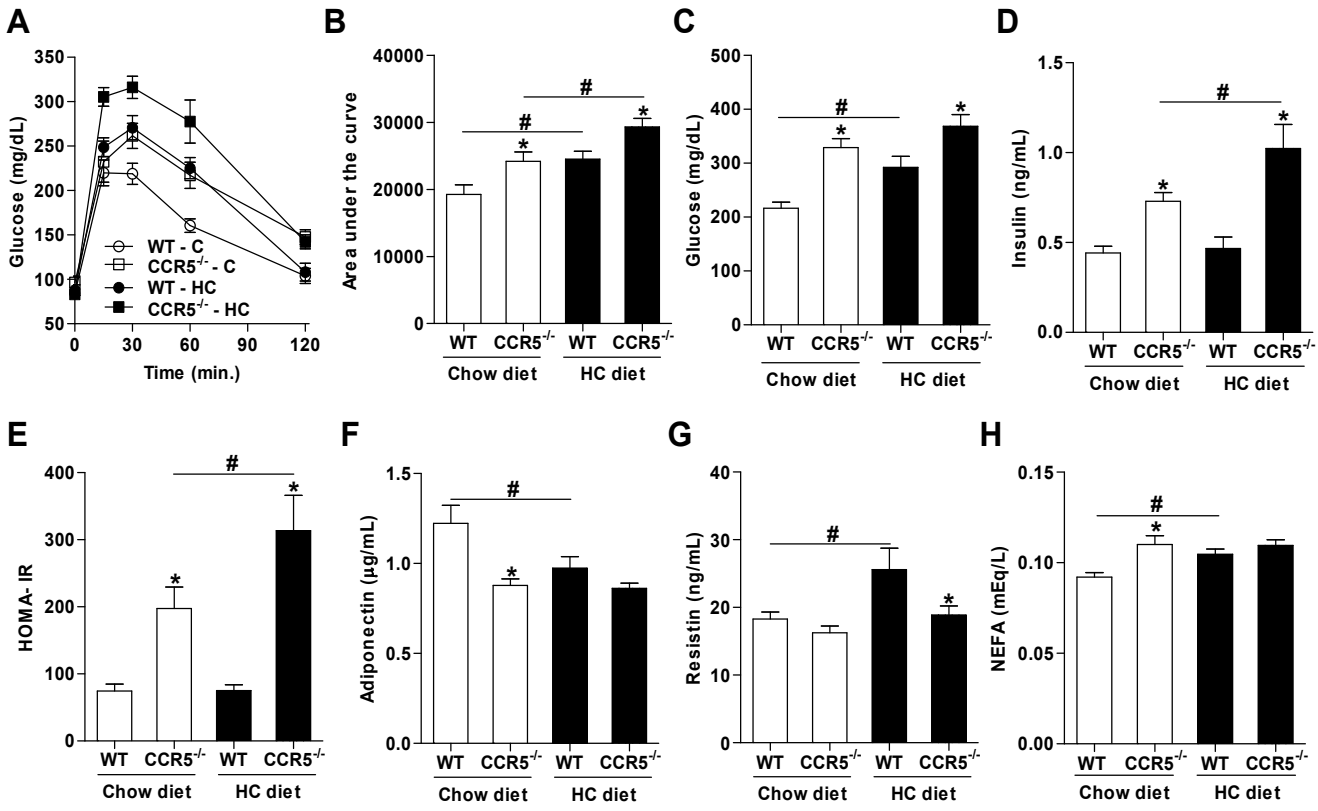


**Figure 3** - (A) Oral glucose tolerance test (OGTT), glucose curve in relation to baseline (time 0 - fasting) at 15, 30, 60 and 90 minutes after gavage of 2 mg / g body weight of glucose. (B) Representation of the area under the OGTT curve. Metabolic analyses in the serum of (C) glucose, (D) insulin, (E) HOMA-IR index, (F) adiponectin, (G) resistin and (H) non-esterified fatty acids (NEFA) of wild-type (WT) and CCR5 knockout mice ($CCR5^{-/-}$) fed with chow diet or high refined carbohydrate-containing (HC) diet during 8 weeks. The bars represent the mean ± standard error of the mean, n = 5-9. * $P < 0.05$ vs. WT; # $P < 0.05$ vs. chow diet.

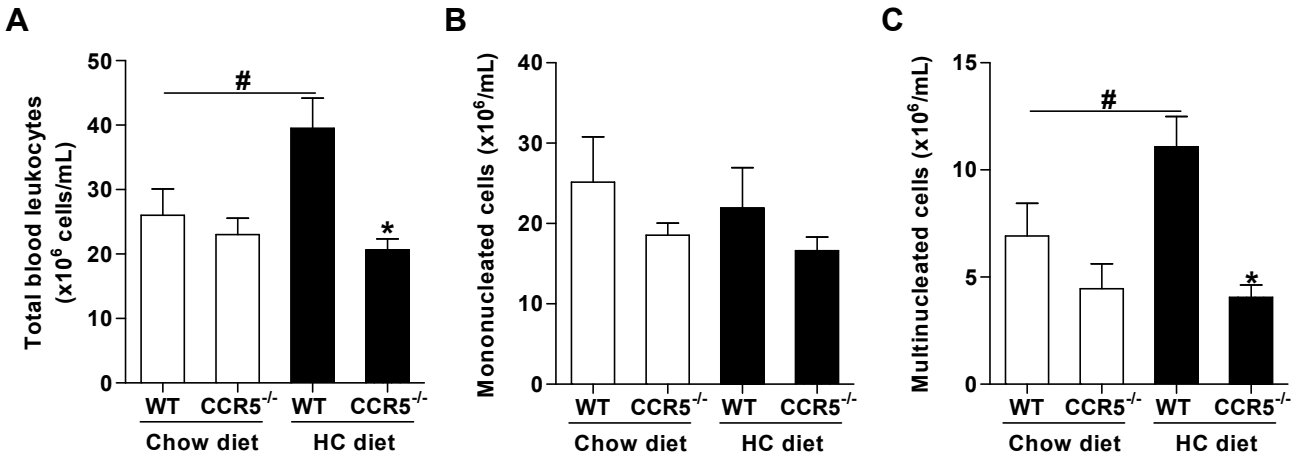


**Figure 4** - (A) Total leukocyte count; differentiation leukocyte count of (B) multinucleated cells and (C) mononucleated cells of wild-type (WT) and CCR5 knockout mice (CCR5$^{-/-}$) fed with chow diet or high refined carbohydrate-containing (HC) diet during 8 weeks. The bars represent the mean ± standard error of the mean, n = 5-9. * $P < 0.05$ vs. WT; # $P < 0.05$ vs. chow diet.

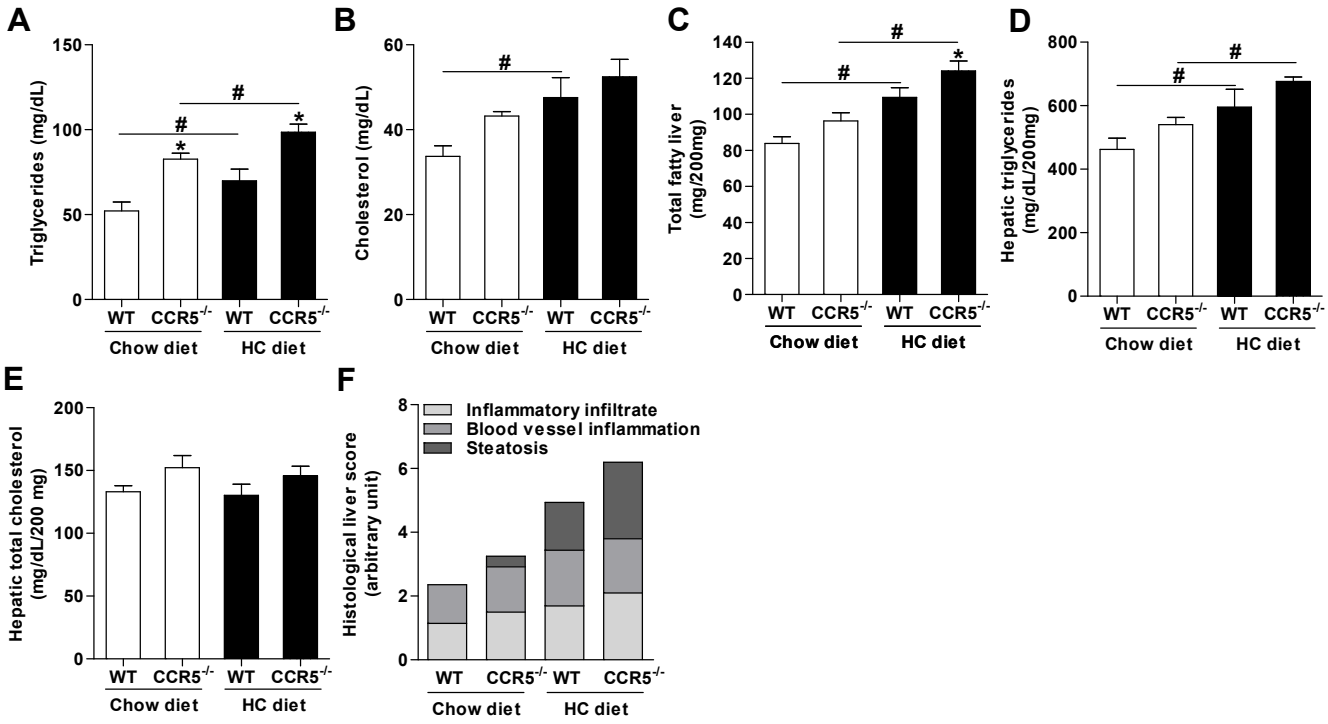


**Figure 5** - Lipid metabolism represented by serum (A) triglycerides and (B) total cholesterol. In the liver was evaluated (C) total fatty, the content of (D) triglycerides and (E) total cholesterol. (F) Histopathological score considering steatosis and inflammation in the liver of wild-type (WT) and CCR5 knockout mice ($CCR5^{-/-}$) fed with chow diet or high refined carbohydrate-containing (HC) diet during 8 weeks. The bars represent the mean ± standard error of the mean, n = 5-9. * $P < 0.05$ vs. WT; # $P < 0.05$ vs. chow diet.

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# 8. DISCUSSÃO

A obesidade está relacionada ao aumento local e sistêmico de diversos metabólitos inflamatórios, como adipocitocinas e citocinas, o que a caracteriza como estado de inflamação crônica de baixo grau. Nesse cenário, além do próprio adipócito alterar seu padrão de secreção de adipocitocinas, a inflamação no tecido também contribui para o aumento do recrutamento de células imunológicas, por meio da secreção de moléculas quimioatraentes, como as quimiocinas. O acúmulo dessas células imunológicas, tais como monócitos, neutrófilos e linfócitos, exacerba a inflamação, favorecendo ainda maior produção de citocinas. Apesar de muitos estudos terem mostrado o papel deletério da inflamação no contexto crônico da obesidade (HOTAMISLIGIL, GS; SHARGILL; SPIEGELMAN, 1993; UYSAL et al., 1997; WEISBERG, STUART P et al., 2003; XU; BARNES; YANG, 2003), também tem sido proposto que a resposta inflamatória pode ter papel fisiológico de forma a controlar o acúmulo excessivo de gordura corporal (BHERING MARTINS et al., 2017; MENEZES-GARCIA et al., 2013; OLIVEIRA, MARINA C. et al., 2013). Assim, o objetivo do presente trabalho foi avaliar o papel de duas importantes citocinas pró-inflamatórias (TNF e IL-18), bem como de uma via de recrutamento de leucócitos (via do receptor de quimiocinas CCR5) nas alterações metabólicas e inflamatórias relacionadas à obesidade no tecido adiposo e no fígado.

Como se sabe, para além das alterações no tecido adiposo, a obesidade está associada também à resposta inflamatória sistêmica, impactando tecidos periféricos, como músculo esquelético e fígado. Para avaliar o impacto da inflamação nesse último, foram utilizados camundongos knockouts para a citocina IL-18 e para o receptor TNFR1. A ablação de ambas as vias culminou na redução das concentrações de importantes citocinas inflamatórias, como IL-6 e TNF, bem como de citocinas pró-fibrogênicas, como IL-4 e IL-13 no tecido hepático. Como tanto a IL-18 quanto o TNF estão relacionados à ativação do fator de transcrição NFkB, uma menor ativação dessa importante via inflamatória poderia justificar a menor secreção de citocinas. O menor conteúdo hepático de citocinas observado associou-se ao maior acúmulo de triglicérides no fígado, mesmo sem o estímulo de dieta obesogênica. Em congruência com esses dados, o trabalho de Yamanishi et al. (2016) também mostrou que camundongos IL-18$^{-/-}$ apresentaram doença hepática gordurosa não-alcoólica, além de dislipidemia. O trabalho de Oliveira et al. (2015) com camundongos com hiporresponsividade inflamatória devido a ablação do receptor do fator de ativação plaquetárias (PAFR) (CAMUSSI; TETTA; BAGLIONI, 1990) também mostrou que esses camundongos apresentaram aumento no conteúdo de gorduras no fígado mesmo quando alimentados com dieta controle. Apesar da redução na resposta inflamatória estar relacionada à melhora de algumas alterações decorrentes de obesidade, como melhora da resistência à insulina (HOTAMISLIGIL, GS; SHARGILL; SPIEGELMAN, 1993), o presente trabalho bem como o de Oliveira et al. (2015) não mostraram efeito protetor com relação

ao acúmulo de gordura hepático, uma vez que tanto a ablação da IL-18, quanto do TNFR1 ou do PAFR levaram a esteatose. Além disso, apesar dos camundongos IL-18$^{-/-}$ e TNFR1$^{-/-}$ apresentarem menores concentrações das citocinas IL-13 e IL-4, e alguns estudos associarem a ablação dessas citocinas à proteção contra fibrose hepática (CHEEVER et al., 1994; KAVIRATNE et al., 2004), o presente trabalho não mostrou redução da área de colágeno no fígado dos camundongos supracitados.

Devido às alterações observadas no armazenamento de gordura no fígado, o próximo objetivo foi avaliar o papel do TNF nas alterações metabólicas e inflamatórias no tecido adiposo de camundongos alimentados com dieta rica em carboidratos refinados. Assim como observado no fígado, a ablação do TNFR1 levou à menor secreção de TNF e IL-6 no tecido adiposo, e essa menor inflamação foi associada à maior deposição de gorduras nesse tecido, mesmo em camundongos tratados com dieta controle. Observou-se também nos camundongos TNFR1$^{-/-}$ alimentados com dieta rica em carboidratos refinados melhora na sensibilidade à insulina, concomitante à menor secreção de resistina no tecido adiposo. A expressão de TNF está aumentada no tecido adiposo de camundongos obesos, e esse aumento está relacionado à menor sensibilidade à insulina (HOFMANN, 1994; HOTAMISLIGIL, GS; SHARGILL; SPIEGELMAN, 1993), uma vez que o TNF participa da ativação de importantes vias inflamatórias como c-Jun N-Terminal Kinases (JUNK) e do NFkB (BENNETT; SATOH; LEWIS, 2003; HIROSUMI *et al.*, 2002; LACKEY; OLEFSKY, 2016). A ativação dessas vias culmina na maior secreção de metabólitos inflamatórios, como IL-6 e o

próprio TNF, intimamente relacionados à gênese da resistência à insulina. O aumento do TNF também está diretamente relacionado ao aumento da lipólise (ZHANG, HUI H. *et al.*, 2002), sendo assim a menor secreção dessa citocina observada no presente trabalho pode justificar o maior conteúdo de gorduras no tecido adiposo, bem como a menor concentração de ácidos graxos livres circulantes observados nos camundongos $TNFR^{-/-}$ alimentados com dieta controle.

O próximo objetivo então foi avaliar o papel da IL-18 no remodelamento do tecido adiposo, uma vez que alguns autores sugerem que essa apresente papel relevante na homeostase metabólica (LINDEGAARD *et al.*, 2013; NETEA, MG; KULLBERG, 2000; NETEA, MIHAI G *et al.*, 2006; ZILVERSCHOON *et al.*, 2008). O presente trabalhou mostrou então que a deficiência da IL-18 levou à maior secreção de citocinas pró-inflamatórias, como IL-6, TNF e IFN-γ no tecido adiposo, diferentemente do observado no fígado. Esse aumento da inflamação associou-se à maior expansão do tecido, mesmo quando esses animais foram alimentados com dieta controle. Em consistência com nossos dados, outros autores também mostraram que a deficiência da IL-18 ou do seu receptor estavam associadas com disfunção metabólica e aumento de gordura corporal (LINDEGAARD *et al.*, 2013; NETEA, MIHAI G *et al.*, 2006). A maior inflamação no tecido adiposo também associou-se à menor secreção de adiponectina e à menor tolerância à glicose. Além das citocinas IL-6, TNF e IFN-γ, a citocina IL-1β, que compartilha a mesma via de ativação dependente de caspase-1 da IL-18, também estava aumentada no tecido adiposo dos camundongos *knockouts*

alimentados com dieta controle, e alguns trabalhos associam também a IL-1β à inflamação e resistência à insulina no contexto de obesidade (BALLAK *et al.*, 2015; GAO *et al.*, 2014; LAGATHU *et al.*, 2006; VANDANMAGSAR *et al.*, 2011). O estudo de Esser *et al.* (2013) mostrou que a expressão gênica da IL-1β no tecido adiposo visceral está correlacionada positivamente com o índice de massa corporal. Gao *et al.* (2014) e Labrecque *et al.*(2019) também mostraram que o tratamento com IL-1β induz aumento da expressão de moléculas inflamatórias, como a subunidade 1 do NF-κB, a quimiocina CCL-5, e da IL-6. Sendo assim, o aumento dessa citocina também pode ter contribuído para aumento da inflamação e intolerância à glicose observadas nos camundongos IL-18$^{-/-}$. Além do aumento das citocinas pró-inflamatórias, outro mecanismo relevante a se mencionar no contexto do metabolismo de glicose e resistência à insulina é a fosforilação do transdutor de sinal e ativadores de transcrição 3 (STAT3) pela IL-18. A fosforilação STAT3 é conhecida por modular a resistência à sinalização da insulina, levando à redução da expressão de proteínas gliconeogênicas como a Fosfoenolpiruvato carboxiquinase e Glicose-6-Fosfatase (AGATI; YEAGLEY; QUINN, 1998; GROSS, DANIELLE N.; WAN; BIRNBAUM, 2009; WANG, RUI-HONG *et al.*, 2011). O trabalho de NETEA, MIHAI G *et al.* (2006) associou a menor fosforilação de STAT3 devido a ablação da IL-18 com o desenvolvimento de resistência à insulina e obesidade.

O aumento da massa adiposa observado nos camundongos IL-18$^{-/-}$ alimentados com dieta controle também foi observado por Lindegaard *et al.*(2013) em camundongos IL-18R$^{-/-}$. Nesse trabalho os autores também

mostraram que esses camundongos apresentaram acúmulo ectópico de gordura em músculo esquelético, acompanhado por prejuízo na ativação de AMP-activated protein kinase (AMPK). Os autores ainda mostraram que a IL-18 pode ativar AMPK em músculo esquelético tanto *in vitro* como *ex vivo*. A AMPK funciona como regulador da homeostase energética, respondendo às baixas concentrações de ATP, sendo que sua ativação regula positivamente as vias para restituição dos suprimentos celulares de ATP, incluindo a oxidação de ácidos graxos (HARDIE; ROSS; HAWLEY, 2012), relacionando então a deficiência de IL-18 ao aumento da deposição de gorduras. De forma congruente, Murphy *et al.* (2016) mostraram que camundongos *knockouts* para o inflamassoma NLRP1 desenvolveram obesidade e síndrome metabólica e que esse fenótipo foi devido à diminuição da produção de IL-18 e da lipólise.

De forma paradoxal, apesar de em modelos experimentais a deficiência da IL-18 ou do seu receptor, culminarem no desenvolvimento de fenótipo semelhante a síndrome metabólica, pacientes com síndrome metabólica apresentam elevadas concentrações séricas dessa citocina (FISCHER *et al.*, 2005; HUNG *et al.*, 2005; ZIRLIK *et al.*, 2007), ao passo que há redução dessa concentração após perda de peso decorrente de cirurgia bariátrica (SCHERNTHANER *et al.*, 2006). Dessa forma, acredita-se que a IL-18 possa ter tanto papel regulador quanto inflamatório, dependendo do perfil de citocinas expressas no tecido.

Até aqui, foi mostrado o importante papel da IL-18 na manutenção da homeostase metabólica, uma vez que sua ablação levou a desenvolvimento de

obesidade, mesmo na ausência de sobrecarga de nutrientes. Assim, o próximo passo foi tentar elucidar em quais células a ausência de IL-18 teria maior impacto para desenvolvimento de obesidade, uma vez que essa citocina pode ser produzida tanto por células imunológicas, como macrófagos, quanto por adipócitos e pré-adipócitos. Para elucidar esse aspecto, no presente trabalho foi realizado um modelo de quimeras. Assim, nossos dados sugerem que a IL-18 tenha papel importante tanto em células imunológicas quanto em adipócitos, uma vez que o transplante de células provenientes da medula óssea de camundongos WT, expressando IL-18, para camundongos IL-18$^{-/-}$ não foi suficiente para suprimir o ganho de peso observado nesses animais. Isso evidencia papel importante da IL-18 em adipócitos para manutenção da homeostase metabólica e remodelamento do tecido adiposo. Em contrapartida o transplante de células derivadas da medula óssea de animais IL-18$^{-/-}$ para camundongos WT, levou à maior secreção de citocinas no tecido adiposo desses animais, com concomitante redução na tolerância à glicose, evidenciando que a IL-18 também tem papel importante em células imunológicas para manutenção da homeostase.

Até aqui, nossos dados sugerem que as citocinas IL-18 e TNF estão envolvidas no controle do armazenamento de gordura tanto no fígado quanto no tecido adiposo, independente de estímulo de dieta, porém a atuação parece se dar de forma diferente no tecido adiposo. Mostramos também que, além de adipócitos, células imunológicas expressando mais ou menos essas citocinas participam da resposta inflamatória e metabólica decorrente de obesidade.

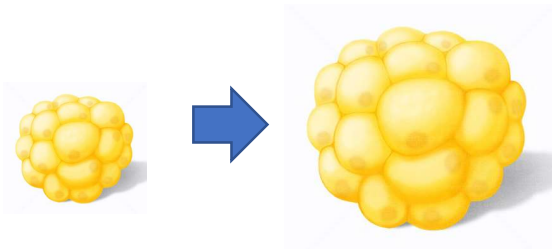
Assim, o próximo passo foi avaliar o papel do recrutamento de leucócitos durante obesidade. Diversos trabalhos associam o eixo receptor CCR2/quimiocina CCl2 ao desenvolvimento e progressão da obesidade (REF). Da mesma forma há evidencias de que o receptor CCR5 e seus ligantes também estejam envolvidos nesse contexto. Assim, o presente trabalho avaliou o papel do recrutamento de leucócitos por meio da ablação de uma via de recrutamento, sendo essa a via do receptor de quimiocinas CCR5. A ablação desse receptor levou à aumento da massa adiposa somente no compartimento inguinal, com menor inflamação neste sítio. Apesar de não apresentarem diferenças no conteúdo de gordura no compartimento visceral quando comparados aos WT, os camundongos *knockouts* tratados com dieta HC apresentaram menor concentração de citocinas nesse tecido. Em congruência, Huhet al. (2018), utilizando um antagonista de CCR2 e CCR5, mostraram que a atenuação dessas duas vias acarreta diminuição de citocinas pró-inflamatórias no tecido adiposo. Os camundongos $CCR5^{-/-}$ ainda apresentaram intolerância à glicose e resistência à insulina, mesmo quando alimentados com dieta controle. Kennedy et al. (2013) também mostraram em seu trabalho que camundongos $CCR5^{-/-}$ tratados com dieta rica em lipídeos apresentavam intolerância à glicose, muito embora não tenham observado o mesmo efeito nos mesmos animais tratados com dieta controle. A resistência à insulina esteve associada à menor secreção de adiponectina e maior concentração de ácidos graxos livres circulantes. Os camundongos $CCR5^{-/-}$ também apresentaram maior concentração de triglicérides circulantes e maior deposição de gorduras no fígado. Assim, nossos

dados evidenciam o papel da resposta inflamatória induzida por leucócitos no metabolismo glicídico e lipídico.

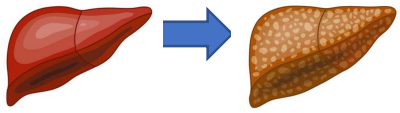
## 9. CONCLUSÃO

A resposta inflamatória desempenha papel crucial no desencadeamento da obesidade e também na manutenção da homeostase metabólica por meio da integração de diferentes vias do sistema imunológico. Apesar de muitos estudos associarem essa inflamação à agravos metabólicos, no presente trabalho a inibição de importantes vias inflamatórias levou a maior acumulo de gordura em órgãos como tecido adiposo e fígado, mostrando seu papel na, manutenção da homeostase metabólica. Assim, apesar de seu efeito deletério a longo prazo, a inflamação parece ser um mecanismo de controle do ganho de peso excessivo.

INFLAMAÇÃO



Resisistência à insulina



**TNFR1$^{-/-}$ IL-18$^{-/-}$ CCR5$^{-/-}$**

POSSÍVEL EFEITO PROTETOR DA ATIVAÇÃO DESSAS VIAS

INFLAMAÇÃO

Desenvolvimento de doença

Homeostase metabólica

# 10. REFERÊNCIAS DA INTRODUÇÃO

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