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Área de Fisiopatologia

DALILA CUNHA DE OLIVEIRA

Avaliação do controle da expressão gênica de citocinas pró inflamatórias mediado pela IL-10. Participação da IL-10 na modulação da resposta inflamatória exercida pela glutamina e na restrição alimentar.

Orientador:
Prof. Dr. Ricardo Ambrósio Fock

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Resumo

Oliveira, D.C**. Avaliação do controle da expressão gênica de citocinas pró inflamatórias mediado pela IL-10. Participação da IL-10 na modulação da resposta inflamatória exercida pela glutamina e na restrição alimentar.** 2017. 125 f. Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, 2017.

O desenvolvimento de uma resposta imune adequada é um processo extremamente importante para a manutenção da homeostase do organismo. Uma série de processos são desencadeados a partir do primeiro contato com micro-organismos patógenos até a efetivação da resposta imune de memória. Todos esses processos envolvem a participação e a complexa atuação de mediadores como as citocinas inflamatórias e também citocinas regulatórias, que exercerão efeitos controlando o processo inflamatório. Diversos mecanismos moleculares, subjacentes à resposta inflamatória, ainda não estão totalmente compreendidos, como por exemplo o controle da expressão de genes inflamatórios exercido pela IL-10. Os processos envolvidos na resposta inflamatória são mantidos às custas do consumo de nutrientes, dentre eles podemos destacar o aminoácido glutamina, que atua em nível molecular, fornecendo nitrogênio para a formação do material genético e fonte energética para determinadas células do sistema imunológico como os macrófagos. Portanto, neste trabalho, investigamos os efeitos da IL-10 na modificação de nucleossomos, evidenciando o papel dessa citocina em regular a expressão de genes inflamatórios em macrófagos. Avaliamos também a função da glutamina, modulando a expressão de RNAm de citocinas inflamatórias e regulatórias dessas células. E por último, desenvolvemos um modelo de restrição alimentar em camundongos, nos quais avaliamos os efeitos desse modelo considerando-se alguns aspectos hematológicos e estudamos as alterações na resposta inflamatória em células esplênicas e do peritônio, bem como avaliamos a suplementação de glutamina *in vitr*o na produção das citocinas (IL-12, TNF-alfa, IL-10) e a expressão do fator de transcrição NFkB. Os resultados compilados mostraram que a IL-10 leva a uma rápida redução da acetilação de nucleossomos, modulando a arquitetura da cromatina de genes inflamatórios como a IL-12. A glutamina modula a expressão de citocinas inflamatórias, regulando positivamente a expressão de IL-10 e Interferon beta. E a restrição alimentar induz a redução de citocinas proinflamatórias (IL-12 e TNF-α), influenciadas pelo aumento da produção de IL-10 e finalmente a suplementação com glutamina não interfere nesses parâmetros nas células peritoneiais e esplênicas do grupo submetido à restrição alimentar. Conclusão: a IL-10 modula a expressão gênica através da modificação de nucleossomos em macrófagos derivados da medula; a glutamina modula a expressão de IL-10 inibindo a resposta inflamatória, e a restrição alimentar modula alguns aspectos hematológicos e possui propriedades anti-inflamatórias.

Palavras chave: resposta imune; epigenética; glutamina; restrição alimentar; citocinas.

Abstract

Oliveira, D.C**. Proinflammatory cytokines gene expression control mediated by IL-10. Participation of IL-10 on inflammatory response exerted by glutamine and dietary restriction 2017**. 125 f. Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, 2017.

The development of an appropriate immune response is an important process to the organism's homeostatic maintenance. A series of processes are triggered upon the very first contact with pathogens, up to the immunological memory establishment. These processes implicate in the participation of complex mediators, such as inflammatory and regulatory cytokines that will control the inflammatory process. Some mechanisms underlying the inflammatory response are not totally understood, the control of inflammatory genes exerted by IL-10 is an example. The processes involved in the inflammatory response are kept with nutrients expense, among these nutrients we can highlight the amino acid glutamine. It acts in a molecular level, supplying nitrogen to genetic material formation and as an energy supply for immune cells such as macrophages. Thus, we investigated the IL-10 effects on nucleosome modifications evidencing this cytokine role regulating inflammatory genes expression in macrophages. We also evaluated glutamine functions modulating inflammatory and regulatory cytokines mRNA expression on these cells. Ultimately, we developed a dietary restriction animal model where we evaluated it's effects on selected haematological aspects, analyzing the alteration in the inflammatory response of splenic and peritoneal cells. We also evaluated *in vitro* glutamine supplementation assessing cytokines production (IL-12, TNF-α, and IL-10) and the expression of NFkB transcription factor. The compiled results a expressive reduction in nucleosome acetylation modifying the chromatin architecture of inflammatory genes such as IL-12 and IL-6. Glutamine modulates inflammatory cytokines gene expression upregulating the expression of IL-10 and interferon beta. The dietary restriction reduces proinflammatory cytokines production (IL-12 and TNF-α), these results are influenced by the upregulated IL-10 production, glutamine supplementation have no effect on these parameters in the dietary restriction group. In conclusion, we can infer that IL-10 modulates gene expression trough nucleosome modification in bone marrow derived macrophages, glutamine has a potential effect on IL-10 expression, inhibiting the inflammatory response and dietary restriction modifies hematological parameters, presenting anti-inflammatory properties.

Key words: immune response; epigenetics; glutamine; dietary restriction; cytokines.

LISTA DE ABREVIATURAS E SIGLAS

APC antigens presenting cells

BCG Bacillus Calmette-Guérin

BMDM bone marrow derived macrophages

ChIP chromatin immunoprecipitation

DNA desoxirribonucleic acid

DR dietary restriction

GLN glutamine

HAT histone acetil transferase

HDAC histone deacetilases

IFNB1 interferon beta 1

IFN-γ Interferon gamma

IL- interleucina 8

IL-1 interleucina 1

IL-10 interleucian 10

IL-10R1 interleucin 10 receptor 1

IL-10R2 Interleucin 10 receptor 2

Il-12 Interleucina 12

IL-1β interleucina 1 beta

IL-6 interleucina 6

IRF3 independent Interferon regulatory factor 3

IκB inhibitor of kappa B

JAK Janus Kinase

KO knock out

LPS lipopolissacarides

MCP-1 Monocyte Chemoattractant Protein-1

mRNA messenger ribonucleic acid

mTOR mechanistic target of rapamycin

MyD88 myeloid differentiation primary-response gene 88

NFkB nuclear factor kappa B

PCR polymerase Chain reaction

pNFkB phosphorylated nuclear factor kappa B

pSTAT3 phosphorilated signal transducers and activators of transcription

PVDF Polyvinylidene Difluoride

qPCR quantitative polymerase chain reaction

RNA ribonucleic acid

RNAm RNA mensageiro

STAT3 signal transducers and activators of transcription

TBD Tris Buffered Saline

TBST Tris Buffered Saline and Tween

TCR T-cell receptor

TLR-4 Tool Like Receptor-4

TNF-α Tumor necrose factor alfa

TRAM TRIF related adaptor molecule

TRIF TIR-domain containing adaptor protein inducing

VCAM-1 vascular cell adhesion molecule 1

WB Western Blotting

WT wild type

Sumário

1. INTRODUÇÃO E JUSTIFICATIVA

A resposta inflamatória é formada por mecanismos complexos. Mediante o contato com patógenos, as células do sistema imunológico, como macrófagos e linfócitos, produzem mediadores como citocinas que atuarão no processo inflamatório.

O controle da extensão e da duração da inflamação é um passo relevante no processo inflamatório, uma das citocinas mais relevantes atuantes no controle da resposta inflamatória é a IL-10.

Diversos mecanismos, a nível molecular e epigenético, são propostos para explicar a ação da IL-10 no controle da expressão de genes pró-inflamatórios, contudo, até o momento nenhum dos mecanismos propostos explicam na sua totalidade os efeitos exercidos por essa citocina.

A glutamina e um relevante aminoácido envolvido no fornecimento de substrato para as células do sistema imunológico. É bem estabelecido que a glutamina modula a resposta inflamatória, sobretudo influenciando na produção de citocinas, dentre elas a IL-10.

No entanto, os efeitos da suplementação com a glutamina e a consequente modulação exercida em macrófagos bem como o envolvimento da IL-10 ainda não são completamente compreendidos.

A restrição alimentar induz a diversas alterações no organismo, incluindo a resposta inflamatória. Não obstante, o papel da restrição alimentar influenciando aspectos da resposta inflamatória em estado basal necessitam de ser elucidados.

Sendo assim, esta pesquisa teve como objetivo avançar nos conhecimentos acerca do controle epigenético da resposta inflamatória exercido pela IL-10. Além disso, averiguamos os efeitos da glutamina e a estreita correlação com a IL-10, bem como evidenciamos os efeitos da restrição alimentar em relevantes aspectos da resposta inflamatória.

2. REVISÃO DA LITERATURA

2.1 Sistema imune e a regulação da expressão gênica

O sistema imune se refere a um conjunto de células e mediadores que interagem de maneira complexa e integrada, protegendo o organismo como um todo incluindo a pele, vias respiratórias e trato intestinal contra bactérias, fungos, vírus, toxinas entre outros antígenos externos (WARRINGTON *et al.*, 2011).

A resposta inflamatória é um componente da resposta imune, sendo essencial ao hospedeiro para a defesa e manutenção da homeostase do organismo. As células mononucleares sanguíneas fazem parte da resposta imune inata, são originadas na medula óssea. Os monoblastos são os precursores mais imaturos derivados da célula progenitora pluripotente hematopoiética e se diferenciam em pró-monócito e monócito (CALDER, 1995; ZAGO *et al*., 2004).

Os monócitos circulam pelo sangue periférico e migram através dos vasos sanguíneos para os vários órgãos e sistemas teciduais, onde se transformam em macrófagos, constituindo uma fase mais avançada na vida da célula mononuclear fagocitária (CALDER, 1995; ZAGO *et al*., 2004).

Essas células são responsáveis pelo mecanismo de fagocitose, pinocitose, produção de citocinas, apresentação de antígenos e produção de radicais livres (NEWSHOLME *et al*., 1999; GREENBERG & GRINSTEIN, 2002).

Os linfócitos T são células que também constituem o sistema imunológico. Esses, por sua vez, são derivados de células tronco hematopoéticas da medula óssea. O desenvolvimento final ocorre no timo. Após o completo desenvolvimento, elas migram para o sangue periférico (IKAWA, 2014; LIU *et al*., 2014).

Os linfócitos T podem ser ativadas por antígenos provenientes de microrganismos. Essa ativação pode ocorrer pelo reconhecimento de antígenos apresentados pelas células apresentadoras de antígenos (APCs) via TCR e vale ressaltar o envolvimento de moléculas de co-estimulação, CD28, presentes na superfície das células T e moléculas de B7 na superfície das APCs ativadas (Figura 1) (LIU *et al*., 2014).

As células T ativadas proliferam, dividem-se e secretam citocinas que desempenham funções específicas. O processo de apresentação de antígenos estimula a diferenciação dessas células em células T citotóxicas (CD8+) ou em célula T auxiliares (CD4+) (Warrington *et al.*, 2011).

As células T citotóxicas (CD8+) estão envolvidas em processos como a destruição de células infectadas. As células T auxiliares (CD4+) desempenham funções como produção de citocinas que atuarão ativando outras células do sistema imune. Essas células não possuem a capacidade de fagocitar ou matar células; no entanto, fazem a mediação da resposta imune direcionando outras células para realizar essas funções (WARRINGTON *et al*., 2011).

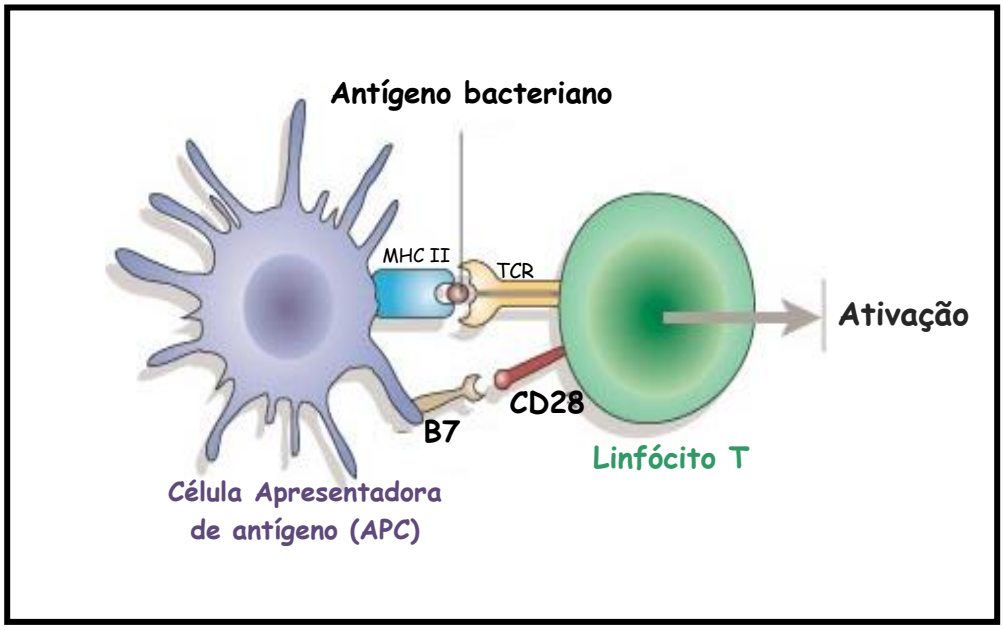

Figura 1: Ativação de Linfócitos T mediada por células apresentadora de antígenos via moléculas de co-estimulação B7 e CD28. Adaptado de Feldmann e Steinman, 2005.

Além disso, as células T ativadas são altamente anabólicas e demonstram um aumento importante na glicólise, assim como um aumento da absorção de glicose e de aminoácidos como a glutamina (LIU *et al*., 2014).

Os linfócitos, quando ativados, realizam processos como proliferação celular, biossíntese e atividades secretórias. Para atender a esse dramático aumento no metabolismo essas células necessitam da obtenção de substratos. A reserva insignificante de nutrientes obriga os linfócitos a aumentar a captação dos substratos metabólicos. Embora os linfócitos sejam capazes de usar glicose, glutamina, corpos cetônicos e ácidos graxos, é estabelecido que a glicose e a glutamina são os alimentos quantitativamente mais importantes para linfócitos ativados (WASINSKI *et al*., 2014).

As células da resposta imune, como os macrófagos, respondem a infecção bacteriana ou viral levando a uma ativação coordenada que consequentemente ativa genes que induzem a rápida produção de citocinas inflamatórias, iniciando a defesa contra o microrganismo invasor (IYER, GHAFFARI & CHENG, 2010).

As citocinas são importantes moléculas moduladoras da inflamação, participantes na inflamação aguda e crônica, através de uma rede complexa e, às vezes, de interações aparentemente contraditórias (TURNER *et al.,* 2014).

As citocinas se dividem em duas grandes classes: pró-inflamatórias e anti-inflamatórias. Na resposta imune clássica, uma antagoniza a outra de uma maneira cuidadosa e precisamente orquestrada, regulando o vigor e a duração da resposta imune (JAFFER, WADE & GOURLAY, 2010).

Ações de diversas citocinas podem ser antagônicas, sinérgicas ou redundantes. Determinadas citocinas podem influenciar a síntese de outras, levando a uma cascata na qual a segunda ou terceira citocina pode mediar a ação biológica da primeira. A capacidade de uma citocina em aumentar ou inibir a produção de outras citocinas constitui um importante sistema regulatório positivo e negativo para a resposta imune e inflamatória (ARAI, *et al*., 1990; SMALE, TARAKHOVSKY & NATOLI, 2014).

A secreção de citocinas é uma resposta rápida, um evento autolimitante. Em geral, as citocinas não são estocadas como moléculas pré-formadas e sua síntese inicia-se pela transcrição de um gene. Tal ativação transcricional é geralmente transitória e as moléculas de RNAm são geralmente instáveis (QUESENBERRY, 1995; SMALE, TARAKHOVSKY & NATOLI, 2014; WALKER *et al*., 1995).

A associação entre um período curto de transcrição e uma meia vida biológica curta do RNAm faz com que a secreção de citocinas seja transitória e dependente da interação ligante-receptor. Uma vez sintetizada, as citocinas são rapidamente secretadas (QUESENBERRY, 1995; SMALE, TARAKHOVSKY & NATOLI, 2014; WALKER *et al*., 1995).

2.2 Resposta inflamatória associada ao lipopolissacarídeo

A produção de citocinas ocorre após o reconhecimento de moléculas presentes nos patógenos e consequentemente ocorre a ativação de vias de sinalização. O Lipopolissacarídeo (LPS) é um padrão molecular associada a microrganismos, presente na parede de bactérias Gram-negativas. Ele pode iniciar a resposta imune e serve como um sinal de alerta precoce de infecção bacteriana (PARK & LEE, 2013).

Um dos receptores que reconhece padrões em patógenos mais bem descritos é o *Tool Like Receptor-4* (TLR-4), esse receptor reconhece o LPS presente na parede de bactérias gram-negativas. Em consequência de estimulo com LPS, na ativação da via clássica, o *Tool Like receptor*-4 ativa a cascata de sinalização dependente de MyD88, induzindo a ativação do NFκB, que culmina na indução de genes inflamatórios, como as citocinas TNF, IL-6, and IL-1β (IYER, GHAFFARI & CHENG, 2010; SMALE, TARAKHOVSKY & NATOLI, 2014).

O fator de transcrição NFκB encontra-se no citoplasma na forma inativa, devido a sua associação com proteínas denominadas inibidores κB (IκB). A fosforilação dos IκB, em função de estímulos externos como LPS, resulta na sua poliubiquitinação, a qual acarreta na sua degradação mediada pelo proteasoma 26S, o que permite, que o fator de transcrição Fator Nuclear- κB (NFκB) transloque para o interior do núcleo celular e ative a transcrição de diversos genes dependentes do κB, como genes de citocinas pró-inflamatórias (FUJIHARA *et al*., 2003; DE OLIVEIRA et al., 2016).

Dentre as citocinas liberadas em resposta a inflamações e injúrias teciduais, a interleucina 6 (IL-6) é produzida prontamente e transitoriamente, exercendo efeitos pleiotrópicos na inflamação, resposta imune e hematopoese. Essa citocina, produzida no estágio inicial da inflamação, no local da lesão, é transportada para o fígado através

da corrente sanguínea, e desencadeia uma produção rápida de uma ampla gama de proteínas de fase aguda como a proteína C reativa e amiloide sérica A (TANAKA, NARAZAKI & KISHIMOTO, 2014).

O TNF-α é uma das citocinas que exercem relevante papel no sistema imune. Essa molécula aumenta a expressão de receptores de superfície em células do endotélio vascular, aumentando a adesividade de leucócitos, inicialmente de neutrófilos e subsequentemente de monócitos e linfócitos. Assim como o TNF-α, a IL-1 é uma citocina primordialmente inflamatória, produzida por macrófagos, envolvida com reações de fase aguda (COTRAN; KUMAR; ROBBINS, 2000) e de importância estratégica no processo inflamatório (ALLEVA *et al*., 1997; VENKATESH *et al.*, 2013).

A interleucina 12 (IL-12) é uma citocina pró-inflamatória produzida por macrófagos ativados, que exerce um papel pró-inflamatório como indutor do desenvolvimento de células T helper 1. A produção de IL-12 é regulada por várias vias de sinalização incluindo os fatores de transcrição NFκB e c-Rel (GRAZIA CAPPIELLO *et al*., 2001; LADERACH *et al*., 2003; SANJABI *et al*., 2000; ZHAO et al. 2012).

Os linfócitos também possuem a capacidade de secretar citocinas, dentre elas destaca-se o Interferon gama (IFN-γ). Essa citocina exerce efeitos pleiotrópicos, apesar de ter sido originalmente definida como um agente antiviral, sabe-se que ela atua tanto na imunidade inata, induzindo a ativação de macrófagos, quanto na imunidade adquirida (GOZALBO, MANEU & GIL, 2014).

As citocinas da família do Interferon (IFN) são relevantes moléculas na resposta imune. Essa família de citocinas tem sido descrita como responsáveis por exercer vasta ação biológica no sistema imune incluindo a iniciação da síntese de proteínas antivirais, promovendo atividades citotóxicas e atuando na diferenciação e maturação de certos leucócitos (SIN *et al*., 2012).

O Interferon beta é uma citocina que pertence à família dos interferons. A produção dessa citocina se inicia no momento do reconhecimento do LPS pelo TLR- 4, que por sua vez estimula a sinalização através das proteínas adaptadoras TRIF (TIR-domain

containing adaptor protein inducing) e TRAM (TRIF related adaptor molecule). Isso leva à ativação do fator inibidor nuclear kB (IKK-i), que desencadeará a fosforilação do resíduo de serina no IRF3, induzindo a dimerização e entrada no núcleo, e realizará a indução da transcrição de genes codificadores do Interferon beta (DECKER *et al.,* 2005, YEN *et al*., 2015) (Figura 2).

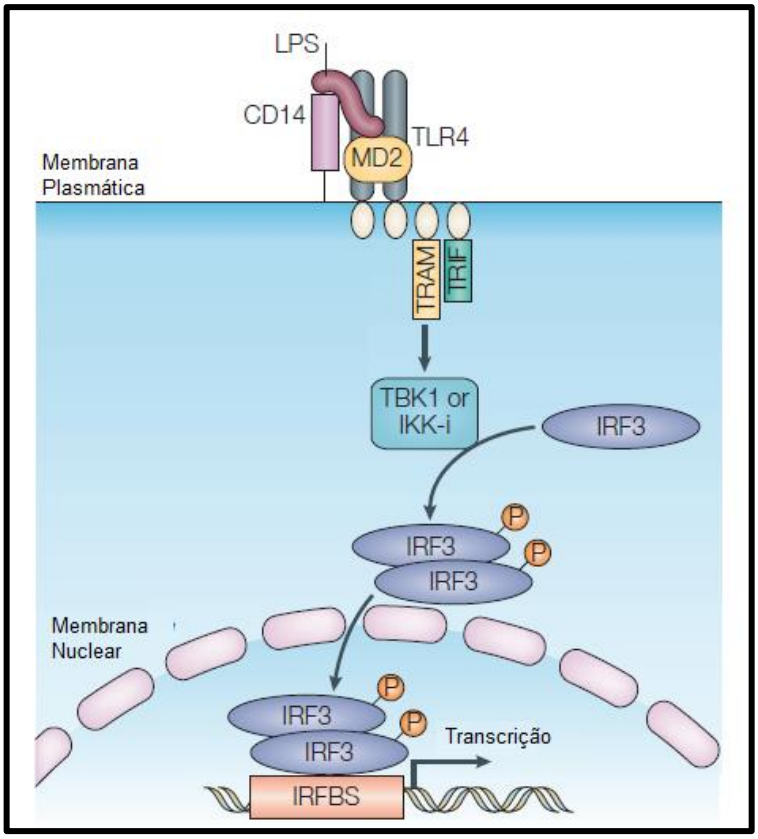

Figura 2: Esquema ilustrativo da indução de Interferon beta (IFN-β) Toll-like receptor 4 (TLR4), estimulando a via de sinalização do IRF3 através das proteínas adaptadoras TRIF e TRAM, levando ao recrutamento do IKK-i, que por sua vez realizará a mediação da fosforilação do resíduo de serina no IRF3, induzindo a dimerização e a entrada no núcleo, em que induzirá a transcrição do gene do IFN-β. Adaptação de Decker et al., 2005, e Iyer & Cheng, 2012.

O interferon também tem a capacidade de exercer função imunorregulatória, tanto na resposta imune inata quanto na resposta imune adaptativa, influenciando a expressão de IL-10 (IYER, GHAFFARI & CHENG, 2010; IYER & CHENG, 2012).

Além dos mecanismos pró-inflamatórios, existem mecanismos para controle dessa resposta: a Interleucina 10 (IL-10) é uma citocina imunossupressora, produzida pelos leucócitos e por células não hematopoiéticas. Essa citocina desenvolve um papel regulatório controlando a produção de citocinas pró-inflamatórias, como TNF-α, IL-6 e IL-12 (JAFFER, WADE & GOURLAY, 2010).

Numerosos estudos demonstram que o tratamento com IL-10 *in vivo* pode reduzir a intensidade do processo inflamatório. Além disso, camundongos *knock out* para a IL-10 desenvolvem uma inflamação semelhante à Doença de Crohn, exibindo uma resposta inflamatória desregulada. Essa citocina exerce funções chave na manutenção da homeostase do sistema imune e protege o hospedeiro contra inflamação excessiva. A IL-10 exerce seus efeitos bloqueando os sinais dependentes de NFκB inibindo a produção de citocinas pró-inflamatórias (JAFFER, WADE & GOURLAY, 2010; SHOUVAL *et al*., 2014; ZHOU, NAZARIAN & SMALE, 2004).

Em resposta a injúrias e ativação inflamatória, o balanço entre as sub-populações de macrófagos se descolam do fenótipo M1(pró-inflamatório) para o fenótipo M2 (anti-inflamatório). Os fenótipos M1 e M2 são percebidos como dois extremos de um estado funcional contínuo, consequentemente, em um cenário biológico real, o espectro das subpopulações, variando de M1 para o M2, serão encontrados. O balanço dessas subpopulações será, portanto, determinado pelo ambiente prevalecente no tecido (ALVAREZ *et al.* 2016).

No momento que uma célula é ativada, em decorrência da presença de um microrganismo patógeno, diversas vias de sinalização controlam a expressão de genes desencadeando processos que efetivam a resposta inflamatória. Novas tecnologias permitiram ampliar a capacidade de entendimento com relação ao funcionamento do sistema imune, interrogando o papel dos genes e de alterações epigenéticas, propondo modelos para explicar a elaboração e o desenvolvimento da resposta inflamatória (SMALE, TARAKHOVSKY & NATOLI, 2014).

A estimulação por LPS é capaz de induzir ou reprimir genes alvo numa cascata temporal. Genes de resposta imediata, incluindo os que codificam diversas citocinas

pró-inflamatórias e quimiocinas, são induzidos transitoriamente, com picos de 2 a 4 horas, e são reprimidos progressivamente. Outros genes são induzidos tardiamente, com um período de até 24 horas de indução após o estímulo. Aung e colaboradores (2006) determinaram que o LPS está envolvido na indução da expressão de genes, seguido pela expressão de reguladores negativos de transcrição, como genes alvo para a inativação por histonas deacetilases (AUNG *et al.*, 2006).

Centenas de genes são ativados em resposta a estímulos inflamatórios de uma maneira cineticamente complexa. Alguns genes são induzidos imediatamente após o estímulo, e outros muitas horas após o estímulo. O recrutamento induzível a genes alvo por fatores de transcrição como NFκB é influenciado por estados de cromatina pré-existente (NATOLI, GHISLETTI & BAROZZI, 2011). Após a entrada no núcleo, o NFκB se liga a cis-regulatórias do DNA em *enhancers* e promotores iniciando a transcrição de citocinas pró-inflamatórias e outros processos celulares como apoptose e proliferação celular (BROWN *et al*., 2014; XIAO, 2004; OSTUNI *et al*., 2013).

Dentre as moléculas que exercem um efeito no controle da resposta inflamatória a citocina IL-10 é uma das mais potentes e significantes. Essa citocina pode fazer a regulação negativa de cerda de 23% dos genes induzidos pelo LPS incluindo IL-12, IL-6 e VCAM e induzir genes que exercem ações controlando a resposta inflamatória como SOCS3, Bcl-3, NF-IL3, DUSP1, TTP e TNIP3 (DILLOW *et al.,* 2014; LANG *et al.,* 2002; ZHOU, NAZARIAN & SMALE, 2004).

A citocina IL-10 age de maneira seletiva inibindo genes pró-inflamatórios específicos através de mecanismos que incluem a repressão da transcrição e desestabilização do RNAm (DILLOW *et al*., 2014). A cascata de sinalização da IL-10 se inicia após a ligação dessa citocina ao seu receptor heterodimérico (composto pelo IL-10R1 e IL-10R2, que são expressos principalmente em macrófagos e células dendríticas), acarretando na fosforilação e ativação do STAT3 (*signal transducers and activators of transcription*) via JAK1 (fig 3). O STAT3 migra então para o núcleo onde desencadeia a transcrição de genes específicos, que por sua vez atenuam a resposta inflamatória por repressão da transcrição de genes de citocinas pro-inflamatórios, como IL-1, IL-6, IL-12 e TNF-α. (HUTCHINS, POULAIN & MIRANDA-SAAVEDRA, 2012).

A resposta regulatória IL-10/STAT3 é indispensável no controle da extensão da inflamação, apesar de o mecanismo completo do STAT3 ainda não ter sido completamente elucidado (HUTCHINS, TAKAHASHI & MIRANDA-SAAVEDRA, 2015). Os genes dependentes de STAT3 são responsáveis por executar algumas das respostas regulatórias associadas a IL-10. Contudo, um modelo baseado unicamente na ativação do STAT3, incluindo todos os genes alvos, não explica suficientemente a resposta anti-inflamatória característica induzida pela IL-10. No entanto, a perda de STAT3 em macrófagos mimetiza a perda de IL-10 (LANG *et al.,* 2002).

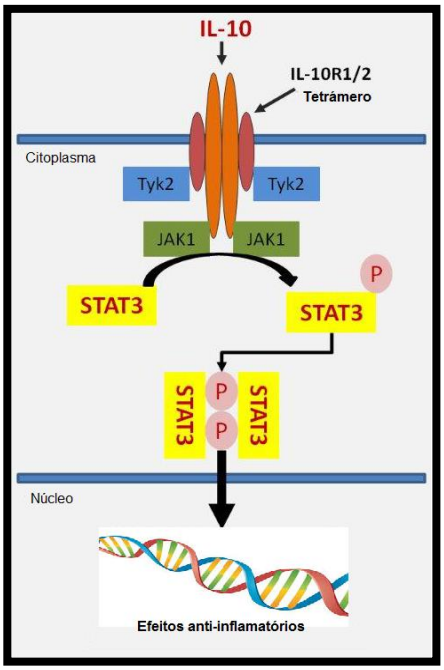

Figura 3: Esquema ilustrativo do mecanismo de interação da IL-10 com o seu receptor específico. A ligação da IL-10 a esse receptor inicia a ativação de uma via de sinalização envolvendo o STAT3, um fator nuclear chave que induz a ativação de genes específicos que codificam fatores da resposta regulatória. Adaptado de Fioranelli e Grazia, 2014.

2.3 Regulação epigenética da resposta inflamatória

A literatura descreve amplamente a respeito dos mecanismos moleculares que levam à ativação da transcrição de genes. No entanto, faltam dados consistentes, demonstrando os mecanismos pelos quais os eventos moleculares levam a um controle

e regulação da expressão de genes pro-inflamatórios (FUREY, 2012; NATOLI, GHISLETTI & BAROZZI, 2011; SMALE, TARAKHOVSKY & NATOLI, 2014).

Modificações epigenéticas são modificações que resultam em alterações da expressão gênica sem mudar a sequência de nucleotídeos, remodelando a estrutura da cromatina, afetando a regulação dos genes reprimindo ou aumentando a ligação de fatores de transcrição através de diferentes mecanismos. Essas modificações epigenéticas são relevantes para o normal desenvolvimento celular, quando interrompidos podem resultar em modificação não desejadas na expressão gênica (HENNESSY & McKERNAN, 2016).

A unidade básica da cromatina, o nucleossomo, consiste de um segmento curto de DNA, envolto em torno de histonas nucleares constituído de duas cópias de H2A, H2B, H3 e H4. Essa organização garante uma estrutura estável à cromatina. A modificação covalente de histonas é um mecanismo epigenético essencial na regulação de genes (BAYARSAIHAN, 2011). A cauda N terminal do nucleossomo fornece uma afinidade reduzida ao DNA, permitindo que a cromatina adote uma estrutura mais relaxada para o recrutamento da maquinaria básica de transcrição. Para exemplificar, as marcações de acetilação de histonas H3K4ac, H3k27ac H3K39ac são associadas com a ativação da transcrição. As histonas deacetilases (HDAC) revertem a atividade das histonas acetil transferase (HAT), tornando a cromatina mais condensada e promovendo a repressão de genes (ANDERSSON, 2015; BAYARSAIHAN, 2011).

A acetilação de histonas induz uma conformação de cromatina aberta que permite que a maquinaria de transcrição acesse os promotores, enquanto que a desacetilação de histonas é correlacionada com o silenciamento de genes (KOBAYASHI *et al.*, 2012).

Em contraste, a metilação das histonas pode manter a cromatina tanto num estado ativo ou reprimido. A trimetilação da histona (H3) na lisina 4 e 36 (H3K4me3 e H3K36me3) facilita a abertura da cromatina para uma transcrição ativa (BARSKI *et al*., 2007). Por outro lado, a metilação das histonas na lisina 9 e 27 (H3K9me3 e H3K27me3) são geralmente associados ao condensamento de cromatina e o silenciamento gênico (LAN & SHI, 2009).

A regulação da transcrição gênica requer a cooperação de regiões regulatórias como promotores e *enhancers*, e proteínas regulatórias como fatores de transcrição e enzimas. Experimentos com enzimas de restrição demonstraram que o *enhancer* e o promoter do gene IL12b possuem remodelamento de nucleossomo após estímulo com LPS, apesar de acreditar-se que essas regiões possuam fatores de transcrição ligados previamente ao estímulo (SMALE, TARAKHOVSKY & NATOLI, 2014).

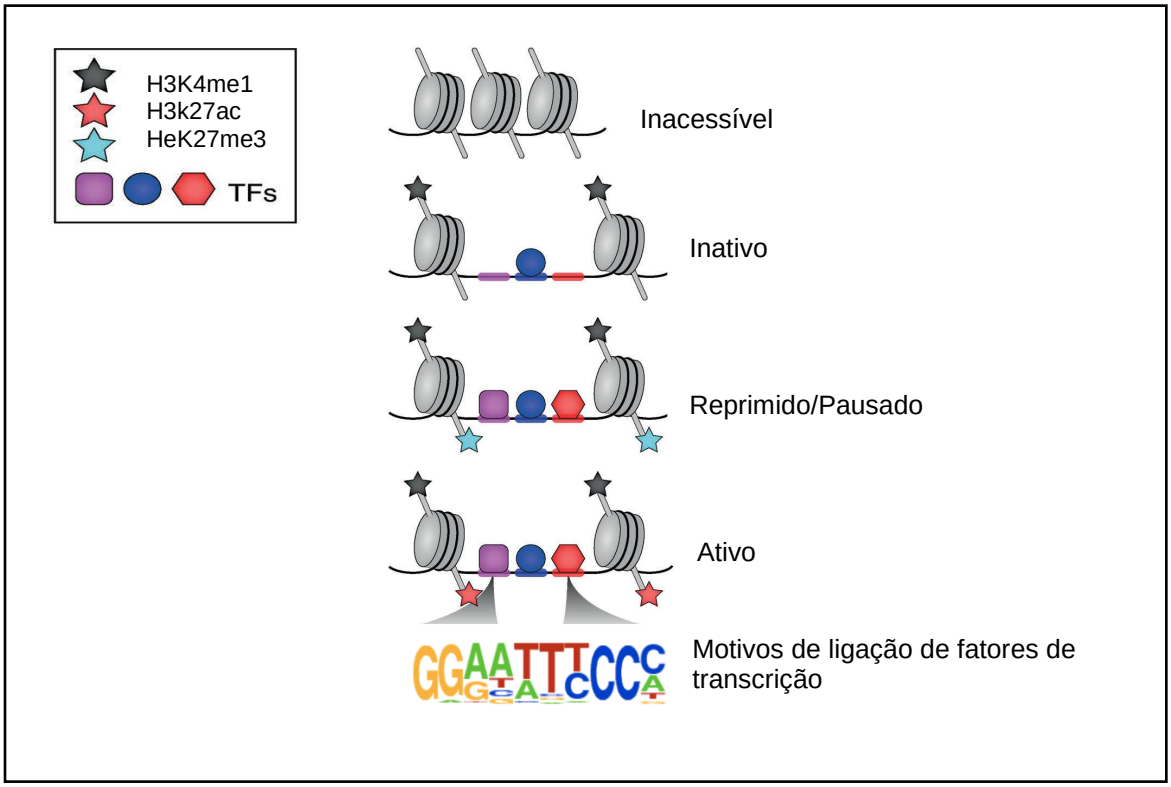

Figure 4: Esquema ilustrativo do estado dos *enhancers* (de cima para baixo): cromatina inacessível bloqueia a ligação de fatores de transcrição impedindo a atividade nos *enhancers. Enhancers* inativos marcados para uma cromatina aberta e H3K4me1. *Enhancers* reprimidos/pausados com a atividade de polycomb repressivo (H3K27me3); e *enhancers* ativos com uma cromatina favorável (aberta, H3K4me1 e H3K27ac) bem como a ligação necessária de fatores de transcrição a locais específicos de ligação modelados por sequencias de DNA preferenciais nos motivos dos fatores de transcrição (Adaptado de Andersson, 2015).

Os *enhancers* são elementos no DNA que estimulam a transcrição através do recrutamento de fatores de transcrição específicos do tecido, RNA polymerase II, e outros cofatores envolvidos na ativação da transcrição. O *enhancer* pode atuar estimulando a transcrição de genes com promotor fraco ou atua fornecendo

informações adicionas e essenciais não codificadas no próprio promotor. Os *enhancer* são tradicionalmente difíceis de identificar devido a sua distância variável com relação ao gene alvo. Apesar do enorme esforço por quase 30 anos, o mecanismo pelo qual o *enhancer* estimula a ativação da transcrição em uma região distante do promotor e do local de início da transcrição permanece pouco compreendido (ANDERSSON, 2015; MARSMAN & HORSFIELD, 2012; SMALE, TARAKHOVSKY & NATOLI, 2014).

A presença de H3K4me1 pode ser observada em muitos *enhancers*, mas por si só não distingue entre *enhancer* ativo e aqueles marcados para serem ativados tardiamente. A H3K27ac (depositado pelo CBP/P300) é um marcador geral de *enhancers* ativos (Figura 4). *Enhancers* silenciados (estabilizados) são marcados com H3K27me3 e/ou H3K9me3 e após a ativação, o H3K27ac substitui esses marcadores durante a diferenciação ou ativação inicial (MARSMAN & HORSFIELD, 2012; NATOLI, 2011; OSTUNI *et al.*, 2013).

Um importante passo a ser dado em direção ao avanço do conhecimento acerca da regulação desse complexo processo se dá pela interrogação dos mecanismos moleculares e epigenéticos, pelos quais a expressão gênica é regulada mediante estímulos regulatórios. Portanto, avaliar os efeitos da IL-10 na regulação epigenética de genes inflamatórios proporciona um progresso singular acerca de relevantes indagações dos mecanismos pelos quais se efetiva a regulação e o controle da resposta inflamatória.

2.4 Glutamina

Aminoácidos são requeridos para a síntese de proteínas e possuem importante papel na resposta imune. Uma dieta adequada, com todos os aminoácidos, é necessária para a manutenção do sistema imune, prevenindo doenças e infecções. Quando o suprimento de aminoácidos é insuficiente, o impacto é negativo sobre a imunidade (LI *et al*., 2007).

Alguns aminoácidos como glutamina, arginina, leucina, metionina e cisteína não apenas participam da síntese de proteínas no organismo, mas também regulam o metabolismo e o sistema imune. A glutamina pode melhorar a barreira intestinal, reduzir a reação inflamatória e promover a recuperação da imunidade, mas ainda faltam conclusões mais claras a respeito dos efeitos clínicos em diferentes pacientes em diversas patologias (TIAN & WANG, 2013, COËFFIER *et al*. 2001).

A glutamina, essencial para função de células como neutrófilos, linfócitos e macrófagos, possui diversas funções no organismo na biossíntese de nucleotídeos, eliminação de amônia, síntese de glutationa, manutenção do equilíbrio ácido-base e transferência de nitrogênio entre os órgãos (NEWSHOLME, 2001).

As células do sistema imune como linfócitos e macrófagos são células caracterizadas por alta taxa de proliferação e secreção de proteínas, que depende do aumento da síntese de RNAm, a qual utiliza purinas, pirimidinas e ribose-5-fosfato, o que caracteriza papel relevante da glutamina na manutenção de elevadas taxas de transcrição (LIU *et al*., 2014; NEWSHOLME *et al.*, 1999).

Segundo Frisina *et al*. (1994) e Rowbottom *et al*. (1995), a taxa de utilização de glutamina por macrófagos é similar ou superior à de glicose. Contudo, apenas pequena parte dos carbonos da molécula de glicose (<10%) e do aminoácido glutamina (5-25%) são completamente oxidados por macrófagos em meio de cultura. Sendo assim, a maioria das moléculas de glicose é convertida em lactato (glicólise), enquanto grande parte da glutamina é convertida em glutamato, aspartato e lactato (glutaminólise) (NEWSHOLME *et al*., 1999).

Após a internalização na célula, a glutamina pode ser degradada para produzir glutamato e α-ketoglutarato, sendo que o segundo entra no ciclo de Krebs. Além de alimentar vias de sinalização energéticas a glutamina também facilita a captação de outros aminoácidos como a leucina e promove a ativação de quinases de crescimento como o mTOR. Em alinhamento com essas observações, quando a concentração de glutamina está baixa, a ativação do mTOR é suprimida, e processos catabólicos como a autofagia estão aumentados. Portanto a glutamina e os seus metabólitos alteram a

resposta celular ao estresse (HE, WEBER & SCHILLING, 2016; JEWELL *et al*., 2015; MEIJER *et al*., 2015).

Em quadros inflamatórios como sepses e injúrias, as células do sistema imune aumentam a demanda por glutamina. As consequências do aumento do requerimento do consumo de glutamina, gera uma condição na qual a demanda pelo nutriente é maior do que o fornecimento, levando a um desequilíbrio que resulta num decréscimo nas concentrações plasmáticas. Essa baixa na concentração limita a habilidade de vários tecidos em atingir a sua total funcionalidade, especialmente as células do sistema imunológico. Sendo assim ocorre um comprometimento das células do sistema imune devido a baixas concentrações de glutamina (ARDAWI & NEWSHOLME, 1983; NEWSHOLME, GORDON & NEWSHOLME, 1987; De OLIVEIRA *et al*., 2016).

Macrófagos peritoneais de camundongos, ativados com BCG, e incubados em meio com ausência de arginina e presença de 2 mM de glutamina apresentam a capacidade de sintetizarem altas taxas de TNF-α. Além disso, WALLACE e KEAST (1992) e YASSAD *et al*. (1997) demonstraram, respectivamente, que o aumento da secreção de IL-1 e IL-6 por macrófagos, estimulados por LPS, foi dependente da concentração extracelular de glutamina (NEWSHOLME *et al*., 1999).

O metabolismo de aminoácidos também está envolvido na ativação de células T, especialmente a glutaminólise. A falta de glutamina bloqueia a proliferação e produção de citocinas (IFN-γ e IL-2), e mesmo que ocorra a suplementação com outros aminoácidos ou precurssores biossintéticos da glutamina esses processos não são recuperados (CARR *et al*., 2010).

Dados mais recentes envolvendo assinatura de expressão gênica revelam que a glutamina é essencial para o desenvolvimento de macrófagos M2. A privação de glutamina em macrófagos polarizados com IL-4 atenua a expressão da assinatura dos genes para M2. O mecanismo subjacente sugere uma conexão entre a maquinaria transcricional necessária para a polarização M2 e glutamina – propondo, portanto, que a glutamina possui um papel na regulação da expressão de genes (MURRAY, 2015; JHA *et al*., 2015).

Sikalidis (2015) é enfático, afirmando existe uma relação aos efeitos da ausência ou decréscimo na concentração de glutamina, em situações em que ocorre queda nos níveis plasmáticos de glutamina num estado de deficiência; ou seja, concentrações de 0,4mM, comparado com a concentração considerada normal 0,6mM, o sistema imune é severamente comprometido. Isso revela a importância desse aminoácido em vários tecidos e principalmente no que concerne a modulação da resposta inflamatória. Portanto espera-se que o fornecimento desse aminoácido na forma de suplementação venha ser benéfico para o organismo (De OLIVEIRA, 2016)

No entanto, a suplementação com glutamina pode interferir na produção de TNF-α, IL-6 e IL-10 de uma forma dose dependente e tempo dependente em macrófagos, provando que a suplementação com diferentes concentrações de glutamina exerce efeitos moduladores em relevantes vias de sinalização, modificando a resposta inflamatória. Gerando informações sobre os possíveis efeitos controversos da suplementação com altas doses de glutamina (Da SILVA LIMA *et al*., 2013; WANG *et al*., 2008).

Em 2013, Heyland e colaboradores, avaliaram os efeitos da glutamina em pacientes hospitalizados em estado crítico, com falência múltipla de órgãos, em diversos países. O intuito desse ensaio clínico era elucidar se a suplementação com glutamina exerceria efeitos terapêuticos benéficos para esses pacientes. A conclusão do estudo trouxe um alerta de que os pacientes que receberam o suplemento com glutamina apresentaram maior taxa de mortalidade. No entanto, o estudo não abordou mecanismos moleculares específicos de ação da glutamina que poderiam estar gerando o aumento da mortalidade e ainda menciona que mais estudos se fazem necessários para assegurar os efeitos da suplementação de glutamina em pacientes hospitalizados (CHEN *et al*., 2014; HEYLAND *et al*., 2013; 2015).

Soma-se a isso a argumentação de Chen e colaboradores de que a efetividade da suplementação de glutamina em pacientes em estado crítico permanece incerta e que altas dosagens de glutamina estão associadas a alta taxas de mortalidade dos pacientes (CHEN *et al.*, 2014; HEYLAND *et al*., 2013).

A suplementação oral com glutamina é capaz de modular importantes moléculas presentes em células do sistema imune e no músculo esquelético. Comprova-se que ocorre atenuação da expressão de NFκB p65 total e fosforilado bem como a expressão de IKK-α/β no músculo esquelético gastrocnêmico de camundongos após o estímulo com LPS e suplementados com glutamina, num modelo de sepsis, promovendo efeitos anti-inflamatórios através da via de sinalização do NF-κB (CRUZAT *et al*., 2014). Além disso, reportou-se que a glutamina reduz a resposta inflamatória em modelos experimentais e em biopsias de pacientes com doença de Crohn através da modulação da via de sinalização do NFkB (LECLEIRE *et al*., 2008; LECHOWSKI *et al*., 2013).

O tratamento com glutamina num modelo de doença hepática não alcoólica, reduziu a ativação de NFκB, sendo capaz de mediar a redução da transcrição de fatores inflamatórios, reduzindo a injúria ou inflamação hepática, atenuando a geração de espécies reativas de oxigênio, aliviando o estresse oxidativo nas células hepáticas (LIN *et al*., 2014).

A combinação de restrição proteica e administração intravenosa de glutamina levou a uma redução nos níveis séricos de TNF-α, após estímulo com LPS, tanto nos animais controle quando nos animais desnutridos. Além disso, o estímulo com LPS induz um aumento do número de polimorfonucleres na cavidade peritoneal dos animais, após a administração de glutamina houve redução do número de células polimorfonucleares na cavidade peritoneal dos animais controles, demonstrando que a glutamina exerce efeitos na produção de citocinas inflamatórias e essa modulação da resposta inflamatória reflete no recrutamento de células polimorfonucleres para os tecidos infectados (SANTOS *et al*., 2016).

Portanto, considerando a modulação exercida pela glutamina no sistema imunológico, uma melhor compreensão dos mecanismos subjacentes a essa modulação proporciona um avanço, fornecimento de informações mais concisas a respeito da suplementação com esse aminoácido

2.5 Restrição alimentar

O termo restrição alimentar é frequentemente utilizado como sinônimo de restrição calórica ou restrição energética. Alguns modelos experimentais induzem a diminuição do consumo calórico, sem indução de desnutrição, através da redução do consumo de alimentos. Dessa forma, assume-se que certos modelos de restrição calórica podem ser chamados intercambiavelmente de modelos de restrição alimentar (JOLLY, 2007).

Os primeiros estudos, induzindo restrição calórica, demonstraram um aumento do tempo de vida de animais em modelos experimentais. Diversos mecanismos explicam os efeitos da restrição alimentar na longevidade, incluindo redução nas espécies reativas de oxigênio, melhora em mecanismos de reparo e funções endócrinas além de manutenção do controle imunológico na idade avançada. Para exemplificar; um modelo com restrição alimentar de 35% da quantidade de alimento *ad libitum* e enriquecido de vitaminas e mineiras promoveu um tempo de vida médio de 53 meses em camundongos, comparado com os respectivos controles que tiveram vida média de 35 meses quando foram alimentados *ad libitum* (DURIANCIK & GARDNER, 2016; GILLESPIE, PICKERING & ESKIW, 2016; McDONALD *et al.*, 2013; MERRY, 2002; WEINDRUCH *et al.*, 1986).

Contudo, qu**e**stionam-se os efeitos da restrição alimentar sobre a longevidade conjecturando que os animais utilizados como controle nos experimentos de restrição alimentar foram alimentados *ad libitum*, gerando uma tendência ao surgimento de obesidade - e consequentemente o sobrepeso levaria a uma morte prematura se comparado aos animais que sofreram restrição alimentar (McDONALD *et al.*, 2013; SOHAL & FORSTER, 2014).

Portanto, dada a ampla gama de resultados, provenientes dos mais variados modelos de restrição alimentar, mais pesquisas se fazem necessárias para articular um conhecimento mais consistente acerca dos efeitos da restrição alimentar no organismo.

Os resultados de diversos modelos de pesquisa com animais induzindo restrição calórica e restrição alimentar demonstraram, sobretudo, efeitos benéficos associados a restrição alimentar, como a prevenção do surgimento de câncer e doenças autoimunes,

redução da produção de radicais livres, níveis de glicose e resistência à insulina e prevenção do surgimento de doenças relacionadas ao envelhecimento (JOLLY, 2007; McDONALD *et al.*, 2013; MUKHERJEE, 2002; ROBERTSON & MITCHELL, 2013; SUAREZ-SOUTO, 2012).

Dessa forma, o sistema imune também foi objeto de pesquisa no contexto da restrição alimentar: há cerca de 15 anos, pesquisadores constataram que a restrição calórica levava a um aumento da produção de prostaglandina E_2 e óxido nítrico em macrófagos peritoneais desafiados com LPS, revelando que a restrição calórica exercia um efeito sobre esses aspectos do sistema imune (STAPLETON *et al.,* 2001*).*

Com o passar dos anos, muitos pesquisadores se engajaram em avaliar os efeitos da restrição alimentar sobre o sistema imune e construir conhecimento acerca dos efeitos dessa intervenção nutricional em diversos aspectos da complexa e integrada rede da resposta inflamatória. A restrição de nutrientes no período da amamentação possui um impacto tardio no sistema imune. Foi observado, num modelo animal de restrição de nutrientes no período da amamentação, a involução do timo, que por sua vez está associado ao adiamento do processo de envelhecimento, demonstrando que a nutrição no início do período pós-nascimento pode impactar tardiamente no sistema imune (CHEN *et al.,* 2010; HEPPOLETTE *et al*., 2016).

Existe uma relação entre a resposta imunológica em alergias e indução de restrição alimentar, pois há redução da produção de IFN-γ e aumento da produção de IL-4 em camundongos submetidos à restrição alimentar. Este estudo revelou que a restrição alimentar alivia a resposta inflamatória desencadeada por estímulos alergênicos em função da menor produção de IL-4 nos camundongos submetidos concomitantemente a estímulos alergênicos e à restrição alimentar quando comparados aos camundongos controle nas mesmas condições experimentais que se alimentaram *ad libitum* (YAMAZAKI *et al*., 2009).

Marcadores sistêmicos de inflamação resultantes de doença periodontal inflamatória em primatas não humanos demonstraram-se reduzidos após a indução de um longo período de restrição calórica. Houve redução de IL-1β e IL-8 no fluido gengival crevicular nos animais machos submetidos à restrição calórica. O mesmo estudo ainda relata redução de IgG nos animais fêmeas e machos submetidos à restrição alimentar.

Atribuindo um efeito benéfico associado à redução do risco à doença periodontal inflamatória na restrição calórica (REYNOLDS *et al.*, 2009).

Biomarcadores inflamatórios estão associados ao surgimento de patologias como a aterosclerose a as suas complicações. Modelos experimentais induzindo restrição alimentar (50% de restrição) leva a uma redução da expressão de RNAm de IL-1β, TNF-α e MCP-1 em diversos tecidos como rim, fígado e baço, causando um efeito protetor do organismo contra acidentes vasculares cerebrais e ataques cardíacos. Levantando-se a possibilidade de indicação de restrição alimentar juntamente com drogas para a redução da hipertensão como tratamento preventivo em pacientes que possuem inflamação crônica como na obesidade (CHIBA & EZAKI, 2010; ROBERTSON & MITCHELL, 2013).

Antígenos específicos de linfócitos T CD8+ estão envolvidos na malária experimental cerebral *(Plasmodium berghei),* esse parasita induz o rompimento da barreira hematoencefálica na presença de eritrócitos infectados com esse parasita; afirma-se que há redução acentuada dos linfócitos no cérebro dos animais infectados e submetidos à restrição alimentar. Esses resultados sugerem um efeito benéfico associado à restrição alimentar devido ao menor recrutamento de células para o cérebro e à preservação da barreira hematoencefálica. Além disso, existe aumento de linfócitos ativos que permanecem no baço ao invés de migrar para o cérebro, permitindo um maior clearence de células vermelhas infectadas, somando-se a isso o acúmulo de macrófagos e linfócitos naive no baço (MEJIA *et al.* 2015).

A restrição alimentar de curto prazo está associada à melhora da sobrevivência após endotoxemia. Com atenuação da resposta inflamatória, houve redução dos níveis plasmáticos de interleucina-6 e redução na população de macrófagos; essa redução de mediadores inflamatórios está associada a alterações tanto qualitativas quanto quantitativas no tecido adiposo com atenuação significativa da resposta inflamatória (STARR *et al*., 2016).

É nítido que a redução do consumo de alimentos é capaz de modular diversos órgãos e sistemas enfatizando os efeitos exercidos pela restrição alimentar na resposta inflamatória nas mais diversas perspectivas fisiopatológicas.

No entanto, não existem muitas informações acerca da indução de restrição alimentar concomitantemente com suplementação de nutrientes, como aminoácidos, e os possíveis efeitos moduladores no sistema imune (WASINSKI *et al.*, 2013).

Sendo assim, uma melhor compreensão dessa estratégia nutricional em condições basais e a compreensão da modulação induzida no sistema imunológico se faz necessária para proporcionar o desenvolvimento do conhecimento acerca dos efeitos da restrição alimentar.

CAPÍTULO I

3. INIBIÇÃO DA TRANSCRIÇÃO DE GENES INFLAMATÓRIOS PELA IL-10 É ASSOCIADA COM RÁPIDA SUPRESSÃO DE ENHANCERS

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Inhibition of Inflammatory Gene Transcription by IL-10 Is Associated with Rapid Suppression of Lipopolysaccharide-Induced Enhancer Activation

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IL-10 limits the magnitude of inflammatory gene expression following microbial stimuli and is essential to prevent inflammatory disease; however, the molecular basis for IL-10–mediated inhibition remains elusive. Using a genome-wide approach, we demonstrate that inhibition of transcription is the primary mechanism for IL-10–mediated suppression in LPS-stimulated macrophages and that inhibited genes can be divided into two clusters. Genes in the first cluster are inhibited only if IL-10 is included early in the course of LPS stimulation and is strongly enriched for IFN-inducible genes. Genes in the second cluster can be rapidly suppressed by IL-10 even after transcription is initiated, and this is associated with suppression of LPS-induced enhancer activation. Interestingly, the ability of IL-10 to rapidly suppress active transcription exhibits a delay following LPS stimulation. Thus, a key pathway for IL-10–mediated suppression involves rapid inhibition of enhancer function during the secondary phase of the response to LPS. ***The Journal of Immunology*****, 2017, 198: 000–000.**

The response of macrophages, even to a single inflammatory stimulus, is remarkably complex. Stimulation of macrophages with LPS rapidly induces the activation of canonical transcription factors, such as NF-κB and IRF3, in a protein synthesis–independent manner, and this is quickly followed by rapid induction of mRNA for a number of primary response genes (1–3). Following this initial wave, there are subsequent waves of mRNA induction that are sensitive to inhibition of protein synthesis, indicating the secondary nature of the response (4). It was documented that a significant proportion of secondary response genes are, in fact, responding to the primary induction of IFN-β, but several other factors are involved in driving secondary response genes, including the atypical nuclear IκB-like molecule IκBζ (5–7). Although mRNA induction following LPS-stimulation of macrophages is likely regulated at multiple levels, detailed studies evaluating changes in newly synthesized mRNA strongly suggest that induction of new gene transcription is the driving force behind the observed global alterations in gene expression (1, 2).

Recently, it was demonstrated that LPS-induced transcription in macrophages is associated with enhancer activation (8). Recruitment of stimulus-dependent transcription factors, such as NF-κB, to genomic sites termed poised enhancers is marked by binding of the pioneer transcription factor PU.1 and monomethylation of histone H3 at lysine 4 (H3K4me1). Activation of these enhancers is associated with increases in acetylation of histone H3 at lysine 27 (H3K27Ac) and increased transcription of *cis*-located genes (8–10). Although there is increased appreciation for the role of enhancer activation in initiating LPS-induced transcription, there is much less known regarding the processes that terminate LPS-induced transcription, and we do not know how factors that limit LPS-induced transcription influence the activation state of LPS-induced enhancers.

One key factor that limits LPS-induced inflammatory gene expression in macrophages is the stimulus-induced production of IL-10, a potent anti-inflammatory cytokine (11). The receptor for IL-10 activates STAT3 through a JAK1-dependent pathway (12). Global profiling experiments comparing mRNA levels in IL-10–deficient macrophages treated with LPS alone or LPS and IL-10 identified a wide range of primary and secondary response genes that are inhibited by IL-10, and studies in STAT3-deficient macrophages indicate that STAT3 is required for IL-10–mediated inhibition (11). Understanding the mechanism of STAT3-mediated inhibition in response to IL-10 has proven enigmatic. In most systems, STAT3 is a transcriptional activator, and there is little evidence that it has direct inhibitory function or binds to regulatory regions of IL-10–inhibited genes (13). This led to the

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E.A.C. designed and performed experiments, collected and analyzed data, and wrote the manuscript; D.C.d.O. and C.M.M. designed and performed experiments and collected and analyzed data; S.B.S. provided critical revision of the manuscript for important intellectual content; and B.H.H designed experiments, supervised experimentation and data collection, and wrote the manuscript.

The data presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/genbank) under accession number GSE86170.

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; ChIP-seq, chromatin immunoprecipitation followed by DNA sequencing; FDR, false discovery rate; H3K4me1, monomethylation of histone H3 at lysine 4; H3K27Ac, acetylation of histone H3 at lysine 27; IP, immunoprecipitation; pre-mRNA, precursor mRNA; RNA-seq, RNA sequencing; WT, wild-type.

hypothesis that, following IL-10R engagement, STAT3 induces genes that secondarily inhibit inflammatory gene expression. A number of STAT3-induced transcriptional inhibitors have been identified, including *Bcl3* and *Nfil3*; however, studies in genetically deficient macrophages failed to demonstrate that these factors are required for IL-10–mediated inhibition (14, 15). A firm understanding of the kinetics of IL-10–mediated inhibition could contribute significantly to delineating potential inhibitory mechanisms; however, knowledge is lacking in this area.

Inhibitory functions for IL-10 in macrophages have been proposed at the level of transcription, mRNA stability, and translation of individual genes (16–19). With regard to transcription, Aste-Amezaga et al. (20) used nuclear run-on assays to demonstrate that addition of IL-10 1 h prior to LPS stimulation inhibited transcription of *IL12B* in human PBMCs. Murray (16) used RT-PCR of primary transcripts to demonstrate that IL-10 added concomitantly with LPS inhibited transcription of *Il1a*, *Cxcl1*, *Il6*, and *Tnf* in IL-10–deficient bone marrow–derived macrophages (BMDMs). Further, studies of the *Il12b* promoter demonstrated that IL-10 inhibited histone H4 acetylation and prevented PolII association, consistent with inhibition of transcription (21). However, we do not yet understand the kinetics of IL-10–induced transcriptional inhibition or the scope of IL-10–mediated inhibition of the LPS-induced transcriptional program. Further, we do not understand how IL-10 influences the activation status of LPS-induced enhancers (22) and whether suppression of active enhancers by IL-10 occurs in a time frame compatible with suppression of active transcription. Answering these questions would increase our understanding of mechanisms responsible for IL-10–mediated inhibition and potentially lead to novel insights regarding mechanisms that terminate activation of inducible enhancers.

Delineating the kinetics of transcriptional inhibition using assays that measure mRNA is problematic, because the varying stability of mRNA makes it difficult to discern rapid changes in underlying transcriptional rates. Therefore, we used precursor mRNA (pre-mRNA) as a surrogate of transcriptional rate to provide a detailed analysis of the kinetics of IL-10–mediated transcriptional suppression. Further, using an RNA-sequencing (RNA-seq) protocol that allowed us to separately analyze mRNA and pre-mRNA at the global level, we identified two clusters of IL-10–inhibited genes that exhibit different kinetics of suppression and provide a mechanism for these differences. Finally, using chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq), we find that IL-10 induces highly dynamic alterations in enhancer activation that are associated with alterations in transcription of IL-10 targets. These studies significantly increase our understanding of mechanisms that regulate LPS-induced inflammatory gene expression.

Materials and Methods

Experimental animals

All mouse strains were maintained on the 129S6/SvEvTac background. Generation of $Rag2^{-/-}$ (wild-type [WT]) and $Il10^{-/-}Rag2^{-/-}$ ($Il10^{-/-}$, IL-10–deficient) mice was described previously (23). All animal procedures were approved by the Harvard Medical Area Standing Committee on Animals.

BMDM preparation and stimulation

BMDMs were grown as previously described (24) and split into 24-well plates on the day prior to stimulation. BMDMs were cultured in 500 μl of 10% FBS DMEM supplemented with penicillin/streptomycin, HEPES, and GlutaMAX at a density of 2.5×10^5 cells per well for RNA extraction or at a density of 1×10^6 cells per well for Western blotting. For stimulation, medium was removed and replaced with fresh medium for unstimulated samples or medium containing 1 ng/ml LPS from *Escherichia coli* serotype 0127:B8 (Sigma, St. Louis, MO). IL-10 (PeproTech, Rocky Hill, NJ) was used at a final concentration of 1 ng/ml. Actinomycin D (Sigma) was added 2 h after LPS stimulation to a final concentration of 5 μg/ml.

RNA extraction and RT-PCR

Cell culture medium was aspirated, and cells were lysed in 500 μl of TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA). RNA was isolated per the manufacturer's instructions with the addition of GlycoBlue Coprecipitant (Thermo Fisher Scientific) and 1-bromo-3-chloropropane (Molecular Research Center, Cincinnati, OH) in lieu of chloroform. cDNA was synthesized from 1 μg of DNase-treated RNA using random hexamers with TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific). RT-PCR was performed using the StepOnePlus System with TaqMan probes or with SYBR Green Master Mix (both from Thermo Fisher Scientific) and custom primers. Primer sequences are available on request. Expression was normalized to GAPDH, and differences between samples were calculated using the ΔΔ cycle threshold method. Fold-change is reported relative to levels observed in untreated macrophages. In graphs of RT-PCR data, each line represents BMDM from an individual mouse; each point on the line represents an individual well. Within the figure legends, *n* indicates the size of the experimental groups in the displayed experiment, and a statement regarding the number of times the experiment was performed is provided.

Western blot

Cells were lysed in 100 μl of RIPA buffer and 6.5 μl of lysate loaded onto Novex 4–12% Bis-Tris gels (all from Thermo Fisher Scientific). Gels were transferred to polyvinylidene difluoride membranes and blotted with anti-STAT3 (4904), anti-pSTAT3 (Y705; 9145), and anti-GAPDH (5174; all from Cell Signaling Technology).

ELISA

IL-12 p40 was measured in culture supernatants using capture Ab C15.6 (2 μg/ml; BD Pharmingen, San Jose, CA) and detection Ab C17-8 (0.5 μg/ml; Thermo Fisher Scientific). IL-10 was measured using capture Ab JES5-16E3 (4 μg/ml) and detected using Ab JES5-2A5 (0.5 μg/ml; both from eBioscience, San Diego, CA). IFN-β was measured with the VeriKine Mouse IFN Beta ELISA Kit, per the manufacturer's instructions (PBL Assay Science, Piscataway, NJ). Each data point represents the results from BMDMs isolated from an individual mouse.

Total RNA-seq

BMDMs were cultured in six-well plates at 1×10^6 cells per well. Cells were rinsed once with PBS, and RNA was isolated following the addition of TRIzol Reagent directly to the plate. RNA was further purified using an RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany). rRNA was depleted using a NEBNext rRNA Depletion Kit, and libraries were made with a NEBNext Ultra Directional RNA Library Prep Kit for Illumina (both from New England Biolabs, Ipswich, MA). Samples were sequenced using the Illumina HiSeq or NextSeq Platforms. Data were analyzed as in Gaidatzis et al. (25). Briefly, reads were aligned to mm10 using Rbowtie. Counts were generated using the QuasR package using qCount with only nonoverlapping genes, and differential analysis was carried out using DESeq. To focus on genes that were strongly induced by LPS, we used a false discovery rate (FDR) of 0.1 and only evaluated genes whose intronic signal increased by ≥100-fold. Further, we only evaluated genes that had ≥50 reads upon LPS stimulation. For genome level display, one replicate was normalized with deepTools (26) using the size factor generated by DESeq and displayed in the Integrative Genomics Viewer. The heat map was generated in R. All data are accessible through the gene expression omnibus accession number GSE86170.

ChIP-seq

Macrophages were plated at 2×10^7 cells per condition on 15-cm plates and, after stimulation, were fixed for 10 min in 1% formaldehyde. Chromatin was isolated and sheared by sonication (10 cycles, 20 s on, 30 s rest). After aliquoting an input fraction, 4×10^6 cells were incubated overnight at 4°C with Protein A Dynabeads (Thermo Fisher Scientific) precoupled with 5 μg of anti-H3K27Ac Ab (ab4729; Abcam) or 300 ng of anti-STAT3 Ab (12640; Cell Signaling Technologies). After incubation, the beads were washed sequentially with a low-salt buffer, high-salt buffer, lithium chloride buffer, and TE buffer. DNA–protein complexes were eluted from the beads with 1% SDS TE buffer at 65°C for 15 min. Input and immunoprecipitation cross-links were reversed by overnight incubation at 65°C. DNA was treated with proteinase K and RNase A (Thermo Fisher Scientific) before purification with a QIAquick PCR Purification Kit (QIAGEN).

Immunoprecipitation and input libraries were prepared using a NEBNext ChIP-Seq Library Prep Master Mix Set for Illumina (New England Biolabs). Samples were sequenced using the Illumina NextSeq platform. Samples were aligned to mm10 using bowtie2 (27), with default parameters and peaks called using MACS2 with the broad flag on for H3K27Ac and H3K4me1. Differential peak calling for H3K27Ac was done with three biological replicates using the DiffBind (28) package and the DESeq algorithm specifying an FDR < 0.1. For genome-level display, one representative replicate's libraries were normalized down to the depth of the least sequenced library and displayed as reads per base pair. STAT3 ChIP-seq data were analyzed in MACS using default parameters. Data were normalized to reads per million for display. For data from Ostuni et al. (8), the untreated H3K4me1 sample was normalized to reads per million. Mean acetylation plots were made using deepTools (26). Plots and statistical analysis in Fig. 6 were done in R. All data are accessible through the gene expression omnibus accession number GSE86170.

Statistical analyses

The Fisher exact test was used to calculate significant overrepresentation of *Ifnar1*-dependent genes (Fig. 3D). ANOVA with the Dunnett multiple-comparisons test was used to identify significant differences in IFN-β secretion (Fig. 5B). The Mann–Whitney *U* test was used to identify significant differences in H3K27 enhancer acetylation ratios (Fig. 6A). Two-way ANOVA with the Tukey multiple-comparisons test was used to identify significant differences in *Cxcl2* pre-mRNA (Fig. 8A). For all analyses, $p < 0.05$ was considered statistically significant.

Results

Endogenous IL-10 limits duration of IL-12 transcription

Il12b is an LPS-induced secondary response gene that is strongly inhibited by IL-10. Addition of exogenous IL-10 inhibits *IL12B* transcription in human PBMCs stimulated with LPS, and it inhibits *Il12b* transcription in murine peritoneal macrophages stimulated with LPS and IFN-γ (11, 29). However, the kinetic relationship between expression of endogenous IL-10 and transcription of *Il12b* has not been fully explored. To evaluate this issue, we stimulated WT and IL-10–deficient BMDMs with LPS and evaluated expression of *Il12b* mRNA every 15 min from 0 to 4 h. In WT BMDMs, *Il12b* mRNA was first detected 60 min after stimulation; it increased until reaching a plateau at 120 min that lasted for the duration of the experiment (Fig. 1A). In IL-10–deficient BMDMs, *Il12b* expression appeared to begin at the same time as in WT cells, but rather than reaching a plateau at 2 h, it continued to rise over the course of the next several hours. The difference in *Il12b* mRNA levels between WT and IL-10–deficient BMDMs was reflected in increased IL-12 p40 secretion by IL-10–deficient macrophages (Fig. 1B). These results suggest that endogenously produced IL-10 limits *Il12b* mRNA production and that this phenomenon begins 2 h after LPS stimulation. To determine whether this is consistent with the kinetics of endogenous IL-10 production, we performed an ELISA to measure IL-10 levels within the supernatants of LPS-stimulated WT BMDMs and found that IL-10 was first detected between 1 and 2 h after LPS stimulation, indeed coinciding with the plateau phase of *Il12b* production observed in WT BMDMs (Fig. 1C).

The observation that secretion of endogenous IL-10 results in a plateau in *Il12b* mRNA levels suggests that the rates of transcription and degradation of *Il12b* are matched or that transcription has ceased, and the remaining *Il12b* message is highly stable. To assess the stability of *Il12b* mRNA, we added the transcriptional inhibitor actinomycin D 2 h after LPS treatment of WT BMDMs and measured the levels of *Il12b* mRNA over the next 2 h. We found very little change in *Il12b* mRNA levels over the course of this experiment, indicating the mRNA was stable and suggesting that transcription of *Il12b* had ceased following expression of IL-10 (Fig. 1D). It was demonstrated that transcriptional activity can be evaluated through measurements of unspliced pre-mRNA, because they typically have a very short lifespan (16, 30). Therefore, we designed RT-PCR primers that selectively recognized *Il12b* pre-mRNA but not mRNA. As predicted, levels of pre-mRNA fell very rapidly after administration of actinomycin D, indicating that measurement of pre-mRNA was an accurate surrogate for transcription (Fig. 1D). Following LPS stimulation of WT BMDMs, levels of *Il12b* pre-mRNA increased earlier than what we had observed for levels of *Il12b* mRNA but decreased rapidly when *Il12b* mRNA reached the plateau phase (Fig. 1E), concomitant with accumulation of IL-10 within the supernatant of WT BMDMs. In contrast, *Il12b* pre-mRNA levels continued to increase for several additional hours in IL-10–deficient BMDMs (Fig. 1E). These results demonstrate that secretion of endogenous IL-10 rapidly inhibits transcription of *Il12b*.

IL-10 rapidly inhibits active transcription of Il12b

The results above suggest that endogenous IL-10 rapidly suppresses transcription of *Il12b* in WT BMDMs stimulated with LPS. To more accurately assess the kinetics of IL-10–mediated suppression, we added exogenous IL-10 to IL-10–deficient BMDMs at a time of active *Il12b* transcription (1.75 h after LPS stimulation). We observed significant suppression of *Il12b* transcription as early as 15 min after exposure and nearly complete suppression by 30 min (Fig. 2A). Further, we found that addition of IL-10 led to a rapid plateau in levels of *Il12b* mRNA (data not shown). Consistent with these results, the timing of IL-10 addition strongly influenced the accumulation of IL-12 p40 within the culture medium (Fig. 2B). These results indicate that IL-10 rapidly inhibits transcription of *Il12b* in LPS-stimulated BMDMs and that this profoundly influences accumulation of secreted IL-12 p40.

Inhibition of transcription is a central mechanism of IL-10–mediated suppression

To further examine the transcriptional regulation of *Il12b* in response to IL-10 we sequenced ribosome-depleted total RNA from IL-10–deficient macrophages that were left untreated, stimulated with LPS alone for 3.25 h (LPS), stimulated with LPS and IL-10 for 3.25 h (continuous IL-10), or stimulated with LPS for 3.25 h with IL-10 added for the last 30 min (acute IL-10). As expected, although we found no signal in untreated cells, we detected strong signals over the exons of *Il12b* following treatment with LPS (Fig. 3A), indicating the accumulation of spliced mRNA. In addition, we observed signal within the introns of *Il12b* (Fig. 3B), indicating the presence of pre-mRNA and, therefore, active transcription. In the samples treated with LPS and continuous IL-10, we saw no signal in exonic or intronic regions, indicating a lack of mRNA accumulation and lack of active transcription. In contrast, in samples treated with LPS and acute IL-10 (final 30 min of the experiment), although the exonic signal exhibited little change in intensity compared with the samples treated with LPS alone, there was marked inhibition of the intronic signal. This suggests that, consistent with RT-PCR results, IL-10 rapidly inhibits transcription of *Il12b*.

The recognition that RNA-seq of total RNA allowed us to observe rapid alterations in *Il12b* transcription by monitoring pre-mRNA suggested that we could use this technique to evaluate the transcriptional effects of IL-10 at the global level. To accomplish this, we calculated expression levels of mRNA and pre-mRNA for all protein-coding genes across all four conditions (untreated, LPS, continuous IL-10, acute IL-10) using exon–intron split analysis with minor alterations (25). Using this approach, we identified 138 genes whose transcription was increased ≥100-fold (FDR < 0.1) by LPS. We grouped these genes into three clusters using a k-means algorithm (Fig. 3C, 3D). Transcription of genes in

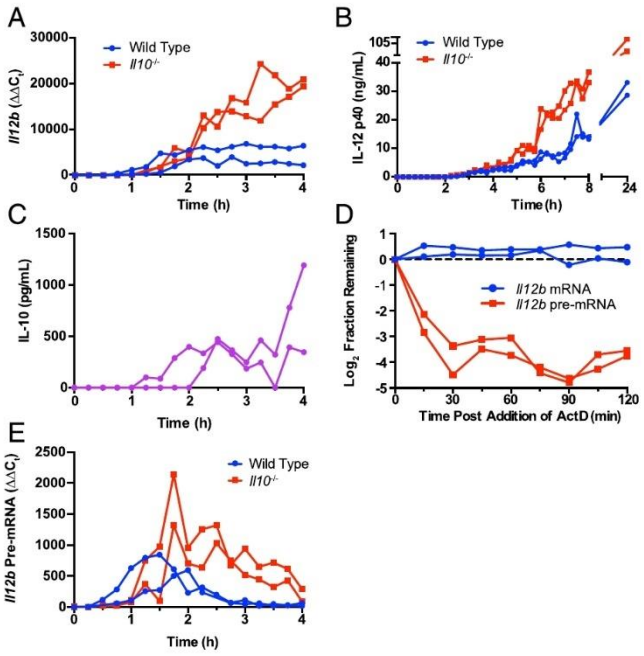


FIGURE 1. IL-10 rapidly inhibits *Il12b* transcription. (**A**) WT and $Il10^{-/-}$ BMDMs were stimulated with LPS (1 ng/ml). RNA was harvested every 15 min for 4 h. *Il12b* mRNA was measured by RT-PCR, and expression levels are displayed relative to unstimulated levels using the ΔΔ cycle threshold method ($n = 2$). (**B**) WT and $Il10^{-/-}$ BMDMs were stimulated with LPS, and supernatants were collected every 15 min for up to 8 h, as well as at 24 h. IL-12 p40 levels were measured by ELISA ($n = 2$). (**C**) IL-10 levels in supernatants of WT BMDMs were measured by ELISA ($n = 2$). (**D**) Actinomycin D (5 μg/ml) was added to WT BMDMs 2 h after LPS stimulation, and RNA was harvested every 15 min for the ensuing 2 h. *Il12b* mRNA and pre-mRNA were measured by RT-PCR and displayed as the $\log_2$ fraction remaining compared with the time of actinomycin D addition ($n = 2$). (**E**) *Il12b* pre-mRNA was measured by RT-PCR in RNA harvested from WT and $Il10^{-/-}$ BMDMs and is displayed relative to the unstimulated levels ($n = 2$). All experiments were performed twice.

Cluster 1 was strongly inhibited by continuous IL-10 but not by acute IL-10. Transcription of genes in Cluster 2 was strongly inhibited by continuous and acute IL-10. Transcription of genes in Cluster 3 was induced by IL-10. Excluding genes in Cluster 3, these data indicate that continuous IL-10 inhibits the transcription of the vast majority of LPS-induced genes, whereas acute IL-10 inhibits the transcription of a subset of these genes.

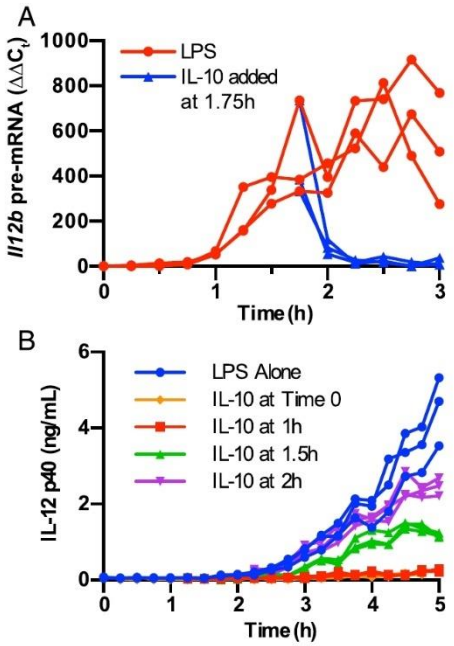


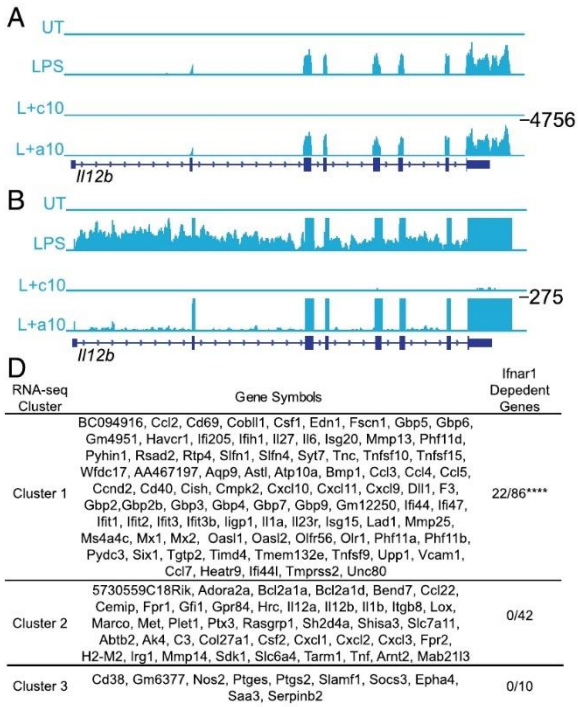
FIGURE 2. Exogenous IL-10 rapidly inhibits *Il12b* transcription. (**A**) $Il10^{-/-}$ BMDMs were stimulated with LPS and then treated with IL-10 (1 ng/ml) at 1.75 h or left untreated. RNA was collected every 15 min, and *Il12b* pre-mRNA was measured by RT-PCR ($n = 3$). This experiment was performed three times. (**B**) $Il10^{-/-}$ BMDMs were stimulated with LPS, with or without the addition of IL-10 at the indicated time points. IL-12 p40 levels in the supernatants were measured by ELISA every 15 min ($n = 3$). This experiment was performed once.

Cluster 1 is enriched for IFN-responsive secondary response genes

We demonstrated markedly different kinetics for the transcriptional response to IL-10 in genes assigned to Clusters 1 and 2. We hypothesized that differential responses could indicate alternative modes of transcriptional regulation. To examine this hypothesis, we scanned the promoters (500 bp upstream of the transcription start site) of all 138 LPS-induced genes for the presence of known transcription factor–binding motifs. Cluster 1 genes were significantly enriched for IRF/ISRE consensus elements, and scanning of genes in Cluster 2 identified strong enrichment for NF-κB binding without the enrichment for IFN-responsive elements observed in Cluster 1 (Fig. 4).

The observation that promoters for Cluster 1 genes were significantly enriched for IRF/ISRE consensus elements suggested that this cluster could represent IFN-β–induced secondary response genes. To examine this possibility further, we compared the proportion of genes in each cluster whose response to LPS required the presence of *Ifnar1*, based on data presented in Tong et al. (4). We found that Cluster 1 was significantly enriched for genes that required the presence of *Ifnar1* compared with Cluster 2 (class 1 genes [22/86], Cluster 2 genes [0/42], $p < 10^{-4}$, Fig. 3D).

Previous results demonstrate that expression of *Ifnb1* is induced early in the response to LPS, whereas *Ifnb1*-responsive genes exhibit delayed kinetics (5). Therefore, a potential explanation for the failure of acute, but not continuous, IL-10 to inhibit genes in Cluster 1 is that rather than directly inhibiting Cluster 1 genes, IL-10 is required earlier in the time course to inhibit expression of *Ifnb1* itself. To examine this possibility, we evaluated expression of *Ifnb1* and the IFN-induced secondary response gene *Mx1* in LPS-stimulated IL-10–deficient BMDMs. *Ifnb1* mRNA expression



D

RNA-seq Cluster	Gene Symbols	Ifnar1 Depedent Genes
Cluster 1	BC094916, Ccl2, Cd69, Cobll1, Csf1, Edn1, Fscn1, Gbp5, Gbp6, Gm4951, Havcr1, Ifi205, Ifih1, Il27, Il6, Isg20, Mmp13, Phf11d, Pyhin1, Rsad2, Rtp4, Slfn1, Slfn4, Syt7, Tnc, Tnfsf10, Tnfsf15, Wfdc17, AA467197, Aqp9, Astl, Atp10a, Bmp1, Ccl3, Ccl4, Ccl5, Ccnd2, Cd40, Cish, Cmpk2, Cxcl10, Cxcl11, Cxcl9, Dll1, F3, Gbp2,Gbp2b, Gbp3, Gbp4, Gbp7, Gbp9, Gm12250, Ifi44, Ifi47, Ifit1, Ifit2, Ifit3, Ifit3b, Iigp1, Il1a, Il23r, Isg15, Lad1, Mmp25, Ms4a4c, Mx1, Mx2, Oasl1, Oasl2, Olfr56, Olr1, Phf11a, Phf11b, Pydc3, Six1, Tgtp2, Timd4, Tmem132e, Tnfsf9, Upp1, Vcam1, Ccl7, Heatr9, Ifi44l, Tmprss2, Unc80	22/86****
Cluster 2	5730559C18Rik, Adora2a, Bcl2a1a, Bcl2a1d, Bend7, Ccl22, Cemip, Fpr1, Gfi1, Gpr84, Hrc, Il12a, Il12b, Il1b, Itgb8, Lox, Marco, Met, Plet1, Ptx3, Rasgrp1, Sh2d4a, Shisa3, Slc7a11, Abtb2, Ak4, C3, Col27a1, Csf2, Cxcl1, Cxcl2, Cxcl3, Fpr2, H2-M2, Irg1, Mmp14, Sdk1, Slc6a4, Tarm1, Tnf, Arnt2, Mab21l3	0/42
Cluster 3	Cd38, Gm6377, Nos2, Ptges, Ptgs2, Slamf1, Socs3, Epha4, Saa3, Serpinb2	0/10

FIGURE 3. Global modulation of LPS-induced gene expression by IL-10. *Il10*$^{-/-}$ BMDMs were left untreated (UT), were stimulated with LPS for 3.25 h (LPS), were stimulated with LPS and IL-10 together for 3.25 h (L+c10), or were stimulated with LPS for 3.25 h with the addition of IL-10 for the last 30 min of the stimulation (L+a10). Total RNA was harvested and sequenced following ribosomal depletion. Normalized distribution of RNA-seq reads is shown at the *Il12b* locus (**A**) and with an expanded *y*-axis to better demonstrate pre-mRNA (**B**). (**C**) Heat map showing k-means clustering of pre-mRNA signal for the 138 genes induced 100-fold by LPS in *Il10*$^{-/-}$ BMDMs. (**D**) Individual genes that make up each cluster and the number of genes in each cluster deemed *Ifnar1*-dependent by Tong et al. (4). ****$p < 0.0001$, Fisher exact test.

peaked 90 min after stimulation before rapidly declining (Fig. 5A). This peak in *Ifnb1* mRNA expression was followed by induction of *Mx1* pre-mRNA (Fig. 5B). Addition of IL-10 at the time of LPS stimulation significantly inhibited expression of *Ifnb1* mRNA and *Mx1* pre-mRNA (Fig. 5A, 5B), as well as secretion of IFN-β into the culture medium (Fig. 5C). In contrast, addition of IL-10 2 h after LPS stimulation (and after the peak of *Ifnb1* mRNA expression) had little effect on transcription of *Mx1* or on the amount of secreted IFN-β (Fig. 5B, 5C). These results strongly suggest that *Ifnb1* itself, rather than an *Ifnb1*-responsive genes, is the primary target for IL-10. Because addition of IL-10 following the peak of *Ifnb1* mRNA expression does not reduce the amount of IFN-β secreted into the culture media, these results explain why addition of IL-10 2 h and 45 min after LPS stimulation (acute IL-10) fails to inhibit transcription of many of the genes in Cluster 1. This is consistent with previous results demonstrating that IL-10 inhibits LPS-induced antiviral activity, presumably IFN-β, but is unable to inhibit gene expression induced in direct response to stimulation with IFN-β (31).

IL-10 rapidly inhibits LPS-induced enhancer activation of genes in Cluster 2

It was shown that LPS induces H3K27 acetylation of a group of poised enhancers marked by H3K4me1 following stimulation of BMDMs, but it is not known whether IL-10 inhibits LPS-induced transcription by interfering with LPS-induced enhancer acetylation (8). Further, because we showed above that IL-10 rapidly suppresses LPS-induced transcription of genes in Cluster 2, we were interested in determining whether rapid inhibition of transcription was associated with rapid suppression of enhancer acetylation. To study the effect of IL-10 on LPS-induced enhancer acetylation, we performed ChIP-seq for H3K27Ac in IL-10–deficient macrophages. We found that treatment with LPS for 3.25 h significantly induced 8417 acetylation peaks (FDR < 0.1). To identify acetylation peaks in proximity to LPS-induced genes, we filtered on LPS-induced H3K27Ac peaks that were located within 50 kb upstream of the transcriptional start site, as well as within 50 kb downstream of the transcription termination site of Cluster 1 and 2 genes. We found that many of these peaks overlapped with sites of H3K4me1 defined by Ostuni et al. (8), indicating that these peaks likely represented bona fide enhancers (data not shown). We found that 49 of 86 genes in Cluster 1 were associated with at least one LPS-induced acetylation peak, and 34 of 42 genes in Cluster 2 were associated with at least one LPS-induced peak. Next, we compared the magnitude of these H3K27Ac peaks in BMDMs stimulated with LPS alone with those stimulated with LPS and continuous IL-10 or with LPS and acute IL-10. Treatment with continuous IL-10 markedly reduced H3K27Ac at peaks associated with genes from Clusters 1 and 2 (Fig. 6A). However, following treatment with acute IL-10, H3K27Ac was significantly lower at peaks associated with genes from Cluster 2 than at peaks associated with genes from Cluster 1 ($p < 0.01$, Mann–Whitney *U* test, Fig. 6A). Examples of Cluster 1 and Cluster 2 enhancers are shown in Fig. 6B and 6C, respectively. Interestingly, this analysis identified a peak inhibited by acute IL-10 that encompasses a DNase hypersensitivity site 10 kb upstream of *Il12b* that was

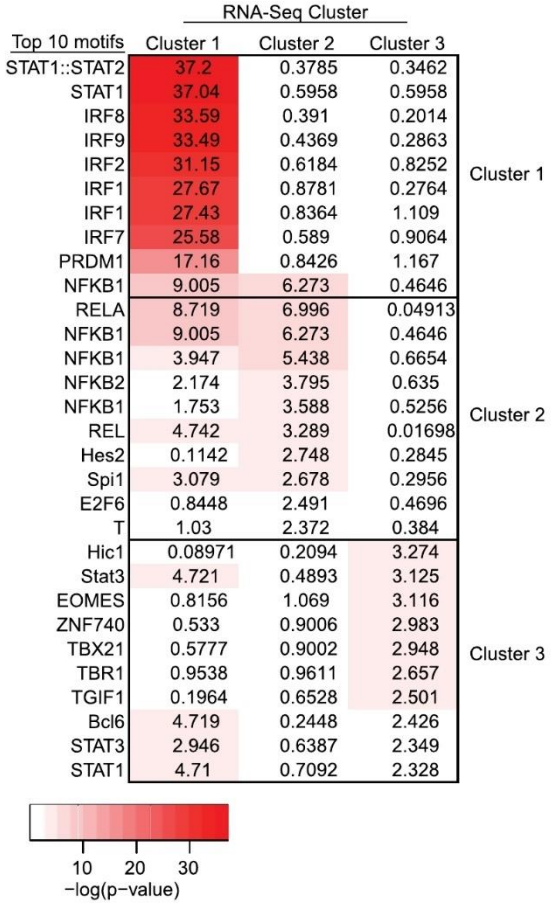


FIGURE 4. Transcription factor binding motifs in LPS-induced promoters. PSCAN analysis of promoters for LPS-induced genes clustered as in Fig. 3C. The top 10 motifs for each cluster are shown on the left. Columns represent enrichment *p* values for each motif organized by cluster (top). The color intensity is proportional to the negative log of the *p* value.

previously demonstrated in a reporter assay to exhibit enhancer activity (Fig. 6C) (22). These data indicate that IL-10 can rapidly suppress enhancers associated with genes in Cluster 2, suggesting that the ability of IL-10 to rapidly suppress transcription of genes in Cluster 2 may be based on suppression of enhancer function.

IL-10–mediated inhibition of Cluster 2 genes is not associated with direct STAT3 binding

STAT3 is required for IL-10–mediated inhibition of LPS-induced inflammatory gene expression (11). However, the function of STAT3 in the inhibitory process is not clear. STAT3 is a transcriptional activator, and binding sites for STAT3 in the proximity of genes inhibited by IL-10 have not been identified (13). This led to the hypothesis that STAT3-dependent induction of inhibitory factors mediates the inhibitory function of IL-10. However, the rapidity with which IL-10 is able to inhibit genes in Cluster 2 argues that dependence on new protein synthesis is unlikely, although certainly not impossible. Therefore, we sought to consider alternative mechanisms that might explain rapid STAT3-mediated inhibition. One possibility is that STAT3 binding directly suppresses activation of enhancers associated with genes in Cluster 2. To address this possibility, we performed ChIP-seq with an anti-STAT3 Ab to identify STAT3 binding sites. After treatment of IL-10–deficient BMDMs with IL-10 alone for 30 min, we identified 31 STAT3-binding peaks within 50 kb upstream of the top 138 LPS-induced genes; strikingly, we found an additional 54 peaks within these regions when BMDMs were stimulated with LPS prior to the addition of IL-10. This indicates that prior LPS treatment reveals IL-10–induced STAT3 binding sites that were not present in unstimulated cells. Interestingly, virtually all identified STAT3 binding sites were located within H3K4me1 peaks, suggesting that STAT3 binds to enhancers. As anticipated, we found strong STAT3 binding near Cluster 3 genes induced by IL-10, such as *Socs3* (Fig. 7, lower left panel), and IL-10 induced an increase in average mean acetylation at STAT3 binding sites associated with genes in Cluster 3, consistent with enhancer activation (Fig. 7, lower right panel). Interestingly, prior LPS treatment

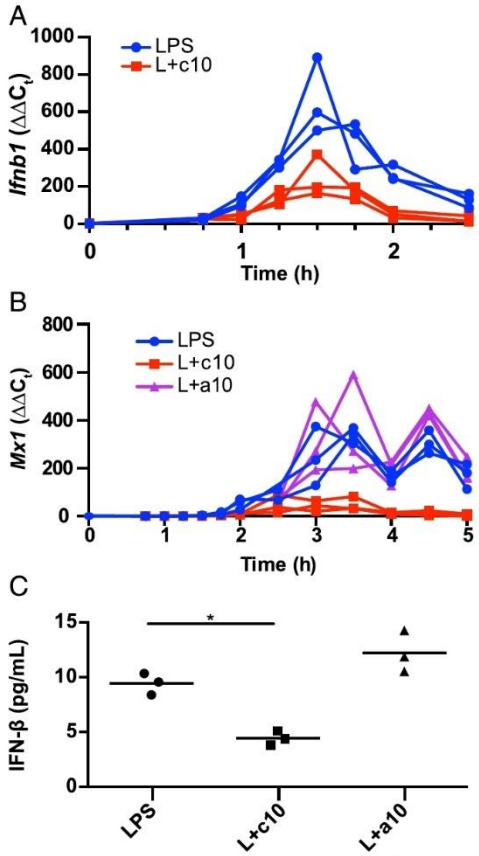


FIGURE 5. IL-10 inhibits IFN-β production. (**A**) *Il10*$^{-/-}$ BMDMs were stimulated with LPS or with LPS and IL-10 (L+c10). RNA was harvested at the indicated time points, and *Ifnb1* mRNA was measured by RT-PCR ($n = 3$). This experiment was performed three times. (**B**) *Il10*$^{-/-}$ BMDMs were stimulated with LPS, stimulated with LPS and IL-10 (L+c10), or stimulated with LPS with the addition of IL-10 2 h after LPS stimulation (L+a10). RNA was harvested at the time points indicated, and *Mx1* mRNA was measured by RT-PCR ($n = 3$). This experiment was performed three times. (**C**) *Il10*$^{-/-}$ BMDMs were stimulated with LPS for 4 h (LPS), simulated with LPS and IL-10 for 4 h (L+c10), or stimulated with LPS for 4 h with addition of IL-10 for the last 2 h (L+a10). Culture supernatants were harvested and analyzed for IFN-β protein secretion by ELISA. This experiment was performed once. $*p < 0.05$, ANOVA, with Dunnett multiple-comparisons test ($n = 3$).

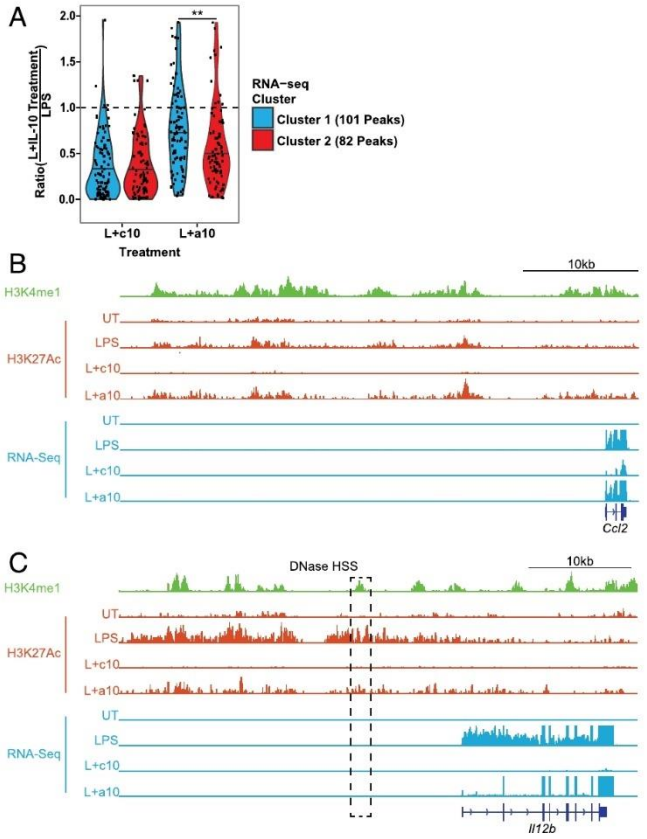


FIGURE 6. IL-10 causes rapid changes in enhancer H3K27Ac status. *Il10*$^{-/-}$ BMDMs were left untreated (UT) or were stimulated with LPS for 3.25 h (LPS), stimulated with LPS and IL-10 for 3.25 h (L+c10), or stimulated with LPS for 3.25 h with addition of IL-10 for the last 30 min of the stimulation (L+a10). Chromatin was harvested, and ChIP-seq was performed with an Ab specific for H3K27Ac. (**A**) The H3K27Ac signal intensity was determined for each LPS-induced acetylation peak within 50 kb upstream or downstream of Cluster 1 and Cluster 2 genes, and a ratio between the signal with LPS alone and with continuous or acute IL-10 treatment was calculated for each peak. These ratios are represented on a violin plot where each dot represents an individual enhancer, and the width of the contours represents a smoothened density of these values. The horizontal line within each violin plot indicates the median value. Representative locus shown for Cluster 1 gene *ccl2* (**B**) and Cluster 2 gene *Il12b* (**C**). H3K4me1 ChIP-seq data from Ostuni et al. (8) normalized to reads per million (green). H3K27Ac ChIP-seq data from macrophages treated as in (A) normalized to the depth of the least-sequenced library (red). Normalized RNA-seq data from macrophages treated as in Fig. 3C (blue). The boxed region in (C) represents the *Il12b* −10 kb enhancer described by Zhou et al. (22). $^{**}p < 0.01$, Mann–Whitney *U* test.

revealed STAT3 binding sites near Cluster 1 genes that were not present with IL-10 treatment alone, and these sites also demonstrated an increase in mean acetylation in response to IL-10, although these peaks had a much weaker STAT3 signal relative to Cluster 3 gene peaks (Fig. 7). Lastly, IL-10 had little effect on mean acetylation at STAT3 binding peaks located within the 50 kb upstream of genes in Cluster 2, and STAT3 binding sites were not identified in the 50 kb upstream of *Il12b* (Fig. 7). Thus, there is little evidence that STAT3 binding directly suppresses activation of LPS-induced enhancers. These results raise the possibility that IL-10–induced STAT3 may have an inhibitory function that does not depend on direct DNA binding.

Cluster 2 gene Cxcl2 is inhibited during the secondary response phase to LPS

Inspection of genes in Cluster 2 revealed many, including *Il12b*, that were previously characterized as secondary response genes in LPS-stimulated BMDMs. However, there were also many genes that have been characterized as primary response genes (*Cxcl1*, *Cxcl2*) (Fig. 3D) (4). Because we had only examined the transcriptional effects of IL-10 at relatively late time points (105 and 165 min following LPS stimulation), we wondered whether the ability of IL-10 to rapidly inhibit transcription was operational at an early time point following LPS stimulation. To evaluate this issue, we compared induction of *Cxcl2* transcription in IL-10–deficient macrophages stimulated with LPS alone, or LPS and IL-10, or stimulated with IL-10 for 1 h prior to stimulation with LPS. As predicted, LPS rapidly induced *Cxcl2* pre-mRNA within 1 h of stimulation. Surprisingly, addition of IL-10 at the time of LPS stimulation or addition 1 h prior to LPS stimulation had little influence on *Cxcl2* pre-mRNA at 1 h post-LPS stimulation, although significant suppression was observed at 2 h post-LPS stimulation (Fig. 8A). This difference was not caused by an inability of macrophages to respond to IL-10 prior to LPS stimulation because similar induction of STAT3 Y705 phosphorylation was observed when cells were treated with IL-10 in parallel with LPS or at later time points (Fig. 8B). These results strongly suggest that IL-10 interferes with a process that is unique to the secondary phase of the response to LPS.

Discussion

We investigated the kinetics of IL-10–mediated inhibition of LPS-induced gene expression. We found that IL-10 rapidly inhibits LPS-induced transcription of *Il12b* in WT cells and that addition of IL-10 to IL-10–deficient macrophages leads to the rapid termination of transcription. Using a novel approach to evaluate global changes in transcription of LPS-induced genes, we found that, although administration of IL-10 at the time of LPS stimulation of IL-10–deficient BMDMs had broad inhibitory effects, only a subset of these genes (Cluster 2) was rapidly inhibited when IL-10 was given 2.75 h after LPS stimulation. Rapid inhibition of transcription of genes in Cluster 2 was accompanied by rapid inactivation of putative *cis*-acting enhancer-like elements, suggesting that IL-10 was acutely influencing enhancer function. Taken together, these results indicate that rapid inhibition of enhancer function may be a key mechanism of IL-10–mediated inhibition.

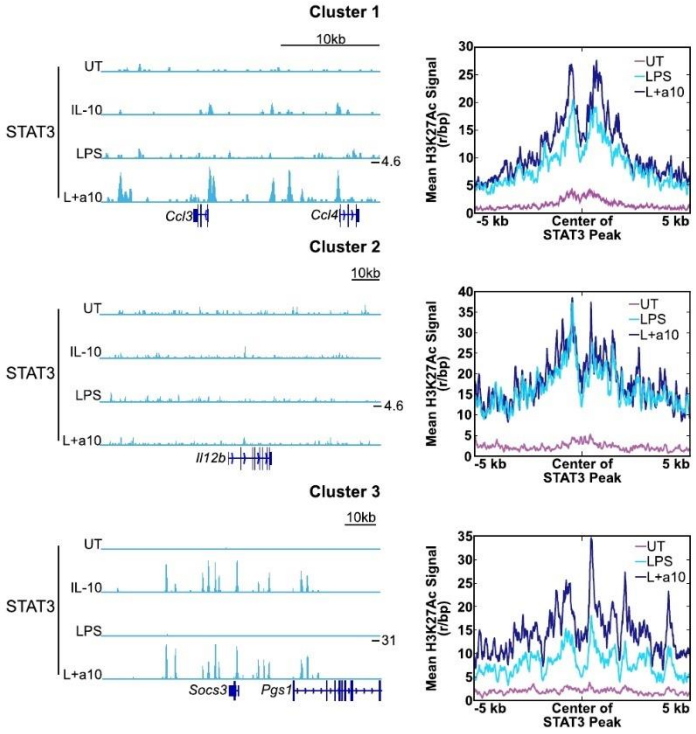


FIGURE 7. STAT3 binding is associated with gene induction. Left, *Il10*$^{-/-}$ BMDM were left untreated (UT) or were stimulated with IL-10 for 30 min (IL-10), stimulated with LPS for 3.25 h (LPS), or stimulated with LPS for 3.25 h with addition of IL-10 for the last 30 min of the stimulation (LPS+a10). Chromatin was harvested, and ChIP-seq was performed for STAT3. STAT3 ChIP-seq signal in reads per million shown for representative locus for Cluster 1 genes *Ccl3* and *Ccl4* (top panel), Cluster 2 gene *Il12b* (middle panel), and a Cluster 3 gene *Socs3* (bottom panel). *Y*-axis maximum for each locus marked in L+a10 track. Right, Mean H3K27Ac ChIP-Seq signal intensities from *Il10*$^{-/-}$ BMDMs treated as in Fig. 6 for STAT3 peak centers identified within 50 kb upstream of genes in each cluster. Signal is normalized by library size to reads per base pair.

It was demonstrated previously that there is a delay in *Il12b* expression following LPS stimulation of WT BMDMs (21), but we were quite surprised to find that the period of active *Il12b* transcription was extremely short due to rapid inhibition by endogenously produced IL-10. Despite the short period of active transcription, *Il12b* mRNA remained present for hours after transcription had terminated, and small differences in the timing of addition of exogenous IL-10 led to large differences in the amount of IL-12 p40 protein measured in the supernatant 24 h after stimulation. Because we expect that similar kinetics will be observed in human cells, these results emphasize that relatively small variations in the timing of induction of *Il12b* transcription or production of IL-10 among individuals could lead to quite large differences in overall levels of Il-12 p40 secretion and biological function. Whether this is an important factor controlling differences in immune and inflammatory responses among individuals remains to be determined.

Although global induction of LPS-induced genes has been widely evaluated (2, 4, 11), the results presented in this article using exon–intron split analysis of total RNA-seq data provide a unique perspective on the kinetics of transcription termination that cannot be fully appreciated from evaluation of mRNA levels alone. This simple method to estimate transcription rates relies on sequencing of ribosome-depleted total RNA and does not require purification of chromatin, as is required for nascent transcript RNA-seq, or IP of chromatin that is required for PolII ChIP-seq. Further, this method simultaneously provides quantification of mRNA and relative rates of active transcription. Application of this technique to other systems where strict temporal regulation of gene expression is critical could reveal dynamic regulation that has not been observed previously.

Use of the exon–intron split analysis allowed us to determine that, although transcription of virtually all LPS-induced genes is inhibited when IL-10 is added at the time of LPS stimulation, transcription of only a subset is rapidly inhibited when IL-10 is administered to IL-10–deficient macrophages 2 h and 45 min following LPS stimulation (Cluster 2). Although we do not yet understand the biochemical basis for IL-10–mediated inhibition, we demonstrated the rapid loss of H3K27 acetylation at LPS-induced enhancer elements associated with Cluster 2 genes following IL-10 treatment. This suggests that the mechanism of IL-10–mediated inhibition involves rapid modulation of LPS-induced enhancer function. It was shown previously that IL-10 can reduce total histone 4 acetylation at the *Il12b* promoter and potentially enhance the function of HDACs, but the exact nature of IL-10–mediated alterations in chromatin acetylation have not been described at the genome-wide level (21). Interestingly, our observation that IL-10 reverses LPS-induced H3K27 acetylation at many putative enhancer elements raises the possibility that previous genome-wide evaluations of LPS-induced enhancer elements may have significantly underestimated their number, because these studies were performed on WT macrophages at time points where one would predict the presence of significant amounts of IL-10 in the culture supernatant (8). Although we believe that rapid modulation of enhancer function by IL-10 is a key pathway mediating suppression of genes in Cluster 2, an important limitation to these conclusions is the variability noted in the response of genes in this cluster to IL-10. This variability may indicate the presence of alternative mechanisms of suppression for selected genes in this cluster.

Although evidence from the exon split analysis suggests rapid inhibitory function for IL-10, we were quite surprised that addition of IL-10 at the time of LPS stimulation did not inhibit the initial transcription of an early response gene, *Cxcl2*, despite clear evidence for suppression at later time points. Although we initially considered the possibility that this was evidence for a delayed effect of IL-10, we do not believe that this is the case, because

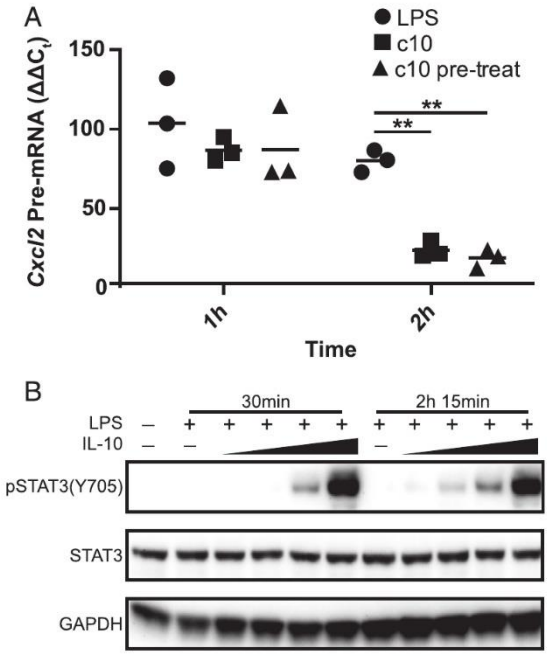


FIGURE 8. The ability of IL-10 to inhibit *Cxcl2* transcription exhibits a delay following LPS stimulation. (**A**) *Il10*$^{-/-}$ BMDMs were stimulated with LPS alone, treated with IL-10 concurrently with LPS stimulation, or pretreated with IL-10 for 1 h prior to LPS stimulation. RNA was harvested at 1 and 2 h after LPS stimulation. *Cxcl2* pre-mRNA levels were measured by RT-PCR and are displayed relative to levels in unstimulated cells. This experiment was performed three times. (**B**) *Il10*$^{-/-}$ BMDMs were stimulated with LPS for 30 min and 2.25 h, with or without addition of IL-10 in increasing doses of 0.0625, 0.25, 1, and 4 ng/ml for the last 30 min of the experiment. Cell extracts were prepared and assayed for pSTAT3 (Y705) or total STAT3 by Western blot. This experiment was performed twice. $^{**}p < 0.01$, two-way ANOVA with Tukey multiple-comparisons test ($n = 3$).

administration of IL-10 1 h prior to LPS stimulation did not reduce the time to the point where inhibition was first observed. This delay was not based on a requirement for LPS to induce components of the IL-10R apparatus, because exogenous IL-10 activated STAT3, even in the absence of prior LPS stimulation. Thus, we prefer the possibility that IL-10 is unable to inhibit the first wave of LPS-induced gene expression but rather functions, at least in part, by inhibiting an LPS-induced positive regulator that is required for the induction of a subset of delayed-response genes and the sustained expression of immediate-response genes. One potential candidate for this positive regulator is IκBζ, which is induced in response to LPS and is required for LPS-induced expression of *Il12b* (7). Interestingly, it was reported that IκBζ interacts with activated STAT3 (32), but it remains to be determined whether this interaction is necessary for IL-10–mediated suppression.

It is tempting to speculate that genes inhibited by continuous, but not acute, IL-10 (Cluster 1) are inhibited more slowly than genes in Cluster 2; however, we believe that our data support a model in which a substantial proportion of genes in Cluster 1 are in fact responding to secreted IFN-β, because Cluster 1 is strongly enriched for LPS-induced genes that require the presence of the type-1 IFNR (4). Although we confirmed that IFN-β is inhibited by IL-10, peak expression of IFN-β occurs at 90 min, thus explaining why IL-10 administration at the time of stimulation, but not after 2 h and 45 min, inhibits Cluster 1 genes. Interestingly, previous work suggested that LPS-induced IFN-β expression may have a role in the regulation of IL-10 itself (33). However, it is important to note that IFN-β priming of IL-10 expression cannot explain the results obtained in these experiments, because the BMDMs used in these experiments lack endogenous IL-10. An important caveat to our conclusions is that select secondary response genes within Cluster 1 are independent of IFNAR; therefore, inhibition of *Ifnb1* expression is unlikely responsible for IL-10–mediated inhibition of all genes within Cluster 1. However, because several of the INFAR-independent secondary response genes identified in Cluster 1, including IL-6 and IL-27, are TRIF dependent, it may be possible that a common IL-10–mediated pathway that inhibits TRIF function plays an important role in inhibiting genes in this cluster (4). Identifying the IL-10–responsive mediators of these effects could have important implications for understanding the overall regulation of the response to LPS.

It is somewhat paradoxical that genes in Cluster 1 only exhibit responsiveness to IL-10 suppression at times points prior to robust secretion of endogenous IL-10 by LPS-stimulated macrophages. However, there are multiple sources of IL-10 in vivo, including regulatory T cells, and it has been demonstrated that non-macrophage sources of IL-10 are essential to prevent inflammatory diseases, such as inflammatory bowel disease (34). Although it is difficult to completely define when a macrophage is first exposed to IL-10 in vivo, we believe that it is plausible that macrophages at sites of pathogen interface, such as the intestine, are exposed to IL-10 at varying times relative to the receipt of an inflammatory stimuli. The critical window of IL-10 responsiveness for genes in Cluster 1, revealed by our study, could have a central role in defining how macrophages respond based on temporal variability in receipt of the IL-10 signal.

What then have we learned regarding the mechanism of IL-10–mediated inhibition? Results from this study suggest that inhibition is based on the rapid suppression of active transcription and deacetylation of LPS-induced enhancer-like elements. This mechanism extends to most LPS-induced genes but is not operational without prior LPS stimulation. The rapid nature of IL-10–mediated suppression would seem to indicate that STAT3-driven induction of a secondary transcriptional inhibitor is unlikely (although certainly not impossible), given that inhibition of active transcription can be observed within 15 min of IL-10 treatment. Although experiments using the protein synthesis inhibitor cycloheximide suggested that IL-10–mediated inhibition requires new protein synthesis, in our hands cycloheximide treatment rapidly inhibits ongoing transcription of *Il12b* (data not shown), making it quite difficult to discern any additional inhibitory effects of IL-10. Thus, we believe that the question of whether IL-10–mediated inhibition requires new protein synthesis remains open. Alternative possibilities to explain IL-10–mediated inhibition that could potentially function on the more rapid time scale observed in this study include the induction of an inhibitory RNA species or a direct inhibitory function for STAT3. However, STAT3 ChIP-seq failed to uncover IL-10–induced STAT3 binding sites associated with suppression of LPS-induced enhancer activation. Thus, further directed experiments geared toward understanding IL-10–mediated signaling pathways that rapidly inhibit transcription are required. Delineating these mechanisms could have important implications for understanding the basis for inflammatory disease, as well as the development of novel therapeutics.

Disclosures

The authors have no financial conflicts of interest.

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CAPÍTULO II

4. A GLUTAMINA MODULA A EXPRESSÃO GÊNICA DE CITOCINAS EM MACRÓFAGOS

Title: Glutamine Modulates Cytokines Gene Expression in Macrophages via IL-10

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ABSTRACT

Glutamine (GLN) is the most abundant free amino acid in the body. In addition, GLN is considered a conditionally essential amino acid under stress conditions, acting as an important modulator of macrophage functions and being able to act in the modulation of the immune responses, specially influencing cytokine production. Macrophages play a key role in inflammatory and immune responses, producing pro-inflammatory cytokines such as TNFα, IL-1, and IL-12, as well as regulatory cytokines such as IL-10, which can down-regulate the production of pro inflammatory cytokines. Interleukin 10 (IL-10) is a multifunctional cytokine that mediates anti-inflammatory response controlling the degree and duration of inflammation. Considering that GLN is an immunonutrient, this study evaluated different glutamine concentration effects on *Tnfa*, *Il12*, *Cxcl2*, *Ifnb1* and *Il10* mRNA expression by Bone Marrow-Derived Macrophages (BMDM). The compiled results showed that GLN supplementation modulates the expression of these cytokines and the up-regulation of *Il10* and *Ifnb1* mRNA after LPS stimulus is remarkable. GLN inhibits pNFκB/NFκB and increases pSTAT3/STAT3 and IRF3 expression. Furthermore, experiments on BMDM from IL-10 KO mice revealed that the inhibitory effects of GLN on pro-inflammatory cytokine gene expression and NFκB family proteins are IL-10-dependent. We also observed that *Ifnb1* mRNA up-regulation occurs even in the presence of exogenous IL-10. In conclusion, we can infer that supplementation with high concentrations of GLN modify the inflammatory response, inhibiting NFκB activation; there is strong involvement with IL-10 on these effects.

Keywords: glutamine, macrophages, cytokines, interleukin-10, interfron beta, inflammatory response.

1. Introduction

Glutamine (GLN) is an amino acid that is highly utilized by immunologic tissues and cells. Increased GLN consumption, in inflammatory states, leads to an overall negative GLN balance in the blood, muscle and immunologic tissue, leading to a limited function, especially on immune cells [1-4].

Taking into consideration the GLN metabolic requirement in high turnover cells and tissues, the absence or low concentration of GLN can impair relevant biological mechanisms, disrupting some physiological processes. In this context, the administration of exogenous GLN in conditions of high GLN demand, such as those observed during inflammation, should modulate inflammatory/immunologic responses [4-6].

The modulation of the inflammatory environment induced by GLN supplementation could perhaps act in a favorable and protective manner in some conditions where there is a lack of the pro-inflammatory control, such as on inflammatory diseases. Some studies have demonstrated that GLN supplementation down-regulated the intestinal inflammatory response in experimental models and in colonic biopsies of patients with Crohn's Disease by modulating the nuclear factor-kappa B (NFκB) pathway [7,8]. However, GLN supplementation can potentially be harmful in critically ill hospitalized patients [9].

A recent meta-analysis study revealed that GLN supplementation induces the modulation of C-reactive protein, TNF-α, and IL-6 release, reducing the levels of these mediators in patients with abdominal surgery, showing that GLN effectively reduces the

inflammatory response and intestinal mucosal permeability in these patients [6]. GLN decreases the production of pro-inflammatory cytokines IL-1, IL-6 and IL-8 and increases the production of IL-10 in the duodenal mucosa of humans [10,11].

Nonetheless, there are no abundant data available associating IL-10 and GLN with modulation of the immunity system, especially concerning the mechanisms involved in cytokine gene expression and the modulation of transcription factors such as IRF3, STAT-3 and NFκB, which participate in relevant immune response pathways.

Taking into consideration that glutamine can alter molecular pathways, modulating pro-inflammatory cytokines such as TNF-a as well as modulating anti-inflammatory cytokines such as IL-10, this mechanism in turn is able to modulate, exacerbate or attenuate the inflammatory response [11, 12]. It may also provide additional information and explain how this mechanism is controlled. Thus, this work aims to investigate, in bone marrow-derived macrophages, modulation of the gene expression of inflammatory and modulatory cytokines under the influence of different concentrations of GLN as well as the participation of IL-10 in this modulation.

2. Material and Methods

2.1 Animals

The mice *Rag2*$^{-/-}$ *(WT)* 129 SvEv were used as wild type *and* the mice double knockout for *Rag2*$^{-/-}$*Il10*$^{-/-}$ *(KO)* 129 SvEv *background was used as an experimental knockout.* All of the animals were used to produce bone marrow-derived macrophages (BMDM) as previously described [13]. Briefly, the femur and tibia were removed and flushed with PBS. After centrifuging (1500 rpm, 5 minutes) the cells were cultured in

non-treated (15 cm) Petri dishes with 25 mL of DMEM, 10% Fetal Bovine Serum, 10000 Units/ml penicillin, 10,000 µg/ml streptomycin, 5% Horse Serum, 2mM Glutamine and 10% L cell-conditioned medium, at 37°C in 5% CO_2. After 24 hours, non-adherent cells were removed and the medium replaced; after 4 days, the dish was washed once with PBS and the cells were gently scraped from the plate and counted using Trypan Blue. BMDM were platted 1 x10^6 cells per well in 24-well polystyrene culture plates with RPMI medium and glutamine free or RPMI supplemented with 0.6mM or 2mM or 10mm of glutamine.

The reasoning for using 0.6, 2 and 10 mmol/L of GLN are as follows: 0.6 mmol/L of GLN represents the concentration normally found in the plasma of healthy rodents and humans; 2 mmol/L is the standard concentration used in cell culture media and preserves cell functions for a prolonged period of incubation (24–72 h) [14]; and 10 mmol/L was used because previous research showed that this concentration provided effects on cytokine genes expression [12,15].

After 24 hours of incubation with GLN, the cells were stimulated with 1 ng/mL of LPS (Serotype 0127:B8, Sigma Aldrich) for different time points. The BMDM from *$Rag2^{-/-}$(WT) mice* were stimulated with LPS for 30, 60, 120, 180, 240 minutes and *the bone marrow from $Rag2^{-/-}IL10^{-/-}$ (KO)* was stimulated with LPS or LPS plus IL-10 (1 ng/ml) for 30 and 60 minutes. The entire procedure was executed under aseptic conditions, and all of the materials used were previously sterilized and pyrogen-free. All animal procedures were approved by the Harvard Medical Area Standing Committee on Animals and by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences at the University of São Paulo.

2.2 RNA extraction

The BMDM from *Rag2$^{-/-}$ (WT) mice or from Rag2$^{-/-}$IL10$^{-/-}$ (KO)* treated as previously described had their RNA isolated in Trizol (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. RT-PCR analysis was performed using probes from Applied Biosystems (Foster City, CA), as per the manufacturer's instructions.

The probes used in the experiments were purchased from Applied *Biosystems, Foster City, CA, USA and* included Tnfa (Mm00443258_m1), Cxcl2 (Mm00436450_m1), Il1b (Mm00434228_m1), Il12b (Mm01288990_m1), Il10 (Mm00439616_m1), Ifnb1 (Mm00439552_s1) and the endogenous control GAPDH (ref number 4351309). Expression for each sample was analyzed and normalized to GAPDH using the ΔΔCt method [16]. The qPCr were determined in a triplicate of animals in all experiments.

2.3 Western blotting

Western blotting was performed to determine the protein levels of NFκB and phosphorylated NFκB, STAT-3 and phosphorylated STAT-3 and also IRF3 in cells from *Rag2$^{-/-}$ (WT) mice. The* cells were *untreated or treated with 10mM of GLN for* 24hours and stimulated or not with LPS (1ng/mL) for 60 minutes. *This set of experiments was performed because gene expression experiments showed that cells treated with GLN (10mM) showed pronounced differences in comparison to untreated cells (0mM).* Primary antibodies p-NFκB (3031s, Cell Signaling), NFκB (8242s, Cell Signaling), p-

STAT3 (9134s, Cell Signaling), STAT3 (4904s, Cell Signaling) and IRF3 (sc-9082, Santa Cruz) were diluted in TBS-Tween buffer to 1:1,000 and incubated overnight.

In *$Rag2^{-/-}IL10^{-/-}$ (KO) mice,* the cells were *untreated or treated with 10mM of GLN for* 24hours and stimulated with LPS (1ng/mL) or LPS plus IL-10 (1 ng/ml) for 60 minutes. The antibodies were diluted in TBS-Tween buffer (1:1000) and incubated overnight. p-NFκB (3031s, Cell Signaling), NFκB (8242s, Cell Signaling), p-IKBα (2859s, Cell Signaling), IKBα (4812s, Cell Signaling) and c-Rel (sc-70 Santa Cruz Biotechnology) were diluted in TBS-Tween buffer (1:1,000) and incubated overnight.

After treated as described previously, the cells were washed with cold PBS and lysed with RIPA buffer (0.1% SDS, 1% Igepal CA-630, 1% sodium deoxycholate,10 mM Tris–HCl (pH 7.5), 150 mM NaCl, to inhibit the activity of proteases and phosphatases, a protease and phosphatase inhibitor cocktail was added. After centrifugation at 14,000 rpm and 4 ºC for 15 min, the supernatant was collected, mixed with LDS sample buffer (Invitrogen) and heated for 5 minutes 70ºC. Equal amounts of each sample were loaded on a NuPAGE Gel (4-12% Bis-Tris Gel 1.0 mm x 17 wells) and transferred to a PVDF membrane (Invitrolon PVDF Filter Paper Sandwich). The membranes were blocked with 5% non-fat milk in TBST; after 1 hour, the membranes were incubated in the appropriate primary antibody at 4ºC overnight, at room temperature overnight, the membranes were incubated with a secondary antibody (1:10000) conjugated to horseradish peroxidase. After three washes with TBST, the immunoreactivity bands were visualized using the Super signal West Pico (Thermo scientific). The images were acquired using the FluorChem HD2 system (Alpha Inotec). The results were expressed in relation to the intensity of β-actin (1:40,000 for anti-β-actin, Cell Signaling Technology, Inc., Beverly,

MA, USA) or GAPDH (1:40,000, 14C10, #2118L, Cell Signaling Technology, Inc., Beverly, MA, USA) and as a percentage of the control value.

2.4 *Statistical analysis*

All data analysis was performed using GraphPad Prism software (GraphPad Software, inc., San Diego, CA). Two Way ANOVA with Bonferroni post-tests was used to identify significant differences in gene expression. Comparisons between 0 mM groups and GLN 10mM groups were made using the Student's t test. The data are expressed as the mean values with their **standard error of the mean**. * indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$.

3. Results

3.1 Glutamine modulates Tnfa, Il12b, Cxcl2, Ifnb1 and Il10 gene expression in BMDM

To evaluate the effects of different concentrations of GLN in regulatory and pro inflammatory cytokine gene expression in BMDM, cells were cultivated for 24 hours with GLN (0, 0.6, 2 or 10 mM); after that, the cells were unstimulated or stimulated with LPS (1ng/mL) for 30, 60, 120, 180, 240 minutes (**Figure 1**).

The results showed reduced *Tnfa* mRNA expression in cells cultivated in 10mM GLN for 120, 180 and 240 minutes and stimulated with LPS in comparison to cells that did not receive GLN. Comparisons between the cells that received 0.6mM and the cells that received 10mM also showed statistical differences, however only after 180 and 240

minutes of LPS stimulation (**Figure 1A**). In addition, the results of mRNA expression Cxcl2 showed reduced values in cells cultured with 10mM of GLN for 240 minutes compared to cells cultivated without GLN (0mM) (**Figure 1B**).

Il12b mRNA expression did not show any differences when comparing cells cultured with 0mM and 10mM of GLN. In addition, cells cultured with 0.6 and 2 mM of GLN showed increased values, and also showed that GLN supplementation with 0.6 and 2 mM up-regulated *Il12b* mRNA expression when comparing cells that did not receive GLN at the time points of 180 and 240 minutes. However, cells that received 10mM of GLN showed reduced *Il12b* expression in comparison to cells that received 0.6 or 2mM of GLN (**Figure 1C**).

The *Ifnb1* mRNA expression was up-regulated in cells cultivated with 10 mM of GLN, especially after 120 of LPS stimulus. At the time point of 180 minutes, we did not detect any difference in *Ifnb1* mRNA expression for any of the glutamine concentrations evaluated. However, after 240 minutes of LPS stimulation, it was possible to evidence increased *Ifnb1* mRNA expression in cells cultivated with 10mM of GLN (**Figure 1D**).

The most interesting result was related to *Il10* mRNA expression, where the expression was up-regulated in cells cultured with 10mM of GLN and stimulated with LPS for 120, 180 or 240 minutes and compared to the other groups studied (**Figure 1E**).

3.2 Glutamine modulates transcription factors (NFκB, STAT-3 and IRF3) expression on BMDM

To better understand the transcription modulation and provide insights into the induction of cytokines, we performed western blotting to quantify transcription factors

directly involved in pro inflammatory and inhibitory cytokines production. We compared groups cultivated without glutamine (0 mM) and groups cultivated with 10mM of GLN due to the results obtained on the first set of experiments, as shown in **Figure 1**.

Our results revealed that the ratio of p-NFκB/NFκB increased in cells cultured without GLN and stimulated with LPS in comparison to the other groups. Interestingly, cells cultured with 10mM of GLN and stimulated with LPS showed reduced p-NFκB/ NFκB ratio in comparison to cells cultured without GLN (0mM) and stimulated with LPS (**Figure 2A**). In relation to the ratio p-STAT-/STAT-3, the results showed that this ratio is up-regulated in cells cultured with 10mM of GLN and stimulated with LPS in comparison to the other groups (**Figure 2B**). It is well established that IL-10 increases STAT-3 signaling pathway activation; because of that we can infer that there is consistency between our findings regarding *Il10* mRNA expression, showed in the first set of our results, influencing STAT-3 expression.

Furthermore, we proceeded to evaluate IRF3 expression in cells treated with different GLN concentrations, to gain insight into the modulation exerted by this amino acid on *Ifnb1* gene expression. The results showed that IRF3 presented increased expression in cells stimulated with LPS. Moreover, the results showed that cells cultured with 10mM of GLN and stimulated with LPS presented increased expression in comparison to the other groups (**Figure 2C**). This result reveals that GLN can increase the expression of this transcription factor and establish a correlation with the up-regulation of IFN beta gene expression induced by GLN, and shown in the previous results.

3.3 GLN gene expression modulation in the absence of IL-10 (knockout animals)

Considering the established regulatory effects exerted by IL-10 and the up regulation on *Il10* gene expression revealed on our results we proceeded evaluating the role of glutamine influencing the cytokines mRNA expression in the cells from Knockout animals for IL-10.

BMDM from *Rag2$^{-/-}$Il10$^{-/-}$(KO) mice were cultivated* in different concentration of glutamine (0; 0.6; 2 and 10 mM) for 24 hours; after that, cells were unstimulated or stimulated with LPS (1ng/mL) for 30 or 60 minutes and IL-10 (1ng/mL) was or was not added (Figure 3).

We did not observe any statistically significant difference in *Tnf, Il12b* and *Il1b* mRNA expression in any of the experimental conditions evaluated on this essay; this result could be explained by the absence of IL-10 modulating *Tnf*, *Il12b* and *Il1b* gene expression (**Figure 3A, B and C**). However, an up-regulation of *Ifnb1* mRNA expression was observed when cells from KO mice were pre-treated with higher concentrations of glutamine (2mM and 10mM) and compared to cells under the other experimental conditions (**Figure 3D**). These results reveal that the absence of IL-10 did not interfere with the up-regulation on *Ifnb1* mRNA induced by GLN.

3.4 GLN cytokines mRNA expression modulation is IL-10 dependent

Going a step further, we decided to investigate the cytokine mRNA expression in the absence of IL-10, focusing on the supplementation of 10 mM GLN and compared with those cells that were cultivated without GLN (0 mM).

BMDM from knockout mice for IL-10 were pretreated with GLN for 24 hours, after that the cells was stimulated with LPS or LPS plus exogenous IL-10 for 1 hour. Our results revealed that there was decreased mRNA expression for *Tnf, Il12b* and *Il1b* when the cells were cultivated with LPS plus IL-10 compared with the cells that received only LPS (**Figure 4A, B and C**). These results provided evidences that GLN inhibitory effects is IL-10 dependent.

We also observed that *Ifnb1* mRNA is up-regulated when the cells were supplemented with 10 mM of GLN and compared to the cells that did not receive GLN (0 mM), providing some extra information that GLN is able to modulate the expression of IFNβ independent of IL-10 (**Figure 4D**).

Following this, we assessed p-IKBα/IKBα ratio, p-NFκB/ NFκB ratio and c-Rel expressions in cells from *$Rag2^{-/-}IL10^{-/-}$ (KO) mice in the same conditions as the previous experiment. The results revealed that there is slightly decreased expression of* p-IKBα/IKBα ratio in cells cultivated with 10mM of GLN and stimulated with LPS plus IL-10 when compared to cells cultivated without GLN and stimulated with LPS plus IL-10 (**Figure 5A**). Moreover, the p-NFκB/ NFκB ratio and c-Rel showed reduced values in cells cultivated with 10mM of GLN and stimulated with LPS plus IL-10 in comparison to the other groups (**Figure 5B and C**).

The cells which were cultured with 10mM of GLN plus LPS and IL-10 showed an expressive inhibition of p-NFκB/ NFκB and c-Rel expression at these time points studied, when compared to the cells cultured with 10mM of GLN plus LPS, but did not receive IL-10. These results provide strong evidences that supplementation with GLN can directly modulate the IL-10 production influencing relevant inflammatory pathways.

4. Discussion

Glutamine (GLN) plays an essential role, promoting and maintaining the function of various organs and cells, such as lymphocytes, macrophages, and neutrophils [3,11]. Although it is well acknowledged that GLN is largely consumed by immune cells, especially macrophages, the specific means whereby this amino acid regulates immune aspects requires further elucidation. Thus, this study aimed to provide additional insight into how variations in GLN concentrations *in vitro* would affect some of the immune properties of macrophages, especially related to the modulation of IL-10.

In the current work, bone marrow-derived macrophages (BMDM) were isolated and expanded, in accordance with the literature. Once properly isolated, BMDM were cultured in medium without GLN (0mM) or supplemented with this amino acid at three concentrations (0.6mM, 2mM or 10mM) and the influence of this amino acid on the pro and anti-inflammatory cytokine gene expression was evaluated after LPS stimulation.

Stimulation of macrophages with LPS induces the activation of relevant transcription factors, such as NFκB and IRF3, in a protein synthesis–independent manner; this is followed by the induction of mRNA for several primary response genes such as TNFα, CXCL-2, IL-12 as well as IFN-β [17,18]. Previous data in the literature states that GLN can interfere with the NFκB pathway, modulating its effects [12,19]. Notwithstanding, our data reveal that GLN can also modulate IRF3 augmenting this expression.

The high rate of GLN utilization by macrophages and its increase when these cells are stimulated shows the importance of this amino acid to the immunologic system; also, the literature reports that GLN level variations can positively or negatively modulate

the immune/inflammatory response [5]. Our results agree with this statement; the data presented in this study showed that the BMDM cultivated with 10mM of GLN and compared to cells cultivated without GLN, presented down-regulated mRNA expression for *Tnf-α, Il-12, Cxcl-12* and on the other hand increased mRNA expression of *Il-10* and *Ifn-β*. These data, reinforcing the fact that GLN depletion can compromise the inflammatory response. In this context, the administration of exogenous GLN in conditions of a high GLN demand, such as those observed during inflammation, should be able to modulate the immune/inflammatory responses [3].

In agreement with the results observed in the RNA expression of cells cultivated with 10mM of GLN, we observed reduced expression of the p-NFκB/NFκB ratio and increased expression of the p-STAT-3/STAT-3 ratio, as well as increased expression of IRF3. Some studies showed that higher concentration of GLN (8-10mM) could have effects on the NFκB pathway. Recent data showed that supplementation with alanyl-L-glutamine dipeptide was capable to attenuate total NFκB and phosphorylated IKK-α/β expression after LPS stimulation in an animal sepsis model promoting anti-inflammatory effects [12].

Moreover, the inhibition of oxidative stress and reduced expression of pro-inflammatory cytokines is highlighted as GLN effects in the model, highlighting the inhibitory effects of GLN on the NFκB pathway and activating the STAT signaling pathways [19].

The increased ratio of p-STAT-3/STAT-3 observed in the current work shows an interesting result, considering the increased *Il-10* mRNA expression. IL-10

predominantly activates STAT-3; once activated, STAT-3 travels to the nucleus where it stimulates the transcription of specific genes, which in-turn are thought to abrogate the inflammatory response by transcriptionally repressing pro-inflammatory cytokine genes [20,21]. In this way, activated STAT-3 is primarily involved in the negative regulation of macrophage activation, and IL-10-induced STAT-3 activation also results in the decreased expression of the inflammatory cytokines, such as TNF-α [22,23].

Among the many biological molecules that control the inflammatory response, the cytokine IL-10 is one of the most potent and significant, as numerous studies have shown that IL-10 treatment can decrease the severity of inflammatory processes *in vivo*. Furthermore, IL-$10^{-/-}$ mice develop an inflammatory Crohn's-like disease and exhibit dysregulated inflammatory responses. This cytokine exerts key functions in maintaining homeostasis of the immune system and to protect the host from excessive inflammation [24].

Likewise, the up-regulated *Il10* mRNA expression revealed in our results could explain the down-regulation of pro-inflammatory mRNA expressions observed in the cells that receive 10mM of GLN treatment. The IL-10 up-regulation in immune cells function exerts anti-inflammatory roles, reducing pro-inflammatory cytokine production [25], proving that GLN can interfere on the imbalance between pro-inflammatory versus regulatory cytokines gene expression [7,26,27]. In human intestinal mucosa, increased IL-10 expression is able to reduce pro-inflammatory cytokines [25], and in mice it protects against endotoxic shock [10,28], as well as attenuating LPS-induced intestinal inflammation in rats [29].

Advancing, we decided to investigate the modulatory effects of GLN on IRF3, a transcription factor activated by LPS and involved of IFN-β expression [30]. Our result showed that IRF3 protein expression was increased in cells that received 10mM of GLN. Surprisingly, this result shows that the mechanisms by which GLN increases IFN-β gene expression is by augmenting IRF3 protein expression, revealing a relevant pathway modulated by GLN. This is the first time that this correlation has been reported.

IFN-β can exert immunomodulatory effects on both innate and adaptive immune cells, in large part by inducing expression of IL-10 [17,30,31]. The mechanisms by IFN-β induces IL-10 is not totally clear, some authors claim that this induction is signal transducer and activator of transcription 1 (STAT1) dependent [17]. However, there is no data in the literature correlating the relationship between IFN-β and IL-10 with GLN supplementation.

Furthermore, our results lead us to question whether there was a direct correlation between up-regulated *Il 10* mRNA expression and GLN. To address this question, we evaluated GLN supplementation effects on pro-inflammatory mRNA expression in the absence of IL-10 using a *$Rag2^{-/-}Il10^{-/-}$ KO model [18]*. As observed in the current work, the results showed that GLN did not directly affect the expression of pro-inflammatory genes such as TNF-α, IL-1β and IL-12 and the reduction of gene expression is dependent on IL-10, which was proven when IL-10 was added to the cell cultures and the expression of these genes was reduced.

To reinforce these results, the increased expression of the p-NFκB/NFκB ratio and p-IKBα/IKBα ratio was also observed in cells from KO animals even with GLN treatment. However, when IL-10 was added to the cell cultures, a reduction in the p-

NFκB/NFκB ratio and p-IKBα/IKBα ratio was observed. In addition, similar results were observed for c-Rel expression. C-Rel is a transcription factor from the NFκB family and has also been shown to play an important role in the induction of cytokines, including the production of IL-12 in macrophages [32].

The current work showed IRF3 level modulation following LPS stimulation, suggesting that the ability of GLN to enhance IRF3 may be responsible for augmenting IFN-β; this is the first step that would lead to increased IL-10 release which in turn would culminate in the inhibitory effects associated with GLN supplementation. In addition, this current work proves that the anti-inflammatory effects of GLN are dependent on IL-10 in the vast majority while GLN is able to modulate IL-10 gene expression in a time-dependent manner.

Figure 1

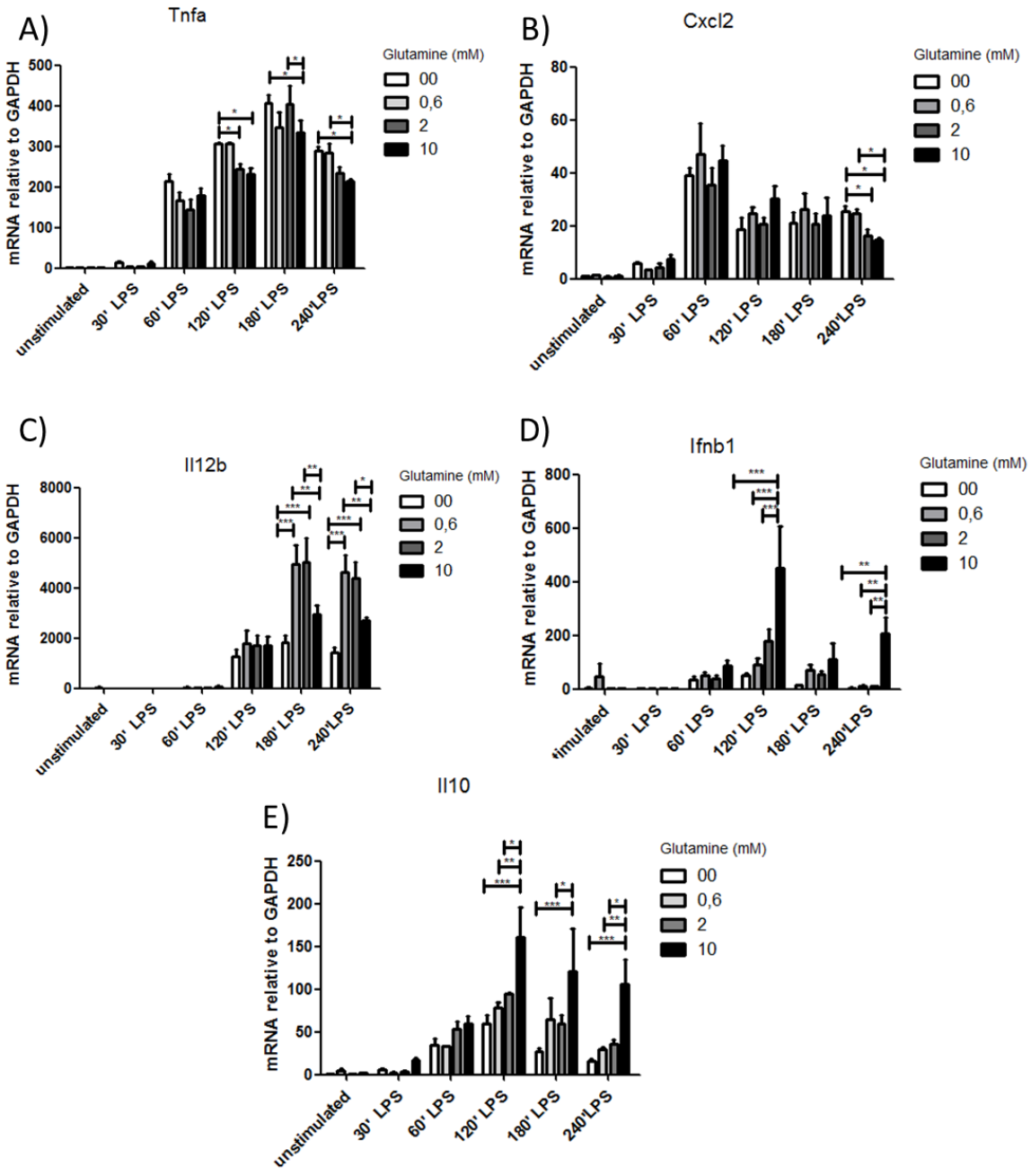

Figure 1: Expression of **A)** *Tnfa,* **B)** *Cxcl2*, **C)** *Il12b*, **D)** *Ifnb1* and **E)** *Il10* mRNA in BMDM supplemented with different glutamine concentrations (0, 0.6mM, 2mM or 10 mM) for 24 hours and unstimulated or stimulated with LPS for 30, 60, 120, 180 or 240 minutes The results are expressed as the mean ± SEM (n=3). The number in brackets denotes the number of animals used in the experiment. *p < 0.05, **p < 0.01, ***p<0,001

Figure 2

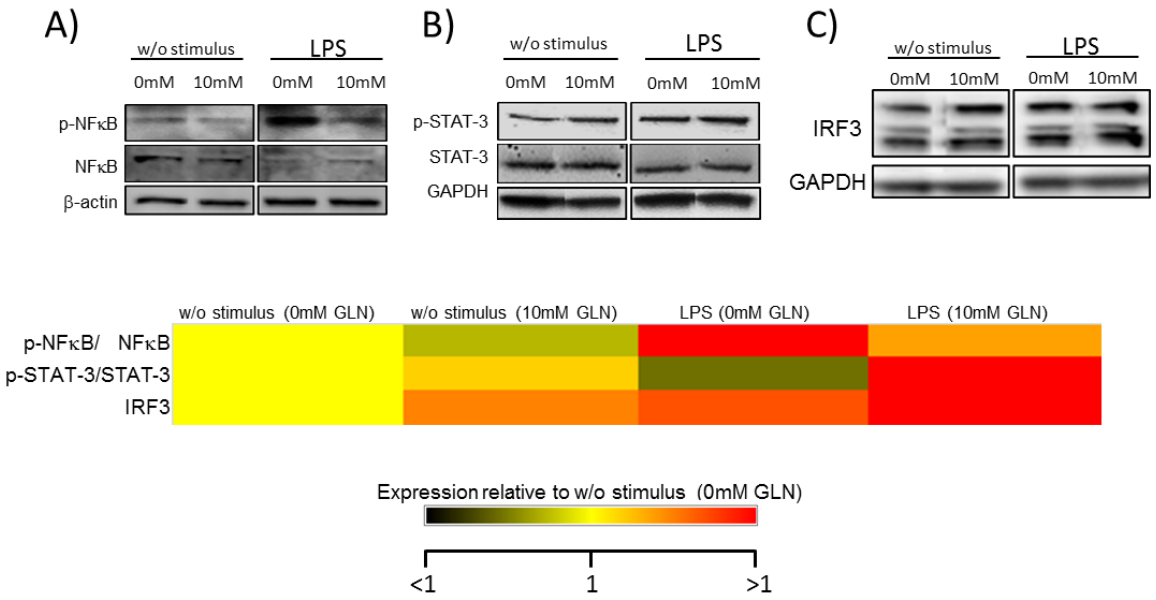

Figure 2: Expression of **A**) the ratio p-NFκB/NFκB, **B**) p-STAT-3/STAT-3 and **C**) IRF3 on BMDM on WT mice. The cells were *supplemented with glutamine (0, 0,6, 2 10 mM) for 24 hours and unstimulated or stimulated with LPS for* 60 minutes (n=3). The number in brackets denotes the number of animals used in the experiment. Heat map of the GLN effects on BMDM stimulated or not with LPS. Results are expressed as normalized values from the group cultured without GLN and not stimulated with LPS. *GAPDH or β-actin were used as loading control in all experiments.*

Figure 3

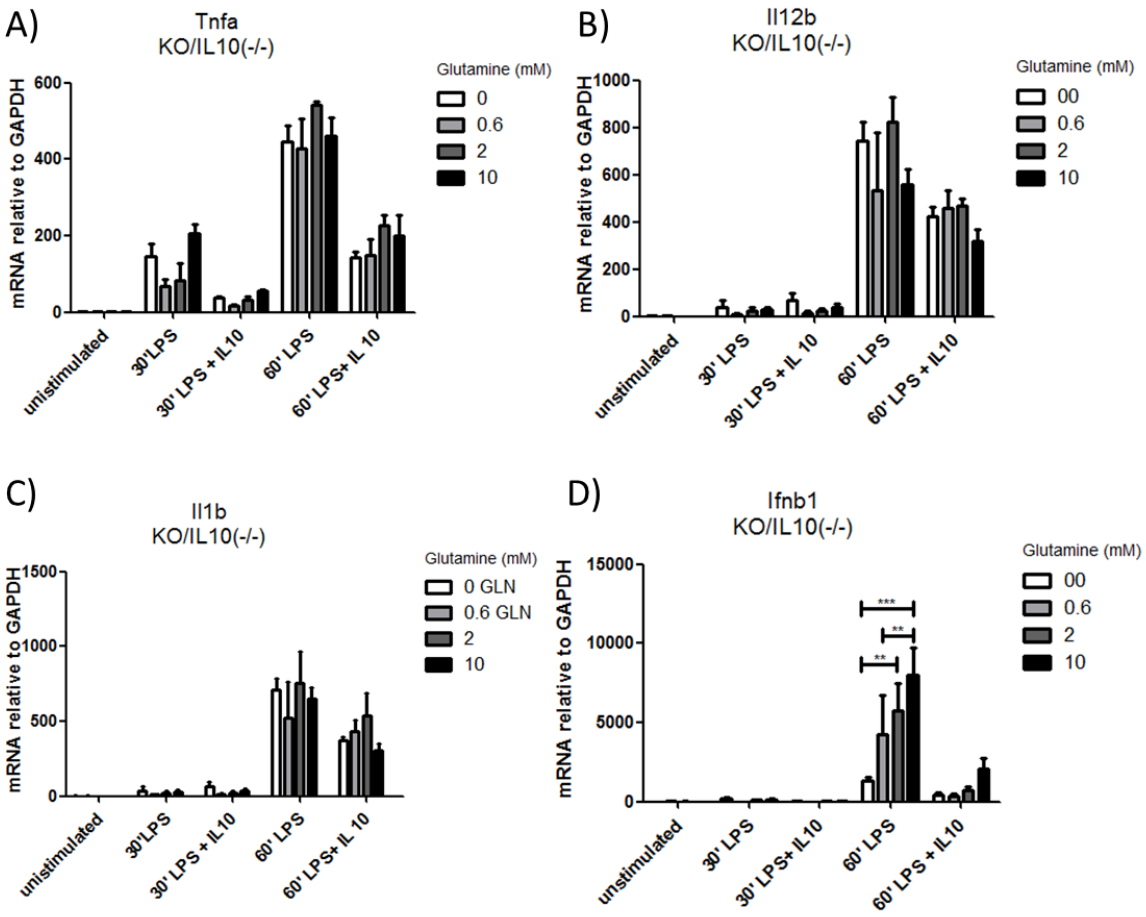

Figure 3: Expression of **A)** *Tnfa*, **B)** *IL12b*, **C)** *Il1b* and **D)** *Ifnb1* mRNA in BMDM from *Rag2*$^{-/-}$*IL10*$^{-/-}$ *(KO) mice cultivated* with different glutamine concentrations (0, 0.6mM, 2mM or 10 mM) for 24 hours and unstimulated or stimulated with LPS for 30 or 60 minutes in the presence or not of IL-10. The results are expressed as the mean ± SEM (n=3). The number in brackets denotes the number of animals used in the experiment. **p < 0.01, ***p<0,001

Figure 4

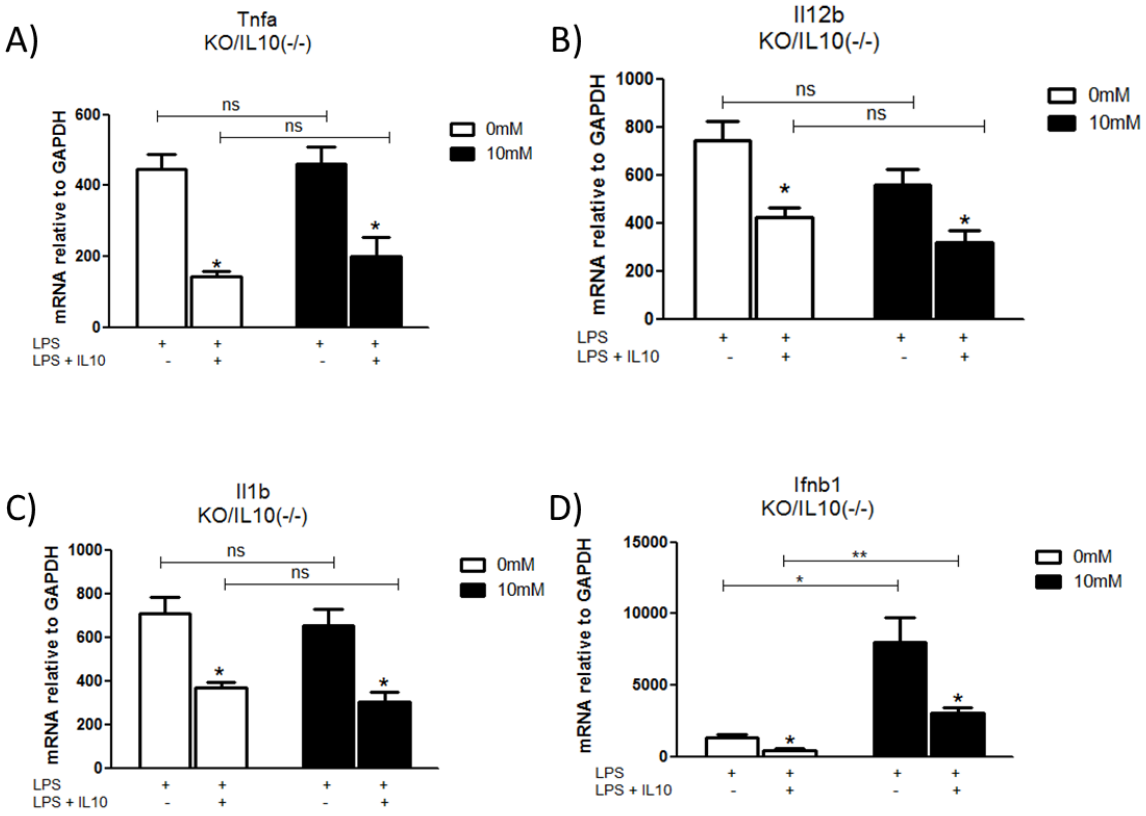

Figure 4: Expression of **A)** Tnfa, **B)** IL12b, **C)** Il1b and **D)** Ifnb1 mRNA in BMDM from *Rag2$^{-/-}$IL10$^{-/-}$ (KO) mice cultivated* without or with GLN 10 mM for 24 hours and stimulated with LPS 60 minutes without or with IL-10. The results are expressed as the mean ± SEM (n=3). The number in brackets denotes the number of animals used in the experiment. *p<0,05 **p < 0.01,.

Figure 5

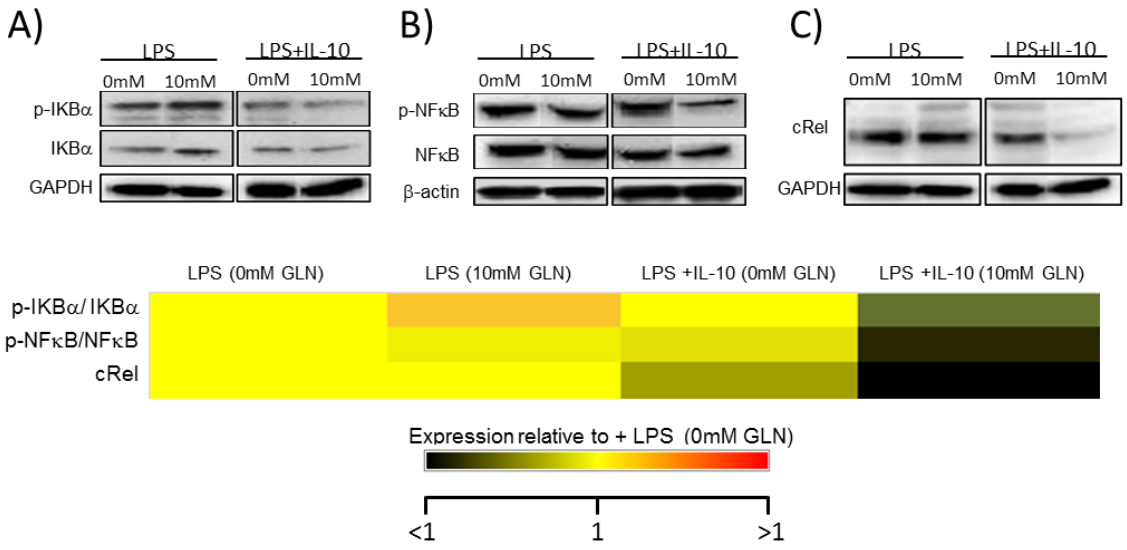

Figure 5: Expression of **A**) p-IKBα/IKBα, **B**) p-NFκB/NFκB and **C**) c-Rel, on BMDM from KO mice. *The cells were* cultured without or with 10mM GLN for 24 hours and stimulated with LPS for 60 minutes with or without IL-10 (n=3). The number in brackets denotes the number of animals used in the experiment. Heat map of the GLN effects on BMDM stimulated with LPS or not. Results are expressed as normalized values from the group cultured without GLN and not stimulated with LPS. *GAPDH or β-actin were used as a loading control in all experiments.*

Figure 6

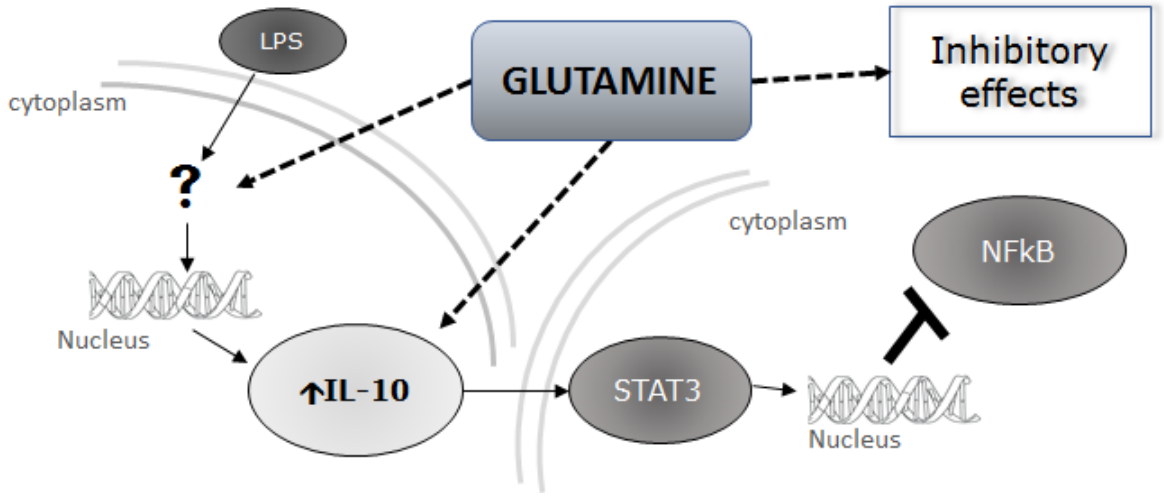

Figure 6: Scheme showing immune response gene, *Il10* and protein STAT3 are modulated by the high glutamine concentration, inhibiting NFκB activation, leading to the inhibitory effects described in this study.

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CAPÍTULO III

5.0 OS EFEITOS DA RESTRIÇÃO ALIMENTAR EM CURTO PERÍODO E A SUPLEMENTAÇÃO COM GLUTAMINA NA MODULAÇÃO DE PROPRIEDADES INFLAMATÓRIAS

Effects of short-term dietary restriction and glutamine supplementation on the modulation of inflammatory properties

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ABSTRACT

Dietary restriction (DR) is a nutritional intervention that exerts profound effects on biochemical and immunological parameters, modulating some inflammatory properties. Gutamine (GLN) is a conditionally essential amino and can also modulate inflammatory properties. However, there is a lack of data evaluating the effects of DR and GLN supplementation, especially in relation to inflammatory cytokine production and the expression of transcription factors such as NFκB. Three-month old male Balb/c mice were subjected to DR by reducing their food intake by 30%. DR animals lost weight and showed reduced levels of triglycerides, glucose, cholesterol and calcium as well as a reduction in bone density. In addition, blood, peritoneal and spleen cellularity were reduced, lowering the total number of peritoneal F4/80 and CD86 positive cells and the total number of splenic CD4 and CD8 positive cells. The production of IL-10 and the expression of NFκB in splenic cells were not affected by DR or by GLN supplementation. However, peritoneal macrophages from DR animals showed reduced IL-12 and TNF-α production and increased IL-10 production with reduced p-NFκB/NFκB expression. In addition, GLN was able to modulate cytokine production by peritoneal cells from the control (CON) group, although no effects were observed in cells from the DR group. These data led us to conclude that DR induces biochemical and immunological changes, in particular by reducing IL-12 and TNF-α production by macrophages and clearly upregulating IL-10 production, whereas GLN supplementation did not modify these parameters in cells from DR animals.

Keywords: Dietary Restriction, Glutamine, Macrophages, Lymphocytes, Cytokines and NFκB.

INTRODUCTION

Dietary restriction (DR) involves reducing the consumption of macronutrients, especially carbohydrates, without leading to malnutrition. DR is correlated with clinical improvements in age-associated diseases and resistance to a variety of acute oxidative stressors and has effects on metabolic fitness such as ameliorating insulin resistance, inducing the upregulation of cryoprotective antioxidants and counterbalancing immune senescence in lymphocytes and macrophages [1,2] as well as modulating inflammatory cytokines [3].

Glutamine (GLN) is known as a conditionally essential amino acid and it has been demonstrated that GLN metabolism also influences cytokine synthesis, the production of which is dependent on the extracellular concentration of GLN [4, 5].

Although plenty of data indicate that DR is beneficial and useful for a variety of pathophysiological situations in several organisms, there are some data that suggest that some aspects of DR are detrimental for the immune/inflammatory system [6]. Moreover, there is a lack of data describing the effects of dietary restriction on basal and physiological conditions [1] as well as the impact of GLN supplementation on cells from DR individuals.

Considering that DR as well as GLN impact a number of functions of the immune system, including the modulation of inflammatory cytokines, in the current work we investigated the effects of 30% DR for 10 days on haematological and biochemical parameters in mice, as well as the impact on the number of peritoneal F4/80 and CD86

positive cells and lymphocyte CD4 and CD8 positive cells. Further, we investigated the effects of *in vitro* GLN supplementation on the production of inflammatory and regulatory cytokines such as TNF-α, IL-12 and IL-10 and on the modulation of the expression of a relevant transcription factor, NFκB.

MATERIALS AND METHODS

Animals

Two-month-old male outbred BALB/c mice were obtained from the animal laboratory at the School of Pharmaceutical Sciences at the University of São Paulo**.** The mice were individually housed and daily food consumption (commercial *Nuvilab CR1***-** Nuvital®) by each animal was monitored for 10 days. After this period of adaptation, the animals were randomly assigned into two groups (control and dietary restriction). The mice assigned to the control group (CON) (n=10) received diet *ad libitum* and the mice assigned to the dietary restriction group (DR) (n=10) received 30% less diet each day than the amount determined during the adaptation period. Mice were subjected to dietary restriction for a period of 10 days.

The CON and DR groups received water *ad libitum.* Body weight and food consumption were monitored every 48 h. During the experimental period the animals were maintained under a regular 12 h light/dark cycle at a temperature of 22–25°C and a relative humidity of 55 ± 10%. The nutritional status was assessed in both groups. A nutritional evaluation was performed by measuring body weight, food consumption, protein, albumin concentrations and haematological parameters. The body weight variation was calculated using the initial (after the adaptation period) and final weight

(day of euthanasia) of the animals in both groups. The Lee index and bone mineral density were also determined. The bone mineral density was assessed using MSFXPro equipment and Bruker Molecular Imaging software (Bruker BioSpin Corporation, Billerica, MA, USA) in association with dual energy X-ray absorptiometry. This study was approved by the Ethics Committee of the School of Pharmaceutical Sciences at the University of São Paulo.

Total blood, serum and plasma

Mice were anaesthetized with xylazine chlorohydrate (Rompum®, 10 mg/kg, Bayer S.A., São Paulo, SP, Brazil) and ketamide chlorohydrate (Ketamina®, 100 mg/kg, Cristália Ltda., Itapira, SP, Brazil), and whole blood samples were obtained. The blood samples were collected in tubes with a final concentration of 1 mg/mL EDTA as an anticoagulant (EDTA, Sigma Chemical Company, St. Louis, MO, USA). The haemogram parameters were determined by automatic methods using an ABC Vet (Horiba *ABX* Diagnostics, *Montpellier*, France). Differential leukocyte counts were performed on blood smears stained with the standard May-Grunwald and Giemsa solutions (Sigma Chemical Co., St. Louis, MO, USA).

Plasma was separated by centrifugation (1,000x*g* for 10 min, 4°C), and the total protein content and albumin concentrations were determined using standard methods [18,19]. In addition, samples collected without EDTA were centrifuged (1,000x*g* for 10 min, 4°C) and the serum separated for the evaluation of biochemical parameters such as cholesterol, calcium, glucose and triglycerides in animals from both groups.

Peritoneal and splenic cells

After being anaesthetized, the animals were exsanguinated, and the peritoneal cavities were washed with 5 mL of sterile RPMI medium without GLN (VitroCell, Campinas, SP, Brazil) supplemented with fetal bovine serum (10% v/v, VitroCell), penicillin, streptomycin (100 units/mL) in an aseptic ambient. The resulting cell suspensions were centrifuged for 10 min at 400x*g* and 4°C and re-suspended in 1 mL of sterile RPMI medium without GLN. Cell viability was determined by trypan blue exclusion. Macrophages were obtained by incubating $1x10^6$ cells per dish in 24-well polystyrene culture plates for 2 h. Non-adherent cells were removed by three vigorous washes with RPMI medium without GLN. The entire procedure was executed under aseptic conditions, and all of the materials used were previously sterilized and pyrogen-free. Total cell counts were determined using a Neubauer chamber, and the different types of cells present were quantified in cytocentrifuge smears stained with the standard May-Grunwald-Giemsa solutions.

Spleen was removed from mice in the CON and DR groups after euthanasia. Cells were obtained by gentle dissociation using needles and tweezers in 5 mL of RPMI medium without GLN (Vitrocell, Campinas, Brazil) medium with EDTA (1 mg/mL). The recovered spleen cells were centrifuged (170x*g* for 10 minutes at 20°C) and resuspended in 1 mL of RPMI medium. The entire procedure was executed in aseptic conditions, and all the material used was previously -sterilized and pyrogen free. Total cells were determined using a Neubauer chamber.

Histological analysis

For histological analysis, the spleen was removed after euthanasia. The spleen from CON and DR animals was weighed, immediately immersed in 4% paraformaldehyde for 1 h, and processed using standard histological techniques (paraffin embedding). Five-micrometre spleen sections were stained with haematoxylin-eosin (H/E) and evaluated by conventional optical microscopy.

Peritoneal and splenic cell cultures

Peritoneal cells were collected as described above. After isolating peritoneal macrophages, $1x10^6$ cells per well were plated in 24-well polystyrene culture plates and cultivated in 1 mL of RPMI medium with GLN at concentrations of 2 or 10 mM for 24 h. Spleen cells were collected as described above. Once isolated $1x10^6$ cells per well were plated in 24-well polystyrene culture plates in 1 mL of RPMI medium without or with GLN at concentrations of 2 or 10 mM for 24 h. The plates were incubated for 24 h at 37^oC, under a 5% CO_2 atmosphere. Cell viability for peritoneal and splenic cells was determined by trypan blue exclusion test (0.1%). Total cell counts were determined using a Neubauer chamber.

The reasoning for using 2 or 10 mmol/L of GLN was as follows: 2 mmol/L is the standard concentration used in cell culture media and preserves cell functions for a prolonged period of incubation (24–72 h); 10 mmol/L was used because previous research showed that this concentration had effects on cytokine gene expression [7, 8, 9].

Flow cytometry

Flow cytometry was used to determine the percentages of peritoneal cells positive for F4/80 and CD86. The splenic population was also used to determine the percentage of positive CD4 and CD8 cells in CON and DR. The antibodies included in this study were anti CD86-PE (Cat#553692, Becton & Dickinson, San Jose, CA, USA), F480-APC (Cat#17-4801-82 eBioscience, San Diego, CA, USA), anti CD4-FITC (Cat#553047, Becton & Dickinson, San Jose, CA, USA) and anti CD8a-PE (Cat#553033 Becton & Dickinson, San Jose, CA, USA).

For the immunophenotyping experiments, 10^5 cells/mL of peritoneal or spleen cell suspension were used. The cells were incubated at 37^oC for 50 minutes in the presence or absence of 1.25 μg/mL of LPS (*Escherichia coli* - B5:055, Sigma, Chemical Company, USA) [9]. Then the tubes were centrifuged at 400*g* for 10 minutes and the supernatant was discarded and the pellet was resuspended and incubated with the respective antibodies for 30 minutes, protected from light. After this period, the samples were washed twice with PBS (400*g* for 3 min). The sediment was resuspended in 300 mL of PBS, and the cells were analysed in a flow cytometer. Flow cytometry was performed using a FACSCanto II (Becton & Dickinson, San Jose, CA, USA). The data were analysed using the software package FlowJo 7.6 (Tree Star, Ashland, OR, USA). The results were expressed as a percentage of a population. FMO was performed to set up the ideal thresholds of each fluorochrome [10].

ELISA and Western blot analysis

After 24 h of incubation at 37°C in a humidified atmosphere of 5% CO_2 with or without GLN the peritoneal or splenic cells were stimulated with 1.25 μg/mL of LPS (Serotype 026:B6, Sigma Aldrich) for 30 minutes. Following stimulation the supernatant was collected and used to quantify the levels of cytokines IL-12, TNF-α and IL-10 by ELISA. The tests were performed using the commercially available reagent Quantikine M Mouse (R&D Systems, Inc., Minneapolis, MN, USA), following the procedures recommended by the manufacturer. In addition, the protein levels of phosphorylated NFκB and total NFκB in stimulated peritoneal and splenic cells were measured using the Western Blot technique. The cells were washed three times with PBS and lysed with RIPA buffer (0.1% SDS, 1% Igepal CA-630, 1% sodium deoxycholate, 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 μg/mL aprotinin, 1 μg/mL leupeptin, 100 μg/mL PMSF, and 0.5 mM EDTA). To inhibit the activity of proteases and phosphatases, a protease and phosphatase inhibitor cocktail was added (Sigma Chemical Co., St. Louis, MO, USA). After centrifugation at 14,000 rpm and 4ºC for 15 min, the supernatant was collected, mixed with 4x Laemmli buffer (1 M Tris HCl (pH 6.8), 10% 2-mercaptoethanol, 10% SDS, 50% glycerol, and 0.01% bromophenol blue) and boiled for 5 min. The protein content of the cell homogenates was determined using a BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA), and equal amounts of protein (10 μg per well) were separated on 10% SDS–polyacrylamide mini-gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA). After incubation with the appropriate primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), including antibodies against NFκB (5:1,000, SC-372) and p-

NFκB (10:1,000, SC-33039), at room temperature overnight, the membranes were incubated with a secondary antibody (1:1,000) conjugated to horseradish peroxidase (Cat. 7074S; Cell Signaling, Inc., Danvers, MA, USA) for 1 h. After three washes with TBST, the immunoreactive bands were visualized using the ECL detection system Amersham ECL™ Advance Western Blotting Detection Kit (Piscataway, NJ, USA). To standardize and quantify the immunoblots, a digital detection system (ImageQuant™ 400 version 1.0.0, Amersham Biosciences, Pittsburgh, PA, USA) was used. The results were expressed in relation to the intensity of b-actin (1:40,000 for anti-β-actin; Cell Signaling Technology, Inc., Beverly, MA, USA) and as a percentage of the control value.

Statistical analysis

The dependent variables were normally distributed. For simple comparisons of two groups (CON and DR) the results were subjected to Student's *t*-test. For multiple comparisons, the results were subjected to two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. All statistical analyses were performed using Graph Pad Prism® software (Graph Pad Prism version 5.03 for Windows; Graph Pad Software, San Diego, CA, USA), and the data are expressed as the mean values with their standard deviations. Statistically significant differences were considered at $p \leq 0.05$.

RESULTS

Body weight, Lee index, biochemical parameters and bone density

Animals fed with 30% less diet lost 14% of their initial weight (**Table 1**). The body mass index (BMI), measured by the Lee index (weight ($g^{0.33}$)/naso-anal measured (mm)), also showed a significant reduction in animals from DR groups in comparison to the CON group (**Table 1**). Moreover, as shown in Table 1, animals from the DR group showed reduced serum levels of cholesterol, glucose, triglycerides, and calcium. However, albumin and protein plasma levels did not differ between groups.

Bone density was assessed to estimate the effect of calcium reduction, and a notable reduction in bone density was observed in animals from the DR group (**Figure 1**). This result proves that a reduction in the amount of food, even for a short period, can cause global alterations in diverse biological parameters in the mouse.

Blood, spleen and peritoneal cellularity

The animals in the DR group presented a reduced red blood cell count, haemoglobin and haematocrit in comparison to animals from the CON group. Animals from the DR group also presented reduced numbers of leucocytes as well as granulocytes. The number of lymphocytes and monocytes did not differ between the DR and the CON groups **(Table 2)**.

Despite the fact that the blood cell counts did not show significant differences in blood lymphocyte number (although it had decreased), a reduction in splenic cellularity in animals from the DR group was observed (**Figure 2A**). However, the histological

analyses in DR animals showed cellular depletion with **rarefaction of the** red pulp cords as well as lower cellularity in the marginal zone with a predominance of immature **lymphoid cells in** the white pulp, suggesting a maturative commitment (**Figure 2C**). The marginal zone, which marks the transition between the white and red pulps, is dedicated to the development of the humoral immune response to circulating infectious agents, and particularly the primary immune response to poorly imunogenic antigens present in the bloodstream.

The reduction in splenic cellularity was also confirmed by the change in spleen weight (**Figure 2B**). The results, as shown in figure 2D, demonstrated a reduced total number of cells positive for CD4 and CD8, however no differences in the percentage of CD4 and CD8 positive cells were observed between the DR and CON groups (**Figure 2E**). The peritoneal cavity of animals from the DR group also showed reduced cellularity (**Figure 3A**) with fewer mononuclear cells (**Figure 3B**) as well as granulocytes (**Figure 3C**) than the CON group. Due to the reduced peritoneal cellularity observed in animals from the DR group, the expression of F4/80 and CD86 markers was evaluated, as CD86 is a relevant co-stimulatory molecule in the antigen-presenting process. The results showed a reduced number of CD86 positive cells in the DR group (**Figure 3E**), however the percentage of CD86 positive cells did not differ significantly between the DR and CON groups (**Figure 3F**).

Dietary restriction and glutamine did not affect CD4/CD8 expression, IL-10 production or NFκB expression after LPS stimulus

To test whether DR induces any modification in the expression of CD8 and CD4 in splenic cells after LPS stimulus flow cytometry was performed. The results demonstrated a reduced total number of $CD4^+$ and $CD8^+$ cells (**Figure 4 A**), but no differences in the percentage expression of CD4 and CD8 were observed between the DR and CON groups (**Figure 4B**). In addition, DR had no effect on IL-10 production or NFκB expression in splenic cells stimulated with LPS. Likewise 10 mM GLN supplementation did not affect IL-10 production or NFκB expression in either group (**Figure 4C and D**).

Dietary restriction and glutamine supplementation influence inflammatory and anti-inflammatory cytokine production and NFκB expression in peritoneal cells after LPS stimulus

CD86 expression in peritoneal cells after LPS stimulation was evaluated, but no differences in the expression of this receptor in $F4/80^+$ cells were observed in either the DR or CON groups (**Figure 5B**). However, when cultured with 1.25 μg of LPS for 24 hours, peritoneal macrophages from DR animals showed altered production of TNF-α and IL-12. As can be seen in **Figure 5C,** the concentration of TNF-α in the supernatant of peritoneal cell cultures from DR mice was significantly lower ($p \leq 0.05$) than that from the CON mice.

IL-12 production was also reduced in the supernatants of peritoneal cells from the DR group in comparison to the CON group (**Figure 5D**). On the other hand, IL-10

production was increased in the supernatants of peritoneal cells from the DR group (**Figure 5E**). Furthermore, the ratio p-NFκB/NFκB was lower in the DR group (**Figure 5F**).

Next, we investigated whether 10 mM of GLN supplementation was able to modulate the production of inflammatory and anti-inflammatory cytokines. The results showed that 10 mM of GLN did not affect TNF-α production (**Figure 5C**), but did reduce IL-12 production (**Figure 5D**), increase IL-10 production (**Figure 5E**) and reduce the p-NFκB/NFκB ratio (**Figure 5F**) in peritoneal cells from the CON group. However, there were no effects of 10 mM of GLN supplementation on TNF-α (**Figure 5C**), IL-12 (**Figure 5D**) or IL-10 (**Figure 5E**) production or on the p-NFκB/NFκB ratio (**Figure 5F**) in peritoneal cells from the DR group.

DISCUSSION

Although it is well known that DR is a highly effective metabolic modulator, the specific means by which this nutritional intervention regulates immune aspects requires further clarification [1,11].

Thus, this study intended to provide additional information on how DR modulates some aspects of the immune/inflammatory response and also to provide insights into whether GLN supplementation in combination with DR would affect some properties of the immune/inflammatory response. In the current work, a DR mouse model was established by reducing their food intake by 30% for 10 days. These animals were used to evaluate various biochemical parameters and were a source of

macrophages and lymphocytes that were used to evaluate some immune/inflammatory properties.

The correlation between restriction of food consumption and alterations in biochemical parameters is well established [1, 12-15]. In the current study, DR mice showed reductions in weight and reduced glucose, cholesterol and triglyceride levels. The reduction of these biochemical parameters is often reported to be associated with beneficial outcomes in the case of obesity and metabolic syndrome, however, there are few data in the literature showing that the reduction of these metabolites is beneficial in basal health conditions [6].

DR mice also showed reduced calcium serum levels in association with reduced bone density, questioning whether DR is beneficial in healthy individuals, at least in terms of this parameter. Previous data in the literature indicates that dietary restriction can induce haematological alterations [16-20]. Our data reveal that only 10 days of DR induced a reduction in the number of all blood cells counted without alteration of cell morphology.

The literature reports altered leucocyte counts in DR individuals [1, 2, 21, 22]; it has also been reported that DR in obese individuals is able to reduce the number of macrophages in adipose tissue [6, 23]. Our data shows a striking reduction in the number of cells in the peritoneal cavity, especially of mononuclear cells, consequently affecting the total number of $CD86^+$ but not its overall percentage. Given that CD86 is an important protein and due to the relevance of this co-stimulatory molecule in the antigen-presenting process [24], a reduction in the number of $CD86^+$ cells could be one of the elements leading to changes in the immune response.

Spleen also showed reduced weight and cellularity without differences in the percentage of CD4 and CD8 positive cells. However, a reduction in the total number of CD4 and CD8 cells was observed. This result is important when attempting to understand how DR can affect the immune response, as a reduction in number of CD86 potently inhibited the $CD4^+$ T cell response and reduced numbers of CD8 affected binding to a major histocompatibility complex (MHC) [24, 25].

Given that a reduction in the number of cells can have a negative impact on the immune system [1, 2, 21] these results lead us to question whether, beyond the quantity, the quality of the immune response produced by those cells could also be affected. Nonetheless, there are few data explaining the specific mechanisms by which DR influences macrophage and lymphocyte production and function.

It has been established that 40% DR over a short period leads to the modulation of relevant pro-inflammatory cytokines [3]. In the current work, we isolated macrophages from the peritoneal cavity and lymphocytes from spleen and stimulated these cells with LPS. Lipopolysaccharide (LPS), a major constituent of the outer membrane of Gram negative bacteria, strongly stimulates host immune responses and the secretion of several pro- and anti-inflammatory cytokines [26].

In splenic lymphocytes, we did not observe any effect of DR on IL-10 production or NFκB expression. However peritoneal macrophages from DR mice showed reduced TNF-α and IL-12 production and increased IL-10 production in association with reduced NFκB expression after the LPS stimulus.

TNF-α and IL-12 are produced by cells from the innate immune system, such as macrophages. TNF-α is a pro-inflammatory cytokine that acts in the acute phase reaction, its primary role being the regulation of NFκB while IL-12 is an important link between innate and adaptive immunity, driving the immune system towards a T helper (Th)1 response, and TNF-α is an important positive regulator of IL-12 [2].

On the other hand, IL-10 is an anti-inflammatory cytokine involved in regulating the intensity and duration of inflammation, exerting this control at a molecular level by inhibiting the activation of transcription factors such as NFκB, and exacerbating IL-10 production, which is also associated with immunosuppression [27-30].

Our results were consistent with the literature, where it is stated that DR can downregulate inflammatory mediators such as inflammatory cytokines [27, 28, 31]. To go further, we decided to evaluate the effect of 10 mM GLN supplementation in DR cells, evaluating cytokine production and NFκB expression induced by LPS. This concentration of GLN was chosen because previous data from our group and the literature suggest that supplementation with a high GLN concentration exerts modulatory effects on cells of the innate immune system, including the production of cytokines [9, 20, 32].

Our results showed that GLN did not affect any parameter evaluated in splenic cells from either group. However, in peritoneal macrophages from CON animals, 10 mM GLN reduced IL-12 production and interestingly increased IL-10 production as well as reducing the p-NFκB/NFκB ratio. However, GLN did not change the production of TNF-α, IL-12 or IL-10 or the expression of NFκB in cells from DR animals. If GLN affected these parameters in cells from DR animals they would be driven to an extremely

immunosuppressive condition, as cells from the DR group already have reduced TNF-α and IL-12 production and increased IL-10 in comparison to cells from the CON group.

This is the first time that the association between nutrient supplementation and DR has been evaluated, highlighting that the combination of such could be harmful. The current work clearly demonstrates that DR and GLN supplementation affect some immune/inflammatory properties, but that the combination of DR and GLN supplementation did not produce synergistic effects.

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Table 1.

Variables	C (n=10)	DR (n=10)
Body weight (g)	29.39± 2.1	21.97± 1.8***
Body weight variation (%)	3.07 ± 2.5	-14.16 ± 7.2 ***
Diet consumption (g/day/animal)	4.68±0.6	3.49±0.3 ***
Lee index	58.38 ± 1.4	54.32 ± 1.2 ***
Protein (g/dL)	4.65 ± 0,3	4.45 ± 0.6
Albumin (g/dL)	1.77 ± 0.1	1.79 ± 0.2
Cholesterol (g/dL)	139.9 ± 20.1	108.6 ± 29.3 **
Glucose (mg/dL)	202.4 ± 64.8	106.9 ± 53.0 *
Triglycerides (g/dL)	120.1± 47.2	37.33 ± 21.2 ***
Calcium (mg/dL)	8.19 ± 0.5	7.16± 0.7 **

Table 1. The results are expressed as the mean and standard deviation of body weight and body weight variation; food consumption; Lee index; the concentrations of plasma protein and albumin; serum cholesterol, glucose, triglycerides, and calcium in control (CON) and dietary restriction (DR) groups. The numbers in parentheses denote the numbers of animals used in the experiments. *($p \leq 0.05$), **($p \leq 0.01$), and ***($p \leq 0.001$) where there was a significant difference between the CON and DR groups.

Table 2.

Variables	C (n= 10)	DR (n=10)
Red Blood Cells (x10 ^{6}/mm^{3})	8.31 ± 0.5	7.42 ± 0.9*
Hemoglobin (g/dL)	12.82± 0.8	11.42 ± 1.3*
Hematocrit (%)	41.1 ± 3,1	36.9 ± 4,8*
Leucocytes (mm^{3})	2,020±0.82	1,270±0.73*
Lymphocytes (mm^{3})	1,770 ± 0.73	1,157 ± 0.67
Granulocytes (mm^{3})	0,216 ± 0.09	0,100 ± 0.07**
Monocytes (mm^{3})	0,036± 0.02	0,024 ± 0.02

Table 2. The results are expressed as the mean ± standard deviation of red blood cell count, haemoglobin, haematocrit, leucocytes, lymphocytes, granulocytes and monocytes in control (CON) and dietary restriction (DR) groups. The numbers in parentheses denote the numbers of animals used in the experiments. *($p \leq 0.05$) and **($p \leq 0.01$) where there was a significant difference between the CON and DR groups.

Figure 1.

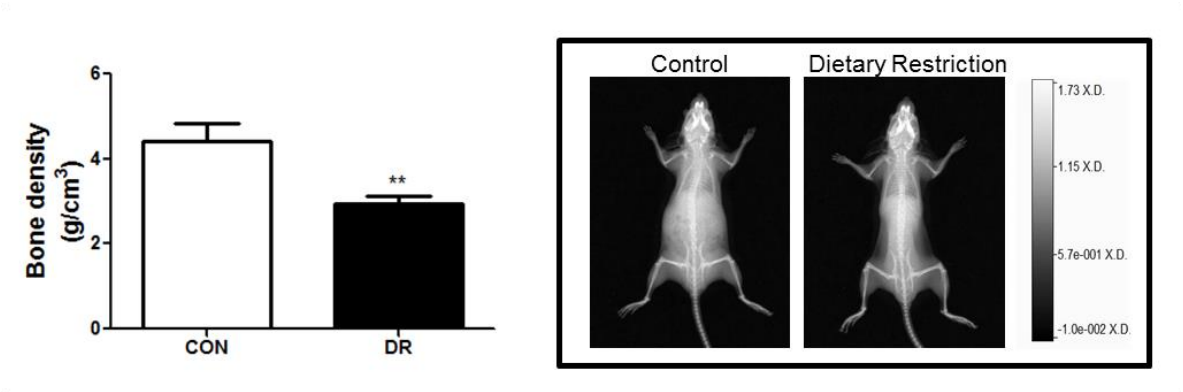

Figure 1. The results are expressed as the mean and standard deviation of bone density, animals from control (CON) (n=3) and dietary restriction (DR) (n=3) groups. *($p \leq 0.05$) where there was a significant difference between the CON and DR groups.

Figure 2.

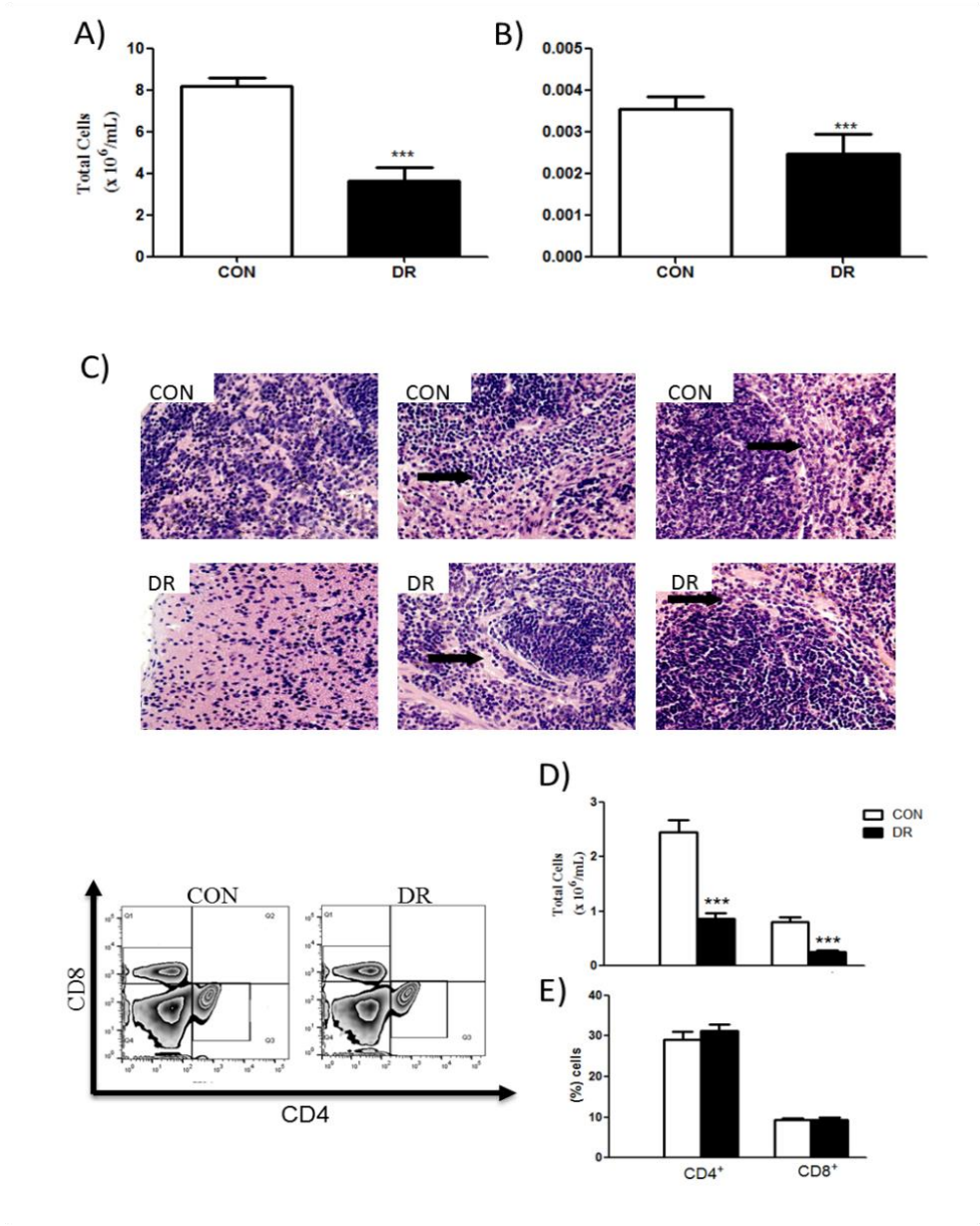

Figure 2. (A) total number of splenic cells, (B) spleen weight/body weight, (C) spleen biopsy section (HE stain, x 40) from a CON and DR animal showing cellular depletion in the DR group; arrows show reduced cellularity in marginal zone with predominance of immature **lymphoid cells in** white pulp in DR group; (D) total number of splenic cells positive for CD4 and positive for CD8, (E) percentage of splenic cells positive for CD4 and positive for CD8 from control (CON) (n=6) and dietary restriction (DR) (n=6) groups. The results are expressed as the mean ± standard deviation. ***($p \leq 0.001$) where there was a significant difference between the CON and DR groups. Histological sections of the spleens from control and dietary restriction animals were prepared in haematoxylin-eosin stain (magnification X 40).

Figure 3.

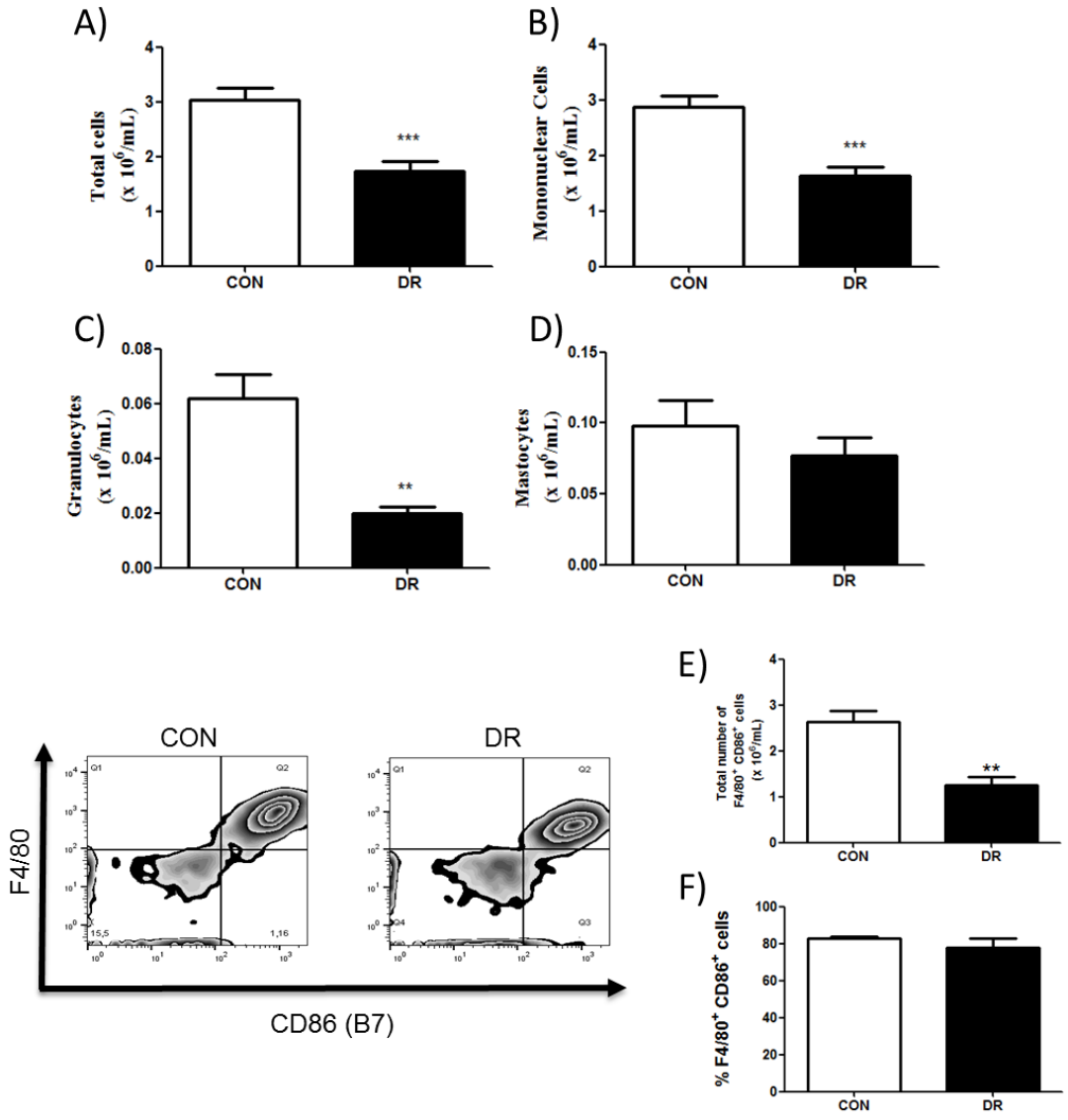

Figure 3. (A) total number of peritoneal cells, (B) peritoneal mononuclear cells, (C) peritoneal granulocytes, (D) peritoneal mastocytes, (E) total number of peritoneal cells positive for F4/80 and CD86, (F) percentage of peritoneal cells positive for F4/80 and CD86 from control (CON) (n=6) and dietary restriction (DR) (n=6) groups. The results are expressed as the mean ± standard deviation. **($p \leq 0.01$) and ***($p \leq 0.001$) where there was a significant difference between the CON and DR groups.

Figure 4.

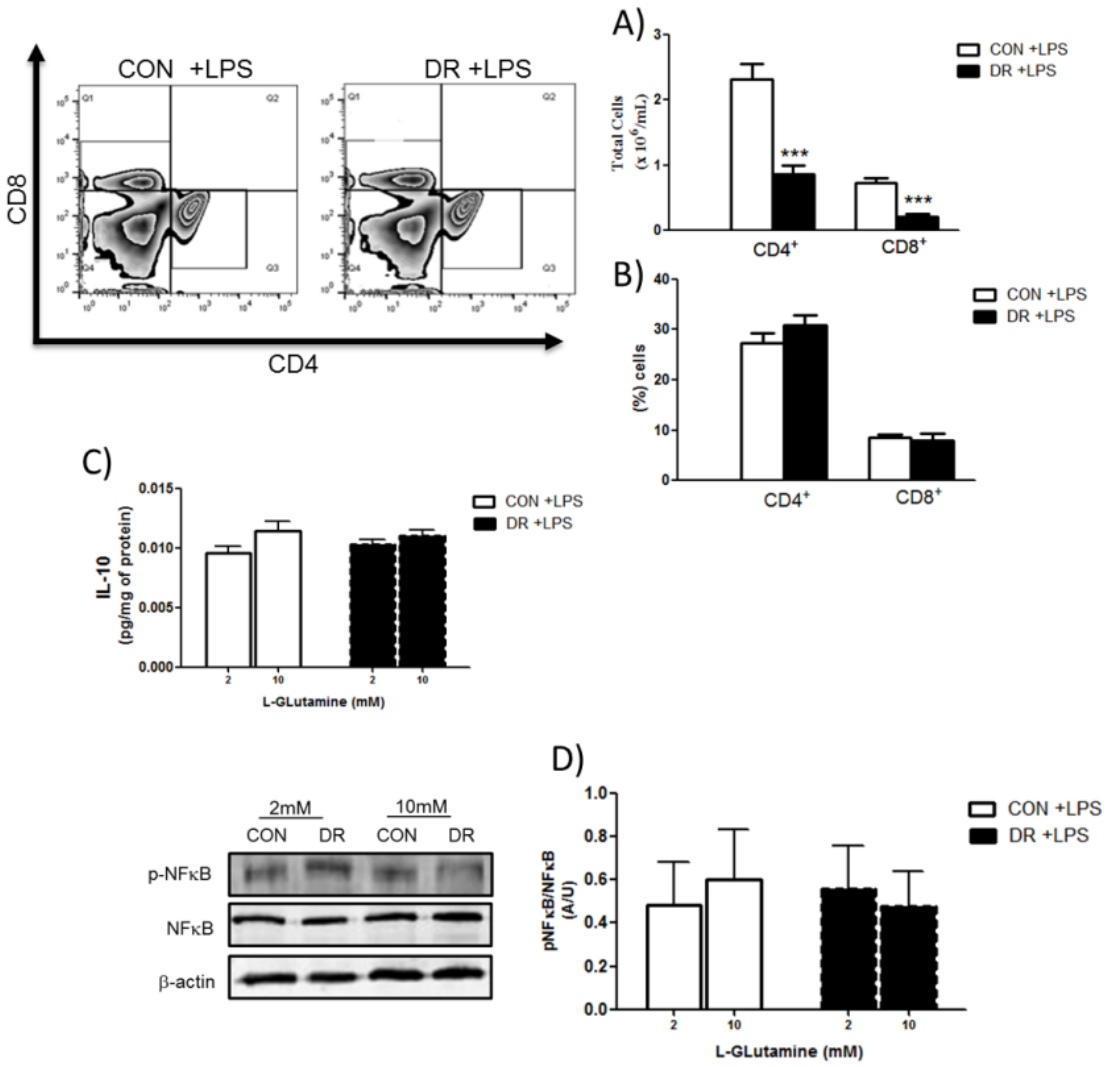

Figure 4. (A) total number of splenic cells positive for CD4 and positive for CD8, (B) percentage of splenic cells positive for CD4 and positive for CD8, (C) IL-10 level in the supernatant of splenic cell culture, (D) the ratio between phosphorylated NFκB and total NFκB from splenic cells determined by western blot analysis from control (CON) (n=6) and dietary restriction (DR) (n=6) groups. Cells were cultivated with 2 mM or 10 mM of GLN and stimulated with LPS. Flow cytometry analyses of splenic cells obtained from CON and DR groups and stimulated with LPS. Representative plots from cytometry data acquisition. Western blot analysis of p-NFκB and NFκB presented in relation to the intensity of β-actin. The results are expressed as the mean ± standard deviation. ***($p \leq 0.001$) where there was a significant difference between the CON and DR groups.

Figure 5.

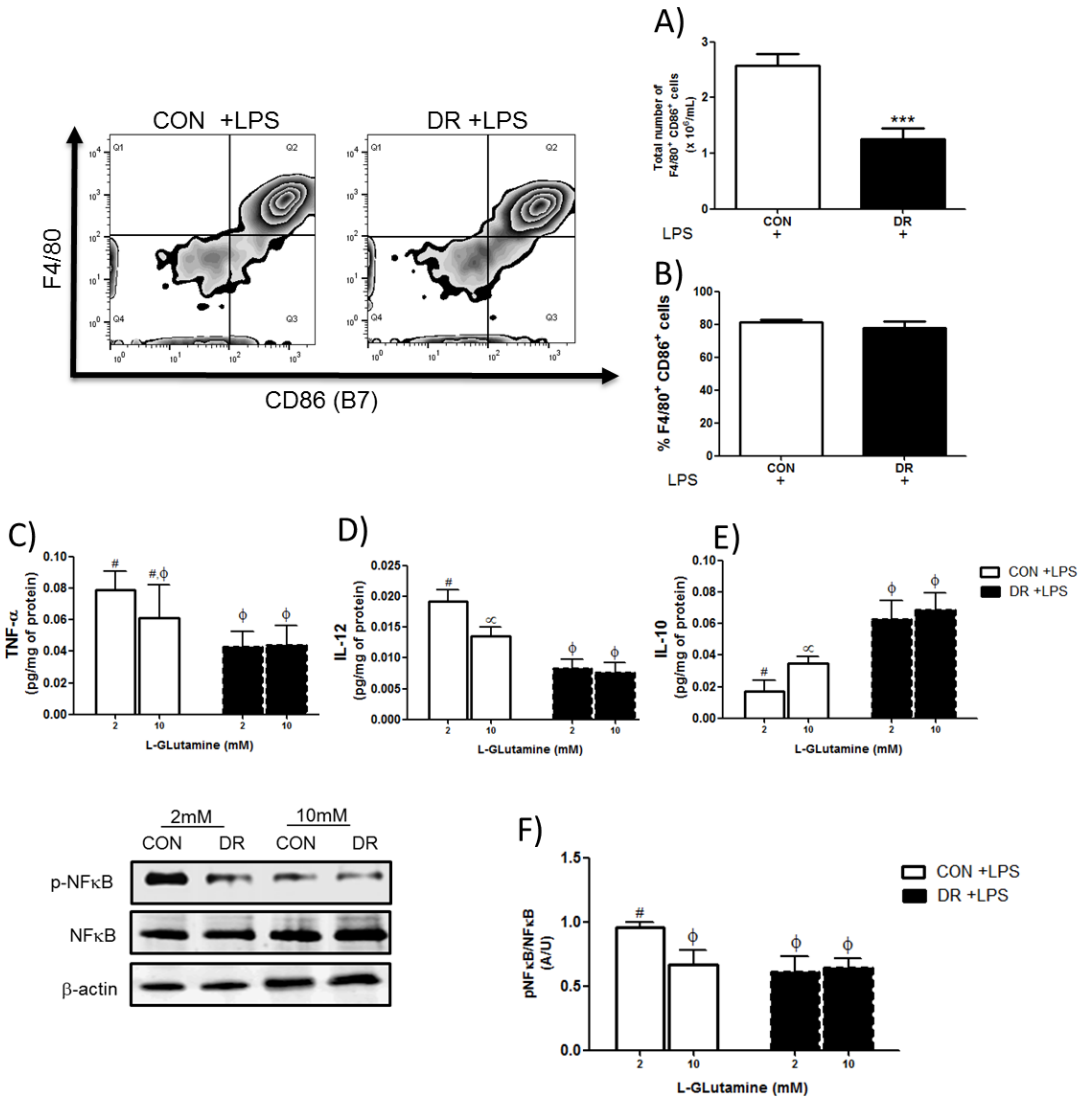

Figure 5. (A) total number of peritoneal cells positive for F4/80 and CD86, (B) percentage of peritoneal cells positive for F4/80 and CD86, (C) TNF-α, (D) IL-12, and (E) IL-10 levels in the supernatant of peritoneal cell culture, (F) the ratio between phosphorylated NFκB and total NFκB from peritoneal cells determined by western blot analysis from control (CON) (n=6) and dietary restriction (DR) (n=6) groups. Cells were cultivated with 2 mM or 10 mM of GLN and stimulated with LPS. Flow cytometry analyses of peritoneal cells obtained from CON and DR groups and stimulated with LPS. Representative plots from cytometry data acquisition. Western blot analysis of p-NFκB and NFκB presented in relation to the intensity of β-actin. The results are expressed as the mean ± standard deviation. ***($p \leq 0.001$) where there was a significant difference between the CON and DR groups. Mean values with different superscript symbols were significantly different between groups ($p \leq 0.05$; ANOVA).

6. DISCUSSÃO FINAL

6.1 Regulação epigenética induzida pela IL-10

A IL-10 é uma proeminente citocina responsável pelo controle da resposta inflamatória; apesar dos esforços da comunidade científica para desvendar os mecanismos subjacentes ao controle da resposta inflamatória exercido pela IL-10, ainda não está bem descrito como essa citocina age molecularmente ao induzir o controle da resposta inflamatória.

Novas tecnologias como a imunoprecipitação de cromatina conjugada ao sequenciamento de alto rendimento e abordagens de bioinformática são recursos valiosos que abrem novas possibilidades para uma melhor compreensão e descrição da fisiologia em nível molecular. Neste trabalho, contamos com a imunoprecipitação de cromatina acoplada ao sequenciamento de alta eficiência (ChIP-seq); essa abordagem nos possibilitou dar passos em direção a uma melhor descrição dos eventos moleculares que geram o controle da resposta inflamatória através da investigação de mecanismos epigenéticos e modificação de nucleossomos induzidos pelo IL-10 (BAILEY *et al.*, 2013).

O ChIP-seq é uma técnica é altamente dispendioso em tempo e requer uma precisão meticulosa nas etapas experimentais. Após a conclusão de todas as etapas dos processos de validação, obtivemos resultados reprodutíveis e bem-sucedidos para a essa técnica que possibilitou agregar conhecimento a respeito dos mecanismos epigenéticos do controle da resposta inflamatória em macrófagos (LANDT *et al.,* 2012; JUNG *et al*., 2014).

A epigenética se refere a modificações induzidas no desenvolvimento ou pelo ambiente que não altera o código genético, mas ao invés disso controlam a forma como a informação codificada no DNA é expressa em determinado tecido inserido em um determinado contexto. Dessa forma, a detecção de modificações de histona, um mecanismo epigenético muito utilizado, fornece informações úteis para uma melhor compreensão da regulação de processos celulares como a regulação da resposta inflamatória (IVASHKIV, 2013).

Recentemente se tornou claro que os marcadores epigenéticos de cromatina são regulados dinamicamente em resposta a estímulos ambientais. Isso resultou em uma mudança da utilização da epigenética para incluir mudanças transitórias na cromatina e/ou no DNA em resposta a estímulos externos que podem exercer o controle da expressão gênica (IVASHKIV, 2013).

Os *enhancers* são regiões regulatórias relevantes caracterizadas por nucleossomos com níveis relativos elevados de histona 3 com um único grupo metil na cauda terminal na lisina 4 (H3K4me1). O papel funcional da H3K4me1 ainda é pobremente definido, mas possivelmente esteja envolvido na interação proteína-proteína que estabiliza a ligação de algumas acetiltransferase de histonas para a cromatina (HEINTZMAN *et al*., 2007; JEONG *et al*., 2011; SMALE, TARAKHOVSKY & NATOLI, 2014).

Existem evidências mostrando que alguns marcadores de nucleossomo são associados a uma cromatina aberta e apresentam forte ligação de PU.1 e especialmente significantes regiões regulatórias como promotores e *enhancers*. Durante a ativação, os macrófagos sofrem remodelamento expressivo da arquitetura dos *enhancers* e promotores; sendo que a transcrição e a rede de regulação epigenética em camundongos é similar às observações obtidas com macrófagos ativados de humanas (SCHMIDT, KREBS & ULAS, 2016; OSTUNI *et al.*, 2013).

O controle da resposta inflamatória vem sendo descrito e desvendado ao mesmo tempo em que novas tecnologias e a bioinformática evoluem; apesar de muito ter sido descrito, ainda há muitas questões a serem respondidas sobre o controle transcricional da resposta inflamatória. A citocina IL-10 desencadeia a expressão de diversos reguladores negativos de genes induzidos pelo LPS. Um importante mecanismo pelo qual a IL-10 inibe a inflamação ocorre em nível transcricional, revelado pela habilidade do tratamento com cicloheximida em bloquear a inibição de genes de resposta primária mediados pelo IL-10 (MEDZHITOV & HORNG, 2009; MURRAY, 2005).

A interleucina 12 (IL-12) é representativa de um grande número de citocinas pró-inflamatórias e outros mediadores da inflamação; o heterodímero bioativo da IL-12 p70 contém as subunidades p35 e p40, produzidas por monócitos/macrófagos, seguidos da

interação com produtos bacterianos. A função central da IL-12 é prover uma ponte crítica entre a imunidade inata e adaptativa (ZHOU *et al.*, 2007).

Dados na literatura mostram um aumento de H3K27ac no *enhancer* do IL-12b após o estimulo com LPS e subsequente recrutamento da enzima Polymerase II e o fator de transcrição NFkB para o *enhancer* e o promotor do gene IL12b (XU *et al*., 2009; GJIDODA *et al.,* 2014; SMALE, TARAKHOVSKY & NATOLI, 2014).

Os resultados obtidos neste trabalho estão de acordo com os dados disponíveis na literatura, confirmando que há uma grande quantidade de acetilação na histona 3 lysina 27 nas regiões do *enhancer* e do promotor do gene IL-12 após o estímulo com LPS, em que se observou acetilação de vastas regiões do gene com picos no *enhancer* do gene do IL-12 (KOBAYASHI *et al*., 2012).

Enfatizando os efeitos da IL-10 na arquitetura dos nucleossomos do gene IL12b, os nossos resultados demonstraram que o tratamento com IL-10 reverte a acetilação induzida pelo LPS no promotor, *enhancer* e sítio de início de transcrição do gene IL12b. Estudos anteriores sugerem que as modificações de nucleossomos observadas no gene do Il12b induzidas pela IL-10 estão associadas ao recrutamento e ação de histonas deacetilases e consequente inibição do recrutamento da enzyma Polymerase II em determinada região do gene (KOBAYASHI *et al*., 2012; ZHOU, NAZARIAN & SMALE, 2004).

6.2 GLUTAMINA

Em virtude dos efeitos exercidos pela glutamina na modulação da produção de citocinas decidimos investigar os possíveis mecanismos subjacentes a essa modulação exercida por esse amino ácido. Um dado intrigante é o fato da suplementação com altas concentrações de glutamina elevar a produção da citocina IL-10 tanto em macrófagos quanto em linfócitos (DA SILVA LIMA *et al.*, 2013; DILLOW *et al.,* 2014; JAFFER, WADE & GOURLAY, 2010; ZHOU, NAZARIAN & SMALE, 2004).

A suplementação com glutamina vem sendo demonstrada como um potencial modulador da produção de citocinas inflamatórias, como o IL-6 e o IL-8, além de aumentar a produção de IL-10 em condições inflamatórias e não inflamatórias,

salientado que resultados semelhantes foram observados em camundongos e humanos (COËFFIER *et al*., 2001; COËFFIER *et al.*, 2003; LECHOWSKI *et al.,* 2013).

Não há vasta literatura demonstrando os mecanismos subjacentes exercidos pela glutamina na modulação da produção de citocinas e a transcrição de genes inflamatórios avaliando-se especialmente no que concerne a essa influência de modo tempo dependente. Por isso decidimos investigar os efeitos da glutamina avaliando a produção de citocinas em macrófagos, estudando diferentes concentrações de glutamina em diferentes tempos de estímulo com LPS.

A IL-10 é uma citocina responsável por modular e regular a produção de citocinas inflamatórias. Essa citocina desempenha diversos mecanismos moleculares no controle de citocinas inflamatórias, alguns deles ainda não estão totalmente elucidados, contudo a inibição do fator de transcrição do NFkB está descrita como um dos mecanismos de ação dessa citocina (DILLOW *et al.,* 2014; JAFFER, WADE & GOURLAY, 2010; ZHOU, NAZARIAN & SMALE, 2004).

Baseando-se nos resultados deste trabalho e de dados disponíveis na literatura, podemos inferir que a produção elevada de IL-10 exercida pela glutamina reflete na inibição da produção de citocinas inflamatórias. Essa inibição reduz a fosforilação e consequentemente diminui a ativação do NFκB, que por sua vez incide na redução da translocação desse fator de transcrição para o núcleo das células, reduzindo a transcrição de genes inflamatórios, modulando a produção de citocinas inflamatórias como o IL-1, IL-12 e TNF-α (ROGERO *et al*., 2008; 2010; SANTOS *et al*., 2016).

Evidenciamos que células suplementadas com alta concentração de glutamina (10mM) expressa elevada quantidade de RNAm de IL-10 sendo que essa expressão se encontrava elevada por algumas horas. Esses resultados estão de acordo com a os nossos resultados do nosso grupo, obtidos em diferentes células. Isso nos possibilita afirmar enfaticamente que existe uma reprodutibilidade nesse resultado, o que demonstra que a modulação exercida pela glutamina elevando a produção de IL-10 é um dos fatores que induzem a inibição da resposta inflamatória na presença de altas concentrações de glutamina (DOS SANTOS *et al*., 2017; SANTOS *et al.*, 2016; DA SILVA LIMA *et al.,* 2013).

Supreendentemente, os nossos resultados mostraram que a suplementação com alta concentração de glutamina (10 mM) eleva a expressão do RNAm de Interferon beta. As citocinas da família dos interferons são capazes de exercer efeitos imunomodulatórios tanto na resposta imune inata, quanto na resposta imune adaptativa em grande parte pela indução da expressão de IL-10. Está bem estabelecido que as células do sistema imune podem produzir elevadas quantidades de IL-10 após o tratamento com interferon beta (IYER & CHENG, 2012; ZHANG *et al*., 2011).

Prosseguindo com as averiguações dos efeitos moduladores da glutamina na ativação de macrófagos, avançamos avaliando a expressão gênicas de citocinas em camundongos *knock out* para o IL-10, Surpreendentemente, observamos novamente aumento na expressão do RNAm do IFN-β, mostrando que há reprodutibilidade na expressão aumentada de IFNβ em células suplementadas com alta concentração de glutamina, e até o momento não há outros relatos de que a suplementação de glutamina module a expressão de interferon beta (YEN *et al.*, 2015).

O IFN-β é uma citocina produzida pelas células do sistema imune em resposta a estímulos biológicos e químicos. Essa citocina regula uma pletora de genes, possuindo efeitos pleiotrópicos promovendo efeitos antiviral, antiproliferativo e imunomodulatório em inúmeros tipos celulares (HAJI ABDOLVAHAB, MOFRAD & SCHELLEKENS, 2016).

Além disso, estudos *in vitro* revelam que o IFN-β induz ao acúmulo de RNAm de IL-10, além de estimular a secreção dessa citocina em cultura de mononucleares do sangue periférico. Esses resultados são reforçados pelas conclusões obtidas com estudos *in vivo* que demonstram aumento da produção de IL-10 após administração de Interferon beta (ERSOY *et al*., 2005; RUDICK *et al.,* 1996; YEN *et al*., 2015).

O mecanismo pelo qual o IFN beta induz a estimulação e regulação positiva na produção de IL-10 ainda não está totalmente elucidado, alguns pesquisadores acreditam haver um envolvimento parcial da molécula PI3K com subsequente inibição do GSK3. Contudo, a confirmação desse mecanismo permanece elusiva (WANG *et al.*, 2012; YEN *et al*., 2015).

Baseando-se nos resultados de que altas concentrações de glutamina podem induzir elevação da expressão de interferon beta, levantamos a hipótese de que essa citocina por sua vez acarreta a indução de expressão de IL-10, que consequentemente promove os efeitos inibitórios observados na suplementação de altas concentrações de glutamina (MELLO *et al.*, 2014; YEN *et al*., 2015).

E isso explicaria a ação da glutamina como moduladora da resposta inflamatória. De fato, a produção elevada de IL-10 está frequentemente associada à regulação negativa de citocinas inflamatórias como a redução da expressão de RNAm de IL12 observada nesse trabalho, levando a uma imunossupressão justificando os efeitos inibitórios observados após a suplementação com glutamina (MELLO *et al.*, 2014; YEN *et al*., 2015).

Os efeitos inibitórios da suplementação com glutamina foram reforçados pelo fato que a concentração de 10mM de glutamina reduziu a ativação do fator de transcrição do NFkB que está diretamente relacionado com a indução de diversas citocinas pró inflamatórias (GARRETT-COX *et al.,* 2009; CRUZAT, *et al.,* 2014).

O aumento da expressão de STAT3 fosforilado é um dado que corrobora a afirmação de que a suplementação com alta concentração de glutamina inibe a resposta inflamatória. Sabe-se que a IL-10 induz a ativação de STAT3 e essa por sua vez está associada à indução de sinais inibitórios nas células do sistema imune. Sendo assim, os resultados obtidos nos possibilitam inferir que a glutamina está diretamente relacionada à inibição da resposta inflamatória em macrófagos. Esses efeitos se dão pela modulação de relevantes elementos envolvidos na resposta inflamatória como a produção de citocinas e fatores de transcrição (DOS SANTOS *et al*., 2017; SANTOS *et al.*, 2016; DE OLIVEIRA *et al.*, 2016; CRUZAT *et al.,* 2014).

Com o intuito de avançar no entendimento da ação da glutamina e a indução de IFN-β, seguimos investigando o IRF3 que é um fator de transcrição ativado por LPS, sua ativação induz a transcrição do gene do IFN-beta (YEN *et al.* 2015).

A expressão do fator de transcrição IRF3 estava aumentada nas células que foram tratadas com 10mM de glutamina e desafiadas com LPS por 2 horas. O que nos leva a

pensar que os efeitos da suplementação de glutamina modulam a expressão desse fator de transcrição e induzem uma variação na resposta dessas células mediante ao estímulo com LPS. Contudo, mais experimentos se fazem necessários para melhor compreender se existe uma correlação entre a suplementação com glutamina e a indução do IRF3, bem como melhor descrever o envolvimento do Interferon beta induzido pela glutamina.

Os dados obtidos neste trabalho apontaram para efeitos inibitórios associadas à suplementação com glutamina; face a um desafio imunológico como infecções esse efeito inibitório poderia vir a ser deletério para o organismo. No entanto, em algumas condições em que há uma desregulação da resposta inflamatória pendendo para um ambiente pró-inflamatório, a glutamina seria um potencial agente terapêutico. Sendo assim, mais estudos se fazem necessários para determinar os efeitos positivos e deletérios associados à glutamina bem como a dosagem segura (AKOBENG, ELAWAD & GORDON, 2016).

6.3 RESTRIÇÃO ALIMENTAR

O primeiro estudo científico que defendia a restrição calórica como benéfica para organismo foi publicado no ano de 1935 por Mc Cay e colaboradores, que constataram aumento da longevidade em animais submetidos a uma dieta restritiva em calorias. Desse modo, nesse primeiro relato, afirmaram enfaticamente esse aspecto positivo na restrição alimentar; estudos posteriores estavam de acordo e também relataram aumento do tempo de vida dos animais submetidos à restrição alimentar; já em outros modelos, evidenciou-se que os efeitos da restrição alimentar nesse aspecto foi neutro e nos demais modelos ocorreu o oposto, o tempo de vida dos animais foi reduzido devido a indução de restrição alimentar (FONTANA, PARTRIDGE & LONGO, 2010; HARPER, LEATHERS & AUSTAD, 2006; LORENZINI, 2014; TANG *et al*., 2016).

A restrição alimentar na forma de restrição calórica vem sendo associada a efeitos positivos no retardamento de doenças associadas ao envelhecimento e melhora do estado do metabolismo. Alguns pesquisadores sugerem que a indução de restrição

alimentar em curto prazo possui relevância clínica para o tratamento e prevenção de patologias associadas desde ao metabolismo, como a resistência à insulina, bem como quimioterapia e inflamação (FABBIANO, SUÁREZ-ZAMORANO & RIGO, 2016; ROBERTSON & MITCHELL, 2013).

O modelo de indução de restrição alimentar induzido neste trabalho foi padronizado com redução em 30% de ração num período de dez dias. Esse tempo e quantidades foram estipulados devido às observações realizadas no desenvolvimento do projeto piloto, que demonstrou alto índice de mortalidade dos animais quando a oferta de ração foi reduzida em 50% ou 70% do consumo diário de cada animal (dados não mostrados).

Com essa breve observação, pode-se dizer que a redução de diferentes proporções do alimento gera uma linha tênue entre sobrevivência e mortalidade, e os impactos da restrição alimentar nesses animais podem vir a ser negativos; sendo assim, estudos mais acurados nos aspectos bioquímicos e imunológicos são relevantes para a elucidação dos efeitos exercidos pela restrição alimentar no organismo como um todo.

Lorenzini (2014) descreveu em seu trabalho um fenômeno muito observado e relatado nas pesquisas envolvendo restrição alimentar, a diminuição no peso corpóreo. A perda de peso corpóreo pode variar de 60 a 85% nos animais que tiveram uma redução no consumo de calorias quando comparado aos animais que consomem a dieta *ad libitum*. Os nossos resultados também demonstraram diminuição do peso corpóreo, o que leva à conclusão de que a redução no peso nos animais foi devido ao consumo de ração limitado, induzido experimentalmente.

A redução do peso é o resultado de perda de massa magra e tecido adiposo, essa perda reflete nos resultados obtidos de índice de Lee que se mostrou diminuídos nos animais do grupo restrição alimentar (LORENZINI, 2014).

A literatura descreve alterações metabólicas significativas em função da redução de peso decorrente da restrição de alimentos. Essas alterações podem ser detectadas em dosagens bioquímicas no plasma (BADONNEL *et al*., 2012; ROBERTSON & MITCHELL, 2013).

No modelo experimental de restrição induzido nesse trabalho observamos redução da densidade óssea. A dosagem de cálcio sérico revelou que ocorre uma diminuição na concentração desse mineral na circulação dos camundongos do grupo restrição alimentar. A literatura estabelece uma correlação entre situações em que há perda de peso com perda de massa óssea, sendo que, para cada 10% de perda de peso corpóreo, é possível que ocorra redução de 1 a 2% de massa óssea também detectada em humanos (GAFFNEY-STOMBERG *et al*., 2014; JOLLY, 2007).

Os níveis séricos de insulina são correlacionados positivamente com a formação de osteoblastos, células que produzem a matriz óssea. A restrição alimentar induz à redução de insulina, criando uma correlação em que a redução de insulina resulta na diminuição da densidade óssea. Essa seria uma explicação plausível para a redução da densidade óssea observada no nosso modelo experimental (HUANG & ABLES, 2016).

Em estudos realizados com diabéticos e obesos submetidos à restrição alimentar, constatou-se uma redução dos níveis de glicose circulante no sangue. Em nosso estudo, também detectamos a redução de glicose nos animais submetidos à restrição alimentar. A restrição calórica exerce um efeito benéfico no que diz respeito à resistência à insulina em indivíduos obesos; no entanto, a literatura não é clara a respeito da glicemia em indivíduos saudáveis submetidos à restrição alimentar. Alguns pesquisadores defendem que a melhora na sensibilidade à insulina associada à restrição alimentar é atribuída em parte à supressão de produção da glicose hepática (COLETTE *et al.*, 2005; FABBIANO, SUÁREZ-ZAMORANO & RIGO, 2016; JOLLY, 2007; LEE *et al*., 2014).

Pesquisas com camundongos e humanos demonstraram que a restrição alimentar induz a redução dos níveis de colesterol e triglicérides. Os nossos resultados evidenciaram redução nos níveis plasmáticos de colesterol e triglicérides em apenas dez dias de restrição alimentar (JOLLY, 2007; LEE *et al*., 2014).

Partindo-se do ponto de vista de que o excesso de colesterol e triglicérides associados a doenças metabólicas podem trazer prejuízos para a saúde, a redução desses metabólitos se torna benéfica para o organismo. Contudo, não existem dados

suficientes na literatura para conceituar estritamente em condições basais se a redução desses valores é benéfica ou adversa para o organismo (JOLLY, 2007).

Na avaliação dos parâmetros hematológicos, mais precisamente da série eritrocitária, evidenciou-se redução do número de hemácias bem como redução dos valores de hemoglobina e hematócrito. Baseando-se nos valores de referência publicados recentemente por Santos *et al*. (2016b), é possível afirmar que a restrição alimentar induzida nesse modelo desencadeou anemia. Não detectamos alterações morfológicas, portanto contatamos que a anemia apresentada pelos animais se mostrou normocítica e normocrômica.

Modelos de privação de alimento apresentaram resultados similares nesses parâmetros hematológicos avaliados mostrando que a restrição alimentar leva a alterações dos parâmetros hematológicos da série vermelha induzindo anemia (HISHINUMA & KIMURA, 1989).

Além disso, na década de 1980, pesquisadores observaram redução no tempo de vida das hemácias de camundongos submetidos à restrição alimentar. Soma-se a isso o fato de ocorrer um aumento de eritropoietina, com elevado número de células precursoras eritroides no fêmur dos animais submetidos à restrição alimentar. Esse aumento da eritropoese não é acompanhado pela síntese de hemoglobina e posteriormente afetaria o tempo de vida das hemácias. No entanto, uma explicação plausível dos mecanismos subjacentes a esse quadro hematológico induzido pela restrição alimentar permanece a ser desvendados (HISHINUMA & KIMURA, 1989; HISHINUMA & KIMURA, 1990; TANG *et al*., 2016; WOJCIAK, 2014).

As nossas análises mostraram redução de leucócitos no sangue periférico, bem como redução da celularidade do peritônio e baço. Esses dados nos levam a pensar que a restrição alimentar modula quantitativamente a produção de células do sistema imune, sendo que essa redução foi observada e descrita em diversos modelos de restrição alimentar. Ainda, a restrição alimentar é capaz de reduzir drasticamente o tamanho do timo em animais idosos, o que é considerado um efeito negativo associado a essa intervenção nutricional (ROBERTSON & MITCHELL, 2013; MENEGUELLO-COUTINHO *et al*., 2014; WASINSKI *et al.*, 2013).

A redução da celularidade do baço é reforçada pelos resultados obtidos nas análises histopatológicas, em que se observou redução da celularidade linfoide no baço, com redução nas regiões foliculares e PALS (periarterial linfática bainha). Em 2006, Suttie correlacionou a restrição calórica a alterações esplênicas associadas à redução de peso corpóreo, dados que corroboram a afirmação de que intervenções nutricionais levam a expressivas modificações em relevantes órgãos, influenciando relevantes processos fisiológicos (SUTTIE, 2006).

Evidenciou-se que a restrição alimentar de longo prazo melhora o fenótipo do envelhecimento das células tronco hematopoiéticas, o que foi comprovado pelo aumento de células tronco hematopoiéticas na medula de animais submetidos a 30% de restrição alimentar, bem como tendência para um comprometimento com a linhagem mieloide durante o processo de envelhecimento. Sendo assim, a restrição alimentar melhora a manutenção da capacidade proliferativa e aumenta o número de células tronco em estado quiescente (TANG *et al*. 2016).

Por outro lado, as alterações induzidas pela restrição alimentar a longo prazo comprometem a diferenciação das células tronco hematopoiéticas com a linhagem linfoide, particularmente inibindo a proliferação de progenitores linfoides e suprimindo a produção de fatores de crescimento e citocinas relevantes para a funcionalidade das células troco hematopéticas. Dessa forma, esse comprometimento impacta na quantidade de células encontradas em órgãos linfoides como o baço, explicando a redução na celularidade que evidenciamos no presente trabalho. Isso nos leva a inferir que a restrição alimentar pode levar a uma imunossupressão por interferir na produção de células da linhagem linfoide (TANG *et al.* 2016).

No que concerne à funcionalidade do sistema imune, a literatura demonstra que a restrição alimentar exerce efeitos moduladores da imunidade inata (JOLLY, 2004; 2007). Suarez-Souto (2012) detectou um aumento da expressão de mediadores da resposta imune inata, de células em estado basal, de camundongos submetidos à restrição alimentar (SUÁREZ-SOUTO *et al.*, 2012).

Alguns trabalhos defendem a restrição alimentar como benéfica num contexto em que patologias estão associadas à desregulação da resposta imune; por exemplo, em

doenças autoimunes, essa intervenção nutricional induziu a redução da produção de IFN-γ e autoanticorpos, além da redução dos efeitos de colite experimental após a restrição alimentar em modelos animais (JOLLY, 2004).

A literatura mostra que modificações na quantidade e na qualidade da dieta interfere diretamente em diversos aspectos do sistema imune, com redução dos componentes do sistema do complemento e redução de imunoglobulinas (IgG e IgM) em cabras submetidas a uma dieta de restrição calórica. Avaliações do sistema imune dos filhotes dessas mesmas cabras demonstraram alterações irreversíveis quando o sistema imune dos filhotes foi desafiado com LPS, provando que alterações na alimentação durante a gravidez refletem alterações no organismo do filhote contribuindo para o surgimento de doenças tardiamente nos filhotes (HE *et al*., 2014).

Shushimita *et al* (2014) reportou que a restrição alimentar tem um grande impacto na redução da população de células B da medula óssea e aumento de linfócitos B, recirculantes no sangue periférico de camundongos submetidos a uma dieta com restrição alimentar por 2 semanas. Além disso, evidenciou redução no tamanho do timo e do baço, sendo que essas últimas alterações estão de acordo com os resultados obtidos neste trabalho. A mesma autora ainda elucidou efeitos da restrição alimentar na maturação de linfócitos T com depleção dessas células no baço e no timo e o impacto no recrutamento das mesmas para a medula óssea (SHUSHIMITA *et al.* 2014, TANG 2016).

A expressão das moléculas CD8 (linfócitos T citotóxicas) e a expressão de células CD4 (linfócitos T auxiliares) sofre uma dramática redução em organismos submetidos a restrição alimentar. Contudo estudos constataram um aumento na relação CD4/CD8 nos linfócitos extraídos do tecido adiposo inguinal proveniente de animais submetidos à restrição alimentar. Provando mais uma vez que a redução do consumo de alimentos também modula as células da linhagem linfoide (MENEGUELLO-COUTINHO *et al.*, 2014).

F4/80 é uma molécula presente na superfície de macrófagos, frequentemente utilizadas para identificação dessa linhagem. Há evidências de que a restrição alimentar leva à redução da expressão do marcador F4/80 nas células do tecido adiposo inguinal.

Esse resultado pode ser correlacionado aos nossos achados que evidenciaram redução na quantidade de macrófagos peritoneais (MENEGUELLO-COUTINHO et al., 2014).

Prosseguindo, decidimos avaliar a produção de citocinas e a influência da suplementação com glutamina em macrófagos peritoneiais estimulados com LPS. Ocorreu uma modulação negativa na produção das citocinas IL-12 e TNF-α nas células provenientes dos animais submetidos à restrição alimentar. A modulação negativa na produção desses relevantes mediadores inflamatórios não foi recuperada após a suplementação com 10mM de Glutamina (Da SILVA LIMA *et al*., 2013).

Os nossos resultados estão de acordo com pesquisas publicadas anteriormente, que mostraram que a restrição alimentar leva a uma redução na produção de IL-6 e IL-12, o que nos possibilita afirmar que a restrição alimentar modula a resposta inflamatória podendo comprometer a defesa do organismo e a suplementação com glutamina não interfere nesses parâmetros (SUN *et al*., 2001).

A literatura relata que a fagocitose em macrófagos peritoneais provenientes de camundongos submetidos a uma dieta com restrição alimentar também se mostrou comprometida. Os autores expõem que os animais submetidos à restrição alimentar estariam protegidos contra o estresse oxidativo gerado em condições de infecção, contudo há um retardamento no processo de maturação de macrófagos e aumento da suscetibilidade infecções (SUN *et al*., 2001).

Esses resultados nos levaram a indagar se as vias de sinalização envolvidas na produção de citocinas inflamatórias estariam alteradas tanto em condições de restrição alimentar quanto na suplementação com glutamina. De fato, evidenciamos que a expressão de NFkB e IKBalfa estão reduzidas nas células submetidas à restrição alimentar. No entanto, a suplementação com 2 mM ou 10 mM de glutamina não interferiu na expressão dessas proteínas nas condições experimentais avaliadas (CRUZAT *et al.*, 2014; Da SILVA LIMA *et al*., 2013; ROGERO *et al*., 2010).

Os macrófagos suplementados com glutamina demostraram uma regulação positiva na produção de IL-10. Associa-se a IL-10 um feito inibitório sobre o fator de transcrição

do NFkB, esses efeitos foram explorados no capítulo II desse trabalho (LECLEIRE *et al.*, 2008).

Analisando o agrupamento dos dados obtidos neste trabalho nos permite reconhecer que a restrição alimentar exerce efeitos negativos no sistema imunológico em condições basais, o que viria a ser deletério face a uma infecção. No entanto, mais estudos se fazem necessários para determinar os efeitos benéficos e adversos da restrição alimentar em outras condições fisiopatológicas (JOLLY, 2007; MEJIA et al. 2015; ROBERTSON & MITCHELL, 2013).

Considerando que a literatura traz dados sobre a glutamina e restrição alimentar separadamente e esses resultados são considerados tanto positivos quanto deletérios sem diferentes condições fisiopatológicas, seria possível traçar um paralelo em que os nossos dados apontam para efeitos negativos associados a ambas intervenções nutricionais num contexto basal no qual não há uma desregulação prévia instalada no organismo.

7. CONSIDERAÇÕES FINAIS

A citocina IL-10 exerce um papel na regulação de genes pró-inflamatórios promovendo a redução da acetilação da lisina 27 da histona 3. Essa atuação ocorre de modo tempo dependente em diferentes regiões como enhancers de genes como Il12b. Esse resultado aborda um mecanismo de regulação epigenética no controle da expressão gênica, propondo um mecanismo inédito pelo qual alguns genes inflamatórios são regulados pela IL-10.

A glutamina regula positivamente a produção de IL-10 em macrófagos de modo dose e tempo dependente; além disso, altas concentrações de glutamina também exercem efeito regulador positivo para IFN-beta e o seu respectivo fator de transcrição IRF-3, de forma que os resultados aqui obtidos demostram um complexo mecanismo de regulação exercido pela suplementação de glutamina.

A restrição alimentar induz a redução de peso corpóreo, alterando a concentração de relevantes componentes bioquímicos como colesterol, cálcio, glicose e triglicérides.

A restrição alimentar induz a uma redução de células da cavidade peritoneal e redução da celularidade esplênica mostrando que a restrição alimentar tem um efeito negativo sobre o sistema imunológico.

A combinação de restrição alimentar com a suplementação com glutamina não alterou os status da produção de citocinas induzidos pela restrição alimentar.

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ANEXO I - Protocolo do comitê de ética no uso de animais

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Comissão de Ética no Uso de Animais - CEUA

Ofício CEUA/FCF **8.2015-P485**

CERTIFICADO

A Comissão de Ética no Uso de Animais, da Faculdade de Ciências Farmacêuticas, da Universidade de São Paulo, **CERTIFICA** que o Projeto de Pesquisa "**Avaliação da glutamina como moduladora do fator de transcrição c-Rel e NFκB em macrófagos e linfócitos em um modelo de restrição alimentar**" (Protocolo CEUA/FCF/485), de responsabilidade do(a) pesquisador(a) **Dalila Cunha de Oliveira**, sob orientação do(a) **Prof. Dr. Ricardo Ambrósio Fock**, está de acordo com as normas do Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi APROVADO em reunião de **6 de fevereiro de 2015**. Conforme a legislação vigente, deverá ser apresentado, no encerramento deste Projeto de Pesquisa, o respectivo **relatório final**.

São Paulo, 10 de fevereiro de 2015.

Profa. Dra. Neuza Mariko Aymoto Hassimotto
Vice-Coordenadora da CEUA/FCF/USP

ANEXO II- Ficha do Auno

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
FICHA DO ALUNO

9142 - 7746697/1 - Dalila Cunha de Oliveira

Email:	dalilafcf@usp.br
Data de Nascimento:	02/06/1985
Cédula de Identidade:	RG - 41.118.629-2 - SP
Local de Nascimento:	Estado de São Paulo
Nacionalidade:	Brasileira
Graduação:	Bacharel em Farmácia - Faculdade Campo Limpo Paulista - São Paulo - Brasil - 2011
Mestrado:	Mestra em Ciências - Área: Análises Clínicas - Faculdade de Ciências Farmacêuticas - Universidade de São Paulo - São Paulo - Brasil - 2013

Curso:	Doutorado
Programa:	Farmácia (Fisiopatologia e Toxicologia)
Área:	Fisiopatologia
Data de Matrícula:	17/10/2013
Início da Contagem de Prazo:	17/10/2013
Data Limite para o Depósito:	17/10/2017
Orientador:	Prof(a). Dr(a). Ricardo Ambrosio Fock - 10/08/2016 até o presente. Email: hemato@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 17/10/2013
Data de Aprovação no Exame de Qualificação:	Aprovado em 14/11/2014
Estágio no Exterior:	Harvard Medical School, Estados Unidos da América - Período de 01/07/2015 até 30/06/2016
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor a partir de 20/04/2013).
Última ocorrência: Matrícula de Acompanhamento em 17/07/2017
Impresso em: 26/07/2017 11:49:24

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
FICHA DO ALUNO

9142 - 7746697/1 - Dalila Cunha de Oliveira

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBA5753-1/1	Nutrigenômica e Programação das Doenças Crônicas Não-Transmissíveis	29/10/2013	04/11/2013	30	2	100	A	N	Concluída
FBA5899-2/3	Biodisponibilidade de Nutrientes e de Substâncias Bioativas em Alimentos e Dietas	10/03/2014	20/04/2014	90	6	100	A	N	Concluída
BIF5707-3/1	Regulação da Expressão Gênica em Processos Fisiológicos (Instituto de Biociências - Universidade de São Paulo)	11/03/2014	24/04/2014	120	8	100	A	N	Concluída
FBC5792-3/1	Tópicos em Análises Clínicas III	11/03/2014	23/06/2014	15	1	90	A	N	Concluída
QBQ5781-7/1	Fundamentos da Citometria de Fluxo – Aplicações em Biologia Celular, Molecular e Bioquímica (Instituto de Química - Universidade de São Paulo)	26/05/2014	01/06/2014	30	2	100	A	N	Concluída
FBC5766-4/1	Tópicos em Análises Clínicas IV	05/08/2014	17/11/2014	15	1	100	A	N	Concluída
MPT5780-2/2	Genética Forense (Faculdade de Medicina - Universidade de São Paulo)	09/09/2014	13/10/2014	75	5	100	A	N	Concluída
BIO5788-3/1	Inglês em Ciência (Instituto de Biociências - Universidade de São Paulo)	04/03/2015	16/06/2015	120	8	90	B	N	Concluída
FBC5707-6/1	Biologia de Sistemas para Ciências da Vida	26/10/2016	08/12/2016	60	0	-	-	N	Matrícula cancelada

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	20	33
Estágios:			
Total:	0	20	33

Créditos Atribuídos à Tese: 167

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula de Acompanhamento em 17/07/2017
Impresso em: 26/07/2017 11:49:24