Universidade do Estado do Rio de Janeiro
Centro Biomédico
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Aleida Soraia Oliveira Dias

Relação entre obesidade, perfil fenotípico e funcional das células T $CD4^+$ e gravidade da asma alérgica: estudo centrado no papel da leptina

Rio de Janeiro
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Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-Graduação em Microbiologia, da Universidade do Estado do Rio de Janeiro. Área de concentração: Microbiologia Médica Humana.

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2023

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Si ka badu ka ta biradu

Eugênio Tavares

RESUMO

DIAS, Aleida Soraia Oliveira. **Relação entre obesidade, perfil fenotípico e funcional das células T CD4$^+$ e gravidade da asma alérgica**: estudo centrado no papel da leptina. 2023. 170 f. Tese (Doutorado em Microbiologia) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2023.

A asma alérgica (AA) é uma doença crônica, caracterizada pela hiperreatividade brônquica a alérgenos, como a glicoproteína Fel d1 dos gatos. A obesidade e a hipovitaminose D têm sido associados a gravidade da AA. O objetivo do estudo foi avaliar o impacto da obesidade e sua relação com os efeitos da leptina e da vitamina D no perfil funcional das células T CD4$^+$ e B de pacientes com AA. Para tanto, em diferentes condições de estimulação, culturas de células mononucleares totais do sangue periférico, ou de células T CD4$^+$ purificadas, os níveis de citocinas foram dosados via ELISA ou Luminex, enquanto a imunofenotipagem foi avaliada por citometria de fluxo. Ainda, os níveis séricos da IgE, leptina e 25(OH)D_3 foram determinados por ELISA e os títulos da IgE Fel d1-específicos foram mensurados pelo ImmunoCAP. No estudo, maiores níveis de IL-5, IL-6 e IL-17 e menor concentração de IL-10 foram quantificados nas culturas das células T CD4$^+$ dos pacientes com a AA grave. A gravidade da AA, especialmente nos obesos, foi diretamente associada a frequência das células Th17 e as das células Th2/Th17 híbridas. Em contraste, a frequência de linfócitos Tr-1/Treg e Br-1 foi menor quando comparado aos eutróficos. A Hiperleptinemia foi diretamente correlacionada com os níveis de IL-5, IL-6 e IL-17, e inversamente associada à IL-10 dosados em culturas de células TCD4$^+$. Ademais, os níveis da leptina sérica correlacionaram-se diretamente com a proporção das células Th17 e Th2/Th17 híbridas, mas foram negativamente correlacionados com a porcentagem de linfócitos Tr-1/Treg e Br-1. Adicionalmente, níveis altos de leptina foram negativamente associados a capacidade da 1,25(OH)$_2D_3$ em inibir, *in vitro*, a produção de citocinas pelas células T CD4$^+$ efetoras dos pacientes com AA. Nos alérgicos a gatos, a Fel d1 induziu a produção de IgE e de citocinas relacionadas aos fenótipos celulares Th2, Th9 e Th17, bem como dos linfócitos T_{FH} IL21$^-$ capazes de sintetizar IL-4, IL-5 e IL-13. A adição da leptina favoreceu a expansão dos fenótipos acima referidos, com exceção das células Th17 e, em contrapartida, reduziu a proporção das células T CD4$^+$ reguladoras convencionais (Tr-1/Treg) e foliculares (T_{FR}). A capacidade da leptina em aumentar a produção da IgE *in vitro* correlacionou-se diretamente com a porcentagem das células T_{FH} IL21$^+$IL5$^+$ e T_{FH} IL21$^-$(IL4$^+$, IL5$^+$e IL13$^+$), e foi inversamente associado à frequência dos linfócitos Treg e T_{FR}. Finalmente, muitos dos desequilíbrios entre as células T CD4$^+$ específicas para a Fel d1 foram associados a elevados níveis séricos de leptina e de IgE específica para Fel d1. Esses achados sugerem que a obesidade, em parte pelo quadro associado à hiperleptinemia e hipovitaminose D, deve impactar negativamente no prognóstico da AA por favorecer a expansão das células Th17 e Th2/Th17 híbridas e diminuir a proporção de linfócitos Tr-1/Treg e Br-1. Por fim, altas doses da leptina devem exacerbar o quadro de hipersensibilidade por reduzir a frequência das células Tregs e induzir a expansão de subtipos das T_{FH} relacionados à produção da IgE de alta afinidade envolvida em reações anafiláticas.

Palavras-chave: Asma alérgica. Gatos. Fel d1. Obesidade. Vitamina D. Leptina. T CD4$^+$. Células B reguladoras

ABSTRACT

DIAS, Aleida Soraia Oliveira. *Relationship between obesity, phenotypic and functional profile of CD4⁺ T cells and severity of allergic asthma: a focused study on the role of leptin.* 2023. 170 f. Tese (Doutorado em Microbiologia) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2023.

Allergic asthma is a chronic disease characterized by bronchial hyperreactivity to allergens, such as the glycoprotein Fel d1 found in cats. Obesity and hypovitaminosis D have been associated with the severity of AA.The aim of the study was to evaluate the impact of obesity and its relationship with the effects of leptin and vitamin D on the functional profile of $CD4^+$ T cells and B cells in patients with AA. Therefore, under different stimulation conditions, cultures of total peripheral blood mononuclear cells, or purified $CD4^+$ T cells, cytokine levels were measured via ELISA or Luminex, while immunophenotyping was evaluated by flow cytometry. Furthermore, serum levels of IgE, leptin and $25(OH)D_3$ were determined by ELISA and Feld1-specific IgE titers were measured by ImmunoCAP. In the study, high levels of IL-5, IL-6 and IL-17 and low concentration of IL-10 were quantified in $CD4^+$ T cell cultures from patients with severe AA. The severity of AA, especially in the obese, was directly associated with the frequency of Th17 cells and hybrid Th2/Th17 cells. In contrast, the frequency of Tr-1/Treg and Br-1 lymphocytes was lower when compared to eutrophic ones. Hyperleptinemia was directly correlated with IL-5, IL-6 and IL-17 levels, and inversely associated with IL-10 measured in CD4+ T cell cultures. Furthermore, serum leptin levels correlated directly with the proportion of Th17 and Th2/Th17 hybrid cells, but were negatively correlated with the percentage of Tr-1/Treg and Br-1 lymphocytes. Additionally, high leptin levels were negatively associated with the ability of $1,25(OH)_2D_3$ to inhibit, *in vitro*, cytokine production by effector CD4+ T cells from patients with AA. In those allergic to cats, Fel d1 induced the production of IgE and cytokines related to Th2, Th9 and Th17 cell phenotypes, as well as T_{FH} IL21- lymphocytes capable of synthesizing IL-4, IL-5 and IL-13. The addition of leptin favored the expansion of the aforementioned phenotypes, with the exception of Th17 cells and, on the other hand, reduced the proportion of conventional regulatory CD4+ T cells (Tr-1/Treg) and follicular (T_{FR}) cells. The ability of leptin to increase IgE production *in vitro* correlated directly with the percentage of T_{FH} IL21+IL5+ and T_{FH} IL21- cells (IL4+, IL5+ and IL13+), and inversely correlated with the frequency of Treg and T_{FR} lymphocytes. Finally, many of the Fel d1-specific CD4+ T cell imbalances have been associated with elevated serum levels of leptin and Fel d1-specific IgE. These findings suggest that obesity, partly due to the condition associated with hyperleptinemia and hypovitaminosis D, must negatively impact the prognosis of AA by favoring the expansion of hybrid Th17 and Th2/Th17 cells and decreasing the proportion of Tr-1/Treg and Br-1 lymphocytes. Finally, high doses of leptin should exacerbate the hypersensitivity condition by reducing the frequency of Treg cells and inducing the expansion of T_{FH} subtypes related to the production of high-affinity IgE involved in anaphylactic reactions.

Keywords: Allergic asthma. Cats. Fel D1. Obesity. Vitamin D. Leptin. T $CD4^+$. Regulatory B cells.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AA	asma alérgica
ACQ-7	questionário de controle da asma com 7 itens
ACT	teste de controle da asma
Ag	antígeno
ANVISA	agência nacional de vigilância sanitária
APC	célula apresentadora de antígeno
Bcl6	*B-cell lymphoma 6*
Br-1	células B reguladoras produtoras de IL-10
Br-3	células B reguladoras produtoras de TFG-β
CCL	ligante com motivos C-C
CD25	subunidade alfa do receptor de interleucina 2
CI	corticoide inalatório
CTLA-4	antígeno citotóxico 4 de linfócitos t
CXCL	ligante com motivos C-X-C
CXCR	receptor com motivos C-X-C
CYP24A1	24-hidroxilase vitamina D
CYP27A1	Monoenzima 25-hidroxilase vitamina D
CYP27B1	1-α-hidroxilase
DATASUS	departamento de informática do sistema único de saúde
DC	célula dendrítica
EAO	asma de início precoce
ECP	proteína catiônica eosinofílica
EET	armadilha extracelular eosinofílica
EMA	*european medicines agency*
EPO	peroxidase eosinofílica
FcεRI	receptor da IgE de alta afinidade
FDA	*food and drug administration*
Fel d1	*Felis domesticus* 1
FeNO	fração exalada de óxido nítrico
FoxP3	*forkhead box P3*

GATA-3	*GATA binding protein 3*
GC	centro germinativo
GCR-α	receptor α dos glicocorticoides
GINA	*global initiative for asthma control*
GITR	*glucocorticoid-induced tumor necrosis factor-related receptor*
GM-CSF	fator estimulador de colônias de granulócitos-macrófagos
IUIS	união internacional de sociedades imunológicas
IgE	imunoglobulina da classe IgE
IgG1	imunoglobulina da classe Ig1
IL	interleucina
IL-7Rα	cadeia alfa do receptor da IL-7
IL-12R	receptor da IL-12
IL-13Rα1	cadeia α-1 do receptor da IL-13
ILC2	célula linfoide inata do tipo 2
ILC3	célula linfoide inata do tipo 3
ILT3	Immunoglobulin-like transcript 3
IMC	índice de massa corporal
ITA	imunoterapia alérgeno específica
iTreg	célula T reguladora induzida
LABA	β2 agonista de longa duração
LAMA	β2 agonista de curta duração
LAO	asma de início tardio
LepR	receptor da leptina
LTD4	leucotrieno D4
M1	macrófagos do tipo 1
M2	macrófagos do tipo 2
MBP	proteína básica
MHC II	molécula de histocompatibilidade principal da classe II
NRLP3	*nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3*
nTreg	Células T reguladora natural
OMS	organização mundial da saúde
OVA	ovalbumina

PAI-1	Inibidor do ativador de plasminogênio tipo 1
PAR2	*protease-activated receptor 2*
PCR	proteína C reativa
PD1	proteína de morte celular programada 1
PD-L1	Ligante da proteína de morte celular programa 1
PGD2	prostaglandina D2
RORγt	*RAR-related orphan receptor - γ*
ROS	espécies reativas de oxigênio
SABA	antagonista muscarínico de longa duração
SBPT	sociedade brasileira de pneumologia e tisiologia
SIgE	imunoglobulina IgE sérica
STAT-6	*signal transducer and activator of transcription 6*
SUS	sistema único de saúde
TAB	tecido adiposo branco
TAM	tecido adiposo marrom
TCR	receptor das células T
T_{FH}	célula T auxiliar folicular do tipo 2
Th1	célula T auxiliar do fenótipo 1
Th2	célula T auxiliar do fenótipo 2
Th9	célula T auxiliar do fenótipo 9
Th17	célula T auxiliar do fenótipo 17
TLR	receptor do tipo toll
TNF-α	fator α de necrose tumoral
TNFR1	receptor 1 do TNF-α
Treg	célula T reguladora
Tr1	linfócitos t reguladores do tipo 1
TSLP	linfopoetina estromal do timo
T2	inflamação do tipo 2
UVB	radiação ultravioleta B
VCAM-1	proteína 1 de adesão celular vascular
VDR	receptor da vitamina D
VLA-4	antígeno 4 de expressão tardia
β2-AR	receptor β2 adrenérgico

SUMÁRIO

INTRODUÇÃO

A asma alérgica: considerações gerais

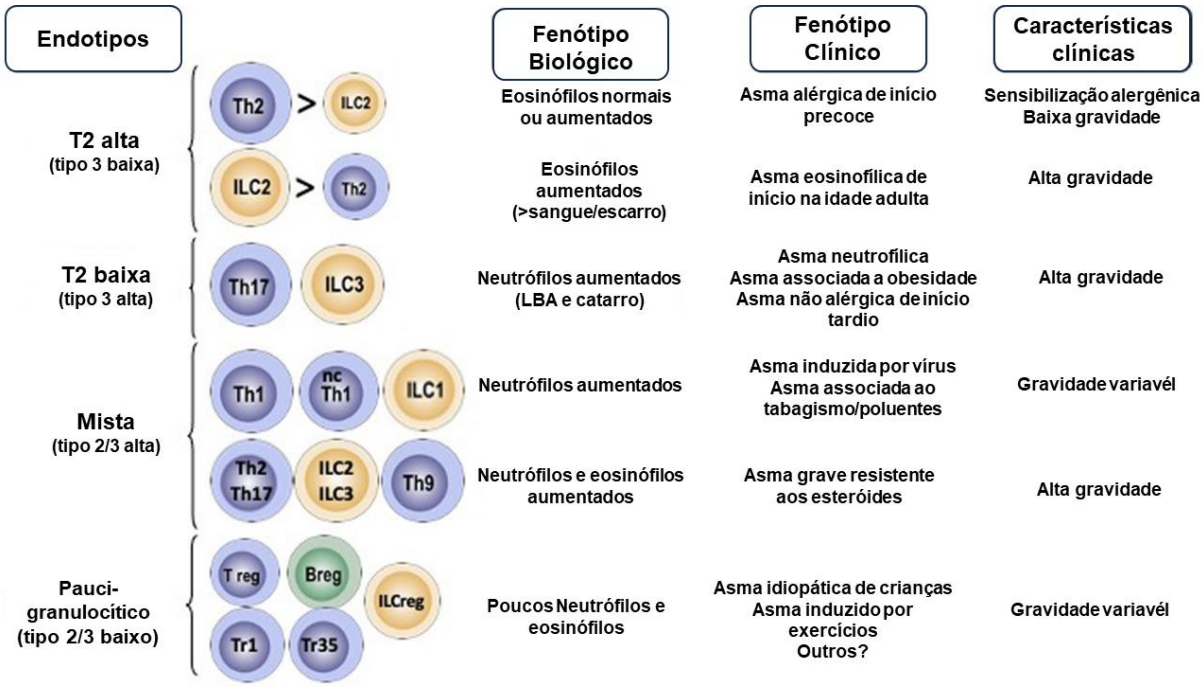
A asma alérgica (AA) é caracterizada por uma hiper-reatividade brônquica a substâncias ambientais, conhecidas como alérgenos. Os alérgenos mais comumente inalados são derivados de proteínas, e são particularmente encontrados em pólen, ácaros, pêlos de animais e fungos (ABBAS; LICHTMAN; PILLAI, 2019; BOSNJAK et al, 2011; COCKCROFT, 2010; HOLGATE et al., 2015; STERK, 2009). Na AA uma série de eventos inflamatórios desencadeiam recorrentes broncoespasmos, hipersecreção de muco nas vias aéreas, o que pode ocasionar a obstrução das vias aéreas (BOONPIYATHAD et al., 2019). Ademais, persistente inflamação pode levar à remodelação das vias aéreas caracterizada por uma hiperplasia das células caliciformes, hipertrofia do músculo liso, fibrose subepitelial e angiogênese (AKDIS, 2012; MEYER et al., 2012; WANG et al., 2018). A limitação variável do fluxo aéreo dos pacientes está associada a sintomas respiratórios, tais como: dispneia, tosse e opressão torácica retroesternal (BUSSE; LEMANSKE, 2001; COOKSON, 1999; FINOTTO, 2019; PIZZICHINI et al., 2020; REDDEL et al., 2015). Cerca de 70%-80% dos casos da AA são encontrados em indivíduos jovens (menos 40 anos) (ROMANET-MANENT et al., 2002; WENZEL, 2012). Em um subgrupo de paciente, no entanto, a AA se manifesta após os 40 anos e ocorre principalmente em mulheres com obesidade (FARZAN et al., 2022).

Uma fonte de alérgenos são os gatos domésticos, sendo a alergia a estes felinos classificada como hipersensibilidade mediada pela imunoglobulina da classe E (IgE). Nas últimas décadas registrou-se um aumento da sua incidência e estima-se que afeta aproximadamente 1 em cada 5 adultos no mundo (CHAN; LEUNG, 2018). Os sintomas da alergia a gatos variam de rinoconjuntivite leve a exacerbações da asma potencialmente fatais (GRÖNLUND et al., 2008). Cerca de 20% a 30% dos pacientes asmáticos desenvolvem sintomas graves ao entrarem em contato com gatos (DÁVILA et al., 2018). O diagnóstico da alergia a gatos é feito com base no histórico clínico, sintomatologia, conhecimento da sensibilização e reatividade positiva a alérgenos de gatos comprovada pela detecção dos níveis séricos da IgE-específica

e/ou teste cutâneo (DÁVILA et al., 2018; SINGH; HAYS, 2016). Segundo a Organização Mundial da saúde (OMS) e a União Internacional de Sociedades Imunológicas (IUIS), há 8 alérgenos *Felis domesticus* (Fel d1 à Fel d8), sendo o Fel d1 o alérgeno mais potente e onipresente (BONNET et al., 2018; CHANG; LEUNG, 2018; SATYARAJ; WEDNER; BOUSQUET, 2019). Encontrado principalmente na pele dos gatos e folículos capilares, o Fel d1 é produzido pelas glândulas sebáceas e os sacos anais, assim como pelas glândulas salivares e lacrimais, e é transportado por pequenas partículas, permanecendo em suspensão no ar por longos períodos (BONNET et al., 2018; CHARPIN et al., 1991; LICCARDI et al., 2003).

Independente do gatilho biológico, a asma é uma doença altamente heterogênea, sendo atualmente dividida em diferentes fenótipos (com base nas apresentações clínicas e histórico, propriedades patofisiológicas, características morfológicas, diagnóstico e resposta ao tratamento) e em diversos endotipos (baseado nos mecanismos moleculares ou fisiopatológicos subjacentes aos fenótipos) (figura 1) (AGACHE; AKDIS, 2019; LOTVALL et al., 2011; SOCIEDADE BRASILEIRA DE PNEUMOLOGIA e TISIOLOGIA, 2020; TAN et al., 2019).

Figura 1 – Endotipos/subendotipos e fenótipos definidos da asma



Legenda: lavado broncoalveolar (LBA); célula B reguladora (Breg); célula linfoide inata reguladora (ILCreg); célula T reguladora do tipo 1 induzida (Tr1); célula T reguladora induzida produtora

de IL-35 (Tr35); célula T auxiliar do tipo 1 não convencional (ncTh1); células T auxiliares (Th); células T reguladora (Treg); inflamação do tipo 2 (T2); células linfoides inatas (ILC)

Nota: Nos endotipos, as proporções de diferentes populações de células efetoras podem culminar em padrões subclínicos adicionais (ex: endotipo T2 alto). Cada endotipo (ou subendotipo) está associado à presença de eosinófilos ou neutrófilos no LBA ou no escarro, ao grau de gravidade da doença ou à características clínicas adicionais. Desta forma, a maioria dos endotipos ou subendotipos podem ser relacionados a maioria dos fenótipos da asma.

Fonte: Traduzido e adaptado de Maggi et al., 2022.

Segundo a OMS (2022), a asma é a doença crônica mais comum entre crianças e adolescentes, com prevalência de aproximadamente 262 milhões de pessoas em 2019 e mortalidade de 455 mil casos. Os países pobres e subdesenvolvidos possuem as maiores taxas de mortalidade, subdiagnóstico e subtratamento. O Brasil é o oitavo país com maior prevalência da asma, onde a doença é a quarta principal causa de internação pelo Sistema Único de Saúde (SUS) (ASSOCIAÇÃO BRASILEIRA DE ALERGIA E IMUNOLOGIA, 2019; SOCIEDADE BRASILEIRA DE PNEUMOLOGIA E TISIOLOGIA, 2012). Em um estudo observacional, descritivo e epidemiológico, realizado por Marques e colaboradores (2022), com dados secundários ao Departamento de Informática do Sistema Único de Saúde (DATASUS), do período de 2016 a 2020, coletados em 2021, foi constatado uma expressiva redução do número de internações por asma no Brasil em 2020 (o maior índice de internação foi observado na região Nordeste). Embora o número de hospitalização e a mortalidade estejam diminuindo na maioria das regiões, ambos associados a um maior acesso ao tratamento, os custos com o manejo da doença continuam elevados (BARRETO et al., 2014; CARDOSO et al., 2017; MARQUES et al., 2022). Entre 2008 e 2013 o custo médio das hospitalizações foi de 170 milhões de reais para o SUS, sendo os maiores custos observados nas regiões Nordeste e Sudeste (CARDOSO et al, 2017; MARQUES et al., 2022). Estima-se que os gastos com a asma grave consomem 25% da renda familiar, sendo que, segundo a OMS o ideal é que não exceda 5% (COSTA et al., 2018; FRANCO et al., 2009).

Não existe um padrão ouro para o diagnóstico da AA. O diagnóstico é baseado na histórico/avaliação clínica e na limitação variável ao fluxo aéreo expiratório (PAPI, et al., 2018). Para tal, diferentes testes de diagnóstico estão disponíveis, todavia o curso clínico da AA e a utilização de medicamentos ou não pelos pacientes no momento do teste afetam a sua sensibilidade e especificidade (BRIGHAM; WEST, 2015). De acordo com GINA (2021), em pacientes que não estão em tratamento, o

diagnóstico da AA é baseado na identificação de um padrão de sintomas respiratórios característico e nos testes de espirometria, reversibilidade do broncodilatador, variabilidade do pico do fluxo e teste de broncoprovocação. Ainda, testes alérgenos-específicos, tais como testes cutâneos e a dosagem da imunoglobulina E (IgE) no sague, podem ser aplicados, apesar da atopia não ser específico para a asma e não ser encontrada em todos os fenótipos.

No Brasil, apenas 12,8% dos asmáticos têm a doença controlada (CANÇADO et al., 2019). O controle da asma, segundo à Sociedade Brasileira de Pneumologia e Tisiologia (SBPT) (2012 e 2020), depende de um conjunto de medidas que reduzam as limitações clínicas, assim como, a melhora nas ações de prevenção. Infelizmente, nem todos os pacientes recebem a medicação adequada no tempo crítico de controle da doença, que deve ser logo após o início da limitação do fluxo aéreo (GINA, 2019; SOCIEDADE BRASILEIRA DE PNEUMOLOGIA E TISIOLOGIA, 2012).

Quanto a gravidade, a asma pode ser classificada em intermitente ou persistente leve, moderada e grave (*GLOBAL INITIATIVE FOR ASTHMA*, 2019). No Brasil, o monitoramento da asma é feito através do questionário GINA, o Questionário de Controle da Asma com 7 itens (ACQ-7) e o Teste de Controle da Asma (ACT) (SOCIEDADE BRASILEIRA DE PNEUMOLOGIA E TISIOLOGIA, 2020). Dado ao conhecimento limitado acerca da variedade de resposta ao esquema terapêutico, o manejo da asma continua sendo um grande desafio. Os diferentes fenótipos parecem implicar no desenvolvimento de diferentes endotipos. Atualmente, o endotipo mais comum e mais conhecido é a asma alérgica eosinofílica com inflamação do tipo 2 (T2) alta (LICARI et al., 2018; WENZEL, 2012).

Imunopatogenia da AA: inflamação T2 alta

Os asmáticos com inflamação T2 alta, usualmente, apresentam doença com início precoce associada a uma atopia mediada por IgE com eosinofília nas vias aéreas e sistêmicas. São mais responsivos ao tratamento com corticoides e a agentes biológicos que inibem a inflamação T2. Os níveis séricos da IgE (sIgE), eosinofilia no escarro e no sangue periférico e a fração exalada de óxido nítrico (FeNO) são amplamente utilizados como biomarcadores da inflamação T2 alta (BRUSSELLE;

KOPPELMAN, 2022; FAHY, 2015; SOCIEDADE BRASILEIRA DE PNEUMOLOGIA E TISIOLOGIA, 2020).

Classicamente, a AA, assim como todas as reações de hipersensibilidade do tipo I, induz resposta imune mediada por mastócitos e a IgE, embora seja crítico o papel das células T auxiliares do fenótipo 2 (Th2) (ABBAS; LICHTMAN; PILLAI, 2019; BURKS et al., 2013). A indução das células Th2 alérgeno-específicas depende de um processo de duas etapas: a sensibilização e o desafio (figura 1) (ABBAS; LICHTMAN; PILLAI, 2012; BOSNJAK et al., 2011; KOMLÓSI et al., 2021).

A resposta imune alérgica: sensibilização ao alérgeno

A fase da sensibilização (figura 2) inicia-se após o reconhecimento primário do alérgeno, que penetra a submucosa, pelas células apresentadoras de antígenos (APC, *antigen presenting cells*) profissionais, particularmente as células dendríticas (DCs, *dendritic cells*). Assim, após a sua internalização, o alérgeno é processado nos compartimentos subcelulares das DCs, e os peptídeos gerados acoplados às moléculas de histocompatibilidade principal da classe II (MHC, *major histocompatibility complex*) são apresentados às células T $CD4^+$ naïves via receptor da célula T (TCR, *T-cell receptor*). As DCs também fornecem um conjunto de sinais que culminam na ativação, proliferação e diferenciação dessas células em fenótipo Th2 efetor (EPSTEIN, 2006; FERREIRA, 2004).

Embora, as DCs possam ser diretamente ativadas pelo alérgeno, estudos demonstraram que a maturidade funcional dessas APCs depende da interação do alérgeno com as células do epitélio pulmonar (COLLISON et al., 2013; HAMMAD et al., 2009; LAMBRECHT; HAMMAD, 2015; WILLART et al, 2012). No estudo realizado por Gandhi e Vliagoftis (2015), a ativação dos receptores do tipo Toll (TLR, *toll like receptor*) 2 e TLR4, ou do receptor ativado por protease 2 (PAR2, *protease-activated receptor 2*), nas células epiteliais das vias aéreas inferiores induziu a liberação das quimiocinas (CXCL-1 e CCL-20) e das citocinas, Interleucina 1β (IL-1β), IL-1α, IL-8, IL-33, IL-25, linfopoetina estromal do timo (TSLP, *thymic stromal lymphopoietin*) e fator estimulador de colônias de granulócitos-macrófagos (GM-CSF, *granulocyte-macrophage coloby stimulating growth factor*), todas envolvidas na migração e

ativação das DCs nos locais de contato com os alérgenos inalados. A IL-1α, TSLP, IL-33 e GM-CSF, via supressão da IL-12, reprogramam a expressão gênica das DCs pulmonares para induzirem células Th2 (DECKERS et al., 2017; DE KLEER et al., 2016, WILLART et al., 2012).

Na presença da IL-4, as DCs induzem a diferenciação das T naives em linfócitos Th2 por ativar fatores de transcrição, o transdutor de sinal e ativador da transcrição 6 (STAT-6, *signal transducer and activator of transcription 6*) e o fator de transcrição de ação "trans" específico de células T (GATA-3, *GATA binding protein 3*) (JINFANG, 2015; SCHLITZER et al., 2013; WALFORD; DOHERTY, 2013). O GATA-3 é responsável por regular positivamente a síntese das citocinas características das células Th2, à IL-4, IL-5 e IL-13 (KRABBENDAM et al., 2018; KURUVILLA; LEE, F.E.; LEE, G.B., 2019; ZOSKY; SLY, 2007). Adicionalmente, o GATA-3 é responsável pela estabilização do fenótipo Th2 por impedir o desenvolvimento das células T auxiliares do fenótipo 1 (Th1), através da repressão do receptor da IL-12 (IL-12R) (RAYEES et al., 2013). Entre as funções biológicas exercidas pelas citocinas relacionadas ao fenótipo Th2, a IL-4 e IL-13 aumentam a infiltração de leucócitos no local de deposição dos alérgenos por elevar, nas células endoteliais locais, a expressão de moléculas de adesão, como por exemplo a proteína 1 de adesão celular vascular (VCAM-1, *vascular cell adhesion molecule 1*), que facilita a transmigração dos eosinófilos e das células Th2, que expressam o antígeno 4 de expressão tardia (VLA-4, *very late antigen-4*). Ainda, a IL-5 não apenas aumenta a sobrevida dos eosinófilos, como também é responsável pela sua formação a partir de precursores na medula-óssea (ABBAS; LICHTMAN; PILLAI, 2019; ROTHENBER, 1998).

Na resposta imune humoral associada às células Th2, a produção de IgE pelas células B foliculares alérgeno-específicas é dependente das células T auxiliares foliculares do tipo 2 (T_{FH2}), caracterizadas pela expressão superficial do receptor de quimiocina CXCR5 e da proteína de morte celular programada 1 (PD-1, *programmed death 1*), além da expressão intracelular da proteína do linfoma 6 de células B (Bcl6, *B-cell lymphoma 6*) e secreção das citocinas IL-4 e IL-21 (BURKS et al., 2013; CROTTY, 2011, 2015; KIM et al., 2001).

Nos centros germinativos (GC), local de interação entre as células T_{FH2} com os linfócitos B, as células T_{FH2} auxiliam nos eventos de seleção das células B de alta afinidade, mudança de classe de anticorpo, além de fornecerem sinais primordiais para a ativação e diferenciação das células B em plasmócitos e células B de memória,

ambos subtipos de células de vida longa (CROTTY, 2011; CYSTER; ALLEN, 2019; FERREIRA, 2004; MESIN; ERSCHING; VICTORA, 2016). No contexto das AA, as citocinas IL-4, IL-13 e IL-21 produzidas pelas células T_{FH2} induzem produção de altos níveis de IgE alérgeno-específico (AKDIS, M.; AKDIS, C. 2012; GOULD; RAMADAMI, 2015). A IgE é capaz de se ligar ao receptor IgE de alta afinidade (FcεRI) presente na superfície dos mastócitos e basófilos, conduzindo essas células a um estado de sensibilização (XIE; DENT, 2019). Levando-se em consideração que a AA é uma apresentação crônica das reações de hipersensibilidades do tipo 1, a fase de sensibilização do indivíduo também envolve a ligação de IgE aos eosinófilos recrutados para o epitélio das vias aéreas inferiores (CAPRON et al., 1995; KITA, 2011).

Apesar do reconhecimento do papel das células $T_{FH}2$ na indução da IgE, Gowthaman e colaboradores (2019) descreveram uma nova subpopulação das T_{FH} relacionada à anafilaxia dependente de IgE, denominada $T_{FH}13$. Quando comparada às células $T_{FH}2$, as células $T_{FH}13$ produzem elevados níveis de IL-13 e muito pouca, ou nenhuma, IL-21. De forma interessante, enquanto as células $T_{FH}2$ induzem a produção da IgE de baixa afinidade, a produção da IgE de alta afinidade aos alérgenos pelas células B depende da ajuda das células $T_{FH}13$ (GOWTHAMAN et al., 2019; GOWTHAMAN; CHEN; EISENBARTH, 2020). Níveis elevados do receptor da IL-13, IL-13Rα1, são encontrados nas células B IgE^+ e B $IgG1^+$ do GC (GOWTHAMAN et al., 2019). Em humanos, elevada frequência das células $T_{FH}13$ circulantes foi associada a altos níveis da IgE alérgeno-específicas. Curiosamente, as células $T_{FH}13$ e a IgE de alta afinidade não são produzidos durante e infecção por helmintos, apesar do desenvolvimento da resposta Th2 robusta (GOWTHAMAN et al., 2019; XIE; DENT, 2019), justificando, ao menos em parte, porque infecções por helmintos não garante proteção total a novas reinfecções por esses parasitas.

A resposta imune alérgica: desafio

Após a sensibilização, a subsequente exposição ao mesmo alérgeno, conhecido como desafio (figura 2), provoca uma resposta imune rápida e robusta. Na AA, essa representa a fase clínica da doença e é disparada pela ativação dos

mastócitos e eosinófilos previamente sensibilizados após o reconhecimento dos alérgenos inalados (GALLI; TSAI, 2012; HAMMAD et al., 2009).

Nos mastócitos, a ligação cruzada de FcεRI/IgE aos alérgenos provoca uma série de eventos intracelulares que culminam na liberação dos mediadores pré-formados, como as histaminas, triptase e proteases, alinhados à síntese e liberação de mediadores lipídicos, tais como os leucotrienos D4 (LTD4), prostaglandina D2 (PGD2) e as citocinas IL-4, IL-5, IL-13 e o fator alfa de necrose tumoral (TNF-α, *tumoral necrosis factor-α*). A ação desses mediadores pró-inflamatórios é responsável pela broncoconstrição intensa, hipersecreção de muco e edema nas paredes das vias aéreas (AMIN, 2012; BOSNJAK et al., 2011; FAHY; DICKEY, 2010; MURDOCH; LLOYD, 2010; MORGAN et al., 2021). Esses mediadores levam a alterações na vasculatura do músculo, amplificando a infiltração de eosinófilos, células que marcam a fase tardia das reações atópicas e que contribuem para a inflamação crônica e o remodelamento das vias aéreas (MURDOCH; LLOYD, 2010).

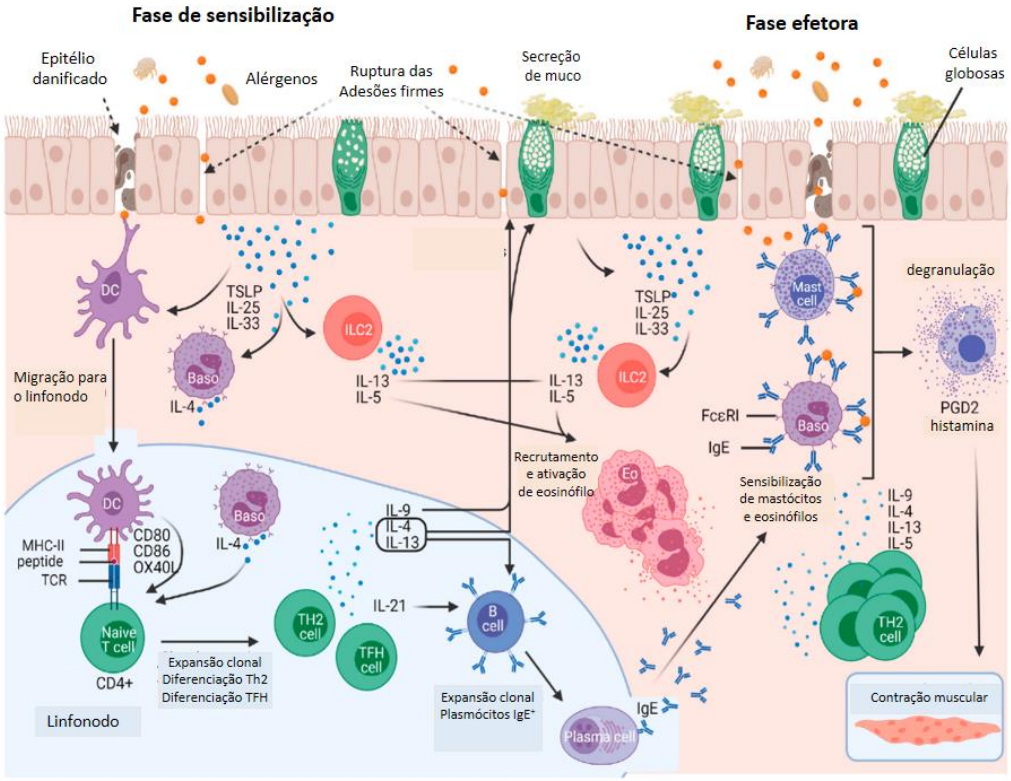
Além dos mastócitos, outras células do sistema imune inato são importantes na patogênese da asma alérgica, como as células linfoides inatas do tipo 2 (ILC2, *innate lymphoid cell 2*) no pulmão (HELFRICH et al., 2019). Essas células são encontradas na borda da barreira epitelial e estão envolvidas na inflamação da mucosa, homeostase e reparo tecidual (KRABBENDAM et al., 2018; WALLRAPP et al., 2018). As ILC2 expressam o GATA-3 e secretam IL-5, IL-9 e IL-13, sendo consideradas a principal fonte inicial da IL-13 (LICONA-LIMON et al., 2013). São ativadas e proliferam em resposta às alarminas IL-25, IL-33 e TSLP produzidas pelas células epiteliais danificadas. Provavelmente, a produção sustentada de IL-5 e IL-13 pelas ILC2 deve ser capital para a indução das células Th2, potencialização da hiperprodução de muco e hiper-reatividade das vias aéreas e aumento no recrutamento de eosinófilos para as áreas em contato com os alérgenos inalados (HALIM et al., 2012, 2014; HALIM; MCKENZIE, 2013). Ainda, em um modelo animal de sensibilização ao amendoim, a expansão de ILC2 IL-13^{+} foram primordiais para o desenvolvimento das células T_{FH} e produção da IgE (KREMPSKI et al., 2020).

Como comentado anteriormente, a sensibilização alérgica na asma já envolve o recrutamento de eosinófilos, subtipo de leucócito que caracteriza a fase tardia da reação de hipersensibilidade do tipo imediata. A ativação dos eosinófilos pelo complexo IgE/alérgeno resulta na sua desgranulação e consequente liberação de proteínas tóxicas, secreção de citocinas inflamatórias, mediadores lipídicos e a

produção de espécies reativas de oxigênio (ROS, *reactive oxigen species*). A proteína básica principal (MBP, *major baisc protein*), proteína catiônica eosinofílica (ECP, *eosinophilic cationic protein*), a peroxidase eosinofílica (EPO, *eosinophilic peroxidation*) e as ROS são tóxicos para as células epiteliais e estão associados ao remodelamento brônquico e hiperresponsividade brônquica (CAÑAS et al., 2018; DRAKE et al., 2018). As citocinas GM-CSF e a IL-13 potencializam as funções eosinofílicas, tais como a citotoxicidade, produção de superóxido, produção de leucotrienos da série 4 e a desgranulação induzida por complexos de FcεR/IgE/Alérgeno (NAGATA et al., 1999). Ainda, eosinófilos ativados liberam armadilhas extracelulares eosinofílicas (EET, *eosinophil extracelular traps*), compostas por DNA e proteínas granuladas tais como MBP e ECP, induzindo a inflamação e hiperresponsividade das vias aéreas (CHOI; SIM; PARK, 2020; DWORSKI et al., 2011). *In vivo*, as EETs são capazes de ativar as ILC2s do parênquima pulmonar por estimular as células epiteliais das vias aéreas há secretarem a IL-33 e TSLP (CHOI et al., 2020). Através de um mecanismo de retroalimentação positivo, as células Th2 aumentam a produção de quimiocinas eosinofílicas, eotaxina-1 e eotaxina-2, nos pulmões, perpetuando a infiltração destas para as vias aéreas (GREENFEDER et al., 2001; STAUDT et al., 2010; TRAVERS; ROTHENBERG, 2015). Vale a pena ressaltar, que a maioria dos pacientes com AA persistente também apresentam rinite alérgica envolvendo os mesmos mecanismos biológicos. Nesse cenário, o papel dos basófilos é pouco explorado (MIYAKE; KARASUYAMA, 2017).

A fase tardia da reação de hipersensibilidade do tipo imediata é caracterizada também pela presença de DCs IL-4^{+}GM-CSF^{+} que ativam e induzem a migração de células Th2 de memória central e efetora para a lâmina própria do pulmão, onde aumentam o recrutamento e a atividade não apenas dos eosinófilos como também dos monócitos (BELLINI et al., 1993; MAES; JOOS; BRUSSELLE, 2012). Ainda, nos pacientes cronicamente expostos ao alérgeno, estruturas semelhantes aos folículos linfoides dos linfonodos podem ser encontradas nas submucosas do trato respiratório inferior. Essas estruturas permitem uma produção local de IgE alérgeno-específico devido a colaboração produtiva entre as células $T_{FH}2$, e provavelmente células $T_{FH}13$, com as células B (CHVATCHKO et al.,1996).

Figura 2 - Eventos envolvidos na imunopatogênese da asma alérgica T2 alta



Legenda: células dendríticas (DCs); célula T auxiliar (Th); célula T auxiliar folicular (T_{FH}); interleucina (IL); imunoglobulina (Ig); receptor da Ig de alta afinidade (FcεRI); células linfoides inatas do tipo 2 (ILC2); linfopoetina estromal do timo (TSLP); basófilo (Baso); mastócito (Mast cell); eosinófilo (Eo); prostaglandina D2 (PGD_2); plasmócitos (Plasma cell); células B (B cell).

Nota: Durante a sensibilização alérgica, as DCs capturam, processam e apresentam peptídeos alergênicos via MHCII, promovendo a ativação e diferenciação das células T $CD4^+$ naïves em células Th2 efetoras alérgeno-específicas, um processo dependente, parcialmente, da IL-4. Para auxiliar esse processo, as células epiteliais das vias aéreas secretam um conjunto de moléculas pró-inflamatórias (TSLP, IL-25, IL-31, IL-33) que favorecem a ativação e a migração das DCs. Observa-se a expansão clonal e a ativação das células Th2 específicas para o alérgeno, e a partir da secreção da IL-4 e IL-13, promovem a produção de IgE. A IgE específico ao alérgeno liga-se a superfície de células efetoras (principalmente mastócitos e eosinófilos) através do FcεRI, causando a sensibilização do paciente. Nesta fase também são gerados um conjunto de células B e Th2 de memória. Durante o desafio, em pacientes sensibilizados, a nova exposição ao alérgeno, e sua ligação às regiões variáveis das IgEs ligadas aos FcεRI expressos nas superfícies dos mastócitos e eosinófilos, culmina na liberação imediata da histamina (mastócitos) e outros mediadores da inflamação, particularmente os leucotrienos, IL-4 e IL-13. Esses mediadores pró-inflamatórios são responsáveis pelos sintomas da fase aguda da asma atópica. O acúmulo desses mediadores e a ativação das Th2 alérgeno-específicas, em cooperação com as ILC2, desempenham um papel central na manutenção dos níveis da IgE especifico ao alérgeno, eosinofília e o recrutamento de células inflamatórias para os tecidos inflamados. Também, são importantes na produção de muco, inflamação e dano tecidual, resultando nas manifestações clínicas mais graves e a inflamação crônica. O papel dos basófilos na AA humana é muito pouco explorado. O envolvimento das células Th9 na AA parece envolver a capacidade da IL-9 em potencializar vários eixos biológicos implicados na gravidade da doença de base celular Th2.

Fonte: Traduzido e adaptado de Komlósi et al., 2021

Recentemente, as células T auxiliares 9 (Th9) foram implicadas na patogenia da asma alérgica T2 alta (KOCH; SOPEL; FINOTTO, 2017). A diferenciação das Th9 ocorre na presença da IL-4 e TGF-β e depende da expressão do fator de transcrição PU.1 (JABEEN et al., 2013; KAPLAN, 2013; LICONA-LIMON, 2013; NEURATH; KAPLAN, 2017). A combinação da IL-4 e TGF- β promove a regulação positiva da expressão de IL-9 sem induzir a transcrição do GATA-3 (VELDHOEN, 2008). A IL-9, a citocina de assinatura desse fenótipo, é responsável pela ativação do fator de crescimento dos mastócitos para induzir a ativação das células B e pela metaplasia das células globulares através da ILC2 (KOCH; SOPEL; FINOTTO, 2017). Por induzir a produção da eotaxina, a IL-9 facilita a infiltração dos eosinófilos e amplifica a capacidade da IL-5 e IL-13 em aumentar a sobrevida dos eosinófilos e a produção de muco, respectivamente (KOCH; SOPEL; FINOTTO, 2017). Estudo em humanos revelou que a contagem das células Th9 e os níveis séricos de IL-9 estão aumentados no sangue periférico de pacientes com AA (HOPPENOT, 2015).

Os mediadores inflamatórios liberados pelas células Th2, células B e os eosinófilos ativados desencadeiam a contração do músculo liso, vasodilatação, exsudação plasmática e secreção de muco. Esse processo inflamatório, juntamente com as mudanças estruturais existentes na parede brônquica, causa uma redução significativa no diâmetro interno das vias aéreas (FERREIRA, 2004). A perpetuação da resposta imediata e da tardia leva ao desenvolvimento da doença crônica, pois os mastócitos, eosinófilos e os linfócitos Th2/Th9 contribuem para a manutenção das citocinas envolvidas na inflamação T2 alta (GORDON et al., 2016; WAKAHARA et al., 2013).

A inflamação persistente das vias aéreas dos pacientes com AA pode levar a sua remodelagem (HOLGATE; POLOSA, 2006; MALMBERG et al., 2020). A remodelagem é resultante da extensa deposição de componentes da matriz, com hipertrofia da musculatura lisa brônquica, angiogênese e a secreção de muco e, tem como consequência a obstrução das vias aéreas associada ao dano do epitélio ciliado (ANDERSSON et al., 2018; HEIJINK et al., 2020). Em longo prazo, essas alterações aumentam a hiperresponsividade a uma variedade de estímulos externos e maior suscetibilidade a infecções (HELBY et al., 2017). O grau da hiperresponsividade, aferido pela espirometria após provocação com metacolina, ajuda a determinar a gravidade da asma nesses pacientes. A frequência dos episódios de exacerbação e a resposta aos broncodilatadores e corticoides também são parâmetros usados para

determinar formas leves, moderadas e graves da asma persistente (BECKER; ABRAMS, 2017; REDDEL et al., 2015).

A base do tratamento farmacológico da AA consiste no uso de corticoide inalatório (CI) isolado ou associado a um β2- agonista de longa duração (LABA) (MANTIS; ROL; CORTHÉSY, 2011; SOCIEDADE BRASILEIRA DE PENUMOLOGIA E TISIOLOGIA, 2020). Para fins de controle da doença, a dose da CI é aumentada progressivamente, podendo também ser associado ao uso de outros fármacos, tais como β2- agonista de curta duração (SABA) e antagonista muscarínico de longa duração (LAMA) (SOCIEDADE BRASILEIRA DE PENUMOLOGIA E TISIOLOGIA, 2020). O controle das crises nos pacientes com asma grave requer altas doses de CI e de LABA (HOLGATE; POLOSA, 2006; REDDEL et al., 2015). Em casos mais graves, muitos pacientes precisam ser internados para fazer o tratamento farmacológico, envolvendo, por exemplo, manejo da exacerbação com corticoides intravenosos e sulfato de magnésio (REDDEL et al., 2015).

Pacientes com AA eosinofílica persistente podem beneficiar-se do uso de imunobiológicos (BUSSE et al., 2019). Cinco anticorpos monoclonais, aprovados pela *Fod and Drug Administration* (FDA) e *European Medicines Agency* (EMA), são usados como fármacos para o tratamento da asma grave: (i) omalizumabe, anti-IgE, que inibe a ligação da IgE ao FcεRI e FcεRII; (ii) benralizumabe, antagonista do receptor da IL-5; (iii) dupilumabe, anti-IL4Rα, inibe a sinalização via IL-4 e IL-13; (iv) mepolizumabe e (v) reslizumabe, inibem a ligação da IL-5 ao seu receptor IL5Rα. Recentemente, o anticorpo anti-TSLP, tezepelumabe, foi aprovado pelo FDA para o tratamento da asma grave em adultos e adolescentes acima e 12 anos de idade (PAPAPOSTOLOU & MAKRIS, 2022; SOCIEDADE BRASILEIRA DE PNEUMOLOGIA E TISIOLOGIA, 2020). No Brasil, o omalizumabe, mepolizumabe e benralizumabe são aprovados pela Agência Nacional de Vigilância Sanitária (ANVISA) para o tratamento da asma grave, sendo que o omalizumabe e mepolizumab estão disponíveis no SUS. Diversos estudos demonstraram que esses fármacos melhoraram a função pulmonar e os sintomas, reduziram as exacerbações, resultando no aumento, de forma significativa, da qualidade de vida e a resposta à terapêutica convencional (BEECKER et al., 2017; CHUPP et al., 2017; HALDAR et al., 2009; RUBIN et al., 2012).

Aproximadamente 10% dos asmáticos são refratários aos tratamentos atuais. Esses pacientes, no entanto, não respondem bem a imunobiológicos para AA padrão, sugerindo uma nova entidade endotípica da doença. De fato, estudos apontam o

envolvimento de células T auxiliares do fenótipo 17 (Th17). Nesses pacientes, elevado número de neutrófilos são frequentemente encontrados nos pulmões, assim como, níveis aumentados da IL-17 foram detectados em amostras do lavado broncoalveolar (LBA) e em biópsias pulmonares (AL-RAMLI et al, 2009; CHANG et al., 2017; MOLET et al., 2001; TREVOR; DESHANE, 2014).

Gravidade da AA: potencial papel das células Th17

As células Th17 são promotoras da inflamação neutrofílica e são associadas à resposta protetora contra às bactérias extracelulares e fungos. Classicamente, as Th17 sintetizam a IL-17F, IL-17A (IL-17), sua citocina de assinatura, a IL-21 e IL-22. A diferenciação desse fenótipo celular nas células T $CD4^+$ depende da indução dos fatores de transcrição receptor γt órfão relacionado à RAR (RORγt, *RAR-related orphan receptor-γ*) e STAT-3, induzidos pela combinação de TGF-β, IL-6, IL-1β e IL-23 (BEDOYA et al., 2013; GHORESCHI et al., 2011; JOERGER et al., 2016; REPPERT et al., 2011; KAKKAR; LEE, 2008; RAMAK-RISHNAN; AL HEIALY; HAMID, 2019; XU et al., 2018). A IL-23 garante a estabilidade do fenótipo e, curiosamente, elevadas concentrações plasmáticas desta citocina foram detectadas em pacientes asmáticos, sendo seus níveis correlacionados diretamente com pior função pulmonar (MCGEACHY et al., 2009; ZHANG et al., 2016). A IL-17 induz as células da imunidade inata e parenquimatosas a secretarem o ligante 8 da quimiocina CXC (CXCL8), a principal quimiocina envolvida no recrutamento de neutrófilos. A inflamação neutrofílica está associada a maior produção de citocinas pró-inflamatórias (TNF-α, IL-1, IL-6, IL-18, IL-17, IL-21 e IL-23) (LOURENÇO et al., 2022). Em resposta à IL-17, as células epiteliais das vias aéreas e do parênquima do pulmão produzem a IL-6, IL-1, TNF-α e IL-8, amplificando os processos patológicos associadas à asma Th17-dependente (BHAKTA et al., 2011; RAY; KOLLS, 2017).

Numerosos estudos têm relatado um aumento das células Th17 e IL-17 nos pacientes asmáticos. Segundo Breton e colaboradores (2018), altos índices da IL-17 foram encontrados em biópsias brônquicas da asma grave. Esses pacientes têm sido enquadrados no endotipo neutrofílico, um subtipo da asma T2 baixa (AKDIS, et al., 2012; ANO et al.,2013; CHOY, et al., 2015). Em estudo publicado por Wang e

colaboradores (2010), intensa infiltração de neutrófilos foi observada nas vias aéreas de pacientes asmáticos, principalmente os com pobre resposta aos corticoides. Ademais, segundo Alcorn, Crowe e Kolls (2010), a transferência de células Th17 alérgeno-específicas, secretoras de IL-17A e IL-17F para camundongos induziu, após sensibilização ao alérgeno, infiltração de neutrófilos e linfócitos Th17 nos pulmões. A infiltração dos neutrófilos foi associada à produção da IL-1β, IL-6 e GM-CSF pelas células epiteliais das vias aéreas e pelas células endoteliais e fibroblastos, sendo todos esses eventos dependentes da IL-17. Nos pacientes com AA, expansão das células inatas linfoides do tipo 3 (ILC3), também produtoras da IL-17 e IL-22, tem sido descrita e correlacionada com o número de neutrófilos e com elevada taxa de exacerbação no teste com metacolina (HEKKING et al., 2018; YU et al., 2014).

Além das células Th17, a ativação do inflamossoma NLRP3 (*nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3)* parece contribuir para a inflamação neutrofílica em pacientes asmáticos (SIMPSON et al., 2014). O NRLP3 é responsável pela produção das formas biologicamente ativas da IL-1β e da IL-18. O papel da IL-1β na asma envolve a sua capacidade de favorecer a diferenciação das células Th17 (LEE; SONG; PARK, 2014). Uma direta associação entre a presença de IL-1β com os níveis da IL-8 no escarro dos asmáticos neutrofílicos foi observada por Simpson e colaboradores (2014). A presença da IL-8, com aumento no recrutamento e ativação de neutrófilos, é um evento central na gravidade desse endotipo da asma.

A asma grave está associada ao aumento da remodelação das vias aéreas, caracterizada pelo aumento da metaplasia das células das mucosas e aumento da massa muscular lisa das vias aéreas. Acredita-se que a presença das Th17 imprima um endotipo mais agressivo a asma. Estudos sugerem que o aumento das $Th17^{+}IL\text{-}17^{+}$, em humanos e camundongos, resulta na hipersecreção de muco, hiperresponsividade e aumento da espessura da musculatura lisa e o remodelamento das vias aéreas (FAHY, 2009; PETERS et al., 2016b; KOCH; SOPEL; FINOTTO, 2017; KUDO; MELTON; CHEN, 2012; WANG et al., 2010; ZIJLSTRA; TEN HACKEN; HOFFMANN, 2012)

As células Th2 não são uma linhagem estável e totalmente diferenciada. Foi descrito um subtipo de célula $TCD4^{+}GATA\text{-}3^{+}ROR\gamma t^{+}$ de memória capaz de produzir em simultâneo a IL-4 e a IL-17 (COSMI et al., 2010; IRVIN; ZAFAR; GOOD, 2014; OHNMACHT; PARK; CORDING, 2015). Wang e colaboradores (2010) demonstraram

que em camundongos as citocinas IL-1β, IL-6 e IL-21 podem diretamente induzir a expressão de RORγt e a produção da IL-17 por células de memória Th2 clássica, sugerindo uma plasticidade fenotípica em resposta aos processos inflamatórios. Essas células Th2/Th17 híbridas já foram encontradas no LBA de alguns pacientes com asma grave (IRVIN; ZAFAR; GOOD. 2014; OHNMACHT; PARK; CORDING, 2015; WANG et al., 2010). Esse endotipo de AA é conhecido como misto, pois é caracterizado pela infiltração abundante de neutrófilos e eosinófilos nos pulmões. É possível que esse endotipo seja responsável pelas respostas sub-ótimas à terapia padrão em alguns pacientes anteriormente considerados apenas T2 alto.

De forma interessante, os mastócitos produzem a IL-17 em resposta ao TNF-α, que é geralmente sintetizada durante a resposta mediada pelas células Th17 (HUEBER et al., 2010). Em um modelo animal da AA induzida por ovalbumina (OVA), a deleção do gene da IL-17 reduziu significativamente a infiltração de eosinófilos para os pulmões, o que foi correlacionado a melhora da função pulmonar. Esses achados sugerem que a IL-17 é capaz de potencializar o recrutamento dos eosinófilos, como também revelam a existência de um endotipo da asma alérgica que envolve células $TCD4^{+}IL\text{-}4^{+}IL\text{-}17^{+}$ (OHNMACHT et al., 2015).

Devido ao seu papel na asma alérgica grave, estudos têm sido desenvolvidos com intuito de bloquear as moléculas envolvidas na indução e função das células Th17 (YAMASAKI; OKAZAKI; HARADA, 2022). O inibidor de STAT-3, C188-9, foi utilizado com sucesso no controle da asma induzida por ácaros em camundongos. Ademais, o C188-9 reduziu o infiltrado de células Th2/Th17 nesses animais. A deficiência na expressão da IL-6, uma citocina chave envolvida na diferenciação das células Th17, reduziu a hiperreatividade das vias aéreas, sendo esse fenômeno associado a queda do infiltrado das células $TCD4^{+}IL\text{-}4^{+}IL\text{-}17^{+}$ nos pulmões dos camundongos (GAVINO et al., 2016).

Além da gravidade da AA envolvendo a presença de neutrófilos, a produção de citocinas inflamatórias relacionadas ao fenótipo Th17, tais como IL-6 e IL-21, devem impactar profundamente na gravidade das exacerbações por danificar vários mecanismos de regulação imune envolvidos na homeostasia.

Regulação da resposta imune na AA

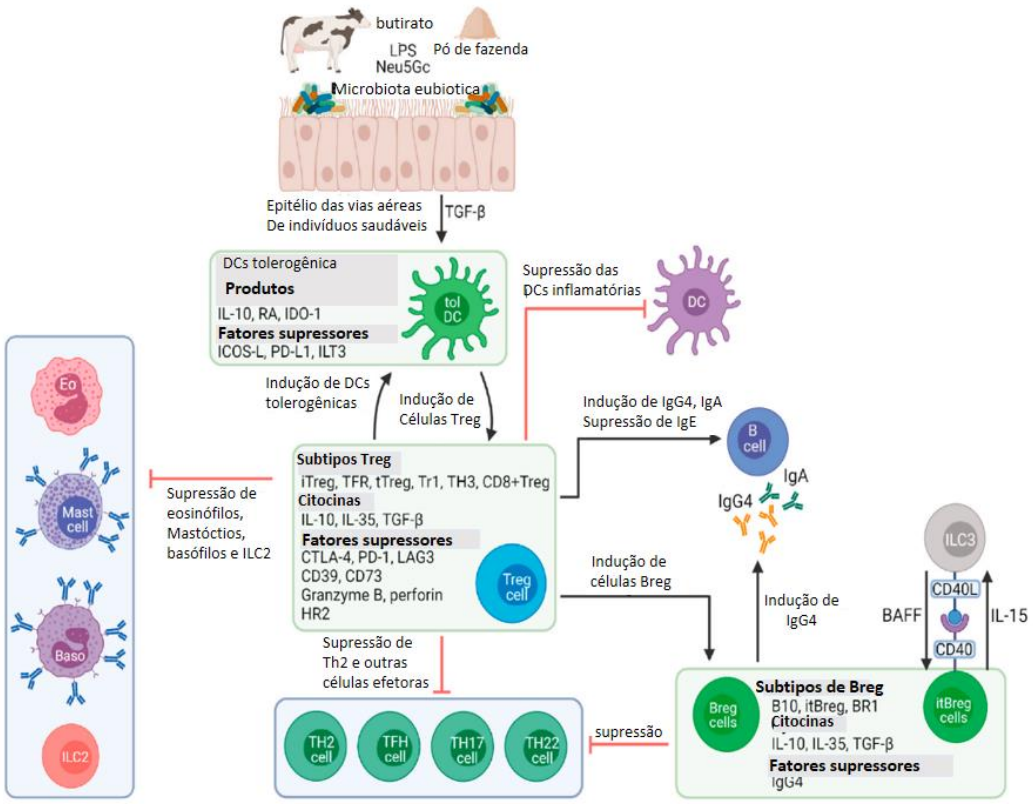
As células T reguladoras (Treg) constituem cerca de 5-15% das células T $CD4^+$ periféricas e são responsáveis pela supressão da resposta imune exacerbada por diferentes mecanismos (figura 3) (CLEMENTE et al., 2019; KOMATSU et al., 2009). Existem duas populações principais de células Tregs: os linfócitos T reguladores naturais (nTregs) e os linfócitos T reguladores induzidos (iTregs) (figura 3) (CLEMENT et al., 2019; KOMATSU et al., 2009; LANGIER; SADE; KIVITY, 2010; XYSTRAKIS et al., 2007).

As nTregs originam-se do timo e obtém a estabilidade fenotípica e funcional durante a seleção e maturação no timo, e respondem aos antígenos próprios. Expressam os marcadores Foxp3 (Fator de transcrição *forkhead box* P3), elevados níveis da CD25 (subunidade alfa do receptor de IL-2) na superfície e são negativas para IL-7Rα/CD127 (cadeia alfa do receptor da IL-7). Outras moléculas de superfície, não exclusivas dessa população, são expressas, a citar: PD1, antígeno citotóxico 4 de linfócitos T (CTLA-4, *cytotoxic T lymphocyte antigen 4*), CD45RO (isoforma da tirosina fosfatase CD45) e proteína relacionada ao TNFR induzida por glicocorticoide (GITR, *glucocorticoid-induced tumor necrosis factor-related receptor*). Quando ativadas podem produzir grandes quantidades de IL-10, TGF-β e IL-35 (BAECHER-ALLAN et al., 2001; BODEN et al., 2003; CARTER et al., 2002; SEDDIKI et al., 2006; ZHAO; WANG, 2018).

As iTregs são derivados dos tecidos linfoides periféricos e são gerados a partir de células T $CD4^+Foxp3^-$ convencionais naïves após entrar em contato com um antígeno apresentado por CDs $IL\text{-}10^+TGF\text{-}\beta^+$ tolerogênicas. Com ampla especificidade, as iTregs são encontradas em grande número nas mucosas do trato digestório e do pulmão. A partir da expressão diferencial de marcadores, as iTregs são divididas em três subpopulações: iTregs convencionais ($CD25^+Foxp3^+TGF\text{-}\beta^+IL\text{-}10^+$), as células Th3 ($CD25^{low}Foxp3^+TGF\text{-}\beta^+$) e os linfócitos T reguladores do tipo 1 (Tr-1, $CD25^{low}Foxp3^-TGF\text{-}\beta^-IL\text{-}10^+$) (CHEN et al., 2003; FARIA; WEINER, 2005; KOMATSU et al.,2009; NOVAL; CHATILA, 2016; RONCAROLO et al., 2006; ZHAO; WANG, 2018). No contexto das reações atópicas, os linfócitos Tr1 inibem a proliferação e a produção de citocinas relacionadas ao fenótipo Th2 (COTTREZ et al., 2000; MATYSZAK et al., 2000; HAMZAOUI et al., 2014; WU et al., 2007).

Além de produzir citocinas anti-inflamatórias, a capacidade das células T reguladoras, que são principalmente do tipo CD4, de expressar PD-1, CTLA-4 (CD152), LAG-3 e CD39/CD73 auxiliam no controle das reações de hipersensibilidades por inibir diretamente células da imunidade inata, particularmente as CDs (PD-L1^{+}CD80^{+}HLA-DRII^{+}), e os linfócitos T efetores (PD-1^{+}A2AR^{+}). Ainda, células B reguladoras produtoras de IL-10 (Br-1) e TGF-β (Br-3) podem também auxiliar na tolerância imune por controlar reações inflamatórias exacerbadas (KESSEL et al., 2012; MA et al., 2020) .As Br-1 são capazes de inibir as APCs, comprometendo assim o processo de apresentação de antígenos, bem como suprimem a produção da IL-4 pelas células Th2, o que resulta na diminuição da produção da IgE. Adicionalmente, a IL-10 aumenta a sobrevida, proliferação, diferenciação e mudança de classe de imunoglobulina dos linfócitos B para IgA e IgG4 (JASPER et al., 2017).

Figura 3 - Mecanismos de tolerância aos alérgenos



Legenda: célula dendrítica tolerogênicas (tol DC); célula dendrítica (DC); eosinófilo (Eo); mastócito (Mast cell); basófilo (Baso); célula linfoide inata do tipo 2 (ILC2); célula T reguladora (Treg cell); células T auxiliar (Th); células B (B cells); células B reguladora (Breg cells);

imunoglobulina (Ig); célula linfoide inata do tipo 3 (ILC3). células B reguladoras induzidas (itBreg); *B-cell activating fator* (BAFF).

Nota: As células dendríticas tolerogênicas induzem a diferenciação das células Treg. As Treg são capazes de inibir eosinófilos, mastócitos, basófilos, ILC2 e as células T efetoras pró-inflamatórias, através da produção das citocinas anti-inflamatórias e outras moléculas inibitórias. A IL-10, derivada das Tregs, promove a diferenciação das células Breg e a produção da IgA e IgG4. As próprias Breg produzem citocinas anti-inflamatórias, assim inibem as células T efetoras: as Br1 produzem a IgG4 e as itBreg, através da produção da IL5, ativam as ILC3 $CD40L^{+}$, que por sua vez regulam a expansão das itBreg, através do BAFF.

Fonte: Traduzido e adaptado de Komlósi et al., 2021.

Atualmente, evidências científicas revelam que a desregulação das Tregs é um fator importante na gravidade da AA. Estudos em pacientes asmáticos, desenvolvidos por Baatjes e colaboradores (2015) e Hartl e colaboradores (2007), identificaram uma frequência menor de Tregs no sangue periférico de pacientes com asma moderada em comparação aos controles saudáveis e poucos Tregs no LBA de crianças asmáticas em comparação aos não-asmáticos, respectivamente. Entre os pacientes AA, a frequência das Tregs foi significativamente menor nos pacientes com asma moderada à grave em comparação aos com asma leve (ABDULAMIR et al., 2009). Adicionalmente, em asmáticos, a expressão do FoxP3 nas células Tregs é reduzida em comparação a controles saudáveis, o que impacta diretamente em sua menor capacidade de inibir resposta inflamatórias (MARQUES et al., 2015; LIN et al., 2008; THUNBERG et al., 2010). Em modelo animal, a remoção das Tregs, após a sensibilização ao alérgeno, agravou a inflamação e a hiper responsividade das vias aéreas (LEWKOWICH et al., 2005). O resgate funcional e o aumento do número das Treg parecem estar associados ao melhor controle das exacerbações da asma, assim as Treg têm sido alvo da imunoterapia alérgeno-específica (ITA).

A ITA consiste na administração repetida de doses crescentes do alérgeno, geralmente por injeção subcutânea ou sublingual, com o intuito de induzir um estado de tolerância permanente após o término do tratamento (BURKS et al., 2013; DURHAM; PENAGOS, 2016). O sucesso da ITA está relacionado à expansão de células Treg e Breg alérgeno-específicos com inibição das respostas Th2 e redução do infiltrado de células inflamatórias (SUAREZ-FUEYO et al., 2014; SWAMY et al., 2012). *In vitro*, a eficiência da ITA foi associada a supressão da síntese da IgE alérgeno-específico e a expansão de células Breg $IgG4^{+}$ (MEILER; KLUNKER; ZIMMERMANN, 2008; YING et al., 2001). De forma interessante, a dessensibilização alérgica aumentou o número das células T $CD4^{+}$ foliculares reguladoras (T_{FR},

$CD4^+CXCR5^+FoxP3^+IL\text{-}10^+$) (SCHULTEN et al., 2018; VARRICCHI et al.,2016). Recentemente, Eljaszewicz e colaboradores (2021) demonstraram que a ITA foi capaz de aumentar a frequência de ILCs, monócitos e DCs com características tolerogênicas. Apesar de reduzir os sintomas e dependência de medicamentos no controle da doença em indivíduos monossensibilizados e com asma leve/moderada, o benefício da ITA é menor nos pacientes com a asma grave e polissensibilizados (SOCIEDADE BRASILEIRA DE PNEUMOLOGIA E TISIOLOGIA, 2020). Esse menor benefício pode estar associado a ocorrência de fatores de risco ambientais que favorecem danos no compartimento regulador do sistema imune, tal como hipovitaminose D e obesidade.

Fatores de risco para a Asma Alérgica

Em indivíduos geneticamente predisponentes, o desenvolvimento e a gravidade da AA estão amplamente relacionados a fatores ambientais, tais como a deficiência de vitamina D (Vit D) e a obesidade.

A vitamina D

Inicialmente, os estudos sobre as funções da vitamina D eram relacionadas exclusivamente ao seu papel na homeostase do cálcio, do fosfato e no metabolismo ósseo. Porém, diferentes estudos têm sugerido que essa molécula exerce diversos efeitos imunomodeladores (BOZZETO et al., 2012; CHUN et al., 2014; HANSDOTTIR; MONICK, 2011; HOLICK, 2007).

As principais isoformas da vitamina D são a vitamina D_2 (ergocalciferol) e a vitamina D_3 (colecalciferol), cujas principais fontes são, respectivamente, dietas baseadas em fonte vegetal e animal. Por ser encontrada em um grupo seleto de alimentos, 90% da vitamina D é obtida através da síntese endógena induzida pela exposição à radiação ultravioleta B (UVB), presente na luz solar (BATTAULT et al., 2013; BOZZETTO et al., 2012).

A síntese da vitamina D ocorre principalmente através da síntese dérmica, que leva a produção da pró-vitamina D_3, que posteriormente é hidrosilada no fígado pela CYP27A1 (monoenzima 25-hidroxilase vitamina D) em $25(OH)D_3$ (25-hidroxi-vitamina D_3). Finalmente, nos rins, a $25(OH)D_3$ é convertida na forma ativa da vitamina D, à $1,25(OH)_2D_3$ (1,25-diidroxi-vitamina D3), pela CYP27B1 (1-α-hidroxilase) (BOUILLON, 2011; MIRZAKHANI et al., 2015). A $1,25(OH)_2D_3$ tem uma meia vida de 4 a 15 horas, sendo sua atividade controlada pela CYP24A1 (24-hidroxilase vitamina D), e liga-se ao receptor intracelular da vitamina D presente nas células (VDR, *vitamin D receptor*) (CHRISTAKOS et al., 2010; FELDMAN; PIKE; ADAMS, 2011; VIETH, 2011).

Na prática clínica, os níveis séricos da $25(OH)D_3$, e não de $1,25(OH)_2D_3$, são usados na identificação da deficiência da vitamina D. Segundo Gupta e colaboradores (2012) essa medição reflete a vitamina D total proveniente da dieta e da exposição ao sol, bem como a conversão da vitamina D das reservas de gordura no fígado. Usualmente, a deficiência é definida como um nível sérico de 25(OH)D menor que 20 ng/mL, a insuficiência com valores compreendidos entre 20-30 ng/mL, a suficiência entre 30-100 ng/mL e a intoxicação com valores maiores que 150 ng/mL (BAEKE et al., 2008; DAWSON-HUGHES et al., 2005; HOSSEIN-NEZHAD; HOLICK, 2013; VIETH et al., 2007).

As propriedades anti-inflamatórias da vitamina D: Papel na AA

Além da função hormonal, a vitamina D participa na modulação imune. O VDR, receptor da vitamina D, é expresso em todas as células da imunidade inata e adaptativa. Ademais, as células T, por expressarem a enzima CYP27B1, são capazes de formar a $1,25(OH)_2D_3$ (BATTAULT et al., 2013; JEFFERY et al., 2012; LANGE et al., 2009; MAES et al., 2020; UMAR; SASTRY; CHOUCHANE, 2018).

A $1,25(OH)_2D_3$, inibe a produção de citocinas pró-inflamatórias e induz a síntese de peptídeos antimicrobianos por monócitos e macrófagos. Também, modula a função das APCs e a ativação e diferenciação das células T (ADAMS. HEWISON. 2008; BOZZETTO et al., 2012). Estudos epidemiológicos em pacientes asmáticos demonstraram que a deficiência da vitamina D é comum e foi associada a diminuição da função pulmonar e hospitalizações (BOZZETTO et al., 2012; BREHM et al., 2010;

HYPPONEN et al., 2004; JACKSON; JOHNSTON, 2010; MANN et al., 2014; OGEYINGBO et al., 2021; WEISS; LITONJUA, 2008). Por outro lado, de acordo com Chinellato e colaboradores (2010), os níveis da vitamina D foram correlacionados positivamente com o melhor controle da AA e negativamente com a broncoconstrição induzida por exercício físico. Não menos importantes, estudos genéticos sugerem que polimorfismos do gene VDR aumentam o risco à AA, desde que genes sabidamente relacionados à asma são regulados pela vitamina D (BOSSÉ et al., 2009; HE et al., 2022; RABY et al., 2004; VOLLMERT et al.,2004).

Apesar do potencial efeito benéfico da vitamina D no controle da asma, alguns autores sugerem que esta pode favorecer o fenótipo asmático. De acordo com dados de Hypponen e colaboradores (2004), a suplementação da vitamina D (2000 UI/dia) nos primeiros anos de vida resultou no aumento do risco de desenvolvimento da rinite alérgica, atopia e asma. Em outro estudo, observou-se que os filhos de mulheres grávidas com altos níveis da vitamina D tinham um maior risco ao desenvolvimento da asma (GALE et al., 2007). Infelizmente, os mecanismos básicos dessa relação adversa não foram ainda explorados, mas podem estar relacionados ao desequilíbrio nos eletrólitos em vigência de intoxicação com doses suprafisiológicos da vitamina D.

Um dos problemas emergentes no controle da asma é a resistência aos corticoides. A resistência tem sido associada à diminuição das células Tregs e redução na expressão de receptores para os corticoides, especificamente a isoforma CGα (DIMELOE et al., 2010; ROBINSON et al., 2009; SEARING et al., 2010; XYSTRAKIS et al., 2006). Estudos *in vitro* têm demonstrado que a vitamina D é capaz de aumentar a produção das células Treg, enquanto que na sua ausência essas células têm o seu número e funções diminuídas (CHAMBERS; HAWRYLOWICZ, 2011; SEVILLA et al., 2021; URRY et al., 2009; XYSTRAKIS et al., 2006).

Imunomodulação das células imunes do fenótipo asmático pela vitamina D

A vitamina D tem efeitos imunomodeladores nas vias inflamatórias induzidas por alérgenos em função da expressão do VDR nos leucócitos (MCKINLEY et al., 2008; TANG et al, 2009). Ensaios *in vivo* demonstraram a capacidade da vitamina D em suprimir a função das células Th2 classicamente envolvidas na patogênese da AA

(LANGE et al., 2009; LLOYD; HESSEL, 2010). Em modelos animais de AA, os níveis da vitamina D foram inversamente correlacionados com o número de eosinófilos e os títulos da IgE (MATHEU et al., 2003). Ainda, em modelo experimental de AA, o controle da hiperreatividade das vias aéreas após suplementação com vitamina D foi associado a uma diminuição da IL-4 e IL-13 (HEINE et al., 2014; TOPILSKI et al., 2004).

Sob influência da vitamina D, as DCs adquirem um fenótipo mais tolerogênico, comprovado pela diminuição da expressão das moléculas coestimuladoras e aumento na liberação de citocinas anti-inflamatórias. A $1,25(OH)_2D_3$ induz a expressão do ligante 1 do PD1 (PD-L1, *Programmed death-ligand 1*) e do receptor inibitório ILT3 **(***Immunoglobulin-like transcript* 3**)** na superfície das CDs, sendo que ambas as moléculas suprimem as células T efetoras e favorecem a expansão das células Treg (DURÁ-TRAVÉ et al., 2017; MCMILLAN; XANTHOU; LLOYD, 2005; STAMPFLI et al., 1999). *In vitro*, o pré-tratamento de CDs com vitamina D e subsequente cocultura com células T $CD4^+$ gerou células Tregs e Tr-1 (BARRAT et al., 2002; PENNA et al., 2005). De forma interessante, em culturas de células T $CD4^+$ de pacientes refratários ao tratamento com glicocorticoides, a adição da $1,25(OH)_2D_3$ aumentou a síntese da IL-10 e restaurou a resposta clínica e imunológica dessa citocina face aos corticoides (XYSTRAKIS et al., 2006).

A vitamina D também inibe as respostas celulares mediadas pelas células Th17, sendo esse mecanismo importante nos casos de pacientes asmáticos com endotipo neutrofílico, considerados os mais graves (CHANG; CHUNG; DONG, 2010; LITONJUA; WEISS, 2007; NANZER et al., 2013). No trabalho publicado por Subramanian e colaboradores de (2017) a vitamina D reduziu a produção de citocinas pró-inflamatórias por neutrófilos ativados, e aumentou a atividade antimicrobiana de macrófagos por favorecer a secreção de peptídeos antimicrobianos.

Além do seu efeito sobre as células T, diferentes estudos têm descrito o papel benéfico sobre os linfócitos B. Em estudos *in vitro*, foi observado que a vitamina D favoreceu a expansão de linfócitos Bregs e diminuiu a produção da IgE (HARTMANN et al., 2011; HEINE et al., 2008). Adicionalmente, a $1,25(OH)_2D_3$ inibe a liberação de mediadores promotores de inflamação liberados pelos mastócitos (BIGGS et al., 2010; LIU et al., 2016; YIP et al., 2014).

A obesidade: um panorama do excesso de massa gorda

A obesidade é uma doença crônica, caracterizada por um acúmulo excessivo e anormal de gordura no organismo. A prevalência da obesidade aumentou em todos os países, e é considerado atualmente uma epidemia global (ORGANIZAÇÃO MUNDIAL DA SAÚDE, 2022).

Atualmente, o Índice de massa corporal (IMC), obtido pela razão entre o peso e altura ao quadrado, é o indicador mais utilizado para avaliar o estado nutricional dos adultos, permitindo assim estratificá-los de acordo com as categorias preconizadas pela OMS (quadro 1) (JAVED et al., 2015; SMITH, K; SMITH, M, 2015). Apesar da praticidade, o IMC possui algumas limitações. Infelizmente, não prediz a composição dos compartimentos de gordura e massa magra. Portanto, a utilização do IMC não é a ferramenta mais adequada quando há a necessidade de avaliação do tecido adiposo individual (ANJOS, 1992; WOOLCOTT; SEURING, 2022).

Quadro 1 - Classificação do índice de massa corporal segundo a organização mundial da saúde

IMC (kg/m^2)	Classificação
< 16,0	Magreza grau III
16,0 - 16,9	Magreza grau II
17,0 - 18,4	Magreza grau I
18,5 - 24,9	Eutrofia (peso normal)
25 - 29,9	Sobrepeso
30 - 34,9	Obesidade grau I
35 - 39,9	Obesidade grau II
≥ 40,0	Obesidade grau III

Legenda: índice de massa corporal (IMC); quilogramas (kg); metros ao quadrado (m^2): Organização Mundial da Saúde (OMS)
Nota: Os pontos de corte utilizados no diagnóstico da obesidade propostos pela organização mundial da saúde.
Fontes: OMS, 2022.

De acordo com a OMS, aproximadamente 2 bilhões de adultos estão com sobrepeso, sendo 650 milhões obesos (BOUTARI; MANTZOROS, 2022). Estima-se que, em 2030, 57,8% (3,3 bilhões de adultos) da população mundial adulta estará acima do peso (FINKELSTEIN et al., 2012; KELLY et al., 2008). Seguindo a tendência mundial, o Atlas da Obesidade Mundial elaborado pela OMS em 2022 especula que, até 2030, 1 em cada 5 mulheres e 1 em cada 7 homens serão obesos, o equivalente à 1 bilhão de pessoas no mundo. O Brasil ocupa a 4ª posição na lista dos países com maior prevalência da obesidade em adultos (ORGANIZAÇÃO MUNDIAL DA SAÚDE, 2022). A maior parte da população obesa vive nos países desenvolvidos e juntos, a obesidade e o sobrepeso, são responsáveis pelo maior número de mortes quando comparados à desnutrição (CABALLERO, 2007).

De natureza multifatorial, a obesidade comum está relacionada a uma vasta gama de doenças crônicas não transmissíveis, tais com as doenças cardiovasculares, diabetes mellitus do tipo 2, hipertensão, apneia do sono, câncer e doenças mentais, o que resulta na perda da qualidade de vida e aumento dos custos aos sistemas de saúde (FIELD, 2001; HU, 2008; KJELLBERG et al., 2017)

No Brasil, de acordo com a Pesquisa Nacional de Saúde - PNS, (2020), 60% dos adultos (cerca de 96 milhões) estão com sobrepeso, entre os quais 41,2 milhões são obesos. O índice da obesidade aumentou 72% no período de 2006 a 2019. No SUS, 3,45 bilhões de reais foram gastos com o tratamento da obesidade, diabetes e hipertensão (NILSON et al., 2020).

Apesar da obesidade estar relacionada a fatores genéticos, a importante influência do sedentarismo e de padrões alimentares inadequados, que resulta em um desequilíbrio entre a ingesta e o gasto calórico, contribui para o aumento de pessoas acometidas. Dietas do mundo moderno, tal como a ingestão de alimentos com baixa concentração de antioxidantes e elevado teor de gorduras saturadas contribui não apenas para um aumento de peso como também para o caráter inflamatório da obesidade.

A Obesidade como um estado inflamatório: O endotipo asma-obeso

O tecido adiposo é dividido em dois subtipos principais: o tecido adiposo branco (TAB) e o tecido adiposo marrom (TAM) (GIRALT; VILLARROYA, 2013). O TAM é importante na manutenção da temperatura corpórea, é amplamente encontrado em

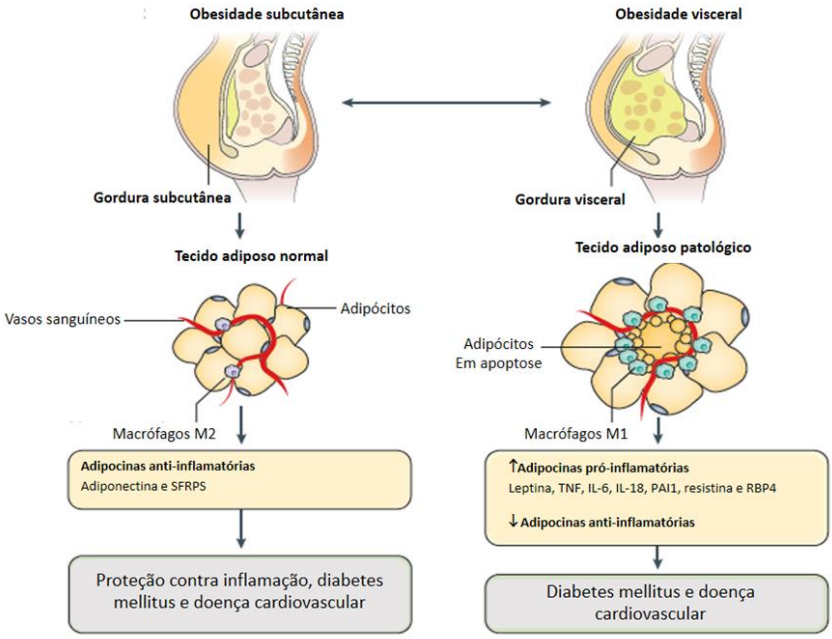
recém-nascidos e, em menor escala, nos adultos (SANTHANAM et al., 2015). O TAB representa a maior parte do tecido adiposo e é responsável pelo armazenamento de energia (GONZALEZ-MUNIESA et al., 2017; HERSOUG; LINNEBERG, 2007). Atualmente, o TAB não é considerado um tecido inerte unicamente de reserva, mas sim um local de regulação de vários processos fisiológicos e patológicos. Com propriedades endócrinas, o TAB executa um papel central na modulação do gasto de energia e na regulação do metabolismo da glicose e do sistema imune (FANTUZZI, 2005; GNACINSKA et al., 2009; MATSUZAWA, 2006). Apesar de ser composto, majoritariamente, pelos adipócitos, no TAB encontramos outras células incluídas na sua fração estromal, a exemplo os fibroblastos pré-adipócitos e diferentes tipos de células imunes (FANTUZZI, 2005; KOCH; SOPEL; FINOTTO, 2017).

A obesidade é caracterizada por um estado de inflamação crônica de baixo grau, onde observamos uma alta expressão de mediadores inflamatórios, tais como IL-6, IL-8, TNF-α, leptina, proteína C reativa (PCR), inibidor-1 do ativador do plasminogênio (PAI-1) e haptoglobina, e a baixa expressão de marcadores anti-inflamatórios, tais como a adiponectina e IL-10 (BAPAT et al., 2022; CHIELLINI et al., 2004; ENGSTROM et al., 2003; ESPOSITO et al., 2002; FESTA et al., 2001; SHORE, 2008). O TNF-α aumenta a expressão das citocinas Th2, a IL-4 e IL-5, pelas células epiteliais brônquicas (WEISS, 2005). Ademais, o TNF-α pode interagir com o seu receptor no músculo liso das vias aéreas e aumentar a contratilidade e induzir inflamação (ATHER; POYNTER; DIXON, 2015). Curiosamente, o receptor 1 do TNF-α (TNFR1) parece estar envolvido no mecanismo de proteção da reatividade das vias aéreas em obesos. Esse evento deve envolver a capacidade do TNFR1 em sequestrar o TNF-α circulante (ZHU et al., 2012).

Em indivíduos eutróficos, o tecido adiposo é composto, majoritariamente, pelos macrófagos do tipo 2 (M2) e poucas células T, principalmente as células Tregs. Os M2, por meio da remoção das células mortas e a secreção dos mediadores anti-inflamatórios IL-10, TGF-β e PGE_2, controlam a inflamação (figura 4) (CHYLIKOVA et al., 2018; CIPOLLETTA, 2014; GONZALEZ-MINUESA et al., 2017; MCCUBBREY; CURTIS, 2013). Em contrapartida, em indivíduos obesos observa-se um aumento dos macrófagos do tipo 1 (M1) e das células T $CD4^+$ efetoras. Os M1 produzem as citocinas pró-inflamatórias (IL-6, TNF-α, IL-1β, IL-23 e IL-12), derivados de oxigénio e adipocinas. Os macrófagos do tipo M1 são particularmente vistos ao redor dos adipócitos hipertrofiados, alguns formando estruturas típicas de células gigantes

multinucleadas e ricas em lipídeos oxidados, uma marca registrada na inflamação crônica local (figura 3) (CABALLERO, 2007; ; CHYLIKOVA et al., 2018; HERSOUG; PARK; PARK; YU, 2005; MATHIS, 2013; SHAPIRO et al., 2013; VISSER et al., 2001).

Figura 4 - Mudança patológica no tecido adiposo



Legenda: Interleucina (IL); inibidor 1 do ativador de plasminogênio (PAI1); proteína de ligação ao retinol 4 (RBP4); proteína 5 relacionada ao frizzled secretada (SFR5) e fator de necrose tumoral α (TNF-α);

Nota: Nos eutróficos, o tecido adiposo é uma fonte de mediadores anti-inflamatórios implicados na homeostase imune (adiponectina e SFRP5). O acúmulo excessivo de gordura visceral, entanto, é uma rica fonte de mediadores pró-inflamatórios (leptina, TNF-α, IL-6, IL-8, PAI1, resistina e RBP4) que mantém o indivíduo em persistente estado inflamatório de baixo grau. Esse perfil mais inflamado associado à obesidade contribui para o comprometimento metabólico sistêmico e risco elevado para doenças cardiovasculares e diabetes do tipo 2. As mudanças patológicas viscerais demonstram maiores níveis da necrose dos adipócitos, devido, em parte, a anormal tensão de oxigênio nos depósitos de gordura e o recrutamento de macrófagos do tipo M1. Muitas dessas células M1 são vistas formando estruturas semelhantes às células gigantes multinucleadas em resposta a produção sustentada de citocinas produzidas pelas células Th1.

Fonte: Traduzido e adaptado de Gonzalez-Muniesa et al., 2017.

Quanto aos fenótipos das células T, a obesidade está associada à diminuição das células Th2 e Treg e o incremento das células T $CD4^+$ do tipo Th1 e Th17 e células T $CD8^+$ citotóxicas (ENDO; YOKOTE; NAKAYAMA; 2017; FEUERER et al., 2009; MATARESE et al., 2010; WANG et al., 2018). Essas alterações têm sido vistas como gatilhos para os processos de exacerbação nas reações de hipersensibilidades.

Observa-se também, a expansão de CDs IL-6^{+}IL-23^{+} (CHO et al., 2016). Por fim, em relação aos linfócitos B, o impacto da obesidade é menos descrito, entretanto, estudos demonstraram que esses linfócitos são capazes de apresentar antígenos às células T do tecido adiposo branco, bem como, secretar citocinas pró-inflamatórias e anticorpos patogênicos (FRASCA; BOMBERG, 2017). Em estudo publicado por Frasca e Bomberg (2017), em camundongos, as células B acumulavam primeiro no tecido adiposo branco quando comparadas às células T, aparentemente promovendo a ativação dessas células. Apesar da descrição clássica entre hipertrofia do tecido adiposo e redução na fração de células Th2 locais, vale a pena destacar que, sistemicamente, a produção de citocinas relacionadas às reações atópicas encontram-se elevadas (PETERS et al., 2016; PINKERTON et al., 2022). Nós acreditamos que esse efeito adverso esteja associado ao dano nas células T e B reguladoras observada nos obesos (DEIULIIS et al., 2011; FABBRINI et al., 2013; GYLLENHAMMER et al., 2016; WAGNER et al., 2013).

Nos últimos 20 anos, a prevalência da AA e da obesidade aumentou, e estudos epidemiológicos prospectivos indicam que a asma e a obesidade coexistem em muitos pacientes (BEUTHER; SUTHERLAND, 2007; FLEGAL; CARROLL; OGDEN, 2010; JACKSON; SYKES; MALLIA, 2011). Distintos fenótipos da asma são reconhecidos em pacientes obesos. Podemos encontrar dois grupos principais: asma de início precoce (EAO, *early asthma onset*) e asma de início tardio (LAO, *late asthma onset*) (DIXON et al., 2011; PETERS-GOLDEN et al., 2006). A EAO, os pacientes tendem a ser jovens no momento do diagnóstico (menor que 12 anos), apresentam maior prevalência de alergia, cuja obesidade agrava a asma pré-existente e, como tal, a asma não desaparece com a perda de peso, embora haja uma melhora nos sintomas (BATES et al., 2017; DYXON; POYNTER, 2016; SIDELEVA; DIXON, 2014). Em contrapartida, a LAO ocorre em pessoas mais velhas (a partir de 12 anos), afetando principalmente as mulheres. A asma do tipo LAO é comumente descrita como não alérgica, apresentando sintomas mais exuberantes e menos responsiveis ao tratamento com corticoides inalatórios (BATES et al., 2017; SIDELEVA; DIXON, 2014; MOORE et al., 2014). Adicionalmente, por se resolver após a perda de peso, nos de início tardio a doença parece ser dependente do ganho excessivo de massa gorda e não é associada a asma clássica (BATES et al., 2017; DIXON et al., 2011).

Os mecanismos por trás da associação adversa entre a asma e a obesidade ainda não são bem elucidados, porém, devem envolver o quadro de metainflamação

no qual os indivíduos obesos são condicionados. No estudo publicado por Han e colaboradores (2014), nos pacientes asmáticos e com altos níveis do óxido nítrico exalado, a obesidade foi associada ao aumento da gravidade da doença. Scott e colaboradores (2011) detectaram elevados níveis de neutrófilos nas vias aéreas de mulheres asmáticas obesas. A neutrofilia vem sendo, de fato, descrita nos casos graves da asma, o que nos leva a crer que a obesidade se relaciona ao pior prognóstico das alergias, com maior risco de admissões hospitalares e menor resposta aos corticosteroides (CARROLL et al., 2007; LANG et al, 2008; ORDONEZ; SHAUGHNESSY; MATTHAY, 2000; QUINTO; ZURAW; POON, 2011).

A vigência de sobrepeso foi associada a um maior número de ILC-2 e ILC-3, assim como maior quantidade IL-17 no LBA dos pacientes (EVERAERE et al., 2016; KIM et al., 2014). Todos esses parâmetros foram associados com o comprometimento da função pulmonar e pior controle terapêutico, que parece estar associado a uma reduzida expressão de β2-AR (receptor β2 adrenérgico) e GCR-α (receptor α dos glicocorticoides) (EVERAERE et al., 2016). No que diz respeito às citocinas, elevado IMC foi associado aos níveis circulantes de IL-6, IL-5 e IL-13 (PETERS et al., 2016a; PINKERTON et al., 2022). Quanto aos eosinófilos, a maioria dos estudos relatam uma diminuição desses leucócitos, ou mesmo, nenhuma relação entre este e a obesidade (LESSARD et al., 2008; VAN VEEN et al., 2008). No entanto, Desai e colaboradores (2013) encontraram um aumento dos eosinófilos na submucosa de pacientes obesos, apesar de nenhuma diferença ter sido observada no escarro, sugerindo que a obesidade possa alterar o tráfego dos eosinófilos da asma clássica Th2. De fato, estudos *in vitro*, indicam que a quimiotaxia e a adesão dos eosinófilos parecem estar aumentadas na obesidade, sendo muitos desses efeitos creditados a ação imune da leptina (GROTTA et al., 2013; KATO et al., 2011).

A leptina: papel na imunidade da asma alérgica

A leptina é uma proteína de 16 kD codificada pelo gene LEP e é encontrada predominantemente nos adipócitos (ZHANG et al., 1994; WANG; CHENGPING,

2022). Ela age nos neurônios hipotalâmicos sinalizando a sensação de saciedade e, muitas vezes, age como um antagonista ao aumento do efeito da fome mediado pela grelina (BLÜHER; MANTZOROS, 2015; JEQUIER, 2002). As concentrações da leptina variam de acordo o *status* nutricional. É produzida de acordo com a massa de tecido adiposo, como tal, as suas concentrações são maiores em indivíduos obesos (KLOK et al, 2007; MAPFEI et al., 1995; SOLIMAN et al., 2000). Além da ingesta alimentar, fatores fisiológicos como o sexo, idade e o início da puberdade influenciam a sua concentração, e a sua síntese também pode ser regulada por outros hormônios, tais como a insulina e glicocorticoides (CORRÊA; PIMENTEL; CORTEZ, 2012). Mulheres pós-púberes apresentam níveis dessa adipocina 40% a 200% maiores do que os seu homólogos masculinos (BLUM et al., 1997; LICINIO et al, 1998). Os seus efeitos no sistema nervoso central são sujeitos à indução de resistência, sendo esse fenômeno associado a múltiplos mecanismos, tais como falhas em atravessar a barreira hematoencefálica, redução de sua expressão ou da transdução do sinal do seu receptor (CORRÊA; PIMENTEL; CORTEZ, 2012).

Todas as células do sistema imune expressam o receptor da leptina (LepR) (KATO et al., 2008; WONG; CHEUNG; LAM, 2007). Nos macrófagos e monócitos, a leptina aumenta a fagocitose e a secreção de TNF-α, IL-6, IL-1β (CARBONE; ROCCA; MATARESE, 2012). Em adição, a leptina estimula a quimiotaxia de neutrófilos e a produção de ROS (GROTTA et al., 2013; KAMP et al., 2013; TILG; MOSCHEN, 2006; UBAGS et al., 2014). Essa adipocina também é capaz de aumentar a sobrevida e função dos eosinófilos (CONUS; BRUNO; SIMON, 2005; WONG; CHEUNG; LAM, 2007).

Relativo à imunidade adaptativa, a leptina é capaz de reduzir o limiar de ativação e aumentar a resistência de linfócitos T à morte por apoptose (BEUTHER; WEISS; SUTHERLAND, 2006; FANTUZZI, 2005; PUCINO et al., 2014). A leptina parece induzir a proliferação das células T $CD4^+$ naïves ($CD45RA^+$) e diminuir a frequência de células T $CD4^+$ de memória ($CD45RO^+$) (LORD et al., 1998; TILG; MOSCHEN, 2006). De forma interessante, deficiência de leptina em animais e em humanos foi associada a uma redução no número total de células T $CD4^+$ e uma mudança do fenótipo Th1 para o Th2 (FERNÁNDEZ-RIEJOS et al., 2011; PROCACCINI et al., 2012). Esses achados foram corroborados por Saucillo e colaboradores (2014), no qual, a leptina exógena foi capaz recuperar a proliferação das células T $CD4^+$. Fenotipicamente, a leptina aumenta a produção das citocinas Th1

e inibe as citocinas Th2 (BRESTOFF et al., 2015; LORD et al., 1998; MARTIN-ROMERO et al., 2000; RASTOGI et al., 2015). Coletivamente esses dados sugerem que a leptina pode auxiliar o hospedeiro na resposta imune contra uma vasta gama de patógenos. Todavia, o excesso dessa adipocina tem sido associado a diversas doenças autoimunes em modelos animais (FANTUZZI, 2005). De forma interessante, altos níveis da leptina foram inversamente correlacionados com a função pulmonar em pacientes asmáticos (KALMARZI, et al., 2017).

Na obesidade, a leptina, aparentemente, pode induzir a um estado pró-inflamatório por favorecer a expansão de células T $CD4^+$ envolvidos nas reações de hipersensibilidades, por aumentar a produção de TNF-α, IL-6 e IL-17, e por comprometer o *status* funcional e sobrevida das células Treg (MATARESE et al., 2010; NAYLOR; PETRI, 2016). Estudos demonstraram uma correlação direta entre os níveis da leptina e a reatividade das vias aéreas e os níveis da IgE em pessoas asmáticas (CAREY et al., 2007; SHORE et al., 2005; SIDELEVA; DIXON, 2014). Em adolescentes obesos, altos níveis da leptina foram inversamente correlacionados a função pulmonar, e a expressão da leptina na gordura visceral foi associada a reatividade das vias aéreas em adultos (EISING et al., 2014; HUANG et al., 2017; SIDELEVA et al., 2012).

Finalmente, a leptina pode suprimir as funções das células T reguladoras (CONUS; BRUNO; SIMON, 2005). Curiosamente, uma importante fonte de leptina são as próprias células Treg, que secretam tanto essa adipocina, quanto expressam receptores para a mesma, podendo ser atraídos para o tecido adiposo pela leptina secretada pelos adipócitos (PAPATHANASSOGLOU et al., 206). A neutralização *in vitro* da leptina aumentou a proliferação das células Tregs. Em modelo experimental, camundongos deficientes para a leptina, ou para o seu receptor, apresentaram maior número de células Tregs (DE ROSA et al. 2007). De acordo com esses achados, o aumento dos níveis de leptina contribui negativamente na proliferação, e provavelmente na função, das células Tregs.

Nas últimas décadas, a prevalência da AA e da obesidade têm aumentado em todo o mundo. A obesidade vem sendo descrita como um fator ambiental importante no desenvolvimento e na gravidade da asma. Adicionalmente, os pacientes com AA obesos respondem mal às terapias comumente utilizadas e apresentam altos índices de exacerbações, sendo responsáveis por gastos significativos com cuidados de saúde. Os motivos pelos quais a obesidade impacta o curso da AA não são

conhecidos, porém devem envolver em parte o estado de hipovitaminose D e hiperleptinemia em que esses pacientes estão condicionados. Nestas condições, provavelmente há formação de mediadores únicos da inflamação que modulam o comportamento das células envolvidas na patogênese da asma.

1.**OBJETIVOS**

1.1 **Geral**

Avaliar o perfil imune das células T CD4+ e células Br1 de pacientes com asma alérgica e sua relação com a ocorrência de obesidade e *status* da leptina e da vitamina D.

1.2 **Específicos**

Artigo 1 - Serum leptin levels correlate negatively with the capacity of vitamin D to modulate the in vitro cytokine production by CD4$^+$ T cells in asthmatic patients
Aleida S. O. Dias, Isabele C. L. Santos, Letícia Delphim, Gabriel Fernandes, Larissa R. Endlich, Marcos Octávio S. D. Cafasso, Ana Lúcia Maranhão, Sonia Regina da Silva, Regis M. Andrade, Anshu Agrawal, Ulisses C. Linhares, Cleonice A. M. Bento. Clinical Immunology. 2019; 205: 93-105. doi 10.1016/j.clim.2019.06.001

a) Quantificar a produção de citocinas nos sobrenadantes das culturas das células mononucleares do sangue periférico (CMSP) e das culturas de células T CD4$^+$ purificadas ativadas com anti-CD3 e anti-CD28 em indivíduos saudáveis e em pacientes com AA;
b) Determinar a capacidade da 1,25$(OH)_2D_3$ em modular, *in vitro*, as citocinas produzidas pelas células T ativadas (CMSP e T CD4$^+$ isoladas) do grupo controle e dos pacientes com a AA;
c) Dosar os níveis séricos da leptina e correlacioná-los com a produção de citocinas em cultura de células T CD4$^+$ de pacientes asmáticos;
d) Correlacionar os níveis plasmáticos da leptina com a capacidade da 1,25$(OH)_2D_3$ em inibir a síntese, *in vitro*, das citocinas produzidas pelas células T CD4$^+$ purificadas e ativadas obtidas de pacientes com AA;
e) Correlacionar os achados imunes com a gravidade da AA e ocorrência de obesidade.

Artigo 2 - Leptin favors Th17/Treg cell subsets imbalance associated with allergic asthma severity

Carolina M. Vollmer, Aleida S O Dias, Lana M. Lopes, Taissa M. Kasahara, Letícia Delphim, Júlio César C. Silva, Lucas Paulo Lourenço, Hilary Cesário Gonçalves, Ulisses C. Linhares, Sudhir Gupta, Cleonice A M Bento. Clinical and Translational Allergy. 2022; e12153. doi 10.1002/clt2.12153

a) Determinar a frequência de diferentes subtipos de células T $CD4^{+}IL\text{-}4^{+}$ e células T $CD4^{+}IL\text{-}4^{-}$ em pacientes com a asma alérgica;
b) Avaliar a proporção de diferentes fenótipos das células T $CD4^{+}$ ativadas em pacientes asmáticos agrupados de acordo com a gravidade da AA e ocorrência de obesidade;
c) Analisar, nos pacientes com AA, a frequência de células T $CD4^{+}$ e células B reguladores de acordo com a ocorrência de obesidade e gravidade da doença;
d) Mensurar os níveis séricos da leptina no grupo dos pacientes asmáticos e obesos, e nos controles saudáveis, e correlacioná-los com diferentes subtipos dos linfócitos T $CD4^{+}$ e os linfócitos B reguladores circulantes
e) *In vitro*, quantificar as citocinas secretadas pelas células T $CD4^{+}$ purificadas e ativadas policlonalmente na presença de dose de leptina associada à obesidade;
f) Na presença da leptina, avaliar a capacidade das Treg em inibir a proliferação das T efetoras, *in vitro*.

Artigo 3 - Leptin favors imbalance of antigen-specific $CD4^{+}$ T-cells associated with severity of cat allergy

Carolina Vollmer, Aleida Dias, Marisa Sales, Priscila Sacramento, Júlio Cesar Silva, Hugo A. A. Oyamada, Ulisses C. Linhares, Sudhir Gupta, Taissa M. Kasahara, Cleonice A. M. Bento. Journal of Immunology Research. 2023

a) Avaliar o papel da leptina, em dose relacionada à obesidade, em modular o perfil de citocina secretado por culturas de CMSP policlonalmente ativadas obtidas de indivíduos alérgicos a gatos;

b) Quantificar os títulos da IgE produzidos nas culturas das CMSP ativadas, e correlacioná-los com as citocinas liberadas pelas células estimuladas com mitógenos;
c) Avaliar o papel da leptina em modular a frequência de diversos subtipos de linfócitos T $CD4^+$ efetores e reguladores em resposta à Fel d1;
d) Correlacionar os níveis de IgE produzidos em culturas de CMSP com a proporção de subtipos das T_{FH} Fel d1-específicos na presença, ou não, da leptina;
e) Determinar se a ocorrência de obesidade impacta na frequência de subtipos de células T $CD4^+$ efetoras e reguladoras em resposta à Fel d1;
f) Avaliar se os níveis plasmáticos de leptina têm alguma relação com o perfil de citocinas e a frequência de células T_{FH} e Treg nas culturas das CMSP ativadas com Fel d1 obtidas de pacientes alérgicos a gatos;
g) Investigar se existe alguma relação entre os níveis circulantes de leptina com os títulos de IgE, assim como desse anticorpo com a produção de citocinas e a frequência de células T_{FH} e Treg Fel d1-específicas.

2. **ARTIGOS CIENTÍFICOS**

2.1 **Artigo 1 - Serum leptin levels correlate negatively with the capacity of vitamin D to modulate the in vitro cytokine production by CD4⁺ T cells in asthmatic patients (Artigo publicado)**

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Serum leptin levels correlate negatively with the capacity of vitamin D to modulate the *in vitro* cytokines production by CD4$^+$ T cells in asthmatic patients

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ABSTRACT

Both obesity and low vitamin D levels have been associated with allergic asthma (AA) severity. In the present study, severity of AA was associated with obesity but to the *in vitro* IgE production. In those patients, higher levels of IL-5, IL-6 and IL-17 were quantified in CD4$^+$ T-cell cultures as compared with patients with mild and moderate AA. In addition, the lowest IL-10 levels were detected in the cell cultures from patients with a worse prognosis. Interestingly, the occurrence of AA elevates the plasma levels of leptin, and this adipokine was positively correlated with the release of IL-5, IL-6 and IL-17, but inversely correlated with IL-10 production, by CD4$^+$ T-cells from patients. In AA-derived CD4$^+$ T-cell cultures, 1,25(OH)2D3 was less efficient at inhibiting IL-5, IL-6 and IL-17 production, and up regulating IL-10 release, as those from healthy subjects. Interestingly, the *in vitro* immunomodulatory effects of vitamin D were inversely correlated with serum leptin levels. In summary, our findings suggested that obesity, probably due to the overproduction of leptin, negatively impacts AA as it favors imbalance between Th2/Th17 and regulatory phenotypes. The deleterious effects of leptin may also be due to its ability to counter-regulate the immunosuppressive effects of vitamin D.

1. Intriduction

Allergic asthma (AA) is an immune-mediated reaction of the airways to inhaled allergens characterized by recurrent episodes of breathing difficulty that affects approximately 300 million people worldwide [1]. The disease may significantly impair the patient's quality of life and lead to very high healthcare costs, thus representing a major public health problem worldwide [1,2]. The acute symptoms of asthma include wheezing, coughing and shortness of breath that can lead to airway tissue remodeling that is characterized by increased smooth muscle mass, fibrosis, and mucus production. This airway hyperreactivity (AHR) leads to airflow obstruction that can be life threatening.

The prevalence of AA has been increasing over recent decades, and it is believed that risk factors associated with modern-life style, such as obesity, may contribute to the increase of allergic diseases in genetically predisposed and epigenetically regulated individuals [3].

Obesity increases asthma severity and compromises pharmacological treatment [4–7]. Given that obesity is associated with pro-inflammatory status [8], it is possible that cytokines and adipokines modulate the behavior of immune cells involved in the immune-pathogenesis of AA.

Classical AA is associated with allergen-specific Th2 cells that produce high IL-4, IL-5 and IL-13 levels [9,10]. The differentiation of this phenotype depends on dendritic cell (DC) subsets able to present processed peptides from allergens associated with MHC class II molecules

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in association with IL-4 release [11,12]. Furthermore, epithelial cells (EC) from asthmatic patients, by releasing high levels of IL-25 [13], IL-33 [14] and thymic stromal lymphopoietin protein (TSLP) [15], favor the development of a pro-allergic DC phenotype [16,17]. These EC-derived cytokines also potentiate IL-5 and IL-13 production by local Group 2 innate lymphoid cells (ILC2) [18–20]. A hallmark of this response is the production of allergen-specific IgE by B cells [21]. The clinical symptoms of AA are particularly a result of biological actions of pre-formed and newly synthetized pro-inflammatory mediators released by IgE-sensitized mast cells and mainly eosinophils after cross-linking of these antibodies by allergens. In the context of AA, sulphidopeptide leukotrienes (LTC4, LTD4, and LTE4) and platelet-activating factor (PAF) [22,23] play a pivotal role in the pathogenesis of acute attacks due to their ability to provoke local vasodilatation, edema formation, local neurogenic stimulation, smooth muscle contraction and mucus hypersecretion.

The control of acute asthma attacks involves the use of β2 agonists (bronchodilators) and inhaled corticoids (ICs) [24,25]. Unfortunately, in a proportion of patients, symptoms are not adequately despite high doses of these drugs [26], and Recent studies have suggested an involvement of another $CD4^+$ T cell phenotype in those patients, the Th17 cells.

In humans, IL-17-producing $CD4^+$ T cell differentiation can be induced by IL-6 and IL-23 produced by DCs [27]. An increased level of both IL-17 and IL-23 in the serum and lungs of patients with severe asthma were reported [28]. The immunological pattern of this type of asthma involves neutrophilic airway inflammation associated with intense local damage and resistance to ICs [29,30]. Moreover, a mixed pattern of Th2 and Th17 cells appears to co-exist in some asthmatic patients, which also tends to be refractory to ICs therapy than patients with the classical Th2 pattern [31,32]. Corticoid resistance mechanisms are not well understood but they probably involve the ability of IL-17 to up-regulate GRβ, while attenuating the expression of the functional corticoid receptor, GRα isoform [33].

Obesity complicates eosinophilic and neutrophilic AA. Obese asthmatic patients do not respond as well to ICSs and adrenergic β2 agonists [34–36], a phenomenon probably related to increased production of inflammatory cytokines [34]. Obesity is characterized by an increased pro-inflammatory M1 to anti-inflammatory M2 macrophage ratio in adipose tissue [37]. While M2 is linked to wound healing by attenuating inflammation through release of IL-10 and TGF-β, M1 cells produce high levels of pro-inflammatory cytokines (IL-12, IL-6, TNF-α, IL-23) and free radicals derived from oxygen (ROS) and nitrogen (NO) [38,39]. In addition to its local pro-inflammatory pattern, adipose tissue can modulate systemic immune response through release of adipokines, such as leptin and adiponectin [8]. While adiponectin favors expansion of M2 and Tregs cells, leptin plays a pro-inflammatory role, damaging regulatory T cell function, which compromises the production of anti-inflammatory cytokines, such as IL-10 [40]. An imbalance in the expression of receptors for both adipokines in T cells of obese individuals, with up regulation of leptin receptor, being reported [41,42].

By inducing the production of TNF-α and IL-6, obesity-associated levels of leptin favor activation of effector T cells, mainly Th17 phenotype, and damage Treg function, which reduces the production of anti-inflammatory cytokines such as IL-10 [40,43–48]. Interestingly, high leptin levels were inversely correlated with lung function in asthmatic patients [49].

In addition to promoting high leptin levels, lower circulating vitamin D levels observed in obese patients [50] could also negatively impacts the immune-pathogenesis of asthma. In a study by Santos et al. [50], the prevalence of vitamin D deficiency was 35% higher in obese individuals compared to the eutrophic group. More severe asthma symptoms in children have been associated with poor maternal intake of vitamin D during fetal development [51]. There is a growing literature on the association between vitamin D deficiency and the risk of exacerbated asthma [52,53] and corticosteroid resistance [54–59] in patients.

Apart from its regulatory role in calcium homeostasis, vitamin D appears to play a protective role in the context of inflammatory disorders, which is probably due to its ability to modulate many aspects of the immune response. In $CD4^+$ T cells from healthy individuals, the active form of 1,25 dihydroxyvitamin D [1,25(OH)2D3] has been shown to elevate liberation of IL-10, and reduce production of IFN-γ and IL-17 [60–62]. The effect of 1,25(OH)2D on T cells may be indirect, by down-regulating the immunogenic function of antigen presenting cells, such as DCs. Indeed, 1,25(OH)2D not only reduces the expression of CD80, but also diminishes the production of pro-inflammatory cytokines by LPS-maturated DCs [63]. Whether the levels of circulating leptin correlates with the ability of vitamin D to regulate the cytokine profile of T cells in asthmatic patients is still unclear. Therefore, the objective of the present study was to investigate the immunomodulatory affects of 1,25(OH)2D3 on the *in vitro* cytokine profile of T cells from lean and overweight/obese asthmatic patients are related to the *in vivo* leptin levels.

2. Methods

2.1. Subjects

Sixty patients with AA (40 women and 20 men) were recruited from March 2017 to November 2018 from Federal University of the State of Hospital/UNIRIO (Rio de Janeiro, Brazil). Pulmonary functions were assessed by spirometry according to American Thoracic Society standards [64]. Asthma severity was evaluated on the basis of the Global Initiative for Asthma criteria [65]. Asthmatic subjects were subdivided into 3 groups: mild ($n = 20$), moderate ($n = 20$) and severe ($n = 20$) asthma. Patients were allowed to receive treatment with ICs, but not systemic steroids for 2 months prior to the study. All patients had a positive skin prick test, defined as a > 5-mm diameter skin wheal response to at least 1 of 8 common allergens (*Dermatophagoides pteronyssinus*, *D. farinae*, *Alternaria*, mixed grass pollen, mixed tree pollen, dog and cut hairs and cockroach). The occurrence of infectious or other autoimmune diseases were excluded by clinical and serological tests. Sixty healthy subject, matched by age, gender and body mass index (BMI) were recruited for the control group. BMI is calculated from the mass (weight in kg) and height (in meters) of an individual taking in account the formula ($BMI = kg/m^2$). Then the subjects were stratified as lean (BMI from 18.5 to 24.9), overweight (BMI from 25 to 29.9) and obese class I (BMI from 30 to 35). In the present study, all subjects included were nonsmokers, with no history of upper or lower airway infectious diseases 4 months prior to recruitment in the study, no chronic heart or pulmonary diseases; and did not receive oral or intravenous steroids, theophylline, long-acting β2-agonists, leukotriene antagonists or antihistamines 1 month prior to the study. The Ethics Committee for Research on Human Subjects at the Federal University of the State of Rio de Janeiro (UNIRIO) approved the study and blood was collected only after written informed consent was obtained from each individual. (CAAE 44951215.6.0000.5258).

2.2. Cell cultures and stimuli

Peripheral blood mononuclear cells (PBMCs) from 30 mL of blood were separated by centrifugation on Ficoll–Hypaque gradients. The viable cells, measured by trypan blue exclusion, were adjusted to a concentration of 1×10^6 cells/mL and cultured in 24-well flat bottom microtiter plates in 1 mL RPMI 1640 adding 2 mM L-glutamine, 10% fetal calf serum, 20 U/mL penicillin, 20 μg/mL streptomycin, and 20 mM HEPES buffer (GIBCO, Carlsbad, California, USA). In some experiments, enriched $CD4^+$ T cells and B cells were obtained *via* negative selection using magnetic columns according to manufacturer's instructions (EasySep™, StemCell Technology, Canada). Briefly, 50 μL of the

isolation cocktail was added to a cell suspension (1×10^7 cells/1 mL) in a 14 mL tube. After rapidly mixing, the suspension was incubated for 10 min at room temperature. Then, already homogenized RapidSphere suspensions were added to the cell suspension at 100 µL for $CD4^+$ T cells and 150 µL for $CD19^+$ cells (B cells). After rapidly mixing, the cell suspension was incubated at room temperature for 5 min. Finally, 4 mL of HBSS solution was added to the cell suspension and, after pipetting, the tube was then placed on the magnet for 5 min and supernatants were recovered. The purity of $CD4^+$ T cells and B cells was > 98%, as measured by flow cytometry (data not shown). Both PBMC (1×10^6/mL) and enriched $CD4^+$ T cell (1×10^5) cultures were maintained for 72 h in the absence (medium alone) or presence of anti-CD3/anti-CD28 beads (10 µl/mL) at 37 °C and 5% CO_2. To evaluate the *in vitro* production of IgE in co-culture system, $CD4^+$ T cells (1×10^4/500 µL) and B cells (1×10^4/500 µL) were stimulated with 1 µg/mL of Staphylococcal enterotoxin B (SEB) from *Staphylococcus aureus* (Sigma-Aldrich Co) for 6 days. The *in vitro* effect of vitamin D was evaluated after adding 10 or 20 ng/mL of 1,25 dihydroxyvitamin D3 [1,25(OH) 2D] at the beginning of cell cultures. The dose of vitamin D used here was based on the ability of different 1,25 (OH)2D concentrations (1, 5, 10 and 20 ng/mL) to modulate the release of IL-6 by polyclonally-activated T cell cultures from healthy subjects (data not shown). Notably, the 1,25(OH)2D did not affect cell survival, determined by both trypan blue exclusion and Propidium iodide. The cell cultures were maintained at 37 °C in a humidified 5% CO_2 incubator.

2.3. Quantification of cytokines

The quantification of cytokines secreted by polyclonally-activated T cells was performed in by using OptEIA ELISA kits (BD, Pharmigen, San Diego, CA), according to manufacturer's instructions. Briefly, each assay was performed using pairs of mAbs directed to human IL-10, IL-6, IFN-γ, IL-4, IL-5 and IL-17. The reaction was revealed with streptavidin horseradish peroxidase, using 3,3′,5,5′-tetramethyl-benzidine (TMB) as substrate. Recombinant human cytokines, at concentrations ranging from 3.5 to 500 pg/mL, were used to construct standard curves.

2.4. Plasmatic vitamin D measurement

Plasma 25(OH) vitamin D concentration was measured using a commercially available ELISA kit (Immunodiagnostik, Bensheim, Germany) as per the manufacturer's instructions. The 25(OH)D3 levels are normally measured since 1,25(OH)2D is more stable (44). According to the classification proposed by Holick & Chen (2008), normal vitamin D levels were defined as being higher than 30 ng/mL.

2.5. Leptin and IgE quantification

Circulating leptin levels were measured from the harvested plasma and quantified by ELISA using Leptin kit (Human) following manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY). The concentration of IgE secreted by B cells in the co-culture system was determined by human IgE ELISA kit (ab108650) (Abcam co). Briefly, 100 µL of the samples from plasma (leptin) or supernatants of co-cultures (IgE) were added to each well containing anti-leptin or anti-IgE primary antibody, respectively. Then, the wells were incubated for 1 h, washed and treated with 100 µL of the biotinylated secondary IgG antibody for anti-Leptin or for anti-IgE. Finally, 100 µL of the streptavidin-horseradish peroxidase conjugate enzyme was added to the wells and then the TMB substrate (3,3,5,5′-tetramethylbenzidine). Plates were read at 450 nm in ELISA reader (Dynex Technologies, USA). Lyophilized leptin ranging from 31.3–2000 pg/mL was used to construct the standard curve. Regarding IgE, the standard curve was constructed from 10 to 800 IU/mL of recombinant human IgE.

2.6. Statistical analysi

Statistical analysis was performed using Prism 5.0 software (GraphPad Software). All immunological evaluations were performed in triplicate or quadruplicate in each individual and the intra-assay variability ranged from 8.7 to 15.1% (median value of 10.1%) as calculated by the software. To compare > 2 groups, we used one-way ANOVA followed by the Turkey test for data with Gaussian. The non-parametric Mann-Whitney *U* test and the Student's *t*-test were applied to determine whether the two groups were statistically different for nonparametric and parametric variables, respectively. Correlations between variables were investigated using Pearson's correlation. Significance in all experiments was defined as $p < .05$.

3. Results

3.1. Characteristics of AA patients and impact of obesity and severity of disease on the production of cytokines by T cells

For our study, 60 patients (40 female and 20 male) with mild ($n = 20$), moderate ($n = 20$) and severe ($n = 20$) allergic asthma (AA) were recruited and stratified by body mass index (BMI). It is worth noting that patients with severe asthma were overweight ($n = 4$) or obese class I ($n = 16$). In patients with moderate AA, 6 were lean, 10 overweight and 4 obese (class I). Among patients suffering from mild AA, 12 were lean, 6 overweight and 2 had obesity class I. Insufficient mean values of circulating 25(OH)2D3, defined as values < 30 ng/mL, were detected in healthy subjects and AA patients, without any statistical difference (Table 1).

The first immune assay performed was the analysis of the cytokine profile of activated T cells in PBMC cultures in response to mitogen. As can be observed in Fig. 1A, significantly higher levels of IL-6, IL-17, IL-4 and IL-5 were measured in AA patients when compared with the control group. In contrast, IFN-γ production was higher by activated T cells from healthy subjects. No significant difference was observed regarding IL-10 secretion in both experimental groups (Fig. 1A). Notably, no cytokine was detectable in unstimulated PBMC cultures (medium alone) from any subject. When cytokine production was stratified by BMI, no statistical difference was observed concerning almost cytokines secreted in the control cultures, except IL-6 that was produced in higher

Table 1
Characteristics of subjects.

	Control[a]	Asthma[b]
No of subjects (n)	60	60
Gender, female/male (n)	40/20	40/20
Age [(years), mean ± SD]	39.1 ± 17.1	38.9 ± 16.3
Plasmatic vitamin D [mean (range)][c]	25.9 (8.1–117)	22.1 (5.3–67)
BMI (n)[d]		
Lean	18	18
Mild	ND	12
Moderate	ND	6
Severe	ND	0
Overweight	20	20
Mild	ND	6
Moderate	ND	10
Severe	ND	4
Obese class I	22	22
Mild	ND	2
Moderate	ND	4
Severe	ND	16

[a]Healthy individuals and [b]patients with mild ($n = 20$), moderate (n = 20) and severe (n = 20) allergic asthma. [c]Plasmatic 25(OH) vitamin D concentrations in ng/L were measured using commercially available ELISA kit. [d]Body mass index: is a value derived from the mass (weight in kg) and height (in meters) of an individual (lean: 18.5–24.9, overweight: 25–29.9 and obese class I: 30–35). ND = no determined.

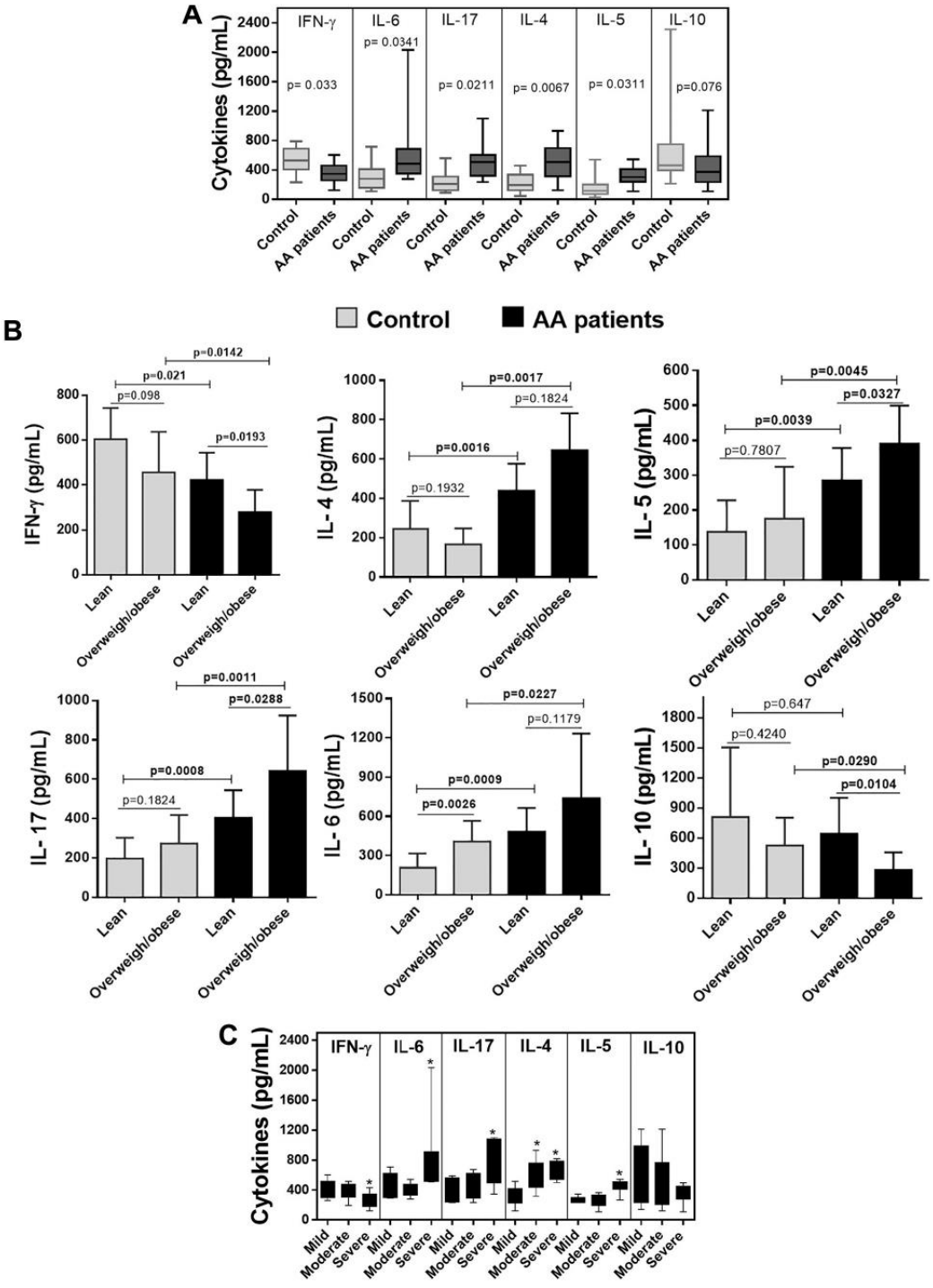


Fig. 1. Cytokine production by polyclonally-activated T cells from asthmatic patients as a function of obesity and clinical status. In (**A**), the *in vitro* production of cytokine by PBMC (1×10^6/mL) from healthy ($n = 60$) and asthmatic patients ($n = 60$) was evaluated by ELISA after activating T cells with anti-CD3/anti-CD28 beads (10 μL/mL) for 3 days. In (**B**) and (**C**), cytokine levels were stratified by BMI and severity of allergic asthma (AA), respectively. In (**A**) and (**B**), the mean values were compared and the *p* values are shown in the figure, In (**C**), (*) indicates $p < .05$.

levels by T cells from overweight subjects (Fig. 1B). In AA patients, the secretion of IFN-γ was lower, especially among overweight/obese subjects. By contrast, higher IL-4, IL-5, IL-6 and IL-17 levels were detected in the supernatants of activated T cell cultures from AA patients, mainly among overweight/obese ones (Fig. 1B). Elevated BMI reduces the ability of polyclonally-activated T cells from patients to produce IL-10 (Fig. 1B). Concerning clinical status, elevated levels of IL-5, IL-6 and IL-17 were observed in cell cultures from patients with severe asthma (Fig. 1C). Moreover, IL-4 levels were significantly higher in those with moderate and severe AA (Fig. 1C). IFN-γ release was compromised in cell cultures from patients suffering from severe AA (Fig. 1C). No difference was observed between disease severity and IL-10 production (Fig. 1C).

3.2. The role of 1,25 (OH)2D3 in modulating cytokine production by T cells from AA patients

The active form of vitamin D, 1,25(OH)2D3, is known to favor the production of IL-10 and reduce pro-inflammatory cytokines by human T cells (60–63). As compared to the control group, the active form of vitamin D was less efficient at reducing the production of cytokines related to the Th1 (IFN-γ), Th2 (IL-4 and IL-5) and Th17 (IL-6 and IL-17) phenotypes in AA patients. Moreover, 1,25(OH)2D3 was less potent at increasing IL-10 secretion by AA-derived activated T cells (Fig. 2A). Among the patients, the effect of 1,25(OH)2D3 for modulating cytokine production was impaired in overweight/obese patients, mainly those suffering from severe asthma (Fig. 2B).

3.3. Relationship between systemic leptin levels and in vitro production of cytokines by T cells of AA patients

Although both experimental groups were matched for BMI, significantly higher concentrations of leptin were quantified in the plasma of AA patients (Fig. 3A). A positive and significant correlation was observed between circulating leptin levels with IL-5, IL-6 and IL-17 concentrations released by AA-derived activated T cell cultures (Fig. 3B). Interestingly, among patients, the leptin levels correlated inversely with the ability of 1,25(OH)2D3 to inhibit the *in vitro* production of IFN-γ, IL-6, IL-5 and IL-17 (Fig. 3C). On the other hand, the ability of the active form of vitamin D to elevate *in vitro* IL-10 production was inversely correlated with leptin levels (Fig. 3C). No correlation was observed with regard to IL-4 production and this adipokine.

3.4. Relationship between plasma levels of leptin and 1,25(OH)2D3 effects on cytokine secretion by $CD4^+$ T cells and IgE production by B cells in AA patients

AA may be mediated not only by Th2 cells but also by the Th17 phenotype [29–32], and similar to what was observed in PBMC cultures, the production of IL-4, IL-5, IL-6 and IL-17 by activated $CD4^+$ T cells were higher in patients than in the control group (Fig. 4A). In this system, the secretion of IL-10 (Fig. 4A) and IFN-γ (data not shown) by mitogen-stimulated $CD4^+$ T cells was lower in patients (Fig. 4A). Moreover, the highest levels of IL-5, IL-6 and IL-17 were detected among overweight/obese patients with severe AA (data not shown). Notably, no cytokine production was observed in unstimulated cultures.

Previous results displayed a lower efficiency of 1,25(OH)2D3 in controlling the overproduction of cytokines related to Th2 and Th17 cell phenotypes in PBMC cultures from AA patients. Here, even in the presence of highest concentration (20 ng/mL), 1,25(OH)2D3 was less efficient at reducing the production of IL-4, IL-5, IL-6 and IL-17 in samples from AA patients (Fig. 4A). Additionally, in these cultures, the active form of vitamin D was also less efficient at increasing IL-10 production (Fig. 4A). As demonstrated in the Fig. 4B, dramatic resistance to the *in vitro* vitamin D effects was seem among overweight/obese patients with severe AA.

With regard to the leptin, a positive and significant correlation was observed between this adipokine and IL-4, IL-5, IL-6 and IL-17 production by $CD4^+$ T cells in AA patients (Fig. 4C). On the other hand, lower IL-10 levels were oberved in cell cultures from patients with high plasma levels of leptin (Fig. 4C). Finally, the ability of 1,25(OH)2D3 in reducing pro-inflammatory cytokines and increasing IL-10 secretion in these cultures was inversely correlated with leptin levels (Fig. 4D).

Classically, in most patients, exacerbation of allergic asthma is accompanied by an increase in IgE production [21–23]. Here, IgE production in SEB-activated $CD4^+$ T/B cells co-cultures was higher in AA subjects when compared with the control group (Fig. 5A). Vitamin D, at the highest concentration (20 ng/mL), was less also efficient at reducing the production of this antibody in patient samples (Fig. 5A). Although the titers of IgE was not correlated with the clinical status (Fig. 5B), B cells from overweight patients trend to secrete more IgE than lean ones (Fig. 5C). No correlation was observed between plasma leptin levels and the *in vitro* production of IgE (Fig. 5D).

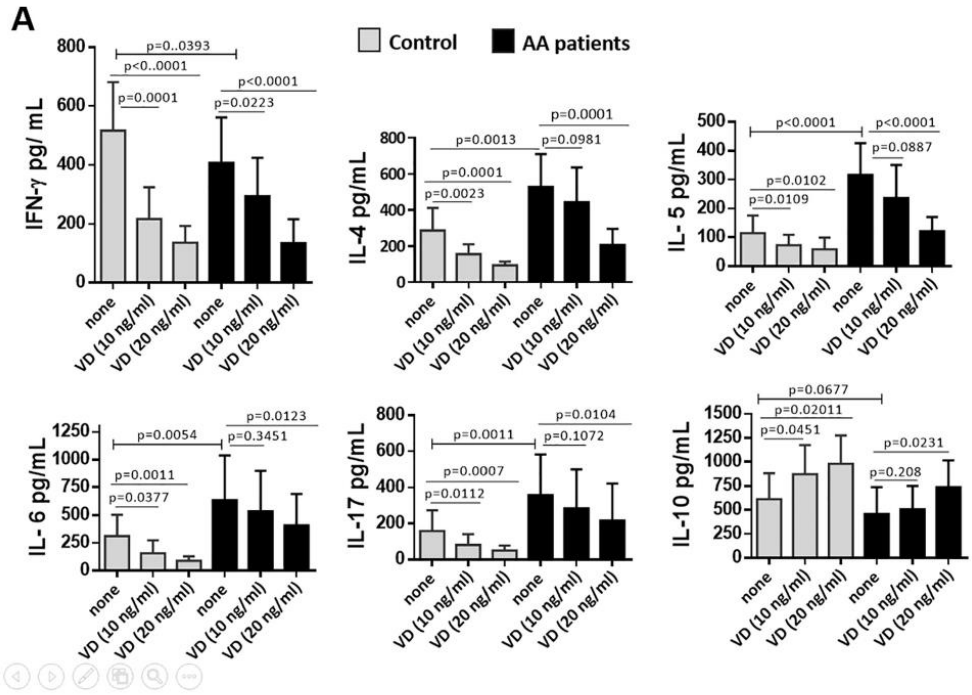
4. Discussion

The prevalence of allergic asthma (AA) has increased dramatically over the last three decades around the world. A similar scenario has been observed for obesity. According to the World Health Organization, in 2016, > 1.9 billion adults were overweight, and, of those, over 650 million were obese (WHO, 2018). Obesity has been described as an important environmental factor that plays a key role in the development and severity of the asthma [34–36]. In addition, obese AA patients account for significant health care expenditures as many of them respond poorly to commonly available therapies. Our findings indicate that being overweight/obese favors the expansion of $CD4^+$ T cells that produce cytokines implicated in the pathogenesis of AA. Furthermore, the immunomodulatory actions of vitamin D on the production of both T cell-derived cytokines and IgE was altered when individuals were overweight/obese and have higher circulating levels of leptin. To our knowledge, this is the first report showing a relationship between obesity, plasma leptin and the *in vitro* effects of vitamin D on AA patients.

In the present study, the majority of AA patients were women, which was expected since the disease is more common in females [66]. It is noteworthy that patients with severe asthma were overweight ($n = 4$) or obese ($n = 16$), and this was related to cytokine imbalance and hypo-responsiveness to vitamin D in $CD4^+$ T cell cultures.

Classically, AA is mediated by Th2 cytokines, such as IL-4 and IL-5, which coordinate an immune response involving allergen-specific IgE, mast cells and eosinophils [9,10]. These cytokines, along with lipid mediators of inflammation released by IgE-activated eosinophils, promote mucus overproduction and bronchoconstriction [21,22]. In the present study, and in line with the classical literature, higher IL-4 and IL-5 levels were detected in the supernatants of both PBMC cultures containing activated T cells and purified $CD4^+$ T cells from AA patients when compared with the control group. Although higher titers of IgE had been secreted in $CD4^+$ T cells/B cells co-cultures from AA patients, their levels were not associated with clinical status. There are at least three reasons to explain why *in vitro* IgE levels were not associated with disease severity. Firstly, as did not measured allergen-specific IgE, it is possible that its levels increase only during an acute asthma episode; secondly, specific IgE can be synthesized and produced locally by B cells within the respiratory mucosa [67]; and thirdly, not all cases of AA involve the production of Th2-related cytokines and detectable IgE. Indeed, allergic asthma is recognized as a heterogeneous disease with different inflammatory profiles. It is well known that Th17 cells can also mediate asthma, and in this case, it tends to be more severe, and is poorly controlled by inhaled corticoids (ICs) and beta-2-adrenergic agonists [68].

An increased IL-17 level has been reported in the peripheral blood



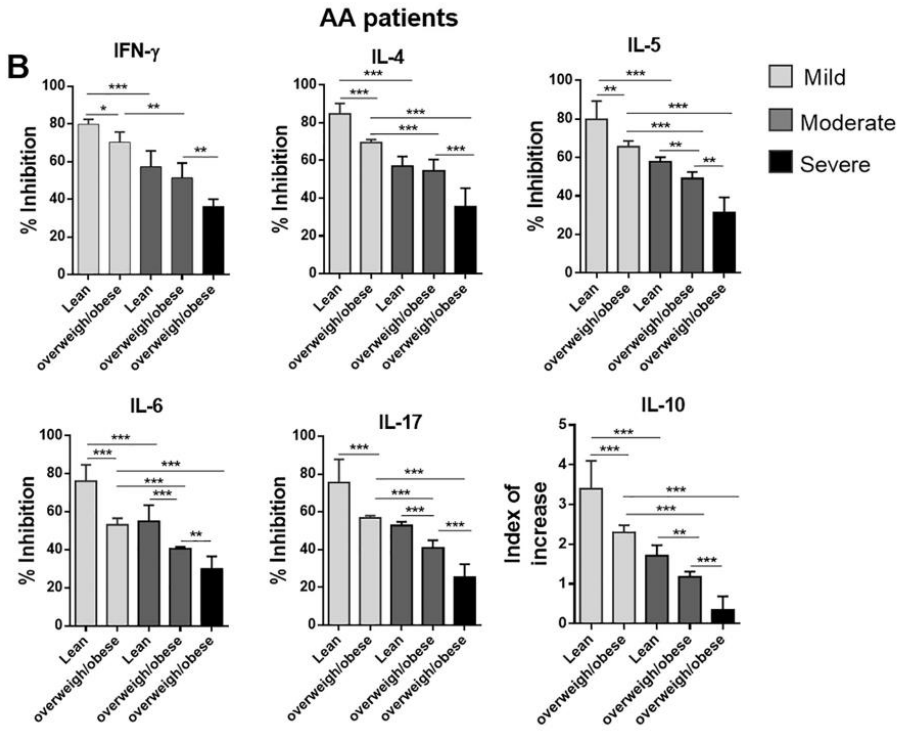


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Fig. 2. Impact of 1,25 (OH)2D3 on the *in vitro* T cell-derived cytokine production of asthmatic patients. In (**A**), the PBMC cultures (1×10^6/mL) from asthmatic patients ($n = 60$) and control group ($n = 60$) were stimulated with anti-CD3/anti-CD28 beads (10 μL/mL), for 3 days. In some wells, different concentrations of 1,25 (OH)2D3 (10 and 20 ng/mL) were added at the beginning of cell cultures. In (**B**), the capacity of 1,25 (OH)2D3 (20 ng/mL) in modulating the release of cytokines by activated T cells from AA patient is showed in function of BMI and clinical status. The levels of cytokines were determined by ELISA. The mean values were compared and the p values shown in the fig. A. In B, (*), (**) and (***) indicates $p < .05$, $p < .01$ and $p < .0001$, respectively.

and lungs of patients with severe asthma [28]. By inducing IL-8 and TNF-α production, IL-17 favors the recruitment and activation of fibroblast and neutrophilic granulocytes to the lower airway [33,34]. The massive infiltration of neutrophils in the lung is a well-known clinical feature of steroid resistant asthma [29,30]. IL-17 also directly affects the airway smooth muscle leading to allergen-induced airway hyperresponsiveness [69]. In the present study, both activated T cells and purified $CD4^+$ T cells from AA patients produced elevated quantities of Th17-related cytokines IL-17 and IL-6 when compared with the control group. The serum IL-6, another Th17-related cytokine, is a marker of asthma severity [70]. In addition, we observed a relationship between asthma severity and IL-17 and IL-6 cytokines produced by T cells. The highest levels of IL-17 were detected in 14 from 20 AA patients with severe AA, and 11 of them were obese. On the other hand, IL-4 was

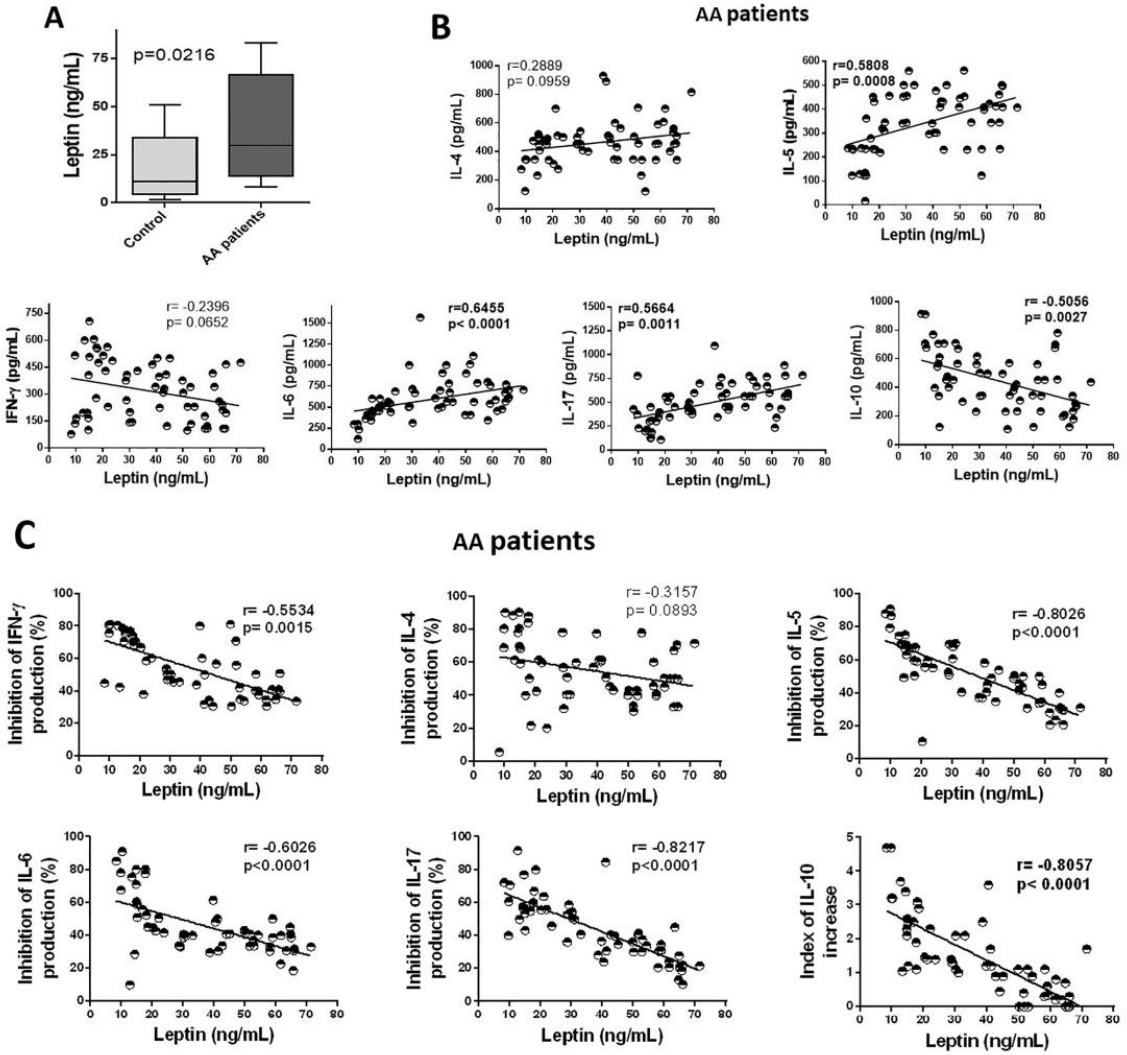

Fig. 3. Leptin dosage and its correlation with *in vitro* cytokine production by activated T cells from asthmatic patients in response to 1,25 (OH)2D3. In (**A**), the plasma levels of leptin were dosed in the control group ($n = 60$) and asthmatic subjects ($n = 60$). The concentrations of leptin were correlated with *in vitro* cytokine production (**B**) and the ability of 1,25 (OH)2D3 (20 ng/mL) (**C**) to modulate the release of those cytokines by T cells from AA patient following activation with anti-CD3/anti-CD28 beads (10 μL/mL). Both leptin and cytokine quantification were determined by ELISA.

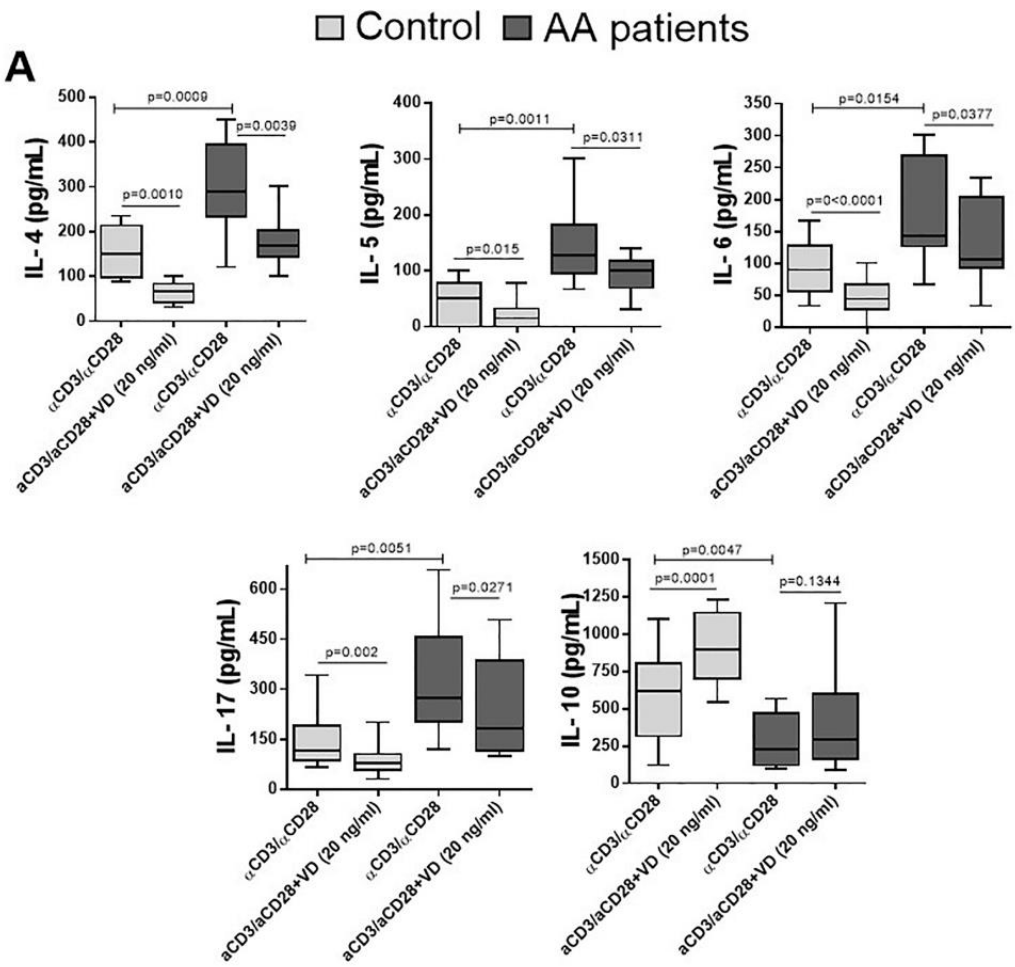

Fig. 4. Effects of 1,25(OH)2D3 on CD4$^+$ T cell-derived cytokine production by asthmatic patients with different plasma leptin levels. In (A), CD4$^+$ T (1×10^6/mL) from both control group ($n = 40$) and asthmatic patients ($n = 44$) were anti-CD3/anti-CD28 beads (10 μL/mL) for 3 days in the presence of 20 ng/mL of 1,25(OH)2D3. The levels of cytokines were determined by ELISA. In (B), all cytokines were stratified in function of obesity and clinical status of AA patients [mild (10 leans, 6 overweight and 2 obese), moderate (6 leans, 5 overweight and 5 obese) and severe (5 overweight and 5 obese)]. The correlation between *in vivo* leptin levels with (C) cytokine production and (D) the ability of 1,25(OH)2D3 at modulating cytokine secretion is also showed at the figures.

dominant among the other 6 patients with severe AA. Although the current literature affirms the existence of at least 2 asthma subtypes, Th2 and Th17 patterns, the levels of IL-5 in our present study varied widely among all severe AA patients. Nevertheless, mean values for IL-5 were invariably higher than those quantified in the cell cultures from patients with mild and moderate AA. More recently, some studies have also described a mixed Th2/Th17 pattern in some AA patients [31,32]. This phenotype is characterized by concomitant production of IL-5 and IL-17 cytokines, pulmonary infiltration of eosinophils, worse lung function and increased steroid requirement [31,32]. Unfortunately, the techniques used in the present study do not allow us to determine the presence of these hybrid Th2/Th17 cells, but this issue will be evaluated by our group using flow cytometry.

An interesting finding was with respect to IFN-γ production. This cytokine is classically secreted by Th1 and Tc-1 cells, both T cell phenotypes mainly implicated in protection against intracellular microorganisms and viruses [71,72]. The production of IFN-γ was significantly lower in AA patients. This phenomenon was particularly evident among overweight/obese patients with severe asthma. In addition to structural changes patients with severe AA favor infections of the lower airways. This is probably due to the decreased capacity of T cell to produce IFN-γ which increases susceptibility to respiratory viral infection, such as influenza and rhinovirus [73]. Increased risks of pneumococcal pneumonia and invasive pneumococcal disease has also been reported in AA patients [74]. The lower serotype-specific pneumococcal antibody titers in these patients were related to an elevated IL-5 to IFN-γ ratio secreted by PBMC after stimulation by allergens [75]. Moreover, those patients show a significantly higher risk of community acquired *E. coli* infection [76]. Altogether, these findings reveal a negative impact of AA on immune resistance against pathogens,wis worsened by obesity.

When obesity occurs with asthma, it is associated with more severe asthma, poor pharmacological control and increased asthma exacerbation [34–36]. This deleterious relationship is probably due to the generation of unique inflammation mediators that modulate the behavior of T cell phenotypes involved in asthma. It is known that obesity

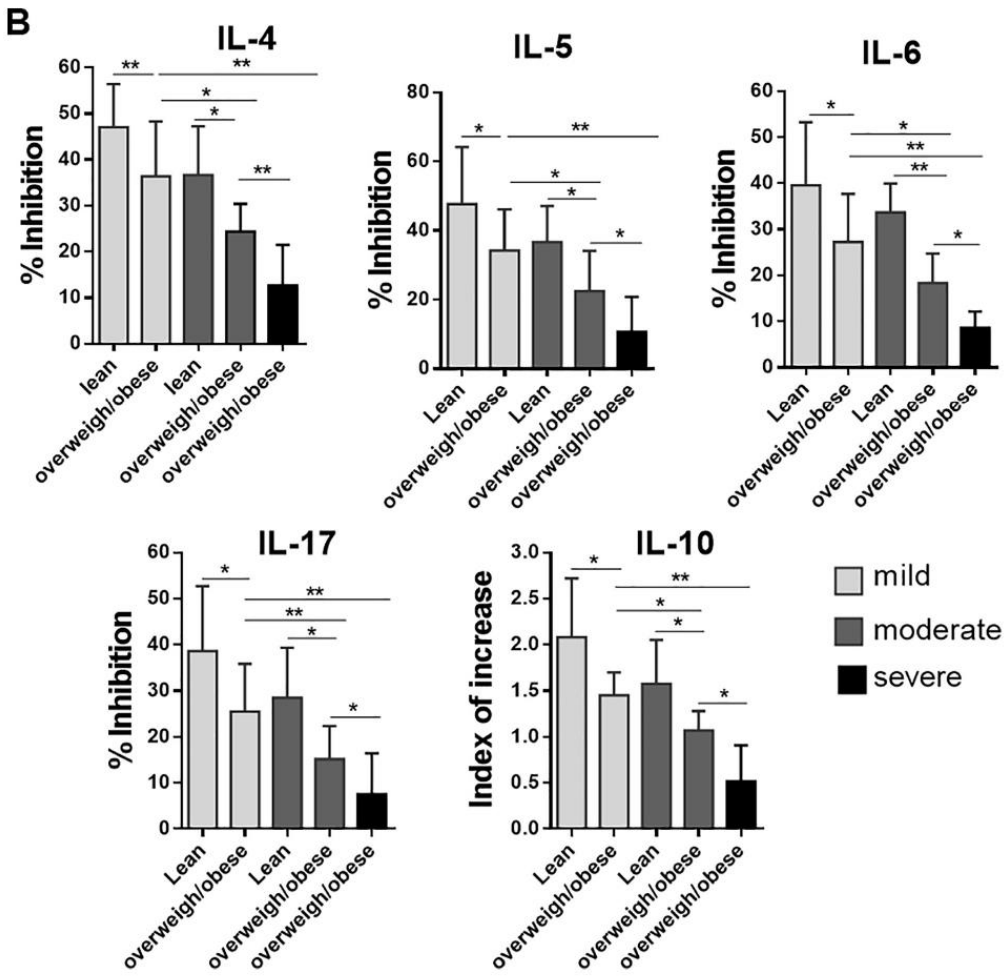

Fig. 4. (*continued*)

complicates both Th2 and Th17-associated asthma, and the deleterious impact of weight gain on allergies may be linked to overproduction of leptin.

Elevated expression of leptin receptors has been observed on T cells from obese subjects [41,42]. On $CD4^+$ T cells, this adipokine favors the expansion of effector T cells, like Th17 cells [40,43–48]. On the other hand, high leptin levels damage regulatory T cell function by inducing IL-1β, TNF-α and IL-6 production [77]. Leptin effects can be direct or indirect, by up regulating the secretion of pro-inflammatory cytokines (TNF-α, IL-6, IL-12) and the expression of co-stimulator molecules by dendritic cells [78]. These immunogenic antigen presenting cells are involved in the induction of hypersensitivity disorders [79,80].

In our study, occurrence of obesity favors an increase of IL-6 production by activated T cells in the control group. In the asthma group, the release of IL-6, IL-17, IL-4 and IL-5 by whole T cells and $CD4^+$ T cells were significantly higher in overweight/obese patients. Interestingly, although both experimental groups were matched for BMI, significantly higher leptin levels were quantified in the plasma of AA patients. This could be explained by elevated inflammatory status in those patients in comparison with the control group, since pro-inflammatory cytokines enhance leptin production [81]. A positive and significant correlation was observed between levels of this adipokine and the concentrations of IL-5, IL-6 and IL-17 quantified in the supernatants of PBMC containing activated T cells as well as purified $CD4^+$ T cell cultures. By contrast, a negative correlation was observed between *in vivo* leptin and IL-10 produced by $CD4^+$ T cells. This latest finding is in line with the literature that correlates hypersensitive disorders, like asthma, with failures in regulatory mechanisms mediated by regulatory T cells, such as IL-10 production [82,83].

In the present study, although no difference was observed concerning IL-10 released by T cells from AA patients from different clinical subgroups, the levels of this anti-inflammatory cytokine were lower in overweight/obese individuals. Interestingly, the proportion of IL-10-secreting $CD4^+$ T cells was significantly lower in overweight/obese patients with severe asthma. This result suggests that obesity may negatively affect IL-10 synthesis by $CD4^+$ T cells in patients with severe asthma. Knowledge about the role of regulatory T cells in allergy comes from immunotherapy (AIT) studies [84]. Successful AIT has been associated with up regulation of functional Tregs and Tr-1 cell subsets, all of them able to produce high levels of anti-inflammatory cytokines, such as IL-10 and TGF-β [85–88]. IL-10 and TGF-β inhibit IgE released by decreasing the production of Th2 cytokines [89], inhibiting the

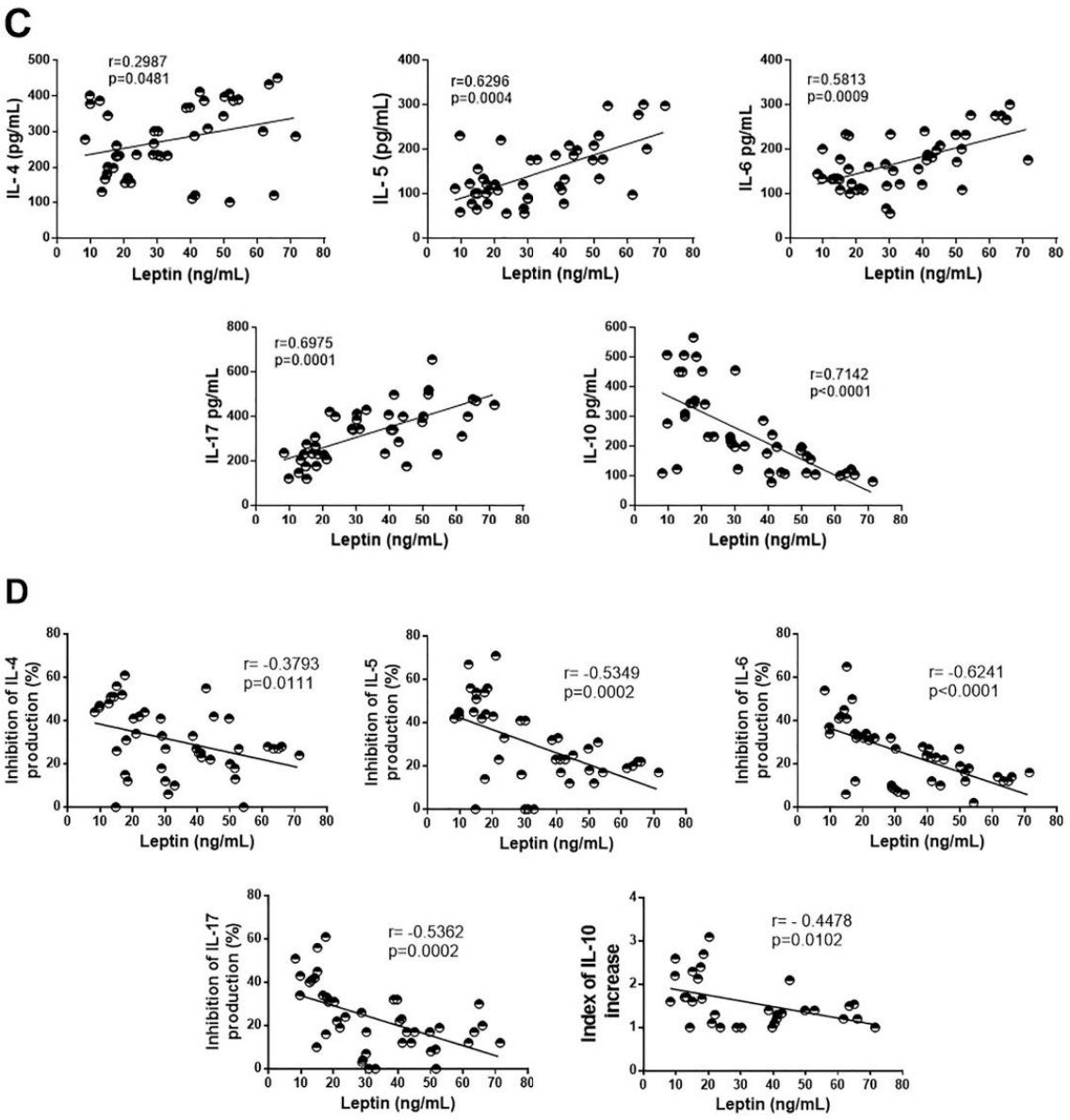

Fig. 4. (*continued*)

degranulation of effector mast cells and eosinophils [90–92]. This is associated with downregulation of FcεRI expression on these cells [84,93,94], and modulating allergen-driven effector T h17 cells as well as neutrophil activation.

Disturbances in regulatory mechanisms in allergic asthma could also be associated with vitamin D deficiency. Although vitamin D deficiency is common in the general population, obese individuals present a higher risk of developing it than eutrophic individuals [50]. Vitamin D deficiency has been reported to be associated with greater risk of asthma [52–59]. Additionally, low serum 25(OH) vitamin D levels increase corticosteroid requirements for controlling inflammation during asthmatic episodes [95]. Further, a study by Zhang et al. [96] demonstrated the ability of the active form of vitamin D, 1,25(OH)2D3, to enhance corticoid's capacity to elevate the production of IL-10 by LPS-activated monocytes from asthmatic patients. 1,25(OH)2D3 also potentiates the ability of corticoid to generate IL-10-producing Tr1 cells [97,98]. These induced Tr1 cells are able to impair proliferation and cytokine production by human effector T cells, including allergen-specific Th2 cell lines [99]. Moreover, 1,25(OH)2D3 inhibits *in vitro* Th17-related cytokine production in severe asthmatic patients regardless of their clinical responsiveness to corticosteroids [100]. In the present study, the active form of vitamin D was less effective at controlling the

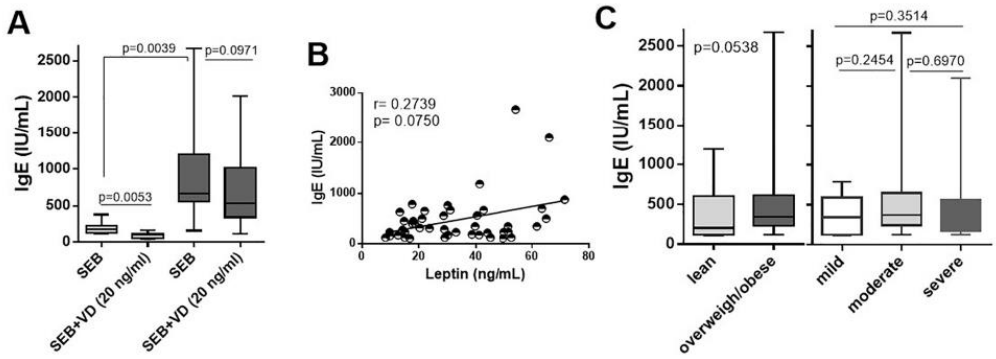

Fig. 5. The *in vitro* IgE production in allergic asthma patients in the presence of vitamin D. In (**A**), $CD4^+$ T (1×10^4/500 μL) and B cell (1×10^4/500 μL) co-cultures from control ($n = 40$) and asthmatic patients (n = 44) were stimulated with SEB (1 μg/mL) for 6 days in the presence of 20 ng/mL of 1,25(OH)2D3. The IgE contents in the supernatants were dosed by ELISA. Among patients with mild (10 leans, 6 overweight and 2 obese), moderate (6 leans, 5 overweight and 5 obese) and severe (5 overweight and 5 obese) asthma, IgE titers are showed in function of either leptin levels (**B**) or obesity and clinical status (**C**).

in vitro overproduction of Th2- and Th17-related cytokines in activated PBMC and $CD4^+$ T cell cultures from AA patients in comparison with the control group. Higher *in vitro* 1,25(OH)2D3 resistance was observed in cell cultures from overweight/obese patients with severe asthma. Moreover, in AA patients, the active form of vitamin D was also less efficient at elevating *in vitro* IL-10 production, with this effect being more marked in activated $CD4^+$ T cells. Finally, the circulating leptin levels were inversely correlated with the ability of 1,25(OH)2D3 to diminish pro-inflammatory cytokines and increase IL-10 released by $CD4^+$ T cell cultures from AA subjects.

5. Conclusions

In summary, our findings suggested that obesity negatively impacts the clinical course of allergic asthma by favoring an imbalance between Th2/Th17 phenotypes and regulatory T cell ratio. These deleterious effects may at least in part be due to lower $CD4^+$ T cell responsiveness to vitamin D and higher circulating levels of leptin.

Declaration of competing interests

All authors declare that there are no conflicts of interest.

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2.2 **Artigo 2 - Leptin favors Th17/Treg cell subsets imbalance associated with allergic asthma severity (Artigo publicado)**

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ORIGINAL ARTICLE

Leptin favors Th17/Treg cell subsets imbalance associated with allergic asthma severity

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Abstract

Background: Obesity has often been associated with severe allergic asthma (AA). Here, we analyzed the frequency of different circulating $CD4^+$T-cell subsets from lean, overweight and obese AA patients.

Methods: Mononuclear cells from peripheral blood were obtained from 60 AA patients and the frequency of different $CD4^+$T-cell subsets and type 1 regulatory B cells (Br1) was determined by cytometry. The effect of obese-related leptin dose on cytokine production and Treg cell function in AA-derived $CD4^+$ T cell cultures was evaluated by ELISA and 3H thymidine uptake, respectively. Leptin levels were quantified in the plasma by ELISA. According to the BMI, patients were stratified as lean, overweight and obese.

Results: AA severity, mainly among obese patients, was associated with an expansion of hybrid Th2/Th17 and Th17-like cells rather than classic Th2-like cells. On the other hand, the frequencies of Th1-like, Br1 cells and regulatory $CD4^+$ T-cell subsets were lower in patients with severe AA. While percentages of the hybrid Th2/Th17 phenotype and Th17-like cells positively correlated with leptin levels, the frequencies of regulatory $CD4^+$ T-cell subsets and Br1 cells negatively correlated with this adipokine. Interestingly, the obesity-related leptin dose not only elevated Th2 and Th17 cytokine levels, but also directly reduced the Treg function in $CD4^+$ T cell cultures from lean AA patients.

Conclusion: In summary, our results indicated that obesity might increase AA severity by favoring the expansion of Th17-like and Th2/Th17 cells and decreasing regulatory $CD4^+$T cell subsets, being adverse effects probably mediated by leptin overproduction.

KEYWORDS
allergic asthma, leptin, obesity, regulatory T cells, Th17 cells

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1 | INTRODUCTION

Asthma is a chronic condition of the lower airways classified as allergic and non-allergic.[1] This clinical condition can significantly reduce the patient's quality of life, in addition to generating economic impacts on health care systems.[2,3] Depending on the number of episodes and the therapeutic treatment required to control exacerbations, persistent asthma can be classified as mild, moderate or severe, with the last being potentially fatal due to the irreversibility of bronchial hyperresponsiveness,[4] even with the standard treatment applying long-acting β2 agonists (bronchodilators) and oral corticoids.

Asthma is a not a single entity, it comprises of complex disease involving different immune mechanisms (endotypes) and variable clinical presentations (phenotypes). Classically, allergic asthma (AA) is endotype triggered by innocuous environmental substances called allergens and involves the activation of allergen-specific T helper 2 (Th2) cells and production of immunoglobulin E (IgE).[5,6] The hallmark of this endotype is the production of high levels of interleukin (IL)-4, IL-5 and IL-13 that favor not only IgE production, but also activation of mast cells and eosinophils in the respiratory tract of AA patients following allergen exposure. Moreover, IL-5 and IL-13 produced by local group 2 innate lymphoid cells (ILC-2) in response to epithelial cell-derived IL-25, IL-33 and thymic stromal lymphopoietin protein (TSLP) amplify Th2-mediated AA.[6,7] In this endotype, the symptoms are particularly a result of biological actions of newly synthetized sulphidopeptide leukotrienes (LTC4, LTD4, and LTE4) and platelet-activating factor (PAF),[8] mainly produced by activated eosinophils. These pro-inflammatory lipids play a pivotal role in the pathogenesis of acute attacks due to their ability to provoke local vasodilatation, edema formation, local neurogenic stimulation, smooth muscle contraction and mucus hypersecretion. However, as aforementioned, the pathogenesis of asthma is even more complex, and some patients, particularly those with resistance to inhaled corticosteroids, present an endotype non-T2 of the disease characterized by intense infiltration of neutrophils in the respiratory airways during exacerbation,[1,4,9] suggesting the involvement of Th17 cells in severe forms of asthma.[10]

In humans, pathogenic Th17 differentiation appears to be induced by IL-23^{high}DCs,[11] with increased levels of both IL-17 and IL-23 being found in the serum and lungs of patients with severe asthma.[10,12-15] Moreover, the mixed-granulocytic endotype, characterized by elevated levels of eosinophils and neutrophils in bronchial-alveolar lavages, involves the induction of dual IL-17 and IL-4-secreting CD4$^+$ T-cells in some patients with severe asthma.[16,17] Interestingly, in vitro studies have indeed revealed a greater sensitivity of Th2 cells to glucocorticoids when compared to Th17 and IL-4$^+$ Th17 cells.[18] Furthermore, in addition to effector CD4$^+$ T cell subsets, asthma severity should also be associated with functional impairment of regulatory lymphocyte compartment capable of producing IL-10, such as T cells that express (Tregs), or not (Tr1), the FoxP3 marker, as well as type 1 regulatory B cells (Br1).[19-21] The existence of several endotypes of the disease with different responses to therapy might be associated with a complex and poorly understood relationship between genetic factors and environmental events, such as obesity.

Obesity can be defined as a chronic disease mediated by abnormal and excessive accumulation of fat in the body, leading to adverse effects on physical, social and mental wellbeing.[22] The prevalence of both asthma[23] and obesity[24] has increased over the past 20 years, and prospective epidemiological meta-analysis studies indicate that asthma and obesity coexist in many patients.[25] In these patients, obesity is an important risk factor for greater frequency and severity of asthma exacerbation and poor response to therapy.[26] This adverse association may involve increased production of different adipokines, such as leptin.

In addition to the increased frequency of both pro-inflammatory M1 macrophages and effector CD4$^+$ and CD8$^+$ T-cells in the adipose tissue, visceral obesity is normally characterized by hyperleptinemia.[27] Apart from its role in regulating balance energy expenditure and nutritional status, leptin is also critical for normal T cell response.[28] Nonetheless, elevated leptin levels have been associated with hypersensitive reactions mediated by both Th2[29] and Th17[30] phenotypes. Moreover, by inducing tumor necrosis factor-α (TNF-α) and IL-6 production, obesity-associated leptin levels also damage Treg function, which reduces the production of anti-inflammatory cytokines such as IL-10.[31,32] In asthmatic patients, high leptin levels were inversely correlated with lung function in asthmatic patients.[32] In a recent study published by our group,[30] a direct correlation between AA severity with both plasma leptin concentrations and levels of IL-5, IL-6 and IL-17, released by purified CD4$^+$ T-cells, was observed. Nonetheless, this study did not analyze the relationship between different CD4$^+$ T cell phenotypes and AA severity. Therefore, the objective of the present study was to characterize the proportions of different effector and regulatory CD4$^+$ T cell subsets, as well as Br1, according to AA severity. Also, the direct effect of obese-related leptin dose on the cytokine production by CD4$^+$ T cells was additionally investigated. It is possible that immune imbalance associated with obesity and elevated leptin levels favor the expansion of different CD4$^+$ T cell subsets associated with AA severity.

2 | MATERIAL AND METHODS

2.1 | Subjects

Sixty patients with AA (47 women and 13 men) were recruited from March 2017 to November 2019 from the Federal University of the State of Rio de Janeiro Hospital/UNIRIO (Rio de Janeiro, Brazil). Persistent asthma was diagnosed by a history of recurrent wheezing, dyspnea and chest tightness, and confirmed by methacoline bronchial hyperresponsiveness [mild (PC20 ≥ 1 but ≤4 mg/ml) or moderate to severe (PC20 < 1 mg/ml)], when FEV1 was ≥70%, or bronchial reversibility after salbutamol inhalation (when

FEV1 was <70%). According to the Global Initiative for Asthma criteria,[5] AA patients were subdivided into 3 groups: mild [n = 20, FEV1 (% predicted) 81–101; FEV1 reversibility (%) 11–18], moderate [n = 20, FEV1 (% predicted) 61–85; FEV1 reversibility (%) 3.9–14] and severe [n = 20, FEV1 (% predicted) 39–77; FEV1 reversibility (%) 2.6–17]. Patients were allowed to receive treatment with inhaled corticosteroids for 2 months prior to the study, but not with systemic steroids. All patients had a positive skin prick test, defined as a >5-mm diameter skin wheal response to at least 1 of 6 common allergens (*Dermatophagoides pteronyssinus*, *D. farinae*, *Alternaria*, mixed grass pollen, dog and cat hair). The great majority of patients are polysensitized (15% of patients had positive reaction to 1 or 2 allergens and 85% had a positive reaction to 3 or more allergens). The presence of rhinitis was observed in 65% (n = 39) and the severity of symptoms was determined by using total nasal symptom score (TNSS) (sneezing, congestion, itching, and rhinorrhea)[33] (Table 1). Of note, relevant clinical allergens were recorded to dust mites (n = 31), cat dander (n = 12), dust mites and cat dander (n = 13), *Altemaria* (n = 2) and mixed grass pollen (n = 2). The occurrence of infectious or other autoimmune diseases were excluded by clinical and serological tests. Twenty healthy subjects (15 women and 5 men), matched by age, gender and body mass index (BMI) were also recruited for the control group. BMI is calculated from the mass (weight in Kg) and height (in meters) of an individual adopting the formula (BMI = kg/m^2). Subjects were stratified as lean (BMI from 18.5 to 24.9), overweight (BMI from 25 to 29.9) and obese class I (BMI from 30 to 35) according to BMI. In the present study, all subjects included were nonsmokers, with no history of upper or lower airway infectious diseases 4 months prior to recruitment in the study. We also excluded individuals taking oral or intravenous steroids, theophylline, long-acting β2-agonists, leukotriene antagonists or antihistamines 2 months prior to the study. The Ethics Committee for Research on Human Subjects at the Federal University of the State of Rio de Janeiro (UNIRIO) approved the study, and blood was collected only after written informed consent was obtained from each individual.

2.2 | Flow cytometry analysis

Peripheral blood was collected in heparin-containing tubes (BD Vacutainer, Franklin Lakes, NY) and peripheral blood mononuclear cells (PBMC) were obtained by centrifugation on the Ficoll–Hypaque density gradient. Fresh viable PBMC (1×10^6/ml) were cultured in 24-well flat-bottomed microplates with 2 ml of RPMI medium (ThermoFisher Scientific Inc.) supplemented with 2 μM of L-glutamine (GIBCO, Carlsbad, CA, USA), 10% of fetal calf serum, 20 U/mL of penicillin, 20 μg/ml of streptomycin and 20 mM of HEPES buffer. The cells were stimulated with phorbolmyristate acetate (PMA, 20 ng/ml; Sigma-Aldrich) plus Ionomycin (600 ng/ml; SigmaAldrich) for 4 h in the presence of brefeldin A (10 μg/ml) (BD Biosciences, San Diego, CA, USA). The cell cultures were maintained at 37°C in a humidified 5% CO2 incubator. After 4 h, different CD4$^+$ T cell subsets and Br1 cells were identified by staining the PBMC with mouse anti-human monoclonal antibodies (mAbs) for CD4-FITC, CD19-FITC, CD39-PE-Cy7, IL-4-APC, IL-10-APC, IL-17-

TABLE 1 Subject characteristics

		AA[b]		
	Control[a]	Mild	Moderate	Severe
No. of subjects (n)	20	20	20	20
Gender (female/male) (n)	15/5	14/6	16/4	17/3
Age [(years), mean ± SD]	31.9 ± 13.1	40.7 ± 18.1	40.2 ± 19.7	42.9 ± 16.8
BMI (n)[c]				
Lean	7	7	6	7
Overweight	6	6	6	5
Obese class I	7	7	8	8
Rhinitis, symptoms severity, n[d]				
None	0	7	4	6
Mild	0	9	2	1
Moderate	0	3	5	8
Severe	0	1	9	5

[a]Healthy individuals.
[b]Patients with mild (n = 20), moderate (n = 20) and severe (n = 20) allergic asthma (AA).
[c]Body mass index: a value derived from the mass (weight in Kg) and height (in meters) of an individual (lean: 18.5–24.9, overweight: 25–29.9 and obese class I: 30–35).
[d]TNSS,[34] total nasal symptom score [Rhinorrhea, nasal itching, nasal obstruction, and sneezing [scored as 0 (none), 3–6 points (mild), 7–9 points (moderate), and 10–12 points (severe)].

PE-Cy7, IFN-γ-PE, and FoxP3-PE. These mAbs and all isotype control antibodies were purchased from BD Biosciences (San Diego, CA, USA). Briefly, whole blood cells were incubated with various combinations of mAbs for surface markers (CD4, CD19 and CD39), for 30 min at room temperature in the dark, according to manufacturer's instructions. The cells were washed with PBS +2%FBS, then the red blood cells were lysed with Fix/Lyse solution (eBiosciences) for 10 min at room temperature before cell permeabilization, which was performed by incubating cells with Cytofix/Cytoperm solution (BD Pharmigen, San Diego, CA) at 4°C for 20 min. After washing, the mAbs for intracellular staining (IL-4-APC, IL-17-PE-Cy7, IFN-γ-PE, IL-10-APC, and FoxP3-PE) were added in different combinations and incubated for 30 min at 4°C. The cells were acquired on Accuri C6 (Accuri™, Ann Arbor, MI, USA) or Attune NxT flow cytometers (Thermo Fisher Corporation) and analyzed using Cflow (Accuri™, Ann Arbor, MI, USA). Isotype control antibodies and single-stained samples were used to periodically check the settings and gates on the flow cytometer. After acquisition of 200,000–300,000 events, lymphocytes were gated based on forward and side scatter properties after the exclusion of dead cells, by using propidium iodide, and doublets. Additionally, gated cells were negative for CD14 marker.

2.3 | Leptin quantification

Circulating leptin levels were measured using a commercial ELISA kit following manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY). Plates were read at 450 nm in ELISA reader (Dynex Technologies, USA). Lyophilized leptin ranging from 31.3 to 2000 pg/ml was used to construct the standard curve.

2.4 | The effect of leptin on cytokine production by $CD4^+$ T cells

The $CD4^+$ T cells were obtained from PBMC via negative selection using magnetic columns according to manufacturer's instructions (EasySep™, StemCell Technology, Canada). Briefly, 50 µL of the isolation cocktail was added to a cell suspension of 1×10^7 cells in 1 ml of HBSS in a 15 ml tube. After 10 min incubation at room temperature, a volume of 100 µL for CD4 of the RapidSpheres suspensions were added to the cell suspension, followed by further incubation at room temperature for 5 min. Subsequently, 4 ml of HBSS were added to the cell suspension and the tube was then placed on a magnet for 5 min. Finally, the supernatants were recovered. The purity of $CD4^+$ T cells was >98%, as measured by flow cytometry (data not shown). The $CD4^+$ T cells (1×10^5/ml) were suspended in RPMI-1640 medium supplemented with 2 µM of L-glutamine (GIBCO, Carlsbad, CA, USA), 10% fetal calf serum, 20U/ml of penicillin, 20 µg/ml of streptomycin and 20 mM of HEPES buffer, cultured in 24-well flat-bottomed microplates in the presence or absence of anti-CD3/anti-CD28 beads (10 µL/ml) for 3 days, which corresponds to the period of maximal T cell activation by polyclonal stimuli. In some cultures, an obesity-related leptin dose (Lep; 50 ng/ml; Figure S1) (Sigma Chemicals, St Louis, MO) was added. After 3 days, the supernatant from different T cell cultures was collected and the cytokines were quantified by ELISA technique using OptEIA ELISA kits (BD, Pharmigen, San Diego, CA), according to manufacturer's instructions. Each ELISA was performed using pairs of antibodies against IL-4, IL-5, IL-13, IFN-γ, IL-6, IL-17A (IL-17), and IL-10. The reaction was revealed with streptavidin-horseradish peroxidase, using 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate. Recombinant human cytokines, at concentrations ranging from 3.5 to 500 pg/ml, were used to construct standard curves.

2.5 | The role of leptin in modulating Treg function

In another set of experiments, following manufacturer's instructions, PBMC cultures were enriched with $CD4^+$ $CD127^{low}$ $CD25^+$ regulatory T cells (Tregs) and $CD4^+CD25^-$ responder T cells (Tresp) by using EasySep™ kits (EasySep™, StemCell Technology, Canada). As determined by cytometry, the purity of the Tregs and Tresp cells was >91.4% and >93.9%, respectively (data not shown). For the suppression assay, $CD4^+CD25^+CD127^{neg/low}$ T cells (Tregs, 1×10^5/ml) from AA patients were maintained for 24 h in the presence of medium alone or leptin (50 ng/ml). At the end of the incubation period, the cells were washed with medium (RPMI at 5% CFS), to remove residual adipokine, and then co-cultured with autologous $CD4^+CD25^-CD127^+$ Tresp at 1:8 and 1:4 Treg/Tresp ratios. Co-cultures were set up in triplicate, incubated for 3 days at 37°C in the presence of anti-CD3/anti-CD28 beads, and cell proliferation was evaluated through 3H thymidine uptake after addition of 1 µCi/well 8 h to the cell cultures before the end of the incubation period. The cells were harvested in glass fiber filters in an automatic cell harvester and radioactive incorporation was measured using a liquid-scintillation counter.

2.6 | Statistical analyzes

All statistical analyzes were conducted using the Prism 8.0 program (GraphPad Software). Immunological evaluations were performed in triplicate or quadruplicate for each individual with the intra-assay variability ranging from 8.9% to 13.7% (median value of 11.2%) as calculated by the software. Comparisons between immune assays in cell cultures from the different groups were performed with ANOVA followed by Tukey test for data with Gaussian distribution and by Kruskal-Wallis followed by Dunn's test for data without Gaussian distribution. The results were also corrected by Bonferroni. The analysis of correlations between plasma leptin levels and different lymphocyte subtypes was conducted using Spearman correlation. Significance in all experiments was defined as $p < 0.05$.

3 | RESULTS

3.1 | Impact of clinical status and obesity on different effector CD4$^+$ T-cell subsets in AA patients

For our study, 60 patients (47 females and 13 male) with mild (n = 20), moderate (n = 20) and severe (n = 20) allergic asthma (AA) were recruited and subsequently stratified by body mass index (BMI). As expected, most patients also suffered from rhinitis (Table 1). Twenty healthy subjects (15 females and 05 male) were also recruited as control (Table 1). Of note, no difference in the CD4$^+$ T cell and B cell counts was observed between healthy subjects and AA patients in the different clinical subgroups. Our primary objective was to evaluate the proportion of total IL-4$^+$CD4$^+$ T cells in AA patients. Following the gating strategy shown in Figure 1A, a higher proportion of IL-4$^+$CD4$^+$ T-cells was detected in AA patients when compared to individuals from the control group, as expected (Figure 1B). Additionally, among patients, the highest percentage of these cells was detected in samples from individuals with severe AA (Figure 1B).

Based on the combination of cytokines used to identify classical Th1 (IL-4$^-$IL-17$^-$IFN-γ$^+$), Th2 (IL-4$^+$ IL-17$^-$IFN-γ$^-$) and Th17 (IL-4$^-$IL-17$^+$IFN-γ$^-$) phenotypes, we observed that, in the control group, the great majority of total IL-4$^+$CD4$^+$ T-cells expressed neither IL-17 nor IFN-γ (Figure 1C). Interestingly, in patients, the severity of the disease was associated with an expansion of IL-4$^+$IL-17$^+$IFN-γ$^-$ CD4$^+$ T-cells (Figure 1D) rather than classic Th2-like cells (Figure 1C). This hybrid Th2/Th17 cell subtype was practically absent in the control group (Figure 1D). Among CD4$^+$ T cells negative for IL-4, the proportion of IFN-γ$^+$IL-17$^-$ (type Th1) was significantly higher in healthy individuals in comparison to patients (Figure 1E). In the AA group, an important decrease in the frequency of Th1 cells was observed in patients with severe AA (Figure 1E). In contrast, a higher frequency of Th17-like cells was detected in patients with severe AA when compared to all other individuals (Figure 1F). No difference was observed in the proportion of this phenotype between the control group and patients with mild or moderate AA (Figure 1F). Finally, the percentage of IL-4$^-$CD4$^+$ T cells capable of co-expressing IL-17 and IFN-γ was low and no difference between the different groups of subjects was observed (Figure 1G).

Regarding the BMI of patients, although the percentage of total CD4$^+$ T cells had not been associated with fat mass, the frequency of Th2-like cells was significantly lower in obese individuals with severe disease when compared to mild and moderate AA (Figure 2A). No significant difference was observed in the groups with mild and moderate AA in terms of Th2 cells (Figure 2A). On the other hand, a higher percentage of the hybrid Th2/Th17 phenotype was observed in obese patients with severe AA, with no significant difference between patients with mild or moderate forms of the disease (Figure 2B). Similarly, the highest percentages of Th17-like cells were observed in obese AA patients, especially those with severe disease (Figure 2C). In contrast, both the severity and excessive weight gain negatively impacted the frequency of Th1-like cells in AA patients (Figure 2D).

3.2 | Obesity amplifies damage in the regulatory lymphocyte compartment in patients with AA

Following the gating strategy for identifying CD4$^+$ T cells that express the FoxP3, IL-10 and CD39 markers demonstrated in Figure 3A, we observed a lower frequency of IL-10$^+$Foxp3$^+$CD4$^+$ T cells (Tregs) in patients with severe AA when compared to the control group. Among IL-10$^+$FoxP3$^-$CD4$^+$ T cells, known as Tr1 cells,[34] a lower percentage was observed in patients with moderate and severe AA when compared to the control group (Figure 3B), without statistical difference between healthy individuals and mild AA patients. Amongst the patients, the lowest percentage of Tr1 cells was observed among those with severe AA (Figure 3B). The acquisition of the CD39 marker has been associated with high functional capacity of FoxP3$^+$ CD4$^+$ T-cells.[35] Figure 3C shows that the percentage of these cells was significantly higher in healthy individuals when compared with patients with moderate and severe AA, but not when compared to those with mild AA. Amongst patients, the frequency of this subtype of regulatory T cell was highly variable with no significant difference according to AA severity (Figure 3C). As for Br1 cells, identified as IL-10$^+$CD19$^+$ cells (Figure 3D), a lower percentage was identified in the samples obtained from patients with moderate and severe AA when compared to the control group (Figure 3E). Among patients, the frequency of Br1 cells was significantly higher in those with mild AA compared to individuals with severe forms of the disease (Figure 3E).

Regarding BMI, obesity was associated with a lower percentage of IL-10$^+$FoxP3$^+$CD4$^+$ T-cells only in patients with severe AA when compared to other patients and the control group (Figure 4A). In addition, the percentage of CD39$^+$FoxP3$^+$CD4$^+$ T-cells was significantly lower in overweight or obese patients who presented moderate or severe forms of AA (Figure 4C). As for Tr1 cells, a lower frequency of this cell subtype was observed only in obese patients with moderate and, mainly, severe AA (Figure 4B). As demonstrated in Figure 4D, the presence of obesity was also associated with a lower percentage of IL-10$^+$CD19$^+$ cells among patients with mild, moderate and, mainly, severe AA.

3.3 | Elevated plasma leptin levels are related to an imbalance of CD4$^+$ T-cell subsets and Br1 cells implicated in AA severity

Many of the immune disorders in obese patients have been associated with high production of certain adipokines, particularly leptin.[28-32,36,37] As expected, plasma leptin levels were significantly lower in lean subjects from the control group and AA patients when compared to their overweight/obese counterparts (Figure 5A). However, and interestingly, among lean subjects, leptin concentrations were higher in severe

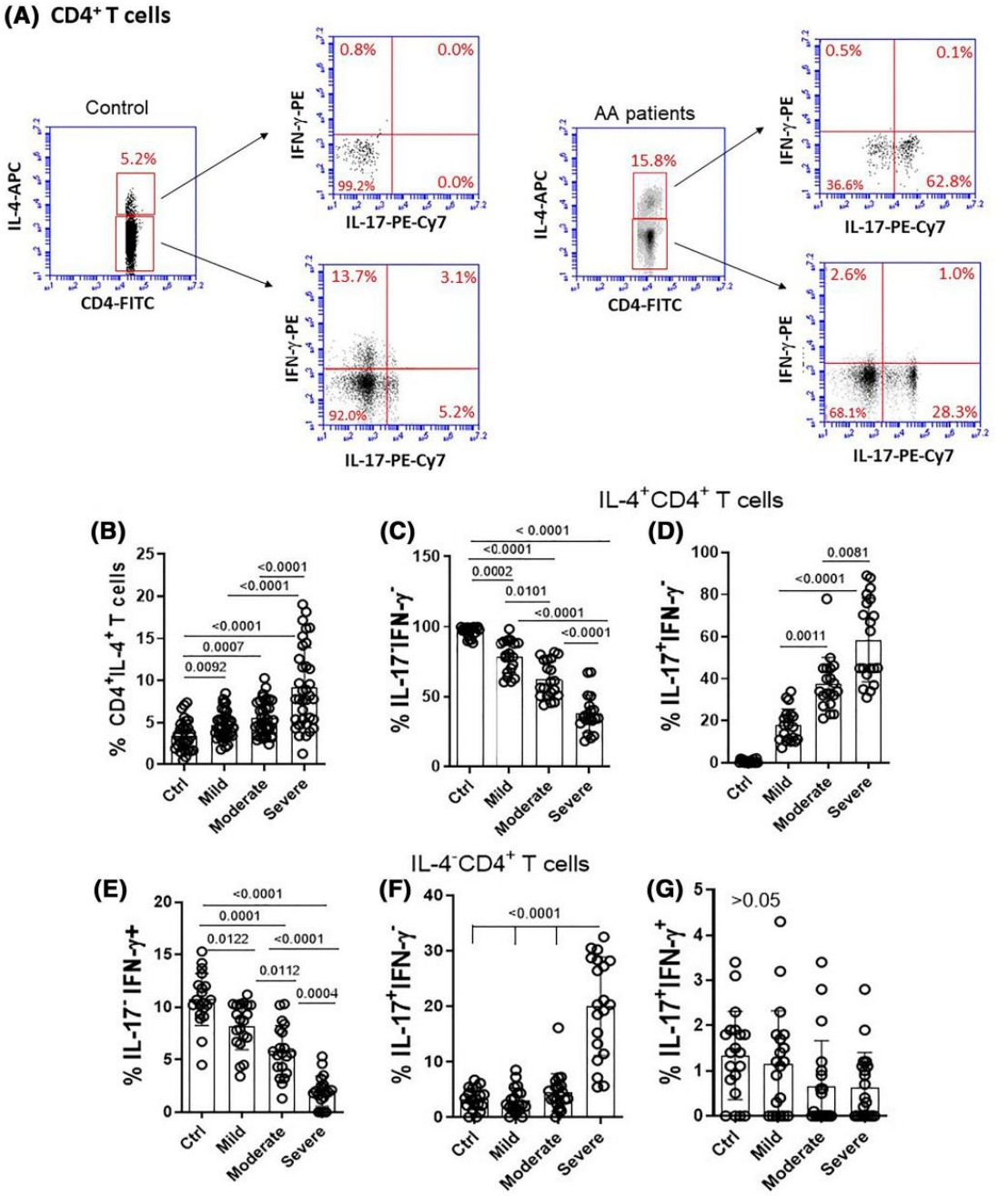

FIGURE 1 Frequency of circulating CD4+ T cell subtypes capable of producing IL-4, IL-17 and IFN-γ in AA patients according to clinical status. PBMC (1×10^6/ml), obtained from healthy subjects ($n = 20$) and patients with mild (= 20), moderate ($n = 20$), and severe ($n = 20$) AA were stimulated for 4 h with PMA (20 ng/ml) and ionomicina (600 ng/ml). Taking into consideration the gating strategy shown in the panel A, the percentage of (B) all IL-4+CD4+T cells, as well as (C) Th2-like (IL-4+IL-17−IFN-γ-), (D) hybrid Th2/Th17 phenotype (IL-4+IL-17+IFN-γ-), (E) Th1-lie (IL-4−IL-17−IFN-γ+), (F) Th17-like (IL-4−IL-17+IFN-γ-) and (G) dual Th1/TH17 phenotype were identified by cytometry. The mean values were compared and analyzed between the groups using the one-way ANOVA and the p value shown in the figure

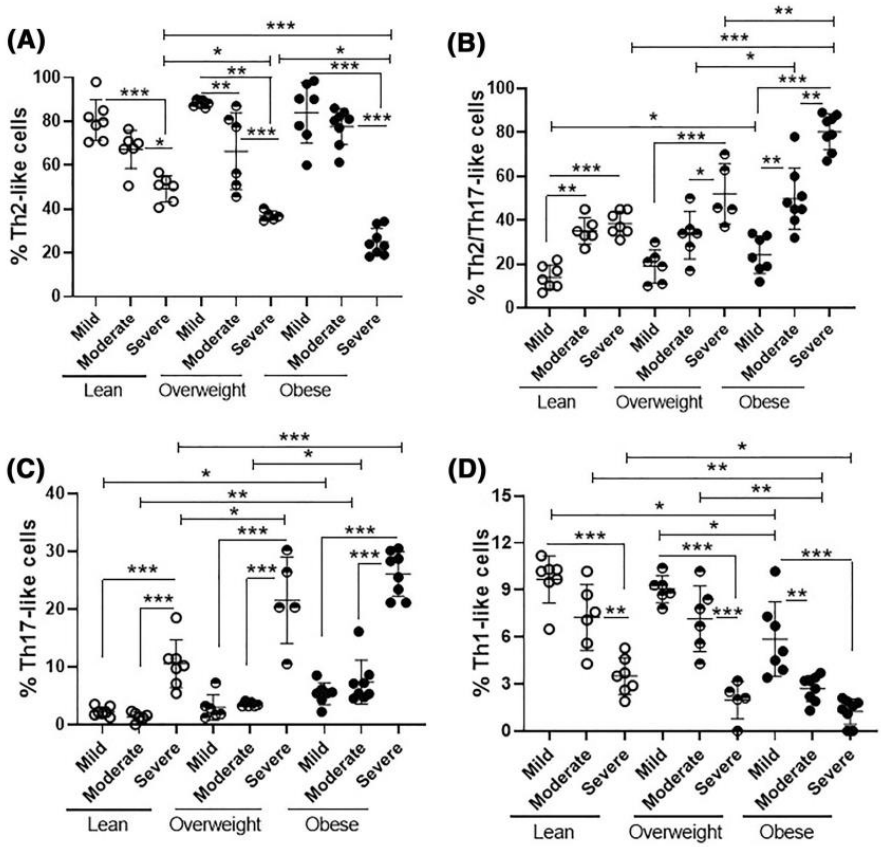

FIGURE 2 Frequency of different subtypes of circulating $CD4^+$ T cells capable of producing IL-4, IL-17 and IFN-γ in AA patients according to BMI. PBMC (1×10^6/ml), obtained from healthy subjects ($n = 20$) and patients with mild (= 20), moderate ($n = 20$) and severe ($n = 20$) AA were stimulated for 4 h with PMA (20 ng/ml) and ionomicina (600 ng/ml), and the percentage of $CD4^+$ T cells related to (A) Th2 ($IL\text{-}4^+IL\text{-}17^-IFN\text{-}\gamma^-$), (B) Th2/Th17 ($IL\text{-}4^+IL\text{-}17^+IFN\text{-}\gamma^-$), (C) Th17 ($IL\text{-}4^-IL\text{-}17^+IFN\text{-}\gamma^-$) and (D) Th1 ($IL\text{-}4^-IL\text{-}17^-IFN\text{-}\gamma^+$) phenotypes from patients were stratified by BMI within each clinical subgroup of AA (mild, moderate or severe). The mean values were compared and analyzed between the different groups using the two-way ANOVA and (*), (**), (***) indicate $p < 0.05$, <0.001 and <0.0001

AA patients (Figure 5A). Similarly, among the overweight/obese subjects, circulating levels of this adipokine were also significantly higher in AA patients with moderate and severe disease (Figure 5A). Regarding the different $CD4^+$ T-cell phenotypes, a positive and significant correlation was observed between plasma leptin levels and the percentages of hybrid Th2/Th17 phenotype (Figure 5C) and Th17-like cells (Figure 5E). On the other hand, an inverse and significant correlation was observed between this adipokine and the proportion of Th1-type cells (Figure 5D). No correlation was observed between plasma leptin concentrations and the percentage of Th2-type cells (Figure 5B). With regard to regulatory phenotypes, a significant decrease in the percentage of Tr1 cells (Figure 5G) and $CD39^+FoxP3^+CD4^+$ T-cells (Figure 5H), as well as Br1 cells (Figure 5I), was observed in patients with higher circulating levels of this adipokine in patients with AA, with no significant difference in the proportion of $IL\text{-}10^+FoxP3^+CD4^+$ T-cells (Figure 5F). In the control group, similar results were observed. While leptin levels positively correlated with the percentage of Th17-like cells, a negative correlation was observed between this adipokine and the frequency $CD39^+FoxP3^+CD4^+$ T-cells and Tr1 cells (Figure S2).

3.4 | Effect of leptin on in vitro cytokine production and Treg function in $CD4^+$ T cells from AA patients

Previous findings demonstrated the relationship between the plasma leptin levels and the frequency of different effector and regulatory T cells in AA patients. As demonstrated in Figure 6, higher levels of IL-5 (Figure 6B), IL-6 (Figure 6C), IL-13 (Figure 6E), and IL-17 (Figure 6F), associated with a lower release of IL-10 (Figure 6D), were observed

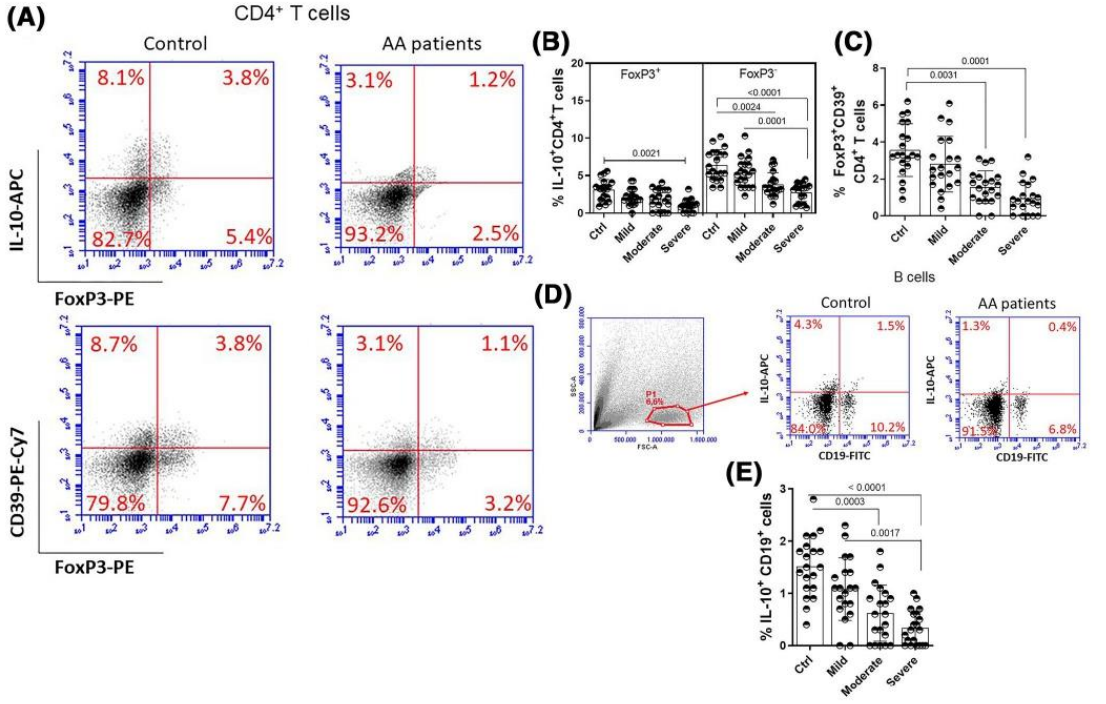

FIGURE 3 Frequency of regulatory CD4⁺ T cells and Br1 cells in AA patients according to clinical status. PBMC (1×10^6/ml), obtained from healthy subjects ($n = 20$) and patients with mild (= 20), moderate ($n = 20$) and severe ($n = 20$) AA were stimulated for 4 h with PMA (20 ng/ml) and ionomicina (600 ng/ml), and, following the gating strategy shown in the panel A, the percentage of IL-10⁺ CD4⁺ T cells able to express or not FoxP3 protein (B) as well as FoxP3⁺CD39⁺ CD4⁺ T cells (C) was evaluated by cytometry. In (D) and (E), the identification strategy and mean values of regulatory B cells (CD19 + IL-10 ⁺) are shown, respectively. The mean values were compared and analyzed between the groups using the one-way ANOVA and the *p* value shown in the figure

in purified CD4⁺ T cells from more obese than leaner AA patients. Interestingly, obesity-related leptin concentration significantly increased the ability of purified CD4⁺ T cells from lean AA patients to produce IL-5, IL-13, IL-17 and IL-6, but diminished IL-10 release. No significant difference was observed with regard to IL-4 (Figure 6A) and IFN-γ (data not shown). Although no significant difference was observed for pro-inflammatory cytokines released by obese-derived cell cultures in response to leptin, this adipokine reduced IL-10 levels (Figure 6D). Interestingly, in addition to reducing the proportion of IL-10-secreting CD4⁺ T cells, leptin significantly damages the ability of Tregs to inhibit Tresp proliferation (Figure 7) in cell cultures from lean ($n = 5$) and obese ($n = 5$) AA patients.

4 | DISCUSSION

Classically, AA has been described as an adverse immune event mediated by allergen-specific Th2 cells, which involves the release of IL-4, IL-5 and IL-13, IgE production and mast cell and eosinophil activation.[5] These granulocytes release different pro-inflammatory mediators involved in AA immunopathogenesis, particularly leukotrienes (C4, D4 and E4) and PAF.[5] In the present study, and in agreement with the literature, a higher proportion of total IL-4-producing CD4⁺ T cells was detected in the peripheral blood of AA patients when compared to the group of healthy individuals (control group), mainly among patients with severe AA. Nonetheless, and interestingly, we have found that most IL-4-secreting CD4⁺ T-cells in patients with severe AA were IL-17 positive and IFN-γ negative. In addition, a higher proportion of typical Th17 cells was also observed in patients with severe AA. This finding is in line with other studies of asthma that demonstrated the complex immunopathogenesis of the disease, which involves other CD4⁺ T-cell subtypes in lung disease, such as Th17 and IL-17⁺IL-4⁺CD4⁺ T-cells.[10,12-17] Moreover, in addition to IL-17, higher levels of IL-5, IL-6 and IL-13 were released by activated CD4⁺ T cells from obese AA patients. Regarding IL-4, the lack of significance in the levels of this Th2-related cytokine between the two experimental groups may indicate that only IL-4-producing Th17-like cells are associated with AA severity. Notable association was observed between obesity and expansion of circulating hybrid Th2/Th17 and Th17cells in our AA patients, mainly

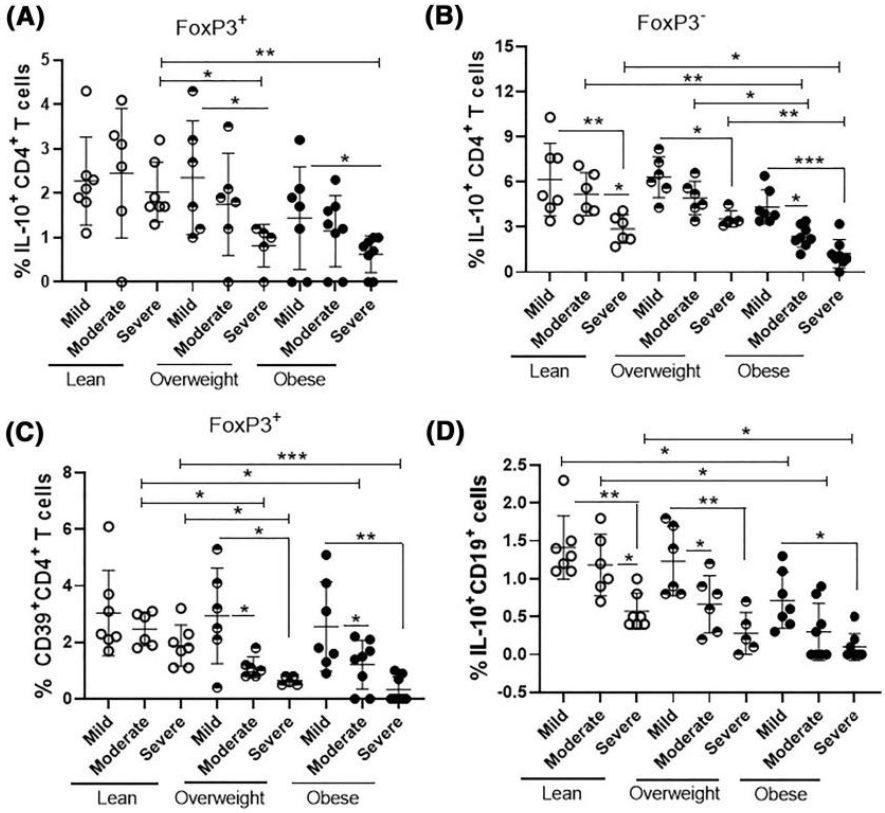

FIGURE 4 Frequency of $CD4^+$ T lymphocyte subtypes and Br1 cells in AA patients according to BMI. The proportion of $IL\text{-}10^+CD4^+$ T cells capable (A) or not (C) of expressing FoxP3, as well as (B) $FoxP3^+CD39^+CD4^+$ T cells and (D) $IL\text{-}10^+$B lymphocytes was stratified by BMI for each clinical group of patients with mild, moderate or severe AA. The percentage of different cell types was evaluated after stimulating PBMC (1×10^6/ml) for 4 h with PMA (20 ng/ml) and ionomicina (600 ng/ml) by cytometry using anti-CD4, anti-CD39, anti-CD19, anti-FoxP3 and anti-IL-10. The mean values were compared and analyzed between the groups using the two-way ANOVA and (*), (**), (***) indicate $p < 0.05$, <0.001 and <0.0001

those with severe forms of the disease. These findings corroborate the hypothesis that obesity is linked to worse clinical outcomes for asthma. This adverse association must be related to chronic state of systemic low-grade inflammation in which the obese subject is conditioned by a greater production of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNFα, and overproduction of some adipokines, particularly leptin.[27,28]

Elevated leptinemia aggravates allergic diseases.[29,30] Kalmarzi et al.[32] demonstrated an inverse correlation between plasma leptin levels and lung function in asthma patients. In the present study, even when paired with body mass index, higher plasma leptin levels were quantified in moderate and severe AA patients when compared with the control group. This phenomenon should be associated with the ability of cytokines related to Th17 (IL-1β, IL-6 and IL-17) and Th2 (IL-4 and IL-5) cells in up-regulating the production of this adipokine.[36-38] Concerning $CD4^+$ T cell phenotypes, we have found a positive correlation between plasma leptin levels with the frequency of hybrid Th2/Th17 cells and Th17-like cells. Other authors have already demonstrated the relationship between IL-17 and leptin,[38] however, the correlation between this adipokine and hybrid Th2/Th17 cells is new in the literature, since this double phenotype is still little explored.

Regard to the classic Th2-like cells ($IL\text{-}4^+IFN\text{-}\gamma^-IL\text{-}17^-$) no correlation was observed with leptin concentrations and this phenotype. Nonetheless, obesity-related dose of leptin elevated not only the release of IL-6 and IL-17, but also IL-5 and IL-13 by $CD4^+$ T cells in lean-derived cell cultures from patients. Ciprandi et al.[39] demonstrated a direct relationship between leptin levels and IgE titers and eosinophil counts in allergic patients. Therefore, there is a possibility that hyperleptinemia amplifies lung damage during acute exacerbations of asthma by directly up regulating the production of cytokines by Th2/Th17 cell subsets.

Different to Th2 cells, classical Th1 cells are not associated with AA, and, in the present study, the percentage of these cells

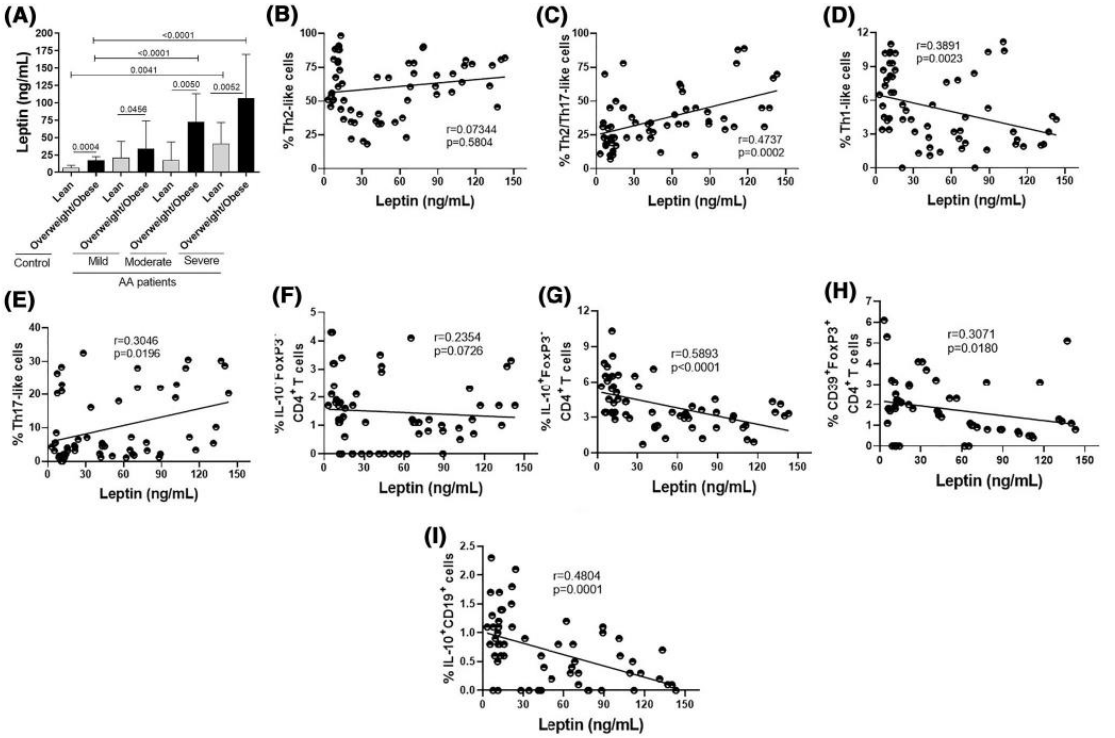

FIGURE 5 Plasma leptin dosage and its correlation with the frequency of different $CD4^+$ T cell subtypes and circulating Br1 cells in AA patients. In (A), plasma from healthy subjects ($n = 20$) and patients with mild ($n = 20$), moderate ($n = 20$) and severe ($n = 20$) AA with different BMI values, were submitted to leptin dosage by ELISA and mean values were compared between the different groups. In (B-E), the leptin levels were correlated with the percentage of different $CD4^+$ T cell subsets related with (B) Th2 ($IL\text{-}4^+IL\text{-}17^-$ $IFN\text{-}\gamma^-$), (C) Th2/Th17 ($IL\text{-}4^+IL\text{-}17^+IFN\text{-}\gamma^-$), (D) Th1 ($IL\text{-}4^-IL\text{-}17^-$ $IFN\text{-}\gamma^+$), and (E) Th17 ($IL\text{-}4^-IL\text{-}17^+IFN\text{-}\gamma^-$) phenotypes, as well as $IL\text{-}10^+$ $CD4^+$ T cells, expressing (F) or not FoxP3 (G), $CD39^+FoxP3^+CD4^+$ T cells (H) and Br1 cells (I). The variables were submitted to Spearman's correlation and the *p* values indicated in the figure

was dramatically reduced in obese AA patients with severe forms of the disease. In addition, the proportion of this $CD4^+$ T cell subset was negatively correlated with plasma leptin levels. Classically, by releasing IFN-γ, Th1 cells are fundamental in the response against intracellular pathogens, since this cytokine elevates microbicidal power of phagocytes and the cytotoxic function of both NK and $CD8^+$ T cells.[40] Thus, this lower frequency of Th1 cells in our patients should help explain, at least in part, why severe AA elevates susceptibility to infections by intracellular pathogens.[41]

In addition to the involvement of different effector $CD4^+$ T cell subtypes, development of AA is also associated with damage in IL-10 production by $CD4^+$T and B cells.[42,43] Here, both AA severity and obesity negatively affected the frequency of Tregs and Tr1 cells. Moreover, higher plasma leptin levels correlated inversely with those cells. Also, obesity-related leptin level not only decreased IL-10 production by $CD4^+$ T cells, but also reduced the in vitro suppressive function of these lymphocytes from lean and obese AA patients.

Like IL-10, a cytokine that plays a potent anti-inflammatory role, the CD39 molecule has also been described as a marker of Treg cell function.[35] CD39, in association with CD73, metabolizes the extracellular adenosine triphosphate (ATP) molecule into adenosine monophosphate (AMP), a potent immune inhibitor.[35] Here, lower frequency of $CD39^+FoxP3^+CD4^+$ T-cells was observed in patients with moderate and severe AA. Further, a lower frequency of these cells was found in patients with higher plasma levels of leptin. This finding is new for AA, but a study by Cortez-Espinosa et al.[44] observed a negative correlation between the proportion of $CD39^+$ Treg cells with IBM in patients suffering from type 2 diabetes.

In addition to T cells, IL-10-secreting B (Br1) have been associated with protection against allergic diseases.[42,43] In the present study, we observed a decrease in the proportion of Br1 cells in patients with moderate and, mainly, severe AA. Among these patients, the lowest proportion of Br1 was seen in obese patients with high plasma leptin levels.

Although preliminary, our findings showed that obesity favors the expansion of circulating Th17-like cells and hybrid Th2/Th17

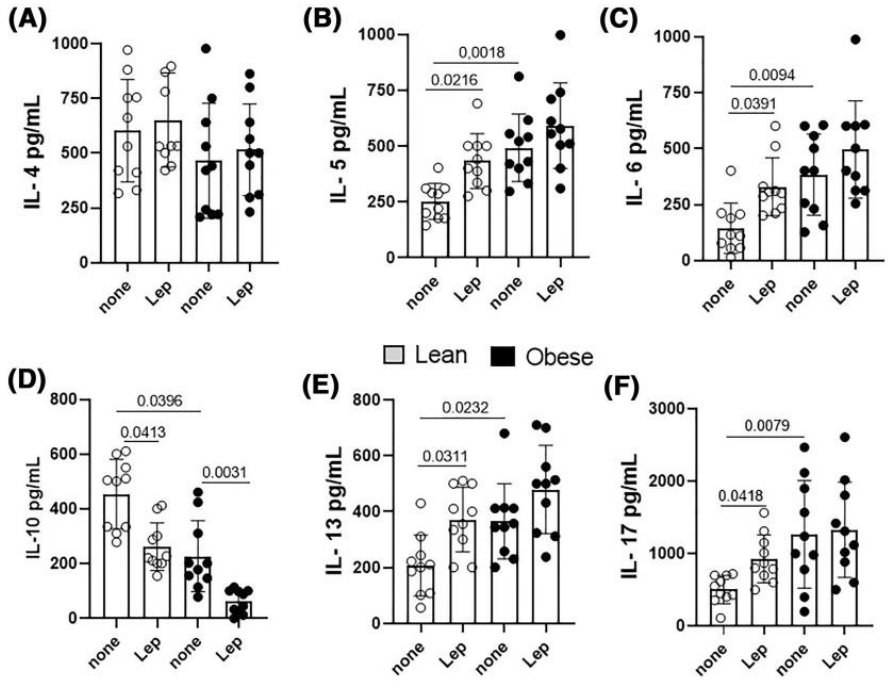

FIGURE 6 The role of leptin in cytokine production by $CD4^+$ T cells from AA patients. $CD4^+$ T cells (1×10^6/ml) from lean ($n = 10$) and obese ($n = 10$) AA patients were stimulated with anti-CD3/anti-CD28 beads (10 µL/ml) in the presence of leptin (50 ng/ml). After 3 days, the supernatants were collected and the cytokine contents assayed by ELISA. Data are shown as mean ± SD of 5 independent experiments with 1 lean and 1 obese AA sample per experiment. Significance was calculated by comparing the different groups and the p values indicated in the figure

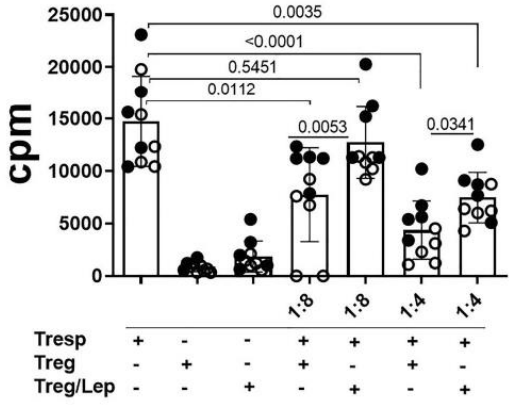

FIGURE 7 Leptin directly reduces in vitro Treg function. Treg cells (2.5×10^4), previously maintained with medium alone or with leptin, were co-cultured with Tresp at 1:8 and 1:4 Treg/Tresp ratio in the presence of anti-CD3/anti-CD28 beads. After 3 days, cell proliferation was determined through [3 H] thymidine up take. The mean values of cpm were compared using Student's t test and the p values are indicated in the figure. The effect of leptin on Treg function was investigated in five independent experiments with two donors each (total 10 donors)

phenotype associated with AA severity, together with damage to regulatory $CD4^+$ T cell subsets and Br1 cells. Our findings also suggest a role for the hyperleptinemic state in this complex immune imbalance, which might help to explain why weight loss positively affects allergy outcomes.[45]

AUTHOR CONTRIBUTIONS

Patient monitoring and sample collection by Ulisses C. Linhares and Letícia Delphim. Cleonice A. M. Bento, Sudhir Gupta and Carolina Melo Vollmer designed and wrote the paper. Carolina Melo Vollmer, Aleida S. O. Dias, Lana M. Lopes, Taissa M. Kasahara, Júlio Cesar C. Silva and Hilary Cesário Gonçalves performed the experiments. Carolina Melo Vollmer and Lucas Paulo Lourenço analyzed the data. Cleonice A. M. Bento and Sudhir Gupta contributed with vital reagents. All authors participated in critical revision of the manuscript, provided important intellectual input and approved the final version. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

All authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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2.3 **Artigo 3 - Leptin favors imbalance of antigen-specific CD4+ T-cells associated with severity of cat allergy (Artigo submetido)**

Leptin favors imbalance of antigen-specific CD4$^+$ T-cells associated with severity of cat allergy

Leptin, CD4$^+$ T cell subsets and cat allergy

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Abbreviations: allergic asthma (AA), allergic rhinitis (AR), B cell lymphoma 6 (Bcl-6). body mass index (BMI), germinal center (GC), healthy subjects (HS), Leptin (Lep), monoclonal

antibodies (mAbs), non-follicular helper T (non-T_{FH}), peripheral blood mononuclear cells (PBMC), programmed cell death protein-1 (PD-1), follicular helper T (T_{FH}), follicular regulatory T cells (T_{FR}).

Abstract

Obesity can complicate IgE-mediated allergic diseases. We aimed to investigate the ability of obesity-related leptin dose to modulate Feld1-specific $CD4^+$ T-cells in patients allergic to cat, considered the third most common cause of human respiratory allergies. Here, Feld1 induced IgE and the production of cytokines related to Th2, Th9 and Th17 cell phenotypes. Feld1 was more efficient at increasing the frequency of $T_{HF}IL\text{-}21^-$ cells positive for IL-4, IL-5 and IL-13 than $T_{HF}IL\text{-}21^+$ cells. Leptin favored the expansion Th2-like and Th9-like cells and $T_{FH}IL\text{-}21^-$ cells positive for IL-4, IL-5 and IL-13, but reduced the proportion of conventional (Treg/Tr-1) and follicular (T_{FR}) regulatory $CD4^+$ T-cell subsets expressing or not CD39 marker. Finally, many of the imbalances between Fel d1-specific $CD4^+$ T-cells were also correlated with plasma leptin and anti-Fel d1 IgE titers. In summary, hyperleptinemia leptin dose should negatively impact the severity of cat allergies by favoring the expansion of pathogenic Fel d1-specific $CD4^+$ T-cell phenotypes and damaging the functional status of regulatory $CD4^+$ T-cell subsets.

Keywords: Leptin, Fel d1, Th2/Th9, T_{FH} cells, Treg/Tr-1 cells, T_{FR} cells.

1. Introduction

Cat allergies are the most common mammalian-origin allergy in humans, affecting approximately 1 in 5 adults worldwide [1, 2]. The most common clinical presentations in these patients are rhinitis, asthma, and/or conjunctivitis. When persistent, the clinical symptoms may impair quality of life [3, 4]. Furthermore, severely allergic patients may present an anaphylactic reaction, requiring emergency medical care. Although eight allergens derived from cats have been described, designated Fel d1 to d8, only Fel d1 has clinical significance, accounting for up to 96% of human allergic sensitization to cats [5, 6]. Primarily produced by salivary and sebaceous glands [5]. Fel d1 can easily become and remain airborne in dander and dust particles for extended periods [5, 6].

The hallmark of cat sensitization and symptom severity is the production of high-affinity Fel d1-specific IgE [6, 7]. Although Th2 cytokines, IL-4 and IL-13, can increase IgE production, the synthesis of this antibody is critically dependent on B cell collaboration with follicular helper T (T_{FH}) cells [8]. In the germinal center (GC) of lymphoid follicles, T_{FH} cells are characterized by high expression of CXCR5, programmed cell death protein (PD-1), B cell lymphoma 6 (Bcl-6), and IL-21 production [9]. In the GC, T_{FH} cells not only mediate the selection of high-affinity and isotype switched B cells, but also promote differentiation of these cells into plasma cells and memory B cells [9]. Although T_{FH} cells in peripheral blood are Bcl-6 negative and express low PD-1 levels, they are able to induce antibody production from peripheral B cells [9]. Based on cytokines, human circulating T_{FH} cells have been classified as $T_{FH}1$ (IL-21^{+}IFN-γ^{+}), $T_{FH}2$ (IL-21^{+}IL-4^{+}), $T_{FH}17$ (IL-21^{+}IL-17^{+}) and, more recently, $T_{FH}13$ (IL-21^{low}L-4^{hi}IL-5^{hi}IL-13^{hi}) [8]. Interestingly, while $T_{FH}2$ cells induce low-affinity IgE production, the production of IgE with high-affinity to allergens critically depends on

$T_{FH}13$ cells [8, 10]. The binding of high-affinity IgE/allergen to FcεRI on mast cells and basophils immediately triggers histamine release, quickly causing a cluster of typical cat allergic symptoms [11]. Further, in addition to mast cells, eosinophils activated by the same Fel d1/IgE complexes contribute to allergy pathogenesis by producing lager amounts of sulphidopeptide leukotrienes (LTC4, LTD4, and LTE4) and platelet-activating factor (PAF), pro-inflammatory lipids that provoke local vasodilatation, edema formation, local neurogenic stimulation, smooth muscle contraction and mucus hypersecretion [12]. Moreover, IL-9-secreting $CD4^+$ T (Th9) cells have also been implicated in atopic allergy [13]. IL-9 prolongs the survival of mast cells, potentiates IgE production and amplifies the ability of IL-5 and IL-13 to increase eosinophil survival and mucus production [13].

As well as inducing the Th2/$T_{FH}2$/$T_{FH}13$ axis, the severity of IgE-mediated allergies has been associated with functional impairment of non-follicular [Treg ($CXR5^-$ $FoxP3^+IL\text{-}10^+$) and Tr-1 ($CXCR5^-FoxP3^-IL\text{-}10^+$)] and follicular [$T_{FR}$ ($CXCR5^+$ $FoxP3^+IL\text{-}10^+$)] regulatory $CD4^+$ T cells [14, 15]. While T_{FR} cells control IgE production by B cells in GCs, Treg and Tr-1 cells are essential to reducing inflammatory cytokine release by local mast cells, eosinophils and Th2 cells [14, 15]. Therefore, any adverse event that favors Th2/T_{FH} cell expansion and damages regulatory $CD4^+$ T cell phenotypes should affect the severity of atopic diseases, such as obesity.

Obesity has been related to severity of allergy symptoms and to higher levels of total and allergen-specific IgE in atopic individuals [16, 17]. In cat allergic patients, obesity was associated with total and allergen-specific IgE levels [18]. This adverse relationship must be, at least partially, associated with high leptin production, an adipokine known to modulate the functional status of T cells [19].

Leptin is a 16 kDa peptide encoded by the OB gene. At physiological concentrations, leptin plays an adjuvant role in the immune response Against different

pathogens [20]. However, hyperleptinemia, as observed in obese individuals, has been associated with the severity of allergic reactions [21] Ciprandi et al. [22] demonstrated a direct relationship between leptin levels, IgE titers and eosinophil counts in patients with allergic rhinitis. In immediate allergic reactions, studies regarding the effects of leptin on the composition of different allergen-specific $CD4^+$ T-cells have not been conducted to date. Therefore, the main objective of the present study was to investigate the ability of obesity-related leptin doses to modulate the *in vitro* different effector and regulatory Fel d1-specific $CD4^+$ T cells from patients with persistent cat allergies.

2. Material and Methods

Subjects

Twenty-eight patients with allergic rhinitis (AR) and/or asthma (AA) to cat dander were recruited from March 2020 to September 2021 from the Federal University of the State of Rio de Janeiro Hospital/UNIRIO (Rio de Janeiro, Brazil). All patients had a skin-prick test and IgE positive for cat dander extract (Table 1). Persistent asthma was diagnosed by a history of recurrent wheezing, dyspnea and chest tightness, and confirmed by methacholine bronchial hyperresponsivenes, when FEV1 was ≥ 70%, or bronchial reversibility after salbutamol inhalation (when FEV1 was <70%). AA patients were subdivided according to the Global Initiative for Asthma criteria [46] into 3 groups: mild, moderate and severe. With regard to AR, symptom severity was determined by using the total nasal symptom score (TNSS) (sneezing, congestion, itching and rhinorrhea) [47] (Table 1). We excluded patients taking oral or intravenous steroids, theophylline, long-acting β2-agonists, leukotriene antagonists or antihistamines 1 month prior to the study. As control group, twenty healthy subjects (HS), matched for age and sex and with no history of allergic diseases, were also recruited into the study. According to the body mass index (BMI), subjects were stratified as lean (BMI from 18.5 to 24.9), overweight (BMI from 25 to 29.9) and class I obesity (BMI from 30 to 35). Regardless of experimental group, smoking individuals and those with history of upper or lower airway infectious disease 2 months prior to recruitment were also excluded of the study. The Ethics Committee for Research on Human Subjects at the Federal University of the State of Rio de Janeiro (CAAE 44951215.6.0000.5258), approved the study, and blood was collected only after written informed consent was obtained from each individual.

Cell cultures

Peripheral blood was collected in heparin-containing tubes (BD Vacutainer, Franklin Lakes, NY) and peripheral blood mononuclear cells (PBMC) were obtained by centrifugation on the Ficoll–Hypaque density gradient. Fresh viable PBMCs (1×10^6 /mL) were cultured in 24-well flat-bottomed microplates with 2 mL of RPMI medium (ThermoFisher Scientific Inc.) supplemented with 2 µM of L-glutamine (GIBCO, Carlsbad, CA, USA), 10% fetal calf serum, 20 U/mL of penicillin, 20 µg/mL of streptomycin and 20 mM of HEPES buffer. As positive control, PBMC cultures were stimulated with phytohaemaglutinine (PHA, 1 µg/mL) (Sigma-Aldrich Co) for 3 days in a humidified 5% CO_2 incubator. In order to evaluate the antigen-specific response, the cells were stimulated with Fel d1 (10 µg/mL) (MyBioSource, San Diego, CA, USA) for 6 days. This concentration of Fel d1 was chosen from a previous study that evaluated T cell response to this antigen [6]. In these cultures, the role of leptin (Sigma-Aldrich Co) was determined after the addition of 50 ng/mL of this adipokine. This leptin concentration was determined after a dose-response curve (10, 50 and 100 ng/mL) of cytokine-secreting $CD4^+$ T cells from healthy subjects (HS) and cat-allergic patients (CAP) in PBMC cultures activated with PHA (1 µg/mL) (Sigma-Aldrich Co) (FigS.1). Notably, this leptin concentration is related to the levels of this adipokine in obese subjects [48]. After 6 days of culturing, the supernatants were collected, frozen at -20°C for further analysis of cytokine production (Luminex) and IgE levels (ELISA). The PBMC was also used to identify different $CD4^+$ T cell phenotypes using flow cytometry.

Flow cytometry analysis

Different $CD4^+$ T cell subsets in response to Fel d1 were identified by staining the PBMCs with mouse anti-human monoclonal antibodies (mAbs) for CD3-APC-H7 (SK7 clone), CD4-BV605 (T4 clone), CXCR5-PerCP.eF710 (mu5ubee clone), PD-1-APC (MIH4 clone), CD39-FITC (TU66 clone), FoxP3-PECy5.5 (PGH101 clone), IL-4-PECy7 (8D48 clone), IL-5-eFluor450 (TRFK5 clone), IL-9-BV4211 (MH9A3 clone), IL-10-BV722 (JES3-9D7 clone), IL-13-APC (JES10-5A2 clone), IL-17-AF488 (eBio64DEC17 clone) and IL-21-PE (3A3-N2.1 clone). These mAbs and all isotype control antibodies were purchased from Thermo Fischer (San Diego, CA, USA). Briefly, PBMCs were incubated with various combinations of mAbs for surface markers (CD3, CD4, CXCR5, PD-1, and CD39) for 30 min at room temperature in the dark, according to manufacturer's instructions. The cells were washed with PBS + 2%FBS, then submitted to permeabilization by incubating the PBMCs with Cytofix/Cytoperm solution (BD Pharmigen, San Diego, CA) at 4°C for 20 min. After washing, the mAbs for intracellular staining (FoxP3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17, and IL-21) were added in different combinations and incubated for 30 min at 4°C. The stained cells were acquired on Attune NxT flow cytometers (Thermo Fisher Corporation) and analyzed using FlowJo (Tree Star, Inc). Isotype control antibodies and single-stained samples were used to periodically check the settings and gates on the flow cytometer. After acquisition of 200,000 to 300,000 events, lymphocytes were gated based on forward and side scatter properties after the exclusion of dead cells, by using propidium iodide, and doublets.

Luminex, ImmunoCAP and ELISA assays

The titers of plasma IgE anti-cat was determined by florescence enzyme immunoassay with capsulated cellular polymer solid-phase (ImmunoCAP) coupled with cat dander (REF 14.451201, Therm Fischer Sicentific Inc.) with the detection limit ranging from 0.1 to 100 Ku/L. Circulating leptin levels were measured using a commercial ELISA kit following manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY). Plates were read at 450 nm in ELISA reader (Dynex Technologies, USA). Lyophilized leptin ranging from 31.3-2000 pg/mL was used to construct the standard curve. The levels of different cytokines and IgE in the supernatants from cell cultures were determined using the "Th1/Th2/Th9/Th17 Cytokine 18-plex human Panel" kit (InvitroGen, San Diego, CA, USA) and human IgE ELISA kit (88-50610-22) (Invitrogen, Thermo Fisher Scientific Co), respectively.

Statistical analyzes

All statistical analyzes were conducted using the Prism 8.0 program (GraphPad Software). Comparisons between immune assays in non-stimulated (none) or activated PBMC cultures with Fel d1 and Fel d1/leptin were performed with one-way ANOVA, followed by Tukey test for data with Gaussian distribution, and by Kruskal-Wallis, followed by Dunn's test for data without Gaussian distribution. The nonparametric Mann-Whitney U test and Student's t test were applied to determine whether the two groups were statistically different for nonparametric and parametric variables, respectively. Pearson's and Spearman's correlation were applied for variables with or without normal distribution, respectively. Significance for all experiments was defined as $p<0.05$.

3. Results

3.1 Leptin modulates cytokine and IgE production by Fel d1-stimulated PBMCs from cat-allergic patients

Table 1 shows that most cat allergic patients were overweight/obese women who presented moderate or severe symptoms of rhinitis (AR) and asthma (AA). As no statistical difference among patients with different clinical symptoms (AR x AA x AR/AA) was observed with regard to immunological assays, they were all included together as a single patient group (CAP- cat allergic patients). For the control group, some experiments were additionally performed in 20 age- and gender-matched healthy subjects (HS). Higher levels of IL-4, IL-5, IL-6, IL-13 and IL-17 were observed in CAP-derived PBMC cultures containing plolyclonlly-activated T cells, as compared with HS (Fig. S2). In those cell cultures, leptin elevated the release of IL-6, IFN-γ and IL-17 in HS group and the secretion of IL-5, IL-6, IL-13 and IL-17 in CAP group. In contrast, this adipokine reduced the levels of IL-10 secreted by mitogen-activated T cells from both experimental groups (Fig. S2). Concerning the cytokine profile in response to Fel d1, this major cat antigen induced not only the production of IL-4, IL-5, IL-13, IL-9, IL-6, IL-17, IL-21 and IL-10 (Fig. 1A), but also the secretion of IgE (Fig. 1B). The addition of leptin increased the release of IL-5, IL-13, IL-6, IL-17, IL-21 (Fig. 1A) and IgE (Fig. 1B), but reduced IL-10 production (Fig. 1A). Of note, neither medium nor leptin alone were not able to induce detectable cytokine (data not shown). Concerning the control group, Fel d1 only significantly elevated the production of IL-10, with no difference after leptin addition (Fig. S3). In patients, *in vitro* IgE production directly correlated with both IL-4 and IL-5, released by Fel d1-stimulated cells (Table 2), and IL-4, IL-5 and IL-9, secreted by Fel d1/Lep-activated PBMC cultures (Table 2). In contrast, in Fel d1/Lep-stimulated cells, IL-10 secretion inversely correlated to IgE (Table 2).

3.2 Leptin alters the frequency of effector and regulatory Ag-specific CD4$^+$ T cell subsets in CAP

From identification of CXCR5 and PD-1 markers on CD4$^+$ T cells, and using the gating strategy shown in figure 2A, no difference in the percentage of non-T_{FH} cells (CXCR5$^-$) (Fig. 2B), whole T_{FH} (Fig. 2B) and T_{FH} PD-1$^+$ (Fig. 2C) cells was observed in the cell cultures stimulated with Fel d1, with or without leptin. In contrast, taking into account the representative dot-plots shown in figure 2D, Fel d1 elevated the proportion of Th2-like cells [IL-4$^+$ (Fig. 2E), IL-5$^+$ (Fig. 2F) and IL-13$^+$ (Fig. 2H)] and Th9 (IL-9$^+$) cells (Fig. 2G), with no change in the percentage of Th17-like cells (IL-17$^+$ and IL-21$^+$) (Fig. 2I and 2J). Leptin elevated the frequency of Fel d1-specific Th2-like cells [IL-5$^+$ (Fig. 2F) and IL-13$^+$ (Fig. 2H)] and Th17-like cells [IL-17$^+$ (Fig. 2I)]. With regards to the classical T_{FH} cells (CXCR5$^+$IL-21$^+$), and following the gating strategy shown in figure 2K, Fel d1 upregulated the proportion of T_{FH}IL-21$^+$IL-17$^+$ (Fig. 2P). Notably, Fel d1 more efficiently upregulated the frequency of T_{FH}IL-21$^-$ cells positive for IL-4 (Fig. 2L), IL-5 (Fig. 2M), IL-9 (Fig. 2N), and IL-13 in comparison with T_{FH}IL-21$^+$cells (Fig. 2O). Leptin not only enhanced the proportion of T_{FH}IL-21$^+$ IL-5$^+$ (Fig. 2M) and T_{FH}IL-21$^+$ IL-9$^+$ (Fig. 2N), but also that of T_{FH}IL-21$^-$ cells positive for IL-4 (Fig. 2L), IL-5 (Fig. 2M), and IL-13 (Fig. 2O). The ability of leptin to upregulate non-T_{FH} and T_{FH} cell phenotypes was observed in cell cultures from lean, overweight and obese patients (data not shown).

Concerning regulatory T cells, through the expression of FoxP3, IL-10 and CD39 on CD4$^+$ T cells, we determined the impact of leptin on modulating the proportion of Fel d1-specific Treg/Tr-1 cells (Figs. 3A to 3C) and T_{FR} cells (Figs. 3D to 3F). Taking into account the gating strategy shown in figures 3A and 3D, Fel d1 increased the proportion of Treg (CXCR5$^-$FoxP3$^+$IL-10$^+$) (Fig. 3B) and T_{FR} (CXCR5$^+$FoxP3$^+$IL-10$^+$)

(Fig. 3E), expressing or not CD39. This allergen also upregulated the frequency of Tr-1 ($CXCR5^{-}FoxP3^{-}IL\text{-}10^{+}CD39^{-}$ and $CXCR5^{-}FoxP3^{-}IL\text{-}10^{+}CD39^{+}$) (Fig. 3C) and follicular Tr-1-like cells ($CXCR5^{+}FoxP3^{-}IL\text{-}10^{+}CD39^{-}$ and $CXCR5^{+}FoxP3^{-}IL\text{-}10^{+}CD39^{+}$) (Fig. 3F). Regardless of cell subtype, leptin significantly reduced the proportion of IL-10-secreting $CD4^{+}$ T cell subsets (Fig. 3).

In vitro IgE production directly correlated with the percentage of $T_{FH}IL\text{-}21^{+}IL\text{-}5^{+}$ and $T_{FH}IL\text{-}21^{-}$ positive for IL-4 and IL-5 in Fel d1- and Fel d1/Lep-stimulated cell cultures (Table 2). Similarly, higher IgE production was observed in Fel d1/Lep-activated cell cultures with a higher proportion of $T_{FH}IL\text{-}21^{-}IL\text{-}9^{+}$ and $IL\text{-}21^{-}IL\text{-}13^{+}$ (Table 2). By contrast, IgE negatively correlated with the proportion of allergen-specific $FoxP3^{+}IL\text{-}10^{+}$ T_{FR} cells that express, or not, CD39 marker, mainly after leptin addition (Table 2). No relationship was observed for the frequency of allergen-specific non-T_{FH} and Treg cells and IgE levels after leptin addition (Table 2).

Finally, according BMI, higher frequency of Th2-like cells ($IL\text{-}5^{+}$ and $IL\text{-}13^{+}$) (Fig. 5A), $T_{FH}IL\text{-}21^{+}$ ($IL\text{-}5^{+}$ and $IL\text{-}17^{+}$) (Fig. 5B) and $T_{FH}IL\text{-}21^{-}$ cell subsets ($IL\text{-}5^{+}$, $IL\text{-}9^{+}$ and $IL\text{-}13^{+}$) (Fig. 5C), was observed in obese patients. Conversely, obesity negatively impacted the ability of Fel d1 to elevate Treg (Fig. 5D) and T_{FR} cells (Fig. 5E), expressing or not CD39, as well as Tr-1 $CD39^{+}$ cells (Fig. 5D).

3.3 Correlation between plasma leptin levels, anti-cat IgE titers and the in vitro *cytokine profile in CAP.*

As demonstrated in Table 3, leptin levels positively correlated with IL-5, IL-6 and IL-17 secretion by Fel d1-stimulated cells, as well as the frequency of both non-T_{FH} ($IL\text{-}5^{+}$, $IL\text{-}13^{+}$ and $IL\text{-}17^{+}$) and $T_{FH}IL\text{-}21^{-}$ cells positive for IL-5, IL-9 and IL-13. In contrast, a negative correlation was observed between circulating levels of this adipokine and

the proportion of Treg and T_{FR} cells, expressing or not CD39 marker. Moreover, the proportion of $CD39^{+}$Tr-1 cells inversely correlated with leptin concentration (Table 3). Although no significant correlation was observed between plasma leptin and anti-cat IgE (r=0.4054, p=0.0845), titers of this antibody positively correlated with both IL-5 release and the percentage of T_{FH}IL-21^{-}IL-4^{+} and IL-21^{-}IL-13^{+} cells in Fel d1-stimulated PBMC cultures (table 4). On the other hand, higher levels of this antibody were observed in patients with lower Treg and T_{FR} cell proportions, expressing CD39 or not, and Tr-1 $CD39^{+}$ cells (Table 4).

4. Discussion

Obesity can complicate IgE atopic diseases [16, 23]. Here, in cat allergic patients, this adverse relationship should involve, at least in part, increased leptin production that promotes an imbalance between different $CD4^+$ T cell phenotypes specific to Fel d1, the major cat allergen.

In the present study, Fel d1 not only increased the production of cytokines related to Th2 and Th9 cells, but also the proportion of antigen-specific T_{FH} cell subsets, mainly IL-21^-IL-4^+, IL-21^-IL-5^+ and IL-21^-IL-13^+. Despite the small sample size in this study, a higher percentage of Fel d1-specific Th2-like cells and $T_{FH}2$/$T_{FH}13$ cell phenotypes was observed among obese patients and directly correlated with plasma leptin levels. *In vitro*, this adipokine directly favored the expansion of Fel d1-specific Th2- and Th9-related phenotypes, as well as the percentage of T_{HF}IL-21 (IL-5^+ and IL-9^+) and T_{HF}IL-21^- (IL-4^+, IL-5^+ and IL-13^+) cell subsets. Additionally, leptin elevated IgE production. This finding agrees with a study that demonstrated a direct relationship between leptin levels and IgE production in atopic patients [18]. Regarding cell phenotypes, IgE levels in PBMC cultures stimulated with Fel d1/Lep directly correlated with the frequency of T_{FH}IL-21^+IL-5^+ and T_{HF}IL-21^- negative for IL-4, IL-5, IL-13 and IL-9, but not Th2-like cells. Furthermore, plasma titers of anti-Fel d1 IgE positively correlated with T_{FH}IL-21^- positive for IL-5 and IL-13. This finding agrees with studies that demonstrate that T_{FH} cells, but not Th2 cells, are critical for IgE production [8, 10]. Among T_{FH} cells, while the $T_{FH}2$ cell subset induces the production of low-affinity IgE [8]. the $T_{FH}13$ cell subset is responsible for producing high-affinity IgE [10]. $T_{FH}13$ cells are characterized by high IL-4, IL-5 and IL-13 expression associated with very low IL-21 production [18]. Yang et al. [24] demonstrated that IL-21 is a negative regulator of IgE class-switch recombination in human B cells. Although we did not evaluate either T_{FH} cells that

simultaneously express IL-4, IL-5 and IL-13, nor IgE affinity, we believe that the ability of leptin to increase the frequency of Fel d1-specific T_{FH}IL- 21^{-} able to produce Th2-related cytokines is one of the mechanisms that this adipokine uses to intensify cat allergy severity. Indeed, the formation of high affinity IgE:FcεRI complexes on mast cells, by activating Lyn/Syk/LAT-1 axis, promotes intense and immediate histamine release and leukotriene synthesis [11, 24, 25], resulting in associated allergic symptoms, such as airway constriction, increased mucus production, and coughing.

Despite not directly inducing IgE production, Th2 cytokines participate in the pathogenesis of atopic allergic reactions. IL-4 and IL-13 amplify eosinophil and Th2 cell transmigration to the allergen exposure site [26]. IL-5 is responsible for increasing eosinophil formation and survival [27]. IL-13 increases B cell survival [28] and compromises respiratory function by increasing mucus production in the airways [29]. Therefore, leptin ability to increase the frequency of Fel d1-specific Th2-like cells should impact the severity of allergic reactions to cats. Indeed, leptin, by potentiating Th2-mediated response, has been associated with atopic diseases [30]. Moreover, here, leptin also favored expansion of Fel d1-specific Th9-like cells. IL-9, along with IL-5 and IL-13, prolong mast cell and eosinophil survival, and increase mucus production [13, 31]. Interestingly, despite the lack of data about human $T_{FH}9$ cells, in murine allergy models, these cells support memory IgE^{+} B cell generation [32, 33]. Finally, the ability of leptin to upregulate Fel d1-induced IL-6 production may also contribute to IgE synthesis, since IL-6 acts as a potent stimulant for B-cell proliferation, plasma cell survival, and antibody production [34-36].

Recently, the severity of mite-allergic asthma has been associated with Der f3-specific Th17 cells [37]. Furthermore, IL-17 directly promoted IgE production by human B cells [38] and favors eosinophil accumulation in mucosa of atopic patients [39]. In

the present study, although Fel d1 has induced $T_{FH}IL\text{-}17^{+}$, and leptin amplified this cell subtype, no relationship was observed with either *in vitro* IgE production or plasma anti-Fel d1 IgE. However, it is possible that during disease exacerbation, this cell phenotype may contribute to cat allergy immunopathogenesis by promoting eosinophil infiltration into the airway of patients.

Regarding regulatory $CD4^{+}$ T cells, the severity of allergic reactions has been associated with functional damage of allergen-specific Treg cells ($CXCR5^{-}FoxP3^{+}IL\text{-}10^{+}$), Tr-1 ($CXCR5^{-}FoxP3^{-}IL\text{-}10^{+}$) and T_{FR} ($CXCR5^{+}FoxP3^{+}IL\text{-}10^{+}$) [14. 15]. In the present study, leptin reduced the frequency of Fel d1-specific Treg/Tr-1 and T_{FR} cells, most of them expressing CD39. Furthermore, the proportion of those cells inversely correlated with plasma leptin levels. Through the release of IL-10, a net anti-inflammatory cytokine, and CD39 expression, these cells control hypersensitivity reactions [40]. CD39 degrades the extracellular adenosine triphosphate (ATP)/adenosine diphosphate (ADP) into adenosine monophosphate (AMP) that is subsequently hydrolyzed, by CD73, to adenosine (ADO), a metabolite which inhibits effector T cells [41]. A study by Li et al. observed the role of $CD39^{+}$Treg cells in controlling airway inflammation in the murine model of allergic asthma [42]. Notably, the frequency of Treg and T_{FR} cells, expressing CD39 or not, inversely correlated with both IgE production in Fel d1/Lep-stimulated cell cultures and plasma anti-Fel d1 IgE titers. In agreement with our findings, a study by Martin-Orozco et al. [43], demonstrated an inverse relationship between FoxP3 expression in the regulatory $CD4^{+}$T cell compartment with serum IgE levels and eosinophilia.

5. Conclusions

Although preliminary, our findings suggest that hyperleptinemia, by favoring expansion of pathogenic Fel d1-specific $CD4^+$ T cells and impairing the functioning of regulatory $CD4^+$ T cell subsets, would not only exacerbate disease severity, but also negatively impact the success of allergen-specific immunotherapies against cat allergies [44, 45].

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Data Availability Statement:

All data generated or analyzed during this study are included in this article and its supplementary material. Further enquiries can be directed to the corresponding author.

Conflict of Interest Statement

All authors declare that there are no conflicts of interest.

Ethics approval and patient consent

This study was approved by the Ethics Committee for Research on Human Subjects at the Federal University of the State of Rio de Janeiro (CAAE 44951215.6.0000.5258) and blood was collected only after written informed consent was obtained from each individual.

Author contributions

Patient monitoring and sample collection by UCL. CAMB and SG designed and wrote the paper. CMV, ASOD, MSC, PMS, JCCS, HAO and TMK performed the experiments. CMV and TMK analyzed the data. CAMB and SG contributed with vital reagents. All authors participated in critical revision of the manuscript, provided important intellectual input and approved the final version. All authors read and approved the final manuscript.

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Figures

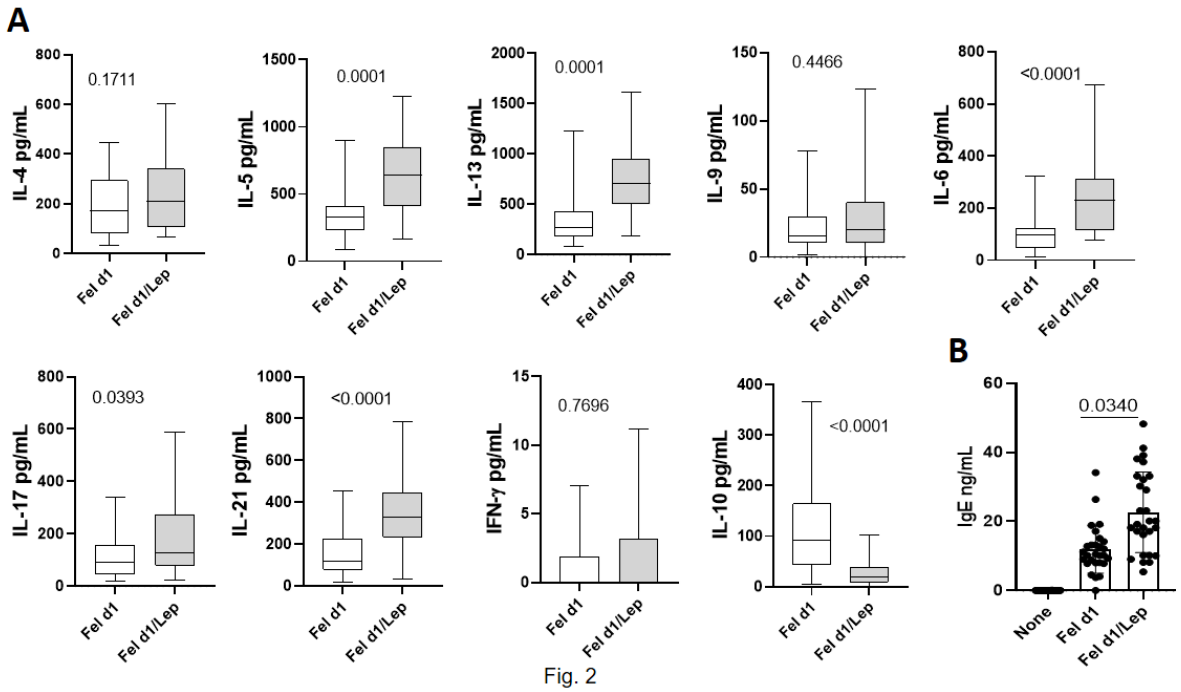


Figure 1. Leptin modulates the cytokine profile and IgE production by PBMCs from cat-allergic patients in response to Fel 1d. PBMC cultures (1 x 10^6/mL) from cat-allergic patients were maintained for 6 days in the presence of culture medium alone (without) or with 10 µg/mL of Fel 1d, with or without 50 ng/mL of leptin (Lep). At the end of the culture time, the supernatants were harvested and the (**A**) cytokine (IL-4, IL-5, IL-13, IL-6, IL-17, IL-21, IFN-γ and IL-10) and (**B**) IgE levels were determined by Luminex and ELISA, respectively. Mean values were compared using one-way ANOVA and *p* values are shown in the graphs. All data are shown as mean ± SD of six independent experiments with 4 and 6 samples per experiment.

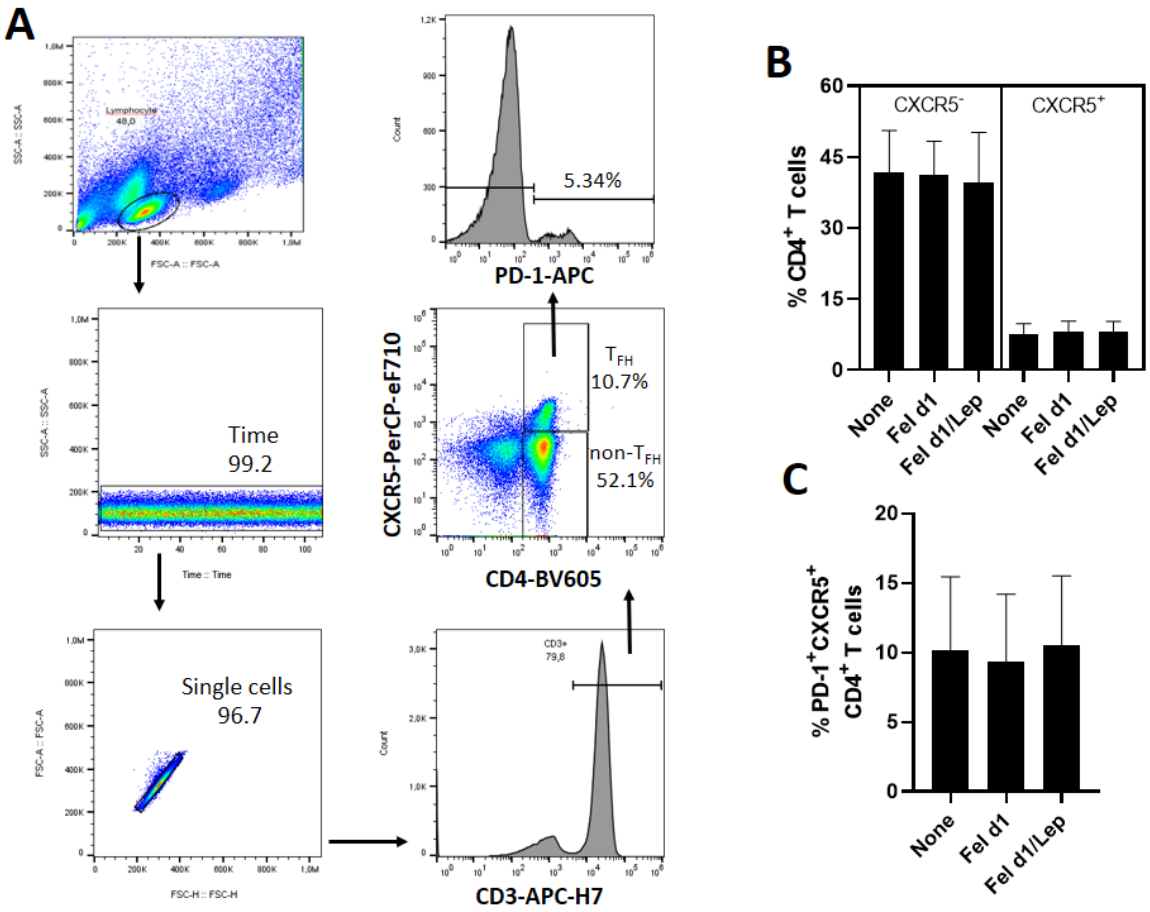

A
Lymphocyte
48,0
SSC-A :: SSC-A
FSC-A :: FSC-A
Time
99.2
Time :: Time
Single cells
96.7
FSC-A :: FSC-A
FSC-H :: FSC-H
Count
5.34%
PD-1-APC
CXCR5-PerCP-eF710
T_FH
10.7%
non-T_FH
52.1%
CD4-BV605
CD3+
79,8
Count
CD3-APC-H7
B
CXCR5⁻
CXCR5⁺
% CD4⁺ T cells
None
Fel d1
Fel d1/Lep
C
% PD-1⁺CXCR5⁺ CD4⁺ T cells
None
Fel d1
Fel d1/Lep

Fig. 2A to 2C

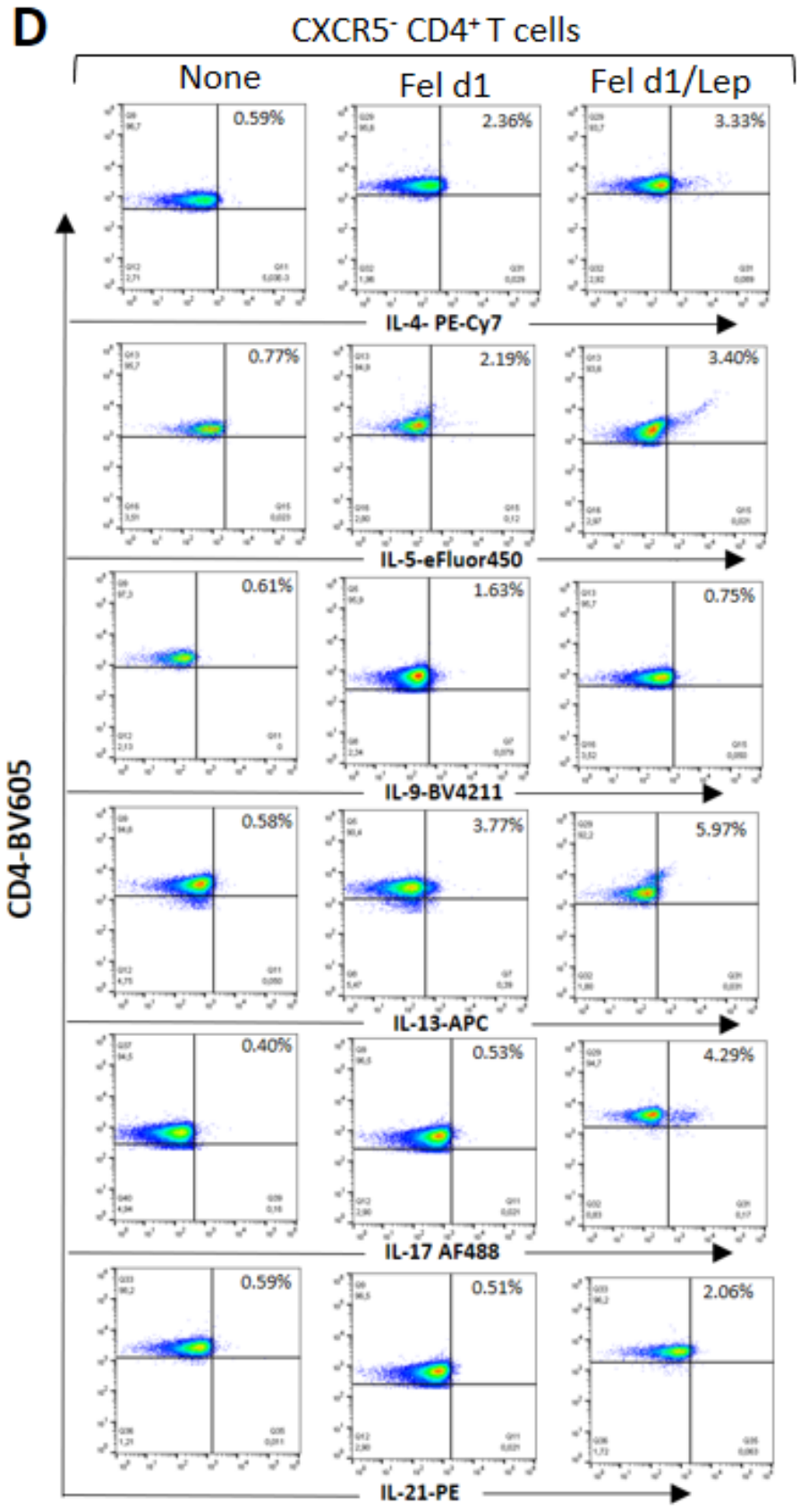

D
CXCR5⁻ CD4⁺ T cells
None
Fel d1
Fel d1/Lep
0.59%
2.36%
3.33%
IL-4- PE-Cy7
0.77%
2.19%
3.40%
IL-5-eFluor450
0.61%
1.63%
0.75%
IL-9-BV4211
0.58%
3.77%
5.97%
IL-13-APC
0.40%
0.53%
4.29%
IL-17 AF488
0.59%
0.51%
2.06%
IL-21-PE
CD4-BV605

FIG. 2D

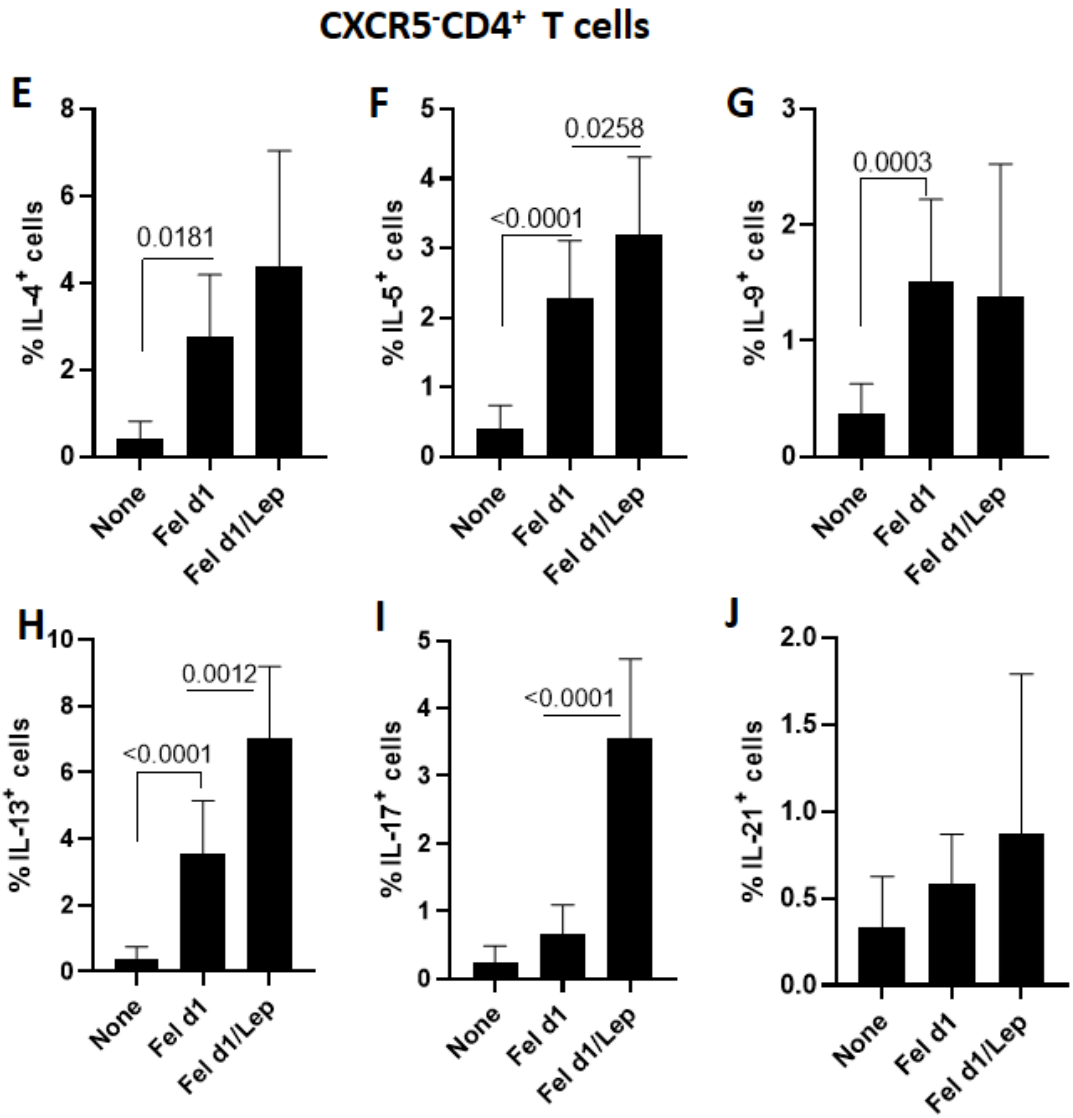

CXCR5-CD4+ T cells
E
% IL-4+ cells
0.0181
None
Fel d1
Fel d1/Lep
F
% IL-5+ cells
<0.0001
0.0258
G
% IL-9+ cells
0.0003
H
% IL-13+ cells
<0.0001
0.0012
I
% IL-17+ cells
<0.0001
J
% IL-21+ cells

Fig. 2E to 2J

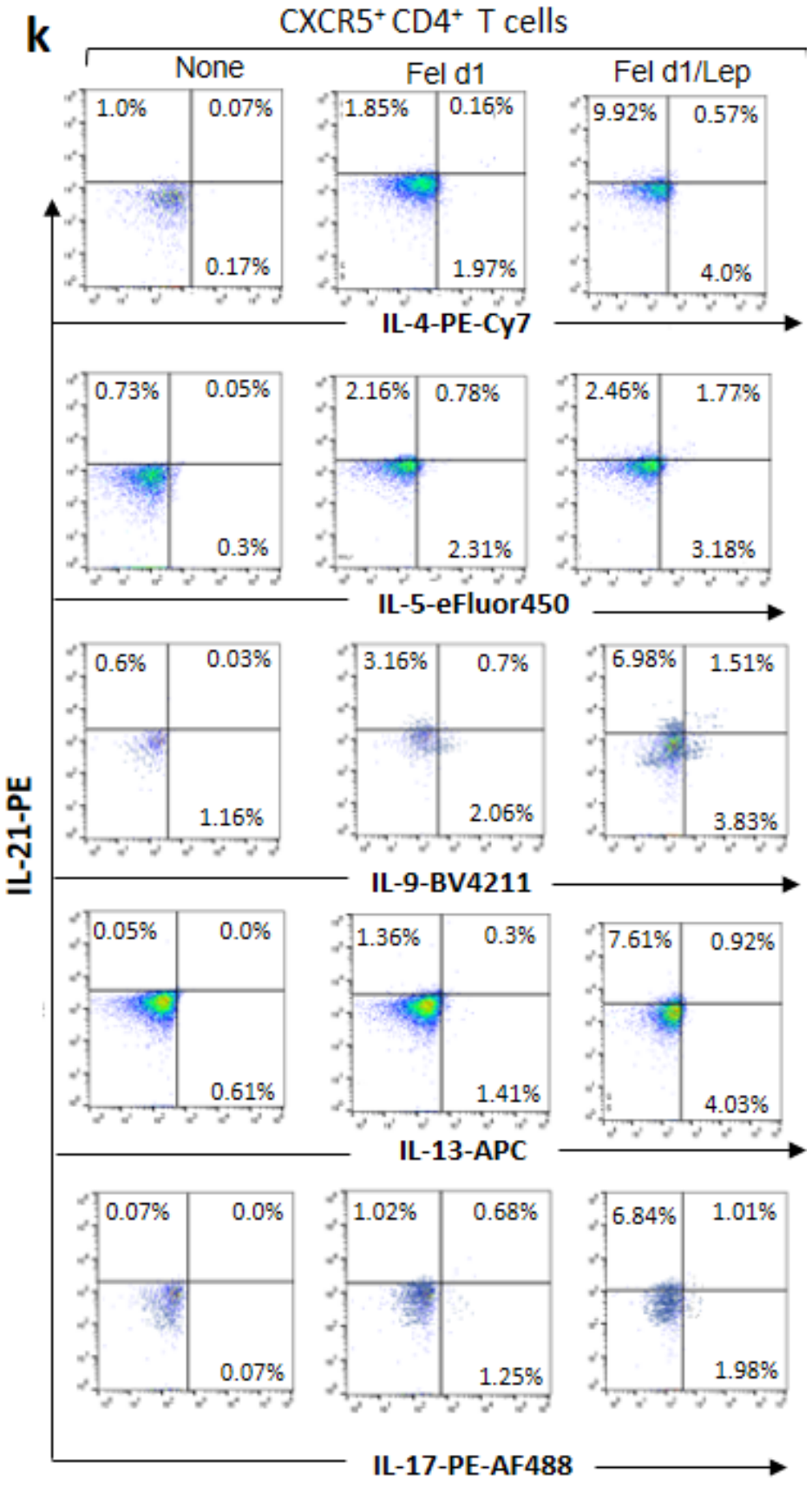

k
CXCR5⁺ CD4⁺ T cells
None
Fel d1
Fel d1/Lep
1.0%
0.07%
0.17%
1.85%
0.16%
1.97%
9.92%
0.57%
4.0%
IL-4-PE-Cy7
0.73%
0.05%
0.3%
2.16%
0.78%
2.31%
2.46%
1.77%
3.18%
IL-5-eFluor450
0.6%
0.03%
1.16%
3.16%
0.7%
2.06%
6.98%
1.51%
3.83%
IL-9-BV4211
IL-21-PE
0.05%
0.0%
0.61%
1.36%
0.3%
1.41%
7.61%
0.92%
4.03%
IL-13-APC
0.07%
0.0%
0.07%
1.02%
0.68%
1.25%
6.84%
1.01%
1.98%
IL-17-PE-AF488

Fig. 2K

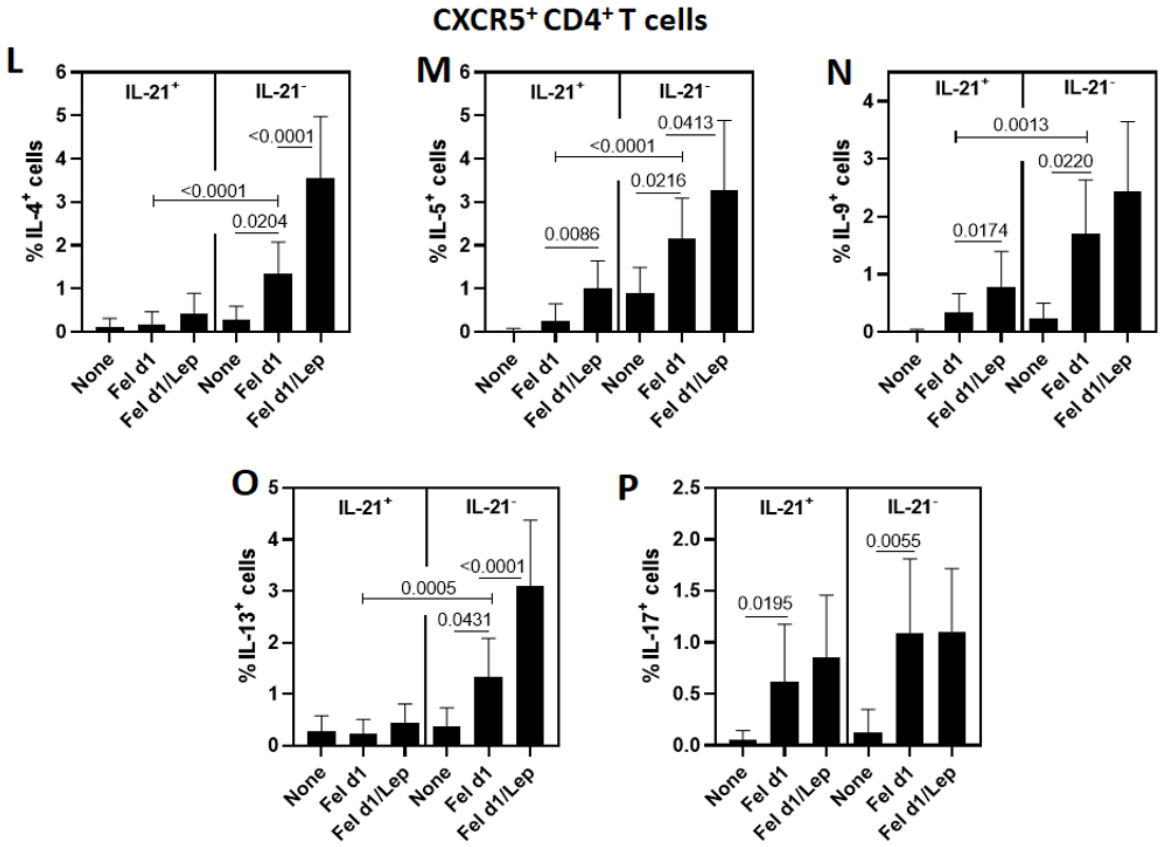
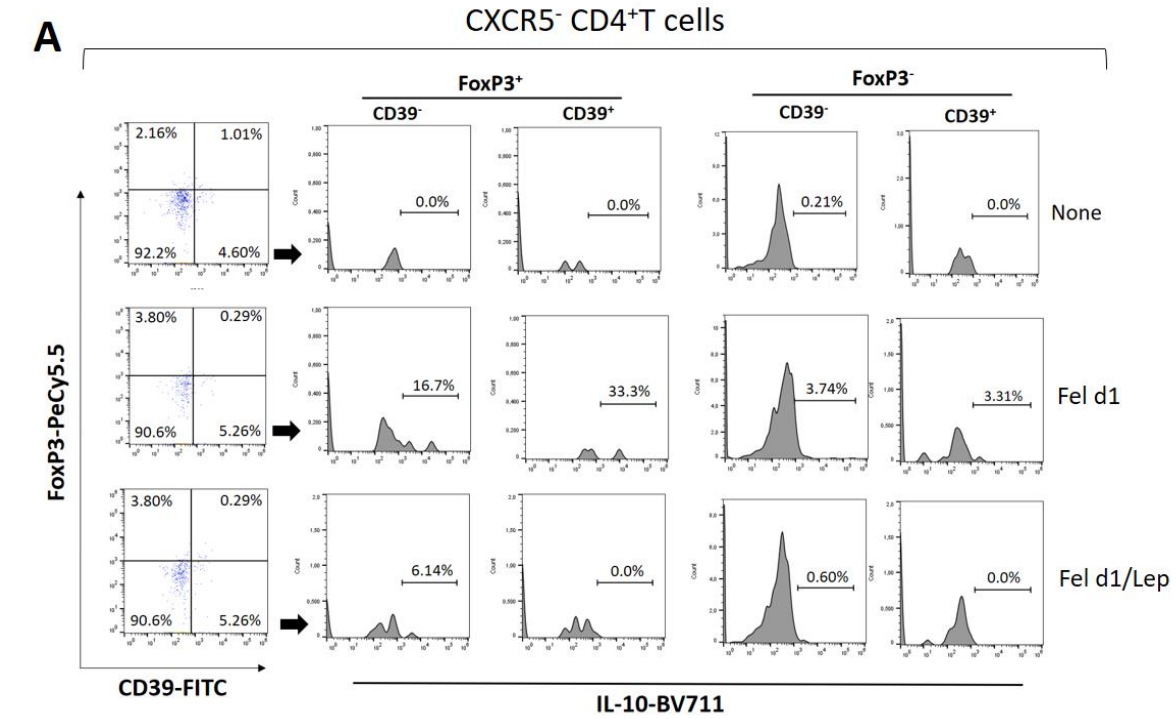


Figure 2. Leptin effect on the frequency of different Fel d1-specific T_{FH} and non-T_{FH} cell subsets in cat-allergic patients. PBMCs (1 x 10^6/mL) from cat-allergic patients were cultured for 6 days in the presence of culture medium alone (none) or with 10 µg/mL Fel 1d, with or without 50 ng/mL leptin (Lep). At the end of the culture time, and adopting the gating strategy shown in graph **A**, the mean ± SD of conventional $CD4^+$ T cells (non-T_{FH}, $CXCR5^-$) and total T_{FH} cells (T_{FH}, CXCR5+) (**B**), as well as the $T_{FH}PD\text{-}1^+$ cell subset (**C**) were analyzed by cytometry. In (**D**) and (**K**), representative dot-plots of cytokine-producing non-T_{FH} and T_{FH} cells were shown, respectively. In (**E** to **J**), the mean ± SD of percentage of (**E**) IL-4^+, (**F**) IL-5^+, (**G**) IL-9^+, (**H**) IL-13^+, (**I**) IL-17^+ and (**J**) IL-21^+ among non-T_{FH} cells, while (**L** to **P**) showed the mean ± SD values for T_{FH}IL-21^+ and T_{FH}IL-21^- cells able to produce IL-4 (**L**), IL-5 (**M**), IL-9 (**N**), IL-13 (**O**), and IL-17 (**P**). Data are shown as mean ± SD of five independent experiments with 2 and 6 samples per experiment. Significance was calculated using one-way ANOVA and *p* values are shown in the graphs.


A
CXCR5⁻ CD4⁺T cells
FoxP3⁺
FoxP3⁻
CD39⁻
CD39⁺
CD39⁻
CD39⁺
2.16%
1.01%
92.2%
4.60%
0.0%
0.0%
0.21%
0.0%
None
3.80%
0.29%
90.6%
5.26%
16.7%
33.3%
3.74%
3.31%
Fel d1
3.80%
0.29%
90.6%
5.26%
6.14%
0.0%
0.60%
0.0%
Fel d1/Lep
FoxP3-PeCy5.5
CD39-FITC
IL-10-BV711

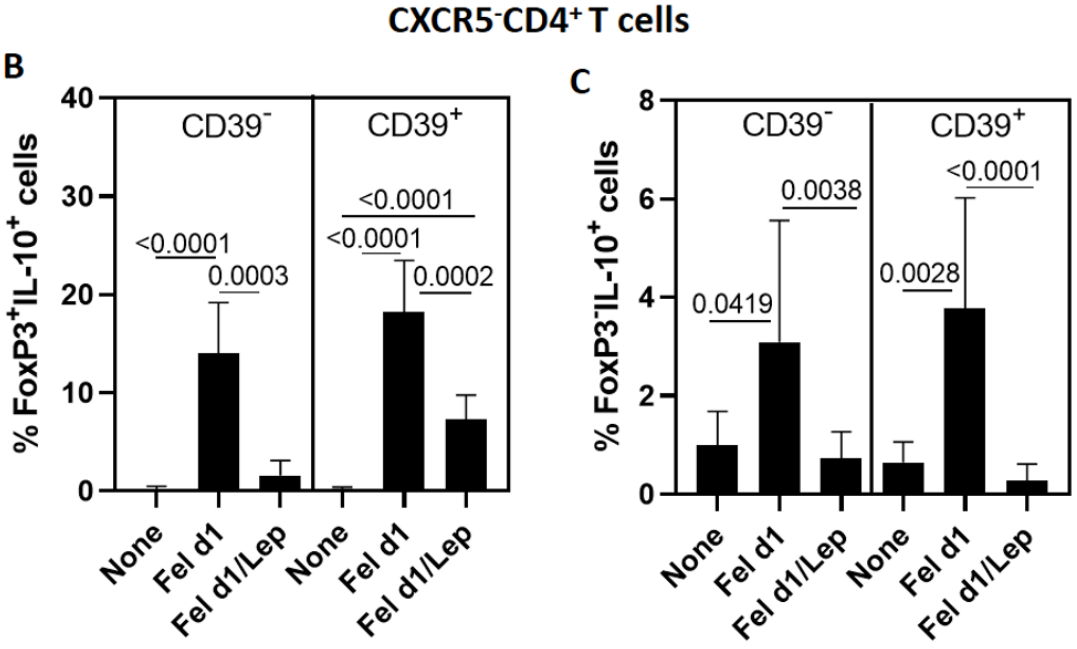

CXCR5⁻CD4⁺ T cells
B
% FoxP3⁺IL-10⁺ cells
CD39⁻
CD39⁺
<0.0001
0.0003
<0.0001
<0.0001
0.0002
None
Fel d1
Fel d1/Lep
None
Fel d1
Fel d1/Lep
C
% FoxP3⁻IL-10⁺ cells
CD39⁻
CD39⁺
0.0038
0.0419
<0.0001
0.0028
None
Fel d1
Fel d1/Lep
None
Fel d1
Fel d1/Lep

Fig. 3A and 3B

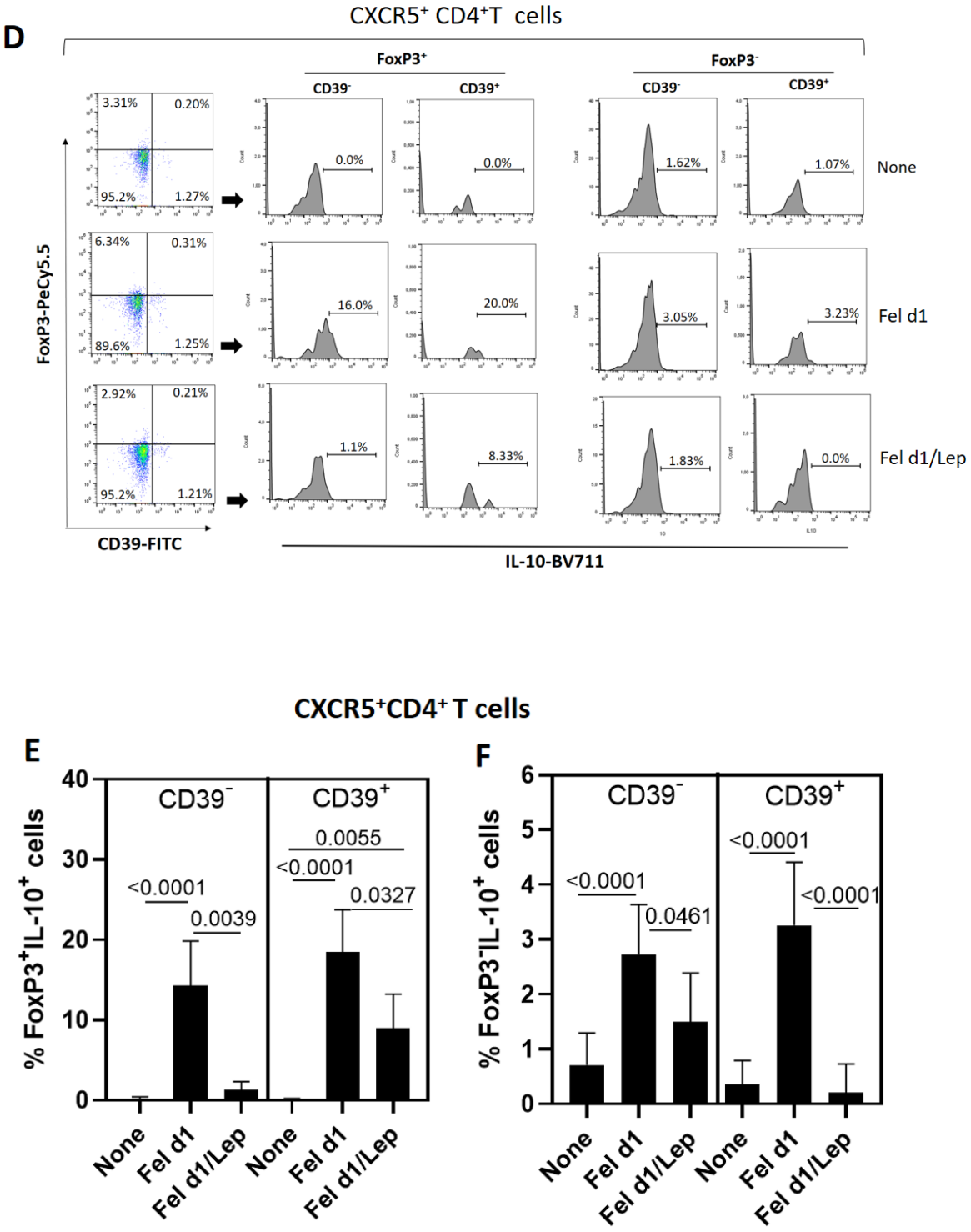


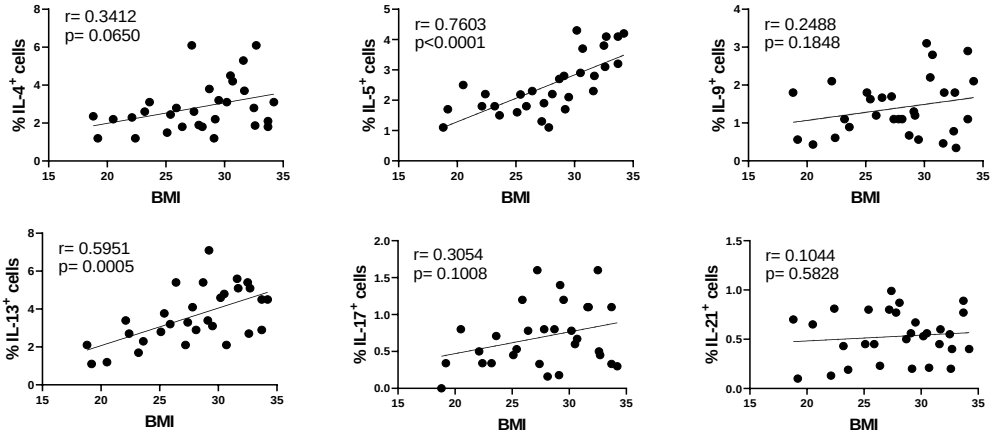
Fig. 3D to 3F

Figure 3. Leptin reduced the frequency of Fel d1-specific Tregs and T_{FR} cell subsets in PBMC cultures from cat-allergic patients. Taking into account the expression of CXCR5, FoxP3, IL-10 and CD39, and following representative dot-plots

and histograms shown in graphs **A** and **D**, the frequency of (**A**) conventional (Tregs/Tr-1, $CXCR5^-$) and (**D**) follicular (T_{FR}/T_{FR}-1, $CXCR5^+$) regulatory $CD4^+$ T cells was determined in PBMC cultures from cat-allergic patients after stimulation for 6 days with Fel d1 and Fel d1/Lep. The mean values (±SD) of $FoxP3^+IL\text{-}10^+CD39^-$ and $FoxP3^+IL\text{-}10^+CD39^+$ (**B** and **E**), and $FoxP3^-IL\text{-}10^+CD39^-$ and $FoxP3^-IL\text{-}10^+CD39^+$ (**C** and **F**), on Fel D1-specific Treg and T_{FR} cell subsets was determined. Data are shown as mean ± SD of five independent experiments with 2 and 6 samples per experiment. Significance was calculated using one-way ANOVA and *p* values are shown in the graphs.

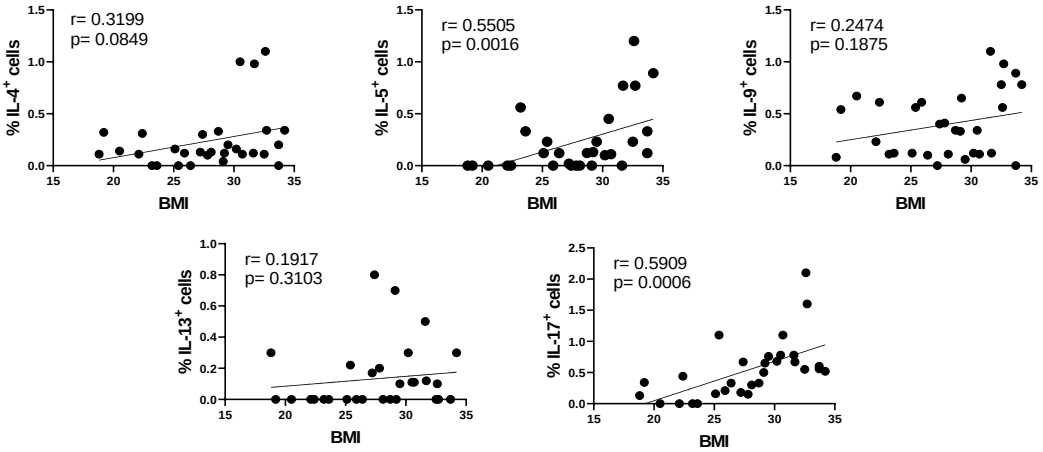
A

Non-T_{FH} cells



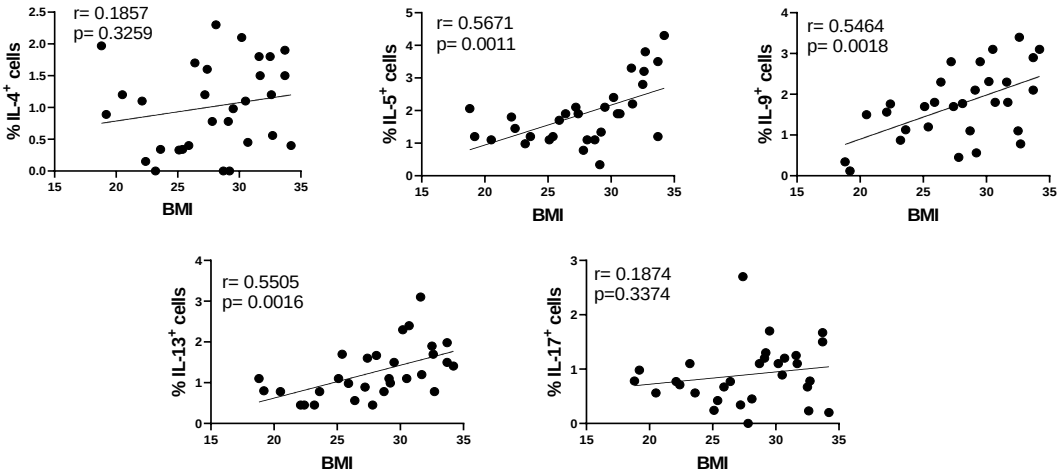
B

T_{FH}IL-21^{+} cells



C

T_{FH}IL-21^{-} cells



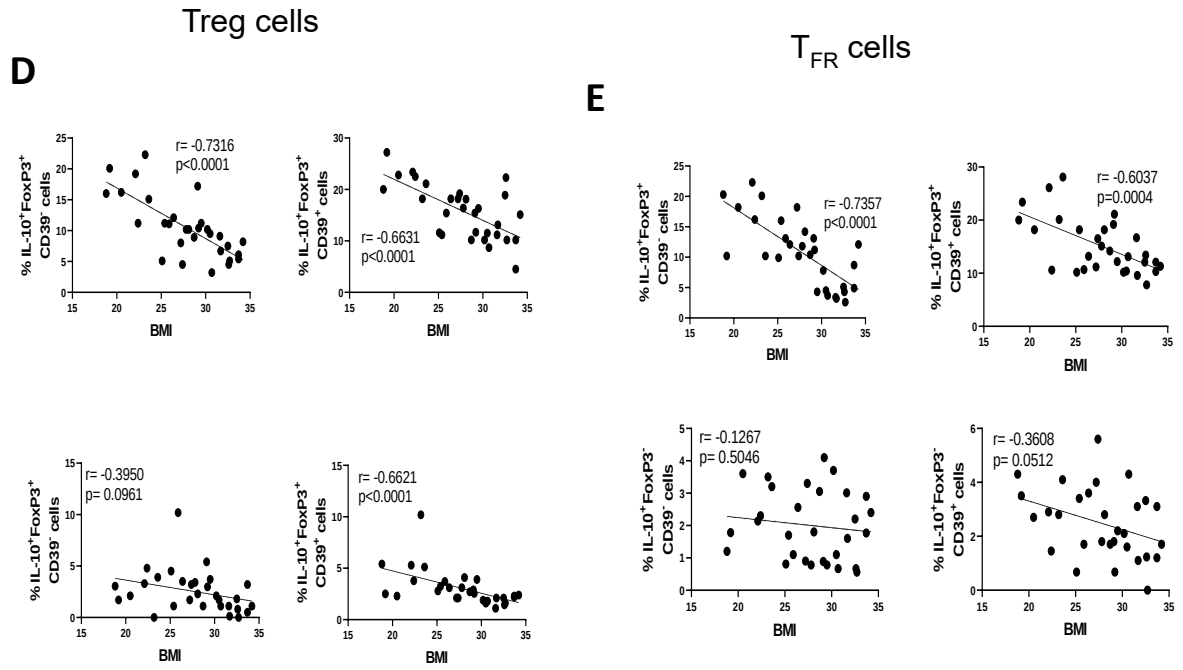


Figure 4. The frequency of effector and regulatory Fel d1-specific CD4$^+$ T cell subsets according BMI. The frequency of cytokine-producing non-T_{FH} cells (**A**), IL-21$^+$ (**B**) and IL-21$^-$ T_{FH} cells (**C**), as well as Treg/Tr-1 (**D**) and T_{FR} (**E**) cells in response to Fel d1 was correlated with BMI by using Pearson's correlation.

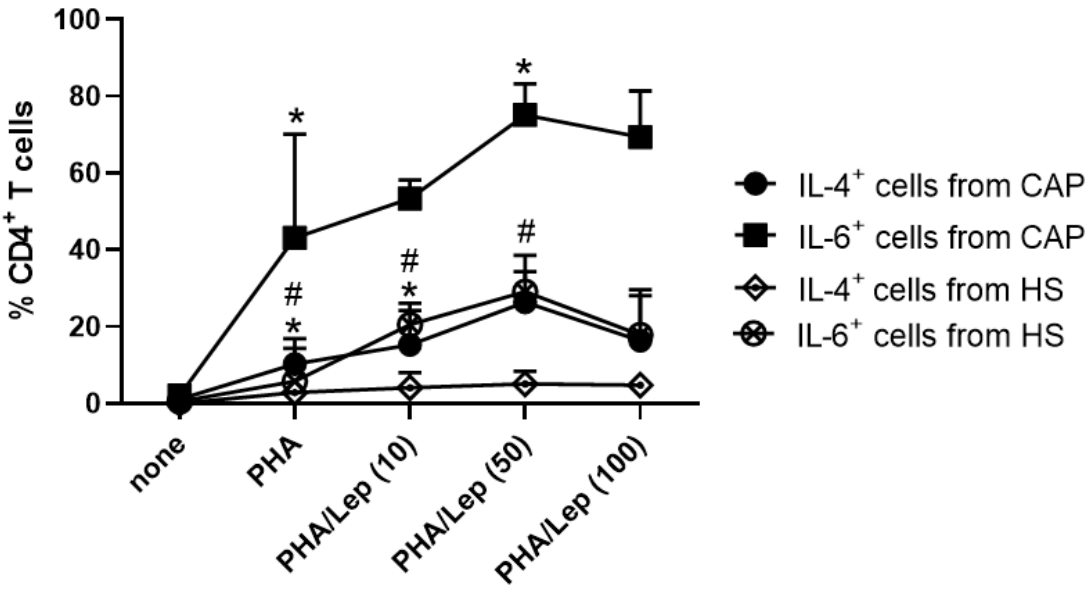


Figure S1. The impact of different leptin doses on the frequency of IL-4 and IL-6-producing CD4⁺ T cells from healthy subjects and patients with allergy to cat. PBMC cultures (1 x 10^6/mL) from 10 healthy subjects (HS) and 10 cat allergic patients (CAP) to cat were stimulated with PHA (1 µL/mL) in the absence (none) or in the presence of leptin (10, 50 and 100 ng/mL). After 3 days, the percentage of IL-4⁺ and IL-6⁺CD4⁺ T cells was quantified by cytometry. In the figure, (*) and (#) indicate $p<0.05$ in CAP and HS groups, respectively.

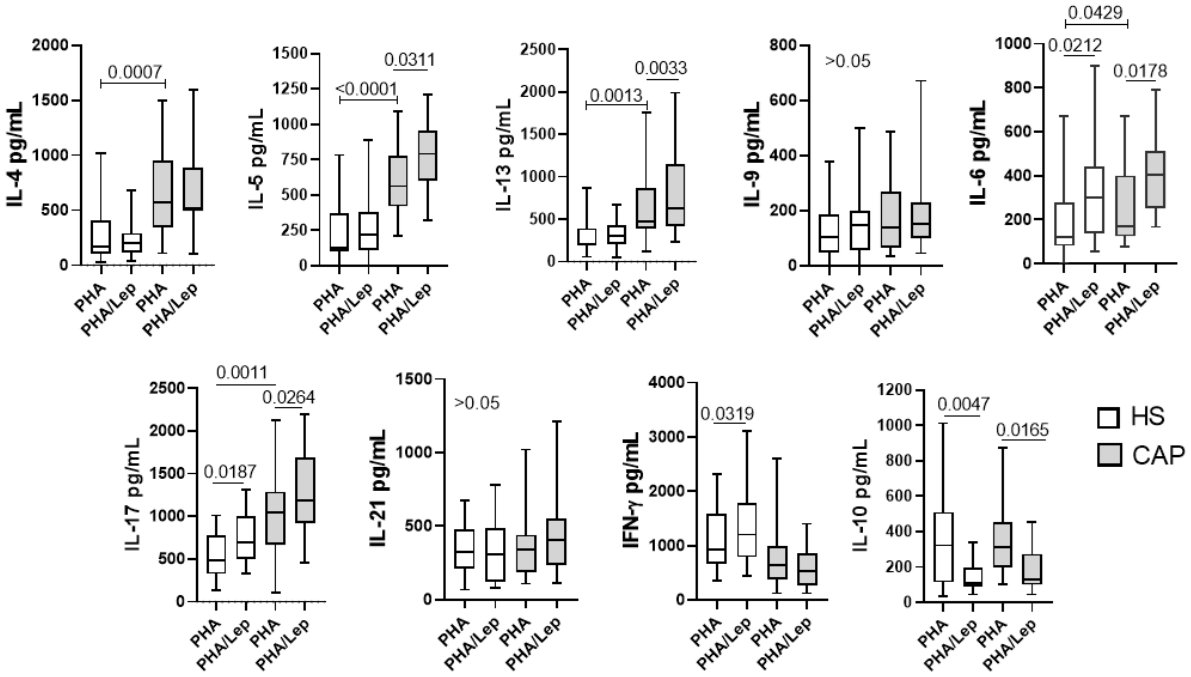


Figure S2. The *in vitro* cytokine production by polyclobally-activated T cells from healthy subjects and cat-allergic patients. PBMC cultures (1 x 10^6/mL) from healthy subjects (HS) and cat-allergic patients (CAP) were maintained for 3 days in the presence PHA (1 µg/mL), with or without 50 ng/mL of leptin (Lep). At the end of the culture time, the supernatants were harvested and the release of IL-4, IL-5, IL-13, IL-6, IL-17, IL-21, IFN-γ and IL-10 were determined by Luminex. Data are shown as mean ± SD of five independent experiments with 2 and 6 samples per experiment. Significance was calculated using one-way ANOVA and *p* values are shown in the graphs.

Figure S2. The in vitro cytokine production by PBMCs from healthy subjects in response to Fel 1d. PBMC cultures (1 x 10^6/mL) from non allergic subjects (Control group, n=10) were maintained for 6 days in the presence of culture medium alone (none) or with 10 µg/mL of Fel 1d, with or without 50 ng/mL of leptin (Lep). At the end of the culture time, the supernatants were harvested and the levels of IL-4, IL-5, IL-13, IL-6, IL-17, IL-21, IFN-γ and IL-10 were determined by Luminex. Data are shown as mean ± SD of two independent experiments with 4 and 6 samples per experiment. Significance was calculated using one-way ANOVA and *p* values are shown in the graphs.

Table 1. The characteristics of subjects.

		CAP[2]		
	Control[1]	*Rhinitis*	*Asthma*	*Asthma and rhinitis*
N[0] of subjects (n)	20	8	6	16
Gender (female/male) (n)	15/5	6/2	3/2	10/5
Age [(years), mean ± SD]	29.1 ± 13.8	28.8 ± 7.9	31.2 ± 10.1	30.3 ± 8.7
Clinical presentation (n)[3]				
Mild	ND	1	2	1
Moderate	ND	4	3	7
Severe	ND	3	1	8
BMI (n)[4]				
Lean	5	2	2	3
Overweight	10	3	3	6
Obese class I	5	3	1	7

[1]Healthy subjects. [2]Cat allergic patients suffering from rhinitis, asthma alone or rhinitis and asthma to cat dander. [3]The severity of rhinitis and asthma symptom was determined by TNSS (total nasal symptom score) and GINA (Global Initiative for Asthma) criteria, respectively. [4]Body mass index: a value derived from the mass (weight in Kg)

and height (in meters) of an individual (lean: 18.5-24.9, overweight: 25-29.9 and obese class I: 30-35). ND: no determined.

Table 2. Correlation between *in vitro* IgE production and cytokine profile in Fel d1-stimulated PBMC cultures from cat-allergic patients.

	IgE (ng/mL)			
	Fel d1		Fel d1/Lep	
Cytokines (pg/mL)	*r*	*p*	*r*	*p*
IL-4	0.7324	**0.0002**	0.5343	**0.0152**
IL-5	0.7715	**0.0001**	0.4618	**0.0404**
IL-6	0.1533	0.5187	0.1026	0.6610
IL-9	0.2980	0.2020	0.5648	**0.0095**
IL-13	0.4045	0.0769	0.3488	0.1320
IL-17	0.3263	0.1603	0.2286	0.3323
IL-21	0.1064	0.6553	0.2211	0.3488
IL-10	-0.3143	0.1772	-0.4547	**0.0440**
Non-T_{FH} cells (%)				
IL-4^{+}	0.3177	0.1017	0.2120	0.2451

IL-5^{+}	0.2809	0.2012	0.3223	0.1013
IL-9^{+}	0.4001	0.1003	0.3673	0.1765
IL-13^{+}	0.3181	0.1091	0.1983	0.3412
IL-17^{+}	0.3019	0.1288	0.2883	0.2651
IL-21^{+}	0.2711	0.2188	0.1577	0.4018
IL-21^{+}T$_{FH}$ cells (%)				
IL-4^{+}	0.4011	0.1122	0.4283	0.0657
IL-5^{+}	0.5891	**0.0073**	0.7848	**0.0001**
IL-9^{+}	0.4571	0.1011	0.4122	0.1617
IL-13^{+}	0.4109	0.1891	0.2512	0.4041
IL-17^{+}	0.3491	0.2019	0.2947	0.3239
IL-21^{-}T$_{FH}$ cells (%)				
IL-4^{+}	0.6012	**0.0085**	0.6966	**0.0023**
IL-5^{+}	0.5901	**0.0091**	0.6818	**0.0031**
IL-9^{+}	0.4112	0.0981	0.6474	**0.0085**
IL-13^{+}	0.4191	0.1187	0.6928	**0.0021**

IL-17^{+}	0.3191	0.2018	0.2729	0.3654
Treg cells (%)				
IL-10^{+}*FoxP3*$^{+}$ *CD39*$^{-}$	- 0.1134	0.6019	- 0.2759	0.3589
IL-10^{+}*FoxP3*$^{+}$ *CD39*$^{+}$	-0.2133	0.3891	-0.1653	0.5870
Tr-1 cells (%)				
IL-10^{+}*FoxP3*$^{-}$ *CD39*$^{-}$	-0.1411	0.6781	-0.1320	0.6653
IL-10^{+}*FoxP3*$^{-}$ *CD39*$^{+}$	-0.3712	0.1891	-0.4097	0.1335
T_{FR} cells (%)				
IL-10^{+}*FoxP3*$^{+}$ *CD39*$^{-}$	- 0.5781	**0.0212**	- 0.7135	**0.0011**
IL-10 FoxP3^{+} *CD39*$^{+}$	-0.6011	**0.0116**	-0.7552	**0.0003**
IL-10^{+}*FoxP3*$^{-}$ *CD39*$^{-}$	-0.3019	0.2711	-0.4009	0.1645
IL-10^{+}*FoxP3*$^{-}$*CD39*$^{+}$	-0.1092	0.6012	-0.2944	0.3259

In PBMC cultures from cat-allergic patients stimulated with Fel d1 or Fel d1/Lep, the levels of cytokines, determined via Luminex, and the frequency of different effector and regulatory $CD4^{+}$ T cells subsets, evaluated by cytometry, was correlated with the *in vitro* IgE concentration, assayed through ELISA.

Table 3. Correlation between plasma leptin levels and cytokine profile of cells in Fel d1-stimlated PBMC cultures from cat-allergic patients.

	Leptin (ng/mL)	
Cytokines (pg/mL)	*r*	*p*
IL-4	0.1998	0.3982
IL-5	0.6438	**0.0022**
IL-6	0.5476	**0.0124**
IL-9	0.1674	0.4805
IL-13	0.3117	0.1810
IL-17	0.4989	**0.0251**
IL-21	0.3013	0.1968
IL-10	-0.2360	0.3165
Non-T_{FH} cells (%)		
IL-4$^+$	0.2822	0.3472
IL-5$^+$	0.7354	**0.0004**
IL-9$^+$	0.2296	0.4475
IL-13$^+$	0.6960	**0.0011**
IL-17$^+$	0.7868	**0.0002**
IL-21$^+$	0.1816	0.5498
IL-21$^+$$T_{FH}$ cells (%)		
IL-4$^+$	0.3877	0.1897
IL-5$^+$	0.3052	0.2096
IL-9$^+$	0.1429	0.6428
IL-13$^+$	0.1447	0.6341

IL-17^{+}	0.3714	0.1605
IL-21^{-} T_{FH} cells (%)		
IL-4^{+}	0.4014	0.0595
IL-5^{+}	0.6611	**0.0165**
IL-9^{+}	0.7675	**0.0001**
IL-13^{+}	0.8748	**<0.0001**
IL-17^{+}	0.3780	0.1014
Treg cells (%)		
IL-10^{+}*FoxP3*$^{+}$*CD39*$^{-}$	-0.8116	**<0.0012**
IL-10^{+}*FoxP3*$^{+}$*CD39*$^{+}$	-0.7331	**0.0007**
Tr-1 cells (%)		
IL-10^{+}*FoxP3*$^{-}$*CD39*$^{-}$	-04924	0.0893
IL-10^{+}*FoxP3*$^{-}$*CD39*$^{+}$	-0.8186	**<0.0001**
T_{FR} cells (%)		
FoxP3^{+}*CD39*$^{-}$	-0.7015	**0.0024**
FoxP3^{+}*CD39*$^{+}$	0.7845	**<0.0001**
FoxP3^{-}*CD39*$^{-}$	0.3011	0.2659
FoxP3^{-}*CD39*$^{+}$	0.4018	0.0950

The levels of cytokines secreted, determined via Luminex, and frequency of different conventional (non-$T_{FH,}$ CXCR5^{-}) and follicular helper (T_{FH}, CXCR5^{+}) CD4^{+} T cell subsets, evaluated by cytometry, in Fel d1-stimulated PBMC cultures from cat-allergic patients were correlated with the plasma leptin concentration, assayed through ELISA.

Table 4. Correlation between IgE titers and cytokine profiles of Fel d1-stimlated PBMC cultures from cat-allergic patients.

	IgE (Ku/L)	
Cytokines (pg/mL)	*r*	*p*
IL-4	*0.2431*	*0.3016*
IL-5	*0.6642*	***0.0014***
IL-6	*0.3848*	*0.0939*
IL-9	*0.1853*	*0.4342*
IL-13	*0.1070*	*0.2160*
IL-17	*0.3757*	*0.1036*
IL-21	*0.3473*	*0.1335*
IL-10	*-0.3304*	*0.1547*
Non-T_{FH} cells (%)		
IL-4$^+$	0.3928	0.1841
IL-5$^+$	0.1107	0.7176
IL-9$^+$	0.3737	0.1736
IL-13$^+$	0.2311	0.4441
IL-17$^+$	0.3407	0.2318
IL-21$^+$	0.3465	0.2671
IL-21$^+$$T_{FH}$ cells (%)		
IL-4$^+$	0.3736	0.2070
IL-5$^+$	0.4101	0.0987
IL-9$^+$	0.1484	0.6298
IL-13$^+$	0.1621	0.5938

IL-17^{+}	0.3301	0.2686
IL-21^{-}T$_{FH}$ cells (%)		
IL-4^{+}	0.6298	**0.0024**
IL-5^{+}	0.4170	0.0601
IL-9^{+}	0.3809	0.1526
IL-13^{+}	0.6823	**0.0078**
IL-17^{+}	0.3791	0.2025
Non-T$_{FH}$ cells (%)		
IL-10^{+}*FoxP3*$^{+}$*CD39*$^{-}$	-0.8473	**<0.0001**
IL-10^{+}*FoxP3*$^{+}$*CD39*$^{+}$	- 0.7414	**0.0009**
Tr-1 cells		
IL-10^{+}*FoxP3*$^{-}$*CD39*$^{-}$	- 0.3643	0.1017
IL-10^{+}*FoxP3*$^{-}$*CD39*$^{+}$	- 0.7986	**0.0001**
T$_{FR}$ cells (%)		
IL-10^{+}*FoxP3*$^{+}$*CD39*$^{-}$	-0.7070	**0.0016**
IL-10^{+}*FoxP3*$^{+}$*CD39*$^{+}$	-0.8177	**<0.0000**
IL-10^{+}*FoxP3*$^{-}$*CD39*$^{-}$	0.1648	0.5089
IL-10^{+}*FoxP3*$^{-}$*CD39*$^{+}$	0.1956	0.5189

The titer of plasma IgE was correlated with both cytokines levels, evaluated by Luminex, and the frequency, determined by cytometry, of different conventional (non-T$_{FH,}$ CXCR5^{-}) and follicular helper (T$_{FH}$, CXCR5^{+}) CD4^{+} T cell subsets in Fel d1-stimulated PBMC cultures from cat-allergic patients.

3 **DISCUSSÃO**

A asma alérgia é uma doença crônica das vias aéreas, caracterizada pela limitação do fluxo aéreo como consequência do seu estreitamento, resultante da inflamação crônica secundária ao influxo de células inflamatórias e o extravasamento do plasma, espessamento das paredes das vias aéreas e o aumento da secreção de muco (GINA, 2021). Não muito raro, os eventos inflamatórios podem resultar em comprometimento da função pulmonar, com obstrução persistente do fluxo aéreo devido ao remodelamento das vias aéreas e o aumento da produção de muco (AKAR-GHIBRIL et al., 2020; JOHANSSON et al., 2001). A AA é um dos problemas da saúde respiratória mais recorrentes no Brasil, onde afeta 20 milhões de pessoas, além do seu tratamento ser altamente dispendioso para o sistema de saúde (MINISTÉRIO DA SAÚDE, 2022). Trata-se de uma doença altamente heterogênea e complexa (TUAZON et al., 2022). Atualmente, baseado nas características observáveis e nos mecanismos moleculares, diferentes fenótipos e endotipos são propostos com o intuito de compreender os eventos envolvidos na imunopatogenia da AA (AL HEIALY; RAMAKRISHNAN; HAMID, 2022; LOURENÇO et al., 2022).

A AA é desencadeada pela sensibilização a alérgenos ambientais, tais como plantas, ácaros e animais domésticos, principalmente a gatos. Nas séries de trabalhos de nosso grupo sobre o assunto, dois artigos publicados foram conduzidos numa coorte de pacientes com AA causada por diferentes alérgenos. Em nosso último artigo (submetido), que investiga o perfil da resposta imune antígeno específica, foi conduzida em indivíduos com AA alérgicos a gatos em resposta à glicoproteína Fel d1. A alergia a gatos afeta aproximadamente 1 em cada 5 adultos, sendo considerada a alergia de origem em animais mais frequente (BOUSQUET et al.,2007; ZAHRADNIK, E. N., 2017). Entre os 8 alérgenos de gatos reconhecidos pela OMS e a IUIS, apenas a Fel d1 tem importância clínica. A Fel d1 é responsável por 96% das sensibilizações humana aos gatos, uma vez que pode ficar disponível e permanecer no ar em forma de caspas e partículas de poeira por longos períodos (BONNET et al., 2018; LEYNAERT et al., 2000; REEFER et al., 2004).

Independente do gatilho, a ocorrência e gravidade da AA é influenciada pela interação entre fatores genéticos e ambientais, como por exemplo a obesidade. A obesidade é um problema de saúde global, pois aproximadamente 2 bilhões de

adultos estão com excesso de peso, entre os quais 650 milhões são obesos (OBRADOVIC et al., 2021; ORGANIZAÇÃO MUNDIAL DA SAÚDE, 2019). É considerado um estado inflamatório crônico de baixo grau, que envolve a ativação de macrófagos pró-inflamatórios (M1) e linfócitos T $CD4^+$ e T $CD8^+$ efetores capazes de produzir citocinas inflamatórias, tais como IL-1β, TNF-α, IL-6 e IFN-γ (ROUJEAU; JOCKERS; DAM, 2014). Um conjunto de estudos indicam que a obesidade está correlacionada à gravidade da AA e menor responsividade aos esquemas terapêuticos atuais (ABATE et al., 1995; BARROS et al., 2017; CRUJEIRAS et al., 2015; HOLGUIN, 2012; RAY; ORISS: WENZEL, 2015; ZHANG et al., 1994). Em geral, asma associada à obesidade é mais comum em mulheres e, de fato, em nosso estudo a maioria dos pacientes com AA é do sexo feminino e a prevalência da asma grave associada ao sobrepeso ou obesidade.

Classicamente, a resposta aos alérgenos induz a ativação e diferenciação das células Th2. Os linfócitos Th2, por secretarem IL-4, IL-5 e IL-13, coordenam os eventos biológicos responsáveis pelos sintomas e características da inflamação T2 alta, caracterizada pela produção de IgE alérgeno-específico, recrutamento dos eosinófilos, hipersecreção de muco e remodelamento das vias aéreas (KURUVILLA; LEE, F.E.; LEE, G. B., 2019; MCBRIEN, MENZIES-GOW, 2017; WENZEL, 2012). Recentemente, foi proposto que as células Th9 agem em sinergismo com as células Th2 clássicas na fisiopatogenia da AA. A IL-9 potencializa a produção da IgE e aumenta a habilidade da IL-5 e da IL-13 de elevar a sobrevida dos eosinófilos e a produção de muco, respectivamente (ANGKASEKWINAI, 2019).

No primeiro artigo, quando comparado a indivíduos saudáveis (grupo controle), maiores níveis da IL-5 e da IL-4 foram produzidos por células T $CD4^+$ de pacientes com AA a diferentes alérgenos quando ativadas *in vitro* por mitógenos. Esses achados foram corroborados pelos resultados do segundo artigo, em que maior proporção de células T $CD4^+IL\text{-}4^+$, determinada pela citometria de fluxo, foi identificada em culturas de células mononucleares do sangue periférico (CMSP) de pacientes com AA, principalmente os com doença grave, se comparado ao grupo controle. A citometria de fluxo é uma técnica mais sofisticada e permite acessar a grande plasticidade dos diferentes subtipos de linfócitos T. De fato, como será demonstrado mais a frente, os subtipos de células T $CD4^+IL\text{-}4^+IL\text{-}17^+$, e não o fenótipo celular Th2 clássico, é que foram associados à gravidade da AA no segundo artigo. Ainda, no terceiro artigo, usando o marcador para determinar células T $CD4^+$ convencionais (não-$T_{FH;}$ $CXCR5^-$

) e foliculares ($T_{FH;}$ $CXCR5^+$), a adição de Fel d1 aumentou a frequência de células Th2, positivas para IL-4, IL-5 e IL-13, e células Th9 em pacientes com AA e rinite alérgica (RA) a gatos. Entretanto, dentre esses subtipos celulares, apenas a proporção de células T $CD4^+$ capazes de produzir IL-5 e IL-13 correlacionaram-se com obesidade. Por outro lado, a obesidade impactou positivamente na porcentagem de diferentes subtipos células T_{FH}, o principal subtipo de células T $CD4^+$ implicado na produção de anticorpos.

Apesar de elevados títulos da IgE terem sido detectados em coculturas de células B e T $CD4^+$ de pacientes asmáticos no artigo 1, estes não foram correlacionados com o estado clínico. Diferentes motivos podem explicar a falta de relação. Em primeiro lugar, nesse estudo, nós dosamos apenas IgE total e não alérgeno-específicos. Ainda, é possível que, durante as exacerbações clínicas, esse anticorpo seja produzido localmente por plasmócitos da submucosa do trato respiratório. Finalmente, sabemos que nem todos os casos de AA são mediados por citocinas relacionadas ao fenótipo Th2 e IgE (DIAS et al., 2019).

Como previamente mencionado, a síntese da IgE alérgeno-específica depende da interação entre os linfócitos B e as células T_{FH}. No CG dos folículos linfoides, as células T_{FH} são fenotipicamente identificadas como $CXCR5^+Bcl6^+PD1^+IL\text{-}21^+$. Na periferia, apesar das células T_{FH} não expressarem o Bcl6 e o PD1, esses linfócitos são capazes de auxiliar as células B na resposta imune humoral de alta eficiência (LEYNAERT et al., 2000; SCHMIT; BENTEBIBEL; UENO, 2014). Baseado no perfil de citocinas, as células T_{FH} são divididas em 3 grupos: (i) células $T_{FH}1$ ($IL\text{-}21^+IFNy^+$); (ii) células $T_{FH}2$ ($IL\text{-}21^+IL\text{-}4^+$); (iii) células $T_{FH}17$ ($IL\text{-}21^+IL\text{-}17^+$). Em 2019, um estudo de Gowthaman e colaboradores demonstrou que a produção da IgE de alta afinidade foi associada a uma nova população de células T_{FH}, conhecida como células $T_{FH}13$. As células $T_{FH}13$ produzem quantidades minutas de IL-21, mas expressam IL-4, IL-5 e elevados níveis de IL-13. Dados apresentados no artigo 3, revelam que uma maior proporção de células $T_{FH}2$ e $T_{FH}13$ Fel d1-específicas foi observada entre os pacientes obesos com AA e RA a gatos. A frequência desses subtipos de células T_{FH} se correlacionou diretamente com os níveis circulantes de leptina. Ainda nesse artigo, em culturas de CMSP estimuladas com Fel d1 na presença de dose de leptina associada à obesidade, os níveis de IgE Feld1-específicas secretados foram diretamente correlacionados com a frequência de diferentes populações das células T_{FH}, mas não de linfócitos Th2 clássicos. De forma interessante, os títulos séricos da

IgE contra a Fel d1 foram diretamente associadas a porcentagem de células semelhantes ao fenótipo $T_{FH}13$ (IL-2^{-} IL-5^{+}IL-13^{+}). Estes resultados fortalecem a teoria de que a produção da IgE é dependente dos linfócitos T_{FH}. Enquanto o subtipo $T_{Fh}2$ parece ser responsável pela produção da IgE de baixa afinidade, as células $T_{FH}13$ (IL-$21^{-/+}$IL-4^{+}IL-5^{+}IL-13^{++}) têm sido associadas a síntese de IgE de alta afinidade (GOWTHAMAN et al., 2019; GOWTHAMAN et al., 2020). Infelizmente, devido a limitação do nosso painel, não foi possível aferir a porcentagem de células T_{FH} capazes de produzir simultaneamente IL-4, IL-5 e IL-13, nem tão pouco determinar a afinidade da IgE que nós quantificamos. Levando-se em consideração que a gravidade da alergia a gatos tem sido relacionada a produção de IgE (CRACK et al., 2011; REEFER et al., 2004), e que a ligação de complexo IgE/Fel d1 de alta afinidade ao receptor FcεRI induza intensa ativação dos mastócitos e eosinófilos (MITA, YASUEDA, AKIYAMA, 2000; YANG et al., 2020), nós acreditamos que a capacidade da leptina elevar a frequência de células T_{FH} IL-21^{-}IL-13^{+} Fel d1-específica seja um dos mecanismos pelos quais a obesidade agrava as exacerbações clínicas em pacientes com AA e RA a gatos.

A imunopatogênese da asma é heterogênea e em alguns casos, especialmente os refratários ao tratamento com os corticoides, apresentam um endotipo T2 baixa, caracterizado por intensa neutrofilia nas vias aéreas. Esses dados apontam para a participação das Th17 na asma grave (BARROS et al., 2016; BOUSQUET et al., 1998; BURKS et al., 2013; SCHATZ; ROSENWASSER, 2014). As Th17 diferenciam-se na presença da IL-6 e IL-23 e sintetizam as citocinas IL-17, IL-21 e IL-22. A IL-17 promove a infiltração de neutrófilos, produção de muco, hiperatividade brônquica e remodelamento das vias aéreas (KUDO et al., 2012; MOLET et al., 2001). Sabidamente, a neutrofilia é um indicativo da resistência maciça aos corticoides (EIFAN et al., 2015). Diversos estudos relatam um aumento das Th17 e IL17 no sangue periférico e nas vias aéreas de pacientes asmáticos graves (BARCZYK; PIERZCHA; SOZANSKA, 2003; KERZEL et al., 2012; WEI et al., 2021). De forma interessante, a IL-17 tem sido relacionada à síntese da IgE, além de favorecer o acúmulo dos eosinófilos na mucosa de pacientes alérgicos (AMIN et al., 2020; MILOVANOVIC et al., 2010).

Dados apresentados no artigo 1 demonstraram que, nos pacientes com AA, as células T $CD4^{+}$ ativadas por mitógenos produziram altos níveis da IL-6 e da IL-17 em comparação com o grupo controle. Adicionalmente constatamos uma associação

entre a gravidade da doença e a produção dessas citocinas. Segundo Rincon e Irvin (2012), a IL-6 sérica é um marcador da gravidade da asma e os nossos resultados corroboram esse achado. Posteriormente, no artigo 2, usando como ferramenta a citometria de fluxo, a frequência das células Th17 foi maior nos pacientes com a asma grave. Ainda na população $IL17^+$, a Fel d1, principalmente na presença de elevada dose de leptina, favoreceu a expansão das $T_{FH}17$, porém essas células não foram associadas aos níveis plasmáticos *in vitro* da IgE. Acreditamos que, apesar desses resultados, a expansão de células $T_{FH}17$ Fel d1-específicas contribua com a gravidade da RA e AA a gatos por favorecer o recrutamento de eosinófilos para as vias aéreas dos pacientes durante as exacerbações agudas.

Entre as citocinas Th2 dosadas no primeiro trabalho, os níveis da IL-5 foram associados à gravidade da AA, e esse resultado está de acordo com os achados de Greenfeder e colaboradores (2001) e Rijavec e colaboradores (2021). Através do seu receptor, a IL-5 regula a promoção, migração e sobrevivência dos eosinófilos (MENZIES-GOW et al., 2003; STONE; PRUSSIN; METCALFE, 2010). Recentemente foi descrita uma população das Th2 com fenótipos mistos, conhecida como Th2/Th17. As células híbridas Th2/Th17 são $GATA\text{-}3^+RORyt^+$, produzem IL-5 e IL-17 e detectadas nas vias aéreas de asmáticos graves. Ademais, a presença desse fenótipo misto é comumente associada à intensa infiltração de eosinófilos, perda da função pulmonar e o pior controle da doença (IRVIN et al., 2014; WANG et al., 2010). Em muitos desses pacientes, o controle da AA depende de altas doses de corticoides orais sistêmicos (BHAKTA, WOODRUFF, 2011; FROIDURE et al., 2016; RAY; ORISS; WENZEL, 2015). Em acordo com esses achados, dados apresentados no artigo 2 revelaram que a maioria das células T $CD4^+IL4^+$ dos pacientes com a AA grave produziam IL-17. Esse fenótipo foi mais prevalente entre os pacientes obesos. A maior proporção das células Th17 e Th2/Th17 observadas nos dois artigos publicados estão de acordo com outros estudos (AGACHEL et al., 2010, AL-RAMLI et al., 2009; CHIEN et al., 2013; IRVIN et al., 2014; WANG et al., 2010), o que revela complexa rede de endotipos implicados na doença. O fato de termos observado uma associação entre a obesidade e a expansão das células Th17 e Th2/Th17 hibridas nos asmáticos, sugere que este seja um provável mecanismo que liga o excesso de massa gorda com pior prognóstico da AA.

Com relação ao fenótipo celular Th1 estudado no artigo 1, a produção do IFN-γ foi significativamente menor nos pacientes asmáticos, em especial entre os com

sobrepeso/obesos e asma grave. Células Th1 são centrais em coordenar a resposta imune protetora contra diferentes patógenos intracelulares e vírus (KIDD, 2003; WODARZ; JANSEN, 2001). O IFN-γ aumenta o poder microbicida dos fagócitos e a citotoxicidade das células NK e dos linfócitos T $CD8^+$ (MCKINSTRY; STRUTT; SWAIN, 2010). Vale destacar ainda que a porcentagem das células Th1 demonstrada no segundo artigo foi inversamente correlacionada com os níveis plasmáticos da leptina. Esses achados podem ajudar a explicar, ao menos em parte, porque pacientes com a asma grave são mais propensos a infecções das vias aéreas baixas pelo vírus da influenza, rinovírus e *Escherichia coli* (OLIVER et al., 2014). A ocorrência de obesidade deve ainda complicar mais esse dano no perfil Th1 por mecanismos adversos envolvendo o estado de inflamação de baixo grau nos pacientes com AA (JUHN, 2014).

Quando presente, a obesidade imprime um carácter mais patogênico a asma. Como previamente mencionado, o endotipo asma-obeso, que apresenta maior refratariedade à terapêutica convencional, é encontrado com maior frequência no sexo feminino acima dos 40 anos (BARROS et al., 2017; HOLGUIN, 2012; RAY; ORISS, WENZEL, 2015). O estado de inflamação sistêmica de baixo grau observada na obesidade é caracterizado não apenas pela produção de citocinas inflamatórias, como também a secreção de adipocinas, tal como na hiperleptinemia (APOSTOLOPOULOS et al., 2016).

Nos obesos, os níveis circulantes de leptina são 4 à 6 vezes maiores quando comparados aos eutróficos (MUELLER et al., 1998; PAPATHANASIOU et al., 2018; PINTO et al., 2004; TODA et al., 2017). A leptina desempenha as suas funções ao ligar-se ao seu receptor presente em uma vasta gama de células, incluindo as do sistema imunológico (BHATT; GULERIA; KABRA, 2021; HE et al., 2022; WANG et al., 2022). Elevada expressão do receptor foi detectada em linfócitos T de pessoas obesas (DE ROSA et al., 2007; MARTIN-ROMERO et al., 2000). A hiperleptinemia modula várias funções das células da imunidade inata e adaptativa. Elevados níveis dessa adipocina aumenta a quimiotaxia e a sobrevida dos neutrófilos (FREDERICH et al., 1995; MYERS et al., 2012). Por aumentar a expressão de moléculas co-estimuladoras nas DCs, a leptina favorece reações de hipersensibilidade (AL-HASSI et al., 2012; CHEN et al: 2014; FRIEDMAN, 2014; KOLACZYNSKI et al., 1996). Também, induz a diferenciação e proliferação das células efetoras, tais como as células Th1 e Th17 (AHMED; GAFFEN, 2010; CALDEFIE-CHEZET et al., 2001; KANEMARU et al., 2015;

LOFFREDA et al., 1998; PINI, FANTUZZI, 2010; WAGNER et al., 2013). Em contrapartida, por induzir a produção da IL-1β, IL-6 e IL-12, a leptina inibe as células Treg (MATARESE et al., 2010). Nos dados apresentados no primeiro artigo observamos que, de fato, a obesidade favoreceu a produção de IL-6 pelas células T policlonalmente ativadas em culturas de CMSP obtidas tanto do grupo controle quanto de pacientes com AA. Corroborando esses achados, no segundo artigo, níveis elevados não apenas de IL-6, como também de IL-5 e IL-13, foram liberados pelas células T $CD4^+$ dos pacientes obesos com AA e, no artigo 3, a leptina aumentou a produção da IL-6 em culturas de CMSP em resposta à Fel d1. A contribuição da IL-6 na patogênese das reações alérgicas envolvendo IgE deve envolver a capacidade desta citocina de estimular a proliferação dos linfócitos B, a sobrevida dos plasmoblastos e a produção de anticorpos (KOPF et al., 1994; TOSATO et al., 1988).

De forma interessante, apesar dos grupos controle e de pacientes estudados nos artigos 1 e 2 terem sido pareados segundo o IMC, os níveis séricos da leptina foram significativamente mais elevados nos pacientes com asma alérgica, especialmente nos pacientes com AA moderada e grave. Essa diferença pode ser explicada pela maior produção de certas citocinas inflamatórias nos pacientes com AA que têm habilidade de induzir a produção de leptina (EL-WAKKAD et al., 2013; IIKUNI et al., 2008; ZHENG et al., 2018). De fato, nossos dados revelaram uma correlação direta e significativa entre os níveis da leptina com as concentrações da IL-6, IL-17 e IL-5 secretados por células T $CD4^+$ policlonalmente ativadas. Em contraste, no segundo artigo, uma correlação inversa e significativa foi vista entre os níveis da leptina e a frequência de células T $CD4^+IL10^+$. Esse resultado está de acordo com outros estudos que associam a asma com a diminuição da produção da IL10 pelas células Treg (LLOYD et al., 2009; SMITH et al., 2008). Por outro lado, uma correlação direta entre os níveis plasmáticos da leptina e a porcentagem de células Th17 e Th2/Th17 híbridas foi observada, mas não com a frequência das células Th2 clássicas. A relação entre a obesidade e a gravidade de asma atópica deve estar ligada à capacidade da leptina em aumentar a produção de IgE e eosinofilia nos pacientes (CONUS: BRUNO: SIMON, 2005; CIPRANDI et al., 2009; WONG: CHEUNG: LAM, 2007). Em nosso último trabalho, dados a partir de ensaios *in vitro* alérgeno-específico, a leptina favoreceu a produção da IgE e a expansão dos fenótipos Th2, Th9, e as células $T_{FH}IL\text{-}21^-$ capazes de produzir IL-4, IL-5 e IL-13 em resposta à Fel d1. Esses achados estão de acordo com Visness e colaboradores

(2009), no qual a leptina foi correlacionada com a produção da IgE. Essa relação entre hiperleptinemia e pior desfecho da AA deve envolver também a capacidade dessa adipocina em modular negativamente a função dos linfócitos reguladores.

As células T reguladoras têm um importante papel na manutenção da tolerância e controle da inflamação na asma. Na superfície das células Treg são encontradas algumas moléculas fundamentais para determinar a sua funcionalidade, como por exemplo: (i) CD25, também conhecida como cadeia α do receptor de alta afinidade para a IL-2, que ao se ligar a IL-2 exógena, impede o seu consumo pelas células T efetoras pró-inflamatórias; (ii) CTLA-4 (CD152), inibe a resposta inflamatória por desativar as APCs após reconhecimento das moléculas da família B7; e (iii) CD39, que junto com CD73, reduz a inflamação ao degradar a adenosina trifosfato (ATP) em adenosina (ADO), um potente inibidor das células T efetoras (CHEN et al., 2016; AFSHAR et al.,2019; OCHOA-REPÁRAZ; KASPER, 2017). Uma série de estudos indicam que, na asma, o compartimento das células reguladoras está comprometido. Alguns pesquisadores evidenciaram, em humanos, uma menor frequência das células Treg (HARTL et al., 2007; HUANG et al., 2017b; KINOSHITA et al., 2014; LIN; SHIEH, WANG, 2008; MAMESSIER et al., 2008; PROVOOST et al., 2009). O trabalho de Weathington e colaboradores (2019) demonstrou uma menor expressão dos genes associados as funções das células Tregs no pulmão de pacientes com asma grave.

Em nosso segundo artigo, a ocorrência de obesidade e da AA grave afetaram a frequência de células Treg e Tr1. Ademais, a hiperleptinemia foi negativamente correlacionada aos níveis dessas células e a produção da IL-10. Resultados similares foram observados em pacientes obesos com asma por alguns autores (MATARESE et al., 2010; VAN DE VEEN et al., 2016). Ainda em nosso segundo artigo, a obesidade foi ligada a uma menor frequência das células T $CD4^{+}FoxP3^{+}IL10^{+}$ apenas nos pacientes com asma alérgica grave, quando comparados aos outros grupos e os controles. Na AA, a IL-10, por inibir a produção de IL-4 e IL-13, tem sido relacionada a uma redução na produção de IgE, inibição da desgranulação dos eosinófilos e mastócitos e da ativação dos neutrófilos e das células Th17 efetoras (BURTON et al., 2014; SHAMJI; DURHAM, 2017; WING et al., 2008). Nós ainda observamos que a porcentagem das células T $CD4^{+}FoxP3^{+}CD39^{+}$ não apenas foi menor nos pacientes com AA moderada e grave, como também foi inversamente correlacionada com os níveis circulantes de leptina. Cortez-Espinosa e colaboradores (2015) encontraram uma correlação negativa entre a frequência das células Treg$CD39^{+}$ com o IMC de

pacientes com diabetes mellitus do tipo 2, contudo, a identificação dessa população no contexto da AA é nova. Ainda no compartimento regulador, dados publicados no segundo artigo revelaram uma diminuição na frequência das células B secretoras da IL-10 (Br1) nos pacientes com a asma moderada e, em especial, com asma alérgica grave. As concentrações plasmáticas da leptina foram inversamente correlacionadas com a porcentagem das células Br1, indicando que, possivelmente, a leptina possui um efeito deletério nessas células por comprometer a produção da IL-10.

A gravidade das reações alérgicas tem sido associada à diminuição de diferentes subpopulações de células T reguladoras convencionais [nTregs ($CXCR5^-FoxP3^+IL\text{-}10^+$) e Tr1 ($CXCR5^-FoxP3^-IL\text{-}10^+$)] e células T foliculares reguladoras (T_{FR}; $CXCR5^+FoxP3^+IL\text{-}10^+$) dirigidas contra diferentes alérgenos (CLEMENT et al., 2019, MARTÍN-OROZOCO; NORTE-MUÑOZ; GARCÍA, 2017). No presente estudo, dados apresentados no artigo 3 demonstraram a capacidade da leptina de diminuir a porcentagem das células Treg/Tr1 e células T_{FR} específicas para Fel d1, principalmente no subtipo celular que expressa CD39. Em um modelo animal de AA as células Treg $CD39^+$ foram primordiais no controle da inflamação das vias aéreas (LI et al., 2015). Assim como na pesquisa de Martin-Orozco e colaboradores (2017), no terceiro artigo, a frequência das células Treg e células T_{FR}, positivas ou não para o CD39, correlacionaram-se negativamente com os títulos de IgE plasmáticas Fel d1-especifícas e com os níveis de IgE secretado em culturas de PBMC estimuladas com a Fel d1 na presença de leptina.

Além das células Treg $IL\text{-}10^+$, a perda dos mecanismos regulatórios na AA pode estar associada à deficiência da vitamina D. A hipovitaminose D é comum em qualquer idade e é majoritariamente detectada em indivíduos do sexo feminino (PALACIOS & GONZALEZ, 2014). Ademais, pessoas obesas são mais propensas ao desenvolvimento da hipovitaminose D do que os eutróficos (PEREIRA-SANTOS et al.,2015). Nos últimos tempos, a deficiência dessa vitamina tem sido atrelada há um maior risco de desenvolvimento da asma (BOZZETTO et al., 2012; CARROLL et al., 2011; DEVEREUX et al., 2010; HALL; FISCHER; AGRAWAL, 2016). Adicionalmente, baixas concentrações séricas da vitamina D foram associadas à necessidade de maior dose de corticoides para controle das exacerbações clínicas (PENNA & ADORINI, 2000). Em condições de suficiência, a vitamina D atua como um importante imunomodulador, controlando a produção de citocinas inflamatórias associadas ao fenótipo Th17, enquanto aumenta a proporção de células Treg e produção das

citocinas anti-inflamatórias IL-10 e TGF-β (AFZALI et al., 2007; ARANOW, 2011). Estudos indicam que a forma ativa da vitamina D, a $1,25(OH)_2D_3$, aumenta a capacidade dos corticoides em induzir a produção de IL-10 por monócitos de pacientes asmáticos ativados pelo lipopolissacarídeo (LPS) e potencializar a geração de células Tr1 (COTTREZ et al., 2000; MATYSZAK et al., 2000; HAMZAOUI et al., 2014; WU et al., 2007). Além disso, no estudo de HAMZAOUI e colaboradores (2014), a $1,25(OH)_2D_3$ inibiu, *in vitro*, a produção das citocinas chaves do fenótipo Th17 em pacientes com a asma grave, de forma independente à resposta aos corticoides. Entretanto, dados apresentados em nosso primeiro artigo demonstraram que, em comparação com o grupo controle, a $1,25(OH)_2D_3$ foi menos eficiente em diminuir, *in vitro*, a produção de IL-4, IL-5, IL-6 e IL-17 nas culturas de células T $CD4^+$ dos pacientes com AA. Elevada resistência à forma ativa da vitamina D foi observada, *in vitro*, na cultura das células de pacientes com excesso de peso, ou obesidade, e com asma grave. Nos pacientes asmáticos, a $1,25(OH)_2D_3$ também foi menos eficiente em aumentar a produção da IL-10 pelas células T $CD4^+$ policlonalmente ativadas. Por fim, os níveis plasmáticos da leptina foram negativamente correlacionados com a capacidade da forma ativa da vitamina D em diminuir a produção de citocinas inflamatórias e aumentar a síntese da IL-10 pelas culturas das células T $CD4^+$ dos asmáticos.

Até a data da publicação do artigo 1, em 2019, até onde sabíamos, este foi o primeiro artigo a estabelecer uma relação entre a obesidade, níveis plasmáticos de leptina e os efeitos da forma ativa da vitamina D em culturas de células T $CD4^+$ em pacientes com a AA. Em suma, os nossos resultados indicam que o panorama do excesso de gordura induz a expansão de células T $CD4^+$ secretoras de citocinas envolvidas na patogênese da AA, as células Th17 e o fenótipo híbrido Th2/Th17. Além do mais, o sobrepeso/obesidade comprometeu as propriedades imunomoduladoras da vitamina D na produção das citocinas relacionadas aos fenótipos Th2 e Th17 e secreção *in vitro* da IgE.

Em relação ao perfil imune das células T $CD4^+$ seguindo a estimulação *in vitro* com alérgeno de gatos, os resultados sugerem que a hiperleptinemia aumentou a síntese da IgE Fel d1-específica. Além disso, o excesso de leptina induziu a expansão das células Th2, Th9 e subpopulações de células T_{FH} Fel d1-específicas. Contudo, a presença dessa adipocina, em dose relacionada à obesidade, impactou

negativamente a frequência de células T $CD4^+$, especialmente os subtipos mais funcionais que expressam o marcador CD39.

CONCLUSÃO

Os resultados apresentados nos dois primeiros artigos, apesar de preliminares, sugerem que a obesidade impacta negativamente o curso da asma alérgica por favorecer o desequilíbrio entre as células Th2 e subtipos de linfócitos Th17 ao promover a expansão de células Th17 clássicas e do fenótipo híbrido Th2/Th17, ambos subtipos celulares associados à gravidade da AA. As exacerbações agudas da AA devem ser mais graves nos obesos devido a danos funcionais em subtipos de células T $CD4^+$ reguladoras e às células Br1. Ainda nesses estudos, muitos dos eventos adversos descritos nos primeiros dois artigos foram associados ao estado de hiperleptinemia no qual os obesos são condicionados, que podem amplificar seus efeitos adversos por diminuir a capacidade da vitamina D de reduzir a produção de citocinas inflamatórias associadas à AA grave.

No contexto da resposta ao principal alérgeno de gatos, a glicoproteína Fel d1, a hiperleptinemia, de fato, atua diretamente diminuindo a proporção de diferentes subtipos de células T $CD4^+$ reguladoras e induzindo a expansão de células Th2, Th9 e, principalmente, células $T_{FH}IL\text{-}21^-$ capazes de produzir IL-4, IL-5 e IL-13, fenótipo folicular que tem sido ligado a produção de IgE de alta afinidade e anafilaxia. Acreditamos que esses efeitos da leptina no desequilíbrio de diferentes subtipos de células T $CD4^+$ Fel d1-específica podem impactar negativamente o sucesso das imunoterapias alérgeno-específicas (ITA) em indivíduos alérgicos a gatos.

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ANEXO A - Carta do Comitê de Ética do Hospital Universitário Gaffrée e Guinle

HOSPITAL UNIVERSITÁRIO
GAFFREE E
GUINLE/HUGG/UNIRIO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Impacto da obesidade no perfil imune de pacientes atópicos
Pesquisador: Cleonice Alves de Melo Bento
Área Temática:
Versão: 2
CAAE: 44951215.6.0000.5258
Instituição Proponente: Hospital Universitário Gaffree e Guinle/HUGG/UNIRIO
Patrocinador Principal: Financiamento Próprio
FUN CARLOS CHAGAS F. DE AMPARO A PESQUISA DO ESTADO DO RIO DE JANEIRO - FAPERJ

DADOS DO PARECER

Número do Parecer: 1.164.594
Data da Relatoria: 25/06/2015

Apresentação do Projeto:
IMPACTO DA OBESIDADE NO STATUS FUNCIONAL DAS CÉLULAS T E B DE PACIENTES ATÓPICOS

Objetivo da Pesquisa:
O objetivo geral deste trabalho será correlacionar a frequência de células Th2/Th17/Th1 e Treg/Breg com o índice de massa corpórea e os níveis
periféricos de leptina em pacientes com alergias imediatas.

Avaliação dos Riscos e Benefícios:
O procedimento usado para colher o seu sangue é o mesmo utilizado nos exames de rotina. Portanto, trará risco mínimo.
Benefícios:
Os resultados do estudos não irão beneficiar diretamente. Eles irão nos ajudar a compreender como a obesidade pode exercer efeitos
adversos no sistema imune de pacientes alérgicos.

Comentários e Considerações sobre a Pesquisa:
Projeto apresentou de forma clara todas as pendências anteriores, descritas por este comitê de ética. O métodos foram descritos, o orçamento detalhado e explicitada sua origem (finaciamento

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HOSPITAL UNIVERSITÁRIO
GAFFREE E
GUINLE/HUGG/UNIRIO

prórprio e da própria FIOCRUZ) e os testes estatísticos foram também descritos pelo pesquisador.

Considerações sobre os Termos de apresentação obrigatória:

Completos

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

Não há.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

RIO DE JANEIRO, 30 de Julho de 2015

Assinado por:
Pedro Eder Portari Filho
(Coordenador)

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ANEXO B – Comprovante de submissão do terceiro artigo

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cbento _ <cbento@unirio.br>

Manuscript submitted to Journal of Immunology Research
1 mensagem

Journal of Immunology Research <jir@hindawi.com> 15 de junho de 2023 às 10:21
Responder a: Journal of Immunology Research <sathya.priya@hindawi.com>
Para: Cleonice A Bento <cbento@unirio.br>

Hindawi

Dear Dr. Bento ,

Congratulations, the manuscript titled "Leptin favors imbalance of antigen-specific CD4+ T cells associated with severity of cat allergy" has been successfully submitted to Journal of Immunology Research.

We will confirm this submission with all authors of the manuscript, but you will be the primary recipient of communications from the journal. As submitting author, you will be responsible for responding to editorial queries and making updates to the manuscript.

In order to view the status of the manuscript, please visit the manuscript details page.

Thank you for submitting your work to Journal of Immunology Research.

MANUSCRIPT DETAILS

APÊNDICE – Termo de consentimento livre e esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

"Impacto da Obesidade no status funcional das células T e B de pacientes atópico"

EXPLICAÇÃO PARA A PACIENTE SOBRE A PROPOSTA DO ESTUDO

1- ***Objetivos do estudo***

O objetivo desse projeto será avaliar se a gravidade das reações alérgicas está associada aos níveis sistêmicos de leptina, uma proteína produzida pelos adipócitos, células que acumulam gordura no organismo.

2- ***Procedimentos***

Para o nosso estudo, iremos colher uma (01) amostra do seu sangue periférico no volume de 10 mL cada coleta. Nenhuma coleta de sangue a mais será necessária. Toda a coleta do material biológico será conduzida com material adequado e estéril. Seu sangue não será usado nem para estudos genéticos nem tampouco com propósito comercial, e apenas os pesquisadores irão ter acesso a este material. Além disso, nós precisamos de algumas informações: peso e altura, para que possamos calcular o seu índice de massa corpórea. Finalmente, um questionário será enviado para o seu endereço eletrônico contendo perguntas referentes, o que possam estar implicadas, no grau de severidade de sua reação alérgica.

3- ***Riscos e DEsconfortos***

O procedimento usado para colher o seu sangue é o mesmo utilizado nos exames de rotina. Portanto, este lhe trará risco mínimo. Ademais, nenhuma pergunta no questionário irá lhe causar qualquer tipo de constrangimento.

4- ***Benefícios***

Os resultados de nossos estudos não irão beneficiar diretamente. Eles irão nos ajudar a compreender como a obesidade pode exercer efeitos adversos no sistema imune de pacientes alérgicos. Portanto, nós não podemos lhe dar nenhuma garantia que você será beneficiado por participar dessa pesquisa.

5- ***Alternativa de participação***

A sua participação nesse estudo é voluntária. Você pode desistir a qualquer momento.

6- ***Custos e compensações***

Você não irá pagar nem receber nada para participar desse estudo.

7- ***Confidencialidade***

Todas as informações referentes a você, assim como todos os resultados obtidos, serão mantidas sob sigilo. Seu nome não será revelado, exceto para o grupo envolvido na pesquisa. Nenhuma publicação científica irá identificar você.

8- ***Questões e problemas***

Em caso de dúvida sobre a pesquisa ou direito dos pacientes poderá ser contatado a Drª CLEONICE BENTO por meio dos telefones (21) 2531-7908 ou do e-mail cbento@unirio.br, bem como o CEP do HUGG, por meio do telefone (21) 2264-5177 ou do email cephugg@gmail.com, no endereço Rua Mariz e Barros, nº 775 - 2º andar (Prédio da Direção do HUGG), Tijuca, Rio de Janeiro, RJ, nos seguintes horários: de segunda a sexta-feira, de 8:30 às 12:00 horas.

9- ***Consentimento***

Caso você tenha lido e entendido todas as informações previamente descritas, e você ESPONTANEAMENTE concorde em participar desse estudo, favor assinar na linha a baixo.

Assinatura do Paciente:_____

Eu certifico que expliquei a proposta do estudo a paciente, e parece que ela entendeu os objetivos, procedimentos, riscos e benefícios do estudo.

Assinatura do pesquisador:_____

Testemunha:_____

Assinatura da testemunha:_____

Data: _____