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Função da *Aspirin-triggered* RvD1 (AT-RvD1) em células epiteliais brônquicas estimuladas com a IL-4

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Revisão da literatura apresentada ao Curso de Pós-Graduação em Ciências Fisiológicas da Universidade Federal do Triangulo Mineiro, como requisito para obter o titulo de Mestre em Ciências Fisiológicas. Área de concentração II: Imunologia, Parasitologia e Microbiologia.

Orientador: Prof. Dr. Alexandre de Paula Rogério

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Curso de Pós-Graduação em Ciências Fisiológicas

ATESTADO DE CONCLUSÃO

ATESTAMOS para os devidos fins que **Jhony Robison de Oliveira,** matriculado(a) no Curso de Pós-Graduação em Ciências Fisiológicas da Universidade Federal do Triângulo Mineiro (CPGCF/UFTM), Área de Concentração II – Parasitologia, Imunologia e Microbiologia concluiu o Curso de Mestrado em Ciências Fisiológicas, tendo defendido, no dia 24 de junho de 2014, a dissertação intitulada **"Função da Aspirin-triggered RvD1 (AT-RvD1) em células epiteliais brônquicas estimuladas com a IL-4"**, perante a Banca Examinadora constituída pelos(as) Professores(as) Doutores(as): Fernanda de Freitas Anibal – Laboratório de Parasitologia, Centro de Ciências Biológicas e da Saúde, UFSCar, São Carlos, SP; David Nascimento Silva Teixeira – Disciplina de Laboratório Clínico, Departamento de Clínica Médica, ICS/UFTM, MG; Alexandre de Paula Rogério - Orientador, o(a) qual foi **APROVADO(A)** por unanimidade. O processo foi encaminhado para o Setor de Registro de Diploma.

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VALDO JOSÉ DIAS DA SILVA
Coordenador do CPGCF/UFTM

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RESUMO

As células epiteliais brônquicas contribuem para o início e/ou manutenção da resposta inflamatória das vias aéreas como na asma. A ativação de células epiteliais brônquicas induz a produção de quimiocinas, expressão de moléculas de adesão e citocinas que podem influenciar a modulação do processo inflamatório. A asma é uma doença inflamatória das vias aéreas caracterizada pela migração de leucócitos, principalmente eosinófilos, hipersecreção de muco e hiperreatividade das vias aéreas (HRA). A IL-4 é a principal citocina envolvida na resposta do tipo Th2 e modula, dentre outras, a produção da quimiocina CCL2 e IL-8 que estão envolvidas na patofisiologia de algumas doenças inflamatórias, como, a asma. Na resolução da inflamação aguda, mediadores lipídicos como a resolvina D1 (RvD1) e seu epímero AT-RvD1 são produzidos no local da inflamação. Estes mediadores demonstram atividades pró-resolução, acelerando a resolução da inflamação e a restituição da homeostasia do tecido. No modelo de asma experimental tanto a RvD1, quanto o AT-RvD1 demonstraram atividade pró-resolução reduzindo algum dos principais fenótipos da asma (hiperreatividade das via aéreas, produção de citocinas/quimiocinas e inflamação pulmonar). No presente projeto estes resultados foram estendidos e avaliou-se a modulação do AT-RvD1 na ativação de células epiteliais brônquicas humanas (linhagem BEAS-2B) estimuladas com a IL-4 através da análise da produção de CCL2, IL-8 e a expressão das vias de sinalização dos fatores de transcrição STAT6 e NF-κB. Além disso, avaliou-se a expressão de SOCS1 e 3. IL-4 aumentou a produção de CCL2 e IL-8, assim como, aumentou a fosforilação de STAT6, NF-κB, SOCS1 e SOCS3 quando comparado com o grupo controle. AT-RvD1 (100 nM) reduziu a produção de CCL2 e IL-8 quando comparado com células tratados somente com IL-4. Este efeito foi dependente do receptor ALX uma vez que, o antagonista deste receptor (BOC1) reverteu o efeito do AT-RvD1. AT-RvD1 reduziu também a fosforilação de STAT6 e NF-κB. Adicionalmente AT-RvD1 reduziu a expressão gênica de SOCS1 e aumentou SOCS3 quando comparado com células estimuladas com IL-4. Uma vez que estas quimiocinas e estas vias de sinalização estão envolvidas na modulação da resposta neutrofílica e/ou eosinofílica da asma, o AT-RvD1 pode ser usado como terapia alternativa assim como, fornecer subsídios para o desenvolvimento de novas estratégias terapêuticas para controle das doenças inflamatórias das vias aéreas, como a asma.

Palavras-Chave: Células Epiteliais Brônquicas. AT-RvD1. IL-4

ABSTRACT

Bronchial epithelial cells represent the first line of defense against microorganisms and allergens in the airways and play an important role in chronic inflammatory processes such as asthma. In an experimental model, both RvD1 and AT-RvD1, lipid mediators of inflammation resolution, ameliorated some of the most important phenotypes of experimental asthma. Here, we extend these results and demonstrate the effect of AT-RvD1 on bronchial epithelial cells (BEAS-2B) stimulated with IL-4. AT-RvD1 (100 nM) decreased both CCL2 and IL-8 production, in part by decreasing STAT6 and NF-κB pathways. Furthermore, the effects of AT-RvD1 were ALX/FRP2 receptor dependent, as the antagonist of this receptor (BOC1) reversed the inhibition of these chemokines by AT-RvD1. In addition, AT-RvD1 decreased SOCS1 and increased SOCS3 expression, which play important roles in Th1 and Th17 modulation, respectively. In conclusion, AT-RvD1 demonstrated significant effects on the IL-4-induced activation of bronchial epithelial cells and consequently the potential to modulate neutrophilic and eosinophilic airway inflammation in asthma. Taken together, these findings identify AT-RvD1 as a potential pro-resolving therapeutic agent for allergic responses in the airways.

Keywords: Bronchial Epithelial Cells. AT-RvD1. IL-4

LISTA DE ABREVIATURAS E SIGLAS

EPA: Ácido Eicosapentaenóico

DHA : Ácido Docosahexaenóico

15-LO : 15-lipoxigenase

COX - Ciclooxigenase

AT-RvD1: Resolvina D1 formada pela via da aspirina

RvD1: Resolvina D1

ALX/FPR2: Receptor de Lipoxina A_4

IgE: Imunoglobulina E

IL: Interleucina

IFN: Interferon

PARs: Receptores Ativados por Proteases

CCL: Quimiocina Ligante CC

CCR: Receptor de Quimiocina Ligante CC

CXCR: Receptor de Quimiocina CxC

JAK : Janus Quinase

STAT: Sinal Transdutor Ativador da Transcrição

NF-κB: Fator Nuclear kappa B

SOCS: Supressor da Sinalização de Citocinas

SUMÁRIO

1. INTRODUÇÃO

Nas patologias inflamatórias, como as doenças das vias aéreas[1], vasculares[2] e neurológicas[3] uma resposta imunológica descontrolada promove lesão tecidual e danos irreversíveis aos órgãos. Na evolução da inflamação aguda para a crônica, uma resposta inflamatória limitada é consequência da ação de mecanismos endógenos que controlam a magnitude e a duração da resposta aguda[4]. A resolução natural da inflamação aguda é um processo ativo[5, 6] e envolve a interação entre células (hematopoiéticas e estruturais), assim como, processos celulares (apoptose, fagocitose, dentre outros)[7]. As etapas da resolução incluem: a) inibição da infiltração de polimorfonucleares (neutrófilos, eosinófilos e basófilos); b) retorno da permeabilidade vascular ao normal; c) morte de polimorfonucleares (principalmente por apoptose); d) infiltração de monócitos/macrófagos não inflamatórios; e) remoção de neutrófilos apoptóticos, microrganismos e agentes estranhos por macrófagos. Durante o processo de resolução da inflamação aguda, quatro novas famílias de mediadores lipídicos foram descobertas no local inflamatório incluindo as lipoxinas (derivadas do ácido graxo ω-6) as resolvinas, protectinas e maresinas (derivadas do ácido graxo ω-3)[8-10]. Estes mediadores, além de acelerar a resolução do processo inflamatório e a restituição da homeostasia do tecido sem a concomitante imunossupressão, estimulam também a fagocitose de macrófagos, o que difere dos anti-inflamatórios clássicos[11].

Muitas enzimas, além de metabolizar o ácido araquidônico (derivado do ácido graxo ω-6) para formar as prostaglandinas, leucotrienos e as lipoxinas. Também podem metabolizar outros ácidos graxos como os membros da família do ácido graxo ω-3, particularmente o ácido eicosapentaenóico (EPA) e o ácido docosahexaenóico (DHA), para formar potentes compostos com propriedade anti-inflamatória e pró-resolução[4]. O DHA é bastante conhecido por sua função essencial no desenvolvimento neuronal[12], além disso, a mucosa das vias aéreas de indivíduos saudáveis é enriquecida com este lipídio[13]. O DHA está incorporado nas membranas celulares e é rapidamente liberado através da ativação celular para a conversão de mediadores lipídicos locais com atividades de promover a resolução da inflamação. Desta forma estes mediadores foram denominados de “resolvinas” (formadas durante a resolução via interações célula-célula)[14]. Durante as interações entre células que contêm 15-lipoxigenase (15-LO) (por exemplo, o epitélio das vias aéreas)[15] e os leucócitos, o DHA é convertido primeiramente em protectina D1 e na presença de 5-LO dos leucócitos é convertido nas resolvinas da série D (D1-4). Além das resolvinas os seus epímeros (configuração *R* no carbono 17) também podem ser formados no local inflamatório. Por exemplo, o epímero da

resolvina D1 é denominado de AT-RvD1 (*aspirin-triggered-resolvin D1*- configuração 17 *R*) uma vez que, a sua produção endógena pode ser iniciada pela ação da aspirina (via de reações dependentes da enzima ciclooxigenase-2)[16]. No entanto, sua formação também pode ocorrer na ausência da aspirina utilizando somente substratos endógenos catalizados pelo citocromo p450[6]. Estes epímeros (configuração *R*) demonstram potentes ações anti-inflamatórias e pró-resolução equivalentes às resolvinas (configuração *S*). Além disso, os epímeros são menos inativados localmente por enzimas que as resolvinas, demonstrando assim ações mais prolongadas e protetoras no órgão[17, 18]. As resolvinas da serie E (derivadas do EPA), as resolvinas da serie D (derivadas do DHA) e seus epímeros demonstram potentes efeitos biológicos em vários modelos experimentais de inflamação, como os modelos gastrointestinais, renais, vasculares, pulmonares (como a asma), dentre outros[4,19].

Nos últimos anos foram descritos os receptores da RvD1 e do AT-RvD1: o receptor de lipoxina A4 (ALX/FRP2) e o receptor órfão GPR32, ambos receptores com sete domínios transmembranares ligados a proteína G. Estes receptores foram identificados em células epiteliais do pulmão e em macrófagos alveolares[20,21], além de outras células[22].

A asma é uma doença inflamatória das vias aéreas caracterizada pela migração e acúmulo de leucócitos, principalmente eosinófilos, hipersecreção de muco, elevada produção de imunoglobulina E (IgE) e hiperreatividade das vias aéreas. A fisiopatologia da asma é coordenada por uma resposta imunológica de células T $CD4^+$, especificamente de fenótipo Th2, com liberação de citocinas (IL-4, IL-5 e IL-13)[1]. A IL-4 é o maior fator de diferenciação da resposta imune do tipo Th2, além de bloquear a diferenciação de células do tipo Th1 pela inibição da transcrição de interferon-γ (IFN-γ)[23]. A IL-4 em associação com a IL-13 induz em células B a mudança de classe de imunoglobulinas para a IgE[24]. A maioria dos pacientes com asma apresenta sintomas intermitentes ou persistentes que são controláveis por terapias padrões incluindo agonistas β_2-adrenérgico, baixas doses de corticosteróides[25] ou inibidores da síntese de leucotrienos[26]. No entanto, alguns indivíduos asmáticos são refratários a estas terapias, ocasionando exacerbações que requerem tratamentos intensivos em consultórios médicos e hospitais. Diferente da inflamação das vias aéreas da asma estável (com predomínio de eosinófilos e linfócitos)[27], nas exacerbações da asma é observada uma resposta inflamatória neutrofílica, o qual em alguns casos é a principal célula infiltrante[28]. Durante a exacerbação da asma os neutrófilos, eosinófilos e outras células inflamatórias recrutadas para as vias aéreas tornam-se ativadas por alérgenos, liberam mediadores pró-inflamatórios e compostos como as espécies reativas de oxigênio e as proteases com potencial de danificar o epitélio pulmonar [29].

O epitélio das vias aéreas, além da função na manutenção da condução de ar para os alvéolos e proteger o pulmão contra alérgenos, patógenos e partículas inaladas do ambiente, possui também capacidade de influenciar as células dendríticas na modulação da resposta imune inicial e efetora nos processos inflamatórios[30-32]. As células epiteliais expressam receptores de reconhecimento padrão como, por exemplo, os receptores do tipo *Toll* e os receptores ativados por proteases (PARs), que reconhecem microrganismos e alérgenos respectivamente[33, 34]. A ativação desses receptores em células epiteliais induz a produção de quimiocinas, expressão de moléculas de adesão[35] e citocinas que podem influenciar a maturação de células dendríticas e a modulação do processo inflamatório[36- 38]. Hammad et al[39] demonstraram que a inflamação alérgica das vias aéreas induzidas pelo extrato de ácaro (o qual contém o lipopolissacarídeo, ligante do receptor do tipo *Toll 4*) é reduzida na ausência do receptor do tipo *Toll 4* em células estruturais (incluindo as células epiteliais brônquicas), mas não é reduzida na ausência desse receptor em células hematopoiéticas (incluindo as células dendríticas). Os resultados obtidos deste estudo sugerem uma comunicação entre essas células para o desenvolvimento da alergia e identifica uma função imune natural das células epiteliais brônquicas, as quais direcionam a resposta inflamatória alérgica das vias aéreas, via ativação de células dendríticas de mucosa. Além disso, da mesma forma que as células dendríticas residentes no pulmão, as células epiteliais brônquicas também expressam o receptor de IL-4 (IL-4R), podendo influenciar a polarização da resposta para o fenótipo Th2, assim como, as alterações epiteliais que podem ser mais pronunciadas durante o processo inflamatório[40]. Ainda, além das células do tipo Th2, a IL-4 também pode ser liberada por diversas células envolvidas na inflamação das vias aéreas como, por exemplo, os eosinófilos[41] e mastócitos[42]. A IL-4, IL-13 ou CCL11 (quimiocina que recruta seletivamente os eosinófilos) induzem em células epiteliais das vias aéreas a produção de citocinas (TGF-β2, TSLP e/ou GM-CSF) e quimiocinas (CCL2, IL-8 e/ou CCL-11)[33,43-46]. O CCL2, conhecido como proteína quimiotática de monócito-1 (MCP-1), cujo receptor é o CCR2, é um potente quimiotático de monócitos e é produzido constitutivamente após estímulos inflamatórios em diversos tipos de células incluindo as células epiteliais brônquicas. O CCL2 também está envolvido no recrutamento de basófilos, eosinófilos e células Th2[47]. Além disso, o CCL2 também está envolvido na polarização das células Th2[48] e por isso, está envolvida na patogênese de doenças inflamatórias alérgicas, como a asma. A IL-8 é uma quimiocina principalmente envolvida no recrutamento de neutrófilos e exerce este efeito através da ligação em dois receptores na superfície celular, os receptores de quimiocinas CXCR1 e CXCR2[49]. Além de neutrófilos, pode recrutar também Linfócitos B e T, células NK e células dendríticas, têm

caráter pro-inflamatório também por ativar degranulação de neutrófilos, basófilos e macrófagos[50].

Muitas moléculas de sinalização intracelular podem ser alvos para o desenvolvimento de estratégias terapêuticas na asma alérgica. A IL-4, assim como a IL-13 utiliza a cinase Janus (JAK) para iniciar a cascata de sinalização e ativar o transdutor de sinal e ativador da transcrição 6 (STAT6)[51]. Além da STAT6, outros fatores de transcrição (NF-AT, GATA-3, AP-1 e NF-κB) também têm sido implicados como alvos terapêuticos relevantes na asma[52- 55]. Esses fatores são regulados por proteínas cinases demonstrando assim, a importância dessas proteínas na expressão e ativação de mediadores inflamatórios durante a asma. Outro alvo na pesquisa da resolução do processo inflamatório é a família de proteínas envolvidas na regulação negativa da sinalização de citocinas, denominadas supressores de sinalização de citocinas (SOCS). A família das proteínas SOCS possuem oito membros a CIS e SOCS 1, 2 e 3 pertencem a um grupo por serem proteínas menores e atualmente são as mais pesquisadas e controlam a via JAK/STAT e podem influenciar diretamente em perfis de resposta Th1 e/ou Th2. Além disso, são envolvidas também com processos inflamatórios e canceres[56- 59]. No outro grupo estão as SOCS 4, 5, 6 e 7 por serem proteínas de cadeia maior. Pouco se sabe sobre suas funções, apesar delas terem inúmeros alvos[56]. As proteínas SOCS3 e SOCS5, por exemplo, são predominantes expressas em células Th2 e Th1 respectivamente, inibindo reciprocamente o processo de diferenciação Th1 e Th2[56, 60]. A SOCS3 tem papel crucial na formação fetal, ratos com deleção gênica para SOCS3 morrem ainda na forma de embrionária. Esta proteína ainda regula citocinas como IL-1, IL-4, IL-6, IL-11, IL-12, IL27 e inibe sinalização de vários receptores[56, 57]. SOCS3 também pode induzir a inibição de Th1 e Th2 por induzir a produção de IL-10 e células T reguladoras e estão sendo correlacionadas com a diferenciação de timócitos em linfócitos T γδ[58]. Camundongos com deleção gênica para SOCS1 morrem logo após o desmame devido a uma grave inflamação monocítica hepática causada pelos altos níveis de IFN-γ[56]. A SOCS1 está aumentada em células epiteliais das vias aéreas estimuladas pelo IFN-γ inibindo assim as vias de sinalização induzida pela IL-4[61].

A prevalência de asma é maior em populações com baixo consumo alimentar de ácidos graxos ω-3[62], enquanto populações com alto consumo de ácidos graxos ω-3 demonstraram menor prevalência[63]. Curiosamente, não houve evidências consistentes da melhora da asma com a suplementação experimental de ácidos graxos ω-3[64]. A falta de sucesso clínico pode estar associada com a dose, o tempo de administração e a dificuldade de tolerar a ingestão de grande quantidade de óleo de peixe por períodos de tempos

prolongados[65]. Indivíduos com asma ou com fibrose cística demonstram redução da quantidade do DHA (ácido docosahexaenóico, derivado do ácido graxo ω-3) em células epiteliais da mucosa das vias aéreas quando comparados a indivíduos saudáveis[13]. Recentemente, modelos experimentais de inflamação das vias aéreas também foram utilizados para demonstrar as ações das resolvinas (derivadas dos ácidos graxos ω-3) no processo inflamatório pulmonar[66, 67]. No modelo de asma alérgica experimental (induzido pela ovalbumina) foi demonstrado à atividade anti-inflamatória e pró-resolução da resolvina da série E[66]. Da mesma forma, a resolvina D1 e de seu epímero AT-RvD1 também demonstraram estes efeitos inibindo alguns dos mais importantes fenótipos da asma: o recrutamento de eosinófilos e a produção de citocinas Th2 no espaço broncoalveolar, a produção de muco e a hiperreatividade das vias aéreas[68]. No entanto, devido a maior resistência a inativação metabólica as ações farmacológicas do AT-RvD1 foram superiores as ações da RvD1, tanto que, somente AT-RvD1 promoveu aumento da fagocitose à alérgenos em macrófagos das vias aéreas de camundongos (linhagem AMJ2-C8)[68]. Sendo assim, pretende-se neste projeto estender estes resultados focando o efeito do AT-RvD1 em células epiteliais brônquicas humanas estimuladas com a IL-4.

2. OBJETIVOS

2.1. OBJETIVO GERAL

O presente projeto se propõe a investigar o efeito do AT-RvD1 na modulação da ativação das células epiteliais brônquicas humanas estimuladas pela IL-4.

2.2. OBJETIVOS ESPECÍFICOS

2.2.1. Determinar o efeito dose-resposta do AT-RvD1 na produção de CCL2 e IL-8 em células epiteliais brônquicas humanas estimuladas pela IL-4.

2.2.2. Determinar o efeito do AT-RvD1 na produção de CCL2 e IL-8 em células epiteliais brônquicas humanas tratadas com antagonista do recepto ALX e posteriormente estimuladas com IL-4.

2.2.3. Determinar as alterações do tratamento com AT-RvD1 nas vias de sinalização dos fatores de transcrição STAT6 e NF-κB em células epiteliais brônquicas humanas estimuladas pela IL-4.

2.2.4. Determinar o efeito do AT-RvD1 na expressão gênica de SOCS-1 e SOCS-3 em células epiteliais brônquicas humanas estimuladas pela IL-4.

3. JUSTIFICATIVA

Nas doenças das vias aéreas, as interações entre as células estruturais, como as células epiteliais brônquicas e as células hematopoiéticas, como as células dendríticas contribuem para patogênese de doenças inflamatórias das vias aéreas como a asma. Atualmente, a maioria das estratégias das pesquisas terapêuticas é destinada a um único alvo seletivo, por exemplo, atuação de anticorpos neutralizantes. Sendo assim, é pouco provável que este tipo de enfoque seja bem sucedido, uma vez que, há um grau considerável de interação entre a rede de mediadores pró-inflamatórios e os tipos celulares nas vias aéreas, como ocorre na asma. Além disso, as terapias atuais para asma e outras doenças das vias aéreas não são direcionadas para promover a resolução da inflamação pulmonar. A resolução da inflamação é um processo ativo e envolve a produção e ação de mediadores lipídicos locais como as lipoxinas (derivadas do ácido graxo essencial ω-6) e as resolvinas (derivadas do ácido graxo essencial ω-3) que aceleram o término da inflamação, estimulam a fagocitose em macrófagos, assim como, à restituição da homeostasia do tecido. De interesse, indivíduos com asma demonstram redução da quantidade do DHA (ácido docosahexaenóico, derivado do ácido graxo ω-3) em células epiteliais da mucosa das vias aéreas quando comparados a indivíduos saudáveis. A resolvina D1 e seu epímero AT-RvD1 (ambos derivados do DHA) demonstram potentes atividade anti-inflamatória e pró-resolução acelerando a resolução do processo inflamatório. No modelo de asma alérgica experimental (induzido pela ovalbumina) em camundongos, a resolvina D1 e principalmente o AT-RvD1 demonstraram potentes efeitos pró-resolução, reduzindo o processo inflamatório eosinofílico pulmonar, a produção de citocinas Th2, a hiperreatividade das vias aéreas e a produção de muco. Desta forma, o presente projeto pretende estender estes resultados avaliando o efeito do AT-RvD1 em células epiteliais brônquicas humanas estimuladas com a IL-4, podendo representar uma abordagem terapêutica alternativa para o controle das respostas inflamatórias das vias aéreas.

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The role of aspirin-triggered RvD1 (AT-RvD1) in bronchial epithelial cells stimulated with IL-4

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Abstract

Bronchial epithelial cells represent the first line of defense against microorganisms and allergens in the airways and play an important role in chronic inflammatory processes such as asthma. In an experimental model, both RvD1 and AT-RvD1, lipid mediators of inflammation resolution, ameliorated some of the most important phenotypes of experimental asthma. Here, we extend these results and demonstrate the effect of AT-RvD1 on bronchial epithelial cells (BEAS-2B) stimulated with IL-4. AT-RvD1 (100 nM) decreased both CCL2 and IL-8 production, in part by decreasing STAT6 and NF-κB pathways. Furthermore, the effects of AT-RvD1 were ALX/FRP2 receptor dependent, as the antagonist of this receptor (BOC1) reversed the inhibition of these chemokines by AT-RvD1. In addition, AT-RvD1 decreased SOCS1 and increased SOCS3 expression, which play important roles in Th1 and Th17 modulation, respectively. In conclusion, AT-RvD1 demonstrated significant effects on the IL-4-induced activation of bronchial epithelial cells and consequently the potential to modulate neutrophilic and eosinophilic airway inflammation in asthma. Taken together, these findings suggested AT-RvD1 as a potential pro-resolving therapeutic agent for allergic responses in the airways.

Keywords: Bronchial Epithelial Cells. AT-RvD1. IL-4

Introduction

Asthma is an inflammatory disease of the airways characterized by the migration and accumulation of leukocytes, particularly eosinophils, mucus hypersecretion and bronchial hyperreactivity. The pathophysiology of asthma is coordinated by the immune response of $CD4^+$ T cells, specifically the Th2 phenotype. IL-4 is the major cytokine involved in the Th2 immune response. IL-4 uses Janus kinases (JAKs) to initiate the signaling cascade and activate signal transducer and activator of transcription 6 (STAT6), consequently modulating allergic airway inflammation in asthma and other diseases [2]. Most patients with asthma have symptoms that are readily controllable by standard asthma therapies, including β2-adrenergic agonists, low doses of inhaled corticosteroids or leukotriene modifiers [1]. However, 5–10% of asthmatic individuals have poorly controlled disease with frequent exacerbations or symptoms that are refractory to current therapy [3]. Th1 and Th17 cells promote neutrophil recruitment and have been associated with both severe and steroid-resistant asthma [4].

Bronchial epithelial cells are involved in the homeostasis and coordination of immune responses in the airways and represent the first line of defense against microorganisms and allergens in the lungs [5, 6]. These cells express pattern recognition receptors, such as Toll-like receptors (TLR), and protease-activated receptors (PARs), which recognize microorganisms and allergens, respectively [7, 8]. The activation of these receptors on epithelial cells induces the production of chemokines and the expression of adhesion molecules and cytokines [9, 10] that can influence dendritic cell maturation, T cell differentiation and airway inflammation modulation [11- 14]. Bronchial epithelial cells also express the receptor for IL-4 (IL-4RA), and the activation of these cells by IL-4 induces, among other inflammatory parameters [15], the production of chemokines (for example CCL2, IL-8 and/or CCL-11) [7, 13, 14, 16, 17], which modulate leukocyte traffic and consequently airway inflammation in asthma.

During inflammation, the essential omega-3 fatty acid docosahexaenoic acid (DHA; C22:6) is available for enzymatic transformation into several anti-inflammatory and pro-resolving mediators, including the class of molecules termed resolvins [18]. Resolvin and its epimer, Aspirin-Triggered-Resolvin D1 (AT-RvD1, R configuration at carbon 17), are enzymatically derived from DHA and demonstrate anti-inflammatory and pro-resolving effects in several experimental models, including in the airways in acute lung injury [19] and

experimental airway allergic inflammation induced by ovalbumin [20] in mice. In this study, we investigated the role of AT-RvD1 on bronchial epithelial cells stimulated with IL-4.

Materials and Methods

Bronchial epithelial cells

The human bronchial epithelial cell line BEAS-2B (ATCC, Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (DMEM-F12/Gibco-Life Technologies, Carlsbad, Calif., USA) supplemented with 10% fetal bovine serum (Gibco-Life Technologies) and 1% penicillin + streptomycin (Gibco-Life Technologies, Carlsbad, Calif., USA) and incubated at 37 °C in a humidified atmosphere with 5% CO_2 and 95% ambient air.

Stimulus and treatment

AT-RvD1 was donated by Dr David Bruce Levy of the Harvard Medical School. BEAS-2B (4×10^4 cell/mL) cells were cultivated in 96-well plates and treated with AT-RvD1 (1-100 nM) or vehicle (absolute alcohol) for 30 minutes prior to IL-4 (25-100 ng/mL) stimulation. The use of BOC1 (10 μM), an ALX receptor antagonist, followed the same experimental procedure described above but was added 15 min before treatment with AT-RvD1 [21].

CCL2 and IL-8 production in the supernatant of cells treated with AT-RvD1

The supernatant was collected at 24 h after IL-4 stimulation, and the CCL2 and IL-8 concentrations were measured by enzyme-linked immunosorbent assays (ELISA) according to the manufacturers' instructions (BD Pharmingen,San Diego, Calif., USA).

Expression of NF-κB and STAT6 in cells treated with AT-RvD1

The effect of AT-RvD1 on the NF-κB and STAT6 pathways was assessed by cytometry according to Cao et al. [23]. Briefly, 15 min after IL-4 stimulation, cells were fixed with pre-warmed BD Cytofix Buffer (4% paraformaldehyde) for 10 min at 37 °C. After centrifugation, the cells were permeabilized in ice-cold methanol for 30 min and then stained with mouse monoclonal antibodies against anti-NF-κB (BD Biosciences Pharmingen-Phosflow, USA), anti-STAT6 (BD Biosciences Pharmingen- Phosflow, USA) or their corresponding mouse IgG2b isotype (BD Biosciences Pharmingen- Phosflow, USA) for 60

min followed by an FITC- or PE-conjugated goat anti-mouse IgG2b secondary antibody for another 45 min at 10 °C in the dark. The cells were then washed, resuspended and subjected to analysis. The expression of intracellular phosphorylated signaling molecules in 50,000 viable cells was analyzed by flow cytometry (FACSCalibur; BD Biosciences Pharmingen).

The results for phosphorylated NF-κB and STAT6 are shown as a percentage of fluorescence and are expressed as the arithmetic mean.

SOCS1 and SOCS3 expression

At 1 h after IL-4 stimulation, total RNA was extracted from cells using Pure Linkr RNA Mini Kit (Life Technologies, Carlsbad, Calif., USA). cDNA was synthesized by reverse transcription (RT) from total RNA with SuperScript VILO MasterMix (Invitrogen), Carlsbad, Calif., USA) according to the manufacturer's instructions. Duplicate qPCR reactions were performed with primers for SOCS1 (Forward: 5′-TTTT TCGCCCTTAGCGTGA-3′, Reverse: 5′-AGCAGCTCGAAGAGGCAGTC-3′) and SOCS3 (Forward: 5′-TGAGCGCGGCTACAGCTT-3′, Reverse: 5′-TCCTTAATGTCACGCACGATTT-3′) and control GAPDH (Forward: 5′-CCACCCATGGCAAATTCC-3′, Reverse: 5′-TCGCTCCTGGAAGATGGTG-3′) (Life Technologies) using cDNA-specific TaqMan Gene Expression Assays with an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). In each 5-μL TaqMan reaction, cDNA (corresponding to 100 ng reverse transcribed RNA) was mixed with 0.25 μL TaqMan Gene Expression Assay, 2.5 μL TaqMan Universal PCR Master Mix (Applied Biosystems) and 1.25 μL H_2O. The PCR conditions were 95 °C for 20 s, followed by 50 cycles at 95 °C for 3 s and 60 °C for 30 s. Negative control reactions with no cDNA present and three inter-run calibrator samples were included on each assay plate.

The Ct (cycle threshold) values for SOCS1 and SOCS3 mRNA were normalized to GAPDH to provide the delta Ct values. The relative mRNA expression was determined using the Livak method (the $2^{-\Delta\Delta CT}$ method for real-time PCR).

Statistical analysis

The results were expressed as the mean ± standard error of the mean. An evaluation of the results was performed by an analysis of variance (ANOVA) followed by a Tukey post-test among the means using GraphPad PRISM (Version 6.0; GraphPad Software Inc., San Diego, CA, USA). P values less than 0.05 were considered statistically significant.

Results

Dose response effect of IL-4-induced CCL2 production

First, we evaluated the dose-response effect of IL-4 on CCL2 production by bronchial epithelial cells. The results showed a dose response with all doses (25-100 ng/mL) that significantly increased the production of CCL2 in bronchial epithelial cells when compared to non-stimulated cells (control). The dose of 25 ng/mL was chosen to for use in the ensuing experiments.

AT-RvD1 reduces the concentration of chemokines

The activation of bronchial epithelial cells induces, among others, the release of chemokines [7, 13, 14, 16, 17]. Therefore, we evaluated the role of AT-RvD1 in CCL2 and IL-8 production in bronchial epithelial cells stimulated with IL-4. Our results showed that IL-4 stimulation (25 ng/mL for 24 h) induced a prominent increase in CCL2 and IL-8 concentrations compared to non-stimulated cells (control group; Figure 2A, B, respectively). At all doses (1-100 nM), AT-RvD1 significantly reduced CCL-2 (Figure 2A) and IL-8 (Figure 2B) production when compared with the cells treated with IL-4, whereas no significant difference was observed in cells treated with vehicle compared to cells treated with IL-4 (data not shown).

The inhibitory effect of AT-RvD1 on chemokine production is ALX/FPR2 receptor dependent

The results presented above demonstrated that AT-RvD1 modulated the chemokine production induced by IL-4 in bronchial epithelial cells. Recent findings have shown that AT-RvD1 exerts part of its pro-resolving effects via interactions with the ALX/FPR2 receptor present on bronchial epithelial cells [24, 25]. Accordingly, we verified whether the ALX/FPR2-selective antagonist, BOC1, is capable of blocking the effects of AT-RvD1 on chemokine release by BEAS-2B cells after IL-4 stimulation. As demonstrated above, IL-4 stimulated CCL-2 and IL-8 production, and AT-RvD1 reduced both (Figure 3A, B, respectively). Interesting, BOC1 significantly reversed the inhibitory effect of AT-RvD1 on CCL2 (Figure 3A) and IL-8 (Figure 3B) production. No significant difference was observed in cells stimulated with IL-4 and treated with BOC1 (10 μM) when compared with cells treated with IL-4 (data not shown).

AT-RvD1 downregulates the phosphorylation of transcription factors

We next evaluated the effect of AT-RvD1 on the STAT6 and NF-κB pathways. Signal transducer and activator of transcription 6 (STAT6) and nuclear factor kappa B (NF-κB) have been demonstrated to regulate many pathologic features of asthma, and both are activated by IL-4 [26, 27]. As shown in Figures 4A and 4B, IL-4 induced the significant phosphorylation of NF-κB and STAT6 compared to the control. Of note, AT-RvD1 significantly reduced the activation of NF-κB (Figure 4A) and STAT6 (Figure 4B) when compared to cells treated only with IL-4.

AT-RvD1 acts in modulating the expression of SOCS1 and SOCS3

As the SOCS family is known to inhibit STAT signaling, we next evaluated the effect of AT-RvD1 on SOCS1 and SOCS3. In these experiments, the dose of 50 ng/mL was used for stimulation because it demonstrated better results than the dose of 25 ng/mL (data not shown); this is in agreement with previous results [28]. The results showed that AT-RvD1 significantly reduced the expression of SOCS1 when compared with cells stimulated with IL-4 (Figure 5A); moreover, AT-RvD1 significantly increased SOCS3 expression (Figure 5B).

Discussion

IL-4 coordinates the Th2 immune response, which is associated with the pathophysiology of asthma. Interesting lipids mediators of resolution, such as AT-RvD1, demonstrate significant anti-inflammatory and pro-resolution effects in several experimental models, including in experimental allergic airway inflammation induced by ovalbumin in mice, an “asthma-like model”. Here, we demonstrate for the first time the effect of AT-RvD1 in bronchial epithelial cells stimulated with IL-4. AT-RvD1 significantly reduced CCL2 and IL-8 production when compared to cells treated with IL-4. These effects are ALX/FPR2 receptor dependent and in part associated with the downregulation of STAT6 and NF-κB pathways by AT-RvD1. Therefore, AT-RvD1 decreased SOCS1 and increased SOCS3 expression, which play critical roles in lymphocyte differentiation, maturation and function. These results suggest that AT-RvD1 can modulate the innate and adaptive immune responses of asthma and other diseases, but further studies are needed for confirmation.

IL-4 is the major factor in the differentiation of the Th2-type immune response and blocks the differentiation of Th1 cells by inhibiting interferon-γ (IFN-γ) [22]. Bronchial

epithelial cells express IL-4 receptor (IL-4R), and IL-4 induces the production of chemokines such as CCL2 and IL-8, among other inflammatory parameters, in airway epithelial cells [7, 23, 24- 26]. CCL2, also known as monocyte chemotactic protein-1 (MCP-1), is a potent chemotactic for monocytes and is produced constitutively or after stimulation in various cell types, including bronchial epithelial cells [27]. Indeed, CCL2 is chemotactic to monocytes/macrophages, basophils, eosinophils and Th2 cells. In addition, CCL2 is involved in the polarization of Th2 cells and therefore is associated with the pathogenesis of allergic inflammatory diseases, such as asthma [28- 30]. Most patients with asthma have symptoms that are readily controllable by standard asthma therapies [1]. However, 5–10% of asthmatic individuals have poorly controlled disease with frequent exacerbations or symptoms that are refractory to current therapy [1, 3]. Distinct from the airway inflammation of stable asthma, which has been attributed to ongoing Th2-mediated inflammation, with a predominance of eosinophils and lymphocytes, there is increasing evidence to suggest that the increased inflammation in asthma exacerbation is under different regulation [31]. In addition to the eosinophils and lymphocytes that predominate in Th2-type inflammation, asthma exacerbations are notable for a neutrophil-enriched inflammatory response, which in some cases is the principal cellular infiltrate. Neutrophils are the major inflammatory cell in the airways of individuals dying within several hours of an asthma attack and are found in increasing numbers in patients dying of status asthmaticus [32]. Their numbers are increased in the sputum and bronchial washings of patients intubated for status asthmaticus [33- 35]. There are several chemoattractants for neutrophils, such as the IL-8 [36] and the lipid mediator leukotriene B4 (LTB_4) [37]. IL-8 is a chemokine that is mainly involved in the recruitment of neutrophils and exerts this effect by binding to two cell surface receptors, chemokine receptors CXCR1 and CXCR2 [36]. In addition to neutrophils, IL-8 may also recruit B and T lymphocytes, NK cells and dendritic cells [38- 40]. In addition, IL-8 induces the degranulation of neutrophils, basophils and macrophages [41].

LTB_4 and pro-inflammatory lipids mediators are well known to play important roles in asthma [42], but not all lipid mediators are associated with inflammation. For example, lipoxins and resolvins and their epimers are lipids mediators generated during the resolution phase and demonstrate significant anti-inflammatory and pro-resolution effects [43, 44]. In a previous study, our group demonstrated that AT-RvD1 markedly decreased airway eosinophilia and mucus metaplasia, in part by decreasing IL-5 and IkBα degradation in allergen-sensitized and challenged mice. In addition, AT-RvD1 significantly enhanced the macrophage phagocytosis of IgG-OVA-coated beads in vitro and in vivo, a new pro-resolving

mechanism for the clearance of allergens from the airways [20]. In the present work, AT-RvD1 significantly reduced CCL2 and IL-8 production in bronchial epithelial cells when compared to cells only stimulated with IL-4, demonstrating the potential to reduce both neutrophilic and eosinophilic inflammation in asthma.

AT-RvD1 can serve as an agonist for the ALX/FPR2 receptor to transduce, in part, its pro-resolution action [45- 48]. The ALX/FPR2 receptor is broadly expressed in airway epithelial cells and alveolar macrophages and is dynamically regulated during allergic airway responses, leading to decreased receptor abundance [20, 49]. These changes are similar to those observed in human asthma [50]. We demonstrated that the inhibitory effect of AT-RvD1 on chemokine production by BEAS-2B cells stimulated with IL-4 is ALX/FPR2 receptor dependent, as the antagonist of this receptor reversed its effects.

Several transcription factors have also been implicated in the inflammatory process of asthma, including STAT6 and NF-κB [51- 54]. STAT6 has been demonstrated to regulate many pathologic features of lung inflammatory responses, including Th2 cell differentiation, airway eosinophilia, epithelial mucus production and smooth muscle changes [55, 56]. NF-κB controls the expression of some relevant genes encoding chemokines (CCL11, IL-8), cytokines (IL-5) and adhesion molecules (P-selectin) involved in airway eosinophilic and/or neutrophilic inflammation [57- 60]. AT-RvD1 demonstrated a significant effect in reducing the phosphorylation of both STAT6 and NF-κB in BEAS-2B cells stimulated with IL-4. The downregulation of NF-κB by AT-RvD1 is in agreement with a previous study by our group [19, 20]; however, the present study is the first to demonstrate STAT6 modulation by AT-RvD1.

The JAK/STAT pathways have a pivotal role in the differentiation of helper T cells. The SOCS family, induced by cytokine stimulation, inhibits STAT signaling [59, 60]. SOCS1 has been shown to be a critical negative regulator of IFN-γ and consequently of the Th1 immune response [61]. SOCS3 promotes Th2 differentiation by blocking STAT4 signaling. However, the removal of SOCS3 from T cells inhibits Th1 and Th2 responses [62, 63]. In addition, SOCS3 blocks STAT3 signaling and consequently inhibits Th17 polarization [64]. IL-17 plays an important role in the development of severe asthma due to induced neutrophilic inflammation [65, 66]. Therefore, the inhibition of Th17 cell differentiation or IL-17 production could be beneficial for controlling severe asthma. SOCS plays an important role in the modulation of inflammation and is critical due to its broad spectrum of signaling events. However, the role of SOCS in bronchial epithelial cells is not clear. In our

experiments, IL-4 increased both SOCS1 and SOCS3 expression, with SOCS1 showing higher expression, whereas AT-RvD1 decreased SOCS1 and increased SOCS3 expression compared to cells treated only with IL-4. Thus, it is possible that SOCS1 inhibition and SOCS3 induction, involved in Th1 and Th17 immune responses, respectively, by AT-RvD1 may also negatively regulate JAK/STAT signaling pathways in BEAS-2B cells. However, additional studies are needed to test this hypothesis. Taken together, the results suggested that AT-RvD1 has a potential to modulate the immune response in both stable and severe asthma.

Conclusion

In conclusion, our results demonstrate that AT-RvD1 modulates the activation of bronchial epithelial cells induced by IL-4. AT-RvD1, via the ALX/FPR2 receptor, decreased CCL2 and IL-8 production and downregulated the NF-κB and STAT6 pathways. In addition, AT-RvD1 decreased SOCS1 and increased SOCS3 expression. Together, these results suggest that AT-RvD1 has the potential to control airway inflammation in asthma and c represent a new concept in the development of drugs for use in treating asthma and other inflammatory airway diseases.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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Figures

Figure 1

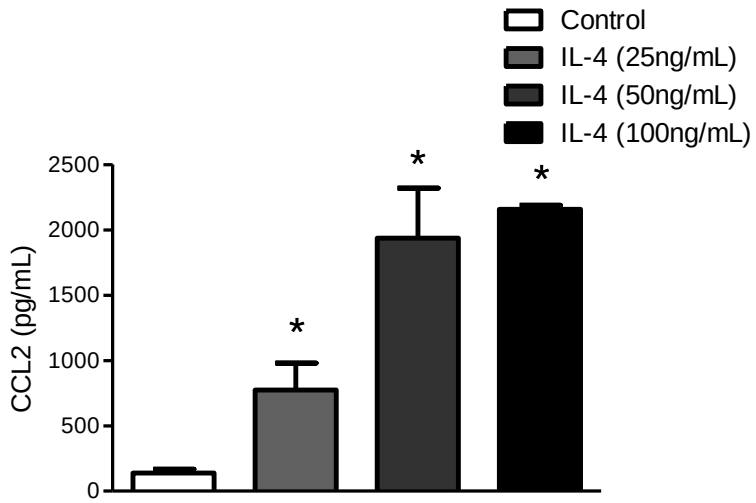

Figure 1- IL-4 (25-100 ng/mL) significantly increases the production of CCL2 in the supernatant of BEAS-2B cells. The analyses were performed at 24 h after stimulation; the CCL2 concentration was determined using an ELISA kit. The data are reported as the means ± SEM (n= 6/group). *$p < 0.05$ versus control group.

Figure 2

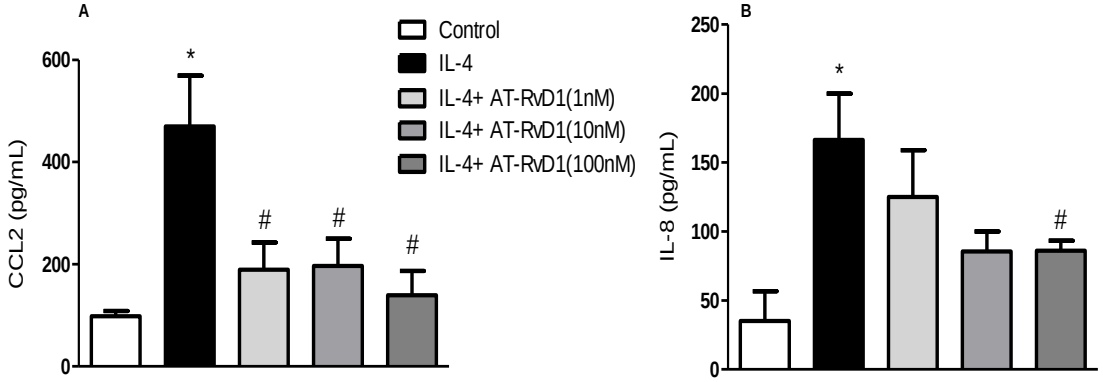

Figure 2- AT-RvD1 reduced the production of CCL2 (A) and IL-8 (B) in bronchial epithelial cells stimulated with IL-4. BEAS-2B cells were stimulated with IL-4 (25 ng/mL) in the presence or absence of AT-RvD1 (1-100 nM) for 24 h, and the culture supernatants were analyzed to determine CCL2 and IL-8 concentrations using an ELISA kit. The data are reported as the means ± SEM (n= 7/group). *$p < 0.05$ versus control group, #$p < 0.05$ versus IL-4-treated group.

Figure 3

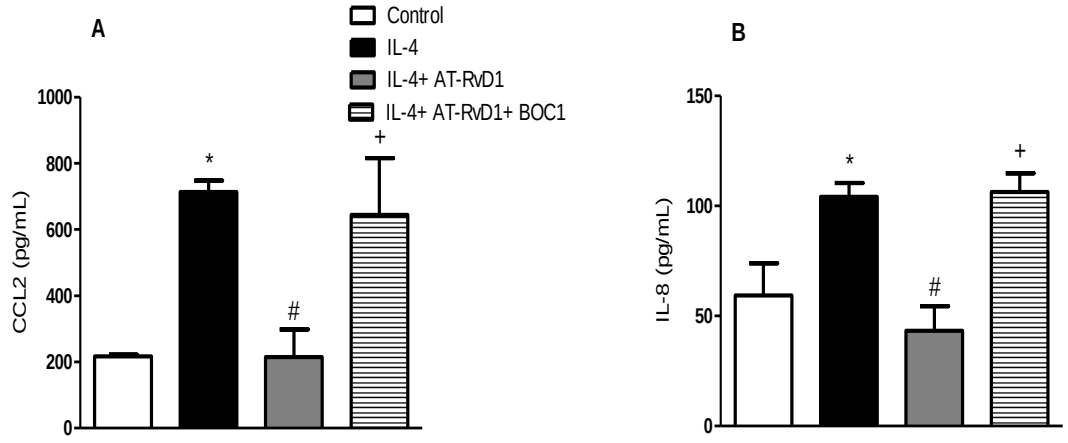

Figure 3- AT-RvD1 reduces CCL2 (A) and IL-8 (B) production in BEAS-2B cells stimulated with IL-4 through ALX/FPR2 receptor activation. BEAS-2B cells were stimulated with IL-4 (25 ng/mL) in the presence or absence of AT-RvD1 (100 nM) or in combination with BOC1, an ALX selective antagonist (10 μM), for 24 h; the culture supernatants were analyzed for CCL2 and IL-8 concentrations using an ELISA kit. The data are reported as the means ± SEM (n= 7/group). * $p < 0.05$ versus control group, # $p < 0.05$ versus IL-4-treated group, + $p < 0.05$ versus IL-4 + AT-RvD1(100 nM)-treated group.

Figure 4

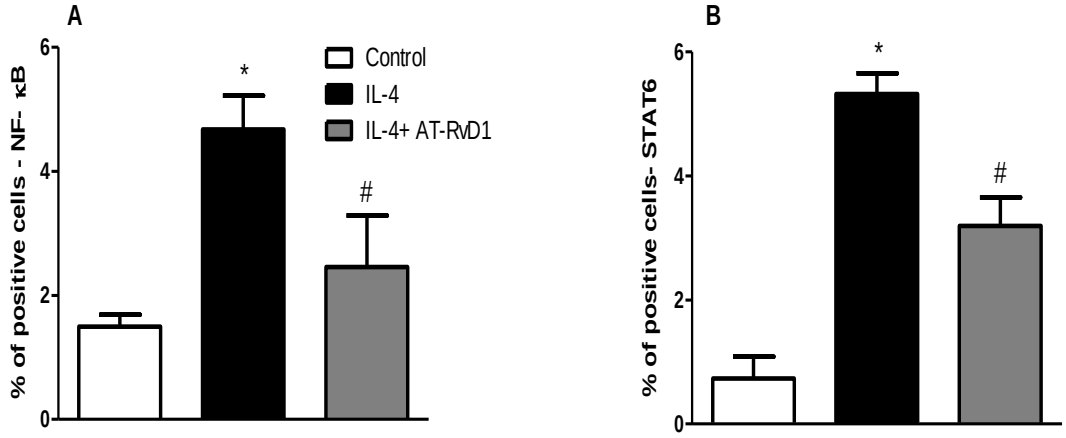

Figure 4- AT-RvD1 downregulates the NF-κB (A) and STAT6 (B) pathways in bronchial epithelial cells stimulated with IL-4. BEAS-2B cells were stimulated with IL-4 (25 ng/mL) for 15 min in the presence or absence of AT-RvD1 (100 nM). The results are expressed as the arithmetic mean plus SEM from three independent experiments. *$p < 0.05$ versus control group, #$p < 0.05$ versus IL-4-treated group.

Figure 5

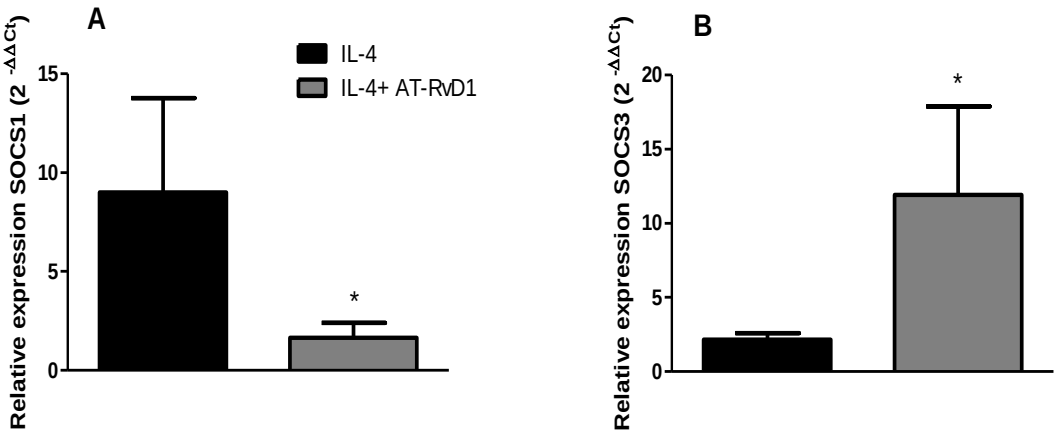

Figure 5- AT-RvD1 decreases SOCS1 (A) and increases SOCS3 (B) expression in bronchial epithelial cells stimulated with IL-4 (50 ng/mL). BEAS-2B cells were treated with AT-RvD1 (100 nM) 30 minutes before IL-4 stimulation. At 1 hour after stimulation, SOCS expression was quantified by qPCR. The results are expressed as the mean ± EPM with n= 4. *p <0.05 versus IL-4.

COMPROVANTE DE SUBMISSÃO

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236250.v1 (Research Article)	The role of aspirin-triggered RvD1 (AT-RvD1) in bronchial epithelial cells stimulated with IL-4 Jhony Robison de Oliveira, Daniely Cornélio Favarin, Tanaka Sarah Cristina Sato Vaz, Balarin Marly Ap. Spadotto, David Nascimento Silva Teixeira, B. Levy, and Alexandre de Paula Rogerio		Under Review

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Daniely Cornelio Favarin

- Ajudou na padronização e realização do qPCR

Sarah C. Sato Vaz Tanaka

- Alem de orientar na padronização e realização do qPCR, também nos ajudou na descrição da metodologia do teste

Dra. Marly Ap. Spadotto Balarin

- Disponibilizou seu laboratório e os aparelhos para realização do qPCR

Dr. David Nascimento Silva Teixeira

- Disponibilizou seu laboratório alem ainda dos aparelhos e alguns reagentes para realização de nosso projeto

Dr. Bruce David Levy

- Cedeu o mediador lipídico AT-RvD1 para a realização dos experimentos.