

Ministério da Saúde

**FIOCRUZ**  
**Fundação Oswaldo Cruz**

**INSTITUTO OSWALDO CRUZ**  
**Pós-Graduação em Biologia Celular e Molecular**

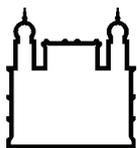
*FABIO PEREIRA MESQUITA DOS SANTOS*

PGD<sub>2</sub> e INFLAMAÇÃO EOSINOFÍLICA: MECANISMOS MOLECULARES E POTENCIAL COMO ALVO TERAPÊUTICO

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Celular e Molecular.

**Orientador (es):** Dra. Christianne Bandeira de Melo  
Dr. Bruno Lourenço Diaz

**RIO DE JANEIRO**  
2011



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POTENCIAL COMO ALVO TERAPÊUTICO**

**ORIENTADORES:** Dra. Christianne Bandeira de Melo  
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**Aprovada em: 17/ 03 /2011**

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Rio de Janeiro, 17 de março de 2011.

***A Deus por tudo que tem feito em minha vida.***

## **AGRADECIMENTOS:**

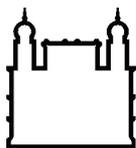
- Aos meus orientadores Christianne Bandeira de Melo e Bruno Lourenço Diaz, pela orientação durante todo esse período e confiança em mim depositada. Chris, obrigado por todas as conversas, por sempre demonstrar energia e empolgação nas discussões sobre experimentos. Tenho muito a agradecer a você, pois acreditou em mim desde o início e, ao longo dessa caminhada, desenvolvi grande admiração pelo seu espírito de trabalho. Agradeço igualmente ao Bruno. A vocês a minha admiração, gratidão e carinho.
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*"Mas esforçai-vos, e não desfaleçam as vossas mãos,  
porque a vossa obra tem uma recompensa."*

II Cr 15:7

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### PGD<sub>2</sub> E INFLAMAÇÃO EOSINOFÍLICA: MECANISMOS MOLECULARES E POTENCIAL COMO ALVO TERAPÊUTICO

#### RESUMO

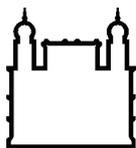
#### TESE DE DOUTORADO

Fabio Pereira Mesquita dos Santos

Durante a resposta alérgica, dentre os vários mediadores inflamatórios de natureza lipídica, a prostaglandina D<sub>2</sub> (PGD<sub>2</sub>) é considerada um mediador-chave. Em adição aos seus conhecidos efeitos quimiotáticos para eosinófilos, recentemente, foi descrito que a PGD<sub>2</sub> é também capaz de promover a ativação dos eosinófilos, induzindo a biogênese de corpúsculos lipídicos e a síntese de leucotrieno C<sub>4</sub> (LTC<sub>4</sub>) nessas organelas recém-formadas. Esses efeitos são atribuídos a ação da PGD<sub>2</sub> sobre seus 2 receptores – DP1 e DP2 – os quais encontram-se expressos de maneira constitutiva na membrana dos eosinófilos. Então, o objetivo principal do estudo foi identificar o receptor específico da PGD<sub>2</sub> envolvido no mecanismo de síntese de LTC<sub>4</sub> por eosinófilos estimulados com PGD<sub>2</sub>.

*In vivo*, num modelo murino de pleurisia alérgica e induzida por PGD<sub>2</sub>, a utilização dos antagonistas seletivos do receptor DP1 (BW A868c) ou do receptor DP2 (CAY10471) inibiu a síntese de LTC<sub>4</sub> nessas respostas inflamatórias. No entanto, somente BWA868C foi capaz de inibir a biogênese de corpúsculos lipídicos nos eosinófilos recrutados para o sítio inflamatório; enquanto que o tratamento com o CAY10471, diminuiu o número de eosinófilos infiltrantes na cavidade pleural, mas não inibiu a biogênese de corpúsculos lipídicos nessas poucas células recrutadas. *In vitro*, eosinófilos humanos purificados estimulados com PGD<sub>2</sub> tiveram a síntese de LTC<sub>4</sub> inibida tanto pelo pré-tratamento com BWA868c, quanto pelo pré-tratamento com CAY10471. Além disso, a ativação do receptor DP1, com seu agonista seletivo (BW245c) e a ativação do receptor DP2 com o agonista seletivo do receptor DP2 (DK-PGD<sub>2</sub>) corroborou a observação de que no processo de síntese de LTC<sub>4</sub> nos eosinófilos, ambos os receptores são necessários, pois somente quando ambos os receptores foram ativados simultaneamente foi observada síntese de LTC<sub>4</sub> nos corpúsculos lipídicos recém-formados (*Eicosacell*). Além disso, caracterizamos que uma das vias de sinalização intracelular envolvida na formação de corpúsculos lipídicos é dependente da ativação de proteína quinase A (PKA).

Em um outro grupo de ensaios, investigamos a PGD<sub>2</sub> como potencial alvo terapêutico em doenças alérgicas. Recentemente, foi descrito que o extrato aquoso de *C. sympodialis* e a warafteína (alcalóide isolado) têm propriedades antialérgicas, visto que não somente reduzem a eosinofilia, mas também, a biogênese de corpúsculos lipídicos, assim como a produção de leucotrienos cisteinados. Dessa forma, aqui demonstramos que os pré-tratamentos tanto com o extrato quanto com o alcalóide isolado, foram capazes de inibir a produção de PGD<sub>2</sub> ocorrida durante a resposta alérgica. *In vitro*, embora a warafteína não tenha inibido a biogênese de corpúsculos lipídicos em eosinófilos induzida por PGD<sub>2</sub>, observamos que é capaz de bloquear a liberação de PGD<sub>2</sub> por mastócitos ativados – mas, não a produção de PGE<sub>2</sub> por macrófagos ativados com A23187 – demonstrando que o mecanismo de ação dos seus efeitos antiinflamatórios não parecem envolver antagonismo de receptores em eosinófilos, e sim inibição da síntese da PGD<sub>2</sub> em sítios alérgicos.



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#### ABSTRACT

#### TESE DE DOUTORADO

**Fabio Pereira Mesquita dos Santos**

During allergic response, among several lipid mediators produced, prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) has emerged as key mediator. In addition to its known eosinophilic effects, recently PGD<sub>2</sub> was described to be able to promote eosinophil activation, inducing lipid bodies biogenesis and LTC<sub>4</sub> synthesis within these newly formed organelles. These effects are attributed to the action of PGD<sub>2</sub> on its 2 receptors – DP1 e DP2 – which are expressed constitutively on eosinophil cell membranes. So, the main objective of this study was to identify the PGD<sub>2</sub> specific receptor involved in LTC<sub>4</sub> synthesis mechanism by stimulated eosinophils with PGD<sub>2</sub>.

*In vivo*, in a murine allergic model of pleurisy and in a pleurisy induced by PGD<sub>2</sub>, the use of selective DP1 receptor (BWA868c) and DP2 receptor (CAY10471) antagonists showed us that both treatments inhibited LTC<sub>4</sub> synthesis during these inflammatory responses. However, only BWA868C treatment was able to inhibit lipid bodies biogenesis within recruited eosinophils to the inflammatory sites, while CAY10471, decreased the number of infiltrated eosinophils in the pleural cavity, but did not inhibit lipid bodies biogenesis within these low number of recruited cells. *In vitro*, pre-treatment with BWA868c or CAY10471 inhibited LTC<sub>4</sub> synthesis by human eosinophils stimulated with PGD<sub>2</sub>. Moreover, the activation of DP1 receptor with its selective agonist (BW245c) and DP2 activation with DP2 selective agonist (DK-PGD<sub>2</sub>) reinforced the observation that during LTC<sub>4</sub> synthesis within eosinophils, activation of both receptors are necessary, because only simultaneous activation of DP1 and DP2, induced LTC<sub>4</sub> synthesis within eosinophilic lipid bodies (Eicosacell). Moreover, we observed that the pathway of cellular signaling involved on lipid bodies biogenesis induced by DP1 activation is dependent on protein kinase A (PKA).

In another set of experiments, we investigated PGD<sub>2</sub> as a therapeutical target of allergic diseases. Recently, it was described that aqueous extract of *C.symphodialis* and warafteine (isolated alkaloid) have antiallergic properties, because of its effects on the reduction of eosinophils recruitment, lipid bodies biogenesis and cysteinyl leukotrienes synthesis. Here, we demonstrated that pre-treatments with extract and its alkaloid were able to inhibit PGD<sub>2</sub> production during allergic response. *In vitro*, warafteine did not inhibit eosinophil lipid bodies biogenesis induced by PGD<sub>2</sub>, but it was capable to inhibit PGD<sub>2</sub> release by activated mast cells – otherwise fail to blockade PGE<sub>2</sub> production by A23187-activated macrophages – suggesting that the action mechanism of its antiinflammatory effects could occur through PGD<sub>2</sub> synthesis inhibition in allergic sites.

## LISTA DE ABREVIATURAS

- . A23187..... Ionóforo de cálcio; “*Calcium ionophore*”
- . AA..... Ácido Araquidônico; “*Arachidonic Acid*”
- . ADRP..... Proteína Relacionada à Diferenciação de Adipócito; “*Adipose Differentiation-Related Protein*”
- . AMPc..... Mono-Fosfato Cíclico de Adenosina; “*Cyclic Adenosine Monophosphate*”
- . AT-56..... Antagonista seletivo da L-PGD<sub>2</sub> sintase; “*L-PGD<sub>2</sub> sintase selective antagonist*”
- . BAL..... Lavado Bronco-Alveolar; “*Bronchoalveolar Lavage*”
- . BAPTA-AM..... Quelante de cálcio intracelular
- . BAYu9773..... Antagonista de receptor CysLT; “*CysLT receptor antagonist*”
- . BW 245c..... Agonista seletivo do receptor DP1; “*DP1 receptor selective agonist*”
- . BWA868c..... Antagonista seletivo do receptor DP1; “*DP1 receptor selective antagonist*”.
- . CAY 10471..... Antagonista seletivo do receptor DP2; “*DP2 selective antagonist*”
- . C5a/C3a..... Componentes do sistema de complemento
- . Ca<sup>+2</sup>..... Íon Cálcio
- . CCR()  
..... “*CC Chemokine Receptor ()*”
- . CCL()  
..... “*CC chemokine ligands ()*”
- . CD()  
..... “*Cluster of Differentiation*”
- . CRTH2..... “*Chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes*”
- . cisLTs..... Leucotrienos Cisteinados; “*Cysteinyl Leukotrienes*”
- . CLC-P..... “*Charcot-Leyden Cristal Protein*”
- . cPLA<sub>2</sub>..... Fosfolipase A<sub>2</sub> citosólica; “*Cytosolic phospholipase A<sub>2</sub>*”
- . CysLT..... Receptor de leucotrieno cisteinado; “*cysteinyl leukotriene receptor*”
- . COX..... Ciclooxygenase; “*Cyclooxygenase*”
- . db-AMPC..... Análogo estrutural do AMPc; “*dibutyl-AMPC*”
- . DP1..... “*D prostanoid receptor 1*”
- . DP2..... “*D prostanoid receptor 2*”
- . DK-PGD<sub>2</sub>..... “*13,14-dihydro-15-keto (DK)-PGD<sub>2</sub>*”
- . ECP..... Proteína Catiônica Eosinofílica; “*Eosinophil Cationic Protein*”
- . EDN..... Neurotoxina Eosinofílica; “*Eosinophil-Derived Neurotoxin*”
- . EDAC..... “*1-ethyl-3-(3-dimethylamino-propyl) carbodiimide*”
- . ELAM-1..... Molécula de Adesão entre Leucócitos e Células Endoteliais-1; “*Endothelial Leukocyte Adhesion Molecule 1*”
- . ELISA..... Ensaio de Imuno-Aderência Ligado à Enzima; “*Enzyme Linked Immuno Sorbent Assay*”
- . EoSVs..... “*Eosinophil sombrero vesicles*”
- . EPO..... Peroxidase Eosinofílica; “*Eosinophil Peroxidase*”
- . ERK ()..... Quinase Regulada por Sinal Extracelular; “*Extracellular Signal-Regulated Kinases*”.
- . FLAP..... Proteína Ativadora da 5-Lipoxigenase(LO); “*5-LO Activating Protein*”
- . FLT3..... Tirosino-quinase 3 Fms-relacionado; “*Fms-like tyrosine kinase 3*”.
- . fmlp..... “*Formyl-Methionyl-Leucyl-Phenylalanine*”

- . GM-CSF ..... Fator Estimulador de Colônias de Granulócitos e Monócitos; “*Granulocyte-macrophage colony-stimulating factor*”
- . GPCRs ..... Receptores acoplados a protein G; “*G protein coupled receptors*”
- . GW9662 ..... Antagonista do PPAR $\gamma$ ; “*PPAR $\gamma$  antagonist*”
- . HBSS ..... “*Hank’s Balanced Salt Solution*”
- . h ..... horas
- . HES ..... Síndrome hiper-eosinofílica; “*Hypereosinophilic Syndrome*”
- . HETE ..... Ácido Hidroxieicosatetraenóico; “*Hydroxyeicosatetraenoic Acid*”
- . HPETE ..... Ácido Hidroperoxieicosatetraenóico; “*Hydroperoxyeicosatetraenoic Acid*”
- . H-PGDS ..... PGD<sub>2</sub> Sintase tipo Hematopoética; “*Hematopoietic PGD<sub>2</sub> Synthase*”
- . HQL-79 ..... Antagonista seletivo da enzima H-PGDS
- . L-PGDS ..... PGD<sub>2</sub> Sintase tipo lipocalina; “*Lipocalin-type PGD<sub>2</sub> synthase*”
- . ICAM-1 ..... Molécula de adesão entre células 1; “*Inter-Cellular Adhesion Molecule 1*”
- . IFN- $\gamma$  ..... Interferon- $\gamma$ ; “*Interferon- $\gamma$* ”
- . Ig() ..... Imunoglobulina(); “*Immunoglobulin*”
- . i.t. ..... Intratorácico;
- . IL-() ..... Interleucina-(); “*Interleukin-()*”
- . IL-()R ..... Receptor da interleucina-(); “*Interleukin-() receptor*”
- . LAM-1 ..... Molécula de Adesão de Leucócitos-1; “*Lymphocyte Adhesion Molecule 1*”
- . LCF ..... Fator Quimiotático para Linfócitos; “*Lymphocyte Chemoattractant Factor*”
- . LDLox ..... Lipoproteínas de baixa densidade oxidadas; “*oxidized Low-Density Lipoproteins*”
- . LDLac ..... Lípidio de baixa densidade acetilado; “*Acetylated low density lipoprotein*”
- . LFA-3 ..... Antígeno Associado à Função de Linfócitos; “*Lymphocyte Function-Associated Antigen 3*”
- . LIR-7 ..... “*Leukocyte immunoglobulin-like receptor 7*”
- . L-PGDS ..... PGD<sub>2</sub> Sintase tipo Lipocalina; “*Lipocalin-type Prostaglandin D Synthase*”
- . LAM ..... Lipoarabinomanana
- . LPS ..... Lipopolissacarídeo; “*Lipopolysaccharide*”
- . LO ..... Lipoxigenase; “*Lipoxygenase*”
- . LT ..... Leucotrieno; “*Leukotriene*”
- . LTB<sub>4</sub> ..... Leucotrieno B<sub>4</sub>; “*Leukotriene B<sub>4</sub>*”
- . LTC<sub>4</sub> ..... Leucotrieno C<sub>4</sub>; “*Leukotriene C<sub>4</sub>*”
- . LTE<sub>4</sub> ..... Leucotrieno E<sub>4</sub>; “*Leukotriene D<sub>4</sub>*”
- . LXA<sub>4</sub> ..... Lipoxina A<sub>4</sub>; “*Lipoxin A<sub>4</sub>*”
- . LY-294002 ..... Inibidor de PI3K; “*PI3K inhibitor*”
- . MAP ..... Proteína Quinase Ativadora de Mitose; “*Mitogen Activated Protein*”
- . MBP ..... Proteína Básica Principal; “*Major Basic Protein*”
- . MCP ..... Proteína Quimiotática para Monócitos-1; “*Monocyte Chemoattractant Protein 1*”
- . MHC ..... Complexo de histocompatibilidade principal; “*Major histocompatibility complex*”
- . MIF ..... Fator Inibidor da Migração de Macrófagos; “*Macrophage Migration Inhibitory Factor*”
- . MIP-1 $\alpha$  ..... Proteína quimiotática para macrófagos 1 $\alpha$ ; “*Macrophage Chemotactic Protein-1 $\alpha$* ”
- . MRP ..... “*Multidrug Resistance Protein*”

. mL	Mililitro
. mg	Miligrama
. µg	Micrograma
. NFκB	Fator Nuclear-kappa B; <i>“Nuclear Factor-kappa B”</i>
. NO	Óxido nítrico; <i>“Nitric oxide”</i>
. OVA	Ovalbumina; <i>“Ovalbumin”</i>
. P2Y12	Receptor purinérgico; <i>“Purinergic receptor”</i>
. PAF	Fator de Ativação Plaquetária; <i>“Platelet-Activating Factor”</i>
. PAFR	Receptor do Fator de Ativação Plaquetária; <i>“Platelet-Activating Factor Receptor”</i>
. PG	Prostaglandina ; <i>“Prostaglandin”</i>
. PGD <sub>2</sub>	Prostaglandina D <sub>2</sub> ; <i>“Prostaglandin D<sub>2</sub>”</i>
. PGE <sub>2</sub>	Prostaglandina E <sub>2</sub> ; <i>“Prostaglandin E<sub>2</sub>”</i>
. PGF	Prostaglandina F; <i>“Prostaglandin F”</i>
. PGH <sub>2</sub>	Prostaglandina H <sub>2</sub> ; <i>“Prostaglandin H<sub>2</sub>”</i>
. PI3K	Fosfoinositol 3-Quinase; <i>“Phosphoinositide 3-Kinase”</i>
. PKA	Proteína quinase A; <i>“Protein kinase A”</i>
. PKC	Proteína quinase C; <i>“Protein kinase C”</i>
. PLC	Fosfolipase C; <i>“Phospholipase C”</i>
. PPAR	<i>“Peroxisome Proliferator-Activated Receptor”</i>
. PTX	Toxina pertussis; <i>“Pertussis toxin”</i>
. RANTES	<i>“Regulated upon Activation Normal T-cell Expressed and Secreted”</i>
. RNA-m	Ácido Ribonucléico Mensageiro; <i>“Messenger Ribonucleic Acid”</i>
. SB-202190	Inibidor de MAPK; <i>“MAPK inhibitor”</i>
. SCF	<i>“Stem Cell Factor”</i>
. TCH	Tiocarbohidrazida
. TGFβ	Fator de Crescimento de Transformação β; <i>“Transforming Growth Factor β”</i>
. Th	célula T auxiliar; <i>“T helper cell”</i>
. TIP47	<i>“Tail-interacting protein of 47 kDa”</i>
. TNF-α	Fator de Necrose Tumoral-α; <i>“Tumor Necrosis Factor-α”</i>
. TXA <sub>2</sub>	Tromboxano A <sub>2</sub> ; <i>“Thromboxane A<sub>2</sub>”</i>
. TXB <sub>2</sub>	Tromboxano B <sub>2</sub> ; <i>“Thromboxane B<sub>2</sub>”</i>
. U-73122	“Inibidor específico de PLC”; <i>“PLC specific inhibitor”</i>
. VCAM-1	Molécula de Adesão das Células Vasculares-1; <i>“Vascular Cell Adhesion Molecule 1”</i>
. VLA-4	Antígeno Muito Tardio-4; <i>“Very Late Antigen-4”</i>

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## 1) INTRODUÇÃO:

### **1.1. Aspectos Gerais da Inflamação Alérgica:**

As doenças respiratórias inflamatórias de fundo alérgico correspondem a um grupo de patologias que atingem a humanidade há muitos anos, tendo os primeiros relatos datados de aproximadamente 2600 (a.C.). Dentre essas patologias, a asma tem papel de destaque por afetar em todo o mundo 10% da população infantil e 5% da população adulta (Capra *et al.*, 2007; Krouse *et al.*, 2007; Boyce, 2008; O'Byrne *et al.*, 2009).

A asma não possui etiologia única e específica, sendo multifatorial e variável de indivíduo para indivíduo (Jarjour *et al.*, 2002). Dessa forma, a asma pode ser induzida:

(i) por antígeno: Pessoas alérgicas quando inalam partículas antigênicas desenvolvem um quadro de broncoconstrição imediata (que também pode ocorrer de 6 a 8 horas após a exposição), e que é consequência da liberação de mediadores inflamatórios específicos dos mastócitos (Aalbers *et al.*, 1993);

(ii) por patógenos ou ar frio: Infecções do trato respiratório provocadas por vírus ou bactérias, assim como a exposição ao ar frio e seco, têm sido associadas ao desenvolvimento da asma (Cockcroft, 2001; Lemanske, Jr. *et al.*, 2003) .

(iii) após atividade física: Caracterizando a “asma induzida por exercício”, onde ocorre uma obstrução transitória nas vias aéreas logo após atividade física intensa, levando a ocorrência de sintomas como a falta de ar, tosse e o chiado (Laitano *et al.*, 2008);

(iv) por sensibilidade a aspirina: Cerca de 10% dos indivíduos adultos asmáticos, apresentam sensibilidade ao ácido acetilsalicílico (AAS), popularmente conhecido como aspirina (e também a outros antiinflamatórios não-esteroidais - AINEs). Especificamente nessa situação, a ingestão do AAS leva ao desenvolvimento de um quadro de asma aguda, podendo ser acompanhada de rinorréia, congestão nasal, irritação conjuntival e/ou rubor da cabeça e pescoço. Esse antiinflamatório atua inibindo a ciclo-oxigenase (COX), assim como os outros AINEs, mas nestes casos parece possibilitar o aumento da síntese de leucotrienos cisteinados (cisLTs) – mediadores inflamatórios responsáveis pela broncoconstrição característica dessa patologia (Horwitz *et al.*, 1998).

Além desses fatores abordados, alguns estudos têm reforçado ainda mais o elo entre a ocorrência dessa patologia e uma provável predisposição genética (fatores hereditários), pois tem sido mostrado que existem polimorfismos específicos relacionados ao aumento na expressão de receptores de citocinas importantes para o estabelecimento da asma, como os receptores da interleucina-5 (IL-5R) e de eotaxina (CCR3) (Park, 2010).

Apesar de ser uma doença de causas multifatoriais, a asma apresenta alterações anatomofisiológicas características, como hipertrofia muscular, hiperplasia das células caliciformes (com conseqüente aumento na produção de muco), hiperplasia vascular (angiogênese) e depósito de colágeno, resultando no remodelamento e obstrução irreversível das vias aéreas. Essas alterações ocorrem devido aos processos de injúria e reparo tecidual, que ocorrem durante o desenvolvimento da mesma e que são recorrentes a cada recidiva do quadro inflamatório (Vignola *et al.*, 2000).

Independente do fator causador da reação inflamatória, durante o desenvolvimento da resposta alérgica, a primeira exposição ao antígeno resultará na síntese de anticorpos da classe IgE, que são produzidos por linfócitos B. Uma vez sintetizados e liberados, esses anticorpos se ligarão aos receptores de alta-afinidade (FcεRI) presentes na superfície dos mastócitos. Numa segunda exposição a esse antígeno, essas células são ativadas, resultando na liberação tanto de mediadores recém-sintetizados, quanto de pré-formados que já estavam estocados nos grânulos citoplasmáticos. Dessa forma, a ativação de mastócitos e linfócitos (principalmente a subpopulação Th2) resultará na secreção de uma ampla variedade de citocinas pró-inflamatórias importantes, dentre as quais se sobressaem as interleucinas (IL)-4, IL-5 e IL-13 (as quais serão responsáveis pela modulação das respostas inflamatórias de patologias de fundo alérgico em geral) (Busse *et al.*, 2001).

Os linfócitos, juntamente com as células epiteliais, macrófagos e mastócitos também irão produzir e secretar quimiocinas, como RANTES e eotaxina/CCL11, que têm um papel de destaque nessa resposta, pois são essenciais para promover o recrutamento para o sítio inflamatório de eosinófilos, assim como na indução da ativação dessas células (Lemanske, Jr. *et al.*, 2003). Também têm sido demonstrado, que a subpopulação linfócitos Th17 exerce um papel modulador no desenvolvimento da asma (McKinley *et al.*, 2008).

Devido as suas características efectoras, os eosinófilos constituem-se como importantes alvos para terapias anti-alérgicas/anti-asmáticas, uma vez que representam uma classe de leucócitos com uma contribuição fundamental tanto para as manifestações agudas, como para as sequelas mais tardias de distúrbios alérgicos em geral (Munitz *et al.*, 2004).

As principais alterações anatomofisiológicas observadas em pacientes asmáticos, parecem ser consequência da presença e da ativação dos eosinófilos infiltrantes no sítio inflamatório. Essas células, uma vez ativadas, são capazes de causar lesão na mucosa e provocar alterações na fisiologia pulmonar devido à liberação e ação de seus produtos celulares (Levi-Schaffer *et al.*, 1999), como as suas proteínas granulares catiônicas específicas, que encontram-se estocadas nos seus grânulos citoplasmáticos (Hernnas *et al.*, 1992; Noguchi *et al.*, 1992; Rochester *et al.*, 1996; Weller *et al.*, 1997); espécies reativas do oxigênio (MacPherson *et al.*, 2001); citocinas e quimiocinas (Lacy *et al.*, 2001); e, mediadores lipídicos, como Fator de Ativação Plaquetária (PAF) (Lee *et al.*, 1984), tromboxano e prostaglandinas (Hubscher, 1975; Kroegel *et al.*, 1993).

Além da produção desses mediadores mencionados, deve ser destacado que, os eosinófilos, correspondem ao principal tipo celular relacionado a produção e liberação de cisLTs: LTC<sub>4</sub>, LTD<sub>4</sub> e LTE<sub>4</sub>. Esses eicosanóides correspondem a potentes mediadores lipídicos, relacionados a ocorrência da sintomatologia observada durante o desenvolvimento da asma, incluindo aumento da permeabilidade vascular, edema, broncoconstrição, hiperreatividade brônquica e, até mesmo, a própria infiltração eosinofílica (Samuelsson *et al.*, 1987; Lewis *et al.*, 1990).

Embora, ao longo dos anos muito tenha sido feito para que se conseguisse uma melhor compreensão da fisiopatologia da asma e, conseqüentemente, aumentar o número de medicamentos disponíveis, atualmente a incidência, a morbidade e a mortalidade associadas aos casos de asma não diminuiu. Muito pelo contrário, existem indícios de aumento na ocorrência da asma no Brasil e em todo o mundo. Segundo informe do GINA (*Global Initiative for Asthma - 2010*), existem 300 milhões de pessoas com asma em todo o planeta. Segundo estudo multicêntrico realizado anteriormente, a prevalência mundial varia entre 1,6% a 36,8% da população geral, dependendo da região e do país, e o Brasil foi classificado em oitavo lugar, apresentando uma

prevalência média de 20% dessa doença (*“International Study of Asthma and Allergies in Childhood” – 1998*).

Assim sendo, todo esforço é importante e novos avanços ainda se fazem necessários para alterar esse quadro atual. A identificação de alvos terapêuticos mais pontuais e específicos, com maior eficácia no bloqueio dos fenômenos envolvidos no desenvolvimento dessa patologia (ou possibilitando a reversão dos mesmos), podem gerar um grande impacto na morbidade associada a essa condição, assim como melhorar a qualidade de vida dos pacientes asmáticos.

Dessa forma, o estudo focado nos mediadores inflamatórios produzidos durante o desenvolvimento da resposta alérgica, assim como os mecanismos que regulam a síntese destes, são pontos críticos para o entendimento das patologias de caráter eosinofílico, além de representar a possibilidade da identificação de novos alvos terapêuticos.

## 1.2. EOSINÓFILOS:

### 1.2.1. Características Gerais:

Os eosinófilos correspondem a uma classe de leucócitos que representam um dos principais tipos celulares recrutados para o sítio inflamatório alérgico, pois uma vez ativadas são fundamentais para a patogênese desse tipo de resposta inflamatória, e especificamente, para resposta inflamatória característica da asma (Wardlaw, 1999; Gleich, 2000; Lemanske, Jr. *et al.*, 2003). Os eosinófilos são leucócitos polimorfonucleares, predominantemente teciduais e encontrados principalmente em regiões de sub-mucosa, e particularmente distribuídas nos tratos respiratório, gastrointestinal e genito-urinário (Weller, 1991). Embora estejam presentes em níveis elevados em infiltrados inflamatórios em reações alérgicas e também sejam muito importantes durante respostas contra helmintos (Sabin *et al.*, 1996; Klion *et al.*, 2004), em condições normais, essas células constituem apenas cerca de 1 a 2 % dos leucócitos totais na circulação periférica de indivíduos não-alérgicos.

Os eosinófilos são produzidos e diferenciados na medula óssea a partir de progenitores mielóides (células pluripotentes que irão originar outros leucócitos, como os neutrófilos), quando estas células são estimuladas por 3 citocinas: o fator de estimulação de colônia macrófago-granulócito (GM-CSF) e as interleucinas (IL)-3 e IL-5. Cada uma dessas citocinas é capaz de promover a eosinopoiese, no entanto a IL-5 tem um papel importante na liberação dos eosinófilos já maduros que ainda estão na medula para a corrente sanguínea, promovendo a diferenciação terminal e a ativação funcional dos mesmos (Yamaguchi *et al.*, 1988).

Na caracterização dos fenômenos associados a diferenciação de eosinófilos, a obtenção de camundongos deficientes da região promotora do fator de transcrição GATA-1 ( $\Delta$ dbl-GATA) – fator de transcrição ativado por IL-5 – possibilitou o desenvolvimento de animais destituídos de eosinófilos (camundongos  $\Delta$ dbl-GATA deficientes) (Yu *et al.*, 2002). No entanto, embora tenha sido verificada a importância dessa via de sinalização para o desenvolvimento de eosinófilos, esse mesmo grupo demonstrou que progenitores mielóides da medula óssea de camundongos  $\Delta$ dbl-GATA deficientes, quando estimulados com as citocinas IL-3, IL-5 e GM-CSF são capazes de se diferenciar *ex vivo* em eosinófilos, provavelmente dependente de um fenômeno

compensatório da deleção de  $\Delta$ dbl-GATA (através da ativação de outro promotor) (Dyer *et al.*, 2007). Além disso, foi demonstrado também ser possível obter eosinófilos (funcionalmente competentes) a partir de células progenitoras mielóides da medula óssea de camundongos selvagens, diferenciadas *ex vivo* com FLT3-L (ligante do receptor FLT3), fator de célula tronco (SCF) e IL-5 recombinante murina (IL-5rm) (Dyer *et al.*, 2008).

### **1.2.2. Características Ultraestruturais:**

Quando atingem seu grau máximo de maturação, essas células apresentam um diâmetro médio de 8  $\mu$ m, apresentam núcleo com número de lóbulos variáveis – a partir de uma média de dois (bi-lobulado) – mas podem atingir um total de quatro lóbulos dependendo do grau de ativação celular (Sokol *et al.*, 1987). Além disso, os seus grânulos citoplasmáticos se coram pela eosina (corante ácido), característica que foi responsável pela adoção do nome “eosinófilo” por Paul Erlich, quando o mesmo identificou essas células no escarro de pacientes asmáticos (McEwen, 1992).

No citoplasma de eosinófilos maduros provenientes da circulação periférica de doadores saudáveis encontram-se os grânulos *primários* que morfológicamente são grandes e esféricos e, estruturalmente, são destituídos de inclusões cristalóides (Figura 1.2.1). Estes grânulos representam cerca de 5% da subpopulação total dos grânulos citoplasmáticos eosinofílicos e possuem “um único compartimento”, que quando maduros, apresentam como constituinte CLC-P (*Charcot-Leyden Cristal Protein*) (Dvorak *et al.*, 1988).

Além desses grânulos, o citoplasma eosinofílico é quase que inteiramente ocupado pelos seus grânulos específicos (ou *secundários*), sendo esta a mais marcante característica morfológica deste tipo celular, visto que correspondem a cerca de 95% dos grânulos da célula. Estes grânulos, sob análise ultraestrutural, se revelaram como um grande núcleo eletrodense e cristalóide, com mais de 0,5  $\mu$ m (na sua maior dimensão) constituído principalmente pela proteína básica principal (MBP), que é uma proteína catiônica rica em resíduos de arginina. A matriz granular mostra-se homogênea, menos densa e é basicamente composta por peroxidase eosinofílica (EPO), proteína catiônica eosinofílica (ECP), neurotoxina derivada do eosinófilo (EDN) e histaminase (Egesten *et al.*, 1986; Sokol *et al.*, 1987).

Além desses dois tipos de grânulos os eosinófilos apresentam ainda no seu citoplasma, os *grânulos menores*, que são morfologicamente e citoquimicamente análogos aos lisossomos em outras células e não apresentam na sua constituição MBP (como os grânulos secundários), nem CLC-P (como os grânulos primários) e os *microgrânulos*. Essas últimas estruturas citoplasmáticas durante muitos anos foram descritos como sendo apenas estruturas “vesiculo-tubulares” (Dvorak *et al.*, 1990; Dvorak *et al.*, 1991; Dvorak *et al.*, 1992; Dvorak *et al.*, 1994a). Recentemente, esses microgrânulos foram caracterizados como *eosinophil sombrero vesicles* (EoSVs), e seu papel na secreção *piecemeal* de citocinas e proteínas granulares eosinofílicas – processo de secreção seletiva dos constituintes granulares, que será abordado ainda nessa seção – vem sendo estudado e cada vez mais melhor caracterizado (Dvorak *et al.*, 2000; Melo *et al.*, 2005; Spencer *et al.*, 2009).



**FIGURA 1.2.1** – Análise ultraestrutural de eosinófilos humanos periféricos por microscopia eletrônica de transmissão – 17500X (Dvorak *et al.*, 1988) (**Flecha fechada** – Grânulos primários; **Flecha aberta** – Grânulos secundários; **Flecha pequena** – Grânulos menores; **Flecha pequena curva** – microgrânulos)

Além dos seus grânulos citoplasmáticos característicos, os eosinófilos apresentam no seu citoplasma retículo endoplasmático e aparelho de Golgi pouco desenvolvidos (Dvorak *et al.*, 1989), e também os corpúsculos lipídicos.

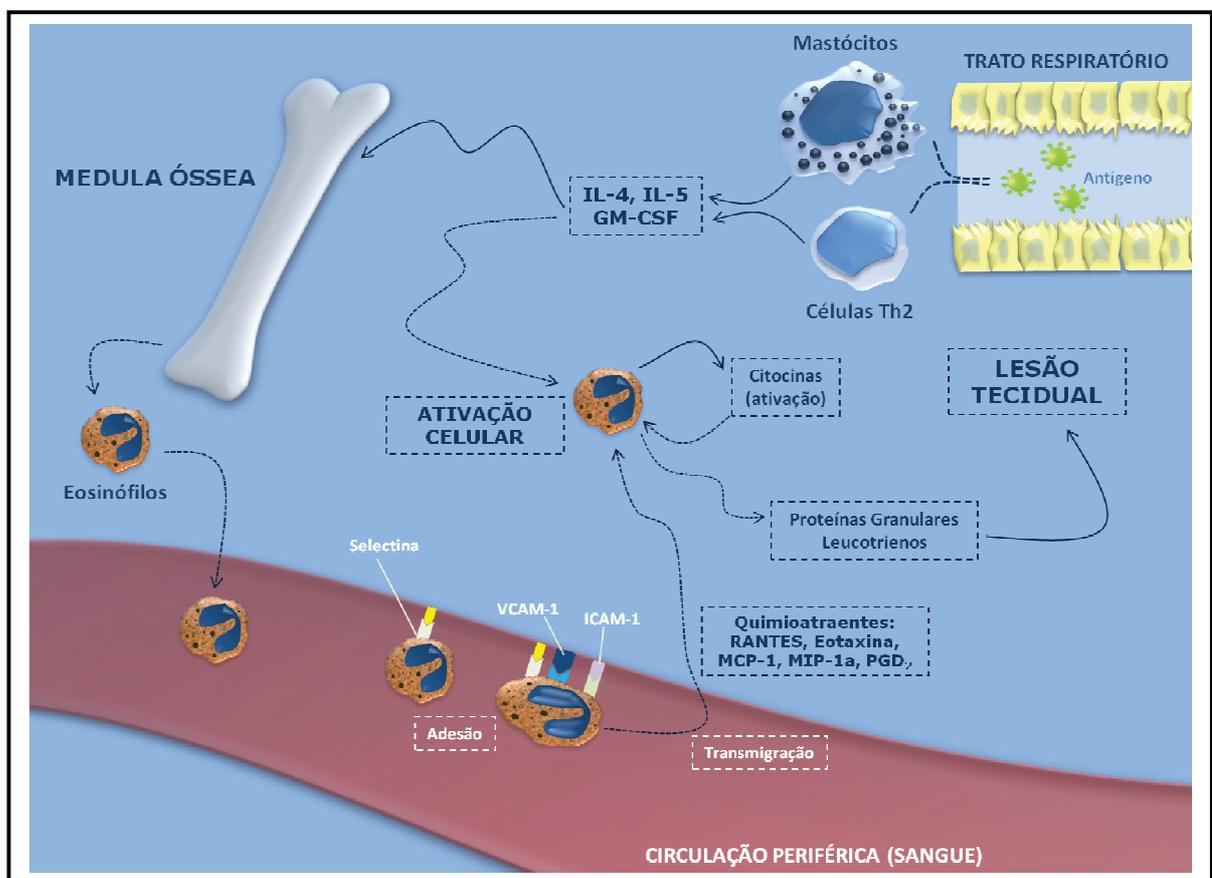
### **1.2.3. Fatores determinantes da eosinofilia tecidual (migração):**

Durante o desenvolvimento de patologias de fundo alérgico – como a asma – a eosinofilia das vias aéreas é a principal característica e tem sido considerada como o centro da patogenia da doença. Dessa forma, o entendimento dos mecanismos que induzam o acúmulo e ativação local deste tipo celular desperta cada vez mais interesse. Para chegar ao sítio inflamatório, o extravasamento de leucócitos inicia-se com a marginação das células circulantes ao longo da microvasculatura, seguida por um tipo de ligação transiente ao endotélio pós-venular (rolamento). Após este reconhecimento inicial, ocorre a adesão firme e a subsequente transmigração dos leucócitos através da monocamada de células endoteliais (diapedese). A etapa final do processo inclui a migração através da matriz extracelular, direcionada por um gradiente de concentração criado por fatores quimiotáticos provenientes do foco inflamatório. A persistência do leucócito recém migrado no tecido é então modulada por componentes da matriz extracelular e citocinas específica produzidas e liberadas durante o desenvolvimento da resposta (Walsh *et al.*, 1991). Como ilustrado na figura 1.2.2, o acúmulo preferencial de eosinófilos nas diversas condições patológicas relacionadas pode ser explicado pelo efeito combinado de vários mecanismos e moléculas, como os destacados abaixo:

Expressão de moléculas de adesão: Os eosinófilos expressam constitutivamente a molécula VLA-4 ( $\alpha_4\beta_1$ ) (“*Very Late Antigen*”), permitindo sua adesão às células endoteliais, através da ligação VLA-4/VCAM-1 (“*Vascular Cell Adhesion Molecule-1*”). Esse mesmo fenômeno não acontece com neutrófilos, visto que essas células não expressam essa molécula (Walsh *et al.*, 1991). A falta de ligação dos neutrófilos à VCAM-1 e o aumento seletivo da expressão endotelial de VCAM-1 induzida por IL-4 (Lee *et al.*, 2004b) parecem contribuir para o acúmulo preferencial de eosinófilos observado em reações inflamatórias mediadas por IgE, como as de origem alérgica e parasitária;

Ação de mediadores inflamatórios quimiotáticos seletivos: Tem sido mostrado que os eosinófilos podem expressar alguns receptores quimiotáticos importantes, como CCR1 e CCR3. Dessa forma, os eosinófilos podem responder a ligantes para esses receptores, como a “*macrophage inflammatory protein*” (MIP)-1 $\alpha$ /CCL3, RANTES/CCL5, “*macrophage chemotactic protein*” (MCP)-2/CCL8, MCP-3/CCL7, MCP-4/CCL-13, eotaxina-1/CCL11, eotaxina-2/CCL24 e eotaxina-3/CCL26 (Ponath *et al.*, 1996; Phillips

*et al.*, 2003; Elsner *et al.*, 2005). Essas células também expressam receptor de PAF (PAFR), e esse mediador lipídico é um importante fator quimiotático para essas células, visto que é produzido durante a resposta alérgica (Wardlaw *et al.*, 1986; Ukena *et al.*, 1989; Barnes, 1991). Além desses receptores, os eosinófilos expressam constitutivamente o receptor DP2, que é responsável pelos efeitos quimiotáticos observados em eosinófilos induzidos pela prostaglandina (PG) D<sub>2</sub>, mediador lipídico que também é produzido em grandes quantidades durante respostas alérgicas (Murray *et al.*, 1986).



**Figura 1.2.2:** Ilustração representativa dos eventos envolvidos no acúmulo tecidual de eosinófilos.

*Aumento seletivo da eosinopoiese:* A produção e liberação de IL-5 em níveis aumentados é responsável pelo aumento da eosinopoiese na medula óssea, resultando

num consequente aumento no número de eosinófilos circulantes, sem afetar os outros tipos de leucócitos sanguíneos (Sanderson, 1992);

Aumento da meia-vida tecidual dos eosinófilos recrutados: Experimentos *in vitro* demonstraram que a apoptose eosinofílica é inibida por IL-5, IL-3 e GM-CSF (“*Granulocyte-Macrophage Colony Stimulating Factor*”). Essas observações sugerem que a produção local destas citocinas aumentaria a persistência tecidual dos eosinófilos no tecido inflamatório (Yamaguchi *et al.*, 1988; Anwar *et al.*, 1993). Vale destacar que os eosinófilos são capazes de secretar essas citocinas, visto que as mesmas encontram-se estocadas nos seus grânulos citoplasmáticos (figura 1.2.3) (Melo *et al.*, 2008b), dessa forma mesmo através de ativação autócrina, os eosinófilos poderiam ser responsáveis por prologar a sua própria sobrevivência tecidual.

Proliferação tecidual: Foi demonstrado que em um modelo experimental de infecção murina por *S.mansoni*, ocorre proliferação extramedular de eosinófilos, fenômeno este dependente da ativação de macrófagos (el-Cheikh *et al.*, 1990). Além disso, foi demonstrado também que, a proliferação tecidual ocorrida após o acúmulo induzido pelo PAF, parece contribuir para o desenvolvimento da resposta inflamatória (Perez *et al.*, 1998).

#### **1.2.4. Ativação eosinofílica e o seu papel no desenvolvimento da resposta inflamatória:**

Nas últimas décadas vários estudos demonstraram uma correlação direta entre a presença dos eosinófilos e a sintomatologia alérgica (Wardlaw, 1999; Gleich, 2000; Lemanske, Jr. *et al.*, 2003). Uma vez no sítio inflamatório, o eosinófilo apresentará sua fisiologia alterada, mostrando sinais de ativação celular. Algumas alterações morfológicas relacionadas a ativação eosinofílica estão representadas na tabela 1.2.1:

**Tabela 1.2.1** – Alterações morfológicas observadas em eosinófilos ativados.

<b>Sítio</b>	<b>Alterações observadas</b>	<b>Referência</b>
<i>Citoplasma</i>	- Densidade diminuída - Vacuolização	De Simone <i>et al</i> , 1982; Fukuda <i>et al</i> , 1985; Vadas <i>et al</i> , 1979; Weller <i>et al</i> , 1991.
<i>Grânulos</i>	- Alteração no número e tamanho - Degranulação	Weller, 1991; Lacy <i>et al</i> , 2001.
<i>Corpúsculos Lipídicos</i>	- Aumento no número	Bozza <i>et al</i> , 2009
<i>Núcleo</i>	- Aumento na segmentação	Sokol <i>et al</i> , 1987

Eosinófilos ativados presentes no sítio inflamatório apresentam ainda outras alterações que caracterizam seu estado de ativação. Já é bem estabelecido que, uma vez ativados, além de secretarem o seu conteúdo granular (citocinas e proteínas tóxicas características), também são capazes de sintetizar e secretar uma gama de mediadores lipídicos, como PAF (Lee *et al.*, 1984; Bartemes *et al.*, 1999), Lipoxina A<sub>4</sub> (Serhan *et al.*, 1987; Bandeira-Melo *et al.*, 2000), TXB<sub>2</sub> (Kroegel *et al.*, 1993), TXA<sub>2</sub> (Hirata *et al.*, 1989; Giembycz *et al.*, 1990), PGE<sub>2</sub> (Bozza *et al.*, 1997) e eoxinas (Feltenmark *et al.*, 2008). Dentre os mediadores lipídicos produzidos, os eosinófilos têm papel de destaque como produtores de grandes quantidades de LTC<sub>4</sub>. Nosso grupo têm demonstrado que, uma vez ativados, tanto no sítio inflamatório alérgico quanto estimulados isoladamente *in vitro*, os eosinófilos apresentam no seu citoplasma aumento no número de corpúsculos lipídicos (Bozza *et al.*, 1997; Vieira-de-Abreu *et al.*, 2005b; Mesquita-Santos *et al.*, 2006), correspondendo a um importante parâmetro de avaliação da ativação eosinofílica. O fenômeno de biogênese dessa organela tem se mostrado altamente regulado e específico, além de essencial para a síntese de eicosanóides, como a PGE<sub>2</sub> (D'Avila *et al.*, 2006) e o LTC<sub>4</sub> (Vieira-de-Abreu *et al.*, 2005b; Mesquita-Santos *et al.*, 2006).

Assim sendo, uma ampla variedade de modelos experimentais, espécies de animais e diferentes intervenções farmacológicas e/ou imunológicas vêm sendo desenvolvidas, para tentar melhor caracterizar as funções dos eosinófilos e, assim, prevenir e/ou impedir o desenvolvimento de patologias de caráter eosinofílico. Dentre

essas intervenções podem ser citadas a utilização de anticorpos monoclonais humanos (IgG1) anti-IL-5 em pacientes com síndrome hipereosinofílica (HES). Esse tratamento vem se mostrando eficaz e possibilitaria a substituição dos corticóides como única estratégia terapêutica, pois apesar de ter efeito antiinflamatório eficaz, pode levar ao surgimento de efeitos secundários não-desejados (Busse *et al.*, 2010). Além disso, também já havia sido demonstrado que o tratamento de primatas com anticorpo contra ICAM-1 preveniu o acúmulo pulmonar de eosinófilos e conseqüentemente a hiperresponsividade das vias aéreas, num modelo de asma experimental (Wegner *et al.*, 1990).

Com o advento do desenvolvimento de animais geneticamente modificados – resultando em camundongos destituídos de eosinófilos (PHIL ou  $\Delta$ dbl-GATA deficiente) – foi possível observar com mais acurácia o papel destas células na patogênese de distúrbios inflamatórios alérgicos, como a asma. Em dois estudos contemporâneos realizados com esses animais, foi observado que ambos animais quando submetidos ao desafio alérgico apresentaram ausência na eosinofilia pulmonar, assim como na hipertrofia epitelial. No entanto, somente na resposta alérgica dos animais PHIL foi demonstrada que a ausência dessas células estava associada a reversão de várias características inflamatórias da resposta alérgica, como hipertrofia epitelial, hiperreatividade brônquica, produção de muco aumentada e hiperplasia das células caliciformes (Lee *et al.*, 2004a). No outro estudo, esses mesmos parâmetros descritos anteriormente se mostraram inalterados, mas foi observado uma redução na deposição de colágeno, com conseqüente diminuição no remodelamento das vias aéreas dos animais  $\Delta$ dbl-GATA deficientes (Humbles *et al.*, 2004). Embora os dois grupos tenham conseguido desenvolver animais destituídos de eosinófilos, o papel exato dessa célula no contexto inflamatório alérgico ainda não fora completamente elucidado, visto que mesmo com animais que não possuíam eosinófilos, os fenômenos observados durante o desenvolvimento da resposta inflamatória alérgica não foram coincidentes.

A utilização de camundongos deficientes de IL-5 havia demonstrado que a diminuição de eosinófilos na região peribrônquica, assim como os níveis reduzidos de TGF $\beta$ , além da própria IL-5, seriam os responsáveis pelo remodelamento das vias aéreas (Cho *et al.*, 2004) . No entanto, a observação de níveis similares de TGF $\beta$  em

animais selvagens e  $\Delta$ dbl-GATA, sugeriu que a redução da fibrose observada nesses animais parece ser independente da expressão de TGF $\beta$  (Humbles *et al.*, 2004).

Outro estudo realizado por Jacobsen e colaboradores usando modelo de alergia induzida por ovalbumina (OVA) nos camundongos PHIL, demonstrou que a ausência de eosinófilos inibiu, além da secreção de citocinas pró-inflamatórias como IL-4, -5 e -13, a proliferação de células T CD4<sup>+</sup> e CD8<sup>+</sup> no pulmão. Nesse estudo, a importância dos eosinófilos é ratificada com a observação de que a transferência adotiva de células Th2-polarizadas OVA específicas antes do estímulo alérgico, não foi capaz de reverter esse quadro de anergia desses animais. Esse quadro só foi revertido quando foi feita a transferência de eosinófilos – em associação com as células OVA-específicas – reforçando o papel dos eosinófilos como células essenciais para o desenvolvimento da resposta alérgica e não somente como células efetoras (Jacobsen *et al.*, 2008).

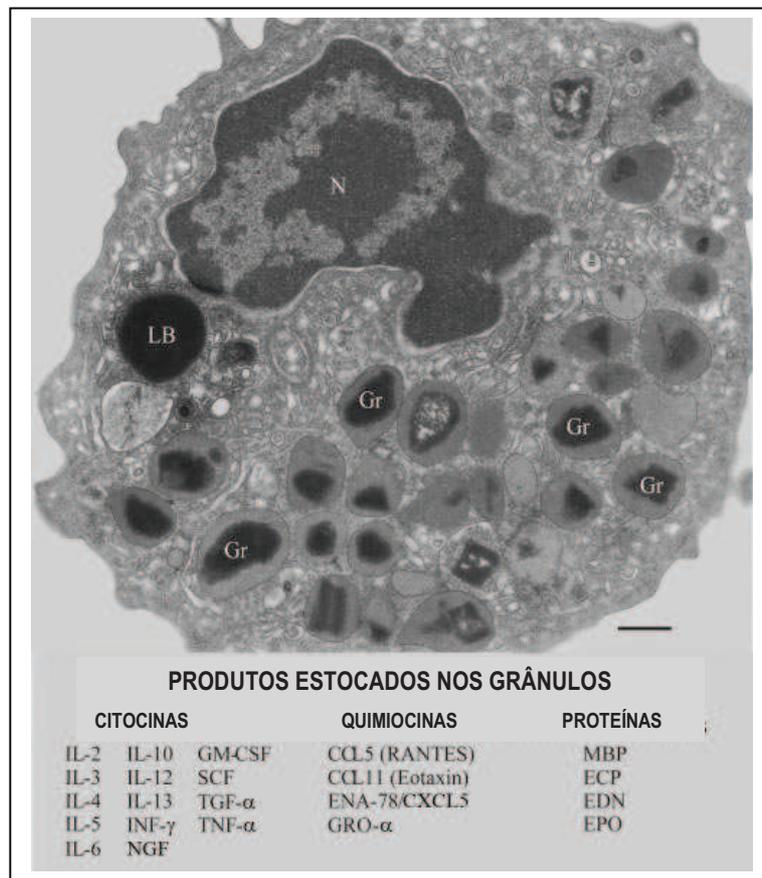
Para poder modular a resposta inflamatória, de acordo com as observações acima abordadas, os eosinófilos são capazes de secretar uma gama de proteínas estocadas em seus grânulos e que não se restringem exclusivamente às proteínas citotóxicas eosinofílicas – mas também citocinas e quimiocinas – que encontram-se estocadas em seus grânulos citoplasmáticos, juntamente com as suas proteínas específicas (Melo *et al.*, 2008a; Melo *et al.*, 2008b). A liberação do conteúdo granular eosinofílico pode acontecer por 3 diferentes maneiras (Lacy *et al.*, 2001):

I) Exocitose: fenômeno que consiste no processo clássico de liberação do conteúdo granular, onde todo conteúdo estocado no grânulo será liberado, após o processo de fusão do grânulo com a membrana plasmática (Moqbel *et al.*, 2006);

II) Citólise: onde todo o conteúdo celular é liberado, após a lise da membrana plasmática, com os grânulos sendo liberados inteiros. Recente estudo demonstrou que os grânulos citoplasmáticos eosinofílicos liberados íntegros nos tecidos inflamatórios, são capazes de responder a estimulação com IFN $\gamma$  e eotaxina, demonstrando que expressam receptores de membrana e que são organelas secretórias capazes de atuar de forma independente da ativação eosinofílica extracelularmente (Neves *et al.*, 2008).

III) Degranulação Piecemeal: que consiste num processo de secreção seletiva do conteúdo granular, onde apenas parte do conteúdo é liberado, sendo um processo dependente de transporte vesicular. Esse parece ser o mecanismo predominante para

a liberação granular na inflamação alérgica (Dvorak *et al.*, 1992; Moqbel *et al.*, 2006). Em recente estudo, Spencer e colaboradores demonstraram que eosinófilos humanos isolados – em face da sua conhecida capacidade de sintetizar e estocar em seus grânulos citoplasmáticos mais de 30 citocinas – são capazes de rapidamente liberar de forma seletiva (e estímulo específica) citocinas com envolvimento característico e bem estabelecido no desenvolvimento das respostas Th1 e Th2, e também com papel imunoregulatório, como IL-4, IL-13, IL-6, IL-10, IL-12, IFN- $\gamma$  e TNF $\alpha$  (Spencer *et al.*, 2009). A *figura 1.2.3* ilustra, os grânulos citoplasmáticos eosinofílicos e lista as possíveis citocinas e quimiocinas estocadas, assim como as suas proteínas tóxicas específicas – MBP, EPO, ECP e EDN – que podem ser liberadas por eosinófilos ativados durante a resposta inflamatória,



**Figura 1.2.3** – Análise ultraestrutural de eosinófilo humano, representando os mediadores inflamatórios e proteínas eosinofílicas armazenados nos grânulos citoplasmáticos (M.E.Transmissão) (**N** – Núcleo / **Gr** – grânulo / **LB** – corpúsculo lipídico) – (Modificado de (Melo *et al.*, 2008b).

Vale ressaltar aqui que, embora tenham sido descritos como sendo células essencialmente efectoras, características de respostas específicas e bem estabelecidas – como na resposta alérgica e na infecção por helmintos – alguns estudos demonstraram que os eosinófilos também são capazes de desempenhar outros papéis durante o desenvolvimento da resposta imunológica.

Foi demonstrado que os eosinófilos são capazes de processar e apresentar antígenos, promovendo a ativação de células T CD4<sup>+</sup>, após migrarem através do sistema linfático para linfonodos pulmonares, promovendo o desenvolvimento de resposta Th2, exercendo assim atividades que são relacionadas às de uma célula apresentadora de antígeno (Lucey *et al.*, 1989; Shi *et al.*, 2000; MacKenzie *et al.*, 2001; Wang *et al.*, 2007). Além disso, foi demonstrado que após a exposição das vias aéreas a um estímulo alérgico, nessas células há aumento na expressão de moléculas de MHC (*Major Histocompatibility Complex*) de classe II (moléculas envolvidas no processo de apresentação e ativação antígeno-específica de células T CD4<sup>+</sup>) além de moléculas co-estimulatórias necessárias para a ativação de células T, como CD40, CD80, and CD86 regulando a ativação dessa resposta (Akuthota *et al.*, 2008). De fato, essa função imunoreguladora da resposta Th2 ocorre não somente durante a resposta alérgica, mas também durante o desenvolvimento da resposta a helmintos, indicando que os eosinófilos podem se comportar como uma célula apresentadora de antígeno (Padigel *et al.*, 2007).

### 1.3. LEUCOTRIENOS CISTEINADOS:

#### 1.3.1 Visão Geral:

Os eosinófilos se caracterizam como as principais fontes dos leucotrienos cisteinados (cisLTs), que correspondem ao leucotrieno C<sub>4</sub> (LTC<sub>4</sub>) e seus derivados extracelulares, os leucotrienos D<sub>4</sub> e E<sub>4</sub> (LTD<sub>4</sub> e LTE<sub>4</sub>) (Weller *et al.*, 1983; Cowburn *et al.*, 1998). Durante o desenvolvimento da asma e de outras inflamações alérgicas, os cisLTs correspondem a uma classe de mediadores inflamatórios de natureza lipídica que é fundamental para a patogênese da asma e de outras inflamações alérgicas (Christie *et al.*, 2002).

Sua produção aumentada está associada ao desenvolvimento de diversos quadros patológicos e processos inflamatórios, como o desenvolvimento do quadro de fibrose pulmonar (Charbeneau *et al.*, 2005) e de doenças cardiovasculares (Funk, 2005). Também são capazes de induzir o acúmulo de células dendríticas para o sítio inflamatório – em um modelo de resposta induzida por antígeno (Parameswaran *et al.*, 2004) – assim como, o acúmulo dessas mesmas células para os linfonodos (Robbiani *et al.*, 2000). Em células polimorfonucleares, são capazes de induzir a geração de NO (Larfars *et al.*, 1999), além de ativar mastócitos, levando a produção de outros mediadores inflamatórios como IL-5, TNF- $\alpha$  e MIP-1 $\beta$  (Mellor *et al.*, 2002). Entretanto, no que tange os objetivos desse estudo, esses mediadores lipídicos destacam-se como um dos principais responsáveis por causar broncoconstrição, aumento da secreção de muco e na permeabilidade vascular, hiperreatividade brônquica e infiltrado de eosinófilos durante a resposta alérgica (Lewis *et al.*, 1990; Laitinen *et al.*, 1993; Henderson, Jr., 1994).

Uma vez sendo produzidos e secretados, os leucotrienos cisteinados são capazes de exercer as suas funções através de dois receptores específicos, que são os receptores CysLT1 e CysLT2. Ambos são receptores que apresentam 7 domínios transmembranares, sinalizando via proteína G $\alpha$ q. O LTD<sub>4</sub> se liga ao receptor CysLT1 com uma afinidade 100 vezes maior do que o LTC<sub>4</sub>, enquanto que o LTC<sub>4</sub> e o LTD<sub>4</sub> se ligam ao CysLT2 com a mesma afinidade (Lynch *et al.*, 1999; Sarau *et al.*, 1999; Heise *et al.*, 2000; Nothacker *et al.*, 2000). Dentre os produtos dessa via metabólica, o produto final dela – que é o LTE<sub>4</sub> – é o mais estável dos leucotrienos cisteinados, mas é o agonista mais fraco desses receptores (Austen *et al.*, 2009; Lee *et al.*, 2009). Em razão

da ausência de ferramentas farmacológicas capazes de exercerem atividades antagonista seletiva para o receptor CysLT2, a droga BAYu9773 tem papel de destaque na caracterização dos mesmos, visto que tem capacidade de antagonizar as ações de ambos os receptores, pois apenas o receptor CysLT1 possui antagonistas seletivos (*por ex.*: montelucaste, zafirlucaste e pranlucaste) (Labat *et al.*, 1992; Tudhope *et al.*, 1994). Ao longo dos anos, evidências da existência de um terceiro receptor têm se acumulado. Este receptor ainda não está completamente caracterizado e tem uma maior afinidade pelo LTC<sub>4</sub> do que pelo LTD<sub>4</sub>, tem localização intracelular e demonstra ter um papel intrácrino na regulação da secreção de citocinas por eosinófilos (Bandeira-Melo *et al.*, 2002c).

Recentemente, fora demonstrado que animais deficientes para os receptores CysLT1 e CysLT2 quando estimulados com LTE<sub>4</sub>, apresentavam edema na orelha maior do que os animais selvagens, demonstrando a existência de um novo receptor que responde preferencialmente ao LTE<sub>4</sub>, chamado de CysLT<sub>E</sub>R. Os efeitos dessa administração foram revertidos com a utilização de PTX e de Y-27632, demonstrando que os efeitos resultantes da ativação desse receptor são dependentes da ativação de proteína G $\alpha$ i e Rho Kinase (Maekawa *et al.*, 2008). Posteriormente, esse receptor veio a ser chamado de CysLT3R e, juntamente com o receptor P2Y12, parece mediar os efeitos inflamatórios do LTE<sub>4</sub> (Austen *et al.*, 2009).

Em relação à distribuição dos receptores CysLT, no pulmão o CysLT1 é predominante na musculatura lisa das vias aéreas (Figuroa *et al.*, 2001), enquanto que o receptor CysLT2 encontra-se majoritariamente expresso em macrófagos intersticiais. No entanto, esses receptores têm uma distribuição variada nos tecidos em geral (tabela 1.3.1), o que determina diferentes consequências biológicas em decorrência da sua ativação:

**Tabela 1.3.1:** Distribuição dos receptores de leucotrienos cisteinados nos diferentes tecidos e organismos e sua modulação farmacológica (Singh *et al.*, 2010):

Receptores	Sinalização	Sítio de Expressão	Agonistas (afinidade)	Antagonistas
CysLT1	Gαq	Traquéia e Íleo de Cobaias	LTD <sub>4</sub> >> LTC <sub>4</sub> > LTE <sub>4</sub>	<i>Seletivos:</i> montelukaste, zafirlucaste e pranlucaste MK571 (murino)
		Brônquios Humanos		
		Pulmão de Ratos		
		Eosinófilos, Linfócitos B, Basófilos, Monócitos e Mastócitos Humanos		
CysLT2	Gαq	Eosinófilos, Macrófagos e Mastócitos Humanos	LTD <sub>4</sub> = LTC <sub>4</sub> > LTE <sub>4</sub>	BAYu9773
		Veia Pulmonar Humana		
		Tráqueia e Íleo de Cobaias		
		Traquéia e Brônquios de Ovelhas		

Em leucócitos, estudos de hibridização *in situ* demonstraram que ambos os receptores CysLT1 e CysLT2 encontram-se expressos em eosinófilos humanos (Heise *et al.*, 2000; Figueroa *et al.*, 2001). A expressão não é seletiva para eosinófilos, visto que outros tipos de leucócitos também expressam o receptor CysLT1, como monócitos/macrófagos, linfócitos B e células CD34<sup>+</sup> (Figueiroa *et al.*, 2001). O receptor CysLT2 precisa ser melhor caracterizado, porém foi encontrado em 20% de monócitos de sangue periférico humano (Heise *et al.*, 2000). Em basófilos humanos, ambos os receptores foram encontrados expressos na superfície celular (Gauvreau *et al.*, 2005). A presença desses receptores na superfície dos eosinófilos (Figueiroa *et al.*, 2001; Heise *et al.*, 2000), basófilos (Gauvreau *et al.*, 2005) e mastócitos (Mellor *et al.*, 2001; Mellor *et al.*, 2003), sugere que pode ocorrer uma ativação autócrina, visto que estas células são capazes de produzir esse mediador inflamatório (MacGlashan, Jr. *et al.*, 1982; MacGlashan, Jr. *et al.*, 1986; Cowburn *et al.*, 1998; Lam *et al.*, 2002). Alguns estudos têm evidenciado alterações na expressão desses dois receptores nos eosinófilos em diferentes quadros patológicos, o que poderia ter impacto funcionalmente importante. Por exemplo, em recente estudo foi demonstrado que ambos os receptores apresentaram-se superexpressos em pacientes com rinosinusite crônica e pólipos

nasais. No entanto, nos pacientes com rinosinusite crônica existia um equilíbrio nessa expressão, enquanto que nos pacientes com pólipos nasais a expressão do receptor CysLT1 foi maior do que a do receptor CysLT2 (Perez-Novo *et al.*, 2006).

Além dessas atividades parácrinas/autócrinas, outro aspecto importante da regulação da atividade de leucócitos se refere ao sítio de síntese do LTC<sub>4</sub>. Já foi demonstrado que o sítio de produção do leucotrieno pode definir a função desse eicosanóide, pois quando produzido em organelas distribuídas no ambiente citoplasmático pode ter funções parácrinas (Lewis *et al.*, 1990; Henderson, Jr., 1994; Lee *et al.*, 2000) e/ou autócrinas (Lee *et al.*, 2000) diversas, além de poder ter função intrácrina (Bandeira-Melo *et al.*, 2002c; Neves *et al.*, 2008).

### **1.3.2. Mecanismos de síntese:**

A síntese dos leucotrienos cisteinados inicia-se a partir da liberação do AA por ação da enzima fosfolipase A<sub>2</sub> (PLA<sub>2</sub>) (Henderson, 1994). Uma vez liberado, o AA se liga a proteína ativadora da 5-LO (FLAP) que irá apresentar o AA para a 5-Lipoxigenase (5-LO). Com isso, o AA sofrerá uma reação de oxidação no carbono 5 (C5) por ação da 5-LO, sendo convertido em ácido 5S-hidroperoxi-eicosatetraenóico (5-HPETE), e por ação dessa mesma enzima, o HPETE será biotransformado no intermediário epóxido ácido 5,6-óxido-7,9-trans-11,14-cis-eicosatetraenóico, que é o LTA<sub>4</sub> (Lewis *et al.*, 1990; Peters-Golden *et al.*, 2000).

Após a conclusão dessa etapa, por ação da enzima LTC<sub>4</sub> sintase, uma glutationa é conjugada ao carbono 6 (C6) do LTA<sub>4</sub>, fazendo com que essa molécula seja convertida em LTC<sub>4</sub>. Após isso, o LTC<sub>4</sub> é transportado ativamente para o meio extracelular através da membrana plasmática, num processo mediado por carreador (Lam *et al.*, 2002), denominado MRP-1 (*Multidrug Resistance Protein-1*) (Leier *et al.*, 1994). Extracelularmente, esse produto sofrerá reações enzimáticas, da enzima  $\gamma$ -glutamil-transpeptidase que clivará o ácido glutâmico do LTC<sub>4</sub> – resultando na formação do LTD<sub>4</sub> – e, posteriormente, da enzima cisteinil-glicina-dispeptidase, que vai clivar a glicina, resultado na formação do LTE<sub>4</sub> (Parker *et al.*, 1980). A figura 1.3.2 ilustra a via de síntese dos leucotrienos cisteínados.

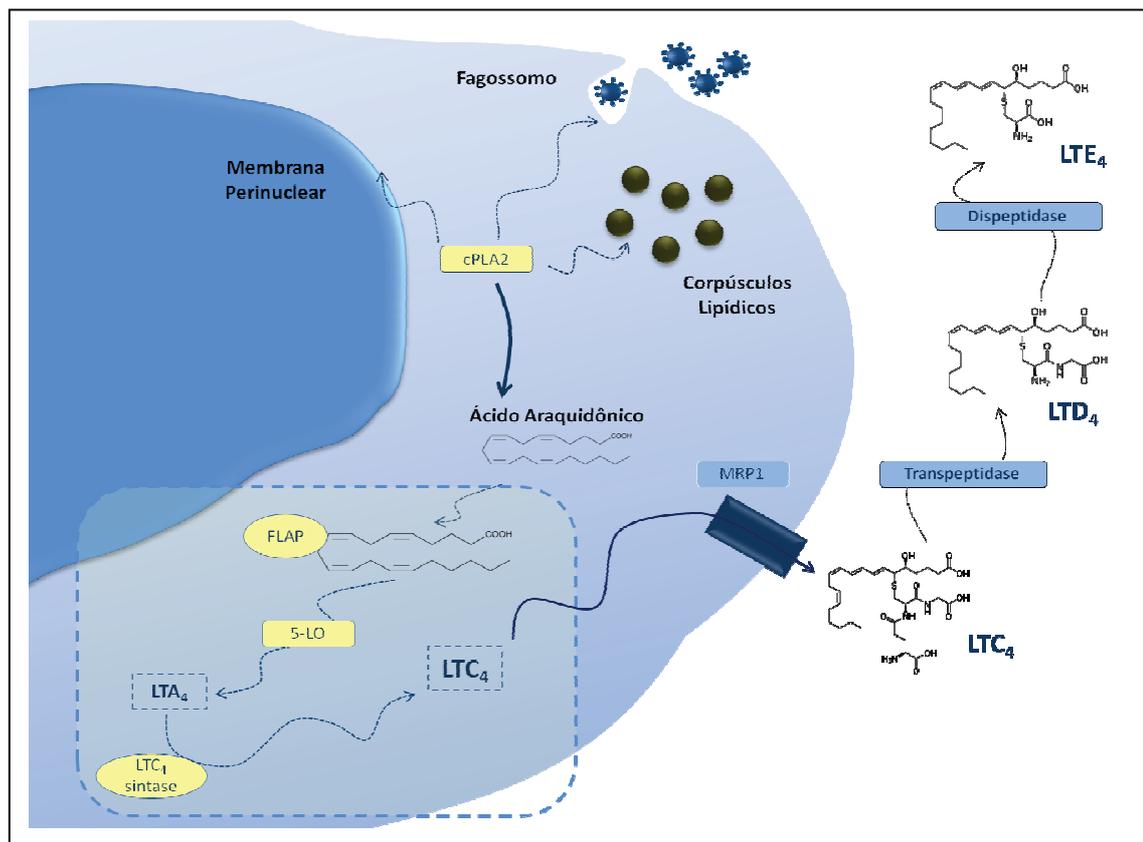
Cada enzima dessa complexa cascata enzimática apresenta características particulares que valem ser abordadas. Foi demonstrado que, em eosinófilos humanos

estimulados com fmlp (“*Formyl-Methionyl-Leucyl-Phenylalanine*”), a liberação de AA de membranas fosfolipídicas é promovida pela PLA<sub>2</sub> citosólica (cPLA<sub>2</sub>), num fenômeno dependente da ativação de ERK1/2 e p38 MAPK (Zhu *et al.*, 2001). Recentes estudos têm demonstrado que na ausência de ativação da cPLA<sub>2</sub>, a PLA<sub>2</sub> secretória grupo V (sPLA<sub>2</sub>-V) é capaz de iniciar a síntese de cisLTs em eosinófilos humanos (Munoz *et al.*, 2003; Munoz *et al.*, 2006). As enzimas LTC<sub>4</sub> sintase e FLAP são proteínas integrais de membrana, sendo a FLAP distribuída nas porções internas e externas da membrana nuclear, assim como na periferia do retículo endoplasmático, enquanto que a LTC<sub>4</sub> sintase encontra-se majoritariamente presente na porção externa da membrana nuclear e na periferia do retículo endoplasmático (Christmas *et al.*, 2002). Além disso, foi demonstrado que essas proteínas podem ser encontradas associadas, como complexos enzimáticos, formando dímeros ou trímeros (Mandal *et al.*, 2004).

A enzima 5-LO é uma proteína solúvel que, em células não estimuladas, pode ser encontrada tanto no citoplasma quanto no núcleo (Brock *et al.*, 1994). Foi demonstrado que em neutrófilos e monócitos, essa enzima está presente no citoplasma, enquanto que em macrófagos alveolares e mastócitos é encontrada no núcleo (Peters-Golden *et al.*, 2000). Além da sua expressão variar entre o citoplasma e núcleo, estudos demonstraram que essa localização intracelular pode se alterar em função do estímulo utilizado. Em eosinófilos humanos foi demonstrado que, estímulos como ionóforo de cálcio (A23187) ou eotaxina, promove aumento nos níveis intracelulares de cálcio, levando a 5-LO a mudar de compartimento, indo respectivamente, do citoplasma para a membrana perinuclear – ou para os corpúsculos lipídicos – num fenômeno denominado de translocação (Bandeira-Melo *et al.*, 2002a). Além disso, vale ressaltar que o processo de translocação de 5-LO dependente de estímulo também já foi observado em outros leucócitos, como por exemplo em basófilos e macrófagos alveolares de rato (Brock *et al.*, 1995), assim como, em macrófagos alveolares humanos (Woods *et al.*, 1995). No entanto, em estudo recente foi demonstrado também que é possível a biogênese de corpúsculos lipídicos já contendo 5-LO (Wan *et al.*, 2007).

Todo esse complexo processo de síntese não ocorre apenas em eosinófilos, mas também em outros tipos celulares como mastócitos (MacGlashan, Jr. *et al.*, 1982), basófilos (MacGlashan, Jr. *et al.*, 1986) e monócitos (Lam *et al.*, 2002). As plaquetas e

as células endoteliais são capazes de sintetizar LTC<sub>4</sub>, num processo de síntese transcelular, pois utilizam o LTA<sub>4</sub> produzido e liberado por neutrófilos para realizar o processo de síntese do LTC<sub>4</sub> (Maclouf *et al.*, 1988; Maclouf *et al.*, 1989). Eosinófilos humanos produzem exclusivamente LTC<sub>4</sub> – em detrimento da produção de LTB<sub>4</sub> – pois não expressam a enzima LTA<sub>4</sub> hidrolase, enzima responsável pela hidrolização do LTA<sub>4</sub> e, conseqüente formação do LTB<sub>4</sub> (Jendraschak *et al.*, 1996).

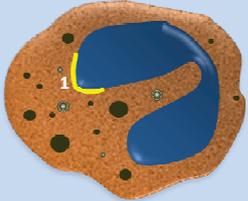
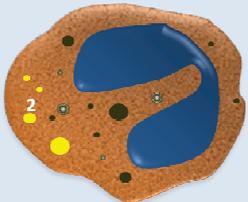
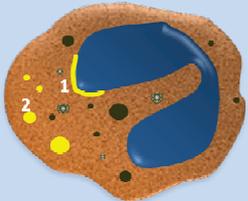


**FIGURA 1.3.2 – Ilustração representativa dos eventos ocorridos na cascata da síntese de leucotrienos a partir do Ácido Araquidônico (AA) liberado pela ação da fosfolipase A<sub>2</sub>.** Os eventos enzimáticos em destaque acontecem nas membranas (perinuclear e fagocítica) e nos corpúsculos lipídicos. Eventos extracelulares representam as transformações enzimáticas necessárias a síntese de LTD<sub>4</sub> e LTE<sub>4</sub> (Transpeptidase =  $\gamma$ -glutamyltranspeptidase / Dispeptidase = cisteinil-glicina-dispeptidase).

Alguns estímulos já foram descritos como sendo capazes de promover a síntese de LTC<sub>4</sub> em eosinófilos. Foi demonstrado que as citocinas IL-5 e IL-3 são capazes de primar eosinófilos humanos *in vitro*, promovendo aumento na síntese de LTC<sub>4</sub> induzida por PAF, C5a ou fmlp (Takafuji *et al.*, 1991). O PAF já havia sido demonstrado ser um

estímulo capaz de promover a síntese de LTC<sub>4</sub> em eosinófilos (Bruijnzeel *et al.*, 1987). O ácido araquidônico também se mostrou um estímulo competente na ativação da 5-LO – num processo dependente de cálcio – levando à síntese de LTC<sub>4</sub> por eosinófilos humanos (Kok *et al.*, 1988). Recentemente, nosso grupo identificou a PGD<sub>2</sub> e o MIF, como estímulos capazes de induzir a síntese desse eicosanóide em eosinófilos humanos (Mesquita-Santos *et al.*, 2006; Vieira-de-Abreu *et al.*, 2010).

Além de todos os fenômenos bioquímicos relacionados à regulação do maquinário enzimático envolvido na síntese de LTC<sub>4</sub> em eosinófilos, a ativação dessas células irá ocasionar alterações organelares e irá fazer com que a síntese desse eicosanóide ocorra em sítios específicos. A localização específica do sítio de síntese de LTC<sub>4</sub> em eosinófilos foi identificada, mostrando que o sítio de síntese é estímulo-dependente (figura 1.3.3). Foi descrito que a ativação de LIR-7 (“*Leukocyte immunoglobulin-like receptor 7*”) e de CD9 – moléculas expressas na superfície eosinofílica – promoveu a síntese de LTC<sub>4</sub> na membrana perinuclear dos eosinófilos (Tedla *et al.*, 2003), assim como quando essas células foram ativadas por A23187 (Bandeira-Melo *et al.*, 2001b); Já a ativação dessas células com eotaxina, RANTES (Bandeira-Melo *et al.*, 2001b), IL-16 (Bandeira-Melo *et al.*, 2002b), PGD<sub>2</sub> (Mesquita-Santos *et al.*, 2006) e MIF (Vieira-de-Abreu *et al.*, 2010) induziu a síntese de LTC<sub>4</sub> em corpúsculos lipídicos; enquanto que a ativação dessas células pela combinação de eotaxina e A23187, revelou que esse fenômeno ocorreu tanto na membrana perinuclear quanto nos corpúsculos lipídicos (Bandeira-Melo *et al.*, 2001b), organelas que vêm se destacando como importantes reguladores de síntese de eicosanóides.

SÍTIO DE SÍNTESE	ESTÍMULO	REFERÊNCIA
	A23187 Anti-CD9 Anti-LIR-7	Bandeira-Melo <i>et al</i> , 2001b Tedla <i>et al</i> , 2003 Tedla <i>et al</i> , 2003
	Eotaxina RANTES MIF PGD <sub>2</sub> IL-16	Bandeira-Melo <i>et al</i> , 2001b Bandeira-Melo <i>et al</i> , 2001b Vieira-de-Abreu <i>et al</i> , 2010 Mesquita-Santos <i>et al</i> , 2006 Bandeira-Melo <i>et al</i> , 2002b
	Eotaxina (+ A23187)	Bandeira-Melo <i>et al</i> , 2001b

**FIGURA 1.3.3** – Ilustração representativa dos sítios de síntese de LTC<sub>4</sub> em eosinófilos em função de estímulos específicos (1 – Membrana Perinuclear / 2 – Corpúsculos Lipídicos).

## 1.4. CORPÚSCULOS LIPÍDICOS:

### **1.4.1. Características morfológicas:**

Os corpúsculos lipídicos são organelas citoplasmáticas que estão presentes em um número variado de células de organismos animais (mamíferos e não-mamíferos), de leveduras, plantas e procariotos. Também são conhecidos como adipossomos, gotas lipídicas, partículas lipídicas, inclusões lipídicas ou vacúolos lipídicos (Murphy, 2001).

São compostos por material amorfo com constituição rica em lipídios apolares (como triacilglicerol e ésteres de colesterol) (Tsuchi-Sato *et al.*, 2002), com conteúdo proteico específico e envolvidos por uma monocamada de fosfolipídios. Essas organelas podem apresentar áreas centrais ou excêntricas, que apresentam vesículas e material particulado escuro (Johnson *et al.*, 1999; Murphy, 2001; D'Avila *et al.*, 2006; Melo *et al.*, 2006).

A análise por microscopia eletrônica, revelou a existência de estruturas membranares internas (Robenek *et al.*, 2004). Embora a origem e a função dessas membranas internas ainda não estejam bem caracterizadas, as mesmas se apresentam como um emaranhado de membranas – provavelmente provenientes do retículo endoplasmático (Wan *et al.*, 2007) – onde se localizam diversas proteínas, como as da família da perilipina (perilipina, ADRP e TIP47) (Robenek *et al.*, 2004) e proteínas integrais de membrana, como as que estão relacionadas com a formação dos eicosanóides, como a FLAP (Silva *et al.*, 2009), cPLA<sub>2</sub> (Yu *et al.*, 2002; Moreira *et al.*, 2009); a LTC<sub>4</sub> sintase (Bozza *et al.*, 1997); 5-LO (Bandeira-Melo *et al.*, 2001b; Silva *et al.*, 2009) e COX (Dvorak *et al.*, 1992; D'Avila *et al.*, 2006).

### **1.4.2. Regulação da biogênese de corpúsculos lipídicos:**

O processo de biogênese dessas organelas ainda não está completamente elucidado e compreendido, embora muitos estudos tenham sido feitos nesse sentido. Dessa forma, muitas hipóteses para explicar esse fenômeno são propostas. A primeira hipótese – e ainda hoje a mais aceita – sugere que o processo biogênico, ocorra a partir da síntese de lipídios neutros, entre os folhetos do retículo endoplasmático, que ao alcançar certo tamanho, saíam por brotamento dessa região, justificando assim a sua

monocamada de fosfolipídios e proteínas sem domínios transmembranares (Brown, 2001; Murphy, 2001; Robenek *et al.*, 2004; Martin *et al.*, 2006).

Essas organelas caracterizam-se por serem estruturas osmiofílicas e que na maioria das preparações para microscopia ótica não são observadas, por serem utilizados fixadores/corantes a base de álcool, o que causariam a dissolução das estruturas lipídicas (Pacheco *et al.*, 2002). Essa osmiofilia é dependente do tipo celular e do estado no qual a resposta inflamatória se encontra. Por exemplo, foi demonstrado que a infecção pelo *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) provocou alterações estruturais nos corpúsculos lipídicos de macrófagos peritoniais de camundongos em função do tempo de infecção, como aumento na eletrodensidade e variação no tamanho (D'Avila *et al.*, 2006).

Durante muito tempo, a importância dessas organelas foi relacionada com o armazenamento de lipídios neutros, funcionando como reservatórios lipídicos para obtenção posterior de energia através da  $\beta$ -oxidação dos mesmos (Ducharme *et al.*, 2008). Entretanto, essas mudanças morfológicas observadas nos corpúsculos lipídicos nos indicam que estas organelas têm um comportamento dinâmico (são funcionalmente ativas), ou seja, são capazes de mudar a sua estrutura em função do tipo de ativação celular a que foram submetidas.

Vários estudos vêm sendo realizados, com o objetivo de compreender a regulação da biogênese de novos corpúsculos lipídicos citoplasmáticos nas células envolvidas com a resposta inflamatória. Observações feitas apontam para um fenômeno agudo, possível de ser detectado dentro de uma hora após o estímulo, além de ser célula e estímulo-dependente (Bozza *et al.*, 2009).

Especificamente em eosinófilos humanos recém-isolados de doadores saudáveis, essas organelas estão presentes em uma quantidade, aproximadamente, três vezes maior do que nos neutrófilos destes mesmos doadores. Seu tamanho e seu número também são observados aumentados nessas células, em quadros patológicos de características eosinofílicas, como por exemplo, em pacientes com síndrome hiper-eosinofílica (HES) (Bozza *et al.*, 1998) e em pacientes asmáticos estimulados por antígeno (comunicação pessoal; Weller PF). Estudos têm demonstrado que, nessas células, diversos estímulos/agonistas específicos podem iniciar os mecanismos de sinalização necessário para promover a formação de novos corpúsculos lipídicos

(tabela 1.4.1). O PAF – um dos primeiros estímulos a ser caracterizado nesse sentido – tem se mostrado capaz de induzir a formação de corpúsculos lipídicos atuando via seu receptor (PAFR). Esse receptor sinaliza através de proteína G inibitória ( $G_{\alpha i}$ ) e a cascata de sinalização é dependente de proteína quinase C (PKC) e fosfolipase C (PLC). Além disso, há ativação de 5-LO, com formação de 5-HETE que também intermedia a biogênese de corpúsculos lipídicos induzida por PAF (Bozza *et al.*, 1996a; Bozza *et al.*, 1997; Bozza *et al.*, 1998).

Outros agonistas seletivos capazes de induzir biogênese de corpúsculos lipídicos em eosinófilos já foram caracterizados, incluindo as quimiocinas RANTES, eotaxina-1, eotaxina-2 e eotaxina-3, através da ativação do receptor CCR3, em eosinófilos e basófilos, mas não em neutrófilos (Bandeira-Melo *et al.*, 2001b; Vieira-de-Abreu *et al.*, 2005b). O CCR3, ao ser ativado, induz a formação de novos corpúsculos lipídicos, numa cascata de sinalização dependente de ativação de fosfoinositol 3-quinase (PI3K), ERK1/2 e p38 MAP quinase. Diferente do visto para a estimulação com o PAF, a via de sinalização promovida por CCR3 não depende da ativação de 5-LO, PKC ou PLC, assim como não depende de PAF (Bandeira-Melo *et al.*, 2001a; Bandeira-Melo *et al.*, 2001b). Recentemente, foi identificado que bradicinina, substância P e  $PGE_2$  são capazes de induzir a biogênese de corpúsculos lipídicos em eosinófilos humanos, através de via de sinalização dependente de PKA (Bakker-Abreu, não-publicado). Dessa forma, essas observações indicam que o processo de formação dos corpúsculos lipídicos é um fenômeno altamente regulado e dependente de estímulo apropriado, além de ser específico para cada tipo celular. Essa conclusão pode ser corroborada, com a observação de que estímulos como  $LTB_4$ , fragmentos do complemento (C5a e C3a) e IL-8 não foram capazes de induzir formação de corpúsculos lipídicos em eosinófilos (Bozza *et al.*, 1996a).

<b>Estímulo</b>	<b>Biogênese de Corpúsculos Lipídicos</b>	<b>Via de Sinalização</b>
<b>Ácidos Graxos</b>		
Insaturados	+	n.d.
Saturados	-	-
<b>Fosfolipídios</b>		
PAF	+	Gαi; PKC; PLC
Liso-PAF	-	-
<b>Eicosanóides</b>		
5-HETE	+	Gαi
PGE <sub>2</sub>	+	PKA
LTB <sub>4</sub>	-	-
<b>Produtos Bacterianos</b>		
LPS / LAM	n.d.	n.d.
Fmlp	n.d.	n.d.
<b>Lipoproteínas</b>		
LDLox / LDLac	n.d.	n.d.
LDL	n.d.	n.d.
<b>Quimiocinas</b>		
Eotaxina / RANTES	+	Gαi; PI3K ; ERK1/2; p38 MAPK
IL-8	-	n.d.
MIF	+	Produção de eotaxina endógena
<b>Citocinas</b>		
IL-5	+	Produção de PAF endógeno
IL-16	+	Produção de RANTES e eotaxina
<b>Peptídeos</b>		
BK	+	n.d.
Substância P	+	n.d.
C3a / C5a	-	-

**Tabela 1.4.1 – Fatores capazes de induzir a formação de corpúsculos lipídicos em eosinófilos humano.**

(n.d.= não determinado; PAF= Fator de Ativação Plaquetária; 5-HETE= Ácido Eicosatetraenóico; PGD<sub>2</sub>= Prostaglandina D<sub>2</sub>; LTB<sub>4</sub>=Leucotrieno B<sub>4</sub> ; LPS= Lipopolissacarídeo; LAM= Lipoarabinomanana; FMLP= Fenil-Metil-Leucil-Fenilalanina; LDLox= Lipídio de baixa densidade oxidado; LDLac= Lipídio de baixa densidade acetilado; BK= Bradicnina) (Bozza et al, 2007; Mesquita-Santos et al, 2010; Vieira-de-Abreu et al, 2010b).

Como todas essas observações no tocante a biogênese de corpúsculos lipídicos em eosinófilos abordadas até agora, foram de estudos realizados em eosinófilos humanos purificados *in vitro*, foi questionado como esse processo estaria sendo modulado numa situação mais complexa de ativação eosinofílica. Dessa forma, em

estudo recente realizado por nosso grupo, foi demonstrado que o desafio alérgico em camundongos sensibilizados, foi capaz de promover a formação de novos corpúsculos lipídicos nos eosinófilos recrutados para o sítio da reação inflamatória alérgica. Esse fenômeno mostrou-se ser dependente de ativação do receptor CCR3, demonstrando que a biogênese de corpúsculos lipídicos nos eosinófilos recrutados durante a resposta inflamatória alérgica é dependente de eotaxina endógena produzida durante esse processo inflamatório (Vieira-de-Abreu *et al.*, 2005b). Mais recentemente, foi demonstrado que o MIF também participa do processo de ativação eosinofílica, pois com a utilização de animais MIF *knockout*, foi possível observar – durante a resposta alérgica – que a ausência da produção dessa citocina levou a uma diminuição no recrutamento de eosinófilos para a cavidade pleural, na biogênese de corpúsculos lipídicos nesses eosinófilos infiltrantes, assim como na síntese de LTC<sub>4</sub> característica dessa resposta. A pleurisia induzida pelo MIF em animais selvagens, mostrou que os corpúsculos lipídicos dos eosinófilos infiltrantes na cavidade pleural correspondiam ao sítio de síntese do LTC<sub>4</sub>. Esses efeitos do MIF também se mostraram ser dependente da produção de eotaxina endógena (Vieira-de-Abreu *et al.*, 2010).

Esses estudos demonstram que a eotaxina, produzida durante a resposta alérgica, desempenha um importante papel na modulação da ativação dos eosinófilos. Observamos também que a PGD<sub>2</sub> produzida durante o desenvolvimento da resposta alérgica, é o mediador responsável por promover o recrutamento de eosinófilos para a cavidade pleural, assim como, induzindo a biogênese de corpúsculos lipídicos e, também, a síntese de LTC<sub>4</sub> nessas organelas citoplasmáticas neo-formadas nas células infiltrantes. E a produção da PGD<sub>2</sub> mostrou-se dependente da eotaxina produzida durante o processo inflamatório alérgico. A importância desses mediadores na ativação eosinofílica, foi corroborada em ensaios *in vitro* com eosinófilos humanos purificados, mostrando que tanto a eotaxina, quanto a PGD<sub>2</sub> cooperam sinergicamente na ativação de eosinófilos (Mesquita-Santos *et al.*, 2006).

#### **1.4.3. Correlação entre a biogênese de corpúsculos lipídicos e síntese de eicosanóides:**

Em leucócitos – e em outras células associadas com o processo inflamatório – os corpúsculos lipídicos aparecem em número caracteristicamente aumentado durante

o desenvolvimento de respostas inflamatórias de origens diversas como, na sepse bacteriana (Pacheco *et al.*, 2002), na artrite (Weinstein, 1980; Bozza *et al.*, 1996b), em infecções por bactérias do gênero *mycobacterium* (Mukherjee *et al.*, 1989; D'Avila *et al.*, 2006) e inflamações alérgicas pulmonares (Vieira-de-Abreu *et al.*, 2005b; Mesquita-Santos *et al.*, 2006). Em patologias de caráter eosinofílico, foi demonstrado que em eosinófilos de pacientes com HES (Solley *et al.*, 1976; Weller, 1991), com doença de Cronh (Beil *et al.*, 1995), com asma (comunicação pessoal; Weller PF), essas células apresentavam o número de corpúsculos lipídicos caracteristicamente aumentado, quando comparado com os eosinófilos circulantes de doadores normais.

Especificamente nos eosinófilos, foi observado que o aumento do número dos corpúsculos lipídicos se correlaciona diretamente com uma produção aumentada de LTC<sub>4</sub> por essas células. Foi demonstrado que os eosinófilos quando estimulados por PAF, ácidos graxos *cis*-insaturados, RANTES ou eotaxinas, primeiro apresentam aumento no número dos corpúsculos lipídicos e subsequente a ocorrência desse fenômeno, quando estimulados com concentração submaximal de ionóforo de cálcio (A23187), são induzidos a aumentar a liberação de LTC<sub>4</sub>. Esses mesmos efeitos relacionados a síntese de LTC<sub>4</sub>, foram observados em relação a síntese de PGE<sub>2</sub>, demonstrando a importância funcional dessas organelas para a síntese de eicosanóides (Bozza *et al.*, 1996b; Bozza *et al.*, 1997; Bandeira-Melo *et al.*, 2001a; Bandeira-Melo *et al.*, 2001b). Essa correlação também foi observada em outros tipos celulares, de modo similar aos eosinófilos. Por exemplo, foi observado que a secreção aumentada de PGE<sub>2</sub> por macrófagos pleurais de camundongos, mostrou-se diretamente associada a um aumento do número de corpúsculos lipídicos, que funcionara como sítio de síntese desse eicosanóide (D'Avila *et al.*, 2006).

#### **1.4.4. Corpúsculos lipídicos eosinofílicos como sítio de síntese de LTC<sub>4</sub>:**

A constituição lipídica e proteica-enzimática dos corpúsculos lipídicos caracterizam essas organelas como sítio de síntese de eicosanóides, corroborando as observações da correlação direta entre a biogênese dessas organelas e a produção de eicosanóides.

Especificamente nos corpúsculos lipídicos de eosinófilos humanos, encontram-se araquidonil fosfolipídios e a fosfolipase A<sub>2</sub> citosólica (cPLA<sub>2</sub>) (Bozza *et al.*, 1997), além

de várias enzimas necessárias a síntese dos eicosanóides como a 5-LO, 15-LO (Bozza *et al.*, 1998), COX (Dvorak *et al.*, 1994b) e LTC<sub>4</sub> sintase (Bozza *et al.*, 1997). Vale destacar que também foi observado nos corpúsculos lipídicos de leucócitos de camundongos, a presença da enzima FLAP (Bozza *et al.*, 2009; Silva *et al.*, 2009).

No entanto, mesmo com todas essas observações, a confirmação definitiva de que a síntese de LTC<sub>4</sub> pode ocorrer nos corpúsculos lipídicos dos eosinófilos, só foi conseguida através da imunolocalização direta desses eicosanóides em seus locais de síntese. Essa imunolocalização foi possível, graças a utilização de uma técnica nomeada de *EicosaCell*. Nessa técnica, a utilização do EDAC (*1-ethyl-3-(3-dimethylamino-propyl) carbodiimide*) para a fixação e permeabilização das células, permite que o eicosanóide recém-sintetizado no seu sítio de síntese, seja fixado – através da ligação cruzada das carboxilas dos lipídios com as extremidades amina das proteínas adjacentes – às proteínas presentes no sítio de síntese (Bandeira-Melo *et al.*, 2001b; Bandeira-Melo *et al.*, 2011). Essa técnica tem se mostrado uma importante ferramenta na identificação dos corpúsculos lipídicos como sítios intracelulares de produção de outros eicosanóides, como a PGE<sub>2</sub>, (D'Avila *et al.*, 2006); e o LTB<sub>4</sub> (Bozza *et al.*, 2009) em macrófagos, por exemplo.

Vale ressaltar que essa técnica possibilitou observar que a produção de LTC<sub>4</sub> pode ocorrer em outros sítios intracelulares, além dos corpúsculos lipídicos, como a membrana perinuclear de eosinófilos, observado quando essas células foram estimuladas com eotaxina em associação com A23187 (Bandeira-Melo *et al.*, 2001b),  $\alpha$ -LIR-7 ou  $\alpha$ -CD9 (Tedla *et al.*, 2003) ou nos fagossomos de macrófagos estimulados com zimozam (Balestrieri *et al.*, 2006).

## 1.5. PROSTAGLANDINA D<sub>2</sub> (PGD<sub>2</sub>):

### 1.5.1. Mecanismos de síntese e fenômenos envolvidos:

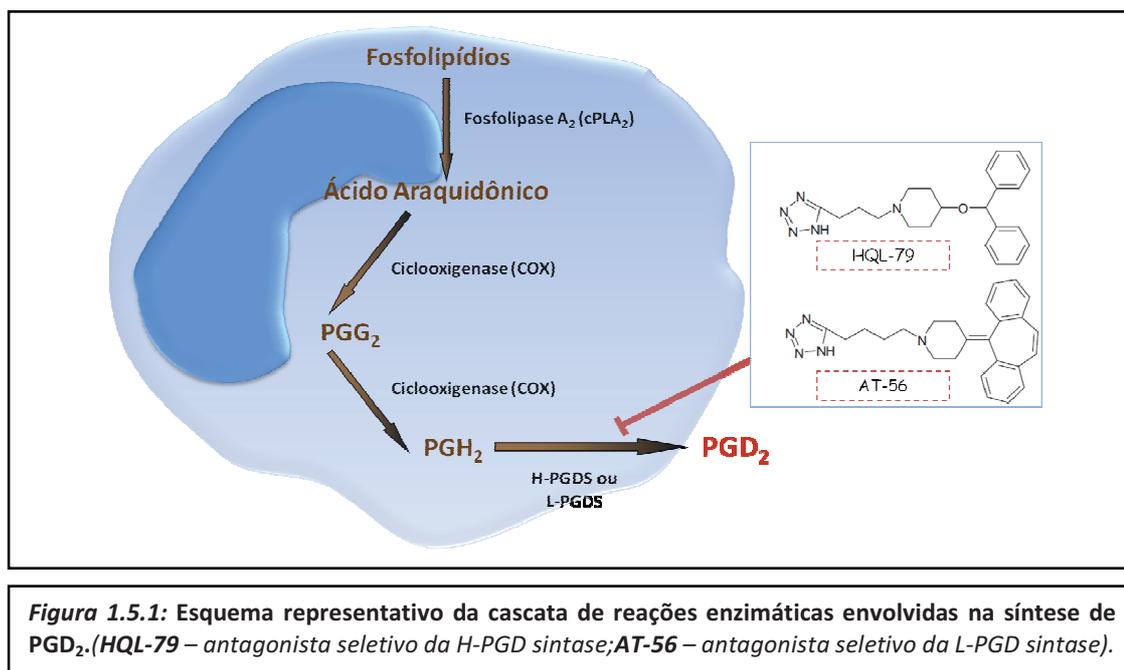
Dentre os mediadores inflamatórios produzidos durante os processos inflamatórios alérgicos, a prostaglandina D<sub>2</sub> (PGD<sub>2</sub>) destaca-se por ser o principal metabólito do ácido araquidônico, produzido pela ciclooxigenase nos mastócitos em resposta a um desafio antigênico. É liberada em grandes quantidades quando estas células estão ativadas, sendo considerado um biomarcador de ativação de mastócitos na asma (Murray *et al.*, 1986).

Na síntese dos prostanóides, o primeiro passo é a liberação do ácido araquidônico esterificado a partir da posição 2 (*sn-2*) dos glicerofosfolípídeos que estão presentes nas membranas celulares, pela ação da fosfolipase A<sub>2</sub> citosólica (cPLA<sub>2</sub>) (Diaz *et al.*, 2003). Nessa via de síntese, o AA liberado sofre ação da enzima ciclooxigenase (COX), que pode ser encontrada sob duas isoformas: COX-1, presente constitutivamente na maioria dos tipos celulares, e a COX-2, que é a forma induzida por estímulos inflamatórios. Ambas são responsáveis pela síntese dos prostanóides como as prostaglandinas (PG) (PGE<sub>2</sub>, PGI<sub>2</sub>, 5-keto-PGF<sub>1α</sub> e PGD<sub>2</sub>) e os tromboxanos (TXA<sub>2</sub>, TXB<sub>2</sub>) (Caughey *et al.*, 2001). O mecanismo de ação da COX consiste na promoção de duas reações na molécula do ácido araquidônico. Na primeira reação, a COX insere 2 (duas) moléculas de oxigênio no AA, para originar a PGG<sub>2</sub>, e depois promove uma reação de endoperoxidase, que reduz a PGG<sub>2</sub> ao seu análogo 15-hidroxi, que é PGH<sub>2</sub>. A PGH<sub>2</sub> irá servir de substrato para a formação dos eicosanóides citados acima, como a prostaglandina D<sub>2</sub> (PGD<sub>2</sub>) (Smith *et al.*, 1991).

Existem dois tipos de enzimas que sintetizam a PGD<sub>2</sub>: (i) PGD<sub>2</sub> sintase tipo lipocalina (L-PGDS) e (ii) PGD<sub>2</sub> sintase hematopoética (H-PGDS). Ambas têm a capacidade de promover a mesma reação que é a isomerização da PGH<sub>2</sub> à PGD<sub>2</sub>. A L-PGDS é uma proteína secretória de 26 kDa expressa por diversos tecidos, como por exemplo células da meninge, células epiteliais do plexo coróide e oligodendrócitos no cérebro, e por células epiteliais do epidídimo e de Leydig no testículo (Urade *et al.*, 2000). Dessa forma, a produção desse eicosanóide por esta enzima está relacionada com a ocorrência de alguns fenômenos fisiológicos, como funções autonômicas e

sensoriais das células nervosas (Goerg *et al.*, 1991; Andreeva *et al.*, 1993), nocicepção (Eguchi *et al.*, 1999) e indução de sono (Hayaishi, 1991; Urade *et al.*, 1999).

Já a H-PGDS é uma proteína citosólica – de mesmo peso molecular que a L-PGDS – também responsável pela biossíntese de  $PGD_2$ , sendo que a sua expressão ocorre promeminentemente em mastócitos (Urade *et al.*, 1990), e também em células apresentadoras de antígeno – como histiócitos, células de Kupfer e células dendríticas de rato (Urade *et al.*, 1989) e humana (Shimura *et al.*, 2010) – e células Th2 (Tanaka *et al.*, 2000; Kanaoka *et al.*, 2003). Dessa forma, a produção de  $PGD_2$  tem sido amplamente associada a patologias de fundo alérgico, como dermatite atópica (Barr *et al.*, 1988; Charlesworth *et al.*, 1989); rinite alérgica (Naclerio *et al.*, 1983) e conjuntivite alérgica (Proud *et al.*, 1990). Especificamente no tocante a resposta inflamatória asmática, esse eicosanóide é responsável por alguns sinais clínicos característicos, como broncoconstrição (Hardy *et al.*, 1984) e vasodilatação (Cheng *et al.*, 2006). A figura 1.5.1 ilustra essas reações seqüenciais da síntese de  $PGD_2$  nos diferentes tipos celulares relacionados a esse evento.



**Figura 1.5.1:** Esquema representativo da cascata de reações enzimáticas envolvidas na síntese de  $PGD_2$ . (HQL-79 – antagonista seletivo da H-PGD sintase; AT-56 – antagonista seletivo da L-PGD sintase).

A síntese de  $PGD_2$  é um ponto importante no desenvolvimento da resposta inflamatória de fundo alérgico. Algumas drogas já foram descritas como sendo capazes de inibir a produção desse eicosanóide. O 4-benzhydryloxy-1-{3-(1H-tetraazol-5-yl)-

*propyl}piperidine* – conhecido como HQL-79, mostrou-se um potente inibidor da H-PGDS, impedindo a síntese de PGD<sub>2</sub> (mas não de PGE<sub>2</sub>) e apresentando efeitos antialérgicos/antiinflamatórios (Matsushita *et al.*, 1998a; Matsushita *et al.*, 1998b). A caracterização bioquímica e funcional do *4-dibenzo[a,d]cyclohepten-5-ylidene-1-[4-(2H-tetrazol-5-yl)-butyl]-piperidine* – composto chamado de AT-56 – o mostrou sendo um inibidor seletivo e oralmente ativo da L-PGDS. Estruturalmente, esses compostos são parecidos, visto que o AT-56 é derivado do HQL-79 (Figura 1.5.1). Mais recentemente, foram demonstradas outras moléculas capazes de inibir tanto a H-PGDS humana quanto a murina (Christ *et al.*, 2010).

Foi demonstrado que a eosinofilia das vias aéreas está correlacionada com aumento nos níveis de PGD<sub>2</sub> no lavado broncoalveolar (BAL) de pacientes com alergia respiratória provocados antigenicamente (Miadonna *et al.*, 1990). Devido a essa correlação, foi investigado qual seria o envolvimento da PGD<sub>2</sub> no acúmulo de eosinófilos *in vivo*. Demonstrou-se inicialmente que a injeção de PGD<sub>2</sub> na traquéia de cachorros induziu aumento no número de eosinófilos (e não de neutrófilos) no sítio inflamatório, fenômeno que foi abolido com o tratamento do antagonista do receptor de PGD<sub>2</sub>, o SKF 88046 (Emery *et al.*, 1989). Estudos posteriores, estabeleceram que a PGD<sub>2</sub> é capaz de ativar diretamente os eosinófilos, pois foi demonstrado que *in vitro* a PGD<sub>2</sub> é capaz de induzir quimiotaxia de eosinófilos com potência similar ao PAF, mas inferior a da eotaxina (Powell, 2003).

Além de induzir a quimiotaxia de eosinófilos, a PGD<sub>2</sub> também é capaz de promover outras respostas associadas à migração e ativação eosinofílica, como o aumento na expressão superficial da molécula de adesão celular CD11b, a remoção de L-selectina, a polimerização de actina e a mobilização de cálcio (Monneret *et al.*, 2001), além de induzir degranulação dessas células (Gervais *et al.*, 2001). Mais ainda, a PGD<sub>2</sub> parece ser o único prostanóide com atividade quimiotática para eosinófilos, visto que PGE<sub>2</sub>, PGF<sub>2</sub>, e o U46619 – agonista do receptor de TXA<sub>2</sub> – não foram capazes de atrair eosinófilos *in vitro* (Monneret *et al.*, 2001). Além disso, a PGD<sub>2</sub> parece mostrar seletividade para eosinófilos, visto que falhou em alterar os níveis intracelulares de cálcio dos neutrófilos e monócitos (Monneret *et al.*, 2002).

### **1.5.2. Caracterização dos Receptores:**

Inicialmente, os fenômenos observados e atribuídos a ação da PGD<sub>2</sub> foram justificados como sendo resultado da ativação do receptor DP (agora chamado de DP1). O desenvolvimento de camundongos deficientes para o receptor DP1 (DP1<sup>-/-</sup>) permitiu a avaliação da importância do mesmo no acúmulo de eosinófilos ocorrido durante a resposta alérgica. Dessa forma, foi visto que, em animais DP1<sup>-/-</sup>, sensibilizados e desafiados com OVA, não houve migração de eosinófilos quando comparado com animais selvagens submetidos ao mesmo modelo de ativação. Além disso, verificou-se que os níveis de IL-4, IL-5 e IL-13 no BAL dos camundongos DP1<sup>-/-</sup> mostravam-se significativamente menores do que dos animais selvagens. Em conjunto, esses dados sugerem que o receptor DP1 quando ativado seja o responsável pela modulação da liberação de mediadores inflamatórios que, conseqüentemente, culminaria com a indução da migração de eosinófilos para o sítio inflamatório, sugerindo assim que esse receptor regulasse esse fenômeno de maneira indireta. Além disso, neste mesmo estudo, também foi observado que este receptor encontrava-se expresso em pequena quantidade no pulmão dos animais selvagens e, que o desafio alérgico foi capaz de aumentar, notadamente, a expressão desse receptor na superfície das células epiteliais ciliadas dos bronquíolos, reforçando ainda mais o papel desse receptor no desenvolvimento da resposta alérgica (Matsuoka *et al.*, 2000).

Estruturalmente, o receptor DP1 encontra-se na membrana plasmática e apresenta 7 domínios transmembranares, sendo assim membro da subfamília dos GPCRs, estando associado a uma proteína G do tipo G<sub>s</sub> (proteína G estimulatória) (Hirata *et al.*, 1994; Monneret *et al.*, 2001). Estudos com células transfectadas com o gene para o receptor DP1 – células HEK293 (expressando o receptor DP1 humano) e células CHO (expressando o receptor DP1 murino) – demonstraram que a conseqüente ativação deste receptor foi capaz de promover a estimulação da adenilato ciclase, levando a um aumento nos níveis de AMPc e a um aumento nos níveis intracelulares de Ca<sup>+2</sup> (Hirata *et al.*, 1994; Boie *et al.*, 1995).

No entanto, o aumento nos níveis de AMPc associado a ativação de eosinófilos é intrigante, pois já foi demonstrado que esse fenômeno está relacionado com inibição da atividade dessas células. Rolipram, droga capaz de inibir fosfodiesterase tipo IV, inibiu a quimiotaxia de eosinófilos humanos induzida por PAF e C5a (componente do sistema

complemento), assim como inibiu eosinófilos murinos de responderem quimiotaticamente ao PAF *in vitro* (Tenor *et al.*, 1996; Alves *et al.*, 1996).

De fato, em concordância com o potencial inibitório do aumento intracelular do AMPc induzido pela ativação de DP1, durante o processo de caracterização dos aspectos envolvidos na modulação da ativação dos eosinófilos pela PGD<sub>2</sub>, foi observado que o agonista seletivo para o receptor DP1 – BW 245c – não conseguiu promover os mesmos efeitos observados pela PGD<sub>2</sub> em eosinófilos *in vitro*. Esta hipótese foi reforçada pela observação de que o antagonista seletivo para o receptor DP1 – BWA 868c – foi incapaz de bloquear a quimiotaxia de eosinófilos induzida pela PGD<sub>2</sub>. Com isso, postulou-se a existência de um segundo receptor, visto que a quimiotaxia de eosinófilos e a expressão de CD11b mostraram-se independentes de DP1 (Monneret *et al.*, 2001).

Já havia sido descrito um receptor órfão expresso seletivamente em basófilos, linfócitos Th2 e eosinófilos, denominado CRTH2 (*“Chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes”*) ou DP2. A análise funcional desse receptor revelou que o sobrenadante da cultura de mastócitos ativados era capaz de induzir influxo de cálcio em células K562/B19 – que expressavam o receptor DP2 – enquanto que o mesmo efeito não foi observado nas células controle, K562/neo, indicando que esse receptor fora ativado por um ligante natural produzido por mastócitos ativados (Nagata *et al.*, 1999a; Nagata *et al.*, 1999b).

Posteriormente, este mesmo grupo – utilizando tratamento com ácido acetil salicílico (aspirina) e com o uso de cromatografia de fase reversa – identificou a PGD<sub>2</sub> como sendo o composto ativo produzido por mastócitos ativados que atuaria via o receptor DP2. Este estudo revelou também a habilidade quimiotática da PGD<sub>2</sub> para eosinófilos, basófilos e linfócitos Th2, mas não para monócitos ou neutrófilos. Assim sendo, o receptor DP2 foi caracterizado como sendo um receptor proteico que também apresenta sete sítios transmembranares – assim como o receptor DP1 – associado a uma proteína G do tipo G<sub>ai</sub> (proteína G inibitória), visto que o tratamento com toxina pertussis (PTX) inibiu a mobilização de cálcio mediada por DP2 e que não houve produção de AMPc em virtude da ativação desse receptor pela PGD<sub>2</sub> (Hirai *et al.*, 2001).

No entanto, ensaios *in vitro* com eosinófilos humanos, demonstrou que a alteração da conformação celular (“*Shape Change*”) induzida por PGD<sub>2</sub> não foi inibida por PTX, mas por U-73122 (inibidor específico de fosfolipase C), assim como essa mesma alteração provocada pela indometacina – agonista DP2 em doses submicromolares (Hirai *et al.*, 2002) – foi inibida por U-73122, LY-294002 (inibidor de PI3 quinase) e SB-202190 (inibidor de MAPK) sugerindo que o receptor DP2 também possa sinalizar via proteína Gαq (Stubbs *et al.*, 2002).

A importância do DP2 na resposta alérgica foi avaliada a partir do desenvolvimento de camundongos deficientes para este receptor (DP2<sup>-/-</sup>). Quando sensibilizados e submetidos ao desafio alérgico, os animais DP2<sup>-/-</sup> apresentaram um aumento na eosinofilia ocorrida no lavado bronco alveolar (BAL). Essa inesperada eosinofilia observada nesses animais, poderia ser justificada a partir da observação de que os níveis de IL-5 produzidos por esplenócitos dos animais DP2<sup>-/-</sup> *in vitro* encontravam-se aumentados (2,5 a 5,0 vezes) em relação as células dos animais selvagens, quando estimulados com anti-CD3/anti-CD8 (Chevalier *et al.*, 2005). Esse resultado é surpreendente, visto que as primeiras observações referentes ao receptor DP2 o descrevem com sendo responsável por induzir a quimiotaxia de eosinófilos (Hirai *et al.*, 2001), mas é condizente com a afirmação de que o papel preciso da PGD<sub>2</sub>, assim como dos seus receptores, na complexa resposta inflamatória alérgica ainda não está bem definido.

Na avaliação das ações da PGD<sub>2</sub> via receptor DP2, o desenvolvimento do ramatroban (BAY-u3405) resulta numa importante ferramenta farmacológica. Inicialmente, esse composto foi caracterizado como sendo antagonista seletivo do receptor TP, antagonizando as ações do tromboxano A<sub>2</sub> (TXA<sub>2</sub>) (McKenniff *et al.*, 1991; Norman *et al.*, 1991). Mas foi demonstrado que o tratamento com ramatroban também é capaz de inibir a migração de eosinófilos induzida por PGD<sub>2</sub> (*in vitro*) de uma maneira dose-dependente, antagonizando as ações desse eicosanóide ao se ligar ao receptor DP2. Enquanto a quimiotaxia induzida por PGD<sub>2</sub> parece depender exclusivamente da ativação de DP2, o influxo de cálcio em eosinófilos induzido por PGD<sub>2</sub> mostrou-se dependente tanto de DP1 como DP2, visto que esse fenômeno foi completamente abolido pelo tratamento com ramatroban (antagonista de DP2) e parcialmente inibido por BWA 868C (antagonista seletivo de DP1) (Shichijo *et al.*, 2003). Pequenas

alterações na estrutura do ramatroban, possibilitou o desenvolvimento do CAY10471, molécula com maior potência e seletividade para o receptor DP2 (Ulven *et al.*, 2005).

A tabela 1.5.1 reúne algumas das características pertinentes a esses receptores.

**Tabela 1.5.1: Tabela comparativa reunindo as principais características dos receptores DP1 e DP2 (Nagata *et al.*, 2003; Sandig *et al.*, 2006) Diaz, BL – comunicação pessoal).**

	DP2 (ou CRTH2)	DP1
<i>Expressão</i>	células Th2, eosinófilos, basófilos e mastócitos	eosinófilos, basófilos, mastócitos, monócitos, células endoteliais, plaquetas, células musculares, entre outras.
<i>Agonistas Comuns</i>	PGD <sub>2</sub> , PGJ <sub>2</sub>	PGD <sub>2</sub>
<i>Agonistas Seletivos</i>	DK- PGD <sub>2</sub> , 15d-PGJ <sub>2</sub> , 15dPGD <sub>2</sub> , PGF <sub>2</sub>	BW245C
<i>Antagonistas Seletivos</i>	CAY 10471	BWA868C
<i>Tipo de Proteína G associada</i>	Gi, Gq	Gs
<i>Efeitos Biológicos</i>	- Quimiotaxia (eosinófilos e basófilos) - Ativação de eosinófilos: polimerização de filamentos de actina, diminuição da expressão de L-selectina (CD62L); “ <i>Shape Change</i> ”; degranulação; Indução da expressão de CD11b	- Inibição da agregação plaquetária, - Vasodilatação, - Relaxamento de músculos estriados, - Promoção de asma alérgica, - Indução ao sono - Inibição de apoptose de eosinófilos
<i>Efeitos intracelulares</i>	Induz aumento na concentração de Cálcio e diminuição na concentração de AMPc	Induz aumento nas concentrações de Cálcio e AMPc

Dentre outras atividades relativas à resposta inflamatória de caráter eosinofílico, a PGD<sub>2</sub> mostrou-se também ser capaz de induzir diretamente células Th2 a produzirem IL-4, IL-5 e IL-13 *in vitro*. Além disso, foi observado também um aumento na quantidade de RNAm de cada um desses mediadores nestas células quando submetidas a estimulação por PGD<sub>2</sub>. Esse fenômeno mostrou-se dependente da ativação do receptor DP2, visto que a estimulação com o seu agonista seletivo DK-PGD<sub>2</sub> foi capaz de induzir efeito similar, mas menos potente que a PGD<sub>2</sub>, enquanto que o BW 245c não foi capaz de promover nenhuma alteração. Mais ainda, o tratamento dos linfócitos com ramatroban inibiu a produção das citocinas Th2 induzido por PGD<sub>2</sub>, o que não foi

observado para o tratamento com o BWA 868c, indicando que a liberação de citocinas induzida por PGD<sub>2</sub> se deve à ativação do receptor DP2 (Xue *et al.*, 2005).

Por conta dessas vias de sinalização diferentes, associadas a diferentes tipos de proteínas G com funções antagônicas (com relação a ativação da adenilato ciclase), vem sendo admitido que esses dois receptores tenham ações antagônicas na ativação eosinofílica (Monneret *et al.*, 2001). No entanto, também foi demonstrado, que esses receptores podem atuar de maneira complementar induzindo a ativação de eosinófilos (Kostenis *et al.*, 2006). Foi demonstrado que a ativação de ambos seria necessária no processo de liberação dessas células a partir da medula óssea (Schratl *et al.*, 2007), e num outro estudo, foi observado que a ativação de DP2 promoveria a quimiotaxia e a degranulação dos eosinófilos, enquanto que a ativação do DP1 seria responsável pelo aumento na sobrevivência dessas células, ao diminuir a apoptose de eosinófilos humanos *in vitro* (Gervais *et al.*, 2001). Apesar dessas observações, recentemente foi demonstrado que o laropirant – antagonista DP1 usado em ensaios clínicos com ou sem a associação com o montelucaste – não foi capaz de reverter os sintomas observados em pacientes asmáticos (Philip *et al.*, 2009). Esses achados reforçam ainda mais a idéia de que o papel desses receptores na ativação eosinofílica precisa ser melhor elucidado.

Atualmente, acredita-se que a PGD<sub>2</sub> esteja, provavelmente, envolvida em múltiplos aspectos e etapas do processo inflamatório de origem alérgica através da ativação desse sistema duplo de receptores. Seu papel na ativação de eosinófilos, assim como na biogênese de corpúsculos lipídicos e síntese de leucotrieno C<sub>4</sub> parece ser um ponto-chave na resposta inflamatória do tipo alérgica.

## 1.6) PERSPECTIVAS TERAPÊUTICAS:

### 1.6.1. Visão Geral:

Nos últimos anos, o tratamento da sintomatologia da asma tem focado na supressão do processo inflamatório das vias aéreas, além de tentar promover o alívio dos sintomas. A utilização de broncodilatadores tem demonstrado que, a utilização de  $\beta$ 2-agonistas sob a forma de aerossol resulta numa ação potente e rápida no tratamento clínico da asma. No entanto, o seu uso prolongado pode levar a ocorrência de efeitos colaterais, como taquicardia por exemplo (Nelson, 1995). É admitido que os broncodilatadores de longa duração, não devem ser utilizados como monoterapia em pacientes que necessitam de medicação diária, podendo ser utilizados em associação com glicocorticóides (Lazarus *et al.*, 2001).

Os glicocorticóides correspondem aos agentes antiinflamatórios mais potentes disponíveis para o tratamento da asma. O seu mecanismo de ação está relacionado a alguns fatores como a diminuição da ativação e função de células inflamatórias, a redução no extravasamento vascular e na diminuição da produção de muco, assim como ao aumento na resposta aos agonistas  $\beta$ -adrenérgicos. No entanto, a utilização de corticóides em altas doses tanto por via oral, quanto por via inalatória, pode levar ao aparecimento de efeitos colaterais, como a síndrome de Cushing (Schraa *et al.*, 1982; Barnes *et al.*, 2003). Dessa forma, a utilização de corticosteróides em associação com  $\beta$ 2-agonistas, teofilina ou inibidores de leucotrienos cisteínados, têm se mostrado eficaz no tratamento da asma, enquanto minimiza os efeitos colaterais observados.

A teofilina é uma droga broncodilatadora que apresenta efeito antiinflamatório satisfatório, ao inibir as fosfodiesterases (Giembycz, 2000). No entanto, a sua associação com os corticosteróides tem se mostrado menos eficiente do que a associação dos corticosteróides com os  $\beta$ 2-agonistas, além de apresentar efeitos colaterais, como alterações gastrointestinais (Davies *et al.*, 1998). Além dessas drogas, uma outra classe de medicamentos antiasmáticos, que vêm sendo bastante utilizada, são os inibidores de leucotrienos cisteinados. Essa drogas podem ser inibidores de síntese (inibidores da 5-LO, como o zileuton) ou antagonistas de receptor CisLTs (como o montelucaste ou zafirlucaste). Os antagonistas de receptor são os mais utilizados no tratamento da asma atualmente, mas são menos efetivos na redução das exarcebações pulmonares, quando comparado com a utilização dos corticosteróides. No entanto, a

associação dos antagonistas de receptor de cisLTs aos corticosteróides (baixas doses) tem se mostrado eficiente no controle da sintomatologia da asma, embora a heterogeneidade farmacogenética existente na população asmática, revele a necessidade de recomendações terapêuticas mais precisas (Horwitz *et al.*, 1998; Drazen *et al.*, 1999).

Contudo, sozinho ou em combinação com outras terapias, os corticosteróides não conseguem reverter completamente toda a inflamação das vias aéreas nos pacientes com asma (Jeffery *et al.*, 1992; Godfrey *et al.*, 1995; Booth *et al.*, 1995). Existem outras terapias já sendo utilizadas, capazes de modular a resposta inflamatória associada a produção de IgE, como o receptor recombinante solúvel de IL-4, usado sob a forma inalatória, por exemplo (Borish *et al.*, 1999), ou a utilização de anticorpos monoclonais humanos (IgG1) anti-IL-5, que provocaria uma redução na eosinofilia característica dessa resposta (Busse *et al.*, 2010). Além disso, o omalizumab – anticorpo humano monoclonal capaz de formar um complexo com a IgE livre – também vem sendo utilizado. Esse anticorpo, impede a interação da IgE com mastócitos e basófilos, sendo capaz de atenuar tanto a fase inicial, quanto a fase tardia da resposta alérgica, impedindo a ativação dessas células, consequentemente suprimindo o acúmulo de eosinófilos nas vias aéreas (Boulet *et al.*, 1997; Fahy *et al.*, 1997).

Talvez toda a complexidade de eventos ocorridos durante o desenvolvimento da resposta inflamatória alérgica, possa justificar a ausência de medidas terapêuticas completamente eficazes, capazes de reverter todo o quadro inflamatório observado durante o desenvolvimento da asma. Dentre todos os fenômenos associados ao desenvolvimento dessas respostas inflamatórias alérgicas, a regulação do recrutamento eosinofílico – assim como a modulação da ativação dessas células – parecem ser pontos-chave no controle da evolução dessas desordens.

Embora ainda não tenha sido completamente elucidado, já fora demonstrada a remissão dos sintomas alérgicos, associada a resolução da eosinofilia tecidual (Gleich *et al.*, 1993; Weller *et al.*, 1997; Rothenberg, 1998). Durante o desenvolvimento dessa resposta, os eosinófilos têm se destacado como células efetoras, visto que uma vez ativadas no sítio inflamatório alérgico parecem ser uma das principais fontes celulares dos cisLTs (Cowburn *et al.*, 1998) e a importância do LTC<sub>4</sub> na sintomatologia da

resposta inflamatória alérgica do tipo asmática já é bem caracterizada (Lewis *et al.*, 1990; Laitinen *et al.*, 1993; Henderson, Jr., 1994).

Nós já demonstramos, que a PGD<sub>2</sub> é um estímulo capaz de ativar eosinófilos, induzindo a produção de LTC<sub>4</sub> nos corpúsculos lipídicos citoplasmáticos recém-formados, tanto em modelos experimentais *in vitro* quanto *in vivo* (Mesquita-Santos *et al.*, 2006). Essas células expressam constitutivamente na membrana plasmática, ambos os receptores da PGD<sub>2</sub> – DP1 e DP2 (Hirata *et al.*, 1994; Nagata *et al.*, 1999a; Nagata *et al.*, 1999b). Identificar o receptor envolvido nesse fenômeno induzido pela PGD<sub>2</sub> em eosinófilos e assim, conseguir modular a ativação dessas células por este eicosanóide, pode se tornar um importante alvo na terapia contra a asma. Alguns estudos têm sido desenvolvidos, com o objetivo de identificar drogas capazes de reverter os efeitos da asma, ao antagonizar a ação dos receptores DP1 e DP2. Embora existam resultados promissores, a modulação da resposta alérgica pela antagonização desses receptores ainda não foi alcançada (Pettipher *et al.*, 2007; Philip *et al.*, 2009; Ulven *et al.*, 2010).

Na tentativa de encontrar novas drogas capazes de exercer atividades anti-inflamatórias, ou imunomoduladoras, para esse quadro patológico, a utilização de produtos naturais pela indústria farmacêutica, tem possibilitado a identificação de novas moléculas com ação anti-inflamatórias (Spelman *et al.*, 2006).

### **1.6.2. *Cissampelos sympodialis* e seu alcalóide isolado: warifteína**

Em face da imensa diversidade de espécies vegetais da flora brasileira, muitas espécies vêm sendo utilizadas para obtenção de moléculas com efeito terapêutico. A *Cissampelos sympodialis* Eichl. é uma espécie da família Menispermaceae encontrada em abundância no nordeste brasileiro, ocorrendo principalmente em áreas de semi-árido (Rhodes DG, 1975) e alguns trabalhos já demonstraram o seu efeito anti-inflamatório na terapia de reumatismos, artrites e doenças do aparelho respiratório, como a asma (Barbosa-Filho *et al.*, 1997; Agra *et al.*, 2007a; Agra *et al.*, 2007b).

A família das Menispermaceae – na qual está incluído o gênero *Cissampelus* – é bem conhecida pela produção de vários tipos de alcalóides, como os bisbenzylquinolínicos. A partir do extrato bruto obtido da *C. sympodialis*, vários alcalóides já foram isolados, dentre os quais destacam-se: milonina (Freitas *et al.*,

1995), warifteína (Cortes *et al.*, 1995), metilwarifteína e laurifolina (Barbosa-Filho *et al.*, 1997), liriodenina e roraimina (de Lira *et al.*, 2002).

Todos esses alcalóides têm atividades farmacológicas identificadas, como atividade antiparasitárias contra *Leishmania sp.* (Fournet *et al.*, 1993), *Trypanosoma cruzi* (Fournet *et al.*, 2000) e *Plasmodium sp.* (Angerhofer *et al.*, 1999). Outros efeitos também já foram atribuídos a esses alcalóides, como atividade espasmolítica e efeito citotóxico (De Freitas *et al.*, 1996; Melo *et al.*, 2003), assim como atividade antidepressiva (Almeida *et al.*, 1998). Além disso, já foram demonstradas também atividades anti-anafilática (Thomas *et al.*, 1997) e antiinflamatória (Lima *et al.*, 2001), modulando inclusive a atividade de macrófagos e linfócitos B (Alexandre-Moreira *et al.*, 2003a; Alexandre-Moreira *et al.*, 2003b).

Em relação aos seus efeitos anti-inflamatórios, foi demonstrado que durante o desenvolvimento da resposta alérgica a utilização do extrato aquoso de *C.symphodialis* por via oral, reduziu os níveis de IgE séricos – assim como o edema de pata – em camundongos estimulados alérgicamente (Bezerra-Santos *et al.*, 2004a). Dentre os alcalóides isolados a partir desse extrato, a warifteína é o mais abundante de todos (Melo *et al.*, 2003) e as atividades anti-inflamatórias da *C.symphodialis* foram atribuídos a esse alcalóide. Foi observado que a utilização do extrato – assim como a warifteína – foi capaz de reverter a eosinofilia pleural, biogênese de corpúsculos lipídicos e a síntese de LTC<sub>4</sub> num modelo murino de inflamação alérgica (Bezerra-Santos *et al.*, 2006). Vale ressaltar que todos esses fenômenos inflamatórios inibidos tanto pelo extrato, quanto pela warifteína, são controlados pela PGD<sub>2</sub> produzida durante a resposta alérgica (Mesquita-Santos *et al.*, 2006). Também já foi demonstrado que a utilização da warifteína foi capaz de inibir o edema de pata e a produção de IgE – além de proteger contra anafilaxia – num modelo de alergia murina, através da inibição da degranulação de mastócitos (Costa *et al.*, 2008).

### 3 – OBJETIVOS:

Essa tese de doutorado pode ser equacionada em 2 partes, que podem ser especificamente descritas como:

**PARTE 1: Identificar o receptor específico da PGD<sub>2</sub> envolvido no mecanismo de síntese de LTC<sub>4</sub> por eosinófilos estimulados com PGD<sub>2</sub>.** Especificamente, focamos em:

- 1) Avaliar especificamente o papel de cada um dos receptores de PGD<sub>2</sub> – DP1 e DP2 – na síntese de LTC<sub>4</sub>;
- 2) Avaliar o efeito da ativação direta de cada um dos receptores da PGD<sub>2</sub> sobre a síntese de LTC<sub>4</sub> nos eosinófilos purificados *in vitro*;
- 3) Identificar e caracterizar as vias de sinalização relacionadas aos fenômenos de biogênese de corpúsculos lipídicos e síntese de LTC<sub>4</sub> em eosinófilos humanos ativados por PGD<sub>2</sub>.

**PARTE 2: Avaliar a síntese/atividade da PGD<sub>2</sub> como alvo da *C.symphodialis* e do seu alcalóide isolado, warifteína.** Especificamente, focamos em:

- 1) Avaliar se a warifteína impediria a PGD<sub>2</sub> de exercer seus efeitos pró-inflamatórios através da sua ligação com os receptores de PGD<sub>2</sub>, impedindo a ligação do eicosanóide aos seus receptores;
- 2) Avaliar se a warifteína estaria inibindo a síntese de PGD<sub>2</sub> durante a resposta alérgica.

#### 4) ARTIGOS ANEXADOS:

Os resultados dessa tese foram compilados em dois artigos. O primeiro deles já foi publicado e o segundo está em fase final de conclusão, para ser submetido à revista “*British Journal of Pharmacology*”. Ambos encontram-se anexados a essa seção.

##### 4.1) Artigo I:

**“Co-operative signaling through DP1 and DP2 prostanoid receptors is required to enhance leukotriene C<sub>4</sub> synthesis induced by prostaglandin D<sub>2</sub> in eosinophils”**

Mesquita-Santos FP, Bakker-Abreu I, Luna-Gomes T, Bozza PT, Diaz BL, Bandeira-Melo C. *Br J Pharmacol.* 2010 Oct 26. doi: 10.1111/j.1476-5381.2010.01086.x. [Epub]

De forma sucinta, este estudo avaliou os mecanismos envolvidos na síntese de LTC<sub>4</sub> por eosinófilos induzida por PGD<sub>2</sub>, através da ativação dos seus receptores DP1 e DP2. Neste trabalho, nós observamos que, embora somente a ativação do receptor DP1 induza a biogênese de corpúsculos lipídicos em eosinófilos, para ocorrer a síntese de LTC<sub>4</sub> em eosinófilos, a ativação simultânea de ambos os receptores se faz necessária, tanto em modelos experimentais *in vivo*, quanto em eosinófilos humanos *in vitro*. Demonstramos também que o sítio de síntese do LTC<sub>4</sub> correspondia aos corpúsculos lipídicos recém-formados.

## RESEARCH PAPER

# Co-operative signalling through DP<sub>1</sub> and DP<sub>2</sub> prostanoid receptors is required to enhance leukotriene C<sub>4</sub> synthesis induced by prostaglandin D<sub>2</sub> in eosinophils

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### Keywords

eosinophils; PGD<sub>2</sub>; LTC<sub>4</sub>; DP<sub>1</sub>;  
DP<sub>2</sub>; cAMP; lipid droplets; lipid  
bodies; allergic inflammation;  
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## BACKGROUND AND PURPOSE

Prostaglandin (PG) D<sub>2</sub> has emerged as a key mediator of allergic inflammatory pathologies and, particularly, PGD<sub>2</sub> induces leukotriene (LT) C<sub>4</sub> secretion from eosinophils. Here, we have characterized how PGD<sub>2</sub> signals to induce LTC<sub>4</sub> synthesis in eosinophils.

## EXPERIMENTAL APPROACH

Antagonists and agonists of DP<sub>1</sub> and DP<sub>2</sub> prostanoid receptors were used in a model of PGD<sub>2</sub>-induced eosinophilic inflammation *in vivo* and with PGD<sub>2</sub>-stimulated human eosinophils *in vitro*, to identify PGD<sub>2</sub> receptor(s) mediating LTC<sub>4</sub> secretion. The signalling pathways involved were also investigated.

## KEY RESULTS

*In vivo* and *in vitro* assays with receptor antagonists showed that PGD<sub>2</sub>-triggered cysteinyl-LT (cysLT) secretion depends on the activation of both DP<sub>1</sub> and DP<sub>2</sub> receptors. DP<sub>1</sub> and DP<sub>2</sub> receptor agonists elicited cysLTs production only after simultaneous activation of both receptors. In eosinophils, LTC<sub>4</sub> synthesis, but not LTC<sub>4</sub> transport/export, was activated by PGD<sub>2</sub> receptor stimulation, and lipid bodies (lipid droplets) were the intracellular compartments of DP<sub>1</sub>/DP<sub>2</sub> receptor-driven LTC<sub>4</sub> synthesis. Although not sufficient to trigger LTC<sub>4</sub> synthesis by itself, DP<sub>1</sub> receptor activation, signalling through protein kinase A, did activate the biogenesis of eosinophil lipid bodies, a process crucial for PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis. Similarly, concurrent DP<sub>2</sub> receptor activation used *tert*-butyl toxin-sensitive and calcium-dependent signalling pathways to achieve effective PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis.

## CONCLUSIONS AND IMPLICATIONS

Based on pivotal roles of cysLTs in allergic inflammatory pathogenesis and the collaborative interaction between PGD<sub>2</sub> receptors described here, our data suggest that both DP<sub>1</sub> and DP<sub>2</sub> receptor antagonists might be attractive candidates for anti-allergic therapies.

## LINKED ARTICLE

This article is commented on by Mackay and Stewart, pp. 1671–1673 of this issue. To view this commentary visit <http://dx.doi.org/10.1111/j.1476-5381.2011.01236.x>

## Abbreviations

5-Lipoxygenase; ADRP, adipose differentiation-related protein; CCR2, chemokine receptor-homologous molecule expressed on T helper type 2 cell (Th2) cells; cysLTs, cysteinyl leukotrienes; DP<sub>1</sub>, D prostanoid receptor 1; DP<sub>2</sub>, D prostanoid receptor 2; EDAC, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide; LTC<sub>4</sub>, leukotriene C<sub>4</sub>; OVA, ovalbumin; PGD<sub>2</sub>, prostaglandin D<sub>2</sub>.

## Introduction

Eosinophil accumulation and subsequent activation at sites of allergic inflammation control the generation and release of diverse lipid and protein mediators critical to the development and perpetuation of allergic processes (Bass and Lemarchand, 2001; Fosterberg and Hogan, 2006). Indeed, eosinophils represent a major source of leukotriene (LT) C<sub>4</sub>, the intracellular parent of the cysteinyl LTs (cysLTs) LTC<sub>4</sub>/L<sub>5</sub>/E<sub>4</sub> (Wetter et al., 1983; Cowman et al., 1998; Bandeira-Melo and Wotter, 2003). Central to the pathogenesis of allergic diseases, cysLTs cause bronchoconstriction, mucus hypersecretion, increased microvascular permeability, bronchial hyperresponsiveness, eosinophil infiltration and airway remodelling (Drazen and Austen, 1987). LTC<sub>4</sub>, as the main eosinophil-derived product of the 5-lipoxygenase pathway of arachidonic acid metabolism in allergic diseases, is formed by the conjugation of LTA<sub>4</sub> with reduced glutathione by LTC<sub>4</sub> synthase. After active transport to the extracellular space, LTC<sub>4</sub> is converted to LTD<sub>4</sub> and LTE<sub>4</sub> through sequential enzymatic removal of glutamic acid and glycine. Even though the eosinophil LTC<sub>4</sub>-synthesizing enzymatic pathways are well known (Bandeira-Melo et al., 2002a), it remains of particular interest to fully characterize endogenous allergy-relevant stimuli, receptors and signalling pathways, as well as to understand the intracellular compartmentalization mechanisms that control allergen-induced, eosinophil-driven, LTC<sub>4</sub> synthesis.

Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) is another key lipid mediator of allergic airway inflammation that is produced following allergen exposure in patients with asthma, atopic dermatitis or allergic rhinitis. Similar to cysLTs, PGD<sub>2</sub> can mimic a number of important features of allergic processes (Penhagar 2008). In regard to the sensitization phase of an allergic response, it has been postulated that PGD<sub>2</sub> modulates cytokine production by dendritic cells, leading to the polarization of T helper type 2 cell (Th2) cells (Theimer et al., 2006; Hammad et al., 2007). In addition to its immune-regulatory role, PGD<sub>2</sub> is a highly effective trigger of blood flow changes, promoting oedema formation and therefore contributing to the nasal congestion symptom of allergic rhinitis (Doyle et al., 1990; Widdicombe, 1996). PGD<sub>2</sub> also mediates the typical cell accumulation of late phase allergic responses by functioning as a selective chemotactant for Th2 cells, basophils and eosinophils (Hira et al., 2001; Monnier et al., 2001).

The immune-modulatory and inflammatory functions of PGD<sub>2</sub> are mediated by high-affinity interaction with two receptors, the prostanoid DP<sub>1</sub> receptor and the chemokine-like receptor-homologous molecule expressed on Th2 cells receptor, now referred to as the DP<sub>2</sub> receptor (receptor nomenclature follows Alexander et al., 2006). Although DP<sub>1</sub> and DP<sub>2</sub> receptors bind the same ligand, there is very little homology between the two receptors, DP<sub>1</sub> receptor being members of the prostanoid receptor family that includes

EP<sub>1,4</sub>, EP<sub>2</sub> and EP<sub>3</sub>, whereas the DP<sub>2</sub> receptors are more closely related to other chemokine receptors such as the LTB<sub>4</sub> receptors, BLT1 and BLT2, and the CCR2 receptor (Thomphat, 2008). Furthermore, while DP<sub>1</sub> receptors are coupled to G<sub>α</sub> proteins and signal through elevation of intracellular levels of cAMP, the DP<sub>2</sub> receptors are coupled to G<sub>βγ</sub> and their activation leads to the elevation of intracellular calcium, reduction in cAMP (Sawyer et al., 2002) and downstream activation of phosphatidylinositol-3-kinase (PI3K) (Xue et al., 2007).

Eosinophils co-express the classical DP<sub>1</sub> receptors coupled to adenylyl cyclase, as well as the recently novel (PTX)-sensitive DP<sub>2</sub> receptors (Monnier et al., 2001). Although the ability of PGD<sub>2</sub> to activate eosinophils while concurrently elevating cAMP levels seems paradoxical, DP<sub>1</sub> receptors appear to be able to co-mediate, with DP<sub>2</sub> receptors, the mobilization of eosinophils from bone marrow as well as chemotaxis (Gheval et al., 2007). Alternatively, it has also been shown that the final chemotaxis-related response of eosinophils to PGD<sub>2</sub> may be, ultimately, determined by a balance between two opposing downstream signalling pathways: the cAMP-dependent, inhibitory pathway activated via DP<sub>1</sub> receptors and the prevailing, stimulatory pathway activated via DP<sub>2</sub> receptors (Monnier et al., 2001; Kossien and Ulven, 2006; Sandig et al., 2007).

Recently, we have shown that, in addition to its chemotactic activity towards eosinophils, PGD<sub>2</sub> controls allergy-relevant eosinophil activation, particularly the increased LTC<sub>4</sub> synthesizing capacity of these cells (Mesquita-Santos et al., 2006). Indeed, other eosinophil chemotactants, including oxazolone (OXA), RANTES (ECL5) and pleural activating factor (PAF) are capable of triggering LTC<sub>4</sub> synthesis within eosinophils through the activation of their cognate G<sub>βγ</sub>-coupled chemokine receptors, such as CCR2 (Botta et al., 1996; Bandeira-Melo et al., 2001). Therefore, we had initially hypothesized that PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis could be mediated by the stimulatory activation of DP<sub>2</sub> receptors while being counter-balanced by a parallel inhibitory cAMP-dependent DP<sub>1</sub> receptor activation. However, here by employing pharmacological strategies, we have uncovered a novel kind of interaction between the PGD<sub>2</sub> receptor types expressed on eosinophils. In contrast to the PGD<sub>2</sub>-driven opposing signalling related to eosinophil chemotactic activities, eosinophil LTC<sub>4</sub> synthesis triggered by PGD<sub>2</sub> appeared to be controlled by the complementary stimulatory events between DP<sub>1</sub> receptor-activated, PKA-driven, lipid bodies and concurrent DP<sub>2</sub> receptor signalling. While PGD<sub>2</sub> emerges as a potent inflammatory mediator of allergic disorders and as an interesting therapeutic target, because of the mandatory dual activation of DP<sub>1</sub> and DP<sub>2</sub> receptors for increasing eosinophil LTC<sub>4</sub> synthesis, either DP<sub>1</sub> or DP<sub>2</sub> receptor antagonists might be highly effective candidates as anti-allergic tools to control cysLTs production regulated by the activation of eosinophils at sites of allergic reactions.

## Methods

### Animals

All animal care and experimental protocols were approved by Oswaldo Cruz Foundation Animal Welfare Committee. For *in vivo* experiments, male Swiss mice of 16–20 g were obtained from Oswaldo Cruz Foundation breeding unit (Rio de Janeiro, Brazil).

### PGD<sub>2</sub>-induced and allergic pleurisy in sensitized mice

As previously described (Mesquita-Santos et al., 2006), mice were sensitized with an s.c. injection (0.2 mL) of ovalbumin (OVA; 30 µg; Sigma, St. Louis, MO, USA) and Al(OH)<sub>3</sub> (5 mg) in 0.9% NaCl solution (saline) at days 1 and 7. Allergic challenge was performed at day 14 by means of an intrapleural injection of OVA (12 µg per cavity; 0.1 mL). Alternatively, sensitized mice were challenged with PGD<sub>2</sub> (25 pmol per cavity), BW245C (25 pmol per cavity), DK-PGD<sub>2</sub> (25 pmol per cavity) or a combination of the latter two agonists (both at 25 pmol per cavity) (all from Cayman Chemicals, Ann Arbor, MI, USA). All stimuli were diluted in sterile saline immediately before use. Control animals were injected with the same volume (0.1 mL) of vehicle. Mice were killed by CO<sub>2</sub> inhalation 24 h after challenge. Pleural fluid was obtained by rinsing cavities with 1 mL of phosphate-buffered saline containing BSA (0.1%). After samples were taken for lipid body analysis, pleural fluid was centrifuged and cell free supernatants were used for the quantification of cystLTs.

### Isolation and *in vitro* stimulation of human eosinophils

Pleural blood was obtained with informed consent from healthy donors under protocols approved by the ethical review boards of both the Federal University of Rio de Janeiro and the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). Eosinophils were isolated by negative selection using Easy-Sep™ system (StemCell Technologies Inc., Vancouver, Canada) (purity >98%; viability >95%) (Bezerra-Santos et al., 2006). Purified eosinophils ( $2 \times 10^6$  cells mL<sup>-1</sup>) in Hank's balanced salt solution without Ca<sup>2+</sup> and Mg<sup>2+</sup> (HBSS<sup>-</sup>) were incubated for 1 h in a water bath (37°C) with PGD<sub>2</sub> (25 nM), BW245C (5–625 nM), DK-PGD<sub>2</sub> (5–625 nM), a combination of BW245C and DK-PGD<sub>2</sub> (both at 25 nM) or PAF (10 µM; from Cayman Chemicals). After samples were taken for lipid body analyses, eosinophils were resuspended in Hank's balanced salt solution with Ca<sup>2+</sup> and Mg<sup>2+</sup> (HBSS<sup>+</sup>) and stimulated with 0.1 µM A23187 (Sigma) for another 15 min (37°C). Cell-free supernatants were then collected for cystLT quantification. Each *in vitro* experiment was repeated at least three times, with eosinophils purified from different donors.

### Treatments

Using the pleurisy models, animals were pretreated with i.p. injections of the DP<sub>1</sub> receptor antagonist BW485C (1 mg kg<sup>-1</sup>; Cayman Chemicals), the dual DP<sub>1</sub>/TP receptor antagonist ranaroban (also known as Bay-u3403; 1 mg kg<sup>-1</sup>; Cayman Chemicals) or the selective DP<sub>2</sub> receptor antagonist Cay10471 (1 mg kg<sup>-1</sup>; Cayman Chemicals) 30 min before either PGD<sub>2</sub> or allergic challenge.

For *in vitro* studies, eosinophils in HBSS<sup>-</sup> were pretreated for 30 min with the DP<sub>1</sub> receptor antagonist BW485C (200 nM), the dual DP<sub>1</sub>/TP receptor antagonist ranaroban (200 nM), the selective DP<sub>2</sub> antagonist Cay10471 (200 nM), two PKA inhibitors H-89 and PKI (both at 10 µM; Calbiochem, La Jolla, CA, USA); PTX (1 µg mL<sup>-1</sup>; Calbiochem) or cell-permeable calcium chelator BAPTA-AM (25 µg mL<sup>-1</sup>; Sigma). Of note, these pretreatments did not modify basal lipid body content found within cytoplasm of non-stimulated eosinophils or adherent eosinophil vacuosity (<50%) (data not shown).

### Quantification of cystLTs

The amount of cystLTs found in cell-free pleural fluid and eosinophil supernatants was measured by the Cysteinyl Leukotriene EIA kit (catalog number 520501; from Cayman Chemicals), according to the manufacturer's instructions.

### EicosoCell for intracellular LTC<sub>4</sub> immuno-detection

As previously described (Bezerra-Melo et al., 2001) to localize LTC<sub>4</sub> at its sites of synthesis, *in vitro* stimulated eosinophils were mixed with a solution of 1-(3-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC; 0.1% final concentration with cells in HBSS<sup>-</sup>), used to crosslink eicosanoid carboxyl groups to amines in adjacent proteins. After 15 min incubation at room temperature with EDAC to promote both cell fixation and permeabilization, eosinophils were then washed with HBSS<sup>-</sup>, cytospan onto glass slides and blocked with HBSS<sup>-</sup> containing 1% BSA for 30 min. Cells were incubated with rabbit anti-LTC<sub>4</sub> antibodies (Cayman Chemicals) or non-immune rabbit IgG (Jackson ImmunoResearch Inc., West Grove, PA, USA) overnight. Finally, cells were co-incubated with goat anti-pig anti-adipose differentiation-related protein (ADRP) antibody (1:200 dilution; Frozenol Industries, North Andover, MA, USA) to distinguish cytoplasmic lipid bodies within eosinophils. The cells were washed 3x from 10 min with HBSS<sup>-</sup> containing 1% BSA and incubated with Alexa488-labelled anti-rabbit IgG and Alexa546-labelled anti-goat-pig secondary antibodies for 1 h.

The specificity of the LTC<sub>4</sub> immuno-labelling was confirmed by the: (i) lack of immunofluorescence within PGD<sub>2</sub>-stimulated human eosinophils incubated with irrelevant IgG (data not shown); and (ii) lack of LTC<sub>4</sub> immuno-labelling within non-stimulated human eosinophils that were incubated with anti-LTC<sub>4</sub> antibody.

Images were obtained using an Olympus BX51 fluorescence microscope at 100x magnification and photographs were taken with the Olympus 72 digital camera (Olympus Optical Co., Tokyo, Japan) in conjunction with Cell Imaging Software for Life Science Microscopy (Olympus Life Science Europe GmbH, Hamburg, Germany). The images were edited using Adobe Photoshop 5.5 software (Adobe Systems, San Jose, CA, USA).

### Lipid body staining and enumeration

To enumerate lipid bodies, cells recovered from pleural cavity or human eosinophils were centrifuged (450 rpm, 5 min) onto glass slides. Cells, while still moist, were fixed in 2.5% formaldehyde (in HBSS<sup>-</sup>, pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained with 1.5% OsO<sub>4</sub> (Sigma)

for 30 min, rinsed in distilled H<sub>2</sub>O, immersed in 1.0% dio-carbohydrazide for 5 min, rinsed in 0.1 M cacodylate buffer, rinsed with 1.5% OsO<sub>4</sub> for 3 min, rinsed in distilled water, and then air dried and analysed. Cell morphology was observed and lipid bodies were examined by light microscopy. Fifty consecutively scanned eosinophils were evaluated by more than one individual and results were expressed as the number of lipid bodies per eosinophil.

### Statistical analysis

Results are expressed as mean  $\pm$  SEM and were analysed statistically by means of analysis of variance followed by the Newman-Keuls *post-hoc* test, with the level of significance set at  $P < 0.05$ .

## Results

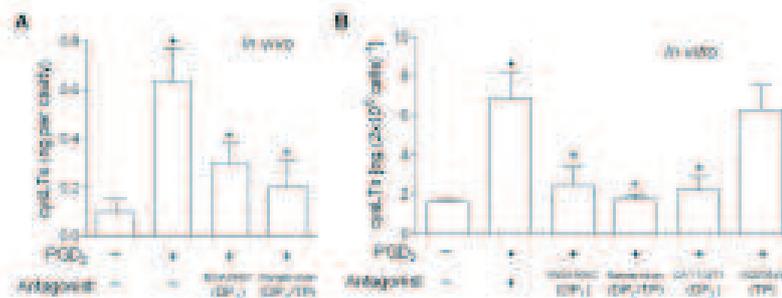
### Both DP<sub>1</sub> and DP<sub>2</sub> receptors contribute to PGD<sub>2</sub>-induced enhanced LTC<sub>4</sub> secretion from eosinophils

We have reported that PGD<sub>2</sub>, formerly recognized only as an eosinophil chemotacticant, is also able to trigger eosinophil activation, characterized by enhanced LTC<sub>4</sub> synthesis (Mesquida-Sanchez *et al.*, 2006). Here, by employing a mouse model of PGD<sub>2</sub>-induced eosinophil activation *in vivo* (Mesquida-Sanchez *et al.*, 2006), as well as human-purified eosinophils stimulated *in vitro* with PGD<sub>2</sub>, we investigated the molecular and cellular mechanisms involved in PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis within eosinophils. Our initial goal was to identify the specific PGD<sub>2</sub> receptor, either DP<sub>1</sub> or DP<sub>2</sub>, the two well recognized, cloned and eosinophil-expressed receptors for PGD<sub>2</sub>, that was involved in this response. *In vivo*, as illustrated in Figure 1, both PGD<sub>2</sub> receptors appeared to contribute to the LTC<sub>4</sub> secretion from eosinophils, inasmuch as pretreatment with either the DP<sub>1</sub> receptor antagonist

BW24558C or the dual DP<sub>1</sub>/TP receptor antagonist rama-rolofan displayed high levels of inhibition. BW24558C and rama-rolofan significantly reduced LTC<sub>4</sub> secretion levels found increased in sites of PGD<sub>2</sub>-triggered eosinophilic inflammation by 63 and 81%, respectively (Figure 1A). Similarly, Figure 1B shows that increased amounts of LTC<sub>4</sub> detected in the supernatants of *in vitro* PGD<sub>2</sub>-stimulated human eosinophils were also inhibited by either the DP<sub>1</sub> receptor antagonist BW24558C, the dual DP<sub>1</sub>/TP receptor antagonist rama-rolofan or by the selective DP<sub>2</sub> antagonist Cay10471 (83, 96 and 89% inhibition, respectively), indicating essential roles for each receptor type, which in turn, perhaps by acting synergistically, evoke PGD<sub>2</sub>-driven secretion of LTC<sub>4</sub> from eosinophils. Of note, by showing that (i) pretreatments with BW24558C and Cay10471 did not interfere with PAF-induced LTC<sub>4</sub> synthesis by human eosinophils (Table 1), or (ii) the pretreatment with the selective TP antagonist SQ29548 failed to alter LTC<sub>4</sub> synthesis triggered by *in vitro* stimulation of eosinophils (Figure 1B; 10% inhibition, respectively), we excluded potential non-specific effects of PGD<sub>2</sub> receptor antagonists and demonstrated that the inhibitory effects of rama-rolofan were dependent on DP<sub>1</sub>, rather than TP receptor antagonism.

### DP<sub>1</sub> and DP<sub>2</sub> receptors cooperate to trigger PGD<sub>2</sub>-driven enhanced LTC<sub>4</sub> secretion from eosinophils

To test the hypothesis that a synergistic mechanism of action between the PGD<sub>2</sub> receptors, DP<sub>1</sub> and DP<sub>2</sub>, controls PGD<sub>2</sub>-driven induction of LTC<sub>4</sub> secretion from eosinophils, selective agonists of DP<sub>1</sub> receptors (BW24558C) or of DP<sub>2</sub> receptors (DE-PGD<sub>2</sub>) were applied to *in vitro* and *in vivo* systems of eosinophil activation. Figure 2 shows that, alone, neither BW24558C nor DE-PGD<sub>2</sub> was able to elicit LTC<sub>4</sub> release from eosinophils at concentrations that PGD<sub>2</sub> by itself is able to trigger LTC<sub>4</sub> secretion both *in vivo* (25 pmol per cavity; Figure 2A) and *in vitro* (25 nM; Figure 2B). In contrast, by co-stimulating



**Figure 1**

Both DP<sub>1</sub> and DP<sub>2</sub> receptors control cycL<sub>4</sub> production triggered by PGD<sub>2</sub>. In A, sensitized mice were pretreated with BW24558C (1 mg kg<sup>-1</sup>) or rama-rolofan (1 mg kg<sup>-1</sup>) and then stimulated with an i.p. injection of PGD<sub>2</sub> (25 pmol per cavity). Analysis of cycL<sub>4</sub> synthesis was performed 24 h after PGD<sub>2</sub> administration; results are expressed as the means  $\pm$  SEM from at least six animals. \* $P < 0.05$  compared with control animals and † $P < 0.05$  compared with PGD<sub>2</sub>-injected mice. In B, for *in vitro* analysis of LTC<sub>4</sub> synthesis, human eosinophils were pretreated for 30 min with BW24558C (200 nM), rama-rolofan (200 nM), Cay10471 (200 nM) or SQ29548 (200 nM), and then stimulated for 1 h with PGD<sub>2</sub> (25 nM). *In vitro* results are expressed as the means  $\pm$  SEM from at least three independent experiments with eosinophils purified from different donors. \* $P < 0.05$  compared with control. † $P < 0.05$  compared with PGD<sub>2</sub>-stimulated eosinophils. cycL<sub>4</sub>, cysteinyl leukotriene; DP<sub>1</sub>, D prostanoid receptor 1; DP<sub>2</sub>, D prostanoid receptor 2; i.p., intraperitoneal; PGD<sub>2</sub>, prostaglandin G<sub>2</sub>.

Table 1

PAI-induced lipid body biogenesis and LTC<sub>4</sub> synthesis by human eosinophils are dependent on PTK-sensitive protein Gα and on intracellular calcium mobilization, but not on activation of DP<sub>1</sub> receptors or PKA activity

Conditions	Treatments	Lipid bodies/ eosinophil	cyst.LTs (ng/2 × 10 <sup>6</sup> cells)
Control		7.7 ± 0.4	1.1 ± 0.6
PAI		16.6 ± 1.4*	4.4 ± 1.4*
	-BW245C	16.0 ± 1.7	4.2 ± 1.1
	+CAY10471	16.7 ± 1.6	4.5 ± 1.2
	-H-89	13.6 ± 2.3	4.5 ± 1.6
	+PTX	10.5 ± 1.4*	1.6 ± 0.8*
	-BAPTA-AM	8.6 ± 0.5*	1.5 ± 0.8*

\*P < 0.05 compared with control group. \*\*P < 0.05 compared with PAI-stimulated eosinophils.

Human eosinophils were pretreated for 30 min with BW245C (200 nM), CAY10471 (200 nM), H-89 (10 μM), Pertussis toxin (PTX, 1 μg/ml) or BAPTA-AM (25 μM), and stimulated with PAI (10 μM). Analysis of lipid body biogenesis and LTC<sub>4</sub> synthesis were performed 1 h after PAI stimulation. Results were expressed as the means ± SEM from at least three different experiments.

cyst.L, cysyle leukotriene; DP<sub>1</sub>, D prostanoid receptor 1; PAI, platelet activating factor.

eosinophils with a mixture of DP<sub>1</sub> and DP<sub>2</sub> agonists, increased cyst.Ls levels were detected in eosinophilic inflammatory sites (Figure 2A) and human eosinophil-free supernatants (Figure 2B), with comparable magnitude to those found *in vivo* (Figure 2A) or *in vitro* (Figure 2B) stimulation with PGD<sub>2</sub>, reinforcing the possibility of a synergism between DP<sub>1</sub> and DP<sub>2</sub> receptors.

#### Cooperation between DP<sub>1</sub> and DP<sub>2</sub> receptors controls LTC<sub>4</sub> synthesis within eosinophil cytoplasmic lipid bodies

Stimulus-dependent extracellular detection of cyst.Ls depends on sequential intracellular events, including: (i) an initial step of activation and proper compartmentalization of both substrate and enzymatic machinery, culminating in intracellular LTC<sub>4</sub> synthesis; (ii) intracellular transport of direct newly synthesized LTC<sub>4</sub> to secretory pathways that is followed by (iii) an active carrier-dependent LTC<sub>4</sub> release through the plasma membrane (see Bandeira-Melo et al., 2002a). Therefore, as virtually no cyst.Ls can be detected in cell-free supernatants of BW245C- or DK-PGD<sub>2</sub>-stimulated eosinophils, one can argue that, under these stimulatory conditions, the intracellular step of LTC<sub>4</sub> synthesis actually occurs, without the subsequent LTC<sub>4</sub> transport/release events, which would only follow after the co-stimulation of both PGD<sub>2</sub> receptors. By employing FicoScaCell technology, a microscopic method that immobilizes and immuno-detects newly synthesized lipid molecules at their sites of synthesis, we have excluded this hypothesis by showing that LTC<sub>4</sub> synthesis itself is elicited

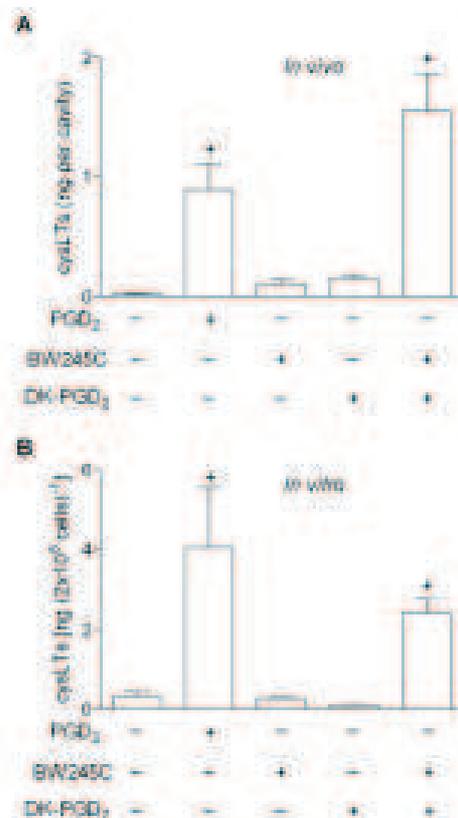
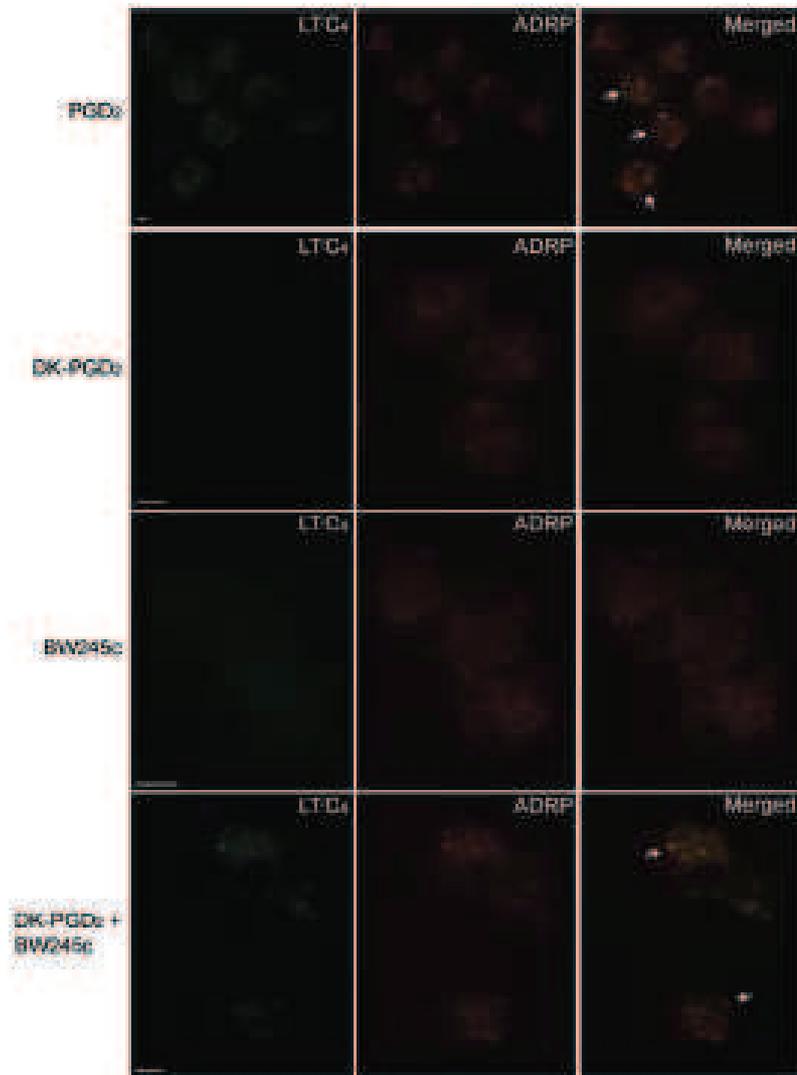


Figure 2

DP<sub>1</sub> and DP<sub>2</sub> receptors cooperate to trigger cyst.Ls production. In **A**, sensitized mice received an i.p. injection of PGD<sub>2</sub> (35 pmol per cavity), BW245C (35 pmol per cavity), DK-PGD<sub>2</sub> (35 pmol per cavity) or BW245C plus DK-PGD<sub>2</sub> (both at 35 pmol per cavity). Analysis of cyst.Ls production within pleural fluids was performed 24 h after i.p. administration. Results are expressed as the means ± SEM from at least six animals. \*P < 0.05 compared with control animals. In **B**, for *in vitro* analysis of LTC<sub>4</sub> production in cell-free supernatants, human eosinophils were stimulated for 1 h with PGD<sub>2</sub> (25 nM), BW245C (25 nM), DK-PGD<sub>2</sub> (25 nM) or with a combination of BW245C plus DK-PGD<sub>2</sub> (both at 25 nM). *In vitro* results are expressed as the means ± SEM from at least three independent experiments with eosinophils purified from different donors. \*P < 0.05 compared with control. P < 0.05 compared with PGD<sub>2</sub>-stimulated eosinophils. cyst.L, cysyle leukotriene; DP<sub>1</sub>, D prostanoid receptor 1; DP<sub>2</sub>, D prostanoid receptor 2; i.p., intraperitoneal; PGD<sub>2</sub>, prostaglandin D<sub>2</sub>.

*in vitro* within human eosinophils only if both DP<sub>1</sub> and DP<sub>2</sub> receptors are simultaneously engaged by either PGD<sub>2</sub> itself (Figure 3; top panel) or by the combination of both PGD<sub>2</sub> receptor agonists BW245C and DK-PGD<sub>2</sub> (Figure 3; bottom panel). Eosinophils activated only with DP<sub>1</sub> or DP<sub>2</sub> agonists show no immuno-fluorescent LTC<sub>4</sub> (Figure 3; middle panels) in FicoScaCell preparations, indicating that the LTC<sub>4</sub> synthesizing machinery was indeed not activated under these conditions of stimulation of a single PGD<sub>2</sub> receptor.



**Figure 3**

LTC<sub>4</sub> synthesis is triggered within eosinophil cytoplasmic lipid bodies by simultaneous activation of DP<sub>1</sub> and DP<sub>2</sub> receptors by either PGD<sub>2</sub> or the combination of BW245c and DK-PGD<sub>2</sub> stimulation of human eosinophils *in vitro*. Fluorescence microscopy images illustrate intracellular immune-detection of newly formed LTC<sub>4</sub> (green) and of ADRP (red) in PGD<sub>2</sub>-stimulated, BW245c-stimulated, DK-PGD<sub>2</sub>-stimulated or BW245c/DK-PGD<sub>2</sub> co-stimulated human eosinophils (as indicated). Overlay images of identical fields are shown in the right column. Arrows indicate co-localization of immunolabelled synthesized LTC<sub>4</sub> with ADRP-bearing lipid bodies. For Fluorescence microscopy analysis, cells were fixed and permeabilized with BDAC and sequentially incubated with anti-LTC<sub>4</sub> and anti-ADRP antibodies and Alexa488-labelled anti-mouse IgG plus Alexa546-labelled anti-guinea pig secondary antibodies. Images are representative of three independent experiments. ADRP, adipose-differentiation-related protein; DP<sub>1</sub>, D prostanoid receptor 1; DP<sub>2</sub>, D prostanoid receptor 2; PGD<sub>2</sub>, prostaglandin D<sub>2</sub>.

A more detailed analysis of these preparations revealed that the intracellular LTC<sub>4</sub>-synthesizing compartment within either PGD<sub>2</sub>- or BW245c/DK-PGD<sub>2</sub>-stimulated eosinophils was in a punctate cytoplasmic pattern, proximate to, but separate from the nucleus, and fully consistent in size and form with eosinophil lipid bodies. In fact, the compartment-

alization of newly formed LTC<sub>4</sub> to eosinophil lipid bodies was confirmed by their co-localization with ADRP (Figure 3; top and bottom panels), a lipid body marker protein. Virtually no LTC<sub>4</sub> was immune-localized within non-stimulated eosinophils (not shown), thus showing that the newly formed lipid bodies of *in vitro* DP<sub>1</sub>/DP<sub>2</sub>-engaged eosinophils

are the enzymatically fully equipped organelles responsible for the effective LTC<sub>4</sub> synthesis. Of note, eosinophils stimulated solely with BW245C, but not with DK-PGD<sub>2</sub>, display a lipid body-enriched cytoplasm as detected by the peroxide cytoplasmic ADRP immunolabelling (Figure 3; middle panel as indicated), suggesting that the initially hypothesized synergistic effect at receptor level between DP<sub>1</sub> and DP<sub>2</sub> does not take place. Instead, PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis by eosinophils appears to be due to distinct mechanistic roles of DP<sub>1</sub> and DP<sub>2</sub> receptors with complementary functional features.

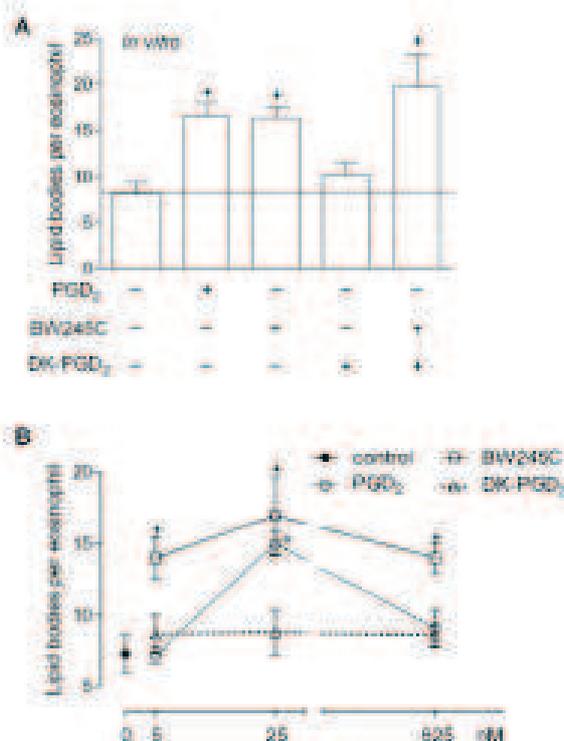
**DP<sub>1</sub> activation, but not that of DP<sub>2</sub>, evokes biogenesis of eosinophil lipid bodies**

To study whether the induction of lipid body assembly driven by DP<sub>1</sub> receptor pathways, contributed to PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis, we employed an osmium-based staining methodology that allows the enumeration of these organelles to check for lipid body biogenesis, under either single or combined receptor stimulation. In agreement with ElectronCell images shown previously (Figure 5), Figure 4 shows that selective activation of DP<sub>1</sub> receptors by BW245C, triggers rapid (within 1 h) assembly of new lipid bodies within human eosinophils in a dose-dependent manner. DP<sub>1</sub> receptor activation by DK-PGD<sub>2</sub>, on the other hand, even when used in high concentrations (Figure 4B) failed to induce eosinophil lipid body biogenesis. No alteration of DP<sub>1</sub> receptor-induced lipid body biogenesis was observed when eosinophils were co-stimulated with both DP<sub>1</sub> and DP<sub>2</sub> receptor agonists (Figure 4A), suggesting that only the DP<sub>1</sub> receptor controls the formation of these LTC<sub>4</sub>-synthesizing organelles.

To further investigate the role of the DP<sub>1</sub> receptor on lipid body biogenesis, we evaluated the participation of each receptor on PGD<sub>2</sub>-induced lipid body formation (osmium-stained cells) by pre-treating PGD<sub>2</sub>-challenged sensitized mice (Figure 5A) and PGD<sub>2</sub>-stimulated human eosinophils (Figure 5B) with either DP<sub>1</sub> or DP<sub>2</sub> receptor antagonists. *In vivo*, while the pretreatment with the DP<sub>1</sub> receptor antagonist ranarotrol did not affect the number of cytoplasmic lipid bodies found within infiltrating eosinophils or PGD<sub>2</sub>-elicited inflammatory reaction, pretreatment with the DP<sub>2</sub> antagonist BW868C nearly abolished this *in vivo* biogenic process. Similarly, *in vitro* pre-treatment of human eosinophils with the DP<sub>1</sub> receptor antagonist BW868C, significantly inhibited PGD<sub>2</sub>-induced eosinophil lipid body biogenesis, while two different DP<sub>2</sub> receptor antagonists, ranarotrol and Cay10471, failed to modify the lipid body assembly triggered by PGD<sub>2</sub>. Thus, it appears reasonable to postulate that the main role of DP<sub>2</sub> receptors in PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis is to initiate the intracellular signalling pathway that leads to the biogenesis of LTC<sub>4</sub>-synthesizing organelles in eosinophils.

**DP<sub>1</sub> receptors signal via PKA activation to trigger eosinophil lipid body biogenesis: a requirement for PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis**

To further evaluate the role of DP<sub>1</sub> receptors in PGD<sub>2</sub>-induced lipid body-driven LTC<sub>4</sub> synthesis, we have studied the contribution of DP<sub>1</sub> receptor-related cAMP-dependent signalling on PGD<sub>2</sub>-induced assembly of new lipid bodies within eosinophils. As shown in Figure 6, *in vitro* lipid body-assembly



**Figure 4** DP<sub>1</sub>, but not DP<sub>2</sub>, activation triggers lipid body biogenesis within human eosinophils *in vitro*. In A, human eosinophils were stimulated with PGD<sub>2</sub> (25 nM), BW245C (25 nM), DK-PGD<sub>2</sub> (25 nM) or with a combination of BW245C plus DK-PGD<sub>2</sub> (both at 25 nM). B shows a dose-response effect of PGD<sub>2</sub> (25 nM), BW245C (25 nM) or DK-PGD<sub>2</sub> (25 nM) on lipid body biogenesis after stimulation of human eosinophils. Analysis of lipid body biogenesis was performed 1 h after stimulation in osmium-stained cells. Results are expressed as means ± SEM from at least three different experiments with eosinophils purified from distinct donors. \*P < 0.05 compared with control. DP<sub>1</sub>, D-prostanoid receptor 1; DP<sub>2</sub>, D-prostanoid receptor 2; PGD<sub>2</sub>, prostaglandin G<sub>2</sub>.

triggered by stimulation with either PGD<sub>2</sub> (Figure 6A) or BW245C (Figure 7) was consistent with the central role of DP<sub>1</sub> receptors. In parallelism with H-89 or PKI (10 μM), two non-structurally related inhibitors of PKA activation, decreased the number of cytoplasmic lipid bodies found within PGD<sub>2</sub>- (Figure 6A; bottom panel) and BW245C-stimulated human eosinophils (Figure 7). While the specificity of PKA involvement in DP<sub>1</sub> receptor-driven effect was strengthened by the lack of effect of H-89 on PAF-induced eosinophil lipid body biogenesis (Table 1), the ability of forskolin, a well-known activator of adenylate cyclase, to trigger rapid formation of lipid bodies within human eosinophils substantiates the role of cAMP/PKA signalling pathway on the regulation of lipid body biogenic process (C. Sandeira-Melo, unpubl. data).

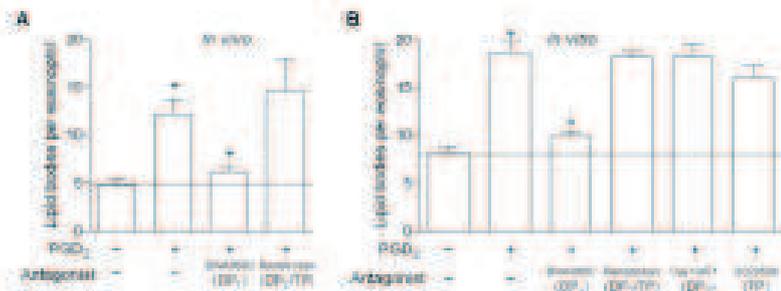


Figure 5

DP<sub>1</sub>, but not DP<sub>2</sub> receptor, control eosinophil lipid body biogenesis triggered by PGD<sub>2</sub> either *in vivo* or *in vitro*. In A, sensitized mice were pretreated with BW286c (1 mg/kg<sup>-1</sup>) or ramelteban (1 mg/kg<sup>-1</sup>), and then stimulated with an i.p. injection of PGD<sub>2</sub> (25 pmol/cavity). Analysis of lipid body biogenesis was performed 24 h after PGD<sub>2</sub> administration in osmium-stained cells. Results are expressed as the means ± SEM from at least six animals. \*P < 0.05 compared with control animals and \*\*P < 0.05 compared with PGD<sub>2</sub>-injected mice. In B, for *in vitro* analysis of lipid body biogenesis, human eosinophils were pretreated for 30 min with BW286c (200 nM), ramelteban (200 nM), Cay10497 (200 nM) or SQ29548 (200 nM), stimulated for 1 h with PGD<sub>2</sub> (25 nM) and subsequently stained with osmium. *In vitro* results are expressed as the means ± SEM from at least three different experiments with eosinophils purified from distinct donors. \*P < 0.05 compared with control. \*\*P < 0.05 compared with PGD<sub>2</sub>-stimulated eosinophils. DP<sub>1</sub>, D-prostanoid receptor 1; DP<sub>2</sub>, D-prostanoid receptor 2; i.p., intraperitoneal; PGD<sub>2</sub>, prostaglandin D<sub>2</sub>.

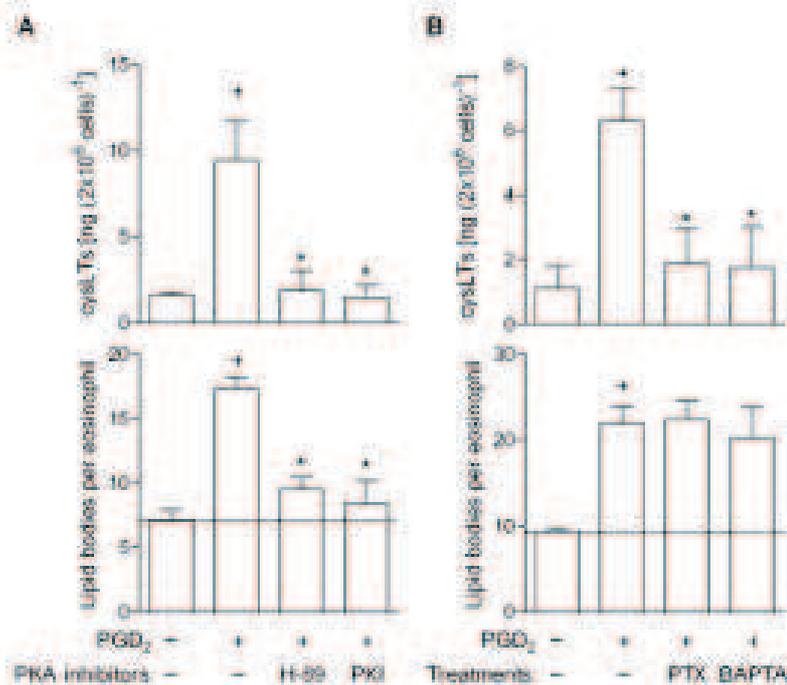
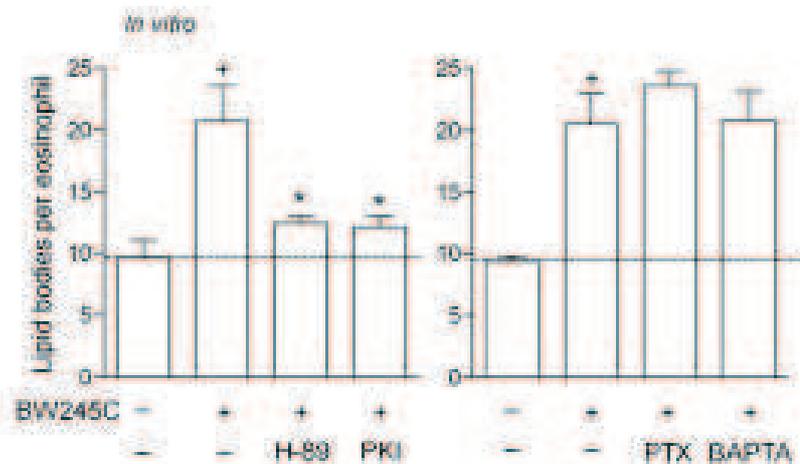


Figure 6

DP<sub>1</sub> receptor-driven PKA activation cooperates with DP<sub>2</sub>-driven G<sub>α</sub> protein activation and calcium influx to mediate lipid body-driven LTC<sub>4</sub> synthesis within human eosinophils triggered by *in vitro* PGD<sub>2</sub>. Human eosinophils were pretreated for 30 min with H-89 (10 μM) and PKI (10 μM) in A, or with PTX (1 μg/ml<sup>-1</sup>) or BAPTA-AM (25 μg/ml<sup>-1</sup>) in B and then stimulated with PGD<sub>2</sub> (25 nM). *In vitro* analysis of LTC<sub>4</sub> production in cell-free supernatants and lipid body biogenesis were analysed 1 h after PGD<sub>2</sub>. Results are expressed as the means ± SEM from at least three different experiments with eosinophils purified from different donors. \*P < 0.05 compared with control group. \*\*P < 0.05 compared with PGD<sub>2</sub>-stimulated eosinophils. DP<sub>1</sub>, D-prostanoid receptor 1; DP<sub>2</sub>, D-prostanoid receptor 2; PGD<sub>2</sub>, prostaglandin D<sub>2</sub>; PTX, Pertussis toxin.



**Figure 7**

PKA activation, but not  $G_{\alpha i}$  protein and calcium influx, mediates lipid body biogenesis within human eosinophils triggered by BW245C *in vitro*. Human eosinophils were pretreated for 30 min with PTX ( $7 \mu\text{g}\cdot\text{mL}^{-1}$ ), BAPTA-AM ( $25 \mu\text{g}\cdot\text{mL}^{-1}$ ), H-89 ( $10 \mu\text{M}$ ) or PKI ( $10 \mu\text{M}$ ), and then stimulated with BW245C ( $25 \text{ nM}$ ). Lipid body biogenesis was analysed 1 h after BW245C stimulation. Results are expressed as the means  $\pm$  SEM from at least three different experiments with eosinophils purified from different donors. \* $P < 0.05$  compared with control group. † $P < 0.05$  compared with  $\text{PGD}_2$ -stimulated eosinophils. PTX, Pertussis toxin.

In agreement with  $\text{DP}_1$ -driven induction of new lipid bodies as a prerequisite to concurrent  $\text{DP}_2$ -elicited signalling required for successful  $\text{LTC}_4$  synthesis, PKA inhibition by either H-89 or PKI pretreatment also consequently reduced  $\text{LTC}_4$  synthesis triggered *in vitro* by the  $\text{PGD}_2$  stimulation of human eosinophils (Figure 6A, upper panel).

#### *DP<sub>2</sub> receptor activation signals via PTX-sensitive G<sub>αi</sub> protein and calcium mobilization to prompt DP<sub>1</sub> receptor-driven newly formed lipid bodies to synthesize LTC<sub>4</sub>*

To establish how the concurrent activation of  $\text{DP}_2$  receptors contributes to  $\text{PGD}_2$ -induced lipid body-driven  $\text{LTC}_4$  synthesis, we have studied the potential role of  $G_{\alpha i}$  activation and cytoplasmic calcium mobilization elicited by  $\text{PGD}_2$ . Lipid body assembly triggered *in vitro* by stimulation with either  $\text{PGD}_2$  (Figure 6B) or BW245C (Figure 7) was not modified by pretreatments with inhibitors of either  $G_{\alpha i}$  activation or cytoplasmic calcium influx, PTX ( $1 \mu\text{g}\cdot\text{mL}^{-1}$ ) and BAPTA-AM ( $25 \mu\text{g}\cdot\text{mL}^{-1}$ ), respectively, ruling out once more the involvement of  $\text{DP}_2$  receptors in lipid body formation triggered by  $\text{PGD}_2$ . As shown in Table 1, PTX and BAPTA-AM were also able to reduce lipid body biogenesis induced by PAF within human eosinophils.

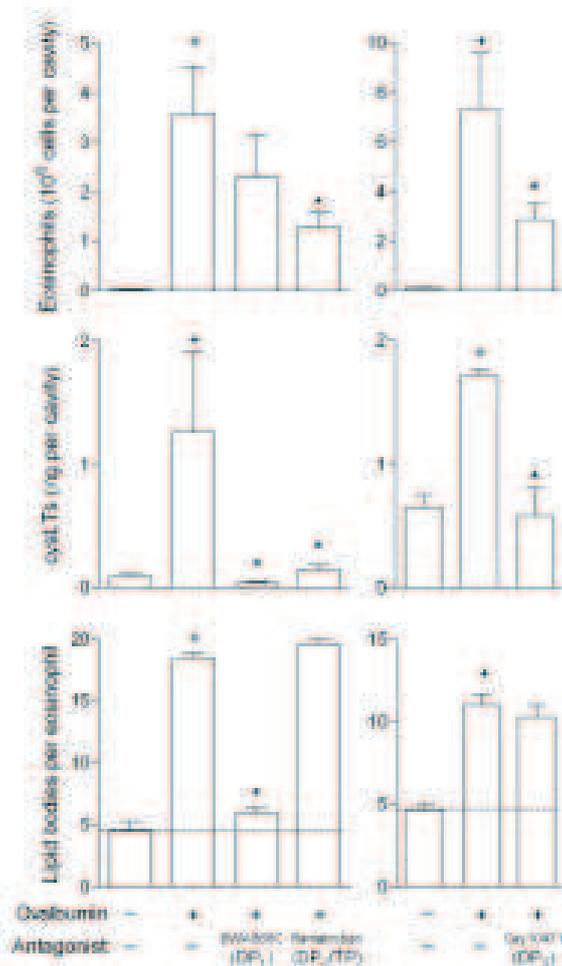
Although playing no role in  $\text{PGD}_2$ -induced lipid body assembly, the inhibition of  $G_{\alpha i}$  protein and calcium mobilization by, respectively, PTX and BAPTA-AM pretreatment, reduced  $\text{LTC}_4$  synthesis triggered *in vitro* by  $\text{PGD}_2$  stimulation of human eosinophils (Figure 6B; upper panel), indicating that  $\text{DP}_2$  receptors, by activating calcium-dependent signalling, converted  $\text{DP}_2$  receptor-induced lipid bodies into enzymatically active organelles capable of  $\text{LTC}_4$  synthesis.

#### *Eosinophil lipid body-driven LTC<sub>4</sub> synthesis elicited in vivo by allergic inflammation is also mediated by a complementary signalling between DP<sub>1</sub> and DP<sub>2</sub> receptors*

To verify whether the cooperative signalling between  $\text{DP}_1$  and  $\text{DP}_2$  receptors also operates under allergic inflammatory conditions *in vivo*, we employed a mouse model of allergic inflammation characterized by eosinophil accumulation and activation. As shown in Figure 8, while the  $\text{DP}_2$  antagonist BW24558C reduced both eosinophil lipid body biogenesis and increased levels of  $\text{cycL2}$  without significantly affecting eosinophil infiltration found in allergic reaction sites, the  $\text{DP}_1$  receptor antagonist ramantetan and Cay10471 decreased the numbers of recruited eosinophils and inhibited allergic  $\text{cycL2}$  production but failed to alter eosinophil lipid body biogenesis; thus, we again, illustrating the mandatory cooperation between the two  $\text{PGD}_2$  receptors to evoke  $\text{LTC}_4$  synthesis, as well establishing the relevance of such cooperation to the molecular mechanisms underlying allergy.

## Discussion

$\text{PGD}_2$  is now emerging as a potential mediator of allergic inflammatory pathologies, because it modulates the polarization of Th2 cells, oedema formation and eosinophil recruitment (Pouliques, 2008). In addition,  $\text{PGD}_2$  is able to directly activate recruited eosinophils, particularly by eliciting the capacity of eosinophils to synthesize  $\text{LTC}_4$  at sites of allergic inflammation. While the enzymic pathway by which eosinophils synthesize  $\text{LTC}_4$  is well characterized, the pathophysiological stimuli and intracellular signalling cascades that



**Figure 8**

Cooperation between DP<sub>1</sub> and DP<sub>2</sub> receptors to trigger lipid body-driven LTC<sub>4</sub> synthesis within human eosinophils also takes place in allergic inflammatory response *in vivo*. Sensitized mice were pretreated with BW284554 (1 mg kg<sup>-1</sup>), montelukast (1 mg kg<sup>-1</sup>) or Cay10471 (1 mg kg<sup>-1</sup>), and then challenged with an i.p. injection of ovalbumin (12 µg per cavity). Analyses of lipid body biogenesis and cyst-L6 production were performed 24 h after allergic challenge. Results are expressed as means ± SEM from at least six animals. \**P* < 0.05 compared with saline-challenged mice and #*P* < 0.05 compared with ovalbumin-challenged mice. cyst-L6, cysteinyl leukotriene; DP<sub>1</sub>, D<sub>1</sub> prostanoid receptor 1; DP<sub>2</sub>, D<sub>2</sub> prostanoid receptor 2; i.p., intraperitoneal.

Given such activity remain to be fully elucidated. Seeking such characterization, it is important to consider the evolving understanding of the potential roles that some eosinophil chemoattractants, which participate in the recruitment of eosinophils to sites of allergic inflammation, have as priming stimuli on eosinophil LTC<sub>4</sub> synthesis.

Our previous studies have demonstrated that, besides PGD<sub>2</sub>, other eosinophil chemoattractants, such as PAF, CCL11

and CCL5, are sensed in response to allergic challenge and, by acting on their specific receptors expressed on eosinophil membranes, initiate an intracellular cascade leading to enhanced LTC<sub>4</sub> synthesis (Borza *et al.*, 1996; Bandoira-Melo *et al.*, 2001). Our attempts to characterize the intracellular signalling pathways committed to chemoattractant-induced enhanced LTC<sub>4</sub> synthesis revealed that diverse stimuli-specific intracellular signalling events control LTC<sub>4</sub> synthesis within eosinophils. For instance, while PAF, acting via its P1R-sensitive G<sub>i</sub>-protein-linked receptor appears to induce LTC<sub>4</sub> synthesis via a downstream signalling involving PKC and phospholipase C (PLC) activation (Borza *et al.*, 1996; 1997; 1998), CCL11 and CCL5, acting via G<sub>i</sub>-protein-linked CCR3 receptors, signal via the activation of mitogen-activated protein kinases and phosphatidylinoside 3-kinase, but not PKC or PLC (Bandoira-Melo *et al.*, 2001). Even though diverse downstream cascades for LTC<sub>4</sub> synthesis can be engaged, common upstream steps triggered by the activation of CCR3 and PAF chemoattractant receptors share G<sub>i</sub>-protein- and calcium influx-regulated cellular activities that, besides culminating in cell polarization/migration, also leads to LTC<sub>4</sub> synthesis. Moreover, the well-documented role of PGD<sub>2</sub> in eosinophil trafficking as well as a variety of migration-related cellular responses, including actin polymerization and increased expression of adhesion molecules, depends on the activation of G<sub>i</sub> proteins and calcium influx (Morrison *et al.*, 2001; Sawyer *et al.*, 2002). Accordingly, we initially hypothesized that the PGD<sub>2</sub> chemoattractant receptor expressed on eosinophils, signalling via the activation of G<sub>i</sub> proteins and calcium influx, would be responsible for PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis. However, our data showed that specific DP<sub>2</sub> receptor stimulation, by itself, was not sufficient to trigger LTC<sub>4</sub> synthesis.

Another shared intracellular event triggered by eosinophil chemoattractants that is essential for the successful LTC<sub>4</sub> synthesis is the rapid assembly of new cytoplasmic lipid bodies – a biological process recognized as an acute, highly regulated cellular event that is stimulus- and cell-specific (Borza *et al.*, 2007). As multifunctional organelles, lipid bodies are a hallmark of leukocyte activation and, together with perinuclear envelope (Bandoira-Melo *et al.*, 2001; Tolla *et al.*, 2003) and phagosomes (Bakstien *et al.*, 2006), represent a potential intracellular domain for LTC<sub>4</sub> synthesis. Within eosinophils, compartmentalized LTC<sub>4</sub> synthesis triggered by eosinophil chemoattractant agents, including PGD<sub>2</sub>, has been located specifically within lipid bodies, thereby explaining why the biogenesis of lipid bodies critically affects the biosynthesis of LTC<sub>4</sub> (Bandoira-Melo *et al.*, 2001, 2002b; Mesquina-Santos *et al.*, 2006). Again, different from the other eosinophil chemoattractant receptors, DP<sub>2</sub> receptor stimulation alone did not promote PGD<sub>2</sub>-induced lipid body biogenesis, which was also not dependent on DP<sub>2</sub> receptor-related G<sub>i</sub> and calcium signalling. Inasmuch as the lipid body biogenic process is mandatory for LTC<sub>4</sub> synthesis, the inability of DP<sub>2</sub> receptors to trigger lipid body biogenesis in part explains its inability to promote enhanced LTC<sub>4</sub> synthesis within eosinophils.

We found that the discrepancy between DP<sub>2</sub> receptors and the other eosinophil chemoattractant receptors, in terms of eliciting LTC<sub>4</sub> synthesis, relies on the more complex PGD<sub>2</sub> receptor system expressed on eosinophils. Besides DP<sub>2</sub> receptors,

eosinophils also express the non-chemotactic DP<sub>2</sub> receptor. By dissecting the specific contributions that each receptor makes to PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis, we demonstrated that the PGD<sub>2</sub>-elicited rapid *de novo* assembly of lipid bodies was insensitive to PTX or a calcium chelator, but was largely dependent on the activation of DP<sub>2</sub> receptor-elicited PKA signalling. Of note, the DP<sub>2</sub> receptor activation by PGD<sub>2</sub> signalling through G<sub>αi</sub> proteins leads to increased cAMP and PKA activity – an intracellular signal transducing cascade that is clinically related to the inhibition of chemoattractant-induced eosinophil motility (Irima et al., 2001; Mosmann et al., 2001), and consistent with the idea of cAMP elevating agents as powerful anti-inflammatory (Toussain et al., 1995; Diaz et al., 1996) or pro-resolution (Souza et al., 2009) agents for the treatment of diseases in which eosinophil accumulation is thought to play a relevant role (Souza et al., 2009). In this context, it was noticeable that DP<sub>2</sub> receptor-driven, PKA-dependent, newly formed lipid bodies were not able to synthesize LTC<sub>4</sub>, as shown in the ficolinA1 preparations. In contrast to BAF- or CCL11-induced LTC<sub>4</sub>-synthesizing lipid bodies, we demonstrated that under PGD<sub>2</sub> stimulation, compartmentalized LTC<sub>4</sub> synthesis within DP<sub>2</sub> receptor-driven eosinophil lipid bodies, which were assembled under cAMP/PKA regulation, demands concurrent DP<sub>2</sub> receptor stimulation, inasmuch as: (i) antagonists for either receptor were equally able to reduce PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis; (ii) DP<sub>1</sub>, but not DP<sub>2</sub> receptor antagonists, inhibited PGD<sub>2</sub>-induced lipid body biogenesis; (iii) only by co-stimulating eosinophils with both DP<sub>1</sub> and DP<sub>2</sub> receptor agonists, was PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis mimicked; (iv) inhibition of PKA inhibited PGD<sub>2</sub>-induced DP<sub>2</sub> receptor-driven lipid body biogenesis and subsequent lipid body-compartmentalized LTC<sub>4</sub> synthesis; and (v) PTX and BAPTA-AM, while failing to interfere with DP<sub>2</sub> receptor-dependent lipid body biogenesis, inhibited PGD<sub>2</sub>-induced lipid body-driven LTC<sub>4</sub> synthesis. Therefore, the molecular mechanisms orchestrating how DP<sub>2</sub> receptor activation converts DP<sub>2</sub> receptor-driven lipid bodies into enzymatically active organelles capable of effective LTC<sub>4</sub> synthesis depend on the coordinated G<sub>αi</sub> activation and calcium mobilization.

Recently, focusing on eosinophil-driven allergic pathologies, we have found that, alongside CCL11, CCL5, BAF and macrophage migration inhibitory factor (Bandiera-Melo et al., 2001; Vieira-de-Almeida et al., 2005; 2010), PGD<sub>2</sub> is as an endogenous and potent biogenic stimulus of enzymatically active lipid bodies, organelles involved in LTC<sub>4</sub> synthesis by eosinophils (Mesquita-Santos et al., 2006). Here, despite the evidence showing that PGD<sub>2</sub>-driven eosinophil migration-related activities are mediated by a balance of opposing intracellular signalling cascades downstream of DP<sub>1</sub> and DP<sub>2</sub> receptor activation within eosinophils (Mosmann et al., 2001), we uncovered that the intracellular mechanisms of receptor-mediated PGD<sub>2</sub>-induced LTC<sub>4</sub> synthesis rely on the collaborative signalling between both PGD<sub>2</sub> receptors. The PGD<sub>2</sub>-elicited LTC<sub>4</sub> synthesis is dependent on the activation of DP<sub>2</sub> receptor-elicited PKA-regulated lipid bodies, in addition to an equally important and concomitant DP<sub>1</sub> receptor-elicited G<sub>αi</sub>/calcium-regulated signalling pathway, which prompts DP<sub>2</sub> receptor-driven, newly formed lipid bodies to synthesize LTC<sub>4</sub>.

Collectively, our findings indicate that PGD<sub>2</sub> binding to DP<sub>2</sub> receptors triggers PKA-driven biogenesis of cytoplasmic lipid bodies, but is incapable of activating the LTC<sub>4</sub>-synthesizing machinery, which is switched on by concurrent DP<sub>1</sub> receptor activation. Furthermore, by using PGD<sub>2</sub> receptor antagonists, we also demonstrated that during allergen-elicited eosinophilic inflammatory reactions, cysts production is also regulated by DP<sub>1</sub>/DP<sub>2</sub>-orchestrated eosinophil activation, thus indicating that either DP<sub>1</sub> or DP<sub>2</sub> antagonists might be highly effective as controlling eosinophil activation-regulated LTC<sub>4</sub> synthesis as sites of allergic reactions. However, considering the disappointing clinical results of the DP<sub>2</sub> receptor antagonist leupipram in asthmatics and allergic rhinitis patients (Phillip et al., 2009), therapies based on dual blockade of DP<sub>1</sub> and DP<sub>2</sub> receptors or PGD<sub>2</sub> synthesis inhibition may display increased beneficial outcome.

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## Conflict of interest

The authors have declared that no competing interests exist.

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#### 4.2) Artigo II:

##### **“Anti-allergic effects of *Cissampelos sympodialis* and its alkaloid warifteine depend on inhibition of PGD<sub>2</sub> synthesis”**

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Neste trabalho demonstramos que, os já descritos efeitos antiinflamatórios do extrato da *C. sympodialis* e do seu alcalóide isolado – a warifteína – capazes de reverter a eosinofilia pleural, a biogênese de corpúsculos lipídicos e a síntese de LTC<sub>4</sub> são mediados pela inibição da síntese de PGD<sub>2</sub> durante os processos inflamatórios alérgicos. Nós demonstramos também que, a warifteína foi capaz de inibir a síntese de PGD<sub>2</sub> – mas não de LTC<sub>4</sub> – por mastócitos ativados *in vitro*, assim como não inibiu a síntese de PGE<sub>2</sub> por macrófagos peritoneais murinos *in vitro* ativados com A23187. Em conjunto esses dados sugerem que provavelmente, o alvo molecular responsável pelas ações antiinflamatórias da warifteína, seja a enzima prostaglandina D sintase hematopoiética (H-PGDS).

## **Anti-allergic effects of *Cissampelos sympodialis* and its alkaloid warifteine depend on inhibition of PGD<sub>2</sub> synthesis**

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### **Abbreviations**

Calcium Ionophore, A23187; COX, ciclooxigenase; cPLA<sub>2</sub>, cytosolic phospholipase; cysLT, cysteinyl leukotriene; DP1, D prostanoid receptor 1; H-PGDS, hematopoietic prostaglandin D synthase; HQL-79, prostaglandin D synthase inhibitor; LTC<sub>4</sub>, leukotriene C<sub>4</sub>; OVA, ovalbumin; PGD<sub>2</sub>, prostaglandin D<sub>2</sub>; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>;

## ABSTRACT:

Medicinal plants are alternative therapeutic options to many diseases. It has been shown that *Cissampelos sympodialis* extract and its isolated alkaloid warifteine display anti-allergic properties, by controlling migration and activation of eosinophils – leukocytes with key roles in the pathogenesis of allergic inflammatory diseases, like asthma. Here, we investigated the mechanism of action involved in the anti-allergic properties of *C. sympodialis* extract and warifteine, focusing on PGD<sub>2</sub> as a potential molecular target. Initially we tested potential direct interference of warifteine on PGD<sub>2</sub>/receptor interaction. PGD<sub>2</sub>-induced eosinophil activation – as *in vitro* induction of lipid body biogenesis within human eosinophils – was not modified by the pre-incubation with warifteine, indicating that antagonism of PGD<sub>2</sub> receptors does not represent warifteine mechanism of action. By employing two mouse model of allergic inflammation, we observed that pretreatment with *C. sympodialis* (400 mg/Kg) or warifteine (50 µg/animal) were capable to inhibit the PGD<sub>2</sub> production during allergic pleurisy. Similarly, *in vitro* pre-incubation with warifteine blocked PGD<sub>2</sub> release triggered by antigen-challenged IgE-sensitized bone marrow-derived mast cells. In contrast, warifteine failed to affect release of mast cell-derived LTC<sub>4</sub>, as well as, PGE<sub>2</sub> production by activated macrophages, showing that both cPLA<sub>2</sub>- and COX-mediated steps of PGD<sub>2</sub> synthesis are not the targeted events of warifteine. Altogether, our findings suggest that the mechanisms involved in the anti-allergic effects of *C. sympodialis* and its alkaloid warifteine depends on inhibition of synthesis rather activity of PGD<sub>2</sub> on their receptors. While the enzymatic step affected by *C. sympodialis* or warifteine are still not defined, hematopoietic prostaglandin D synthase (H-PGDS) emerges as a potential molecular target.

**Keywords:** *C. sympodialis*, warifteine; PGD<sub>2</sub>, LTC<sub>4</sub>, eosinophils, mast cells, allergy; lipid droplet; cysteinyl leukotriene.

## **INTRODUCTION:**

Eosinophils have a critical role in the pathogenesis of allergic inflammatory conditions, like asthma (Wardlaw, 1999; Gleich, 2000; Lemanske et al, 2003). When activated, these cells are capable to elaborate and release several eicosanoids derived from the oxidative metabolism of arachidonic acid, like cysteinyl leukotrienes (cysLTs; LTC<sub>4</sub>/D<sub>4</sub>/E<sub>4</sub>) (Weller et al, 1983; Cowburn et al, 1998). During asthma, cysLTs are involved with reduction of lung function (Arm *et al.*, 1990), mucus secretion from bronchial epithelial cells and increase of pulmonary vascular permeability (Lewis *et al.*, 1990; Laitinen *et al.*, 1993; Henderson, Jr., 1994). Recently, we have demonstrated that LTC<sub>4</sub> released by infiltrating eosinophils depends on highly regulated biogenesis of LTC<sub>4</sub> synthesis-compartmentalizing organelles, named lipid bodies (Mesquita-Santos *et al.*, 2006). In addition, we have also shown that lipid body-driven LTC<sub>4</sub> synthesis within recruited eosinophils is a multi-mediated process controlled by complementary activity of endogenous molecules: RANTES- and eotaxin-driven CCR3 activation, MIF and its receptor CD74 and obligatory cooperation between the PGD<sub>2</sub> receptors DP1 and DP2 (Vieira-de-Abreu *et al.*, 2005b; Mesquita-Santos *et al.*, 2006; Mesquita-Santos *et al.*, 2010; Vieira-de-Abreu *et al.*, 2010). Inasmuch as these mediators act in concert to trigger the remarkable LTC<sub>4</sub> synthesis by eosinophils in sites of allergic inflammation, these molecules emerge as interesting targets to anti-allergic therapies.

Conventional therapies have limited success on asthma control (Firszt *et al.*, 2010). Thus, new therapeutic molecules for allergic diseases treatment should be developed. Medicinal plants are alternative options to many diseases, including allergic

diseases like asthma (Rogerio *et al.*, 2010). *Cissampelos sympodialis* Eichl. (Menispermaceae) is a species found in northeastern and southeast of Brazil. Phytochemical analysis of *C. sympodialis* root extracts identified a group of bioactive alkaloids, including milonine, methylwarifteine, roraimine and warifteine (Barbosa-Filho *et al.*, 1997; de Lira *et al.*, 2002). Popular use has demonstrated anti-inflammatory effects of *C. sympodialis* during inflammatory conditions, like rheumatism and arthritis (Barbosa-Filho *et al.*, 1997; Agra *et al.*, 2007a; Agra *et al.*, 2007b). Concerning cellular impact of *C. sympodialis* and warifeteine, it has been shown that the aqueous fraction of ethanolic extract of *C. sympodialis* and warifteine are capable to inhibit B cells proliferation (Alexandre-Moreira *et al.*, 2003b; Rocha *et al.*, 2010) and macrophage microbicidal activity (Alexandre-Moreira *et al.*, 2003a). In addition, in mouse models of allergic inflammatory response the *C. sympodialis* extract displayed anti-allergic effects being able to inhibit IgE synthesis, oedema formation, eosinophil influx, cysLTs production and lipid body biogenesis within infiltrating eosinophils (Bezerra-Santos *et al.*, 2006). These studies also showed that warifteine, the most abundant alkaloid isolated from *C. sympodialis* extract (Melo *et al.*, 2003), is capable of mimicking *C. sympodialis* extract anti-allergic effects (Bezerra-Santos *et al.*, 2006).

PGD<sub>2</sub> is a major metabolite from arachidonic acid metabolism, produced during allergic response by activated mast cells (Murray *et al.*, 1986). Of note, both *C. sympodialis* extract and warifteine were able to reduce mast cell degranulation (Costa *et al.*, 2008). Allergic parameters including eosinophil influx, cysLTs production and lipid body biogenesis within infiltrating eosinophils are mediated by mast cell-derived PGD<sub>2</sub> produced during allergic response (Mesquita-Santos *et al.*, 2006). While allergen-induced eosinophil influx is mediated by PGD<sub>2</sub> receptor DP2, and lipid body biogenesis

via DP1, allergen-induced production of cysLTs is controlled by cooperation between both PGD<sub>2</sub> receptors. Here, we have hypothesized PGD<sub>2</sub> as the molecular target of *C. sympodialis* and warifteine anti-allergic effect and investigated inhibition of PGD<sub>2</sub> synthesis and/or PGD<sub>2</sub> receptor antagonism as potential mechanisms of action.

## **METHODS:**

### *Preparation of C. sympodialis extract and warifteine purification:*

Leaves from *C. sympodialis* were obtained from the Botanical Garden of the Laboratório de Tecnologia Farmacêutica/ Universidade Federal da Paraíba (voucher specimen Agra 1456). The aqueous extract of *C. sympodialis* was submitted to procedures aimed to isolate warifteine, using column and thin layer chromatography as described at Bezerra-Santos *et al.*, 2006.

### *Animals:*

Swiss male mice (weight 20-25g) were obtained from Oswaldo Cruz Foundation and maintained with food and water *ad libitum* in a room (with temperature ranging from 22 to 24°C and a 12 h light/dark cycle) in the Department of Physiology and Pharmacology. The protocols were approved by the Oswaldo Cruz Animal Welfare Committee.

### *Pleurisy model in actively sensitized mice:*

Each mice was actively sensitized by a subcutaneous (s.c.) injection of a mixture containing 50 µg of ovalbumin (OVA) and 5 mg of Al(OH)<sub>3</sub> in sterile saline (final volume: 0,2mL). In allergic pleurisy model, allergic challenge was performed 14 days after sensitization through by an intrathoracic (i.t.) injection of OVA (12 µg/cavity) dissolved in sterile saline. In prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) pleurisy model, stimuli was performed 14 days after sensitization through by an intrathoracic (i.t.) injection of PGD<sub>2</sub> (25 pmol/cavity). In both model, a control group was stimulated (i.t.) with sterile saline. All i.t. injections were preformed in a final volume of 0,1 ml. Mice were killed under an excess of CO<sub>2</sub> atmosphere 24 hours after challenge/stimuli. Pleural cavities were then rinsed with 1 mL

of PBS to evaluation of pleural eosinophilia, lipid bodies within recruited eosinophils and cysteinyl leukotrienes (cysLTs) levels quantification.

*Asthma model in actively sensitized mice:*

Asthma symptoms in response to OVA was generated in mice as described (Lloyd *et al.*, 2001). Briefly, mice were sensitized with intraperitoneal injection of OVA (10 µg/mouse) and Al(OH)<sub>3</sub> (10 mg/ml) in 0.9% NaCl solution (0.2 ml final solution/animal) on days 1 and 10. From day 19 to day 24 after sensitization, mice were challenged daily for 20 min with OVA (5 %) in phosphate buffered saline (PBS) by aerosol. Aerosolized PBS was administered to sensitized mice as a negative control. These procedures were performed in a 30 x 20 x 10 cm acrylic chamber and the aerosol was generated with an ultrasonic nebulizer.

*Bronchoalveolar lavage (BAL):*

Animals were euthanized by CO<sub>2</sub> and the trachea was surgically exposed and cannulated, 24 h after allergic challenge. The bronchoalveolar lavage (BAL) was collected from the mice by washing the lungs with 1 ml PBS. Samples of BAL were centrifuged at 500 g for 8 min at 4°C to supernatants separation for further PGD<sub>2</sub> quantification.

*In vitro stimulation of human granulocytes:*

Fresh human blood was obtained by venipuncture from healthy adult volunteers and collected into syringes with acidified citrate. After addition of 6% Hetastarch (Fisher Scientific), RBC were allowed to sediment for 1 h at room temperature. The leukocyte-

rich supernatant was overlaid onto an equal volume of Ficoll-Paque (Ficoll-Paque™ Plus - GE Health Care), and centrifuged at 400 g for 20 min. PMN (>95% pure, rest being eosinophils) were recovered from the pellet and washed in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS (HBSS<sup>-/-</sup>). Residual RBC were lysed with hypotonic saline. Cell suspension was adjusted to 2x10<sup>6</sup> cells in medium (HBSS<sup>-/-</sup>) aimed to be stimulated.

*In vitro bone marrow-derived mast cells (BMMC) differentiation and stimulation:*

BMMC were derived in 50% WEHI-3 cell-conditioned medium as a source of IL-3, as previously described (Murakami *et al.*, 1994). After differentiation, concentration of cell suspension (containing approximately 90% BMMC) was adjusted to 10<sup>7</sup> cells/mL in TGB medium and exposed to IgE (1:300). BMMC concentration was adjusted to 8,5x10<sup>5</sup> cells/well and stimulated with IgE-TNP cross-linking (TNP at 100 ng/mL) and A23187 (0,1 µM) during 30 minutes at 37°C to evaluate PGD<sub>2</sub> production.

*In vitro stimulation of mouse peritoneal macrophages:*

Peritoneal macrophages from swiss male mice were recovered from normal mice or from mice stimulated with thioglycolate (3%) intraperitoneal injection. After 72h, animals were euthanized by CO<sub>2</sub> and peritoneal cavities were rinsed with RPMI (without BSF). Cell concentration were adjusted to 2x10<sup>6</sup>/mL and stimulated with A23187 (5µM) during 15 minutes or 2 h at 37°C.

*Treatments:*

In *in vitro* experiments human granulocytes, mouse macrophages and BMMC were incubated with warifteine (0,1; 1; 10 µg/ml) during 30 min (at 4°C) before appropriate

stimulation. In *in vivo* experiments, in both, allergic pleurisy or PGD<sub>2</sub>-induced pleurisy, animals were pre-treated with *C.symphodialis* extracts (400mg/ml) or warifteine (50µg/animal) by a single oral administration – or with H-PGDS inhibitor, HQL-79 (1 mg/kg) through by intraperitoneal injection – 1h before intrapleural stimulation. In *in vivo* asthma model, mice were treated 1 h before (pre-treatment) the last aerosol ovalbumin challenge with warifteine (50 µg/animal) by oral administration.

#### *Evaluation of Leukocytes:*

Total leukocyte counting was made in Neubauer chambers through by an optical microscope analysis, after diluting samples of the pleural fluid in Turk Fluid (2 % acetic acid). Differential leukocyte analysis was performed under an oil immersion objective on cytocentrifuged cells stained with May-Grunwald-Giemsa dye. Counts were reported as total number of cells (x 10<sup>6</sup>) per cavity.

#### *Lipid bodies staining and enumeration:*

Cells obtained from pleural fluid and *in vitro* assay were cytocentrifuged (450 rpm, 5 min) onto glass slides (10<sup>5</sup> cells / slide). While still moist, cells were fixed in 3.7 % formaldehyde (diluted in Ca<sup>2+</sup>/Mg<sup>2+</sup> - free HBSS; pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained with 1.5 % OsO<sub>4</sub> for 30 min, rinsed in distilled H<sub>2</sub>O, immersed in 1.0 % thiocarbohydrazide for 5 min, rinsed in 0.1 M cadodylate buffer, restained with 1.5 % OsO<sub>4</sub> for 3 min, rinsed in distilled water and then dried and mounted. By optical microscopy with an objective lens at 1000X magnification, cell morphology was observed and lipid bodies were enumerated. Twenty-five consecutively scanned

eosinophils were evaluated and results were expressed as the mean numbers of lipid bodies per eosinophil.

*Quantification of cysLTs, PGE<sub>2</sub> and PGD<sub>2</sub> (EIA):*

After take samples for leukocytes evaluation and lipid bodies analysis (*in vivo* and *in vitro* assay), samples were centrifuged (1500rpm / 10 min) and supernatants were collected. Levels of cisLTs (LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>), PGE<sub>2</sub> and PGD<sub>2</sub> produced were measured according to manufacturer's instructions by EIA kits (Cayman Chemical).

*Statistical analysis:*

Data were analyzed by ANOVA followed by Newman Keuls *t* test using GraphPad Prism statistical analysis and graphing software (GraphPad, San Diego, CA). Values given are means  $\pm$  SEM from at least six animals in each group. A value of  $p < 0.05$  was considered significant.

## RESULTS:

### C. sympodialis and warifteine inhibit PGD<sub>2</sub>-induced eosinophilic inflammation

Inasmuch as allergen-induced eosinophil migration and activation, which are mediated by endogenous PGD<sub>2</sub> produced in response to allergic challenge (Mesquita-Santos *et al.*, 2006), are events inhibited by *C. sympodialis* extract and its isolated alkaloid warifteine (Bezerra-Santos *et al.*, 2006), here we tested *C. sympodialis* and warifteine directly against PGD<sub>2</sub> itself. To this end, we initially employed a PGD<sub>2</sub>-induced inflammatory model of eosinophilic reaction triggered by intrapleural challenge with PGD<sub>2</sub> (35 pmol/cavity) in actively sensitized mice. As shown in Figure 1, oral pre-treatment with *C. sympodialis* (400 mg/kg) performed 1 h before PGD<sub>2</sub> administration was effective against PGD<sub>2</sub>-induced eosinophilic inflammation by inhibiting eosinophil accumulation, lipid body biogenesis within recruited eosinophils and cysLT production. Similarly, the pre-treatment with the *C. sympodialis*-derived alkaloid warifteine (0.5 - 50 µg/animal) was also able to inhibit in a dose-dependent fashion PGD<sub>2</sub>-induced eosinophil migration and activation, since attenuated pleural eosinophilia, eosinophil lipid body biogenesis and cysLTs production (Figure 2). These results suggested that the mechanism of action to *C. sympodialis*/warifteine may involve inhibition of PGD<sub>2</sub> agonistic activity during eosinophilic inflammation.

### Warifteine mechanism of action does not involve antagonism of PGD<sub>2</sub> receptor DP1

To test the hypothesis that warifteine functions as a PGD<sub>2</sub> receptor antagonist, we have moved to *in vitro* assays with human eosinophils directly stimulated by either PGD<sub>2</sub> itself or by the selective DP1 agonist BW245c. As shown in Figure 3, pre-incubation of human eosinophils with warifteine failed to affect induction of lipid body

biogenesis triggered by either PGD<sub>2</sub> (Figure 3A) or BW245c (Figure 3B), indicating that a mechanism distinct from antagonism of PGD<sub>2</sub> receptor is responsible by anti-allergic effects of warifteine.

#### PGD<sub>2</sub>-induced eosinophilic inflammation *in vivo* depends on mediation by an endogenous PGD<sub>2</sub>

Similar to *C. sympodialis* and warifteine, the pre-treatment with HQL-79 – an inhibitor of PGD<sub>2</sub> synthesis – was also able to inhibit *in vivo* the parameters of eosinophilic inflammation – eosinophil influx, lipid body biogenesis and cysLT secretion – triggered by PGD<sub>2</sub> administration in actively sensitized animals. These findings indicate that an endogenous PGD<sub>2</sub> produced in response to PGD<sub>2</sub> administration mediates PGD<sub>2</sub>-induced eosinophilic inflammation. Therefore, we can speculate that instead of receptor antagonistic activity, *C. sympodialis* and its alkaloid warifteine may be functioning by a putative inhibitory effect on PGD<sub>2</sub> synthesis (Table 1).

#### *In vivo* C. sympodialis and warifteine inhibits allergen-induced PGD<sub>2</sub> production in mouse models of allergic inflammation.

To test the hypothesis that *C. sympodialis* and warifteine mechanisms of action depend on inhibition of PGD<sub>2</sub> synthesis, the allergic pleurisy and asthma model of pulmonary inflammation were employed. First, we confirmed Bezerra-Santos and coworkers (2006) findings that pre-treatments with *C. sympodialis* and warifteine are capable to inhibit allergen-induced local eosinophilia, lipid body formation within infiltrating eosinophils and cysLTs production (data not shown). In parallel, as shown in Figure 4, *C. sympodialis* and warifteine also blocked PGD<sub>2</sub> production triggered by

allergen challenge, since the PGD<sub>2</sub> levels found either in pleural fluid or BAL of *C. sympodialis*- or warifteine-treated allergen-challenged sensitized animals returned to basal levels in both models of allergic inflammation.

#### Warifteine directly inhibits PGD<sub>2</sub> synthesis by activated mast cells

To attest warifteine ability to directly inhibit PGD<sub>2</sub> synthesis, we studied whether warifteine could affect mast cell ability of synthesizing PGD<sub>2</sub> triggered *in vitro* by antigen challenge. As shown in Figure 5A, pre-treatment of bone marrow-derived mast cells with two concentration of warifteine (1 and 10 µg/mL) significantly reduced PGD<sub>2</sub> amounts found in the cell free supernatants of antigen-challenged IgE-sensitized mast cells. Based on these findings and on PGD<sub>2</sub> well-known mediation of allergic inflammation, we identified inhibition of PGD<sub>2</sub> synthesis as the warifteine mechanism of action.

#### cPLA<sub>2</sub> and COX are not the molecular targets of warifteine capability of inhibiting PGD<sub>2</sub> synthesis

Even though our previous data unveiled PGD<sub>2</sub>-synthesizing process as the target event of warifteine anti-allergic effect, the specific molecule of PGD<sub>2</sub> synthesis cascade affected by warifteine still needs characterization. PGD<sub>2</sub> synthesis, like the synthesis of any other eicosanoid, is a multi-step enzymatic event which comprises several potential molecular targets, including for instance cPLA<sub>2</sub> – a key enzyme of eicosanoid synthesis responsible for mobilizing arachidonic acid from membrane phospholipids. In attempt to find such specific target, we have initially hypothesized cPLA<sub>2</sub> as a molecular candidate and analyzed the effect of warifteine on LTC<sub>4</sub> synthesis by mast cells. Experimental rational was based on the fact that PGD<sub>2</sub> and LTC<sub>4</sub> synthesizing cascades only share the cPLA<sub>2</sub>-mediated step. As shown in Figure 5B, warifteine failed to affect LTC<sub>4</sub>

synthesis, while inhibited PGD<sub>2</sub> synthesis (Figure 5A), triggered by antigen-challenge in IgE-sensitized bone marrow-derived mast cells, indicating that cPLA<sub>2</sub> does not represent the enzyme of PGD<sub>2</sub> synthesis targeted by warifteine.

Following release from membrane phospholipids by cPLA<sub>2</sub>, AA may be catalyzed by cyclooxygenase (COX) enzymes to form prostanoids, like PGD<sub>2</sub> and PGE<sub>2</sub>. After discarding cPLA<sub>2</sub>, we focused on COX as another potential warifteine molecular target by evaluating warifteine effect on PGE<sub>2</sub> production by macrophages. To this end, peritoneal macrophages recovered from normal (Figure 6A) or thioglycolate-injected mice (Figure 6B) were pre-treated with warifteine *in vitro* for 30 min and then stimulated with A23187 (5 mM) for 15 min to induction of PGE<sub>2</sub> production. Both concentrations of warifteine used (1 and 10 µg/mL), which are capable of inhibiting PGD<sub>2</sub> synthesis by mast cells, did not modify PGE<sub>2</sub> synthesis by A23187-stimulated macrophages, indicating that inhibition of COX-regulated enzymatic step of PGD<sub>2</sub> synthesis also does not correspond to warifteine mechanism of anti-allergic effect.

## DISCUSSION:

Modulation of eosinophil migration and activation during allergic inflammatory diseases represent an interesting mechanism to the development of new therapeutical strategies. Indeed, it was demonstrated remission of the allergy-related symptomatology by resolving tecidual eosinophilia (Gleich *et al.*, 1993; Weller *et al.*, 1997; Rothenberg, 1998). Our previous observations showing that pre-treatments with either *C. sympodialis* or warifteine reduce tissue eosinophilia and eosinophil activation, characterized by increased lipid body biogenesis and LTC<sub>4</sub> synthesis (Bezerra-Santos *et al.*, 2006), identified these phyto-treatments as appealing therapeutic alternatives. However, the mechanisms of action operating *C. sympodialis*/warifteine anti-allergic impact were still unknown. Because PGD<sub>2</sub> is a mediator controlling the very same parameters of allergen-triggered eosinophilic inflammation targeted by *C. sympodialis* and warifteine (Mesquita-Santos *et al.*, 2006), here we investigated modulation of PGD<sub>2</sub> as the potential target of inhibitory effects of *C. sympodialis* and warifteine.

Initially, we observed that *C. sympodialis* pre-treatment was able to inhibit PGD<sub>2</sub>-induced inflammatory effects, blocking eosinophil accumulation to the pleural cavity, lipid body biogenesis within recruited eosinophils and cysLT production. As *C. sympodialis* anti-inflammatory effects were attributed to its isolated alkaloid (Bezerra-Santos *et al.*, 2006), we pre-treated actively sensitized mice with warifteine to clarify whether it would also interfere with PGD<sub>2</sub>-induced inflammatory response. In fact, similar to *C. sympodialis*, warifterine also inhibited PGD<sub>2</sub>-induced *in vivo* eosinophilic migration and activation. Based on these data, we raised a initial hypothesis that antagonism of PGD<sub>2</sub> receptors correspond to the molecular target of warifteine anti-allergic effect.

Our group has demonstrated that during allergic response, eosinophil activation can be (i) modulated by treatment with natural product extracts (Vieira-de-Abreu *et al.*, 2005a); (ii) is a multi-mediated event controlled synergistically by at least eotaxin, MIF and PGD<sub>2</sub> (Mesquita-Santos *et al.*, 2006; Vieira-de-Abreu *et al.*, 2010); and (iii) that LTC<sub>4</sub> synthesis by eosinophils activated with PGD<sub>2</sub> requires mandatory simultaneous DP1/DP2 activation, while PGD<sub>2</sub>-induced eosinophil lipid body biogenesis depends exclusively on DP1 activation (Mesquita-Santos *et al.*, 2010). Here, warifteine fail to alter either PGD<sub>2</sub>- or BW245c-induced lipid body biogenesis. Although, we do not performed any experiments to prove that warifteine pre-treatment do not interfere on PGD<sub>2</sub> and DP2 receptor interaction, like by performing chemotaxis or shape-change assays (known DP2-mediated phenomena) (Gervais *et al.*, 2001; Monneret *et al.*, 2001; Schratl *et al.*, 2007), we have excluded our initial idea that warifteine would act on PGD<sub>2</sub> interaction with its receptors. While, we do recognize that the role of DP2 receptor in these events needs to be elucidated, our data on inhibition of PGD<sub>2</sub> synthesis redirected our working hypothesis.

Our findings obtained from assays employing an inhibitor of PGD<sub>2</sub> synthesis (HQL-79) on murine model of PGD<sub>2</sub>-induced eosinophilic inflammation, brought out an alternative explanation to *C. sympodialis* and warifteine ability to inhibit *in vivo* PGD<sub>2</sub>-driven eosinophil migration and activation; these treatments could be inhibiting the production of endogenous PGD<sub>2</sub> synthesized in response to stimulation with PGD<sub>2</sub>.

In both clinical and experimental animal models, PGD<sub>2</sub> is released in large amounts during asthmatic inflammatory response, and it has been proposed as a marker of mast cell activation in asthma (Pettipher, 2008). These cells can produce PGD<sub>2</sub> as the major cyclooxygenase metabolite of arachidonic acid in response to

antigen challenge (Murray *et al.*, 1986; Holgate, 1998). Relevant to our study, it has been demonstrated that mast cells are sensitive to *C. sympodialis* treatment (Costa *et al.*, 2008). To test whether anti-inflammatory effects of *C. sympodialis* and warifteine depend on inhibition of PGD<sub>2</sub> production, we have directly evaluated PGD<sub>2</sub> production *in vivo* using two murine models of allergy, and *in vitro* analyzing antigen-challenged mast cells. As hypothesized, *C. sympodialis* and warifteine were able to reduce the amounts of newly formed PGD<sub>2</sub> recovered from both sites of allergic inflammation and mast cell supernatants. But which enzymatic step of PGD<sub>2</sub> synthesis was the definitive target of warifteine inhibitory effect?

Upon cell activation, to initiate eicosanoid synthesis sterified arachidonic acid must be rapidly mobilized from phospholipid stores through a requisite event performed mainly by cPLA<sub>2</sub>- $\alpha$  (Diaz *et al.*, 2003). Oxidative metabolism of free arachidonic acid mediated by either COX or a family of lipoxygenase (LO) enzymes culminate with the generation of a variety of eicosanoids according to cell type and stimulus. Both COX enzymes, nominally the constitutive COX-1 and the inducible COX-2, catalyze the same two reactions: a cyclooxygenase reaction that inserts two molecules of oxygen into substrate to form PGG<sub>2</sub> and a subsequent endoperoxidase reaction that reduces PGG<sub>2</sub> to its 15-hydroxy analogue, PGH<sub>2</sub> (Smith *et al.*, 1991). The specific COX pathway-derived eicosanoids synthesized from PGH<sub>2</sub> include a series of prostanoids that are defined by the terminal enzymes differentially expressed by different cell types. For instance, mast cells generate large amounts of PGD<sub>2</sub> (but not PGE<sub>2</sub>) due to selective expression of haematopoietic-PGD<sub>2</sub> synthase (H-PGDS), while within macrophages PGH<sub>2</sub> is isomerized to PGE<sub>2</sub> by different PGES enzymes that each preferentially couple with COX-1 or the inducible COX-2 to generate PGE<sub>2</sub> under a range of inflammatory

conditions (Simmons *et al.*, 2004; Kudo *et al.*, 2005). Taking advantage of this sequence of enzymatic steps of eicosanoid synthesis, we have searched for the specific molecule targeted by warifteine by studying the effect of warifteine treatment on production of other eicosanoids. First, since warifteine failed to interfere with mast cell LTC<sub>4</sub> production triggered by IgE cross-linking, cPLA<sub>2</sub> was discarded as target of warifteine mechanism of action. Second, exclusion of cPLA<sub>2</sub> as molecular target was corroborated by the lack of inhibitory effect of warifteine on A23187-induced PGE<sub>2</sub> production by mouse peritoneal macrophages. Of note, other bisbenzilquinoline compounds, tetrandrine and berbamine, distinct from warifteine, are also capable of inhibiting leukocyte activation *in vitro*, reducing A23187-induced production of cysLTs and PGE<sub>2</sub> (Teh *et al.*, 1990) by a mechanism dependent on PLA<sub>2</sub> inhibition. Finally, such lack of effect on PGE<sub>2</sub> synthesis also excluded potential involvement of COX enzymes, mainly COX-1 since the expression of COX-2 under these stimulatory conditions was not confirmed by us.

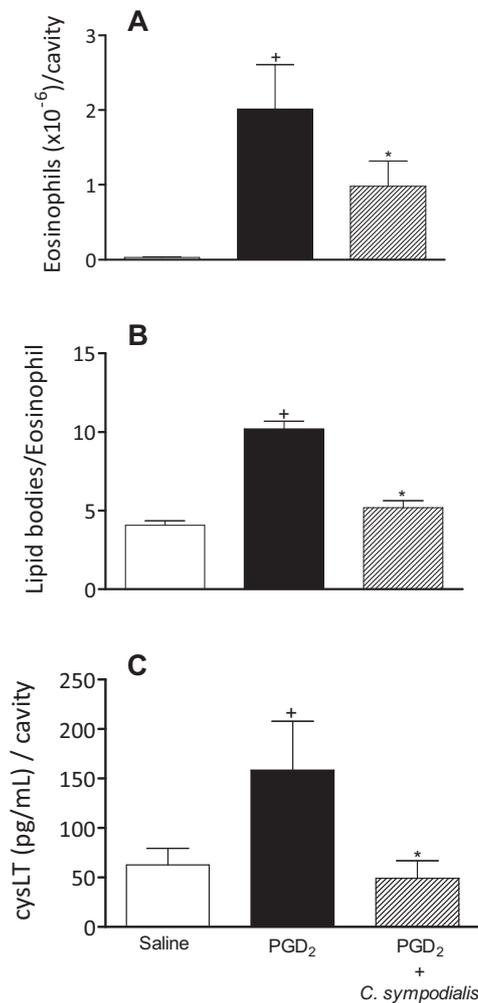
Our study unveiled the mechanism of action of the orally active anti-allergic treatment with *C. sympodialis* and its active alkaloid warifteine. Specifically based on our observations, inhibitory effects of warifteine upon synthesis of PGD<sub>2</sub> – a key mediator of allergic inflammation that regulates eosinophil-driven LTC<sub>4</sub> synthesis – indicates warifteine as a promising therapeutic alternative. While characterization of specific molecular target of warifteine is still pending and will be further investigated in future studies, H-PGDS emerges as a likely candidate that would also favor selectivity to the drug mechanism of action.

**TABLE 1:** HQL-79-driven inhibitory effect reveals that an endogenous PGD<sub>2</sub> mediates eosinophil influx, lipid body biogenesis within recruited eosinophils and cysLTs synthesis triggered by PGD<sub>2</sub> administration in actively sensitized mice.

Stimuli	Treatment	Eosinophils (x10 <sup>6</sup> / cavity)	Lipid Bodies (number / eosinophil)	cysLTs (ng / cavity)
Saline		0,01 ± 0,01	4,69 ± 0,41	160 ± 50
PGD <sub>2</sub>		0,32 ± 0,07 <sup>+</sup>	16,4 ± 0,87 <sup>+</sup>	800 ± 100 <sup>+</sup>
	+ HQL-79	0,05 ± 0,04 <sup>*</sup>	6,38 ± 0,46 <sup>*</sup>	400 ± 90 <sup>*</sup>

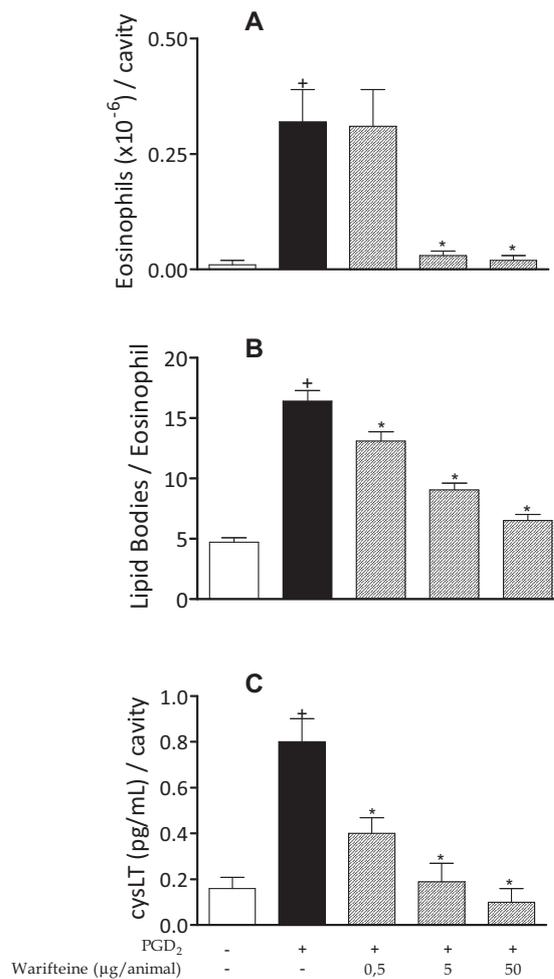
<sup>+</sup>  $p < 0,001$  compared with saline group; <sup>\*</sup>  $p < 0,001$  compared with PGD<sub>2</sub>-stimulated animals.

Intraperitoneal pretreatment with HQL-79 (1mg/Kg) was performed 1 h before PGD<sub>2</sub> (35 pmol/cavity) administration *in* actively sensitized mice. After 24 h, pleural eosinophilia, eosinophil lipid bodies and pleural cysLTs levels were evaluated. Results were expressed as the mean ± SEM from at least six animals. <sup>+</sup> Significantly different from saline-challenged group ( $p \leq 0.05$ ); <sup>\*</sup> Significantly different from PGD<sub>2</sub>-challenged group ( $p \leq 0.05$ ).



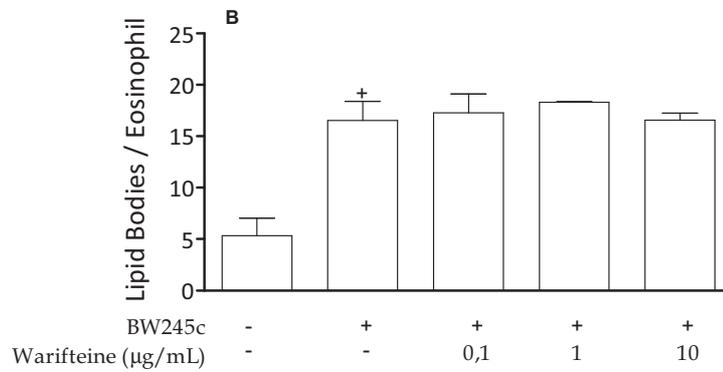
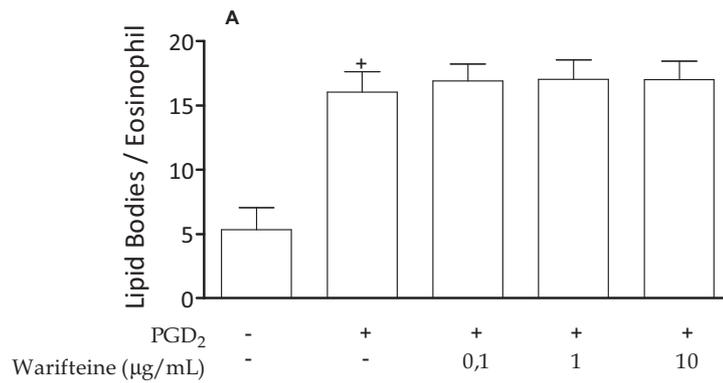
*FIGURE 1: Pre-treatment with C. sympodialis extract inhibited eosinophil influx, biogenesis of eosinophil lipid bodies and cysLT production triggered by PGD<sub>2</sub>.*

Oral treatment with *C. sympodialis* (400 mg/Kg) was performed 1 h before PGD<sub>2</sub> (35 pmoL/cavity) administration *in* actively sensitized mice. After 24 h, pleural eosinophilia (A), eosinophil lipid bodies (B) and cysLT levels (C) were evaluated. Results were expressed as the mean  $\pm$  SEM from at least six animals. <sup>+</sup> Significantly different from saline-challenged group ( $p \leq 0.05$ ); \* Significantly different from PGD<sub>2</sub>-challenged group ( $p \leq 0.05$ ).



**FIGURE 2:** Pre-treatment with warifetine inhibited eosinophil influx, biogenesis of eosinophil lipid bodies and LTC<sub>4</sub> production triggered by PGD<sub>2</sub>.

Oral treatment with warifetine (0.5, 5 or 50 µg/animal) was performed 1 h before PGD<sub>2</sub> (35 pmoL/cavity) administration *in* actively sensitized mice. Twenty four hour after challenge, pleural eosinophilia (A), eosinophil lipid bodies (B) and cysLT levels (C) were evaluated. Results were expressed as the mean ± SEM from at least six animals. <sup>+</sup> Significantly different from saline-challenged group ( $p \leq 0.05$ ); \* Significantly different from PGD<sub>2</sub>-challenged group ( $p \leq 0.05$ ).



*FIGURE 3: Warifteine displayed no direct inhibitory effect on eosinophil activation triggered by in vitro stimulation with PGD<sub>2</sub> or its DP1 receptor agonist BW245c.*

Human granulocytes were pre-treated (30 min) with different concentrations of warifteine (0.1 - 10 µg/mL) and then stimulated *in vitro* with PGD<sub>2</sub> (25nM) (**A**) or BW245c (25nM) (**B**). Lipid body enumeration within eosinophils was analyzed 1 h after stimulation. Results were expressed as the mean ± SEM from three independent experiments. <sup>+</sup> Significantly different from non-stimulated cells ( $p \leq 0.05$ ).

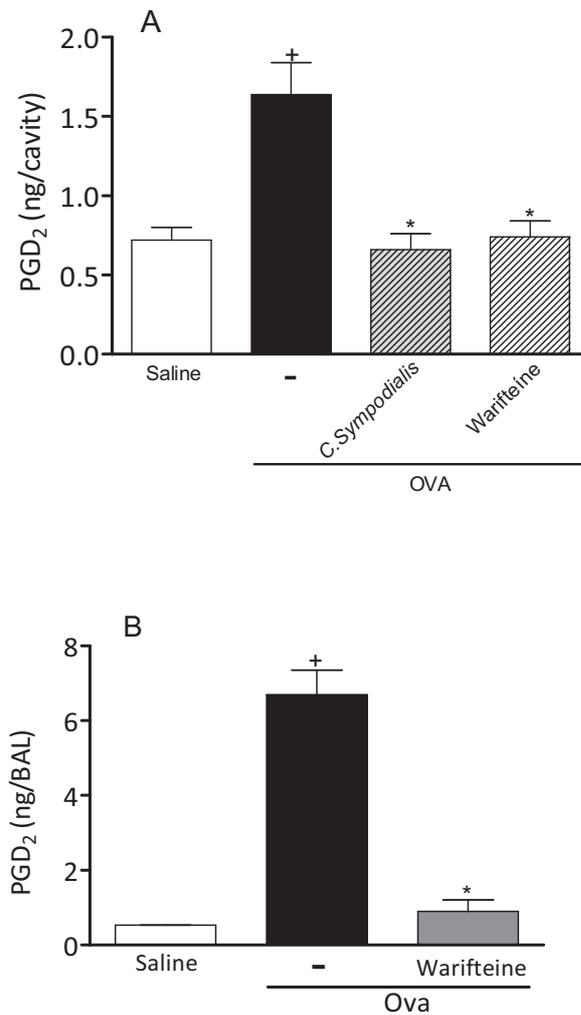
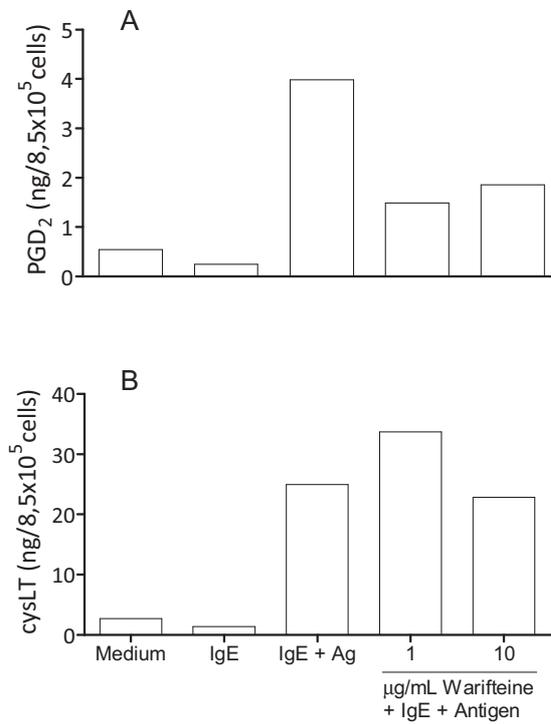


FIGURE 4: Pre-treatments with *C. sympodialis* or warifteine inhibits allergen-induced PGD<sub>2</sub> production in pleurisy and pulmonary models of allergic inflammation.

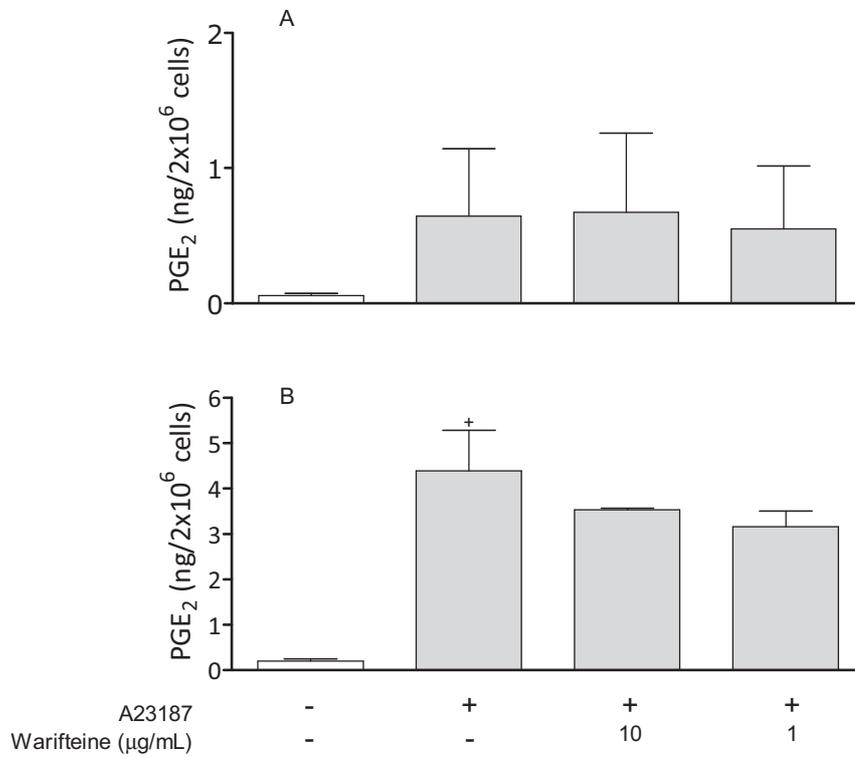
Oral pre-treatments with *C. sympodialis* (400 mg/Kg) or warifteine (50 µg/animal) were performed 1 h before allergic intrapleural challenge (A) or the last aerosol ovalbumin challenge (B) in actively sensitized mice. PGD<sub>2</sub> levels were evaluated in pleural fluid (A) or BAL (B) 24 h after last allergic challenge. Results were expressed as the mean ± SEM from at least six animals.

<sup>†</sup> Significantly different from saline-challenged group ( $p \leq 0.05$ ); \* Significantly different from PGD<sub>2</sub>-challenged group ( $p \leq 0.05$ ).



*FIGURE 5: Warifteine pre-treatment reduced production of PGD<sub>2</sub>, but not LTC<sub>4</sub>, triggered in vitro by antigen-challenge within IgE-sensitized bone marrow-derived mast cells.*

*In vitro* pre-treatment with warifteine (1 or 10 µg/mL) was performed 30 min before antigen challenge in IgE-sensitized bone marrow-derived mast cells (BMMC; 8,5 x 10<sup>5</sup> cells/well). Thirty minutes after challenge, PGD<sub>2</sub> and LTC<sub>4</sub> levels were evaluated in cell free supernatants. Results show a representative assay (n = 2).



*FIGURE 6: Warifteine did not modify PGE<sub>2</sub> production by macrophages activated with A23187. Mouse peritoneal macrophages (2 x 10<sup>6</sup>/mL) recovered from normal (A) or thioglycolate-stimulated mice (B) were stimulated with A23187 (5 µM) for 15 min. Pre-treatment with warifteine (1 or 10 µg/mL) were performed for 30 min before stimulation. Results were expressed as the mean ± SEM from three independent experiments. <sup>+</sup> Significantly different from non-stimulated cells ( $p \leq 0.05$ ).*

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## 5) DISCUSSÃO:

Anteriormente ao desenvolvimento desse trabalho demonstramos que a PGD<sub>2</sub> foi capaz de ativar os eosinófilos induzindo a biogênese de corpúsculos lipídicos e síntese de LTC<sub>4</sub> tanto *in vitro*, quanto *in vivo*. Foi observado também que o sítio de síntese do LTC<sub>4</sub> produzido durante a resposta inflamatória desenvolvida *in vivo* foram os corpúsculos lipídicos recém-formados nos eosinófilos infiltrantes (Mesquita-Santos *et al.*, 2006). Dessa forma, os resultados dessa tese visam a identificação dos mecanismos envolvidos na síntese de LTC<sub>4</sub> por eosinófilos, promovida pela ativação simultânea dos receptores da PGD<sub>2</sub> – os receptores DP1 e DP2 – tanto *in vitro* quanto *in vivo*.

Com o objetivo de conseguir essa caracterização, é importante ressaltar os papéis de alguns estímulos com capacidade quimioatraente para eosinófilos, visto que controlam o *priming* dessas células para a síntese de LTC<sub>4</sub>, além de promoverem o recrutamento das mesmas para o sítio de inflamação alérgica. Esses estímulos quimiotáticos ao atuarem via seus receptores específicos – presentes na superfície eosinofílica – são capazes de iniciar uma cascata de sinalização intracelular, levando a um aumento na síntese desse eicosanóide.

Em eosinófilos, estímulos eosinofilotáticos clássicos da resposta alérgica – como PAF e eotaxina – são capazes de promover uma série de alterações morfo-funcionais relacionadas com o processo inflamatório. Por exemplo, foi demonstrado que o PAF ao promover a ativação do seu receptor de membrana – que é um GPCR associado a uma proteína G $\alpha$ i – é capaz de ativar essas células promovendo a formação de superóxido (Zoratti *et al.*, 1991), ativação de proteínas quinases (Chao *et al.*, 1993), a degranulação (Kroegel *et al.*, 1989), além de induzir a quimiotaxia dessas células (Wardlaw *et al.*, 1986). Em consonância com as atividades quimiotáticas, foi observado que a estimulação desse receptor foi capaz de promover a ativação de uma cascata de sinalização intracelular – dependente da ativação de PKC e fosfolipase C (PLC) – culminando com a síntese de LTC<sub>4</sub> (Bozza *et al.*, 1997; Bozza *et al.*, 1998).

As quimiocinas eotaxina (CCL11) e RANTES (CCL5) – correspondem a mediadores inflamatórios produzidos em grandes quantidades durante a resposta alérgica e – atuando através da ativação do receptor quimiotático CCR3 – são capazes

de promover o aumento nos níveis intracelulares de cálcio, o rearranjo do citoesqueleto, além de induzirem a quimiotaxia dos eosinófilos (Rothenberg *et al.*, 1999). Além desses fenômenos observados, a estimulação do receptor CCR3 está relacionada com a ativação de uma cascata de sinalização intracelular, que também promove a síntese de LTC<sub>4</sub>, de maneira dependente da ativação de proteína G $\alpha$ i e da ativação de MAPK e PI3K, mas não PKC e PLC (Bandeira-Melo *et al.*, 2001b). Embora a ativação dos receptores CCR3 e PAFR promovam a ativação de cascatas de sinalização intracelulares diversas, essas vias compartilham a regulação das atividades celulares, através da promoção da ativação de proteína G $\alpha$ i e influxo de cálcio, culminando com a ativação/migração celular, assim como com a síntese de LTC<sub>4</sub>.

Um outro fator quimiotático que também se mostrou capaz de promover a ativação dos eosinófilos induzindo a síntese de LTC<sub>4</sub> foi a interleucina(IL)-16. Essa citocina, com propriedades quimioatraentes para os eosinófilos (Rand *et al.*, 1991; Cruikshank *et al.*, 2000), foi capaz de ativar eosinófilos humanos (via CD4) promovendo a síntese de LTC<sub>4</sub>, num fenômeno dependente de ativação de CCR3, pois foi observado que houve produção de eotaxina e RANTES, resultando em uma ativação autócrina/parácrina dessas células (Bandeira-Melo *et al.*, 2002b).

Mais recentemente, foi demonstrado também que a PGD<sub>2</sub> e o MIF, mediadores inflamatórios produzidos durante a resposta alérgica, além das suas propriedades eosinofilotáticas (Nagata *et al.*, 1999a; Monneret *et al.*, 2001; Mizue *et al.*, 2005; Kobayashi *et al.*, 2006; Magalhaes *et al.*, 2007), foram capazes de ativar eosinófilos induzindo a síntese de LTC<sub>4</sub>. Foi observado que, durante a resposta alérgica, tanto o MIF quanto a PGD<sub>2</sub> sinergizam com a eotaxina, promovendo a ativação dos eosinófilos (Mesquita-Santos *et al.*, 2006; Vieira-de-Abreu *et al.*, 2010). Essas observações demonstram a participação de mediadores quimiotáticos promovendo, não somente o recrutamento dessas células para o sítio inflamatório, como também a ativação das células recrutadas, sendo assim um ponto importante no desenvolvimento da resposta alérgica. Isso ocorreria através da ativação de seus receptores específicos ou através da produção de outros mediadores (permitindo uma interação sinérgica ou complementar) possibilitando assim a ativação completa dessas células,

Assim sendo, no início desse trabalho, hipotetizamos que a PGD<sub>2</sub> estaria promovendo esse fenômeno através da ativação do receptor DP2. Vale ressaltar, que

assim como os outros receptores quimiotáticos, a ativação desse receptor está envolvida com uma gama de outros fenômenos, como a polimerização de filamentos de actina e o aumento na expressão de moléculas de adesão, que são dependentes da ativação de proteína G $\alpha$ i e influxo de cálcio, além do próprio fenômeno de quimiotaxia (Monneret *et al.*, 2001; Sawyer *et al.*, 2002).

No entanto, ao contrário do observado com o PAF e com eotaxina, a ativação específica do receptor DP2 não foi capaz de promover a ativação eosinofílica induzindo a síntese de LTC<sub>4</sub>. Embora tenhamos observado que a utilização do agonista seletivo do receptor DP2, num modelo *in vivo* de pleurisia murina, tenha promovido o recrutamento de eosinófilos para a cavidade pleural, observamos também que os eosinófilos infiltrantes não eram competentes para produzir as mesmas quantidades de LTC<sub>4</sub> observadas quando na estimulação somente com PGD<sub>2</sub>. Essas observações foram corroboradas *in vitro*, onde eosinófilos humanos estimulados somente com o agonista DP2, também não foram capazes de sintetizar LTC<sub>4</sub>. Esses resultados acabaram por contrariar as expectativas em torno da ativação desse receptor, pois comparado aos demais receptores quimiotáticos, esse receptor poderia ser o principal responsável pela produção de LTC<sub>4</sub> nesta situação de ativação eosinofílica. No entanto, a utilização do antagonista seletivo desse receptor, antes da estimulação dos eosinófilos *in vitro* ou antes da estimulação *in vivo* com PGD<sub>2</sub> nos revelou que, a inibição seletiva da ativação desse receptor é requisito indispensável à síntese aumentada de LTC<sub>4</sub> por eosinófilos ativados por PGD<sub>2</sub>.

Esses resultados corroboram as observações do envolvimento dos receptores quimiotáticos com a síntese de LTC<sub>4</sub>. Embora nesse caso provavelmente, a ativação somente desse receptor não seja suficiente para promover a síntese de LTC<sub>4</sub>, mesmo que a ocorrência dessa ativação promova o desenvolvimento de eventos quimiotáticos (Nagata *et al.*, 1999a).

Dessa forma, nos questionamos, qual seria o mecanismo envolvido no processo de ativação eosinofílica, que culminaria com a síntese de LTC<sub>4</sub> ou o que poderia estar faltando – nesse processo de ativação eosinofílica induzida pela ativação seletiva do receptor DP2 – para que a síntese de LTC<sub>4</sub> pelos eosinófilos não estivesse ocorrendo. Vale ressaltar que o complexo processo de síntese de LTC<sub>4</sub> envolve múltiplas etapas e uma delas é a biogênese de corpúsculos lipídicos. Assim sendo, também foi levantado

o questionamento sobre a correlação entre a ativação de DP2 e o processo de biogênese de corpúsculos lipídicos.

A associação entre a ativação de um receptor quimiotático, levando a biogênese de corpúsculos lipídicos e a consequente síntese de eicosanóides também já se mostrou importante em eosinófilos – estimulados com RANTES ou eotaxina (Bandeira-Melo *et al.*, 2001b) – ou neutrófilos estimulados com PAF, por exemplo (Bozza *et al.*, 1996a).

No entanto, nossos dados mostraram que somente a ativação do receptor DP2 também não foi capaz de promover a biogênese de corpúsculos lipídicos em eosinófilos. Esse fenômeno mostrou-se independente da proteína  $G\alpha_i$  e do influxo de cálcio, pois este fenômeno não foi inibido pela utilização de PTX e BAPTA-AM, antes da estimulação dos eosinófilos. Além disso, a utilização do antagonista DP2 – tanto *in vitro* quanto *in vivo* – também não inibiu esse mesmo fenômeno induzido pela  $PGD_2$ .

Assim a incapacidade em induzir a biogênese de corpúsculos lipídicos, somente através da ativação do receptor DP2, provavelmente explique a inabilidade de promover o aumento na síntese de  $LTC_4$  em eosinófilos, através da ativação do receptor DP2 por si só. Já é bem estabelecido, em leucócitos, que o processo biogênico de corpúsculos lipídicos se correlaciona com a síntese de eicosanóides (Bozza *et al.*, 2009), sendo fundamental para a síntese de  $LTC_4$  em eosinófilos (Bozza *et al.*, 1997; Bandeira-Melo *et al.*, 2001b). Os corpúsculos lipídicos que servem de sítio de síntese de  $LTC_4$  são organelas que apresentam o maquinário enzimático necessário a síntese desses eicosanóides, como 5-LO (Bozza *et al.*, 1998) e  $LTC_4$  sintase (Bozza *et al.*, 1997). Dessa maneira, embora a ativação do receptor DP2 seja capaz de promover uma cascata de sinalização intracelular – induzindo o recrutamento dessas células para o sítio inflamatório, entre outros fenômenos – acreditamos que, por mais complexos que possam ser, esses fenômenos intracelulares não sejam capazes de induzir a biogênese de corpúsculos lipídicos citoplasmáticos, evento já demonstrado ser indispensável a síntese de  $LTC_4$  (Bozza *et al.*, 2009).

Já foi demonstrada a translocação da enzima 5-LO do citoplasma para o corpúsculo lipídico, em eosinófilos ativados com eotaxina (Bandeira-Melo *et al.*, 2002a). Além disso, já fora proposto, um modelo de formação de corpúsculos lipídicos, onde essas organelas recém-formadas, apresentam constitutivamente, algumas proteínas,

entre elas a 5-LO, caracterizando assim esse corpúsculo lipídico recém-formado, como uma molécula competente na síntese de leucotrienos (Wan *et al.*, 2007).

A utilização de PTX e BAPTA-AM antes da estimulação dos eosinófilos com PGD<sub>2</sub>, nos confirmou que a síntese de LTC<sub>4</sub> observada é dependente da ativação do receptor DP2. Embora a ativação desse receptor por si só, não seja suficiente para promover todos os eventos intracelulares associados a síntese de LTC<sub>4</sub>, a via de sinalização intracelular ativada contribui, para que os corpúsculos lipídicos formados possam ser competentes para sintetizar LTC<sub>4</sub>. Uma hipótese ainda não investigada por nós para explicar tal fenômeno, seria que sem a ativação do receptor DP2, os corpúsculos lipídicos recém-formados não sejam enzimaticamente competentes para sintetizar LTC<sub>4</sub>. Isso ocorreria porque estariam destituídos das enzimas necessárias a esse fenômeno ou, caso essas enzimas estejam presentes nessas organelas, poderiam faltar os estímulos complementares necessários a ativação das mesmas. Uma outra alternativa, também não comprovada, é a de que a ativação do receptor DP2 seja responsável por promover a translocação da enzima 5-LO do citoplasma para o corpúsculo lipídico.

Esses resultados nos sugerem que, a ativação dos eosinófilos com a PGD<sub>2</sub> induza a biogênese de uma organela funcionalmente diferenciada, daquela presente em número basal nessas células, visto que a estimulação apenas do receptor DP2 com o seu agonista seletivo, não foi capaz de induzir a síntese de LTC<sub>4</sub>, mesmo com os eosinófilos já apresentando um número de corpúsculos lipídicos basal pré-existente. Dessa forma, especulamos que a ativação dos eosinófilos pela PGD<sub>2</sub> esteja induzindo a biogênese de um corpúsculo lipídico funcionalmente diferente do corpúsculo lipídico já existente, possibilitando assim ocorrer a síntese do LTC<sub>4</sub> nessa organela recém-formada. Essa discrepância entre a ativação do receptor DP2, comparada a ativação de outros receptores de estímulos quimiotáticos para eosinófilos – com relação a indução da síntese de LTC<sub>4</sub> – estaria relacionada a uma regulação mais complexa, mediada pelo sistema de receptores da PGD<sub>2</sub> expresso nessas células, do que a regulação do fenômeno de ativação observado com os outros receptores.

Como já fora mencionado anteriormente, o receptor DP2 é o receptor associado com as ações quimiotáticas da PGD<sub>2</sub> sobre os eosinófilos e possui sete sítios

transmembranares sinalizando através da ativação de uma proteína G do tipo  $G_{\alpha i}$ , sendo capaz de promover influxo de cálcio (Hirai *et al.*, 2001), assim como quimiotaxia e degranulação de eosinófilos (Gervais *et al.*, 2001). Embora esse receptor esteja associado a proteína  $G_{\alpha i}$ , estudos têm demonstrado que esse mesmo receptor pode sinalizar intracelularmente através de cascatas de sinalização intracelulares distintas, pois como é um GPCR, pode formar dímeros (heterodímeros). E esse rearranjo vem sendo proposto para explicar as mudanças na seletividade de alguns receptores associados a proteína G de diferentes sub-famílias (por exemplo,  $G_{\alpha s}$ ,  $G_{\alpha i}$  e  $G_{\alpha q}$ ) (Terrillon *et al.*, 2004). Também já foi demonstrado que as subunidades  $\beta\gamma$  da proteína G, após se dissociarem da subunidade proteína  $G_{\alpha i}$ , também podem ativar PLC (Smrcka *et al.*, 1993). Em eosinófilos foi demonstrado que o *shape change* induzido por  $PGD_2$  não foi inibido por PTX, mas sim por inibidores específicos de PLC, PIP3K e p38 MAPK, sugerindo que a cascata de sinalização envolvida na ativação do receptor DP2 depende – além da proteína  $G_{\alpha i}$  – da ativação de proteína  $G_{\alpha q}/11$ , propondo que esse receptor possa estar sinalizando através dessas duas proteínas G:  $G_{\alpha q}$  e  $G_{\alpha i}$  (Stubbs *et al.*, 2002). Vale ressaltar que já havia sido demonstrado que eosinófilos humanos expressam tanto a proteína  $G_{\alpha i}$ , quanto a proteína  $G_{\alpha q}/11$  (O'Flaherty *et al.*, 2000).

Dessa forma, já fora proposto um modelo de auto-regulação da ativação eosinofílica pelos receptores DP, onde a ativação do receptor DP2 – que está relacionada a uma via de sinalização intracelular associada à ativação da proteína  $G_{\alpha i}$  – resultaria na inativação da produção de AMP cíclico (AMPc) induzida pela ativação do receptor DP1 (Monneret *et al.*, 2001).

No entanto, a possibilidade desse receptor estar formando heterodímeros, talvez possa explicar a possibilidade do receptor DP2 estar sinalizando através de outras proteínas G –  $G_{\alpha q}$  e/ou  $G_{\alpha s}$ , como já fora demonstrado por Stubbs e colaboradores (2002) – ativando assim cascatas enzimáticas intracelulares distintas, das que estão classicamente relacionadas a ativação desse receptor, permitindo assim cooperar com a sinalização induzida pela ativação do receptor DP1, resultando na síntese de  $LTC_4$ . Na realidade a sinalização intracelular promovida pela ativação do receptor DP2 em eosinófilos ainda é controversa, necessitando ser completamente elucidada, visto que a ativação desse receptor promoveu *shape change* de eosinófilos dependente da

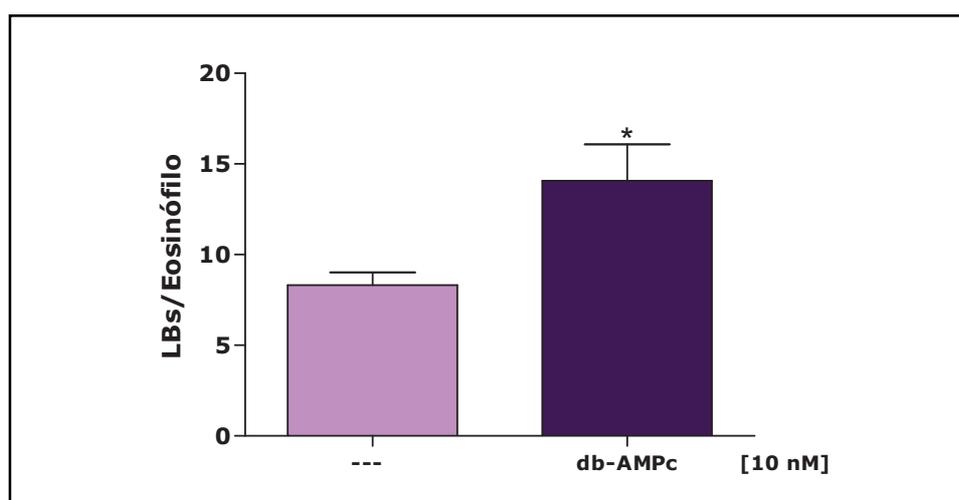
ativação de proteína  $G\alpha_q$  (Monneret *et al.*, 2001; Stubbs *et al.*, 2002), enquanto que a quimiotaxia foi dependente de  $G\alpha_i$  (Sugimoto *et al.*, 2003).

Neste ponto vale mencionar que, além do receptor DP2, os eosinófilos também expressam o receptor não-quimiotático DP1 (Nagata *et al.*, 2003). A análise das contribuições de cada receptor na síntese de  $LTC_4$  induzida por  $PGD_2$ , nos revelou que a  $PGD_2$  foi capaz de promover uma rápida síntese *de novo* de corpúsculos lipídicos independente da ativação do receptor DP2, visto que este fenômeno foi insensível ao antagonista DP2, assim como a PTX ou a quelantes de cálcio. Mas foi extremamente dependente da ativação do receptor DP1, visto que tanto a utilização de antagonista seletivo e inibidores de PKA – via de sinalização relacionada ao receptor DP1 – foram capazes de inibir o fenômeno biogênico induzido por  $PGD_2$ .

Durante a análise da biogênese de corpúsculos lipídicos em eosinófilos induzida por  $PGD_2$ , observamos que a utilização do antagonista do receptor DP2 (antes da estimulação com  $PGD_2$ ), assim como somente a ativação do receptor DP1, embora permita a biogênese de corpúsculos lipídicos pela  $PGD_2$ , não resulte em síntese de  $LTC_4$ . Mesmo resultado observado quando houve somente a ativação do receptor DP2, sugerindo um papel complementar desses receptores no fenômeno de síntese de  $LTC_4$  pelos eosinófilos.

Já é bem estabelecido que a ativação de DP1 por  $PGD_2$  promove uma sinalização através da ativação de proteína  $G\alpha_s$ , levando ao aumento de AMPc e também a ativação de PKA, correspondendo a uma cascata intracelular de transdução de sinal, que está classicamente associada a inibição da mobilidade eosinofílica induzida por estímulos quimioatraentes (Kaneko *et al.*, 1995; Hirai *et al.*, 2001; Monneret *et al.*, 2001). Essas observações são consistentes com a idéia de que agentes/drogas capazes de elevar os níveis de AMPc possuem poderosa capacidade anti-inflamatória (Teixeira *et al.*, 1995; Diaz *et al.*, 1996) ou pró-resolutiva (Sousa *et al.*, 2009), podendo ser utilizados como tratamento para doenças nas quais o acúmulo de eosinófilos desempenha um importante papel (Sousa *et al.*, 2009). Varias observações relacionam a existência de níveis intracelulares elevados de AMPc à inibição eosinofílica, incluindo achados com eosinófilos de rato (Alves *et al.*, 1996) e humanos (Kaneko *et al.*, 1995), que tiveram suas propriedades quimiotáticas inibidas, quando tratados com dibutyryl-AMPc (db-AMPc) – análogo estrutural do AMPc.

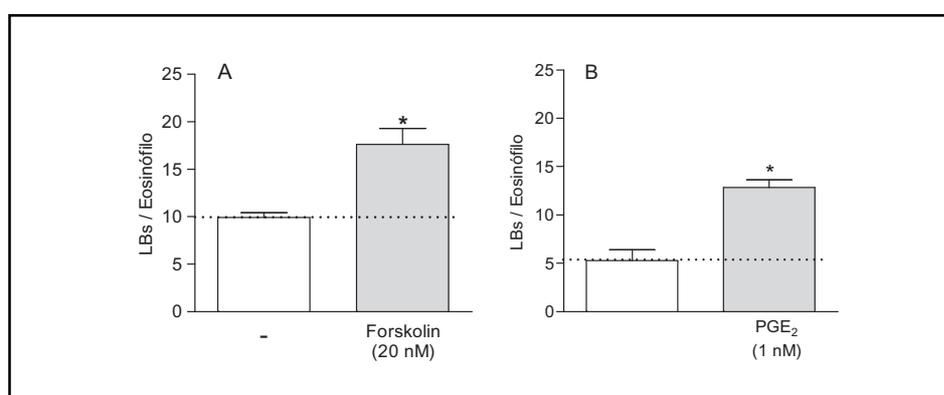
No nosso modelo de ativação do receptor DP1 pela PGD<sub>2</sub> – ou pelo agonista seletivo DP1 – pode ser que esteja acontecendo uma elevação nos níveis de AMPc a uma concentração que não seja capaz de promover efeitos inibitórios aos eosinófilos e, talvez assim, possa ser explicado os efeitos estimulatórios da ativação dessa via de sinalização. Embora não tenhamos quantificado, os níveis intracelulares de AMPc nos eosinófilos estimulados com agonista DP1 – ou com a própria PGD<sub>2</sub> – em estudo recente realizado por nosso grupo, foi revelado que a estimulação direta dessas células *in vitro* com db-AMPc, numa concentração de 10nM, é capaz de induzir a biogênese de corpúsculos lipídicos nessas células (Bakker-Abreu, dados não-publicados – Quadro complementar 1). Vale destacar também que a concentração do db-AMPc utilizada nos nossos experimentos foi 10<sup>4</sup> vezes menor do que a concentração que foi utilizada nos estudos que demonstraram efeitos inibitórios do db-AMPc sobre os eosinófilos (Kaneko *et al.*, 1995; Alves *et al.*, 1996).



**Quadro complementar 1 – Análogo de AMPc (db-AMPc) induz a biogênese de corpúsculos lipídicos (LBs) em eosinófilos.** Avaliação da quantidade de corpúsculos lipídicos em eosinófilos humanos, purificados e estimulados por 1h/37°C com db-AMPc (10 nM). Os citoesfregaços foram corados com tetróxido de ósmio (n=3, \*p≤0,05).

Além disso, quando ativamos diretamente a adenilato ciclase com forskolin – clássico ativador dessa enzima – em eosinófilos humanos *in vitro*, observamos um aumento na formação de corpúsculos lipídicos (Quadro complementar 2A), assim como

quando ativamos essas células com PGE<sub>2</sub>. Embora a PGE<sub>2</sub> já tenha um reconhecido papel antiinflamatório – via aumento de AMPc – sendo capaz de inibir a secreção de citocinas e eicosanóides por macrófagos, mastócitos e eosinófilos (Sestini *et al.*, 1996; Tenor *et al.*, 1996; Aronoff *et al.*, 2005; Feng *et al.*, 2006), nós observamos que a estimulação direta dessas células *in vitro* com PGE<sub>2</sub>, foi capaz de induzir aumento no número de corpúsculos lipídicos citoplasmáticos (Quadro complementar 2B), indicando que a ativação dos receptores EP2 e EP4 – sinalizando através da formação de AMPc – é capaz de induzir a biogênese de corpúsculos lipídicos em eosinófilos.

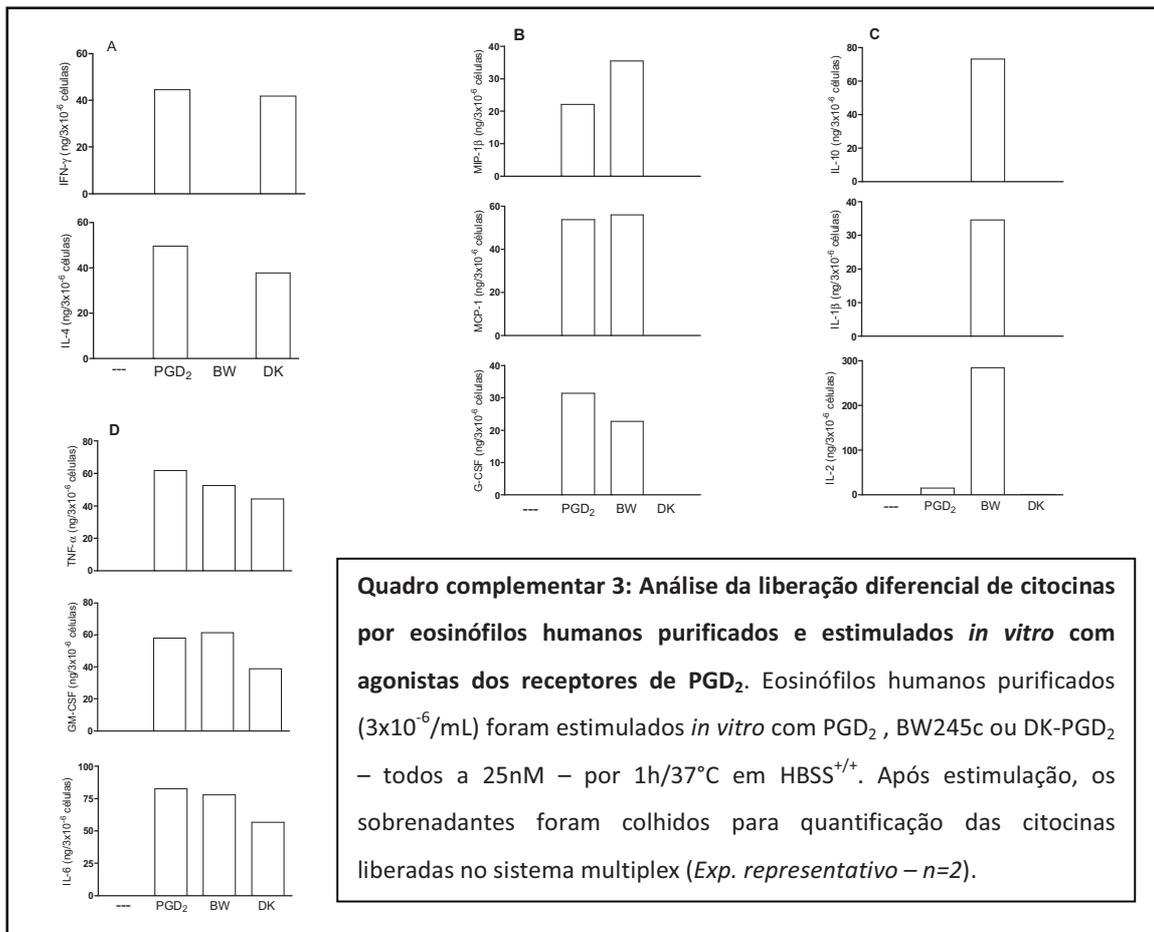


**Quadro complementar 2 – Aumento nos níveis de AMPc é capaz de induzir biogênese de corpúsculos lipídicos (LBs) em eosinófilos.** Eosinófilos humanos purificados estimulados por 60 min/37°C com Forskolin (20nM) (A) ou PGE<sub>2</sub> (1nM) (B) e corados com tetróxido de ósmio para quantificação de LBs. \*  $p \leq 0.05$  com relação ao controle (n=3).

Com esses dados, especulamos que num primeiro momento, a ativação do receptor DP1 possa ativar adenilato ciclase, levando a produção de AMPc em níveis baixos para as suas ações antiinflamatórias, mas capazes de promover a ativação celular ou, até mesmo, ajudar nos eventos envolvidos na ativação eosinofílica em conjunto com a sinalização induzida pelo receptor DP2. E num momento mais tardio do processo de ativação dessas células, os níveis de AMPc se elevariam a um patamar tal, que resultaria na inibição da ativação dessas células, reforçando a hipótese de que esses receptores poderiam se contra-balancear durante a ativação eosinofílica (Monneret *et al.*, 2001).

Nós observamos que a ativação do receptor DP1 induz – dependente da ativação de PKA – a formação de novos corpúsculos lipídicos os quais não são capazes de sintetizar LTC<sub>4</sub>, como observado nas análises com *EicosaCell*. Em função da ativação isolada de cada um dos receptores, a ausência de eosinófilos capazes de sintetizar LTC<sub>4</sub> – ou a não detecção de LTC<sub>4</sub> no sobrenadante das células estimuladas *in vitro* (ou *in vivo*) – já nos dava fortes indícios para permitir a especulação sobre um papel cooperativo entre esses dois receptores. No entanto, os estudos desenvolvidos visando a caracterização funcional de cada um desses receptores têm demonstrado que, na ativação eosinofílica ambos funcionam em um modelo de auto-regulação, onde os fenômenos associados a ativação de um receptor seria modulado negativamente pela ativação do outro receptor (Monneret *et al.*, 2001; Sawyer *et al.*, 2002).

No entanto, alguns trabalhos têm demonstrado que esses receptores podem trabalhar de maneira complementar no tocante a sinalização eosinofílica, resultando na ativação dessas células. Foi demonstrado que a PGD<sub>2</sub> é capaz de promover a ativação do eosinófilos induzindo, além da quimiotaxia e a degranulação dessas células – através do receptor DP2 – um aumento na sobrevivência *in vitro*, através do aumento nos níveis intracelulares de AMPc – via receptor DP1 – inibindo a apoptose dessas células (Gervais *et al.*, 2001). Mais recentemente, fora demonstrado também que a PGD<sub>2</sub> é capaz de promover a liberação de eosinófilos da medula óssea de camundongos, num fenômeno que foi inibido por antagonistas de ambos os receptores DP1 e DP2 (Schratl *et al.*, 2007). Em concordância com esses trabalhos, nós observamos também que eosinófilos humanos são capazes de – dentre a gama de mediadores inflamatórios pré-formados e estocados nos seus grânulos citoplasmáticos – secretar citocinas de forma seletiva, ao terem os dois receptores ativados simultaneamente (Quadro complementar 3). Esses dados, não estão relacionados diretamente com o artigo desenvolvido visando a caracterização do papel dos receptores na síntese de LTC<sub>4</sub>, mas servem para demonstrar que, durante o desenvolvimento da resposta inflamatória, esses receptores podem atuar de maneira complementar sob vários aspectos, contribuindo para a ativação eosinofílica.



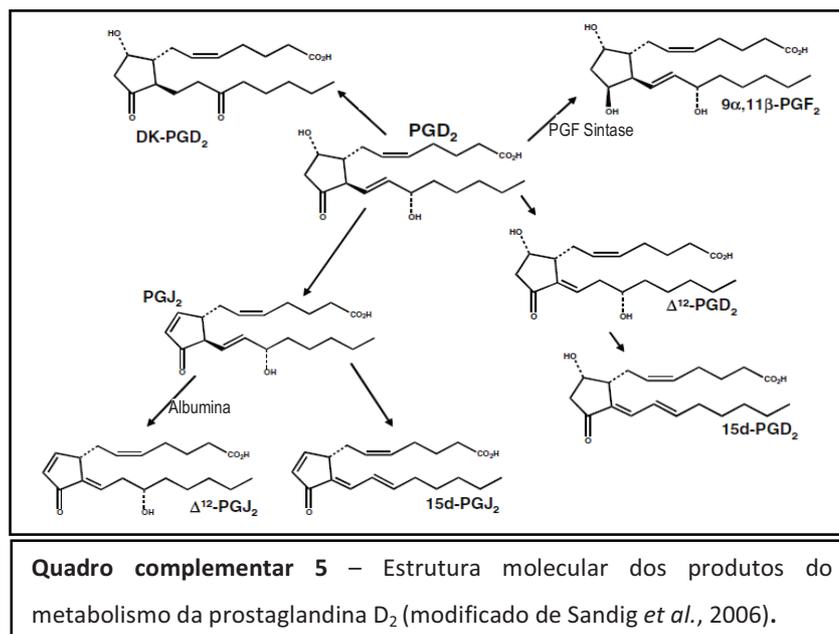
Nós observamos que dentre todas as citocinas estocadas nos grânulos citoplasmáticos eosinofílicos – detectadas nos lisados celulares (Quadro complementar 4) – a ativação somente do receptor DP2 foi capaz de induzir a liberação de IL-4 e IFN $\gamma$  (Quadro complementar 3A), mas não induziu a liberação de MIP-1 $\alpha$ , MCP-1 ou G-CSF (Quadro complementar 3B), assim como a liberação de IL-10, IL-2 e IL-1 $\beta$  (Quadro complementar 3C), que parecem depender somente da ativação de DP1. No caso específico dessas citocinas (IL-10, IL-2 e IL-1 $\beta$ ), os receptores parecem ter papéis complementares, pois a ativação de ambos, pela PGD<sub>2</sub>, não foi capaz de promover a secreção dessas citocinas. Em contrapartida, a secreção de IL-6, GM-CSF e TNF- $\alpha$  induzida pela PGD<sub>2</sub>, ocorre independente do receptor ativado. Embora não tenhamos feito a estimulação desses receptores simultaneamente com os agonistas seletivos, para esclarecer a regulação da secreção diferencial de citocinas pelos receptores DP1 e DP2, os dados em questão mostram que esses receptores regulam o fenômeno de

secreção diferencial das citocinas eosinofílicas. Vale ressaltar que, em estudo recente, já fora demonstrada a capacidade de eosinófilos humanos secretarem as suas citocinas pré-formadas e armazenadas nos seus grânulos citoplasmáticos, em função da sua ativação com outras citocinas, reforçando o potencial papel imunomodulador dessas células (Spencer *et al.*, 2009).

<b>CITOCINA</b>		<b>LISADO (ng/mL)</b>	
<b>IL-1<math>\beta</math></b>	84.4	<b>IL-13</b>	134.25
<b>IL-2</b>	981.42	<b>IL-17</b>	349.05
<b>IL-4</b>	183.79	<b>G-CSF</b>	204.93
<b>IL-5</b>	100.15	<b>GM-CSF</b>	896.98
<b>IL-6</b>	551.56	<b>IFN-<math>\gamma</math></b>	875.42
<b>IL-7</b>	374.02	<b>TNF-<math>\alpha</math></b>	207.9
<b>IL-8</b>	142.61	<b>MCP-1</b>	338.55
<b>IL-10</b>	205.26	<b>MIP-1<math>\beta</math></b>	95.93
<b>IL-12p70</b>	614.01		

**Quadro complementar 4: Tabela representativa das citocinas estocadas nos grânulos citoplasmáticos eosinofílicos.** Lisado celular obtido a partir da lise de  $3 \times 10^6$  eosinófilos em 100 $\mu$ L de solução de lise com inibidores de protease. Análise realizada através do sistema multiplex (*Experimento representativo – n=2*).

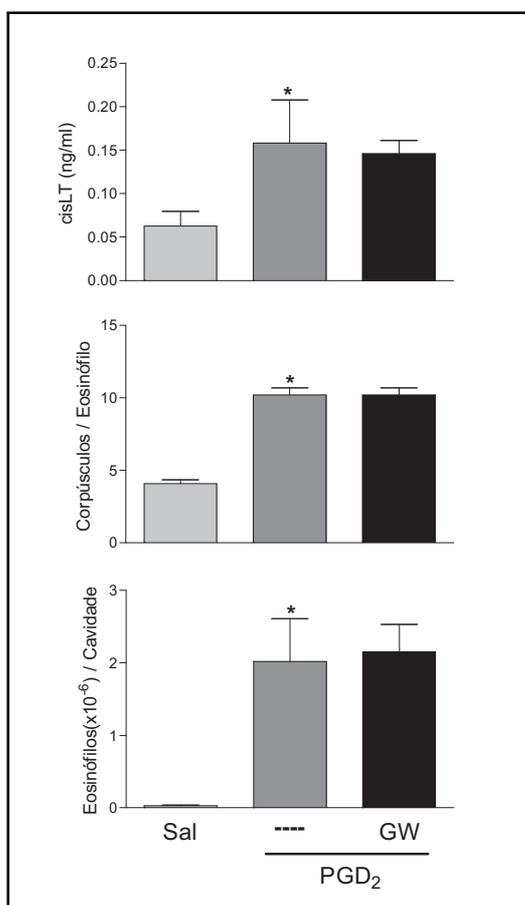
Embora todos os resultados demonstrados até aqui tenham deixado claro a participação dos dois receptores da PGD<sub>2</sub>, alguns estudos têm demonstrado o envolvimento dos metabólitos desse eicosanóide no desenvolvimento da resposta inflamatória. Já foi demonstrado que, num modelo *ex vivo* de estimulação de linfócitos T, um metabólito da PGD<sub>2</sub>, o 15d-PGJ<sub>2</sub> – que é um ligante do receptor nuclear PPAR $\gamma$  (Peroxisome Proliferator-Activated Receptors) (Forman *et al.*, 1995) – que pareceu estar atuando via inibição do fator de transcrição NF $\kappa$ B, promoveu a proliferação dessas células, assim como a produção de IL-2 (Trivedi *et al.*, 2006). Já há algum tempo, vem sendo discutido o papel dos metabólitos da PGD<sub>2</sub> (15-deoxy-prostaglandinas) no processo inflamatório. Esses compostos são produtos da degradação da PGD<sub>2</sub> (Sandig *et al.*, 2007), que podem ser formados por via enzimática ou não (Quadro complementar 5).



Vários estudos têm abordado o papel desses metabólitos, em tipos celulares distintos, como linfócitos T, monócitos/macrófagos, neutrófilos e eosinófilos (Sandig *et al.*, 2007). Especificamente em eosinófilos, de maneira similar a estimulação vista com a PGD<sub>2</sub>, o 15d-PGJ<sub>2</sub> induziu aumento na concentração de cálcio, estimulou a polimerização de actina e estimulou a expressão de CD11b em eosinófilos humanos via receptor DP2 (Monneret *et al.*, 2002), assim como primou eosinófilos humanos para quimiotaxia induzida por eotaxina, de maneira dependente de PPAR $\gamma$  (Heinemann *et al.*, 2003; Kobayashi *et al.*, 2006).

Dessa forma, para descartar que algum dos efeitos da PGD<sub>2</sub> pudesse estar sendo mediado pela ligação de algum metabólito ao PPAR $\gamma$ , utilizamos num modelo murino de pleurisia induzida por PGD<sub>2</sub>, o GW9662 – antagonista do PPAR $\gamma$  (Bendixen *et al.*, 2001) – como pré-tratamento antes da estimulação com PGD<sub>2</sub>. Observamos que tanto a eosinofilia pleral, quanto o número de corpúsculos lipídicos, assim como o LTC<sub>4</sub> produzido nessa resposta não foram afetados. Esses resultados sugerem que, aparentemente, independente da ativação do receptor PPAR $\gamma$ , a ativação dos receptores DP1 e DP2 *in vivo* é o mecanismo através da qual a PGD<sub>2</sub> é capaz de promover o acúmulo de eosinófilos para o sítio inflamatório, assim como ativar essas células induzindo a biogênese de corpúsculos e a síntese de LTC<sub>4</sub> (Quadro complementar 5), embora reconhecemos que analisar o papel do PPAR $\gamma$ , através da

utilização do GW9662 antes da estimulação dos eosinófilos com PGD<sub>2</sub> *in vitro* ainda se faça necessária.



**Quadro complementar 6: Avaliação do PPAR $\gamma$  no recrutamento e na biogênese de eosinófilos *in vivo* induzido por PGD<sub>2</sub>.** Camundongos *swiss* machos sensibilizados com injeção sub-cutânea de 0,2 ml de solução de ovalbumina (50 $\mu$ g/animal) e hidróxido de alumínio (5 mg/ml) em salina estéril (0,9%), foram estimulados com administração (i.t.) de PGD<sub>2</sub> (25nM/100 $\mu$ L em salina estéril). Os animais tratados receberam injeção intra-peritoneal de GW9662 (1mg/kg) 30 minutos antes da injeção de PGD<sub>2</sub>. Celularidade analisada em citoesfregaços corados com *May-Grünwald-Giemsa*. Corpúsculos lipídicos quantificados em lâminas coradas com tetróxido de ósmio. Resultados expressos em média  $\pm$  EPM de pelo menos 8 animais (n=8) (\* $p \leq 0,05$  em relação ao grupo controle).

Para o desenvolvimento da resposta inflamatória induzida pela pleurisia induzida por PGD<sub>2</sub>, foram utilizados camundongos sensibilizados com injeção subcutânea de solução de hidróxido de alumínio e OVA. Esse procedimento foi necessário pois, já foi demonstrado que camundongos não-sensibilizados, não respondem a estimulação pleural com PGD<sub>2</sub> (Mesquita-Santos *et al.*, 2006). Especulamos que esse procedimento

de sensibilização promova, além de outros fenômenos, o aumento na expressão de algum (ou de ambos) os receptores da PGD<sub>2</sub> (nas células residentes) ou aumento no número de mastócitos, que já foi sugerido (Rossi *et al.*, 2005).

Nesse trabalho, nós demonstramos que, por conta da estimulação com a PGD<sub>2</sub>, a síntese compartimentalizada de LTC<sub>4</sub> nos corpúsculos lipídicos formados a partir da ativação do DP1, os quais foram formados sob a regulação de AMPc/PKA, há a necessidade da estimulação/ativação simultânea do receptor DP2, visto que: (1) os antagonistas para cada receptor foram igualmente capazes de reduzir a síntese de LTC<sub>4</sub> induzida por PGD<sub>2</sub>; (2) o antagonista do receptor DP1, mas não do receptor DP2 inibiu a biogênese de corpúsculos lipídicos; (3) somente a co-estimulação dos eosinófilos com ambos os agonistas dos receptores DP1 e DP2, foi capaz e de mimetizar a síntese de LTC<sub>4</sub> induzida por PGD<sub>2</sub>; (4) a inibição de PKA inibiu a biogênese de corpúsculos lipídicos induzida pela ativação do DP1 e a subsequente síntese de LTC<sub>4</sub> compartimentalizada nos corpúsculos lipídicos e; (5) PTX e BAPTA-AM, inibiram a síntese de LTC<sub>4</sub> dependente da formação de corpúsculos lipídicos induzida por PGD<sub>2</sub>, mas não interferiram no processo biogênico dessas organelas.

Portanto, os mecanismos moleculares através dos quais a ativação do receptor DP2 converte os corpúsculos lipídicos formados a partir da ativação do DP1 em organelas enzimaticamente ativas capazes de efetivamente sintetizar LTC<sub>4</sub>, depende da ativação coordenada de G $\alpha$ i e mobilização de cálcio.

Apesar das evidências de que a migração eosinofílica orientada pela PGD<sub>2</sub>, assim como as atividades a ela relacionadas, são mediadas por um balanço das cascatas de sinalização intracelular opostas dos receptores DP1 e DP2 em eosinófilos (Monneret *et al.*, 2001), nós mostramos que os mecanismos intracelulares da síntese de LTC<sub>4</sub> induzida por PGD<sub>2</sub> necessita de uma sinalização cooperativa entre ambos os receptores. A síntese de LTC<sub>4</sub> promovida pela PGD<sub>2</sub> é dependente da biogênese de corpúsculos lipídicos regulada por PKA promovida pela ativação do receptor DP1, em adição a uma igualmente importante e concomitante ativação da via de sinalização mediada por cálcio intracelular/proteína G $\alpha$ i consequentes da ativação do receptor DP2, levando assim ao corpúsculo lipídico recém-formado – por conta da ativação de DP1 – a sintetizar LTC<sub>4</sub>.

Na literatura o papel desses receptores ainda é um tanto quanto confuso, visto que o desenvolvimento de camundongos deficientes do receptor DP1 mostrou uma redução no desenvolvimento da resposta alérgica induzida por ovalbumina (Matsuoka *et al.*, 2000; Kabashima *et al.*, 2003). Já a resposta alérgica desenvolvida em camundongos deficientes no receptor DP2, demonstrou uma eosinofilia aumentada, assim como uma maior capacidade de produção de IL-3 e IL-5, indicando um papel antiinflamatório para esse receptor (Chevalier *et al.*, 2005). Nós também demonstramos que durante a reação inflamatória alérgica, os receptores DP1/DP2 promovem a ativação eosinofílica e também regulam a produção de cisLTs, indicando que os antagonistas de cada um dos receptores DP1 ou DP2.

Em conjunto, os dados obtidos têm uma alta relevância, pois ensaios clínicos realizados com antagonista do receptor DP1 – laropiprant – revelou que em pacientes asmáticos e alérgicos, o uso desse antagonista não se mostrou eficaz (Philip *et al.*, 2009), embora o mesmo tenha se mostrado importante na asma (Matsouka *et al.*, 2000), e com antagonistas do receptor DP2 (Ulven *et al.*, 2010), sugerem que o completo entendimento do papel de cada um desses receptores no desenvolvimento da resposta alérgica, pode trazer grande benefícios terapêuticos para o tratamento dessas patologias.

A utilização de produtos naturais pela indústria farmacêutica, cada vez mais tem possibilitado a identificação de novas moléculas com ação anti-inflamatórias (Spelman *et al.*, 2006). Especificamente relacionado a ampla diversidade de espécies vegetais da flora brasileira, muitas espécies vêm sendo utilizadas para obtenção de moléculas com efeito terapêutico. Nesse contexto a *Cissampelos sympodialis* Eichl. (Menispermaceae) é uma espécie que emerge com promissoras possibilidades de desenvolvimento de medicamentos, visto que alguns trabalhos já demonstraram o seu efeito anti-inflamatório na terapia de reumatismos, artrites e doenças do aparelho respiratório, como a asma (Barbosa-Filho *et al.*, 1997; Agra *et al.*, 2007a; Agra *et al.*, 2007b).

Tem sido demonstrado que esse extrato possui efeitos imunomoduladores, como a inibição da proliferação de linfócitos T, além de inibir a produção de IFN $\gamma$  *in vitro* (Piuvezam *et al.*, 1999). Além disso, foi demonstrado também que esse mesmo extrato inibiu a proliferação de células B e a atividade microbicida *in vitro* de macrófagos

peritoneais sobre *T.cruzi*, permitindo um aumento na proliferação desse protozoário (Alexandre-Moreira *et al.*, 2003a; Alexandre-Moreira *et al.*, 2003b).

Em relação aos seus efeitos antiinflamatórios capazes de modular a resposta alérgica, já foi demonstrado que o pré-tratamento com o extrato aquoso de *C.symphodialis* foi capaz de inibir a produção de IgE, além de reduzir o edema de pata, num modelo murino de inflamação alérgica. Além disso, neste mesmo trabalho o papel imunomodulador desse extrato foi através do aumento nos níveis de citocinas Th1 *in vitro* (Bezerra-Santos *et al.*, 2004b).

Mais recentemente, foi demonstrado que o pré-tratamento com *C.symphodialis* e warifteína realizados 1 h antes do desafio alérgico em camundongos ativamente sensibilizados, foi capaz de reduzir o acúmulo de eosinófilos, a biogênese de corpúsculos lipídicos e a produção de LTC<sub>4</sub> (Bezerra-Santos *et al.*, 2006) e, como a PGD<sub>2</sub> é um mediador inflamatório capaz de controlar esses mesmo parâmetros da inflamação alérgica eosinofílica (Mesquita-Santos *et al.*, 2006), aqui nós investigamos a modulação da PGD<sub>2</sub> como um potencial mecanismo de ação para os efeitos inibitórios da *C.symphodialis* e warifteína.

Inicialmente, nós observamos o que pré-tratamento com o extrato aquoso da *C.symphodialis* foi capaz de inibir os efeitos inflamatórios induzidos por PGD<sub>2</sub>, bloqueando o acúmulo de eosinófilos para a cavidade pleural, a biogênese de corpúsculos lipídicos nos poucos eosinófilos recrutados e a produção de cisLTs. Como os efeitos antiinflamatórios da *C.symphodialis* foram atribuídos ao seu alcalóide isolado – warifteína (Bezerra-Santos *et al.*, 2005) – nós pre-tratamos com warifteína (dose-resposta) camundongos ativamente sensibilizados 1 h antes da estimulação com PGD<sub>2</sub>, afim de esclarecer se a warifteína também poderia estar interferindo na resposta inflamatória induzida por PGD<sub>2</sub>. De fato, de maneira similar a observada pela *C.symphodialis*, o pre-tratamento com a warifteína também inibiu a ativação de eosinófilos *in vivo*. Com esses resultados, nós elaboramos uma hipótese inicial de que o antagonismo dos receptores da PGD<sub>2</sub> corresponderia ao alvo molecular dos efeitos anti-alérgicos da warifteína.

Nosso grupo já demonstrou que a ativação eosinofílica – recrutamento de eosinófilos para a cavidade pleural, a biogênese de corpúsculos lipídicos nessas células recrutadas e a síntese de LTC<sub>4</sub> – podem ser modulados por extratos de produtos

naturais (Vieira-de-Abreu *et al.*, 2005a). Já demonstramos também que a síntese de LTC<sub>4</sub> por eosinófilos ativados com PGD<sub>2</sub> *in vitro* requer uma ativação simultânea obrigatória dos receptores DP1/DP2 e que a biogênese de corpúsculos lipídicos induzida por PGD<sub>2</sub>, depende exclusivamente da ativação do receptor DP1 (Mesquita-Santos *et al.*, 2010). Assim, nessa parte do trabalho, nós observamos que a warifteína não foi capaz de inibir a biogênese de corpúsculos lipídicos induzida por PGD<sub>2</sub> – ou por BW245c *in vitro*. Embora, não tenhamos realizado nenhum experimento para demonstrar que o pré-tratamento com a warifteína não interfere na interação do receptor DP2 com a PGD<sub>2</sub>, como ensaio de quimiotaxia ou ensaio de *shape change*, visto que são fenômenos reconhecidamente mediados pela ativação do receptor DP2 (Monneret *et al.*, 2001; Gervais *et al.*, 2001; Schratl *et al.*, 2007), nós excluimos a possibilidade da warifteína estar exercendo seus efeitos antiinflamatórios através da interação direta com os receptores da PGD<sub>2</sub>. Nós reconhecemos que o papel do receptor DP2 neste cenário, necessita ser elucidado, mas os dados observados com relação a inibição da síntese de PGD<sub>2</sub> redirecionou nossa hipótese de trabalho.

Nossos achados, obtidos com ensaios empregando um inibidor da síntese de PGD<sub>2</sub> (HQL-79) em um modelo murino de inflamação eosinofílica induzida por PGD<sub>2</sub>, trouxe a tona uma alternativa, para explicar a habilidade da *C. sympodialis* e da warifteína em inibir a migração e a ativação de eosinófilos induzida por PGD<sub>2</sub>. Essa hipótese, seria através da inibição da produção de PGD<sub>2</sub>, sintetizada em resposta a estimulação com PGD<sub>2</sub>.

Esse eicosanóide é produzido em grandes quantidades durante a resposta inflamatória asmática, sendo inclusive proposto como um marcador da ativação de mastócitos na asma (Murray *et al.*, 1986). Os mastócitos são células que podem produzir a PGD<sub>2</sub> como o principal produto do metabolismo do ácido araquidônico (AA) pela COX em resposta ao desafio antigênico (Holgate, 1998). Para testar então, se os efeitos antiinflamatórios da *C. sympodialis* e da warifteína dependeriam da inibição da produção de PGD<sub>2</sub>, nós avaliamos os níveis de PGD<sub>2</sub> produzidos *in vivo* usando dois modelos murinos de alergia, e *in vitro* pela análise da síntese de PGD<sub>2</sub> por mastócitos ativados por antígeno. Como postulado, tanto a *C. sympodialis* quanto a warifteína foram capazes de reduzir as quantidades da PGD<sub>2</sub> recém-formada de ambos os sítios inflamatórios alérgicos, assim como a warifteína reduziu os níveis de PGD<sub>2</sub> no

sobrenadante dos mastócitos estimulados com antígeno. Mas, qual o ponto exato na cascata enzimática da síntese de  $\text{PGD}_2$  que poderia ser o alvo das atividades inibitórias da warifteína?

Durante ativação celular, para iniciar a cascata de síntese de eicosanóides, o AA esterificado deve ser rapidamente mobilizado das membranas intracelulares, através de um evento realizado principalmente pela  $\text{cPLA}_2\text{-}\alpha$  (Diaz *et al.*, 2003). O metabolismo oxidativo do AA livre mediado por cada COX ou pela família das enzimas lipoxigenases (LO), culmina com a geração de uma variedade de eicosanóides de acordo com o tipo celular e com o estímulo. Ambas as enzimas COX, nominalmente a constitutiva COX-1 e a induzida COX-2, catalizam as mesmas duas reações: uma reação de ciclooxigenase que insere duas moléculas de oxigênio no substrato AA para formar a  $\text{PGG}_2$  e uma reação subsequente de endoperoxidase que reduz a  $\text{PGG}_2$  ao seu análogo 15-hidroxi – a  $\text{PGH}_2$  (Smith *et al.*, 1991). A via específica da síntese de eicosanóides a partir da  $\text{PGH}_2$  inclui uma série de prostanóides que são definidos pela enzima terminal que é diferencialmente expressa por tipos celulares diversos. Por exemplo, mastócitos geram grandes quantidades de  $\text{PGD}_2$  – mas não de  $\text{PGE}_2$  – devido a expressão seletiva de  $\text{PGD}_2$  sintase hematopoiética (H-PGDS) (Kanaoka *et al.*, 2003), enquanto que nos macrófagos  $\text{PGH}_2$  é isomerizada a  $\text{PGE}_2$  por diferentes enzimas PGE sintases, que cada uma se acopla preferencialmente com COX-1 ou com a isoforma induzida, COX-2, para gerar  $\text{PGE}_2$  em condições inflamatórias diversas (Simmons *et al.*, 2004; Kudo *et al.*, 2005).

Baseado nesta sequência de eventos enzimáticos existentes na cascata de síntese de eicosanóides, nós temos procurado identificar a molécula específica afetada pela warifteína, através da análise dos efeitos do pré-tratamento com a warifteína na produção de outros eicosanóides. A warifteína é o principal alcalóide bisbenzilquinolínico, obtido a partir do extrato alcoólico da *C. sympodialis*, juntamente com outros alcalóides (Porto *et al.*, 2008) e já foi demonstrado que outros alcalóides bisbenzilquinolínicos (tetrândrina e berbamina) são capazes de inibir a síntese de  $\text{LTB}_4$  e  $\text{PGE}_2$  por neutrófilos e monócitos humanos, estimulados *in vitro*, aparentemente através da inibição de  $\text{cPLA}_2$  (Teh *et al.*, 1990).

Como a warifteína não interferiu na produção de  $\text{LTC}_4$  promovida pela ativação com complexo IgE-antígeno, nós descartamos a enzima  $\text{cPLA}_2$  como eventual alvo do

mecanismo de ação inibitório da warifteína e, essa exclusão foi corroborada, com a ausência de efeito inibitório da warifteína na produção de PGE<sub>2</sub> por macrófagos peritoneais murinos *in vitro* induzida por A23187. Esse último resultado foi importante para descartar também um potencial envolvimento da COX, principalmente COX-1 (visto que a expressão de COX-2, sob essas condições estimulatórias não foi detectada).

Nosso estudo desvendou o mecanismo de ação de um tratamento ativo oralmente com *C. sympodialis* e seu composto ativo, a warifteína. Por inibir especificamente a síntese de PGD<sub>2</sub> – um mediador chave na inflamação alérgica que regula a ativação eosinofílica e também a síntese de LTC<sub>4</sub> nos sítios inflamatórios alérgicos – a warifteína emerge como uma alternativa terapêutica promissora. Enquanto a caracterização do alvo molecular específico da warifteína está ainda necessitando ser completamente elucidado – e será futuramente investigado – H-PGDS aparece como um candidato provável para ser o alvo do mecanismo de ação dessa droga.

## 5) CONCLUSÕES:

- √ A síntese de LTC<sub>4</sub> por eosinófilos induzida por PGD<sub>2</sub> é dependente da ativação conjunta de ambos os receptores de PGD<sub>2</sub>: DP1 e DP2.
- √ Os corpúsculos lipídicos recém-formados nos eosinófilos estimulados com PGD<sub>2</sub> servem como sítio de síntese de LTC<sub>4</sub> e o evento de biogênese dessas organelas é controlado pela ativação do receptor DP1, num fenômeno dependente da ativação de PKA;
- √ A ativação do receptor DP2 é condição *sine qua non* a síntese de LTC<sub>4</sub> em eosinófilos induzida por PGD<sub>2</sub>, contribuindo para esse fenômeno através da ativação de proteína G $\alpha$ i e aumento nos níveis de Ca<sup>2+</sup> intracelular.
- √ A warifteína parece exercer seus efeitos antiinflamatórios durante o desenvolvimento de respostas inflamatórias alérgicas, através da inibição da síntese de PGD<sub>2</sub>, potencialmente através do bloqueio da ação da enzima H-PGDS.

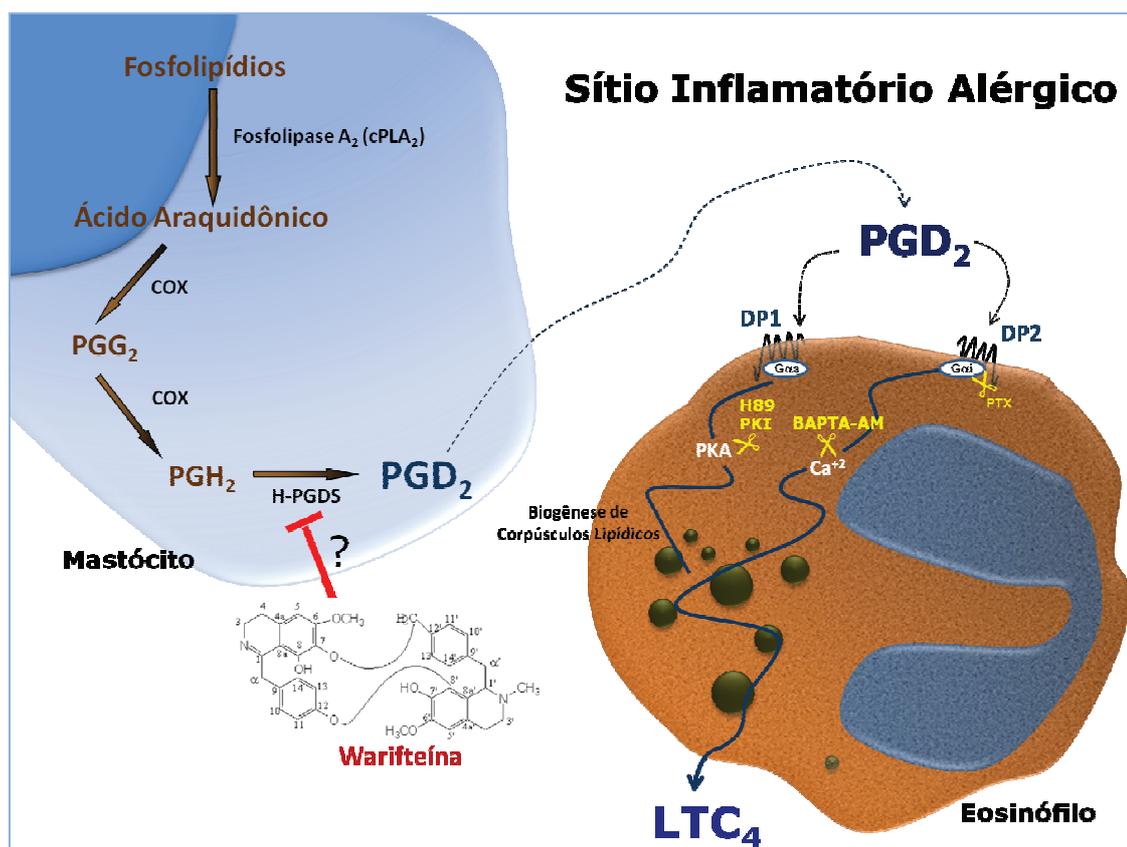


Figura 5.1 – Esquema representativo dos eventos intracelulares ocorridos nos eosinófilos em decorrência da ativação por PGD<sub>2</sub> produzida durante a resposta alérgica e indicação de possível alvo molecular das ações antiinflamatórias da warifteína.

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## 7. ANEXOS:

## Crosstalk between MIF and Eotaxin in Allergic Eosinophil Activation Forms LTC<sub>4</sub>-Synthesizing Lipid Bodies

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**Key words:** Eosinophils, MIF, LTC<sub>4</sub>, lipid droplets, lipid bodies, allergic inflammation, asthma

**Abbreviations:** 5-LO, 5-lipoxygenase; ADRP, adipose differentiation-related protein; EDAC, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide; LTC<sub>4</sub>, leukotriene C<sub>4</sub>; OVA, ovalbumin; MIF, macrophage migration inhibitory factor

### Abstract

Recent studies have demonstrated an essential and non-redundant role for macrophage migration inhibitory factor (MIF) in asthma pathogenesis. Here we investigate the mechanisms involved in MIF-induced eosinophil activation. By using a model of allergic pulmonary inflammation, we observed that allergen challenge-elicited eosinophil influx, lipid body (also known as lipid droplets) biogenesis and leukotriene (LT) C<sub>4</sub> synthesis are markedly reduced in *Mif*<sup>-/-</sup>

compared to wild-type mice. Likewise, *in vivo* administration of MIF induced formation of new lipid bodies within eosinophils recruited to the inflammatory reaction site that corresponded to the intracellular compartment of increased LTC<sub>4</sub> synthesis. MIF-mediated eosinophil activation were at least in part due to a direct effect on eosinophils, since MIF was able to elicit lipid body assembly within human eosinophils *in vitro*, a phenomenon that was blocked by neutralization of the MIF receptor CD74. MIF-induced eosinophil

lipid body biogenesis, both *in vivo* and *in vitro*, was dependent on the cooperation of MIF and eotaxin acting in a positive feed-back loop, since anti-eotaxin and anti-CCR3 antibodies inhibit MIF-elicited lipid body formation, while eotaxin-induced lipid body formation is affected by anti-CD74 and MIF expression deficiency. Therefore, allergy-elicited inflammatory MIF acts in concert with eotaxin as a key activator of eosinophils to form LTC<sub>4</sub>-synthesizing lipid bodies via cross-talk between CD74 and CCR3. Due to the effect of MIF on eosinophils, strategies that inhibit MIF activity might be of therapeutic value in controlling allergic inflammation.

### Introduction

Macrophage migration inhibitory factor (MIF) is a pleiotropic molecule that acts as a critical mediator of both innate and acquired immune responses (1, 2). Recently, a role for MIF in pathogenesis of Th2-driven inflammatory reactions was reported. A central, non-redundant function of MIF in asthma pathogenesis was established using Th2-driven animal models of allergic pulmonary inflammation where endogenous MIF was found to regulate bronchial hyper-responsiveness, mucus secretion and eosinophilic inflammation (3-5). Supporting such role in pulmonary inflammation, asthmatic patients were found to have increased concentrations of MIF in the bronchoalveolar lavage fluids, sputum and sera (6, 7). Similarly, a role for MIF was also described in the Th2 response induced by parasitic infections, such as helminthic infections by *Taenia crassiceps*, *Schistosoma japonicum* and *Schistosoma mansoni* (8-10).

Even though studies concur that MIF participates in the pathogenesis of

Th2-driven diseases, such as asthma and parasitic infestations, the MIF-targeted mechanisms involved in regulation of Th2 pathologies are still incompletely understood. Although polarization towards a Th2 response was initially postulated as the targeted pathway, there is no consensus in the literature on this topic (3-5, 10). In regards to the development of Th2-driven disturbances, we hypothesized that MIF acts as a modulator of eosinophil activation. Indeed, MIF has emerged as a potent inflammatory mediator capable of regulating eosinophil differentiation and chemotaxis, as well as LTC<sub>4</sub> production triggered by allergic challenge (3, 10).

Eosinophil recruitment and subsequent activation at sites of allergic inflammation has the potential to generate and release diverse lipid and protein mediators critical to the development and perpetuation of the allergic process (11-13). Indeed, eosinophils represent a major source of LTC<sub>4</sub> and its extracellular derivatives (LTD<sub>4</sub> and LTE<sub>4</sub>) products of the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism in allergic diseases (14-16).

Central to the pathogenesis of allergic diseases, these cysteinyl leukotrienes (cysLTs) cause bronchoconstriction, mucous hypersecretion, increased microvascular permeability, bronchial hyper-responsiveness, eosinophil infiltration and airway remodeling (17-19). Although the enzymatic pathways by which eosinophils synthesize LTC<sub>4</sub> are well established, the key regulatory mechanisms of allergen-induced LTC<sub>4</sub> synthesis, such as its specialized intracellular compartmentalization and the relevant endogenous allergy-

triggered stimuli, remain uncharacterized.

The formation of LTC<sub>4</sub> requires the functional interaction of at least four proteins acting in cascade: cytoplasmic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), 5-LO-activating protein (FLAP), 5-LO and LTC<sub>4</sub> synthase (20, 21). Studies from several groups suggest that dynamic compartmentalization of these biosynthetic proteins is a primary mechanism for the regulated synthesis of LTC<sub>4</sub> (for review see (22, 23)). Upon leukocyte activation three distinct intracellular domains may compartmentalize such macromolecular organization for LTC<sub>4</sub> synthesis: the nuclear membrane (24), phagosomes (25) and/or lipid bodies (26).

Lipid bodies, also known as lipid droplets or adiposomes, are cytoplasmic lipid domains surrounded by a phospholipid monolayer with a unique fatty acid composition characterized by a neutral lipid rich core and variable protein composition (27, 28). Characterization of lipid body protein content in different cell types has suggested that lipid bodies are heterogeneous, dynamic and multi-functional organelles. Proteins compartmentalized within lipid bodies include enzymes involved in lipid metabolism, cytokines, proteins of the Rab family, specific kinases and small GTPases, thus positioning lipid bodies as potential regulators of lipid metabolism, membrane trafficking and intracellular signaling (for review see (28, 29)). Likewise, by uncovering that eicosanoid-forming enzymes are compartmentalized alongside esterified arachidonic acid (AA) within lipid bodies (26, 30, 31), an additional lipid body function as an inflammatory mediator producer was postulated. This was then

confirmed by the direct detection of newly synthesized LTC<sub>4</sub> in eosinophil lipid bodies (32, 33).

Lipid body biogenesis is a biological process that has been intensively studied over the past few years. Assembly of new lipid bodies is recognized as an acute, highly regulated cellular event that is stimulus- and cell-type specific that takes place at specialized regions of endoplasmic reticulum (ER), and ultimately determines the specific functions of the newly formed lipid bodies. Moreover, increased biogenic activity is a constant feature of leukocyte activation elicited by inflammatory process (for review (28)), as observed in eosinophils from patients with Crohn's disease (34), hyper-eosinophilic syndrome (35) and from animals undergoing allergic inflammation (33, 36, 37).

Even though it is recognized that the allergic inflammatory response increases the number of LTC<sub>4</sub>-synthesizing lipid bodies within activated eosinophils, the detailed molecular mechanisms controlling their biogenic process remain largely unclear. It was determined by *in vitro* assays that specific agonists, particularly eotaxin and PGD<sub>2</sub>, could trigger signaling mechanisms that rapidly stimulate the formation of new LTC<sub>4</sub>-synthesizing lipid bodies within human eosinophils (32, 33). Accordingly, *in vivo* studies conducted by our group showed that both the biogenesis of lipid bodies and the enhanced synthesis of LTC<sub>4</sub> at newly formed lipid bodies triggered by allergic inflammation are partially mediated by eotaxin/RANTES acting via CCR3 receptors (33) and by PGD<sub>2</sub> (37).

As discussed above, MIF mediated the enhancement of LTC<sub>4</sub> production triggered by allergic

challenge in a mouse asthma model (3). Therefore, we aimed to investigate the mechanisms involved in MIF-mediated allergen-elicited LTC<sub>4</sub> synthesis, focusing on the ability of MIF to activate LTC<sub>4</sub>-synthesizing machinery in eosinophils by analyzing three key regulatory aspects: (i) the intracellular LTC<sub>4</sub>-synthesizing compartment; (ii) the induced biogenesis of such cytoplasmic compartments, named lipid bodies; and (iii) the potentially stimulatory crosstalk with eotaxin. Our results show that at sites of eosinophilic inflammation, crosstalk between MIF and eotaxin activates eosinophils and results in the assembly of lipid bodies with ability to synthesize LTC<sub>4</sub>.

## **Material and Methods**

### **Reagents**

Chicken egg ovalbumin (OVA), bovine serum albumin (BSA), phosphate-buffered saline (PBS), 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC) and mouse IgG1 were purchased from Sigma Chemical Co. (St. Louis, MO). Neutralizing antibodies to murine and human eotaxin, rmMIF, rmeotaxin, rheotaxin, rhMIF and CCR3 were purchased from R&D Systems (Minneapolis, MN), whereas anti-CD74 (clone LN2) was from BD Pharmingen (San Diego, CA). MK 886 was purchased from Biomol (Plymouth Meeting, PA).

### **Animals**

C3H/He, C3H/HeJ, BALB/c and MIF deficient mice (*Mif*<sup>-/-</sup>) (3, 38) (BALB/c background) ranging from 6 - 8 weeks old of both sexes were obtained from the Oswaldo Cruz Foundation breeding unit (Rio de Janeiro). Protocols were approved by the Fundação Oswaldo Cruz animal welfare committee.

### **Model of allergic pulmonary eosinophilia**

Allergic inflammation in the lungs of actively sensitized mice was generated as previously described (2). Briefly, mice were sensitized with intraperitoneal injection of OVA (10 µg/mouse) and AHOH3 (10 mg/ml) resuspended in sterile saline (0.2 mL) on days 1 and 10. From days 19 to 24 after sensitization, mice were challenged daily for 20 min with OVA (5% in sterile saline) by aerosol. Sterile saline was aerosolized to sensitized mice as a negative control. Aerosolization was performed in a 30 x 20 x 10 cm acrylic chamber and the aerosol was generated with an ultrasonic nebulizer. Twenty-four hours after the last aerosolization, animals were euthanized by CO<sub>2</sub> inhalation, and the tracheas were surgically exposed and cannulated. The bronchoalveolar lavage (BAL) was collected by washing the lungs with PBS (1 mL).

### **Pleurisy triggered by MIF or eotaxin**

Naive mice received intrapleural injections of either rmMIF (0.1 – 1 µg/cavity) or rmeotaxin (30 pmol/cavity), each diluted in sterile saline immediately before use. Control animals were injected with the same volume (0.1 mL) of saline. Six or twenty-four hours after stimulation, mice were euthanized by CO<sub>2</sub> inhalation and pleural cavities were rinsed with 1 mL of PBS.

### **Leukocyte counts**

Total leukocytes (diluted with Turk's 2% acetic acid fluid) were counted using Neubauer chambers. Differential counts were performed in cytopins stained by the May-Grunwald-Giemsa method. Counts are reported as total number of eosinophils per BAL fluid or pleural cavity.

#### **Quantification of cysteinyl leukotrienes and eotaxin**

Amount of cysteinyl leukotrienes found in BAL or pleural fluid were measured by a commercial EIA kit according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI). Eotaxin were measured in the pleural fluid using commercially available ELISA kit according to the manufacturer's protocol (R&D Systems).

#### **EicosalCell for LTC<sub>4</sub> immunolocalization**

LTC<sub>4</sub> was localized to the sites of formation as previously described (32, 33). Briefly, leukocytes were recovered from the pleural cavity 6 h following MIF or saline administration and 1 h following zileuton by washing the cavity with 500  $\mu$ L of HBSS. Cells were then immediately mixed with 500  $\mu$ L of EDAC (1% in HBSS), which is used to crosslink eicosanoid carboxyl groups to amines of adjacent proteins. After a 30 min incubation at 37°C with EDAC to promote both cell fixation and permeabilization, pleural leukocytes were then washed with HBSS, cytopspin onto glass slides and blocked with HBSS containing 2% normal mouse serum and normal goat serum for 10 min. The cells were then incubated with rabbit anti-LTC<sub>4</sub> antibodies (Cayman Chemicals, Ann Arbor, MI) for 45 min or non-immune rabbit IgG. The anti-ADRP antibody was added for 45 min to distinguish cytoplasmic lipid bodies within leukocytes. Cells were washed three times with HBSS for 10 min and incubated with Cy2-labeled anti-rabbit IgG in addition to Cy3-labeled anti-guinea pig secondary antibodies for 1 h. The slides were analyzed by confocal laser scanning microscopy on a Zeiss LSM 510-META.

#### **Isolation and stimulation of human eosinophils**

Peripheral blood was obtained with informed consent from normal donors and eosinophils were isolated by negative selection using the StemSep™ system (StemCell Technologies Inc.) resulting in a final population that was >99% pure and >95% viable (39). The protocol used was approved by the Fundação Oswaldo Cruz ethical review board. Purified eosinophils ( $2 \times 10^6$  cells/ml in HBSS<sup>+</sup>) were incubated for 1 h in a water bath (37 °C) with rhMIF (0.05 - 5 ng/mL), rheotaxin (1 - 100 ng/mL) or a combination of MIF (0.05 ng/mL) and eotaxin (1 ng/mL). Each *in vitro* experiment was performed in triplicate and repeated at least three times with eosinophils purified from different donors.

#### **Lipid body staining and enumeration**

To quantify lipid bodies, eosinophils were cytocentrifuged (450 rpm, 5 min) onto glass slides. Cells, while still moist, were fixed in 3.7% formaldehyde (diluted in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS, pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained with 1.5% OsO<sub>4</sub> for 30 min, rinsed in distilled H<sub>2</sub>O, immersed in 1.0% thiocarbohydrazide for 5 min, rinsed in 0.1 M cacodylate buffer, restained with 1.5% OsO<sub>4</sub> for 3 min, rinsed in distilled water, and then dried and mounted. Cell morphology was observed and lipid bodies were quantified by microscopy. Fifty consecutively scanned eosinophils were then subjected to blinded evaluation by more than one individual. Results are expressed as the number of lipid bodies per eosinophil.

#### **Treatments**

Using the pleurisy model, animals were pre-treated with intrapleural injections of anti-murine eotaxin mAb

(10 µg/cavity) 30 min prior to allergic challenge. Animals pre-treated with an irrelevant Ab showed no alteration of MIF-induced response (not shown).

For *in vitro* mechanistic studies, eosinophils were pre-treated for 30 min with 10 µg/mL of neutralizing Abs against human CD74 (clone LN2 as described in (40)), eotaxin or CCR3. Isotype matched non-immune Abs were used as controls. The anti-CD74 antibody has azide, however at the concentrations used (final concentration ≤ 0.002%) it did not affect viability (>95%) or lipid body formation.

#### **Statistical analysis**

Results from *in vivo* assays are expressed as mean ± SEM and were analyzed statistically by means of ANOVA followed by the Newman-Keuls Student test with the level of significance set at  $P < 0.05$ .

## **RESULTS**

### *MIF mediates assembly of eosinophil lipid bodies and synthesis of LTC<sub>4</sub> triggered by allergic pulmonary inflammation*

We have previously reported that, in addition to the well-known induction of LTC<sub>4</sub> production, allergic challenge also triggers the biogenesis of lipid bodies, which function as intracellular compartments for LTC<sub>4</sub> synthesis within recruited eosinophils (33). The mechanisms involved in eliciting the biogenesis of cytoplasmic lipid bodies and priming infiltrating eosinophils for enhanced LTC<sub>4</sub> production in allergic inflammatory sites are not completely known but involve, at least, the mediation by eotaxin and PGD<sub>2</sub>, two well-known eosinophilotactic agents (33, 37).

Recently, we and others have identified MIF as a key mediator of

allergic inflammation (3-5, 28). Here to investigate whether MIF has any role in eosinophil lipid body-driven LTC<sub>4</sub> synthesis triggered by allergic challenge, a model of acute allergic pulmonary inflammation in WT versus *Mif*<sup>-/-</sup> mice (BALB/c background) was used. As previously demonstrated (3) and shown in Figure 1A, BAL eosinophilia triggered by aerosol allergic challenge is drastically reduced in *MIF*<sup>-/-</sup> mice compared to the response observed in WT mice. Similarly, both formation of new lipid bodies within recruited eosinophils and cysLTs generation were significantly decreased in allergen-challenged *MIF*<sup>-/-</sup> mice when compared to WT mice (Figure 1B and 1C). Together these data indicate that endogenous MIF, found at an allergic inflammatory site is capable of eliciting lipid body assembly within infiltrating eosinophils, in parallel to enhancing the production of cysLTs.

### *MIF triggers in vivo biogenesis of functional LTC<sub>4</sub>-synthesizing lipid bodies within recruited eosinophils*

Because MIF appears to contribute to eosinophil activation during allergic responses, we examined whether MIF was able to elicit eosinophil LTC<sub>4</sub>-synthesizing machinery in recruited eosinophils *in vivo*. Intraperitoneal administration of MIF (0.1 or 1 µg/cavity) in naive mice induced accumulation of eosinophils in a dose-dependent manner (Figure 2A). These eosinophils showed an increased number of cytoplasmic lipid bodies (Figure 2B), thereby demonstrating the ability of MIF to activate the formation of lipid body biogenesis machinery *in vivo*. Of note, MIF-induced eosinophil activation *in vivo* was not dependent on LPS contamination because C3H/HeJ mice,

an LPS hypo-responsive strain that has an inactivating point mutation within the signal transducing domain of TLR4 (41), also respond to i.pl. MIF (1 µg/cavity) with eosinophil recruitment and lipid body formation of similar magnitude to that observed in C3H/HeN mice, which express functional TLR4 (Table 1).

Like in allergic inflammation and concurring with MIF-induced *in vivo* lipid body biogenesis, there was also significant production of cysLTs in MIF-injected animals (Figure 2C). It was determined that this parallel increase in numbers of lipid bodies and levels of secreted cysLTs, was the result of a functional relationship between lipid bodies and cysLTs. As illustrated in Figure 3, EicosaCell, a methodology that cross-links and immuno-labels LTC<sub>4</sub> at the original sites of synthesis, identified infiltrating eosinophils as the cell population responsible for LTC<sub>4</sub> production during MIF-elicited inflammatory reactions. In addition, a more detailed analysis revealed that the intracellular LTC<sub>4</sub>-synthesizing compartment displayed a punctate cytoplasmic pattern proximal to, but separate from, the nucleus, which is fully consistent in size and form to eosinophil lipid bodies. In fact, the compartmentalization of newly formed LTC<sub>4</sub> in eosinophil lipid bodies was ascertained by the co-localization with ADRP (Figure 3). Virtually no LTC<sub>4</sub> was immuno-localized within cells of saline-injected or MIF-stimulated MK886-treated animals (not shown), thus demonstrating that the newly formed lipid bodies of *in vivo* MIF-stimulated eosinophils are enzymatically suited for effective LTC<sub>4</sub> synthesis.

*MIF activates eosinophils directly through its receptor CD74*

To investigate whether MIF was indeed capable to directly activating eosinophils and sufficient to trigger the formation of new lipid bodies, we stimulated *in vitro* purified human eosinophils with rMIF. Figure 4A shows that rMIF (0.05 - 50 ng/mL) induced a rapid (1 h) and dose-dependent increase in the numbers of cytoplasmic lipid bodies within human eosinophils isolated from healthy donors. Moreover, rMIF-induced lipid body biogenesis appeared to be a direct effect of MIF on specific receptors expressed on eosinophil surface, since the neutralizing antibody against CD74, which recognizes the MHC class-II invariant chain identified as the MIF receptor (40), significantly inhibited the effects of MIF. Together, this indicates that eosinophils respond directly to MIF stimulation, which, at least in part, depends on CD74 binding. Eosinophils pre-treated with isotype matched non immune Ab displayed expected lipid body biogenesis under MIF stimulation (Figure 4 and not shown). Moreover, the specificity of the treatment was confirmed by the observation that CD74 antibody neutralization failed to inhibit PAF induced lipid body formation (from 19.8±1.6 in to 17.4±2.8 lipid bodies/eosinophil, n=3; when non-treated and anti-CD74 treated PAF-stimulated eosinophils were compared).

*Eotaxin mediates MIF-driven eosinophil activation*

The mechanisms involved in MIF-induced lipid body formation were further investigated by focusing on the putative crosstalk between MIF and eotaxin. It is well established that CCR3 activation by eotaxin in eosinophils stimulates both potent chemoattractant and lipid body biogenic responses,

which control allergen-driven LTC<sub>4</sub> synthesizing machinery (for review see (32)). Here several of our findings show that MIF-induced eosinophil activation, both *in vivo* and *in vitro*, appeared to be dependent on eotaxin-driven autocrine and/or paracrine activity: (i) as shown in Figure 5A, *in vivo* pre-treatment with a neutralizing anti-murine eotaxin mAb significantly inhibited both eosinophil influx and lipid body biogenesis induced by the i.p.l. administration of rMIF; (ii) increased levels of eotaxin were found in the pleural fluid of mice injected with MIF (1 µg/cavity) (Figure 5B); and (iii) *in vitro* rMIF-elicited CD74-dependent lipid body assembly within human eosinophils was blocked by neutralizing anti-human eotaxin mAb and is significantly inhibited by anti-human CCR3 mAb (Figure 5C), suggesting that an endogenous eosinophil-derived eotaxin found at the site of the inflammatory reaction could mediate part of the MIF/CD74-induced eosinophil activation response.

#### *MIF mediates eotaxin-driven eosinophil activation*

To better characterize the role of MIF and eotaxin cross-talk on regulating eosinophil responses, the input of MIF/CD74 on eotaxin-induced eosinophil activation was evaluated. Similar to the MIF-triggered eosinophil activation response mediated by eotaxin, MIF autocrine/paracrine activity appears to mediate the ability of eotaxin to trigger lipid body biogenesis. Although *Mif*<sup>-/-</sup> mice stimulated with eotaxin (30 pmol/cavity, i.p.l.) *in vivo* showed eosinophil influx levels comparable to WT mice, *Mif*<sup>-/-</sup> mice displayed negligible eosinophil lipid body biogenesis after eotaxin stimulation (Figure 6). This result was

confirmed when *in vitro* neutralization of human CD74 on human eosinophils was able to inhibit lipid body formation triggered by rheotaxin (Figure 7A), indicating that eotaxin-stimulated eosinophils rapidly secrete MIF that, acting autocrinally and/or paracrinely on CD74 expressed on eosinophils, triggers assembly of new lipid bodies. Finally, as shown in Figure 7B, suboptimal concentrations of rheotaxin (1 ng/mL) and rhMIF (0.05 ng/mL) acted in a cooperative manner to induce assembly of new lipid bodies. Therefore, in addition to mediating eosinophil activation, physiological levels of eotaxin and MIF found *in vivo* at sites of eosinophilic inflammation may also synergize to effectively activate recruited eosinophils.

## DISCUSSION

Recent studies have demonstrated that MIF has an essential role in modulating several aspects of the physiopathology of allergic pulmonary inflammation, including eosinophil recruitment and the production of inflammatory mediators in the lung (3). In addition, it has been previously shown that MIF can activate distinct intracellular signaling pathways, which can enhance cell eicosanoid synthetic capacity (42, 43). Our study investigates the mechanisms involved in the interplay of MIF and cystLTs in allergic pulmonary inflammation, whereby MIF may directly activate and thereby enhance the capacity of eosinophils to synthesize LTC<sub>4</sub>. By employing *in vivo* and *in vitro* assays, we characterized the ability of MIF to directly activate eosinophils, eliciting rapid biogenesis of LTC<sub>4</sub>-synthesizing lipid bodies, organelles considered as markers of leukocyte activation. Moreover, we

demonstrated that MIF functions via activation of its receptor CD74 and acts in concert with eotaxin in a positive feed-back loop to activate eosinophils in response to allergic pulmonary inflammation.

Biogenesis of cytoplasmic lipid bodies has been noted within cells engaged in inflammatory disorders, and even though they were long viewed as lipid storage depots within cells, it has now become increasingly accepted that these organelles compartmentalize a diverse set of proteins and, therefore, participate in different cell functions including eicosanoid synthesis (reviewed in (28)). In regards to lipid bodies found in eosinophils, *in vivo* and *in vitro* studies conducted by our group have shown that an induced highly orchestrated phenomenon, that is mediated in part by CCR3 activation induced by endogenously produced eotaxin and RANTES (32, 36), controls the biogenesis of eosinophil lipid bodies - sites of 5-LO-driven LTC<sub>4</sub> synthesis - which contributes to the characteristic enhancement of cysLTs production in response to allergic challenge (33). In this study, we found that MIF has, as with CCR3 activating chemokines, a potent and direct effect on eosinophils to stimulate lipid body biogenesis. Furthermore, the capacity of MIF to regulate lipid body assembly within infiltrating eosinophils during allergic pulmonary pathogenesis was found to impact the ability of eosinophils to generate LTC<sub>4</sub>, thereby contributing to enhanced cysLTs generation *in vivo*.

The mechanisms involved in MIF-induced eosinophil activation, which culminates with biogenesis of LTC<sub>4</sub>-synthesizing lipid bodies, emerged as a two-player receptor engagement system. In regards to allergic

inflammation, eosinophil-activating mediators including PAF (26, 35), IL-5 (35, 44), CC chemokines (32) and PGD<sub>2</sub> (37) act directly on receptors on the eosinophil surface and initiate intracellular signaling cascades that culminate with *de novo* formation of lipid bodies and prime LTC<sub>4</sub> synthesis.

Recently CD74, the cell-surface form of the MHC class-II-associated invariant chain, was demonstrated to be a high affinity MIF receptor with signaling transducing ability (40). We observed a significant inhibition of MIF-induced lipid body formation in human eosinophils *in vitro* using neutralizing anti-human mAb CD74, suggesting that MIF directly activates eosinophils to form lipid bodies in part by interacting with its specific membrane receptor. Even though MIF may have direct effects on eosinophils via CD74 activation, our results demonstrate that MIF cooperates with eotaxin to trigger robust eosinophil activation and form new lipid bodies.

We recently showed that endogenously generated eotaxin partially mediates allergen- and PGD<sub>2</sub>-induced formation of eosinophil lipid bodies (36, 37). Moreover, either exogenously administered MIF or endogenously generated MIF in the course of allergic reaction are able to elicit eotaxin generation (3). Complementary to this, we verified that inhibition of eotaxin or its receptor CCR3, either *in vivo* or *in vitro*, substantially reduced MIF-triggered eosinophil lipid body formation. This suggests that part of the mechanism of MIF-induced lipid body formation is due to an indirect effect on CCR3 by eosinophil-derived eotaxin, which is rapidly released in response to MIF stimulation and contributes to the

process in an autocrinally/paracrinally manner. Of note, eotaxin-driven eosinophil lipid body biogenesis were also partially inhibited by blocking MIF and CD74, suggesting that MIF and eotaxin cooperate to fully activate eosinophils.

Together, our data, as schematized in Figure 8, demonstrate that during eosinophilic allergic reactions, MIF controls eosinophil activation by binding to its receptor and triggers both LTC<sub>4</sub> synthesis within newly formed lipid bodies as well as the rapid release of preformed eotaxin. In response, such eosinophil-derived chemokine, acting paracrinally and/or autocrinally on eosinophils, binds to the CCR3 receptor to induce both lipid body-driven LTC<sub>4</sub> synthesis as well as secretion of stored MIF from eosinophils. Therefore, after an initial increase of cell-free eotaxin and/or MIF concentrations within sites of allergic reaction characterized by local eosinophilia, a system of eosinophil retro-activation appears to take place that results in lipid body-driven LTC<sub>4</sub> production by infiltrating eosinophils. Importantly, among other cytokines, eosinophils are known to store pre-formed pools of both MIF and eotaxin (reviewed in (45)). Moreover, it is also recognized that properly stimulated eosinophils can rapidly select and

secrete small, but biologically active amounts of eotaxin, which can function in an autocrine manner (39).

Eosinophil activation evoked by MIF launches the rapid but highly regulated biogenic process of lipid body assembly, which forms organelles committed to synthesizing large amounts of LTC<sub>4</sub>, a typical result of allergic inflammatory responses. CysLTs are known to be an essential mediator of bronchial hyper-responsiveness, mucus secretion, the production of inflammatory mediators and eosinophil recruitment; the same allergic features now recognized to be regulated in part by MIF. Our data also unveil a cross-talk mechanism between eosinophil-derived eotaxin and MIF that controls eosinophil activation and regulates the amplification of allergy-related LTC<sub>4</sub> synthesis. Therefore, such MIF/eotaxin-elicited, eosinophil lipid body-driven LTC<sub>4</sub> synthesis rises as an interesting candidate for new anti-allergic therapeutic approaches.

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## Legends

**Figure 1.** MIF mediates eosinophil influx (A), lipid body formation within recruited eosinophils (B) and *cysLTs* synthesis (C) induced by allergen-driven pulmonary inflammation. Analysis was performed on bronchoalveolar fluids harvested 24 h following the final ovalbumin aerolization. Control groups were immunized and aerolized with sterile saline. Results are expressed as the mean  $\pm$  SEM calculated from at least eight animals. \*,  $P \leq 0.05$  compared to saline control group. †,  $P \leq 0.05$  compared to WT stimulated mice.

**Figure 2.** *rmMIF* evokes eosinophil influx (A), lipid body formation within recruited eosinophils (B) and *cysLTs* synthesis (C) in a pleurisy model. Analysis was performed on pleural fluids harvested 24 h following intrapleural injection of *rmMIF* (0.1 and 1  $\mu$ g/cavity). The control group was injected with sterile saline. Results are expressed as the mean  $\pm$  SEM calculated from at least eight animals. \*,  $P \leq 0.05$  compared to control group.

**Figure 3.** Eosinophil *LTC<sub>4</sub>* synthesis in MIF-stimulated animals occurs in lipid bodies. EcosaCell images illustrate intracellular immuno-detection of newly formed *LTC<sub>4</sub>* (green) and ADRP (red) in cells from MIF-stimulated (upper and middle panels) and saline-stimulated animals (lower panel). Overlaid immunofluorescence microscopy images of identical fields are shown in the right column.

**Figure 4.** MIF induces lipid body biogenesis in human eosinophils *in vitro*. (A) Dose-dependent response of rhMIF (from 0.05 to 50 ng/mL) on lipid body biogenesis after stimulation of human eosinophils *in vitro* for 1 h. (B) Effect of 30 minutes pretreatment with anti-CD74 or isotype control IgG1 on rhMIF-stimulated (50 ng/mL) human

eosinophils. Results are expressed as mean  $\pm$  SEM calculated from triplicate results from one donor and are representative of results from eosinophils isolated from at least three distinct donors. \*,  $P \leq 0.05$  compared to HBSS control group. †,  $P \leq 0.05$  compared to MIF-stimulated untreated cells.

**Figure 5.** Role of eotaxin in MIF-driven eosinophil activation. (A) Mice received an i.pl. injection of rmMIF (1  $\mu$ g/cavity). Analysis of eosinophil influx and lipid body formation were performed 24 h following MIF administration. (B) Pleural levels of eotaxin were analyzed 6 h following rmMIF i.pl. injection (1  $\mu$ g/cavity). Results are expressed as the mean  $\pm$  SEM calculated from at least six animals. †,  $P \leq 0.05$  compared to control animals. \*,  $P \leq 0.05$  compared to rmMIF-injected mice. For *in vitro* analysis of lipid body biogenesis shown in (C), purified human eosinophils were pre-incubated with anti-eotaxin or anti-CCR3 and then stimulated with rhMIF (5 ng/mL) for 1 h. *In vitro* results are expressed as mean  $\pm$  SEM calculated from triplicate results from one donor and are representative of results from eosinophils isolated from at least three distinct donors. †,  $P \leq 0.05$  compared to HBSS control group. \*,  $P \leq 0.05$  compared to MIF-stimulated untreated cells.

**Figure 6.** MIF mediates eotaxin-driven eosinophil activation. WT and *Mif*<sup>-/-</sup> mice received an i.pl. injection of eotaxin (30 pmol/cavity). Analysis of eosinophil influx and lipid body formation were performed 24 h following eotaxin administration. Results are expressed as the mean  $\pm$  SEM calculated from at least six animals. †,  $P \leq 0.05$  compared to non-stimulated WT mice. \*,  $P \leq 0.05$  compared to eotaxin-injected WT mice.

**Figure 7.** MIF mediates eotaxin-driven eosinophil activation *in vitro*. Lipid body formation in purified human eosinophils that were (A) pre-incubated for 30 minutes with anti-CD74 or isotype control IgG1 and then stimulated with eotaxin (100 ng/mL) or (B) stimulated with rhMIF (0.05 or 5 ng/mL), eotaxin (1 or 100 ng/mL) or co-stimulated with both eotaxin (1 ng/mL) and rhMIF (0.05 ng/mL) *in vitro*. Analysis of lipid body formation was performed 1 h following stimulation. Results are representative of three independent experiments. †,  $P \leq 0.05$  compared to HBSS control group. \*,  $P \leq 0.05$  compared to eotaxin-stimulated untreated cells.

**Figure 8.** Cross-talk between MIF and eotaxin in regulating eosinophil activation. In sites of allergic inflammatory reactions, physiological concentrations of endogenous MIF and eotaxin (potentially derived from infiltrating eosinophils) act on their cognate receptors expressed on recruited eosinophils (CD74 and CCR3, respectively) and cooperate in an autocrine/paracrine fashion to elicit eosinophil activation, which is characterized by lipid body biogenesis and compartmentalized LTC<sub>4</sub> synthesis.

**Table 1.** *In vivo* effects of rmMIF on eosinophil influx and lipid body biogenesis are not due LPS contamination, since activation of TLR4 is not involved in these phenomena.<sup>a</sup>

Condition	Animal strain	Eosinophils/cavity	Lipid Bodies/cell
saline	C3H/He	0.01 ± 0.01	0.50 ± 0.01
rmMIF	C3H/He	0.18 ± 0.05*	4.54 ± 0.05*
saline	C3H/HeJ	0.01 ± 0.01	0.20 ± 0.01
rmMIF	C3H/HeJ	0.16 ± 0.03*	4.08 ± 0.05*

<sup>a</sup> TLR4 sensitive (C3H/HeN) and non-sensitive (C3H/HeJ) mice were stimulated *in vivo* with rmMIF (1 µg/cavity) for 24 h. Results were expressed as mean ± SEM calculated from at least six distinct animals. \*,  $P \leq 0.01$  compared to saline-stimulated cells.

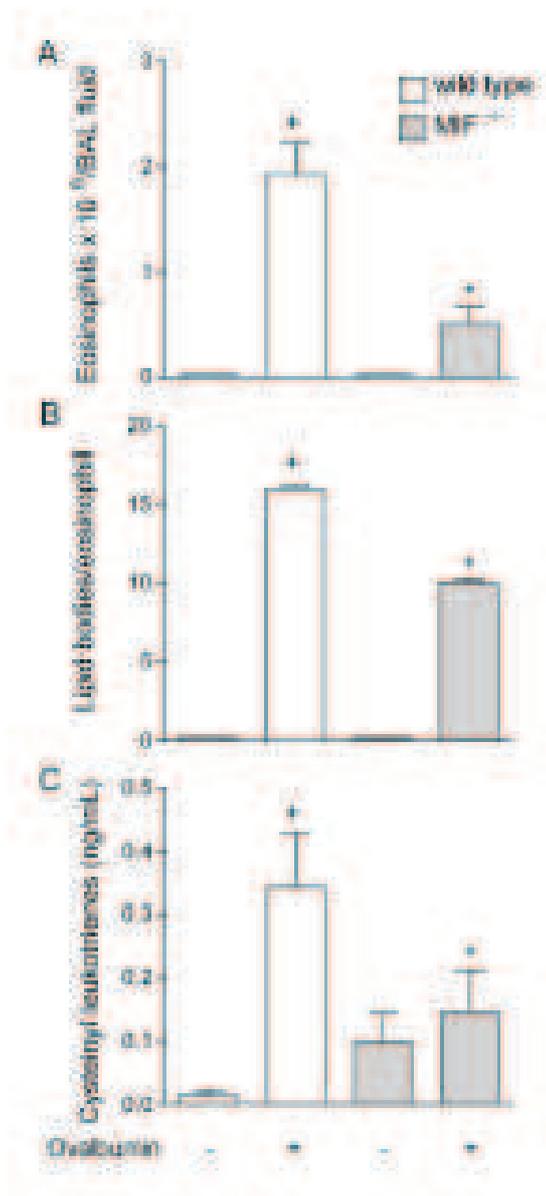


Figure 1

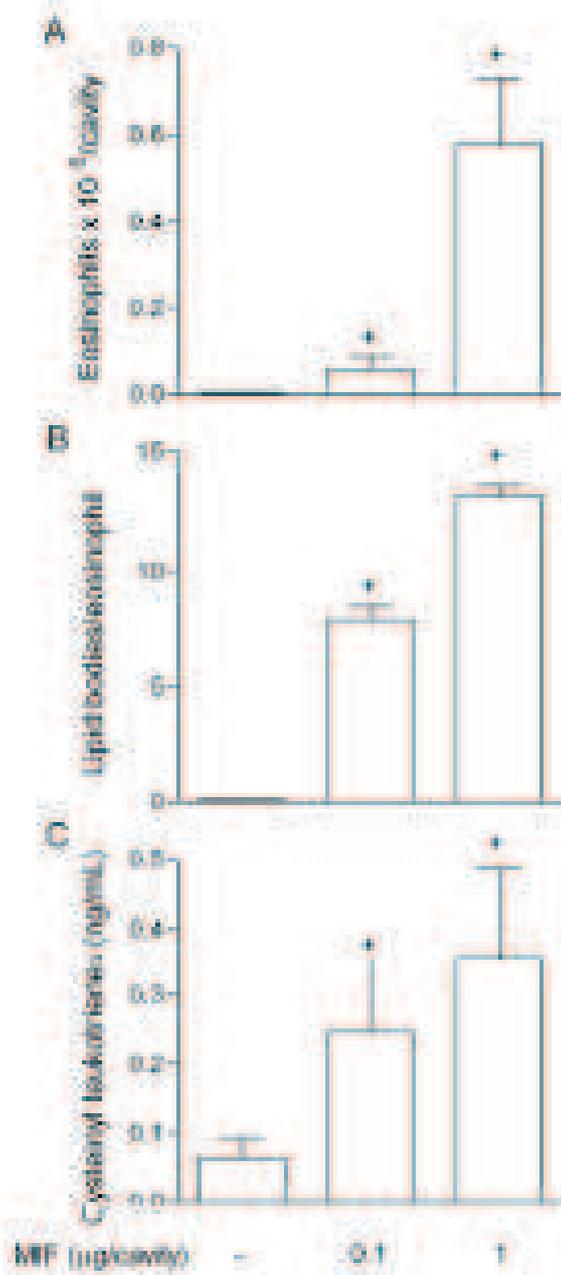
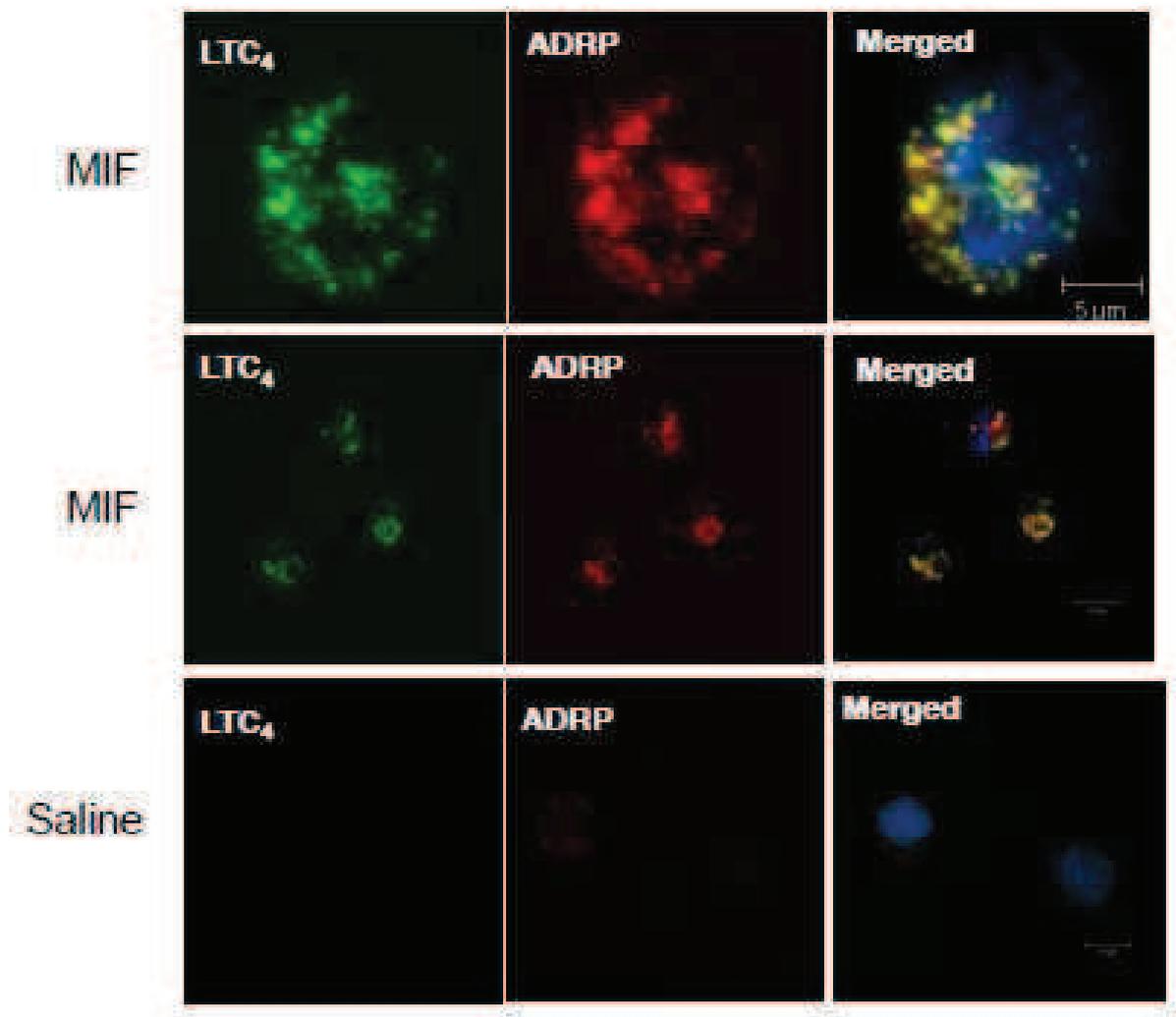


Figure 2



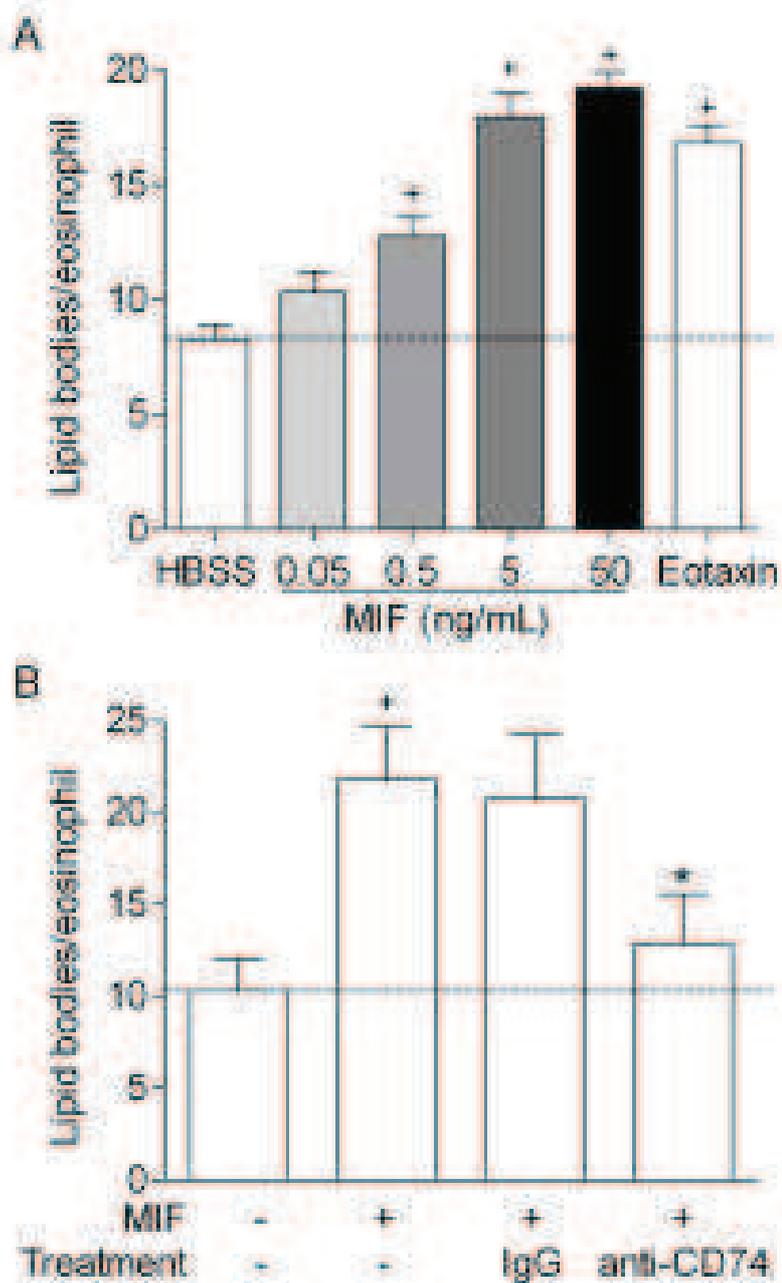


FIGURE 4  
148x222mm (300 x 300 DPI)

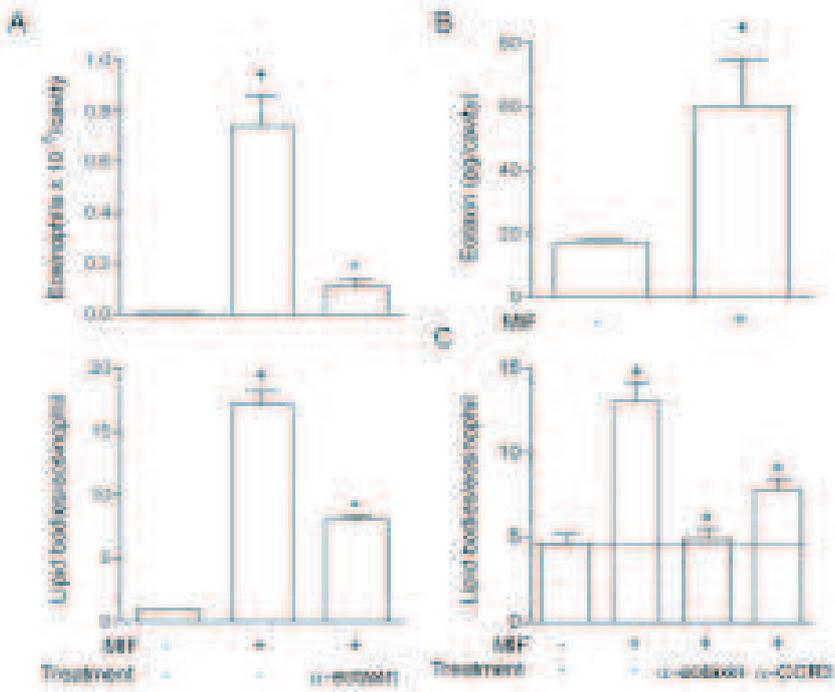


Figure 5:

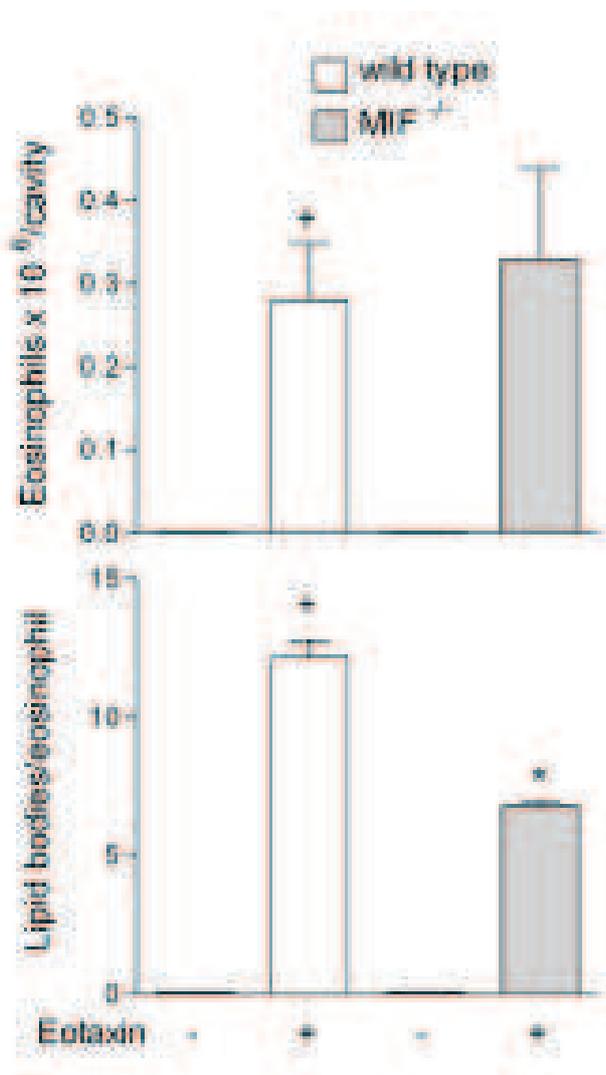


Figure 6

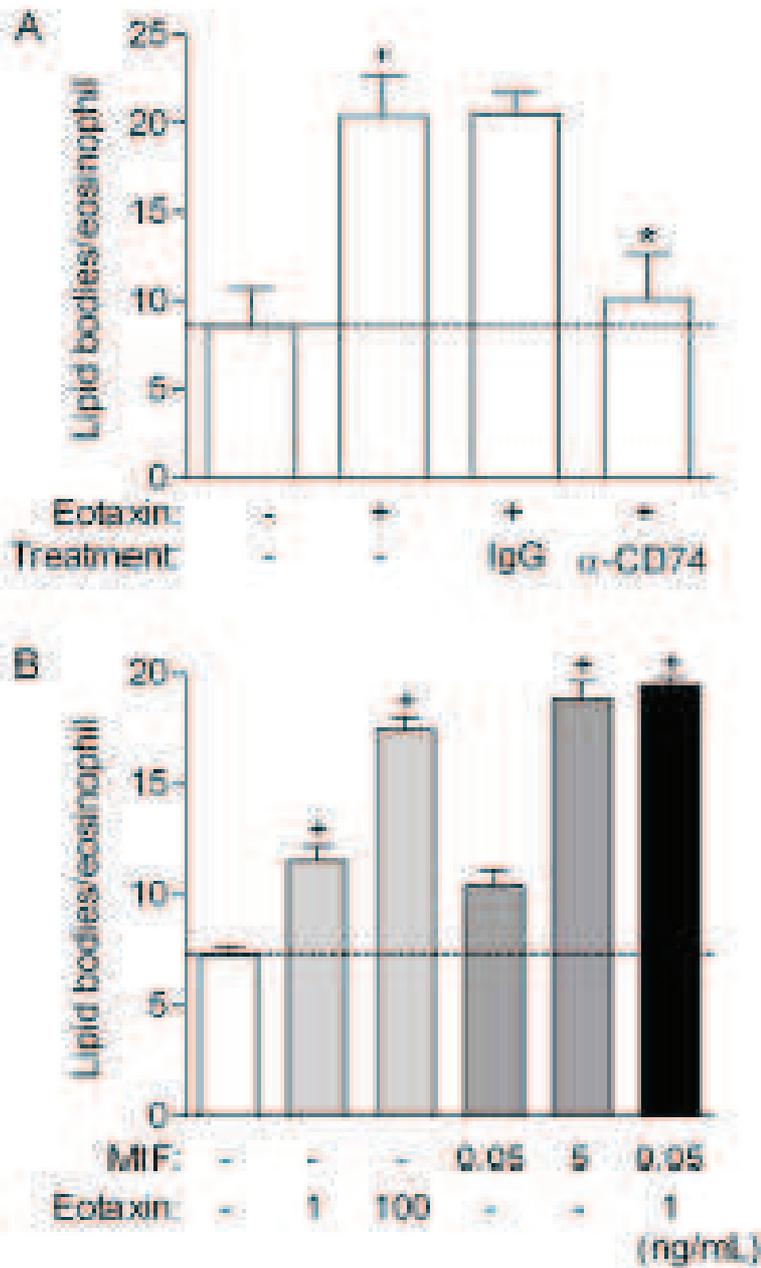
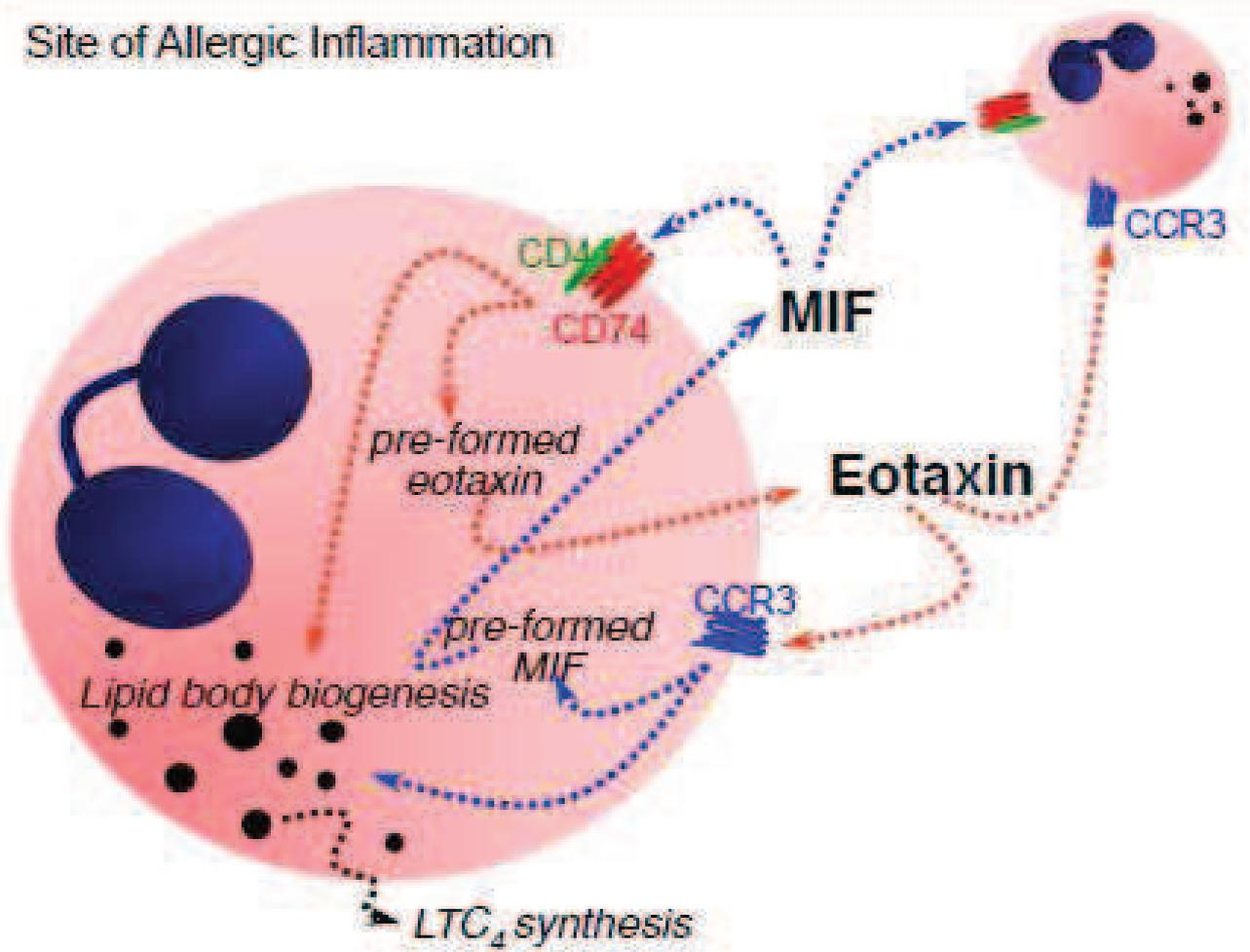


FIGURE 7  
120x190mm (300 x 300 DPI)

Site of Allergic Inflammation





type and activating status of cell [for review see [21]]. Characterization of lipid bodies in different cell types has shown that they are particularly active sites for metabolism of arachidonic lipids [22–25], while Yu et al. [25] demonstrated the compartmentalization of PLA<sub>2</sub> at arachidonic enriched-lipid body. Lipid bodies are also inducible organelles. Rather than a simple co-fatty acid esterification phenomenon, studies investigating the mechanisms of lipid body biogenesis demonstrated that new lipid bodies are assembled in ER membranes by a complex biogenic process that is rapid, but highly regulated, and stimulus- and cell-specific [21].

Lipid body biogenesis and lipid/protein compartmentalization appear to be mutually influenced. Based on that, it has been speculated that for a specific cell type, distinct lipid body biogenic events may determine lipid body composition, and therefore different functional properties. It has been reported that a variety of epithelial cell types display lipid bodies within their cytoplasm [26,27]. Here, we investigated the molecular mechanisms involved in triggering lipid body assembly within intestinal epithelial cells and their functions in cPLA<sub>2</sub>-driven AA-mobilizing activity and cytokine and PGE<sub>2</sub> synthesis.

## 2. Methods

### 2.1. Reagents

Arachidonic acid (AA), Oleic acid (OA), non-esterifiable arachidonic acid analogs, methyl arachidonic trifluoroethyl ketone (ATR), and Prostaglandin (PGE<sub>2</sub>) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Phorbol activating factor (PAF), Palmitic acid (PA), Stearic acid (SA), LPS, Phorbol 12-myristate 13-acetate (PMA), and PMA were purchased from Sigma Chemical Co. (St. Louis, MO), all lipids were diluted in absolute ethanol (Merk) with exception of PAF that was diluted in PBS/BSA 0.1%, same diluent of D-15 (RAD systems, Minneapolis, MN). The inhibitors for COX-2, NO-394, and COX-1, valeryl salicylate were purchased from Cayman. MAP kinase inhibitor SB203580 (p38), SP60125 (JNK), U0126 (MEK1/2) were all from BIOMOL (Plymouth Meeting, PA) and diluted in DMSO (Sigma). cPLA<sub>2</sub>-α selective inhibitor, pyrenyl-2-(N-(2S,4R)-4-(8-oxo-5-ylideneoctyl)-2-ylmethyl-oxobutyl-amine)-1-(2-(2,4-difluorobenzoyl)-4-oxo-1-phenyl-3-ylmethyl)-3-(4-(2,4-difluorobenzoyl)-5-ylideneoctyl)-phenyl)acrylamide, HD1, was purchased from Calbiochem (San Diego, CA), [5633,31,32,34,35-<sup>3</sup>H] Arachidonic Acid with specific activity of 214 Ci/mole was obtained from American Biochemical (Buckinghamshire, England). Monoclonal antibodies for immunoblot assays and immunofluorescence studies were mouse IgG<sub>2b</sub> anti-cPLA<sub>2</sub>-α (clone 4-48-3C) at 1 μg/ml, and mouse IgG<sub>1</sub> anti-GAPDH (clone R69) at 3 ng/ml, both from Santa Cruz Biotechnology (Santa Cruz, CA). Guinea Pig polyclonal anti-ADP from BSA (Flanders, NJ) was used at 1:1000 dilution. Rabbit polyclonal antibody against phosphorylated p38 (pTyr 180/182) was purchased from Biomol and used at 1:2000 dilution. Secondary antibodies were goat polyclonal against mouse IgG (Santa Cruz) or rabbit IgG, and donkey anti-goat anti IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The goat HRP-linked secondary antibodies were anti-mouse IgG (Santa Cruz) diluted at 1:4000, and anti-rabbit IgG from Jackson ImmunoResearch Laboratories diluted at 1:10,000. BodyP<sup>®</sup>-493509 was from Molecular Probes.

### 2.2. Cell culture and treatments

IEC-6 [28], a non-transformed rat intestinal epithelial-derived cell line (Rio de Janeiro Cell Bank, Brazil) was maintained in Dulbecco Modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS, Gibco, Brazil), 100 U/ml penicillin, and 100 μg/ml streptomycin (all cell culture reagents from Invitrogen, Carlsbad, CA) in culture flasks (TPP, Switzerland). Cells were cultured by 0.25% trypsin and 0.38 g/L EDTA in HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> and 1 × 10<sup>6</sup> cells/well were plated over glass coverslips in 24-well plates (TPP) for

lipid body enumeration, and 2 × 10<sup>6</sup> cells/well in 6-well plates for all other experiments. Cells were grown in supplemented DMEM at 37 °C in a 5% CO<sub>2</sub> atmosphere and all experiments were performed after cultures reached confluency (2–3 days after plating). All pharmacological inhibitors were added after culture medium was changed and 30 min before stimulation. Cells were stimulated for different periods with PAF (1 μM), D-15 (10 ng/ml), PMA (10 nM), LPS (1 μg/ml), AA (1–30 μM), OA (10 μM), PGE<sub>2</sub> (10 μM), PA (10 μM), SA (10 μM), in the presence of inhibitor or vehicle. In each experiment, DMSO or ethanol was always below 0.1% and did not modify cell adhesion when compared to untreated cells. The cell viability, determined by Trypan blue dye exclusion at the end of each experiment, was >95% for all stimulatory or treatment conditions.

### 2.3. Lipid body staining and enumeration

Analysis of lipid body numbers was performed in osmium-stained cells. Briefly, whole cell mass, confluent IEC or IEC6 cells were fixed in 1.7% formaldehyde in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS pH 7.4, rinsed in 0.1 M cacodylate buffer, incubated with 1.5% OsO<sub>4</sub> (30 min), rinsed in dH<sub>2</sub>O, immersed in 1.0% thiocarbonylsulfide (5 min), rinsed in 0.1 M cacodylate buffer, treated in 1.5% OsO<sub>4</sub> (3 min), rinsed in dH<sub>2</sub>O, and then dried and mounted. The morphology of fixed cells was observed and lipid bodies were measured by light microscopy with a 100× objective lens in 50 consecutively scanned cells.

Alternatively, the measurement of the area of lipid bodies used as a different approach for lipid body quantification, was performed by staining IEC-6 cells with BODYP<sup>®</sup>-493509 (4,4'-difluoro-1,3,5,8-tetraacetyl-4-chloro-2,4-diaminobenzene). BODYP staining shows the accumulation of neutral lipids in lipid bodies. Images were obtained using an Olympus BX-FLA fluorescence microscope equipped with a Plan Apo 60× objective (Olympus Optical Co., Japan) and CoolSNAP-Pro CF digital camera in conjunction with Image-Pro Plus version 4.5.13 software (Media Cybernetics, San Diego, CA). The images (at least five fields per slide) were transformed into black and white pictures and analyzed with Image 2D (GE Healthcare). The BODYP-labeled cytoplasmic spots (lipid bodies) were determined by automatic spot detection, and the total area of fluorescent spots was obtained for each field and divided by the number of cells in the respective field. Values were expressed as lipid body fluorescent area/cell.

### 2.4. Arachidonic acid release

Determination of AA release was performed as previously described [20] with some modifications. Briefly, IEC-6 cells were collected and plated at 2 × 10<sup>6</sup> cells/well in 6-well tissue culture plates in 2 ml of supplemented DMEM as described above. After 2 days, cells were labeled for 18 h with 1 μCi/well of [<sup>3</sup>H]-AA in 2 ml of supplemented DMEM. After labeling, IEC-6 cells were washed twice with PBS and IEC-6 cells in fresh medium were stimulated with OA (10–30 μM) for 4 h for induction of lipid bodies. Radiation released during stimulation with OA was measured as described below and found to be numerically different from what was released by cells incubated with medium alone (not shown). After cell culture medium was replaced, lipid body-bearing IEC-6 cells were then challenged for 1 h with 5 μM A23187 for sub-optimal stimulation of AA release. Culture supernatants were then harvested and centrifuged at 300g for 10 min, while cells were washed once with PBS and lysed in 2 ml of 0.1% Triton X (Sigma). Samples were stored at -20 °C until 0.2 ml of sample were mixed with 1.8 ml of Ultima Gold scintillation cocktail (Perkin Elmer, Shelton, CT) and then were analyzed in a 156010 IL scintillation counter (Beckman, Fullerton, CA). The percentage of arachidonic acid released for each condition was calculated by separate and subtractive counts divided by total radioactivity (supernatant plus cellular lysate) counts multiplied by 100.

## 2.5. Immunoblot analysis

Cells were collected after stimulation by cell scraper (CORNING) and addition of 120  $\mu$ l of sample buffer (1% Triton X, 0.5% sodium dodecyl sulfate, 0.2% SDS, 150 mM NaCl, 10 mM HEPES, 2 mM EDTA, 2 mM sodium orthovanadate, 20 mM NaF, 5  $\mu$ g/ml Peptatin, 10  $\mu$ g/ml Leupeptin, 1  $\mu$ g/ml Aprotinin, 1 mM PMSF), 40  $\mu$ l of loading buffer (20%  $\beta$ -mercaptoethanol, 170 mM Tris base, 150  $\mu$ M bromophenol blue, 6% glycerol, 0.5% SDS, pH 6.8) was added to the cell lysate. The cellular lysates were immediately heated at 100  $^{\circ}$ C for 5 min. Alternatively,  $10 \times 10^6$  cells were disrupted by nitrogen cavitation and the nuclei-free homogenates were separated by sucrose gradient as described before [27]. The top 3 fractions containing the lipid bodies and proven to be free of cytosol contamination by lack of lactate dehydrogenase activity (Cytosol 96 Kit, Progenia, Madison, WI) were used for precipitation and resuspension in 30  $\mu$ l of 1:1 sample buffer to loading buffer (5  $\mu$ l of samples were used for electrophoresis on a SDS-PAGE 10% polyacrylamide gel at 20 mA/gel for 1 h. After electrophoresis, separated proteins were transferred to nitrocellulose membranes (Sera Cruz) at 250 mA for 2 h at 4  $^{\circ}$ C. Membranes were blocked with TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4) and 5% of non-fat dry milk for 2 h at room temperature. Membranes were washed 5 $\times$  with TBST and incubated with primary antibodies (above) diluted in TBST for 16 h at 4  $^{\circ}$ C under gentle agitation. After washing 5 $\times$  with TBST membranes were incubated with secondary antibodies for 1 h at room temperature. ECL Western Eotting chemiluminescent substrate (Amersham) was added after membranes were washed as before and bands were visualized after exposure of X-ray film autoradiography film (Kodak).

## 2.6. dFA, immunofluorescence

Stimulated and non-stimulated confluent cultures of EC-6 cells on glass coverslips were fixed in 3.7% formaldehyde for 10 min, permeabilized with 0.2% Triton X-100/PBS solution (5 min). After washing, slides were incubated for 1 h with 40  $\mu$ g/ml mouse IgG<sub>1</sub> anti-rPLA<sub>2</sub>- $\alpha$  monoclonal antibody (Sera Cruz) diluted in 0.2% Triton X-100/PBS solution. Same concentration of irrelevant mouse IgG<sub>1</sub> was used as control. After three washes of 5 min, the preparations were incubated 1 h with 1:1000 dilution of Cy3-labeled donkey anti-mouse IgG secondary antibody. After washing with PBS for 10 min (3 $\times$ ), BODIPY (4  $\mu$ g/ml) was added for 5 min to label cytoplasmic lipid bodies within EC-6 cells. Coverslips were then washed with HBSS and an aqueous mounting medium (Polymount, Warrington, PA) was applied to each slide before coverslip attachment. Images were obtained and analyzed as described above, but using a 100 $\times$  objective. Images were edited using Adobe Photoshop CS software (Adobe Systems, San Jose, CA).

## 2.7. Immunolocalization of PGE<sub>2</sub> at its site of production

Immunolocalization of PGE<sub>2</sub> was performed as previously described [28]. In brief, stimulated and non-stimulated confluent cultures of EC-6 cells on glass coverslips were fixed and permeabilized during 30 min at 37  $^{\circ}$ C with water-soluble 1-(3-dimethylamino-propyl) carbodiimide (EDAC, 0.5% in HBSS). Then, cells were washed with HBSS and blocked with 1% normal donkey serum for 30 min before incubation with anti-PGE<sub>2</sub> mAb (Cayman Chemical) plus gamma pig polyvinyl anti-ADHP (1:1000) in 0.1% normal donkey serum for 1 h at room temperature. Cells were washed twice and incubated simultaneously with secondary Ab, Cy3-conjugated donkey anti-mouse and Cy2-conjugated donkey anti-gamma pig (1:1000) for 1 h at room temperature. The cells were washed with HBSS, then mounted in an aqueous mounting medium, and visualized with 100 $\times$  objective by fluorescence microscopy. The specificity of the PGE<sub>2</sub> immune-labeling was ascertained by: (i) the

incubation (1 h) non-before-EDAC with the EDAC inhibitor hydroxybenzotriazole (1  $\mu$ g/ml), (ii) 1 h treatment (before EDAC) with rPLA<sub>2</sub>- $\alpha$  inhibitor pyrenilone-3 (1  $\mu$ M) to avoid the synthesis of PGE<sub>2</sub>, and (iii) the use of an irrelevant antibody control.

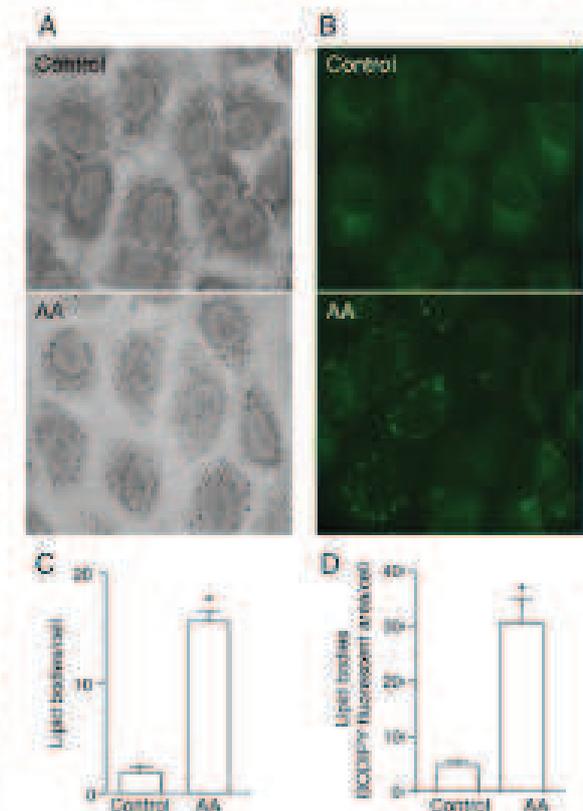
## 2.8. Statistical analysis

Data are expressed as mean  $\pm$  standard error of the mean (SEM) of at least three independent experiments. Multiple comparisons among groups were performed by one-way ANOVA followed by Bonferroni's or Dunnett's test. \* and \*\* represent  $P$  values <0.05 when compared to control non-stimulated group or stimulated group respectively and were considered statistically significant.

## 3. Results

### 3.1. Epithelial cells assemble new lipid bodies under AA stimulation

In order to investigate whether epithelial cells can mount an inducible lipid body biogenic process, we have directly stimulated *in vitro* a non-transformed rat intestinal epithelial cell line (EC-6 cells) with acute lipid body-relevant stimuli [21]. As illustrated in Fig. 1A (top panel), confluent EC-6 cells stained with cerium showed a nonfluorescent cytoplasm with virtually no assembled lipid body. In con-



**Fig. 1.** Lipid body biogenesis is triggered by AA within EC-6 cells. Microscopy images obtained from non-stimulated or AA-stimulated EC-6 cells were counterstained with cerium (A) or labeled with BODIPY (B). (C) New lipid body content after stimulation (4 h) with AA (5  $\mu$ M) or AA (10  $\mu$ M) lipid body were measured including control staining. Results were expressed as mean  $\pm$  SEM from at least three experiments. In (C) BODIPY-labeled lipid bodies were quantified by the measurement of the area of fluorescence per cell. Data on the graph correspond to the mean  $\pm$  SEM from at least three experiments.

contact, confluent EC-6 cells stimulated with AA (10  $\mu$ M) for 4 h, showed a cytoplasm packed with omegaphilic organelles (seen as dark punctate structures), named lipid bodies (Fig. 1A, bottom panel). Fig. 1B shows a different technical approach to visualize and quantify lipid body biogenesis. Similar to contact-seeded cells, 800PS-labeled non-stimulated EC-6 cells showed a clear cytoplasm, while cells stimulated with AA (10  $\mu$ M) showed an increased number of cytoplasmic lipid bodies that appeared as green fluorescent dots (Fig. 1B).

Quantitation of omegase-4-labeled dark inclusions within confluent EC-6 cells showed that cells stimulated with AA (10  $\mu$ M) contain at least 15 times more lipid bodies than non-stimulated cells (Fig. 1C). Densitometric image analysis of 800PS-labeled cells, as a measure of lipid body area as a measure of vesicular lipid accumulation per cell, confirmed the stimulatory effect of AA in inducing lipid body biogenesis within EC-6 cells (Fig. 1D). These findings indicate that epithelial cells can respond with lipid body biogenesis, leading to an increased content of these organelles within their cytoplasm. AA (1 to 30  $\mu$ M)-induced increase in the numbers of cytoplasmic lipid bodies within confluent EC-6 cells was a dose-dependent (Fig. 2A) and lipid peroxidation, which was apparent within 1 h, significant within 2 h and maximum within 4 h at the concentration of 10  $\mu$ M (Fig. 2B). From 8 to 24 h of AA stimulation, number of newly formed lipid bodies declined (Fig. 2B), a phenomenon that did not reflect cytotoxicity inasmuch as cells remained viable (data not shown).

To further identify potential stimulatory signals of epithelial lipid body biogenic process with roles in epithelial inflammatory pathologies, we have also tested the ability of other inflammatory signals, which are known agonists of lipid body biogenesis in leukocytes and relevant to epithelial functions [21], to induce assembly of new lipid bodies within EC-6 cells. As shown in Table 1, stimulation of confluent EC-6 cells for 4 h by a variety of epithelial cell agonists, including IL-1 (10 ng/ml), PMA (30 nM), TNF (1,000 ng/ml) or PAF

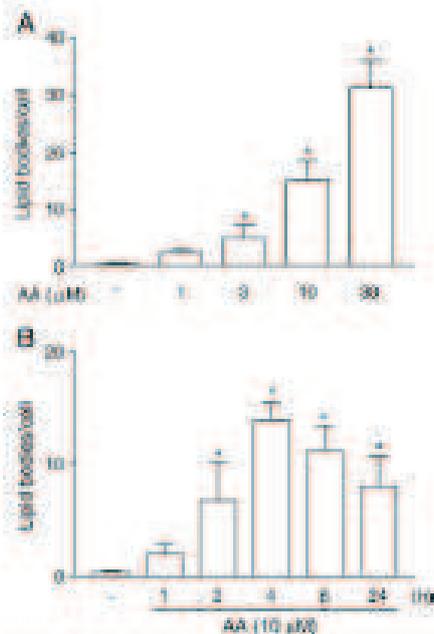


Fig. 2. AA-induced lipid body assembly is a rapid and dose-dependent phenomenon. In (A) dose-response curve of lipid body biogenesis up to 4 h after stimulation with AA (1–30  $\mu$ M). (B) Time course of lipid body formation after stimulation with AA (10  $\mu$ M). Lipid bodies were visualized using omegaphilic labeling. Results were expressed as mean  $\pm$ SD from at least three experiments.

Table 1. Arachidonic acid, tumor necrosis factor- $\alpha$  (TNF), interleukin-1 (IL-1) and platelet activating factor (PAF) induced lipid body biogenesis within EC-6 cells.

Condition	Concentration	Lipid bodies/cell
Control	-	1.7 $\pm$ 0.4
Arachidonic acid	10 $\mu$ M	12.1 $\pm$ 0.8*
IL-1	10 ng/ml	2.5 $\pm$ 0.2
IL-6	1 ng/ml	3.0 $\pm$ 0.5
PMA	30 nM	2.9 $\pm$ 0.7
PAF	1 $\mu$ M	2.0 $\pm$ 0.5

Confluent EC-6 cells were stimulated with AA, IL-1, TNF, PMA or PAF (100 nM) for 4 h (control as indicated). Analysis of lipid body formation was performed 4 h after incubation in contact-seeded cells. Results were expressed as the mean  $\pm$ SD from at least three different experiments.

\* Results are compared to control group.

(1  $\mu$ M) failed to trigger lipid body formation within EC-6 cells, indicating that assembly of new lipid bodies is a cell- and stimulus-specific phenomenon.

**3.2. Molecular mechanisms involved in unstimulated fatty acid-driven assembly of new lipid bodies depend on agonistic activity, G $\alpha$  protein-coupled receptor, and p38 kinase activation**

The biogenic process of lipid bodies represents a complex cellular outcome triggered by a variety of distinct signaling pathways. Here, our attempts to characterize the molecular signals committed to AA-induced biogenesis of cytoplasmic lipid bodies within epithelial cells revealed that the exogenous AA added to EC-6 cells, does not represent a substrate for enzymatic conversion into oleoylethanol or a exclusive source of irreparable fatty acid, but also functions as a positive mediator of cell activation with G protein-coupled receptors-driven downstream signaling through PI3-kinase, PKC and p38.

First, we verified that lipid body formation induced by AA – a unsaturated fatty acid – did not appear to depend entirely on direct incorporation of fatty acids into nascent lipid bodies, but on a complementary specific agonistic activity that depend on fatty acid structural characteristics, since (i) stimulation with either palmitic acid (PA; 10  $\mu$ M) or stearic acid (SA; 10  $\mu$ M) – two saturated fatty acids – did not trigger lipid body assembly within EC-6 cells (Table 2), even though they are available for esterification into newly-assembling lipid bodies [24], (ii) EC-6 cells stimulated with another unsaturated fatty acid (oleic acid; OA; 10  $\mu$ M) showed increased number of cytoplasmic lipid bodies (Table 2), and (iii) ATN (10  $\mu$ M) – a non-esterifiable AA analogue – did induce lipid body biogenesis within confluent EC-6 cells (not shown).

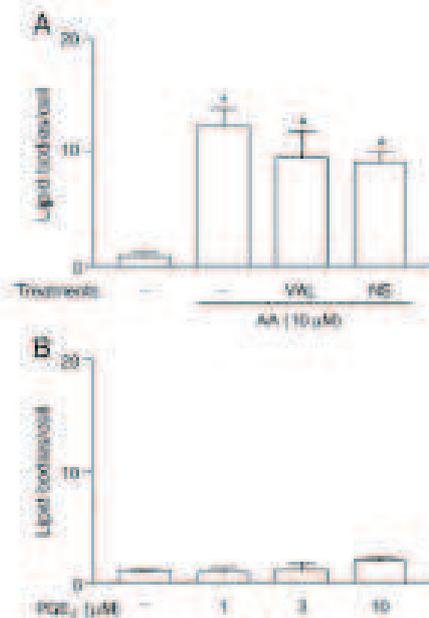
Second, the hypothesis that endogenous PGE<sub>2</sub> synthesized from stimulatory AA was mediating AA-induced lipid body assembly within EC-6 cells was ruled out since (i) pre-treatment with specific inhibitors of either COX-1 (cyclooxygenase; 10  $\mu$ M) or COX-2 (NS-398; 1  $\mu$ M) failed to affect AA-induced lipid body biogenesis within

Table 2. Unsaturated fatty acids (AA and OA), saturated fatty acids (PA and SA), and triglyceride (ATN) induced lipid body biogenesis within EC-6 cells.

Condition	Concentration	Lipid bodies/cell
Control	-	0.7 $\pm$ 0.2
Arachidonic acid	Yes	1.1 $\pm$ 0.3*
Oleic acid	Yes	9.1 $\pm$ 0.8*
Palmitic acid	No	0.7 $\pm$ 0.2
Stearic acid	No	0.4 $\pm$ 0.3

Confluent EC-6 cells were stimulated with AA, OA, PA or SA (10  $\mu$ M). Analysis of lipid body formation was performed 4 h after incubation with fatty acids in contact-seeded cells. Results were expressed as the mean  $\pm$ SD from at least three different experiments.

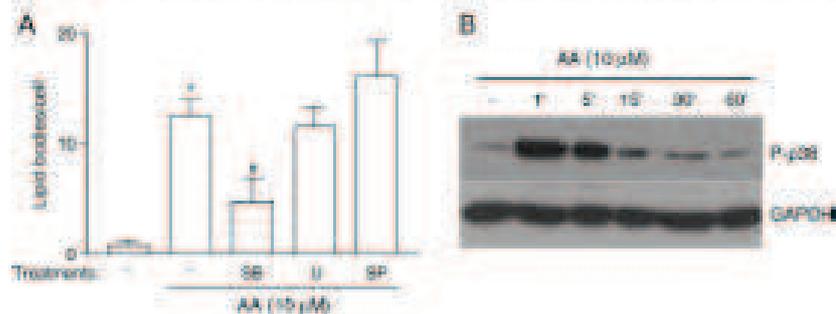
\* Results are compared to control group.



**Fig. 3.** AA-induced PGE<sub>2</sub> does not reverse AA-induced lipid body biogenesis. (A) EC-6 cells were pre-treated with anti-cytP inhibitors (0 μM) or SB-203 (1 μM) 30 min before stimulation with AA (10 μM). It shows that different concentrations of exogenous PGE<sub>2</sub> (1–10 μM) were unable to trigger lipid body formation within 4 h. Lipid bodies were visualized using confocal imaging. Results were reported as the mean ± S.D. from at least three experiments.

EC-6 cells (Fig. 3A), and (iv) stimulation with exogenous PGE<sub>2</sub> (1 to 10 μM) was unable to limit or dysregulate numbers of lipid bodies within EC-6 cells (Fig. 3B).

First, AA stimulation initiates a rapid but specific intracellular signaling that leads to lipid body biogenesis, since: (i) AA-induced lipid body biogenesis within EC-6 cells was significantly inhibited by the pre-treatment with a specific MAP kinase p38 inhibitor (SB20325) (Fig. 4A); (ii) such p38-dependent lipid body biogenesis was preceded by an acute (apparent within 1 min) and transient phosphorylation of p38 detected after stimulation with AA (10 μM) (Fig. 4B); (iii) two other MAP kinases, ERK1/2 and JNK, are not involved in AA-induced lipid body assembly, since inhibitors (U0126 and SP20199, respectively) did not alter AA-induced lipid body formation; and (iv) while inhibitors of tyrosine kinases (genistein)



**Fig. 4.** MAP kinase p38 activates AA-induced lipid body formation in EC-6 cells. (A) EC-6 cells, stimulated *in vitro* with AA (10 μM) were pre-treated with inhibitors SB20325 (1 μM), U0126 (10 μM) or SP20199 (20 μM) of, respectively, three distinct MAP kinases, p38, ERK1/2 and JNK. The experiments were performed at least in duplicate using confocal. Analysis of lipid body formation was performed 4 h after *in vitro* stimulation. Results were reported as the mean ± S.D. from at least three experiments. \* *P* < 0.05 compared to control groups. \*\* *P* < 0.05 compared to AA-stimulated cells. (B) A kinetic of AA-induced phosphorylation of p38 is shown. Total EC-6 cell lysates (2 × 10<sup>6</sup> cells/lane) were separated by SDS-PAGE and subjected to Western blotting for phosphorylated p38 or GAPDH as indicated. The image is representative of a typical assay of three trials.

**Fig. 5.** Effect of PD kinase and PKC on AA-induced lipid body biogenesis within EC-6 cells.

Condition	Treatment	Lipid body/cell
Control	-	0.0 ± 0.0
AA-stimulated	-	1.12 ± 0.09
	+ LY294002	0.0 ± 0.0*
	+ Calphostin C	0.5 ± 0.0*

Control EC-6 cells were pre-treated for 30 min with PD kinase or PKC inhibitors, LY294002 (50 μM) or Calphostin C (1 μM), and stimulated with AA (10 μM). Analysis of lipid body formation was performed 4 h after AA stimulation in control-stimulated cells. Results were reported as the mean ± S.D. from at least three different experiments. \* *P* < 0.05 compared to control group. \*\* *P* < 0.05 compared to AA-stimulated cells.

(Table 1) did not alter AA-induced rapid lipid body biogenesis, inhibitors of PD kinase (LY294002) and PKC (calphostin C) blocked formation of lipid bodies induced by AA within EC-6 cells (Table 3). Of note, all pharmacological inhibitors of kinases used (LY294002, calphostin C, genistein, U0126 and SB20325) did not alter the cytoplasmic local numbers of lipid bodies found in non-stimulated EC-6 cells (not shown).

Fourth, as shown in Table 4 the intracellular signaling controlling AA-induced lipid body biogenesis was similar to that involved in OA-induced lipid body assembly. While ERK1/2- and JNK-activated pathways were not involved in OA stimulation, since their specific inhibitors did not modify OA-induced lipid body formation, p38 activation appeared as a key step of OA-induced lipid body biogenesis in EC-6 cells, inasmuch as SB20325 blocked assembly of new lipid bodies within OA-stimulated EC-6 cells. Of note, also like AA, OA was able to trigger p38 phosphorylation (not shown).

Finally, by pre-treating EC-6 cells with PDK (10 μg/ml; 30 min before stimulation), a classical inhibitor of receptor activation, we observed a significant reduction of epithelial lipid body numbers found within the cytoplasm of either AA- and OA-stimulated cells (Table 5), indicating that OA may be acting on G-protein-coupled receptors expressed on EC-6 cells surface. Therefore, rapid receptor-activated and specific intracellular signaling events, comprising p38 activation, are involved in lipid body biogenesis in unstimulated fatty acid-stimulated EC-6 cells.

### 3.3. Newly formed lipid bodies function as intracellular platforms of p38-driven mobilization of endogenous AA within epithelial cells

The potential functions of the newly assembled lipid bodies within epithelial cells were then investigated. It is well established that at least three distinct intracellular domains may compartmentalize the molecular organization for vicinal synthesis, the nuclear membrane [9–10], phagosomes [10] and lipid bodies [20,31–35]. On newly

**Table 1**  
Role of AMPK on OA-induced lipid body biogenesis within EC-6 cells

Condition	Treatment	Lipid body <sup>a</sup>
Control	-	1.00 ± 0.0
OA-stim.	-	10.6 ± 1.2 <sup>b</sup>
	+ GSK320368	2.00 ± 0.7 <sup>c</sup>
	+ OA 20	8.0 ± 0.5
	+ GSK320368	0.5 ± 0.0

Condition EC-6 cells were pre-treated for 30 min with GSK320368 (2 μM), OA 20 (20 μM) or GSK320368 (20 μM) of respectively, followed with AMP kinase, p38, ERK 1/2 and PKC and stimulated with OA (10 μM). Analysis of lipid body formation was performed 4 h after OA-stimulation in control-treated cells. Results were expressed as the mean ± SEM from at least three different experiments.

<sup>a</sup> Data are expressed as control group.

<sup>b</sup> P < 0.05 compared to OA-stimulated cells.

formed lipid bodies have functional roles in AA metabolism within epithelial cells? We hypothesized that, in unsaturated fatty acid-stimulated epithelial cells, even the first step of enzymatic synthesis takes place within newly formed lipid bodies. Therefore, we evaluated stimulatory capability of exogenous unsaturated fatty acids to control the expression, location and function of AA-mobilizing enzyme cPLA<sub>2</sub>-α within EC-6 cells. Stimulation with either AA (not shown) or OA (Fig. 5B) was unable to increase cPLA<sub>2</sub>-α expression in EC-6 cells. Although stimulation with lipid body-triggering unsaturated fatty acids did not alter total protein content of cPLA<sub>2</sub>-α in EC-6 cells, AA (not shown) or OA (Fig. 5B and C) triggered the translocation of cPLA<sub>2</sub>-α from the cytosol to the newly formed lipid bodies. As shown in Fig. 5B, while major fractions of non-stimulated EC-6 cells did not contain cPLA<sub>2</sub>-α, lipid body-enriched fractions of OA-stimulated cells showed compartmentalized cPLA<sub>2</sub>-α. Fig. 5C (top panel) shows that in non-stimulated EC-6 cells immunofluorescent cPLA<sub>2</sub> was found homogeneously distributed in the cytoplasm that colocalized to BODIPY-labeled lipid bodies. In contrast, in OA-stimulated (10 μM) EC-6 cells, which were packed with newly formed lipid bodies (Fig. 5A), part of cPLA<sub>2</sub>-α immunofluorescence staining shifted from EC-6 cytoplasm to a clear punctate cytoplasmic pattern, proximate to, but separate from the nucleus, and fully co-localized in size and form with lipid bodies of EC-6 cells (Fig. 5C, bottom panel). Lipid body compartmentalization of translocated cPLA<sub>2</sub>-α within OA-stimulated EC-6 cells was accentuated by the co-localization with BODIPY (Fig. 5C, middle panel). Differently from the lipid punctate immune-staining of BODIPY, immunofluorescent cPLA<sub>2</sub>-α was found as an internal ring-like immune-staining, placing cPLA<sub>2</sub>-α as a surface lipid body protein. Addition of A23187 (calcium ionophore, 5 μM) for 10 min to cells previously exposed to unsaturated fatty acids, when cPLA<sub>2</sub>-α is already lipid body-associated, did not change such localization of cPLA<sub>2</sub>-α, i.e. it remained associated with lipid bodies (data not shown). Differently, in resting EC-6 cells (not exposed to exogenous fatty acids) that contain virtually no lipid bodies, activation by A23187 (5 μM; 10 min) induced a partial translocation of cPLA<sub>2</sub>-α from the cytoplasm to the perinuclear envelope of cells (Supplemental Fig. 1A). Of note, stimulation of EC-6 cells for 10 min (not shown) or even for 4 h with A23187 (5 μM) does not trigger lipid body biogenesis in EC-6 cells (Supplemental Fig. 1B). No immunoreactivity was detected when a monoclonal anti-cyclo-G<sub>q</sub> was used as a control in the anti-cPLA<sub>2</sub> antibody in OA-stimulated cells, although the BODIPY-labeled lipid bodies were strongly visualized (not shown).

Finally, we hypothesized that if unsaturated fatty acid-driven newly formed lipid bodies of epithelial cells are indeed involved in AA metabolism, increased numbers of lipid bodies should correlate with higher levels of free AA. Indeed, Fig. 5D shows that, under proper stimulation, EC-6 cells bearing increased numbers of cytoplasmic lipid bodies showed enhanced AA mobilization. Of note, to avoid potential confounding effects of analyzing AA release in AA-stimulated cells, in this set of experiments, only OA was employed as

stimulus. Stimulation for 4 h with different concentrations of OA (10–100 μM) caused a dose-dependent increase of lipid body numbers (Fig. 5D, left panel) within cytoplasm of EC-6 cells with a stimulatory potency similar to AA (Fig. 2A). While, in parallel to such increased number of newly assembled lipid bodies (4 h OA per se was unable to trigger AA release, not shown), OA did prime EC-6 cells in a concentration-dependent manner for an enhanced AA mobilization triggered by subsequent stimulation with sub-optimal concentration of A23187 (Fig. 5D, right panel), indicating a role for cPLA<sub>2</sub>-α-bearing lipid bodies in AA mobilization.

**3.4. OA-induced epithelial lipid bodies compartmentalize cPLA<sub>2</sub>-α-dependent PGE<sub>2</sub> synthesis**

As illustrated in Fig. 6, we identified newly formed lipid bodies as the cytoplasmic organelles compartmentalizing PGE<sub>2</sub> synthesis in OA-stimulated EC-6 cells by using a more sensitive methodology that cross-links and directly immunolabels concentrated micro-analytes at its site of synthesis [31], even though, levels of PGE<sub>2</sub> in supernatants of AA- or OA-stimulated EC-6 cells were not detectable by OA (data not shown). A detailed analysis revealed that virtually all immunofluorescent newly synthesized PGE<sub>2</sub> was in a punctate cytoplasmic pattern, proximate to, but separate from the nucleus, and fully co-localized in size and form with epithelial cell lipid bodies. Specific compartmentalization of newly formed PGE<sub>2</sub> in EC-6 cells lipid bodies was accentuated by the co-localization with ADRP, a protein marker of lipid bodies (Fig. 6). Virtually no PGE<sub>2</sub> immunolabeling (Fig. 6, bottom panel) was observed within EC-6 cells treated with pyrrolidine-2—a specific inhibitor of cPLA<sub>2</sub>-α activity, evidencing the role of this phospholipase in OA-induced lipid body-localized PGE<sub>2</sub> synthesis. It is noteworthy that pyrrolidine-2 (10 or 1 μM) did not inhibit AA- or OA-induced lipid body formation (as assessed in untreated cells, data not shown), indicating that cPLA<sub>2</sub>-α is involved on lipid body function (e.g. PGE<sub>2</sub> synthesis) but not on lipid body biogenesis. The specificity of the PGE<sub>2</sub> immune-labeling was accentuated by the lack of PGE<sub>2</sub> immune-labeling within: (i) non-stimulated OA-stimulated EC-6 cells (Fig. 6, middle panel); (ii) non-stimulated EC-6 cells (not shown); or (iii) OA-stimulated EC-6 cells stained with non-immune IgG (not shown). Therefore, newly formed lipid bodies of OA-stimulated EC-6 cells are inducible and enzymatically skilled organelles for effective PGE<sub>2</sub> synthesis, which depends on the free AA mobilized by the functionally active cPLA<sub>2</sub>-α expressed on lipid bodies.

**4. Discussion**

Lipid body biogenesis is a critical cellular event in whole body lipid metabolic homeostasis, as well as, to several important human diseases [36], in chronic inflammatory pathologies, such as cancer

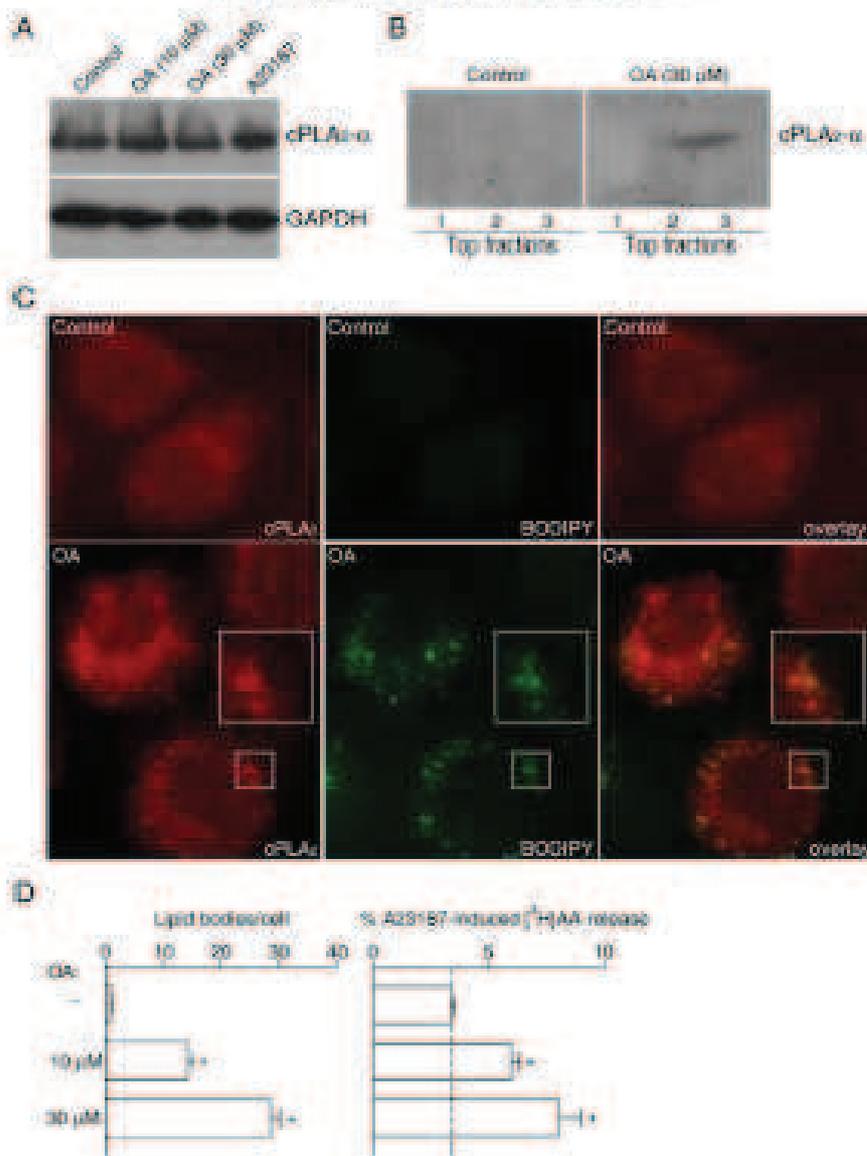
**Table 2**  
AA- and OA-driven induction of lipid body biogenesis within EC-6 cells involve activation of a PD-α-mediated G<sub>q</sub> protein-coupled receptor

Condition	Lipid body <sup>a</sup>
Control	1.00 ± 0.0
Arachidonic Acid	22.8 ± 1.8 <sup>b</sup>
Arachidonic Acid+PTX	12.6 ± 1.0 <sup>c</sup>
OA 10	15.3 ± 1.2 <sup>b</sup>
OA 10+PTX	7.5 ± 0.7 <sup>c</sup>

Condition EC-6 cells were pre-treated for 30 min with pertussis toxin (PTX, 0.1 μg/ml), and stimulated with AA (10 μM) or OA (10 μM). Analysis of lipid body formation was performed 4 h after AA- or OA-stimulation in control-treated cells. Results were expressed as the mean ± SEM from at least three different experiments.

<sup>a</sup> Data are expressed as control group.

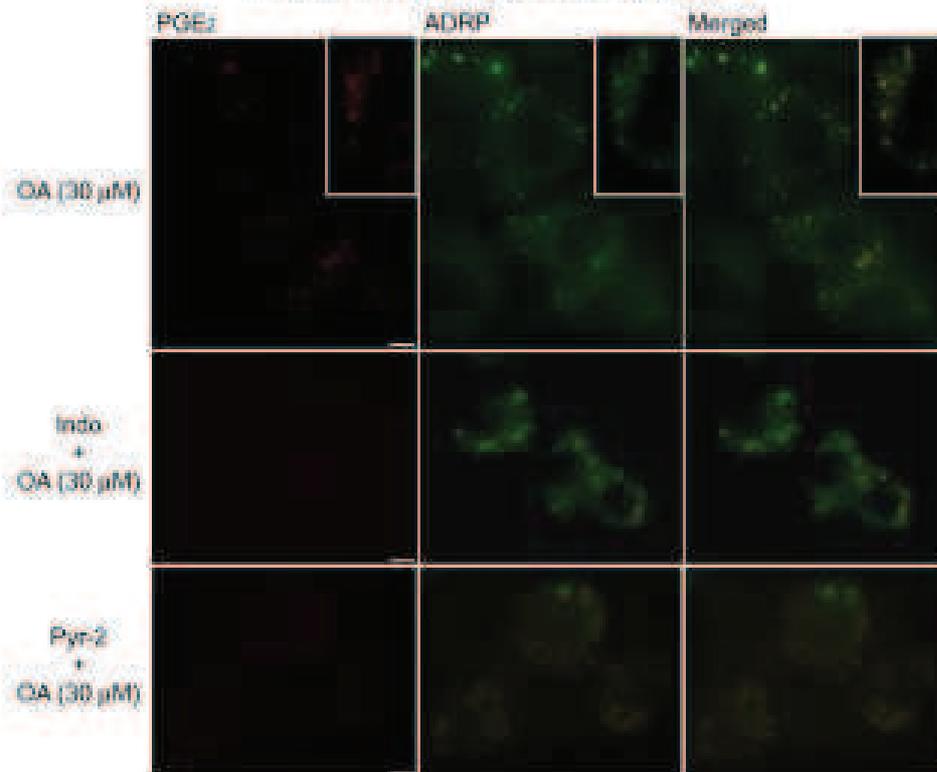
<sup>b</sup> P < 0.05 compared to AA- or OA-stimulated cells.



**Fig. 3.** OA-induced lipid bodies function as a compartment of cPLA $\alpha$ -driven AA metabolism. In (A) EC cells undergo a 4 h incubation with OA or A23187 and are then cPLA $\alpha$  expression in EC cells. Top 100–400 kDa fraction ( $>10^6$  collection) were separated by SDS-PAGE and stained in Western blotting for cPLA $\alpha$  or GAPDH as indicated. The images represent typical of four three different blots. (B) Shows that cPLA $\alpha$  is found in lipid body-enriched top fraction of OA-treated EC cells (see in using fractions of non-stimulated (control) cells. Samples fraction 1, 2 and 3 of sucrose gradients were separated by SDS-PAGE and subjected to Western blotting for cPLA $\alpha$ . In (C) non-stimulated (control) or OA-treated EC cells (10 or 30  $\mu$ M), cells were labeled with BODIPY for lipid body detection (green) and with anti-cPLA $\alpha$  (red). The original image of OA-treated cell is shown on the bottom right. Overlaid show enlarged image of cPLA $\alpha$  (red) co-localizing surrounding BODIPY-labeled lipid bodies. Images are representative of nine independent experiments. In (D) EC cells were stimulated with 0 or 30  $\mu$ M OA for 4 h to assess the new lipid bodies (shown in left panel) and measure enhanced metabolism of AA (right panel). To measure AA release, [ $^3$ H]AA was incorporated for 16 h within EC cells before stimulation with OA, both non- and OA-stimulated. [ $^3$ H]-AA-labeled EC cells were challenged with sub-optimal concentration of A23187 (2  $\mu$ M) for 1 h, and released [ $^3$ H]-AA was measured in the supernatant. Each bar represents the mean  $\pm$  SD from at least three independent experiments.

[27] and Crohn's disease[28], epithelial cells constantly show increased numbers of cytoplasmic lipid bodies. Advances on the mechanisms behind the enhanced assembly of lipid bodies within epithelium are of paramount importance for understanding the pathogenesis of epithelial diseases. Here, employing confluent cultures of normal intestinal epithelium-derived cell line – EC-6 cells – we have unveiled that the biogenic process of lipid bodies within epithelial cells happens very rapidly and in a highly regulated

fashion. Unstimulated fatty acids, AA and OA, appeared as potent biogenic stimuli of cPLA $\alpha$ -bearing PC $\alpha$ -synthesizing lipid bodies, placing epithelial lipid bodies as key organelles involved in AA metabolism and metabolism. The mechanism involved in unstimulated fatty acid-induced lipid body biogenic process appears to be independent of activation to PGE $_2$  and entirely dependent on direct incorporation of exogenous AA, but involve activation of a G-coupled receptor, as well as activation of PI3 kinase, PKC and p38.



**Fig. 6.** Cytoplasmic lipid bodies of OA-stimulated EC-6 cells compromised by dPLA-2α-dependent PGE<sub>2</sub> synthesis in top panels. anti-PGE<sub>2</sub> (red) was merged with control level of anti-ADRP (green) fluorescence images of EC-6 cells stimulated with OA (30 μM, 4 h). Inset panels show enlarged images of PGE<sub>2</sub> (red) and marker lipid bodies (as identified by anti-ADRP)-OA-stimulated EC-6 cells. In middle and bottom panels, OA-stimulated EC-6 cells were incubated for 30 min with indomethacin or pyrroloquinoline quinone after inhibition of lipid body formation by OA stimulation (30–4 h), time=0 μm.

EC-6 cells appeared as an excellent epithelial cell model for lipid body biogenic studies since, different from normal lipid storing cells, confluent EC-6 cells have virtually no lipid bodies. Notably, it has been recently described that transformed epithelial cells, as in colon cancer, present highly increased lipid body numbers through a yet not defined molecular biogenic process [27]. Concerning lipid body biogenic characteristics, it is accepted that: (i) lipid bodies are endoplasmic reticulum (ER)-derived organelles; (ii) growth of new lipid bodies can be evoked by a variety of inflammation-related stimuli; (iii) lipid bodies can be rapidly assembled, thus can be detected within an hour after stimulation; (iv) the biogenic process is cell- and stimulus-specific, and (v) specific signaling events, which may involve activation of phosphatases and/or kinases, elicit stimulus-triggered assembly of lipid bodies (for review see [2]). We verified that some, but not all, general features of lipid body biogenesis are shared by epithelial cells. For instance, among stimuli tested, including AA, OA, L-1, LPS, PMA, or TNF-α well-known biogenic stimuli of lipid bodies [3,8] – only the unsaturated fatty acids were able to trigger assembly of lipid bodies within EC-6 cells, including the concept of lipid body biogenesis as a cell- and stimuli-specific process.

It is often assumed that AA bioreactivity is attributable to its conversion into eicosanoids or other bioactive products. Although PGE<sub>2</sub>, a cyclooxygenase product of AA metabolism, has a key role on maintenance of epithelial cells homeostasis [1] and prostanoids like PGE<sub>2</sub> and PGE<sub>1</sub> are able to trigger rapid lipid body biogenesis in other cell types [33] and Barlowe-Mein, unpublished data), here we

showed that the AA capability to evoke lipid body assembly in EC-6 cells was not mediated by AA-derived cyclooxygenase products.

The notion that unsaturated fatty acids bioreactivity, in evoking lipid body biogenesis, is dependent on a direct activity on epithelial cells functioning as agonists of lipid body assembly was reinforced by a number of observations. First, both AA- and OA-induced lipid body assembly in EC-6 cells was an acute phenomenon apparent within 1 h of stimulation. Second, such rapid lipid body biogenesis elicited in AA-stimulated EC-6 cells was a concentration-dependent phenomenon, suggesting an agonistic activity for AA. Cell activation mediated by unsaturated fatty acids functioning as agonists has been observed in other cell systems, although specific receptors for saturated fatty acids were yet not fully described. Of note, although with little specificity, a variety of molecular targets to AA have been identified, including PPAR receptors and multi-component protein complex, such as NADPH oxidase and ion channels (for review see [3]). Third, AA-induced lipid body biogenesis within epithelial cells was mimicked by another unsaturated fatty acid (OA) but not by saturated fatty acids (PA and SA). The ability of AA and OA versus the inability of PA and SA to trigger lipid body biogenesis indicates that, while saturated fatty acids provide a source of lipids for incorporation into newly forming lipid bodies, the immediately restricted capacities of different fatty acids to induce lipid bodies depend on mechanisms other than simple availability of lipid precursors. Fourth, OA stimulatory effect is inhibited by PKC, indicating that fatty acid-elicited lipid body biogenesis is mediated by a Gα-coupled receptor, potentially a member of a family of

formally express GPCRs (GPC40 and GPR120) [37]. Fifth and finally, fatty acids-driven activation of EC-6 cells that culminates in concentration-dependent lipid body biogenesis depended on rapid activation of p38 MAP kinase, as well as PKA kinase and PKC. Altogether, direct bioactivity of unsaturated fatty acids on epithelial cells triggers a rapid but tightly regulated process of lipid body biogenesis comprising a specific signaling cascade.

Function of newly formed lipid bodies may vary according to cell type, specific biogenic stimulus and subsequent intracellular signaling that set off its biogenic machinery. Intracellular compartmentalization of AA metabolism has emerged as a key feature that controls a variety of inflammatory cell functions [21]. If epithelial cells lipid bodies have indeed arise in AA metabolism, then the AA present in these epithelial lipid-rich structures should be readily hydrolyzable from phospholipids by PLA<sub>2</sub>s, which should be first compartmentalized within lipid bodies. Stimulation of epithelial cells with unsaturated fatty acids, which failed to increase the expression of cPLA<sub>2</sub>- $\alpha$ , triggered the compartmentalization of cPLA<sub>2</sub>- $\alpha$  from its resting cytoplasmic location to newly assembled lipid bodies within epithelial cells. Of note, Wooten and coworkers, by using stably transfected HES-203 cells expressing a GFP-cPLA<sub>2</sub>- $\alpha$  fusion protein, recently showed that AA and other unsaturated fatty acids induced translocation of cPLA<sub>2</sub>- $\alpha$  to pre-existing lipid bodies independent on activation of PKC- $\alpha$ -coupled G-protein coupled receptors [38], which is required for lipid body formation within AA- or OA-stimulated EC-6 cells. The differences observed between our results and Wooten et al.'s may reflect different sources of the studied lipid bodies contrasting the pre-existing lipid bodies in HES-203 cells with the inducible and transient lipid bodies in stimulated EC-6 cells.

Successful AA mobilization is not merely determined by AA availability and proper localization of the relevant enzyme cPLA<sub>2</sub>- $\alpha$ , but also depends on cPLA<sub>2</sub>- $\alpha$  activation by specific down-stream signaling, such as p38 MAP kinase activity [14,29]. Co-competition between cPLA<sub>2</sub>- $\alpha$  with MAP kinases (JNK1, ERK2, p38 and p39) within lipid bodies has been described [25]. Therefore, besides their role in fatty acid-induced lipid body biogenesis within epithelial cells showed here, activated p38 MAP kinase may also modulate within the lipid body-compartment other aspects of epithelial cell physiology, like AA mobilization by regulating activity of lipid body cPLA<sub>2</sub>- $\alpha$ . Indeed, in our EC-6 cells model of epithelial cell activation, all these components of AA mobilizing molecular complex are elicited by unsaturated fatty acid stimulation, including activation of p38 and lipid body localization of cPLA<sub>2</sub>- $\alpha$ . Lipid body-translocated cPLA<sub>2</sub>- $\alpha$  appears to be enzymatically active, inasmuch as in agreement to actively placing cPLA<sub>2</sub>- $\alpha$  close to the lipid body content of esterified AA, unsaturated fatty acids-treated epithelial cells showed an enhanced AA release from cPLA<sub>2</sub>- $\alpha$ -bearing stores in response to intracellular calcium influx. Therefore, the first step of AA mobilization – mobilization of free AA from phospholipids by p38-activated cPLA<sub>2</sub>- $\alpha$  – appears to take place within newly formed lipid bodies of activated epithelial cells. Furthermore, OA-driven cPLA<sub>2</sub>- $\alpha$ -containing lipid bodies also were able to generate PGE<sub>2</sub>, reflecting that besides AA mobilizing activity, OA-induced newly formed lipid bodies are also cyclooxygenase-competent organelles in epithelial cells.

In conclusion, our results indicate that inflammationally relevant molecules, like AA and OA, control lipid body biogenesis and cPLA<sub>2</sub>- $\alpha$ -driven PGE<sub>2</sub>-synthesizing function in epithelial cells. Epithelial cells lipid body biogenesis is a highly regulated phenomenon mediated by activation of a not yet defined G-coupled receptor and p38 kinase. Based on that, we postulate lipid bodies a vital organelle involved in PGE<sub>2</sub>-regulated physiopathological processes and/or epithelial cell-driven inflammatory functions, and therefore, inhibition of lipid body biogenesis may provide a novel targeted anti-inflammatory therapy for epithelial pathologies.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bba.2019.01.003.

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