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**ALLAN BOTINHON ORLANDO**

# MECANISMO PELO QUAL PROSTAGLANDINA $E_2$ ORIUNDA DA EFEROCITOSE INIBE A EXPRESSÃO DO IL-1R E A DIFERENCIAÇÃO DE TH17

**Araraquara, SP**

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Dissertação apresentada ao Programa de Pós-Graduação em Biociências e Biotecnologia Aplicadas à Farmácia da Faculdade de Ciências Farmacêuticas da Universidade Estadual Paulista "Júlio de Mesquita Filho" como pré-requisito para obtenção do Título de Mestre em Biociências e Biotecnologia Aplicadas à Farmácia.

Orientadora: Profª. Drª. Alexandra Ivo de Medeiros

Coorientadora: Dr.ª Naiara Naiana Dejani

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Dissertação de mestrado apresentada à Faculdade de Ciências Farmacêuticas da Universidade Estadual Paulista – UNESP, Campus de Araraquara como requisito para a obtenção do título de Mestre em Biociências e Biotecnologia aplicadas à Farmácia.

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BANCA EXAMINADORA

ALEXANDRA IVO DE MEDEIROS
(orientadora)

CLESLEI FERNANDO ZANELLI

FABIANI GAI FRANTZ
(participante por vídeo-conferência)

Faculdade de Ciências Farmacêuticas – Seção de Pós-graduação
Rodovia Araraquara-Jaú, Km 1 - CEP 14800-903 - Araraquara - SP. - Brasil
tel 16 3301-6900 - fax 16 3301.6901 - posgrad@fcfar.unesp.br

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## Resumo

A resolução de reações inflamatórias é caracterizada pela fagocitose de células mortas, processo denominado eferocitose. Como consequência deste processo, há a produção dos mediadores anti-inflamatórios, fator de transformação de crescimento (TGF-β), prostaglandina $E_2$ ($PGE_2$) e a interleucina-10 (IL-10). No entanto, atualmente sabe-se que a fagocitose de células apoptóticas infectadas direciona a produção de mediadores inflamatórios interleucina-6 (IL-6), interleucina-23 (IL-23) e TGF-β, Resultados obtidos recentemente por nosso grupo demonstram que a fagocitose de células apoptóticas infectadas (iAC) com *Escherichia coli* por células dendríticas promove, além da produção de TGF-β, interleucina-1β (IL-1β) e IL-6, a síntese de altos níveis de $PGE_2$. Além disso, observamos que a $PGE_2$, via receptor de prostaglandina E2 4 (EP4), inibe a diferenciação de linfócitos Th17 por meio da modulação da expressão do receptor de IL-1 (IL-1R) em linfócitos Th. Desta forma, o objetivo deste trabalho é elucidar o mecanismo pelo qual $PGE_2$, oriunda da eferocitose de células apoptóticas infectadas, inibe a expressão de IL-1R e a diferenciação de linfócitos Th17. Os resultados obtidos demonstram que $PGE_2$, via receptor EP4, induz a ativação de adenilato ciclase/PKA/EPAC, e que esta via de sinalização promove além da inibição de IL-1R, a inibição de um importante fator de transcrição envolvido na diferenciação de linfócitos Th17, STAT3. Sabe-se que a $PGE_2$ pode exercer suas funções supressoras na diferenciação de células Th17 pela ativação de SOCS, bem como promover a ativação de PI3K. No entanto, apesar da presença de $PGE_2$ aumentar a expressão de *socs1*, a inibição da fosforilação de STAT3 não parece ser mediada por SOCS1, tão pouco PI3K está envolvida na inibição da diferenciação de células Th17. O conjunto de resultados sugere que a inibição na diferenciação de linfócitos Th17 por $PGE_2$, durante a eferocitose de células infectadas, ocorre através da ativação do eixo EP4/adenilato ciclase/PKA/EPAC, que resulta na inibição direta ou indireta da forforilação de STAT3.



**Palavras-chave: Eferocitose, Prostaglandina E2, Th17.**

## Abstract

The expression of inflammatory reactions is characterized by phagocytosis of dead cells, a process called eferocytosis. As a consequence of this process, there is the production of anti-inflammatory mediators, transforming growth factor (TGF-β), prostaglandin E2 ($PGE_2$) and an interleukin-10 (IL-10). However, it is now known that phagocytosis of infected apoptotic cells is one of the responsible for the production of interleukin-6 (IL-6), interleukin-23 (IL-23) and TGF-β lung mediators. The results obtained by our group demonstrate that the phagocytosis of infected apoptotic cells (iCA) with Escherichia coli by dendritic cells promotes, in addition to the production of TGF-β, interleukin-1β (IL-1β) and IL-6, high levels of $PGE_2$. In addition, $PGE_2$, via the prostaglandin E2 receptor (EP4), inhibits differentiation of Th17 lymphocytes by modulating IL-1 receptor (IL-1R) expression in Th lymphocytes. Thus, the objective of this work is to elucidate the mechanism by which $PGE_2$, derived from the ectocytosis of infected apoptotic cells, inhibits the expression of IL-1R and differentiation of Th17 lymphocytes. The results obtained demonstrate that $PGE_2$, via the EP4 receptor, induces the activation of adenylate cyclase / PKA / EPAC, and that this signaling pathway promotes in addition to the inhibition of IL-1R, the inhibition of an important transcription factor involved in the differentiation of Th17 lymphocytes, the STAT3. $PGE_2$ is known to exert its suppressor functions in the differentiation of Th17 cells by the activation of SOCS, as well as to promote the activation of PI3K. However, despite the presence of PGE2 enhancing *socs1* expression, inhibition of STAT3 phosphorylation does not appear to be mediated by SOCS1, so little PI3K is involved in inhibiting Th17 cell differentiation. The set of results suggests that the inhibition in differentiation of Th17 lymphocytes by PGE2 during the eferocytosis of infected cells occurs through the activation of the EP4 / adenylate cyclase / PKA / EPAC axis, which results in the direct or indirect inhibition of STAT3 forforylation.

**Keywords: Efferocytosis, Prostaglandin E2, Th17.**

# Lista de Figuras

**LISTA DE ABREVIAÇÕES E SIGLAS**

| | |
|---|---|
| **AC** | *Apoptotic Cell* |
| **AMPc** | Adenosina Monofosfato Cíclica |
| **BATF** | *Basic leucine zipper transcription factor, ATF-like* |
| **COX** | Ciclooxigenase |
| **cPLA2** | *Cytosolic phospholipases A2* |
| **DC** | Célula Dendrítica |
| **CM** | Meio Condicionado |
| **EAE** | *Experimental Autoimmune Encephalomyelitis* |
| **EP Receptor** | Receptor de Prostaglandina E |
| **FOXP3** | *forkhead box P3* |
| **GPCR** | Receptor Associado à Proteína G |
| **GM-CSF** | *Granulocyte-macrophage colony-stimulating factor* |
| **HIF1α** | *Hypoxia-inducible factor 1-alpha* |
| **iAC** | *infected apoptotic cell* |
| **IFN-γ** | Interferon gama |
| **IL** | Interleucina |
| **IL-1R** | Receptor de Interleucina-1 |
| **IRF** | *Interferon-Regulatory Factor* |
| **JAK** | *Janus kinase* |
| **Myd88** | *Myeloid Differentiation Primary Response 88* |
| **PAMP** | *Pathogen-associated molecular patterns* |
| **PG** | Prostaglandina |
| **PKA** | Fosfocinase A |
| **PS** | Fosfatidilserina |

| | |
|---|---|
| **RORα** | *RAR-related orphan receptor alpha* |
| **RORγt** | *RAR-related orphan receptor gamma* |
| **RUNX1** | *Runt-related transcription factor 1* |
| **SFB** | Soro Fetal Bovino |
| **SOCS** | *Suppressor of cytokine signaling* |
| **STAT** | *Signal transducer and activator of transcription* |
| **TCR** | *T Cell Receptor* |
| **TGF-β** | *Transforming growth factor beta* |
| **Th** | Célula T *helper* |
| **Treg** | Célula T reguladora |
| **TRIF** | *TIR-domain-containing adapter-inducing interferon-β* |

# Sumário

# Capítulo 1

# Introdução

## *Apoptose e eferocitose*

A morte celular por apoptose é caracterizada por uma sequência de alterações moleculares, como ativação de caspases e modificações em proteínas da membrana celular, bem como por alterações morfológicas, que incluem: fragmentação nuclear, condensação da cromatina e consequente diminuição do volume celular [1].

Uma das principais alterações celulares no início do processo de apoptose ocorre mediante a exposição de fosfolipídeos na porção externa da membrana. A fosfatidiletanolamina, a fosfatidilcolina e a fosfatidilserina são os principais fosfolipídeos envolvidos nesse processo, sendo a fosfatidilserina (PS) a principal molécula envolvida no reconhecimento das células apoptóticas por fagócitos como macrófagos e células dendríticas [2].

Após o reconhecimento das células apoptóticas pelos fagócitos, ocorre então a fagocitose dessas células, e esse processo é denominado eferocitose, palavra oriunda do latim "*efferre*" que significa "levar corpos ao túmulo". Este processo tem se mostrado cada vez mais essencial para a manutenção da homeostase e prevenção de patologias [3,4].

Para que ocorra a eferocitose, é necessário que ocorra uma sequência de eventos, que envolvem tanto o reconhecimento, quanto o englobamento das células apoptóticas pelos fagócitos [5]. Para isso, inicialmente os corpos apoptóticos liberam os chamados sinais *find-me*, como por exemplo ATP, UTP, lisofosfatidilcolina e algumas quimiocinas, os quais exercem a quimioatração dos fagócitos até as células apoptóticas. Uma vez atraídos, os fagócitos reconhecem o corpo apoptótico por meio dos sinais *eat-me*, moléculas expostas na membrana da célula, que permitem a sua identificação como apoptótica. Essas moléculas podem ser PS, bem como sua forma oxidada e ICAM-3. Por

fim, a célula fagocitada é então degradada, e ocorre a liberação de mediadores de perfil principalmente anti-inflamatórios pelo fagócito [6].

***Biologia de Linfócitos Th17***

Os linfócitos T *helper* 17 (Th17) possuem uma atuação importante nos mecanismos de defesa do hospedeiro contra microrganismos, em especial fungos e bactérias, porém também é uma das principais células envolvidas na patogenia de algumas doenças autoimunes [7,8]. A diferenciação de células Th17 em camundongos ocorre a partir de linfócitos T CD4 *naive* ativados por células dendríticas produtoras principalmente de TGF-β e IL-6 [9].

Os linfócitos Th *naive* expressam o receptor funcional de IL-6, ou seja, a subunidade α do receptor de IL-6 (IL-6R), assim como a subunidade sinalizadora, gp130. A interação da IL-6 ao complexo gp130 e IL-6R promove a dimerização do mesmo e a ativação de *janus kinase* 1/2 (JAK1/2) resultando na fosforilação de resíduos de tirosinas. A fosforilação destes resíduos permite a interação com STAT1 e/ou STAT3 (*Signal transducer and activator of transcription)* favorecendo a fosforilação, a dimerização dessas proteínas que translocam-se para o núcleo e exercem sua função de fatores de transcrição [10]. Além disso, a interação de TGF-β ao seu respectivo receptor induz o aumento da expressão de IL-6Rα o que auxilia no processo de ativação e diferenciação de células Th17. A ativação de STAT3, por IL-6R, juntamente com TGF-β, promove a indução dos fatores de transcrição *RAR-related orphan receptor gamma* (RORγt), *RAR-related orphan receptor alpha (*RORα) e *Runt-related transcription factor 1* (RUNX1), que resulta na diferenciação de células Th17 [11,12]. RORγt é considerado o fator de transcrição imprescindível na diferenciação de Th17, entretanto, apesar da importância de RORγt na expressão de IL-17, a deficiência deste fator de transcrição, parece não impedir totalmente a síntese de IL-17, sugerindo que outros fatores de transcrição poderiam auxiliar diretamente no aumento tanto da expressão de RORγt, como de IL-17 [13-16]. Nesse contexto, outros fatores de transcrição como IRF4, *Basic leucine zipper*

*transcription factor, ATF-like* (BATF), STAT3 e *Hypoxia-inducible factor 1-alpha* (HIF1α) têm sido descritos durante o processo de ativação, auxiliando na estabilização e na fase final de diferenciação de células Th17 [17,18].

Ainda, a sinalização via IL-6 e TGF-β promove o aumento da expressão dos receptores para IL-21 e IL-23 [9]. A citocina IL-21 é produzida pela própria célula Th17 e age autocrinamente potencializando a diferenciação em Th17, enquanto que a citocina IL-23, produzida principalmente por células dendríticas, está associada com a manutenção e a expansão desse fenótipo. [11,16,19]. Adicionalmente, a ativação de STAT3, via sinalização de IL-6, parece ser a principal via de sinalização envolvida no aumento da expressão de IL-1R. Entretanto, embora IL-23 e IL-21 isoladamente não tenham demonstrado efeito na expressão do IL-1R, na presença de IL-6, a sinalização destas vias potencializam a expressão desse receptor. IL-1β, em associação com IL-6, induz a expressão de fatores de transcrição envolvidos na diferenciação de células Th17, como o RORγt e IRF4, estando estes diretamente envolvidos na expressão de IL-17. A importância do IL-1β na diferenciação de linfócitos Th17 foi demonstrada utilizando linfócitos Th *naive* obtidos de animais deficientes do receptor de IL-1β, cuja a deficicência desse receptor inibiu a diferenciação de Th17. Ainda, a citocinas IL-1β tem sido descrita como um importante mediador envolvido na diferenciação de células Th17 patogênicas, ou seja, produtoras de IL-17 e Interferon gama (IFN-γ) [20]. Além disso, o envolvimento de IL-1β na diferenciação de Th17 também tem sido demonstrado em modelos de doenças autoimunes. Ou seja, a ausência de receptor (animais *Il1r1*-/-) impossibilitou o desenvolvimento de encefalomielite autoimune experimental (EAE). Além disso, a sinalização via IL-1R mostrou-se essencial na indução da expressão de IRF4 e RORγt, visto que linfócitos isolados de animais Il1r1-/- não expressam estes fatores de transcrição [21,22].

***Efeito da eferocitose na diferenciação de linfócitos Th17***

O *clearence* de células apoptóticas pode ocorrer tanto na ausência de infecção, em uma situação de homeostase, ou durante um processo infeccioso em que há um grande acúmulo de células apoptóticas infectadas, ou seja, possuem no seu interior *Pathogen-Associated Molecular Patterns* (PAMP). Sabe-se que a eferocitose de células infectadas com *E. coli* induz a produção de IL-23, TGF-β e IL-6 por células dendríticas. O papel desses mediadores solúveis na diferenciação de células Th17 foi comprovado através da estimulação de células T "*naive*", com anti-CD3 e anti-CD28, na presença do meio condicionado oriundo de células dendríticas (DC) incubadas com células apoptóticas infectadas [23]. A importância da presença do PAMP no interior dessas células apoptóticas foi confirmada utilizando meio de cultura oriundo de DC deficientes em *TIR-domain-containing adapter-inducing interferon-β* (TRIF) e *Myeloid Differentiation Primary Response 88 (*MyD88). Nessas condições o meio de cultura condicionado, oriundo de DC deficientes em TRIF e MyD88, impediu a diferenciação de células Th17 e promoveu a diferenciação de células Treg [23]. Apesar da vasta literatura sobre os mediadores envolvidos na diferenciação de células Th17, esse foi o primeiro estudo que propôs um modelo fisiológico de diferenciação de células Th17, no qual a fagocitose de células apoptóticas infectadas por bactéria, como fonte de citocinas anti-inflamatória (TGF-β) e pró-inflamatória (IL-6), atua gerando condições ideais para a diferenciação de células Th17.

***Prostaglandina E2 ($PGE_2$) e vias de sinalização mediados pelos receptores EP***

As prostaglandinas (PGs) são mediadores lipídicos oriundos do metabolismo do ácido araquidônico liberado de fosfolipídeos de membranas pelas fosfolipases (cPLA2) [24]. O ácido araquidônico liberado pode ser metabolizado pela cicloxigenase 1 (COX-1) e cicloxigenase 2 (COX-2), associadas às membranas nuclear e do retículo endoplasmático, com sua porção de ligação ao substrato orientada para o citoplasma e geram o metabólito

intermediário, denominado PGH2. Enquanto a COX-1 é uma enzima constitutiva, a enzima COX-2 é induzida em situações inflamatórias e atua potencializando a produção de prostaglandinas [25]. As sintases responsáveis pela metabolização da PGH2 determinam o destino da mesma, podendo ocorrer a formação de PGI2, PGF2, PGD2, $PGE_2$ ou tromboxanos A2. Esses prostanóides são liberados pela célula através de um transportador de membrana e, devido sua curta meia vida, logo exercem sua função efetora de forma autócrina e parácrina [26].

A $PGE_2$ é uma das mais abundantes PGs produzidas pelas células de nossos tecidos e exerce sua função através de 4 subtipos de receptores: EP1, EP2, EP3 e EP4. Esses subtipos são receptores associados à proteína G (GPCR) e variam quanto a propriedades de ligação à $PGE_2$, distribuição tecidual, expressão e tipo de proteína G [27]. Dentre esses, EP1, EP2 e EP4 são expressos em altos níveis em células Th *naive* em camundongos, enquanto EP3 apresenta uma baixa ou inexistente expressão nesses linfócitos [28,29]. Boniface e Bak-Jensen descreveram que a ativação de linfócitos Th *naive* humanos CD4+CD45RO- aumenta a expressão dos receptores EP2 e EP4.

EP1 é um receptor acoplado a proteína Gαq/p e promove o aumento de Ca2+ intracelular, EP2 e EP4 estão acoplados a subunidade α estimuladora da proteína G (Gαs) e promovem o aumento da concentração intracelular de adenosina mono fosfato cíclica (cAMP) [30,31]. Os níveis intracelulares de cAMP são regulados pela atividade de dois tipos de enzimas: a adenilato ciclase (AC) e as fosfodiesterases (PDE). O aumento intracelular de cAMP, um importante segundo mensageiro, leva a ativação de duas proteínas efetoras: a proteína quinase A (PKA) e a proteína de troca diretamente ativada por cAMP (EPAC) [32,33].

A PKA é um tetrâmero constituído de duas subunidades reguladoras e de duas subunidades catalíticas. A ligação do cAMP à subunidade reguladora de PKA promove uma mudança conformacional que leva à dissociação das subunidades catalíticas, que se difundem para dentro do núcleo e promovem a fosforilação de seus substratos. A

fosforilação de substratos nucleares e citoplasmáticos mediada por PKA é importante para múltiplas funções celulares, incluindo metabolismo, diferenciação, transmissão sináptica, atividade de canais iônicos, crescimento e desenvolvimento celular.

A EPAC contém um domínio de ligação ao cAMP semelhante à subunidade reguladora de PKA, além de um domínio de fator de troca (GEF). A EPAC se liga ao cAMP, ativando uma proteína da superfamília Ras, chamada Rap1, que tem sido relacionada no controle da adesão celular e formação de junções celulares. Assim, o aumento nos níveis intracelulares de cAMP pode resultar na ativação tanto de PKA quanto de EPAC, o que depende da concentração e localização dessas duas enzimas dentro da célula [32].

Sabe-se que a sinalização de $PGE_2$ através do receptor EP4, é capaz de ativar além da via que envolve adenilato ciclase/PKA/EPAC, a via de sinalização da fosfatidilinositol-3-quinase (PI3K) [34]. A PI3K exerce um papel importante, contribuindo com a diferenciação de linfócitos Th17, como foi demonstrado em linfócitos T que expressavam uma PI3K inativa, foram incapazes de se diferenciar em Th17 [35].

***Envolvimento da $PGE_2$ na diferenciação de linfócitos T* helper**

A participação de $PGE_2$ em células da imunidade adaptativa vem sendo desvendada nos últimos anos e, diferente dos efeitos supressores previamente descritos na ativação de células T *naive*, recentes trabalhos demonstram uma importante função imunoestimuladora desse prostanóide no desenvolvimento de células Th17 e Th1 em vários modelos de infecção e autoimunidade [24,25]. O papel supressor da $PGE_2$, via EP2, foi demonstrado pela inibição da capacidade proliferativa de linfócitos T usando um modelo de reação linfocitária mista. Esse efeito supressor da $PGE_2$, assim como de um agonista de EP2 (8-CPT-cAMP), em células T periféricas parece ser mediado por PKA-Csk, que atua antagonizando a sinalização de TCR e impedindo a ativação da quinase da família Src (Lck) [36].

Ainda em uma visão imunossupressora, trabalhos destacam o papel da $PGE_2$ na diferenciação de células Treg. A $PGE_2$ pode promover a diferenciação de células Treg pelo aumento da expressão do fator de transcrição FOXP3, aumentando a função supressora dessas células [37]. Contradizendo esses efeitos, outros estudos demonstram que os efeitos supressores da $PGE_2$ em células T podem ser revertidos pela forte interação mediada por TCR e o complexo CD3. A ativação de linfócitos na presença de altas concentrações de anticorpos anti-CD28/anti-CD3, na presença de $PGE_2$, resulta no aumento da porcentagem de linfócitos Th1. Curiosamente, essa sinalização facilitadora da $PGE_2$ ocorre via EP2 e EP4, porém é mediada pela ativação de PI3K [34].

No âmbito das células Th17, a participação de $PGE_2$ na diferenciação deste subtipo celular parece ser dependente dos receptores EP2 e EP4 e foi comprovada em diferentes modelos experimentais [29]. Em modelo murino de encefalomielite autoimune experimental (EAE) foi demonstrado que a $PGE_2$ atua via receptores EP2 e EP4, e sinaliza via cAMP–PKA, agindo sinergicamente com IL-23 na expansão de células Th17. Além disso, a adição exógena da $PGE_2$ pode aumentar a produção de IL-23 por DCs colaborando indiretamente na expansão de Th17. Por outro lado, foi demonstrado que a $PGE_2$ inibe a diferenciação de células Th17 e a resposta imune antifúngica contra *Cryptococcus neoformans* [7].

***Suppressor of cytokine signaling (SOCS)***

A interação da citocina ao seu receptor resulta na ativação de JAK quinases que fosforilam resíduos de tirosinas, criando locais de ancoragem para as STATs, seguido da ativação e dimerização dessas STATs permitindo a translocação para o núcleo e a interação as suas respectivas regiões promotoras[38]. A duração e a intensidade desses sinais são regulados pela ação de proteínas com funções supressoras que interferem na sinalização JAK/STAT, denominadas SOCS, *Suppressor of cytokine signaling* [39]. Estas, constituem uma família de proteínas composta de sete membros (SOCS1-SOCS7). A princípio, acreditava-se que cada representante dessas proteínas exercia uma função

inibitória intracelular específica para as diferentes STATs. Por exemplo, a SOCS3 inibe a STAT3 impedindo desta forma a translocação deste fator de transcrição para o núcleo e a expressão de genes específicos [40]. No entanto, foi comprovado que a ação inibidora de algumas SOCS pode ocorrer em diferentes STATS. Por exemplo, SOCS1 que inicialmente era descrita pela inibição da ativação de STAT1, também possui ações inibitórias em STAT3 [41], como foi demonstrado em camundongos, que após o tratamento com inibidor de SOCS1, apresentaram maior ativação de STAT3 em células peritoneais [42].

Uma vez que SOCS3 apresenta esse efeito supressor sobre STAT3, essa proteína também exerce um efeito supressor na diferenciação de linfócitos Th17. Sabe-se que as células tronco mesenquimais são capazes de induzir a expressão de SOCS3 em linfócitos T"*naive*", via IFN-γ, e isso inibiu a diferenciação em linfócitos Th17, por meio da inibição de STAT3 [41].

Tem sido demonstrado que a $PGE_2$, juntamente com IL-6 ou IL-10, é capaz de diminuir a atividade de STAT3 em macrófagos, e isto se deve ao aumento na expressão de SOCS3 [43]. Recentes dados obtidos por nosso grupo demonstram que a eferocitose de células apoptóticas infectadas por células dendríticas promove a liberação de TGF-β, IL-6, assim como a síntese de IL-1β e $PGE_2$. A presença deste prostanóide inibe a expressão do IL-1R e a diferenciação de Th17 [44], no entanto os mecanismos e as vias de sinalização pelos quais essa inibição ocorre ainda não foram investigados. Desta forma, talvez o aumento de SOCS3 poderia ser um dos mecanismos pelos quais a $PGE_2$ inibiria a diferenciação de linfócitos Th17 no contexto da eferocitose.

Estudos demonstram que, em alguns tipos celulares, SOCS3 é regulada positivamente pela ação da $PGE_2$ [45], no entanto nada se sabe quanto ao efeito deste prostanóide, tão pouco o envolvimento de SOCS3, durante o processo de inibição da diferenciação para Th17 no contexto da eferocitose. Considerando que STAT3 é um importante fator de transcrição envolvido na expressão do IL-1R e na diferenciação de

células Th17 e a $PGE_2$ é capaz de aumentar a expressão de SOCS3, a hipótese deste estudo é que $PGE_2$, oriunda da fagocitose de células apoptóticas infectadas, induziria a expressão de SOCS3 levando a modulação negativa da expressão do IL-1R e da diferenciação de linfócitos Th17.

**Figura 1. Hipótese do Problema**

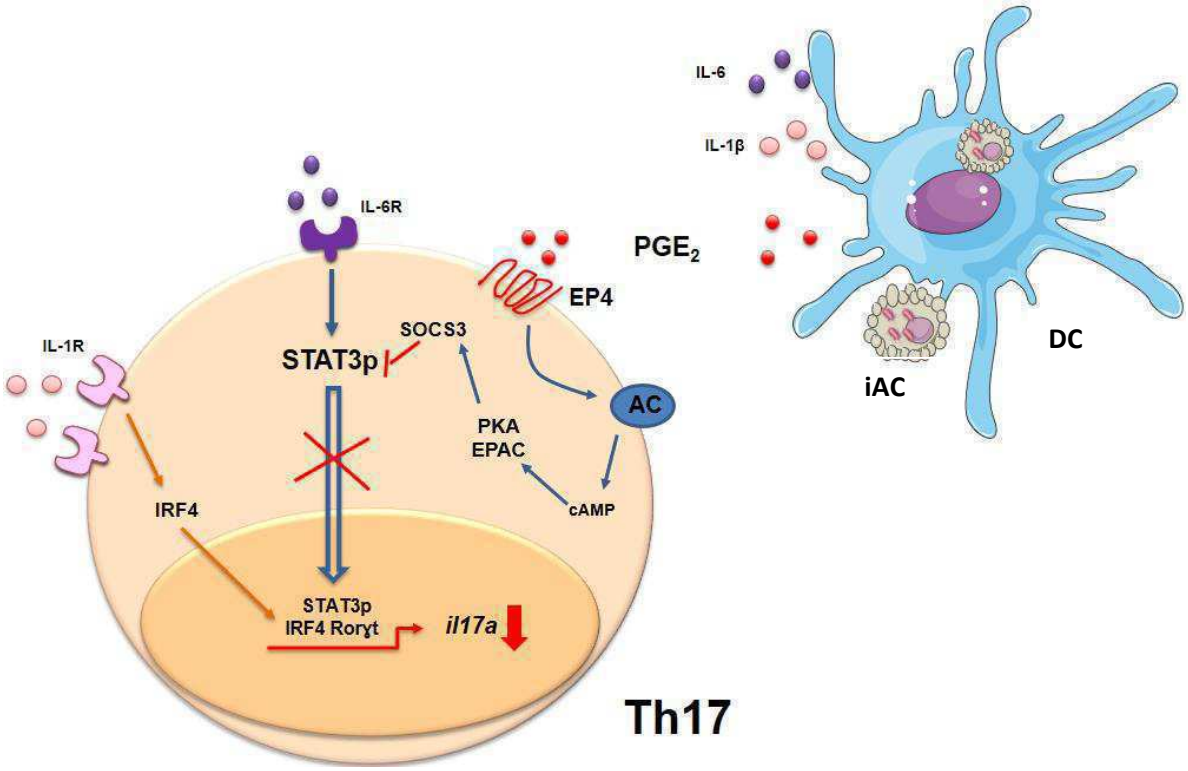


**Fig 1. Mecanismo proposto sobre a ação da $PGE_2$ durante diferenciação de linfócitos Th na presença de citocinas provenientes da eferocitose de células apoptóticas infectadas**. A $PGE_2$, via EP4, age aumentando cAMP (*cyclic adenosine monophosphate*) pela ativação de adenilato ciclase (AC) que, por sua vez, ativa PKA, induzindo SOCS3, a qual leva a inibição de STAT3, e essa cascata culmina na modulação negativa da expressão do il17a e do fator de transcrição IRF4, comprometendo a diferenciação de Th17. iAC = célula apoptótica infectada; DC= Célula dendrítica; Th17= Linfócito T helper 17.

## Objetivos Específicos

I) Determinar a participação de EP4-PKA/EPAC e ou PI3K na indução de expressão de SOCS1/3 durante a diferenciação de células Th17;

II) Caracterizar o envolvimento de SOCS1/3 na inibição da via de sinalização mediada por STAT3 e na expressão do IL-1R durante a diferenciação de linfócitos Th17; em linfócitos T em processo de diferenciação na presença do meio condicionado oriundo da fagocitose de células apoptóticas infectadas.

## Materiais e Métodos

***Animais***

Foram utilizados camundongos fêmeas, de 6 a 14 semanas de idade, pertencentes à linhagem selvagem C57BL/6 adquiridos no Biotério da Unicamp – CEMIB, Campinas-SP. Os animais foram mantidos em mini isoladores com temperatura, umidade, fluxo de ar, ciclo de luz claro/escuro controlado e livre acesso à água e ração esterilizadas. Todos os procedimentos experimentais foram julgados e autorizados pelo comitê de ética local.

***Diferenciação de DC a partir de precursores de Medula Óssea***

As DC foram diferenciadas a partir de precursores da medula óssea do fêmur e tíbia dos animais C57BL/6 segundo protocolo preconizado por Lutz et al. (1999)[46]. $1x10^6$ células precursoras foram diferenciadas em placa de petri, na presença de 20ng/mL de GMCSF (Peprotech), em 20ML de meio RPMI suplementado com 10% de soro bovino fetal (FBS) e 10 µg/mL de antibiótico gentamicina. No sétimo dia de cultura as células semi-aderentes foram coletadas e utilizadas nos ensaios de fagocitose de células apoptóticas infectadas.

***Geração das células apoptóticas infectadas***

Como fonte de células apoptóticas infectadas, células da linhagem RAW 264-7 foram incubadas com *Escherichia coli* (ATCC 259992), na proporção 1:10, por 2 h para fagocitose. As bactérias foram centrifugadas a 300g, durante 5 minutos, lavadas em PBS, e então ressuspensas em meio DMEM contendo 10% de FBS para a fagocitose. Após as 2 horas, as células foram lavadas com PBS contendo gentamicina (10 μg/mL), para morte de bactérias não fagocitadas, e irradiadas com 350 mJ UVC para a indução de apoptose, como descrito por Torchinsky et al. (2009)[23].

***Geração de Meio de cultura condicionado (CM) e Meio de cultura condicionado Indo (CM/Indo)***

As células dendríticas (DC) imaturas diferenciadas de medula óssea foram co-cultivadas com células apoptóticas infectadas (IAC) na proporção de 3:1 (IAC:DC). Após 18 h o sobrenadante foi coletado, centrifugado e filtrado para remoção de células e alíquotas de 1 mL foram geradas e armazenadas em freezer à -80ºC. As amostra foram utilizadas para o ensaio de diferenciação de células T *naïve*. Para a obtenção de CM baixa concentração de $PGE_2$, DC foram previamente tratadas com indometacina (10μM) antes da adição das células apoptóticas infectadas.

***Obtenção de células T naive***

Linfócitos Th *naive* foram isolados a partir de células de baço de animais C57BL/6 pela utilização de *beads* magnéticas ($CD4^+CD62L^+$ T Cell Isolation Kit II mouse (#130-093-227 - Miltenyi Biotech) de acordo com instruções do fabricante.

***Ensaio de diferenciação de células T na presença do CM ou CM/Indo***

As células T CD4+ *naive* isoladas de animais WT, foram diferenciadas na presença de CM ou CM/Indo, como descrito previamente por Torchinsky et al. (2009)[23]. Resumidamente, $10^6$ linfócitos T *naive*/poço foram adicionados em placas de 48 poços na presença de CM ou CM/Indo e tratados com 4 μg/mL de anti-CD3 e 2 μg/mL anti-CD28. Como controle positivo, linfócitos foram cultivados na presença de um coquetel de diferenciação para Th17 (IL-6, TGF-β, anti-IFN-γ, anti-IL-4, anti-IL-2) diluído em IMDM suplementado com 10% de FBS, 10uM gentamicina, 2 mM L-glutamina, 10 mM HEPES e 1 nM piruvato de sódio. Para avaliar o envolvimento da $PGE_2$, bem como de suas vias de sinalização, as células T"*naive*" foram cultivadas na presença de 5μM do agonista do receptor EP4 (Cay10598) (Cayman), 5μM do antagonista de EP4 (L-161,982) (Tocris), 5μM dos ativadores de adenilato ciclase, PKA e Epac (Forskolin, 8-Bromo-cAMP, 8-CPT-2Me-cAMP) (Tocris), 5μM do inibidor de EPAC (ESI-09) (Tocris); 500, 100 ou 10nM do inibidor de PI3K (Wortmanina) bem como com 40, 20 ou 5μM de iKir SOCS1.

***Análise da expressão de marcadores de superfície e intracelulares por citometria de fluxo***

Após a diferenciação, as células T obtidas dos diferentes protocolos propostos foram estimuladas por 4h com 0.1 μg/ml de PMA (Sigma) e 0,5 μg/mL de ionóforo do cálcio (A23187) e 10 μ/mL de Brefeldina A (Sigma). Posteriormente, as células foram marcadas com anticorpos anti-IL-17A-PE-Cy7, anti-CD4-PE (BB) e $1x10^5$ eventos foram adquiridos por tubo em citômetro FACSCantoTM (Becton & Dickinson, San Diego, CA, USA) e analisados pelo programa FCS Express (FCS Express 4.0).

***Real Time PCR quantitativo (qPCR)***

O RNA foi isolado de linfócitos T naive durante o processo de diferenciação na presença CM ou CM/Indo. Em seguida, o mesmo foi transcrito para cDNA e amplificado por real time qPCR quantitativo utilizando os primers específicos para os genes de interesse, *il17a, socs3 e socs1*. A expressão relativa do gene de interesse, utilizando a formula 2-ΔΔCT, foi calculada através do método de controle (CT) comparativo. Para realização do qPCR, foi utilizado o termociclador ABI Prim 7300 (Applied Biosystems, Foster City, CA).

Quadro 1. Primers utilizados

| | | |
|---|---|---|
| ***gapdh*** | F-AACTTTGGCATTGTGGAAGG | R-ACACATTGGGGGTAGGAACA |
| ***socs3*** | F- ACCAGCGCCACTTCTTCACG | R-GTGGAGCATCATACTGATCC |
| ***socs1*** | F- ACTTCTGGCTGGAGACCTCA | R- CCCAGACACAAGCTGCTACA |
| ***il17a*** | F- AGGCAGCAGCGATCATCC | R- GTGGAACGGTTGAGGTAGTC |

***Phosflow por citometria de fluxo***

$5x10^5$ linfócitos foram cultivados com CM ou CM/Indo, e tratados com agonista EP4 (Cay 10598); antagonista EP4 (L-161,982); ativador de PKA (8-Bromo-cAMP); ativador EPAC (8-CPT-2Me-cAMP). Como controle positivo, linfócitos $TCD4^+$ *naive* foram cultivados na presença de 20 ng/mL de IL-6; como controle negativo linfócitos $TCD4^+$ *naïve* foram cultivados em meio de cultura IMDM. Após 15, 30 e 60 min, as células foram obtidas e lavadas com PBS. As células foram fixadas com Fixation Buffer (BD Cytofix) e incubadas a 37°C por 12 min. As células foram lavadas novamente, tratadas com Perm Buffer III (BD Phosflow) previamente resfriado e incubadas no gelo por 30 min. Por fim, foram lavadas com PBS e marcadas com anticorpo anti-STAT3p-AlexaFluor 647, anti-CD4-PE (BD) e anti-IL-17A-PE-Cy7 (BD).

***Inibição da expressão do mRNA de socs1 e socs3 – Silenciamento gênico***

Para a inibição da expressão de *socs1* e *socs3*, os linfócitos foram transfectados com lipofectamina RNAiMAX (ThermoFisher) como agente de transfecção conjugado com as sequências de silenciamento para *socs1 e socs3*, ou com a sequência controle, para o controle negativo. $5x10^4$ células foram cultivadas por 48h na presença de 100 µL do meio Opti MEM, e tratadas com 10 µL do conjugado, conforme as orientações do fabricante.

***Inibição de SOCS1 por meio de iKir SOCS1***

Para a inibição de SOCS1 durante a diferenciação de linfócitos T CD4+, foram utilizadas duas sequencias peptídicas conjugadas com um grupo palmitoil na porção N-terminal, para penetração celular. Sendo uma sequência controle (DTHFARTFARSHSDYRRI), e uma sequência inibidora de SOCS1 (DTHFRTFRSHSDYRR) (Genscript). Os linfócitos foram tratados com 5, 20 ou 40µM dos peptídeos solubilizados em PBS por 20 minutos antes de serem diferenciados na presença de CM.

***Análise estatística***

Os resultados foram apresentados como média ± SD e foram analisados utilizando o programa estatístico Prism 5.0 (GraphPad Software, San Diego, CA). Foi realizada a análise de variância (ANOVA) seguida do pós-teste Bonferroni. Foram consideradas diferenças estatisticamente significativas se $p<0,05$.

## Resultados e discussão

***Produção de Meio Condicionado oriundo da fagocitose de células apoptóticas infectadas***

Para estudar o mecanismo pelo qual $PGE_2$ inibe a diferenciação de linfócitos Th17, vários lotes de **Meio Condicionado (CM)**, sobrenadante oriundo da co-cultura de DC e células apoptóticas infectadas, foram gerados para execução dos todos os experimentos subsequentes. As condições experimentais para obtenção destes Meios Condicionados foram: I) meio Condicionado oriundo da DC co-cultivadas com células apoptóticas infectadas (iAC), denominado **CM**; II) Meio Condicionado oriundo da DC tratadas com inibidor de COX (Indometacina) e co-cultivadas com iAC, denominado **CM/Indo.**

Os novos lotes de sobrenadantes oriundos da co-cultura de DC+iAC foram validados quanto aos níveis de $PGE_2$ nos respectivos CM. A condição CM contém altos níveis de $PGE_2$ e CM/Indo possui baixos níveis de $PGE_2$, como pode ser observado na Figura 2A e já demonstrado previamente por trabalhos do grupo [47].

Todos os lotes de CM e CM/Indo gerados foram também avaliados e validados quanto a eficiência na diferenciação de células Th17, garantindo desta forma a reprodutibilidade experimental para os subsequentes experimentos. Ou seja, os linfócitos T *naive* diferenciados na presença de CM ou CM/Indo foram avaliados quanto à expressão de $CD4^+$ e $IL\text{-}17A^+$ utilizando citometria de fluxo. Foi observado que na presença do CM, contendo, portanto, altos níveis de $PGE_2$, ocorreu a diferenciação de Th17 (23%). No entanto as células T *naive* diferenciadas na presença de CM/Indo houve um aumento drástico na porcentagem de células Th17 (43%) (Fig. 2B). Ou seja, a presença de altos níveis de $PGE_2$ inibe a diferenciação de células Th17. Sendo assim, os CMs gerados a partir da co-cultura celular foram validados em relação ao padrão de

diferenciação de linfócitos Th17 já descrito em prévios trabalhos do nosso grupo [1]. Portanto, após a obtenção e validação dos CM, os mesmos foram utilizados para os experimentos subsequentes visando determinar os mecanismos envolvidos na inibição da diferenciação de linfócitos Th17 pela ação da $PGE_2$.

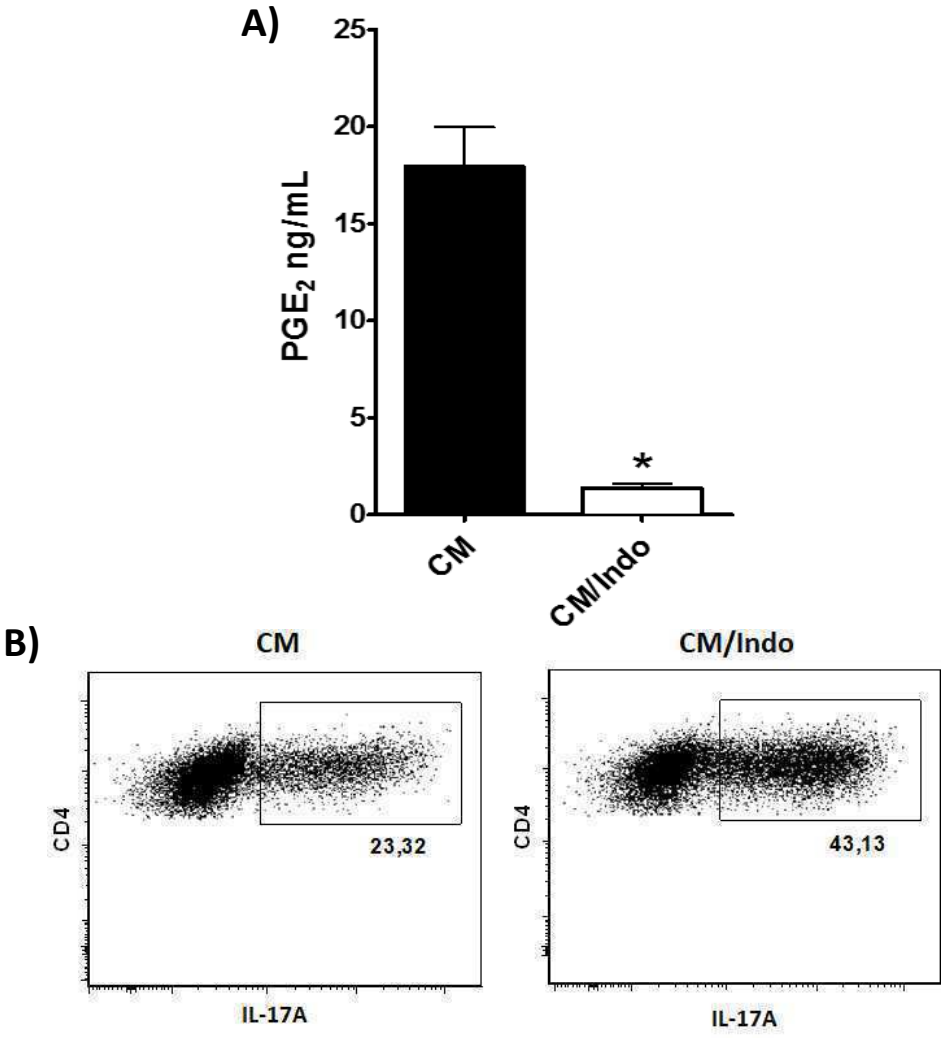


**Fig 2. $PGE_2$ presente em CM inibe a diferenciação de linfócitos T CD4 naive em Th17. (A)** A concentração de $PGE_2$ em CM e CM/Indo foi determinada após 18 horas de co-cultura, por ELISA. Os resultados estão representados como média ± SEM de 3 experimentos independentes realizados em triplicata. ** p<0,05 vs CM; # p<0,05 vs CM/Indo.* **(B)** Linfócitos T CD4 *naive* foram ativados com 4µg/mL de anti-CD3 e 2µg/mL de anti-CD28, na presença de CM ou CM/Indo. A porcentagem de linfócitos T $CD4^+IL\text{-}17^+$ foi avaliada por citometria de fluxo, após estímulo com PMA/Ionomicina/GolgiStop. Resultado representativo de 3 experimentos independentes.

***$PGE_2$ inibe a diferenciação de linfócitos Th17 via ativação de adenilato ciclase e PKA.***

Uma vez que resultados prévios do grupo mostram que a $PGE_2$ oriunda da fagocitose de células apoptóticas infectadas com *E. coli* é capaz de inibir a diferenciação de linfócitos Th17 via receptor EP4, os primeiros experimentos foram conduzidos na tentativa de se determinar a via de sinalização envolvida neste processo de inibição via $PGE_2$/EP4

Sabe-se que os receptores EP1 e EP3 são pouco expressos em linfócitos Th17 [48], e que o aumento na expressão de RORγt, essencial para diferenciação desse tipo celular, está relacionado à inibição do receptor EP2, enquanto EP4 não é afetado [49]. EP4 é um receptor acoplado a proteína G com uma subunidade α estimuladora, portanto sua ativação está relacionada com o aumento de cAMP intracelular [30]. A ativação desse mensageiro secundário, por sua vez, pode levar a ativação de PKA e EPAC [32]. Para avaliar a importância dessa via de sinalização, os linfócitos T $CD4^+$ *naive* foram cultivados nas seguintes condições: CM na presença do antagonista de EP4 (L161,982), ou CM/Indo na presença do agonista de EP4 (Cay10598). As células foram avaliadas quanto à expressão de *il17a* e à produção de IL-17A, por qPCR e citometria de fluxo, respectivamente.

Confirmando os resultados previamente obtidos por nosso grupo [44], o bloqueio da ação de $PGE_2$, assim como com o tratamento com o antagonista de EP4 foram capazes de restaurar a expressão de *il17a* (Fig. 3A), bem como a diferenciação de linfócitos Th17 (Fig. 3B). O tratamento com o agonista de EP4, por sua vez, foi capaz de inibir a expressão de *il17a* e a diferenciação de células Th17, condizendo com o fato de EP4 ser o principal receptor de $PGE_2$ expresso em linfócitos Th17.

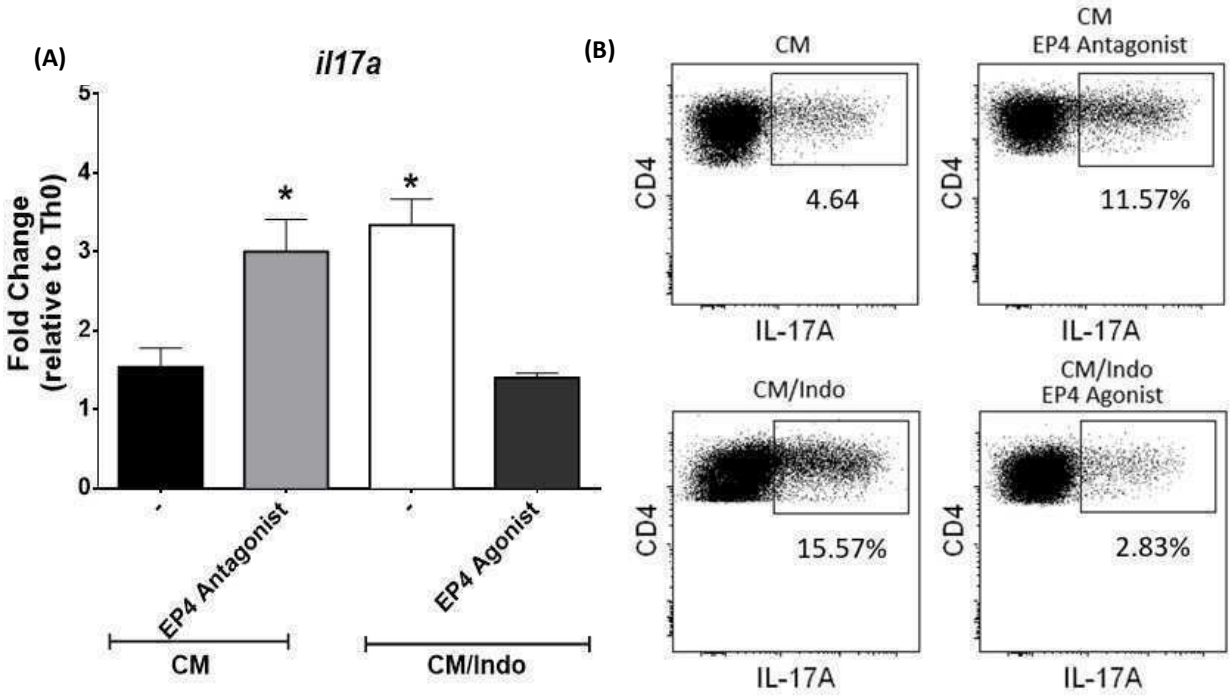


**Fig 2. $PGE_2$ inibe a diferenciação de linfócitos via EP4.** Os linfócitos T CD4+ *naive* foram cultivados com anti-CD3 e anti-CD28 na presença de CM, CM + 10µM antagonista de EP4 (L161,982), CM/Indo apenas ou CM/Indo na presença de 5µM agonista de EP4 (Cay10598). (A) Após 48 horas de cultura, as células foram lisadas e a expressão de il17a foi quantificada por qPCR. Os resultados estão representados como média ± SEM de 3 experimentos independentes. * $p<0,05$ vs CM. Análise da variância ANOVA, pós-teste Bonferroni. (B) Após 72 horas de cultura, as células foram marcadas e analisadas por citometria de fluxo. Resultado representativo de 3 experimentos independentes.

Visto que a $PGE_2$ é capaz de ativar a via de sinalização PKA/EPAC em macrófagos[50], o próximo passo foi investigar a cascata de sinalização "downstream" ao receptor EP4. Para tanto, os linfócitos T $CD4^+$ *naive* foram cultivados na presença do ativador de adenilato ciclase (forskolin), ou o ativador de PKA (8-bromo-cAMP), ou ativador de EPAC (8CPT-2Me-cAMP), ou inibidor de EPAC (ESI-09). O uso de ativadores de adenilato ciclase, PKA e EPAC, proteínas importantes na via de sinalização de EP4, resultou no efeito inibitório em relação a expressão de *il17a* (Fig. 4A), da mesma forma que o agonista de EP4. No entanto, ao avaliarmos a porcentagem de células produtoras de IL-17A por citometria de fluxo, apenas o ativador de PKA foi capaz de inibir a diferenciação de Th17 de maneira tão eficaz quanto aos valores obtidos na condição CM (Fig. 4B). No entanto, nem o ativador de EPAC tão pouco o inibidor de EPAC foram capazes de inibir a ação supressora da $PGE_2$ na diferenciação dos linfócitos Th17. Essa diferença pode ter ocorrido em função de mecanismos de controle pós-transcricionais na tradução de IL-17A. Enquanto os ativadores de adenilato ciclase, PKA e EPAC são capazes de inibir a transcrição do gene para *il17a,* apenas o ativador de PKA foi capaz de inibir a tradução da proteína, resultando assim na inibição na diferenciação de Th17 observada na citometria de fluxo.

Portanto, esses resultados sugerem que $PGE_2$ é capaz de inibir a diferenciação de linfócitos Th17 via receptor EP4, culminando no aumento de cAMP intracelular pela ativação da adenilato ciclase que, por sua vez, leva a ativação de PKA, e inibe a expressão de genes relacionados à diferenciação de células Th17.

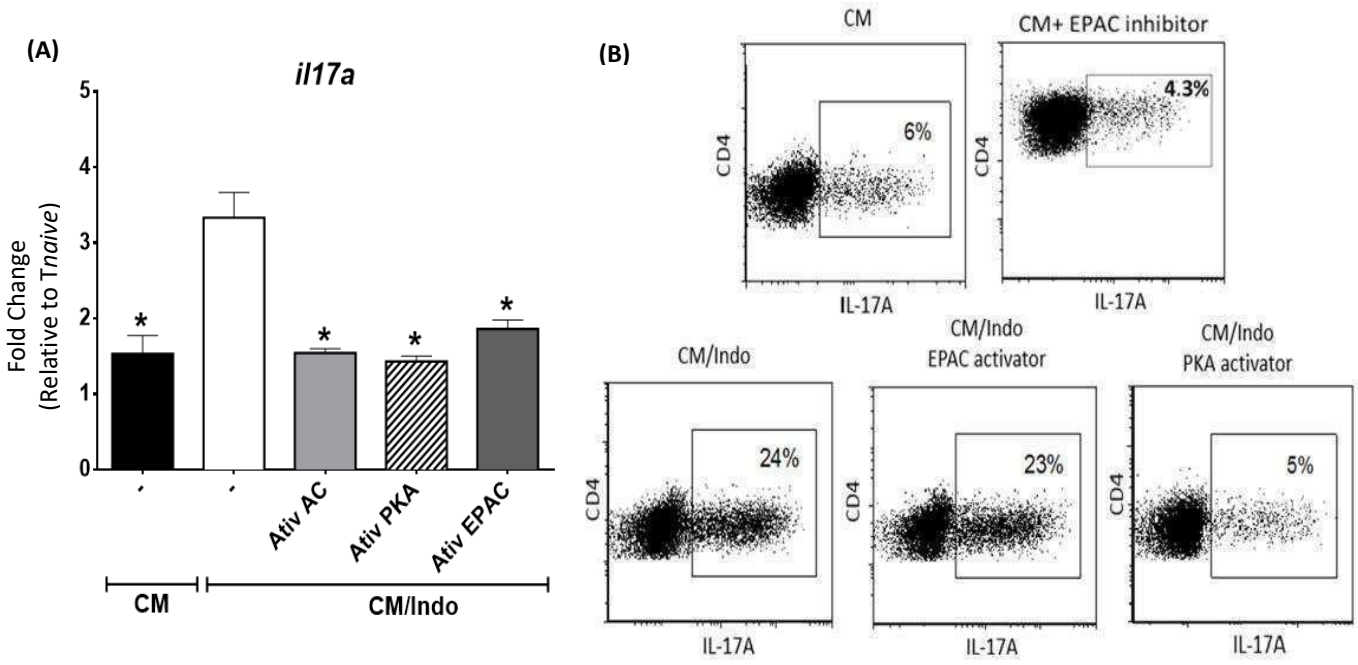


**Fig 3. Ativação de PKA inibe a diferenciação de linfócitos.** Os linfócitos T CD4+ *naive* foram cultivados com anti-CD3 e anti-CD28 na presença de CM, CM na presença de 5µM inibidor de EPAC (ESI-09), CM/Indo apenas ou CM/Indo na presença de 5µM de ativador de AC (Forskolin), CM/Indo na presença de 5µM de ativador de PKA (8-Bromo-cAMP) ou CM/Indo na presença de 5µM de ativador de EPAC (8CPT-2Me-cAMP). (A) Após 48 horas de cultura, as células foram lisadas e a expressão de *il17a* foi quantificada por qPCR. Os resultados estão representados como média ± SEM de 3 experimentos independentes. * p<0,05 vs CM/Indo. Análise da variância ANOVA, pós-teste Bonferroni. (B) Após 72 horas de cultura, as células foram marcadas e analisadas por citometria de fluxo. Resultado representativo de 3 experimentos independentes.

### *Altos níveis de $PGE_2$ no CM inibe a expressão de IL-1R durante a diferenciação de células Th17*

Resultados prévios obtidos por nosso grupo demonstram que a eferocitose de células apoptóticas infectadas por DC leva a síntese de altos níveis de $PGE_2$ e a presença desse prostanóide leva a inibição da diferenciação de linfócitos Th17. Além disso, por meio do uso de uma plataforma qPCR *array*, foi também observado que a presença de altos níveis de $PGE_2$ resulta em uma drástica inibição da expressão do gene *il1r,* assim como na expressão de mais de 21 genes em linfócitos T [6]. Além disso, através do uso de um anticorpo bloqueador de IL-1R, bem como um antagonista desse receptor (aIL-1R),

Dejani. N. (2015), demonstrou que a sinalização deste receptor está diretamente relacionada com a capacidade dos linfócitos T CD4+ "*naive*" diferenciarem-se em linfócitos Th17.

Mediante a importância de IL-1β na diferenciação de células Th17 no contexto de eferocitose de células apoptóticas infectadas e o efeito inibitório de $PGE_2$ na expressão do gene *il1r*, nosso próximo passo foi confirmar o resultado do qPCR *array* através da técnica de qPCR convencional, bem como avaliar este efeito inibitório na proteína IL-1R. Para isso, linfócitos T CD4+ "*naive*" foram diferenciados na presença de CM ou CM/Indo, foram analisados quanto a expressão de *il1r* por qPCR, ou quanto a expressão da proteína IL-1R por citometria de fluxo. A análise por qPCR confirmou o resultado do *array*, uma vez que as células diferenciadas na presença de CM/Indo (baixas concentrações de $PGE_2$) apresentaram uma maior expressão de *il1r* (Fig. 5A). O mesmo efeito inibitório de $PGE_2$ foi observado na expressão da proteína IL-1R, como pode ser observado pela menor intensidade de fluorescência exibido pelas células diferenciadas na presença de CM, ou seja, contendo altos níveis de $PGE_2$ (Fig. 5B).

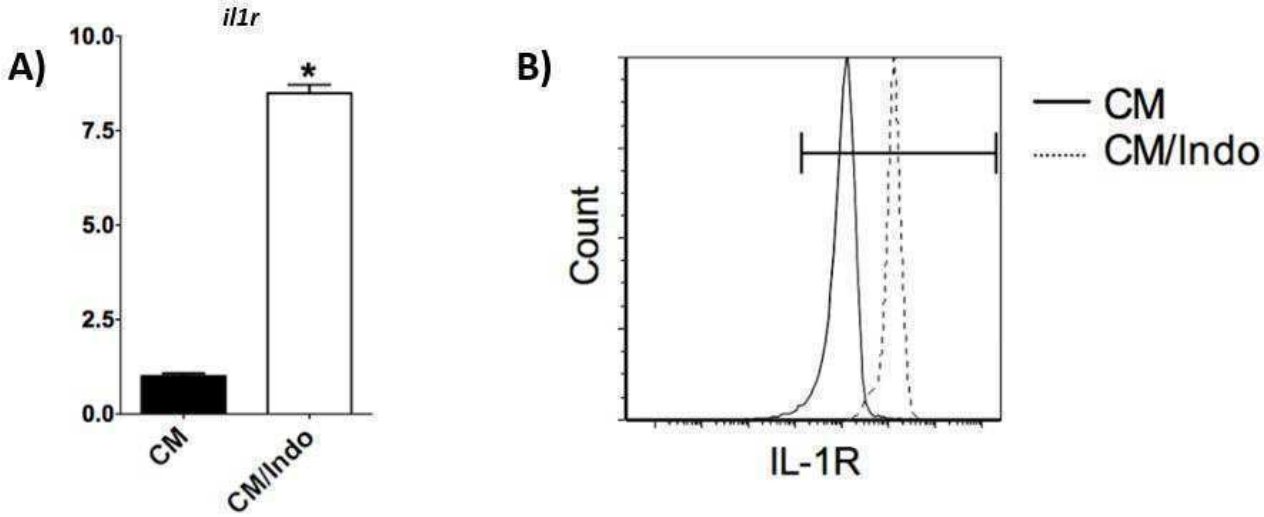


**Fig 5. $PGE_2$ inibe a expressão de IL-1R em linfócitos T CD4+ "naive".** Os linfócitos T $CD4^+$ *naive* foram cultivados com anti-CD3 e anti-CD28 na presença de CM, ou CM/Indo. (A) Após 48 horas de cultura, as células foram lisadas e a expressão de *Il1r* foi quantificada por qPCR. Os resultados estão representados como média ± SEM de 3 experimentos independentes. * $p<0,05$ vs CM. Análise da variância ANOVA, pós-teste Bonferroni. (B) Após 72 horas de cultura, as células foram marcadas e analisadas por citometria de fluxo. Resultado representativo de 2 experimentos independentes.

***O eixo adenilato ciclase/PKA inibe a expressão de IL-1R***

Considerando que houve a confirmação do efeito inibitório de $PGE_2$ sobre a expressão de IL-1R, o próximo passo foi avaliar se essa inibição também ocorre através do eixo adenilato ciclase/PKA, o mesmo eixo que leva a inibição da diferenciação de linfócitos Th17. Para isso, linfócitos T CD4+ "naive" foram diferenciados na presença de CM, CM/Indo, ou CM/Indo e tratados com os ativadores de adenilato ciclase ou PKA, e então a expressão de IL-1R foi analisada por citometria de fluxo (Fig. 6).

Foi observado que os linfócitos T CD4+ diferenciados na presença de CM, ou CM/Indo na presença dos ativadores de adenilato ciclase ou PKA apresentaram a inibição na expressão de IL-1R, quando comparados aos linfócitos diferenciados na presença de CM/Indo, sendo que o ativador de adenilato ciclase foi o que proporcionou a maior inibição na expressão desse receptor.

O IL-1R vem sendo descrito como um fator importante durante a diferenciação de linfócitos Th17. Sabe-se que linfócitos T CD4+ obtidos de animais deficientes para IL-

1R apresentaram uma expressiva inibição na diferenciação de células Th17 quando comparado a linfócitos de animais WT [51]. Além disso, a sinalização da citocina IL-1β é essencial na diferenciação de linfócitos Th17 com um perfil patogênico, uma vez que sua presença reduz a produção de IL-10, e contribui para produção de IFN-γ pelos linfócitos [52]. Dessa forma, sugerimos que a inibição da expressão de IL-1R durante a diferenciação de células Th17, na condição de CM, ocorre via $PGE_2$-adenilato ciclase/PKA.

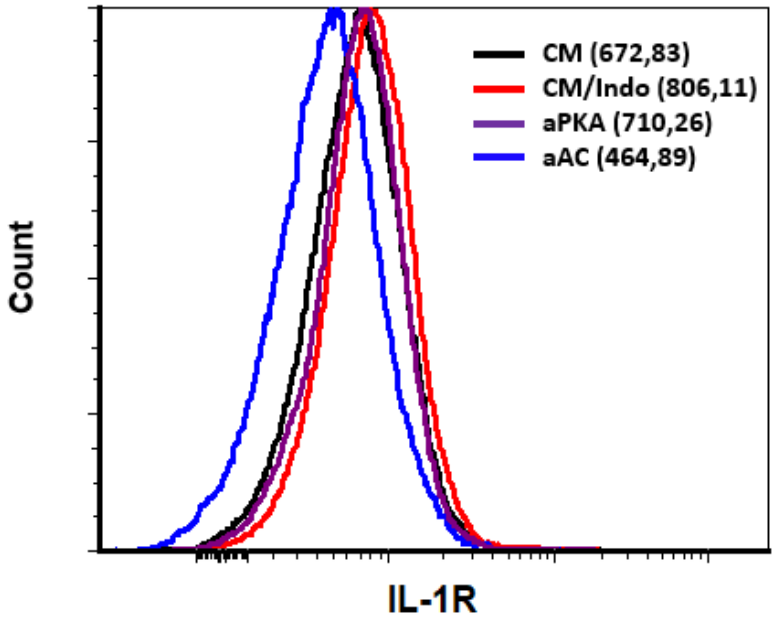


**Fig 6. Ativação de adenilato ciclase e PKA inibe a expressão de IL-1R.** Os linfócitos T $CD4^+$ *naive* foram cultivados com anti-CD3 e anti-CD28 na presença de CM, CM/Indo apenas ou CM/Indo na presença de 5μM de ativador de AC (Forskolin), CM/Indo na presença de 5μM de ativador de PKA (8-Bromo-cAMP). Após 72 horas de cultura, as células foram marcadas e analisadas por citometria de fluxo. Resultado representativo de 2 experimentos independentes.

***Sinalização de $PGE_2$ via EP4/Adenilato Ciclase/PKA inibe a ativação de STAT3.***

Uma vez que a ativação de PKA está relacionada com a inibição da expressão de genes diretamente relacionados a diferenciação de células Th17, o próximo passo foi a determinar a via de sinalização pela qual PKA induz essa inibição de IL-1R e diferenciação de células Th17. Sabe-se que durante a diferenciação de linfócitos Th17 há o aumento na expressão de genes como *il17a*, *il1r e irf4* que são regulados por um

complexo de fatores de transcrição[13,14]. Dentre os fatores de transcrição envolvidos na diferenciação de Th17, tem-se descrito a participação de RORγt, RORα, RUNX1 e STAT3, sendo esses ativados pela sinalização dos receptores de IL-6 e TGF-β [11].

Uma vez que a sinalização de IL-1R é importante para a expressão de genes relacionados a diferenciação de linfócitos Th17 [40,53], e sua expressão é induzida pela ativação de STAT3 [54], os experimentos subsequentes foram conduzidos com foco na ativação de STAT3, para avaliar se o mecanismo pelo qual $PGE_2$ inibe a expressão de IL-1R e a diferenciação de células Th17 é através da inibição de STAT3p

Sabe-se que a sinalização pelo receptor de IL-6 resulta na ativação de JAK-quinases que fosforilam tirosinas no complexo receptor intracelular, favorecendo locais de ancoragem para as STATs, que resultam na fosforilação e dimerização dessas proteínas. As proteínas STAT3, uma vez fosforiladas, translocam-se para o núcleo e irão se complexar a outros fatores de transcrição, como IRF4 e RORγt. Esse complexo de proteínas liga-se à regiões promotoras, induzindo assim a expressão de genes como *il17a* e *il1r* [38,55]. Portanto, o próximo passo foi avaliar se a sinalização de $PGE_2$, via EP4/PKA seria capaz impedir a fosforilação de STAT3 e inibir dessa forma a expressão de *il17a* e *il1r*.

Para tanto, os linfócitos T $CD4^+$ *naive* foram cultivados na presença de CM ou CM/Indo e a fosforilação de STAT3 foi determinada por PhosFlow. Pode-se observar que, na condição CM, a fosforilação de STAT3 foi inibida em comparação com linfócitos cultivados em CM/Indo (Fig. 7A). Para comprovar que esse efeito ocorre em função da sinalização de $PGE_2$ via EP4, foram utilizados agonista e antagonista de EP4, bem como os ativadores de PKA e EPAC. Os resultados mostram que, na condição CM, o tratamento com antagonista de EP4 foi capaz de restaurar a fosforilação de STAT3p. Por outro lado, na presença de CM/Indo, tanto o tratamento com agonista de EP4 (Fig. 7B), como a

presença de agonistas de PKA e EPAC tiveram efeito inibitório na fosforilação de STAT3 (Fig 7C).

Esses resultados indicam que altas concentrações de $PGE_2$, produzida por DC durante a eferocitose de células infectadas, inibem a fosforilação de STAT3 e, consequentemente, a expressão de *il17a* em linfócitos T $CD4^+$ *naive*, via EP4/PKA. EM relação aos tratamentos com os agonistas de PKA e EPAC, a presença do ativador de PKA foi capaz de suprimir a fosforilação de STAT3 de maneira mais acentuada. A inibição da fosforilação de STAT3 corroboram com o resultado apresentado previamente, visto que apenas o ativador de PKA foi capaz de inibir a diferenciação de linfócitos de células Th17 (Fig. 4B). Portanto a inibição de diferenciação de Th17 por $PGE_2$ via ativação de EP4/PKA, mas não de EPAC, parece ser a via mais preponderante neste processo de inibição tanto da fosforilação de STAT3, como da ativação de células Th17.

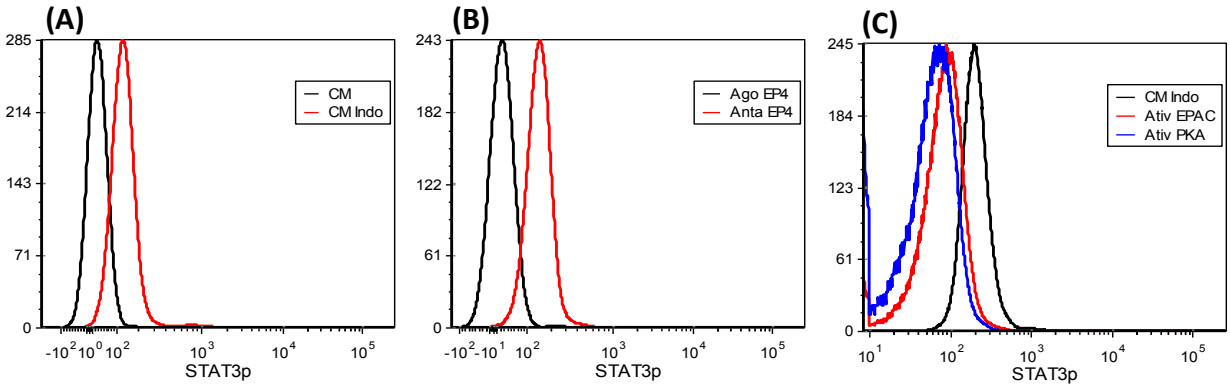


**Fig 7. Inibição de STAT3p via EP4/PKA.** Os linfócitos T $CD4^+$ *naive* foram cultivados com anti-CD3 e anti-CD28 na presença de: **(A)** CM ou CM/Indo; **(B)** CM + 5µM Antagonista de EP4 (L161,982) ou CM/Indo + Agonista de EP4 (Cay10598); **(C)** CM/Indo; CM/Indo + 5µM de ativador de AC (Forskolin); CM/Indo + 5µM de ativador de PKA (8-Bromo-cAMP) ou CM/Indo + 5µM de ativador de EPAC (8CPT-2Me-cAMP). Após 15 minutos de cultura as células foram marcadas e analisadas por PhosFlow. Gráfico representativo de 3 experimentos independentes.

***$PGE_2$ induz a expressão de SOCS1, mas não de SOCS3***

Considerando o efeito inibitório de $PGE_2$, via sinalização EP4/PKA, na fosforilação de STAT3, o próximo passo foi investigar o mecanismo pelo qual PKA poderia influenciar na fosforilação de STAT3p, resultando na inibição da diferenciação de linfócitos Th17.

Sabe-se que a sinalização das citocinas, via STATs, é regulada por uma família de proteínas chamadas SOCS (*Suppressor of cytokine signaling*), proteínas essas capazes de interferir nessa sinalização. Algumas proteínas SOCS atuam na via JAK/STAT por meio de um impedimento alostérico, como por exemplo SOCS1 e SOCS3. SOCS1 liga-se diretamente no sítio de ativação de JAK, e SOCS3, em resíduos de tirosina fosforilados presentes nos receptores de citocinas ativados, ambas interferindo na fosforilação STAT3, que é essencial na diferenciação de linfócitos Th17 [56].

Estudos anteriores demonstraram que a $PGE_2$, juntamente com IL-6 ou IL-10, é capaz de diminuir a atividade de STAT3 em macrófagos, e isto deve-se ao aumento na expressão de SOCS3 [45]. Dessa forma, os próximos experimentos foram conduzidos com o intuito de avaliar se a presença de $PGE_2$, no contexto da fagocitose de células apoptóticas infectadas, seria capaz de induzir a expressão das proteínas SOCS1 e SOCS3, durante a diferenciação em linfócitos Th17.

Para tanto, os linfócitos T $CD4^+$ *naive* foram diferenciados na presença de CM ou CM/Indo, e a expressão de *socs1* e *socs3* foi quantificada. Os linfócitos T CD4+ diferenciados na presença de CM ou CM/Indo, não apresentaram diferenças em relação a expressão de *socs3* (Fig. 8A). No entanto, quando avaliamos a proteína *socs1* foi observado o aumento expressão desta proteína na presença de CM quando comparado a condição CM/Indo (Fig.8B).

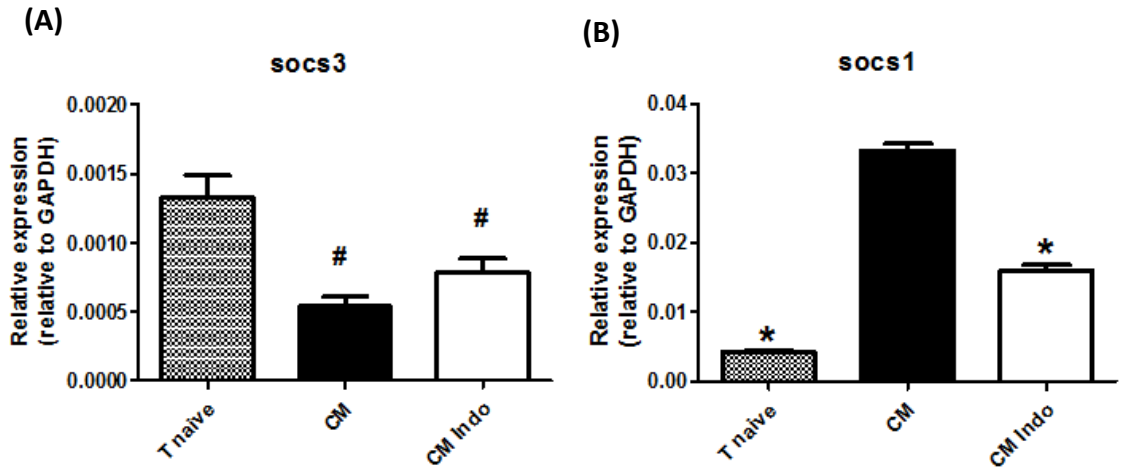


**Fig 8. Aumento na expressão de socs1 em células tratadas com CM.** Linfócitos T CD4$^+$ *naive* foram cultivados com anti-CD3 e anti-CD28 na presença de CM ou CM/Indo. Após 24h, as células foram lisadas para extração de RNA e a expressão de *socs1* e *socs3* foi analisada por qPCR. Os resultados estão representados como média ± SEM de 3 experimentos independentes. ** p<0,05 vs CM; # p<0,05 vs CM/Indo.* Análise da variância ANOVA, pós-teste Bonferroni

***Padronização da técnica de silenciamento utilizando a técnica de "small interference" - siRNA***

Visto que a presença de altos níveis de $PGE_2$ na condição CM, induz o aumento da expressão de *socs1,* mas não de *socs3*, o próximo passo foi tentar comprovar se a inibição da fosforilação de STAT3 estaria diretamente envolvida com a inibição mediada por *socs1*. Para tanto, foi utilizada a técnica de silenciamento gênico, utilizando como ferramenta o silenciamento por siRNA.

Os linfócitos T "*naive*" foram transfectados com lipofectamina contento a sequência siRNA, na presença de meio Opti-MEM e, após 48h, as células foram lisadas analisadas por qPCR. Nesta primeira tentativa, não foi observado a diminuição na expressão de *socs1* quando comparado com a sequência a sequência controle (*scrambled* siRNA) (Fig. 9). Uma possível explicação para o resultado negativo deve-se ao fato de ter sido utilizado a transfecção de siRNA via lipofectamina que é bem estabelecido em células aderente, e protocolo sugerido de acordo com as instruções do fabricante. No

entanto, um dos fatores limitantes desse ensaio parece ser a utilização desse protocolo de transfecção em células não aderentes.

A padronização da transfecção está sendo realizada novamente, não apenas para *socs1*, mas também para *socs3,* a partir de algumas otimizações no protocolo sugerido pelo fabricante, bem como o uso de outra técnica de transfecção, a eletroporação. No entanto, não obtivemos sucesso no silenciamento dos genes devido à alta taxa de morte celular.

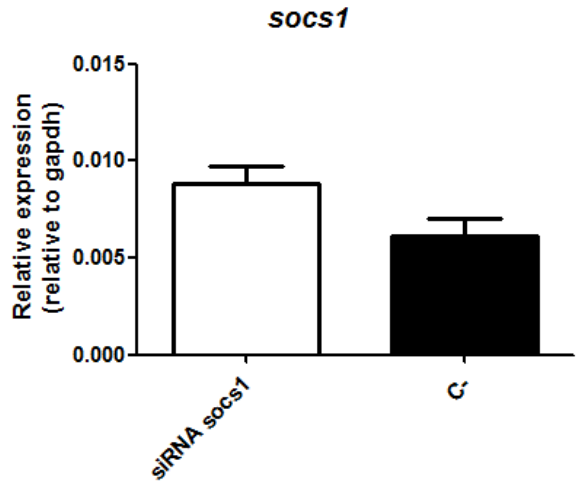


**Fig 9. Padronização do silenciamento gênico de socs1.** Linfócitos T CD4+ *naive* foram cultivados com anti-CD3 e anti-CD28 na presença do conjugado de lipofectamina + siRNA para *socs1*, ou do conjugado de lipofectamina + siRNA controle. Após 48h, as células foram lisadas para extração de RNA e a expressão de *socs1* foi analisada por qPCR.

### *Silenciamento funcional de SOCS1 - peptídeo inibidor de SOCS1 "Kinase inhibitory region" (iKir SOCS1)*

Uma vez que a padronização do silenciamento gênico de *socs1*, utilizando a técnica a transfecção de siRNA via lipofectamina não foi possível, paralelamente optamos por um protocolo alternativo utilizando como ferramenta um peptídeo inibidor de SOCS1, denominado *iKir* SOCS1. Esse peptídeo é capaz de se ligar a *Kinase inhibitory region* (*Kir*), presente na SOCS1, e assim, inibir a função que a proteína exerce sobre a JAK [57].

Os linfócitos T CD4+ "*naive*" foram diferenciados na presença de CM, e tratados com 20µM de *iKir* SOCS1 ou com 20µM de um peptídeo controle e então avaliados quanto à expressão de *il17a* por qPCR. Os linfócitos diferenciados na presença de CM e tratados com 20µM de *iKir* SOCS1, não apresentaram diferença na expressão de *il17a*, quando comparados com CM sem tratamento (Fig 10). Surpreendentemente, o tratamento de linfócitos T CD4 cultivados com CM na presença do peptídeo controle resultou no aumento na expressão de *il17a*. Uma das possíveis explicações seria possíveis interações inespecíficas desse "peptídeo irrelevante" como consequência da alta concentração (20µM) utilizada na cultura celular. Com isso, novos experimentos foram realizados utilizando diferentes concentrações dos peptídeos com o intuito de determinar o efeito da inibição de SOCS1 durante o processo de diferenciação de linfócitos Th17.

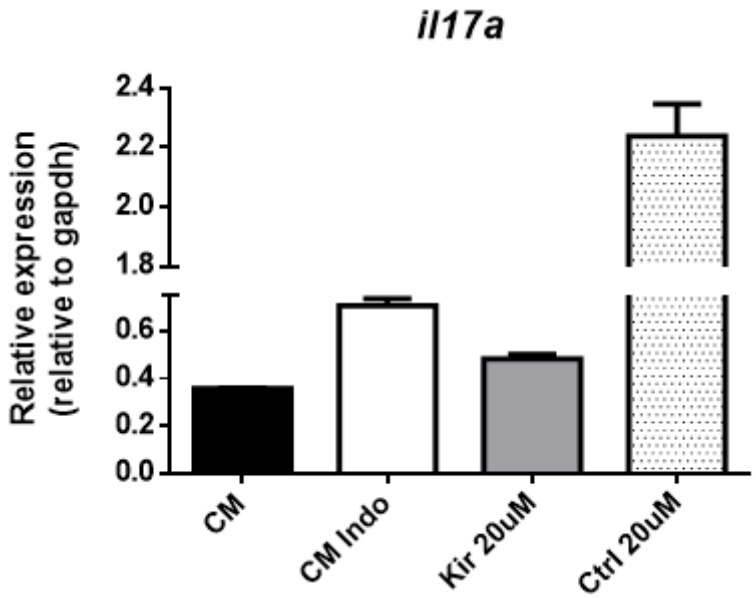


**Fig 10. Tratamento de linfócitos com iKir SOCS1 - qPCR.** Linfócitos T CD4$^{+}$ *naive* foram cultivados com anti-CD3 e anti-CD28 na presença de CM, e tratados com 20µm de iKir SOCS1 ou de peptídeo controle. Após 48h, as células foram analisadas por qPCR quanto à expressão de *il17a.*

Para definir qual a melhor concentração de iKir SOCS1 a ser utilizada, os linfócitos T CD4 cultivados na presença de CM foram incubados com diferentes concentrações dos peptídeos (5, 20 ou 40μM) e a diferenciação de células Th17 foi avaliada por citometria de fluxo. As concentrações 20 e 40μM induziram uma alta taxa de morte celular, como pode ser observado nas imagens de perfil celular, quando comparado aos linfócitos diferenciados apenas com CM ou tratados com 5μM iKir SOCS1 (Fig. 11). Diferente do esperado, o tratamento com 5μM SOCS1 promoveu uma discreta diminuição na diferenciação de Th17 (~13%) quando comparados ao controle CM (20%), e os tratamentos com o peptídeo controle bem como iKir, não apresentaram diferenças entre si (Fig. 11). Uma possível explicação seria que a concentração 5μM deste peptídeo pode estar fora da janela terapêutica, dessa forma, a baixa concentração impossibilita que o peptídeo desempenhe sua função inibidora de maneira eficaz. Apesar da presença de $PGE_2$ resultar no aumento na expressão de *socs1*, através das técnicas utilizadas para a análise da atividade funcional, não foi possível concluir se SOCS1 está envolvida no mecanismo de inibição de Th17.

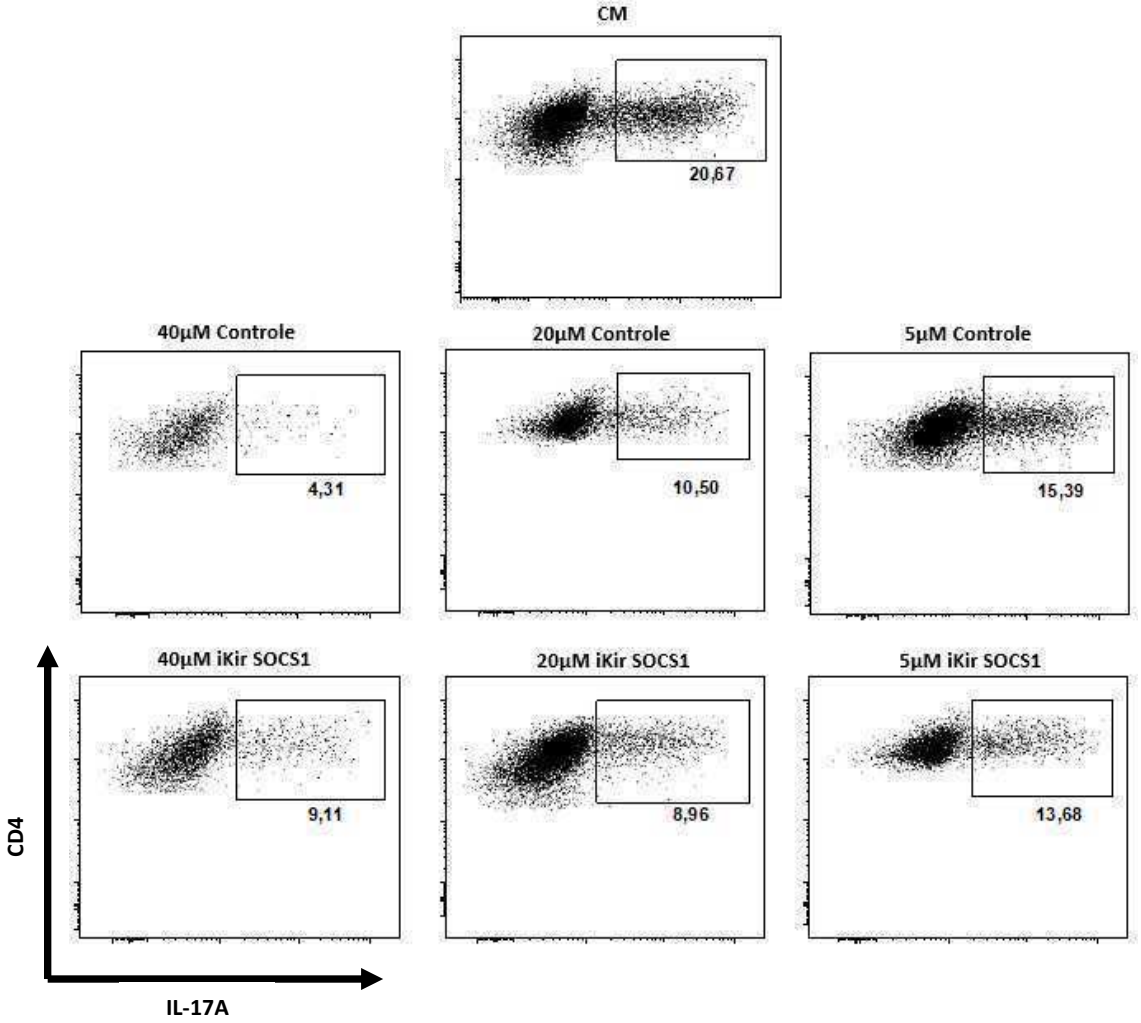


**Fig 11. Tratamento de linfócitos com iKir SOCS1:** Linfócitos T CD4$^+$ *naive* foram cultivados com anti-CD3 e anti-CD28 na presença de CM, e tratados com 5, 20 ou 40µM de iKir SOCS1 ou de peptídeo controle. Após 72h, as células foram analisadas por citometria de fluxo quanto à diferenciação em linfócitos Th17. Resultado representativo de 2 experimentos independentes.

### *PI3K não participa da inibição de Th17 pela ação de $PGE_2$*

Já se tem descrito que a sinalização de $PGE_2$ via receptor EP4 é capaz de induzir a ativação da enzima PI3K [58]. Além disso, sabe-se que essa quinase é essencial para a diferenciação de linfócitos Th17, visto que os linfócitos T CD4+ que expressam uma PI3K não funcional são incapazes de se diferenciar em linfócitos Th17 [35]. Considerando esse papel importante da PI3K para os linfócitos Th17, e sua ativação através do receptor de $PGE_2$, nosso próximo questionamento foi avaliar se PI3K, induzida pela sinalização de $PGE_2$-EP4, estaria envolvida na inibição da diferenciação de células Th17. Para isso,

os linfócitos T CD4+ "naive" foram diferenciados na presença de CM e tratados 500, 100 e 10nM de wortmanina, um inibidor de PI3K, e analisados quanto a expressão de *il17a* por qPCR, e quanto a diferenciação em linfócitos Th17 por citometria de fluxo.

O tratamento com o inibidor de PI3K foi capaz de inibir a expressão de *il17a* (Fig. 12A), bem como a diferenciação de linfócitos Th17, e essa inibição foi mais pronunciada quando comparado a condição de linfócitos T CD4+ diferenciados na presença de CM (Fig. 12B).

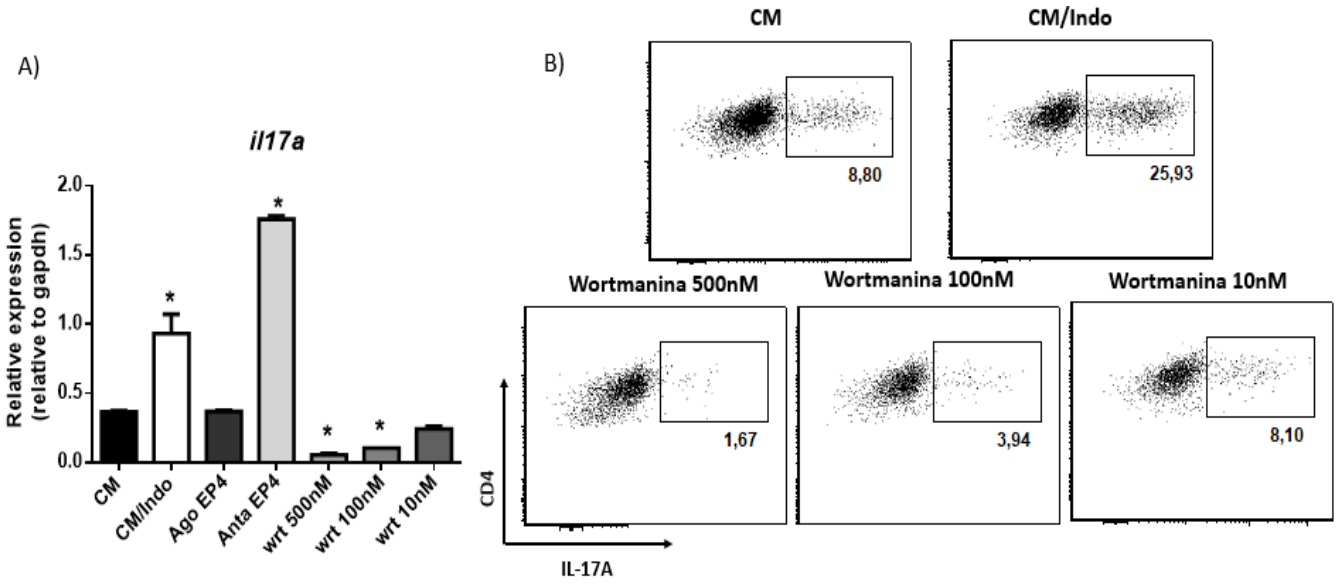


**Fig 12. Sinalização de PI3K é essencial para a diferenciação de linfócitos Th17.** Os linfócitos T CD4+ *naive* foram cultivados com anti-CD3 e anti-CD28 na presença de CM, CM na presença de 500, 100 e 10nM de inibidor de PI3K (Wortmanina), CM/Indo. (A) Após 48 horas de cultura, as células foram lisadas e a expressão de *il17a* foi quantificada por qPCR. Os resultados estão representados como média ± SEM de 3 experimentos independentes. * p<0,05 vs CM. Análise da variância ANOVA, pós-teste Bonferroni. (B) Após 72 horas de cultura, as células foram marcadas e analisadas por citometria de fluxo. Resultado representativo de 3 experimentos independentes.

Como já demonstrado previamente por Haylock-Jacobs (2011) PI3K é essencial para a diferenciação de linfócitos Th17, além disso, também é essencial para ativação de células T CD4+. A PI3K, uma vez ativada, pode fosforilar outras proteínas, como ERK quinases e Akt, que por sua vez, modulam a expressão de genes relacionados com a

ativação e proliferação dos linfócitos T. Portanto, ao utilizarmos o inibidor de PI3K tanto a sinalização mediada por $PGE_2$, bem como àquela induzida pela sinalização via TCR (Fig. 13) foram interrompidas, e indiretamente, a diferenciação de células Th17 foi afetada.

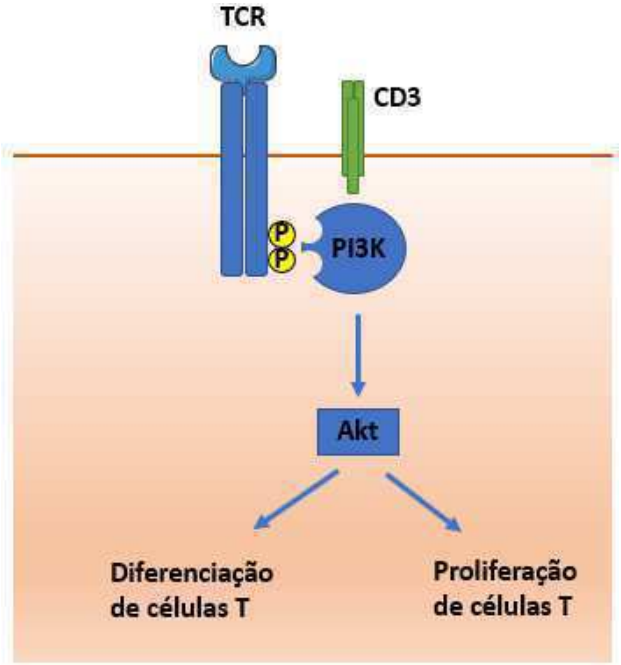


Figura adaptada de Srivastava, *et al.* 2013

**Fig 13. Ativação de PI3K via TCR.** PI3K é capaz de interagir com o domínio SH2 na porção intracelular do TCR, onde é ativada, e passa então a fosforilar seus substratos, os quais estão envolvidos com a diferenciação e proliferação de linfócitos Th17 [59].

Com isso, é possível concluir que apesar possível ativação de PI3K pela $PGE_2$ presente em CM, o qual contribui com a diferenciação de linfócitos Th17, a sinalização via EP4 também ativa a via da adenilato ciclase/PKA/EPAC, o qual se sobressai, inibindo a expressão de genes essenciais para a diferenciação deste subtipo celular, e consequentemente a diferenciação de linfócitos Th17.

## Conclusão

Os resultados apresentados indicam que a $PGE_2$, oriunda da fagocitose de células apoptóticas infectadas, é capaz de inibir a diferenciação de linfócitos T $CD4^+$ *naive* em Th17, através do receptor EP4 resultando no aumento de cAMP intracelular (Fig. 14A). O aumento deste mensageiro secundário leva a ativação PKA e inibição da fosforilação de STAT3 (Fig. 14B), impedindo desta forma, a translocação desse fator de transcrição ao núcleo. Além disso, a ativação do eixo EP4/adenilato ciclase/PKA promove a inibição da expressão de IL-1R, receptor que exerce grande importância na diferenciação de linfócitos Th17, e sua inibição repercute na inibição desse subtipo linfocitário. No entanto, apesar de $PGE_2$ induzir a expressão de *socs1* (Fig. 14C), bem como de PI3K (Fig. 14D), não foi possível concluir se ambas estão envolvidas no mecanismo de inibição de Th17 através das estratégias farmacológicas utilizadas.

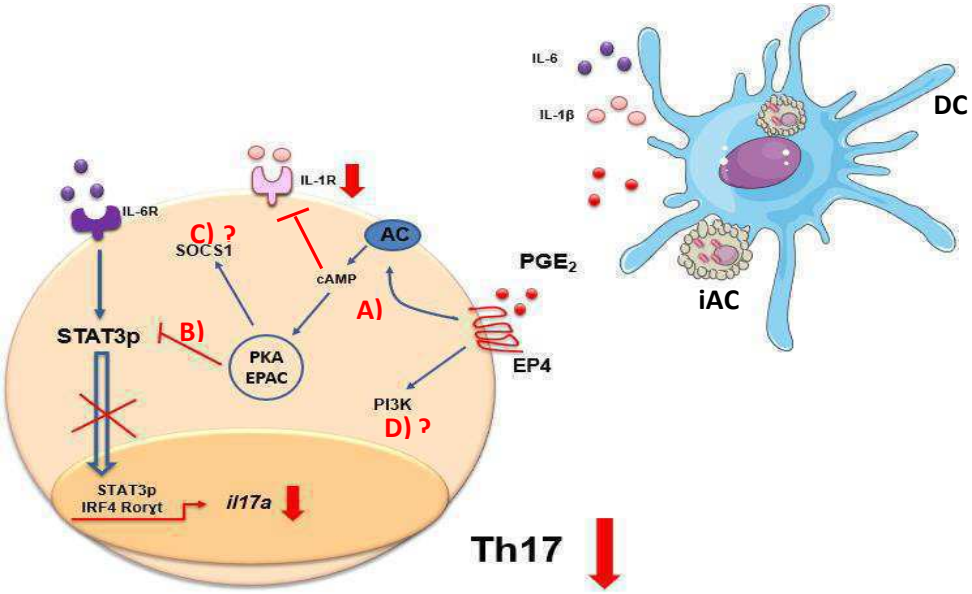


**Fig 14. $PGE_2$ derivada da eferocitose de células apoptóticas infectadas inibe STAT3p e a diferenciação de Th17 via receptor EP4.** A) $PGE_2$, ativa o receptor EP4, desencadeia a sinalização via adenilato ciclase/PKA/EPAC. B) $PGE_2$ via adenilato ciclase/PKA/EPAC inibe STAT3p. C) e D) $PGE_2$ induz o aumento na expressão de SOCS1 e PI3K, no entanto o envolvimento de ambas durante a inibição de linfócitos Th17 ainda requer mais estudos. iAC = célula apoptótica infectada; DC= Célula dendrítica; Th17= Linfócito T *helper* 17; AC = Adenilato Ciclase.

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# Capítulo 2

# Intestinal host defense outcome is dictated by $PGE_2$ production during efferocytosis of infected cells

Naiara Naiana Dejani[1,2] (Dejani, NN), Allan Botinhon Orlando[1] (Orlando, AB), Victoria Eugenia Niño[1] (Ninõ, VE), Letícia de Aquino Penteado[1] (Penteado, LA), Felipe Fortino Verdan[1,2] (Verdan, FF), Júlia Miranda Ribeiro Bazzano[1] (Bazzano, JMR), Ana Campos Codo[1] (Codo, AC), Ana Carolina Guerta Salina[1,2] (Salina, ACG), Luis Carlos Spolidorio[3] (Spolidorio, LC), C. Henrique Serezani[4] (Serezani, CH), Alexandra Ivo de Medeiros[1,2] (Medeiros, AI)

[1]Department of Biological Sciences, School of Pharmaceutical Sciences, UNESP - Univ Estadual Paulista, Araraquara, São Paulo, Brazil. [2]Department of Biochemistry and Immunology, Faculty of Medicine of Ribeirão Preto, USP - University of São Paulo, Ribeirão Preto, São Paulo, Brazil. [3]Department of Physiology and Pathology, School of Dentistry, UNESP - Universidade Estadual Paulista, Araraquara, São Paulo, Brazil. [4] Department of Medicine, Division of Infectious Diseases, Vanderbilt University Medical Center, Nashville, TN, USA.

**Corresponding author:**
Prof. Dr. Alexandra Ivo de Medeiros. Department of Biological Sciences, School of Pharmaceutical Sciences, UNESP - Univ Estadual Paulista, Araraquara, São Paulo, Brazil. Rodovia Araraquara-Jaú Km 1, 14801-902, Araraquara, São Paulo, Brazil. 55-16-3301-6049. E-mail: medeirosai@fcfar.unesp.br

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**Abstract**

Inflammatory responses are terminated by the clearance of dead cells, a process termed efferocytosis. A consequence of dead cell removal is the synthesis of the anti-inflammatory mediators TGF-β, $PGE_2$, and IL-10. However, the efferocytosis of *Citrobacter rodentium*-infected cells favors Th17 responses by eliciting the synthesis of TGF-β, IL-6 and IL-23. Recently, we showed that the efferocytosis of *Escherichia coli*-infected macrophages by dendritic cells also triggers $PGE_2$ production in addition to pro-Th17 cytokine expression. We therefore examined the role of $PGE_2$ during Th17 differentiation and intestinal pathology. The efferocytosis of *E. coli*-infected cells impaired IL-1R expression and inhibited Th17 differentiation via $PGE_2$-EP4-PKA. The outcome of murine intestinal *C. rodentium* infection was greatly dependent on the EP4 receptor. EP4 antagonist-treated infected mice showed enhanced intestinal host defense compared to vehicle-treated infected mice. These data suggest that therapeutically targeting EP4 signaling during infectious colitis may be promising to enhance Th17 immunity and host defense.

**Significance Statement**

The present study provided the first evidence of a novel regulatory mechanism by which $PGE_2$, produced by efferocytosis of infected cells, suppresses Th17 differentiation and compromises adaptive immunity. An important finding of this study is that $PGE_2$ is produced by DCs during the uptake of infected apoptotic cells *in vitro* and during enteric *C. rodentium* infection *in vivo*. Targeting $PGE_2$ markedly improved Th17 differentiation and intestinal host defense against *C. rodentium*. The suppressive effect of $PGE_2$ was mediated via the EP4-cAMP-PKA pathway, which impaired IL-1R expression in T cells and compromised the Th17 phenotype. Moreover, selective impairment of EP4 signaling increased the colonic Th17 population and antimicrobial peptide expression, resulting in the reduction of *C. rodentium* load in the colon.

## Introduction

A variety of microorganisms are able to trigger different types of cellular death, such as apoptosis (1, 2). The ingestion of dead cells, a process termed efferocytosis, is critical to maintain homeostasis and prevent autoimmune disorders (3). Efficient clearance of non-infected apoptotic cells, induced by stress, damage or cellular turnover, by macrophages or dendritic cells (DCs), is essential to inhibit inflammatory responses (4). Efferocytosis induces the production of transforming growth factor-β (TGF-β), interleukin-10 (IL-10), prostaglandin E2 (PGE2) and platelet-activating factor (PAF) and inhibits the secretion of inflammatory mediators, such as TNF-α, IL-1, CXL1, IL-8 and leukotriene C4 (5, 6). However, during some infections, the recognition of infected apoptotic cells by DCs induces a proinflammatory program that leads to T cell immunity (7) and antimicrobial responses (8, 9). The efferocytosis of Mycobacterium tuberculosis-infected macrophages favors pathogen killing (9) and promotes adaptive immunity by cross-priming CD8+ T cells (10). Moreover, the phagocytosis of herpes simplex virus-1-infected cells leads to viral antigen-specific CD8+ T cell activation in vitro and in vivo (11). Upon uptake of Escherichia coli-infected apoptotic cells, DCs release IL-23, TGF-β, and IL-6 and induce Th17 cell differentiation (7). In addition to Th17-polarizing cytokines, we recently reported that the efferocytosis of E. coli-infected cells leads to PGE2 production by DCs (12, 13). PGE2 is one of the most abundant lipid mediators produced by both immune and structural cells and acts through four different G protein-coupled receptors (14). EP1 is coupled to Gαq and promotes the increase of intracellular Ca2+; EP3 is coupled to Gαi proteins and its activation inhibits cyclic adenosine monophosphate (cAMP) formation (15). However, EP2 and EP4 are Gαs-coupled receptors that lead to increased cAMP concentrations (15). Changes in cAMP induce pleiotropic cellular responses, via the activation of protein kinase A (PKA), and protein exchange directly activated by cAMP (EPAC)-dependent and cAMP (EPAC)-independent pathways (16).

While all four EP receptors are detected in naive CD4+ T cells (17), EP2 and EP4 are the most abundant and more potent EP receptors expressed on effector Th17 cells (18). PGE2 plays both an important and controversial role in the activation, differentiation and expansion of Th17 cells (19, 20). Despite the well-known effects of PGE2 in Th17 development, the relevance of PGE2 produced during the efferocytosis of infected dead cells in Th17 cells in the gut host defense remains undetermined.

IL-6 and TGF-β are critical for the activation of the transcription factors RORγt and STAT3 to promote Th17 commitment. Moreover, the upregulation of IL-1R, IL-23R,

IL-21R expression and IL-21 production by early Th17 cells favors the expansion of these cells and their subsequent expression of IL-17 (21). Additionally, PGE2 acts synergistically with IL-23 and favors the expression of IL-17A, (18) (22) and it directly improves IL-17 expression in human Th17 cells in vitro (23). Moreover, exogenous PGE2 increases IL-23 production in DCs and promotes IL-1 and IL-23 receptor expression, which further drives the expansion of Th17 cells (24, 25). However, Valdez et al 2014 demonstrated that PGE2 via EP2/4 inhibits IRF4 activation and impairs Th17 cell differentiation and immunity against Cryptococcus neoformans infection in mice (26).

The pathogen Citrobacter rodentium causes mouse intestinal infection and has similar virulence factors as the human pathogen enteropathogenic and enterohemorrhagic E. coli (27). Additionally, C. rodentium infection is a model used to study chronic human intestinal diseases, such as ulcerative colitis and Crohn's disease (28), and intestinal host defense (27). IL-17 and IL-22 play an important role during the resolution of C. rodentium infection (29). This infection causes the apoptosis of intestinal epithelial cells, which is critical for Th17 cell differentiation in vivo, since inhibiting apoptosis during C. rodentium infection drastically impairs typical colonic Th17 responses (7). Thus, the role of PGE2 produced during the efferocytosis of infected cells in Th17 cell differentiation and intestinal host defense remains unknown; therefore, we set up experiments to determine whether PGE2 affects Th17 cell commitment to drive host defenses against intestinal infection in mice. These data revealed a novel regulatory mechanism by which PGE2 suppresses Th17 cell differentiation during the efferocytosis of infected cells and compromises adaptive immunity, suggesting that therapeutically targeting PGE2 actions during infectious colitis may greatly induce microbial clearance and restore intestine homeostasis.

## Results

### Th17 cell differentiation is controlled by PGE2 produced during the efferocytosis of E. coli-infected cells

Recognition of apoptotic Escherichia coli-infected cells leads to TGF-β, IL-6 and IL-23 production and triggers Th17 cell differentiation (7). In addition, we previously described that PGE2 and IL-1β are also produced during efferocytosis of E. coli-infected cells (12, 13). Consistent with previous studies (7, 12), we detected the production of PGE2, IL-1β, IL-6, IL-23 and TGF-β in the supernatant (also called conditioned medium, CM) of DCs co-cultured with apoptotic E. coli-infected cells (IAC) (Fig. 1A and B). The differentiation of naive CD4+ T cells in the presence of CM drove predominantly IL-17A expression and Th17 cell differentiation (Fig. 1C), whereas the low expression of IFN-γ and Foxp3 was observed (Supplementary Fig. 1A and C). Next, we confirmed that the products released from the efferocytosis of E. coli-infected cells by DCs were the crucial step to generate a microenvironment suitable for Th17 cell differentiation. We observed that only CM from co-culture was capable of inducing high Th17 cell differentiation, while supernatants from isolated infected apoptotic cells or DCs alone were not great inducers of IL-17-producing CD4+ T cells, indicating that the efferocytosis of infected cells by DCs is the critical player for Th17 induction (Supplementary Fig. 1B).

To determine whether PGE2 regulates DC-dependent Th17 generation, we treated DCs with the COX inhibitor, indomethacin (CM/Indo) or ibuprofen (CM/Ibup). The data showed that COX inhibition decreased PGE2 production correlated with reduced IL-1β and IL-23 synthesis, during the uptake of apoptotic E. coli-infected cells (Fig. 1B and C). Surprisingly, the incubation of naïve CD4+ T cells with CM/Indo or CM/Ibup, showed enhanced differentiation into Th17 cells compared to the CM, from untreated DCs (Fig. 1C and Supplementary Fig. 2). Although PGE2 may inhibit the proliferation of T cells (30), we did not observe any effects on Th17 cell proliferation when cells were cultured in either CM or CM/Indo (Supplementary Fig. 3). These data indicate that reduced Th17 cell differentiation in CM was not promoted by impaired proliferation of naive CD4+ T cells.

The specific role of PGE2 in the reduction of Th17 cell differentiation was further evidenced when we performed “add-back” experiments. The addition of PGE2 into CM/Indo reduced the differentiation of Th17 cells compared to CM/Indo alone (Fig. 1C). Moreover, when PGE2 was specifically depleted from CM, using a prostaglandin E2 affinity column (CM-PGE2), Th17 cell differentiation was higher compared to CM

(Supplementary Fig. 2). These data further confirm that PGE2 is indeed the main soluble mediator secreted during the efferocytosis of infected apoptotic cells that impairs Th17 cell differentiation.

**PGE2-EP4 signaling impairs Th17 cell differentiation**

To study which EP receptor could be mediating the PGE2 inhibitory effect, we initially determined the expression profile of EP receptors in T cells during differentiation into Th17 cells in the presence of CM. The data showed and further confirmed previous reports that EP4 was the most abundant receptor expressed in Th17 cells during differentiation when compared to EP1, EP2 and EP3 expression (Fig. 2A and B). In addition, we determined which receptor was involved in the suppression of Th17 cell differentiation by treating naïve CD4+ T cells with different EP agonists and antagonists. EP4 antagonist prevented CM-inhibited Th17 cell differentiation, while EP1 and EP2 antagonists did not show any effect on Th17 cell differentiation (Fig. 2C and D, Supplementary Fig. 4).

To further address the intracellular mechanism by which EP4 engagement compromises Th17 cell differentiation, naïve CD4+ T cells were treated with different concentrations of forskolin (adenylyl cyclase activator), 8-Bromo-cAMP (PKA activator) and 8-CPT-2Me-cAMP (EPAC activator) in the CM/Indo group. The incubation of naïve CD4+ T cells with forskolin and the 8-Bromo-cAMP impaired IL-17A production, while 8-CPT-2Me-cAMP did not affect CD4+ T cell differentiation into Th17, in the presence of CM/Indo (Fig. 2E). In addition, naïve CD4+ T cells treated with the PKA peptide inhibitor (PKI 14-22 amide-myristoylated) in CM showed enhanced IL-17A production compared to untreated cells (Supplementary Fig. 5).

Given that STAT3 dictates Th17 cell differentiation, we determined whether the PGE2-EP4-PKA axis could affect STAT3 phosphorylation and Th17 cell differentiation. Naïve CD4+ T cells cultured in the presence of CM showed reduced phosphorylated STAT3 compared to cells in CM/Indo (Fig. 2F). Moreover, EP4 antagonist restored STAT3 phosphorylation in cells in CM, while the EP4 agonist and PKA activator decreased STAT3 phosphorylation in cells in CM/Indo (Fig. 2F). Therefore, during the recognition of infected apoptotic cells, PGE2 produced by DCs inhibits Th17 cell differentiation via STAT3.

**PGE2 generated during clearance of infected apoptotic cells downregulates IL-1R expression in naïve CD4+ T cells**

Next, we investigated the gene expression profile by which PGE2 influences Th17 cell commitment by employing a Th17 focused qPCR gene array. In the presence of CM, we observed the downregulation of 21 genes during Th17 cell differentiation compared to CD4+ T cells differentiated in CM/Indo (Supplementary Table 1). The mRNA expression of il17f, il17a, il1r1, ccl2 and ccl7 was at least 4-fold higher in CD4+ T cells differentiated in CM/Indo or CM/Ibup compared to CM (Fig. 3A and Supplementary Fig. 6). The expression of il1r1 was also confirmed by individual qPCR and FACS analysis (Fig. 3A and B). The pretreatment of naïve CD4+ T cells with EP4, but not EP2 antagonist, further enhanced il1r1 and il17a expression in CD4+ T cells cultured with CM alone (Fig. 3C). Additionally, we observed that the EP4 agonist and forskolin (adenylyl cyclase activator) or 8-Bromo-cAMP (PKA activator) decreased il1r1 and il17a expression in cells cultured in CM/Indo.

IL-1R signaling is critical during the differentiation, commitment and maintenance of Th17 cells (31). To determine the cross-talk between the PGE2-EP4-IL-1R axis in Th17 fate, we treated naïve CD4+ T cells with the IL-1R antagonist (IL-1Ra, interleukin-1 receptor antagonist) or CM and CM/Indo with IL-1β neutralizing antibodies (anti-IL-1β) to block IL-1β actions. CD4+ T cells differentiated in CM in the presence of EP4 antagonist plus anti-IL-1β or IL-1Ra markedly showed a reduced percentage of IL-17A-producing lymphocytes (Fig. 4A). Indeed, the frequency of Th17 cells cultured in CM/Indo was significantly decreased when we blocked IL-1β actions, indicating that IL-1R signaling is required for Th17 cell differentiation in this context. These results suggest that during the efferocytosis of E. coli-infected cells PGE2 generated in the microenvironment might control Th17 cell differentiation by downregulating IL-1R expression via the EP4-PKA axis.

***Citrobacter rodentium* intestinal infection impairs Th17 cell differentiation through the PGE2-EP4 signaling pathway**

The pharmacological inhibition of apoptosis during C. rodentium infection drastically impairs Th17 responses (7). Although Th17 responses are crucial to control C. rodentium infection, the role of PGE2 in the pathogenesis and intestinal host defense against C. rodentium remains unknown. Therefore, to investigate whether PGE2 controls Th17 cell differentiation in vivo, we induced infectious colitis by C. rodentium in mice.

The mice were infected with C. rodentium and treated with indomethacin, EP4 antagonist or vehicle, as shown in Fig. 5A. C. rodentium infection markedly increased PGE2 production, whereas treatment with indomethacin significantly reduced the levels of PGE2 in the colons of infected mice compared to those of vehicle-treated infected mice (Fig. 5B). As expected, the EP4 antagonist did not affect the PGE2 levels in infected mice compared to those in vehicle-treated infected mice (Fig. 5B). The frequency of TCRβ+CD4+IL-17+ cells in the colonic lamina propria of indomethacin or EP4 antagonist-treated infected mice was at least 2-fold higher compared to that in vehicle-treated infected mice (Fig. 5C). In addition, infected mice treated with EP4 antagonist showed decreased weight loss and colon length reduction compared to vehicle-treated infected mice (Fig. 5D and E). The treatment of control noninfected mice with indomethacin or EP4 antagonist had no significant alteration in weight loss, colon length and Th17 cell population in the colonic tissue (Fig. 5C-E). However, infected animals treated with indomethacin or EP4 antagonist, showed increased Th17 cell population in the colon, consistent with the in vitro data (Fig. 5C). Moreover, il17a and il1r expression was higher in the colons of EP4 antagonist-treated infected mice compared to that in vehicle-treated infected mice (Fig. 6B).

Interestingly, infected mice that received indomethacin or EP4 antagonist showed a decreased number of C. rodentium bacteria in the colon compared to that of vehicle-treated infected mice (Fig. 6A). In addition, EP4 antagonist-treated infected mice showed enhanced antimicrobial peptide expression in the colon compared to that of vehicle-treated infected mice (Fig. 6C). We also investigated morphological changes in the colonic tissue of antagonist-treated C. rodentium-infected mice. In the representative image of the colon from vehicle-treated, and indomethacin and EP4-infected mice, we observed localized mononuclear cell infiltration and areas of epithelial tissue injury, whereas the integrity of the muscularis mucosae was preserved (Fig. 6D). Treatments with indomethacin or EP4 antagonist attenuated mononuclear cell infiltration and partially maintained parenchyma compared with vehicle-treated infected mouse (Fig. 6D). Based on these findings, PGE2, produced by the efferocytosis of infected cells in vivo, also impairs Th17 cell differentiation and intestinal host defense by acting through the EP4 receptor.

**Discussion**

Microbial infections that cause the apoptosis of host cells have been shown to trigger Th17 immune responses (32). Clearance of infected apoptotic cells by DCs induces the synthesis of Th17-inducing cytokines, such as TGF-β and IL-6 (7). Indeed, C. rodentium is an intestinal pathogen that causes enhanced apoptosis of epithelial cells, which is a critical process to trigger Th17 cell differentiation (7). Here, we demonstrated that PGE2 is also produced by DCs during uptake of infected apoptotic cells in vitro and during enteric C. rodentium infection in vivo. Targeting PGE2 in vitro and in vivo markedly improved Th17 cell differentiation and intestinal host defense against C. rodentium. The suppressive effect of PGE2 was mediated via the EP4-cAMP-PKA pathway, which impaired IL-1R expression in T cells and compromised the Th17 cell phenotype. Moreover, selective impairment of EP4 signaling increased the colonic Th17 cell population and antimicrobial peptide expression, resulting in the reduction of the C. rodentium load in the colon.

The uptake of apoptotic cells by phagocytes has been described as capable of modulating different cells of the immune system, resulting in either the suppression or activation of these cells (33). The efferocytosis of non-infected cells by DCs or macrophages leads to the synthesis of anti-inflammatory mediators, such as TGF-β, PAF and PGE2 and favors Treg generation (5, 34). However, the capture of apoptotic E. coli-infected cells promotes TGF-β, IL-6 and IL-23 release and triggers inflammation and Th17 cell differentiation (7). Herein, we demonstrated that PGE2, produced during the uptake of infected apoptotic cells, impairs Th17 cell differentiation and intestinal host defense. A similar effect has been described in the Leishmania donovani infection, in which PGE2 inhibited IL-17 synthesis and compromised the host response against infection, while treatment with COX inhibitors reversed this effect (35). Additionally, during Cryptococcus neoformans infection in mice, treatment with indomethacin enhanced Th17 responses and promoted the survival of infected mice (26).

The treatment of DCs with indomethacin, during the uptake of apoptotic E. coli-infected cells, also decreased the production of IL-6, IL-23 and IL-1β cytokines, which are essential for Th17 cell differentiation. This result is consistent with previous findings demonstrating that PGE2 acts in an autocrine manner, favoring the synthesis of these mediators (36, 37). Reduced levels of these cytokines in CM/Indo did not affect naïve CD4+ T cell differentiation into Th17, while the exogenous addition of PGE2 in CM/Indo decreased the frequency of Th17 cells obtained in CM.

EP1, EP2 and EP4 are expressed in human naive CD4+ T cells and, EP2 and EP4 are mainly expressed in Th17 cells (18, 38). EP1 signaling increases intracellular Ca2+, whereas EP2 and EP4 pathways enhance intracellular cAMP levels, resulting in the activation of PKA and EPAC proteins (39). RORγt expression, during Th17 cell differentiation, impairs EP2 expression, while EP4 expression is not affected (40). Consistent with these reports, we observed that EP4 was the prevalent PGE2 receptor expressed in Th17 cells. EP1 and EP2 antagonists did not influence the inhibitory effect of PGE2 on Th17 cell differentiation in vitro, in the context of the efferocytosis of infected cells. Although these data indicate that PGE2 mainly acts via EP4-cAMP-PKA to suppress Th17 cell differentiation and intestinal host defense, the relevance of EP1 or EP2 actions in vivo remains elusive.

IL-6, IL-21 and IL-23 receptors signaling induces IL-17 expression via STAT3 activation, which binds the promoter with RORγt and activates RORα and IRF4 expression (41, 42). Indeed, IL-6R signaling promotes IL-1R expression (43). IL-1β signaling plays a critical role during Th17 cell differentiation and is also related to the maintenance and expansion of Th17 cells (31, 44). Interestingly, the present results demonstrated that PGE2, produced by efferocytosis of infected cells, impaired STAT3 phosphorylation and the expression of IL-1R in T cells via the EP4-PKA pathway. Suppressor of cytokine signaling (SOCS) binds to Janus kinase and suppresses STAT activation (45). Interestingly, PGE2 and misoprostol, a PGE analog, induces SOCS1 expression in bone marrow cells during peritonitis (46). Indeed, SOCS3 and SOCS1 can inhibit STAT-1 and STAT-3 phosphorylation (47, 48). Therefore, the early inhibition of STAT3 phosphorylation may have been a critical point for impaired IL-1R expression, although the specific mechanism by which EP4 signaling affects STAT3 phosphorylation (via SOCS or others), and hence impairs IL-1R expression remains to be further elucidated.

IL-1β receptor deficient lymphocytes have reduced IL-23R expression and less ability to produce IL-17A (31). This finding is consistent with the present qPCR data showing that, in addition to il1r, the expression of il23r was also decreased, which may have contributed to the reduced Th17 cell differentiation in CM. Similar data have been reported for autoimmune encephalomyelitis (EAE), as the IL-1R KO had a lower capacity to induce Th17 responses compared to WT animals and showed resistance to development of EAE (49).

Th17 cells are important to host defenses against pathogens, such as fungi and extracellular bacteria (41). Th17 cytokines increase granulopoiesis and the expression of chemokines, which coordinate cellular recruitment and neutrophil chemotaxis to the inflammatory site, and IL-17A and IL-22 induce the expression of defensins and cathelicidins by epithelial cells (29). Furthermore, IL-26, also produced by Th17, acts as an antimicrobial peptide, capable of destroying different bacteria, such as Pseudomonas aeruginosa, E. coli, Klebsiella pneumonia and Staphylococcus aureus (50). Several studies have shown that the protection against C. rodentium infection correlates with the production of IL-17A and IL-22 (28, 51). Additionally, Th17 cells and other cells such as innate lymphoid cells (ILCs), γδ T, NK, and NK-T, can also produce IL-17A (52). However, CD3+ cells depletion during C. rodentium infection drastically reduces host defense and aggravates infection (53), which demonstrates the relevance of CD3+ lymphocytes in this context. The present data demonstrated that C. rodentium infection enhances colonic Th17 responses, compared to noninfected control mice. Indeed, the treatment of infected mice with indomethacin or EP4 antagonist markedly increased IL-17A and antimicrobial peptide expression while drastically reducing the bacterial load in colonic tissue compared to that in vehicle-treated infected mice.

The recognition of different microorganisms induces PGE2 synthesis by phagocytes and may modulate the function of immune cells (19). In addition, some pathogens trigger the apoptosis of host cells, and the clearance of infected apoptotic cells greatly increases the levels of PGE2 in the microenvironment. Therefore, the ability of some pathogens to induce apoptosis and consequently, favor PGE2 synthesis, brings an intriguing possibility of these microorganisms to use this pathway to manipulate or suppress host defense. For example, PGE2 impairs the phagocytosis and killing of some bacteria, virus and fungi by macrophages (54-56). Moreover, PGE2, derived from efferocytosis, compromises the clearance of S. pneumoniae infection by alveolar macrophages (6). However, the effects of PGE2 or EP4 signaling on macrophage effector functions in colonic tissue or during the efferocytosis of infected cells remain unclear.

Different findings have demonstrated the controversial roles of PGE2 in Th17 cell differentiation and expansion, in vitro or in vivo. PGE2 facilitates DC migration to lymph nodes (57, 58) and the production of IL-23 (59) and directly improves the expansion of Th17 cells (23). However, a suppressive effect of this prostanoid on Th17 cells has also been reported (26, 60, 61). For example, using a model of C. neoformans infection in mice, Valdez (2012) demonstrated that PGE2, in the early stages of infection, negatively

influenced the fate of Th17 cells, although high IL-17 production from memory Th17 cells was observed (26). The majority of studies describing the effect of exogenous PGE2 in Th17 cell differentiation or expansion have used higher concentrations (1-10 μM), in mainly pre-activated CD4+ T cells (18, 23). However, in the present study, the amount of endogenous PGE2 produced by DCs during efferocytosis of E. coli-infected cells was between 40-50 nM. Therefore, the mechanism that dictates the opposite effect of PGE2 may be complex and integrate a variety of factors. For example, discrepancies may be related to distinct cellular microenvironments (homeostasis or inflammation); maturation and activation states of CD4+ T cells, or type of EP receptor activated and concentration of exogenous or endogenous PGE2. Herein, in the context of efferocytosis of infected cells, endogenous levels of PGE2 associated with an inflammatory microenvironment and the type of receptor engaged (EP4) on naïve CD4+ T cells may have contributed to the inhibitory actions of PGE2 on Th17 cell differentiation.

Immune and non-immune cells express EP4 (62), hence the modulation of EP4 signaling might also affect a variety of cellular functions. Considering that the EP4 antagonist enhanced Th17 cell differentiation in vivo indicates an interesting strategy to target specific PGE2 actions and immune responses to promote host defense. However, exacerbated Th17 cell responses are also related to chronic inflammation and autoimmune disorders, such as rheumatoid arthritis, psoriasis and multiple sclerosis (63). Indeed, a recent study has demonstrated that the engulfment of infected apoptotic cells by DCs may promote the exposure of self and non-self antigens to naïve CD4+ T cells in the inflammatory microenvironment, allowing the activation of autoreactive clones (64). Therefore, further investigations are needed to understand the late effects of enhancing Th17 cell responses and its link to increased autoimmunity risk.

The non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX-1/2 enzymes, which are involved in the synthesis of prostanoids and are frequently used to relieve pain and treat inflammation. Although chronic use has been described to increase the risk of small intestinal damage and bleeding(65, 66), epidemiological evidence indicates that long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDs) may prevent colorectal cancer (67).

Thus, the present study provided the first evidence of a novel regulatory mechanism by which PGE2, produced by the efferocytosis of infected cells, suppresses Th17 cell differentiation and compromises adaptive immunity. Herein, we demonstrated that EP4 antagonist improved Th17 cell differentiation and intestinal host defense, without

affecting colonic PGE2 levels. Considering the diversity of prostaglandin actions and receptors in different organs and cells types, the use of an EP4 receptor antagonist may be relevant to improve selectivity and avoid unwanted reactions. Therefore, targeted EP4 signaling inhibition during infections that trigger host apoptosis and PGE2 synthesis, may be promising to enhance Th17 immunity and host defense.

## Methods

### Mice

Female wild-type C57BL/6 (6 to 8 weeks old) were obtained from Centro Multidisciplinar para Investigação Biológica, Universidade de Campinas (CEMIB/UNICAMP). Mice were kept in the animal facility at School of Pharmaceutical Sciences, São Paulo State University (UNESP). Animals were maintained under pathogen-free conditions in mini-isolators with controlled temperature, dark/light cycle, humidity, airflow and with free access to sterilized water and food. Experimental procedures were approved by the Institutional Animal Care and Committee from School of Pharmaceutical Sciences – UNESP.

### Generation of conditioned medium from co-cultures of DCs with infected apoptotic cells

Bone-marrow derived dendritic cells were differentiated with GM-CSF (PeproTech) for 7 days (12, 13). As a source of infected cells, RAW 264.7 cells were cultured with Escherichia coli (ATCC 259992) (ratio 1:10) for 2 h to allow phagocytosis. Then, the cells were washed with PBS (Lonza) to remove bacteria and cellular debris. Infected cells were exposed to UVC radiation and maintained in a humidified 37°C, 5% CO2 incubator for 4 h, as previously described (12, 13). DCs were treated for 20 min with 10 μM of COX inhibitor indomethacin or ibuprofen (Cayman) or left untreated. For conditioned medium (CM), DCs were co-cultured for 18 h with apoptotic E. coli-infected cells (IACs) at ratio 1:3, and the supernatants from each condition were collected for cytokines/PGE2 quantification and used in naïve CD4+ T cells differentiation assay. For experimental controls, DCs were left in resting conditions in the absence of IACs; cultured with E. coli ATCC 259992 (ratio 1:3) or treated with LPS. PGE2 was also removed from CM by Prostaglandin E2 affinity column (Cayman). 10 nM of PGE2 (Cayman) was added exogenously to some cultures.

**Differentiation of naïve CD4+ T cells**

Naïve CD4+ T cells were purified from spleen of C57BL/6 mice using CD4+CD62L+ T Cell Isolation Kit II mouse (Miltenyi Biotech) according to the manufacturer's protocol. Approximately 5x105 naïve CD4+ T cells were cultured in the presence of 250 μL of supernatant from DCs, CM or CM/INDO plus 250 μL of fresh IMDM medium supplemented with 10% FBS, 1 nM of non-essential amino acids, 1 mM of L-glutamin, 1 nM of sodium pyruvate and 55 μM of 2-β mercaptoethanol, anti-CD3 (4 μg/mL), anti-CD28 (2 μg/mL), 5 μg/mL of anti-IL-2, anti-IL-4 and anti-IFN-γ (BD). After 72 h, the cells were stimulated for flow cytometry analysis or the supernatant was collected for cytokine quantification by ELISA.

**Reagents**

Naïve CD4+ T cells were differentiated in CM or CM/Indo in the presence of PGE2 receptors agonists and antagonist: Butaprost (EP2 agonist) (Cayman), AH6869 or PF04418948 (EP2 antagonist) (Tocris); Cay 10598 (EP4 agonist) (Cayman), L-161,982 (EP4 antagonist) (Tocris); activators or inhibitors of adenylyl cyclase, PKA and EPAC: Forskolin (adenylyl cyclase activator), 8-Bromo-cAMP (PKA activator), 8-CPT-2Me-cAMP (EPAC activator), SQ22536 (adenylyl cyclase inhibitor), H89 (PKA inhibitor), KT520 (PKA inhibitor) e ESI-09 (EPAC inhibitor) (all Tocris) and neutralizing IL-1β antibody (BD) and IL-1Ra agonist (Peprotech) for 72 h. The cells were stimulated as previously described, and the supernatants were collected for IL-17A quantification. The cells were obtained for analysis of IL-17A expression by flow cytometry and transcription factors related to Th17 cell differentiation.

**Flow cytometry analysis**

After stimulation with 0.1 μg/mL of phorbol 12-myristate 13-acetate (PMA - Sigma) and 0.5 μg/mL of calcium ionophore (A23187) in the presence of brefeldin A (10 μg/mL - Sigma) for 4 h, the cells were labeled with Fixable Viability Dye (eBioscience), permeabilized and incubated with anti-IL-17A-PECy7 or APC, anti-CD4-FITC or PE, anti-Foxp3-APC and anti-IFN-γ-APC or FITC (BD and eBioscience). The cells were acquired by flow cytometry (FACS Canto - Becton & Dickinson, San Diego, CA, USA) and analyzed by software FCS 4 Express Flow Cytometry (De Novo Software).

**Phosflow assay**

Approximately 5x105 lymphocytes were cultivated in CM or CM/indo or treated with EP4 agonist (Cay 10598) or antagonist (L-161,982); PKA activator (8-Bromo-cAMP) and EPAC activator (8-CPT-2Me-cAMP). After 15 min, the cells were harvested. The cells were fixed with Fixation Buffer (BD Cytofix) and incubated in 37°C for 12 min. The cells were washed again and treated with chilled Perm Buffer III (BD Phosflow) and incubated on ice for 30 min. The cells were stained with antibody anti-phosphorylated STAT3-AlexaFluor 647 and anti-CD4-PE (BD).

**Enzyme-linked immunosorbent assay (ELISA)**

Supernatants were evaluated for the presence of cytokines and PGE2. The minimum detectable concentrations were: 31.25 pg/mL for IL-6 and IL-1β and IL-10 (BD Pharmingen); 15.6 pg/mL for IFN-γ and TGF-β (R&D System); 7.8 pg/mL for PGE2 (PGE2 EIA Kit, Cayman Chemicals); 8 pg/mL for IL-23 (Biolegend) and 4 pg/mL for IL-17A (eBioscience). For the in vivo experiments, tissue was stored in PBS in the presence of protease inhibitor and indomethacin. Tissue homogenate was used for PGE2 quantification. PGE2 amounts were normalized by total protein concentration (Protein Assay kit, Bio-Rad).

**Quantitative Real Time PCR (qPCR)**

RNA from CD4+ T cell cultures or tissue homogenate was isolated according to manufacturer's instruction (RNAspin Mini, GE Healthcare) and transcript in cDNA (iScript cDNA Synthesis Kit, BioRad). Gene expression was determined by amplification and quantification by SybrGreen (Life) and specific primers. The relative gene expression was calculated by the 2-ΔCt method. For qPCR, thermocycler ABI Prim 7300 (Applied Biosystems, Foster City, CA) was used. The sequences of primers used are available at supplementary information.

**qPCR Array**

Naïve CD4+ T cells were differentiated in the presence of CM, CM/Indo or CM/Ibup. Antibodies, anti-CD3 (4 μg/mL), anti-CD28 (2 μg/mL), anti-IL-2, anti-IL-4 and anti-IFN-γ (5 μg/mL) (BD), were added into the cultures. After 48 h, cells were harvest and RNA was extracted (RNeasy, Qiagen) followed by cDNA synthesis (RT2 microRNA First Strand Kit, Qiagen). Gene expression was evaluated by PCR array (Th17 Response PCR

Array, Qiagen). Plates were processed in an ABI Prim 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Normalization of RNA expression was performed using SABiosciences Online PCR Array Data Analysis Web Portal. RNA expression was compared between cells differentiated in the presence of CM and CM/Indo or CM/Ibup. Fold change was calculated by 2-ΔCt method. Data were analyzed using Qiagen-RT2 Profile PCR Array analysis tool.

**Citrobacter rodentium-induced infectious colitis model**

C57BL/6 mice were infected by gavage with Citrobacter rodentium (ICC168) (2x109 UFC) in 200 µL of PBS. For control, animals received PBS alone by the same via. Animals were divided into experimental groups: Controls: vehicle-treated noninfected mice, noninfected mice treated with indomethacin or EP4 antagonist; Infected: vehicle-treated infected mice, infected mice treated with indomethacin or EP4 antagonist. Animals were treated intraperitoneally (i.p.) with vehicle (PBS+2% DMSO), indomethacin (5 mg/kg) on the 1st, 3rd, 5th and 7th days after infection or EP4 antagonist (L-161,982) (10 mg/kg) daily, for 7 days. Animals were weighted daily. On 8th day of infection, colon and feces were collected after euthanasia. Feces were stored in LB medium for CFU determination and colon was photographed, measured and divided for CFU determination, PGE2 quantification, genes expression, lamina propria lymphocytes (LPLs) isolation and histopathological evaluation of colitis. Tissues and feces homogenate was strewed in LB solid agar containing nalidixic acid (NAL) (50 µg/mL – Sigma) for specific selection of NAL resistant C. rodentium bacteria in plates at 37°C for 24 h.

**Lamina propria lymphocytes (LPLs) isolation**

C. rodentium-infected C57BL/6 mice were euthanized on day 8, and LPLs were isolated from colon according to Torchinsky (2009) (Torchinsky, Garaude et al. 2009). First, the tissue cut longitudinally was washed with PBS, processed in small fragments and incubated for 20 min at 37°C with PBS containing 2% FBS and 1 mM of 1,4-Dithiothreitol (DTT) (Sigma) in agitation. This procedure was repeated twice, and a wash with PBS was performed. Tissue was incubated for 1 h at 37°C with RPMI 5% FBS containing 1.6 mg/mL of collagenase D (Roche). After that, tissue was homogenized using a 20G syringe and 70 µm cell strainer. Wells and strainers were washed with RPMI medium to reduce cell loss. Cell pellets were resuspended in 15-mL tubes with 4 mL of

a 44% Percoll solution and then transferred to 6 mL of a 66% Percoll solution. Tubes were centrifuged at 2800 rpm for 20 min. Interface cells were collected using a plastic pipet, washed with PBS and resuspended in IMDM with 10% FBS, PMA, ionomycin and brefeldin A. Cells were stimulated for 4 h at 37°C before staining for phenotypic analysis by flow cytometry.

**Histopathological evaluation of colitis**

Colon tissues were fixed in 10% buffered formalin, embedded in paraffin, stained with hematoxylin-eosin and analyzed as previously described (68).

**Statistical Analysis**

Data are presented as ± SEM and analyzed by Prism 5.0 (GraphPad Software, San Diego, CA). For comparison between the experimental groups, One-Way ANOVA analysis was performed, followed by Bonferroni or Tukey's multiple comparisons post-test. Statistical significant differences were indicated for P values $\leq 0.05$.

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**Author Contributions**

Conceived and designed the experiments: Dejani, NN; Orlando, AB; Verdan, FF; Serezani, CH; Medeiros, AI. Performed the experiments: Dejani, NN, Verdan, FF; Orlando, AB, Castanõ, VN; Penteado, LA; Verdan, FF; Codo, A.; Bazzano, J.; Salina, AG. Analyzed the data: Dejani, NN; Orlando, AB; Spolidorio, LC; Medeiros, AI. Drafted the manuscript: Dejani, NN; Medeiros, AI; Serezani, CH.

**Disclosures**

The authors declare no conflicts of interest.

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**Figure Legends**

**Figure 1. $PGE_2$ inhibits Th17 cell differentiation in the context of the efferocytosis of *E. coli*-infected cells by DCs.** DCs were previously treated or not with indomethacin (10 μM) and then co-cultured in the presence of apoptotic *E. coli*-infected cells (IAC), at the ratio 1:3 for 18 h. **(a)** The concentration of $PGE_2$ and cytokines were measured by ELISA in the supernatants derived from resting DCs (-), DCs co-cultured with IACs (CM) or indomethacin-treated DCs co-cultured with IACs (CM/Indo). Data represent mean ± SEM of at least 3 independent experiments performed in triplicate. *p<0.05 compared to (-) DC; #p<0.05 compared to (CM) DC+IAC. **(b)** Naïve $CD4^+$ T cells were activated and differentiated with anti-CD3 and anti-CD28 in the presence of supernatant from resting DC (-), and in the presence of the conditioned media CM, CM/Indo or CM/Indo with addition of exogenous $PGE_2$ (10 nM) for 72 h. The percentage of $CD4^+IL\text{-}17A^+$ T cells was determined by flow cytometry and showed by representative dot plots and bar graph as well as the levels of IL-17A released by lymphocytes was measured in the supernatant of cultures by ELISA. Data represent mean ± SEM of at least 5 independent experiments performed in triplicate. *p<0.05 compared to CM; #p<0.05 compared to CM/Indo.

**Figure 2. $PGE_2$ impairs STAT3 phosphorylation and Th17 cell differentiation through EP4 activation.** Naïve $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of CM from DC co-cultured with apoptotic *E. coli*-infected cells (IAC) or CM/Indo from indomethacin pre-treated DC incubated with (IAC). **(a)** The expression of EP1, EP2, EP3 and EP4 receptors on T cell differentiated in the presence of CM were measured by qPCR after 48 h of culture. Data represent mean ± SEM of 2 independent experiments performed in triplicate. *p<0.05 compared to EP1; #p<0.05 compared to EP2. **(b)** The expression of EP2 (left) and EP4 (right) receptor was assessed by flow cytometry on naïve $CD4^+$ T cells or T cells after 72 h of differentiation in the presence of CM or CM/Indo. Data are shown in representative histograms of 2 independent experiments. **(c)** Naïve $CD4^+$ T cells were treated with AH6869 or PF04418948 (EP2 antagonist) or L-161,982 (EP4 antagonist) and differentiated in the presence of CM or cultured treated with Butaprost (EP2 agonist) or Cay10598 (EP4 agonist) and differentiated in the presence of CM/Indo. After 72 h, the expression of IL-17A was analyzed by flow cytometry and data are shown by representative dot plots from at least 5 independent experiments as well as **(d)** by bar graph showing the percentage of $CD4^+IL17A^+$ T cells (left) and the levels of IL-17A detected by ELISA in the cultures supernatants (right). Data represent mean ± SEM of 3 independent experiments performed in triplicate. *p<0.05 compared to CM (-

); #p<0.05 compared to CM/Indo (-); #p<0.05 compared to CM (EP2 antagonist). **(e)** Naïve $CD4^+$ T cells were treated with Forskolin (adenylyl cyclase activator), 8-Bromo-cAMP (PKA activator) or 8-CPT-2Me-cAMP (EPAC activator) and differentiated in the presence of CM/Indo. After 72 h, IL-17A released in the supernatant of the cultures was measured by ELISA. Data represent mean ± SEM of 3 independent experiments performed in triplicate. *p<0.05 compared to CM/Indo (-). **(f)** Naïve $CD4^+$ T cells were cultured with CM in the presence or absence of L-161,982 (EP4 antagonist) or with CM/Indo in the presence or absence of Cay 10598 (EP4 agonist) or 8-Bromo-cAMP (PKA activator) or 8-CPT-2Me-cAMP (EPAC activator). After 15 min, cells were stained for STAT3 phosphorylation detection by phosflow assay. Data are shown in representative histograms of 2 independent experiments.

**Figure 3. $PGE_2$ compromises the expression of Th17-related genes during the efferocytosis of *E. coli*-infected cells.** Naïve $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of supernatant from efferocytosis of *E. coli*-infected cells (IAC) by untreated DC (CM) or indomethacin-treated DC (10 μM) (CM/Indo). **(a)** After 48 h, RNA was extracted and the expression of Th17 related genes was measured by qPCR array. Representative scatter plot of expressed genes in T lymphocytes cultured in CM/Indo (Y-axis) or CM (X-axis). Genes 4-folder over or downregulated are highlighted in the scatter plot. Bar graphs are presenting the most expressed genes in the array (*il17f, il17a, ccl2, ccl7* and *il1r*) confirmed by conventional qPCR. Data represent mean ± SEM of 3 independent experiments performed in triplicate. *p<0.05 compared to CM. **(b)** After 72 h of differentiation, the expression of IL-1R was analyzed by flow cytometry on $CD4^+$ T cells cultured in CM or CM/Indo condition. Data are shown in a representative histogram of 2 independent experiments. **(c)** Naïve $CD4^+$ T cells were differentiated in CM condition in the presence or not of L-161,982 (EP4 antagonist), or cultured in CM/Indo condition in the presence or not of Cay10598 (EP4 agonist), Forskolin (adenylyl cyclase activator) or 8-Bromo-cAMP (PKA activator). After 48 h of culture RNA was extracted and the expressions of *il1r* and *il17a* genes were measured by qPCR. Data represent mean ± SEM of 3 independent experiments. *p<0.05 compared to CM (-); #p<0.05 compared to CM/Indo (-).

**Figure 4. IL-1R signaling is critical for Th17 cell differentiation in the context of the efferocytosis of *E. coli*-infected cells.** Naïve $CD4^+$ T cells activated with anti-CD3 and anti-CD28 were differentiated in CM condition in the presence of L-161,982 (EP4 antagonist), L-161,982 plus anti-IL-1β or L-161,982 plus IL-1Ra; or in CM/Indo condition in the presence or not of Cay10598 (EP4 agonist), anti-IL-1β or IL-1Ra. After 72 h, cells were stimulated and

stained to determine the percentage of $CD4^+IL17A^+$ T cells by flow cytometry. **(a)** Data are shown on representative dot plots (left) and bar graph by mean ± SEM of at least 3 independent experiments. *p<0.05 compared to CM (-); #p<0.05 compared to CM/Indo (-).

**Figure 5. *In vivo* inhibition of $PGE_2$ synthesis or signaling via EP4 improves Th17 cell population in the colonic tissue of *Citrobacter rodentium*-infected mice.** Mice were orally infected or not infected with *C. rodentium* and treated every other day with indomethacin (5 mg/kg), daily with L-161,982 (EP4 antagonist) (10 mg/kg) or vehicle-PBS during 7 days. On 8th day of infection colons were harvested. **(a)** Scheme of treatment. **(b)** $PGE_2$ levels quantified by ELISA in the colonic tissue. **(c)** The percentage of $TCR\beta^+CD4^+IL17A^+$ T cells in the colon were assessed by flow cytometry and demonstrated by representative dot plots and bar graph. **(c)** Measurement of body weight throughout the experiment. **(d)** The colon length of different groups of animals was measured after euthanasia. Data represent mean ± SEM of 2 independent experiments. N=7-10. *p<0.05 compared to Control (Vehicle); #p<0.05 compared to Infected (Vehicle).

**Figure 6. Indomethacin or EP4 antagonist treatment improves host defense against *Citrobacter rodentium* infection in mice.** Mice were infected with *C. rodentium* and treated i.p. every other day with indomethacin (5 mg/kg) or daily with L-161,982 (EP4 antagonist) (10 mg/kg) or vehicle-PBS for 7 days. On 8th day of infection colons were harvested. **(a)** The colonic tissue homogenates were plated for CFU counting per gram of tissue. **(b)** The expression of *il1r, il17a, il22* and **(c)** antimicrobial peptides genes were analyzed by qPCR in the colonic tissue. **(d)** Representative hematoxylin-and-eosin staining sections of distal colonic tissues from untreated non-infected and vehicle-, indomethacin- and EP4 antagonist-treated infected mice are shown. Data represent mean ± SEM of 2 independent experiments. N=7-10. *p<0.05 compared to vehicle-treated infected mice group.

**Figure 1**

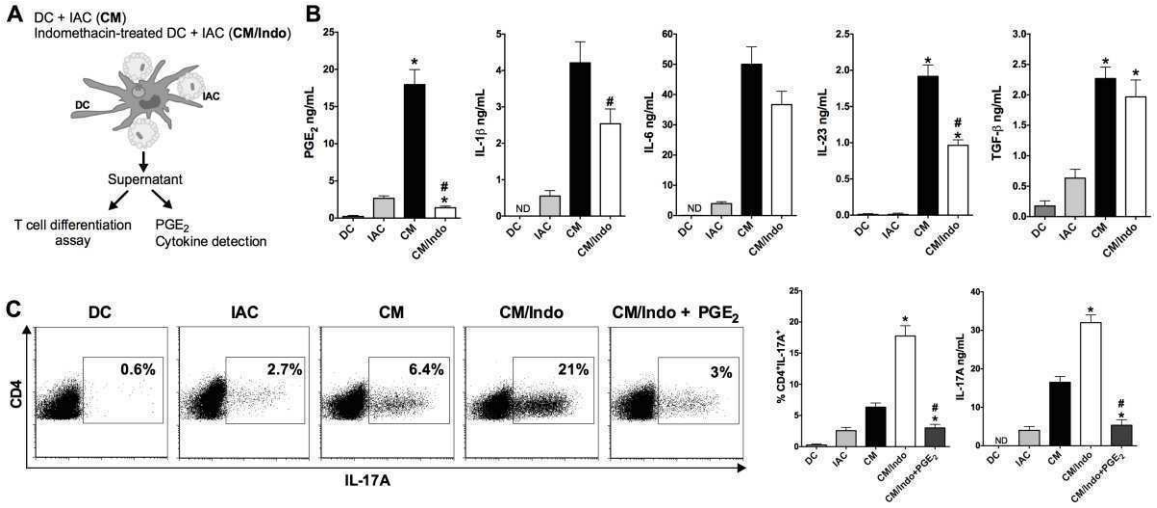

A
DC + IAC (CM)
Indomethacin-treated DC + IAC (CM/Indo)
DC
IAC
Supernatant
T cell differentiation assay
PGE2
Cytokine detection
B
PGE2 ng/mL
IL-1β ng/mL
IL-6 ng/mL
IL-23 ng/mL
TGF-β ng/mL
ND
DC
IAC
CM
CM/Indo
C
DC
IAC
CM
CM/Indo
CM/Indo + PGE2
0.6%
2.7%
6.4%
21%
3%
CD4
IL-17A
% CD4+IL-17A+
IL-17A ng/mL
CM/Indo+PGE2

**Figure 2**

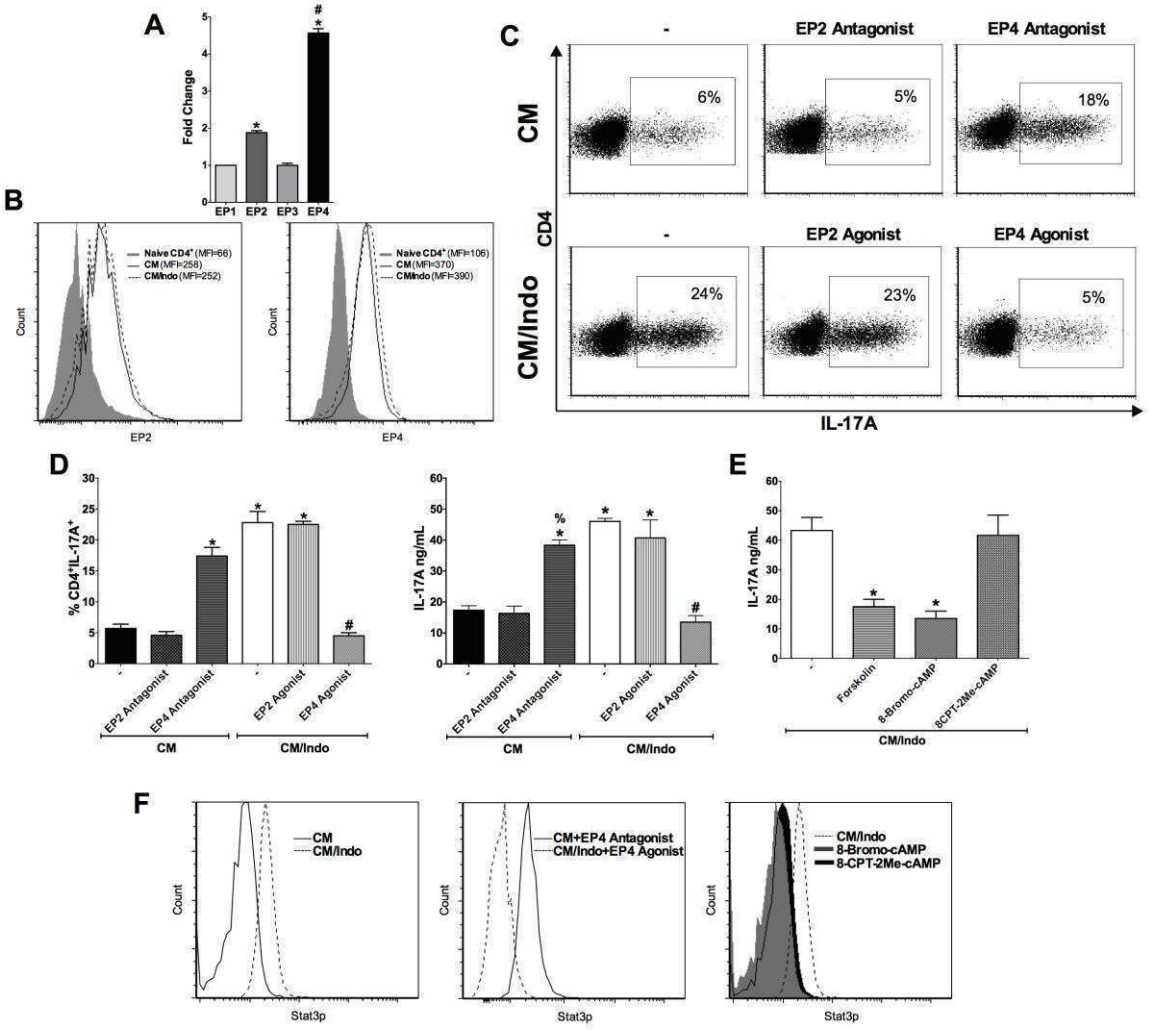

A
Fold Change
EP1 EP2 EP3 EP4
B
Naive CD4+ (MFI=66)
CM (MFI=258)
CMIndo (MFI=252)
Count
EP2
Naive CD4+ (MFI=106)
CM (MFI=370)
CMIndo (MFI=390)
Count
EP4
C
-
EP2 Antagonist
EP4 Antagonist
CM
6%
5%
18%
CD4
-
EP2 Agonist
EP4 Agonist
CM/Indo
24%
23%
5%
IL-17A
D
% CD4+IL-17A+
EP2 Antagonist
EP4 Antagonist
EP2 Agonist
EP4 Agonist
CM
CM/Indo
IL-17A ng/mL
EP2 Antagonist
EP4 Antagonist
EP2 Agonist
EP4 Agonist
CM
CM/Indo
E
IL-17A ng/mL
Forskolin
8-Bromo-cAMP
8CPT-2Me-cAMP
CM/Indo
F
CM
CM/Indo
Count
Stat3p
CM+EP4 Antagonist
CM/Indo+EP4 Agonist
Count
Stat3p
CM/Indo
8-Bromo-cAMP
8-CPT-2Me-cAMP
Count
Stat3p

**Figure 3**

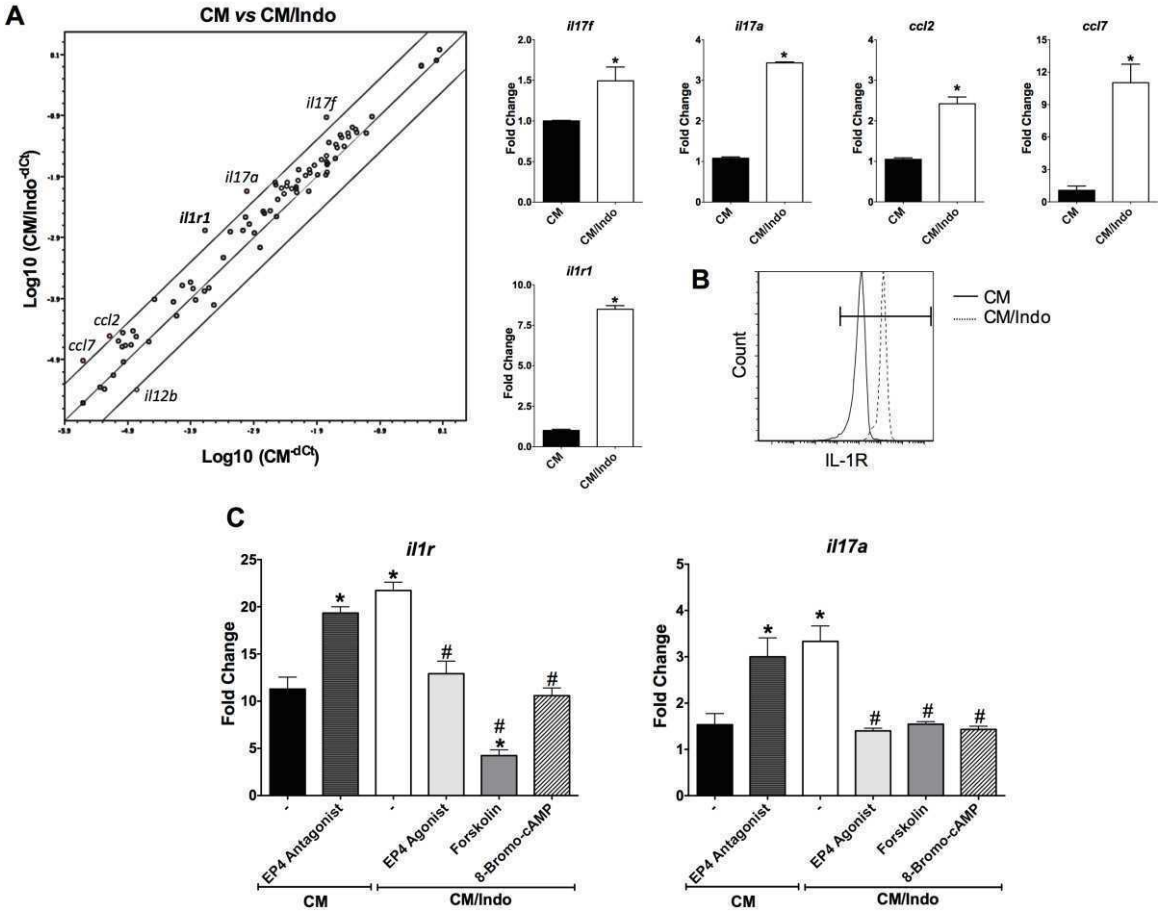

A
CM vs CM/Indo
Log10 (CM/Indo-dCt)
Log10 (CMdCt)
il17f
il17a
il1r1
ccl2
ccl7
il12b
il17f
il17a
ccl2
ccl7
il1r1
Fold Change
CM
CM/Indo
B
Count
IL-1R
CM
CM/Indo
C
il1r
il17a
Fold Change
EP4 Antagonist
EP4 Agonist
Forskolin
8-Bromo-cAMP
CM
CM/Indo

**Figure 4**

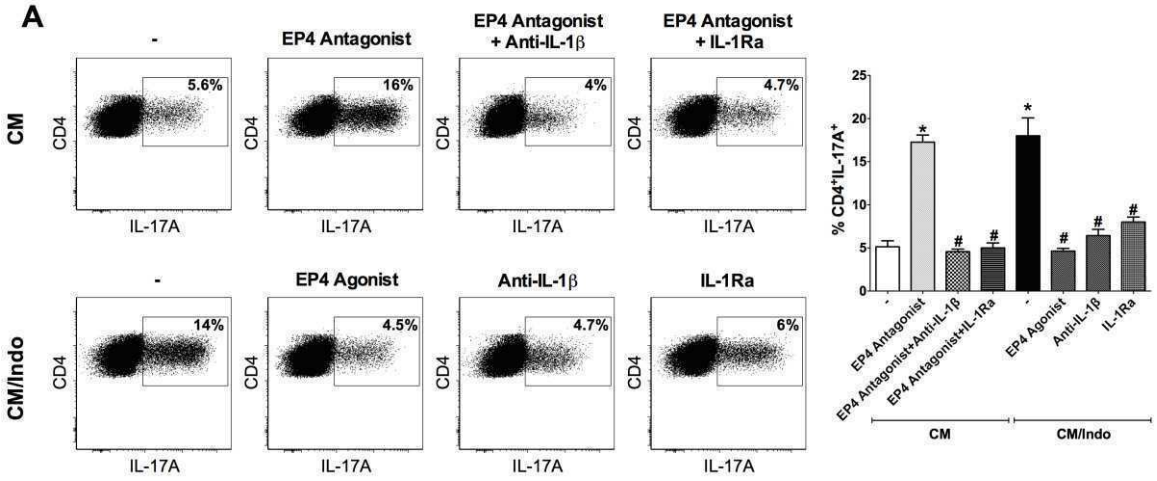

A
-
EP4 Antagonist
EP4 Antagonist + Anti-IL-1β
EP4 Antagonist + IL-1Ra
CM
CM/Indo
EP4 Agonist
Anti-IL-1β
IL-1Ra
5.6%
16%
4%
4.7%
14%
4.5%
4.7%
6%
CD4
IL-17A
% CD4+IL-17A+
EP4 Antagonist
EP4 Antagonist+Anti-IL-1β
EP4 Antagonist+IL-1Ra
EP4 Agonist
Anti-IL-1β
IL-1Ra
CM
CM/Indo

**Figure 5**

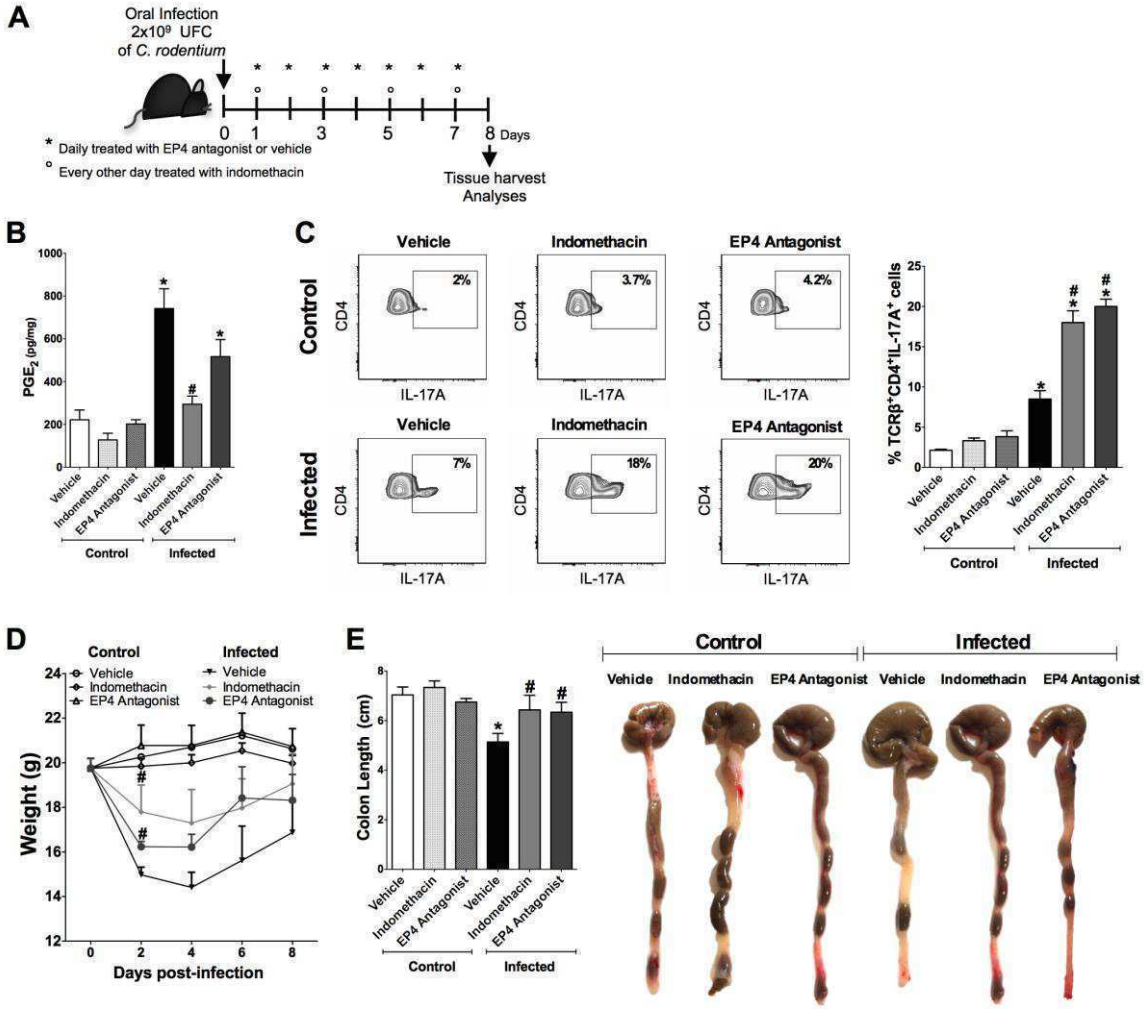
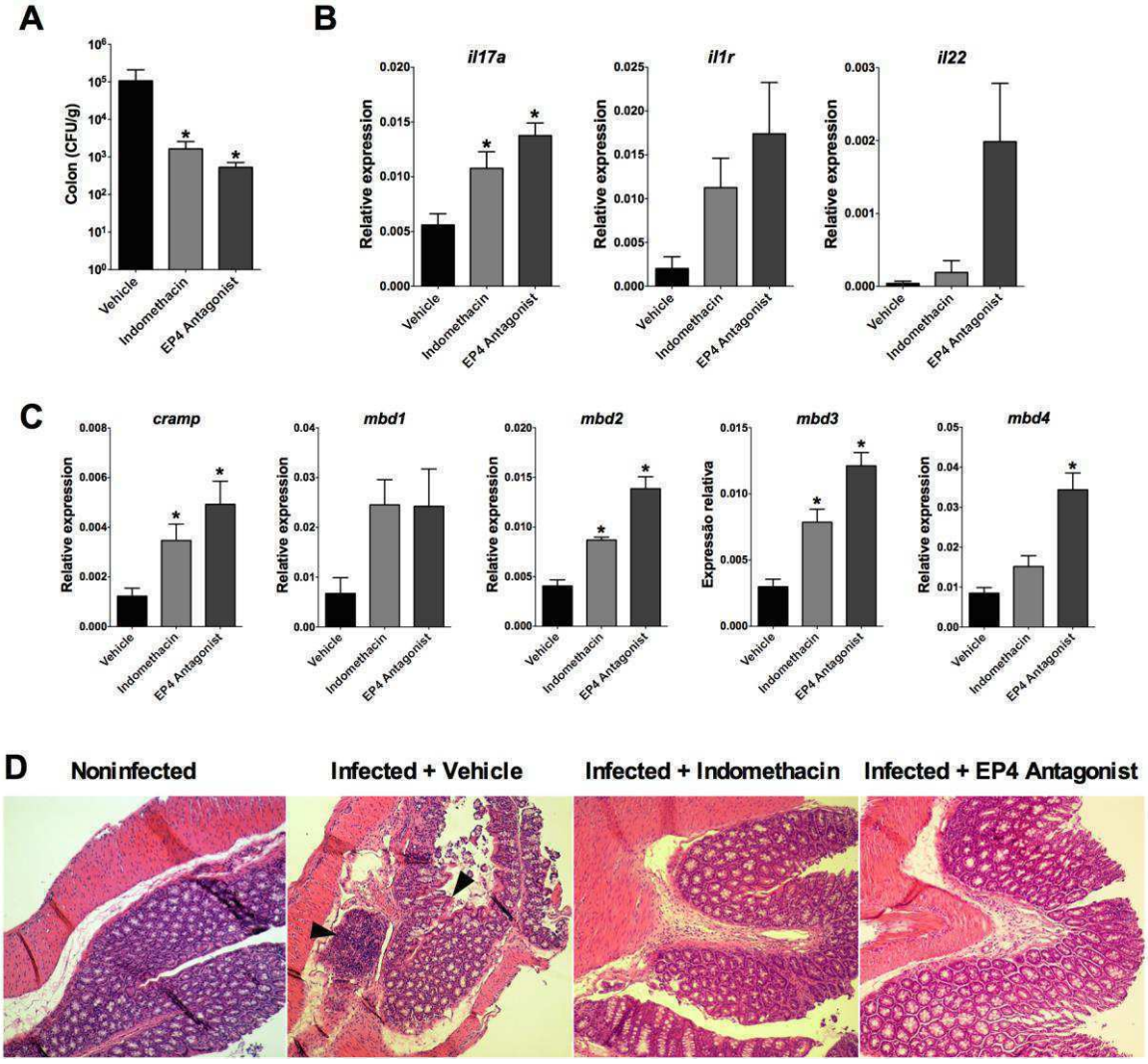

A
Oral Infection
2x10⁹ UFC
of C. rodentium
0 1 3 5 7 8 Days
* Daily treated with EP4 antagonist or vehicle
° Every other day treated with indomethacin
Tissue harvest
Analyses
B
PGE2 (pg/mg)
Vehicle
Indomethacin
EP4 Antagonist
Control
Infected
C
Vehicle
Indomethacin
EP4 Antagonist
Control
Infected
CD4
IL-17A
2%
3.7%
4.2%
7%
18%
20%
% TCRβ+CD4+IL-17A+ cells
D
Control
Infected
Vehicle
Indomethacin
EP4 Antagonist
Weight (g)
Days post-infection
E
Colon Length (cm)
Control
Infected
Vehicle
Indomethacin
EP4 Antagonist

**Figure 6**


A
Colon (CFU/g)
Vehicle
Indomethacin
EP4 Antagonist
B
il17a
il1r
il22
Relative expression
C
cramp
mbd1
mbd2
mbd3
mbd4
Expressão relativa
D
Noninfected
Infected + Vehicle
Infected + Indomethacin
Infected + EP4 Antagonist

# Distinctive role of efferocytosis in dendritic cell maturation and migration in sterile or infectious conditions

**Short title**
**IAC efferocytosis triggers DC maturation**

**Letícia de Aquino Penteado[1]; Naiara Naiana Dejani[1,2]; Felipe Fortino Verdan[1,2]; Allan Botinhon Orlando[1]; Victoria Ninõ [1]; Fernanda De Nuzzi Dias[1]; Ana Carolina Guerta Salina[1,2]; Alexandra Ivo Medeiros[1]**

[1]Department of Biological Sciences, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, São Paulo, Brazil. [2]Department of Biochemistry and Immunology, Faculty of Medicine of Ribeirão Preto, USP - University of São Paulo, Ribeirão Preto, São Paulo, Brazil.

**Corresponding author:**

Prof. Dr. Alexandra Ivo de Medeiros. [1]Department of Biological Sciences, School of Pharmaceutical Sciences, UNESP - Univ Estadual Paulista, Araraquara, São Paulo, Brazil. Rodovia Araraquara-Jaú Km 1, 14801-902, Araraquara, São Paulo, Brazil. 55-16-3301-6049. E-Mail: medeirosai@fcfar.unesp.br

**Key words:** Efferocytosis, dendritic cells, prostaglandin E2, infected apoptotic cell, CCR7

**Abbreviation:**

AC – Apoptotic cell

DC – Dendritic cell

IAC – Infected apoptotic cell

CCR7 – CC-Chemokine receptor type 7

PS – Phosphatidylserine

DAMPs – Danger-associated molecular patterns

PAMPs - Pathogen-associated molecular patterns

MHC – Major histocompatibility complex

BMDC - Bone marrow-derived dendritic cells

GM-CSF - Granulocyte–macrophage colony-stimulating factor

GFP – Green fluorescence protein

CFSE – Carboxyfluorescein succinimidyl ester

COX – Cyclooxygenase

qPCR - Quantitative real time PCR

ELISA - Enzyme-linked immunosorbent assay

MFI – Median fluorescence intensity

**Summary**

Efferocytosis, or clearance of apoptotic cells (ACs), by dendritic cells (DCs) leads to immune response suppression and tolerance to self-antigens. However, efferocytosis of infected cells (IACs) leads to the production of a mixed pro- and anti-inflammatory cytokine milieu. We examined the DC phenotype and ability to migrate after phagocytosis of ACs or IACs and observed higher levels of CD86 and CCR7 expression in DCs, as well as enhanced migration capacity following efferocytosis of infected cells. Interestingly, higher levels of IL-1β, IL-10 and PGE2 were also produced in this context. Blockage of IAC recognition led to an impaired maturation profile and PGE2 production, which may have contributed to reduced CD86 and CCR7 expression and migration capacity. These data contribute to the understanding of how efferocytosis of sterile or infected cells may regulate the adaptive immune response, although the precise role of PGE2 in this process requires further investigation.

## Introduction

Billions of cells die daily via apoptosis in different tissues during homeostatic processes. Resident macrophages, dendritic cells (DCs) and tissue neighboring "nonprofessional" phagocytes play important roles in the removal of these cells, a process termed efferocytosis 1, 2. Efficient clearance of apoptotic cells (ACs) depends on important steps described as "find-me" and "eat-me" signals. ACs release different soluble 'find-me' signals such as adenosine triphosphate nucleotides (ATP) and the chemokine fractalkine (CX3CL1), thus promoting the recruitment of phagocytes such as macrophages and DCs 3, 4. Moreover, surface exposure of phosphatidylserine (PS) molecules in ACs is an 'eat me' signal that plays a major role during efferocytosis 5. The recognition of PS is mediated by several receptors expressed in the cell membrane, such as T-cell immunoglobulin mucin protein 4 (TIM-4) and brain angiogenesis inhibitor 1 (BAI1), and this recognition results in the efficient engulfment of ACs 6, 7.

Some reports have shown that efferocytosis may affect innate and adaptive immune responses. For example, during sterile inflammation caused by chemical exposure or cigarette smoke, there is an intense recruitment of neutrophils and massive accumulation of sterile ACs into the tissue 8. Efferocytosis of sterile cells induces the production of anti-inflammatory mediators such as IL-10, TGF-β, platelet-activating factor (PAF) and prostaglandin E2 (PGE2), which leads to suppression of the immune response 9-11. Efferocytosis by macrophages and DCs has been described as an anti-inflammatory and immunosuppressive event involved in tissue remodeling, repair and tolerance 12-14. Engulfment of ACs is also related to cross-presentation of antigens and activation of both CD4+ and CD8+ T cell responses during viral infection 15, 16 and tumor growth 17, 18. Moreover, during some microbial infections, bacterial products can promote neutrophil death. Phagocytosis of Escherichia coli-infected ACs by DCs results in the production of

both pro- and anti-inflammatory mediators, such as TGF-β, IL-6 and IL-23 19. Although phagocytosis of infected ACs has been characterized as an important innate immunity effector function to impair the proliferation of microorganisms, recent studies have described it as a dangerous process that can promote autoimmunity 20. Phagocytosis of Mycobacterium tuberculosis-infected ACs by macrophages is involved in the killing of the pathogen 21, 22, while capture of Citrobacter rodentium-infected ACs by DCs leads to bacterial and self-peptide presentation to T cells and development of autoimmune disorder 20.

In addition to the recognition of ACs, DCs are the most important phagocytes in orchestration of the adaptive immune response. Through a vast repertoire of pattern recognition receptors (PRRs), DCs recognize and process danger- and pathogen-associated molecular patterns (DAMPs and PAMPs) along the endocytic pathway 23. Then, DCs undergo morphological, phenotypical and functional changes, acquiring an activated status represented by three typical features: (i) high levels of extracellular class II MHC molecules; (ii) increased expression of CD80 and CD86; and (iii) inflammatory mediator production 24. Moreover, to migrate from the peripheral site to draining lymph nodes (LNs) and initiate a proper adaptive immune response, DCs upregulate CC-Chemokine receptor type 7 (CCR7), which favors migration to LNs in response to chemotactic gradients of CCL19 and/or CCL21 25. The prostanoid PGE2, which is produced during infections, inflammation and efferocytosis, can enhance DC migration to LNs in a CCR7-dependent manner 26-28.

Contradictory effects of engulfment of ACs on DC maturation have been described. The uptake of apoptotic tumor cells increases CD86 and class II MHC expression as well as migration to LNs 18. By contrast, efferocytosis of β-pancreatic cells leads to DC immunosuppression caused by PGE2 production 12. Given the important role of DCs in

the clearance of ACs and the lack of studies comparing the effect of phagocytosis of sterile ACs and E. coli infected-ACs (IACs) on differential migration behavior and soluble mediator production by DCs, in this study, we evaluated whether phagocytosis of those different sources of ACs promotes differential phenotypes of DC activation. Here, we demonstrate the distinct effects of phagocytosis of ACs or IACs on the maturation and migratory capacity of DCs and on PGE2 and inflammatory cytokine production.

## Materials and Methods

### Mice

C57BL/6 female mice (8 to 10-week old) were purchased from Centro Multidisciplinar para Investigação Biológica, Universidade de Campinas (CEMIB/UNICAMP). The animals were maintained in mini-isolators with controlled temperature, humidity, airflow and dark/light cycle with free access to sterilized water and food. BALB/c mice were obtained from the University of São Paulo (USP) at Ribeirão Preto School of Medicine, Brazil. All animal experiments performed were approved by the Institutional Animal Care and Use Committee of the School of Pharmaceutical Sciences, São Paulo State University (UNESP).

### Generation of bone marrow-derived dendritic cells (BMDCs)

DCs were differentiated from bone marrow precursor cells of C57BL/6 mice according to the protocol described by Lutz et al. 1999 29 with a few modifications. BMDCs were cultured in 100 × 20 mm tissue culture plates (BD FalconTM) with 20 mL of complete RPMI-1640 medium (LonzaTM) (supplemented with 10% fetal bovine serum and 10 µg/mL gentamicin (GibcoTM)) containing 40 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTechTM). On days 3 and 6, the RPMI-1640 medium was replaced with fresh complete RPMI-1640 medium containing 40 ng/mL GM-CSF.

On day 7, the supernatant was removed, and the BMDCs were resuspended in RPMI-1640 (LonzaTM) medium.

**Generation of sterile ACs and IACs**

As a source of sterile ACs, RAW 264.7 cells cultured in DMEM medium (LonzaTM), supplemented with 10% fetal bovine serum were exposed to UVC radiation (0.35 J) and maintained in a humidified 37°C, 5% CO2 incubator for 4 h. For IAC generation, RAW 264.7 cells were cultured with Escherichia coli (E. coli) (ATCC 259992) (ratio 1:10) for 2 h for phagocytosis. Then, the cells were washed with PBS (LonzaTM) to remove the bacteria and cellular debris. The infected cells were exposed to UVC radiation (0.35 J) and maintained in a humidified 37°C, 5% CO2 incubator, for 4 h. The ACs and IACs were then collected and resuspended in RPMI-1640 medium, counted and adjusted to the desired cell suspension to perform efferocytosis. Apoptosis was evaluated by staining with Annexin-V conjugated with FITC following the manufacturer's protocol. Cells were acquired by flow cytometry (FACS CantoTM, Becton & Dickinson, San Diego, CA, USA) (Supplementary Fig S1 b, S1 c) and analyzed by the software FCS 4 Express Flow Cytometry.

**Evaluation of the infected cell rate**

RAW 264.7cells were cultured with green fluorescent protein (GFP)-expressing E. coli (ratio 1:10) for 2 h in DMEM medium (LonzaTM) supplemented with 10% fetal bovine serum. To confirm the phagocytosis rate, cells were collected and washed twice with PBS to remove free bacteria and cell debris. Cells were labeled with CD11b, and the percentage of infected cells was confirmed by flow cytometry (Supplementary Fig S1 a).

**Efferocytosis assay**

DCs were co-cultivated with ACs or IACs at a 1:3 ratio (DC: AC) in a 6-well plate (BD FalconTM) with 4 mL of RPMI-1640 serum-free medium supplemented with 10μg/mL

gentamicin. Cells were maintained in a humidified 37°C, 5% CO2 incubator for 13 h. The efficiency of efferocytosis by DCs and the percentage and median fluorescence intensity (MFI) of CD11c+ AC-CFSE+ cells were evaluated by flow cytometry (Supplementary Fig S1 d). As a positive control, DCs were cultured with E. coli (1:1 ratio), and as a negative control, DCs were cultured with RPMI-1640 medium only. After 13 h, the cells and supernatant of each culture were collected. The supernatant was stored in a -80°C freezer until ELISA analysis.

**Blockage of AC and IAC recognition**

ACs and IACs were incubated with Annexin-V microbeads (Miltenyi Biotec®) in the presence of 2.5 mM Ca2+ according to the manufacturer's protocol. To confirm the impairment of efferocytosis, DCs were co-cultured with AC-CFSE+ that was previously incubated with Annexin-V microbeads. The percentage of CD11c+AC-CFSE+ cells was evaluated by flow cytometry (Supplementary Fig S1e).

**CFSE staining**

The CFSE stock solution was diluted in pre-warmed PBS to a working concentration of 1.5 μM for the in vitro efferocytosis assay and 2 μM for the in vitro migration assay. Briefly, CFSE at the desired concentration was added to the cells, which were incubated at 37°C for 15 min. The cells were centrifuged and resuspended in fresh pre-warmed medium, incubated at 37°C for 30 min, and then washed once more, according to the manufacturer's protocol (CellTrace CFSE Kit, Life).

**In vitro migration assay**

DCs were isolated by magnetic separation with magnetic CD11c+ microbeads (Miltenyi Biotec®) according to the manufacturer's protocol. After isolation, the DCs were labeled with CFSE. To evaluate the migration capacity of DCs, 5-μm-pore Transwell membranes (Transwell® Permeable Supports,Corning Incorporated) were placed in a 24-well plate,

and 2.5x105 DCs of each condition in 100 μL of RPMI-1640 serum-free medium was added to the upper chamber. In the lower chamber, 300 ng/mL CCL19 (Recombinant Murine MIP-3β, PeprotechTM) and 250 ng/mL CCL21 (Recombinant Murine Exodus-2, PeprotechTM)chemokines were added in 600 μL of RPMI-1640 serum-free medium. The plate was maintained in a humidified 37°C, 5% CO2 incubator for 6 h. Then, the transmigrated DCs were photographed. The same fields were imaged using a Nikon Eclipse 50i microscope (Nikon, Melville, NY) with a 10x objective lens. In addition, the transmigrated cells were harvested and counted by flow cytometry.

**In vivo migration assay**

To perform the in vivo migration assay, DCs differentiated from C57BL/6 bone marrow precursors were labeled with FarRed (1μM) (CellTrace FarRed Kit, Life) and then co-cultured with ACs or IACs in a humidified 37°C, 5% CO2 incubator for 13 h. Then, 2x106 DCs from each culture were injected into the footpads of BALB/c mice. After 48 h, cells from popliteal LNs were obtained and analyzed by flow cytometry for the presence of IAb+ FarRed+ cells.

**Evaluation of the DC maturation phenotype**

The maturation phenotype of the DCs was assessed using anti mouse-CD11c (BioLegend®, APC), anti-mouse-CD197 (CCR7) (BioLegend®, PE), anti-mouse-IAb, (Class II MHC) (BD PharmingenTM, FITC) and anti-mouse-CD86 (BioLegend®,PE/Cy7) antibodies by flow cytometry. Nonspecific binding was blocked using FcBlock (BD PharmingenTM).

**RNA extraction and quantitative Real-Time PCR (qPCR)**

RNA was extracted from DCs from each condition and isolated with an RNAspin RNA Isolation Kit (GE Healthcare) according to the manufacturer's protocol and transcribed into cDNA. For qPCR, an ABI Prim 7300 thermocycler (Applied Biosystems, Foster

City, CA) was used. The relative quantity of each sample was normalized to the average level of the constitutively expressed housekeeping gene Gapdh. The following primers were used: Gapdh, forward 5′-AACTTTGGCATTGTGGAAGG-3′, reverse 5′-ACACATTGGGGGTAGGAACA-3′; Ptgs1, forward 5'-AGGAGATGGCTGCTGAGTTGG-3', reverse 5'- AATCTGACTTTCTGAGTTGCC-3' and Ptgs2, forward 5′-GGGCCCTTCCTCCCGTAGCA-3′, reverse 5′-TGAGCCTTGGGGGTCAGGGA.

**Enzyme-linked immunosorbent assay (ELISA)**

To determine the microenvironment created by the DCs under each condition, ELISA was performed to determine the production of IL-6, IL-23, TGF-β, IL-10, IL-1β and PGE2. The minimum detectable concentrations were 31.25 pg/mL for IL-6, IL-1β and IL-10 (BD Pharmingen™); 15.6 pg/mL for TGF-β (DuoSet® ELISA, R&D System); 7.8 pg/mL for PGE2 (PGE2 EIA Kit, Cayman Chemicals); and 8 pg/mL for IL-23 (ELISA MAXTM Deluxe Set, Biolegend). All procedures were performed according to the manufacturer's instructions.

**Statistical analysis**

The results were analyzed by Prism 5.0 (GraphPad Software, San Diego, CA). For comparisons among multiple experimental groups, one-way ANOVA analysis was performed followed by Tukey's multiple comparison test. For comparisons between two experimental groups, Student's t test was performed. Statistically significant differences were indicated at P values of $\leq 0.05$.

**Results**

**Phagocytosis of IACs triggers CD86 and CCR7 upregulation on DCs**

It was previously reported that DCs can migrate and reach peripheral LNs following AC phagocytosis 30. However, it is still unclear if efferocytosis affects DC maturation and migration in sterile inflammation or infectious microenvironments. Therefore, as a source of sterile and infected ACs, we induced apoptosis in non-infected and E. coli-infected RAW 264.7 cells, respectively. Almost 90% of RAW 264.7 cells were infected with E. coli, and the percentage of early and late apoptosis was approximately 98% (Supplementary Fig. S1b,c). We then investigated whether the recognition of ACs or IACs distinctly affected the maturation phenotype of DCs. Our results demonstrated that DCs that engulfed ACs showed lower CD86 and CCR7 expression, analogous to unstimulated DCs in resting conditions (Fig. 1a-e). However, phagocytosis of IACs promoted enhanced expression of CD86 and CCR7 on DCs (Fig. 1a-e). Indeed, DCs that interacted with IACs showed higher double positivity for CD86+CCR7+ molecules compared with DC+ACs and a phenotype similar to that of DCs incubated with E. coli (Fig. 1a,b). Regarding MFI analysis, DCs that engulfed IACs presented higher surface expression of CCR7 and CD86 compared to DCs co-cultured with ACs and similar levels compared to DCs incubated in the presence of E. coli (Fig. 1d).

Moreover, to confirm that DC maturation was promoted by efferocytosis of IACs carrying PAMPs, we blocked efferocytosis using purified Annexin-V microbeads (Ann), which are capable of covering exposed PS, thus inhibiting recognition of ACs by phagocytes. Treatment with purified Annexin-V inhibited more than 50% of efferocytosis of IACs (Supplementary Fig. S1d,e) and partially inhibited the expression of CD86+CCR7+ on DCs (Fig. 1a-d), suggesting that recognition of IACs by DCs triggers CD86 and CCR7 upregulation. By contrast, recognition of sterile ACs had no effect on the DC phenotype compared to unstimulated DCs in the resting condition.

**Efferocytosis of IACs promotes anti- and pro-inflammatory cytokine production**

Next, we determined the cytokine environment promoted by the efferocytosis of ACs or IACs. Engulfment of sterile ACs promotes an anti-inflammatory response via the production of IL-10, TGF-β and PGE2 10, whereas DC interaction with IACs is followed by production of higher levels of IL-6, TGF-β and IL-23 to create a proper microenvironment for Th17 cell differentiation 19. In addition to IL-6, TGF-β and IL-23, we also observed greater amounts of IL-1β and IL-10 production by DCs co-cultured with IACs compared to DCs co-cultured with sterile ACs (Fig. 2a-e). Indeed, the blockage of IAC recognition by purified Annexin-V led to lower levels of IL-6 production by DCs (Fig 2b). However, the impairment of phagocytosis of IACs by DCs had no effect on TGF-β, IL-23, IL-10 and IL-1β production (Fig 2a,c-e).

**Phagocytosis of IACs by DCs induces high levels of PGE2**

Macrophages and DCs produce PGE2 during efferocytosis under homeostatic conditions 9, 10. However, there are no data regarding PGE2 production after the recognition and phagocytosis of IACs. Interestingly, our results demonstrated that recognition of IACs promotes an increase in PGE2 production of at least 10-fold compared to recognition of ACs by DCs (Fig. 3a). By contrast, the blockage of PS by Annexin-V microbeads impaired IAC recognition and drastically inhibited PGE2 production by DCs (Fig 3a).

Whereas COX-1 is constitutively expressed in almost all cells, COX-2 expression is induced and enhanced during inflammation stimuli 31. Therefore, we also evaluated whether phagocytosis of ACs and IACs modulated the expression of either COX isoform. Consistent with the increase in PGE2 production by DCs after each stimulus, COX-2 expression was also enhanced when DCs were co-cultured with E. coli, and its expression was even higher after stimulating DC with IACs (Fig 3c). However, when the recognition of IACs was blocked, COX-2 expression and PGE2 production by DCs decreased (Fig

3a,c). By constrast, no significant change in COX-1 expression was observed (Fig 3b), suggesting that PGE2 production during recognition of IACs is probably associated with COX-2 upregulation.

**Efferocytosis of infected cells triggers the maturation and migratory capacity of DCs *in vitro* and *in vivo***

Since we observed increased CCR7 expression on DCs after efferocytosis, we sought to investigate the capability of DCs to migrate following interaction with ACs or IACs. Phagocytosis of IACs by DCs produced higher amounts of PGE2 and also improved migration in a CCR7-dependent manner, compared to DCs that engulfed ACs (Fig. 3a and Fig. 4a,b). This migratory capacity of DCs was drastically inhibited when efferocytosis of IAC was blocked by Annexin-V microbeads (Fig. 4a,b).

As a proof of concept, we investigated the migration of DCs after efferocytosis of ACs or IACs toward draining LNs in vivo. Bone marrow cells from C57BL/6 mice were differentiated into DCs and labeled with FarRed. These cells were co-cultured with ACs or IACs and injected in the footpads of BALB/c mice. After 48 h, cells from popliteal LNs were evaluated by IAb+ FarRed+. According to the in vitro results, DCs that engulfed IACs showed enhanced migration toward draining LNs in vivo compared to DCs that phagocytized ACs (Fig. 4c-e).

**Discussion**

Here, we demonstrated differential migration behavior and soluble mediator production by DCs after efferocytosis of sterile or infected cells. Efferocytosis of infected cells increased CD86 and CCR7 expression on DCs, PGE2 and IL-6 production, and migration capability. By contrast, phagocytosis of sterile ACs had a low impact on the phenotype and function of DCs. The maturation and migration after recognition of IACs may be

related to higher expression of COX-2 and PGE2 production, as well as a result of PRR activation by bacterial components present inside the endosome.

The presence of DCs in peripheral tissues and their ability to mediate efficient efferocytosis create an opportunity to capturenon-self and self-antigens during homeostasis or infection 32. Because DCs can interact with naïve T cells through trafficking to LNs, recognition of ACs by DCs may have an important role in T cell immunity. This event is mainly regulated by the expression of the chemokine receptor CCR7, which promotes migration through lymphatic vessels following a CCL19 and CCL21 chemotactic gradient 25, 33. Although AC-laden DCs have been found in the draining LNs of many tissues 30, 34, here we demonstrated that efferocytosis affects DC activation and migration under sterile and infectious conditions. We found that DCs that recognize either ACs or IACs were able to migrate toward a CCL19/CCL21 chemokine gradient in vitro as well as toward draining LNs in vivo. However, DCs in the presence of IACs showed greater migration capacity and higher amounts of PGE2 and IL-6 production compared with the AC condition.

Recent studies have demonstrated that PGE2 plays an important role in DC migration through CCR7 expression. Hauser and colleagues demonstrated that PGE2 alone does not increase CCR7 expression on human monocyte-derived DCs but induces oligomerization of the CCR7 receptor, leading to an efficient signaling pathway that enhances migration 27. However, in combination with other mediators such as TNF-α, IL-1β, and IL-6, PGE2 increases CCR7 expression 28. Our results show that efferocytosis of IACs promotes PGE2 production, CCR7 expression, and migration of DCs. Moreover, efferocytosis blockage caused low PGE2 production and impaired migration of DCs, demonstrating the importance of efferocytosis to trigger PGE2 synthesis and favor CCR7 expression and the migration machinery.

The expression of class II MHC, CD86 and CD80 is critical during the activation of naïve CD4+ T cells by DCs 35. Indeed, it has been reported that CD86 plays a greater role in naïve CD4+ T cell activation and differentiation than CD80 36. Interestingly, we did not observe differences in CD80 and CD40 expression in DCs activated with ACs or IACs (data not shown), whereas interaction with E. coli or E. coli-infected ACs caused enhanced expression of CD86 on DCs. PGE2 is also an important mediator involved in CD86 expression and induction of IL-6 synthesis 37, 38. Our results support these findings, since the impairment of efferocytosis by blocking PS decreased PGE2 and IL-6 production and drastically inhibited the migration of DCs in vitro. Therefore, our results suggest that high levels of PGE2 production during the efferocytosis of IACs could explain the ability of DCs to migrate toward CCL19/CCL21 chemokines and upregulate CD86 expression.

Phagocytosis of apoptotic tumor cells leads to increased CD86 and class II MHC expression on DCs, and these cells can migrate and present tumor peptides to CD4+ and CD8+ T cells. Indeed, mice vaccinated with DC + apoptotic tumor cells developed protective immunity against tumors (melanoma B16) 18. In addition, Quillien and colleagues (2005) have shown greater maturation and migration of DCs after efferocytosis of apoptotic tumor cells and in the presence of exogenous PGE2, TNF or LPS 39. These findings corroborate our results since phagocytosis of IACs promotes high levels of PGE2 production and induces CD86 and CCR7 expression on DCs. As peptides from ACs can be presented on class I and II MHC molecules 32, 40, 41, the DC maturation state and production of specific cytokines may promote T cell subtype commitment. We observed that phagocytosis of ACs promotes IL-10, TGF-β and PGE2 production. These results corroborate literature data that the recognition of ACs is a silent and anti-inflammatory process that is likely to lead to T cell anergy or regulatory T cell

differentiation 12, 19, 42. By contrast, the presence of IACs induced elevated IL-6, IL-1β and PGE2 production, mediators that have been described to be involved in Th17 differentiation 19.

Th17 cells have been related to host defense against bacterial and fungus infection, as well as in autoimmunity disorders 43. Thus, considering that DCs phagocytosing IACs acquire the capacity of presenting self and nonself peptides, together with the expression of costimulatory molecules and inflammatory mediators 20 such as PGE2, it is important to further address whether the high prevalence of this prostanoid could interfere with T cell differentiation and actually control Th17 commitment or unwanted self-reactive T cell clones.

The partial blockage of efferocytosis using Annexin-V microbeads decreased PGE2 and IL-6 production, whereas synthesis of IL-1β, IL-23, IL-10 and TGF-β was not affected. Since ACs can release soluble mediators such as IL-10 44 and TGF-β 45, we suggest that some of these cytokines were produced by IACs, which were not engulfed by DCs. Another important point to consider is that accumulated ACs may undergo other types of cell death, such as necrosis or pyroptosis, and release DAMPS that can activate DCs.

In summary, our results demonstrated that engulfment of IACs was capable of triggering DC migration and upregulation of CD86 and CCR7 molecules. In addition, IAC-activated DCs produced high levels of Th17-related cytokines, as described previously 19. Moreover, our results demonstrated for the first time that phagocytosis of IACs by DCs induces elevated levels of PGE2, probably due to dual activation of PRR that interact with ACs and bacterial components. Given that contradictory findings have indicated that PGE2 can dampen T cell activation 46, 47 or improve Th17 differentiation 48, further investigation is needed to elucidate the role of PGE2 produced by efferocytosis in triggering T cell immunity activation, suppression or tolerance.

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**Authors Contributions**

Conceived and designed the experiments: Penteado, LA; Dejani, NN; Medeiros, AI. Performed the experiments: Penteado, LA; Dejani, NN, Verdan, FF; Orlando, AB, Niño, V; Dias, FN; Salina, AG. Analyzed the data: Penteado, LA; Dejani, NN; Orlando, AB; Medeiros, AI. Wrote the paper: Penteado, LA; Dejani, NN; Medeiros, AI.

Penteado, LA and Dejani, NN contributed equally to this work.

**Disclosures**

The authors declare no conflict of interest.

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**Figure Legends**

**Fig 1. Expression of maturation markers is enhanced after efferocytosis of infected cells.** DCs were co-cultured with ACs or IACs in the presence or absence of Annexin-V microbeads. As a positive control, DCs were cultured with *E. coli* (ratio 1:1), and as a negative control, DCs were incubated in RPMI-1640 serum-free medium. After 13 h, DCs were isolated by magnetic separation with $CD11c^+$ and assessed by flow cytometry. **a.** Density contour graph showing the percentage of $CCR7^+CD86^+$ DCs. The cells were pre-gated on the $CD11c^+MHC\text{-}II^{high}$ population. The results are representative of three independent experiments. **b.** Bar graphs presenting the percentage of $CCR7^+CD86^+$ DCs. The mean values and error bars represent the SEM from three independent experiments. * $p < 0.05$. **c. d.** Bar graph presenting CD86 **(c)** and CCR7 **(d)** MFI of $CD11c^+$ cells. Fold-change relative to DC. Mean values and error bars represent the SEM from 3 independent experiments. * $P < 0.05$. **e.** Histogram overlays of CCR7 expression on DCs.

**Fig 2. Efferocytosis of infected cells promotes the production of anti and pro-inflammatory mediators.**

After 13 h of culture, the supernatant of each condition was collected and analyzed by ELISA for the presence of **a.** TGF-β, **b.** IL-6, **c.** IL-23, **d.** IL-10 and **e.** IL-1β. The mean values and error bars represent the SEM from three independent experiments. * $P < 0.05$.

**Fig 3. PGE2 production is enhanced by DCs after efferocytosis of infected cells.**

After 13 h of culture, the supernatant of each condition was collected and analyzed by ELISA for the presence of PGE2. **a.** Bar graph representing PGE2 production by DCs in each condition. The mean values and error bars represent the SEM from three independent experiments are shown. * $p < 0.05$. **b, c.** Bar graph representing mRNA expression of

*Ptgs 1* **(b)** and *Ptgs 2* **(c)** after 13 h of culture. The relative mRNA expression was normalized to *Gapdh* expression. The mean values and error bars represent the SEM from three independent experiments. * $P < 0.05$.

**Fig 4. Efferocytosis of infected cells triggers DC migration capacity *in vitro* and *in vivo*.**

To evaluate DC migration capacity *in vitro*, a Transwell assay was performed in which $2.5x10^5$ CFSE-labeled DCs from each condition were added in the upper chamber and CCL19/CCL21 were added in the lower chamber. After 6 h, the DCs were photographed or counted by flow cytometry. **a.** Migrating DC photograph of each condition. The results are representative of four independent experiments. **b.** Bar graph representing *in vitro* migrating DCs counted after efferocytosis. The results are representative of four independent experiments. The *in vivo* migration capacity of DCs was assessed by labeling DCs with FarRed. After co-culture with ACs or IACs, DCs from C57BL/6 were injected in the footpads of BALB/c mice. After 48 h, cells from popliteal LNs were obtained and analyzed by flow cytometry for the presence of $IA^{b+}FarRed^{+}$ cells. **c.** Density contour graph of $IA^{b+}FarRed^{+}$ cells in LNs. **d. e.** Bar graph of the percentage **(d)** and number **(e)** of $IA^{b+}FarRed^{+}$ migrating cells. The mean values and error bars represent the SEM. The results from one experiment are presented. N=5. * $P < 0.05$.

**Supplementary figure**

**Fig. S1. a.** Dot plot graph showing the percentage of $CD11b^{+}$*E. coli*-$GFP^{+}$ cells. To evaluate the percentage of IACs, RAW 264.7 cells were incubated with *E. coli*-GFP (ratio 1:10) for 2 h. **b. c.** Dot plot graph showing the percentage of ACs **(b)** and IACs. After irradiation with UV light and 4 h of rest, the cells were incubated with Annexin-V and 7-

AAD to evaluate apoptotic or necrotic cells, respectively. **d.** Dot plot graph showing the percentage of $CD11c^{+}CFSE^{+}$ cells. To evaluate DC efferocytosis, ACs were labeled with CFSE and co-cultured with DCs for 13 h. **e.** Dot plot graph showing the percentage of $CD11c^{+}CFSE^{+}$ cells after blocking PS recognition. Blockage of AC recognition was performed by labeling ACs with CFSE, following incubation of ACs with Annexin-V microbeads. All cells were acquired by flow cytometry and analyzed by the software FCS 4 Express Flow Cytometry.

**Figure 1**

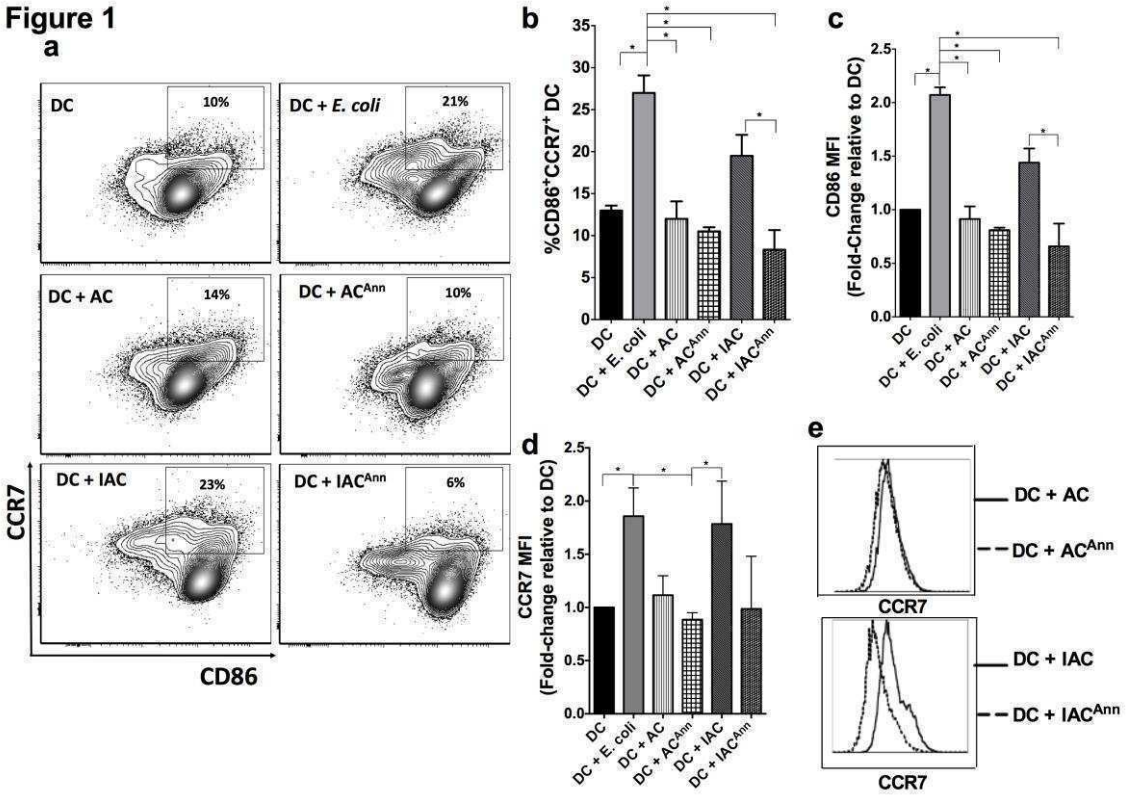

a
DC
10%
DC + E. coli
21%
DC + AC
14%
DC + AC^Ann
10%
DC + IAC
23%
DC + IAC^Ann
6%
CCR7
CD86
b
%CD86+CCR7+ DC
DC
DC + E. coli
DC + AC
DC + AC^Ann
DC + IAC
DC + IAC^Ann
c
CD86 MFI
(Fold-Change relative to DC)
d
CCR7 MFI
(Fold-change relative to DC)
e
DC + AC
DC + AC^Ann
CCR7
DC + IAC
DC + IAC^Ann
CCR7

**Figure 2**

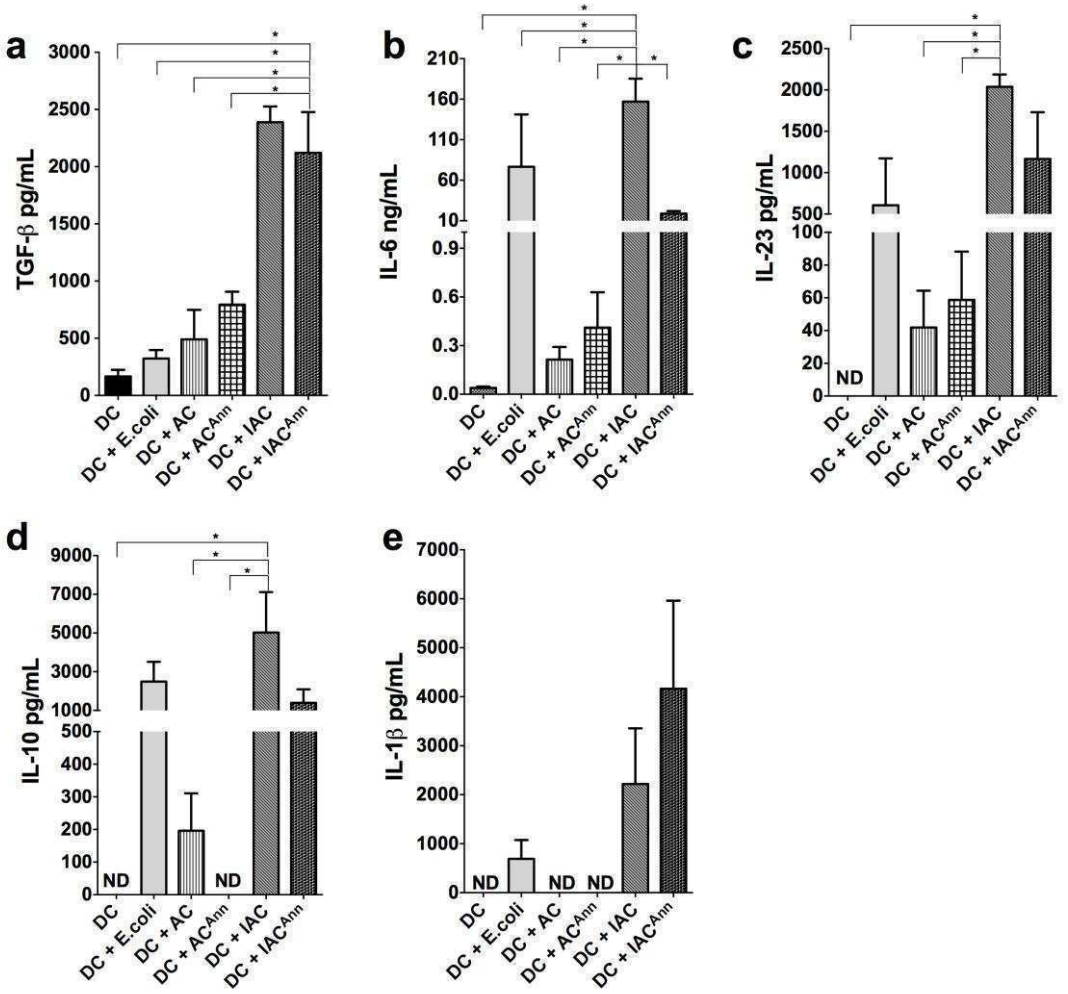
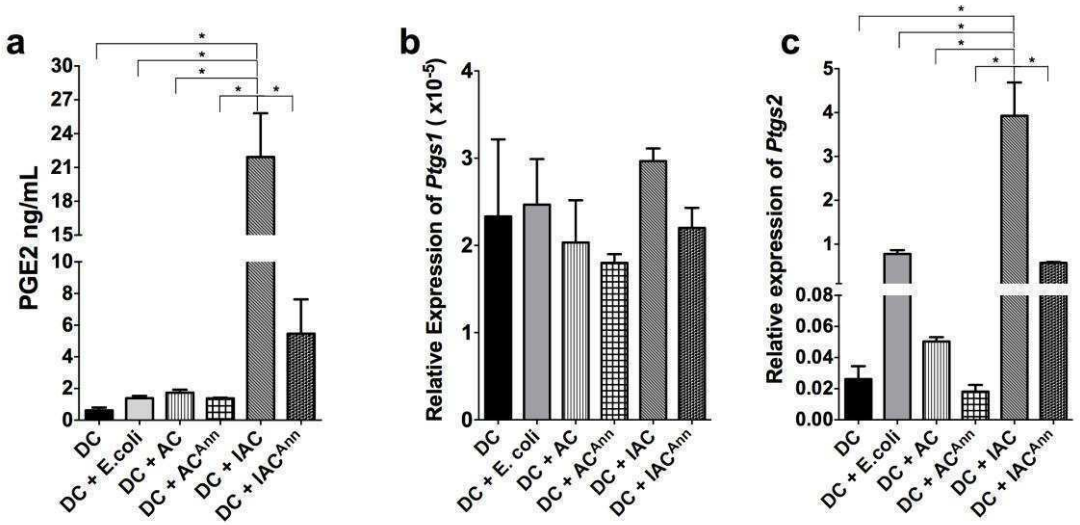
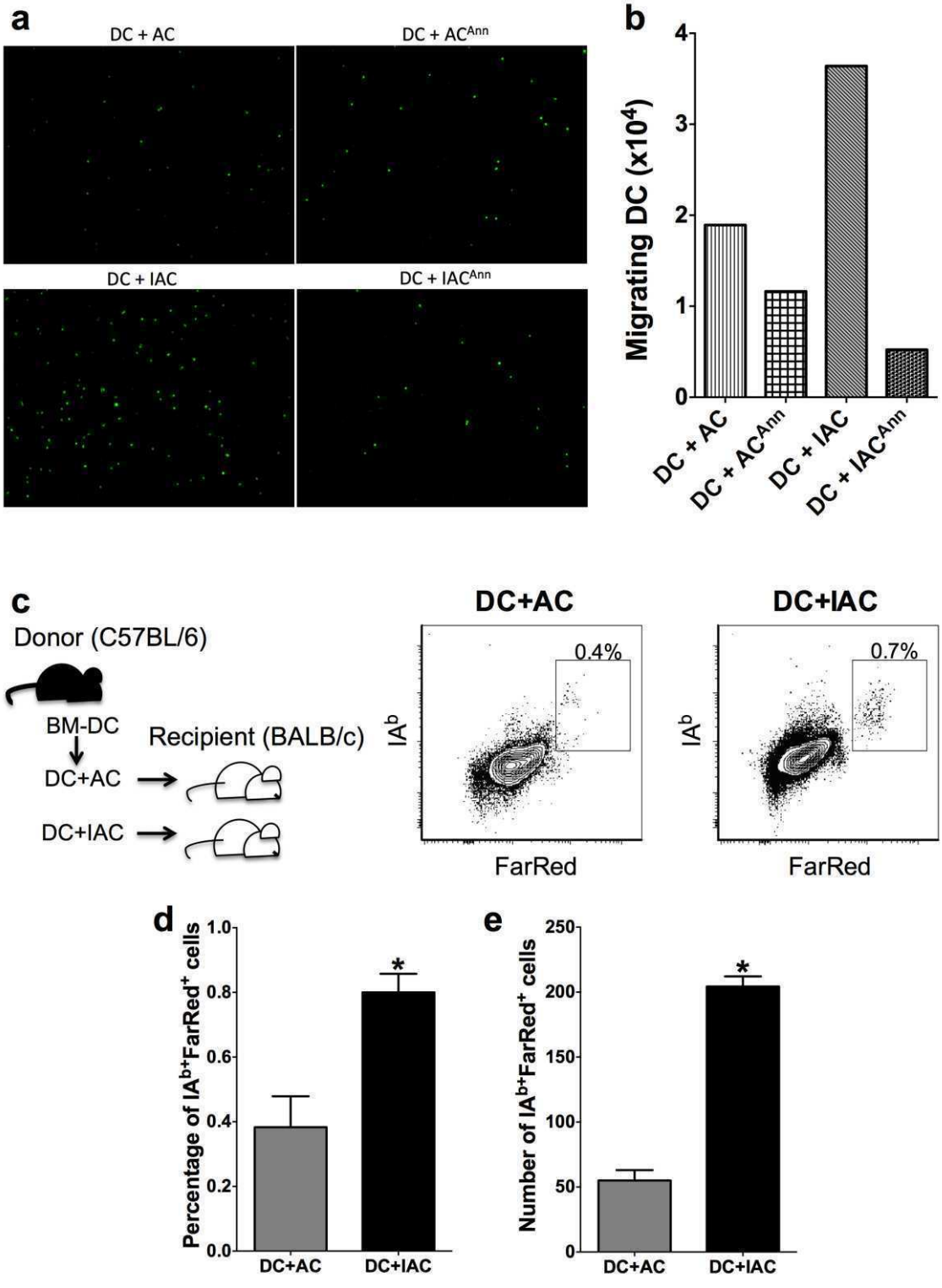

a
TGF-β pg/mL
3000
2500
2000
1500
1000
500
0
b
IL-6 ng/mL
210
160
110
60
10
0.9
0.6
0.3
0.0
c
IL-23 pg/mL
2500
2000
1500
1000
500
100
80
60
40
20
0
ND
d
IL-10 pg/mL
9000
7000
5000
3000
1000
500
400
300
200
100
0
ND
ND
e
IL-1β pg/mL
7000
6000
5000
4000
3000
2000
1000
0
ND
ND
ND
DC
DC + E.coli
DC + AC
DC + AC^Ann
DC + IAC
DC + IAC^Ann

**Figure 3**


a
PGE2 ng/mL
30
27
24
21
18
15
10
8
6
4
2
0
DC
DC + E.coli
DC + AC
DC + AC^Ann
DC + IAC
DC + IAC^Ann
b
Relative Expression of Ptgs1 ( x10^-5)
4
3
2
1
0
DC
DC + E. coli
DC + AC
DC + AC^Ann
DC + IAC
DC + IAC^Ann
c
Relative expression of Ptgs2
5
4
3
2
1
0.08
0.06
0.04
0.02
0.00
DC
DC + E.coli
DC + AC
DC + AC^Ann
DC + IAC
DC + IAC^Ann

# Figure 4


a
DC + AC
DC + AC^Ann
DC + IAC
DC + IAC^Ann
b
Migrating DC (x10^4)
0
1
2
3
4
DC + AC
DC + AC^Ann
DC + IAC
DC + IAC^Ann
c
Donor (C57BL/6)
BM-DC
Recipient (BALB/c)
DC+AC
DC+IAC
DC+AC
DC+IAC
0.4%
0.7%
IA^b
FarRed
d
Percentage of IA^b+FarRed+ cells
0.0
0.2
0.4
0.6
0.8
1.0
*
DC+AC
DC+IAC
e
Number of IA^b+FarRed+ cells
0
50
100
150
200
250
*
DC+AC
DC+IAC

**Supplementary information**