JULIANA PRISCILA VAGO DA SILVA

ESTUDO DE PROTEÍNAS INDUZIDAS POR GLICOCORTICOIDES: PAPEL DE GILZ (*GLUCOCORTICOID-INDUCED LEUCINE ZIPPER*) E ANEXINA A1 NA RESOLUÇÃO DA INFLAMAÇÃO AGUDA

Instituto de Ciências Biológicas
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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular do Departamento de Morfologia, do Instituto de Ciências Biológicas, da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do título de Doutora em Ciências.

Área de concentração: Biologia Celular

Orientadora: Profa. Dra. Lirlândia Pires de Sousa

Instituto de Ciências Biológicas
Universidade Federal de Minas Gerais
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Universidade Federal de Minas Gerais - Instituto de Ciências Biológicas
Programa de Pós-Graduação em Biologia Celular

ATA DA DEFESA DE TESE DE DOUTORADO DE

JULIANA PRISCILA VAGO DA SILVA

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Às **treze horas e trinta minutos** do dia **17 de dezembro de 2015**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado de Programa, para julgar, em exame final, o trabalho final intitulado: **"ESTUDO DE PROTEÍNAS INDUZIDAS POR GLICOCORTICOIDES: PAPEL DE GILZ (GLUCOCORTICOID-INDUCED LEUCINE ZIPPER) E ANEXINA A1 NA RESOLUÇÃO DA INFLAMAÇÃO AGUDA"**, requisito final para obtenção do grau de Doutora em Biologia Celular. Abrindo a sessão, a Presidente da Comissão, **Dra. Lirlândia Pires de Sousa**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Dra. Lirlândia Pires de Sousa	UFMG	Aprovada
Dra. Ester Roffê Santiago	FIOCRUZ - MG	Aprovada
Dra. Sandra Helena Poliselli Farsky	USP	Aprovada
Dr. Caio Tavares Fagundes	UFMG	APROVADA
Dr. Gustavo Batista de Menezes	UFMG	Aprovado

Pelas indicações, a candidata foi considerada: APROVADA
O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 17 de dezembro de 2015.**

Dra. Lirlândia Pires de Sousa (Orientadora) Lirlândia Pires de Sousa

Dra. Ester Roffê Santiago Ester Roffê Santiago

Dra. Sandra Helena Poliselli Farsky Sandra Farsky

Dr. Caio Tavares Fagundes Caio Tavares Fagundes

Dr. Gustavo Batista de Menezes

Profa. Luciana de Oliveira Andrade
Sub-Coordenadora do Programa de
Pós Graduação em Biologia Celular ICB / UFMG

Obs: Este documento não terá validade sem a assinatura e carimbo do Coordenador

Este trabalho foi realizado no Laboratório de Sinalização na Inflamação, Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia e Laboratório de Imunofarmacologia, Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, contando com apoio financeiro do CNPq, CAPES, FAPEMIG, PRPq-UFMG e European Community's Seventh Framework Programme (FP7-2007-2013).

Dedico este trabalho a minha querida família!

Aos meus pais, Nilda e Wallace.

As minhas irmãs, Ludmila e Amanda.

Ao meu amor, Fernando.

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“All truths are easy to understand once they are discovered;
the point is to discover them.”

Galileo Galilei

RESUMO

A resposta inflamatória é caracterizada pela liberação inicial de mediadores pró-inflamatórios e migração de leucócitos para o local da lesão em resposta a um estímulo estéril ou causado por patógenos. No entanto, a inflamação não controlada e persistente pode ser causa de doenças inflamatórias crônicas, sistêmicas ou autoimunes com eventual perda de função do órgão afetado, uma vez que proteases presentes nos grânulos dos neutrófilos podem perpetuar a inflamação por atuarem como agente flogístico. Desta forma, este processo deve ser finamente controlado pela ação de mediadores anti-inflamatórios e/ou pró-resolutivos e de anti-proteases endógenas que auxiliam na resolução do processo inflamatório e no retorno da homeostase tecidual. Nos últimos anos o estudo de agonistas da resolução, objetivando o encontro de moléculas que atuam na fase produtiva e resolutiva da inflamação, tem ganhado muita importância com as descobertas de moléculas pró-resolutivas lipídicas e proteicas. Dentre esses mediadores, destacam-se GILZ (*glucocorticoid-induced leucine zipper)* e anexina A1 (AnxA1), duas proteínas induzidas por glicocorticoides (GCs) que medeiam diversas funções desta classe de fármacos. Assim, o presente trabalho investigou a participação de GILZ e AnxA1, bem como o efeito de anti-proteases naturais e sintéticas, na resolução da resposta inflamatória aguda. Para tal, foi utilizado o modelo murino de pleurisia induzida por LPS em camundongos BALB/c. Após a injeção de LPS na cavidade pleural dos animais, as células presentes no lavado pleural foram coletadas em diferentes intervalos pós-injeção e analisadas. Foi observado um aumento da expressão de GILZ e AnxA1 durante a fase resolutiva da pleurisia, especialmente em macrófagos com fenótipos resolutivos. O tratamento profilático ou terapêutico de camundongos injetados com LPS com o peptídeo TAT-GILZ (permite a liberação de GILZ *in vivo*) melhorou os índices de resolução, diminuiu os níveis de citocinas e promoveu a apoptose de neutrófilos. O peptídeo TAT-GILZ também diminuiu a ativação/acúmulo das proteínas associadas com sobrevivência celular ERK1/2, NF-κB e Mcl-1. Em camundongos deficientes em GILZ ($GILZ^{-/-}$) a resolução da inflamação foi associada a um aumento precoce de AnxA1 sem modificação no influxo de neutrófilos induzida por LPS. Dexametasona (Dex) resolveu a inflamação induzindo a expressão de GILZ, cuja expressão foi dependente AnxA1. A resolução induzida por Dex não foi alterada em camundongos $GILZ^{-/-}$, devido ao aumento compensatório da expressão de AnxA1. Estes resultados mostram que embora deficiência de GILZ seja compensado pelo aumento de AnxA1 a administração terapêutica de GILZ induz um programa pró-apoptótico eficaz em

neutrófilos promovendo a resolução de inflamação neutrofílica induzida por LPS. Quanto ao estudo do balanço de proteases/anti-proteases, durante a cinética de resolução AnxA1 intacta foi detectada nas células desafiadas com PBS, e a sua clivagem foi máxima entre 8-24 horas após LPS, quando o recrutamento de neutrófilos e a expressão e a atividade de elastase foi máxima. A expressãõ de AnxA1 intacta assim como de anti-proteases endógenas (SLPI - *secretory leukocyte protease inhibitor* e Elafina) foi aumentada durante a fase resolutiva da inflamação. O tratamento de camundongos com SLPI e Elafina promoveu a resolução da inflamação associada com aumento de neutrófilos apoptóticos e aumento de AnxA1 intacta no interior da cavidade pleural. De maneira semelhante, o inibidor sintético de elastase, Sivelestat, promoveu a resolução da inflamação associada à prevenção de clivagem de AnxA1 e aumento da apoptose de neutrófilos dependente de caspases. De forma importante, Sivelestat aumentou o número de macrófagos com fenótipos resolutivos e a eferocitose de neutrófilos apoptóticos. A neutralização de AnxA1 utilizando um anticorpo neutralizante ou o bloqueio do seu receptor aboliu o efeito pró-resolutivo induzido por Sivelestat. Estes resultados mostram que a inibição de elastase por anti-proteases promove a resolução da inflamação associada com o aumento dos níveis de AnxA1 intacta e apoptose. Tomados em conjunto, estes dados mostram que proteínas induzidas por GCs ou estratégias terapêuticas que aumentam seus níveis podem se constituir em uma estratégia interessante para o controle de doenças inflamatórias.

ABSTRACT

The inflammatory response is characterized by the initial release of pro-inflammatory mediators and migration of leukocytes to the site of injury in response to sterile stimulus or caused by pathogens. However, persistent or uncontrolled inflammation may be cause of chronic inflammatory, systemic or autoimmune diseases with eventual loss of function of the affected organ, since proteases present in the granules of neutrophils can perpetuate inflammation by acting as phlogistic agent. Thus, this process must be finely controlled by the action of anti-inflammatory and/or pro-resolving mediators and endogenous anti-proteases that assist in resolution of the inflammatory process and the return of tissue homeostasis. In recent years the study of agonists of the resolution, aiming to find molecules that act in the production and termination phase of inflammation, has gained much importance to the findings of lipid and protein pro-resolving molecules. Among these mediators, are highlighted GILZ (*glucocorticoid-induced leucine zipper*) and annexin A1 (AnxA1), proteins induced by glucocorticoids (GCs) that mediate various functions of this class of drugs. The present study investigated the participation of GILZ and AnxA1, and the effect of natural and synthetic anti-proteases in the resolution of the acute inflammatory response. For this purpose, it was used the murine model of LPS-induced pleurisy in BALB/c mice. Subsequently, the cells in the pleural fluid were collected at different post injection intervals and analyzed. It was observed that GILZ and AnxA1 expression was increased during the resolution phase of pleurisy, especially in macrophages with resolutive phenotypes. The prophylactic or therapeutic treatment of mice injected with LPS with the peptide TAT-GILZ (allows the *in vivo* delivery of GILZ), improved resolution indices, decreased cytokine levels, and promoted apoptosis of neutrophils. TAT-GILZ also decreased the activation/accumulation of the survival proteins ERK1/2, NF-κB and Mcl-1. In deficient GILZ ($GILZ^{-/-}$) mice the resolution of inflammation was associated with an early increase of AnxA1 without changing the influx of neutrophils induced by LPS. Dexamethasone (Dex) solved inflammation inducing GILZ expression, which was dependent of AnxA1. The resolution induced by Dex has not changed in $GILZ^{-/-}$ mice due to a compensatory increase AnxA1 expression. These results shows that despite GILZ deficiency are offset of AnxA1 increase, the therapeutic administration of GILZ effectively induces a pro-apoptotic program by promoting resolution of neutrophilic inflammation induced by LPS. As regards the study of the balance of protease/anti-protease during kinetic resolution, intact AnxA1 was detected in cells challenged with PBS, and its

cleavage was maximal between 8-24 hours after LPS, when the recruitment of neutrophils and the expression and elastase activity was maximal. The AnxA1 expression as well as endogenous antiproteases expression (SLPI - *secretory leukocyte protease inhibitor* and Elafin) was increased during the resolution phase of inflammation. Treatment of mice with SLPI and Elafin promoted the resolution of inflammation associated with increased apoptotic neutrophils and intact AnxA1 in the pleural cavity. Similarly, the synthetic inhibitor of elastase Sivelestat, promoted the resolution of inflammation associated with prevention of AnxA1 cleavage and increased neutrophil apoptosis in a caspases dependent manner. Importantly, Sivelestat incresased the number of macrophages with resolutive phenotype and efferocytosis of apoptotic neutrophils. The AnxA1 neutralization using a neutralizing antibody or blocking its receptor abolished the effect induced by Sivelestat. These results show that inhibition of elastase by anti-proteases promote the resolution of inflammation associated with increased levels of intact AnxA1 and apoptosis. Taken together, these data show that proteins induced by GCs or therapeutic strategies which increases their levels may constitute an interesting strategy to the control of inflammatory diseases.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

ABC: *ATP-binding cassette* – transportador ABC

ALXR: *lipoxin* A4 *receptor* – receptor de lipoxina A4

ANOVA: análise de variância

AnxA1: anexina A1

APAF-1: *apoptotic protease activating factor 1* – fator de ativação de protease associado a apoptose 1

AP-1: proteína ativadora 1

ATP: adenosina trifosfato

BALB/c: linhagem de camundongos albinos BALB/c

Bcl-xL: *B-cell lymphoma-extra large*

BSA: *bovine serum albumin* – albumina de soro bovino

Caspase: *cysteine-dependent aspartate-directed* – proteases cisteína-dependente e aspartato-específicas

COX-2: cicloxigenase-2

DAMP: *damage-associated molecular patterns* – padrões moleculares associados ao dano

ERK1/2: *extracellular signal-regulated* kinase – cinase regulada por sinal extracelular

FADD: *Fas-associated protein with death domain* – proteína com domínio de morte associada à Fas

FMLP: N-formil-methionil-leucil-phenilalanina

FPR: receptor de formil peptídeo

GC: glicocorticoide

GR: *glucocorticoid receptor* – receptor de glicocorticóide

GILZ: *glucocorticoid-induced leucine zipper* – proteína induzida por glicocorticóide que possui zipper de leucina

GPCR: *G protein coupled receptors* – receptor transmembrânico acoplado à proteína G

GRE: *glucocorticoid responsive elements* – elementos responsivos aos glicocorticóides

ICAM-I: *intercellular adhesion molecule-I* – molécula de adesão intercelular-I

iNOS: *inducible nitric oxide synthase* – sintase induzida do óxido nítrico

IL-( ): interleucina-( )

i.pl.: intrapleural

i.p.: intraperitonial

JAM: *junctional adhesion molecule* – molécula de adesão juncional

JNK: *c-Jun N-terminal kinase* – cinase c-Jun N-terminal

LPS: lipopolissacarídeo

MAPK: *mitogen-activated protein kinase* – proteína cinase ativada por mitógeno

Mcl-1: *myeloid cell leukemia sequence 1* – leucemia mielóide seqüência 1

MKP-1: *Mitogen-activated protein kinase phosphatase-1* – fosfatase de MAPKs

MPO: mieloperoxidase

Mres: macrófagos resolutivos

M1: macrófagos do tipo M1

M2: macrófagos do tipo M2

NLR: *Nod-like receptor* – receptor do tipo Nod

NFIL-6: *nuclear factor-IL-6* – fator nuclear da interleucina 6

NF-κB: fator nuclear kappa B

PAMP: *pathogen associated molecular pattern* – padrão molecular associado à patógenos

PBS: *phosphate-buffered saline* – Tampão fosfato salina

PECAM: *platelet endothelial cell adhesion molecule* – molécula de adesão plaqueta-célula endotelial-1

PI3K: fosfatidilinositol 3-cinase

PLA2: fosfolipase A2

PMN: polimorfonuclear

PMSF: *phenylmethylsulphonyl fluoride* – fenilmetilsulfonilfluoreto

PR3: proteinase-3

ROS: espécie reativa de oxigênio

rTNF: receptor de fator de necrose tumoral

SIV: Sivelestat

SLPI: *secretory leukocyte protease inhibitor* - Inibidor de protease secretado por leucócitos

TGF: *transforming growth factor* – fator de crescimento transformante

TLR: *toll like receptor* – receptor do tipo Toll

TNF-α: *tumor necrosis factor alpha* – fator de necrose tumoral alfa

VCAM: *vascular cellular adhesion molecule-1* – molécula de adesão celular-vascular 1

VE-caderina: *vascular endothelial cadherin* – caderina vascular-endotelial

SUMÁRIO

1- INTRODUÇÃO

1.1- Inflamação

A inflamação aguda é caracterizada por uma resposta dos tecidos a um estímulo inflamatório, que pode ser de natureza não infecciosa ou causada por um microrganismo. Cornelius Celsus, no século I D.C., descreveu os sintomas clínicos da inflamação, reconhecidos atualmente como os quatro sinais cardinais da inflamação: o rubor (vermelhidão, devido à hiperemia), tumor (edema, causado por aumento da permeabilidade microvascular e extravasamento de proteínas para o espaço intersticial), calor (associado com o aumento do fluxo sanguíneo e hipertermia - aumento da temperatura corporal), e dor (em parte devido a alterações nas terminações nervosas). Um quinto sinal foi adicionado posteriormente por Rudolf Virchow em 1858, a perda da função, associada à disfunção dos órgãos envolvidos neste processo. No final do século XIX, Elie Metchnikoff introduziu conceitos baseando-se em eventos celulares e apontou o papel chave de leucócitos, tanto na defesa do hospedeiro quanto na manutenção da homeostase tecidual (Medzhitov, 2010).

Atualmente, sabe-se que o processo inflamatório é baseado em eventos vasculares e celulares, ocorrendo alterações na microcirculação tais como fenômenos angiogênicos, liberação de moléculas solúveis, acúmulo de leucócitos, inicialmente polimorfonucleares (PMN) seguido pela chegada de monócitos que, no local, se diferenciam em macrófagos. Esse processo pode ocorrer em resposta a diversos agentes lesivos de natureza infecciosa, traumática, tumoral ou autoimune. A inflamação é caracterizada, na sua fase produtiva, principalmente pela liberação de mediadores pró-inflamatórios e migração de leucócitos para o local da lesão, com a finalidade de eliminar o estímulo indutor e restaurar a homeostase tecidual (Nathan, 2002; Norling et al., 2010).

1.1.1 - Recrutamento de leucócitos

Durante o processo inflamatório, a migração de leucócitos é minuciosamente regulada. Um conjunto complexo de moléculas são produzidas e secretadas em resposta ao agente indutor, o que resulta na quimiotaxia dos leucócitos e permite a interação de células circulantes com as células endoteliais, possibilitando a transmigração dos leucócitos para o

sítio inflamatório (Gilroy *et al.*, 2004). A interação entre fatores quimiotáticos e receptores resulta na ativação de inúmeras moléculas pertencentes às vias sinalizadoras intracelulares que são fundamentais para que a resposta inflamatória ocorra (Marinissen *et al.*, 2001). A liberação desses mediadores pró-inflamatórios e quimiotáticos ocorre por células residentes (macrófagos, células dendríticas e células epiteliais) no tecido lesado, ativa os leucócitos circulantes e induz mudanças rápidas nas propriedades de adesão desses e das células endoteliais (Nourshargh *et al.*, 2005; Simon *et al.*, 2005).

O processo de recrutamento pode ser dividido em etapas (Figura 1). Inicialmente, a liberação de fatores pró-inflamatórios e quimiotáticos leva ao aumento da expressão de moléculas de adesão no endotélio. Em seguida, os leucócitos são atraídos do lúmen do vaso sanguíneo para a parede do endotélio e são então capturados, ocorrendo um rolamento dessas células, com consequente ativação celular. Posteriormente, ocorre uma adesão firme entre o leucócito e o endotélio, permitindo a transmigração dos leucócitos para os tecidos intersticiais em direção ao estímulo quimiotático (Simon *et al.*, 2005; Petri *et al.*, 2008; Yuan *et al.*, 2012).

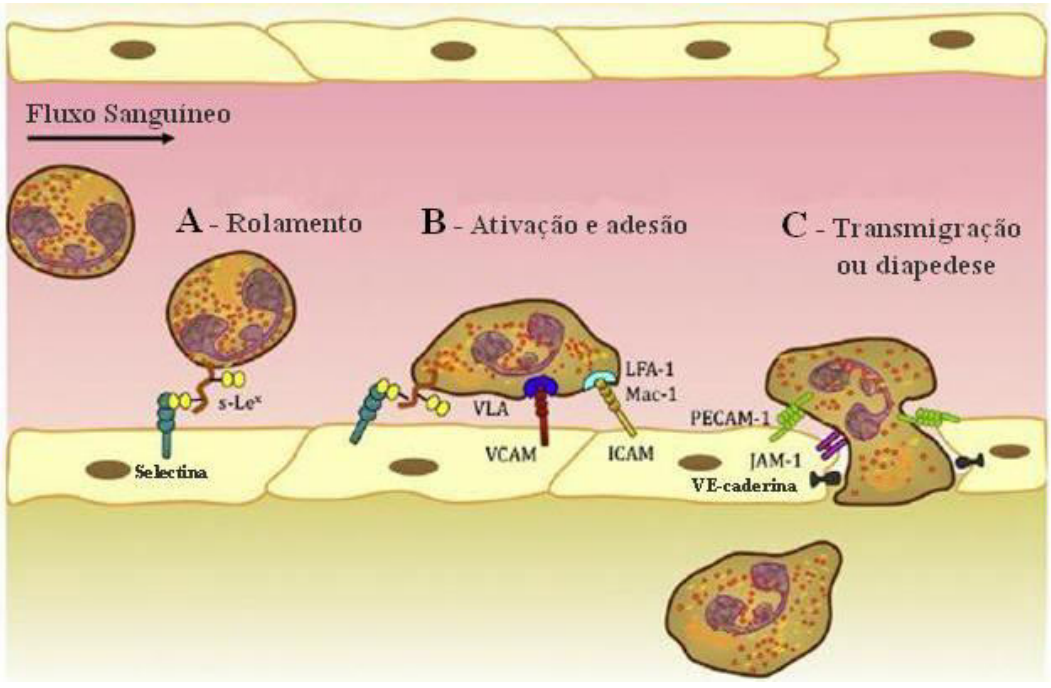

Figura 1. Representação esquemática da adesão de neutrófilos e migração transendotelial. Em resposta a estímulos inflamatórios, ocorre aumento da expressão das moléculas de adesão (selectinas) em neutrófilos e células endoteliais. Os neutrófilos rolam ao longo da parede endotelial vascular através de interações fracas mediadas pelas selectinas (A). Posteriormente, ocorre uma adesão firme dos neutrófilos ao endotélio através de moléculas de adesão (ICAM-1 e VCAM) na superfície da

célula endotelial e integrinas (Mac-1 e VLA) na superfície do neutrófilo (B). Subsequentemente, os neutrófilos transmigram através do endotélio vascular por meio de um processo que envolve interações complexas com moléculas juncionais do endotélio, VE-caderina, JAMs e PECAM-1 (C). ICAM: molécula de adesão intercelular 1; VCAM: molécula de adesão celular-vascular; Mac-1: antígeno macrófago 1; VLA: *very late antigen;* VE-caderina: caderina vascular-endotelial; JAM: molécula de adesão juncional; PECAM-1: molécula de adesão celular vascular-1 .
Fonte: Adaptado de Yuan *et al*., 2012.

1.1.2 – Neutrófilos

Os neutrófilos são células de defesa efetoras da imunidade inata, representando, em humanos, o maior grupo de leucócitos encontrados na circulação sanguínea. Os neutrófilos são os primeiros leucócitos a serem recrutados para os sítios de inflamação aguda (Nathan, 2002). Vários mediadores do recrutamento de leucócitos já estão bem estabelecidos, considerados agentes quimiotáticos clássicos, como as quimiocinas CXCL1 e CXCL2 murinos (CXCL8 humano), leucotrieno B4, fator do complemento C5a, e peptídeos formilados como FMLP (*N-formil-methionil-leucil-phenilalanina*), dentre outros (Zlotnik *et al.*, 2006; Borregaard, 2010). Esses leucócitos polimorfonucleares são produzidos na medula óssea a partir de células tronco mielóides e, na circulação, possuem uma meia vida de 6 a 8 horas, embora recentemente este conceito tenha sofrido modificações (Tak *et al.*, 2013). Após migrar para o tecido, a meia-vida dos neutrófilos pode ser prolongada de 3 a 5 dias pela ação de mediadores inflamatórios produzidos localmente, o que garante tempo suficiente para exercer suas ações antibacterianas e fagocitárias (Summers *et al.*, 2010).

Os neutrófilos, bem como macrófagos, agem no reconhecimento de PAMPs (padrões moleculares associados à patógenos) e DAMPs (padrões moleculares associados ao dano celular), através de receptores do tipo *Toll Like* (TLRs), ou outros receptores da resposta imune inata, desencadeando ativação de cascatas sinalizadoras intracelulares e de genes pró-inflamatórios. Um dos receptores mais estudados é o TLR4, que medeia respostas às bactérias Gram negativos através do reconhecimento do lipopolissacarídeo de membrana, o LPS (Prince *et al.*, 2011).

Os neutrófilos podem ser ativados por mediadores inflamatórios ou por peptídeos bacterianos, aumentando a expressão de moléculas de adesão, com consequente migração

para os tecidos em direção a um gradiente quimiotático. A ativação celular também está relacionada com o aumento da sua capacidade fagocítica e produção de importantes efetores da resposta neutrofílica. Os neutrófilos também apresentam grânulos que contêm produtos tóxicos bactericidas como mieloperoxidase (MPO), peróxido de hidrogênio (H_2O_2), ácido hipocloroso (HOCl), elastases, proteinase-3 (PR3), gelatinases, colagenases, metaloproteinases e fosfolipase A2 (PLA2). Estes grânulos podem se fundir com vacúolos citoplasmáticos formando o fagossomo, onde os microrganismos serão degradados pela liberação de enzimas hidrolíticas e de espécies reativas de oxigênio, ou pode ocorrer a degranulação, onde seus produtos são secretados para o meio extracelular (Burg *et al.*, 2001; Simon *et al.*, 2005; Prince *et al.*, 2011). Além disso, proteases liberadas de neutrófilos agem como agentes flogísticos favorecendo o recrutamento adicional de PMN para o sítio inflamatório (Soehnlein *et al.*, 2010). Desta forma, é importante que haja um controle do número e tempo de permanência dos neutrófilos no sítio inflamatório, uma vez que o acúmulo excessivo pode não ser eliminado de forma eficiente pelos macrófagos e a permanência destas células pode levar ao dano tecidual.

1.1.3 - Macrófagos

Os macrófagos são células fagocíticas muito importantes na modulação da resposta imunológica. São células derivadas de monócitos e têm origem na medula óssea a partir de uma célula tronco hematopoiética comum. Em resposta ao fator estimulante de colônias de macrófagos (M-CSF), células tronco hematopoiéticas se dividem e se diferenciam em monócitos, que deixam a medula óssea, entram na corrente sanguínea e migram para diferentes órgãos e tecidos. Uma vez nos tecidos, essas células podem se diferenciar localmente, dando origem a diferentes tipos de macrófagos que podem ser residentes ou transitórios, e que vão desempenhar papéis importantes na resposta imune inata ou adaptativa (Nathan, 2008; Wynn *et al.*, 2013). Este paradigma declara que os macrófagos teciduais residentes em todo o corpo originam a partir de precursores sanguíneos, os monócitos, os quais continuamente substituem o *pool* tecidual, conforme necessário, durante toda a vida. No entanto, existe um novo paradigma em que a população de macrófagos residentes surge dentro dos órgãos durante a embriogênese, expandindo-se conforme a expansão do órgão, e repopulando para manter o *pool* de macrófagos em um nível constante nos tecidos. Neste

paradigma os monócitos são relegados ao papel de precursores de macrófagos, particularmente durante o processo inflamatório (Randolph, 2014).

Os macrófagos são considerados fagócitos profissionais, ou seja, células cuja função primária é a de fagocitar partículas, que podem ser inertes ou microrganismos. Os macrófagos também estão envolvidos na remoção de restos celulares provenientes de células apoptóticas, que são gerados durante a remodelação de tecidos e resolução dos processos inflamatórios, processo este chamado de eferocitose. Além disso, desempenham um papel importante na resposta imune adaptativa, como apresentadores de antígenos aos linfócitos (Nathan, 2008; Poon *et al.*, 2014). Os macrófagos detectam sinais de perigo através de receptores do tipo *Toll*, receptores de reconhecimento de padrões intracelulares *Nod-like* (NLRs) e do receptor de interleucina-1 (IL-1R) (Park *et al.*, 2004; Kono *et al.*, 2008).

Diferentes estímulos podem desencadear respostas dos macrófagos levando à ativação destas células nos tecidos. As citocinas produzidas por células do sistema imune podem direcionar o fenótipo destas células, dando origem a macrófagos com fisiologias distintas (Mosser *et al.*, 2008). Existe uma classificação que é muito utilizada para determinar os diferentes tipos fenotípicos de macrófagos frente a um determinado tipo de resposta. Os macrófagos podem ser ativados de forma clássica, denominados macrófagos M1 (macrófago de defesa do hospedeiro, induzido principalmente por IFN-γ), ou os macrófagos podem ser ativados de forma alternativa denominados macrófagos M2 (cicatrizadores/reparadores, induzidos principalmente por IL-4). Nesta classificação o grupo M2 compreende todos os outros tipos de macrófagos, os quais possuem uma diferença muito grande em sua fisiologia e bioquímica. Desta forma, foi denominado um terceiro tipo de macrófago, dentro desse grupo, os macrófagos resolutivos (Mres) (Schif-Zuck *et al.*, 2011; Ariel *et al.*, 2012). O Mres desempenha um papel importante na resolução da resposta inflamatória, devido à produção e liberação de mediadores anti-inflamatórios e pró-resolutivos, além de proteínas anti-fibróticas e antioxidantes que limitam o dano tecidual e fibrose (Ariel *et al.*, 2012).

1.1.4 - Resolução da Resposta Inflamatória

A inflamação é uma resposta do organismo às alterações ambientais e é parte de um sistema de defesa que foi aperfeiçoado e conservado evolutivamente ao longo de milhões de

anos (Marchalonis *et al.*, 2002). Esse processo é geralmente protetor e mantém a homeostase tecidual, mas se não controlado se torna deletério ao hospedeiro progredindo para a inflamação crônica, cicatrização e fibrose. Em quase todos os casos, a causa fundamental do dano tecidual é o acúmulo excessivo de leucócitos. Por outro lado, na reação inflamatória limitada pelo organismo, o recrutamento de leucócitos é acoplado à liberação de fatores locais que previnem o recrutamento adicional ou excessivo de leucócitos permitindo a resolução do processo (Norling *et al.*, 2010).

A resolução da inflamação é um processo ativo e contínuo, que envolve a ativação de um programa endógeno, com produção e liberação de diferentes mediadores bioquímicos e ativação de vias de sinalização que garantem a restauração rápida e bem sucedida da homeostase do tecido (Serhan *et al.*, 2007; Alessandri *et al.*, 2013; Sousa *et al.*, 2013). O início da inflamação aguda é caracterizado pela liberação de mediadores pró-inflamatórios, os quais atraem células efetoras para o foco inflamatório. Esse processo normalmente é auto-limitante já que ocorre um balanço entre a produção de mediadores pró e anti-inflamatórios. Em resposta à injúria ou infecções, os neutrófilos migram para o sítio inflamatório, neutralizando e eliminando estímulos potencialmente deletérios. Com o fim do estímulo, ocorre diminuição local dos mediadores pró-inflamatórios, através da diminuição da síntese e aumento do catabolismo dos mesmos. Adicionalmente a esses eventos, ocorre liberação de moléculas anti-inflamatórias e pró-resolutivas que previnem a manutenção do edema e migração de PMN adicionais. Ainda, mediadores pró-resolutivos induzem o recrutamento de monócitos de maneira não flogística tornando mais eficiente o processo de eferocitose (Godson *et al.*, 2000; McArthur *et al.*, 2015). Estes eventos marcam o início do processo resolutivo que irá restabelecer a homeostase tecidual (Figura 2) (Serhan *et al.*, 2007; Alessandri *et al.*, 2013; Sousa *et al.*, 2013).

Uma resolução bem sucedida irá limitar a lesão tecidual, impedindo a progressão da inflamação. No entanto, se o hospedeiro não for capaz de conter o agente agressor ou ocorrerem falhas nos mecanismos pró-resolutivos, a inflamação pode perpetuar-se resultando em diferentes graus de lesão tecidual. Se a lesão tecidual for leve, as células serão substituídas por novas células em um processo conhecido como regeneração. No entanto, se o dano tecidual for extenso e duradouro, como ocorre nas inflamações crônicas, as células lesadas serão substituídas, ocorrendo deposição de colágeno e cicatrização, um processo que muitas vezes leva a perda da função do órgão (Gilroy *et al.*, 2004).

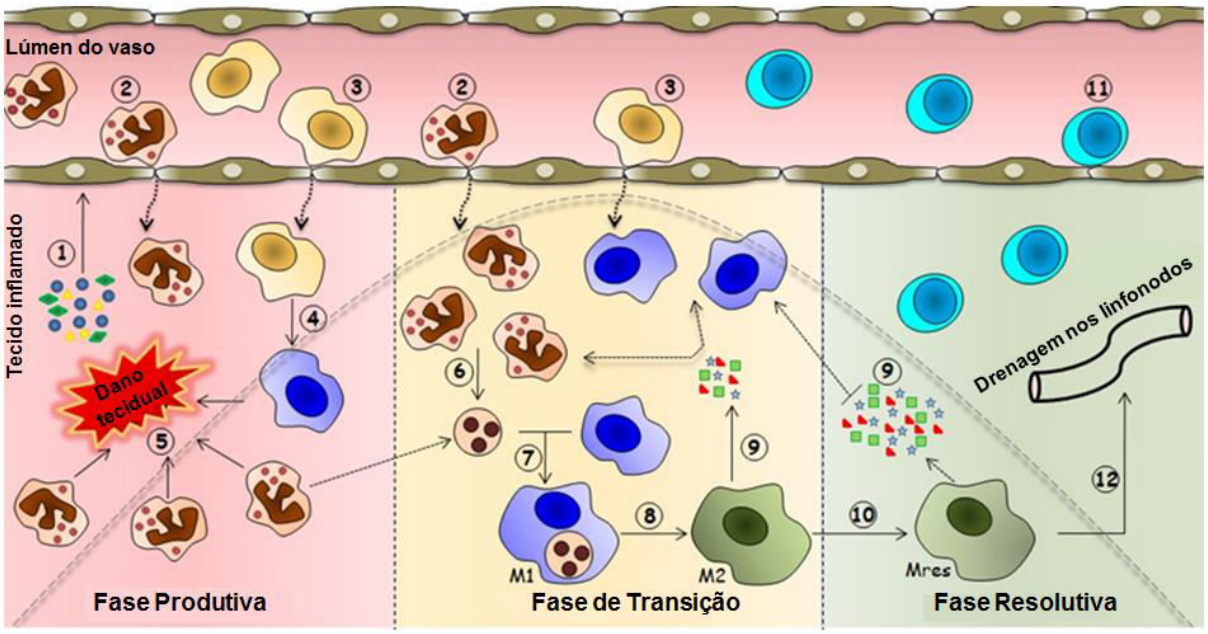

Figura 2 - Série orquestrada de eventos que levam à resolução do processo inflamatório agudo. A lesão tecidual (estéril ou infecciosa) leva ao reconhecimento de padrões moleculares (DAMPs e/ou PAMPs) pelas células residentes (macrófagos teciduais, células dendríticas e células epiteliais), os quais produzem rapidamente vários mediadores pró-inflamatórios (1). Nesta fase produtiva da inflamação, mediadores atuam promovendo vasodilatação e consequente aumento do fluxo sanguíneo local, modificando a permeabilidade do endotélio. Estes eventos são acompanhados por hiperemia e exsudação de proteínas plasmáticas e de líquidos (edema). As células endoteliais também são ativadas e expressam moléculas de adesão celular (selectinas e integrinas) e apresentam mediadores quimioatrativos, que permitam a captura e extravasamento de leucócitos (2-4). Leucócitos polimorfonucleares (sobretudo neutrófilos) são as primeiras células que extravasam para os tecidos inflamados (2), seguidos por células mononucleares (3). Com a progressão da resposta inflamatória, há intenso influxo de leucócitos para o tecido inflamado. Estas células podem ser ativadas e se tornarem uma fonte importante de uma variedade de mediadores, incluindo fatores de crescimento, citocinas, quimiocinas, mediadores lipídicos e espécies reativas de oxigênio (ROS) (5), o que permitirá que os leucócitos exerçam as suas funções efetoras nos tecidos. Na fase de transição da inflamação, apesar do intenso acúmulo de leucócitos PMNs, sob a ação de sinais pró-resolutivos (mediada por mediadores pró-resolutivos e diminuição dos níveis de agentes modificadores da sobrevivência celular), inicia-se a apoptose (6), seguido de fagocitose por macrófagos teciduais (eferocitose) (7). Este processo envolve vários sinais que levam à atração, a ligação e a remoção de células apoptóticas por macrófagos. Durante a eferocitose, macrófagos mudam seu fenótipo de M1 para M2 (8). Macrófagos M2 - (ou M2 *like*) são altamente eferocíticos e produzem moléculas anti-inflamatórias (tais como a IL-10 e TGF-β) e mediadores pró-resolutivos (9). Tais mediadores têm o potencial para inibir o recrutamento adicional de PMN, intensificar a migração de monócitos e amplificar a eferocitose. Macrófagos M2 se

modificam para macrófagos resolutivos (Mres) (10), os quais apresentam capacidade fagocítica reduzida, mas, no entanto, produzem proteínas anti-fibróticas e antioxidantes que limitam o dano tecidual e fibrose. Tais eventos pavimentam o caminho para a fase de resolução da inflamação. O aumento da produção de citocinas anti-inflamatórias, de mediadores pró-resolutivos e anti-fibróticos por macrófagos resolutivos (Mres), a repovoação por linfócitos (11) e apoptose de macrófagos ou drenagem destas células para o linfonodo local (12) encerram o processo inflamatório e restauram a homeostase do tecido. Fonte: Adaptado de Alessandri *et al*., 2013.

A apoptose de neutrófilos seguido pelo reconhecimento e remoção por macrófagos é um processo fundamental para a resolução da inflamação aguda. Após o dano tecidual, os leucócitos PMN são os primeiros a chegarem ao local, seguido pela migração de monócitos, que se diferenciam localmente em macrófagos. Os macrófagos da resposta inicial, chamados de M1, apresentam um perfil pró-inflamatório devido à presença de estímulos como LPS e IFN-γ. Estes macrófagos apresentam alta capacidade fagocítica e baixa capacidade eferocítica. Normalmente estão envolvidos com a liberação de mediadores inflamatórios como citocinas, quimiocinas, espécies reativas de oxigênio (ROS) e óxido nítrico (NO). Após a fagocitose, e, principalmente em presença de IL-4 ou IL-13, os macrófagos sofrem uma alteração de fenótipo para M2 e produzem moléculas anti-inflamatórias (IL-10, TGF-β), liberando também mediadores pró-resolutivos que impedem o recrutamento adicional de PMN e promovem o recrutamento de monócitos, amplificando assim a eficiência do processo de eferocitose. Estes macrófagos estão envolvidos com o reparo tecidual e tem um papel importante no retorno da homeostase. Os macrófagos M2 possuem alta capacidade eferocítica e, uma vez desempenhado seu papel de remoção de células apoptóticas seu fenótipo é novamente alterado para Mres (macrófago resolutivo), devido à ação de moléculas como lipoxinas, resolvinas, protectinas, maresinas, GC, TGF-β e IL-10. O Mres, embora tenha capacidade fagocítica reduzida, está envolvido com aumento da produção de mediadores anti-inflamatórios, pró-resolutivos, anti-fibróticos e anti-oxidantes, os quais limitam o dano tecidual e fibrose, sendo posteriormente drenado pelos vasos linfáticos (Ariel *et al.*, 2012; Alessandri *et al.*, 2013; Murray *et al.*, 2014).

Assim, durante a resolução do processo inflamatório uma série de eventos contribui para o término da resposta inflamatória. A vasodilatação e formação de edema contribuem para a redução das concentrações efetivas do estímulo inflamatório, os leucócitos recrutados

eliminam o agente efetor, os mediadores inflamatórios são desativados espontaneamente ou enzimaticamente, moléculas com função inibitória ou pró-resolutivas são produzidas (incluindo lipoxinas, resolvinas, protectinas, maresinas, prostaglandinas ciclopentenônicas (CyPGs), glicocorticoide, melanocortinas, anexina A1 (AnxA1) e interleucina (IL)-10) e as células inflamatórias são eliminadas por apoptose seguida de eferocitose pelos macrófagos (Gilroy *et al.*, 2004; Serhan *et al.*, 2007; Sousa *et al.*, 2013).

1.2 – Glicocorticoides: aspectos gerais

Glicocorticoides (GCs) são agentes anti-inflamatórios e imunossupressores potentes que são amplamente utilizados na prática clínica para o tratamento de várias condições inflamatórias e autoimunes. A habilidade dos GCs endógenos em suprimir a expressão de uma variedade de genes pró-inflamatórios e induzir certos genes anti-inflamatórios têm sido muito explorada com a utilização de GCs exógenos para o tratamento de doenças inflamatórias. Os GCs são reguladores críticos de respostas inflamatórias e imunes, bem como de uma grande variedade de processos fisiológicos fundamentais, incluindo homeostase metabólica, proliferação celular, desenvolvimento e reprodução, além de regularem o metabolismo de carboidratos, lipídeos e proteínas (Vandevyver *et al.*, 2013).

O primeiro uso clínico dos GCs data de meados de 1940, quando Philip Hench tratou, com sucesso, os sintomas de artrite reumatoide com cortisona (Kadmiel *et al.*, 2013). Os GCs apresentam a capacidade de estimular cascatas de transdução de sinal intracelulares que levam a alterações na rota da transcrição de genes envolvidos na progressão do ciclo celular e apoptose (Gross *et al.*, 2009). Em concentrações terapêuticas, os GCs promovem efeitos anti-inflamatórios potentes, e assim diversas doenças ou desordens de natureza autoimune, inflamatória ou alérgica são frequentemente tratadas com GCs sintéticos, como dexametasona ou prednisolona (Vandevyver *et al.*, 2013). Os GCs são prescritos, ainda, como imunossupressores para pacientes transplantados, além de atuarem também no tratamento contra o câncer, devido às ações anti-proliferativas e anti-angiogênicas (Kadmiel *et al.*, 2013).

Durante a inflamação, os GCs endógenos (cortisol em humanos e corticosterona em camundongos) desempenham um papel crítico na resolução do processo inflamatório. O cortisol é um hormônio produzido e secretado pelo córtex da glândula adrenal, sendo a sua

concentração circulante regulada através do ajuste do eixo hipotalâmico-hipofisário-adrenal (HPA), influenciada por fatores como o *stress* e o *feedback* negativo, tanto na hipófise, quanto no hipotálamo (Kadmiel *et al.*, 2013). Durante o processo inflamatório, citocinas pró-inflamatórias estimulam a produção do Hormônio Liberador de Corticotrofina (CRH) que, consequentemente, estimula a liberação do Hormônio Adrenocorticotrófico (ACTH) pela hipófise, de modo que o ACTH induz a síntese do cortisol pelas células da zona fasciculada do córtex da glândula adrenal (Figura 3) (Gross *et al.*, 2009; Silverman *et al.*, 2012). Como revisto por Taves e colaboradores (2011), além da produção sistêmica, existem evidências experimentais de produção local de corticosteroides, observado em diferentes tecidos (Taves *et al.*, 2011).

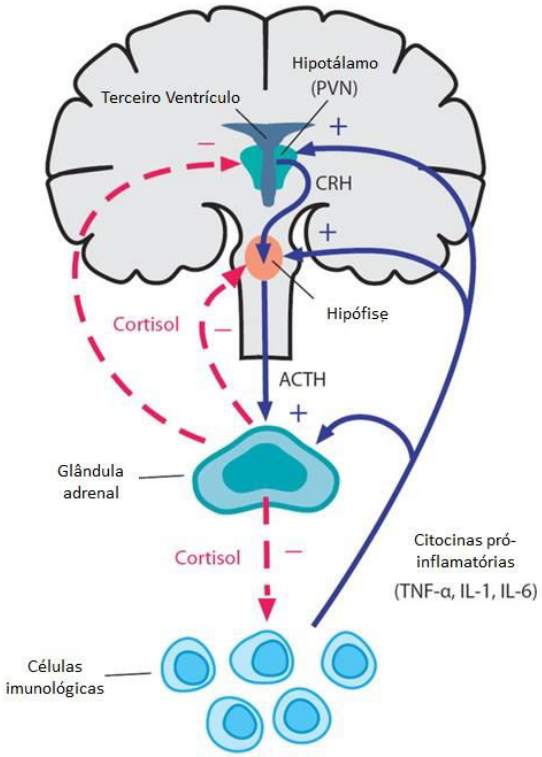

Figura 3 – Comunicação bidirecional entre o sistema imunológico e o eixo HPA. Citocinas pró-inflamatórias estimulam a liberação de cortisol pela glândula adrenal. A regulação da liberação de cortisol por *feedback* negativo pode ocorrer por meio do bloqueio da produção do hormônio liberador de corticotropina (CRH), pelo núcleo paraventricular do hipotálamo, pela inibição da liberação do ACTH pela hipófise ou, ainda, pela inibição direta da liberação de cortisol pela adrenal.
Fonte: Adaptado de Silverman *et al.*, 2012.

1.2.1 - Receptores de glicocorticoides

Tanto a ação fisiológica quanto farmacológica dos GCs são mediadas pelo receptor de glicocorticoide (GR), o qual é um fator de transcrição pertencente à superfamília de receptores nucleares (Oakley *et al.*, 2013). Estruturalmente, os GRs são compostos por três domínios funcionais: 1) o domínio amino-terminal, responsável pela interação com os fatores de transcrição; 2) o domínio central de ligação ao DNA, que consiste de dois importantes domínios dedos de zinco (*Zinc fingers*) para a dimerização do GR, translocação nuclear e ligação ao DNA; e 3) o domínio de ligação Carboxi-terminal, que contém o domínio de ligação aos GCs, bem como de co-reguladores (co-ativadores ou co-repressores) (Oakley *et al.*, 2013; Vandevyver *et al.*, 2013). Assim como todas as proteínas celulares, os receptores nucleares são sintetizados nos ribossomos citoplasmáticos e a migração dessas proteínas para o núcleo requer a existência do sinal de localização nuclear, situado próximo ao domínio de ligação ao DNA (Yudt *et al.*, 2001).

Existem dois tipos de receptores de GCs, os quais medeiam a maioria das ações dos GCs. São eles: os receptores do tipo I, ou receptores de mineralocorticoides (MR), e os receptores do tipo II, ou receptores de glicocorticoides (GR) (Medzhitov, 2010). Os MR apresentam expressão restrita, apesar de terem uma afinidade 10 vezes maior aos GCs, em comparação aos GRs. Dessa forma, a sinalização mediada por MR é limitada devido à baixa expressão deste receptor, e também restrita a apenas alguns tipos celulares (Viengchareun *et al.*, 2007; Vandevyver *et al.*, 2013). Normalmente os MR estão ocupados por níveis basais de GCs, regulando a homeostase de sais e água, enquanto que os GR são ativados durante o pico de secreção do cortisol, observado principalmente em situações de estresse (Viengchareun *et al.*, 2007).

Os GR são proteínas citoplasmáticas constitutivamente expressas que atuam por meio da alteração da expressão dos genes-alvo em resposta a um sinal hormonal específico. Nos últimos anos, o conceito tradicional de que GCs agem por meio de um único tipo de GR tem sido modificado com a descoberta de uma grande variedade de subtipos de receptores, provenientes do processamento alternativo do gene do GR (Oakley *et al.*, 2013). Dentre as isoformas originadas por *splicing* alternativo do transcrito primário de GR, o GRα e o GRβ são isoformas do GR humano que já foram identificadas. O GRα é a isoforma predominante, associada à ligação aos GCs e transdução do sinal no núcleo. O GRβ não é responsivo aos

GCs e se difere do GRα na sequência carboxi-terminal (Oakley *et al.*, 2011; Ligr *et al.*, 2012). Portanto, os GCs ligados ao GRβ não atuam como fatores de transcrição, não ocorrendo ativação ou transcrição gênica. Outras isoformas de GR também já foram identificadas em vários tipos celulares e tecidos relacionados com a resistência aos GCs, como GR-Y, GR-A e GR-P. Os diferentes padrões tecido-específicos levam a efeitos específicos para cada tipo de tecido em diferentes doenças (Vandevyver *et al.*, 2013). Dessa forma, a transcrição gênica pelos GCs é complexa tendo em vista todas essas isoformas de GR, e são necessários, portanto, estudos complementares.

1.2.2- Mecanismo de ação dos glicocorticoides

Por apresentarem alta lipofilia, os GCs são capazes de atravessar a membrana citoplasmática por transporte passivo. Na ausência de ligação ao cortisol ou ao GC sintético, o GR encontra-se inativo no citoplasma, estabilizado por um complexo proteico denominado proteínas do choque térmico (*heat shock protein)*, tais como hsp90, hsp70, hsp90 proteína de ligação p23, imunofilinas (FKBP51, FKBP52), chaperonas e outros fatores que previnem a sua degradação e auxiliam na sua maturação (Vandevyver *et al.*, 2013).

Uma vez alcançado o citoplasma da célula alvo, os GCs podem se ligar aos GR levando a uma alteração conformacional que resulta na dissociação do heterocomplexo com as proteínas estabilizadoras, com consequente exposição do sinal de localização nuclear e translocação do homodímero GC-GR para o núcleo (Oakley *et al.*, 2011). Entretanto, novas evidências experimentais sugerem que proteínas chaperonas são também necessárias para a translocação nuclear (Busillo *et al.*, 2013). No núcleo, o complexo GC-GR se liga aos sítios específicos no DNA chamados elementos responsivos aos glicocorticoides (GREs), atuando como fator de transcrição, mediando o aumento da expressão de inúmeros genes e diminuindo a expressão de outros, de forma coordenada, resultando na indução da síntese de proteínas anti-inflamatórias (anexina A1, GILZ, IκB e IL-10), na indução de proteínas que atuam no metabolismo sistêmico ou na regulação de genes pró-inflamatórios (Clark, 2007; Vandevyver *et al.*, 2013). Este processo é chamado de transativação e a maioria dos efeitos adversos associados aos CGs parece estar relacionada a este mecanismo. Contudo, nem todas as interações GR-DNA induzem expressão gênica. De fato, 50% dos genes regulados por GCs

são repremidos, devido, em parte, à interação do GR com GREs negativos para suprimir ativação gênica (Busillo *et al.*, 2013).

Os GCs também atuam por meio de outro mecanismo genômico chamado de transrepressão em que monômeros de moléculas de GC e receptores de GC interagem com fatores de transcrição envolvidos com a regulação de genes pró-inflamatórios como o NF-κB e AP-1. A inibição desses fatores de transcrição resulta na inibição da síntese de mediadores pró-inflamatórios como: citocinas, quimiocinas, enzimas evolvidas na síntese de prostaglandinas, dentre outros (Clark, 2007; Busillo *et al.*, 2013). Os mecanismos de transrepressão (TR) mediados por GR são mais promíscuos e envolvem ligação ao DNA de GR monoméricos a GREs negativos (nGREs) ou repetições invertidas (IR) com menos de três espaçadores para reprimir de maneira específica a transcrição gênica (Vandevyver *et al.*, 2013).

Apesar de apresentarem uma eficácia anti-inflamatória excelente, o uso de GCs na terapêutica deve ser muito controlado devido a alguns efeitos adversos. Primeiramente, a resistência tecidual desenvolvida em pacientes que fazem uso crônico de GC limita o sucesso de muitas terapias. Muitas doenças inflamatórias, como a Doença Pulmonar Obstrutiva Crônica, fibrose pulmonar e fibrose cística são amplamente resistentes ao tratamento com GCs. Além da resistência à terapia, muitas vezes o uso de GC é acompanhado por efeitos adversos graves que incluem síndrome metabólica, perda óssea, diabetes, obesidade, doenças cardiovasculares e sintomas psiquiátricos, dentre outros (Barnes, 2011; Oakley *et al.*, 2011; Vandevyver *et al.*, 2013). Além disso, os GCs podem aumentar a susceptibilidade às infecções (devido à imunossupressão). Uma vez que o uso prolongado de GCs leva à supressão do eixo hipotálamo-hipófise-adrenal não é recomendado que o tratamento seja interrompido bruscamente (Clark, 2007; Beaulieu *et al.*, 2011).

Os efeitos adversos são mais evidentes nos tratamentos com altas dosagens e por longos períodos. Os efeitos adversos metabólicos dos GCs parecem ser dependentes da indução da expressão gênica, ou seja, da transativação (Vandevyver *et al.*, 2013). Consequentemente, os chamados agonistas seletivos de GR (SEGRAs), que favorecem a transrepressão em detrimento da transativação, foram desenvolvidos como agentes terapêuticos com efeitos adversos limitados. Entretanto, dados recentes apontam que a transativação induzida pelo GR é indispensável para as suas propriedades anti-inflamatórias,

sendo que esta estratégia de melhorar a terapia com GCs não se mostrou bem sucedida (Vandevyver *et al.*, 2013).

Estudos recentes demonstram que diversas proteínas anti-inflamatórias, dentre elas GILZ (*Glucocorticoid Induced Leucine Zipper*), Anexina-A1 (AnxA1) e MAPK (*Mitogen Activated Protein Kinase*) fosfatase-1 (MKP-1) são induzidas por transativação (Cheng *et al.*, 2014). Esses dados sugerem que o paradigma que separa os efeitos anti-inflamatórios e metabólicos tendo por base os mecanismos de transrepressão e transativação é inadequado para explicar os efeitos causados por GCs (Cheng *et al.*, 2014). Para desenvolver uma nova estratégia que mimetiza as funções imunomoduladoras dos GCs, mas que limita os efeitos metabólicos, uma alternativa seria a descoberta de agonistas capazes de induzir os efeitos inibitórios dos GCs no sistema imune por meio de moléculas que independem do GR para suas ações anti-inflamatórias (Figura 4). O conhecimento das propriedades anti-inflamatórias destas e de outras proteínas induzidas por GCs pode levar ao desenvolvimento de fármacos que extrairiam as características benéficas dos GCs excluindo os efeitos deletérios dos mesmos sobre o metabolismo (Perretti *et al.*, 2009; Beaulieu *et al.*, 2011; Cheng *et al.*, 2014).

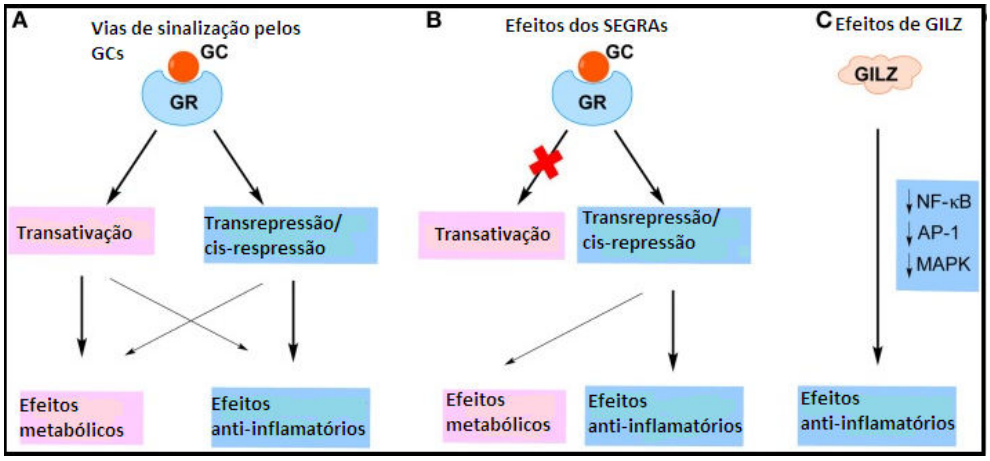

Figura 4 – Representação esquemática dos mecanismos de transativação e transrepressão, e o efeito direto de proteínas induzidas por GCs. Estratégias para melhorar a terapia com glicocorticoides: (A) Efeitos celulares dos GCs; a molécula de GC liga-se ao receptor GR e ativa as vias de sinalização intracelulares. Acreditava-se que a transativação fosse responsável pelos efeitos metabólicos dos GC, enquanto que a transrepressão mediava os seus efeitos anti-inflamatórios. (B) Esse conceito foi tomado como base para desenvolvimento dos SEGRAs, com o objetivo de inibir os efeitos metabólicos dos GC. (C) Os efeitos anti-inflamatórios de GILZ são independentes da ligação ao GR, podendo evitar a grande maioria dos efeitos metabólicos. Fonte: Adaptado de Cheng *et al.*, 2014.

1.3 - GILZ – *Glucocorticoid Induced Leucine Zipper*

A proteína GILZ foi identificada em 1997, durante um estudo com o objetivo de caracterizar genes transcricionalmente induzidos por dexametasona, um GC sintético (D'Adamio *et al.*, 1997). Estudos subsequentes, principalmente de superexpressão de GILZ, verificaram que GILZ tem funções anti-inflamatórias que mimetizam os efeitos anti-inflamatórios dos glicocorticoides associados a interações com as vias de NF-κB e AP-1 (Ayroldi *et al.*, 2001; Mittelstadt *et al.*, 2001). A descoberta de que GILZ interage e inibe a atividade do fator de transcrição NF-κB foi a primeira descrição de mecanismo de ação de GILZ, inicialmente caracterizado em células T (Ayroldi *et al.*, 2001). Subsequentemente, outros alvos de GILZ foram identificados como Raf-1, Ras, MAPK (Proteína cinase ativada por mitógeno) ERK1/2, todos também alvos dos GCs (Ayroldi *et al.*, 2002; Ayroldi *et al.*, 2007; Soundararajan *et al.*, 2007). Devido a grande variedade de interações com proteínas e sua abundância em vários tipos celulares, GILZ exerce, de fato, um papel crucial no controle do tráfego de proteínas e na sinalização.

GILZ, também conhecida como proteína da família de 3 domínios TSC22 (TSC22D3), é uma proteína que contém 137 aminoácidos, apresentando 3 domínios: o N-terminal (1-75 aa), que inclui *tuberous sclerosis complex* TSC-box (61-75 aa); *leucine zipper* (76-97 aa); e o domínio C-terminal (98-137 aa) (Beaulieu *et al.*, 2011). O motivo zíper de leucina de GILZ está localizado na parte central da proteína e medeia principalmente a homodimerização de GILZ, necessária para muitas das suas funções (Di Marco *et al.*, 2007), enquanto que os outros dois domínios são responsáveis por interações proteína-proteína entre GILZ, fatores de transcrição e as moléculas de sinalização. A porção C-terminal de GILZ é uma região rica em prolina necessária para a ligação direta entre GILZ e a subunidade p65 de NF-κB (Riccardi *et al.*, 2001; Berrebi *et al.*, 2003; Di Marco *et al.*, 2007). O domínio N-terminal de GILZ liga-se diretamente a Raf-1, molécula de ativação da via MAPK, inibindo a sua função. A interação entre GILZ e c-Fos e c-Jun (dois constituintes de AP-1) também ocorre através do domínio N-terminal de GILZ. Além disso, GILZ também se liga à Ras através do TSC-box, ou também pode interagir com Ras e Raf em conjunto, para formar um trímero (Figura 5) (Ayroldi *et al.*, 2002; Ayroldi *et al.*, 2007).

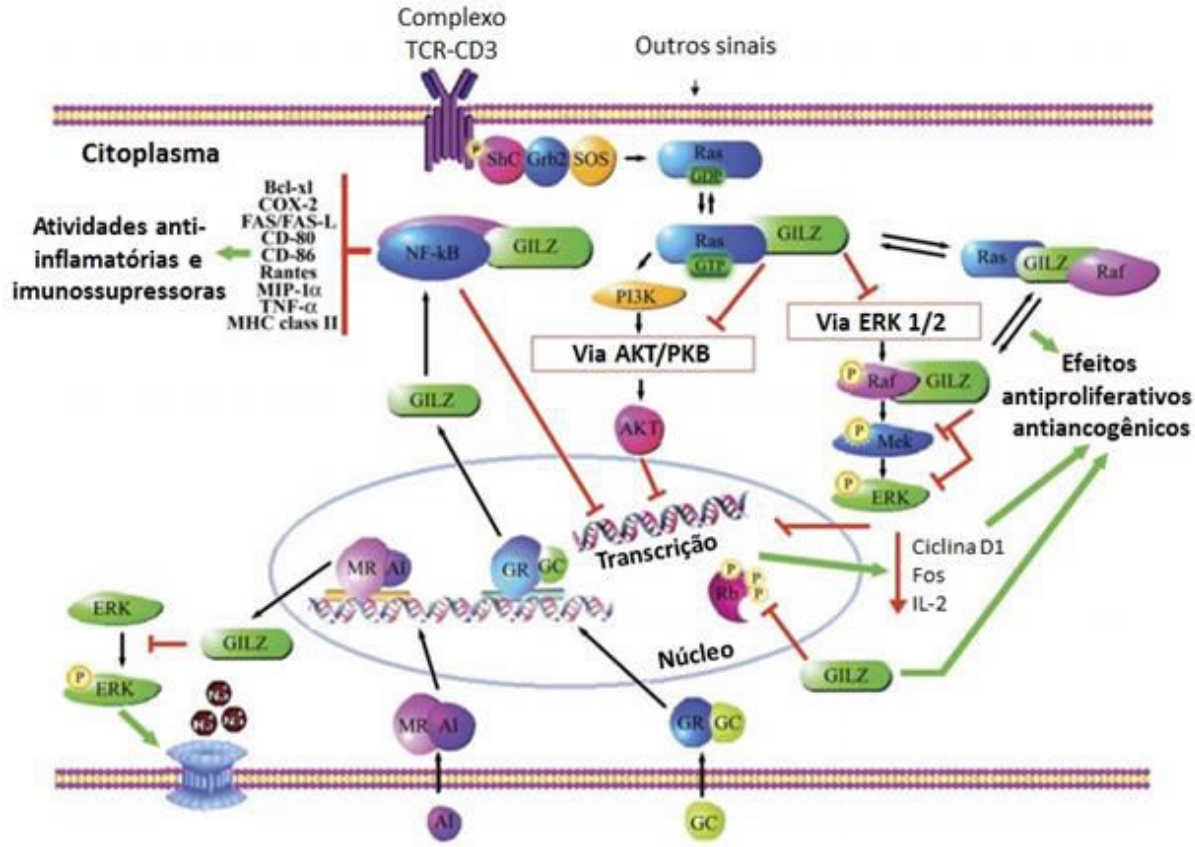

Figura 5 – Mecanismo de ação de GILZ. Ilustração esquemática do papel de GILZ como mediador das atividades anti-inflamatórias e imunossupressoras dos GCs. GILZ, induzido por GCs, interage diretamente com NF-κB e inibe a transcrição dependente de NF-κB, medeia os efeitos anti-inflamatórios e imunossupressores nos compartimentos linfóide e mielóide. O complexo Shc/Grb2/SOS converte Ras à sua forma ativa ligada ao GTP, levando à ativação das vias ERK-1/2 e Akt/PKB. Ao se ligar à Ras e Raf-1, GILZ inibe a fosforilação de MEK e ERK, inibindo, assim, a proliferação celular dependente dessa via. Fonte: Adaptado de Ayroldi e Riccardi, 2009.

O gene Tsc22d está localizado no cromossomo X (Cannarile *et al.*, 2001), e a sequência gênica do promotor de GILZ humano já foi caracterizada. O promotor de GILZ inclui 6 GREs, que inclui sítios de ligação para STAT6, fator nuclear de células T ativadas (NFAT), Oct-1, c-myc, elementos responsivos *forkhead* (FHREs), e proteína de ligação ao elemento de resposta ao cAMP (CREB), além de sequência responsiva ao estrógeno (Asselin-Labat *et al.*, 2004; Tynan *et al.*, 2004; Wang *et al.*, 2004). GILZ é um dos produtos da transcrição gênica mediada por GR. Uma vez dentro da célula, o GC forma um complexo com GR, promovendo transativação da região do gene associada à GILZ, o que resulta na síntese da proteína. Isso significa que a expressão de GILZ é proporcional à disponibilidade e função do GR e de GC. No entanto, já existem evidências de que GILZ exerce efeitos inibitórios

sobre o sistema imunológico, independente da ativação dos GR e, dessa forma, não apresenta efeitos metabólicos adversos associados aos GCs (Cheng *et al.*, 2014).

GILZ parece funcionar como um mediador celular do efeito anti-inflamatório dos GCs e é constitutivamente expresso em muitos tecidos humanos e de camundongos, sendo sua expressão rapidamente regulada por GCs em timócitos, macrófagos, células dendríticas e células T murinas, em células humanas do trato respiratório e células tronco mesenquimais, dentre outras (Ayroldi *et al.*, 2009). GILZ apresenta quatro isoformas (GILZ 1-4), sendo seus nomes atribuídos na medida em que foram sendo descobertas (Soundararajan *et al.*, 2007). GILZ-1 é uma proteína que apresenta peso molecular de 17 kDa e GILZ-2 21 kDa. Foi demonstrado que GILZ-1 é predominante em vários tipos celulares nos quais essa variante de transcrição foi examinada, tais como células T, mastócitos e células epiteliais (Hoppstadter *et al.*, 2012). Foi demonstrado que tanto GILZ-1 quanto GILZ-2 são as isoformas indutíveis por dexametasona, resultando na inibição da ativação de NF-κB (Hoppstadter *et al.*, 2012). É importante ressaltar que dados publicados acerca dos efeitos anti-inflamatórios de GILZ não associam os efeitos adversos dos GCs a essa proteína, sugerindo que GILZ exerça seus efeitos anti-inflamatórios, assim como os GCs, porém por vias distintas (Cheng *et al.*, 2014).

Já foi demonstrado que citocinas, como IL-1β, TNF-α e IFN-γ, reduzem os níveis de mRNA de GILZ em células epiteliais (Eddleston *et al.*, 2007). Além disso, foi demonstrado que GILZ tem expressão baixa ou até mesmo nenhuma expressão em doenças inflamatórias, como a doença de Crohn, tuberculose ou rinossinusite crônica (Berrebi *et al.*, 2003; Zhang *et al.*, 2009). Nestes casos, a maior concentração de GILZ encontra-se nos pulmões, sendo que os macrófagos representam a principal fonte dessa proteína (Hoppstadter *et al.*, 2012). Dados experimentais apontam que células tratadas com agonistas de TLR-4, como LPS, têm os níveis tanto do mRNA de GILZ quanto da proteína diminuídos (Hoppstadter *et al.*, 2012). Como revisto por Thiagarajah e colaboradores (2014), a baixa expressão de GILZ pode estar associada a doenças como a fibromialgia e desordens relacionadas ao estresse e depressão, de modo que o mapeamento das atividades de GILZ tem sido utilizado na caracterização dessas doenças (Thiagarajah *et al.*, 2014).

Estudos prévios sugerem que a proteína GILZ pode exercer um papel de destaque na apoptose de leucócitos, podendo contribuir para a resolução do processo inflamatório. Isso se deve ao fato de que GILZ tem a capacidade de interagir com muitas proteínas celulares

(Figura 5), como por exemplo, o fator de transcrição NF-κB, ao ligar-se à subunidade p65, e AP-1, ao interagir com as subunidades c-fos e c-jun, realizando transrepressão gênica, ou inibindo a via MEK/ERK1/2 através da ligação com Ras e Raf-1. GILZ medeia, portanto, várias funções dos GCs, como por exemplo: modulação do sistema imune, apoptose e proliferação celular (Ayroldi *et al.*, 2009). Contudo, pelo nosso conhecimento, o papel de GILZ na apoptose de neutrófilos não havia sido determinado até a execução deste trabalho.

1.4 - Anexina A1

Descrita por Flower e Blackwell em 1979, Anexina A1 (AnxA1), conhecida também como lipocortina-1, é uma proteína induzida por GCs, que foi inicialmente descrita como inibidora da ação da enzima fosfolipase A2 (Flower *et al.*, 1979). A AnxA1 é um dos membros da superfamília das anexinas, que é constituída por pelo menos 13 proteínas relativamente abundantes e estruturalmente semelhantes (Gerke *et al.*, 2002).

Estruturalmente, as anexinas são constituídas por dois domínios: uma extremidade amino terminal apresentando características variáveis de comprimento e composição de acordo com o tipo de proteína (N-terminal), e uma extremidade carboxílica com maior grau de conservação entre os membros da família das anexinas (C-terminal) (Kim *et al.*, 2001). Esta última região constitui a estrutura primária comum de ligação ao Ca^{2+}, a fosfolipídios e também ao ATP, e contém quatro a oito repetições de uma sequência conservada de 70-80 aminoácidos (Raynal *et al.*, 1994). O domínio N-terminal é específico para cada membro da família das anexinas e interage com os diferentes ligantes destas proteínas ocorrendo fosforilação, glicosilação, ação de peptidases e clivagem proteolítica seletiva. A AnxA1 é uma proteína que apresenta propriedades anti-inflamatórias e a região N-terminal é caracterizada como promotora da ação anti-inflamatória dessa proteína (Lee *et al.*, 1999; Kim *et al.*, 2001). Níveis altos de expressão de AnxA1 são encontrados de forma constitutiva e são particularmente abundantes no citoplasma de células diretamente envolvidas na resposta inflamatória, tais como monócitos, macrófagos e neutrófilos, podendo atingir cerca de 4% das proteínas solúveis totais de neutrófilos (Goulding *et al.*, 1990; Morand *et al.*, 1994; Headland *et al.*, 2015).

1.4.1 - Mecanismos de ação, liberação e clivagem de AnxA1

A proteína AnxA1 é considerada uma mediadora da ação anti-inflamatória dos GCs endógenos e exógenos. A inibição da atividade da PLA2 constitui um mecanismo anti-inflamatório importante, pois tem como consequência a inibição da formação do ácido araquidônico, com os consequentes efeitos inibitórios sobre a geração de prostaglandinas, leucotrienos e fator de agregação plaquetária (Kim *et al.*, 2001). Além deste primeiro efeito, inicialmente descrito na inibição de PLA2, vários outros efeitos na inibição de mediadores inflamatórios, como a inibição da expressão de enzimas e de moléculas de adesão (Perretti *et al.*, 2009) foram descritos para AnxA1.

Um dos principais mecanismos do efeito anti-inflamatório da AnxA1 está relacionado com a inibição da transmigração dos leucócitos. Esse efeito está associado tanto à AnxA1 quanto aos peptídeos sintéticos gerados a partir da porção N-terminal desta proteína, particularmente o peptídeo Ac2-26 (Hayhoe *et al.*, 2006). Estudos evidenciam que o mecanismo de ação da AnxA1 na regulação da migração celular está relacionado com inibição da atividade das moléculas de adesão, principalmente as integrinas e selectinas (Solito *et al.*, 2000). Alguns trabalhos já demonstraram que o peptídeo sintético Ac2-26 também está associado ao desprendimento de L-selectina (Walther *et al.*, 2000), diminuindo o rolamento e adesão de PMN em células endoteliais (Hayhoe *et al.*, 2006). Um trabalho publicado recentemente demonstrou que o peptídeo Ac2-26 reduz a afinidade de neutrófilos ativados para ICAM-1 e VCAM-1 (Drechsler *et al.*, 2015). Além disso, já foi demonstrado que neutrófilos ativados podem externalizar grandes quantidades da AnxA1 citoplasmática (>50%), e esta AnxA1 exposta sobre a membrana plasmática do leucócito aderente exerce uma ação inibitória, reduzindo a transmigração através das células endoteliais (Perretti *et al.*, 2004). Vários estudos em modelos de inflamação aguda, crônica, ou mesmo sistêmica, demonstraram que a proteína AnxA1 é inibidora do extravasamento de leucócitos para o local da inflamação (Yang *et al.*, 2004; Damazo *et al.*, 2005; Souza *et al.*, 2007; Gastardelo *et al.*, 2009; Perretti *et al.*, 2009, Sugimoto *et al*, 2016). AnxA1 também está envolvida com a inibição da enzima ciclo-oxigenase 2 (COX-2) e da enzima sintase do óxido nítrico (iNOS), além de estar relacionada com a liberação de IL-10 em fagócitos, com a indução da apoptose de células inflamatórias e a remoção de células e corpos apoptóticos (Figura 6) (Parente *et al.*, 2004; Vago *et al.*, 2012).

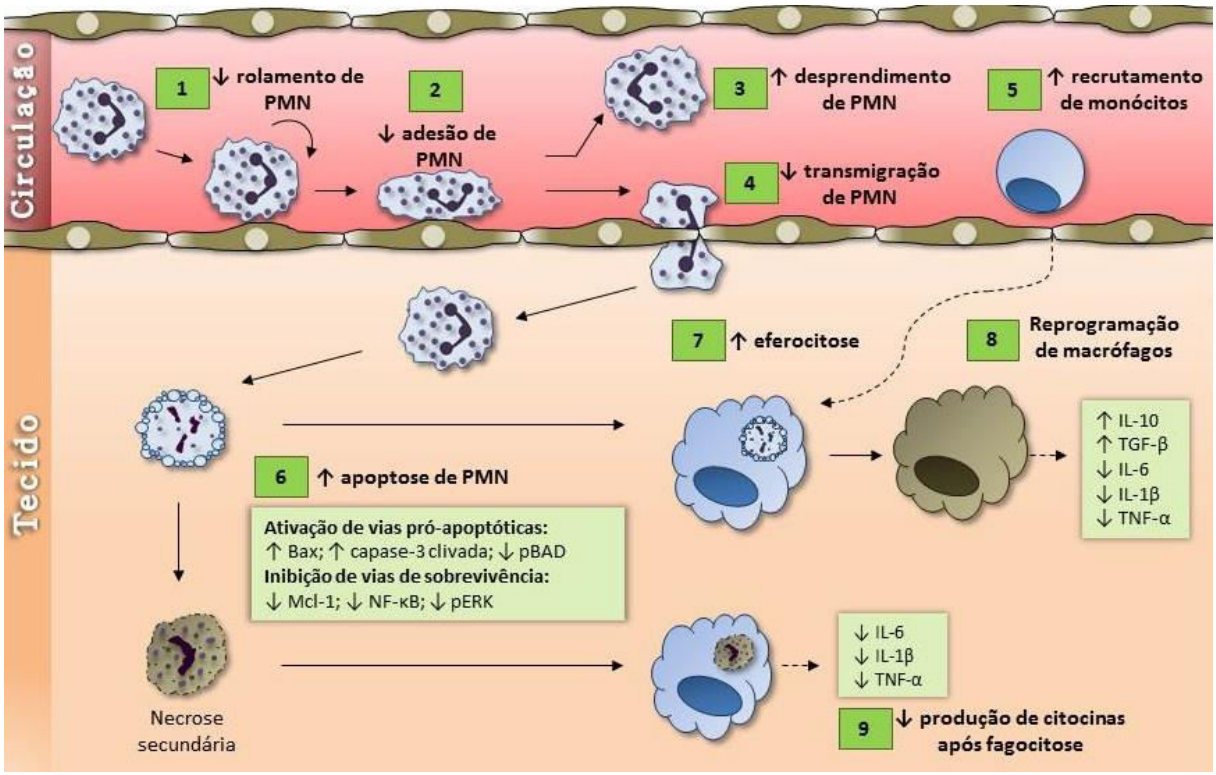

Figura 6. Eventos celulares associados aos efeitos anti-inflamatórios e pró-resolutivos de AnxA1 e seus miméticos derivados da porção N-terminal. AnxA1 modula diversos eventos celulares e moleculares da resposta inflamatória, participando de mecanismos endógenos associados com a indução de uma resolução adequada. A administração farmacológica da AnxA1 resulta na diminuição do rolamento (1) e aderência (2) de neutrófilos ao endotélio, aumenta o desprendimento de células aderentes (3), e inibe a transmigração de neutrófilos (4). Além disso, AnxA1 é capaz de induzir apoptose (6). AnxA1 endógena e exógena também promove o recrutamento de monócitos (5) e a fagocitose de neutrófilos apoptóticos por macrófagos (7). A fagocitose de neutrófilos apoptóticos por macrófagos é acompanhada pela indução de sinais anti-inflamatórios, incluindo produção de TGF-β e diminuição dos níveis de citocinas pró-inflamatórias (8). Além disso, AnxA1 está relacionada com a reprogramação de macrófagos para um fenótipo pró-resolutivo (8), e impede a produção de citocinas pro-inflamatórias após a fagocitose de células em necrose secundária (9).
Fonte: Adaptado de Sugimoto *et al*., 2016.

Os mecanismos moleculares que são responsáveis pela secreção da AnxA1 são célula específicos. Após ativação celular, a AnxA1 intracelular é ativamente mobilizada para a membrana plasmática e é então externalizada e/ou secretada por um dos seguintes mecanismos: ativação do transportador ABC (*ATP-binding cassette*), fosforilação do resíduo de serina na porção N-terminal pela cinase PKC, ou fusão de grânulos de gelatinase com a

membrana plasmática (Perretti *et al.*, 2009).

AnxA1 se liga à membrana plasmática de maneira Ca^{2+} dependente. Na presença de íons Ca^{2+}, em concentrações maiores que 1mM, a AnxA1 extracelular sofre uma mudança conformacional que leva a exposição da região N-terminal e ligação ao seu receptor ALX (também conhecido com FPR2 murino ou FPRL1 humano). O receptor FPR2 faz parte de uma pequena família de receptores FPR (FPR1, FPR2 e FPR3), que são expressos por vários tipos celulares, incluindo neutrófilos, monócitos, macrófagos, células endoteliais e epiteliais. AnxA1 e os peptídeos derivados da sua porção N-terminal competem com lipoxina A_4 e a proteína amilóide sérica A pelo sítio ativo de FPR2. Curiosamente, já foi demonstrado *in vitro* que os peptídeos ativos de AnxA1 ativam todos os três receptores da família FPR. No entanto a relevância biológica deste achado ainda não está clara já que fragmentos bioativos de AnxA1 ainda não foram estudados no contexto *in vivo* (Perretti et al., 2009). AnxA1 pode ativar a sinalização por mecanismos autócrinos, parácrinos ou justácrinos (contato célula-célula), envolvendo interação entre a AnxA1 na superfície da célula secretora e o receptor ALX da célula alvo. Este parece ser o mecanismo de ação mais comum em condições inflamatórias (Perretti *et al.*, 2009).

A AnxA1 intacta (37 kDa) pode ser encontrada no citoplasma de neutrófilos circulantes ou na membrana plasmática dos neutrófilos intravasculares aderidos ao endotélio. Uma vez no espaço extravascular, a maior parte da proteína é clivada na região N-terminal, dando origem a AnxA1 de 33 kDa e outros fragmentos ainda não bem estudados. A região N-terminal é caracterizada como promotora da ação anti-inflamatória da AnxA1. Resultados experimentais utilizando o peptídeo sintético contendo a mesma sequência de aminoácidos, denominado Ac2-26, confirmaram a presença desse sítio ativo nessa região, o qual é efetivo em atenuar vários parâmetros da resposta inflamatória quando utilizado em modelos experimentais de inflamação (Harris *et al.*, 1995; Oliani *et al.*, 2001; Perretti *et al.*, 2003; Souza *et al.*, 2007; Vago *et al.*, 2012).

Como revisto por Perretti e D'Acquisto (2009), vários estudos já demonstraram que os GCs (endógenos e exógenos) induzem a expressão de AnxA1 (Perretti *et al.*, 2009). A transcrição do gene que codifica AnxA1 é regulada por dois sistemas, o constitutivo e o indutível. O sistema constitutivo está relacionado com a manutenção da expressão basal da AnxA1, através da região de regulação constitutiva, que é importante para iniciar o processo

de transcrição. Já o sistema de regulação indutível é bastante complexo. Estudos da região promotora do gene da AnxA1 indicam que este gene contém elementos de resposta aos GCs (GREs), o que poderia explicar o aumento da síntese de AnxA1 em resposta a GCs (Peers *et al.*, 1993; Solito *et al.*, 1998). Vários trabalhos demonstram que a síntese de AnxA1 induzida pelos GCs é mediada por mecanismos que dependem da ligação do complexo GC-GR ao DNA, com consequente aumento da transcrição do gene que codifica AnxA1 (Peers *et al.*, 1993; Suarez *et al.*, 1993; Perretti *et al.*, 1996). No entanto, o envolvimento dos GREs presentes no promotor do gene que codifica a AnxA1 na sua síntese induzida por GCs é ainda discutível (Solito *et al.*, 1998). Outros estudos demonstram que a regulação da transcrição do gene da AnxA1 pelos GCs pode envolver mecanismos moleculares alternativos. Em alguns casos, os GCs podem ativar direta ou indiretamente outros fatores de transcrição, como o CREB (*cAMP responsive element-binding protein*) e o NFIL-6 (*nuclear factor-IL-6*). Na ativação indireta, o promotor de AnxA1 parece não ter um receptor canônico de GCs, mas contém um sítio consenso de ligação parcial que medeia a capacidade de resposta a IL-6, sugerindo que GCs regulam a expressão de AnxA1 indiretamente através de IL-6 (Solito *et al.*, 1998; Antonicelli *et al.*, 2001). Deste modo, mais estudos devem ser realizados a fim de se elucidar os mecanismos pelos quais os GCs regulam expressão de AnxA1, já que esse processo ainda não está bem definido.

1.4.2 - AnxA1 e seus produtos de clivagem

A clivagem proteolítica é uma forma de gerar fragmentos (peptídeos) com diversas atividades biológicas. A proteína AnxA1 possui atividade anti-inflamatória na sua forma intacta (37 kDa). Uma vez externalizada, após ativação celular, AnxA1 pode sofrer ação de proteases como proteinase 3 e elastase, que clivam AnxA1 na sua porção N-terminal, gerando produtos de clivagem como o produto de 33 kDa (Oliani *et al.*, 2001; Vong *et al.*, 2007; Vago *et al.*, 2012). Um dos primeiros estudos que demonstraram a importância dos fragmentos gerados a partir de AnxA1 foi realizado por Huang e colaboradores (1987), os quais observaram que diferentes produtos de clivagem de AnxA1 apresentavam potencial distinto de inibição da enzima PLA2 (Huang *et al.*, 1987). Estudos mais recentes mostram que o produto de clivagem de 33 kDa pode estar relacionado à efeitos pró-inflamatórios (Tsao *et al.*, 1998; Williams *et al.*, 2010; Vago *et al.*, 2012). Williams e colaboradores (2010) relataram

que a AnxA1 clivada de 33 kDa, encontrada nas frações de proteínas solúveis de neutrófilos ativados, apresentavam efeitos pró-inflamatórios através da ativação de ERK1/2 e indução da migração transendotelial de neutrófilos. Neste estudo, nem a proteína intacta de 37 kDa, nem o peptídeo Ac2-26 foram capazes de ativar ERK1/2 (Williams *et al.*, 2010). Além disso, o conceito de que o produto de clivagem de AnxA1 de 33 kDa possa ser pró-inflamatório foi relatado em um estudo onde encontraram a forma de 33 kDa como preferencialmente expressa em amostras de lavado bronco-alveolar (BAL) de pacientes com fibrose cística (Tsao *et al.*, 1998). Recentemente, nosso grupo de pesquisa demonstrou em um modelo de inflamação aguda, um maior acúmulo de AnxA1 clivada (33 kDa) durante o período de maior infiltrado neutrofílico (Vago *et al.*, 2012).

Corroborando com o potencial pró-inflamatório dos produtos de clivagem de AnxA1, já foi demonstrado que AnxA1 resistente a clivagem exibiu um efeito anti-inflamatório maior ao longo do tempo em comparação com a proteína parental (Pederzoli-Ribeil *et al.*, 2010), além de acelerar significativamente a resolução da inflamação em um modelo animal de artrite (Patel *et al.*, 2012). Além disso, um peptídeo derivado da AnxA1 com uma mutação na sua região de clivagem (CR-AnxA1$_{2\text{-}50}$), tornando-o resistente à clivagem pela ação de elastase e PR3, demonstrou ser mais eficaz em induzir resolução da inflamação quando comparado com o peptídeo não mutado (Dalli *et al.*, 2013). Em contraste com esses achados, Blume *et al*. (2012), utilizando PMNs necróticos, demonstrou que AnxA1 clivada funciona como um sinal quimiotático para macrófagos durante a necrose celular secundária, diminuindo assim o potencial pró-inflamatório destas células necróticas (Blume *et al.*, 2012). Tomando todos estes estudos em conjunto e pelo nosso conhecimento, até o presente, ainda não é claro qual a função biológica dos peptídeos gerados a partir da clivagem de AnxA1, uma vez que eles ainda não foram testados *in vivo*. Seria razoável hipotetizar que a clivagem de AnxA1 por proteases de neutrófilos ativados seja um mecanismo que sobrepõe à ação anti-inflamatória da proteína endógena, incluindo a diminuição da transmigração de neutrófilos e o desprendimento de neutrófilos do leito vascular. Assim, a quantidade de proteases existe durante a resposta inflamatória e o balanço entre a clivagem de AnxA1 e a manutenção da proteína ativa pode ditar o desfecho da inflamação.

1.5 – Proteases e Anti-proteases

Proteases são moduladores chave da resposta inflamatória e podem ser encontradas em altas concentrações nos sítios inflamatórios. Essas enzimas são produzidas por uma variedade de células inflamatórias fagocíticas, incluindo os neutrófilos, macrófagos e também células epiteliais (Greene *et al.*, 2009; Mancek-Keber, 2014). As proteases são subdivididas em 4 grupos, que incluem as serina proteases, cisteína proteases, metaloproteinases, e as menos comuns, proteases do ácido aspártico. Geralmente, as serina proteases são submetidas a um processo de modificação pós-traducional de duas fases, dando origem as suas formas ativas maduras (Lopez-Otin *et al.*, 2008; Twigg *et al.*, 2015). A elastase do neutrófilo (NE) e proteinase 3 (PR3) são serina proteases de neutrófilos com uma gama de substratos, causando impacto na função de células e tecidos através de diversos mecanismos, tais como a degradação de patógenos ingeridos e motilidade celular através da matriz extracelular (Henriksen, 2014). As formas maduras das NE e PR3 são armazenadas nos grânulos azurófilos dentro do citoplasma de neutrófilos e secretadas para o meio extracelular após ativação celular (Borregaard *et al.*, 1997). Uma vez no meio extracelular, essas proteases são capazes de degradar uma variedade de proteínas extracelulares causando dado tecidual, além de induzir a produção de citocinas pró-inflamatórias (Bank *et al.*, 2001; Korkmaz *et al.*, 2010).

Por outro lado, em resposta à liberação destas enzimas, anti-proteases endógenas são produzidas a fim de neutralizar o excesso de proteases, protegendo os tecidos do hospedeiro. Estas anti-proteases são classificadas como sistêmicas (produzidas por hepatócitos e propagadas através da circulação) ou de alarme (sintetizadas e segregadas pelas células no local da inflamação) (Sallenave, 2000; Williams *et al.*, 2006). Anti-proteases de alarme incluem SLPI (*secretory leukocyte protease inhibitor*) e Elafina (secretada predominantemente nas mucosas), e são moduladas em vários estados patológicos (Williams *et al.*, 2006; Greene *et al.*, 2009; Mancek-Keber, 2014). SLPI e Elafina podem ser proteoliticamente clivadas pela NE em excesso, o que resulta na inativação dessas anti-proteases (Twigg *et al.*, 2015).

SLPI é uma serina protease catiônica de 11,7 kDa associada com a inibição de um grande espectro de proteases, incluindo NE, PR3, catepsina G, tripsina e quimiotripsina (Williams *et al.*, 2006). SLPI é expressa em resposta a vários estímulos, tais como lipopolissacarídeos bacterianos (LPS), NE e citocinas, como IL1-β e TNF-α (Williams *et al.*,

2006; Twigg *et al.*, 2015). Assim como SLPI, Elafina é também uma serina protease, que pode ser encontrada em sua forma intacta de 9,9 kDa (o qual apresenta maior atividade anti-proteásica), ou na forma clivada de 6 kDa (Williams *et al.*, 2006). Elafina inibe a elastase pancreática porcina, NE e PR3, com um baixo grau de reversibilidade. No entanto, não inibe alguns tipos de proteases como catepsina G ou tripsina, apresentando, portanto, um espectro mais restrito de inibição quando comparado à SLPI (Wiedow *et al.*, 1990). Além da sua atividade anti-proteásica, e de maneira similar à SLPI, Elafina também possui atividade anti-microbiana e anti-inflamatória. Alguns estudos já demonstraram que uma das atividades anti-inflamatórias desempenhadas por SLPI e Elafina está associada à inibição da atividade de NF-κB. Outro fator importante é que ambas anti-proteases são capazes de neutralizar o LPS, impedindo a ativação de receptores do tipo *Toll* (Vachon *et al.*, 2002; Taggart *et al.*, 2005; Butler *et al.*, 2006). Tomados em conjunto, esses trabalhos sugerem que, tanto a SLPI quanto Elafina, exercem efeitos anti-inflamatórios por vias intra ou extracelulares.

Tendo em vista o papel de antiproteases endógenas no equilíbrio da homeostase tecidual, e mais precisamente no controle da inflamação, foi desenvolvido no Japão uma anti-protease sintética. Mais conhecido como Sivelestat (Elaspol®), esse inibidor seletivo de elastase já está disponível no Japão e Coréia do Sul para o tratamento da lesão pulmonar aguda, incluindo síndrome da dificuldade respiratória aguda em pacientes com síndrome da resposta inflamatória sistêmica (Henriksen, 2014). Sivelestat é um inibidor específico da NE não apresentando efeito em outras proteases, como plasmina, trombina, catepsina B ou colagenase I (Morimoto *et al.*, 2008).

Hagiwara e colaboradores (2009) demonstraram *in vitro* que o Sivelestat diminui a ativação de NF-κB pela inibição da fosforilação de IκB (Hagiwara *et al.*, 2009). De fato, estudos pré-clínicos mostraram que o Sivelestat reduz marcadores de lesão tecidual e inflamação sistêmica, incluindo lesão de isquemia e reperfusão (Kambe *et al.*, 2009; Uchida *et al.*, 2010), sepse (Hayakawa *et al.*, 2010) e lesão pulmonar aguda (Yasui *et al.*, 1995; Iba *et al.*, 2006), além de reduzir os níveis de citocinas pró-inflamatórias como IL-8, IL-6 e TNF-α (Yoshimura *et al.*, 2003; Uchida *et al.*, 2010). Vários estudos clínicos recentes mostraram que o Sivelestat melhora parâmetros de lesão pulmonar aguda e síndrome da dificuldade respiratória aguda (Aikawa *et al.*, 2014). Além disso, outros estudos mostraram que o Sivelestat melhora o quadro clínico de pacientes submetidos à esofagectomia (Wang *et al.*, 2015) e cirurgia hepática (Tsujii *et al.*, 2012).

1.6 - Apoptose

Apoptose ou morte celular programada é um tipo de morte celular essencial para a homeostase tecidual e para o desenvolvimento normal dos organismos multicelulares. Algumas doenças como o câncer, doenças autoimunes até mesmo síndromes degenerativas estão associadas a defeitos no controle dessa via (Cory *et al.*, 2002). A apoptose é caracterizada por eventos morfológicos e bioquímicos que agem concomitantemente, ocasionando retração da célula, vacuolização do citoplasma, formação de *blebbing* (bolhas) de membrana, perda de aderência com a matriz extracelular, condensação da cromatina e fragmentação do núcleo associada com clivagem do DNA (Cohen, 1993). Um dos processos iniciais é a exposição da fosfatidilserina, um fosfolipídio de membrana que, em células viáveis é encontrado exclusivamente na porção interna da membrana plasmática e, durante a apoptose é evertido para superfície externa da membrana (Fadok *et al.*, 1992). Durante a apoptose, ocorre a formação de corpos apoptóticos (pequenas vesículas que transportam o conteúdo celular). As células em apoptose, bem como os corpos apoptóticos são reconhecidos, fagocitados e degradados pelas células vizinhas ou fagócitos profissionais (Savill *et al.*, 2000). Assim, nenhuma proteína intracelular ou metabólitos são liberados para o tecido circundante.

A ativação da apoptose pode ser iniciada por duas vias distintas: a via intrínseca (mitocondrial) e a via extrínseca (citoplasmática). A via intrínseca é ativada por estresse intracelular ou extracelular. Os sinais que são transduzidos convergem principalmente para a mitocôndria. Essa organela integra os estímulos de morte celular, os quais induzem a permeabilização mitocondrial e consequente liberação do citocromo c. Esse processo é desencadeado por elevações nos níveis de proteínas pró-apoptóticas da família Bcl-2. Esta família de proteínas citoplasmáticas é caracterizada pela presença de membros que suprimem a apoptose (Ex: Mcl-1, Bcl-2, Bcl-xL, A1) ou promovem apoptose (Ex: Bax, Bak, Bik, Bad, Bid, Bim e Puma). No citosol, o citocromo c forma um complexo com a APAF-1 e caspase-9, chamado apoptossomo, que promove a clivagem e consequente ativação da caspase-9, que posteriormente ativa as caspases-3/7. A via extrínseca é desencadeada pela ligação de ligantes específicos a um grupo de receptores de membrana da superfamília dos receptores de fatores de necrose tumoral (rTNF). Quando os receptores de morte celular reconhecem um ligante específico, os seus domínios de morte interagem com proteínas adaptadoras como a FADD. Essas moléculas têm a capacidade de recrutar a caspase-8 que irá ativar a caspase-3/7,

executando a morte por apoptose (Cory *et al.*, 2002; Best, 2008). A apoptose de neutrófilos seguida pela subsequente remoção por fagócitos é um processo essencial na resolução inflamatória (Rossi *et al.*, 2007; Hallett *et al.*, 2008; Fox *et al.*, 2010).

Os neutrófilos expressam constitutivamente os membros pró-apoptóticos da família Bcl-2, incluindo Bax, Bad, Bak, Bid e Bik. Estas proteínas têm meias-vidas relativamente longas e seus níveis celulares mudam pouco durante a exposição a agentes que aceleram ou retardam a apoptose de neutrófilos. Os neutrófilos humanos também expressam proteínas anti-apoptóticas Mcl-1 e A1, e em menores níveis Bcl-xL e Bcl-2. Mcl-1 e, em menor grau A1, são particularmente importantes para a sobrevida dos neutrófilos em resposta a estímulos pró-inflamatórios (Milot *et al.*, 2011).

1.6.1 - Evidências da participação de AnxA1 e GILZ na apoptose e eferocitose

A primeira evidência do papel de GILZ na apoptose de leucócitos foi observada em experimentos conduzidos em camundongos transgênicos que superexpressam GILZ na linhagem de células T. Os timócitos destes camundongos sofrem apoptose espontaneamente, com ativação de caspase-8 e caspase-3, e inibição de Bcl-xL, sugerindo que GILZ apresenta efeitos semelhantes aos GCs neste tipo celular (Delfino et al., 2004). Em contrapartida, GILZ não induz apoptose em linfócitos T maduros, uma vez que a superexpressão de GILZ com a tecnologia TAT-GILZ induziu apoptose de timócitos, mas não de esplenócitos (Delfino et al., 2004; Ayroldi et al., 2009). Outro trabalho demonstrou que a inibição da via de sobrevivência celular PI3K/Akt resulta na superexpressão de GILZ com consequente apoptose de células de mieloma múltiplo (Grugan et al., 2008). Além disso, já foi demonstrado que GILZ suprime Ras e inibe a ativação de ERK e Akt, com consequente redução da proliferação celular (Ayroldi et al., 2007; Joha et al., 2012). Até o momento não existem evidências experimentais diretas mostrando a participação de GILZ na eferocitose. No entanto, já foi demonstrado que na ausência de GILZ ocorre um aumento na capacidade fagocítica de células dentríticas (Lebson *et al.*, 2011) e que macrófagos M2 e Mres têm a expressão de GILZ aumentada (Vago *et al.*, 2015). Assim, parece que GILZ exerce funções antagônicas nos processos de fagocitose e de eferocitose.

Vários estudos têm mostrado a correlação entre AnxA1 e apoptose de leucócitos. Solito e colaboradores (2001) mostraram que a superexpressão de AnxA1 em células monocíticas U937 induziu a apoptose espontânea dessas células, e esse processo foi associado com a ativação de caspase-3. Também foi demonstrado que AnxA1 exógena aumentou transitoriamente as concentrações de cálcio intracelular acompanhado da desfosforilação da proteína pró-apoptótica Bad e consequente apoptose de neutrófilos humanos (Solito *et al.*, 2003). Após um aumento de cálcio citosólico, a fosfatase ativada calcineurina desfosforila Bad, permitindo sua associação com a mitocôndria, formando um heterodímero com Bcl-xL e promovendo a apoptose (Wang *et al.*, 1999). Estas evidências experimentais sugerem que AnxA1 possa mediar os efeitos pró-apoptóticos dos glicocorticoides em algumas células, ativando caspase-3 e alterando fluxos de cálcio.

Alguns estudos também correlacionam AnxA1 com o *clearance* de células e corpos apoptóticos. De acordo com Scannell e colaboradores (2007), AnxA1 endógena é liberada de neutrófilos apoptóticos e age sobre macrófagos, promovendo a fagocitose e remoção das células apoptóticas (Scannell *et al.*, 2007). Outro estudo mostrou que macrófagos tratados com glicocorticoides secretam AnxA1, agindo de forma autócrina ou parácrina e aumentando a fagocitose de neutrófilos apoptóticos (Maderna *et al.*, 2005). Também já foi demonstrado que AnxA1 liberada de células necróticas pode atuar em macrófagos, promovendo a eferocitose de neutrófilos apoptóticos (Blume *et al.*, 2012). Recentemente, nosso grupo de pesquisa (Vago *et al.*, 2012), bem como do professor Mauro Perretti (Dalli *et al.*, 2013) demonstrou, em experimentos conduzidos *in vivo*, que o aumento dos níveis endógenos de AnxA1 ou administração exógena desta proteína (através do peptídeo Ac2-26 ou Ac2-50) está associado com a apoptose e eferocitose de neutrófilos, com consequente resolução da inflamação neutrofílica. Além do efeito na apoptose de neutrófilos, Dalli e colaboradores (2012) sugerem que AnxA1 controla o *clearance* de neutrófilos apoptóticos em condições não inflamatórias, através de experimentos que mostraram que macrófagos de animais deficientes para AnxA1 ($AnxA1^{-/-}$) têm menor capacidade de fagocitar neutrófilos apoptóticos (Dalli *et al.*, 2012). Recentemente, foi demonstrado o envolvimento de AnxA1 no recrutamento de monócitos durante a fase de resolução da inflamação, reforçando a ideia de que a AnxA1 exerce seus efeitos pró-resolutivos não somente na apoptose de neutrófilos mas também contribuindo com a remoção dessas células do local da inflamação (McArthur *et al.*, 2015).

2 - JUSTIFICATIVA

Existe um grande interesse em entender os mecanismos responsáveis pela eliminação de células no foco inflamatório bem como a inativação dos mediadores secretados localmente, além da ativação de moléculas com propriedades pró-resolutivas. Nosso grupo de pesquisa tem se dedicado ao estudo de vias de sinalização e mediadores importantes para a resolução da resposta inflamatória. Já demonstramos que a modulação da AnxA1 endógena pode ser essencial para a resolução da resposta inflamatória (Vago *et al.*, 2012). Particularmente, foi mostrado que a resolução da inflamação aguda foi associado com a apoptose de neutrófilos e o aumento da expressão de AnxA1 intacta (37 kDa) e que durante o pico da inflamação neutrofílica (8-24h) houve aumento na clivagem da proteína AnxA1 (produto de 33 kDa). Desta forma, nos questionamos se a modulação da clivagem de AnxA1 poderia promover a resolução da inflamação. Já está bem estabelecido que AnxA1 é clivada por algumas proteases, dentre elas elastase e proteinase 3, e que o tratamento de animais com AnxA1 resistente à clivagem aumenta a atividade anti-inflamatória quando comparada à proteína natural.

Assim como AnxA1, já foi demonstrado que GILZ possui atividade anti-inflamatória sendo um mediador de vários dos efeitos anti-inflamatórios dos GCs. Yang e colaboradores (2009) sugeriram, através de experimentos *in vitro* utilizando macrófagos deficientes em AnxA1, que GILZ poderia ser um mediador dos efeitos anti-inflamatórios de AnxA1 (Yang *et al.*, 2009). Outro estudo, utilizando fibroblastos, mostrou o envolvimento de AnxA1 na indução de GILZ de maneira independente de FPR2 (Jia *et al.*, 2013). No entanto, estes achados precisam ser consubstanciados *in vivo*, bem como os mecanismos subjacentes a esses eventos. Além disso, o efeito de GILZ em eventos chave do processo resolutivo como apoptose de neutrófilos seguido de sua remoção por macrófagos (eferocitose) necessita ser mais bem investigado.

Assim, pretendemos com este estudo, avaliar o papel de GILZ como um mediador da resolução da inflamação, validar *in vivo* o papel de GILZ e sua inter-relação com AnxA1 na resposta inflamatória, e avaliar o efeito de inibidores da clivagem de AnxA1 na resolução do processo inflamatório.

3 - OBJETIVOS

3.1 - Objetivo geral

Estudar o papel das proteínas induzidas por glicocorticoides, GILZ e AnxA1, em eventos chave da resolução da inflamação aguda.

3.2 - Objetivos específicos

3.2.1 - Avaliar o papel de GILZ durante a resolução natural da pleurisia induzida por LPS.

3.2.2 - Verificar o efeito da superexpressão e inibição de GILZ na resolução da resposta inflamatória aguda.

3.2.3 - Avaliar o papel de GILZ e sua inter-relação com AnxA1 na resolução da inflamação aguda.

3.2.4 - Avaliar a expressão e atividade de elastase, uma protease que cliva AnxA1 endógena, durante a pleurisia induzida por LPS e sua correlação com a dinâmica de acumulação de AnxA1.

3.2.5 - Avaliar o efeito de anti-proteases endógenas durante a pleurisia induzida por LPS e sua correlação com a dinâmica de acúmulo de AnxA1 e a resolução da inflamação aguda.

3.2.6 - Verificar o efeito da inibição de proteases, especificamente de elastase, no acúmulo de AnxA1 e resolução da resposta inflamatória aguda, e o mecanismo resolutivo envolvido.

4 – CAPÍTULO 1

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The Role and Effects of Glucocorticoid-Induced Leucine Zipper in the Context of Inflammation Resolution

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Glucocorticoid (GC)-induced leucine zipper (GILZ) has been shown to mediate or mimic several actions of GC. This study assessed the role of GILZ in self-resolving and GC-induced resolution of neutrophilic inflammation induced by LPS in mice. GILZ expression was increased during the resolution phase of LPS-induced pleurisy, especially in macrophages with resolving phenotypes. Pretreating LPS-injected mice with *trans*-activator of transcription peptide (TAT)–GILZ, a cell-permeable GILZ fusion protein, shortened resolution intervals and improved resolution indices. Therapeutic administration of TAT-GILZ induced inflammation resolution, decreased cytokine levels, and promoted caspase-dependent neutrophil apoptosis. TAT-GILZ also modulated the activation of the survival-controlling proteins ERK1/2, NF-κB and Mcl-1. GILZ deficiency was associated with an early increase of annexin A1 (AnxA1) and did not modify the course of neutrophil influx induced by LPS. Dexamethasone treatment resolved inflammation and induced GILZ expression that was dependent on AnxA1. Dexamethasone-induced resolution was not altered in GILZ$^{-/-}$ mice due to compensatory expression and action of AnxA1. Our results show that therapeutic administration of GILZ efficiently induces a pro-apoptotic program that promotes resolution of neutrophilic inflammation induced by LPS. Alternatively, a lack of endogenous GILZ during the resolution of inflammation is compensated by AnxA1 overexpression. ***The Journal of Immunology*****, 2015, 194: 000–000.**

Resolution of inflammation is an active and continuous process with production and activation of biochemical mediators and signaling pathways to ensure rapid and successful restoration of tissue homeostasis (1–3). During the resolution phase of inflammation, multiple proresolving molecules are produced to temper the inflammatory response and guarantee the return to homeostasis.

One of the most important endogenous proresolution pathways is that mediated by glucocorticoids (GCs) produced by the adrenal glands. Exploiting these physiological effects, GCs are among the most important drugs that have been developed for the treatment of inflammatory diseases. However, metabolic side effects of GCs limit their therapeutic application (4–6). The mechanisms of GCs are complex and depend on inhibition of transcription factors, such as NF-κB and AP-1, as well as induction of anti-inflammatory regulatory proteins such as annexin A1 (AnxA1), GC-induced leucine zipper (GILZ), and MAPK phosphatase (MKP)-1 (3). Thus, there is a growing interest in understanding the effects of GC-induced proteins that may allow dissociation of GC anti-inflammatory effects from their adverse metabolic effects.

GILZ was first identified in 1997 (7) and has been characterized as a novel GC-induced protein that mediates many anti-inflammatory effects of GC in leukocytes (5, 8). GILZ has been reported to interact with NF-κB and AP-1, and to inhibit the MEK/ERK1/2 pathway by binding to the upstream proteins Ras and Raf-1. These mechanisms are thought to be important for their ability to attenuate inflammation (8). GILZ appears to have a physiological role in the regulation of inflammatory mechanisms; however, there are few reports that explore its role in inflammatory disease (9–13).

AnxA1 is another GC-induced protein that has been shown to be anti-inflammatory and proresolving in various animal models of inflammation and in physiological conditions (4). Indeed, AnxA1

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Abbreviations used in this article: AnxA1, annexin A1; Dex, dexamethasone; GC, glucocorticoid; GILZ, glucocorticoid-induced leucine zipper; i.pl., intrapleural(ly); KO, knockout; MKP, MAPK phosphatase; Mres, resolution-promoting macrophage; TAT, *trans*-activator of transcription peptide; WT, wild-type.

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limits initial steps of inflammation, specifically the recruitment of leukocytes and generation of proinflammatory mediators. AnxA1 also acts on the resolution phase of inflammation by inducing apoptosis of neutrophils (14, 15) and increasing efferocytosis by macrophages (16, 17). Importantly, AnxA1 production and activities are involved in proresolution effects of GCs (18) and histone deacetylase inhibitors (19). Recently, it was demonstrated in vitro that GILZ is a target of the anti-inflammatory effects of AnxA1 (20). However, whether AnxA1 cooperates with GILZ in vivo to convey the anti-inflammatory and proresolving activities of endogenous or synthetic GCs remains unknown.

In this study, we investigated the role of GILZ in natural and GC-driven resolution of inflammation. We demonstrate that GILZ is expressed during the resolving phase of inflammation in macrophages with proresolving phenotypes. Pharmacological treatment with recombinant GILZ protein reduces resolution intervals and promotes resolution of LPS-induced neutrophilic inflammation, whereas self-resolving inflammation was dependent on a compensatory balance between AnxA1 and GILZ expression.

Materials and Methods

Animals

All procedures described in this study had prior approval from the Animal Ethics Committee of Universidade Federal de Minas Gerais, Brazil (Comitê de Ética em Experimentação Animal/Universidade Federal de Minas Gerais protocol no. 15/2011). Male BALB/c mice (8–10 wk) bred in the Animal Facility of Centro de Bioterismo of Universidade Federal de Minas Gerais (Brazil) were housed under standard conditions and had free access to commercial chow and water. GILZ-deficient male mice were generated as described (13), and C57BL/6 littermates were bred in the animal facility of the Immunopharmacology Laboratory.

Drugs, reagents, and Abs

The peptide *trans*-activator of transcription peptide (TAT) and the TAT-GILZ fusion protein (constructed by inserting GILZ cDNA in the TAT-C vector to produce an in-frame fusion protein) were generated as described (9). Briefly, TAT and TAT-GILZ were in-frame cloned into the pGEX-4T2 plasmid (GE Healthcare). The pGEX-4T2 plasmid is a GST fusion vector carrying a tac promoter for chemically (isopropyl β-D-thiogalactopyranoside) inducible high-level expression of the protein. GST fusion protein was expressed in *Escherichia coli* grown at 30°C and induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 90 min. Following lysis by sonication, most of the induced protein was found in the soluble material, which was purified with glutathione–Sepharose 4B beads (GE Healthcare) following the manufacturer's instructions. Eluted proteins were dialyzed for 48 h against PBS. Protein quantification and purity were evaluated by SDS-PAGE and by Coomassie blue staining. LPS contamination in each batch of TAT and TAT-GILZ was evaluated by using a *Limulus* amebocyte lysate chromogenic endotoxin quantification kit (Pierce, catalog no. 88282). LPS contamination was low (<0.5 endotoxin unit/ml or <0.05 ng). For comparison, the amount of LPS injected to induce cell recruitment was 1.250 endotoxin units (250 ng/cavity). ZVAD-fmk and Ac2-26 peptide were from Tocris Bioscience (Ellisville, MO). Rabbit anti–p-ERK1/2, anti–Mcl-1, and mouse anti–p-IκB-α Abs were from Cell Signaling Technology (Beverly, MA). Rabbit anti-GILZ and anti–MKP-1 and secondary anti-rabbit and anti-mouse peroxidase conjugate Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-AnxA1 was purchased from Invitrogen (Carlsbad, CA). Anti–β-actin, LPS (from *E. coli* serotype O:111:B4), and dexamethasone (Dex) were from Sigma-Aldrich (St. Louis, MO). Anti-AnxA1 antiserum was a gift from Dr. Steve Poole (Biotherapeutics Group, National Institute for Biological Standards and Control, U.K.).

Leukocyte migration into the pleural cavity induced by LPS

Mice received an intrapleural (i.pl.) administration of LPS (250 ng/cavity) or PBS as previously described (15, 21). Cells present in the pleural cavity were harvested at different times after LPS injection by washing the cavity with 2 ml PBS. Total cell counts were performed in a modified Neubauer chamber using Turk's stain. Differential cell counts were performed on cytocentrifuge preparations (Shandon III) stained with May–Grünwald–Giemsa using standard morphological criteria to identify cell types. The results are presented as the number of cells per cavity.

Treatment protocols

To evaluate the effect of anti-inflammatory/proresolving agents on LPS-induced pleurisy, mice were treated with the synthetic GC Dex (2.0 mg/kg, i.p.), Ac2-26 peptide (100 μg, i.p.), or TAT-GILZ peptide (0.2 mg/kg, i.p.) 4 h after LPS challenge. The peptide TAT (0.1 mg/kg, i.p.) was used as a control. In other experiments, TAT-GILZ or the control peptide were administered before LPS injection in a pretreatment protocol. To prevent the action of AnxA1 induced by Dex, mice were treated with anti-AnxA1 antiserum (0.1 ml hyperimmune serum diluted in 100 μl PBS/mouse, i.p.) (22). Nonimmune goat serum was used as control. ZVAD-fmk (1 mg/kg), a broad-spectrum caspase inhibitor (21), was given systemically (i.p.) 15 min before TAT-GILZ injection. Drugs were dissolved in DMSO or ethanol and diluted further in PBS. Control mice received vehicle only.

Calculation of resolution indices

We quantified the resolution indices as described (23, 24). Murine pleural exudates were collected at 4, 8, 24, 48, and 72 time points after challenge. The numbers of PMN and mononuclear cells were determined by total and differential leukocyte counting. Using these two cell types, the resolution of acute inflammation was defined in quantitative terms by the following resolution indices: 1) magnitude (ψ_{max} and T_{max}), where ψ_{max} indicates maximal PMN and T_{max} indicates time point when PMN numbers reach maximum; 2) duration (T_{50}), which indicates time point when PMN numbers reduce to 50% of maximum; and 3) resolution interval (R_i), which indicates interval between T_{max} and T_{50}, when 50% PMN are lost from the pleural cavity.

Assessment of leukocyte apoptosis

Apoptosis was assessed morphologically as previously reported (15, 21). Briefly, cells (5×10^4) collected after LPS administration were cytocentrifuged, fixed and stained with May–Grünwald–Giemsa, and counted using oil immersion microscopy (×100 objective) to determine the proportion of cells with distinctive apoptotic morphology (cells with chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies out or inside macrophages). At least 500 cells were counted per slide, and results are expressed as the mean ± SEM of percentage of cells with apoptotic morphology. Assessment of apoptosis was also performed by flow cytometry using FITC-labeled annexin V (ApoDETECT Annexin V^{FITC} kit, Invitrogen) and propidium iodide, as an index of loss of nuclear membrane integrity.

Flow cytometry analysis for leukocyte populations and expression of GILZ and AnxA1

Cells present in the pleural cavity were harvested at 24 and 48 h after administration of LPS or PBS (24 h). The populations of macrophages and neutrophils were analyzed by staining with fluorescent mAbs against F4/80 (PE-Cy7, eBioscience, San Diego, CA), Gr1 (FITC, BioLegend, San Diego, CA), Gr1 (PE, BioLegend), CD11b (PE-Cy5, BD Biosciences, San Jose, CA), CD11c (PE-Cy7, BD Biosciences), GILZ (PE, eBioscience), AnxA1 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-rabbit (Alexa 647, BD Biosciences). After being stained for surface markers, cells were permeabilized with permeabilization buffer (eBioscience) for 30 min. Stained cells were acquired in a BD LSRFortessa cell analyzer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Gating strategy is ilustrated in Supplemental Fig. 2D. Macrophage populations were defined according to F4/80, Gr1, and CD11b expression. Cells selected in the side scatter/forward scatter gate (*first dot plot* in Supplemental Fig. 2D) were analyzed for F4/80 and Gr1 expression (*second dot plot* in Supplemental Fig. 2D). $F4/80^+$ cells were further analyzed for intensity of F4/80 expression (*first row* in Supplemental Fig. 2D); the $F4/80^{med}$ population was then evaluated for CD11b expression, then $F4/80^{med}CD11b^{low}$ cells, considered resolution-promoting macrophages (Mres). The $F4/80^+Gr1^-$ population was further analyzed for intensity of F4/80 expression (*second row* in Supplemental Fig. 2D); the $F4/80^{high}$ population was then evaluated for CD11b expression; the M2 population is $F4/80^{high}$, $Gr1^-$, $CD11b^{high}$. The $F4/80^+Gr1^+$ population was further analyzed for intensity of F4/80 expression (*third row* in Supplemental Fig. 2D); the $F4/80^{low}$ population was then evaluated for CD11b expression; the M1 population is then $F4/80^{low}$, $Gr1^+$, $CD11b^{med}$. The three macrophages populations were evaluated for GILZ and AnxA1 expression (*fifth* and *sixth columns* in Supplemental Fig. 2D). The percentage presented in each dot plot is related to the previous population analyzed. AnxA1 labeling was performed at 1:500 dilution, and cells from AnxA1-deficient mice showed no detectable labeling (not shown). Negative controls were cells stained with fluorochrome-bound secondary Abs only.

Quantitation of mRNA expression by real-time PCR

Total RNA from cells harvested from the pleural cavity was extracted using the RNeasy Mini kit (Qiagen, Crawley, U.K.) according to the manufacturer's instructions. cDNA was synthetized using 1 μg RNA with SuperScript III reverse transcriptase (Invitrogen), following the manufacturer's instructions. Real-time PCR was performed in duplicate, with 1 μl cDNA at a concentration of 100 ng, 0.5 μM primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, U.K.) using StepOne (Applied Biosystems, Foster City, CA, USA). The data were analyzed using StepOne System software with a cycle threshold (Ct) in the linear range of amplification and then processed by the $2^{-\Delta\Delta Ct}$ method. Primers (Integrated DNA Technologies) used are the following: *AnxA1* (5′-ATCAGCGGTGAGCCCCTATC-3′, 5′-TTCATCCAGGGGGCTTTCCTG-3′), *GILZ* (5′-CAGCAGCCACTCAAACCAGC-3′, 5′-ACCACATCCCCTCCAAGCAG-3′), and *Gapdh* (5′-AGAAGACTGTGGATGGCCCC-3′, 5′-TGACCTTGCCCACAGCCTT-3′). A dissociation step was always included to confirm the absence of unspecific products. In each experiment, samples of all groups were run on one plate with two technical replicates. *Gapdh* was used as an endogenous control to normalize the variability in expression levels, and results were expressed as fold increase.

Lysate preparation and Western blot analysis

Inflammatory cells harvested from the pleural cavity were washed with PBS and whole-cell extracts were prepared as previously described (15, 25). Protein amounts were quantified with the Bradford assay reagent from Bio-Rad (Bio-Rad, Hercules, CA). Extracts (50 μg) were separated by electrophoresis on a denaturing 10–15% polyacrylamide-SDS gel and electrotransferred to nitrocellulose membranes, as described (26). Membranes were blocked overnight at 4°C with PBS containing 5% (w/v) nonfat dry milk and 0.1% Tween 20, washed three times with PBS containing 0.1% Tween 20, and then incubated with specific primary Abs (Mcl-1, p-ERK1/2, p–IκB-α, AnxA1, GILZ, MKP-1, or anti β-actin) using a dilution of 1:1000 in PBS containing 5% (w/v) BSA and 0.1% Tween 20. After washing, membranes were incubated with appropriated HRP-conjugated secondary Ab (1:3000). Immunoreactive bands were visualized by using ECL detection system, as described by the manufacturer (GE Healthcare, Piscataway, NJ).

Measurement of cytokines and chemokines

The levels of cytokines IL-1β, TNF-α, and IL-6 and of the chemokines CCL2 and CCL5 were measured in supernatants obtained from pleural cavity washes after TAT-GILZ treatment or at different time points after LPS challenge in GILZ-deficient male mice and C57BL/6 littermates by ELISA, using commercially available Abs according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN).

Corticosterone assay

Blood samples of mice injected with saline or LPS were collected with heparin and centrifuged at 2000 × *g* for 15 min for plasma collection. Samples were stored at −80°C until assayed. Corticosterone was measured using a corticosterone EIA kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions.

Statistical analysis

All results are presented as the means ± SEM. Data were analyzed by one-way ANOVA, and differences between groups were assessed using the Student–Newman–Keuls posttest. When only two groups were evaluated, a Student *t* test was used. A p value <0.05 was considered significant. Calculations were performed using the Prism 5.0 software for Windows (GraphPad Software, San Diego, CA).

Results

Self-resolving inflammation of LPS-induced pleurisy is accompanied by increased expression of GILZ in resolution macrophages

We performed experiments in a well-established model of LPS-induced pleurisy (15, 21). In this model i.pl. injection of LPS induces a time-dependent influx of neutrophils into the pleural cavity of mice that peaks at 8–24 h and decreases thereafter. Resolution of neutrophilic inflammation took place at 48 h, coincident with the mononuclear cell influx into the pleural cavity (Fig. 1A). Next, we investigated whether GILZ expression was associated with resolution phase of neutrophilic inflammation and whether macrophages were a source of this protein. As observed in Western blot (Fig. 1B) and quantitative PCR analysis (Fig. 1C), GILZ was detected in PBS-injected mice, virtually disappeared during LPS-induced neutrophilic infiltration, and was again strongly detected during the resolution phase (48–72 h). The kinetics of AnxA1 expression were quite similar to GILZ as shown by Western blot (15) and quantitative PCR (Supplemental Fig. 1). In the early phase of inflammation macrophages are an important source of cytokines and inflammatory mediators, but at later time points this cell type is crucial for the resolution of inflammatory response (27). Based on a recent description of three macrophage populations, that is, M1 ($F4/80^{low}Gr1^{+}Cd11b^{med}$), M2 ($F4/80^{high}Gr1^{-}Cd11b^{high}$), and Mres ($F4/80^{med}Cd11b^{low}$) (28, 29), we performed evaluation of these populations by flow cytometry in cells from the pleural cavity of LPS-injected mice. As shown in Fig. 1D and 1G, we detected more M2 and Mres macrophages at 48 h. M2 macrophages were detected in PBS-injected mice, were significantly reduced at 8 h, and significantly increased at 48 h after LPS challenge (Fig. 1D). The number of M2 cells was higher than that of Mres (Fig. 1D, 1G). The kinetics of M2 macrophage numbers were quite similar to those of GILZ and AnxA1 expression. In contrast, M1 macrophages were not detected in PBS-injected mice, were abundant at 8 h, and virtually disappeared 48 h after LPS challenge (Supplemental Fig. 2A). In M1 macrophages, GILZ expression was not detected in PBS-injected mice, but it was increased at 8 and 48 h after LPS injection (Supplemental Fig. 2B). In contrast, AnxA1 expression in M1 macrophages was increased at 8 h but virtually disappeared 48 h after LPS challenge (Supplemental Fig. 2C). Significantly, expression of both GILZ and AnxA1 was increased in M2 (Fig. 1E and 1F, respectively) and Mres macrophages (Fig. 1H and 1I, respectively) at 48 h; $AnxA1^{+}$ Mres macrophages were approximately 10-fold more abundant than $GILZ^{+}$ Mres macrophages (Fig. 1H, 1I). The gating strategy used is shown in Supplemental Fig. 2D. These results indicate that changes in GILZ expression in macrophages coincide with self-resolving neutrophilic inflammation.

Pre- and posttreatment of LPS-inflamed mice with the TAT-GILZ peptide induces resolution of neutrophilic inflammation

Next, we evaluated the effects of TAT-GILZ, a GILZ fusion protein containing a TAT peptide to allow the in vivo delivery of the protein, on resolution of inflammation. TAT-GILZ or the peptide control (TAT peptide only) were injected before LPS challenge, and cells were collected at 4, 8, 24, 48, and 72 h after challenge. We quantified the resolution interval (Ri) by defining acute inflammatory parameters (23, 24). Pretreatment with TAT-GILZ significantly reduced the number of PMN recruited to the pleural cavity and shortened R_i by ∼12 h (Fig. 2A). To verify its therapeutic potential, TAT-GILZ was administered 4 h after LPS challenge and cells were obtained from the pleural cavity by washing at 5, 8, and 24 h after LPS administration. Posttreatment of mice with TAT-GILZ greatly decreased neutrophil accumulation in the pleural cavity at 8 and 24 h (Fig. 2C). In both protocols, TAT-GILZ treatment did not significantly alter the number of mononuclear cells (Fig. 2B, 2D), and TAT peptide alone had no significant effect. In keeping with these findings, treatment of mice with TAT-GILZ 4 h after LPS challenge decreased pleural IL-6 and TNF-α levels (Fig. 2E and 2F, respectively) but did not modify pleural IL-1β levels (Fig. 2G). These results indicate that pharmacological treatment with a GILZ fusion protein induces resolution of neutrophilic inflammation.

TAT-GILZ promotes resolution of neutrophilic inflammation by inducing neutrophil apoptosis and inhibiting survival pathways

We queried whether the induction of leukocyte apoptosis was an underlying mechanism of TAT-GILZ–induced resolution of LPS-

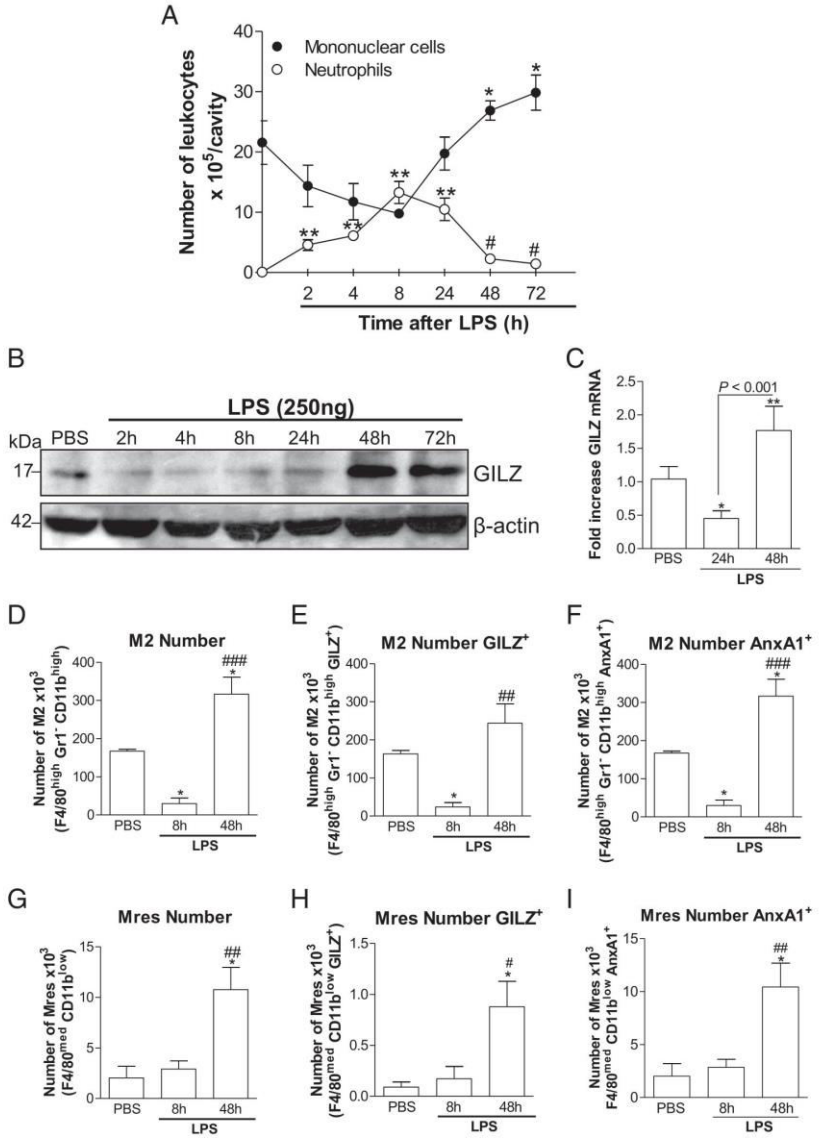

FIGURE 1. Time course of GILZ expression during LPS-induced pleurisy. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and the cells present in the pleural cavity were harvested at several time points and processed for total and differential leukocyte counts of cytospin preparations by (**A**) light microscopy, (**B**) Western blot, and (**C**) quantitative PCR analysis for GILZ expression. Flow cytometry analysis of pleural leukocytes collected after PBS or LPS injection is shown. (**D**) M2 ($F4/80^{high}Gr1^{-}CD11b^{high}$) number, (**E**) M2 expressing GILZ, and (**F**) AnxA1. (**G**) Mres ($F4/80^{med}CD11b^{low}$) number, (**H**) Mres expressing GILZ, and (**I**) AnxA1. Results are expressed as the number of cells per cavity or fold increase and are shown as the mean ± SEM of at least five mice in each group. Quantitative PCR data were performed in samples from control and treated groups of at least five animals for each time point. Analyses of gene expression were performed with two technical replicates with samples of all groups run on one plate. For loading control, membranes were reprobed with anti–β-actin. Blots are representative of three independent experiments using pooled cells from at least five animals in each experiment. $^{*}p < 0.05$, $^{**}p < 0.01$ when compared with PBS-injected mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ when compared with 8 h after LPS-injected mice.

induced pleurisy. To investigate this, TAT-GILZ–injected mice were pretreated with a broad-spectrum caspase inhibitor, zVAD-fmk. The results presented in the Fig. 3A show that the effect of TAT-GILZ on pleural neutrophil numbers was prevented by the pan-caspase inhibitor. Importantly, treatment with zVAD alone did not alter the kinetics of neutrophil recruitment after injection of LPS (21). Accordingly, treatment of mice with TAT-GILZ induced neutrophil apoptosis in the pleural cavity, as assessed using either morphological criteria (Fig. 3B) or annexin V staining (Fig. 3C). In contrast, treatment with TAT-GILZ did not induce apoptosis of macrophages (Supplemental Fig. 3). We also evaluated biochemical markers of survival pathways that control neutrophil lifespan. Using Western blotting of cell extracts of TAT-GILZ–treated mice, we found that treatment with TAT-GILZ reduced

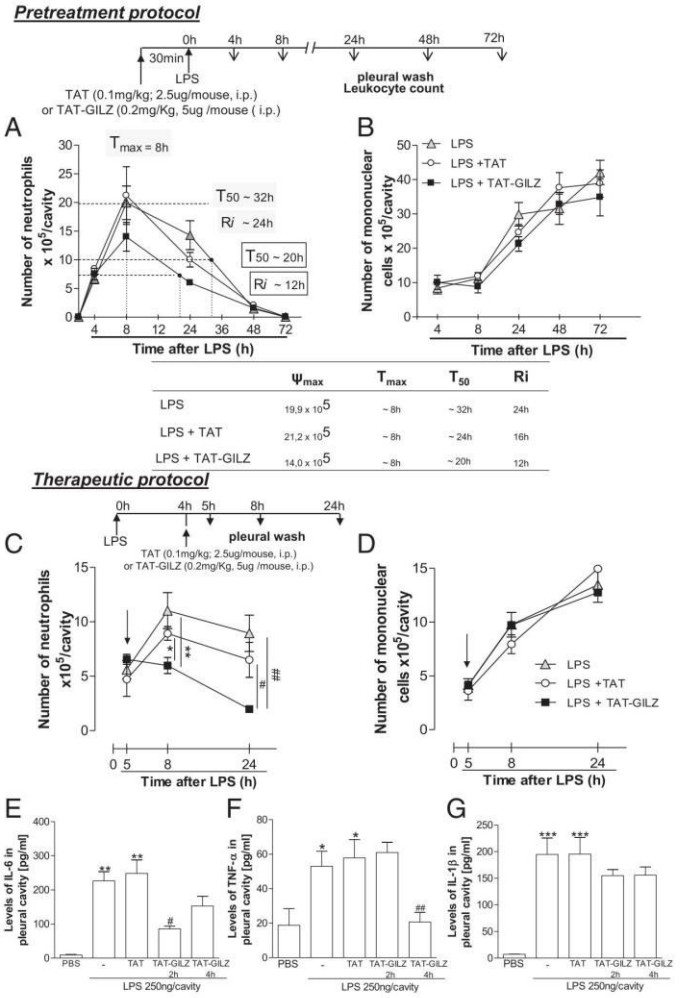

FIGURE 2. Effect of pre- and posttreatment of mice with TAT-GILZ peptide on LPS-induced pleurisy. For pretreatment, mice received an injection of TAT (0.1 mg/kg, i.p.), TAT-GILZ (0.2 mg/kg, i.p.), or vehicle. After 15 min, mice were challenge with LPS (250 ng/cavity, i.pl.). For posttreatment, mice were injected with LPS (250 ng/cavity, i.pl.) and 4 h later received an injection of TAT or TAT-GILZ. (**A** and **C**) The number of neutrophils and (**B** and **D**) mononuclear cells were evaluated at several time points. Of note, T_{max} = 8 h, the time point when PMN numbers reach maximum; $T_{50} \sim 20$ h, the time point when PMN numbers reduce to 50% of maximum; and $R_i \sim 12$ h, resolution interval, the time period when 50% PMN are lost from the pleural cavity. Levels of (**E**) IL-6, (**F**) TNF-α, and (**G**) IL-1β were measured by ELISA in supernatants obtained from pleural cavity washes after 2 and 4 h of TAT or TAT-GILZ treatment of 4 h LPS-injected mice. Results are expressed as the number of cells per cavity or pleural cytokines levels (in pg/ml) and are shown as the mean ± SEM of at least five mice in each group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with PBS-injected mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ when compared with TAT only or 8 h after LPS-challenged mice.

cellular levels of p-ERK1/2, p–IκB-α, and Mcl-1 (Fig. 3D). Taken together, these findings indicate that GILZ plays an important role in the signaling events leading to the neutrophil proapoptotic program during the resolution of acute inflammation.

Resolution of neutrophilic inflammation in GILZ$^{-/-}$ mice was accompanied by increased levels of AnxA1 and corticosterone

In attempt to characterize better the role of endogenous GILZ in resolution of acute inflammation, we conducted a kinetic study of LPS-induced pleurisy in GILZ$^{-/-}$ mice and compared them to wild-type (WT) littermates (C57BL/6). The resolution of inflammation was accompanied by decreased neutrophil accumulation in the pleural cavity in both GILZ$^{-/-}$ and WT mice (Fig. 4A). The number of pleural mononuclear cells at 48 h after LPS was lower in GILZ$^{-/-}$ mice than in WT mice (Fig. 4B). Pleural levels of the cytokines IL-6 and TNF-α and monocyte chemoattractive chemokines CCL2 and CCL5 were similar in WT and GILZ$^{-/-}$ mice (Fig. 4C).

Based on previous studies in which using small interfering RNA strategies to inhibit GILZ resulted in exacerbation of inflammatory response (10, 13), similar resolution of LPS-induced inflammation in GILZ$^{-/-}$ and WT mice (Fig. 4A) was unexpected. We hypothesized the existence of compensatory mechanisms such as the expression of other GC-induced proteins in the setting of GILZ deficiency. Interestingly, GILZ$^{-/-}$ mice showed an early increase of the active intact anti-inflammatory 37-kDa form of AnxA1, but not MKP-1, another GC-induced anti-inflammatory protein that inhibits MAPK activation (Fig. 4D). GILZ$^{-/-}$ mice had increased expression of AnxA1 without stimulation (PBS injected mice) as compared with WT mice (Supplemental Fig. 4). When mice were challenged with LPS, detectable AnxA1 was almost entirely in the cleaved (inactive) form in WT mice, whereas intact AnxA1 remained abundant in GILZ$^{-/-}$ mice and AnxA1 mRNA was increased (Supplemental Fig. 4).

It has been demonstrated that lack of AnxA1 results in increased numbers of corticotrophs (30), and AnxA1$^{-/-}$ mice show increased corticosterone levels after inflammatory stimulus (31). Therefore, we hypothesized that an increase in corticosterone levels could underlie the increased expression of AnxA1 in GILZ-deficient mice. Plasma corticosterone levels in GILZ$^{-/-}$ mice after LPS stimulation were twice those observed in WT mice (WT, 60.7 ± 11.9 ng/ml; GILZ$^{-/-}$, 126.5 ± 30.6 ng/ml; $p < 0.05$).

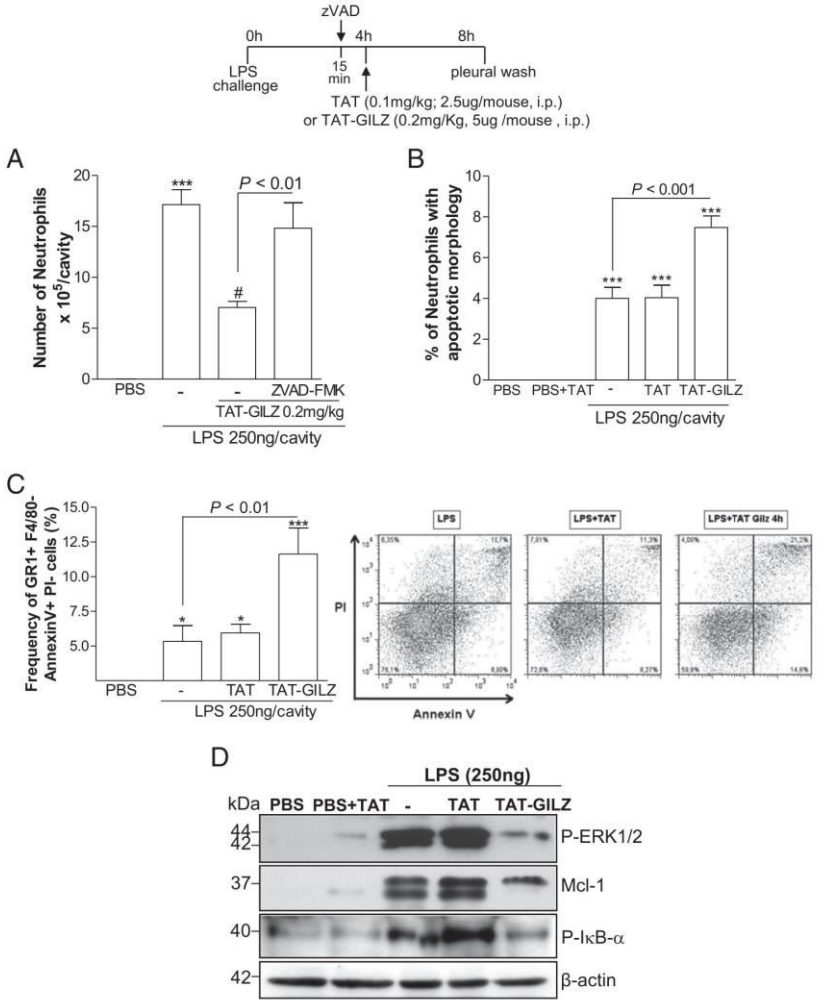

FIGURE 3. Effect of treatment with TAT-GILZ on neutrophil apoptosis in vivo. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 4 h later received an injection of TAT (0.1 mg/kg, i.p.), TAT-GILZ (0.2 mg/kg, i.p.), or vehicle. The pan-caspase inhibitor zVAD-fmk (1 mg/kg, i.p.) was given 15 min before the peptide. (**A**) The number of neutrophils, (**B**) cells with distinctive apoptotic morphology, (**C**) frequency of annexin V^+ neutrophils, and (**D**) Western blot to detection of p-ERK, Mcl-1, and p–IκB-α were evaluated 4 h after peptide treatment. For loading control, membranes were reprobed with anti–β-actin. Blots are representative of three independent experiments using pooled cells from at least five animals in each experiment. $^*p < 0.05$, $^{***}p < 0.001$ when compared with PBS-injected mice. $^{\#}p < 0.05$ when compared with 8 h after LPS-challenged mice.

These data are akin to those observed when comparing AnxA1-deficient mice (31), suggesting a regulatory effect on corticosterone production in absence of either of these GC-induced proteins. Taken together, these findings suggest that self-resolution of inflammation in $GILZ^{-/-}$ mice may depend on a compensatory effect mediated by overexpression of AnxA1 induced by endogenous GC.

Anti-AnxA1 abolished Dex-induced GILZ accumulation

AnxA1 is a GC-induced protein that mediates the anti-inflammatory and proresolving activities of endogenous and exogenous GC (15, 18, 32). It was demonstrated in in vitro experiments using $AnxA1^{-/-}$ macrophages that GILZ is a mediator of the anti-inflammatory effects of AnxA1 (20). Therefore, we asked whether AnxA1 is upstream of GILZ during responses to exogenous GC in our in vivo phlogistic settings. Treatment of LPS-injected mice with Dex decreased pleural neutrophil accumulation (Fig. 5A). Compared to PBS-treated controls, the administration of LPS markedly inhibited GILZ expression in pleural cells, but this effect was reversed by GC treatment, as evidenced by increased GILZ expression in Dex-treated mice (Fig. 5B). To evaluate whether AnxA1 could control GILZ expression in vivo, mice were treated with anti-AnxA1 neutralizing Ab before Dex treatment. Neutralization of AnxA1 abolished the effects of Dex on neutrophil accumulation (Fig. 6C) as previously shown (15) and inhibited the expression of GILZ (Fig. 5C), suggesting that AnxA1 is required for GC induction of GILZ in vivo. Next, we evaluated whether Ac2-26, a synthetic peptide that contains the N-terminal active portion of AnxA1, could induce GILZ expression. By flow cytometry, we found that Ac2-26 peptide treatment increased intracellular GILZ in macrophages ($F4/80^+$ cells) within 1 h (Fig. 5D). This result was not seen in neutrophils, examined by

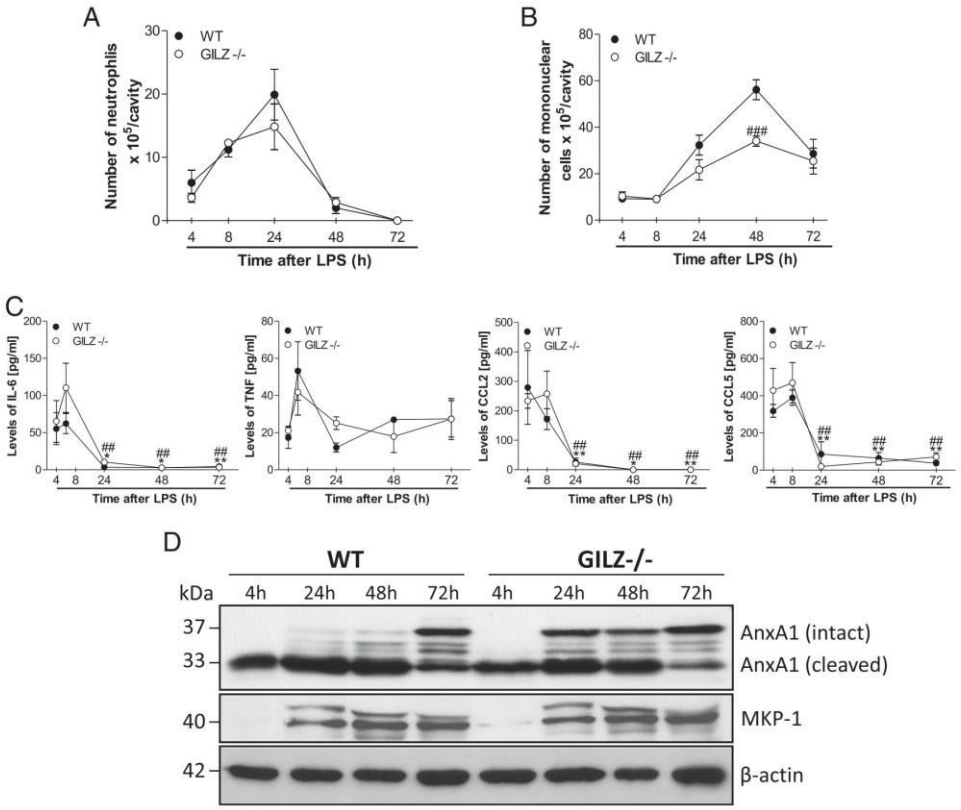

FIGURE 4. Time course of leukocyte influx, cytokine/chemokine, and AnxA1 levels during LPS-induced inflammation in WT and GILZ $^{-/-}$ mice. WT and GILZ$^{-/-}$ mice were injected with LPS (250 ng/cavity, i.pl.) and the cells present in the pleural cavity were harvested at different times and processed for total and differential leukocyte counts of cytospin preparations by (**A** and **B**) light microscopy and (**D**) Western blot analysis for AnxA1 and MKP-1. (**C**) Levels of IL-6, TNF-α, CCL2, and CCL5 (in pg/ml) were measured by ELISA assay in supernatants obtained from pleural cavity washes after LPS injection. Results are shown as the mean ± SEM of at least four mice in each group. For loading control, membranes were reprobed with anti–β-actin. Blots are representative of two independent experiments using pooled cells from at least four animals in each experiment. $^{###}p < 0.01$ when GILZ KO mice were compared with WT mice 48 h after LPS challenge. For levels of IL-6, TNF-α, CCL2, and CCL5: $^{*}p < 0.05$, $^{**}p < 0.01$ when compared with 8 h after LPS-challenged mice WT groups. $^{##}p < 0.01$ when compared with 4 or 8 h after LPS-challenged mice KO groups.

labeling GILZ in cells stained with Gr1^{+} in the same experimental setting (data not shown).

Neutralizing AnxA1 in GILZ$^{-/-}$ mice abolishes resolution of neutrophilic inflammation

Next, we queried whether GC could induce resolution of inflammation in the absence of GILZ. Treatment of LPS-injected mice with Dex promoted resolution of neutrophilic inflammation in both WT and GILZ$^{-/-}$ mice genotypes (Fig. 6A). In WT mice, resolution of inflammation was associated with increased expression of intact AnxA1. As shown before (Fig. 4D, Supplemental Fig. 4A), GILZ$^{-/-}$ mice expressed increased intact AnxA1 in pleural cavity cells after LPS injection (compare *lanes 1* and *3*, Fig. 6B), and this was not further increased by Dex treatment (compare *lanes 3* and *4*, Fig. 6B). Finally, we investigated whether inhibiting AnxA1 in GILZ$^{-/-}$ mice could affect the phenotype of these mice. By using an AnxA1 neutralizing Ab previously used in other studies (15, 22), we showed that mice in which AnxA1 was neutralized were refractory to resolution induced by Dex (Fig. 6C), indicating that by preventing the compensatory effects of AnxA1, GILZ$^{-/-}$ mice lost the ability to resolve inflammation in response to GC treatment. Of note, treatment of mice with a control goat nonimmune serum had no effect on the resolution of inflammation (data not shown). These results clearly suggest that enhanced AnxA1 expression compensates for the lack of GILZ in mediating acute and GC-induced resolution of inflammation.

Discussion

GCs are potent anti-inflammatory and immunosuppressive drugs that are used therapeutically for the treatment of many inflammatory conditions. The broad-spectrum effects of GCs depend on their inhibitory effects on transcription factors, such as NF-κB and AP-1, and their capacity to induce anti-inflammatory regulatory proteins. Our group has recently shown the importance of AnxA1, a GC-induced protein, in driving natural and Dex-induced resolution of inflammation (15). Another GC-induced protein, GILZ, has been reported to interact with the same transcription factors as those of the GC receptor, and thereby to inhibit inflammation (8). However, the role of endogenous GILZ on natural and GC-induced resolution of acute inflammation has not been established. In this work, we studied whether GILZ mediates GC effects in the resolution of inflammation and investigated the proresolving properties of exogenously administrated GILZ. Moreover, because GILZ mediates AnxA1 anti-inflammatory ac-

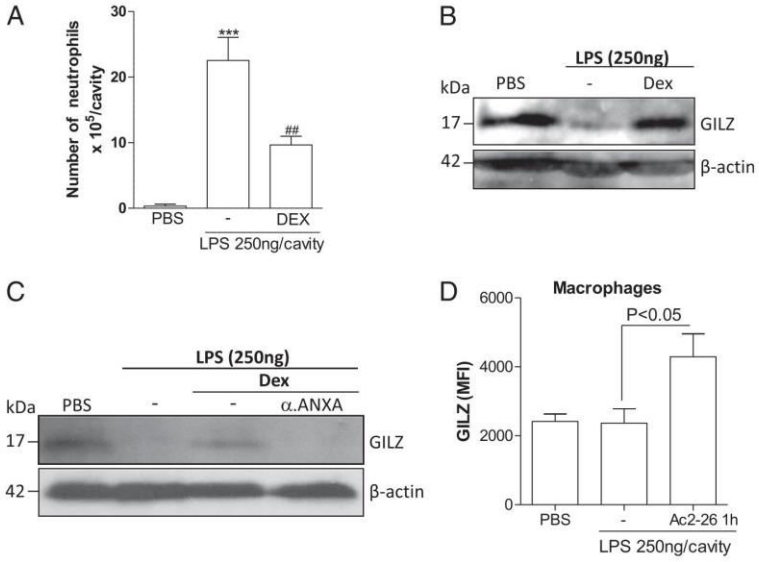

FIGURE 5. Effect of treatment with anti-AnxA1 antiserum on Dex-induced resolution of acute inflammation. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 4 h later received an injection of Dex (2 mg/kg, i.p.) or Ac2-26 (100 μg/cavity, i.pl.). Cells present in the pleural cavity were harvested 4 h after Dex or 1 h after Ac2-26 treatment. (**A**) Neutrophil counts and (**B** and **C**) Western blot for GILZ detection 4 h after Dex treatment. (**D**) Mean fluorescence intensity (MFI) of intracellular GILZ, evaluated by flow cytometry, in macrophages ($F4/80^+$ cells) 1 h after Ac2-26 treatment. Results are shown as the mean ± SEM of at least five mice in each group. For loading control, membranes were reprobed with anti–β-actin. Blots are representative of three independent experiments using pooled cells from at least five animals in each experiment. $^{***}p < 0.001$ when compared with PBS-injected mice, $^{\#\#}p < 0.01$ when compared with 8 h after LPS-challenged mice.

tivity in macrophages (20), we investigated the possible relationship between GILZ and AnxA1 in the resolution of neutrophilic inflammation. We found that GILZ is expressed in resolving Mres and M2 macrophages, suggesting that GILZ could play a key role in macrophage-induced resolution activities. Exogenous administration of a GILZ fusion protein (TAT-GILZ) promoted resolution of acute inflammation by inducing neutrophil apoptosis. In $GILZ^{-/-}$ mice, despite decreased mononuclear cell numbers, natural resolution of inflammation was unaltered, associated with enhanced plasma levels of corticosterone and early appearance of intact AnxA1. These latter results suggest that AnxA1 compensates for the lack of GILZ and permits natural resolution of inflammation in $GILZ^{-/-}$ mice. Finally, we showed that GILZ expression is dependent on AnxA1 during Dex-induced resolution of LPS inflammation, and Dex-induced resolution of inflammation is preserved in $GILZ^{-/-}$ mice, an effect explained by a compensatory increase in expression of AnxA1.

The kinetics of GILZ expression during a self-resolving model of pleurisy were similar to kinetics of AnxA1 expression (and as described in Ref. 15); that is, resident cells of the pleural cavity express GILZ and during the productive phase of LPS-induced pleurisy GILZ expression is inhibited. Indeed, it has been demonstrated that GILZ is downregulated in human alveolar macrophages upon TLR activation (33) and in HUVECs and macrophages upon treatment with TNF-α (34). In contrast, during the resolution phase, 48–72 h after LPS, GILZ expression was upregulated, and cells producing GILZ and AnxA1 were mostly Mres and M2 macrophages.

Macrophages are thought to be important at the onset of inflammation by producing proinflammatory mediators and performing effector functions. As the inflammatory response evolves, macrophages are reprogrammed toward a more resolving/restorative phenotype and are orchestrators of a series of events leading to successful resolution of inflammation (27, 35). One of the main functions of macrophages is elimination of apoptotic granulocytes (efferocytosis). Indeed, efferocytosis itself may reprogram macrophages with change of their phenotype. In models of self-resolving inflammation, various phenotypes of macrophages may coexist (28, 36, 37). M2 (or M2-like) macrophages are highly efferocytic and produce anti-inflammatory molecules such as IL-10 and TGF-β and biologically active amounts of proresolving mediators, including resolvins, protectins, and maresins (29, 38). Such mediators have the potential to inhibit further PMN recruitment, intensify monocyte migration, and amplify efferocytosis. M2 macrophages then switch to Mres phenotype, which display reduced phagocytosis, but instead produce antifibrotic and antioxidant proteins that limit tissue damage

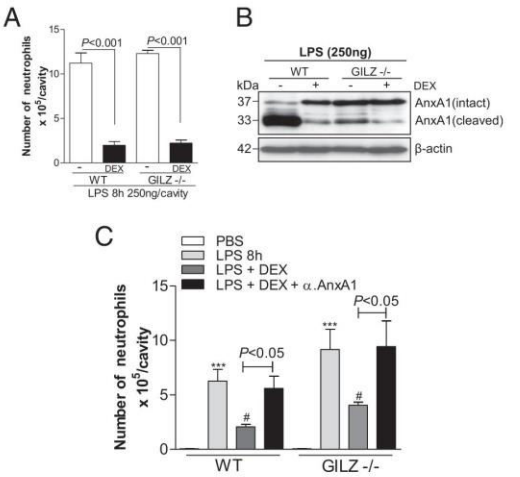

FIGURE 6. Effect of treatment with Dex and anti-AnxA1 antiserum in $GILZ^{-/-}$ mice. WT and $GILZ^{-/-}$ mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 4 h later received an injection of Dex (2 mg/kg, i.p.) or antiserum anti-AnxA1 (200 μl, i.p.) 1 h before Dex. Cells present in the pleural cavity were harvested 4 h after Dex or Dex plus anti-AnxA1 and processed for (**A** and **C**) neutrophil counts and (**B**) Western blot analysis of AnxA1. Results are shown as the mean ± SEM of at least five mice in each group. For loading control, membranes were reprobed with anti–β-actin. Blots are representative of two independent experiments using pooled cells from at least five animals in each experiment. $^{***}p < 0.001$ when compared with PBS-injected mice, $^{\#}p < 0.05$ when compared with 8 h after LPS-challenged mice.

and fibrosis (28). It has been demonstrated that GILZ is constitutively expressed in nonphlogistic conditions in human and murine macrophages (39). In the present study, we demonstrated that GILZ was expressed in M2 and Mres macrophages and its appearance coincides with resolution of inflammation. Indeed, GILZ acts physiologically to balance the inflammatory process (5), and in this study we show, to our knowledge for the first time, a temporal relationship between increases in GILZ and M2/Mres macrophages during the resolution of inflammation. Many mediators can accelerate the clearance of apoptotic granulocytes mediated by macrophages, including AnxA1, IL-10, and proresolution lipids (2). The increased expression of GILZ and AnxA1 (15) observed during the resolution phase of inflammation may contribute to increase the efferocytic capacity of macrophages, an effect already demonstrated for AnxA1 (17, 40).

Similar to AnxA1, GILZ has been shown to mediate several anti-inflammatory effects of GC, including modulation of T lymphocyte activation, apoptosis of thymocytes, and antiproliferative and anti-inflammatory activities, and it is upregulated by IL-10 and TGF-β in several cell types (7, 8). More recently, a study showed that GILZ promoted the induction of regulatory T cells and that lack of GILZ in T cells of GILZ knockout (KO) mice caused the development of spontaneous colitis (41). In this study, we demonstrated that treating mice with a TAT-GILZ fusion protein induced resolution of neutrophilic inflammation in both preventive and therapeutic schedules. TAT-GILZ accelerated resolution of acute inflammation, reducing the magnitude of PMN infiltration and shortening the resolution interval. Additionally, we showed that TAT-GILZ treatment was associated with decreased levels of the important proinflammatory cytokines, IL-6 and TNF-α. Indeed, in more complex models of inflammation where TAT-GILZ was used it was an effective strategy to control inflammation. For instance, TAT-GILZ administration promoted a protective effect in a model of inflammatory bowel disease (9) and spinal cord injury (12). Moreover, recent studies showed that GILZ overexpression inhibits endothelial cell adhesion function (42) and protects against endotoxemia (43) and arthritis (13).

The apoptosis of neutrophils is an important event in resolution of acute inflammation (2, 15, 21). The involvement of GILZ in the process of apoptosis has been suggested by experiments in GILZ transgenic mice, which overexpress GILZ in the T cell lineage. Thymocytes from these mice undergo apoptosis, activate caspase-8 and caspase-3, and downregulate Bcl-x_L, suggesting that GILZ has effects similar those of GCs (44). Alternatively, another study showed that GILZ inhibited thymocyte apoptosis induced by TCR activation by inhibiting NF-κB activity and IL-10 production (45). Additionally, GILZ did not induce apoptosis in mature mouse T cell lymphocytes (8, 44). These results indicate that GILZ regulates T cell apoptosis and, similar to GC, induces apoptosis in resting T cells while protecting activated T lymphocytes. Inhibition of the PI3K/Akt pathway resulted in GILZ upregulation and increased apoptosis of multiple myeloma cells (46). Interestingly, GILZ acts as a Ras signal suppressor and decreases activation of ERK and Akt, leading to reduction of cell proliferation and transformation (47). Furthermore, GILZ interacts with mammalian target of rapamycin complex 2, inhibiting AKT phosphorylation and activating FOXO3a-mediated transcription of the proapoptotic protein Bim in BCR-ABL^+ cells (48). In our study, we demonstrated, to our knowledge for the first time, that TAT-GILZ was able to induce neutrophil apoptosis and inhibit important prosurvival pathways such as p-ERK1/2, NF-κB, and Mcl-1. Altogether, these data clearly do show that GILZ plays an important role in the signaling events underlying the proapoptotic and proresolving effects that lead to resolution of acute inflammation.

A study of Yang et al. (20) showed that GILZ could be a mediator of the anti-inflammatory effects of AnxA1, a known anti-inflammatory and proresolving protein (4, 32). They showed lower levels of GILZ in Dex-treated AnxA1-deficient macrophages, as compared with Dex-treated WT macrophages (20). A follow-up study using lung fibroblasts showed that the involvement of AnxA1 in GC induction of GILZ was independent of formyl peptide receptor 2, suggesting that there was no requirement for engagement of the AnxA1 receptor for effects on GILZ expression (49). Our group has previously shown that by inhibiting AnxA1, the effect of Dex to induce resolution of neutrophilic inflammation was abolished (15). Importantly, in the present study, we show that this effect is associated with decreased GILZ expression, suggesting that in vivo there is a crosstalk between GILZ and AnxA1. Moreover, the inhibition of the compensatory increase of AnxA1 in $GILZ^{-/-}$ mice induced refractoriness to resolution induced by Dex. Indeed, we were able to show in vivo that injection of the AnxA1 peptide Ac2-26 in LPS-inflamed mice increased GILZ expression in macrophages, an effect not shown in vitro (20). Therefore, these findings suggest a regulatory relationship between AnxA1 and GILZ, in which AnxA1 is involved in a mechanism leading to modulation of GILZ expression in in vivo settings.

In the model of LPS-induced pleurisy used in this study, GILZ deficiency resulted in a reduction of the number of total leukocytes and mononuclear cells, but curiously the absence of GILZ did not modify the natural course of resolution of neutrophilic inflammation, that is, neutrophilic inflammation resolved similarly in GILZ-deficient and WT mice. In a self-resolving model of zymosan-induced peritonitis, AnxA1-deficient mice resolved inflammation similarly to WT mice, despite greater numbers of neutrophils and increased amounts of the chemokines KC and IL-1β at early time points (50). Additionally, AnxA1 KO mice showed defective GC suppression of inflammation in carrageenan-induced edema, zymosan-induced peritonitis, and Ag-induced arthritis, when GC was administered previously to the inflammatory stimulus (18, 32). In the context of GILZ deficiency it has been demonstrated that inhibition of endogenous GILZ by small interfering RNA

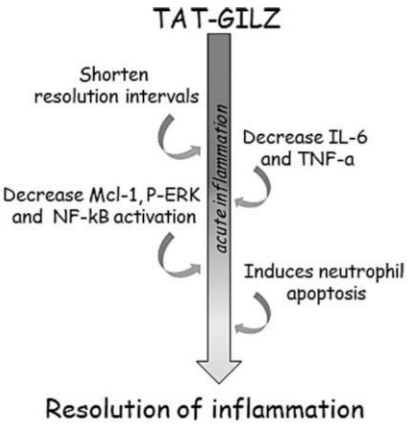

FIGURE 7. Proposed model for TAT-GILZ–induced resolution of inflammation. TAT-GILZ, a GILZ fusion protein containing a TAT peptide to allow the in vivo delivery of the protein, decreases proinflammatory cytokines IL-6 and TNF-α, inhibits prosurvival proteins such as p-ERK1/2, p–IκB-α, and Mcl-1, short resolution intervals, and also promotes neutrophil apoptosis. These sequences of events result in an efficient resolution of acute inflammation.

increased the severity of a mouse model of collagen-induced arthritis, with enhanced production of TNF-α and IL-1β (10). However, GILZ KO mice did not present difference in severity of inflammation as compared with WT mice in a model of arthritis (13), although GILZ treatment using an adenoviral strategy was therapeutically effective. This latter study (13) is in agreement with our findings using LPS-induced inflammation, showing that absence of GILZ does not modify the severity or course of natural resolution of inflammation, but that an exogenous GILZ-based strategy efficiently resolves acute inflammation. Taken together, these studies indicate that GILZ may affect differently the outcome of inflammation resolution in different experimental models, possibly due to the fact that GILZ and AnxA1 influence each other's expression. In contrast to these findings, it has been shown that there was worsening of arthritis and refractoriness to Dex treatment after silencing GILZ expression in vivo (10). In our experiments, the kinetics of cell influx and the effects of Dex were unaltered in GILZ-deficient mice. Because in our experimental conditions there was enhanced expression of AnxA1 in the absence of GILZ, it was reasonable to suggest that compensatory AnxA1 expression in GILZ KO mice could replace the loss of GILZ. Interestingly, increased levels of AnxA1 in GILZ-deficient mice after LPS stimulus were associated with increased plasma levels of corticosterone. More importantly, blockade of AnxA1 with a neutralizing Ab blocked the capacity of GCs to promote resolution in GILZ-deficient mice, suggesting that the increase of AnxA1 in the absence of GILZ could account for the normal resolution phenotype in these mice. The ability of AnxA1 to exert inhibitory effects in the absence of GILZ also likely explains the hitherto perplexing observation that GILZ deficiency does not appear to impair the anti-inflammatory effects of GC (13). In this context, the potential for a GILZ-based therapy to be proresolving during inflammation is fairly clear. There are as yet no data on which to determine whether such an approach would result in GC-like immunosuppressive effects, as well as increased infections, during chronic therapy.

In conclusion, our findings indicate that although endogenous GILZ is redundant for the self-resolving model of acute inflammation used in the present study, therapeutic administration of GILZ efficiently induces a proapoptotic program in neutrophils leading to resolution of acute inflammation (Fig. 7). To our knowledge, this is the first observation that GILZ promotes resolution of neutrophilic inflammation by inducing apoptosis of neutrophils. Moreover, our results suggest that the lack of phenotype of GILZ-deficient mice in some experimental contexts is likely due to compensation mediated by an increase in AnxA1 expression. Hence, these results reinforce the idea that there is a coordinated regulation of GILZ and AnxA1, and that exploitation of the association of these proteins may represent a powerful anti-inflammatory strategy for the treatment of inflammatory diseases.

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Disclosures

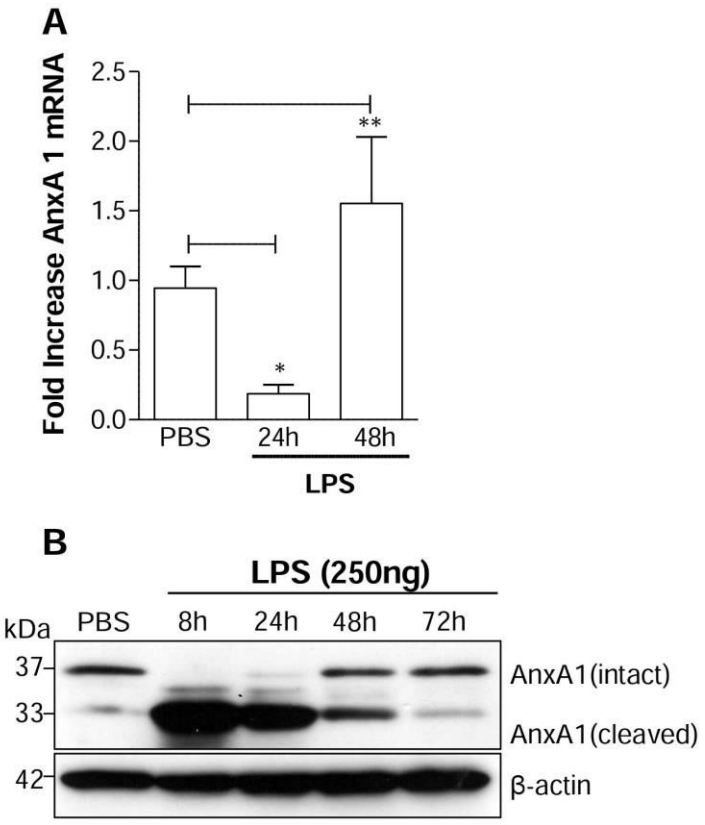
The authors have no financial conflicts of interest.

References

1. Serhan, C. N., S. D. Brain, C. D. Buckley, D. W. Gilroy, C. Haslett, L. A. O'Neill, M. Perretti, A. G. Rossi, and J. L. Wallace. 2007. Resolution of inflammation: state of the art, definitions and terms. *FASEB J.* 21: 325–332.
2. Alessandri, A. L., L. P. Sousa, C. D. Lucas, A. G. Rossi, V. Pinho, and M. M. Teixeira. 2013. Resolution of inflammation: mechanisms and opportunity for drug development. *Pharmacol. Ther.* 139: 189–212.
3. Sousa, L. P., A. L. Alessandri, V. Pinho, and M. M. Teixeira. 2013. Pharmacological strategies to resolve acute inflammation. *Curr. Opin. Pharmacol.* 13: 625–631.
4. Perretti, M., and F. D'Acquisto. 2009. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat. Rev. Immunol.* 9: 62–70.
5. Beaulieu, E., and E. F. Morand. 2011. Role of GILZ in immune regulation, glucocorticoid actions and rheumatoid arthritis. *Nat. Rev. Rheumatol.* 7: 340–348.
6. Clark, A. R., and M. G. Belvisi. 2012. Maps and legends: the quest for dissociated ligands of the glucocorticoid receptor. *Pharmacol. Ther.* 134: 54–67.
7. D'Adamio, F., O. Zollo, R. Moraca, E. Ayroldi, S. Bruscoli, A. Bartoli, L. Cannarile, G. Migliorati, and C. Riccardi. 1997. A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. *Immunity* 7: 803–812.
8. Ayroldi, E., and C. Riccardi. 2009. Glucocorticoid-induced leucine zipper (GILZ): a new important mediator of glucocorticoid action. *FASEB J.* 23: 3649–3658.
9. Cannarile, L., S. Cuzzocrea, L. Santucci, M. Agostini, E. Mazzon, E. Esposito, C. Muià, M. Coppo, R. Di Paola, and C. Riccardi. 2009. Glucocorticoid-induced leucine zipper is protective in Th1-mediated models of colitis. *Gastroenterology* 136: 530–541.
10. Beaulieu, E., D. Ngo, L. Santos, Y. H. Yang, M. Smith, C. Jorgensen, V. Escriou, D. Scherman, G. Courties, F. Apparailly, and E. F. Morand. 2010. Glucocorticoid-induced leucine zipper is an endogenous antiinflammatory mediator in arthritis. *Arthritis Rheum.* 62: 2651–2661.
11. Srinivasan, M., and S. Janardhanam. 2011. Novel p65 binding glucocorticoid-induced leucine zipper peptide suppresses experimental autoimmune encephalomyelitis. *J. Biol. Chem.* 286: 44799–44810.
12. Esposito, E., S. Bruscoli, E. Mazzon, I. Paterniti, M. Coppo, E. Velardi, S. Cuzzocrea, and C. Riccardi. 2012. Glucocorticoid-induced leucine zipper (GILZ) over-expression in T lymphocytes inhibits inflammation and tissue damage in spinal cord injury. *Neurotherapeutics* 9: 210–225.
13. Ngo, D., E. Beaulieu, R. Gu, A. Leaney, L. Santos, H. Fan, Y. Yang, W. Kao, J. Xu, V. Escriou, et al. 2013. Divergent effects of endogenous and exogenous glucocorticoid-induced leucine zipper in animal models of inflammation and arthritis. *Arthritis Rheum.* 65: 1203–1212.
14. Solito, E., A. Kamal, F. Russo-Marie, J. C. Buckingham, S. Marullo, and M. Perretti. 2003. A novel calcium-dependent proapoptotic effect of annexin 1 on human neutrophils. *FASEB J.* 17: 1544–1546.
15. Vago, J. P., C. R. Nogueira, L. P. Tavares, F. M. Soriani, F. Lopes, R. C. Russo, V. Pinho, M. M. Teixeira, and L. P. Sousa. 2012. Annexin A1 modulates natural and glucocorticoid-induced resolution of inflammation by enhancing neutrophil apoptosis. *J. Leukoc. Biol.* 92: 249–258.
16. Maderna, P., S. Yona, M. Perretti, and C. Godson. 2005. Modulation of phagocytosis of apoptotic neutrophils by supernatant from dexamethasone-treated macrophages and annexin-derived peptide Ac2-26. *J. Immunol.* 174: 3727–3733.
17. Dalli, J., C. P. Jones, D. M. Cavalcanti, S. H. Farsky, M. Perretti, and S. M. Rankin. 2012. Annexin A1 regulates neutrophil clearance by macrophages in the mouse bone marrow. *FASEB J.* 26: 387–396.
18. Hannon, R., J. D. Croxtall, S. J. Getting, F. Roviezzo, S. Yona, M. J. Paul-Clark, F. N. Gavins, M. Perretti, J. F. Morris, J. C. Buckingham, and R. J. Flower. 2003. Aberrant inflammation and resistance to glucocorticoids in annexin $1^{-/-}$ mouse. *FASEB J.* 17: 253–255.
19. Montero-Melendez, T., J. Dalli, and M. Perretti. 2013. Gene expression signature-based approach identifies a pro-resolving mechanism of action for histone deacetylase inhibitors. *Cell Death Differ.* 20: 567–575.
20. Yang, Y. H., D. Aeberli, A. Dacumos, J. R. Xue, and E. F. Morand. 2009. Annexin-1 regulates macrophage IL-6 and TNF via glucocorticoid-induced leucine zipper. *J. Immunol.* 183: 1435–1445.
21. Sousa, L. P., F. Lopes, D. M. Silva, L. P. Tavares, A. T. Vieira, B. M. Rezende, A. F. Carmo, R. C. Russo, C. C. Garcia, C. A. Bonjardim, et al. 2010. PDE4 inhibition drives resolution of neutrophilic inflammation by inducing apoptosis in a PKA-PI3K/Akt-dependent and NF-κB-independent manner. *J. Leukoc. Biol.* 87: 895–904.
22. Souza, D. G., C. T. Fagundes, F. A. Amaral, D. Cisalpino, L. P. Sousa, A. T. Vieira, V. Pinho, J. R. Nicoli, L. Q. Vieira, I. M. Fierro, and M. M. Teixeira. 2007. The required role of endogenously produced lipoxin A_4 and annexin-1 for the production of IL-10 and inflammatory hyporesponsiveness in mice. *J. Immunol.* 179: 8533–8543.
23. Bannenberg, G. L., N. Chiang, A. Ariel, M. Arita, E. Tjonahen, K. H. Gotlinger, S. Hong, and C. N. Serhan. 2005. Molecular circuits of resolution: formation and actions of resolvins and protectins. *J. Immunol.* 174: 4345–4355.
24. Chiang, N., M. Shinohara, J. Dalli, V. Mirakaj, M. Kibi, A. M. Choi, and C. N. Serhan. 2013. Inhaled carbon monoxide accelerates resolution of inflammation via unique proresolving mediator-heme oxygenase-1 circuits. *J. Immunol.* 190: 6378–6388.
25. Sousa, L. P., A. F. Carmo, B. M. Rezende, F. Lopes, D. M. Silva, A. L. Alessandri, C. A. Bonjardim, A. G. Rossi, M. M. Teixeira, and V. Pinho. 2009. Cyclic AMP enhances resolution of allergic pleurisy by promoting inflammatory cell apoptosis via inhibition of PI3K/Akt and NF-κB. *Biochem. Pharmacol.* 78: 396–405.
26. De Sousa, L. P., B. S. Brasil, B. M. Silva, M. H. Freitas, S. V. Nogueira, P. C. Ferreira, E. G. Kroon, and C. A. Bonjardim. 2005. Plasminogen/plasmin regulates *c-fos* and *egr*-1 expression via the MEK/ERK pathway. *Biochem. Biophys. Res. Commun.* 329: 237–245.
27. Lichtnekert, J., T. Kawakami, W. C. Parks, and J. S. Duffield. 2013. Changes in macrophage phenotype as the immune response evolves. *Curr. Opin. Pharmacol.* 13: 555–564.

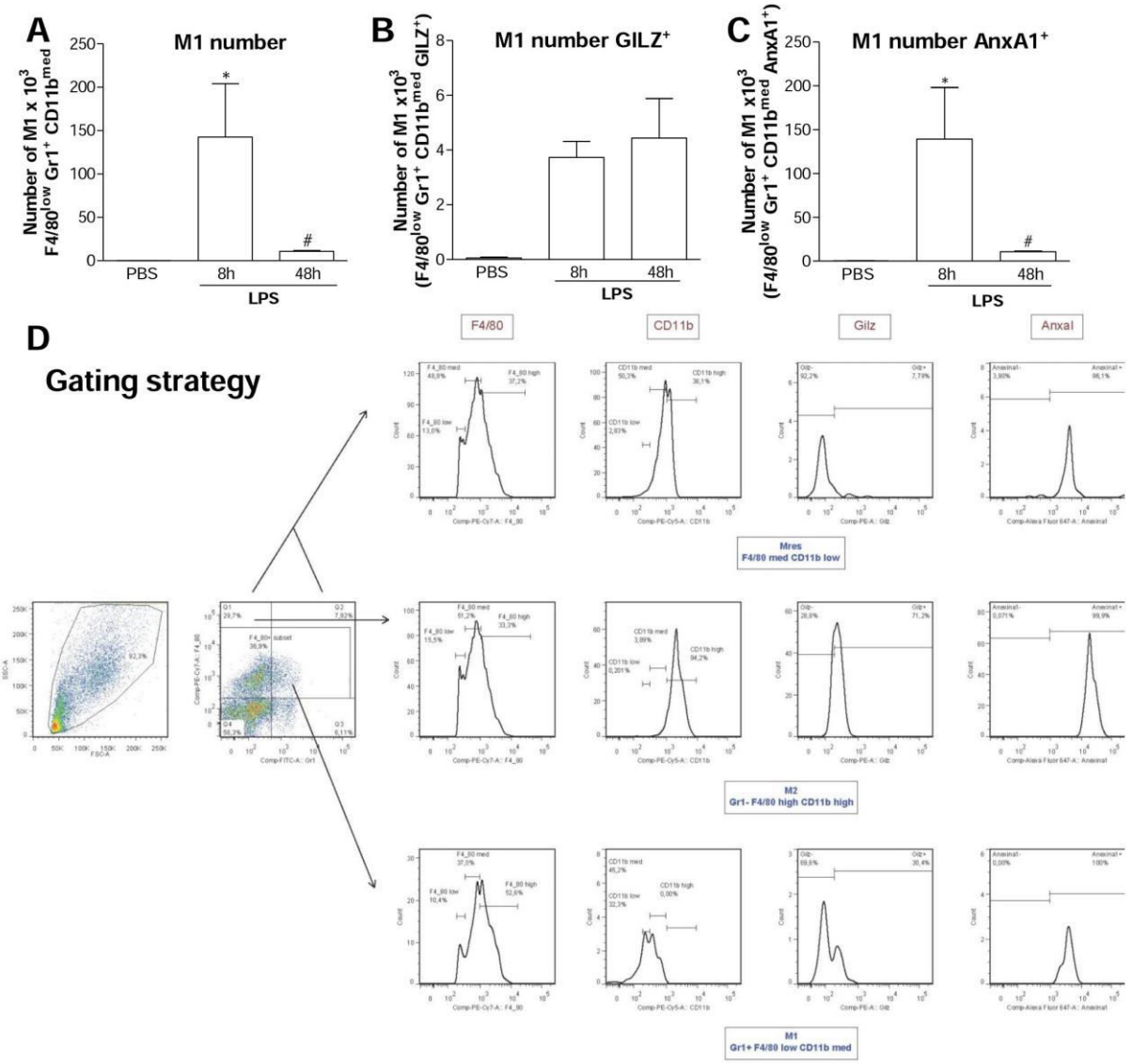
28. Schif-Zuck, S., N. Gross, S. Assi, R. Rostoker, C. N. Serhan, and A. Ariel. 2011. Saturated-efferocytosis generates pro-resolving CD11b low macrophages: modulation by resolvins and glucocorticoids. *Eur. J. Immunol.* 41: 366–379.
29. Ariel, A., and C. N. Serhan. 2012. New lives given by cell death: macrophage differentiation following their encounter with apoptotic leukocytes during the resolution of inflammation. *Front. Immunol.* 3: 4.
30. Morris, J. F., S. Omer, E. Davies, E. Wang, C. John, T. Afzal, S. Wain, J. C. Buckingham, R. J. Flower, and H. C. Christian. 2006. Lack of annexin 1 results in an increase in corticotroph number in male but not female mice. *J. Neuroendocrinol.* 18: 835–846.
31. Akasheh, R. T., M. Pini, J. Pang, and G. Fantuzzi. 2013. Increased adiposity in annexin A1-deficient mice. *PLoS ONE* 8: e82608.
32. Yang, Y. H., E. Morand, and M. Leech. 2013. Annexin A1: potential for glucocorticoid sparing in RA. *Nat. Rev. Rheumatol.* 9: 595–603.
33. Hoppstädter, J., B. Diesel, L. K. Eifler, T. Schmid, B. Brüne, and A. K. Kiemer. 2012. Glucocorticoid-induced leucine zipper is downregulated in human alveolar macrophages upon Toll-like receptor activation. *Eur. J. Immunol.* 42: 1282–1293.
34. Hahn, R. T., J. Hoppstädter, K. Hirschfelder, N. Hachenthal, B. Diesel, S. M. Kessler, H. Huwer, and A. K. Kiemer. 2014. Downregulation of the glucocorticoid-induced leucine zipper (GILZ) promotes vascular inflammation. *Atherosclerosis* 234: 391–400.
35. Soehnlein, O., and L. Lindbom. 2010. Phagocyte partnership during the onset and resolution of inflammation. *Nat. Rev. Immunol.* 10: 427–439.
36. Bystrom, J., I. Evans, J. Newson, M. Stables, I. Toor, N. van Rooijen, M. Crawford, P. Colville-Nash, S. Farrow, and D. W. Gilroy. 2008. Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP. *Blood* 112: 4117–4127.
37. Stables, M. J., S. Shah, E. B. Camon, R. C. Lovering, J. Newson, J. Bystrom, S. Farrow, and D. W. Gilroy. 2011. Transcriptomic analyses of murine resolution-phase macrophages. *Blood* 118: e192–e208.
38. Dalli, J., and C. N. Serhan. 2012. Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators. *Blood* 120: e60–e72.
39. Berrebi, D., S. Bruscoli, N. Cohen, A. Foussat, G. Migliorati, L. Bouchet-Delbos, M. C. Maillot, A. Portier, J. Couderc, P. Galanaud, et al. 2003. Synthesis of glucocorticoid-induced leucine zipper (GILZ) by macrophages: an anti-inflammatory and immunosuppressive mechanism shared by glucocorticoids and IL-10. *Blood* 101: 729–738.
40. Scannell, M., M. B. Flanagan, A. deStefani, K. J. Wynne, G. Cagney, C. Godson, and P. Maderna. 2007. Annexin-1 and peptide derivatives are released by apoptotic cells and stimulate phagocytosis of apoptotic neutrophils by macrophages. *J. Immunol.* 178: 4595–4605.
41. Bereshchenko, O., M. Coppo, S. Bruscoli, M. Biagioli, M. Cimino, T. Frammartino, D. Sorcini, A. Venanzi, M. Di Sante, and C. Riccardi. 2014. GILZ promotes production of peripherally induced Treg cells and mediates the crosstalk between glucocorticoids and TGF-β signaling. *Cell Rep.* 7: 464–475.
42. Cheng, Q., H. Fan, D. Ngo, E. Beaulieu, P. Leung, C. Y. Lo, R. Burgess, Y. G. van der Zwan, S. J. White, L. M. Khachigian, et al. 2013. GILZ overexpression inhibits endothelial cell adhesive function through regulation of NF-κB and MAPK activity. *J. Immunol.* 191: 424–433.
43. Pinheiro, I., L. Dejager, I. Petta, S. Vandevyver, L. Puimège, T. Mahieu, M. Ballegeer, F. Van Hauwermeiren, C. Riccardi, M. Vuylsteke, and C. Libert. 2013. LPS resistance of SPRET/Ei mice is mediated by Gilz, encoded by the *Tsc22d3* gene on the X chromosome. *EMBO Mol. Med.* 5: 456–470.
44. Delfino, D. V., M. Agostini, S. Spinicelli, P. Vito, and C. Riccardi. 2004. Decrease of Bcl-x_L and augmentation of thymocyte apoptosis in GILZ overexpressing transgenic mice. *Blood* 104: 4134–4141.
45. Delfino, D. V., M. Agostini, S. Spinicelli, C. Vacca, and C. Riccardi. 2006. Inhibited cell death, NF-κB activity and increased IL-10 in TCR-triggered thymocytes of transgenic mice overexpressing the glucocorticoid-induced protein GILZ. *Int. Immunopharmacol.* 6: 1126–1134.
46. Grugan, K. D., C. Ma, S. Singhal, N. L. Krett, and S. T. Rosen. 2008. Dual regulation of glucocorticoid-induced leucine zipper (GILZ) by the glucocorticoid receptor and the PI3-kinase/AKT pathways in multiple myeloma. *J. Steroid Biochem. Mol. Biol.* 110: 244–254.
47. Ayroldi, E., O. Zollo, A. Bastianelli, C. Marchetti, M. Agostini, R. Di Virgilio, and C. Riccardi. 2007. GILZ mediates the antiproliferative activity of glucocorticoids by negative regulation of Ras signaling. *J. Clin. Invest.* 117: 1605–1615.
48. Joha, S., A. L. Nugues, D. Hétuin, C. Berthon, X. Dezitter, V. Dauphin, F. X. Mahon, C. Roche-Lestienne, C. Preudhomme, B. Quesnel, and T. Idziorek. 2012. GILZ inhibits the mTORC2/AKT pathway in BCR-ABL$^+$ cells. *Oncogene* 31: 1419–1430.
49. Jia, Y., E. F. Morand, W. Song, Q. Cheng, A. Stewart, and Y. H. Yang. 2013. Regulation of lung fibroblast activation by annexin A1. *J. Cell. Physiol.* 228: 476–484.
50. Damazo, A. S., S. Yona, R. J. Flower, M. Perretti, and S. M. Oliani. 2006. Spatial and temporal profiles for anti-inflammatory gene expression in leukocytes during a resolving model of peritonitis. *J. Immunol.* 176: 4410–4418.

Supplementary Figure 1



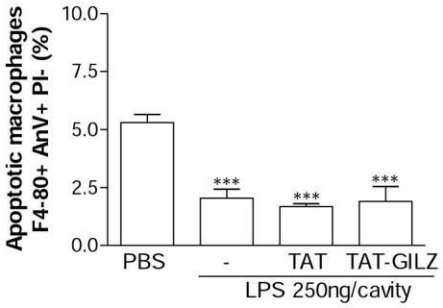
Supplementary Figure 1. AnxA1 expression during LPS-induced pleurisy. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and the cells present in the pleural cavity were harvested at the time points shown and processed for Q-PCR or western blot analysis to detect AnxA1 expression. Results are expressed as fold increase and are shown as the mean ± SEM of at least five mice in each group. *, $P < 0.05$; **, $P < 0.01$; when compared with PBS-injected mice. Q-PCR data were performed in samples from control and treated groups from at least five animals for each group. Analysis of gene expression were performed with two technical replicates with samples of all groups run on one plate. For loading control, membranes were re-probed with anti-β-actin. Blots are representative of three independent experiments using pooled cells from at least five animals.

Supplementary Figure 2



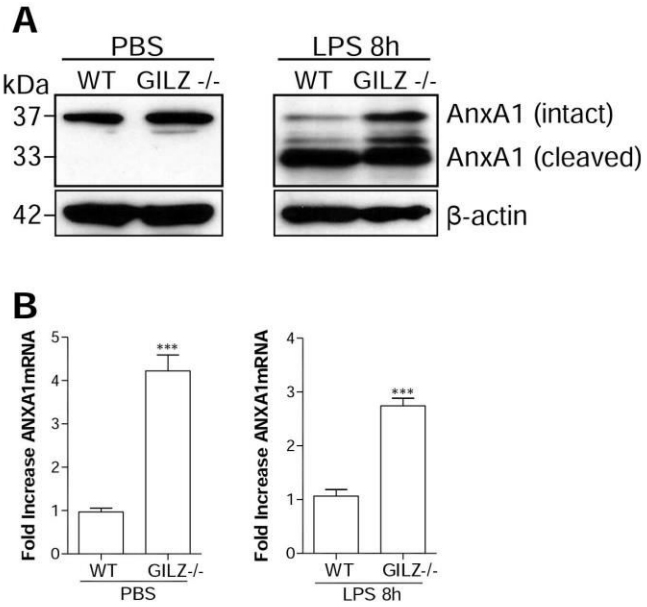
Supplementary Figure 2. Flow cytometry analysis of macrophage populations of pleural leukocytes collected after PBS or LPS-injection. Cells recovered from pleural cavity were stained with specific antibodies and analyzed by flow cytometry. (A) Number of M1 ($F4/80^{low}$ / $Gr1^{+}$/ $CD11b^{med}$), (B) M1 expressing GILZ, (C) M1 expressing AnxA1 and (D) gating strategy for evaluation of cells. Macrophage populations populations were defined according to F4/80, Gr1 and CD11b expression. Cells selected in the SSC x FSC gate (first dot plot) were analyzed for F4/80 and Gr1 expression (second dot plot). $F4/80^{+}$ cells were further analyzed for intensity of F4/80 expression (first row); F4/80med population was then evaluated for CD11b expression, then $F4/80^{med}$ $CD11b^{low}$ cells, considered Mres. $F4/80^{+}$ $Gr1^{-}$ population was further analyzed for intensity of F4/80 expression (second row); $F4/80^{high}$ population was then evaluated for CD11b expression; M2 population is $F4/80^{high}$, $Gr1^{-}$, $CD11b^{high}$. $F4/80^{+}$ $Gr1^{+}$ population was further analyzed for intensity of F4/80 expression (third row); $F4/80^{low}$ population was then evaluated for CD11b expression; M1 population is then $F4/80^{low}$, $Gr1^{+}$, $CD11b^{med}$. The three macrophages populations were evaluated for GILZ and AnxA1 expression (5th and 6th columns, respectively). The percentage presented in each dot plot is related to the previous population analyzed. Results are expressed as the number of cells x 10^3 per cavity and are shown as the mean ± SEM of at least five mice in each group. *, $P < 0.05$; when compared with PBS-injected mice and #, $P < 0.05$ when compared to 8 hours after LPS-injected mice.

Supplementary Figure 3



Supplementary Figure 3. Effect of treatment with TAT-GILZ peptide on macrophage apoptosis *in vivo*. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 4 hours later received an injection of TAT (0.1mg/kg, i.p.), TAT-GILZ (0.2mg/kg, i.p.) or vehicle. Cells were analyzed by flow cytometry to detected the frequency of Annexin-V positive macrophages. The experiments were repeated twice, and results of one representative experiment performed with at least four mice per group are shown (mean ± SEM). *** $P <$ 0.001, when compared with PBS-injected mice.

Supplementary Figure 4



Supplementary Figure 4. AnxA1 expression at baseline and 8 h after LPS-induced inflammation in WT and GILZ $^{-/-}$ mice. Wild-type (WT) and GILZ$^{-/-}$ mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and the cells present in the pleural cavity were harvested at different times and processed for (A) western blot, and (B) Q-PCR analysis for AnxA1 expression. Q-PCR data were performed in samples from control and treated groups from at least four animals for each group. Analysis of gene expression were performed with two technical replicates with samples of all groups run on one plate**.** Results are expressed as fold increase and are shown as the mean ± SEM of at least four mice in each group. ***, $P <$ 0.001; when compared with WT mice. For loading control, membranes were re-probed with anti-β-actin. Blots are representative of two independent experiments using pooled cells from at least four animals in each experiment.

5 – CAPÍTULO 2

Proresolving actions of synthetic and natural protease inhibitors are mediated by Annexin A1

Running Title: Protease inhibitors evoke inflammation resolution

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Footnotes

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2. **Abbreviations used in this article**

AnxA1, annexin A1; SIV, Sivelestat; ELA, Elafin; NE, Neutrophil elastase; PR3, proteinase 3; GC, glucocorticoid; SLPI, secretory leukocyte protease inhibitor; i.pl., intrapleural; i.p., intraperitoneal.

Abstract

Annexin-A1 (AnxA1) is a glucocorticoid-regulated protein endowed with anti-inflammatory and proresolving properties. Intact AnxA1 is a 37 kDa protein that may be cleaved *in vivo* at the N-terminal region by neutrophil proteases including elastase and proteinase-3, generating the 33-kDa isoform that is largely inactive. Here, we investigated the dynamics of AnxA1 expression and the effects of synthetic (Sivelestat, Eglin) and natural (SLPI and Elafin) protease inhibitors on the resolution of LPS-induced inflammation. During the settings of LPS inflammation AnxA1 cleavage associated closely with the peak of neutrophil and elastase expression and activity. SLPI expression increased during resolving phase of the pleurisy. Therapeutic treatment of LPS-challenge mice with rhSLPI or Elafin accelerated resolution, an effect associated with increased numbers of apoptotic neutrophils in the pleural exudates, inhibition of elastase, and modulation of the survival-controlling proteins NF-κB and Mcl-1. Similar effects were observed with Sivelestat, which dose-dependently inhibited neutrophil elastase and shortened resolution intervals. Mechanistically, Sivelestat-induced resolution was caspase-dependent, associated to increased levels of intact AnxA1 and decreased expression of NF-κB and Mcl-1. The proresolving effect of anti-proteases was also observed in a model of monosodium urate crystals-induced inflammation. Sivelestat skewed macrophages towards resolving phenotypes and enhanced efferocytosis of apoptotic neutrophils. A neutralizing antiserum against AnxA1 and a nonselective antagonist of AnxA1 receptors abolished the accelerated resolution promoted by Sivelestat. Collectively these results show that elastase inhibition not only inhibits inflammation but actually promotes resolution, and this response is mediated by protection of endogenous intact AnxA1 with ensuing augmentation of neutrophil apoptosis.

Introduction

The inflammatory process triggered by infection or tissue damage is characterized by microscopic events that include increased vascular permeability and leukocyte accumulation. Leukocyte recruitment, mainly polymorphonuclear leukocytes (PMN), is triggered by a number of pro-inflammatory mediators generated at the site of inflammation acting as chemotactic agents (1). Once recruited, PMNs release several granules rich in proteases that are important against infection. However, neutrophil products can also be harmful to the host leading to intense tissue injury (2).

Proteases are enzymes produced by a variety of phagocytic inflammatory cells, including neutrophils (3, 4). Neutrophil elastase (NE) and proteinase 3 (PR3) are destructive serine proteases with a range of substrates causing impact on cell and tissue function through diverse mechanisms, from degradation of ingested pathogens to favoring cell motility through the extracellular matrix (2). Therefore, it is not surprising that protease activity is tempered by anti-protease molecules which are secreted to neutralize any excess of these enzymes. Anti-proteases are classified as both systemic (produced by hepatocytes and distributed through the circulating) and alarm (synthesized and secreted by local cells to the site of inflammation) (5, 6). Alarm anti-proteases such as SLPI (secretory leukocyte protease inhibitor) and Elafin are secreted predominantly by the mucosal epithelium and their levels are modulated during multiple pathological conditions (3, 4, 6). Recent investigations indicate that SLPI and Elafin are inducible in human alveolar macrophages and neutrophils (7).

Despite the release of anti-proteases as counter-regulatory mechanism for excessive inflammation, the inflammatory response is also coupled to the release of local anti-inflammatory and proresolving factors preventing future or excessive recruitment of neutrophils, tissue damage, and allowing the resolution of inflammation (8). Among these mediators, there are anti-inflammatory and proresolving lipids, such as lipoxins and resolvins (9), and proresolving proteins, such as annexin A1 (AnxA1) (10, 11).

AnxA1 is a glucocorticoid (GC)-regulated protein known as a mediator of several GC functions. The N-terminal region contains the main pharmacophore for the anti-inflammatory properties of AnxA1 (12); therefore, the intact protein of 37-kDa can stimulate multiple activities to help resolve acute inflammation. The regulation is quite unusual, with large amounts of the protein within innate immune cells. However, after cell activation, AnxA1 is externalized on the cell surface, the N-terminal region is exposed and can interact in a paracrine/autocrine fashion with its receptor named FPR2/ALX. However, within this microenvironment AnxA1 is vulnerable to be cleaved at the N-terminal region by proteases including NE and PR3, generating the 33-kDa isoform of poorly known properties (13, 14). Studies have shown that the 33-kDa isoform of AnxA1 may be associated with pro-inflammatory effects (15, 16). Congruently, cleavage-resistant AnxA1 exhibited greater anti-inflammatory effect compared to the parent protein, in different animal models of inflammation (17, 18). In addition, an AnxA1 peptide with mutation on a distinct cleavage site was potently active in promoting resolution, inducing neutrophil apoptosis and efferocytosis (19) and exerting protection in the complex settings of sepsis (20).

The calculation of resolution indices was introduced for the first time by Bannenberg et al. (21) and allows assessment of the proresolving properties of agents by the temporal regulation of leukocyte recruitment at inflammatory sites. These indices chart and take into account 1) magnitude ψ_{max} (maximal of neutrophil numbers that are present in the exudates) and T_{max} (time when ψ_{max} is maximal, i.e., time when neutrophil numbers reach maximum), 2) duration of the resolution interval (R_i) from T_{max}, i.e., the time that it takes for the number of neutrophils to reach half of ψ_{max} (T_{50}). This is an important proresolving parameter that quantifies how efficient a new agent is.

Herein, we investigated the effects of synthetic (Sivelestat, Eglin) and natural (Elafin, SLPI) specific neutrophil protease inhibitors on resolution of LPS-induced pleural inflammation and queried if and how these effects could be associated with preservation of

AnxA1 integrity. Collectively, our data show that strategies aiming at protecting and/or incrementing endogenous AnxA1 levels may be harnessed for the treatment of unresolved inflammation.

Materials and Methods

Animals

Male BALB/c mice (8-10 weeks) were housed under standard conditions and had free access to commercial chow and water. Mice were obtained from the Bioscience Unit of Instituto de Ciências Biológicas (Brazil). All described procedures had prior approval from the Animal Ethics Committee of Universidade Federal de Minas Gerais, Brazil (CETEA/UFMG, *Protocol number: 15/2011*).

Drugs, reagents and antibodies

Sivelestat (#S7198, Sigma-Aldrich, St. Louise, MO), Eglin c (#SP3133b, Cambridge Bioscience), Elafin (AnaSpec, #61641), and recombinant human SLPI (R&D systems #1274-PI-100) were dissolved in DMSO and diluted further in PBS. Endotoxin level in RhuSLPI was < 1.0 EU per 1 μg of the protein by the LAL method (as presented in R&D systems catalog #1274-PI-100). Rabbit anti-P-ERK1/2 (#4377), anti-caspase-3 (#9665), anti-Mcl-1 (#5453), anti GAPDH (#3683) and mouse anti-P-IκB-α (#9246) were purchased from Cell Signaling Technology (Beverly MA, USA). Anti-SLPI, anti-elastase, and secondary anti-rabbit and anti-mouse peroxidase conjugate antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, EUA). Anti-Elafin was from Bioss. Rabbit anti-AnxA1 was from Invitrogen (Carlsbad, CA, EUA). Anti β-actin and LPS (from *Escherichia coli* serotype O:111:B4) were from Sigma Chemicals (St. Louis MO, USA). BOC-1 (N-t-Boc-Met-leu-Phe) was from MP Biomedicals (California, USA). ZVAD-fmk was from Tocris (Ellisville, MO, USA). The peptides $AnxA1_{2-50}$ and CR (cleavage-resistant)-$AnxA1_{2-50}$ were purchased as described (19). Anti-AnxA1 antiserum (D3428) was a donation from National Institute for Biological Standards and Control (NIBSC) (Blanche Lane, Potters Bar Hertfordshire EN6 3QG, UK).

Assessment of leukocyte migration induced by LPS and monosodium urate crystals

Mice received an intrapleural (i.pl.) injection of LPS (250 ng/cavity) or PBS as previously described (22, 23). The cells present in the pleural cavity were harvested by washing the cavity with 2 ml of PBS at different time points after injection of LPS. The monosodium urate (MSU) crystals were prepared as described (24). Mice were placed under anesthesia (150:10 mg/kg ketamine:xylazine; i.p. Syntec, São Paulo, Brazil) and were injected with MSU crystals (100 µg) into the tibiofemoral joint. Knee wash were performed at 18 h after MSU injection. The total cell counts were performed in a Neubauer chamber using Turk's stain. Differential cell counts were performed on cyto-centrifuge preparations (Shandon III) stained with May-Grünwald-Giemsa using standard morphological criteria to identify cell types. The results are presented as the number of cells per cavity.

Treatment protocols

To evaluate the effect of protease inhibitors agents on LPS-induced pleurisy, mice were treated with specifics neutrophil elastase inhibitors Sivelestat (1, 5 and 25mg/kg, i.p.), Elafin peptide (10µg/mouse, i.p.), and a recombinant human secretory leukocyte protease inhibitor, SLPI (10µg/mouse, i.p.); an inhibitor of elastase and cathepsin G, Eglin c peptide (100µg/mouse, i.p.); 4 or 8 h after LPS challenge. We used also a recently described AnxA1 peptide CR (cleavage-resistant)-AnxA1$_{2-50}$ and its control peptide AnxA1$_{2-50}$ (19). To prevent the action of AnxA1 induced by Sivelestat, mice were treated with anti-AnxA1 antiserum (0.1 ml of hyperimmune serum diluted in 100µl of PBS/ mice, i.p.) and with BOC-1 (5.0 mg/kg, i.p.), a nonselective FPR antagonist that blocks the FPR and ALXR receptors (25). Non-immune goat serum was used as control (not showed). ZVAD-fmk (1mg/kg), a broad-spectrum-caspase inhibitor was given systemically (i.p.) 15 minutes before Sivelestat injection, as described (23). Drugs were dissolved in DMSO and diluted further in PBS. Control mice received only vehicle.

Evaluation of leukocyte apoptosis and efferocytosis

Apoptosis was assessed as previously reported (22, 23). Briefly, cells (5 x 10^4) were collected after LPS challenge, cyto-centrifuged, fixed and stained with May-Grünwald-Giemsa. Posteriorly, to determine the proportion of cells with distinctive apoptotic morphology, the cells were counted using oil immersion microscopy (x100 objective). At least 500 cells per glass slide were counted and results are expressed as the mean ± SEM of percentage of cells with apoptotic morphology. Of note: cells are considered apoptotic when presenting chromatin condensation, nuclear fragmentation and formation of apoptotic bodies out or inside macrophages. Assessment of neutrophil ($Ly6G^+/F4/80^-$) apoptosis was also performed by flow cytometry using FITC-labeled annexin-V and 7-Aminoactinomycin D (7-AAD). Efferocytosis was assessed by flow cytometry as previously showed (19), considering the frequency of macrophages containing PMNs ($F4/80^+/Ly6G^+$ cells). Antibodies used were F4/80 (PEcy7, eBioscience, San Diego, CA), Ly6G (BV421, BD Biosciences, San Jose, CA). Analysis of efferocytosis was also performed by preparing cytospin slides and determining the proportion of macrophages that ingested apoptotic bodies (500 cells per slides were counted).

Flow cytometry analysis for leukocyte populations

Cells present in the pleural cavity were harvested 24 hours after LPS injection (LPS 6h + SIV 18h). The leukocyte populations were analyzed by staining with fluorescent monoclonal antibodies against F4/80 (PE, eBioscience), GR1 (BV421, BD Biosciences) and CD11b (FITC, BD Biosciences). After being stained for surface markers, cells were permeabilized with permeabilization buffer (eBioscience) for 30 minutes. Stained cells were acquired in BD FACSCanto II cell analyzer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., USA). Macrophage populations were defined according to F4/80, GR1 and CD11b expression, as previously shown (26, 27).

In vitro experiments to evaluate neutrophil apoptosis

Neutrophils were isolated from human peripheral blood from healthy donors (Ethics Committee of the Universidade Federal de Minas Gerais, Brazil - Institutional Review Board Project #0319.0.203.000-11) by using histopaque gradient (Histopaque 11191 and 10771 – from Sigma Chemicals, St. Louis MO, USA) as described (28). Neutrophils (1 x 10^6 cell per well) were resuspended in RPMI media, seeded in 24 wells culture plates (BD Biosciences) and incubated at 37°C in a 5% CO_2 atmosphere. Cell viability was determined by trypan blue staining and the purity of preparations were over 95%. To evaluate the effect of Sivelestat on LPS-induced prosurvival/delayed apoptosis of neutrophils, isolated neutrophils were cultured in presence of LPS (100 ng/ml) and 1 h after were treated with different concentrations of Sivelestat (10, 30 and 100 µg/ml) for 2 or 5 h, or with Elafin (100 ng/ml), SLPI (100 ng/ml). In some experiments neutrophils were pre-treatment by 1 h with zVAD (100µM) before addiction of anti-proteases. Apoptosis was evaluated as described above. Experiments were performed in triplicates.

Western blot analysis

Whole cell extracts were quantified with the Bradford assay reagent from Bio-Rad (Bio-Rad, USA). After quantification, 50µg of whole protein were separated by electrophoresis on a denaturing polyacrylamide-SDS gel (10-15%) and electro-transferred to nitrocellulose membranes, as described (22). Membranes were blocked with PBS containing 5% (w/v) nonfat dry milk and 0.1% Tween-20 (v/v) overnight at 4ºC, washed with PBS-Tween-20 0.1% (v/v) and then incubated with specific primary antibodies (Elastase, SLPI, Elafin, cleaved caspase-3, AnxA1, or anti β-actin) using a dilution of 1:1000 in PBS-BSA 5% (w/v) and 0.1% Tween-20. After washing with PBS-Tween-20 0.1% (v/v), membranes were incubated with appropriated peroxidase-conjugated secondary antibody (1:3000). Immunoreactive bands were visualized by using ECL detection system, as described by the

manufacturer (GE Healthcare, Piscataway, NJ). The values of intact and cleaved AnxA1 were quantified by using a densitometric analysis software (ImageJ, Image Processing and Analysis in Java; NIH, Bethesda, MD). Changes in protein levels were estimated and results are expressed as cleaved AnxA1, cleaved caspase-3, MCl-1 or P-IκB-α (in arbitrary units - AU), normalized to the values of β-actin in the same sample.

Calculation of resolution indices

We quantified the resolution indices as described (21, 29). Murine pleural exudates were collected at 8, 24, 36 and 48 h after challenge with LPS. The treatment with Sivelestat (5mk/kg) was performed at the peak of inflammation, 8 h after the challenge. The number of PMN and mononuclear cells was determined by total and differential leukocyte counting. The resolution of acute inflammation were defined in quantitative terms by the following resolution indices: 1) magnitude (ψ_{max} and T_{max}), ψ_{max} (maximal PMN), T_{max} (time point when PMN numbers reach maximum); 2) duration (T_{50}), T_{50} (time point when PMN numbers reduce to 50% of maximum) and 3) resolution interval R_i (the interval between T_{max} and T_{50}, when 50% PMN are lost from the pleural cavity).

Elastase activity assay

The elastase activity was measured in cell extracts prepared in the absence of proteases inhibitors by using an in-house procedure that relies on the use of MeO-Suc-AA-Pro-Val-pNA (M4765-Sigma Aldrich, St. Louis, MO, USA) as substrate. Cells obtained from pleural cavity of mice were lysed on appropriated buffer (200 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, pH 8.0). The lysate was centrifuged at 12,000 rpm in a microcentrifuge for 15 minutes at 4°C, and 50µl of supernatant were incubated with 50µl of substrate MeO-Suc-AA-Pro-Val-pNA in a 96-well microplate, at 37°C for 2 h. A standard curve was performed with p-nitroaniline in accordance to the procedures supplied by the manufacturer (BioVision Inc.,

California, USA). The absorbance of samples was analyzed in a spectrophotometer (Spectra Max 190, Molecular Devices) at 405 nm. The results are presented as elastase activity absorbance.

Statistical analysis

Data were analyzed by one-way ANOVA, and differences between groups were assessed using the Student-Newman-Keuls post-test. A P value < 0.05 was considered significant. All results are presented as the mean ± SEM. Calculations were performed using the Prism 5.0 software (GraphPad software, San Diego, CA).

Results

Elastase expression/activity and AnxA1 cleavage is associated with the acute phase of inflammation and inversely correlated with the resolution phase

A well-established model of LPS-induced pleurisy was used (22, 23). In this model the intrapleural injection of LPS induced a time-dependent influx of leukocytes into the cavity (Fig. 1A). The number of neutrophils peaked at 8 and 24 h and decreased thereafter (Fig. 1A). There was a significant increase of mononuclear cells into the pleural cavity that coincided with the resolution phase of inflammation, as seen by decline of neutrophils number (48 and 72 h) (Fig. 1A). Next, we analyzed the kinetics of elastase, an important protease present in neutrophils. The expression and activity of elastase accompanied the kinetics of neutrophil recruitment in the pleural cavity (Figs. 1B and 1C, respectively).

Since elastase can modulate AnxA1 integrity (13, 14), we investigated the kinetics of accumulation for the active/intact (37-kDa) or inactive/cleaved (33-kDa) forms of AnxA1 (Fig. 1B). As previously shown (22) and in the Figure 1B, in PBS-challenged mice, intact AnxA1 protein (37-kDa) was the sole form detected. During the acute phase of LPS-induced neutrophil recruitment (8 and 24 h time-points), intact AnxA1 expression decreased markedly and the cleaved species were strongly detected. Intact AnxA1 expression was regained during the resolution phase of inflammation (48 and 72 h) (22). Interestingly, high elastase activity was associated with increased levels of the cleaved form of AnxA1 (33-kDa), whilst the decline of elastase activity was associated to higher levels of intact AnxA1 (37-kDa) (Fig. 1B, as compared to Fig. 1C).

Endogenous protease inhibitors are increased in the resolving phase of LPS-induced pleurisy and are able to promote resolution when given exogenously

Following these analyses, we queried what could be the profile of kinetic of the endogenous elastase inhibitors, SLPI (secretory leukocyte protease inhibitor). In PBS injected mice (Fig.

2A) SLPI was detected in basal setting, disappeared at the peak of neutrophil influx (8 h) and was strongly expressed at the time points of resolution of inflammation (48 and 72 h). Although it has been argued that there is no mouse ortholog to Elafin (2) we were able to detect a predicted band with molecular weight consistent with Elafin, whose kinetics was quite similar to the SLPI (data not shown). Importantly, augmented expression of endogenous serine protease inhibitors at the 24 h time-point coincided to the early decline of elastase activity (Fig. 1C), suggesting existence of a yin/yang balance between inhibitors and elastase activity.

To verify their therapeutic potential, SLPI (recombinant human) and Elafin (synthetic peptide) were injected at the peak of inflammation (8 h post-LPS) and the inflammatory status was determined at the 24 h time-point. Treatment of mice with both polypeptides decreased neutrophil numbers into the pleural cavity after 24 h of challenge (Fig. 2B). Interestingly, such effect was accompanied by appearance of apoptotic neutrophils in the pleural cavity (Fig. 2C), and associated to inhibition of neutrophils elastase and decrease of the pro-survival pathways NF-κB (evaluated by IκB-α phosphorylation) and Mcl-1 (Fig. 2D). We also evaluated the effectiveness of a short treatment protocol with SLPI and Elafin (4h LPS + 4h anti-protease) and observed that they were also effective in decreasing neutrophils counts in the pleural cavity (PBS: 0.09 ± 0.041; LPS: 8.6 ± 0.99; LPS+SLPI: 4.5 ± 0.61; LPS+Elafin: 5.3 ± 0.19; Number of neutrophils x 10^5/cavity; n=5 mice per group; $P < 0.001$, when comparing LPS *x* PBS; and $P < 0.01$, when comparing LPS *x* LPS+SLPI or LPS+Elafin). Importantly, both treatments were able to prevent AnxA1 degradation (Fig. 2D). The short treatment protocol was also carried out using Eglin c (a synthetic inhibitor of elastase and cathepsin G) and compared it to Elafin peptide. Administration of Elafin or Eglin c similarly decreased neutrophil numbers in the pleural cavity and such event was associated with appearance of apoptotic neutrophil in the pleural cavity, as shown by morphological criteria (Supplementary Figs. 1A-C). Importantly, there were increased numbers of macrophages

containing apoptotic bodies after anti-proteases treatment (indicated by arrowheads in representative images of the Supplementary Fig. 1C). Taken together, these results indicate a temporal expression of anti-elastase at times when resolution begin and thereafter, suggesting that endogenous protease inhibitors function as a control checkpoint to regulate the inflammatory response. In accordance with this possibility, exogenous therapy with elastase inhibitors was able to promote resolution of neutrophilic inflammation and this was associated to a pro-apoptotic program in neutrophils.

Sivelestat, a synthetic specific elastase inhibitor shortens resolution intervals, preserves intact AnxA1 in pleural exudates and promotes caspase-dependent neutrophil apoptosis

Next, we tested whether synthetic small molecule inhibitors could be effective in preventing AnxA1 cleavage, providing a translational potential to these data. The selective inhibitor Sivelestat (SIV) was injected 8 h after LPS (at the peak of inflammation). SIV produced a dose-dependent inhibition of neutrophil counts at 24 h after LPS (Fig. 3A). These effects on cell numbers were associated with a dose-dependent decrease in elastase function (Fig, 3B) together with prevention of AnxA1 cleavage, as monitored by Western blot and quantified by densitometry analysis (Fig. 3C). The dose of 5 mg/kg was selected to establish the resolution indices for SIV in this model. Mice received an injection of LPS and 8 h later a systemic injection of SIV and cells were collected at 8, 24, 36 and 48 h after LPS. The treatment of mice with SIV shortened Resolution intervals (R_i): $R_{i\ LPS}$ ~ 26 h; $R_{i\ LPS+SIV}$ ~ 18 h (Fig. 4A). Noteworthy, short treatment of LPS-inflamed mice with SIV was able to decrease elastase expression, increase intact levels of AnxA1 (37-kDa form) and partially inhibit AnxA1 degradation (33-kDa band) in pleural exudates at 2 and 4 h after injection of the compound (Fig. 4B). Moreover, the measurement of AnxA1 in the supernatant of pleural exudates show increased AnxA1 content after SIV treatment, which may be the result of the increased AnxA1 externalization (PBS: 1 ± 0.49; LPS: 1.9 ± 0.2; LPS+SIV: 3.8 ± 0.4; total AnxA1 in

arbitrary units normalized against PBS-Group, n=3 mice per group; $P < 0.05$, when comparing LPS *x* LPS+SIV). These results indicate that pharmacological treatment with SIV promoted resolution of LPS-induced neutrophilic inflammation probably by increasing AnxA1 expression and preventing its cleavage.

To assess if serine protease inhibitors shows similar effects by accelerating resolution in another experimental model of neutrophilic inflammation, we performed a set of experiments using a murine model of gout. This model is characterized by an intense recruitment of neutrophils after a single injection of MSU crystals into the knee (24, 30). Interestingly, the treatment of mice with serine protease inhibitors (Elafin, SLPI and Sivelestat) decreased the numbers of leukocytes into the knee cavity (Supplementary Fig. 2A) associated with increased numbers of apoptotic neutrophils (Supplementary Fig. 2B).

Next, we evaluated potential mechanism(s) underlying the proresolving effects of SIV. The neutrophil elastase inhibitor was injected 4 h after LPS, and neutrophil numbers determined 4 h later. As shown in Figure 5A, SIV (5mg/kg) efficiently decreased neutrophil numbers in the pleural cavity and this was associated with reduced elastase activity in the cavity (PBS: 0.08 ± 0.02 absorbance; LPS: 1.4 ± 0.1 absorbance; LPS + SIV: 0.7 ± 0.1 absorbance, n=4 mice per group; $P < 0.01$ when comparing LPS *x* LPS+SIV). Such an effect was prevented by using a pan-caspase inhibitor (zVAD-fmk), indicating a caspase-dependency in SIV-induced resolution (Fig. 5A). Treatment with zVAD alone did not alter the kinetics of neutrophil recruitment (Fig. 5A). More importantly, SIV induced dose-dependent apoptosis of neutrophils in the pleural cavity, as quantified by morphological (Fig. 5B) and biochemical criteria, including increase of caspase-3 cleavage, decrease of Mcl-1 and NF-κB (evaluated by P-IκB-α), (Fig. 5C and 5D), and flow cytometry (Fig. 5E).

Neutrophils are exposed to inflammatory mediators at sites of inflammation that may extend their life span by delaying apoptosis (28). As SIV induced apoptosis *in vivo,* in a milieu exposure to pro-survival factors, we investigated the ability of SIV to counteract the

pro-survival effects of LPS. Treatment of human neutrophils with SIV dose-dependently increased levels of intact AnxA1 in neutrophils (Supplementary Figure 3A) and induced neutrophil apoptosis, as evaluated by increased percentage of apoptotic neutrophils (Supplementary Fig. 3B and 3C - representative Figures) and caspase-3 cleavage (Supplementary Fig. 3A), when compare LPS-treated cells with LPS+SIV. Noteworthy, the treatment of human neutrophils with the pro-survival LPS, decrease the spontaneous apoptosis of cultured neutrophil (Supplementary Figure 3B and 3C). In addition, Elafin and SLPI were also able to override the survival-inducing effects of LPS and promoted neutrophil apoptosis *in vitro* (Supplementary Fig. 4A). Apoptosis was abolished by pretreatment with zVAD. Protease inhibitors inhibited total elastase activity in presence or absence of zVAD (Supplementary Fig. 4B). Taken together these findings indicate that elastase inhibitors can effectively induce or accelerate a pro-apoptotic program in neutrophils leading to resolution of inflammation.

Treatment of inflamed mice with Sivelestat skews macrophage towards M2 and Mres phenotype and enhances efferocytosis of apoptotic cells

Next we evaluated the leukocyte population of LPS-challenged mice after treatment with Sivelestat, based on a recent description of three macrophage populations: M1 ($F4/80^{low}$ $Gr1^{+}$ $Cd11b^{med}$), M2 ($F4/80^{high}$ $Gr1^{-}$ $Cd11b^{high}$) and resolution-promoting macrophages Mres ($F4/80^{med}$ $Cd11b^{low}$) (26, 27, 31). The gating strategy was performed as previously shown (26). LPS-injection increased the number of M1 macrophages as previously shown (31), which was decreased with SIV treatment (data not shown). M2 macrophages were detected on PBS-injected mice and at 24 h, but significantly increased after SIV treatment (Fig. 6A). Interestingly, the number of Mres was only increased after SIV treatment (Fig. 6B). In keep with the pro-efferocytic ability of these macrophages, efferocytosis of apoptotic neutrophils was also increased as evaluated by flow cytometry (Fig. 6C), and by counting the percentage

of macrophages contained apoptotic bodies from cytospin preparations (data not shown). These results indicate that the resolution induced by SIV is associated with an accumulation of M2 and Mres macrophages and clearance of apoptotic cells into the pleural cavity.

Sivelestat-induced resolution of inflammation is AnxA1 dependent

Since AnxA1-derived peptides engineered to resist serine protease cleavage promote resolution of inflammation more potently than wild type peptides, next we compared the effects of SIV with those of a cleavage resistant peptide (CR)-AnxA1$_{2\text{-}50}$ (19). Figure 7A shows that at 5 mg/kg SIV decreased neutrophil numbers into the pleural cavity to the same extent of the natural AnxA1 peptide (150μM/mouse). However, CR-AnxA1$_{2\text{-}50}$ was more effective than either treatment. Noteworthy, all treatments decreased Mcl-1 and NF-κB activation, two important survival molecules in neutrophils (32) (Fig. 7B).

Finally, these partially similar pharmacological effects of SIV and AnxA1 peptide and the modulation exerted by elastase on AnxA1 brought us to test whether endogenous AnxA1 could be involved in pro-resolving actions of SIV. Mice were treated with a neutralizing anti-AnxA1 antibody given alongside a prophylactic protocol, ahead of SIV administration. Neutralization of AnxA1 with a blocking antibody abolished the modulation exerted by SIV on neutrophil accumulation (Fig. 7C), and prevented SIV-induced decrease on Mcl-1 accumulation and IκB-α phosphorylation (Fig. 7D). Moreover, we investigated the effect of SIV following inhibition of AnxA1 receptor by using BOC-1 (a nonselective AnxA1 receptor antagonist), and found that under this situation SIV lost its effectiveness on the promotion of inflammation resolution (Fig. 7E). Taken together, these results clearly suggest a direct functional correlation between elastase inhibitors and the dynamic of AnxA1 accumulation, suggesting an engagement of the endogenous proresolving AnxA1 system in the resolution of inflammation promoted by anti-proteases.

Discussion

Proteases regulate a wide variety of essential physiological functions, including protein catabolism, cell growth and migration, blood coagulation, inflammation and modulation of pharmacologically active peptides. Thus, the finely tuned natural equilibrium between proteases and their inhibitors is essential for the maintenance of homeostasis. Hence an imbalance of the function of proteolytic enzymes is a common feature of inflammatory diseases (33). Recent studies have shown that therapeutic inhibition of proteases, including neutrophil-derived elastase, may be a promising therapeutic strategy in view of the powerful anti-inflammatory effects of these inhibitors in various pre-clinical models of diseases (2).

We have reported the importance of AnxA1 in driving the resolution of the acute inflammatory response (12, 32). Importantly, we detected an active process of AnxA1 cleavage to its 33-kDa breakdown product during the peak of acute pleurisy (22). The impact of this phenomenon appears not to be irrelevant, since recent studies have indicated that modulation of AnxA1 cleavage may be a new strategy to control inflammatory diseases, as seen with AnxA1 cleavage resistant mutants and shorter modified peptides (17-19). However, the role of anti-proteases, more specifically, elastase inhibitors, in protecting AnxA1 cleavage *in vivo* and its association to resolution of acute inflammation has not been established. In this follow up study, we investigated the role of synthetic (Sivelestat, Eglin c) and natural (Elafin, SLPI) elastase inhibitors on the resolution of LPS-induced neutrophilic inflammation. Here, we applied a dynamic model of pleural inflammation and reported that pharmacological treatment with both natural and synthetic anti-proteases promoted resolution of inflammation. Such effect was also seen in a model of gout. Mechanistically, treatment with anti-proteases induced AnxA1 expression and caspase-dependent neutrophil apoptosis associated to NF-κB inhibition and Mcl-1 decrease. Moreover, treatment of inflamed mice with Sivelestat (SIV) promotes reprogramming of macrophages to the phenotypes that are more prone to resolution and efferocytosis of apoptotic neutrophils. Finally, the effect of SIV was AnxA1 dependent,

since it was abolished by inhibiting AnxA1 with a neutralizing antibody and by blocking its receptor, suggesting that endogenous AnxA1 is involved in the proresolving actions of anti-proteases.

The regulation of the activity of potentially harmful proteases secreted by leukocytes during inflammation is important for the prevention of excessive tissue injury (34). Secretory leukocyte protease inhibitor (SLPI) is a serine proteinase inhibitor constitutively expressed in mucosal tissues and immune cells, including monocytes, macrophages, and neutrophils (35, 36) that exerts pleiotropic activities in different biological systems (7). For example, SLPI promotes cutaneous wound healing, cell proliferation of epithelial cells, prevents HIV infection, exhibits antimicrobial and antifungal functions, inhibits NF-κB activation, and modulates macrophage functions (6, 37). The protective effect of SLPI as an anti-inflammatory mediator has been documented in inflammatory lung diseases, including chronic obstructive pulmonary disease, cystic fibrosis (38) and allergic asthma (37).

Importantly, SLPI deficient mice have exacerbated susceptibly to endotoxin-induced shock (39). Another endogenous anti-protease, known as skin-derived antileukoprotease (SKALP) or Elafin, has similar anti-inflammatory actions (40). Elafin is a secreted protein expressed in epithelial cells such as skin and lung epithelium, but also by immune cells including neutrophils (41) and macrophages (42). Elafin inhibits the activation of pro-inflammatory transcription factors AP-1 (activator protein 1) and NF-κB and like SLPI, possesses antimicrobial and fungicidal properties (40). The anti-inflammatory effects of Elafin have been established in a number of studies and animal models, including lung inflammatory disease induced by LPS, chronic obstructive pulmonary disease, cardiac dysfunction, and intestinal diseases (3, 6). In the current study, both SLPI and Elafin potently accelerated resolution with significant reduction of neutrophils numbers. The observation that the kinetics of anti-protease expression paralleled that of macrophages, suggest that – in these settings – this cell type is their most likely source. The physiological function of anti-protease

was complemented by the efficacy of exogenous administration of these protease inhibitors with evident positive impact on neutrophil apoptosis and macrophage efferocytosis. These results identify specific cellular processes as major event/target of anti-protease physio-pharmacology. Akin with these findings is the study that indicated higher SLPI production from murine macrophages during the clearance of apoptotic cells (36). A more recent report showed that SLPI is a pivotal mediator of anti-inflammatory response in acetaminophen-induced acute liver failure by modulating the monocyte/macrophage function, and this included a reduced production of pro-inflammatory cytokines and increased phagocytosis of necrotic debris (43). Therefore, our results do show the relevance and effects of anti-proteases in the context of inflammation resolution and add to the literature by suggesting that it may indeed be useful to development of protease inhibitors to control over-exuberant inflammatory reactions.

It is noteworthy that SIV, a synthetic specific neutrophil elastase inhibitor, is clinically used as an anti-inflammatory agent for acute lung injury and acute respiratory distress syndrome (2). In preclinical models, SIV reduces markers of tissue injury and systemic inflammation including ischemia reperfusion injury (44), sepsis (45) and acute lung injury (46, 47). In our set of experiments, treatment of animals with SIV dose-dependently reduced neutrophil accumulation into the pleural cavity, an effect associated to reduced resolution indices and resolution intervals (R_i). Importantly, and for the first time, we showed that SIV induced caspase-dependent neutrophil apoptosis and AnxA1 expression in neutrophils, highlighting the pivotal proresolving protein AnxA1 as an important player in the mechanism of action of this drug. It is likely that engagement of AnxA1 may be a common feature of known anti-inflammatory drugs and data with glucocorticoids (48) and, more recently, chromones (49) appear to corroborate the existence of this shared protective pathway.

Neutrophil lifespan is increased by anti-apoptotic factors, such as Mcl-1 (myeloid cell leukemia-1), which in general have their expression inversely correlated with the degree of

512 neutrophil apoptosis (50). In our work we showed that protease inhibitors were able to induce
513 resolution of inflammation and this is associated with decreased levels of Mcl-1 in pleural
514 exudates. In fact, it is already demonstrated that Mcl-1 down-regulation contributes to anti-
515 inflammatory and pro-resolution effects leading to resolution of inflammation (23, 26, 28, 51,
516 52). Altogether, these data reinforce the idea that intervention on neutrophil survival could be
517 a potential pharmacological strategy to control inflammatory diseases.

518 An important determinant of inflammation resolution is the efficient removal of
519 apoptotic neutrophils by macrophages (53). Macrophages are usually classified as either
520 classically (M1) or alternatively activated (M2). Under a pro-inflammatory environment
521 macrophages usually have pro-inflammatory phenotype (M1) that has little efferocytic
522 capacity and increased capacity to engulf (phagocytose) foreign organisms. Inflammatory
523 macrophages are skewed to the M2 phenotype in a resolution milieu and they produce IL-10
524 and TGF-β, which have anti-inflammatory actions (54, 55). M2 macrophages are prone to
525 efferocytose neutrophils (56) and uptake of apoptotic cells during the resolution of
526 inflammation leads to their conversion to a proresolving CD11b low phenotype (27). It has
527 been shown that murine macrophages secrete an increased amount of SLPI when
528 encountering apoptotic cells, which may help to attenuate potential inflammation during
529 clearance of these cells (36). Moreover, it was shown that Elafin prevented CD14 cleavage by
530 elastase and restored apoptotic cell recognition by macrophages (57). Importantly, our *in vivo*
531 data show that, in addition to inducing apoptosis of neutrophils, anti-proteases were able to
532 decrease macrophages with a pro-inflammatory phenotype and to increase number of
533 proresolving macrophages.

534 Intact AnxA1 (37-kDa) is the biologically active form of the protein endowed with
535 anti-inflammatory properties but, at sites of inflammation, it can be cleaved to its inactive
536 form of 33-kDa by neutrophil-derived proteases (13, 14). Thus, modulation of endogenous
537 AnxA1 pool might be an important mechanism to resolve inflammatory responses. This can

538 be even of more acute importance because the 33-kDa form of AnxA1 displays pro-
539 inflammatory effects by promoting ERK1/2 activation and neutrophil transendothelial
540 migration (16). The first concept that the 33-kDa AnxA1 form might have pro-inflammatory
541 properties has been suggested by findings in fluid samples from cystic fibrosis patients (15).
542 Recently, we demonstrated in a model of acute inflammation, greater accumulation of AnxA1
543 cleaved (33-kDa) during the peak of neutrophilic inflammation (22). Akin to these findings,
544 and as reported above, cleavage-resistant AnxA1 displays a greater anti-inflammatory effect
545 over time compared to the parent protein (17), and accelerated resolution in an animal model
546 of inflammatory arthritis (18).

547 Finally the present study tested whether a peptide derived from the AnxA1 N-terminal
548 region, hence the active portion of the protein with respect to proresolving actions, mutated in
549 its cleavage site, termed cleavage resistant (CR)-AnxA1$_{2\text{-}50}$. This peptide is resistant to the
550 action of elastase and PR3, and is more effective in inflammatory peritonitis and acute
551 myocardial infarct (19). In analogy to SIV – which increased levels of intact AnxA1 in
552 neutrophils - CR-AnxA1$_{2\text{-}50}$ was highly effective in reducing pleurisy. This peptide is also
553 very potent with an active dose in the low nmol range (19, 20). It has been shown that the
554 inhibition of AnxA1 function by protein neutralization or the blockade of AnxA1 receptor are
555 useful strategies to revert anti-inflammatory and proresolving effects mediated by AnxA1 in
556 different inflammatory conditions (22, 25, 49, 58). Here, we showed that the blockade of
557 AnxA1 was able to reverse the resolution induced by SIV, suggesting that the proresolving
558 effects of SIV are dependent on AnxA1. Collectively, these findings suggest that AnxA1 can
559 be a mediator of the SIV proresolving actions and modulation of endogenous pool of AnxA1
560 might be an important approach to impact on on-going or recurrent inflammatory status by
561 driving endogenous pro-resolution pathways and processes.

562

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Authorship

Author contributions: L.P.S., M.M.T. and J.P.V., designed research, analyzed data and wrote the paper. J.P.V., L.P.T. and M.A.S., performed experiments and analyzed data. M.A.S., G.L.N.L., K.M.L., T.R.C., and F.S.C., performed Western blot analysis together J.P.V. A.L.C.R. M.A.A.F., carried out some *in vivo* experiments. F.F.C.N. helped with apoptosis experiments. I.G. carried out *in vitro* neutrophil analysis together J.P.V and performed murine model of gout. V.P. provided expertise; and M.P. provided essential tools, guidance in the study and contributed to manuscript writing.

Conflict of interest Disclosures

The authors declare no competing financial interest.

References

1. Medzhitov, R. 2010. Inflammation 2010: new adventures of an old flame. *Cell* 140: 771-776.
2. Henriksen, P. A. 2014. The potential of neutrophil elastase inhibitors as anti-inflammatory therapies. *Current opinion in hematology* 21: 23-28.
3. Greene, C. M., and N. G. McElvaney. 2009. Proteases and antiproteases in chronic neutrophilic lung disease - relevance to drug discovery. *British journal of pharmacology* 158: 1048-1058.
4. Mancek-Keber, M. 2014. Inflammation-Mediating Proteases: Structure, Function in (Patho)Physiology and Inhibition. *Protein and peptide letters*.
5. Sallenave, J. M. 2000. The role of secretory leukocyte proteinase inhibitor and elafin (elastase-specific inhibitor/skin-derived antileukoprotease) as alarm antiproteinases in inflammatory lung disease. *Respiratory research* 1: 87-92.
6. Williams, S. E., T. I. Brown, A. Roghanian, and J. M. Sallenave. 2006. SLPI and elafin: one glove, many fingers. *Clin Sci (Lond)* 110: 21-35.
7. Weldon, S., N. McGarry, C. C. Taggart, and N. G. McElvaney. 2007. The role of secretory leucoprotease inhibitor in the resolution of inflammatory responses. *Biochemical Society transactions* 35: 273-276.
8. Norling, L. V., and C. N. Serhan. 2010. Profiling in resolving inflammatory exudates identifies novel anti-inflammatory and pro-resolving mediators and signals for termination. *Journal of internal medicine* 268: 15-24.
9. Serhan, C. N. 2014. Pro-resolving lipid mediators are leads for resolution physiology. *Nature* 510: 92-101.
10. Dalli, J., and C. N. Serhan. 2012. Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators. *Blood* 120: e60-72.
11. Sousa, L. P., A. L. Alessandri, V. Pinho, and M. M. Teixeira. 2013. Pharmacological strategies to resolve acute inflammation. *Current opinion in pharmacology* 13: 625-631.
12. Perretti, M., and F. D'Acquisto. 2009. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nature reviews. Immunology* 9: 62-70.
13. Vong, L., F. D'Acquisto, M. Pederzoli-Ribeil, L. Lavagno, R. J. Flower, V. Witko-Sarsat, and M. Perretti. 2007. Annexin 1 cleavage in activated neutrophils: a pivotal role for proteinase 3. *The Journal of biological chemistry* 282: 29998-30004.
14. Oliani, S. M., M. J. Paul-Clark, H. C. Christian, R. J. Flower, and M. Perretti. 2001. Neutrophil interaction with inflamed postcapillary venule endothelium alters annexin 1 expression. *The American journal of pathology* 158: 603-615.
15. Tsao, F. H., K. C. Meyer, X. Chen, N. S. Rosenthal, and J. Hu. 1998. Degradation of annexin I in bronchoalveolar lavage fluid from patients with cystic fibrosis. *American journal of respiratory cell and molecular biology* 18: 120-128.
16. Williams, S. L., I. R. Milne, C. J. Bagley, J. R. Gamble, M. A. Vadas, S. M. Pitson, and Y. Khew-Goodall. 2010. A proinflammatory role for proteolytically cleaved annexin A1 in neutrophil transendothelial migration. *Journal of immunology* 185: 3057-3063.
17. Pederzoli-Ribeil, M., F. Maione, D. Cooper, A. Al-Kashi, J. Dalli, M. Perretti, and F. D'Acquisto. 2010. Design and characterization of a cleavage-resistant Annexin A1 mutant to control inflammation in the microvasculature. *Blood* 116: 4288-4296.
18. Patel, H. B., K. N. Kornerup, A. L. Sampaio, F. D'Acquisto, M. P. Seed, A. P. Girol, M. Gray, C. Pitzalis, S. M. Oliani, and M. Perretti. 2012. The impact of endogenous annexin A1 on glucocorticoid control of inflammatory arthritis. *Annals of the rheumatic diseases* 71: 1872-1880.

19. Dalli, J., A. P. Consalvo, V. Ray, C. Di Filippo, M. D'Amico, N. Mehta, and M. Perretti. 2013. Proresolving and tissue-protective actions of annexin A1-based cleavage-resistant peptides are mediated by formyl peptide receptor 2/lipoxin A4 receptor. *Journal of immunology* 190: 6478-6487.
20. Gobbetti, T., S. M. Coldewey, J. Chen, S. McArthur, P. le Faouder, N. Cenac, R. J. Flower, C. Thiemermann, and M. Perretti. 2014. Nonredundant protective properties of FPR2/ALX in polymicrobial murine sepsis. *Proceedings of the National Academy of Sciences of the United States of America* 111: 18685-18690.
21. Bannenberg, G. L., N. Chiang, A. Ariel, M. Arita, E. Tjonahen, K. H. Gotlinger, S. Hong, and C. N. Serhan. 2005. Molecular circuits of resolution: formation and actions of resolvins and protectins. *Journal of immunology* 174: 4345-4355.
22. Vago, J. P., C. R. Nogueira, L. P. Tavares, F. M. Soriani, F. Lopes, R. C. Russo, V. Pinho, M. M. Teixeira, and L. P. Sousa. 2012. Annexin A1 modulates natural and glucocorticoid-induced resolution of inflammation by enhancing neutrophil apoptosis. *Journal of leukocyte biology* 92: 249-258.
23. Sousa, L. P., F. Lopes, D. M. Silva, L. P. Tavares, A. T. Vieira, B. M. Rezende, A. F. Carmo, R. C. Russo, C. C. Garcia, C. A. Bonjardim, A. L. Alessandri, A. G. Rossi, V. Pinho, and M. M. Teixeira. 2010. PDE4 inhibition drives resolution of neutrophilic inflammation by inducing apoptosis in a PKA-PI3K/Akt-dependent and NF-kappaB-independent manner. *Journal of leukocyte biology* 87: 895-904.
24. Amaral, F. A., V. V. Costa, L. D. Tavares, D. Sachs, F. M. Coelho, C. T. Fagundes, F. M. Soriani, T. N. Silveira, L. D. Cunha, D. S. Zamboni, V. Quesniaux, R. S. Peres, T. M. Cunha, F. Q. Cunha, B. Ryffel, D. G. Souza, and M. M. Teixeira. 2012. NLRP3 inflammasome-mediated neutrophil recruitment and hypernociception depend on leukotriene B(4) in a murine model of gout. *Arthritis and rheumatism* 64: 474-484.
25. Souza, D. G., C. T. Fagundes, F. A. Amaral, D. Cisalpino, L. P. Sousa, A. T. Vieira, V. Pinho, J. R. Nicoli, L. Q. Vieira, I. M. Fierro, and M. M. Teixeira. 2007. The required role of endogenously produced lipoxin A4 and annexin-1 for the production of IL-10 and inflammatory hyporesponsiveness in mice. *J Immunol* 179: 8533-8543.
26. Vago, J. P., L. P. Tavares, C. C. Garcia, K. M. Lima, L. O. Perucci, E. L. Vieira, C. R. Nogueira, F. M. Soriani, J. O. Martins, P. M. Silva, K. B. Gomes, V. Pinho, S. Bruscoli, C. Riccardi, E. Beaulieu, E. F. Morand, M. M. Teixeira, and L. P. Sousa. 2015. The role and effects of glucocorticoid-induced leucine zipper in the context of inflammation resolution. *Journal of immunology* 194: 4940-4950.
27. Schif-Zuck, S., N. Gross, S. Assi, R. Rostoker, C. N. Serhan, and A. Ariel. 2011. Saturated-efferocytosis generates pro-resolving CD11b low macrophages: modulation by resolvins and glucocorticoids. *European journal of immunology* 41: 366-379.
28. Lucas, C. D., K. C. Allen, D. A. Dorward, L. J. Hoodless, L. A. Melrose, J. A. Marwick, C. S. Tucker, C. Haslett, R. Duffin, and A. G. Rossi. 2013. Flavones induce neutrophil apoptosis by down-regulation of Mcl-1 via a proteasomal-dependent pathway. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 27: 1084-1094.
29. Chiang, N., M. Shinohara, J. Dalli, V. Mirakaj, M. Kibi, A. M. Choi, and C. N. Serhan. 2013. Inhaled carbon monoxide accelerates resolution of inflammation via unique proresolving mediator-heme oxygenase-1 circuits. *Journal of immunology* 190: 6378-6388.
30. Martinon, F., and L. H. Glimcher. 2006. Gout: new insights into an old disease. *The Journal of clinical investigation* 116: 2073-2075.
31. Ariel, A., and C. N. Serhan. 2012. New Lives Given by Cell Death: Macrophage Differentiation Following Their Encounter with Apoptotic Leukocytes during the Resolution of Inflammation. *Frontiers in immunology* 3: 4.

685 32. Perez, D. A., J. P. Vago, R. M. Athayde, A. C. Reis, M. M. Teixeira, L. P. Sousa, and
686 V. Pinho. 2014. Switching off key signaling survival molecules to switch on the
687 resolution of inflammation. *Mediators of inflammation* 2014: 829851.
688 33. Turk, B. 2006. Targeting proteases: successes, failures and future prospects. *Nature*
689 *reviews. Drug discovery* 5: 785-799.
690 34. Sallenave, J. M., J. Shulmann, J. Crossley, M. Jordana, and J. Gauldie. 1994.
691 Regulation of secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific
692 inhibitor (ESI/elafin) in human airway epithelial cells by cytokines and neutrophilic
693 enzymes. *American journal of respiratory cell and molecular biology* 11: 733-741.
694 35. Eisenberg, S. P., K. K. Hale, P. Heimdal, and R. C. Thompson. 1990. Location of the
695 protease-inhibitory region of secretory leukocyte protease inhibitor. *The Journal of*
696 *biological chemistry* 265: 7976-7981.
697 36. Odaka, C., T. Mizuochi, J. Yang, and A. Ding. 2003. Murine macrophages produce
698 secretory leukocyte protease inhibitor during clearance of apoptotic cells: implications
699 for resolution of the inflammatory response. *Journal of immunology* 171: 1507-1514.
700 37. Marino, R., T. Thuraisingam, P. Camateros, C. Kanagaratham, Y. Z. Xu, J. Henri, J.
701 Yang, G. He, A. Ding, and D. Radzioch. 2011. Secretory leukocyte protease inhibitor
702 plays an important role in the regulation of allergic asthma in mice. *Journal of*
703 *immunology* 186: 4433-4442.
704 38. Reeves, E. P., N. Banville, D. M. Ryan, N. O'Reilly, D. A. Bergin, K. Pohl, K.
705 Molloy, O. J. McElvaney, K. Alsaleh, A. Aljorfi, O. Kandalaft, E. O'Flynn, P.
706 Geraghty, S. J. O'Neill, and N. G. McElvaney. 2013. Intracellular secretory
707 leukoprotease inhibitor modulates inositol 1,4,5-triphosphate generation and exerts an
708 anti-inflammatory effect on neutrophils of individuals with cystic fibrosis and chronic
709 obstructive pulmonary disease. *BioMed research international* 2013: 560141.
710 39. Nakamura, A., Y. Mori, K. Hagiwara, T. Suzuki, T. Sakakibara, T. Kikuchi, T.
711 Igarashi, M. Ebina, T. Abe, J. Miyazaki, T. Takai, and T. Nukiwa. 2003. Increased
712 susceptibility to LPS-induced endotoxin shock in secretory leukoprotease inhibitor
713 (SLPI)-deficient mice. *The Journal of experimental medicine* 197: 669-674.
714 40. Shaw, L., and O. Wiedow. 2011. Therapeutic potential of human elafin. *Biochemical*
715 *Society transactions* 39: 1450-1454.
716 41. Sallenave, J. M., M. Si Tahar, G. Cox, M. Chignard, and J. Gauldie. 1997. Secretory
717 leukocyte proteinase inhibitor is a major leukocyte elastase inhibitor in human
718 neutrophils. *Journal of leukocyte biology* 61: 695-702.
719 42. Mihaila, A., and G. M. Tremblay. 2001. Human alveolar macrophages express elafin
720 and secretory leukocyte protease inhibitor. *Zeitschrift fur Naturforschung. C, Journal*
721 *of biosciences* 56: 291-297.
722 43. Antoniades, C. G., W. Khamri, R. D. Abeles, L. S. Taams, E. Triantafyllou, L. A.
723 Possamai, C. Bernsmeier, R. R. Mitry, A. O'Brien, D. Gilroy, R. Goldin, M.
724 Heneghan, N. Heaton, W. Jassem, W. Bernal, D. Vergani, Y. Ma, A. Quaglia, J.
725 Wendon, and M. Thursz. 2014. Secretory leukocyte protease inhibitor: a pivotal
726 mediator of anti-inflammatory responses in acetaminophen-induced acute liver failure.
727 *Hepatology* 59: 1564-1576.
728 44. Kambe, M., R. Bessho, M. Fujii, M. Ochi, and K. Shimizu. 2009. Sivelestat reduces
729 myocardial ischemia and reperfusion injury in rat hearts even when administered after
730 onset of myocardial ischemia. *Interactive cardiovascular and thoracic surgery* 8: 629-
731 634.
732 45. Hayakawa, M., K. Katabami, T. Wada, M. Sugano, H. Hoshino, A. Sawamura, and S.
733 Gando. 2010. Sivelestat (selective neutrophil elastase inhibitor) improves the mortality
734 rate of sepsis associated with both acute respiratory distress syndrome and
735 disseminated intravascular coagulation patients. *Shock* 33: 14-18.

46. Iba, T., A. Kidokoro, M. Fukunaga, K. Takuhiro, S. Yoshikawa, and K. Sugimotoa. 2006. Pretreatment of sivelestat sodium hydrate improves the lung microcirculation and alveolar damage in lipopolysaccharide-induced acute lung inflammation in hamsters. *Shock* 26: 95-98.
47. Yasui, S., A. Nagai, K. Aoshiba, Y. Ozawa, Y. Kakuta, and K. Konno. 1995. A specific neutrophil elastase inhibitor (ONO-5046.Na) attenuates LPS-induced acute lung inflammation in the hamster. *The European respiratory journal* 8: 1293-1299.
48. Rhen, T., and J. A. Cidlowski. 2005. Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *The New England journal of medicine* 353: 1711-1723.
49. Yazid, S., G. Leoni, S. J. Getting, D. Cooper, E. Solito, M. Perretti, and R. J. Flower. 2010. Antiallergic cromones inhibit neutrophil recruitment onto vascular endothelium via annexin-A1 mobilization. *Arteriosclerosis, thrombosis, and vascular biology* 30: 1718-1724.
50. Milot, E., and J. G. Filep. 2011. Regulation of neutrophil survival/apoptosis by Mcl-1. *TheScientificWorldJournal* 11: 1948-1962.
51. Rossi, A. G., D. A. Sawatzky, A. Walker, C. Ward, T. A. Sheldrake, N. A. Riley, A. Caldicott, M. Martinez-Losa, T. R. Walker, R. Duffin, M. Gray, E. Crescenzi, M. C. Martin, H. J. Brady, J. S. Savill, I. Dransfield, and C. Haslett. 2006. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. *Nature medicine* 12: 1056-1064.
52. El Kebir, D., L. Jozsef, W. Pan, L. Wang, N. A. Petasis, C. N. Serhan, and J. G. Filep. 2009. 15-epi-lipoxin A4 inhibits myeloperoxidase signaling and enhances resolution of acute lung injury. *American journal of respiratory and critical care medicine* 180: 311-319.
53. Serhan, C. N., S. D. Brain, C. D. Buckley, D. W. Gilroy, C. Haslett, L. A. O'Neill, M. Perretti, A. G. Rossi, and J. L. Wallace. 2007. Resolution of inflammation: state of the art, definitions and terms. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 21: 325-332.
54. Korns, D., S. C. Frasch, R. Fernandez-Boyanapalli, P. M. Henson, and D. L. Bratton. 2011. Modulation of macrophage efferocytosis in inflammation. *Frontiers in immunology* 2: 57.
55. Alessandri, A. L., L. P. Sousa, C. D. Lucas, A. G. Rossi, V. Pinho, and M. M. Teixeira. 2013. Resolution of inflammation: mechanisms and opportunity for drug development. *Pharmacology & therapeutics* 139: 189-212.
56. Michlewska, S., I. Dransfield, I. L. Megson, and A. G. Rossi. 2009. Macrophage phagocytosis of apoptotic neutrophils is critically regulated by the opposing actions of pro-inflammatory and anti-inflammatory agents: key role for TNF-alpha. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 23: 844-854.
57. Henriksen, P. A., A. Devitt, Y. Kotelevtsev, and J. M. Sallenave. 2004. Gene delivery of the elastase inhibitor elafin protects macrophages from neutrophil elastase-mediated impairment of apoptotic cell recognition. *FEBS letters* 574: 80-84.
58. Dalli, J., L. V. Norling, D. Renshaw, D. Cooper, K. Y. Leung, and M. Perretti. 2008. Annexin 1 mediates the rapid anti-inflammatory effects of neutrophil-derived microparticles. *Blood* 112: 2512-2519.

Figure Legends

Figure 1. Time-course of elastase expression and activity during LPS-induced pleurisy. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and the cells present in the pleural cavity were harvested at several time points and processed for total and differential leukocyte counts of cytospin preparations for light microscopy (A), western blot analysis for elastase and AnxA1 (B), or extracts were prepared to measurement of elastase activity (C). Results are expressed as the number of leukocytes per cavity and are shown as the mean ± SEM of at least five mice in each group. *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$ when compared with PBS-injected mice and #, $P < 0.05$; or ###, $P < 0.001$ when compared to 8 and 24 h after LPS-injected mice. For loading control, membranes were re-probed with anti-β-actin. Blots are representative of three independent experiments using pooled cells from at least five animals.

Figure 2. Kinetics of endogenous anti-protease expression and effect of exogenous treatment with SLPI and Elafin on LPS-induced pleurisy. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and the cells present in the pleural cavity were harvested at several time points and the pool of lysed cells processed for western blot analysis for SLPI detection (A). Mice were injected with LPS (250 ηg/cavity, i.pl.) and 8 h later received an injection of human recombinant SLPI (10μg/ml, i.p.) or Elafin peptide (10μg/ml, i.p.). Cells present in the pleural cavity were harvested 24 h after LPS challenge and processed for count of neutrophil numbers (B), cells with distinctive apoptotic morphology (C), and western blot analysis for neutrophils elastase, P-IκB-α, AnxA1 and Mcl-1 detection (D). Results are expressed as the number of neutrophils per cavity and are shown as the mean ± SEM of at least five mice in each group. **, $P < 0.01$ or ***, $P < 0.001$ when compared with PBS-injected mice; and ##, $P < 0.01$ or ###, $P < 0.001$ when compared to 24 h LPS-injected mice.

809 For loading control, membranes were re-probed with anti-β-actin. Blots are representative of
810 three independent experiments using pooled cells from at least five animals.
811
812 **Figure 3. Effect of different doses of Sivelestat on LPS-induced pleurisy.** Mice were
813 injected with LPS (250 ng/cavity, i.pl.) or PBS and 8 h after received an injection of
814 Sivelestat (SIV-1, 5 or 25 mg/kg, i.p.). The numbers of neutrophils were evaluated 24 h after
815 LPS injection (A). In (B), cells from pleural cavity were processed for Western Blot analysis
816 to elastase. Graph in the Figure (C) are densitometry analysis using ImageJ Software of
817 proteins levels seen in the autoradiograms. Data are expressed in arbitrary units. Results are
818 expressed as the number of cells per cavity and are shown as the mean ± SEM of at least five
819 mice in each group. ***, $P < 0.001$ when compared with PBS-injected mice; and #, $P < 0.05$,
820 ##, $P < 0.01$, when compared to 24 h LPS-injected mice. For loading control, membranes
821 were re-probed with anti-β-actin. Blots are representative of two independent experiments
822 using pooled cells from at least five animals.
823
824 **Figure 4. Effect of Sivelestat treatment on resolution indices of LPS-induced pleurisy**
825 **and AnxA1 expression.** Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 8 h
826 after received an injection of Sivelestat (SIV- 5 mg/kg, i.p.). The numbers of neutrophils were
827 evaluated at 8, 24, 36 and 48 h after LPS injection and resolution indices were quantified (A).
828 Of note: T_{max} = 8 h, the time point when PMN numbers reach maximum; T_{50} SIV ~ 26 h, the
829 time point when PMN numbers reduce to 50% of maximum; and R_i SIV ~ 18 h, resolution
830 interval, the time period when 50% PMN are lost from the pleural cavity. In (B), 4 h-LPS
831 challenged mice were treated with SIV by 2 h and 4 h. Pleural lavages were performed at 6 h
832 and 8 h after LPS and cell extracts were processed for Western Blot. For loading control,
833 membranes were re-probed with anti-β-actin. Blots shown in (B) are representative of two
834 independent experiments using pooled cells from at least four animals.

835

836 **Figure 5. Effect of Sivelestat treatment on neutrophil apoptosis *in vivo*.** Mice were
837 injected with LPS (250 ng/cavity, i.pl.) or PBS and 4 h later received an injection of Sivelestat
838 (SIV- 5 mg/kg, i.p.) or vehicle. The pan-caspase inhibitor zVAD-fmk (1 mg/kg, i.p.) was
839 given 15 minutes before SIV. The number of neutrophils (A) was evaluated 4 h after SIV
840 treatment. (B) and (C) are dose-response experiments using SIV at dose of 1, 5 and 25mg/kg
841 for treatment of 4 h-LPS injected mice. Cells from the pleural cavity were harvested 4 h after
842 SIV treatment and processed for count of neutrophils with distinctive apoptotic morphology
843 (B), western blot analysis to detection of cleaved caspase-3, Mcl-1 and P-IκB-α (C).
844 Densitometry analyses are shown (D). The number of Annexin-V positive neutrophils (E)
845 with representative dot plots evaluated by flow cytometry 24 h after LPS-injection. **, $P <$
846 0.01 or ***, $P < 0.001$, when compared with PBS-injected mice; #, $P < 0.05$; ##, $P < 0.01$ or
847 ###, $P < 0.001$, when compared SIV treatment to 8 h after LPS-challenged mice, or when
848 compare the groups treated only with SIV to SIV + zVAD. For loading control, membranes
849 were re-probed with anti-β-actin. Blots are representative of three independent experiments
850 using pooled cells from at least five animals.

851

852 **Figure 6. Macrophage polarization and efferocytosis after treatment with Sivelestat**
853 **during LPS-induced pleurisy.** Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS
854 and 8 h after received an injection of Sivelestat (SIV- 5 mg/kg, i.p.). The cells were harvested
855 24 h after LPS injection. Flow cytometry analysis was performed for M2 ($F4/80^{high}$ $GR1^{-}$
856 $CD11b^{high}$) (A) and Mres ($F4/80^{medium}$ $CD11b^{low}$) (B) number, and frequency of efferocytosis
857 ($F4/80^{+}/Ly6G^{+}$) (C). Results are expressed as the number of cells per cavity and are shown as
858 the mean ± SEM of at least five mice in each group. **, $P < 0.01$; ***, $P < 0.001$, when
859 compared with PBS-injected mice and #, $P < 0.05$; ###, $P < 0.001$ when compared to 24
860 hours after LPS-injected mice.

Figure 7. Comparative effect of treatment with Sivelestat and AnxA1 peptides, and effect of AnxA1 neutralization on Sivelestat-induced resolution of acute inflammation. Mice were injected with LPS (250 ng/cavity, i.pl.) or PBS and 8 h later received an injection of Sivelestat (SIV- 5 mg/kg, i.p.), or the peptides $AnxA1_{2-50}$ and CR (cleavage resistant)-$AnxA1_{2-50}$ (150μM, per mouse, i.p). Cells from the pleural cavity were harvested 24 h after LPS-challenge and processed for count of neutrophils (A) and Western Blot analysis to detection of Mcl-1 and P-IκB-α (B). In (C) and (D), cells from the pleural cavity were harvested 4 h later Sivelestat injection. Anti-AnxA1 (200μl, i.p.) was given 30 min prior LPS-injection and 30 min prior SIV. Evaluation of the neutrophil numbers from cytospin preparations (C) and Western Blot analysis to detection of P-IκB-α and Mcl-1 (D). In (E) BOC-1 (5mg/kg, i.p.) was given 30 min prior SIV for evaluation of the neutrophil numbers from cytospin preparations. Results are shown as the mean ± SEM of at least five mice in each group. ***, $P < 0.001$, when compared with PBS-injected mice; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ when compared to LPS-challenged mice. For loading control, membranes were re-probed with anti-β-actin. Blots are representative of two independent experiments using pooled cells from at least five animals.

Figure 1

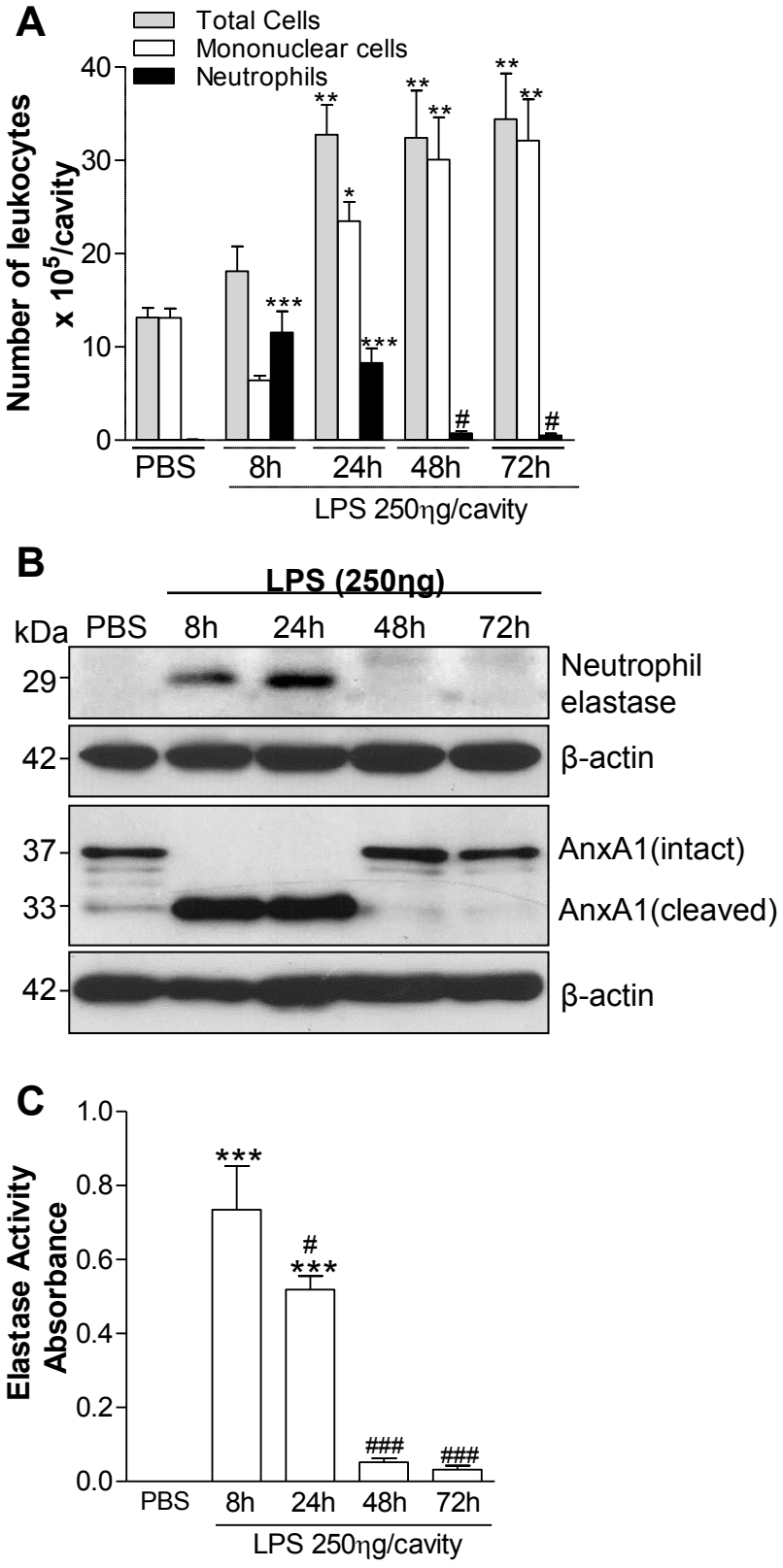

A
Total Cells
Mononuclear cells
Neutrophils
Number of leukocytes x 10^5/cavity
40
30
20
10
0
PBS
8h
24h
48h
72h
LPS 250ηg/cavity
B
LPS (250ηg)
kDa
PBS
8h
24h
48h
72h
29
Neutrophil elastase
42
β-actin
37
AnxA1(intact)
33
AnxA1(cleaved)
42
β-actin
C
Elastase Activity Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
PBS
8h
24h
48h
72h
LPS 250ηg/cavity

Figure 2

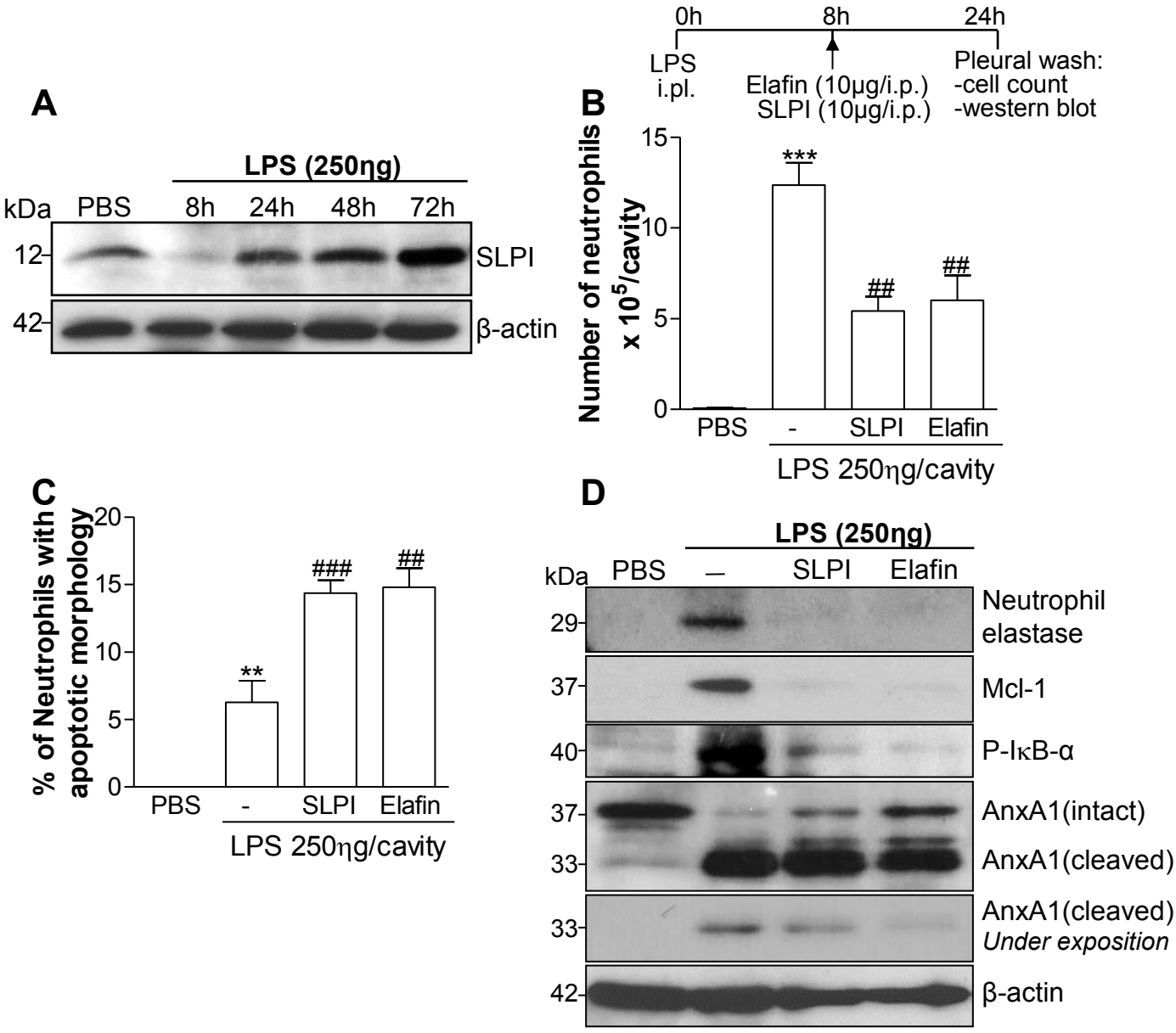

A
LPS (250ηg)
kDa
PBS
8h
24h
48h
72h
12
SLPI
42
β-actin
B
0h
8h
24h
LPS
i.pl.
Elafin (10µg/i.p.)
SLPI (10µg/i.p.)
Pleural wash:
-cell count
-western blot
Number of neutrophils
x 10^5/cavity
15
10
5
0
***
##
##
PBS
-
SLPI
Elafin
LPS 250ηg/cavity
C
% of Neutrophils with
apoptotic morphology
20
15
10
5
0
**
###
##
PBS
-
SLPI
Elafin
LPS 250ηg/cavity
D
LPS (250ηg)
kDa
PBS
—
SLPI
Elafin
29
Neutrophil
elastase
37
Mcl-1
40
P-IκB-α
37
AnxA1(intact)
33
AnxA1(cleaved)
33
AnxA1(cleaved)
Under exposition
42
β-actin

Figure 3

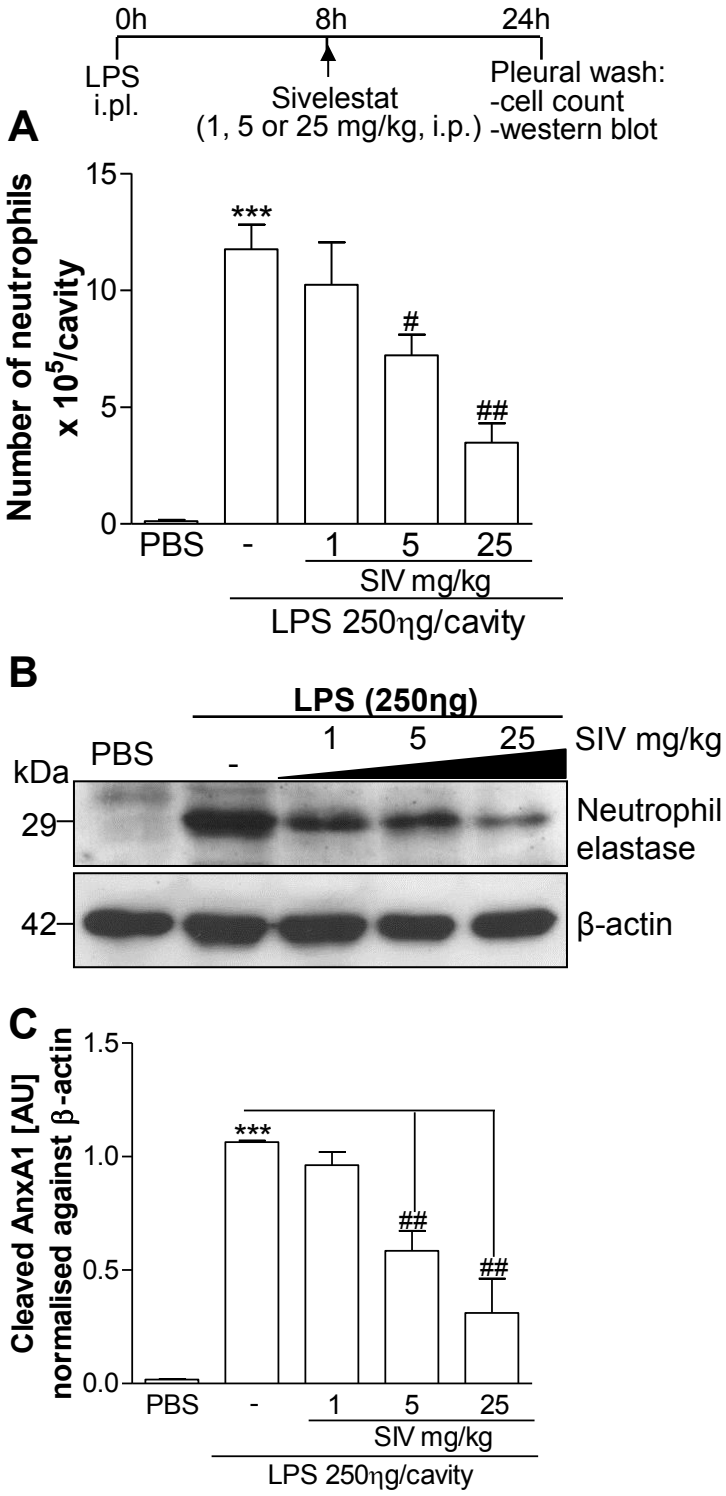
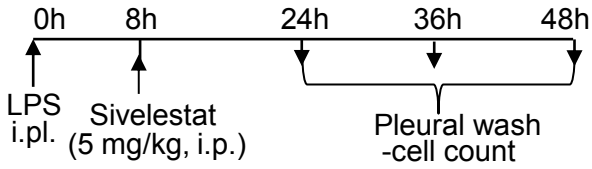
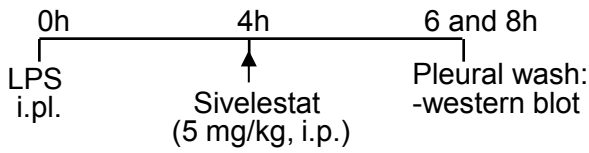
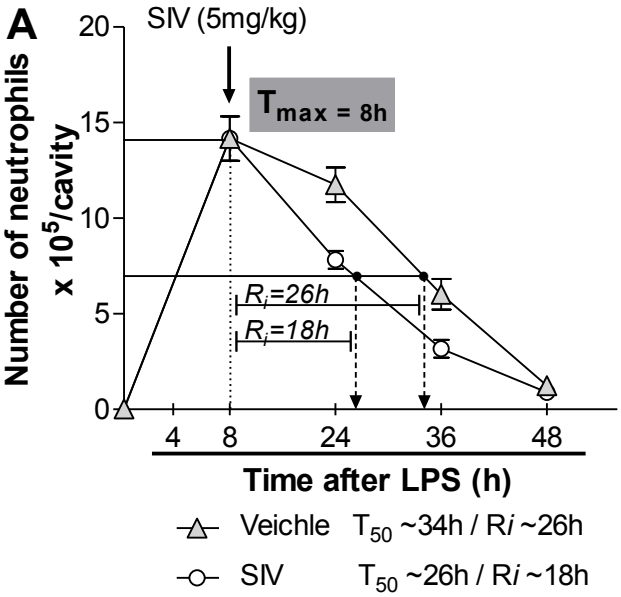

0h
8h
24h
LPS
i.pl.
Sivelestat
(1, 5 or 25 mg/kg, i.p.)
Pleural wash:
-cell count
-western blot
A
Number of neutrophils
x 10^5/cavity
15
10
5
0
***
#
##
PBS
-
1
5
25
SIV mg/kg
LPS 250ηg/cavity
B
LPS (250ηg)
PBS
-
1
5
25
SIV mg/kg
kDa
29
42
Neutrophil
elastase
β-actin
C
Cleaved AnxA1 [AU]
normalised against β-actin
1.5
1.0
0.5
0.0
***
##
##
PBS
-
1
5
25
SIV mg/kg
LPS 250ηg/cavity

Figure 4


0h
8h
24h
36h
48h
LPS
i.pl.
Sivelestat
(5 mg/kg, i.p.)
Pleural wash
-cell count


0h
4h
6 and 8h
LPS
i.pl.
Sivelestat
(5 mg/kg, i.p.)
Pleural wash:
-western blot


A
SIV (5mg/kg)
T_{max} = 8h
R_i=26h
R_i=18h
Number of neutrophils
x 10^5/cavity
20
15
10
5
0
4
8
24
36
48
Time after LPS (h)
Veichle T_{50} ~34h / R*i* ~26h
SIV T_{50} ~26h / R*i* ~18h

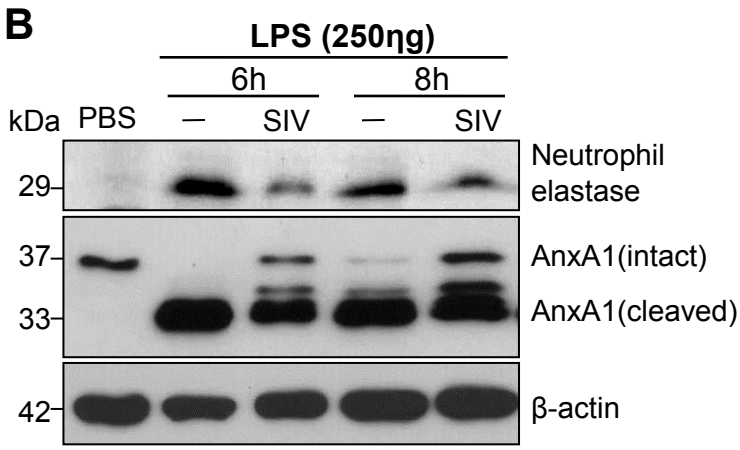

B
LPS (250ηg)
6h
8h
kDa
PBS
—
SIV
—
SIV
29
37
33
42
Neutrophil
elastase
AnxA1(intact)
AnxA1(cleaved)
β-actin

Figure 5

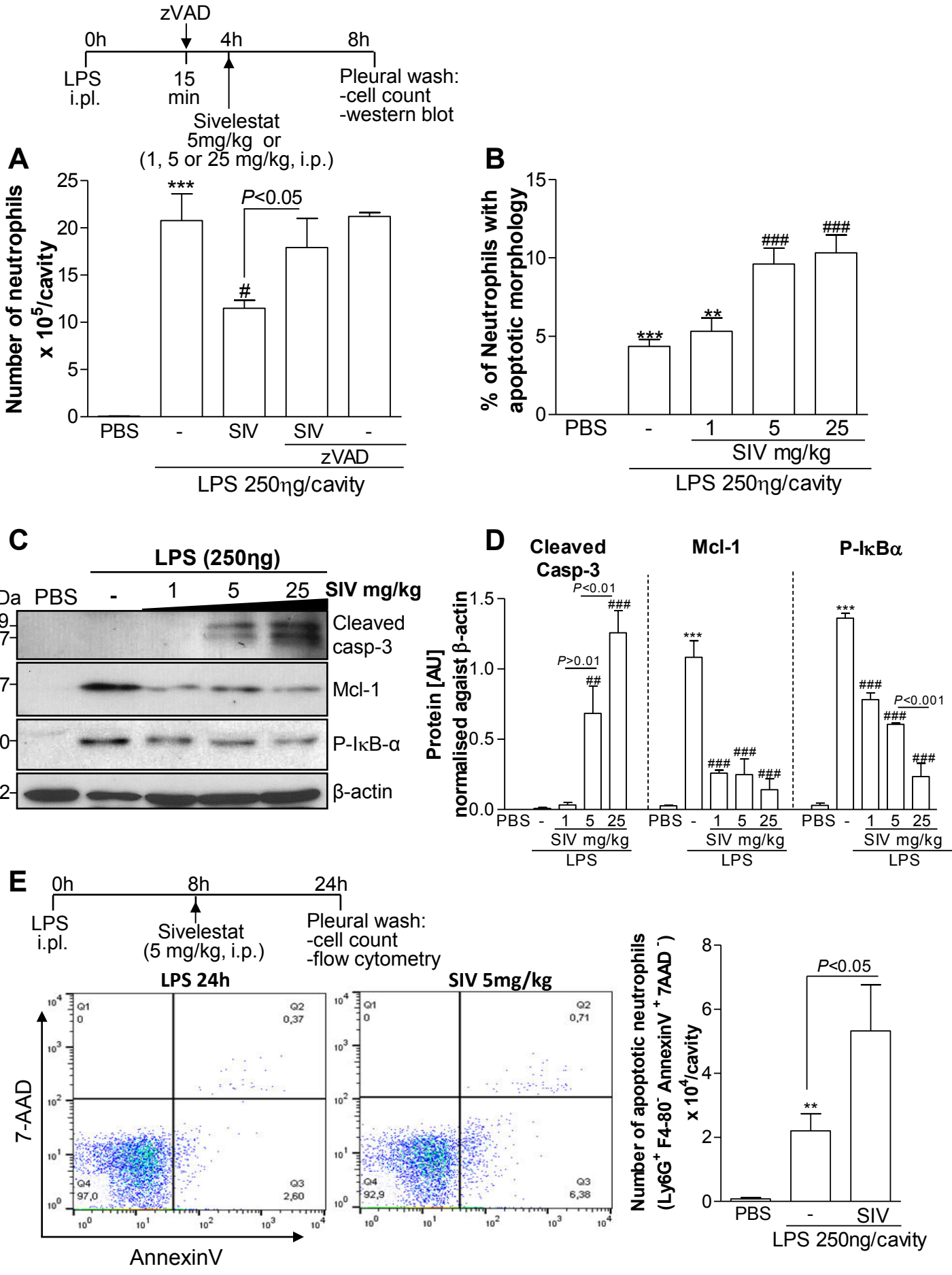

zVAD
0h
4h
8h
LPS i.pl.
15 min
Sivelestat 5mg/kg or (1, 5 or 25 mg/kg, i.p.)
Pleural wash: -cell count -western blot
A
Number of neutrophils x 10^5/cavity
P<0.05
PBS
SIV
SIV + zVAD
LPS 250ηg/cavity
B
% of Neutrophils with apoptotic morphology
SIV mg/kg
LPS 250ηg/cavity
C
LPS (250ηg)
kDa
PBS
SIV mg/kg
Cleaved casp-3
Mcl-1
P-IκB-α
β-actin
D
Cleaved Casp-3
Mcl-1
P-IκBα
Protein [AU] normalised agaist β-actin
P<0.01
P>0.01
P<0.001
LPS
E
0h
8h
24h
LPS i.pl.
Sivelestat (5 mg/kg, i.p.)
Pleural wash: -cell count -flow cytometry
LPS 24h
SIV 5mg/kg
7-AAD
AnnexinV
Q1 0
Q2 0,37
Q4 97,0
Q3 2,60
Q2 0,71
Q4 92,9
Q3 6,38
Number of apoptotic neutrophils (Ly6G+ F4-80- AnnexinV+ 7AAD-) x 10^4/cavity
P<0.05
LPS 250ng/cavity

Figure 6

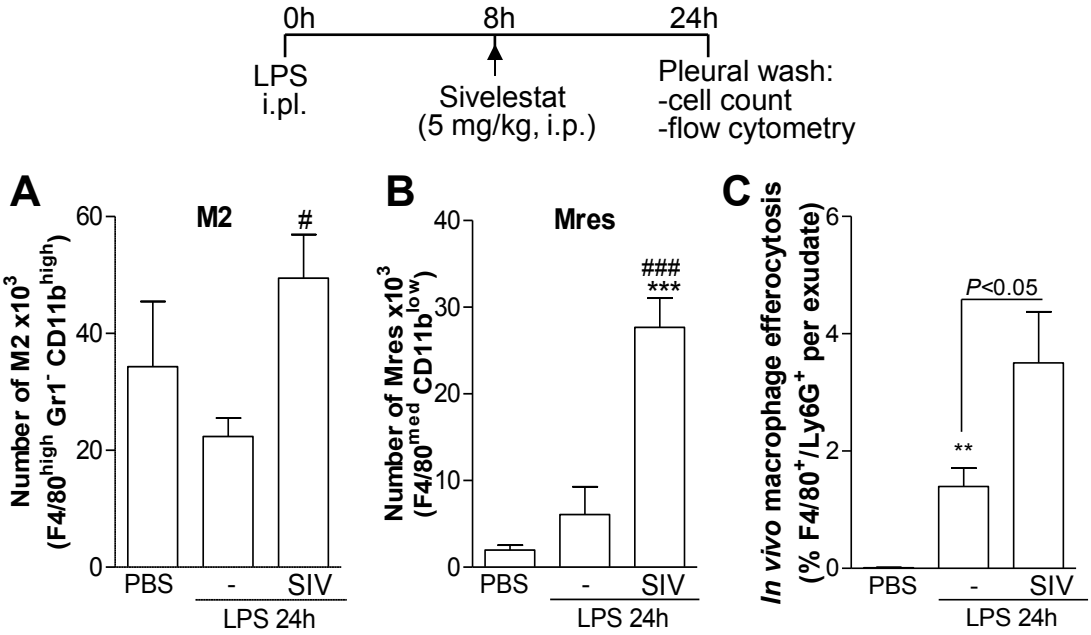
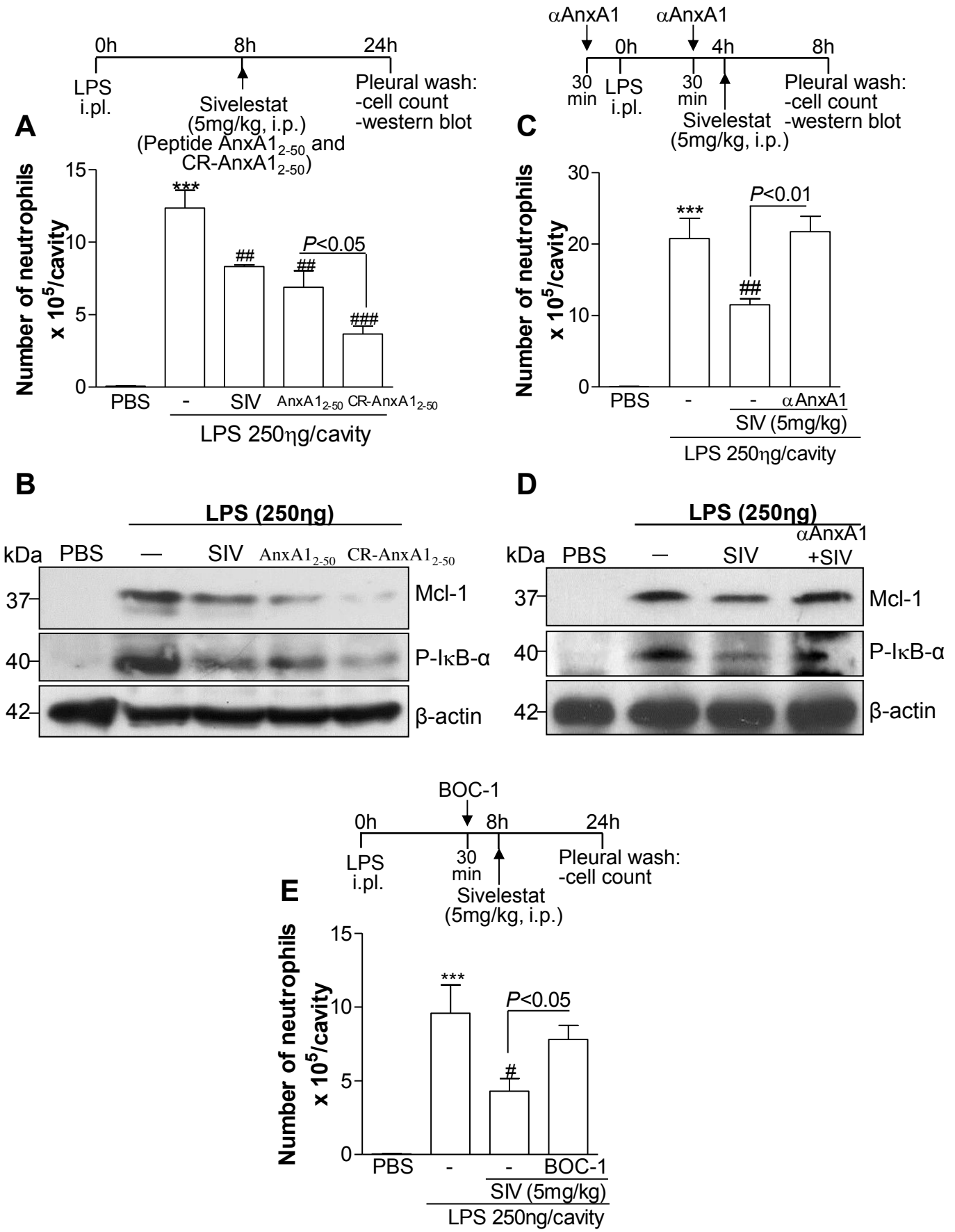
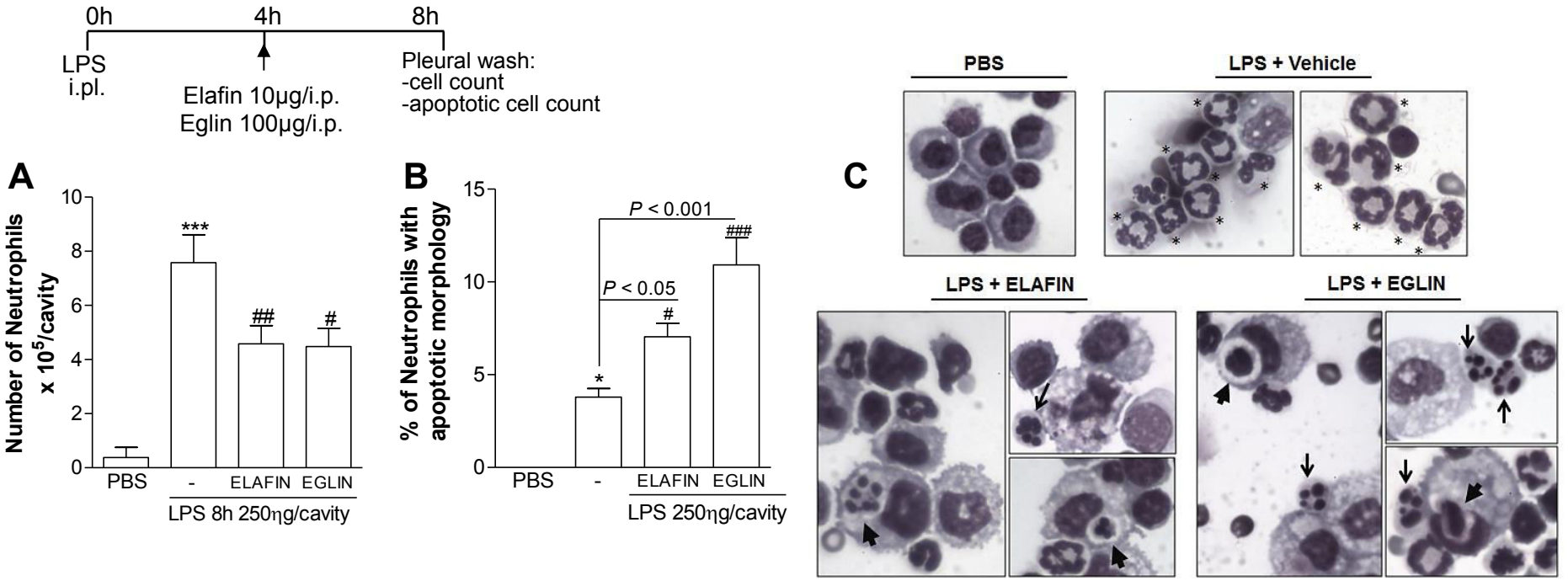

0h
8h
24h
LPS
i.pl.
Sivelestat
(5 mg/kg, i.p.)
Pleural wash:
-cell count
-flow cytometry
A
M2
#
Number of M2 x10^3
(F4/80^high Gr1^- CD11b^high)
60
40
20
0
PBS
-
SIV
LPS 24h
B
Mres
###
***
Number of Mres x10^3
(F4/80^med CD11b^low)
40
30
20
10
0
PBS
-
SIV
LPS 24h
C
In vivo macrophage efferocytosis
(% F4/80^+/Ly6G^+ per exudate)
P<0.05
**
6
4
2
0
PBS
-
SIV
LPS 24h

Figure 7

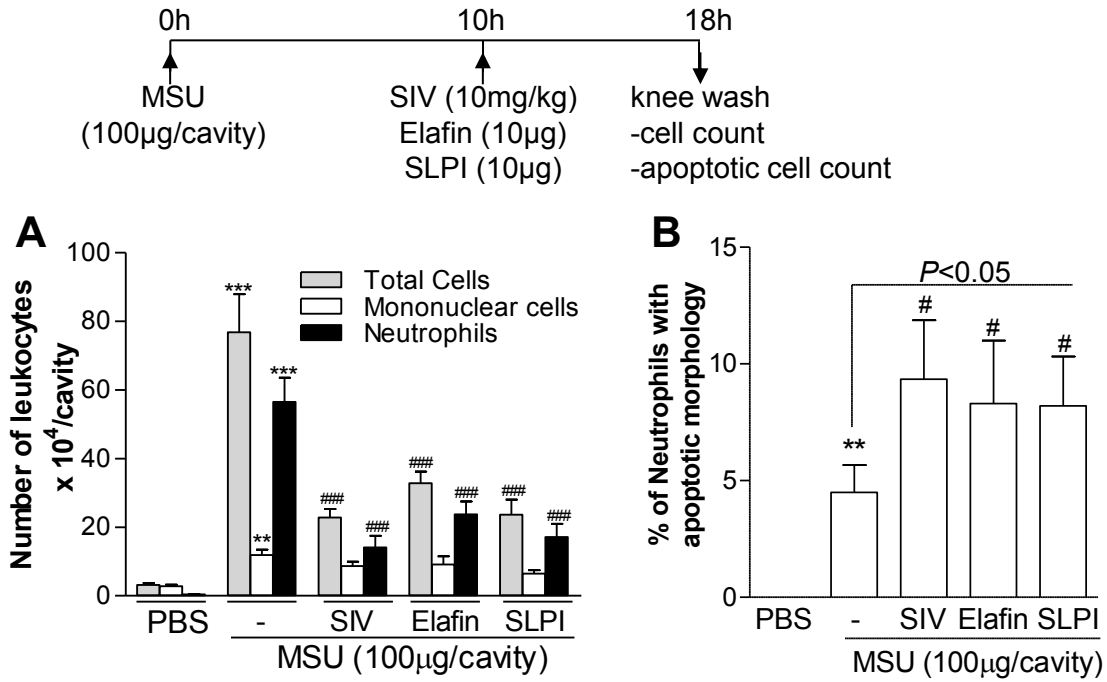

A
0h
8h
24h
LPS i.pl.
Sivelestat (5mg/kg, i.p.) (Peptide AnxA1 2-50 and CR-AnxA1 2-50)
Pleural wash: -cell count -western blot
Number of neutrophils x 10^5/cavity
***
##
##
###
P<0.05
PBS
-
SIV
AnxA1 2-50
CR-AnxA1 2-50
LPS 250ηg/cavity
C
αAnxA1
αAnxA1
0h
4h
8h
30 min
LPS i.pl.
30 min
Sivelestat (5mg/kg, i.p.)
Pleural wash: -cell count -western blot
Number of neutrophils x 10^5/cavity
***
##
P<0.01
PBS
-
-
αAnxA1
SIV (5mg/kg)
LPS 250ηg/cavity
B
LPS (250ηg)
kDa
PBS
—
SIV
AnxA1 2-50
CR-AnxA1 2-50
37
40
42
Mcl-1
P-IκB-α
β-actin
D
LPS (250ηg)
αAnxA1
kDa
PBS
—
SIV
+SIV
37
40
42
Mcl-1
P-IκB-α
β-actin
E
BOC-1
0h
8h
24h
LPS i.pl.
30 min
Sivelestat (5mg/kg, i.p.)
Pleural wash: -cell count
Number of neutrophils x 10^5/cavity
***
#
P<0.05
PBS
-
-
BOC-1
SIV (5mg/kg)
LPS 250ng/cavity

Supplementary Figure 1



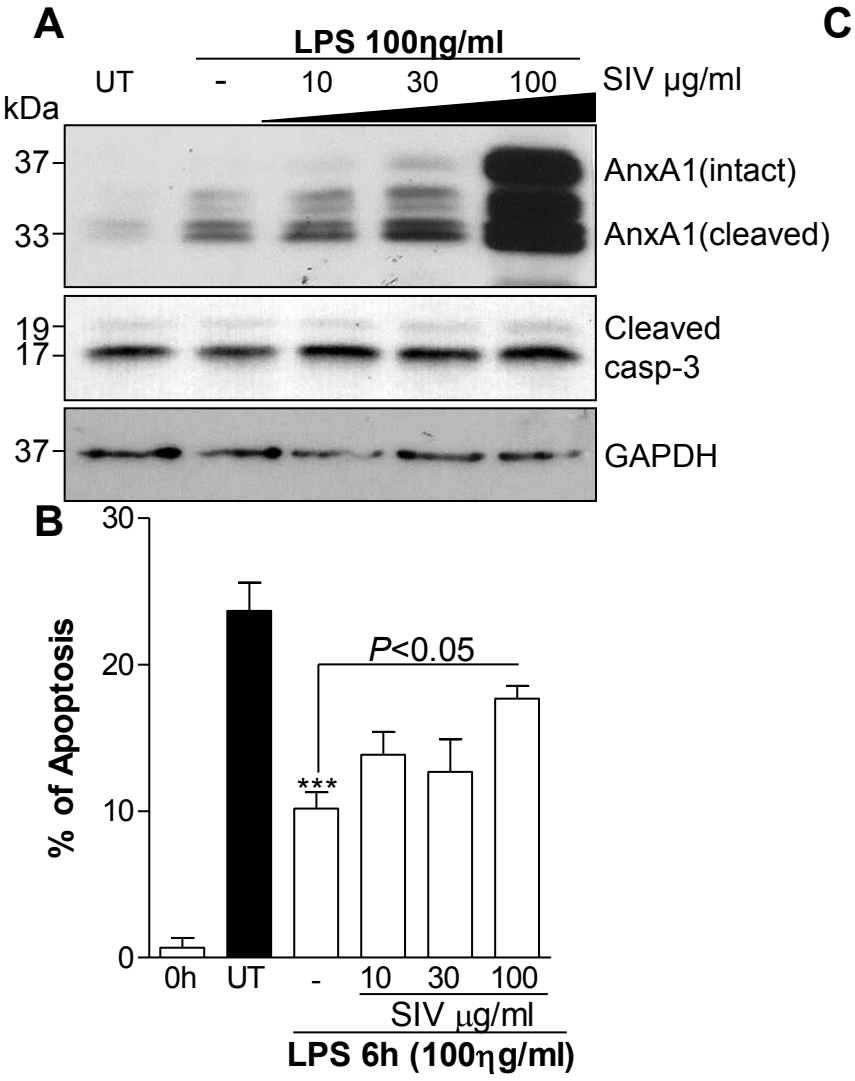
Supplementary Figure 1 – Effect of Elafin and Eglin c on LPS-induced pleurisy. Mice were injected with LPS (250 ng/cavity, i.pl.) and 4 h later received an injection of Elafin peptide (10µg/ml, i.p.) or Eglin c (100µg/ml, i.p.). Cells from the pleural cavity were harvested 8 h after LPS-challenge and processed for count of neutrophils (A) and neutrophils with distinctive apoptotic morphology (B). In (C), representative figures of nonapoptotic (asterisk), apoptotic (arrows) neutrophils, and apoptotic cells inside macrophages (arrowheads). PBS and vehicle (upper panels) and Elafin/Eglin treatments (lower panels) are shown. Original magnifications ×100. Results are expressed as the number of cells per cavity and are shown as the mean ± SEM of at least five mice in each group. *, $P < 0.05$; ***, $P < 0.001$, when compared with PBS-injected mice; and #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, when compared to 8 h after LPS-injected mice.

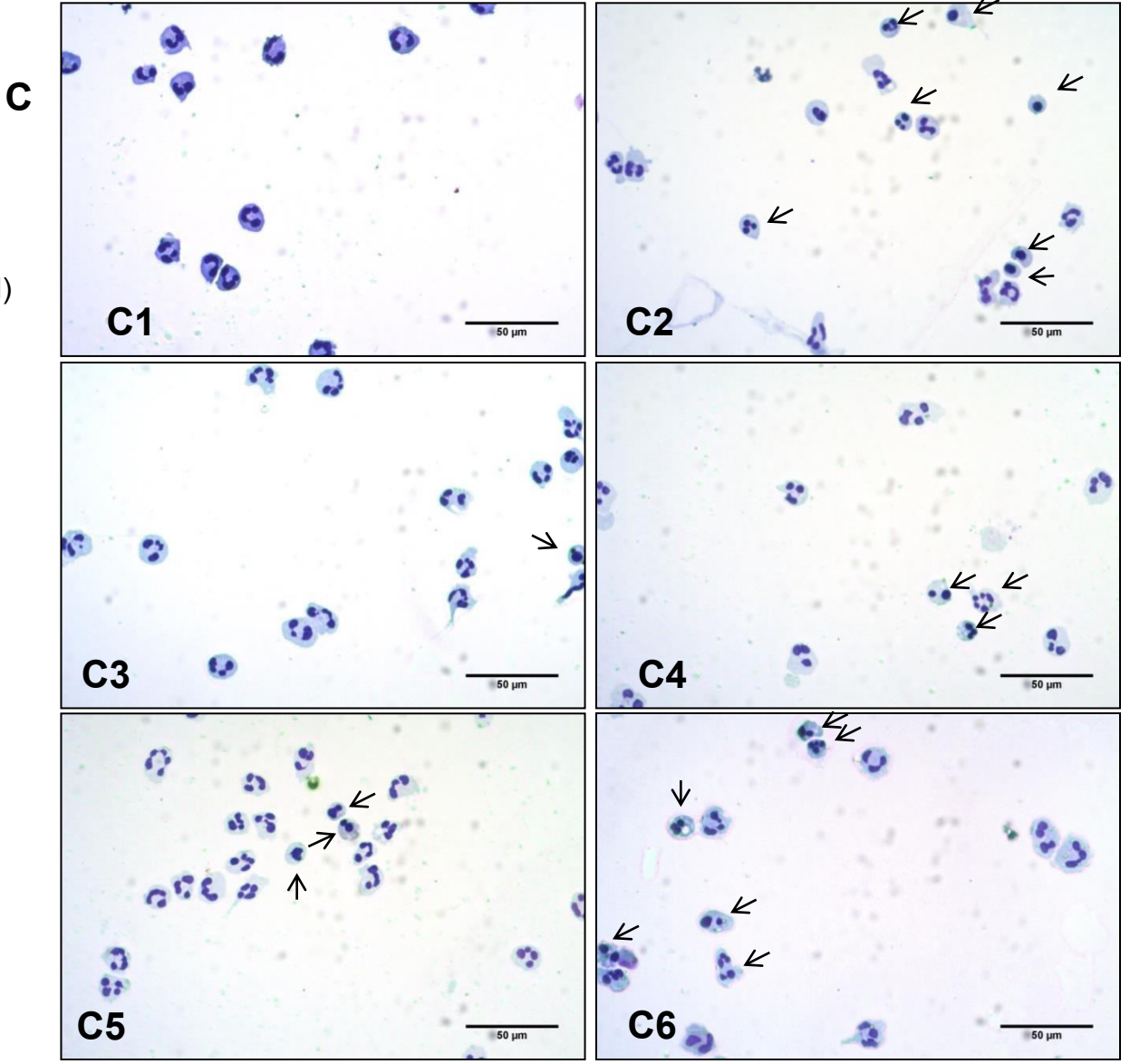
Supplementary Figure 2



Supplementary Figure 2 – Effect of Sivelestat, Elafin and SLPI on MSU-induced inflammation. Mice were injected with MSU crystals (100 µg) into the tibiofemoral joint and 10 h later received an injection of Sivelestat (10mg/kg, i.p.), Elafin (10µg/mouse, i.p.) or SLPI (10µg/mouse, i.p.). The cells from articular cavity were harvested 18 h after MSU-challenge and processed to count of leukocytes (A) and neutrophils with distinctive apoptotic morphology (B). Results are expressed as the number of cells per cavity and are shown as the mean ± SEM of at least five mice in each group. **, $P < 0.01$ or ***, $P < 0.001$ when compared with PBS- injected mice and #, $P < 0.05$ or ###, $P < 0.001$ when compared with 10h-MSU challenged mice.

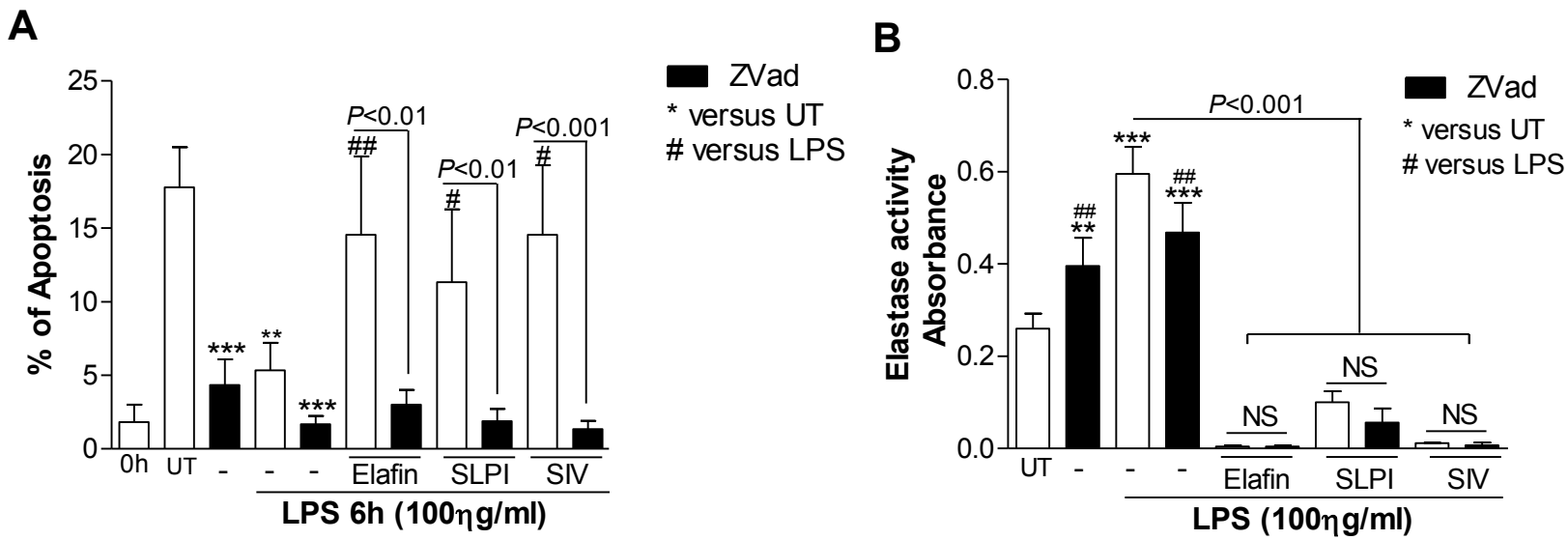
Supplementary Figure 3





Supplementary Figure 3 – Effect of different concentrations of Sivelestat on AnxA1 levels and neutrophil apoptosis. Neutrophils isolated from human peripheral blood (1 x 10^6 cell per well) were cultured with LPS (100 ng/ml) for 1 h, and after with Sivelestat (10, 30 and 100 µg/ml) for 2 h (A) or 5 h (B). Neutrophils were processed for Western Blot analysis for AnxA1 and cleaved caspase-3 detection (A) or cytospin preparations for apoptosis count (B). In (C), representative figures of control 0h (C1); cultured by 6h only with complete medium (C2); LPS 6h (C3); LPS 6h + SIV 10µg/ml (C4); LPS 6h + SIV 30µg/ml (C5); LPS 6h + SIV 100µg/ml (C6). Original magnifications ×20. ***, $P < 0.001$, when compared LPS treated group with untreated (UT) neutrophils. For loading control, membranes were re-probed with anti-GAPDH. The experiments were performed in biological triplicates. Blots are representative of two independent experiments.

Supplementary Figure 4



Supplementary Figure 4 – Effect of protease inhibitors on human neutrophil apoptosis and elastase activity. Neutrophils isolated from human peripheral blood (1 x 10^6 cell per well) were cultured with zVAD (100µM) for 1 h, and after with LPS (100 ng/ml) or LPS plus Elafin (100 ng/ml), SLPI (100 ng/ml) or Sivelestat (100 µg/ml) for further 5 h. Neutrophils were processed for cytospin preparations for apoptosis count (A) or measurement of elastase activity - 1 h after treatments (B). **, $P < 0.01$; ***, $P < 0.001$, when compared with untreated (UT) neutrophils. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, when compared with LPS treated group. The experiments were performed in biological triplicates.

7 – DISCUSSÃO

Os GCs são agentes anti-inflamatórios e imunossupressores potentes amplamente utilizados na clínica médica para o tratamento de várias doenças inflamatórias. No entanto, apesar dos GCs apresentarem uma excelente eficácia terapêutica, um fator importante que deve ser considerado é o fato do tratamento com esses medicamentos estar associado a vários efeitos adversos (Perretti *et al.*, 2009; Beaulieu *et al.*, 2011; Alessandri *et al.*, 2013). Em muitos casos, principalmente para o tratamento de doenças crônicas, é necessário o uso prolongado de GCs e com dosagens altas o que pode resultar em intolerância, e, muitas vezes, é necessário o ajuste de doses (Barnes, 2011; Oakley *et al.*, 2011; Vandevyver *et al.*, 2013). As ações dos GCs não são completamente entendidas e podem depender da indução de proteínas regulatórias anti-inflamatórias. No presente estudo, foi demostrado a relevância de duas proteínas induzidas por GCs, GILZ e AnxA1, na resolução da resposta inflamatória aguda, utilizando um modelo murino de pleurisia induzido por LPS. Nossos principais achados foram: 1) a expressão de GILZ e AnxA1 aumentou na fase resolutiva da inflamação, mais precisamente em macrófagos com fenótipo anti-inflamatório; 2) a superexpressão de GILZ com a proteína de fusão TAT-GILZ promoveu a resolução da inflamação por indução da apoptose de neutrófilos (caspase-dependente) e diminuição dos níveis de NF-κB, P-ERK1/2 e Mcl-1; 3) na ausência de GILZ (animais *knockout*) ocorreu um mecanismo compensatório com aumento de corticosterona e AnxA1 intacta; 4) a expressão de GILZ foi dependente de AnxA1; 5) o aumento da expressão e atividade da protease elastase foi associado à fase de intenso recrutamento de neutrófilos e clivagem de AnxA1, enquanto o tratamento com anti-proteases induziu um acúmulo de AnxA1 intacta, associado à apoptose de neutrófilos (caspase-dependente) e diminuição dos níveis NF-κB e Mcl-1; 6) o tratamento com uma anti-protease sintética específica para elastase, Sivelestat, promoveu reprogramação macrofágica para um fenótipo resolutivo, aumentando a eferocitose de células apoptóticas; 7) o efeito pro-resolutivo de Sivelestat foi dependente de AnxA1.

Nosso grupo de pesquisa tem se dedicado ao estudo de vias de sinalização e mecanismos que possam contribuir para resolução da resposta inflamatória (Sousa *et al.*, 2009; Sousa *et al.*, 2010; Vago *et al.*, 2012; Sousa *et al.*, 2013; Reis *et al.*, 2015). Os mediadores inflamatórios produzidos nas etapas iniciais da inflamação aumentam a sobrevida dos neutrófilos através da

indução de moléculas sinalizadoras associadas à sobrevida celular como NF-κB e Mcl-1 e inibição da ativação de caspases. Além de NF-κB, a proteína anti-apoptótica Mcl-1 (*myeloid cell leukemia-1*) é uma importante mediadora da sobrevivência de células, especificamente em neutrófilos. Em geral, a expressão de Mcl-1 é inversamente proporcional ao grau de apoptose de neutrófilos (Milot *et al.*, 2011). Em nosso trabalho, foi demonstrado que a superexpressão de GILZ com TAT-GILZ e aumento dos níveis de AnxA1 intacta, através da administração de anti-proteases, foram associados à diminuição dos níveis de Mcl-1 nos exsudatos pleurais e resolução da inflamação. De fato, vários trabalhos já demonstram que a regulação dos níveis de Mcl-1 pode contribuir para a indução da resolução da resposta inflamatória (Rossi *et al.*, 2006; El Kebir *et al.*, 2009; Sousa *et al.*, 2010; Lucas *et al.*, 2013; Vago *et al.*, 2015).

Compostos que atuem aumentando a apoptose de neutrófilos são agentes com potencial terapêutico para o tratamento de doenças inflamatórias agudas ou crônicas onde há um acúmulo excessivo dessas células (Duffin *et al.*, 2010). Até o presente estudo, poucos trabalhos mostraram o envolvimento de GILZ na apoptose de leucócitos. Delfino e colaboradores (2004) demonstraram que a superexpressão de GILZ nas linhagens de células T em animais transgênicos resultou na apoptose espontânea de timócitos, com ativação de caspase 8 e 3, e diminuição nos níveis de Bcl-xL (Delfino *et al.*, 2004). Outro estudo demonstrou que a inibição da via PI3K/Akt está associada ao aumento de GILZ e indução de apoptose em células de mieloma múltiplo (Grugan *et al.*, 2008). No presente trabalho, foi demonstrado, pelo nosso conhecimento, de forma pioneira, que a superexpressão de GILZ foi associada à resolução da inflamação aguda, mais precisamente por indução da apoptose de neutrófilos e diminuição de proteínas que controlam a sobrevivência celular. Reforçando nossos achados, foi demonstrado mais recentemente, em estudos *in vitro*, que GILZ está envolvido com a indução da apoptose de neutrófilos humanos por um mecanismo dependente da inibição de Mcl-1 (Espinasse *et al.*, 2015).

Trabalhos publicados previamente já mostraram o papel de AnxA1 na apoptose de leucócitos *in vitro* (Solito *et al.*, 2001; Solito *et al.*, 2003). Posteriormente, nosso grupo de pesquisa demonstrou que a AnxA1 desempenha um papel chave na resolução da inflamação aguda pela indução da apoptose de neutrófilos *in vivo* (Vago *et al.*, 2012). Dados da literatura sugerem que os efeitos anti-inflamatórios da AnxA1 estejam associados à forma intacta da

proteína de 37 kDa, mais precisamente na sua porção N-terminal (Oliani *et al.*, 2001; Vong *et al.*, 2007). Sabendo-se que AnxA1 sofre clivagem por proteases de leucócitos, o grupo de pesquisa do professor Mauro Perretti desenvolveu uma forma de AnxA1 resistente a clivagem, que apresentou efeitos anti-inflamatórios mais proeminentes que a proteína parental (Pederzoli-Ribeil *et al.*, 2010). Além disso, já foi demonstrado, incluindo pelo nosso grupo de pesquisa, que, durante o processo inflamatório, ocorre um acúmulo de AnxA1 clivada (Tsao *et al.*, 1998; Williams *et al.*, 2010; Vago *et al.*, 2012). Portanto, o bloqueio da clivagem de AnxA1, principalmente em um contexto inflamatório, pode ser uma estratégia farmacológica importante que contribui para uma resolução mais efetiva da inflamação. No presente trabalho, foi demonstrado que, durante a cinética da inflamação, ocorre um acúmulo de elastase que coincide com o aumento da AnxA1 clivada. Nos pontos de resolução, os níveis de elastase diminuem, restabelecendo os níveis de AnxA1 intacta. Além disso, a inibição da clivagem de AnxA1, através da administração de anti-proteases endógenas ou sintética específica para elastase, foi associada com a indução da resolução da inflamação neutrofílica. Mecanisticamente, a resolução da inflamação foi associada com a indução da apoptose de neutrófilos e eferocitose. Já foi demonstrado que as anti-proteases desempenham um papel importante do controle da resposta inflamatória (Pham, 2006). Aqui, mostramos pela primeira vez, ao menos no nosso conhecimento, que um dos mecanismos pelos quais essas anti-proteases exercem seus efeitos, é através do aumento da AnxA1 na sua forma intacta, através da inibição da sua clivagem. Vale ressaltar que a AnxA1 é uma importante proteína envolvida no controle do processo inflamatório e que a modulação dessa proteína *in vivo* pode representar uma estratégia terapêutica importante para o controle de doenças inflamatórias.

Um dos aspectos importantes para uma resolução efetiva do processo inflamatório é a apoptose de neutrófilos, seguida pela remoção por fagócitos (Serhan *et al.*, 2007; Duffin *et al.*, 2010; Alessandri *et al.*, 2013). Os macrófagos são classificados como fagócitos profissionais e compõe o sistema fagocitário mononuclear, bem como as células dendríticas (Chow *et al.*, 2011). Durante a resposta inflamatória, os macrófagos podem ser reprogramados transcricionalmente pela ativação de DAMPs, PAMPs e mudanças no microambiente tecidual, tendo papel central na regulação da inflamação local (Lichtnekert *et al.*, 2013; Labonte *et al.*, 2014). Em um ambiente pró-inflamatório os macrófagos têm geralmente um fenótipo pró-inflamatório (M1), que

apresenta pouca capacidade eferocítica e alta capacidade fagocítica. No entanto, o fenótipo desses macrófagos pode ser alterado para M2, com um perfil anti-inflamatório e alta atividade eferocítica (Schif-Zuck *et al.*, 2011; Alessandri *et al.*, 2013). No presente estudo, foi demonstrado que durante a resolução espontânea da inflamação, ocorre uma mudança do fenótipo dos macrófagos. Foi observado que o número de macrófagos M1 estava aumentado durante o pico da inflamação e que os macrófagos anti-inflamatórios/pró-resolutivos (M2 e Mres) tiveram aumento em número nos períodos de resolução. De maneira interessante, foi observado que a expressão de GILZ e AnxA1 estava aumentada nesses macrófagos (M2 e Mres). Além disso, o tratamento de animais inflamados com o inibidor específico de elastase, Sivelestat, diminuiu o número de macrófagos M1, aumentando M2 e Mres. Em conformidade com o aumento de macrófagos propensos à eferocitose, foi observado também um aumento da eferocitose de neutrófilos apoptóticos após o tratamento com Sivelestat. Esses dados sugerem que macrófagos anti-inflamatórios/pro-resolutivos exercem um papel fundamental para a resolução da inflamação e que as proteínas GILZ e AnxA1 possam estar envolvidas no processo de reprogramação macrofágica.

Tradicionalmente, as terapias anti-inflamatórias concentraram-se em estratégias que inibem ou neutralizam mediadores pró-inflamatórios, bem como ativação e o recrutamento de leucócitos. O racional é que a inibição da fase produtiva da resposta inflamatória resultará em abrandamento da progressão da inflamação. Exemplos dessas terapias incluem anticorpos anti-citocinas pró-inflamatórias como TNF-α e IL-1β, anti-inflamatórios não esteroidais e os glicocorticoides sintéticos, os quais inibem a produção local de quimiocinas e moléculas de adesão, resultando em uma diminuição do influxo de leucócitos (Sousa *et al.*, 2013). Já as estratégias pró-resolutivas apresentam-se mais complexas, podendo envolver mediadores bioquímicos e vias de sinalização que controlam: 1) o término da resposta inflamatória (principalmente pela diminuição do recrutamento de granulócitos e reversão da vasodilatação e permeabilidade vascular); 2) mudança na produção de mediadores pró-inflamatórios para pró-resolutivos; 3) inativação de vias de sinalização associadas com a produção de citocinas e a sobrevivência de leucócitos; 4) apoptose de células recrutadas para o local da inflamação; 5) eferocitose de células apoptóticas; 6) mudança do fenótipo de células de pró-inflamatório para pró-resolutivo (especialmente macrófagos) (Alessandri *et al.*, 2013). É importante ressaltar que

determinados agentes anti-inflamatórios como os esteroides, podem ser considerados *resolution-safe* uma vez que promovem a fagocitose de leucócitos apoptóticos e aumentam a expressão e liberação de AnxA1. Em contraste, outros compostos anti-inflamatórios podem ser considerados *resolution-toxic* podendo levar à maior duração da resposta inflamatória (consenso em resolução-Serhan *et al*., 2007). Nesse sentido, o potencial farmacológico de agentes pró-resolutivos, tem despertado o interesse de vários grupos de pesquisa. Um dos métodos para mensurar a efetividade pró-resolutiva de um agente é através da medida dos intervalos de resolução. O cálculo dos intervalos de resolução permite a avaliação das propriedades pró-resolutivas de agentes pela avaliação temporal do recrutamento de leucócitos em locais inflamados (Bannenberg *et al.*, 2005). Este é um parâmetro importante que quantifica o quão eficiente é um novo agente na indução da resolução. Nesse trabalho, foi demonstrado que a superexpressão de GILZ, através da administração do peptídeo TAT-GILZ, e o inibidor específico de elastase, Sivelestat, foram capazes de diminuir os intervalos de resolução num modelo de inflamação aguda.

Alguns estudos sugerem que os efeitos reguladores de AnxA1 sobre a migração de leucócitos pode ser mediada por receptores FPR, uma vez que esses efeitos de AnxA1 sobre a transmigração podem ser revertidos por BOC-1 ou BOC-2, antagonistas não seletivos dos receptores FPR (Perretti *et al.*, 2001; Gavins *et al.*, 2005; Gastardelo *et al.*, 2009; Dufton *et al.*, 2010). Alguns estudos já demonstraram que a administração de BOC-1 está associada à inibição da ação de AnxA1 sobre o recrutamento de neutrófilos para o local da inflamação (Walther *et al.*, 2000; Souza *et al.*, 2007; Vago *et al.*, 2012). Em nosso trabalho, foi utilizado BOC-1 como uma estratégia farmacológica para inibir os efeitos da AnxA1 intacta, associada ao tratamento com anti-proteases. Foi observado que BOC-1 reverte o efeito pró-resolutivo do Sivelestat. Além disso, outra estratégia utilizada para bloquear os efeitos da AnxA1 foi através da administração de um anticorpo neutralizante. De maneira semelhante ao BOC-1, o anticorpo neutralizante foi capaz de inibir a resolução induzida por Sivelestat, uma vez que promoveu a persistência de neutrófilos na cavidade pleural após o tratamento com essa anti-protease. Além disso, ao bloquear a AnxA1 induzida por dexametasona em animais GILZ$^{-/-}$, foi observado que a resolução induzida por dexametasona foi refratária e de maneira semelhante aos animais WT. Esses achados sugerem que AnxA1 desempenha um papel importante na resolução da inflamação e sobrepõe os efeitos do GILZ endógeno.

Recentemente, foi demonstrado *in vitro* que GILZ é um mediador dos efeitos anti-inflamatórios de AnxA1. Yang e colaboradores (2009) mostraram que macrófagos deficientes em AnxA1 apresentavam níveis menores de GILZ quando comparados à macrófagos WT, ambos tratados com GCs (Yang *et al.*, 2009). Mais recentemente, um estudo demonstrou que a indução de GILZ mediada por AnxA1 foi mantida em presença do inibidor específico de FPR2 (utilizando o antagonista WRW4), sugerindo que AnxA1 possa induzir a expressão GILZ independentemente da ativação do seu receptor (Jia *et al.*, 2013). Nossos resultados mostrados aqui reforçam a ideia do envolvimento de AnxA1 na regulação da expressão de GILZ *in vivo*. Animais tratados com Ac2-26, peptídeo derivado da porção N-terminal de AnxA1, tiveram um aumento da expressão de GILZ em macrófagos. De maneira interessante, o bloqueio de AnxA1 através da administração de um anticorpo neutralizante, impediu a resolução induzida por dexametasona, associado à diminuição da expressão de GILZ. Curiosamente, animais GILZ$^{-/-}$, apresentaram níveis elevados de AnxA1 no exsudato inflamatório ou mesmo em condições não flogísticas. Tomados em conjunto, esses dados sugerem o envolvimento de AnxA1 na regulação de GILZ. No entanto, ainda não foi possível determinar exatamente como ocorre essa modulação da expressão de GILZ mediada por AnxA1. Pretendemos avaliar futuramente se AnxA1 está interferindo diretamente na transcrição gênica ou na regulação pós-transcricional de GILZ.

Já foi demonstrado que os efeitos adversos provenientes dos GCs dependem da via de transativação gênica (Vandevyver *et al.*, 2013). No entanto, a indução da expressão de proteínas anti-inflamatórias/pró-resolutivas também depende desse mesmo processo. Vale ressaltar que agonistas do receptor de GCs que não possuem o efeito de transativação, apresentam um efeito anti-inflamatório bem menor quando comparado aos GCs sintéticos já existentes. Neste trabalho, foi demonstrado que GILZ e AnxA1, proteínas induzidas por GCs, desempenham um papel importante na regulação do processo inflamatório, contribuindo para a resolução da resposta inflamatória. Dessa forma, nossa intenção foi investigar possíveis mecanismos alternativos pelos quais os GCs possam exercer seus efeitos anti-inflamatórios, abstraindo-se de seus efeitos adversos. O conhecimento das propriedades anti-inflamatórias/pró-resolutivas destas e de outras proteínas induzidas por GCs pode levar ao desenvolvimento de fármacos que extrairiam as características benéficas dos GCs excluindo os efeitos deletérios dos mesmos como aqueles sobre o metabolismo celular.

8 – CONCLUSÃO

Nossos dados demonstram que as proteínas GILZ e AnxA1 desempenham um papel fundamental na condução da resolução da inflamação neutrofílica. Mecanisticamente, GILZ e os inibidores de proteases resolvem a inflamação por indução da apoptose de neutrófilos, um efeito caspase-dependente, correlacionado com diminuição dos níveis de Mcl-1 e NF-κB. Para o nosso conhecimento é a primeira observação de que GILZ promove a apoptose de neutrófilos e de que os efeitos pró-resolutivos mediados por anti-proteases são dependentes de AnxA1. Assim, os resultados apresentados aqui reforçam a ideia de que GILZ e AnxA1 ou agentes que modulam AnxA1 endógena, como os inibidores de proteases, podem representar uma estratégia anti-inflamatória eficaz para o tratamento de doenças nas quais a acumulação de neutrófilos desempenham um papel relevante.

9 – REFERÊNCIAS BIBLIOGRÁFICAS

Aikawa N, Kawasaki Y (2014). Clinical utility of the neutrophil elastase inhibitor sivelestat for the treatment of acute respiratory distress syndrome. *Therapeutics and clinical risk management* **10:** 621-629.

Alessandri AL, Sousa LP, Lucas CD, Rossi AG, Pinho V, Teixeira MM (2013). Resolution of inflammation: mechanisms and opportunity for drug development. *Pharmacology & therapeutics* **139**(2)**:** 189-212.

Antonicelli F, De Coupade C, Russo-Marie F, Le Garrec Y (2001). CREB is involved in mouse annexin A1 regulation by cAMP and glucocorticoids. *European journal of biochemistry / FEBS* **268**(1)**:** 62-69.

Ariel A, Serhan CN (2012). New Lives Given by Cell Death: Macrophage Differentiation Following Their Encounter with Apoptotic Leukocytes during the Resolution of Inflammation. *Frontiers in immunology* **3:** 4.

Asselin-Labat ML, David M, Biola-Vidamment A, Lecoeuche D, Zennaro MC, Bertoglio J*, et al.* (2004). GILZ, a new target for the transcription factor FoxO3, protects T lymphocytes from interleukin-2 withdrawal-induced apoptosis. *Blood* **104**(1)**:** 215-223.

Ayroldi E, Migliorati G, Bruscoli S, Marchetti C, Zollo O, Cannarile L*, et al.* (2001). Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB. *Blood* **98**(3)**:** 743-753.

Ayroldi E, Riccardi C (2009). Glucocorticoid-induced leucine zipper (GILZ): a new important mediator of glucocorticoid action. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23**(11)**:** 3649-3658.

Ayroldi E, Zollo O, Bastianelli A, Marchetti C, Agostini M, Di Virgilio R*, et al.* (2007). GILZ mediates the antiproliferative activity of glucocorticoids by negative regulation of Ras signaling. *J Clin Invest* **117**(6)**:** 1605-1615.

Ayroldi E, Zollo O, Macchiarulo A, Di Marco B, Marchetti C, Riccardi C (2002). Glucocorticoid-induced leucine zipper inhibits the Raf-extracellular signal-regulated kinase pathway by binding to Raf-1. *Molecular and cellular biology* **22**(22)**:** 7929-7941.

Bank U, Ansorge S (2001). More than destructive: neutrophil-derived serine proteases in cytokine bioactivity control. *Journal of leukocyte biology* **69**(2)**:** 197-206.

Bannenberg GL, Chiang N, Ariel A, Arita M, Tjonahen E, Gotlinger KH*, et al.* (2005). Molecular circuits of resolution: formation and actions of resolvins and protectins. *Journal of immunology* **174**(7)**:** 4345-4355.

Barnes PJ (2011). Glucocorticosteroids: current and future directions. *British journal of pharmacology* **163**(1)**:** 29-43.

Beaulieu E, Morand EF (2011). Role of GILZ in immune regulation, glucocorticoid actions and rheumatoid arthritis. *Nature reviews. Rheumatology* **7**(6)**:** 340-348.

Berrebi D, Bruscoli S, Cohen N, Foussat A, Migliorati G, Bouchet-Delbos L*, et al.* (2003). Synthesis of glucocorticoid-induced leucine zipper (GILZ) by macrophages: an anti-inflammatory and immunosuppressive mechanism shared by glucocorticoids and IL-10. *Blood* **101**(2)**:** 729-738.

Best SM (2008). Viral subversion of apoptotic enzymes: escape from death row. *Annual review of microbiology* **62:** 171-192.

Blume KE, Soeroes S, Keppeler H, Stevanovic S, Kretschmer D, Rautenberg M*, et al.* (2012). Cleavage of annexin A1 by ADAM10 during secondary necrosis generates a monocytic "find-me" signal. *Journal of immunology* **188**(1)**:** 135-145.

Borregaard N (2010). Neutrophils, from marrow to microbes. *Immunity* **33**(5)**:** 657-670.

Borregaard N, Cowland JB (1997). Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89**(10)**:** 3503-3521.

Burg ND, Pillinger MH (2001). The neutrophil: function and regulation in innate and humoral immunity. *Clinical immunology* **99**(1)**:** 7-17.

Busillo JM, Cidlowski JA (2013). The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore. *Trends in endocrinology and metabolism: TEM* **24**(3)**:** 109-119.

Butler MW, Robertson I, Greene CM, O'Neill SJ, Taggart CC, McElvaney NG (2006). Elafin prevents lipopolysaccharide-induced AP-1 and NF-kappaB activation via an effect on the ubiquitin-proteasome pathway. *The Journal of biological chemistry* **281**(46)**:** 34730-34735.

Cannarile L, Zollo O, D'Adamio F, Ayroldi E, Marchetti C, Tabilio A*, et al.* (2001). Cloning, chromosomal assignment and tissue distribution of human GILZ, a glucocorticoid hormone-induced gene. *Cell death and differentiation* **8**(2)**:** 201-203.

Cheng Q, Morand E, Yang YH (2014). Development of novel treatment strategies for inflammatory diseases-similarities and divergence between glucocorticoids and GILZ. *Frontiers in pharmacology* **5:** 169.

Chow A, Brown BD, Merad M (2011). Studying the mononuclear phagocyte system in the molecular age. *Nature reviews. Immunology* **11**(11)**:** 788-798.

Clark AR (2007). Anti-inflammatory functions of glucocorticoid-induced genes. *Molecular and cellular endocrinology* **275**(1-2)**:** 79-97.

Cohen JJ (1993). Apoptosis: the physiologic pathway of cell death. *Hospital practice* **28**(12)**:** 35-43.

Cory S, Adams JM (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nature reviews. Cancer* **2**(9)**:** 647-656.

D'Adamio F, Zollo O, Moraca R, Ayroldi E, Bruscoli S, Bartoli A*, et al.* (1997). A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. *Immunity* **7**(6)**:** 803-812.

Dalli J, Consalvo AP, Ray V, Di Filippo C, D'Amico M, Mehta N*, et al.* (2013). Proresolving and tissue-protective actions of annexin A1-based cleavage-resistant peptides are mediated by formyl peptide receptor 2/lipoxin A4 receptor. *Journal of immunology* **190**(12)**:** 6478-6487.

Dalli J, Jones CP, Cavalcanti DM, Farsky SH, Perretti M, Rankin SM (2012). Annexin A1 regulates neutrophil clearance by macrophages in the mouse bone marrow. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **26**(1)**:** 387-396.

Damazo AS, Yona S, D'Acquisto F, Flower RJ, Oliani SM, Perretti M (2005). Critical protective role for annexin 1 gene expression in the endotoxemic murine microcirculation. *The American journal of pathology* **166**(6)**:** 1607-1617.

Delfino DV, Agostini M, Spinicelli S, Vito P, Riccardi C (2004). Decrease of Bcl-xL and augmentation of thymocyte apoptosis in GILZ overexpressing transgenic mice. *Blood* **104**(13)**:** 4134-4141.

Di Marco B, Massetti M, Bruscoli S, Macchiarulo A, Di Virgilio R, Velardi E*, et al.* (2007). Glucocorticoid-induced leucine zipper (GILZ)/NF-kappaB interaction: role of GILZ homo-dimerization and C-terminal domain. *Nucleic acids research* **35**(2)**:** 517-528.

Drechsler M, de Jong R, Rossaint J, Viola JR, Leoni G, Wang JM*, et al.* (2015). Annexin A1 counteracts chemokine-induced arterial myeloid cell recruitment. *Circulation research* **116**(5)**:** 827-835.

Duffin R, Leitch AE, Fox S, Haslett C, Rossi AG (2010). Targeting granulocyte apoptosis: mechanisms, models, and therapies. *Immunological reviews* **236:** 28-40.

Dufton N, Hannon R, Brancaleone V, Dalli J, Patel HB, Gray M*, et al.* (2010). Anti-inflammatory role of the murine formyl-peptide receptor 2: ligand-specific effects on leukocyte responses and experimental inflammation. *Journal of immunology* **184**(5)**:** 2611-2619.

Eddleston J, Herschbach J, Wagelie-Steffen AL, Christiansen SC, Zuraw BL (2007). The anti-inflammatory effect of glucocorticoids is mediated by glucocorticoid-induced leucine zipper in epithelial cells. *The Journal of allergy and clinical immunology* **119**(1)**:** 115-122.

El Kebir D, Jozsef L, Pan W, Wang L, Petasis NA, Serhan CN*, et al.* (2009). 15-epi-lipoxin A4 inhibits myeloperoxidase signaling and enhances resolution of acute lung injury. *American journal of respiratory and critical care medicine* **180**(4)**:** 311-319.

Espinasse MA, Pepin A, Virault-Rocroy P, Szely N, Chollet-Martin S, Pallardy M*, et al.* (2015). Glucocorticoid-Induced Leucine Zipper Is Expressed in Human Neutrophils and Promotes Apoptosis through Mcl-1 Down-Regulation. *Journal of innate immunity*.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *Journal of immunology* **148**(7)**:** 2207-2216.

Flower RJ, Blackwell GJ (1979). Anti-inflammatory steroids induce biosynthesis of a phospholipase A2 inhibitor which prevents prostaglandin generation. *Nature* **278**(5703)**:** 456-459.

Fox S, Leitch AE, Duffin R, Haslett C, Rossi AG (2010). Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. *Journal of innate immunity* **2**(3)**:** 216-227.

Gastardelo TS, Damazo AS, Dalli J, Flower RJ, Perretti M, Oliani SM (2009). Functional and ultrastructural analysis of annexin A1 and its receptor in extravasating neutrophils during acute inflammation. *The American journal of pathology* **174**(1)**:** 177-183.

Gavins FN, Sawmynaden P, Chatterjee BE, Perretti M (2005). A twist in anti-inflammation: annexin 1 acts via the lipoxin A4 receptor. *Prostaglandins, leukotrienes, and essential fatty acids* **73**(3-4)**:** 211-219.

Gerke V, Moss SE (2002). Annexins: from structure to function. *Physiological reviews* **82**(2)**:** 331-371.

Gilroy DW, Lawrence T, Perretti M, Rossi AG (2004). Inflammatory resolution: new opportunities for drug discovery. *Nature reviews. Drug discovery* **3**(5)**:** 401-416.

Godson C, Mitchell S, Harvey K, Petasis NA, Hogg N, Brady HR (2000). Cutting edge: lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. *Journal of immunology* **164**(4)**:** 1663-1667.

Goulding NJ, Godolphin JL, Sharland PR, Peers SH, Sampson M, Maddison PJ*, et al.* (1990). Anti-inflammatory lipocortin 1 production by peripheral blood leucocytes in response to hydrocortisone. *Lancet* **335**(8703)**:** 1416-1418.

Greene CM, McElvaney NG (2009). Proteases and antiproteases in chronic neutrophilic lung disease - relevance to drug discovery. *British journal of pharmacology* **158**(4)**:** 1048-1058.

Gross KL, Lu NZ, Cidlowski JA (2009). Molecular mechanisms regulating glucocorticoid sensitivity and resistance. *Molecular and cellular endocrinology* **300**(1-2)**:** 7-16.

Grugan KD, Ma C, Singhal S, Krett NL, Rosen ST (2008). Dual regulation of glucocorticoid-induced leucine zipper (GILZ) by the glucocorticoid receptor and the PI3-kinase/AKT pathways in multiple myeloma. *The Journal of steroid biochemistry and molecular biology* **110**(3-5)**:** 244-254.

Hagiwara S, Iwasaka H, Hidaka S, Hasegawa A, Noguchi T (2009). Neutrophil elastase inhibitor (sivelestat) reduces the levels of inflammatory mediators by inhibiting NF-kB. *Inflammation research : official journal of the European Histamine Research Society ... [et al.]* **58**(4)**:** 198-203.

Hallett JM, Leitch AE, Riley NA, Duffin R, Haslett C, Rossi AG (2008). Novel pharmacological strategies for driving inflammatory cell apoptosis and enhancing the resolution of inflammation. *Trends in pharmacological sciences* **29**(5)**:** 250-257.

Harris JG, Flower RJ, Perretti M (1995). Alteration of neutrophil trafficking by a lipocortin 1 N-terminus peptide. *European journal of pharmacology* **279**(2-3)**:** 149-157.

Hayakawa M, Katabami K, Wada T, Sugano M, Hoshino H, Sawamura A*, et al.* (2010). Sivelestat (selective neutrophil elastase inhibitor) improves the mortality rate of sepsis associated with both acute respiratory distress syndrome and disseminated intravascular coagulation patients. *Shock* **33**(1)**:** 14-18.

Hayhoe RP, Kamal AM, Solito E, Flower RJ, Cooper D, Perretti M (2006). Annexin 1 and its bioactive peptide inhibit neutrophil-endothelium interactions under flow: indication of distinct receptor involvement. *Blood* **107**(5)**:** 2123-2130.

Headland SE, Norling LV (2015). The resolution of inflammation: Principles and challenges. *Seminars in immunology* **27**(3)**:** 149-160.

Henriksen PA (2014). The potential of neutrophil elastase inhibitors as anti-inflammatory therapies. *Current opinion in hematology* **21**(1)**:** 23-28.

Hoppstadter J, Diesel B, Eifler LK, Schmid T, Brune B, Kiemer AK (2012). Glucocorticoid-induced leucine zipper is downregulated in human alveolar macrophages upon Toll-like receptor activation. *European journal of immunology* **42**(5)**:** 1282-1293.

Huang KS, McGray P, Mattaliano RJ, Burne C, Chow EP, Sinclair LK*, et al.* (1987). Purification and characterization of proteolytic fragments of lipocortin I that inhibit phospholipase A2. *The Journal of biological chemistry* **262**(16)**:** 7639-7645.

Iba T, Kidokoro A, Fukunaga M, Takuhiro K, Yoshikawa S, Sugimotoa K (2006). Pretreatment of sivelestat sodium hydrate improves the lung microcirculation and alveolar damage in lipopolysaccharide-induced acute lung inflammation in hamsters. *Shock* **26**(1)**:** 95-98.

Jia Y, Morand EF, Song W, Cheng Q, Stewart A, Yang YH (2013). Regulation of lung fibroblast activation by annexin A1. *Journal of cellular physiology* **228**(2)**:** 476-484.

Joha S, Nugues AL, Hetuin D, Berthon C, Dezitter X, Dauphin V*, et al.* (2012). GILZ inhibits the mTORC2/AKT pathway in BCR-ABL(+) cells. *Oncogene* **31**(11)**:** 1419-1430.

Kadmiel M, Cidlowski JA (2013). Glucocorticoid receptor signaling in health and disease. *Trends in pharmacological sciences* **34**(9)**:** 518-530.

Kambe M, Bessho R, Fujii M, Ochi M, Shimizu K (2009). Sivelestat reduces myocardial ischemia and reperfusion injury in rat hearts even when administered after onset of myocardial ischemia. *Interactive cardiovascular and thoracic surgery* **8**(6)**:** 629-634.

Kim S, Ko J, Kim JH, Choi EC, Na DS (2001). Differential effects of annexins I, II, III, and V on cytosolic phospholipase A2 activity: specific interaction model. *FEBS letters* **489**(2-3)**:** 243-248.

Kono H, Rock KL (2008). How dying cells alert the immune system to danger. *Nature reviews. Immunology* **8**(4)**:** 279-289.

Korkmaz B, Horwitz MS, Jenne DE, Gauthier F (2010). Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. *Pharmacological reviews* **62**(4)**:** 726-759.

Labonte AC, Tosello-Trampont AC, Hahn YS (2014). The role of macrophage polarization in infectious and inflammatory diseases. *Molecules and cells* **37**(4)**:** 275-285.

Lebson L, Wang T, Jiang Q, Whartenby KA (2011). Induction of the glucocorticoid-induced leucine zipper gene limits the efficacy of dendritic cell vaccines. *Cancer gene therapy* **18**(8)**:** 563-570.

Lee KH, Na DS, Kim JW (1999). Calcium-dependent interaction of annexin I with annexin II and mapping of the interaction sites. *FEBS letters* **442**(2-3)**:** 143-146.

Lichtnekert J, Kawakami T, Parks WC, Duffield JS (2013). Changes in macrophage phenotype as the immune response evolves. *Current opinion in pharmacology* **13**(4)**:** 555-564.

Ligr M, Li Y, Logan SK, Taneja S, Melamed J, Lepor H*, et al.* (2012). Mifepristone inhibits GRbeta coupled prostate cancer cell proliferation. *The Journal of urology* **188**(3)**:** 981-988.

Lopez-Otin C, Bond JS (2008). Proteases: multifunctional enzymes in life and disease. *The Journal of biological chemistry* **283**(45)**:** 30433-30437.

Lucas CD, Allen KC, Dorward DA, Hoodless LJ, Melrose LA, Marwick JA*, et al.* (2013). Flavones induce neutrophil apoptosis by down-regulation of Mcl-1 via a proteasomal-dependent pathway. *FASEB J* **27**(3)**:** 1084-1094.

Maderna P, Yona S, Perretti M, Godson C (2005). Modulation of phagocytosis of apoptotic neutrophils by supernatant from dexamethasone-treated macrophages and annexin-derived peptide Ac(2-26). *Journal of immunology* **174**(6)**:** 3727-3733.

Mancek-Keber M (2014). Inflammation-Mediating Proteases: Structure, Function in (Patho)Physiology and Inhibition. *Protein and peptide letters*.

Marchalonis JJ, Kaveri S, Lacroix-Desmazes S, Kazatchkine MD (2002). Natural recognition repertoire and the evolutionary emergence of the combinatorial immune system. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **16**(8)**:** 842-848.

Marinissen MJ, Gutkind JS (2001). G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends in pharmacological sciences* **22**(7)**:** 368-376.

McArthur S, Gobbetti T, Kusters DH, Reutelingsperger CP, Flower RJ, Perretti M (2015). Definition of a Novel Pathway Centered on Lysophosphatidic Acid To Recruit Monocytes during the Resolution Phase of Tissue Inflammation. *Journal of immunology* **195**(3)**:** 1139-1151.

Medzhitov R (2010). Inflammation 2010: new adventures of an old flame. *Cell* **140**(6)**:** 771-776.

Milot E, Filep JG (2011). Regulation of neutrophil survival/apoptosis by Mcl-1. *TheScientificWorldJournal* **11:** 1948-1962.

Mittelstadt PR, Ashwell JD (2001). Inhibition of AP-1 by the glucocorticoid-inducible protein GILZ. *The Journal of biological chemistry* **276**(31)**:** 29603-29610.

Morand EF, Jefferiss CM, Dixey J, Mitra D, Goulding NJ (1994). Impaired glucocorticoid induction of mononuclear leukocyte lipocortin-1 in rheumatoid arthritis. *Arthritis and rheumatism* **37**(2)**:** 207-211.

Morimoto N, Morimoto K, Morimoto Y, Takahashi H, Asano M, Matsumori M*, et al.* (2008). Sivelestat attenuates postoperative pulmonary dysfunction after total arch replacement under deep hypothermia. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* **34**(4)**:** 798-804.

Mosser DM, Edwards JP (2008). Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology* **8**(12)**:** 958-969.

Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S*, et al.* (2014). Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* **41**(1)**:** 14-20.

Nathan C (2002). Points of control in inflammation. *Nature* **420**(6917)**:** 846-852.

Nathan C (2008). Metchnikoff's Legacy in 2008. *Nature immunology* **9**(7)**:** 695-698.

Norling LV, Serhan CN (2010). Profiling in resolving inflammatory exudates identifies novel anti-inflammatory and pro-resolving mediators and signals for termination. *Journal of internal medicine* **268**(1)**:** 15-24.

Nourshargh S, Marelli-Berg FM (2005). Transmigration through venular walls: a key regulator of leukocyte phenotype and function. *Trends in immunology* **26**(3)**:** 157-165.

Oakley RH, Cidlowski JA (2011). Cellular processing of the glucocorticoid receptor gene and protein: new mechanisms for generating tissue-specific actions of glucocorticoids. *The Journal of biological chemistry* **286**(5)**:** 3177-3184.

Oakley RH, Cidlowski JA (2013). The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. *The Journal of allergy and clinical immunology* **132**(5)**:** 1033-1044.

Oliani SM, Paul-Clark MJ, Christian HC, Flower RJ, Perretti M (2001). Neutrophil interaction with inflamed postcapillary venule endothelium alters annexin 1 expression. *The American journal of pathology* **158**(2)**:** 603-615.

Parente L, Solito E (2004). Annexin 1: more than an anti-phospholipase protein. *Inflammation research : official journal of the European Histamine Research Society ... [et al.]* **53**(4)**:** 125-132.

Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A*, et al.* (2004). Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *The Journal of biological chemistry* **279**(9)**:** 7370-7377.

Patel HB, Kornerup KN, Sampaio AL, D'Acquisto F, Seed MP, Girol AP*, et al.* (2012). The impact of endogenous annexin A1 on glucocorticoid control of inflammatory arthritis. *Annals of the rheumatic diseases* **71**(11)**:** 1872-1880.

Pederzoli-Ribeil M, Maione F, Cooper D, Al-Kashi A, Dalli J, Perretti M*, et al.* (2010). Design and characterization of a cleavage-resistant Annexin A1 mutant to control inflammation in the microvasculature. *Blood* **116**(20)**:** 4288-4296.

Peers SH, Smillie F, Elderfield AJ, Flower RJ (1993). Glucocorticoid-and non-glucocorticoid induction of lipocortins (annexins) 1 and 2 in rat peritoneal leucocytes in vivo. *British journal of pharmacology* **108**(1)**:** 66-72.

Perretti M, D'Acquisto F (2009). Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nature reviews. Immunology* **9**(1)**:** 62-70.

Perretti M, Flower RJ (1996). Measurement of lipocortin 1 levels in murine peripheral blood leukocytes by flow cytometry: modulation by glucocorticoids and inflammation. *British journal of pharmacology* **118**(3)**:** 605-610.

Perretti M, Flower RJ (2004). Annexin 1 and the biology of the neutrophil. *Journal of leukocyte biology* **76**(1)**:** 25-29.

Perretti M, Gavins FN (2003). Annexin 1: an endogenous anti-inflammatory protein. *News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society* **18:** 60-64.

Perretti M, Getting SJ, Solito E, Murphy PM, Gao JL (2001). Involvement of the receptor for formylated peptides in the in vivo anti-migratory actions of annexin 1 and its mimetics. *The American journal of pathology* **158**(6)**:** 1969-1973.

Petri B, Phillipson M, Kubes P (2008). The physiology of leukocyte recruitment: an in vivo perspective. *Journal of immunology* **180**(10)**:** 6439-6446.

Pham CT (2006). Neutrophil serine proteases: specific regulators of inflammation. *Nature reviews. Immunology* **6**(7)**:** 541-550.

Poon IK, Lucas CD, Rossi AG, Ravichandran KS (2014). Apoptotic cell clearance: basic biology and therapeutic potential. *Nature reviews. Immunology* **14**(3)**:** 166-180.

Prince LR, Whyte MK, Sabroe I, Parker LC (2011). The role of TLRs in neutrophil activation. *Current opinion in pharmacology* **11**(4)**:** 397-403.

Randolph GJ (2014). A macrophage revolution-and beyond. *Immunological reviews* **262**(1)**:** 5-8.

Raynal P, Pollard HB (1994). Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochimica et biophysica acta* **1197**(1)**:** 63-93.

Reis AC, Alessandri AL, Athayde RM, Perez DA, Vago JP, Avila TV, *et al.* (2015). Induction of eosinophil apoptosis by hydrogen peroxide promotes the resolution of allergic inflammation. *Cell death & disease* **6:** e1632.

Riccardi C, Bruscoli S, Ayroldi E, Agostini M, Migliorati G (2001). GILZ, a glucocorticoid hormone induced gene, modulates T lymphocytes activation and death through interaction with NF-kB. *Advances in experimental medicine and biology* **495:** 31-39.

Rossi AG, Hallett JM, Sawatzky DA, Teixeira MM, Haslett C (2007). Modulation of granulocyte apoptosis can influence the resolution of inflammation. *Biochemical Society transactions* **35**(Pt 2)**:** 288-291.

Rossi AG, Sawatzky DA, Walker A, Ward C, Sheldrake TA, Riley NA*, et al.* (2006). Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. *Nature medicine* **12**(9)**:** 1056-1064.

Sallenave JM (2000). The role of secretory leukocyte proteinase inhibitor and elafin (elastase-specific inhibitor/skin-derived antileukoprotease) as alarm antiproteinases in inflammatory lung disease. *Respiratory research* **1**(2)**:** 87-92.

Savill J, Fadok V (2000). Corpse clearance defines the meaning of cell death. *Nature* **407**(6805)**:** 784-788.

Scannell M, Flanagan MB, deStefani A, Wynne KJ, Cagney G, Godson C*, et al.* (2007). Annexin-1 and peptide derivatives are released by apoptotic cells and stimulate phagocytosis of apoptotic neutrophils by macrophages. *Journal of immunology* **178**(7)**:** 4595-4605.

Schif-Zuck S, Gross N, Assi S, Rostoker R, Serhan CN, Ariel A (2011). Saturated-efferocytosis generates pro-resolving CD11b low macrophages: modulation by resolvins and glucocorticoids. *European journal of immunology* **41**(2)**:** 366-379.

Serhan CN, Brain SD, Buckley CD, Gilroy DW, Haslett C, O'Neill LA*, et al.* (2007). Resolution of inflammation: state of the art, definitions and terms. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **21**(2)**:** 325-332.

Silverman MN, Sternberg EM (2012). Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoid receptor dysfunction. *Annals of the New York Academy of Sciences* **1261:** 55-63.

Simon SI, Green CE (2005). Molecular mechanics and dynamics of leukocyte recruitment during inflammation. *Annual review of biomedical engineering* **7:** 151-185.

Soehnlein O, Lindbom L (2010). Phagocyte partnership during the onset and resolution of inflammation. *Nature reviews. Immunology* **10**(6)**:** 427-439.

Solito E, de Coupade C, Canaider S, Goulding NJ, Perretti M (2001). Transfection of annexin 1 in monocytic cells produces a high degree of spontaneous and stimulated apoptosis associated with caspase-3 activation. *British journal of pharmacology* **133**(2)**:** 217-228.

Solito E, de Coupade C, Parente L, Flower RJ, Russo-Marie F (1998). IL-6 stimulates annexin 1 expression and translocation and suggests a new biological role as class II acute phase protein. *Cytokine* **10**(7)**:** 514-521.

Solito E, Kamal A, Russo-Marie F, Buckingham JC, Marullo S, Perretti M (2003). A novel calcium-dependent proapoptotic effect of annexin 1 on human neutrophils. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **17**(11)**:** 1544-1546.

Solito E, Romero IA, Marullo S, Russo-Marie F, Weksler BB (2000). Annexin 1 binds to U937 monocytic cells and inhibits their adhesion to microvascular endothelium: involvement of the alpha 4 beta 1 integrin. *Journal of immunology* **165**(3)**:** 1573-1581.

Soundararajan R, Wang J, Melters D, Pearce D (2007). Differential activities of glucocorticoid-induced leucine zipper protein isoforms. *The Journal of biological chemistry* **282**(50)**:** 36303-36313.

Sousa LP, Alessandri AL, Pinho V, Teixeira MM (2013). Pharmacological strategies to resolve acute inflammation. *Current opinion in pharmacology* **13**(4)**:** 625-631.

Sousa LP, Carmo AF, Rezende BM, Lopes F, Silva DM, Alessandri AL*, et al.* (2009). Cyclic AMP enhances resolution of allergic pleurisy by promoting inflammatory cell apoptosis via inhibition of PI3K/Akt and NF-kappaB. *Biochemical pharmacology* **78**(4)**:** 396-405.

Sousa LP, Lopes F, Silva DM, Tavares LP, Vieira AT, Rezende BM*, et al.* (2010). PDE4 inhibition drives resolution of neutrophilic inflammation by inducing apoptosis in a PKA-PI3K/Akt-dependent and NF-kappaB-independent manner. *Journal of leukocyte biology* **87**(5)**:** 895-904.

Souza DG, Fagundes CT, Amaral FA, Cisalpino D, Sousa LP, Vieira AT*, et al.* (2007). The required role of endogenously produced lipoxin A4 and annexin-1 for the production of IL-10 and inflammatory hyporesponsiveness in mice. *Journal of immunology* **179**(12)**:** 8533-8543.

Suarez F, Rothhut B, Comera C, Touqui L, Marie FR, Silve C (1993). Expression of annexin I, II, V, and VI by rat osteoblasts in primary culture: stimulation of annexin I expression by dexamethasone. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **8**(10)**:** 1201-1210.

Sugimoto MA, Vago JP, Teixeira MM, and Sousa LP (2016). Annexin A1 and the Resolution of Inflammation: Modulation of Neutrophil Recruitment, Apoptosis, and Clearance. *Journal of Immunology Research*, vol. 2016(13):10.1155/2016/8239258.

Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER (2010). Neutrophil kinetics in health and disease. *Trends in immunology* **31**(8)**:** 318-324.

Taggart CC, Cryan SA, Weldon S, Gibbons A, Greene CM, Kelly E*, et al.* (2005). Secretory leucoprotease inhibitor binds to NF-kappaB binding sites in monocytes and inhibits p65 binding. *The Journal of experimental medicine* **202**(12)**:** 1659-1668.

Tak T, Tesselaar K, Pillay J, Borghans JA, Koenderman L (2013). What's your age again? Determination of human neutrophil half-lives revisited. *Journal of leukocyte biology* **94**(4)**:** 595-601.

Taves MD, Gomez-Sanchez CE, Soma KK (2011). Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. *American journal of physiology. Endocrinology and metabolism* **301**(1)**:** E11-24.

Thiagarajah AS, Eades LE, Thomas PR, Guymer EK, Morand EF, Clarke DM*, et al.* (2014). GILZ: Glitzing up our understanding of the glucocorticoid receptor in psychopathology. *Brain research* **1574:** 60-69.

Tsao FH, Meyer KC, Chen X, Rosenthal NS, Hu J (1998). Degradation of annexin I in bronchoalveolar lavage fluid from patients with cystic fibrosis. *American journal of respiratory cell and molecular biology* **18**(1)**:** 120-128.

Tsujii S, Okabayashi T, Shiga M, Takezaki Y, Sugimoto T, Kobayashi M*, et al.* (2012). The effect of the neutrophil elastase inhibitor sivelestat on early injury after liver resection. *World journal of surgery* **36**(5)**:** 1122-1127.

Twigg MS, Brockbank S, Lowry P, FitzGerald SP, Taggart C, Weldon S (2015). The Role of Serine Proteases and Antiproteases in the Cystic Fibrosis Lung. *Mediators of inflammation* **2015:** 293053.

Tynan SH, Lundeen SG, Allan GF (2004). Cell type-specific bidirectional regulation of the glucocorticoid-induced leucine zipper (GILZ) gene by estrogen. *The Journal of steroid biochemistry and molecular biology* **91**(4-5)**:** 225-239.

Uchida Y, Freitas MC, Zhao D, Busuttil RW, Kupiec-Weglinski JW (2010). The protective function of neutrophil elastase inhibitor in liver ischemia/reperfusion injury. *Transplantation* **89**(9)**:** 1050-1056.

Vachon E, Bourbonnais Y, Bingle CD, Rowe SJ, Janelle MF, Tremblay GM (2002). Anti-inflammatory effect of pre-elafin in lipopolysaccharide-induced acute lung inflammation. *Biological chemistry* **383**(7-8)**:** 1249-1256.

Vago JP, Nogueira CR, Tavares LP, Soriani FM, Lopes F, Russo RC*, et al.* (2012). Annexin A1 modulates natural and glucocorticoid-induced resolution of inflammation by enhancing neutrophil apoptosis. *Journal of leukocyte biology* **92**(2)**:** 249-258.

Vago JP, Tavares LP, Garcia CC, Lima KM, Perucci LO, Vieira EL*, et al.* (2015). The role and effects of glucocorticoid-induced leucine zipper in the context of inflammation resolution. *Journal of immunology* **194**(10)**:** 4940-4950.

Vandevyver S, Dejager L, Tuckermann J, Libert C (2013). New insights into the anti-inflammatory mechanisms of glucocorticoids: an emerging role for glucocorticoid-receptor-mediated transactivation. *Endocrinology* **154**(3)**:** 993-1007.

Viengchareun S, Le Menuet D, Martinerie L, Munier M, Pascual-Le Tallec L, Lombes M (2007). The mineralocorticoid receptor: insights into its molecular and (patho)physiological biology. *Nuclear receptor signaling* **5:** e012.

Vong L, D'Acquisto F, Pederzoli-Ribeil M, Lavagno L, Flower RJ, Witko-Sarsat V*, et al.* (2007). Annexin 1 cleavage in activated neutrophils: a pivotal role for proteinase 3. *The Journal of biological chemistry* **282**(41)**:** 29998-30004.

Walther A, Riehemann K, Gerke V (2000a). A novel ligand of the formyl peptide receptor: annexin I regulates neutrophil extravasation by interacting with the FPR. *Molecular cell* **5**(5)**:** 831-840.

Walther A, Riehemann K, Gerke V (2000b). A Novel Ligand of the Formyl Peptide Receptor: Annexin I Regulates Neutrophil Extravasation by Interacting with the FPR. *Molecular Cell* **5**(5)**:** 831-840.

Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F*, et al.* (1999). Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. *Science* **284**(5412)**:** 339-343.

Wang JC, Derynck MK, Nonaka DF, Khodabakhsh DB, Haqq C, Yamamoto KR (2004). Chromatin immunoprecipitation (ChIP) scanning identifies primary glucocorticoid receptor target genes. *Proceedings of the National Academy of Sciences of the United States of America* **101**(44)**:** 15603-15608.

Wang ZQ, Chen LQ, Yuan Y, Wang WP, Niu ZX, Yang YS*, et al.* (2015). Effects of neutrophil elastase inhibitor in patients undergoing esophagectomy: a systematic review and meta-analysis. *World journal of gastroenterology* **21**(12)**:** 3720-3730.

Wiedow O, Schroder JM, Gregory H, Young JA, Christophers E (1990). Elafin: an elastase-specific inhibitor of human skin. Purification, characterization, and complete amino acid sequence. *The Journal of biological chemistry* **265**(25)**:** 14791-14795.

Williams SE, Brown TI, Roghanian A, Sallenave JM (2006). SLPI and elafin: one glove, many fingers. *Clinical science* **110**(1)**:** 21-35.

Williams SL, Milne IR, Bagley CJ, Gamble JR, Vadas MA, Pitson SM*, et al.* (2010). A proinflammatory role for proteolytically cleaved annexin A1 in neutrophil transendothelial migration. *Journal of immunology* **185**(5)**:** 3057-3063.

Wynn TA, Chawla A, Pollard JW (2013). Macrophage biology in development, homeostasis and disease. *Nature* **496**(7446)**:** 445-455.

Yang YH, Aeberli D, Dacumos A, Xue JR, Morand EF (2009). Annexin-1 regulates macrophage IL-6 and TNF via glucocorticoid-induced leucine zipper. *Journal of immunology* **183**(2)**:** 1435-1445.

Yang YH, Morand EF, Getting SJ, Paul-Clark M, Liu DL, Yona S*, et al.* (2004). Modulation of inflammation and response to dexamethasone by Annexin 1 in antigen-induced arthritis. *Arthritis and rheumatism* **50**(3)**:** 976-984.

Yasui S, Nagai A, Aoshiba K, Ozawa Y, Kakuta Y, Konno K (1995). A specific neutrophil elastase inhibitor (ONO-5046.Na) attenuates LPS-induced acute lung inflammation in the hamster. *The European respiratory journal* **8**(8)**:** 1293-1299.

Yoshimura Y, Hiramatsu Y, Sato Y, Homma S, Enomoto Y, Jikuya T*, et al.* (2003). ONO-6818, a novel, potent neutrophil elastase inhibitor, reduces inflammatory mediators during simulated extracorporeal circulation. *The Annals of thoracic surgery* **76**(4)**:** 1234-1239.

Yuan SY, Shen Q, Rigor RR, Wu MH (2012). Neutrophil transmigration, focal adhesion kinase and endothelial barrier function. *Microvascular research* **83**(1)**:** 82-88.

Yudt MR, Cidlowski JA (2001). Molecular identification and characterization of a and b forms of the glucocorticoid receptor. *Molecular endocrinology* **15**(7)**:** 1093-1103.

Zhang XH, Lu X, Long XB, You XJ, Gao QX, Cui YH*, et al.* (2009). Chronic rhinosinusitis with and without nasal polyps is associated with decreased expression of glucocorticoid-induced leucine zipper. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **39**(5)**:** 647-654.

Zlotnik A, Yoshie O, Nomiyama H (2006). The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome biology* **7**(12)**:** 243.

9 – PRODUÇÃO CIENTÍFICA

1- **Vago JP**, Tavares LP, Garcia CC, Lima KM, Perucci LO, Vieira ÉL, *et al.* (2015). The role and effects of glucocorticoid-induced leucine zipper in the context of inflammation resolution *Journal of immunology*. 2015 May 15;194(10):4940-50.

2- Lacerda-Queiroz N, Brant F, Rodrigues DH, **Vago JP**, Rachid MA, Sousa LP, Teixeira MM, Teixeira AL. (2015). Phosphatidylinositol 3-Kinase γ is required for the development of experimental cerebral malaria. *PLoS One*. 2015 Mar 16;10(3):e0119633.

3- Isabel Vieira de Assis Lima, Alline Cristina Campos, Aline Silva Miranda, Érica Leandro Marciano, Flávia Mendes, **Juliana Priscila Vago**, Lirlândia Pires de Sousa, , *et al.* (2015). PI3Kγ deficiency enhances seizures severity and associated outcomes in a mouse model of convulsions induced by intrahippocampal injection of pilocarpine. *Exp Neurol.* 2015 May;267:123-34. doi: 10.1016/j.expneurol.2015.02.021.

4- Alesandra Reis, Ana Letícia Alessandri, Rayssa Athayde, Denise Perez, **Juliana Vago**, Thiago Ávila, *et al.* (2015). Induction of eosinophil apoptosis by hydrogen peroxide promotes the resolution of allergic inflammation. *Cell Death Dis*. 2015 Feb 12;6:e1632. doi: 10.1038/cddis.2014.580.

5- Perez DA, **Vago JP**, Athayde RM, Reis AC, Teixeira MM, Sousa LP*, et al.* (2014). Switching off key signaling survival molecules to switch on the resolution of inflammation. *Mediators of inflammation* 2014: 829851.

6- Carmo AA, Costa BR, **Vago JP**, de Oliveira LC, Tavares LP, Nogueira CR*, et al.* (2014). Plasmin induces in vivo monocyte recruitment through protease-activated receptor-1-, MEK/ERK-, and CCR2-mediated signaling. *Journal of immunology* 193(7): 3654-3663.

7- Barbosa IG, Nogueira CR, Rocha NP, Queiroz AL, **Vago JP**, Tavares LP*, et al.* (2013). Altered intracellular signaling cascades in peripheral blood mononuclear cells from BD patients. *Journal of psychiatric research* 47(12): 1949-1954.

8- **Vago JP**, Nogueira CR, Tavares LP, Soriani FM, Lopes F, Russo RC*, et al.* (2012). Annexin A1 modulates natural and glucocorticoid-induced resolution of inflammation by enhancing neutrophil apoptosis. *Journal of leukocyte biology* 92(2): 249-258.

9- de Miranda AS, Rodrigues DH, Amaral DC, de Lima Campos RD, Cisalpino D, Vilela MC, Lacerda-Queiroz N, de Souza KP, **Vago JP**, Campos MA, Kroon EG, da Glória de Souza D, Teixeira MM, Teixeira AL, Rachid MA. (2012). Dengue-3 encephalitis promotes anxiety-like behavior in mice. *Behavioural brain research* 230(1): 237-242.

10 – ANEXOS

ANEXO A - Comprovante de submissão de trabalho científico

The Journal of Immunology

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This is a revised version of a previously reviewed manuscript.

Manuscript #	15-00886-FLR
Current Revision #	1
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Submission Date	2015-12-02 08:30:45
Current Stage	Under Review
Title	Proresolving actions of synthetic and natural protease inhibitors are mediated by Annexin A1
Running Title	Protease inhibitors evoke inflammation resolution
Manuscript Type	Full Length
Category	Innate Immunity and Inflammation
Corresponding Author	Mauro Teixeira (Universidade Federal de Minas Gerais)
Contributing Authors	Juliana Vago Luciana TAVARES Michelle Sugimoto Graziele Lima Izabela Galvao Thaís de Caux Kátia Lima Ana Luíza Ribeiro Fernanda Carneiro Fernanda Nunes Vanessa Pinho Mauro Perretti Lirlândia Sousa
Abstract	Annexin-A1 (AnxA1) is a glucocorticoid-regulated protein endowed with anti-inflammatory and proresolving properties. Intact AnxA1 is a 37 kDa protein that may be cleaved in vivo at the N-terminal region by neutrophil proteases including elastase and proteinase-3 (PR3), generating the 33-kDa isoform that is largely inactive. In this study, we investigated the dynamics of AnxA1 expression and the effects of synthetic (Sivelestat, Eglin) and natural (secretory leukocyte protease inhibitor [SLPI] and Elafin) protease inhibitors on the resolution of LPS-induced neutrophil inflammation. During the settings of LPS-induced inflammation AnxA1 cleavage associated closely with the peak of neutrophil and elastase expression and activity. SLPI expression increased during resolving phase of the pleural inflammation. Therapeutic treatment of LPS-challenge mice with rhSLPI or Elafin accelerated resolution, an effect associated with increased numbers of apoptotic neutrophils in the pleural exudates, inhibition of elastase, and modulation of the survival-controlling proteins NF-κB and Mcl-1. Similar effects were observed

ANEXO B - Certificado do Comitê de Ética Experimental

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL
- C E T E A -

CERTIFICADO

Certificamos que o **Protocolo CEUA nº 15/2011,** relativo ao projeto intitulado "***ESTUDO DO PAPEL DE PROTEÍNAS INDUZIDAS POR GLICOCORTICÓIDES NA RESOLUÇÃO DA RESPOSTA INFLAMATÓRIA AGUDA***", que tem como responsável(is) **Lirlândia Pires de Sousa** , está(ão) de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo ***Comitê de Ética em Experimentação Animal*** **(CETEA/UFMG),** tendo sido aprovado na reunião de **7/ 12/2011.**

Este certificado expira-se em **7/ 12/ 2016.**

CERTIFICATE

We hereby certify that the **Protocol CEUA nº 15/2011,** related to the project entitled "***ROLE OF GLUCOCORTICOID-INDUCED PROTEINS ON RESOLUTION OF ACUTE INFLAMMATION***", under the supervisiors of **Lirlândia Pires de Sousa,** is in agreement with the Ethical Principles in Animal Experimentation, adopted by the ***Ethics Committee in Animal Experimentation*** **(CETEA/UFMG),** and was approved in **December 7, 2011.**

This certificate expires in **December 7, 2016.**

Belo Horizonte, 12 de Dezembro de 2011.

Profª. Jacqueline Isaura Alvarez-Leite
Coordenadora do CETEA/UFMG