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INVESTIGAÇÃO DOS EFEITOS DO PEPTÍDEO LIBERADOR DE GASTRINA (GRP) E SEU ANTAGONISTA RC-3095 EM CÉLULAS MIELOIDES

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Tese apresentada como requisito para a obtenção do grau de Doutor pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul

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“If I have seen further, it is by standing on the shoulders of giants.

Isaac Newton,1676

“No amount of experimentation can ever prove me right;

a single experiment can prove me wrong”.

Albert Einstein

“Happiness is real...”

Gaetan Barbet, 2014

RESUMO

O microambiente tumoral e as doenças inflamatórias promovem alterações nas células do nosso sistema imune à medida que progridem. Diversas moléculas estão envolvidas nessa modulação, e por isso são alvos terapêuticos. O peptídeo liberador de gastrina (GRP) é produzido por tumores, onde promove proliferação celular. Este também está correlacionado com doenças crônicas como a artrite reumatoide e asma, e em doenças agudas, como a sepse. Recentemente, nosso grupo descobriu ação direta do GRP em neutrófilos, promovendo indução de migração. O presente trabalho se propôs a estudar a interface entre tumores produtores de GRP e o recrutamento celular, assim como aprofundar os estudos celulares sobre os processos de ativação e migração de neutrófilos promovidos pelo peptídeo. Em tumores, observamos que uma linhagem de adenocarcinoma pulmonar não prolifera quando exposto ao GRP, porém é induzida a migrar quando exposta ao peptídeo, estabelecendo um potencial papel deste na promoção de metástases para esse tipo tumoral. Na interface da imunologia tumoral, através do desenvolvimento de um modelo *in vivo* de superexpressão de GRP em melanoma murino (B16F10), observamos que esse aumento do GRP induz a infiltração de monócitos inflamatórios no microambiente tumoral. Em paralelo, verificamos que a produção de espécies reativas de oxigênio e a migração em direção ao GRP são dependentes do complexo NADPH oxidase. Esse estímulo promove ativação intensa, culminando na produção de redes extracelulares de neutrófilos (NETs). Já o antagonista do seu receptor, GRPR, apresentou potencial anti-inflamatório, sendo capaz de inibir a migração neutrofílica via modulação de IL-8 e reduzindo a extensão da lesão hepática induzida por paracetamol (acetaminofeno), alterando a motilidade dos neutrófilos no tecido e a expressão de moléculas de adesão. Assim, os resultados aqui apresentados demonstram um panorama amplo da função do GRP na biologia tumoral e no sistema imune.

PALAVRAS-CHAVE: GRP, GRPR, imunologia, neutrófilos.

ABSTRACT

Tumor microenvironment and inflammatory diseases promote alterations in our immune system along with their development. Several molecules are implicated in this modulation and are therefore considered therapeutic targets. Gastrin-releasing peptide (GRP) is produced in tumors where it promotes cellular proliferation. It is also correlated with chronic diseases, as in rheumatoid arthritis and asthma, and in the acute condition of sepsis. Recently, our group found a direct GRP action over neutrophils, promoting migration. This work aimed to study the interface between GRP-producing tumors and the recruitment of immune cells, as well as extend the cellular studies about neutrophil activation and migration processes promoted by the peptide. In tumors, we observed that a lung adenocarcinoma cell line does not proliferate in response to GRP. Yet, it is induced to migrate when exposed to the peptide, indicating a potential role for GRP in metastasis of this type of cancer. In our tumor immunology studies, we established a novel i*n vivo* model by overexpressing GRP in a melanoma cell line (B16F10). We observed the augment of infiltrating inflammatory monocytes in the tumor microenvironment of these tumors. In parallel, we verified that reactive oxygen species production and migration in response to GRP is dependent of the NADPH oxidase complex. GRP stimulation promotes an intense activation, which culminates in neutrophil extracellular traps (NETs) release. In addition, the GRP receptor (GRPR) antagonist RC-3095 presented anti-inflammatory potential, inhibiting neutrophil migration towards IL-8 and reducing the extent of acetaminophen-induced liver damage. This effect was due to motility alterations in infiltrating neutrophils within the tissue and reduction of cell adhesion molecules. The results presented herein demonstrate the wide panorama of GRP's interactions in tumor and immune biology.

KEY WORDS: GRP, GRPR, immunology, neutrophils.

LISTA DE ILUSTRAÇÕES

LISTA DE SIGLAS

ADAM17	Uma desintegrina e uma metaloproteinase do tipo 17
ALF	Falência hepática aguda
APAP	Acetaminofeno
BBN	Bombesina
BLP	Peptídeos análogos a bombesina
BRS-3	Subtipo do receptor da bombesina do tipo 3
C5a	Complemento do tipo 5a
CCL	Ligante de quimiocina
CCR	Receptor de quimiocina
CGD	Doença granulomatosa crônica
CNS	Sistema nervoso central
CXCR	Receptor de quimiocina
CXCL	Ligante de quimiocina
DAG	Diacilglicerol
DAMPs	Padrões Moleculares Associados a Dano
DC	Células dendríticas
DILI	Lesão hepática induzida por medicamentos
DNA	Ácido desoxirribonucleico
DPI	Cloreto de difenilenoiodônio
Duox	Oxidases Duais
ERK	Quinase regulada por sinais extracelulares
FAK	Quinase de adesão focal

fMLP	N-formil-metionil-leucil-fenilalanina
FPR	Receptor de peptídeos formilados
GDP	Difosfato de guanosina
GPCR	Receptor acoplado à proteína G
GRK	Quinase do Receptor acoplado à proteína G
GRP	Peptídeo liberador da gastrina
GRPR	Receptor do peptídeo liberador da gastrina
GTP	Trifosfato de guanosina
HOCl	Ácido hipocloroso
ICAM	Molécula de adesão intercelular
IL-17	Interleucina 17
IL-1β	Interleucina 1 beta
IL-6	Interleucina 6
INFγ	Interferon gama
IP3	Inositol trifosfato
KC	Células de Kupfer
LFA-1	Antígeno-1 associado à função de linfócito
LPS	Lipopolisacarídeo
LTB4	Leucotrieno B 4
MAPK	MAP quinases
MMP8	Metaloproteinase 8
MMP9	Metaloproteinase 9
Mo-DC	Células dendríticas derivadas de monócitos

MPO	Mieloperoxidase
mtDNA	DNA mitocondrial
mTOR	Alvo mamífero da rapamicina
NAC	N-acetil-cisteína
NADPH	Nicotinamida adenina dinucleótido fosfato
NAPQI	N-acetil-para-benzoquinonaimina
NE	Elastase neutrofílica
NETs	Redes extracelulares de neutrófilos
NFκB	Fator nuclear kappa-B
NK	Células "*natural killers*"
NKT	Assassinas naturais T
NLRP3	Receptor do tipo NOD 3
NMBR	Receptor da neuromedina B
Nox2	NADPH oxidase 2
PAD-4	Peptidil-arginina deiminase tipo 4
PAF	Fator de ativação plaquetária
PAMPS	Padrões moleculares associados a patógenos
PETScan	Tomografia por emissão de pósitrons
PI3-K	Fosfatidilinositol-3-quinase
PIP3	Fosfatidilinositol 3, 4, 5-trifosfato
PKC	Proteína quinase C
PLC-β	Fosfolipase C- β
PMA	Forbol-12-miristato-13-acetato

PMN	Polimorfonuclear
PSGL-1	Glicoproteína ligante-1 da P-selectina
PTEN	Fosfatase homóloga à tensina
PTx	Toxina pertussis
RA	Artrite reumatoide
RhoA	Família de genes homólogos de Ras, membro A
ROCK	Proteína quinase associada a Rho
ROS	Espécies reativas de oxigênio
SCLC	Câncer de pulmão de pequenas células
SHIP	Inositol-fosfatase contendo SH2
SIRS	Síndrome da resposta inflamatória sistêmica
SLE	Lúpus eritematoso sistêmico
SOD	Superóxido desmutase
TACE	Enzima conversora do TNF
TAM	Macrófagos associados ao tumor
TAN	Neutrófilos associados ao tumor
TGF	Fator de crescimento tumoral
TLR	Receptor do tipo Toll
TNF	Fator de necrose tumoral
VCAM	Molécula de adesão de células vasculares
VLA4	Antígeno muito tardio 4

SUMÁRIO

Capítulo 1

1 INTRODUÇÃO

1.1 Peptídeo liberador de gastrina – GRP
1.2 GRP e tumores
  1.2.1 Progressão tumoral
  1.2.2 Imunologia tumoral
  1.2.3 Metástase
  1.2.4 Leucócitos associados ao tumor
  1.2.5 Monócitos
1.3 GRP e o sistema imune
  1.3.1 Neutrófilos
  1.3.2 Migração
  1.3.3 Quimiotaxia
  1.3.4 Sinalização da migração
  1.3.5 ROS e NADPH oxidase
  1.3.6 ROS e migração
  1.3.7 NETs
  1.3.8 Modelo de migração in vivo – lesão hepática

2 JUSTIFICATIVA

3 OBJETIVOS

3.1 Objetivos Gerais

3.2 Objetivos Específicos

1. INTRODUÇÃO

1.1. Peptídeo Liberador de Gastrina – GRP

O peptídeo liberador de gastrina (*Gastrin-releasing peptide*: GRP) é o homologo mamífero da Bombesina (BBN), um peptídeo isolado a partir da pele do anfíbio *Bombina bombina* com características semelhantes (ERSPAMER; ERPAMER; INSELVINI, 1970). O GRP foi denominado como "liberador de gastrina" devido ao seu papel inicialmente proposto na indução da secreção de gastrina pelas células G do antro gástrico (MCDONALD et al., 1979). Os peptídeos apresentam em sua porção carboxi-terminal um grupamento metilamida, e assim como outros neuropeptídeos da sua família, o GRP é formado a partir do processamento de um grande produto traduzido inicial (preproGRP, com 148 aminoácidos). O preproGRP sofre uma sequência de reações de clivagem que culminam no peptídeo de 27 aminoácidos (aa), chamado GRP (GRP_{1-27}, e a partir deste ponto chamado de GRP) e com atividade funcional. Este pode sofrer nova clivagem formando o GRP_{1-17} e o GRP_{18-27} que promove diferentes respostas biológicas (Figura 1).

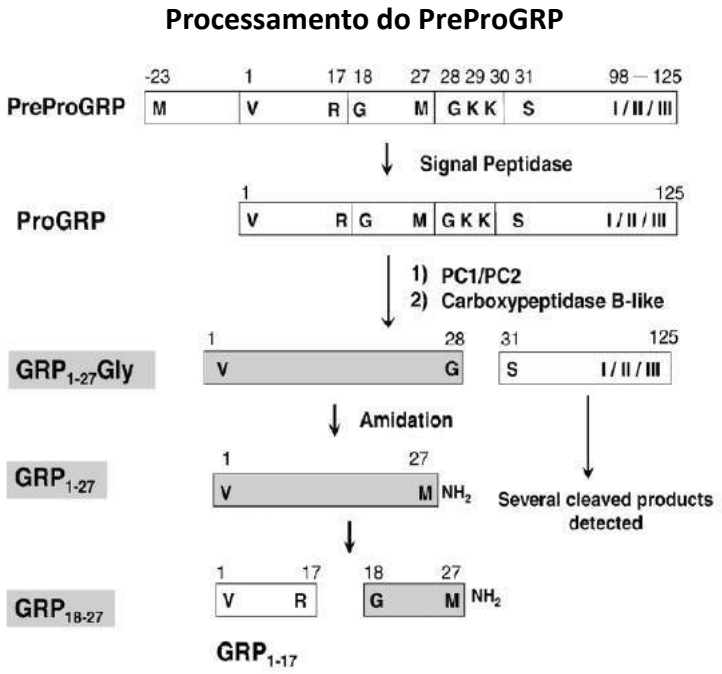


Figura 1 - Processamento do PreproGRP até o peptídeo funcional GRP_{18-27}
Adaptado de Patel et., 2006

Além do controle na liberação da gastrina, o GRP atua em uma série de respostas biológicas, como na contração do músculo liso gastrointestinal, liberação de hormônios e/ou neurotransmissores no estômago, intestino, pâncreas, cólon e órgãos endócrinos, entre outros (RAMOS-ÁLVAREZ et al., 2015). Ele apresenta ainda um importante papel no sistema nervoso central (*Central nervous system*: CNS) e periférico, auxiliando na regulação do ritmo circadiano, ansiedade, resposta ao medo e estresse (DANTAS et al., 2006; GRIDER, 2004; MARTINEZ; TACHÉ, 2000; MARTINS et al., 2005). Nos neurônios, o peptídeo atua junto a neurotransmissores, mostrando um possível papel na modulação da memória (KAMICHI et al., 2005; ROESLER et al., 2006, 2009, 2014) e em doenças neurodegenerativas como Parkinson, Alzheimer e esquizofrenia (ROESLER et al., 2007).

Essa vasta atuação do neuropeptídeo indica sua relevância tanto para função, como para a homeostase tecidual. Sendo assim, estudos que aprofundam conhecimentos sobre a biologia do GRP em diferentes sistemas contribuem para a compreensão dos fenômenos biológicos dos quais o GRP participa. Esse trabalho se propôs a investigar o GRP e a sua via de sinalização principal no que tange à biologia tumoral e ao sistema imune, através da investigação dos processos celulares e moleculares.

1.2. GRP e tumores

Embora o GRP apresente diversas funções sistêmicas (descritas de maneira geral acima), o peptídeo foi principalmente investigado e correlacionado com a biologia tumoral, na sua progressão e agressividade. GRP e a BBN são considerados agentes mitogênicos, induzindo a proliferação e crescimento celular (LEHY et al., 1986; PUCCIO; LEHY, 1989; ROZENGURT; SINNETT-SMITH, 1983; WILLEY; LECHNER; HARRIS, 1984), além de apresentarem efeitos protetores sobre a mucosa gastrointestinal (GÜNAL et al., 2002; MERCER et al., 1998). Estes peptídeos também atuam como importantes fatores de crescimento tumoral em uma diversificada gama de cânceres (CORNELIO; ROESLER; SCHWARTSMANN, 2007; PATEL; SHULKES; BALDWIN, 2006). Esta ação foi primeiramente identificada no câncer de pulmão de pequenas células humano (*Small-cells lung cancer*: SCLC) (CUTTITTA et al., 1985). Neste mesmo trabalho, foi proposta a hipótese autócrina

para o GRP, onde o próprio tumor produziria o fator de crescimento (neste caso o GRP) e seus receptores, gerando uma indução própria de sua proliferação. Sequencialmente, foi descoberto que o GRP também está envolvido no desenvolvimento fetal dos pulmões e com patologias pediátricas no pulmão, o que corrobora com a noção de que tumores promovem a expressão de moléculas importantes na fase embrionária a fim de aumentarem a sua capacidade proliferativa (LI; NAGALLA; SPINDEL, 1994; SHAN et al., 2004; SUBRAMANIAM et al., 2007; SUNDAY et al., 1998).

Além do SCLC, o GRP é encontrado em diversos tipos de tumores primários humanos (PATEL; SHULKES; BALDWIN, 2006). Mais recentemente, foi demonstrada correlação entre a expressão de GRP e seu receptor com a formação de metástases, sendo que metástases primárias apresentam maior expressão de ambas proteínas (FANG et al., 2009; NI et al., 2012; TELL et al., 2011; WELSH et al., 2003). Em vista disso, grande parte da literatura científica contemporânea sobre GRP e BBN explora a sua utilização para fins de diagnóstico de tumores por métodos não invasivos, através de marcadores para PETScan (ZHANG et al., 2012) e como direcionador para compostos antitumorais (CHANDA et al., 2010).

Para exercer todas essas funções, o GRP interage com a família dos receptores de peptídeos similares à bombesina (*Bombesin-like peptides*: BLP) que é composta por receptores com sete domínios transmembrana, acoplados à proteína G (GPCRs). Existem três subtipos principais de receptores descritos e clonados de BLP: GRPR (CORJAY et al., 1991; SPINDEL et al., 1990), NMBR, BRS-3 (JENSEN et al., 2008). O GRPR é também chamado de "*GRP preferring receptor*", apresentando a maior afinidade com o GRP (pelo menos 24 vezes mais afinidade) do que com os outros agonistas naturais dos dessa classe de receptores (RAMOS-ÁLVAREZ et al., 2015).

A alta expressão de GRP e a hipótese autócrina estimularam que muitos estudos fossem realizados sobre a prevalência de GRP e seu receptor GRPR em tumores (MATTEI et al., 2014; PATEL; SHULKES; BALDWIN, 2006; RAMOS-ÁLVAREZ et al., 2015). Essa expressão do GRPR foi confirmada em diversos tumores, estando associada à agressividade, células-tronco tumorais (SARVI et al., 2014), formação de metástases (MATTEI et al., 2014; SANTOS et al., 2015) e

angiogênese tumoral (HEUSER et al., 2005; MARTÍNEZ et al., 2005a). O GRP sinaliza através da interação com GRPR que, por sua vez, promove seu efeito através da ativação de proteína G, mais especificamente através de proteína G α q/11 ou q12/13 (CORJAY et al., 1991; HELLMICH; BATTEY; NORTHUP, 1997).

A ativação das proteínas Gαq leva à estimulação da fosfolipase Cβ (PLC-β), resultando num aumento de cálcio citoplasmático, gerando inositol trifosfato (IP3) e diacilglicerol (DAG) (ROZENGURT, 1998). Essa sinalização promove proliferação celular através de MAPKs (p38 e ERK) e PI3K/AKT e redução de proteína reguladora PTEN (FLORES; LENZ; ROESLER, 2009; PAUL et al., 2013; QIAO et al., 2005; RAMOS-ÁLVAREZ et al., 2015). Já, a sinalização das proteínas G α q 12/13 promove a ativação da *small* GTPase RhoA, que por conseguinte ativa a quinase ROCK, promovendo migração tumoral (PATEL et al., 2014).

O GRP também pode atuar estimulando outras vias. Esse é o caso da ativação de c-Src (ZHANG et al., 2004) que ativa PI3-K resultando na fosforilação da metaloproteinase TACE (ADAM17) por intermédio da quinase PDK-1. Essa enzima promove a clivagem e liberação de proteínas como o TGF-beta e anfirregulina, que promovem a proliferação e agressividade tumoral (ZHANG et al., 2006). Da mesma forma, a sinalização mediada pela proteína Gaq12/13-RhoA pode ativar a via Shh (*Sonic hedgehog*) promovendo proliferação (CASTELLONE et al., 2014). Além disso, o potencial invasivo e metastático induzido por GRP é decorrente da ativação da quinase de adesão focal (FAK) (LEE et al., 2012) e AKT (QIAO et al., 2013a). Soma-se a isso o fato do GRP também ser capaz de fosforilar a quinase alvo da rapamicina humana (mTOR), inibindo fatores pro-autofágicos, e por fim, reduzindo a autofagia e estimulando a proliferação de células endoteliais, promovendo angiogênese tumoral (WOON KIM et al., 2013). Essa rede de sinalizações induzida pelo eixo GRP/GRPR verificada nesses estudos ratifica as diversas funções celulares do GRP.

Devido à soma de todos esses achados, o GRPR foi proposto como um importante novo alvo terapêutico específico em câncer (CORNELIO; ROESLER; SCHWARTSMANN, 2007), e antagonistas seletivos do GRPR, como o RC-3095, foram pesquisados como potenciais novos agentes antitumorais (CHATZISTAMOU et al., 2001; RADULOVIC; MILLER; SCHALLY, 1991; SZEPESHAZI et al., 1997). O

RC-3095 é um pseudononapeptídeo e antagonista seletivo do GRPR (Pinski, Yano, Rekasi *et al.*, 1992). Quando testado em camundongos *nude*, resultou em uma significante redução tumoral de várias linhagens celulares de câncer, incluindo SCLC, de tumores de pâncreas, mama, próstata, gástrico e de ovário (BAJO et al., 2004; CHATZISTAMOU et al., 2001; ENGEL et al., 2005; HOHLA; SCHALLY, 2010; QIN et al., 1994; YANO et al., 1992). A administração subcutânea diária do RC-3095 não apresentou efeitos tóxicos na concentração em que foi testada (10-20 µg por animal) e promoveu a regressão tumoral completa em alguns casos. Após esses resultados, o peptídeo sintético foi testado *in vitro* e *in vivo* para diversos tipos tumorais, apresentando bom prognóstico na redução e desaparecimento do tumor (CORNELIO; ROESLER; SCHWARTSMANN, 2007; STANGELBERGER et al., 2006). Em razão destes resultados promissores, o RC-3095 foi avaliado em um teste clínico de fase I, onde a sua toxicidade foi previamente testada em modelos animais específicos e sua dosagem estabelecida (SCHWARTSMANN et al., 2006). Porém, os resultados clínicos não demonstraram avanço nos pacientes testados e ainda não foi demonstrado qual seria o mecanismo específico responsável pela ação antitumoral do antagonista.

Mais recentemente, alterações na expressão do GRPR e GRP por engenharia genética foram demonstradas como moduladoras da agressividade do tumor, promovendo aumento do crescimento tumoral e metástase quando o receptor ou o peptídeo foram superexpressos em linhagens celulares de neuroblastoma e cólon (NI et al., 2012; QIAO et al., 2005). Entretanto, quando os tumores tiveram a expressão de GRP ou GRPR abolida, ocorreu a redução da malignidade dos tumores ou indução de senescência (MENEGOTTO et al., 2016; PAUL et al., 2011; QIAO et al., 2008). De maneira similiar, o controle transcricional do GRPR por microRNAs se mostrou importante na progressão tumoral (QIAO et al., 2013).

1.2.1. Progressão tumoral

A oncogênese e o desenvolvimento tumoral são processos complexos multifacetados que permitem o surgimento do câncer. Para vencer as barreiras de controle impostas pelos sistemas biológicos, as células neoplásicas devem obter a capacidade de realizar pelo menos as seis "*Hallmarks of Cancer*": (i) resistir à morte celular (apoptose), (ii) manter a sinalização proliferativa, (iii) evadir os supressores

de crescimento, (iv) induzir angiogênese, (v) permitir a imortalidade replicativa, (vi) ativar invasão e metástase (Hanahan e Weinberg, 2000). Mais recentemente, foram consolidados mais quatro alterações que possibilitam o crescimento tumoral: (vii) desregulação energética celular, (viii) fuga da destruição imune, (ix) instabilidade genômica e mutação, (x) inflamação produzida pelo tumor (HANAHAN; WEINBERG, 2011). Essas são alterações celulares intrínsecas e extrínsecas que transformam uma célula normal em homeostase tecidual, em um câncer. Intrínsecamente, para tornar-se um tumor, a célula deve conseguir sobreviver aos diversos mecanismos controladores da homeostase, reparo e erro do material genético, ausência de nutrientes e senescência (HANAHAN; WEINBERG, 2011). Extrinsecamente, a célula pré-tumoral também sofre inúmeras pressões de seleção, competindo com as células naturais do tecido por espaço (WIRTZ; KONSTANTOPOULOS; SEARSON, 2011) e nutrientes, e ainda sendo monitorada pelo sistema imune que busca alterações teciduais, principalmente através de alterações em proteínas de membrana (VESELY et al., 2011). Em razão disso, o estabelecimento de um tumor é um fato raro e que deve ser compreendido como um evento evolutivo, onde várias pressões seletivas distintas (intrínsecas e extrínsecas) continuamente selecionam as células aptas a resistir às condições de estresse antes impostas (NAVIN et al., 2011), além de progredirem e crescerem (GRIVENNIKOV; GRETEN; KARIN, 2010).

Devido às diversas pressões de seleção durante a oncogênese tumoral, os cânceres sólidos não são apenas um agregado de células clones do precursor tumoral. Estudos atuais demonstram uma vasta diversificação e especialização de células pertencentes ao tumor, onde se apresentam com fenótipos e genótipos distintos (DAVIES; LINEWEAVER, 2011; NAVIN et al., 2011). Em vista disso, alguns grupos vêm propondo comparar a dinâmica tumoral aos processos que ocorrerem em populações de bactérias sociais, onde o crescimento e controle da população bacteriana é regido por uma resposta sensorial de quórum (*Quorum sensing*) (HICKSON et al., 2009; NG; BASSLER, 2009), adicionando novas noções sobre a complexidade que a seleção e evolução tumoral podem promover (BEN-JACOB; COFFEY; LEVINE, 2012).

1.2.2. Imunologia Tumoral

O sistema imune desempenha três papéis essenciais na prevenção tumoral: (i) combate aos vírus com potencial oncogênico; (ii) a eliminação de patógenos e resolução de inflamações associadas, prevenindo um ambiente de inflamação crônica; (iii) identificação e eliminação de tumores através de antígenos tumorais específicos e outros mecanismos de reconhecimento de alterações celulares (GRIVENNIKOV; GRETEN; KARIN, 2010; VESELY et al., 2011). Esse controle imune sobre o surgimento de células neoplásicas é essencial para a homeostase dos tecidos. O terceiro processo citado acima (iii) foi denominado de vigilância imunológica, onde principalmente as células do sistema imune adaptativo (células T CD8+, e também NKT e NK) são responsáveis por vasculhar os tecidos em busca de alterações em proteínas de membrana nas células próprias (DUNN et al., 2002).

Em razão dessa vigilância, mais recentemente, foi observado que o sistema imune realiza mais uma pressão de seleção sobre os tumores, acarretando em um processo chamado de edição imune do câncer (*cancer immunoediting*) (HANAHAN; WEINBERG, 2011; SCHREIBER; OLD; SMYTH, 2011). Essa pressão extrínseca mantém as células não eliminadas e possibilita que essas adquiram grandes alterações celulares, especialmente na expressão dos seus antígenos tumorais, tornando o tumor mais agressivo. Esse processo ocorre em três etapas: (i) eliminação, onde as células citotóxicas imunes matam os tumores que reconhecem; (ii) equilíbrio, quando raríssimas células tumorais que não foram eliminadas sobrevivem, sendo mantidas dentro de um controle do crescimento estável pelo sistema imune, em um estado de dormência do crescimento do tumor, mas que permite, através da instabilidade genética, a plasticidade celular – fase onde incide a edição tumoral; (iii) escape, momento em que a edição tumoral possibilita uma fuga dos mecanismos controladores existentes e o câncer progride (SCHREIBER; OLD; SMYTH, 2011; VESELY et al., 2011).

Em função dessa nova visão da progressão tumoral, a compreensão dos mecanismos imunes que promovem ou controlam o câncer vem ganhando importância para a busca de terapias eficazes. Mais recentemente, um grande número de evidências demonstra que o sistema imune, apesar de suas funções de vigilância e prevenção de crescimento tumoral, pode ser influenciado por diferentes

mecanismos para fazer o papel contrário, ou seja, auxiliar a progressão tumoral. Este cenário será explorado seguir neste capítulo.

1.2.3. Metástases

A formação de metástases é o desfecho mais grave do desenvolvimento tumoral. Cerca de 90% das mortes decorrentes de câncer não estão associadas ao tumor primário, e, sim, às metástases formadoras de tumores secundários em órgãos distantes (CHAFFER; WEINBERG, 2011). Apesar de sua relevância, esse processo permanece o menos compreendido dentro da patogênese do câncer. O conhecimento até então se restringe às etapas pelas quais as células metastáticas devem passar até atingir o sítio secundário, dentro do processo chamado de cascata de invasão de metástases (*invasion-metastasis cascade*) (BACAC; STAMENKOVIC, 2008; CHAFFER; WEINBERG, 2011). Nesta cascata, as células tumorais devem adquirir a plasticidade genética necessária para realizar os seguintes passos: (i) desprendimento do tumor primário (como célula isolada ou subpopulação) (FRIEDL; ALEXANDER, 2011), (ii) invasão para o tecido local, (iii) entrada nos tecidos linfáticos ou sanguíneos (*intravasion*), (iv) sobrevivência ao ambiente da circulação fora do microambiente tumoral, (v) ancoragem em um órgão distante, (vi) saída dos vasos e entrada novamente no tecido (*extravasion*), (vi) formação da micro-metástase, (vii) colonização da metástase e desenvolvimento do tumor secundário (BACAC; STAMENKOVIC, 2008; FRIEDL et al., 2012; FRIEDL; GILMOUR, 2009; GUPTA; MASSAGUÉ, 2006; VALASTYAN; WEINBERG, 2011; WIRTZ; KONSTANTOPOULOS; SEARSON, 2011). Para cada um desses passos, a célula ou massa tumoral sofre alterações fenotípicas que as permitam progredir (Bacac e Stamenkovic, 2008). Porém, além da capacidade plástica intrínseca do tumor, fatos externos podem auxiliar a selecionar tumores com potencial invasivo e metastático. Dentre eles estão as células imunes, principalmente as do sistema imune inato (como macrófagos, monócitos e neutrófilos), que são recrutadas ou influenciadas pelo tumor permitindo ao tumor aumentar o seu potencial agressivo e maligno (CONDEELIS; POLLARD, 2006; DE LARCO; WUERTZ; FURCHT, 2004; JOYCE; POLLARD, 2009; WANG et al., 2011a, 2011b).

1.2.4. Leucócitos associados ao tumor

A infiltração de células do sistema imune em tumores pode promover ou retardar o crescimento tumoral, dependendo do fenótipo dessas células infiltrantes (DE VISSER; EICHTEN; COUSSENS, 2006), tendo o tumor a capacidade de influenciar esses fenótipos por diferentes mecanismos (DE SOUZA; BONORINO, 2009, 2012). A identificação desses mecanismos auxilia na revelação de novos alvos terapêuticos para o tratamento do câncer.

A classificação de tumores e o prognóstico para pacientes com câncer são atualmente executadas por análise de amostras biopsiadas do tumor primário e de linfonodos drenantes. A classificação TNM (AJCC-UICC-2009) de estadiamento do tumor, com base nessa análise anatomohistopatológica, é útil para a estimativa de resultados em diferentes formas de câncer (FOX et al., 2011). No entanto, tem limitações devido à variabilidade existente entre os pacientes de câncer. Uma das razões subjacentes a esta limitação é a suposição de que o crescimento do tumor é completamente autorregulado (HANAHAN; WEINBERG, 2011). Os tumores são invariavelmente infiltrados por células do sistema imunológico, e, a fim de crescer, os tumores precisam empregar múltiplas estratégias de escape do sistema imune, não só pela edição expressão de antígenos tumorais (SCHREIBER; OLD; SMYTH, 2011), mas também através do desenvolvimento de diferentes propriedades imunossupressoras (DE SOUZA; BONORINO, 2012).

Estudos forneceram evidências convincentes de que o desenvolvimento do câncer é muito influenciado pela resposta imune do hospedeiro. Estudos que analisaram centenas de amostras de tumores humanos (GALON, 2006; MLECNIK et al., 2011) concluíram que não apenas o número de células imunes infiltrantes, mas também o seu tipo e estado de ativação, podem realmente ser mais precisos para a predição de gravidade e resultado do que o sistema TNM, que classifica os tumores em relação à sua malignidade. Estes estudos propuseram uma pontuação imune a ser calculada com base na infiltração de tumores do cólon por células T CD8+CD45RO +, (células T citotóxicas de memória) que podem prever a sobrevida livre de doença, destacando a relevância de uma resposta imune adaptativa em pacientes com câncer. A importância das células do sistema imune inato também foi mostrada por outros estudos, os quais demonstraram que a infiltração por monócitos

e macrófagos do tipo M2 pode aumentar malignidade (QIAN; POLLARD, 2010) e promover metástase via produção de CCL2 (QIAN et al., 2011). Além disso, as respostas imunes podem desempenhar um papel promotor do desenvolvimento do câncer. A IL-6 produzida por células mieloides da lâmina própria promove a sobrevivência de células epiteliais intestinais pré-malignas e câncer associado à colite (GRIVENNIKOV et al., 2009).

O papel paradoxal de neutrófilos, tanto na supressão de tumores, como na sua promoção, direta ou indiretamente controlando o crescimento do tumor, angiogênese e metástase foi reavaliado em termos da caracterização de diferentes tipos de neutrófilos associados a tumores (TAN), com polarização de fenótipo N1 ou N2 (FRIDLENDER et al., 2009). Estas populações polarizadas são semelhantes ao que foi descrito para macrófagos (M1: macrófago inflamatório - antitumoral; M2: macrófago anti-inflamatório – pró-tumoral) (BISWAS; MANTOVANI, 2010); e o seu desenvolvimento fenotípico é influenciado pelo microambiente e parece ser controlado por produção de TGF-beta no tumor. Neutrófilos N2 são caracterizados por um perfil de expressão que promove angiogênese do tumor e metástase (NOZAWA; CHIU; HANAHAN, 2006; PICCARD; MUSCHEL; OPDENAKKER, 2012). Na verdade, desde 1995 sabe-se que a depleção de neutrófilos pró-tumorais (N2) em camundongos não tratados pode ser suficiente para inibir o crescimento tumoral, mesmo quando células T CD8 + estão ausentes, destacando um potencial imunossupressor de neutrófilos N2 (PEKAREK et al., 1995).

Estes são apenas alguns exemplos sobre as informações de que a análise do microambiente do tumor pode revelar (FRIDMAN et al., 2011, 2012). Contudo, a origem das células recrutadas por tumores, e os mecanismos sistêmicos pelos quais são recrutadas são ainda pouco estudados. É possível que essas células sejam modificadas e atraídas pelos tumores antes mesmo de integrarem o microambiente tumoral. Um estudo propõe o baço, e não a medula óssea, como o sítio hematopoiético de origem dos monócitos e neutrófilos promotores de metástase (CORTEZ-RETAMOZO et al., 2012). Isso seria possível devido a um acúmulo, no baço de animais portadores de tumores, de precursores monocíticos CD11b+Ly6C+ e neutrofílicos CD11b+Ly6G+. Os mecanismos desse recrutamento são ainda desconhecidos.

1.2.5. Monócitos

Os monócitos são células mieloides que representam ~10% dos leucócitos sanguíneos humanos e 4% em camundongos (AUFFRAY; SIEWEKE; GEISSMANN, 2009). Monócitos circulam no sangue, medula óssea e baço, e têm um importante papel durante o desenvolvimento e regulação da homeostase tecidual. Realizam a remoção de células apoptóticas, retirada de compostos tóxicos e têm grande potencial de produzir citocinas e quimiocinas durante a resposta inflamatória. Entretanto, a principal função dos monócitos parece ser a de constituir um reservatório sistêmico de precursores mieloides, permitindo a renovação de macrófagos e células dendríticas (DCs) nos tecidos. Em condições inflamatórias, os monócitos circulantes migram da periferia para os tecidos, onde se diferenciam em células mieloides fagocíticas (macrófagos) e apresentadoras de antígeno (DCs). Por isso, eles são essenciais para o funcionamento pleno do sistema imune, atuando na conexão entre o sistema imune inato e a o adaptativo (GEISSMANN et al., 2010). Existem duas subpopulações principais de monócitos. A população mais rara na circulação expressa altos graus de CX3CR1 (receptor da fraktalquina) e baixos níveis de Ly6C e do receptor quimiotático para CCL2, o CCR2. Já, os monócitos inflamatórios expressam baixos níveis de CX3CR1, e altos de Ly6C (Ly6C^{high}) e CCR2. Nos humanos, esses monócitos são classificados pela expressão diferencial de CD16 e CD14, respectivamente (SHI; PAMER, 2011). Os monócitos inflamatórios contribuem para a defesa imune pelo recrutamento direto para os locais infectados/lesados e a subsequente entrada nos linfonodos. Os sinais pró-inflamatórios provenientes do sítio afetado (como CCL2, entre outros) induzem a saída da medula desses monócitos, que partem para circulação e, após, para o foco infeccioso sendo guiado pela aumento de moléculas de adesão das células endoteliais vicinais. Além da direta atuação no foco inflamatório, essas células são essenciais, liberando uma segunda onde de quimiocinas e citocinas e podem também transferir antígenos às DCs clássicas presentes no tecido ou se diferenciar em células dendríticas derivadas de monócitos (Mo-DC) (AUFFRAY; SIEWEKE; GEISSMANN, 2009; RANDOLPH; OCHANDO; PARTIDA-SÁNCHEZ, 2008; SHI; PAMER, 2011). Entretanto, a ativação excessiva desse subtipo de monócitos pode agravar patologias, como aterosclerose, infarto do miocárdio e hemorragia cerebral (HAMMOND et al., 2014; SHI; PAMER, 2011).

Monócitos inflamatórios são relevantes também na progressão tumoral. Diversos tipos de tumores podem produzir quimiocinas que induzem o recrutamento desse subtipo de monócitos. Em tumores de mama, cólon e melanoma, já foi observado que o recrutamento de monócitos Ly6C^{high} via CCL3, CCL4, CCL5 e, principalmente, CCL2 são responsáveis pelo aumento de macrófagos associados ao tumor (TAM: *tumor-associated macrophages*) no microambiente tumoral e que o bloqueio dessa via reduz os tumores (LEUSCHNER et al., 2011; MURDOCH et al., 2008). Soma-se a isso o fato desses monócitos estarem altamente correlacionados coma promoção de metástases e a progressão tumoral (KITAMURA et al., 2015; KITAMURA; QIAN; POLLARD, 2015; QIAN et al., 2011). Além disso, essas células são encontradas em elevado número no baço de camundongos com tumores. Tais células apresentam marcadores de células imaturas e/ou progenitores da medula, e têm a capacidade de capturar antígenos tumorais, fazendo apresentação cruzada desses antígenos às células T CD8+ de memória, o que pode elevar a quebra do reconhecimento dos antígenos tumorais (BRONTE; PITTET, 2013; UGEL et al., 2012). Sendo assim, a quantificação dos monócitos inflamatórios associados aos tumores pode ser considerada como marcação de progressão tumoral em modelos animais e as terapias visando essas interações, com resultados promissores em testes clínicos (D'INCALCI et al., 2014; PIENTA et al., 2013).

1.3. GRP e o sistema Imune

O GRP foi proposto também como um modulador do sistema imune. Estudos dos anos 80 e 90 mostraram o GRP ativando monócitos, linfócitos e macrófagos peritoneais (DE LA FUENTE et al., 1991; DEGAN et al., 2008; DEL RIO et al., 1994; MEDINA et al., 1998a; RUFF et al., 1985). O peptídeo também foi encontrado estimulando a proliferação de linfócitos (DEL RIO et al., 1994) e a atividade das células NK (*Natural Killers*) (DEL RIO; DE LA FUENTE, 1995; MEDINA et al., 1998b).

Outros trabalhos demostraram a influência do RC-3095 sobre um modelo animal de sepse (DAL-PIZZOL et al., 2006; PETRONILHO et al., 2012). A administração do antagonista promoveu a diminuição da liberação de citocinas pró-inflamatórias, IL-1 β e TNF, tanto *in vitro* como *in vivo*, aumentando a sobrevida dos camundongos e reduzindo o dano inflamatório após a indução com LPS. Níveis aumentados de GRP

foram encontrados em pacientes com sepse e síndrome da resposta inflamatória sistêmica (SIRS) (PETRONILHO et al., 2012). A sobrevida de pacientes sépticos com maiores níveis de GRP no plasma (>10 pg/ml) apresentou-se reduzida.

Foi verificada uma correlação entre os níveis de GRP e os níveis de citocinas pró-inflamatórias IL-6 e TNF no líquido sinovial de pacientes com artrite reumatoide (RA) e a severidade da doença (GRIMSHOLM et al., 2005; ORIGUCHI et al., 2011). Os resultados mostraram o GRP em concentração aumentada em pacientes com quadros mais graves da doença, auxiliando sua manutenção. Outro estudo mais recente também verificou a diminuição de citocinas pró-inflamatórias (INF-γ, IL-6, IL-1β, TNF), promovida pelo tratamento com o antagonista seletivo do GRP (RC-3095) em modelos murinos de artrite, além da diminuição de hiperplasia nas articulações dos animais (OLIVEIRA et al., 2008, 2011).

Nosso grupo identificou uma ligação importante entre o neuropeptídeo e o recrutamento de neutrófilos, mediado pelo o GRPR (CZEPIELEWSKI et al., 2012). Como a contribuição do infiltrado imune é determinante para a progressão tumoral, é possível sugerir que o GRP produzido por tumores influencia também as células do sistema imune que expressam o GRPR. Além disso, a ligação do GRP no GRPR em tumores ativa vias conhecidas como PI3K/AKT, MAPK e FAK (*focal adhesion kinase*). Em neutrófilos, foi demonstrada a ativação de MAP quinases ERK e p38 em resposta ao GRP (GLOVER et al., 2005; KOH; LEYTON; MOODY, 1999; LIU et al., 2007; SCHWARTSMANN et al., 2005; TAGLIA et al., 2007). Recentemente, foi adicionado um mecanismo chave para a migração de células de câncer de cólon reguladas pelo GRPR através da via Gα13-PRG-RhoA-ROCK (PATEL et al., 2014). Rho-quinases também são essenciais para migração de neutrófilos (HASAN et al., 2012; NIGGLI, 2003). Estas são vias que transmitem sinais para importantes funções de diferentes subpopulações de leucócitos (HIRSCH, 2000; NEVES; RAM; IYENGAR, 2002). Soma-se a isso, o trabalho de um outro grupo demonstrando que o GRP pode modular a produção de citocinas de linfócitos Th1 e Th2 em um modelo de asma (ZHOU et al., 2011) e a evidência de que macrófagos associados ao carcinoma renal expressam GRPR e são fonte de GRP no microambiente tumoral (BEDKE et al., 2010). Propomos, portanto, investigar a contribuição do GRP produzido por tumores na modulação das células do infiltrado tumoral.

1.3.1. Neutrófilos

No início do século 20, Paul Ehrilich e Elie Metchnikoff foram condecorados com o prêmio Nobel de Medicina de 1908 pelas suas contribuições no campo de imunologia. Dentre elas, estava a descoberta de um leucócito sanguíneo capaz de fazer fagocitose e combater patógenos. Metchnikoff teorizou que essas células migrariam para os locais onde haveria lesão e digeririam os micróbios que encontrassem (CAVAILLON, 2011). Ele foi o primeiro a identificar os neutrófilos, com seu núcleo lobular, chamando-os de leucócitos polimorfononucleares (PMN) – como são reconhecidos até hoje (Figura 2). Atualmente, os neutrófilos são classificados como as células mieloides mais abundantes na circulação dos mamíferos, correspondendo a 50-70% dos leucócitos sanguíneos em humanos. Entretanto, essas células vivem durante um curto período de tempo em condições homeostáticas, com uma meia-vida de 1,5 e 8 horas em camundongos e humanos, respectivamente, e vivendo cerca de 12 horas nos camundongos (BASU, 2002; KOLACZKOWSKA; KUBES, 2013). A soma dessas características requerem alta regulação do número de neutrófilos, devendo estes serem eliminados em grande número diariamente. Essa taxa é de aproximadamente 10^7 neutrófilos em camundongos e 10^{11} em humanos, e essa retirada de neutrófilos “envelhecidos” ocorre no baço, fígado ou medula óssea, controlando a homeostase hematológica através do ritmo circadiano (FURZE; RANKIN, 2008; VON VIETINGHOFF; LEY, 2008). Os neutrófilos considerados a primeira linha de defesa do nosso sistema imune, controlando infecções bacterianas e fúngicas e, também, a propagação de vírus (AMULIC et al., 2012). Para isso, os neutrófilos dispõem de uma gama de mecanismos efetores para controlar a proliferação de patógenos, e de uma grande capacidade migratória para alcançar os mais diversos sítios infectados. Esses são os principais fatores sensíveis à perturbação que podem gerar ou agravar patologias, apresentando relevância clínica.

Figura 2 - Primeiros desenhos de fagócitos, por Elie Metchnikoff em 1901

1.3.2. Migração

Neutrófilos presentes na circulação podem ser rapidamente recrutados para os sítios inflamatórios durante infecções e lesões estéreis. Macrófagos e mastócitos residentes, e também células do estroma efetuam esse "chamado" quando percebem alterações moleculares da homeostase do tecido (KOLACZKOWSKA; KUBES, 2013). Uma variedade de estímulos, especialmente padrões moleculares associados à patógenos (PAMPs) e padrões moleculares associados à lesão (DAMPs), ativam essas células sentinelas para produzirem mediadores inflamatórios (IL-1β, IL-17, TNF, variadas quimiocinas e mediadores lipídicos) (ZEYTUN et al., 2010). Essas moléculas pró-inflamatórias estimulam as células endoteliais da circulação próxima ao foco inflamatório a aumentar a expressão de moléculas de adesão na sua face luminal, e a expressão dessas proteínas garantirá a localização e fixação do neutrófilo a parede do vaso mais próximo ao foco inflamatório. Sendo assim, o endotélio expressa P-selectina, E-selectina e diversas proteínas da superfamília das integrinas, como ICAMs e VCAMs, e essas proteínas irão participar de diferentes etapas da transmigração vascular (ou diapedese) (BORREGAARD, 2010).

A cascata do recrutamento dos neutrófilos tem as seguintes etapas: captura (*tethering*), rolamento (rolling), adesão, *crawling* e transmigração. Os neutrófilos expressam constitutivamente PSGL-1 (*P-selectin glycoprotein ligand-1*) e L-selectina

(ou CD44) que ligam-se através de P-selectina, E-selectina, respectivamente. Esse é o primeiro contato do neutrófilo com o endotélio ativado, provocando a frenagem do neutrófilo que está sob alta pressão devido ao fluxo vascular. Esse contato ativa no neutrófilo uma série de quinases (PI3K e p38, por exemplo) e prepara o neutrófilo para a interação via integrinas (LEY et al., 2007; MUELLER et al., 2010; YAGO et al., 2010). A diminuição da velocidade do neutrófilo permite que mais contatos de selectinas sejam realizados resultando no literal rolamento do neutrófilo sobre o lúmen vascular. Ao longo do rolamento sobre o endotélio "inflamado", o gradiente de moléculas pró-inflamatórias e quimiotáticas atrai o neutrófilo em direção ao foco, aumentando nas células e no endotélio a expressão de proteínas de adesão. Esse aumento de interação ocorre através da expressão de β2-integrinas nos neutrófilos e a presença de ICAMs e V-CAMs no endotélio. Os neutrófilos expressam β2-integrinas LFA-1 (ou α1β2; CD11a – CD18) e Mac-1 (ou αMβ2; CD11b – CD18) que ligam-se em ICAM1 e ICAM2 (KOLACZKOWSKA; KUBES, 2013).

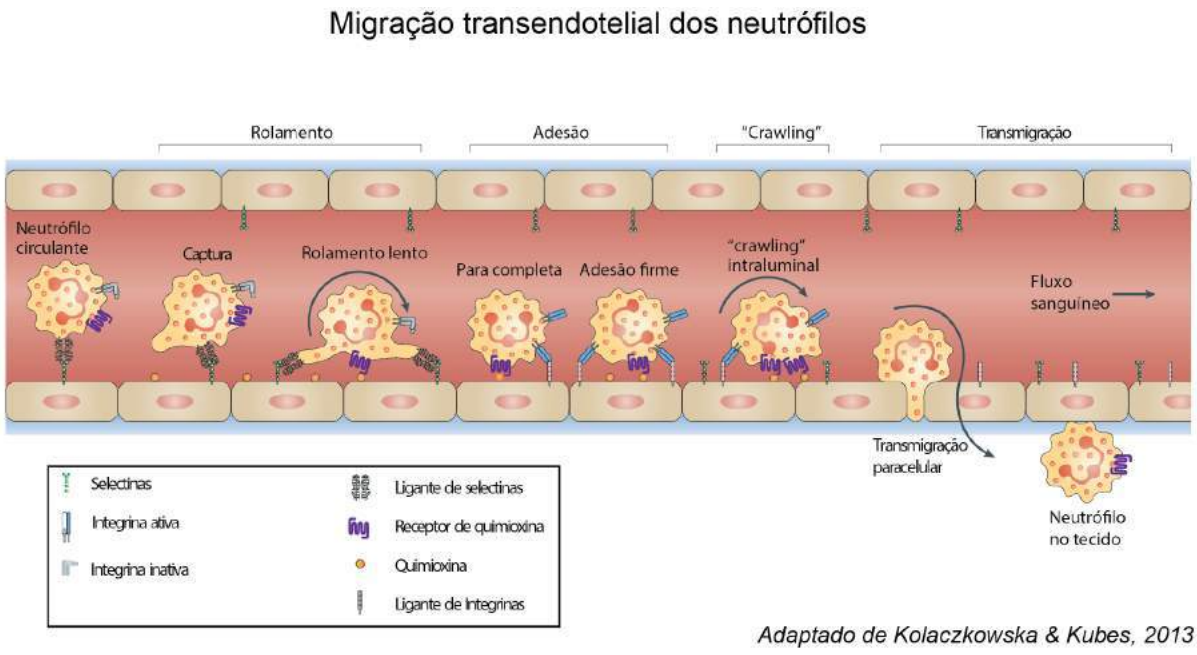


Figura 3 - Sequência de evento da migração transendotelial dos neutrófilos

Após a adesão, o neutrófilo se movimenta (*crawling*) sobre o lúmen endotelial até encontrar um espaço de junção celular entre as células do endotélio ou se guia através dos periócitos, utilizando os seus espaços de inserção da estrutura subendotelial para transmigrar (PROEBSTL et al., 2012). Esses processos são dependentes de β2-integrinas e ICAM (PHILLIPSON et al., 2006). Na sequência, a

passagem através da camada endotelial (diapedese) ocorre paracelularmente (entre células endoteliais) ou transcelularmente (através de uma células endotelial). O primeiro modo é o preferido pelos neutrófilos, demora cerca de 5-15 minutos e depende da liberação de moléculas de adesão, como VCAM. O segundo modo é mais demorado (20-30 minutos), porque a células endotelial tem que emitir uma projeção similar a uma microvilosidade. Essa migração depende de ICAM e VCAM no endotélio e LFA-1 e VLA4 (*very late antigen* 4; ou as integrina α4) nos neutrófilos (CARMAN; SPRINGER, 2004). Após emigrar dos vasos, o neutrófilo tem diferentes proteases (elastase neutrofíflica, mieloperoxidade, MMP8, MMP9, proteinase 3) com capacidade de degradar a matriz extracelular da membrana basal e, assim, seguir sua migração o foco inflamatório. Entretanto, foi demostrado que existem regiões com matrizes menos densas que propiciam a saída do neutrófilo e que esses "caminhos" coincidem com a localização dos periócitos, chamando a atenção para a importância dessa células acessórias do sistema vascular na inflamação (STARK et al., 2013; WANG et al., 2006). Por fim, o neutrófilo pode continuar se guiando por concentrações de quimioatraentes até alcançar o local inflamado e efetuar o seu papel.

Todo o processo de migração neutrofílica é encadeado através de passos sequenciais de ativação dos neutrófilos, seguida do estímulos para aumento da expressão de moléculas de adesão e interação com o endotélio, além de direcionamento através de gradientes quimiotáticos (como exposto acima). Entretanto, essa ativação depende do reconhecimento, por meio de receptores de reconhecimento de padrão (PRRs: *patern-recognition receptors*), de derivados infecciosos ou células lesadas. Para realizar esse reconhecimento, os neutrófilos apresentam receptores para PAMPs e DAMPs, como receptors Toll (TLRs: *Toll like receptors*) para LPS (TLR4), lipopeptídeos bacterianos (TLR2), flagelina (TLR5) e DNA (TLR9). Também expressam receptores para produtos derivados de mitocôndria, como o fMLP (FPR1/2) e de quimiocinas, como CXCL1, CXCL2, CXCL8 (CXCR1/2). Todos esses receptores são essenciais para migração e ação do neutrófilo, ativando alterações no citoesqueleto para migração, mecanismos de degranulação e de produção de espécies reativas de oxigênio (ROS) para o combate à patógenos (ALVES-FILHO et al., 2006, 2009; AMULIC et al., 2012; CASTOLDI et al., 2012; PARKER et al., 2005; TREVELIN et al., 2012).

1.3.3. Quimiotaxia

As quimiocinas são os principais agentes controladores do tráfego de neutrófilos, promovendo quimiotaxia através de receptores acoplados a proteína G (GPCR) (THELEN, 2001). Eles têm como característica possuírem sete domínios transmembrana e sua ativação promove polimerização de actina, comandando mudanças morfológicas necessárias para a movimentação celular, juntamente com influência nas moléculas de adesão expressas (VAN BUUL; HORDIJK, 2004; VICENTE-MANZANARES; SÁNCHEZ-MADRID, 2004). A ativação de GPCRs também pode promover ativação celular, exocitose e apoptose, através de diferentes vias de sinalização (ROSE et al., 2004).

A maioria dos receptores de quimiocinas promovem sua sinalização através de proteínas G sensíveis à toxina *pertussis* (PTx), da classe G-alfa inibitória (Gαi) (SPANGRUDE et al., 1985; WETTSCHURECK; OFFERMANNS, 2005). A ligação de uma quimiocina à porção extracelular do receptor desencadeia uma mudança estrutural e ativa a proteína G. Essa alteração induz a troca de difosfato de guanosina (GDP) presente no estado basal inativo da proteína ligado à subunidade Gαi, por trifosfato de guanosina (GTP), gerando a dissociação do receptor e da proteína heterotrimérica em subunidades Gαi e Gβγ (WETTSCHURECK; OFFERMANNS, 2005). A subunidade Gαi inibe a adenilato ciclase (KATANAEV, 2001) e as subunidades Gβγ prosseguem a sinalização para quimiotaxia (NEPTUNE; BOURNE, 1997), ativando fosfolipases (como PLCβ), e proteínas quinases, como fosfoinositol 3-quinase (PI3K, principalmente a isoforma γ – PI3Kγ). Isto resulta em acúmulo de mediadores intracelulares, como fosfatidilinositol 3, 4, 5-trifosfato (PIP3), fluxo de cálcio e ativação de GTPases monoméricas, as quais coordenam os processos de adesão celular, polimerização de actina e eventos contráteis que polarizam a célula e permitem o movimento celular (CICCHETTI; ALLEN; GLOGAUER, 2002; NIGGLI, 2003; ROT; VON ANDRIAN, 2004; WARD, 2004; WU; HUANG; JIANG, 2000). Acumulação de PIP3 na porção do neutrófilo mais ativada (ou seja, a parte do neutrófilo mais próxima ao ponto de concentração do gradiente quimiotático) forma a parte “frontal” (*leading edge*), posterior, do neutrófilo, gerada pela polimerização da actina através de GTPases (cdc42 e Rac1/2) que promovem o montagem dos filamentos de actina (F-actina). Porém,

para migrar, o neutrófilo também ativa uma via oposta, que recicla o PIP_3 para PIP_2 e desfazendo o citoesquetelo na porção “caudal” (*trailing end*), anterior, gerada por ativação de RhoA e contração do complexo actina-miosina. Essa via complementar é composta pelas fosfatases PTEN e SHIP. Essas duas vias conseguem por fim polarizar o formato ameboide do neutrófilo em função das maiores concentrações do quimioatrator, formando parte frontal (pseudópode, ou *leading edge*) e a parte caudal (urópode, *trailing end*) do neutrófilo (HEIT et al., 2008b; ITTNER et al., 2012)

1.3.4. Sinalização da migração

A via clássica de estimulação de migração ocorre através de proteína Gαi, tendo um papel essencial na indução da migração dependente de quimiocinas. Todavia, receptores quimiotáticos podem funcionar através de outras proteínas G, apresentando vias alternativas da migração de células imunes, principalmente em neutrófilos (SHI et al., 2007). A principal via alternativa funciona com receptores ligados à proteína Gαq; o agonista (fator quimiotático) induz a liberação das subunidades Gαq e βγ. A proteína Gαq ativa diretamente PLCβ2 e β3 a que liberam mensageiros intracelulares trifosfato inositol (IP3) e diacilglicerol (DAG). O IP3 promove a liberação de cálcio dos estoques intracelulares e o DAG ativa a PKC, estimulando outras vias de sinalização, indiretamente ativando de PI3K e terminando por ativar fatores de transcrição como NF-κβ (NEVES; RAM; IYENGAR, 2002). Essa via alternativa é estimulada apenas por alguns fatores quimiotáticos, como fMLP, encontrada atuante em certas populações leucocitárias, como neutrófilos, eosinófilos e células dendríticas (DCs), não apresentando função em células T e B para esses fatores quimiotáticos (BORCHERS et al., 2002; SHI et al., 2007; TAKASHIMA et al., 2008).

Nas proximidades do foco inflamatório, a alta concentração de sinais e a saturação dos gradientes migratórios aumenta a complexidade da motilidade guiada do neutrófilo. Por isso, os receptores quimiotáticos apresentam uma sinalização altamente regulada. Por exemplo, a sinalização de moléculas quimiotáticas derivadas de infecções, como fMLP e complemento C5a, sobrepuja os sinais derivados de estímulos endógenos (como CXCL8 e LTB4), priorizando o combate a patógenos aos sinais gerados por células intermediárias como as endoteliais. Esse processo é guiado através da ativação seletiva de vias de sinalização específicas.

Os derivados de patógenos atuam primariamente através da ativação da p38/MAPK, e os derivados do organismo ativam a via de PI3K/AKT. A ativação da p38 inibe a via da PI3K/AKT, fazendo o neutrófilo a migrar preferencialmente em direção aos estímulos derivados dos patógenos, criando uma hierarquização dos fatores quimiotáticos (HEIT et al., 2008a, 2008b).

Mais recentemente, as MAP quinases (MAPK) ERK e p38 foram mostradas controlando os sinais de "início" e "parada" da migração de neutrófilos para o derivado de patógeno fMLP (LIU et al., 2012). O fMLP ativa seus GPCRs, FPR1 e FPR2; porém, também promove a fosforilação dos receptores via GRK (*G protein-coupled receptor kinases*), induzindo a dessensibilização e internalização do FPR, terminando com migração (REVANKAR et al., 2004). Esse processo foi regulado por ERK e p38, que são ativadas em diferentes concentrações de fMLP. A indução de ERK por fMLP em altas concentrações potencializa a atividade de GRK2, o que inibe a migração neutrofílica. Entretanto, em baixas concentrações de fMLP, p38 atua de maneira alternativa, fosforilando o próprio FPR e bloqueando a GRK2, estimulando a migração do neutrófilo em direção ao derivado mitocondrial (LIU et al., 2012).

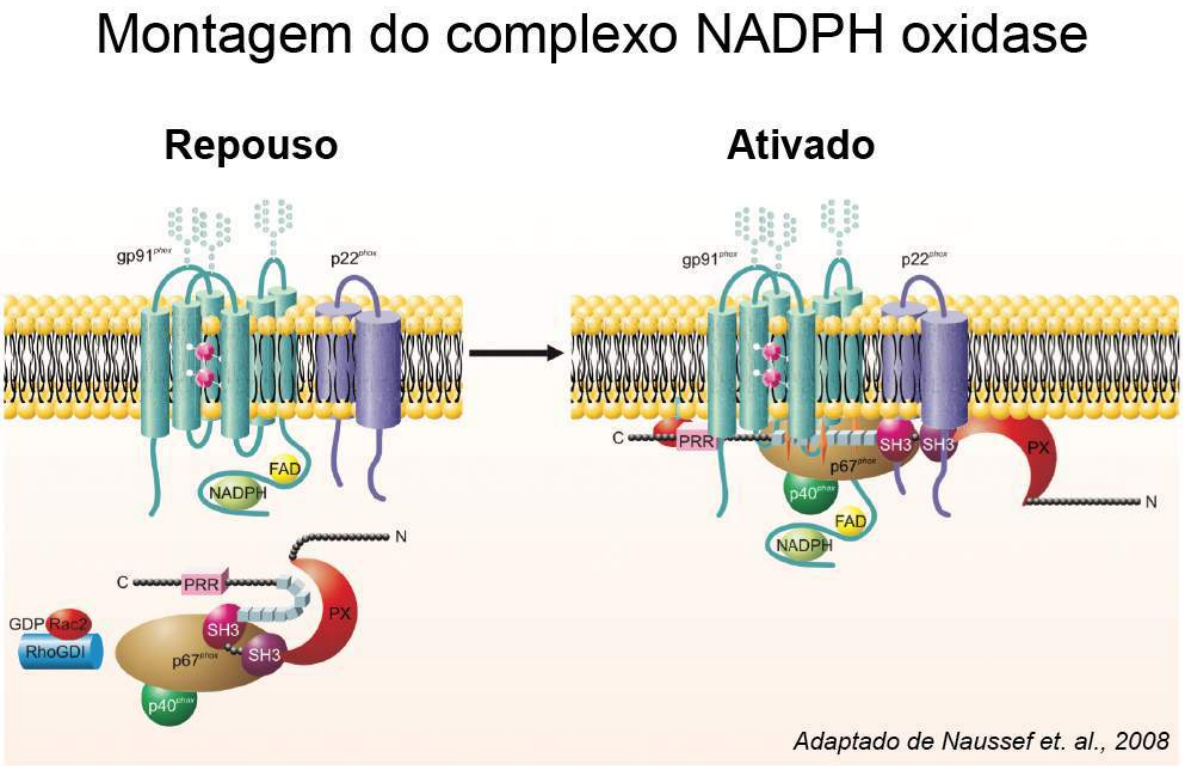
A quimiotaxia em direção a uma molécula também pode ser regulada através dos efeitos sobre os passos da ativação do neutrófilos durante a transmigração. Por exemplo, em baixas concentrações, a IL-8 estimula a retirada (*shedding*) de L-selectina da membrana e o aumento de β2 integrinas, estimulando a migração (LEY, 2002). Enzimas proteases podem promover *shedding* de β2 integrinas na membrana plasmática dos neutrófilos, reduzindo a migração, o que ocorre em alguns casos após a transmigração de neutrófilo (GOMEZ et al., 2012; ZEN et al., 2011).

1.3.5. ROS e NADPH oxidase

O neutrófilo carrega consigo uma grande variedade de substâncias antimicrobianas. E o transporte desse material na circulação é altamente regulado. Como resultado, neutrófilos possuem diversos tipos de grânulos: primários (ou azurofílicos, contendo mieloperoxidase e elastase neutrofílica), secundários (ou específicos, contendo lactoferrina e lisozima) e o terciários (contendo metaloproteinases, como a gelatinase) (FAURSCHOU; BORREGAARD, 2003). Além desse aparato, os neutrófilos têm uma grande capacidade fagocítica e podem

expandi-la ao fusionar seus grânulos e a produção de radicais livres ao fagolisossomo, tornando-o mais eficiente. Sendo assim, a inativação microbiana via fagocitose e a liberação extracelular de grânulos e espécies reativas de oxigênio (ROS) cria um ambiente inóspito para os patógenos (WINTERBOURN et al., 2006). Esse processo acontece no foco inflamatório, após a transmigração e a completa ativação, induzindo o "*burst*" oxidativo e a mobilização dos grânulos azurofílicos e específicos para a membrana. Além disso, a fusão dos grânulos específicos e/ou vesículas secretoras com a membrana plasmática ou fagossomal permite a criação do complexo multienzimático ligado à membrana chamado de NADPH oxidase, responsável pela geração de espécies reativas de oxigênio que atuam dentro do fagolisossomo ou extracelularmente (NATHAN, 2002).

Figura 4 - Montagem do complexo NADPH oxidase para produção de ROS



A NADPH oxidase (ou NOX2) é uma NADPH:O_2 oxidoredutase que transfere elétrons do NADPH para o oxigênio molecular, gerando ânions superóxido (O_2-). A NADPH oxidase é uma proteína transmembrana heterodimérica (flavocitocromo b_{558}) presente nos grânulos específicos e vesículas secretórias, e que consiste de duas subunidades: $gp91^{phox}$/NOX2 e $p22^{phox}$. Quando estimuladas, as quatro subunidades

$p40^{phox}$, $p47^{phox}$, $p67^{phox}$ e a GTPase monomérica Rac1/2 (proteínas presentes no citoplasma) são translocadas para a membrana, onde se associam com o flavocitocromo para formar a NADPH oxidase ativa (Figura 4). O complexo NOX2 produz inicialmente o ânion superóxido e esse pode ser convertido até peróxido de hidrogênio (H_2O_2) através da enzima superóxido dismutase (SOD) presentes no citosol e da mitocôndria. Além disso, o H_2O_2 reage com íons cloreto para formar ácido hipocloroso (HOCl) através da enzima mieloperoxidase (MPO), presente nos grânulos azurofílicos (NATHAN; SHILOH, 2000). Ambos H_2O_2 e HOCl são tóxicos e potentes antimicrobianos, porém também são capazes de agredir o tecido do hospeiro (NATHAN, 2002).

A ativação da produção de espécies reativas de oxigênio pode ser induzida por fagocitose ou contato (através de receptores específicos) com complexos imunes, quimioatraentes como IL-8, LTB4, C5a e PAF, por certos produtos microbianos (como fMLP), componentes da parede celular fúngica (como zimosan) e agonistas de receptores Toll-like (TLRs). Compostos ativadores de PKC (proteína quinase C) como o forbol 12-miristato 13-acetato (PMA) são ativadores potentes da NADPH oxidase e são muito utilizados como controles positivos ou exacerbadores dessa via nos procedimentos experimentais (NAUSEEF; BORREGAARD, 2014). A sinalização intracelular que regula essa via é realizada através da fosforilação das subunidades a fim de montar o complexo proteico. A via das quinase PI3K/AKT fosforila as subunidade citoplasmáticas, ativando o complexo. A PKC fosforila $gp91^{phox}$, aumentando sua ligação com $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ e Rac1/2, gerando e ativando o complexo NOX2 (RAAD et al., 2009). Vale ressaltar que o conhecimento dessas rotas também tem relevância clínica, porque indivíduos com uma mutação *missense* no gene $p40^{phox}$ desenvolvem a doença granulomatosa crônica (CGD), sofrendo de infecções bacterianas e fúngicas graves e recorrentes (AMULIC et al., 2012). Esses neutrófilos deficientes na CGD apresentem uma produção de ROS reduzida quando estimulados por fagocitose ou TNF-α, fMLP e zimosan. Porém, sua produção de ROS em resposta a agonistas solúveis como PMA é normal, demonstrando o papel especializado da $p40^{phox}$ montagem oxidativa do fagossomo (NAUSEEF; BORREGAARD, 2014). Apesar da principal fonte de ROS ser o complexo NOX2 (NADPH oxidade), esses compostos oxidantes podem ser provenientes de outras vias intercelulares. A cadeia transportadora de elétrons da

mitocondrial também é outra importante fonte de estresse oxidativo, assim como a xantina oxidase, que é expressa na superfície luminal do endotélio, a lipooxiganase, ciclooxiganase, entre outras (NATHAN; CUNNINGHAM-BUSSEL, 2013).

1.3.6. ROS e migração

Além da grande importância no combate a patógenos, recentemente, a produção de ROS se mostrou relevante também para a migração do neutrófilos. Como exposto anteriormente, o completo NOX2 pode ser ativado diretamente nos neutrófilos via fatores quimiotático (IL-8, C5a, fMLP e LTB4). Porém, foi mostrado que o próprio H_2O_2 pode atuar de maneira parácrina ou autócrina na indução da migração. No dano tecidual, o peróxido de hidrogênio pode agir como indutor quimiotático direto através da Duox (*Nox isoform dual oxidase*) (NIETHAMMER et al., 2009). Outro mecanismo encontrado foi da oxidação via ROS da LYN tirosina quinase e provavelmente outras quinases da família da SRC quinase que promovem ativação da migração neutrófilos (YOO et al., 2011). Além disso, o ROS produzidas nos neutrófilos auxiliam a migração através do H_2O_2 produzido no *leading edge*, promovendo acumulação de PIP_3 nessa porção celular. NOX2 também é capaz inativar PTEN, reforçando o acúmulo desse inositol na parte frontal do neutrófilo (KUIPER et al., 2011). Consistente com essas informações são os achados de que neutrófilos deficientes em NOX2, tratados com inibidor seletivo DPI (*diphenyle-neiodonium chloride*), ou com anti-oxidante NAC (*N-acetylcysteine*) não conseguem migrar normalmente em direção a gradientes quimiotáticos ou focos inflamatórios *in vivo*, porque perdem a sua capacidade de polarização, emitindo múltiplos pseudópodos. Não obstante, neutrófilos de pacientes com CGD, além de terem dificuldades de acabar com infecções bacterianas, apresentam características de ausência de polarização, culminando em um acúmulo de neutrófilos nas regiões inflamatórias. O que indica que talvez o ROS também tenha um papel relevante para cessar o processo inflamatório (HATTORI et al., 2010; KUIPER et al., 2011).

No pico de concentração do gradiente quimiotático, os neutrófilos devem efetuar sua ação fagocítica e de liberação de grânulos, e, em função disso, a concentração de ROS nestes locais é muito mais elevada e estável do que próxima a poucos neutrófilos que estão iniciando o processo de emigração da corrente sanguínea. Foi observado que altos níveis de ROS suprimem a motilidade celular

através do bloqueio da polimerização de actina em razão do acúmulo de actinas glutationilas (SAKAI et al., 2012). Sendo assim, as ROS podem reter os neutrófilos altamente ativados a ficarem nos locais com grande concentração de ROS, restringindo os efeitos deletérios que essa células poderia gerar se pudesse migrar novamente para outros locais (NATHAN; CUNNINGHAM-BUSSEL, 2013).

1.3.7. NETs

Mais recentemente, um novo mecanismo de controle infecioso dos neutrófilos foi identificado. Brinkmann e colaboradores, em 2004, demonstraram que os neutrófilos podem liberar redes extracelulares de neutrófilos (NETs, *neutrophil extracellular traps*) (BRINKMANN et al., 2004). As NETs são estruturas emitidas extracelularmente pelos neutrófilos com capacidade adesiva (prendendo patógenos) e antimicrobiana (URBAN et al., 2006). Os neutrófilos geram as NETs através da descondensação da cromatina (DNA) e expelem esse material para fora da célula, num processo chamado de NETosis. Assim, uma soma de DNA, proteínas associadas ao DNA (como histonas H3 citrulinada), e as enzimas proteolíticas presentes nos grânulos dos neutrófilos (como NE, MPO, proteinase 3, e outras), são liberadas no meio extracelular, atingindo diversos patógenos. Diferentes estímulos podem induzir produção de NETs, como: LPS, PMA, bactérias Gram-negativas e positivas, fungos, parasitas, plaquetas ativadas e proteínas virais (BRINKMANN et al., 2004; CLARK et al., 2007; FUNCHAL et al., 2015; GUIMARÃES-COSTA et al., 2009; MCCORMICK et al., 2010; URBAN et al., 2006; WARTHA et al., 2007).

Os mecanismos de formação das NETs ainda não estão totalmente compreendidos. Sabemos que os neutrófilos ativados para NETs perdem a integridade de sua membrana nuclear, acabando com a distinção da eucromatina com a heterocromatina (FUCHS et al., 2007). Após, ocorre a perda da integridade das membranas dos grânulos neutrofílicos, fusionando os componentes citoplasmáticos com nucleares, mais o conteúdo dos grânulos (GUIMARÃES-COSTA et al., 2012). Por fim, as NETs são liberadas através da membrana plasmática em um processo nada similar aos outros tipos de morte celular como da necrose e da apoptose (FUCHS et al., 2007).

A formação das NETs depende de NADPH oxidase, MPO e NE. Ativação de vias de sinalização de MAPKs translocam essas proteases (principalmente NE) para o núcleo, onde ocorrerá a degradação das histonas e consequente descondensação da cromatina (HAKKIM et al., 2011; PAPAYANNOPOULOS et al., 2010). Também durante esse processo, ocorre a citrulinação das histonas H3, e essa citrulinação é catalisada por PAD-4 (*peptidylarginine deiminase 4*) em neutrófilos. A inibição da atividade de PAD-4 é suficiente para cessar com a formação de NETs (LEWIS et al., 2015).

Em estudos *in vitro*, o PMA tem sido muito usado como controle positivo para indução de NETs em 3 a 4 horas após o tratamento com PMA. Interessantemente, neutrófilos deficientes em NOX2, tratados com DPI, ou de pacientes com CGD tem a produção de NETs em resposta à PMA desregulada, demonstrando um papel essencial de ROS no mecanismo de *NETosis* (PHILLIPSON; KUBES, 2011). De maneira mais curiosa ainda, apesar dos estudos mostrarem a morte de neutrófilos por *NETosis* (BRINKMANN; ZYCHLINSKY, 2007), existem alguns relatos *in vivo* de neutrófilos lançando NETs e mantendo a sua capacidade fagocítica e migratória (YIPP et al., 2012; YOUSEFI et al., 2009).

Por fim, apesar do seu grande potencial antimicrobicida, a liberação indiscriminada de tanto material com atividade catalítica pode ter efeitos deletérios ao hospedeiro. A exposição de moléculas próprias no meio extracelular pode levar à autoimunidade. Achados mostram correlações entre a cromatina e componentes de neutrófilos presentes nas NETs e os auto-anticorpos encontrados em pacientes com lúpus eritematoso sistêmico (SLE) (GARCIA-ROMO et al., 2011). A liberação dessas redes de DNA no pulmão e no líquido sinovial podem agravar patologias associadas a esses tecidos (BRANZK; PAPAYANNOPOULOS, 2013; KHANDPUR et al., 2013). Além disso, plaquetas podem ser ligar às NETs, estando associadas à formação de tromboses (FUCHS et al., 2010).

1.3.8. Modelo de migração *in vivo* – lesão estéril hepática

Os modelos *in vitro* nos auxiliam a isolar variáveis e testar interações diretas e combinadas em ambientes controlados. Porém, os sistemas vivos são muitíssimo mais ricos e complexos. Durantes nossos estudos anteriores sobre a influência do GRP em neutrófilos, utilizamos a injeção de GRP no peritônio de camundongos como sistema para estudar a migração *in vivo* do peptídeo (CZEPIELEWSKI et al., 2012). Porém, apesar da peritonite gerada, na ausência de infecções, esse sistema não correlaciona diretamente com uma patologia. Portanto, na busca de um modelo mais completo, patológico e onde a migração de neutrófilos para o sítio inflamatório agravasse a doença, chegamos aos modelos de lesão hepática – pela sua relevância fisiológica, imune e patológica.

O maior órgão interno do nosso corpo é o fígado, correspondendo a 2,5% da massa corporal total (VOLLMAR; MENGER, 2009) O fígado tem irrigação única, recebendo grande parte (aproximadamente 80%) do sangue através da veia porta que traz nutrientes do intestino, porém com baixa oxigenação. O restante é sangue oxigenado fornecido pela artéria hepática. Essa combinação de circulações com características distintas flui através da rede de capilares especializados do fígado, chamados sinusoides (JENNE; KUBES, 2013). O fígado é formado por inúmeros agrupamentos poliédricos chamadas lóbulos hepáticos. Nestes, localizam-se as células do parênquima hepático, os hepatócitos. Os hepatócitos se posicionam entre os sinusóides até o vaso central denominado veia centrolobular e compõe aproximadamente 80% do fígado. Sua função vital é a de metabolizar carboidratos, proteínas e lipídeos; secreção de bile e hormônios; produção de albumina, fatores de coagulação e proteínas de fase aguda; armazenamento energético e controle glicêmico, dentre outras. Além disso, essas células são capazes de detoxificar compostos endógenos e exógenos, como amônia, hormônios esteroides, medicamentos, contaminantes ambientais, álcool e drogas (ROSSAINT; ZARBOCK, 2013). Para realizar todas essas funções, o hepatócito apresenta uma alta taxa energética e de transcrição, somado ao grande número de mitocôndrias no seu citoplasma (DUNCAN et al., 2010).

Apesar de toda importância fisiológica do fígado, cada vez mais esse órgão vem sendo compreendido como decisivo para o funcionamento do sistema imune.

No fígado, encontram-se macrófagos residentes chamados de células de Kupffer (KC). Elas representam por volta de 35% de todas células não-parenquimais do órgão e representam cerca de 80% de todos os macrófagos do corpo (JENNE; KUBES, 2013). As KC localizam-se dentro dos sinusóides, permitindo que fagocitem material direto da microcirculação hepática, que está vindo diretamente do intestino. Essa função de filtração protege a circulação sistêmica de PAMPs, DAMPs e debris celulares, auxiliando também a disseminação de patógenos em condições homeostáticas e patológicas (BALMER et al., 2014). O fígado também apresenta um grande número de células *natural killer* (NK) e *natural killer* T (NKT) (CRISPE, 2009). Esses linfócitos também se localizam no lúmen sinusoidal e são responsáveis pela produção de grandes quantidades de interferon gama (IFNγ) (GAO; RADAEVA; PARK, 2009) Sendo assim, doenças que acometem o fígado têm grandes efeitos sistêmicos e com grande relevância clínica (BERNAL; WENDON, 2013).

A falência hepática aguda (ALF) é um exemplo de doença grave, que leva a perda de função do fígado e a sintomas como encefalopatia, coagulopatia, descontrole metabólico, sepse e falência múltipla de órgãos. Suas origens podem provir de infecções virais ou medicamentos. A lesão hepática por medicamentos (DILI) vem aumentando seu número de casos no mundo pela popularização dos fármacos e seu uso indiscriminado (RANGNEKAR; FONTANA, 2011). Dentre os fármacos capazes de promover DILI (antibióticos, antiepiléticos, anti-inflamatórios) o analgésico paracetamol (APAP ou acetaminofeno) é o que leva mais pacientes a procurar ajuda médica por DILI (LARSON et al., 2005). As intervenções são muito limitadas. Quando nos estágios iniciais (dentro das primeiras 24 horas) o N-acetilcisteína (NAC) é a terapia indicada para intoxicação por APAP (LEE, 2010) Entretanto, muito pacientes necessitam de transplante hepático (BERNAL et al., 2010).

Assim como em pacientes, o modelo animal de lesão hepática medicamentosa demonstra lesões extensa no fígado muito similares às de humano, e também são geradas pelo acúmulo do metabólito APAP chamado de N-acetil-para-benzoquinonaimina (NAPQI), derivado da oxidação do citocromo P450. Em excesso, ele se liga covalentemente a biomoléculas, desestabilizando mitocôndrias, membranas, citoesqueleto, os sistemas enzimáticos do hepatócito, e induzindo

fragmentação de DNA e instabilidade genômica, culminando na necrose oncolítica dos hepatócitos (JAMES; MAYEUX; HINSON, 2003). Nesses mesmos modelos, foi observada a dependência de células imunes para o agravamento das lesões hepáticas através da observação do desfecho após a overdose medicamentosa em animais que não possuíam células NK e NKT (LIU; GOVINDARAJAN; KAPLOWITZ, 2004; MASSON et al., 2008), neutrófilos (LIU et al., 2006) e eosinófilos (PROCTOR et al., 2013).

Por conseguinte, o processo inflamatório na DILI começou a ser estudado na busca de alternativas para o tratamento dessa patologia. Como a lesão hepática por APAP é um modelo de lesão estéril, ele é utilizado também como plataforma para o estudo e descoberta de novos DAMPs e PAMPs (ANTOINE et al., 2010; CAI et al., 2014). Dentro desse panorama, observou-se que rapidamente após o início da inflamação aguda, neutrófilos são recrutados para o fígado em grandes número (MARQUES et al., 2012; MCDONALD et al., 2010). Essa migração é induzida por: quimiocinas como a CXCL1 e CXCL2 (homólogos murinos da CXCL8 humana) via o receptor CXCR2 (MARQUES et al., 2012); peptídeos formilados oriundos de mitocôndrias dos hepatócitos mortos, via o receptor FPR (MCDONALD et al., 2010); e DAMPs, como mtDNA e DNA nuclear via TLR9 e TLR4 (MARQUES et al., 2015; MCGILL et al., 2012). As demais células imunes do fígado também reagem a necrose do hepatócitos, produzindo altos níveis, principalmente, de TNF e IL-1β (SAIMAN; FRIEDMAN, 2012), fazendo o reconhecimento de padrão via TLR2, TLR4 e NLRP3, que também estão associados a patologia da DILI (CAI et al., 2014; MCDONALD et al., 2010; XU et al., 2011; YANG et al., 2015).

O conhecimento da influência dessas diversas vias, mediadores e células imunes distintas acumulado nos últimos anos, estabelece um quadro para o desenho de estratégias para bloquear a atividade ou reconhecimento pelo sistema imune a inflamação aguda estéril. Alguns grupos mostraram que a utilização de antagonistas TLR4, TLR9 e DNAse podem ter um potencial efeito protetor na DILI por reduzir o reconhecimento e/ou reduzir a molécula que desencadeia a resposta exacerbada (CAI et al., 2014; IMAEDA et al., 2009; TUJIOS; FONTANA, 2011). Entretanto, como descrito anteriormente, o influxo inicial de neutrófilos é um dos fatores marcantes na DILI. Por isso, tratamentos que inativem a invasão

descontrolada de neutrófilos no sítio inflamatório também poderiam ter efeitos benéficos para o tecido (ISHIDA et al., 2006; MARQUES et al., 2012).

Consequentemente, o modelo de inflamação estéril através de lesão hepática induzida drogas mostra-se uma interessante ferramenta para estudar as interações *in vivo* de mediadores inflamatórios (como CXCL1 e CXCL2), seus receptores (como CXCR2) e células imunes, como no estudo da migração de neutrófilos para focos inflamatórios. E a grande bagagem científica desse modelo auxilia a normalizar o estudos de novos agentes que possivelmente possam influenciar esse sistema, como agentes anti-inflamatórios.

JUSTIFICATIVA

Nossa hipótese é a de que a produção de GRP pelo tumor influencia a quimiotaxia de células imunes, e possivelmente a sua função, recrutando-as para um fenótipo promotor de progressão tumoral e metástase.

A presença de GRP/GRPR em tumores apresenta correlação com a agressividade dos tumores. Sendo assim, estudos sobre a ação do GRP sobre a biologia tumoral e também seus efeitos em células do sistema imune são importantes a fim de determinar o seu papel *in vivo*. O desenvolvimento de novos alvos terapêuticos passa pela compreensão dos mecanismos celulares e moleculares que foram investigados.

2. OBJETIVOS

2.1. Objetivo Geral

Identificar mecanismos pelos quais o GRP e o seu receptor GRPR influenciam inflamação e migração de células tumorais e mieloides imunes.

Esses experimentos foram realizados no Instituto de Pesquisas Biomédicas, Hospital São Lucas, no Laboratório de Imunologia Celular e Molecular da PUCRS, e na Universidade Federal de Minas Gerais, Departamento de Morfologia, no Laboratório de Imunobiofotônica.

2.2. Objetivos Específicos

1. Modular a expressão do GRP em células de melanoma murino B16F10 gerando transfectantes que superexpressam o GRP;
2. Investigar o papel migratório do GRP em tumores;
3. Investigar a capacidade do GRP em estimular neutrófilos através da produção de espécies reativas de oxigênio (ROS);
4. Verificar se a produção de ROS induzida por GRP é dependente da enzima NADPH oxidase;
5. Demonstrar a dependência de ROS e NADPH oxidase na quimiotaxia induzida por GRP.
6. Determinar os efeitos do RC-3095 em células mieloides em um modelo de inflamação.

Os próximos capítulos estão organizados da seguinte forma:

- No **Capítulo 2** consta o manuscrito preparado em formato de artigo científico, aguardando para submissão e é composto pelos resultados obtidos do objetivo específicos número 1.
- No **Capítulo 3** consta o artigo científico, publicado no periódico científico *Tumor Biology* e composto pelos resultados obtidos através do objetivo específicos número 2.
- No **Capítulo 4** está o manuscrito preparado em formato de artigo científico para revista *European Journal of Immunology*, e é composto pelos resultados obtidos do objetivos específicos número 3, 4 e 5;
- No **Capítulo 5** está o artigo publicado no periódico internacional *European Journal of Immunology*, e é composto pelos resultados obtidos do objetivos específicos número 6.
- No **Capítulo 6** são apresentadas as considerações finais;
- No **ANEXO A** encontra-se a introdução do manuscrito preparado em formato de artigo científico referente ao trabalho desenvolvido no período do Doutorado Sanduíche nos EUA;
- No **ANEXO B** e **C** estão artigos publicados em periódicos científicos (*PlosOne* e *Toxicology in Vitro*, respectivamente) onde investigamos efeitos sobre a produção de NETs;
- No **ANEXO D** está sendo apresentada a Carta de Aprovação do Comitê de Ética para o Uso de Animais;

Capítulo 2

"*Tumor-derived GRP modulates inflammatory monocyte infiltration*"

Manuscrito aguardando submissão

Como o GRP é altamente encontrado em tumores e descobrimos que o peptídeo pode recrutar neutrófilos, hipotetizamos que a produção de GRP pelos tumores poderia ser uma maneira encontrada por células tumorais para evadir o sistema imune, trazendo células para o microambiente e modulando suas características para um fenótipo pró-tumoral.

A seguir, encontra-se o manuscrito que contém os achados sobre a expressão do GRP em tumores e a sua influência sobre monócitos e neutrófilos do microambiente tumoral. O trabalho ainda não está concluído. Entretanto, decidimos mostrar os dados em formato de artigo científico para grifar os resultados encontrados e demonstrar mais claramente os pontos fortes e fracos que o projeto apresenta atualmente.

TUMOR-DERIVED GRP MODULATES INFLAMMATORY MONOCYTES INFILTRATION

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Short title: GRP modulates monocyte-tumor infiltration

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INTRODUCTION

Gastrin-releasing peptide (GRP) is a mammalian bombesin-like neuropeptide, with important roles in the regulation of smooth muscle contraction in the bowel, the release of hormones in the gastrointestinal tract, pancreatic enzyme secretion and as a neurotransmitter in the central nervous system (MAJUMDAR; WEBER, 2011). GRP is also considered to be a mitogenic agent capable of inducing proliferation and cell growth, since it is involved in the development of the fetal lungs and acts as a growth factor of human tissues and tumors (PATEL; SHULKES; BALDWIN, 2006). GRP production is elevated in many types of cancer and its mitogenic action of in tumors is well described in the literature, however little is known about its relationship with metastasis (QIAO et al., 2008). Similar to other neuropeptides, GRP is firstly expressed as an 148 amino acid molecule which is later cleaved, giving rise to the high active from GRP18-27 (PATEL et al., 2007). GRP has been described to alter shape, as well as increase cell adhesion through changes in the actin cytoskeleton in neuroblastoma cells (LEE et al., 2012). Therefore, researchers have begun to investigate whether GRP could be involved in regulating other stages of tumorigenesis, in addition to cell proliferation. In fact, some studies showed that the GRP and its receptor (GRPR) can regulate morphology during tumor differentiation, correlating with metastasis (PAUL et al., 2013; QIAO et al., 2013).

In another set of studies, researches have found that increased GRP production is linked with proinflammatory conditions, modulating immune cells in asthma (ZHOU et al., 2011), arthritis (OLIVEIRA et al., 2011), and sepsis

(PETRONILHO et al., 2012). We have demonstrated that GRP can act as a chemotactic molecule for neutrophils, activating specific signaling pathways to induce neutrophil migration both *in vivo* and *in vitro* (CZEPIELEWSKI et al., 2012). In tumors, infiltrating neutrophils (TANs) have been described to polarize in two phenotypes, namely N1 and N2 (FRIDLENDER et al., 2009; SIONOV; FRIDLENDER; GRANOT, 2014), similarly to what has been described for tumor infiltrating macrophages (TAMs) (GALDIERO et al., 2012; MURRAY et al., 2014).

Little information is available on tumor-derived GRP effects over immune cells (QIAO et al., 2007). Therefore, we sought to investigate the influence of GRP on cells infiltrating the tumor microenvironment (TME), using a syngeneic melanoma mouse model. In this study, we developed a new experimental system to study GRP in the TME, overexpressing the peptide in melanoma cells.

Our results show that GRP recruits inflammatory monocytes, rather than neutrophils, to tumor foci. Interestingly, when GRP is injected into untransfected (wild-type) tumors, a subpopulation of monocytes disappears from the tumor. Tumor-derived GRP production does not affect the numbers of circulating monocytes and neutrophils. Recruitment of monocytes to tumor sites did not correlate with tumor progression. Rather, GRP has a direct effect on melanoma cells *in vitro*, acting as a mitogenic agent for melanoma cells. These results illustrate the complexity of GRP interactions taking place in the tumor microenvironment. We discuss the implications of our findings in this context.

MATERIAL AND METHODS

Reagents

Gastrin-releasing peptide (GRP_{1-27}) was obtained from Sigma-Aldrich. RC-3095 was synthesized by Ambiopharm (North Augusta, USA) and provided by Cristália Ltda (Itapira, Brazil). Trizol was obtained from Gibco Invitrogen. Fetal bovine serum was from Cultilab. Primers were synthetized by Taqman. Neomycin was purchased from Novafarma. Qubit, Superscript III kit was obtained from Invitrogen. Trypan blue and RNase were purchased from Sigma-Aldrich.

Cells

Mouse melanoma cell line B16F10 (ATTC CRL-6475) was kindly provided by Dr. Martin H. Bonamino (INCA, Brazil). Cells were cultured with DMEM (4.5 g/L glucose), 10% of FBS, 1% of Pen/Strep (Gibco, Carlsbad, USA), and were maintained standart conditions in humidified chamber at 37ºC and 5% CO_2.

Mice

Female C57BL/6 mice (6-10 weeks old) were from CeMBE, PUCRS, (Brazil). All studies were approved by the Animal Care and Use Committee (CEUA no. 12/00321). Mice were housed in temperature-controlled rooms and given water and food *ad libitum* until use. Care and handling of the animals were in accordance with the National Institute of Health Guidelines.

Trypan blue exclusion assay

Cell proliferation was determined by counting cells that excluded trypan blue with a hemocytometer, and treated under the same conditions described for Ki67 assay.

Ki67 expression

B16F10 cells ($5x10^4$) were plated on 24-well culture plates until reached 50% confluence. Cells were treated with GRP (50 and 100 nM) with 10% FBS, or medium alone for 24 hours in 37°C and 5% CO_2. Cells were detached from the plate using trypsin, centrifuged and resuspended in DMEM. Cells were then stained for Ki67 (anti-Ki67 eFluor 710) and viability (Fixable Viability Dye eFluor 780) following eBioscience protocol for Staining Intracellular nuclear proteins and Viability staining Protocol C. Data were analyzed by flow cytometry.

7-AAD cell cycle analysis

B16F10 cells ($5x10^4$) were plated on 24-well culture plates until reached 50% confluence. Cells were treated with GRP (50 and 100 nM) with 10% FBS, or medium alone for 24 hours in 37°C and 5% CO_2. Cells were detached from the plate using trypsin, centrifuged, resuspended and $5x10^5$ cells/mL were placed in flow cytometry tubes. Cells were fixed with Cytofix/Cytoperm buffer (BD Biosciences) for 30 minutes on ice, washed and centrifuged twice with BD

Perm/Wash. DNA was stained with 20 µL of 7-AAD (BD Biosciences) for 15 min at room temperature. Data were analyzed by flow cytometry.

Plasmids and Cloning

Transposon pT3-NEO (carrying neomycin selection cassette) and transposase SB100X were kindly provided by Dr. Martin H. Bonamino (INCA, Brazil). To construct the pT3-GRP-IRES-NEO transposon, human 27 amino acid gastrin-releasing peptide (GRP_{1-27}) was synthesized with an IRES sequence (internal ribosome entry site), and codon-optimized by Genscript (NJ, USA) based on the sequence provided GenBank (NIH, USA) curated sequences. The sequence was linearized by AgeI digestion and inserted into pT3-NEO backbone.

Transfection

B16F10 cells were transfected with either pT3-GRP-IRES-NEO or control transposon pT3-NEO empty vector. Lonza® Nucleofector® II electroporation system was used for stable transfections according to the manufacturer's protocols. Transfected cells were positively selected with neomycin (2 µg/ml) for 3 weeks and cell lines were obtained.

RT-PCR and real-time RT-PCR (qPCR)

Total DNA was isolated with Qiagen kit according to the protocol of the manufacturer. The primers used were as follows: U3-promotor 5'-GCGAACGC GTATGGTCCC-3' (forward), GRP-IRES 3'- GACCTTCCACCGGTGGAT-5'

(reverse), mouse GRP (mGRP) 5'-TGTATGCGGCTGACAGAGAC-3' (forward), 3'-AACTTAGCGGTTTGAACGTCG-5' (reverse).

For real-time RT-PCR assays, cDNA was obtained with Superscript III Kit following manufecture's protocol. 15 ng of cDNA was amplified with SYBR Green Universal PCR Mastermix (Invitrogen) in triplicate. For sample analysis, the threshold was set based on the exponential phase of products, and CT value for samples was determined. The resulting data were analyzed with the comparative CT method for relative gene expression quantification against house keeping gene GAPDH

***In vivo* tumor model**

B16F10 cell lines ($1x10^6$ in 100µl) were subcutaneously (s.c.) injected in the left leg of anesthetized mice. Tumor volume was measured with a digital caliper every 2 days and tumor size was calculated and reported as volume using the formula ($L1^2$ x L2) / 2, where L1 is the shortest diameter and L2 is the longest diameter. For the single intratumoral injection, mice were anesthetized and 0.6 µg of GRP diluted in 1X PBS was injected into the tumor (50µl) analyzed 24 or 48 hours later. Ovalbumin peptide $_{257-264}$ (OVA) was used as an unrelated protein injection control (0.6 µg).

Blood preparation and cell isolation

Peripheral blood was collect directly from the heart at the right atrium using a 1 mL syringe. Blood was mixed with 10% of EDTA 0.5M to prevent coagulation.

Red blood cells were lysed using BD FACS Lysing Solution according to manufacture's protocol (BD Biosciences, USA). After, cells were rinsed in FACS buffer containing 1% of FBS and 0.01% sodium azide. Blood cells were subsequently stained as described below. Spleen and dLN were harvested and mashed against a cell-strainer in RPMI 2% FBS. For spleen, after single cell isolation, red blood cells were lyzed with BD FACS Lysing Solution according to manufacture's protocol (BD Biosciences, USA). Cells were filtered and counted for FACS analysis.

Tumor-infiltrating cell analysis

Tumors were harvested upon euthanizing mice, and incubated with collagenase D (2 mg/ml) plus DNase I (0.2 mg/ml) solution for 45 minutes, shaking at 37ºC, to produce a single cell suspension. Cells were filtered, counted and stained for FACS.

Flow cytometry

Cellular preparations were incubated for 20 minutes on ice with anti-CD16/32 FcBlock solution. Next, cells were incubated with the myeloid cell antibody cocktail: MHC II (IAb)-FITC, Ly6G-PE, CD11b-PerCP-Cy5.5®, Ly6C-PE-Cy7, F4/80-APC and Viability dye®-APC-Cy7. Data were obtained using FACSCantoII (Beckton Dickinson) and BD FACSDiva software, and analyzed using Flowjo vX.

Statistical analyses

Data are presented as mean ± SE. Results were analyzed using GraphPad Prism 6. Statistical differences among the experimental groups were evaluated by ANOVA with Tukey correction, or with Student's t test when only two groups were compared. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Intratumoral delivery of GRP promotes monocyte clearance in melanoma

We had previously characterized GRP as a neutrophil chemoattractant that induces neutrophil migration *in vitro* and *in vivo* (CZEPIELEWSKI et al., 2012). We asked if GRP inside the TME would lead to the recruitment of neutrophils. To investigate this, we used two different approaches. We first attempted to mimic GRP release inside tumors by performing GRP intratumoral injection in B16F10 tumor-bearing mice at day 13 of tumor growth (Fig. 1A). In parallel, we developed a B16F10 cell line that constitutively overexpressed GRP. B16F10 cell line is a well-establish model for tumor progression and metastasis, valuable for its aggressiveness (DAMSKY; BOSENBERG, 2010), and we have previously determined tumor growth kinetics in our lab (DE SOUZA et al., 2011; MAITO et al., 2012). On day 13, tumors are bigger enough (>800 mm^3) to receive injections, and have not yet metastasize (MAITO et al., 2012; MARTÍNEZ-CORRAL et al., 2012). We excised tumors and quantified infiltrating neutrophils and inflammatory monocytes, as well as determined number of these cells in the draining lymph node (dLN) and spleen of tumor-bearing mice injected with GRP or OVA peptide (ovalbumin, used as a unrelated protein control). After 24 and 48 hour of peptide injection, mice were euthanized; tumors and organs were harvested, and subjected to analysis. We found an increase in percentages of neutrophil infiltration after 48 hours upon GRP injection, however this was not sustained when we analyzed absolute cell numbers (Fig. 1B-C). Rather, we found that another important population of myeloid cells, the inflammatory monocytes, was being modulated by peptide

injection. Although we found just a tendency for reduced percentage of these myeloid cells, they disappear from the tumor infiltrate by 48 hour post-injection (Fig. 1B-C). In addition, tumor growth was unaltered with both peptides injected.

No alterations in the analyzed cell subpopulations were found in dLN and spleen (Fig. 2). We only observed a reduction in spleen neutrophil numbers at 24 hour after injection, suggesting that they may be leaving the organ and moving towards the tumor as reported earlier (CORTEZ-RETAMOZO et al., 2012). As expected, no difference in tumor growth was found due to the short period of evaluation. We could not detect alterations in presence or number of other subtypes of myeloid cells and lymphocytes (data not shown). These results show that GRP can modulate neutrophils in the tumor microenvironment. However, the effect of GRP in the clearance of inflammatory monocytes has to be further investigated.

Gastrin-releasing peptide promotes melanoma cells proliferation

To confirm whether GRP could exert a direct effect over murine melanoma B16F10 cells, we performed *in vitro* experiments of cell proliferation. Cell counts (Fig. 3A), cell cycle analysis (Fig. 3B) and Ki67 expression (Fig. 3C) verified that GRP induces B16F10 proliferation, confirming previous studies (FANG et al., 2009). Moreover, GRPR antagonist, RC-3095, was able to promote cell apoptosis of B16F10 cells (Fig. 3D). Accordingly, this antagonist is known to generate tumor cell death in a variety of cancer cell types (FLORES; LENZ; ROESLER, 2009). High percentage of malignant melanomas expresses GRPR in patients biopsies (MARRONE et al., 2013). Therefore, apart from effects on

immune cells, GRP promotes tumor growth of B16F10 *in vitro*, which complicates the *in vivo* analysis of GRP effects in the tumor microenvironment.

Development of a murine melanoma overexpressing gastrin-releasing peptide

An abrupt influx of a growth factor would dramatically alter the tumor, and the injection itself disturbs the structure of the tumor, generating more questions than answers. Because of this, and to ensure continued and more natural production of GRP in the TME, we sought to develop a model in which GRP peptide is overexpressed in B16F10 cells. For that, we used the Sleeping Beauty transponson/transposase system (ADAMS; VAN DER WEYDEN, 2008) to generate a stable cell line overexpressing GRP. We cloned the GRP sequence inside the pT3 transposon which has a U3 promotor and a neomycin resistance cassette to select the transfected cells, resulting in the pT3-GRP-IRES-Neo plasmid (Fig. 4A). As a control, we used an empty vector plasmid containing only the pT3 transposon and the neomycin cassette, pT3-Neo plasmid. Next, we electroporated B16F10 wild type (B16-WT) with our plasmids. B16F10 cells transfected with pT3-GRP-IRES-Neo (B16-GRP) and with pT3-Neo (B16-pT3) were selected with neomycin for 3 weeks and checked for the presence of the integrated GRP sequence within the construct (Fig. 4B). Since we were overexpressing only the final active peptide and not the whole GRP gene we performed qPCR with specific primers that solely recognize the mouse GRP gene and not the construct sequence. We could thus establish there was no effect of transfection over the intrinsic GRP production of tumor cells, ruling

out or an autocrine negative feedback effect of overexpressed GRP over B16F10 (Fig. 4C).

GRP overexpression modulates infiltrating monocytes in the TME

To test the influence of GRP production by melanoma cells within the tumor microenvironment, mice were injected with B16-pT3 or B16-GRP and we analyzed neutrophil, monocyte tumor infiltration and tumor growth (Fig. 5A). Unexpectedly, overexpression of GRP decreases the percentage and a tendency to reduce the numbers of neutrophils within the tumor (Fig. 5B). Oppositely to what we found with GRP intratumoral injection (Fig. 1), inflammatory monocytes were present in higher numbers and with increased frequencies within the melanoma (Fig. 5B-C). However, tumor growth did not show size alterations. Analysis in blood, dLN and spleen revealed no system immune cell variation in tumor-bearing mice that overexpress GRP (Fig. 6). These results show that GRP presence in the tumor microenvironment can modulate the migration and infiltration of myeloid cells.

CONCLUSION

In this work, we developed a stable melanoma cell line model that overexpresses GRP, allowing the study its influences on tumor microenvironment. Here, we applied different approaches to evaluate the importance of a molecule in the TME. Artificially adding high amounts of GRP via intratumoral injection decreases drastically the presence of inflammatory monocytes, while tumor production of GRP promotes the recruitment of these

cell to the TME. Inflammatory monocytes are known to increase with tumor growth and correlate with worse prognosis (QIAN et al., 2011). Inflammatory monocytes are also recognized migrate from the periphery and differentiate into tumor-associated macrophages (TAMs) (CORTEZ-RETAMOZO et al., 2012; FRANKLIN et al., 2014), which are a well-known myeloid cell enrolled in the tumor progression (MANTOVANI; SICA, 2010; MEDZHITOV, 2008). The relationship between immune cell, remarkably myeloid cells, and the tumor environment is the new frontier in cancer treatment. Novel immune checkpoint blockade regimens are revolutionizing the field, but the limitations to its effectiveness are to set by those infiltrating myeloid cells (HIGHFILL et al., 2014). Novel targets and concepts of how these cells migrate and polarize within the tumor are key to further develop proper treatments for non-responders. Therefore, understanding how GRP increases inflammatory monocyte in the TME is valuable to propose new therapies.

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CONFLICT OF INTERESTS

Cristália Ltda. (Itapira, Brazil) granted a fellowship to R.S.C and supplied the GRPR antagonist for this study. Cristália Ltda. holds a patent related to RC-3095. The other authors disclosed no potential conflicts of interest.

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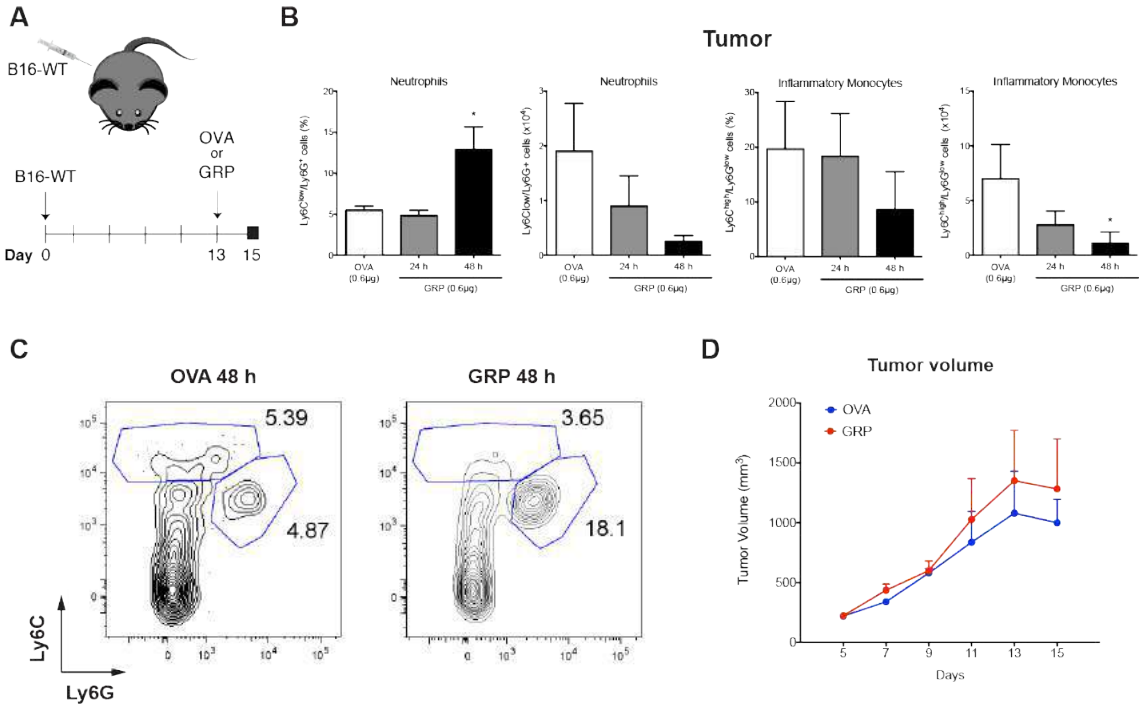
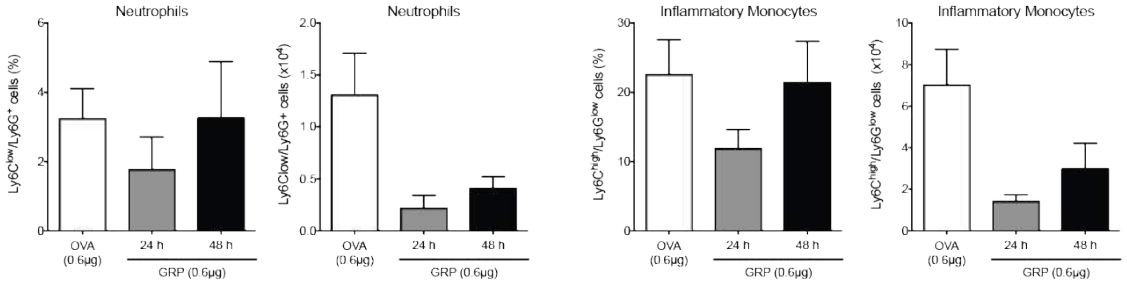


Figure 1 – Intratumoral injection of influences inflammatory monocytes. A. Mice were injected s.c. with B16-WT melanoma cell line and tumors were harvested after 15 days to analyze tumor infiltrating myeloid cell compartment. **B-C.** 24 or 48 hour before the analysis intratumoral injection of GRP (0.6µg) was performed. As control peptide OVA (0.6µg) was injected 48 hours prior harvesting. Infiltrating inflammatory monocytes ($Ly6G^{-}$ / $Ly6C^{high}$) and neutrophil percentages ($Ly6G^{+}$ / $Ly6C^{mid}$) are shown. Alive immune cells were gated based on $CD11b^{+}$/$MHCII^{-}$ expression. **C.** Representative flow cytometry plot showing reduction in the inflammatory monocyte population and increase neutrophil percentage. **D.** Tumor growth was measure every other day. Graph shows the measurements of OVA (blue lines) and GRP (red lines) injected 48 hours prior analysis. Data are representative of two independent experiments, performed with four mice per group for each sample, and expressed as mean ± SE. * $p<0.05$ compared to OVA control.

dLN



Spleen

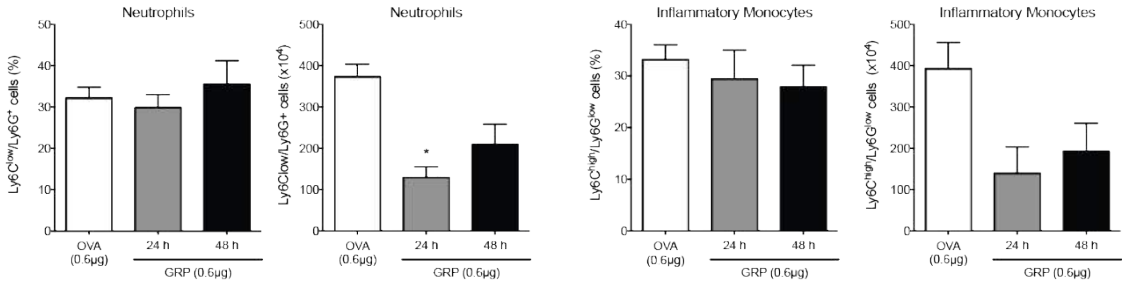


Figure 2 – Intratumoral injection do not alter dLN and spleen neutrophils and monocytes. Mice were injected s.c. with B16-WT melanoma cell line and tumors were harvested after 15 days to analyze immune cell alteration in the draining lymph node (dLN) and spleen. Intratumor injection of GRP (0.6µg) at 24 or 48 hours prior the experiment were compared with injection of control peptide (OVA, 0.6µg) 48 hours before analysis. Inflammatory monocytes ($Ly6G^-$ / $Ly6C^{high}$) and neutrophil ($Ly6G^+$ / $Ly6C^{mid}$) percentages and counts are shown. Alive immune cells were gated based on $CD11b^+$/$MHCII^-$ expression. Data are representative of two independent experiments, performed with four mice per group for each sample, and expressed as mean ± SE. * $p<0.05$ compared to OVA control.

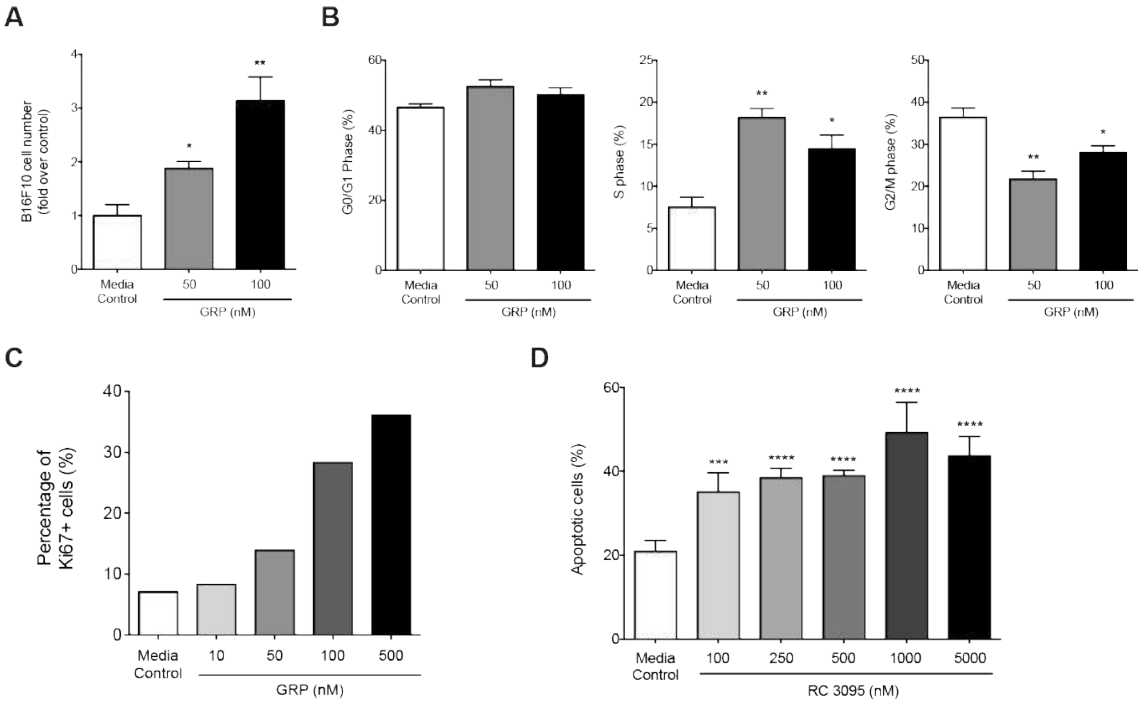


Figure 3 – Proliferative effect of GRP on murine melanoma cells. A. B16F10 cells were treated with GRP 50nM or 100 nM in 10% of FBS medium for 24 hours. Cell count was performed using a hemocytometer and trypan blue exclusion. Fold over the total number of untreated cells is shown. **B.** 7-AAD-cell cycle analysis of B16F10 treated with GRP (50nM and 100nM). Gating strategy based on morphology and 7-AAD staining. Percentage of G0/G1 (left graph), S (middle graph) and G2/M (right graph) cell cycle stages are shown after 24 hours of stimulation. **C.** Ki67+ B16F10 cells treated with GRP (10, 50, 100 and 500nM) after 24 hours post treatment are presented. **D.** Viability of B16F10 was verified by Annexin V/PI assay 24 hours after incubation with control, or RC-3095 (100, 250, 500, 1000 and 5000 nM). Data are representative of two independent experiments, performed in triplicate (A, B and D) or single samples (C) for each sample, and expressed as mean ± SE. ****P < 0.0001; ***P<0.001; **P<0.01; *P< 0.05.

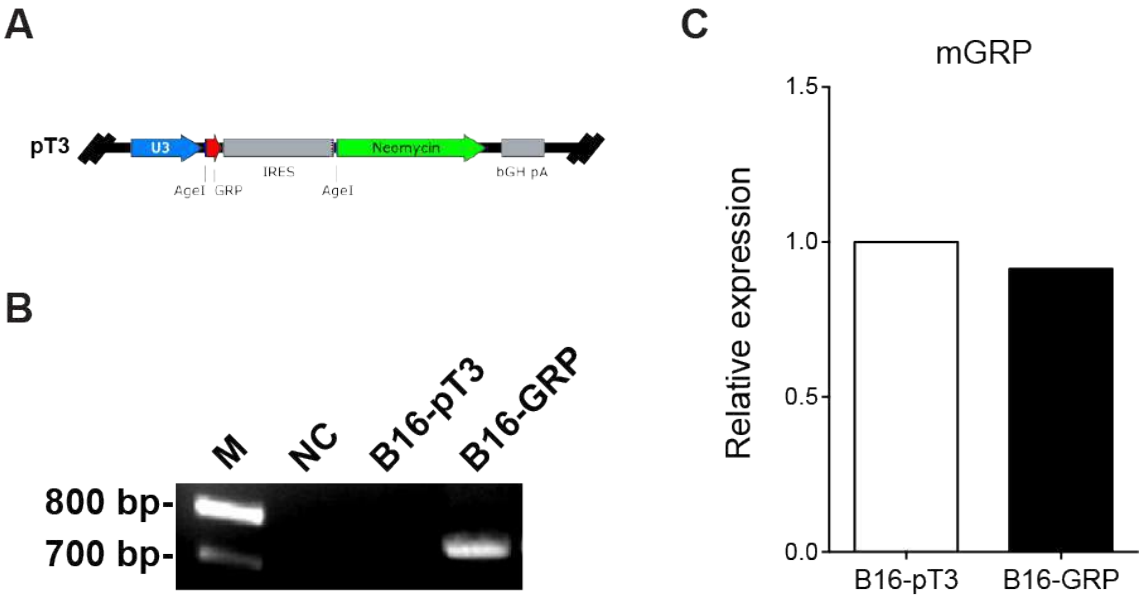


Figure 4 – Overexpression of GRP_{18-27} in B16F10. A. pT3 transposon was constructed to express GRP sequence. Graphic illustration of pT3-GRP-IRES-NEO transposon. **B.** RT-PCR confirming the presence of the GRP sequence in the construct. pT3 transposon plasmid DNA were used as a template and specific primers against U3-promotor (forward) and GRP-IRES (reverse) were amplified. M = Molecular weight marker; NC = Negative control, no DNA; pT3-NEO = pT3 transposon control, empty vector; pT3-GRP-IRES-Neo = experimental transposon for GRP overexpression. **C.** Overexpression of GRP_{18-27} does not alter mouse GRP gene expression in B16F10. qPCR was performed with primers specific for mouse GRP gene. Relative expression is shown with arbitrary units.

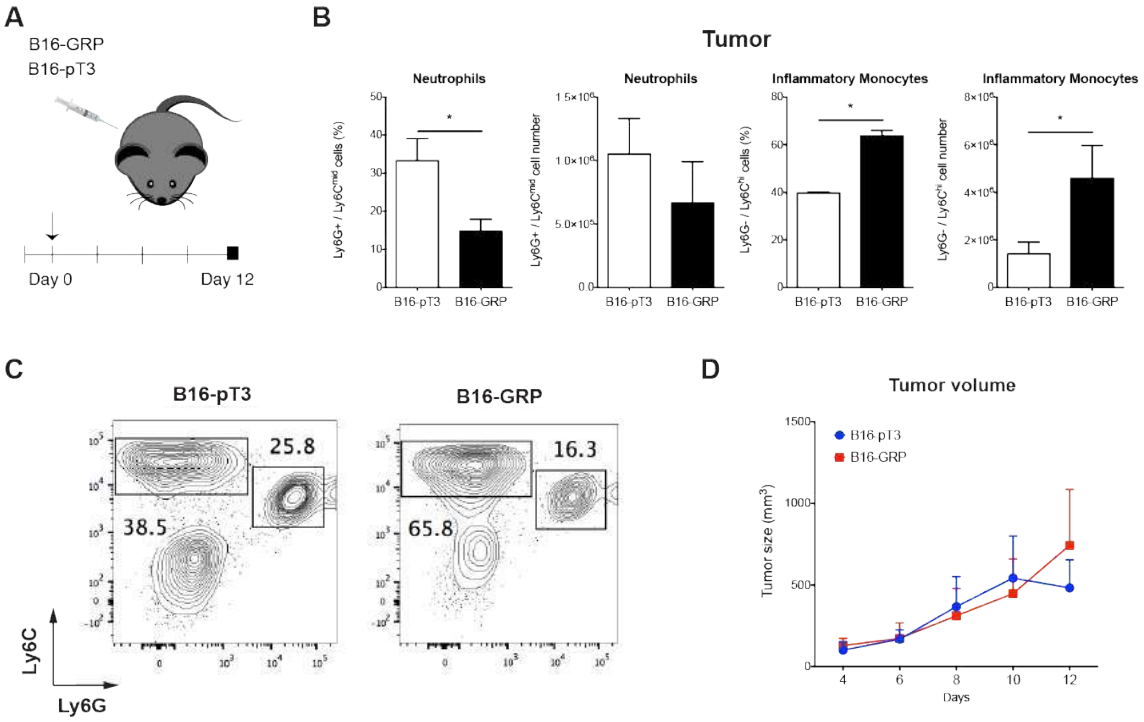


Figure 5 – Melanoma GRP-producing cells recruits inflammatory monocytes to the tumor microenvironment. **A.** Mice were injected s.c with B16-GRP or B16-pT3 and accompanied for 12 days. **B-C.** B16-GRP-bearing mice presented more infiltrating inflammatory monocytes ($Ly6G^{-}$ / $Ly6C^{high}$) and less percentage of neutrophils ($Ly6G^{+}$ / $Ly6C^{mid}$). (n=5 per group; * $p<0.05$ vs B16-pT3 empty vector control). **C.** Representative flow cytometry plot displaying the enlargement of inflammatory monocyte population within tumor microenvironment. **D.** Tumor growth was measure every other day. Graph shows the measurements of B16-pT3 (blue lines) and B16-GRP (red lines). Data are representative of two independent experiments, performed with four mice per group for each sample, and expressed as mean ± SE.

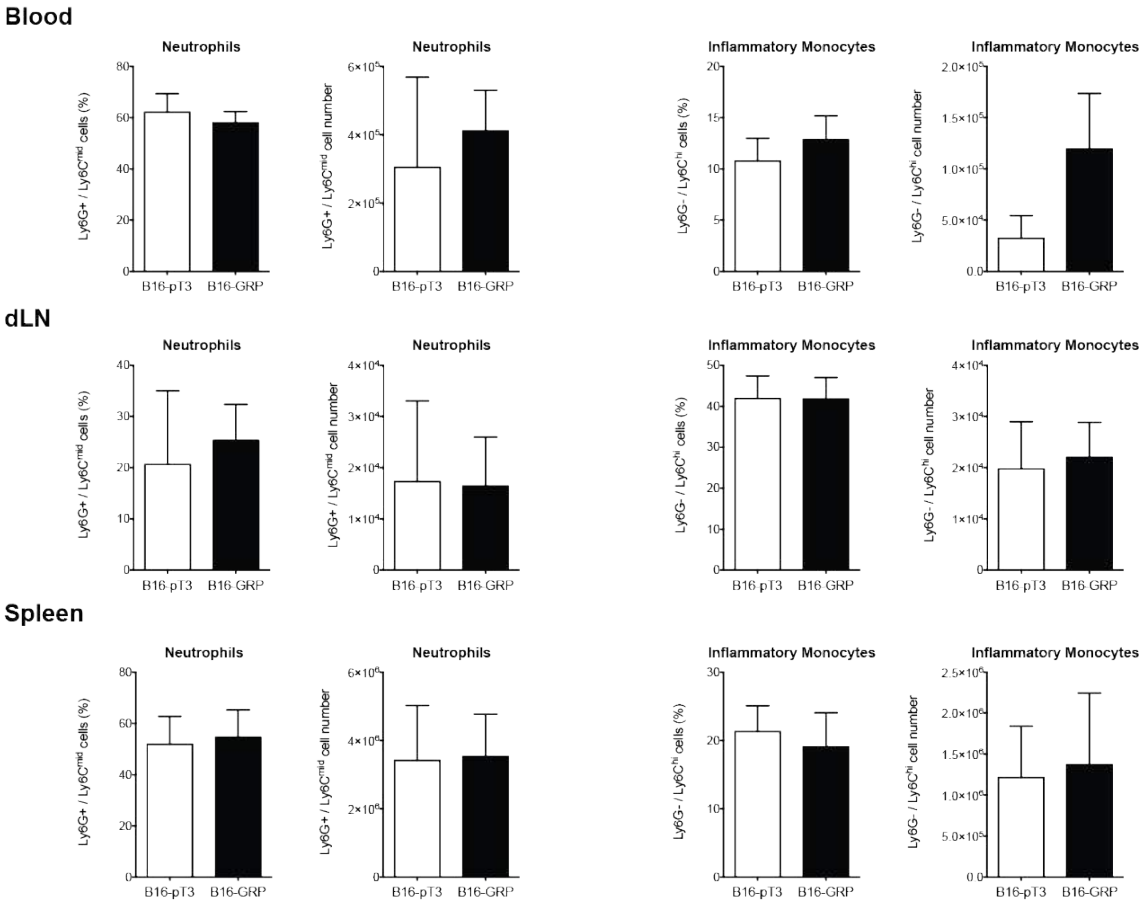


Figure 6 – GRP overexpression does not alter neutrophils and monocytes in the blood, dLN and spleen. Mice were injected s.c. with B16-pT3 (white bars) or B16-GRP (black bars) melanoma cell lines and tumors were harvested after 12 days to analyze immune cell alteration in blood, draining lymph node (dLN) and spleen. Inflammatory monocytes ($Ly6G^-$ / $Ly6C^{high}$) and neutrophil ($Ly6G^+$ / $Ly6C^{mid}$) percentages and counts are shown. Alive immune cells were gated based on $CD11b^+$/$MHCII^-$ expression. Data are representative of two independent experiments, performed with fiver mice per group for each sample, and expressed as mean ± SE.

Capítulo 3

" *Neuropeptide gastrin-releasing peptide induces PI3K/reactive oxigen species-dependent migration in lung adenocarcinoma cells*"

Artigo publicado no periódico científico

Tumor Biology

Além de induzir quimiotaxia de células imunes, o GRP atua na migração de células tumorais. Porém, esses estudos são de complexa interpretação, porque a estimulação proliferativa do GRP mascara muitas vezes os dados da indução da motilidade. Sendo assim, decidimos investigar a ação do GRP sobre ademocarcinomas de pulmão de não pequenas células que não proliferam via GRP na promoção da migração e suas via de sinalização.

A seguir encontra-se o artigo científico mostrando a indução de migração desses tumores na dependência de PI3K e sem a ativação da proliferação. Este artigo foi publicado no periódico científico *Tumor Biology.*

Original Article

Tumor Biology

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Neuropeptide gastrin-releasing peptide induces PI3K/reactive oxygen species–dependent migration in lung adenocarcinoma cells

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Abstract

Nerve fibers and neurotransmitters have increasingly been shown to have a role in tumor progression. Gastrin-releasing peptide is a neuropeptide linked to tumor aggressiveness, acting as an autocrine tumor growth factor by binding to its receptor, gastrin-releasing peptide receptor, expressed by many tumors. Although neuropeptides have been previously linked to tumor cell proliferation, more recent studies have uncovered roles for neuropeptides in chemotaxis and metastasis. Understanding the precise roles of such peptides in cancer is crucial to optimizing targeted therapy design. We have previously described that gastrin-releasing peptide acts directly as a chemotactic factor for neutrophils, dependent on PI3K, ERK, and p38. In this study, we investigated roles for gastrin-releasing peptide in lung adenocarcinoma. We asked if gastrin-releasing peptide would act as a proliferative and/or chemotactic stimulus for gastrin-releasing peptide receptor–expressing tumor cells. In A549 cells, a non-small cell lung carcinoma line, the treatment with gastrin-releasing peptide leads to activation of AKT and ERK1/2, and production of reactive oxygen species. Gastrin-releasing peptide induced migration of A549 cells, dependent on gastrin-releasing peptide receptor and PI3K, but not ERK. However, no proliferation was observed in these cells in response to gastrin-releasing peptide, and gastrin-releasing peptide did not promote resistance to treatment with a chemotherapy drug. Our results suggest that, similar to what happens in neutrophils, gastrin-releasing peptide is a migratory, rather than a proliferative, stimulus, for non–small cell lung carcinoma cells, indicating a putative role for gastrin-releasing peptide and gastrin-releasing peptide receptor in metastasis.

Keywords

Gastrin-releasing peptide, gastrin-releasing peptide receptor, non–small cell lung carcinoma, chemotaxis, gastrin-releasing peptide, metastasis, neuropeptide, cancer

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Introduction

The nervous system influences biological processes beyond the roles originally described, namely, physiologic and motor regulation. Cancer innervation has been shown to benefit tumor growth and its dissemination.[1–3] Recent associations between sympathetic neural fibers and tumors have been described. The neuropeptide Y is expressed in many tumors and linked to increased vascularization, cancer promotion, and progression.[4,5] Substance P activates oncogenes and is associated with DNA damage.[6] Moreover, different studies have linked neural-related factors to the modulation of metastasis, as well as extravasation, colonization, and degradation of base membranes.[7–12]

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The neuropeptide GRP (gastrin-releasing peptide) has various physiological roles, mainly acting as a neurotransmitter.[13] GRP is known to act by interacting with its preferential receptor, gastrin-releasing peptide receptor (GRPR)[14] which is overexpressed in lung, prostate, breast, stomach, pancreas, and colorectal tumors.[15] As a result, GRP is usually thought of as a mitogenic factor in cancer; however, little is known about its effects on tumorigenesis, metastasis, and the pathways involved in mediating this process. Interestingly, a GRPR antagonist, RC-3095, was able to induce regression in several types of tumor cells in vitro and in vivo, including glioblastoma,[16,17] small cell lung cancer (SCLC),[18] pancreatic cancer,[19] and ovarian cancer.[20]

GRP also acts during fetal lung development[21,22] and as an autocrine and paracrine growth factor for other human tissues[23,24] and tumors.[13,15,25] GRP serum levels are elevated in many malignancies especially in SCLC. Pulmonary neuroendocrine cells (PNECs) are the main source of GRP in the lung.[26] In asthma models, GRP is overexpressed in the lung, acting as an immunomodulatory molecule.[27] Furthermore, GRP acts as a chemotactic molecule for neutrophils.[28] To study the relative contribution of GRP to tumor proliferation, survival, and migration, we used the non–small cell lung carcinoma (NSCLC) cell line A549, a type of lung adenocarcinoma cell, the highest mortality related to malignant diseases in the world. Our results report that GRP is a migratory, rather than a mitogenic or pro-survival, stimulus for NSCLC cells.

Materials and methods

Cell culture

Human alveolar epithelial adenocarcinoma (A549) were obtained from ATCC (CCL-185) and maintained in Dulbecco's Modified Eagles Medium (DMEM; Gibco™ Cat. No. 31600-034) supplemented with 10% fetal bovine serum (FBS; Gibco Cat. No. 16000-044) and 0.1 mg/mL of penicillin–streptomycin antibiotic (Gibco Cat. No.15140122). All cells were incubated at 37°C and 5% CO_2 in a humidified incubator with medium being replaced every 48 h.

Trypan blue exclusion assay

Cells (5×10^4) were starved (0.5% FBS) for 24 h then treated with GRP (50 and 100 nM; Sigma-Aldrich Cat. No. G8022) with 10% FBS, or medium alone for 24 h at 37°C and 5% CO_2, cell proliferation was determined by counting cells that excluded Trypan Blue (Sigma-Aldrich Cat. No. T8154) with a hemocytometer.

Viability assays

A549 cells (1×10^4) were plated on 96-well culture plates until reached 50% confluence and later starved (0.5% FBS) for 24 h. For the proliferation assay, cells were treated with GRP (10 nM and 100 nM) or medium alone for 24 h at 37°C under 5% CO_2. For the viability assay, cells were pretreated with GRP (10, 50, 100, and 500 nM) for 15 min in the incubator. Cisplatin (CDDP; Fauldcispla 10 mg/10 mL from Libbs Farmacêutica) 20 µg/mL was then added to each well. Cells were incubated for 24 and 48 h. Proliferation and viability were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in each treatment group described above. Briefly, MTT (5 mg/mL; Sigma-Aldrich Cat. No. M2128) solution was added to each well. Cells were incubated at 37°C for 2 h in a humidified incubator with 5% CO_2. The formed formazan crystals were solubilized by the addition of dimethyl sulfoxide (DMSO). The optical density was measured at a wavelength of 570 nm with an ELISA plate reader (Antros Zenyth 340r). In addition, cells were treated as described above and stained by the Fixable Viability Dye (eFluor® 780; eBioscience Cat. No. 65-0865) following eBioscience Viability Staining Protocol C. Samples were acquired by BD FACSCantoII™ and analyzed using Flowjo v10.

Ki67 expression

A549 cells (5×10^4) were plated on 24-well culture plates until reached 50% confluence. Cells were starved (0.5% FBS) for 24 h then treated with GRP (50 and 100 nM) with 10% FBS, or medium alone for 24 h at 37°C and 5% CO_2. Cells were detached from the plate using trypsin (Sigma-Aldrich Cat. No. T4049), centrifuged, and stained for viability (Fixable Viability Dye eFluor 780) and Ki67 expression (anti-Ki67 eFluor® 710) following eBioscience protocol for Staining Intracellular Antigens for Flow Cytometry (Protocol B) using Foxp3/Transcription Factor Staining Buffer Set (eBioscience Cat. No. 00-5523). Samples were acquired by BD FACSCantoII and analyzed using Flowjo v10.

Propidium iodide cell cycle analysis

A549 cells (5×10^4) were plated on 24-well culture plates until reached 50% confluence. Cells were starved (0.5% FBS) for 24 h then treated with GRP (50 and 100 nM) or RC-3095 (1 µM) with 10% FBS, or medium alone for 24 h at 37°C and 5% CO_2. Cells were detached from the plate and fixed with 70% ethanol for 1 h on ice. Then, cells were washed, centrifuged twice, and 50 µL of RNase A stock solution (10 mg/mL; Sigma-Aldrich Cat. No. R4875) was added to each tube for 15 min at 37°C. After incubation, DNA was stained with 10 µg of Propidium Iodide (PI) Staining Solution (BD Pharmingen™ Cat. No. 556463) for 30 min at room temperature. Samples were acquired by BD FACSCantoII and analyzed using Flowjo v10.

BrdU assay

A549 cells (5×10^4) were plated on 24-well culture plates until reached 50% confluence. Cells were starved (0.5%

FBS) for 24 h then treated with GRP (50 and 100 nM) with 10% FBS, or medium alone for 24 h at 37°C and 5% CO_2. Cell cycle analysis was performed using the APC BrdU Flow Kit (BD Pharmingen™ Cat. No. 552598) and protocol was performed according to the manufacturer's instruction manual. Data were acquired by BD FACSCantoII and analyzed using Flowjo v10.

Clonogenic assay

This assay was performed according to Franken et al.[29] Briefly, 100 cells (A549) were plated on six-well plates and let adhered for approximately 4 h. Cells were washed, then treated with GRP (50 and 100 nM) or medium alone (both 10% FBS) for 15 days in 37°C and 5% CO_2. On the 15th day, cells were washed three times with cold phosphate-buffered saline (PBS) 1× and fixed with freezer-cold methanol for 10 min. Colonies were stained with 1 mL of crystal violet for 10 min, and the excess were washed with running water. Only colonies with 50 cells or more were counted.

Expression of phospho-Akt, phospho-Erk1/2, and phospho-P38

A549 cells (5×10^4) were plated on 24-well culture plates until reached 50% confluence. Cells were starved (0.5% FBS) for 24 h and then treated with GRP 50 nM in DMEM 0% FBS or medium alone for 5 min in 37°C and 5% CO_2. The expression of phospho-AKT, phospho-ERK1/2, and phospho-p38 was measured by flow cytometry following BD Bioscience Phosflow Protocol for Adherent Cells using trypsin to detach cells. Briefly, cells were fixed in pre-warmed Phosflow Buffer I (BD Phosflow™ Cat. No. 557870) for 10 min at 37°C. After washing, permeabilization was performed with Phosflow Perm Buffer II (BD Phosflow™ Cat. No. 558050) for 30 min on ice. Cells were washed twice and stained with phycoerithryn (PE) anti-AKT (pT308; BD Phosflow™ Cat. No. 558275), PE-Cy™7 anti-p38 (pT180/pY182; BD Phosflow™ Cat. No. 560241), and FITC anti-ERK1/2 (BD Phosflow™ Cat. No. 612592) for 30 min on ice. Data were acquired by BD FACSCantoII and analyzed using Flowjo v.10.

Intracellular reactive oxygen species generation assay

For the determination of intracellular reactive oxygen species (ROS) generation, two different probes were used: 5-(and-6)-chloromethyl-2′,7′-dichlorodihydroflurescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes™ Cat. No. C6827) and CellROX® Deep Red Reagent (Molecular Probes Cat. No. C10422). Both are nonfluorescent while in a reduced state and, upon oxidation, yield intracellular fluorescent compounds. For the first probe, cells (1×10^5) were plated on 24-well culture plates for 24 h. Medium was removed and cells were stimulated for 1 h at 37°C and 5% CO_2 with GRP (50 and 100 nM) and H_20_2 (0.03%) in 500 µL of serum-free DMEM. Afterward, cells were detached, centrifuged at 300*g* for 5 min, and incubated in a 2.5 µM CM-H_2DCFDA solution for 1 h at 37°C and 5% CO_2. Cytosolic ROS production was measured by FACSCantoII (Beckon Dickinson) and analyzed by FlowJo v10. As for the later, 10^3 A549 cells were plated on a cover slide for 24 h in DMEM 10% FBS. Cells were treated equally to the previous method. CellROX® Deep Red Reagent was added at a final concentration of 5 µM to cells and then incubated for 30 min at 37°C. Confocal images were taken in a Zeiss LSM 5 Exciter microscope.

Wound healing assay

A549 cells (2×10^6) were seeded on 6-well plates DMEM 10% FBS for 24 h or until reached 90% confluence at 37°C under 5% CO_2. Subsequently, cells were starved (0.5% FBS) for 24 h. Then, using a p200 pipette tip, a straight line was scraped into the well to mimic an injury. Cells were washed three times with PBS to remove the debris and treated with GRP 50 nM in 10% FBS, or medium alone and allowed to migrate for 48 h. Cultures were photographed at day zero, 24 and 48 h via an inverted optical microscope with a capturing digital image system (Olympus IX51), always in the same field. Photographs were further analyzed by Adobe® Photoshop® CS5.

Transwell chemotaxis assay

Protocol described by Shi et al.[30] was followed with minor modifications for the Transwell® system (Corning Cat. No. 3464). Briefly, A549 cells (2×10^6) were seeded on a six-well plate and starved (0.5% FBS) for 24 h at 37°C under 5% CO_2. Cells were then suspended in DMEM 10% FBS (2×10^5 cells/200 µL) and added to top wells of 8 µm-Transwell® inserts. GRP was added to the bottom wells at 50 nM containing 10% FBS, or medium alone with 10% FBS. Cells were incubated for 8 h at 37°C under 5% CO_2. To evaluate the involvement of ROS, ERK, PI3K, and GRPR on GRP-induced cell migration, cells were pretreated in suspension with selective inhibitors *N*-acetyl-l-cysteine (NAC; Fluimucil®), PD98059 (Cell Signaling Technology® Cat. No. 9900), LY294002 (Cell Signaling Technology® Cat. No. 9901), and RC-3095 (provided by Cristália Ltda), respectively, at 37°C under 5% CO_2 for 1 h. At last, inserts were removed from the plate; cells on the bottom were fixed in the membrane and stained with 4′,6-diamidino-2-phenylindole (DAPI; 1:50; Molecular Probes Cat. No. D1306) for 5 min. Using a scalpel, membranes were removed and placed on a glass slide in a mounting medium and covered with a coverslip. Slides were examined at 200× magnification using an optical

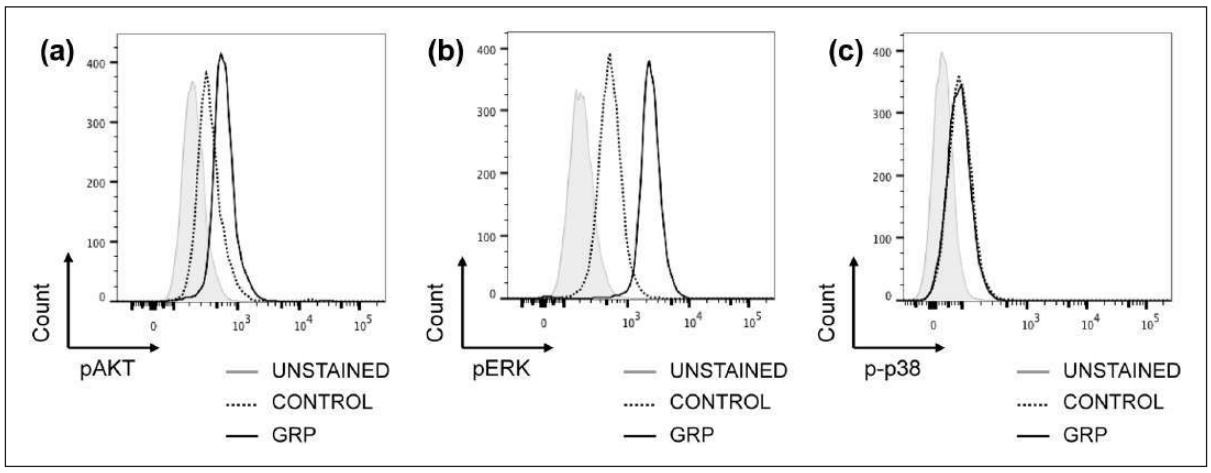

Figure 1. GRP induces AKT/MAPKs phosphorylation. A549 cells were stimulated with GRP (50 nM) for 5 min and (a) AKT, (b) ERK1/2, or (c) p38 activation patterns were monitored by Phosflow analysis. Number of counted cells and expression of pAKT, pERK1/2, and p-p38 is shown in the histograms. Isotype control (tinted histogram) and medium only (black histogram line) were used as negative controls. Data are representative from two independent experiments performed in triplicates.

microscope Olympus BX41 and six random fields were captured and further analyzed using ImageJ software (NIH) to perform cell count.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Results were analyzed using GraphPad Prism 6. Statistical differences among the experimental groups were evaluated by analysis of variance (ANOVA) with Tukey correction or with Student's t test. The level of significance was set at $p < 0.05$.

Results

GRP activates signaling pathways in NSCLC

GRPR is the most commonly expressed GRP receptor in patients with lung cancer,[31] which is of particular interest considering that adenocarcinoma expresses higher levels of this receptor than all other histological types.[32] To further investigate NSCLC responses to GRP in in vitro models, we selected the A549 cell line for its high expression of GRPR.[33] This tumor metastasizes primarily to the liver as well as in bone, adrenal glands, and brain, and yet, little is known about the biology of this disease. Previous studies investigating the effect of GRP on NSCLC cell lines have shown that GRP stimulates AKT pathway.[33] GRP also induces rapid activation of p44/42 MAPK in lung cancer cells through epidermal growth factor receptor (EGFR).[34] When we stimulated cells with GRP and evaluated AKT, p38, and ERK1/2 phosphorylation, we observed not only AKT activation (Figure 1(a)) but also ERK1/2 (Figure 1(b)). No changes were seen in p38 phosphorylation (Figure 1(c)). Because these proteins are involved in several cell-signaling pathways that regulate not only proliferation but also survival and migration, we sought to investigate these outcomes in A549 cells following GRP treatment.

GRP does not stimulate proliferation or survival of NSCLC cells

To evaluate the effect of GRP on lung cancer cell proliferation, we exposed cells to different concentrations of GRP, and analyzed proliferation/survival by Trypan Blue exclusion and MTT assays. No differences in cell counts were observed compared to medium only, in any of the concentrations used, 24 (Figure 2(a)) or 48 h (Figure 2(b)), or in the MTT assay (Figure 2(c)). In addition, a clonogenic assay did not show any increase in colony formation after treatment with GRP (50 and 100 nM; Figure 2(d)). Cells were also analyzed for expression of proliferation marker Ki67 (Figure 2(e) and (f)), cell cycle accumulation (Figure 2(g)), and the ability to incorporate bromodeoxyuridine (BrdU; Figure 2(i)). Results of Ki67 and cell cycle assays were similar to the previous assays.

Because previous studies have seen a decrease in GRP efficiency in the presence of serum,[35] all experiments were performed with either 0.5% or 10% of serum content during stimulation (data on 0.5% FBS experiments are presented on Supplementary Figure 1). As can be observed, results were not different when performed in 0.5% or 10% of FBS during experiments (Figure 2 and Supplementary Figure 1). Especially, proliferation, which can be affected by growth factors present in serum, did not differ in these two culture conditions. Altogether, our results indicated that GRP does not stimulate A549 cell proliferation.

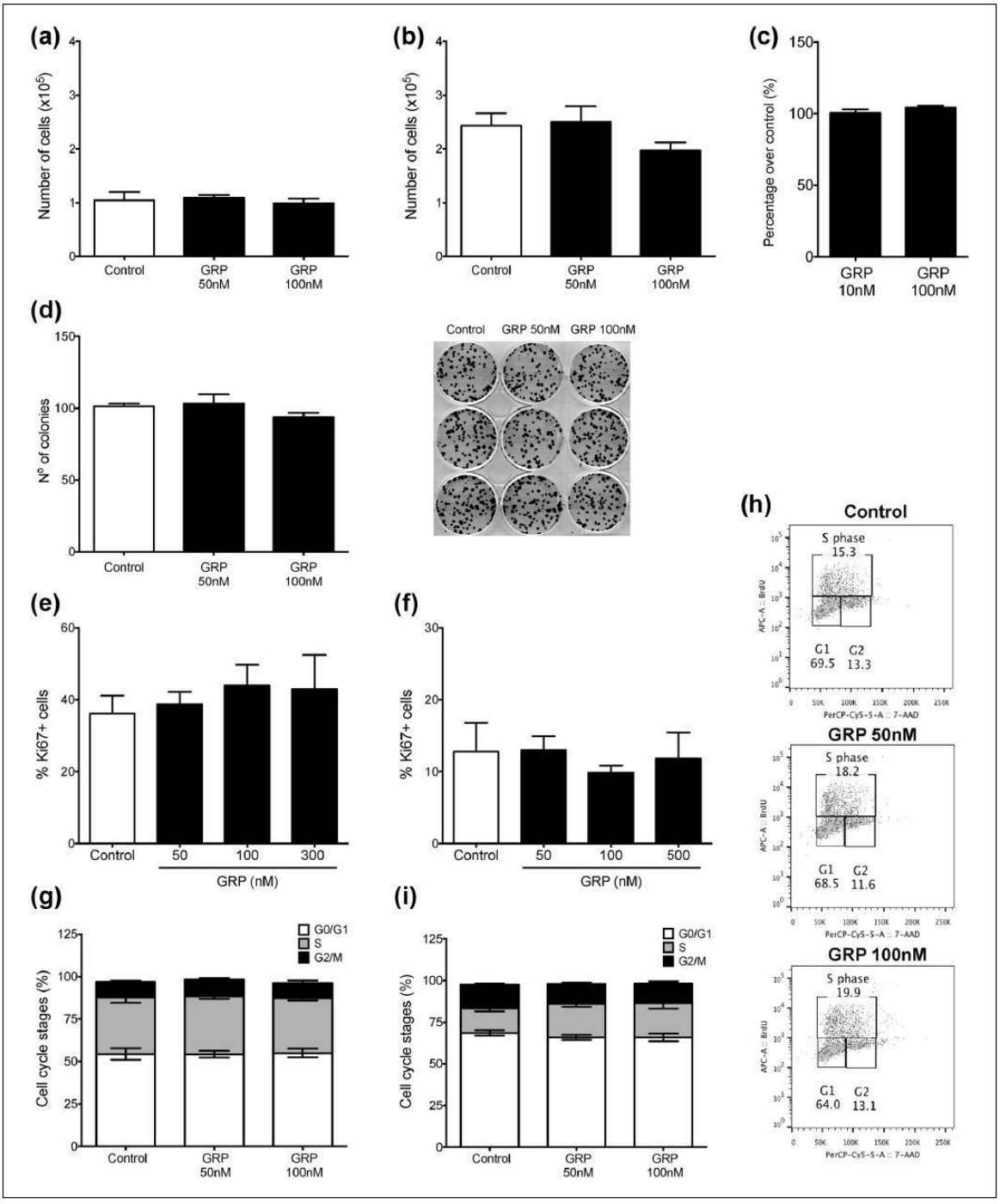

Figure 2. Lack of proliferative effect of GRP on A549 cells. (a and b) A549 cells were treated with GRP 50 or 100 nM in 10% of FBS medium for (a) 24 or (b) 48 h. Cell count was performed using a hemocytometer and Trypan Blue exclusion. Total number (mean ± SEM) of counted cells per group is shown. (c) MTT proliferation assay was performed with treated-cells in two concentrations of GRP (10 and 100 nM) in 10% of FBS for 24 h. Data (mean ± SEM) are expressed as percentage of an untreated control. (d) Clonogenic assay of A549 cells treated with GRP (50 or 100 nM). Number of colonies per well was counted 15 days after stimuli. Data (mean ± SEM) are representative from three independent experiments performed in triplicates. (e and f) Ki67 expression (% of Ki67 + cells) of A549 treated with GRP (50 and 100 nM) in 10% FBS medium after (e) 24 or (f) 48 h. (g) PI–cell cycle analysis of A549 treated with GRP (50 and 100 nM). Percentage of G0/G1 (white bars), S (gray bars), and G2/M (black bars) cell cycle stages are shown for cells cultured for 24 h. (h and i) BrdU incorporation assay of A549 treated with GRP (50 and 100 nM). (h) Representative FACS plots show gate strategy for BrdU/7-AAD staining. (i) Percentage of G0/G1 (white bars), S (gray bars), and G2/M (black bars) cell cycle stages are shown for cells cultured for 24 h. Data (mean ± SEM) are representative from three independent experiments performed in triplicates. Statistics: one-way ANOVA with Tukey post-test or Student's t test ($p < 0.05$).

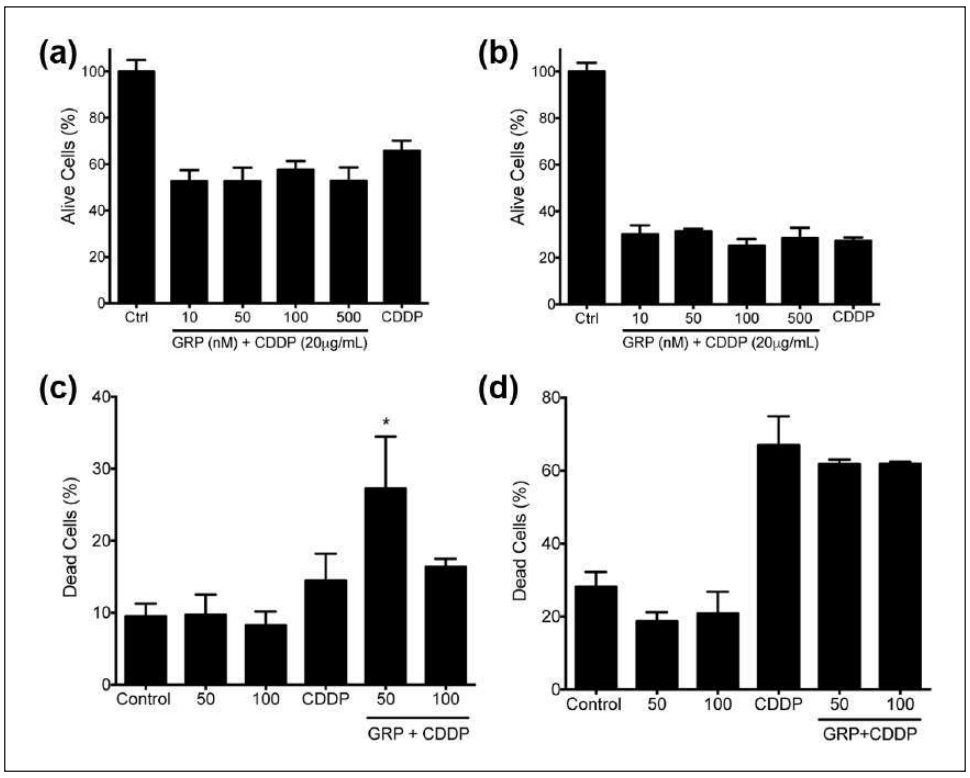

Figure 3. Pre-treatment with GRP does not increase cell survival upon challenge with cisplatin. (a and b) MTT survival assay of A549 cells treated with GRP (10, 50, 100, and 500 nM) for 15 min prior to CDDP (20 μg/mL) for (a) 24 or (b) 48 h. Data (mean ± SEM) are representative from two independent experiments performed in quadruplicates and are expressed as percentage of an untreated control. (c and d) Cells cultured in 10% FBS medium were then treated with GRP (50 and 100 nM) for (c) 24 or (d) 48 h and stained by the Fixable Viability Dye (1:1000) for 30 min. Cell viability was analyzed by flow cytometry. Data (mean ± SEM) are representative from two independent experiments performed in triplicates. Statistics: one-way ANOVA with Tukey post-test (*$p < 0.05$).

To investigate whether the signaling pathways activated by GRP were related to improving survival in NSCLC, we pretreated A549 cells with GRP and incubated them with cisplatin (CDDP) for 24 and 48 h. No difference was observed in cell survival by MTT and viability staining assays in any of the GRP concentrations tested in 24 (Figure 3(a) and (c)) or 48 h (Figure 3(b) and (d)). These findings indicate that although GRP activates proteins involved in survival pathways, it is does not prevent cells from death by CDDP.

GRP induces ROS production

Elevated rates of ROS have been detected in almost all cancers, where they promote many aspects of tumor development and progression. ROS activate proteins involved in cell proliferation, migration, and survival.[36] Here, we report for the first time that GRP stimulates ROS production on A549 cells (Figure 4(a) and (b)) when incubated with both CellROX Deep Red reagent dye and CM-H_2DCFDA probe, respectively.

GRP induces cell migration

It is known that morphogenesis is an important step for cellular motility during the development of invasive nature of many tumors. Recent data suggest that GRP serves primarily as a morphogen and not as a mitogen as usually viewed.[35,37] In order to evaluate the migration potential of GRP on the A549 cell line, we used two established migration assays. In the wound healing assay, we found a significant difference when treating cells with GRP in 24 and 48 h after the wound compared to non-treated cells (Figure 5(a) and (b)). However, some argue this assay does not evaluate migration alone and the effect seen could be due to cell proliferation, which was dismissed in our previous experiments (Figure 2). To support our data, we also performed the transwell assay. We found that GRP 50 nM was able to induce cell migration (Figure 5(c) and (d)). To investigate if GRP-induced cell migration is dependent on ROS, PI3K, ERK, or GRPR activation, cells were pretreated with selective inhibitors, or RC-3095. Pretreating A549 cells with NAC (Figure 5(e)), PI3K inhibitor, or RC-3095 (Figure 5(f)) significantly decreased the migration toward GRP. To

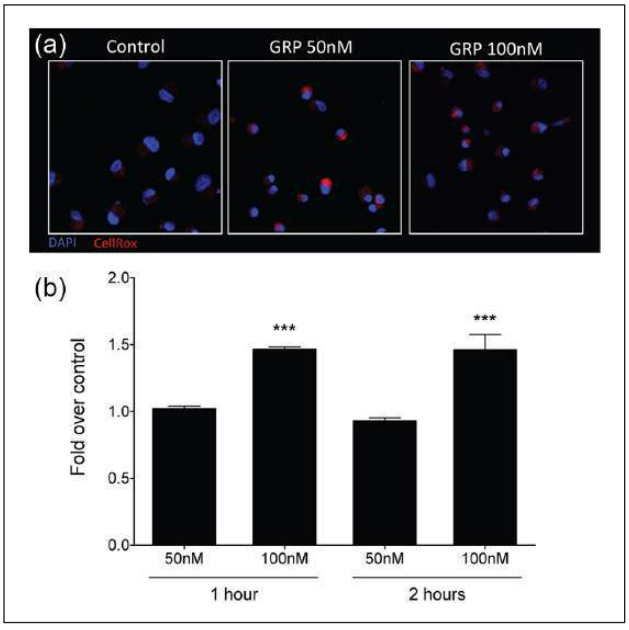

Figure 4. GRP induces ROS production in A549 cells. (a) 10^3 cells were stimulated with GRP (50 and 100 nM) for 1 h and incubated with 5 μM of CellROX Deep Red Reagent for 30 min. Confocal images were taken in a Zeiss LSM 5 Exciter microscope. Data are representative of one experiment. (b) Cells were stimulated with GRP 50 and 100 nM for 1 h and incubated with 2.5 μM of CM-H2DCFDA for 1 h. ROS generation was analyzed by flow cytometry using FACSCantoII. Data are representative of two independent experiments performed in triplicates with similar results. Statistics: Student's t test, ***$p < 0.001$.

discard a possible cytotoxic role for the GRPR antagonist, RC-3095, which could bias our result, we performed some toxicity tests. RC-3095 at 10 μM had no significant cytotoxic effect or in any lower concentration tested (not shown). Altogether, these results demonstrate that the rapid activation of AKT/MAPK pathways via GRPR induces cell migration, rather than proliferation or survival, in A549 cells.

Discussion

Tumors often resemble integrated organs, being composed by different cells, which work coordinately. Although both central and sympathetic nerve elements are present in different types of tumors,[38] little is known regarding the roles they play. Sympathetic nerve fibers that reach tumors can release neurotransmitters and neuropeptides locally,[12] activating signaling pathways that can facilitate tumor growth.[7,8]

There are two basic types of lung cancer. SCLCs comprise about 15%–20% of all cases, while NSCLC is related to 80%–85% of lung cancer cases. Based on histology, NSCLCs are divided in squamous cell carcinoma, large cell carcinoma, or adenocarcinoma, the latter being the most common type of lung cancer.[39] SCLCs express high levels of GRP,[40] which has been shown to influence tumor aggressiveness.[25,41] However, NSCLC does not usually produce GRP[42,43] and NSCLC patients do not have high plasma levels of GRP.[44,45] The cell line selected for this study (A549) is derived from NSCLC adenocarcinoma, and we did not find any GRP production in A549 supernatants (not shown). However, we found a significant ROS- and PI3K-dependent effect for GRP over A549 migratory activity mediated by GRPR.

NSCLC are higher GRPR expressers[32] than SCLCs, indicating that this receptor could have an important role in this tumor's biology. One possibility is that nerve fibers infiltrating the tumor secrete GRP, influencing metabolic processes that contribute to tumor progression.[7] GRP is widely expressed in the brain, spinal cord, and nerve fibers.[46] Moreover, many studies have shown that GRP/GRPR are involved in the mediation of itch, fear memory consolidation, anxiety, and stress.[47–49] The relationship between chronic stress and cancer is well established in the literature.[9,11] Therefore, chronic stress may influence cancer cells by the release of GRP, further activating the sympathetic nerve system and the hypothalamic–pituitary–adrenal axis through the release of stress hormones.[38]

Another possibility is that GRP is provided to tumors by PNECs. PNECs are resident lung cells and have been suggested to be the cell of origin for some SCLCs.[50,51] Lung adenocarcinoma is the most frequent type of cancer in smokers.[52] GRP expression is increased in PNECs during hypoxia[53] and smoking,[54,55] providing a candidate link for stress-induced lung cancers. Therefore, it is possible that NSCLCs use GRP produced by PNECs to stimulate migration, promoting tumor aggressiveness. Nicotine and other tobacco toxins activate PNECs;[56] thus, it could be hypothesized that GRP is connected to lung cancer promotion in smokers.

In our study, we found that both AKT and ERK1/2 were activated after stimulation with GRP. These proteins are involved not only in cell proliferation and migration but also in survival. Previous studies investigating the effect of GRP on NSCLC cell lines showed that GRP stimulates AKT activation primarily via c-Src activation, followed by extracellular release of the EGFR ligand amphiregulin, leading to the activation of EGFR and PI3K. Also, GRP induces rapid activation of p44/42 MAPK in lung cancer cells through EGFR.[34] Nevertheless, no previous study has reported ERK1/2 activation on a non-small cell lung carcinoma cell line. Although AKT and ERK1/2 were activated following GRP treatment, we did not observe any increase on cell proliferation in any of the assays performed, and it was not influenced by serum content (0.5% or 10% FBS). These results are consistent with other studies, which reported that even without the presence of serum in the medium, GRPR activation has an inconsistent, and only modest, effect on tumor growth, leading to the hypothesis that the GRP acts as a weak mitogen on some tumors.[35]

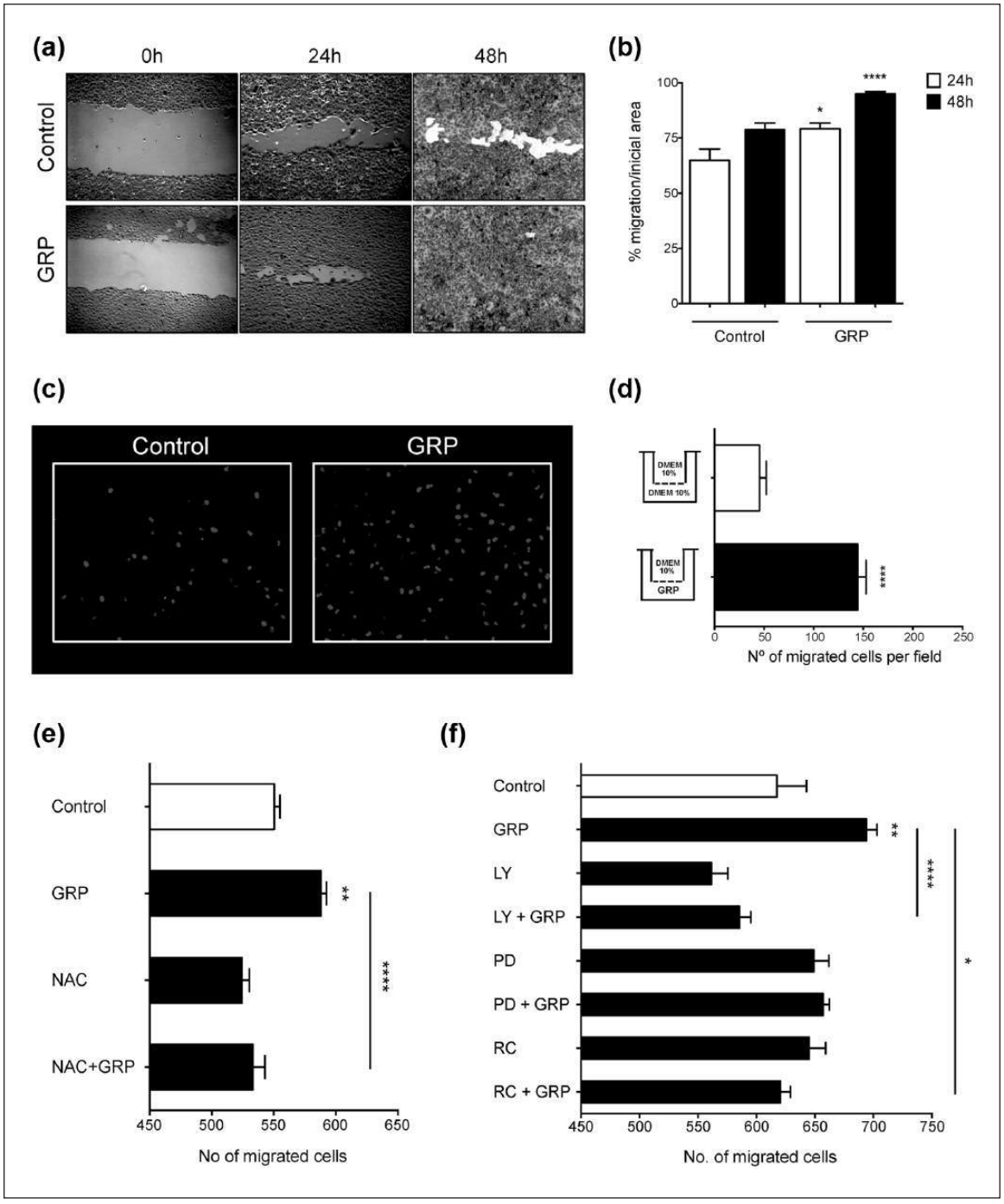

Figure 5. GRP promotes A549 cell migration. (a and b) A549 cells stimulated with GRP (50 nM) in 10% FBS medium were subject to wound healing assay and stained with hematoxylin (blue area). (a) Representative images at 0 (left column), 24 (middle column), or 48 h (right column) after treatment. Microscopy images with 200× magnification. (b) Image quantification of the wounded area size. Migration is expressed as percentage of coverage of the starting wounded area (set as 0%). Nine pictures per sample were taken and analyzed. Data (mean + SEM) representative of three independent experiments performed in triplicates. Statistics: Student's t test, *$p < 0.05$, ***$p < 0.001$. (c)–(f) A549 cells were starved and placed in the upper side of transwell migration chambers and medium alone (as control) or medium plus GRP 50 nM with 10% FBS were added to the lower chamber. Transwell membranes were stained with DAPI and six pictures per sample were taken. (c) Representative microscopy images (at 200× of magnification) of transwell migration assay (quantified in d). (e and f) Cells were pretreated with selective inhibitors of ERK, PI3K, GRPR, and ROS (PD98059, LY294002, RC-3095, and NAC, respectively). (d)–(f) Quantification of cell number per field of each group is shown. Data (mean + SEM) representative of two independent experiments performed in triplicates. Statistics: one-way ANOVA with Tukey post-test ($p < 0.05$).

Liu et al.[33] found that GRP rescues A549 cells exposed to gefitinib, an EGFR inhibitor. In order to verify this survival effect, we investigated if GRP could rescue cells from cytotoxic effects of cisplatin, which is still one of the main treatments in NSCLC cases.[57] However, no difference was observed, possibly due to the mechanism involved in cisplatin-mediated cytotoxicity, which induces apoptosis differently from EGFR inhibitors. Even though GRP activated survival signals, it was not enough to protect cells from CDDP. Consequently, in tumor cells that do not constitutively produce GRP, even though GRP may be provided paracrinally by local nerve cells, that signal alone might not be enough to protect from drug-induced cell death.

Early studies suggested that GRP could play a role on NSCLC migration.[58] Jensen[35] proposed that GRP would rather act as a morphogen. More recent evidence indicated that Caco-2 and HT-29 colon cancer cell lines had increased cell motility when treated with bombesin.[37] A different study demonstrated that motility of the same cell lines was mediated by GRP via upregulation of ICAM-1 via FAK.[59] Others have observed a morphological change in BE(2)-C neuroblastomas with high expression of GRPR, and GRPR knockdown inhibited the metastatic potential both in vitro and in vivo.[60] Our results are in agreement with these findings, further elucidating the mechanisms underlying the migratory stimulation potential of GRP for lung cancer cells.

Finally, this is the first study to demonstrate that the treatment with GRP induces ROS generation in lung adenocarcinoma cells, which can be related to PI3K/AKT activation that leads to cell migration. ROS are known to participate directly or as a second messenger in promoting lung carcinogenesis,[61] facilitating mutagenesis, cell proliferation, survival, differentiation, and motility.[36,62] Future studies will determine the relative contribution of this pathway during in vivo progression and metastatic activity of NSCLC.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Capítulo 4

"*Neutrophil activation by GRP is dependent on reactive oxygen species generation to induce migration and NETs*"

Manuscrito aguardando submissão para a revista *European Journal of Immunology* em formato de *Short Communication*

Moléculas quimiotáticas, ao promoveram quimiotaxia, ativam outros processos celulares em neutrófilos que os preparam para a sua função final no combate de patógenos ou reparo celular. Para seguir caracterizando as funções do GRP diretamente em neutrófilos, hipotetizamos que o peptídeo ativaria mecanismos básicos da biologia dos fagócitos.

A seguir, encontra-se o manuscrito de artigo científico demostrando que o estímulo do GRP em neutrófilos induz a produção de espécies reativas de oxigênio na dependência de NADPH oxidase e, que esse fenômeno de ativação pode culminar da liberação de NETs. Este artigo aguarda experimentos discutidos nas considerações finais e será submetido em formato de *Short Communication* para a revista *European Journal of Immunology*.

NEUTROPHIL ACTIVATION BY GRP IS DEPENDENT ON REACTIVE OXYGEN SPECIES GENERATION TO INDUCE MIGRATION AND NETs

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Short title: GRP-induced ROS and NETs

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ABSTRACT

Neutrophil activation and migration is one of the hallmarks of innate immune inflammatory responses. Production of reactive oxygen species (ROS) upon encounter with chemoattractants is essential for pathogen elimination. We have shown that the neuropeptide gastrin-releasing peptide (GRP) induces neutrophil migration. However, it is still unknown whether GRP activates other neutrophil mechanisms and responses. *In vivo*, GRP release has been proposed to play an inflammatory role in arthritis and sepsis. Here, we report that GRP can promote ROS production in human neutrophils *in vitro* via NADPH oxidase, and that GRP-induced chemotaxis is dependent on NADPH oxidase-derived ROS. Moreover, GRP is able to generate NETs release, confirming its capacity to activate effector neutrophil responses. Our results support the idea that GRP is a proinflammatory mediator, directly inducing neutrophil activation.

INTRODUCTION

Neutrophils are the largest population of innate immune cells surveilling our body. Activation of these cells cause them to migrate from the periphery to inflammatory sites and this is essential to host protection and wound healing (Amulic et al. 2012). Neutrophils in inflammatory foci can destroy pathogens by several mechanisms, which include the release of enzymes and reactive oxygen species, as well as phagocytosis (Amulic et al. 2012). However, neutrophil stimulation, and the control of its migration, are relevant to several diseases (Nathan 2006). Neutrophils migrate upon detecting inflammatory processes, via chemokines (eg. IL-8 and IL-17), produced by tissue resident cells, as well as endothelial cells; cytokines (IL-1beta and TNF); and other chemoattractants (eg. leukotriene B_4 and PGE_2). Direct recognition of pathogen and host-derived chemotactic molecules (eg. fMLP and DNA) also constitutes a powerful migration stimulus for neutrophils (Kolaczkowska & Kubes 2013). All of these chemoattractants are sensed mainly by G-protein couple receptors (GPCRs). Signaling through these receptors also leads to increased expression of adhesion molecules, and prepare neutrophils for the release of granules and oxidative stress proteins upon transendothelial migration, in order to perform end-target functions (Nathan 2006).

One of the essential effector mechanisms of neutrophils is the large production of reactive species of oxygen (ROS). ROS are mainly produced by NADPH oxidase complex (NOX2) in the phagossome or plasma membrane, in the form of superoxide ($[O_2]^{\bullet -}$). This oxidant can be rapidly converted into hydrogen peroxide (H_2O_2), which can be further transformed into hypochlorous acid (HOCl) by myeloperoxidase (MPO). These radicals react with pathogen

proteins, promoting killing (Nathan & Cunningham-Bussel 2013). Recently, the ROS signaling pathway was found to be essential for neutrophil migration by affecting actin polymerization in response to chemoattractant molecules (Hattori et al. 2010, Sakai et al. 2012). Therefore, investigation of chemotactic molecules should cover its dependence of ROS production.

Gastrin-releasing peptide (GRP) is a mammalian bombesin-like peptide produced in large quantities by tumors. Tumor-produced GRP is capable to induce proliferation and cell growth (Patel et al. 2006), where it acts preferentially via GRPR (gastrin-releasing peptide receptor) (Ramos-Alvarez et al. 2015). Different studies correlate the increased production of GRP with immune cells function and, therefore, its involvement in asthma (Zhou et al. 2011), rheumatoid arthritis (Grimsholm et al. 2005, Oliveira et al. 2011) and sepsis (Dal-Pizzol et al. 2006). Interestingly, GRP is also described as a neuropeptide, because it is produced in the central nervous system, promoting a broad spectrum of physiological processes including glicemia, feeding, gastrin and somatostatin release, gastric acid and pancreatic secretion, gastrointestinal motility and memory formation (Ramos-Alvarez et al. 2015, Roesler et al. 2014). Along with its expression in the spinal cord and peripheral nerves, promoting pain perception and itch sensation (pruritus) (Sun & Chen 2007, Sun et al. 2009). We have demonstrated that GRP can act as a chemotactic molecule for neutrophils, activating MAPK and AKT signaling pathways to induce neutrophil migration, both *in vitro* and in a model of peritonitis *in vivo* (Czepielewski et al. 2012). To further extend our knowledge about GRP-induced neutrophil migration, we sought to investigate the direct effect of GRP on neutrophil activation. In this study, we examined whether GRP activates reactive oxygen

species production, and if this production is relevant to neutrophil chemotaxis. Our results indicate that upon GRP stimulation, human neutrophil produce ROS, in a NADPH oxidase-dependent manner. Accordingly, GRP-induced migration is impaired when NADPH is inhibited, or ROS is inactivated with a scavenger. Interestingly, prolonged exposure to GRP could also promote NETosis, a major effector mechanism that have been recently demonstrated to be involved in several neutrophil responses (Papayannopoulos & Zychlinsky 2009). These findings indicate that GRP activates neutrophils, promoting ROS production and ROS-dependent chemotaxis, and activating drastic end-target responses, culminating with NET release.

MATERIAL AND METHODS

Reagents

PMA (Phorbol myristate acetate) was purchased from Promega (Brazil). Gastrin-releasing peptide (GRP), Dextran, Diphenylene iodonium (DPI), N-acetyl-L-cysteine (NAC), and Histopaque-1077 were obtained from Sigma-Aldrich (USA). RC-3095 was synthesized by Ambiopharm (North Augusta, USA) and provided by Cristália Ltda (Itapira, Brazil). ECORI and HINDIII were from Invitrogen. The 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diaceate, acetyl ester (CM-H_2DCFDA) was from Molecular Probes (USA). RPMI 1640 was from Cultilab (Brazil) and FCS was from Gibco (USA).

Human neutrophil isolation

Whole blood (20 mL) was collected from healthy volunteer donors (with a mean age of 29 years, from both sexes) with documented verbal consent into heparin-treated tubes. Erythrocytes were removed using Dextran sedimentation followed by two rounds of hypotonic lysis. Neutrophils were isolated from the resulting cell pellet using Histopaque-1077 density centrifugation and then resuspended in RPMI 1640 medium. Neutrophil purity was evaluated by flow cytometry using FACSCanto II (Becton Dickinson), based on morphology and granulocyte marker expression, resulting in around 90% of purity. Single cells were verified by gating on granulocyte size on the basis of forward scatter (FSC) and side scatter (SSC), followed by CD66b and CD3 expression

discrimination. Cell viability was always higher than 97%, as examined by Trypan Blue exclusion assay.

Assay of intracellular ROS generation

Determination of intracellular ROS generation was based on the oxidation of 0.5 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) to yield an intracellular fluorescent compound. Neutrophils (2 x 10^6 cells/ml) were pre-treated with ROS scavenger NAC (1 mM) or NADPH oxidase inhibitor, DPI (100 μM) and stimulated with GRP for 30 minutes at 37°C under 5% CO_2 atmosphere. Afterwards, cells were incubated with CM-H_2DCFDA for 30 minutes at 37°C under 5% CO_2. Cytosolic ROS production was measured by flow cytometry using FACSCanto II flow cytometer (Becton Dickinson) with the BD FACSDiva software and analyzed with FlowJo vX.

Neutrophils Chemotaxis Assay

Neutrophil chemotaxis was assayed in a Transwell system (Corning) using 5-μm polycarbonate membranes (Czepielewski et al. 2012). GRP (5 nM) was added to the bottom wells, neutrophils (1 x 10^5/100 μL) were added to top wells and incubated for 2 h at 37°C under 5% CO_2. Afterwards, migrated neutrophils were harvested and counted on a Neubauer chamber. To evaluate the involvement of ROS on GRP-induced neutrophil chemotaxis, cells were pretreated with NAC (1 mM) or with DPI (100 μM) for 30 minutes at 37ºC under 5% CO_2.

Chemotactic index was calculated as the ratio of the number of migrated neutrophils in chemoattractant-containing wells divided by the number of neutrophils that migrated to RPMI 1640 medium alone.

Quantification of NETs release

Neutrophils (2 x 10^6/mL) were stimulated with GRP (5, 50 and 100 nM), PMA (50 nM) or medium alone. After 1 h, 20 U/ml of each restriction enzyme (ECORI and HINDIII) was added to cultures, which were kept for 2 h at 37°C, under 5% CO_2 atmosphere. NETs were quantified in culture supernatants using Quant-iT dsDNA HS kit (Invitrogen), according to manufacturer's instructions.

Statistical analyses

Data are presented as mean ± SE. Results were analyzed using GraphPad Prism 5. Statistical differences among the experimental groups were evaluated by ANOVA with Tukey correction or with Student's t test. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

GRP-induced ROS production is dependent on NADPH oxidase

Neutrophils produce ROS upon stimulation by chemoattractants. The mechanisms underlying GRP-induced neutrophil migration have not been fully characterized. To investigate whether GRP could induce ROS production similarly to other neutrophil stimulants, we treated human purified neutrophils with different doses of GRP (5 and 50 nM) known to induce cell migration, and verified neutrophil intracellular ROS production. Accordingly, PMA stimulation resulted in ROS production. Similarly, GRP treatment promoted ROS production in neutrophils in a dose dependent manner (Fig. 1A). To verify whether GRP effect occurs via its receptor GRPR, we pretreat neutrophils with GRPR antagonist (RC-3095) and stimulated them with GRP. As expected, GRPR inhibition suppressed ROS production, demonstrating its direct influence on neutrophil GRPR signaling (Fig. 1B). We also pretreated human neutrophils with ROS scavenger, N-acetylcisteine (NAC) to further confirm ROS generation by GRP (Fig. 1C). Next, we sought to determine the signaling pathway involved in ROS production by GRP. The main source of reactive oxygen species in neutrophils is the engagement of the NADPH complex. To investigate if ROS production upon GRP stimulation is dependent of NADPH oxidase, we pretreated neutrophils with NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI). Pretreatment of neutrophils with DPI abolished GRP-induced ROS production (Fig. 1C) which indicated the neuropeptide acts as a classical inflammatory stimulus in neutrophils, activating oxidative burst through ROS formation (Raad et al. 2009).

We had previously shown that GRP induces neutrophil chemotaxis dependently on PLC- β2, PI3K, ERK, p38 signaling pathways, which are normally activated by major neutrophil stimulants (Czepielewski et al. 2012). To confirm the importance of ROS on GRP-induced neutrophil migration signaling, we pretreated cells with DPI and stimulated them to migrate towards a GRP gradient in a Transwell chemotaxis assay. We found that pretreatment with DPI profoundly inhibited neutrophil chemotaxis to GRP (Fig. 2). The results show that GRP-induced neutrophil migration relies on ROS production by NADPH oxidase. Ability to produce ROS through NADPH oxidase in neutrophils is fundamental for pathogen control, as patients with chronic granulomatous disease (CGD) displaying genetic deficiencies in NADPH complex subunits have impaired bactericidal and fungicidal capabilities (Amulic et al. 2012). Also, CGD patients neutrophils exhibit erratic chemotaxis and odd formation of multiple cellular protrusions (Hattori et al. 2010). Formation of pseudopods at the neutrophil's leading edge (closest side to the chemoattractant source) is based on F-actin polymerization, promoting cellular elongation and posterior retraction. ROS production has been shown to be essential for these activation F-actin polymerization and formation of pseudopodia. However, when following a chemotactic gradient this decreased ROS-induced polymerization leads to loss of cellular polarization and directionality (Sakai et al. 2012). Collectively, our results provide evidence that GRP can act both as a chemotactic factor and an inflammatory peptide.

GRP promotes neutrophil extracellular traps generation

Neutrophils can eliminate pathogens by diverse mechanisms, both intra- and extracellular. To combat microorganisms extracellularly, highly activated neutrophils can release neutrophil extracellular traps (NETs) (Branzk & Papayannopoulos 2013). Recently, besides pathogen-derived compounds, host-derived molecules are also shown to induce NETs release (Brinkmann et al. 2004, Tadie et al. 2013). NADPH oxidase-dependent ROS production is a key mechanism to this process (Nauseef & Borregaard 2014). Thus, we asked whether activation and production of ROS promoted by GRP could lead to NETosis. In order to test this, we treated human neutrophils with PMA, a known NETs activator, or different doses of GRP (5, 50 and 100 nM). Interestingly, after 3 hours in culture with the stimuli, extracellular DNA was found in the culture media of neutrophils treated with this unspecific neutrophil activators (PMA) and GRP, in a concentration-dependent manner (Fig. 3). These results indicate that GRP is one of the endogenous molecules with ability to induce profound activation of neutrophils upon exposure.

GRP release has been correlated with the proinflammatory milieu of sepsis via TL4 signaling and arthritis (Oliveira et al. 2011, Petronilho et al. 2012). These diseases are marked by neutrophil overactivation and oxidative burst alterations, and NETs have been found to play a critic role in the disease (Alves-Filho et al. 2010, Martinod et al. 2015, Wright et al. 2014). In arthritis, neutrophil-derived ROS act directly and as a secondary message, promoting cartilage destruction (Wright et al. 2014), while NETs have been recently shown to be associated with the generation of autoantibodies (Khandpur et al. 2013, Pratesi et al. 2014). In sepsis, ROS participates in the exacerbated neutrophil

activation that promotes systemic inflammatory response syndrome and in the later exhaustion of neutrophil function together with nitric oxide synthases (NOS) (Rios-Santos et al. 2007), while TLR4-induced NETs lead to organ ischemia and damage (Camicia et al. 2014, Tanaka et al. 2014). Taken together, our results show that GRP is not only a chemotactic molecule for neutrophils but also activates the production of ROS through its receptor GRPR.

CONCLUDING REMARKS

Commonly, neutrophils produce ROS in order to eliminate pathogens (Kumar & Sharma 2010). Additionally, studies have demonstrated the importance of ROS on neutrophil migration (Hattori et al. 2010). ROS induction is known to be dependent on NADPH oxidase, and this enzyme is involved in ROS migration induced by GRP. Furthermore, GRP is able to induce NET formation by human neutrophils, which functions as a double-edged sword mechanism during an inflammatory response, protecting the host from invading pathogens but also amplifying tissue damage. We believe that GRP released during an inflammatory process would promote neutrophil recruitment, ROS generation and NET release. The excessive production of these neutrophil effector mediators would contribute to pathogenesis. Chronic release of GRP, as seen in arthritis, could perpetuate this process.

The fact that GRP is produced by central and peripheral neurons (Kamichi et al. 2005, Martinez & Taché 2000, Pereira et al. 2015) and act together with mast cells in itch behavior (Andoh et al. 2011), exemplifies exciting novel

neuroimmune networks. New data is showing how well connected nerves and resident immune cells in the tissue in homeostasis control (Gabanyi et al. 2016). Disease relapse in response to stress is a common aspect of autoimmune comorbidities (Stojanovich 2010), as well as asthma (Frieri 2003). Hence, the possibility that a neuropeptide can activate end-target responses seen as promotion of migration and NETosis in inflammatory conditions is highly relevant. Further studies should be pursued in order to clarify these mechanisms and neuroimmune connections *in vivo* and their relevance to GRP-associated comorbidities.

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CONFLICT OF INTERESTS

Cristália Ltda. (Itapira, Brazil) granted a fellowship to R.S.C and supplied the GRPR antagonist for this study. Cristália Ltda. holds a patent related to RC-3095. The other authors disclosed no potential conflicts of interest.

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Figure 1

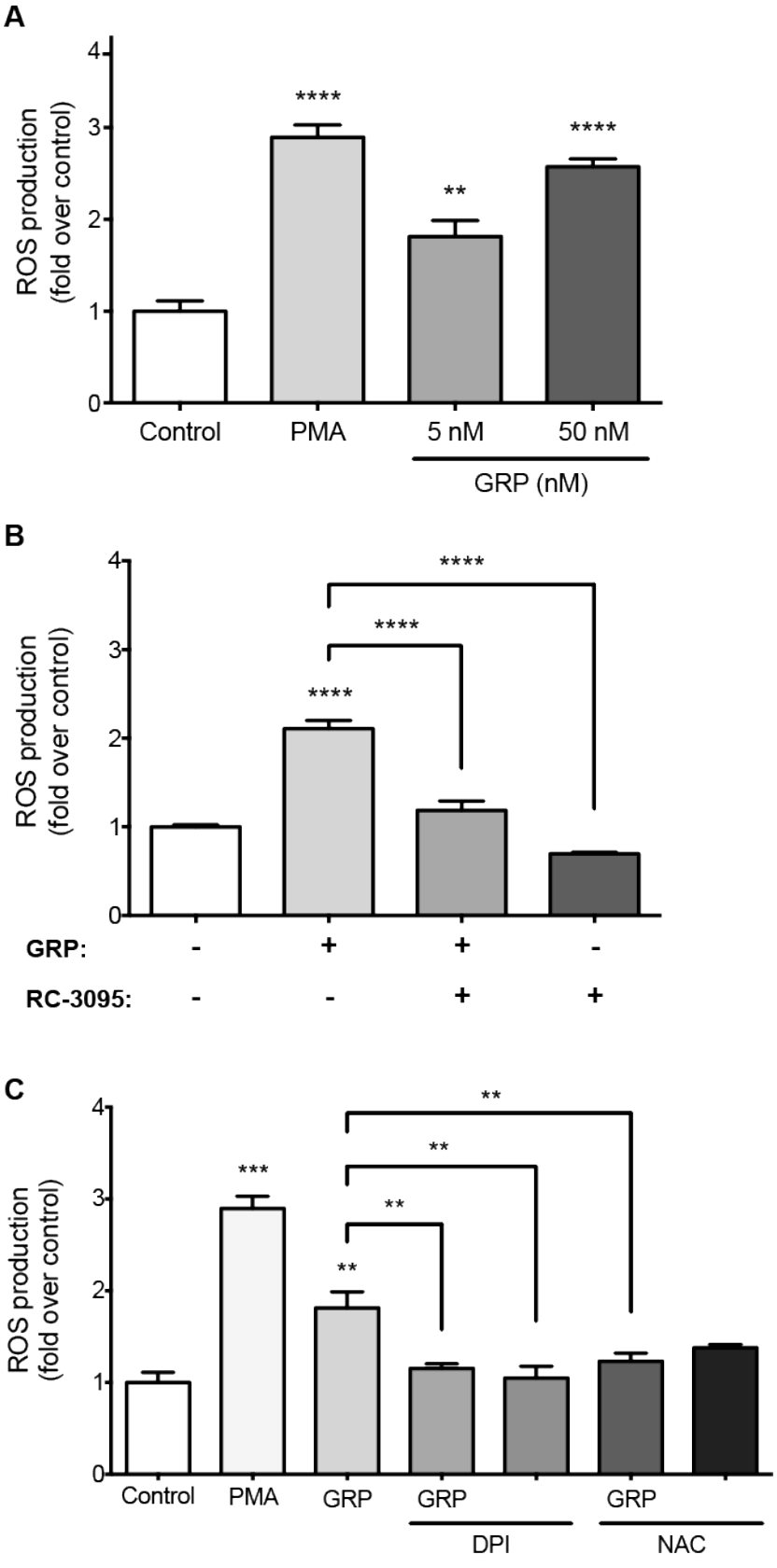
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Fig. 1. GRP promotes ROS production in neutrophils. (A-C) Human neutrophils ($1x10^6$/mL) were treated with different stimuli, incubated with CM-H_2DCFDA for 30 min and ROS production was evaluated by flow cytometry. **(A)** Neutrophils were treated with PMA (50 nM) or GRP (5 or 50 nM) for 30 minutes at 37ºC under 5% CO_2.**(B)** Neutrophils were pretreated with GRPR antagonist (RC-3095, 50nM) for 30 minutes and stimulated with GRP (5nM) for 30 minutes. **(C)** Neutrophils were pretreated with DPI (100 μM) or NAC (1 mM) for 30 min and stimulated with GRP (5 nM) for 30 min at 37ºC under 5% CO_2. Data are representative of two independent experiments, performed in triplicate for each sample, and expressed as mean ± SE. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; compared with control or were indicated.

Figure 2

A

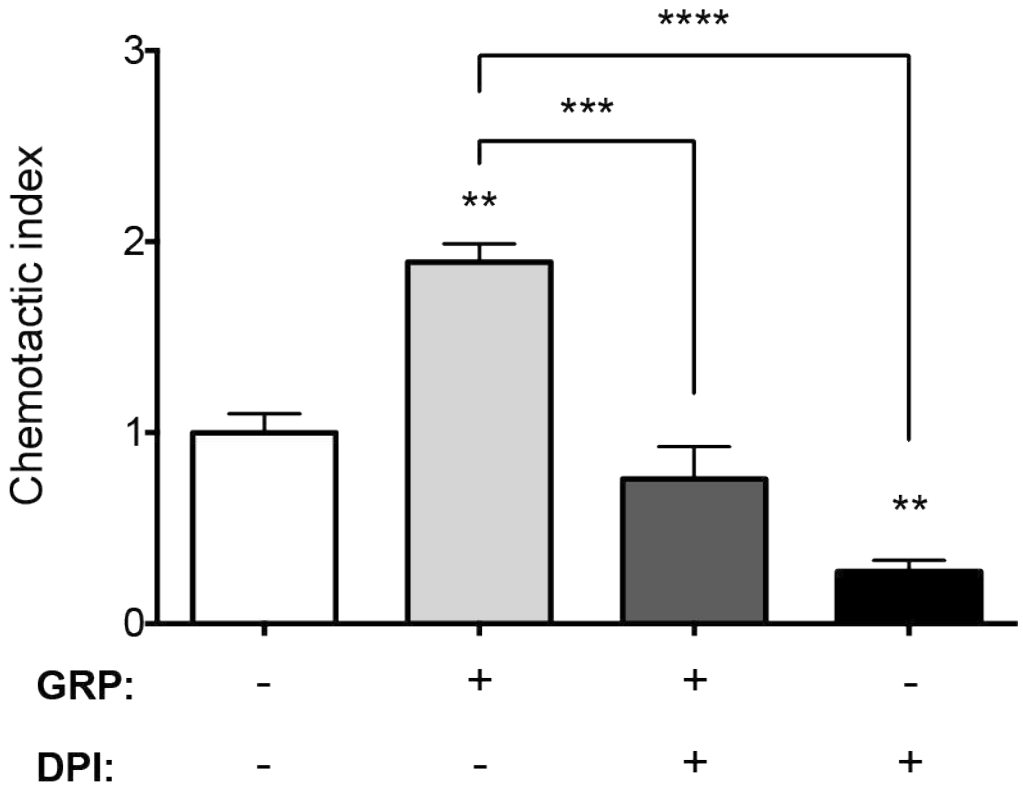

Fig. 2. GRP-induced neutrophil chemotaxis is dependent on NADPH oxidase-derived ROS production. Neutrophils (1 x 10^5/100 µL) were pretreated with DPI (100 µM) for 30 min and allowed to migrate toward GRP (5 nM) for 2h at 37ºC under 5% CO_2. Migrated cells were counted and the chemotactic index was calculated. Data are representative of two independent experiments, performed in triplicate for each sample, and expressed as mean ± SE. ****p < 0.0001; ***p < 0.001; **p < 0.01; compared with control or were indicated.

Figure 3

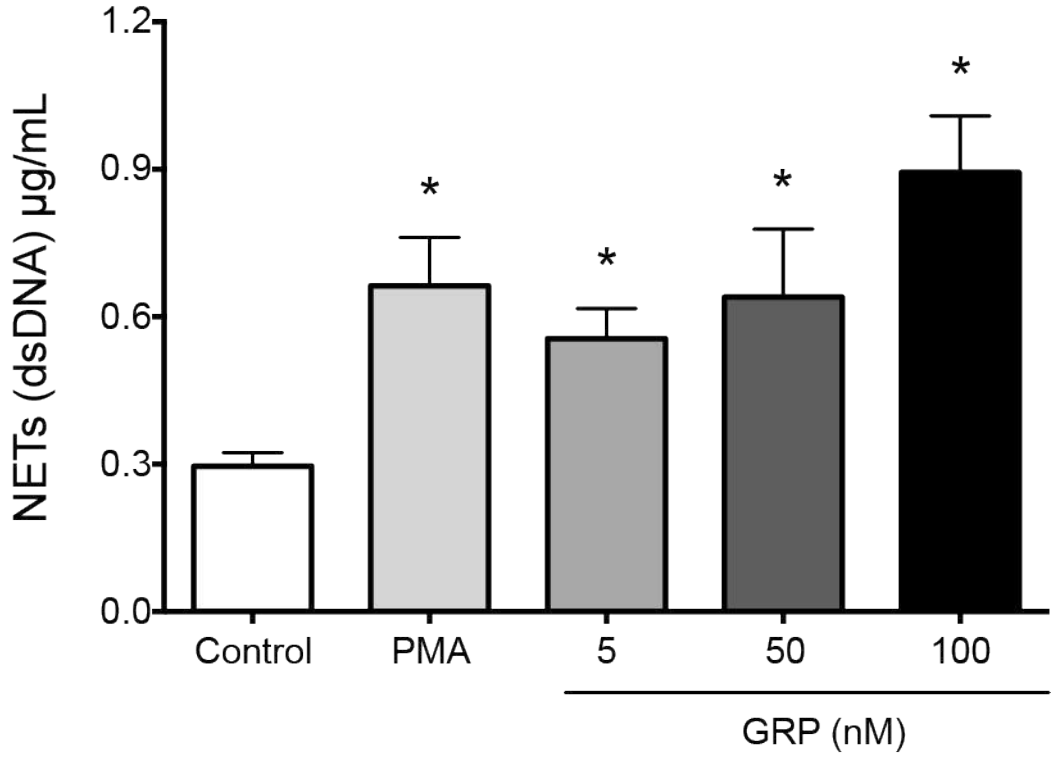

Fig. 3. Gastrin-releasing peptide induces NET release. Human neutrophils ($1x10^6$/mL) were stimulated with GRP (5, 50 and 100 nM), PMA (50 nM) or medium alone for 3 h at 37°C under 5% CO_2. NETs were quantified in culture supernatants using Quant-iT dsDNA HS kit. Data are representative of at least three independent experiments performed in triplicates for each sample for each sample and represent mean ± SE. *$p<0.05$; when compared to negative control (Control).

Capítulo 5

"*GRPR antagonist protects from drug-induced liver injury by impairing neutrophil chemotaxis and motility*"

Artigo publicado no periódico científico *European Journal of Immunology*

Durante nossos estudos de quimiotaxia induzida pelo GRP, observamos que o seu antagonista, RC-3095, detinha um potencial anti-migratório para outros quimioatratores. Sendo assim, hipotetizamos que o antagonista poderia ser empregado em doenças marcadas pelo alto influxo de neutrófilos.

A seguir encontra-se o artigo científico publicado no periódico científico *European Journal of Immunology*, onde verificamos o potencial do RC-3095 no tratamento da lesão hepática induzida por overdose de paracetamol, através da redução da motilidade dos neutrófilos. O artigo ainda ganhou evidência por ter sido escolhido como a capa da edição de Abril de 2017 do periódico *European Journal of Immunology*, como pode ser observado na próxima página. E na página subsequente existe um resumo gráfico que concatena de forma visual os achados desta parte do trabalho.

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European Journal of Immunology

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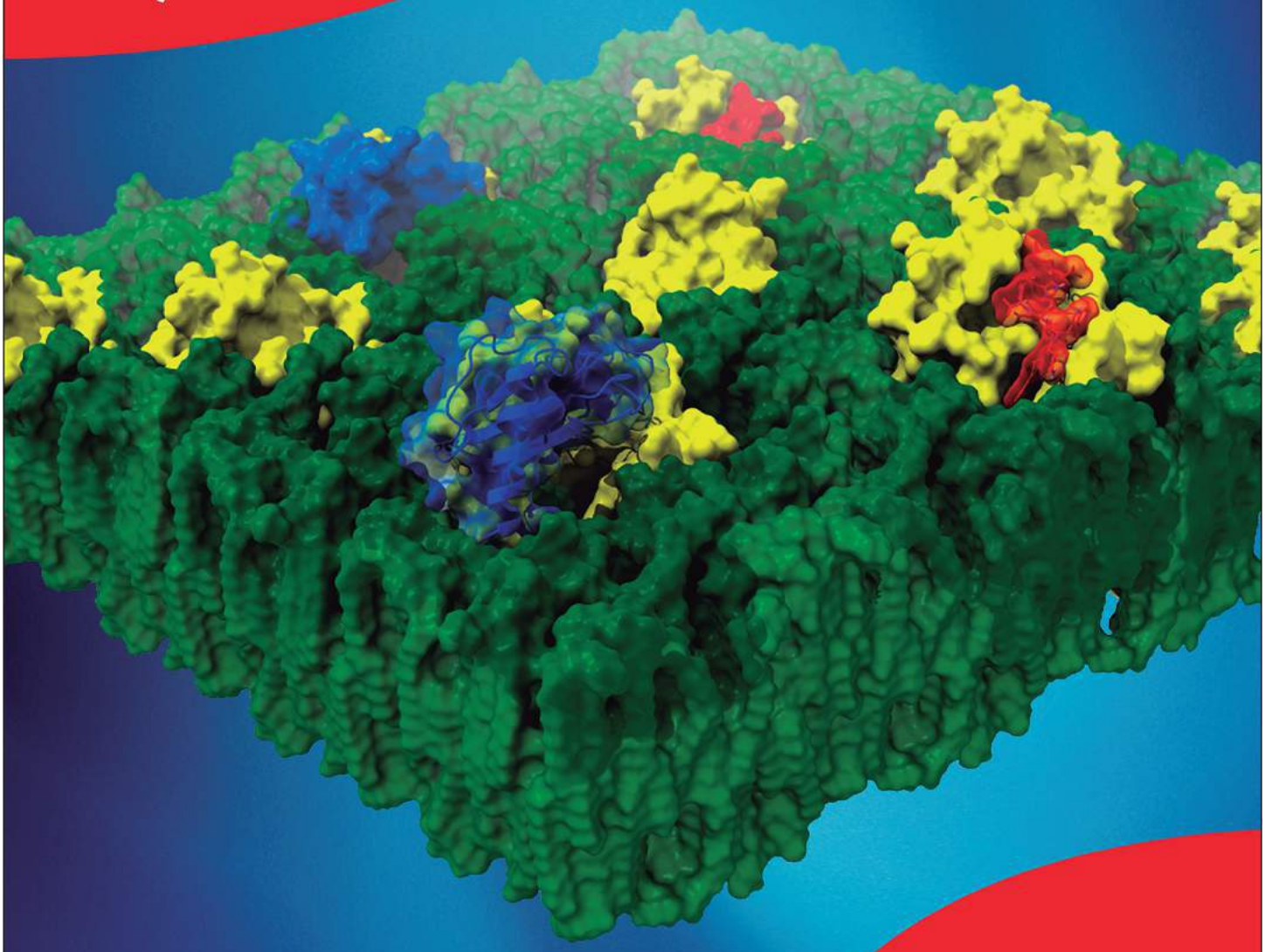

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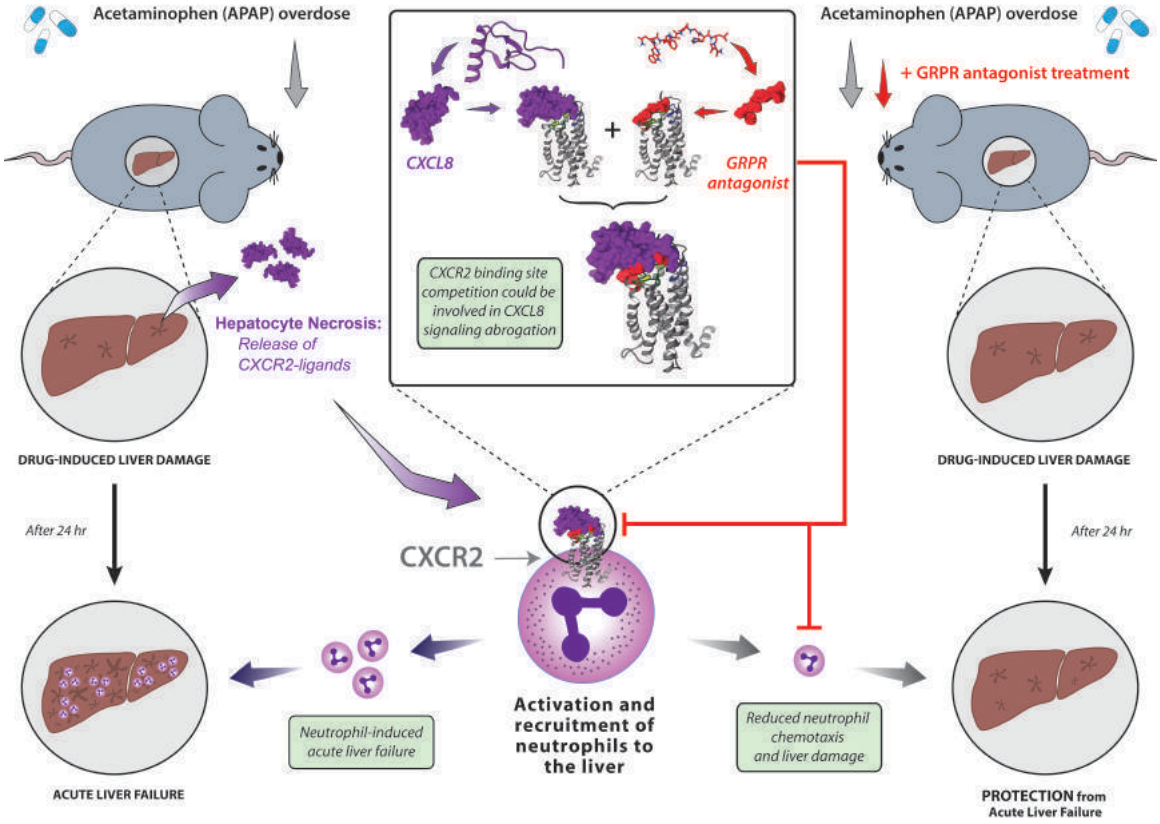
GRPR antagonist protects from drug-induced liver injury by impairing neutrophil chemotaxis and motility (pages 646–657)

Rafael S. Czepielewski, Natália Jaeger, Pedro E. Marques, Maísa M. Antunes, Maurício M. Rigo, Débora M. Alvarenga, Rafaela V. Pereira, Rodrigo D. da Silva, Tiago G. Lopes, Vinícius D. da Silva, Bárbara N. Porto, Gustavo B. Menezes and Cristina Bonorino

Drug-induced liver injury is expanded by neutrophil recruitment. Here, we describe that a GRPR antagonist protects from extended liver damage by reducing neutrophil activation and motility. An unexpected interaction between GRPR antagonist and CXCR2 competes for the binding site of CXCL8, preventing neutrophil chemotaxis.



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Basic

European Journal of Immunology

Innate immunity

Research Article

GRPR antagonist protects from drug-induced liver injury by impairing neutrophil chemotaxis and motility

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Drug-induced liver injury (DILI) is a major cause of acute liver failure (ALF), where hepatocyte necrotic products trigger liver inflammation, release of CXC chemokine receptor 2 (CXCR2) ligands (IL-8) and other neutrophil chemotactic molecules. Liver infiltration by neutrophils is a major cause of the life-threatening tissue damage that ensues. A GRPR (gastrin-releasing peptide receptor) antagonist impairs IL-8-induced neutrophil chemotaxis in vitro. We investigated its potential to reduce acetaminophen-induced ALF, neutrophil migration, and mechanisms underlying this phenomenon. We found that acetaminophen-overdosed mice treated with GRPR antagonist had reduced DILI and neutrophil infiltration in the liver. Intravital imaging and cell tracking analysis revealed reduced neutrophil mobility within the liver. Surprisingly, GRPR antagonist inhibited CXCL2-induced migration in vivo, decreasing neutrophil activation through CD11b and CD62L modulation. Additionally, this compound decreased CXCL8-driven neutrophil chemotaxis in vitro independently of CXCR2 internalization, induced activation of MAPKs (p38 and ERK1/2) and downregulation of neutrophil adhesion molecules CD11b and CD66b. In silico analysis revealed direct binding of GRPR antagonist and CXCL8 to the same binding spot in CXCR2. These findings indicate a new potential use for GRPR antagonist for treatment of DILI through a mechanism involving adhesion molecule modulation and possible direct binding to CXCR2.

Keywords: Acetaminophen · Acute liver failure · Chemotaxis · GRPR antagonist · Neutrophil

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Introduction

Drug-induced liver injury (DILI) is one of the major causes of acute liver failure (ALF) and this threatening condition has limited therapeutic options [1]. Overdosed patients receiving early medical care respond well to antioxidant *N*-acetyl cysteine, however other patients can develop severe liver damage and must be subjected to liver transplantation [2]. Acetaminophen (APAP) is a popular analgesic found in hundreds of medications and is safe in therapeutic doses. However, when used in overdose, it can induce massive hepatocyte necrosis, being the most common cause of ALF in developing countries [3]. APAP-induced ALF (A-ALF) is known to be dependent on local release of mitochondria-derived formyl peptides and DNA, and CXC chemokine receptor 2 (CXCR2) ligands (chemokine (C-X-C motif) ligand 1 (CXCL1), chemokine (C-X-C motif) ligand 2 (CXCL2), CXCL5, and CXCL8) [4]. This response to hepatotoxicity initiates a rapid activation and recruitment of neutrophils into liver necrotic zones, exacerbating damage and liver failure [5]

Neutrophil movement is governed by reaction to environmental molecular cues, sensed by a plethora of receptors. One of the most important neutrophil responses is promoted by CXCL8. CXCL8 in humans (also known as IL-8), and its analogues in mice, CXCL1, CXCL2, and CXCL5 are recognized by G-protein-coupled receptors (GPCRs) CXCR1 (where CXCR1 is chemokine (C-X-C motif) receptor 1) and, more significantly, by CXCR2 [6]. As seen with CXCR2 KO mice, blockage of CXCR2 axis is effective to reduce acute and chronic inflammatory diseases including DILI, tumor progression, and arthritis [4, 7].

A critical mechanism that regulates GPCRs signaling during migration is receptor internalization, resulting in temporary ligand desensitization and cell arrest [8], involving activation of MAPKs p38 and ERK [9]. Cell migration is also controlled by interactions of neutrophils with endothelial cells, allowing neutrophil transmigration from blood. Neutrophil expression levels of β2 integrins CD11a-CD18 (also known as LFA-1) and CD11b-CD18 (also known as Mac-1) control adhesion to ICAM1/2 in the endothelium [10].

We have previously demonstrated that the gastrin-releasing peptide (GRP) is a chemotactic molecule for neutrophils, activating signaling pathways that induce neutrophil migration both in vitro and in vivo through its receptor, GRP receptor (GRPR) using a mechanism dependent on TNF, ERK, p38, and protein kinase B (AKT) [11]. RC-3095, a GRPR-selective antagonist [12], impairs neutrophil chemotaxis toward GRP. Interestingly, we observed this molecule also inhibits neutrophil migration in response to CXCL8. We thus hypothesized that RC-3095 (RC) was not a selective antagonist of GRPR, but rather posesses neutrophil inhibitory properties modulated by CXCR2. In this study, we investigated whether RC could decrease DILI by impairing neutrophil migration in vivo, using the mouse model where APAP overdose induces liver injury. Our results indicate that RC is able to inhibit neutrophil migration toward CXCL8, CXCL2, and to the necrotic foci, resulting in reduction of liver injury. At the damaged site, motility of neutrophils is greatly reduced. This phenomenon is independent of CXCR2 internalization, but rather relates to the modulation of surface expression of adhesion molecule CD11b, CD62L, and the phosphorylation of ERK and p38. In addition, in silico analysis predicted a direct interaction of RC with CXCR2 at the same extracellular loops (ECL) where CXCL8 binds. These findings indicate a new use for RC in the treatment of neutrophil-mediated inflammation, independently of GRP.

Results

GRPR antagonist impairs neutrophil recruitment toward CXCL8 in vitro

Neutrophil migration toward CXCL8 is one of the canonical mechanisms of the innate immune system inflammatory response to maintain tissue homeostasis [13]. To gather more information about the potential anti-inflammatory effect of RC, we tested its inhibitory influences in an in vitro neutrophil migratory assay with the major endogenous chemoattractant, CXCL8. Accordingly, purified human neutrophils migrated toward a CXCL8 gradient and CXCR2 inhibitor impaired this process (Fig. 1A) [11]. Interestingly, pretreatment of cells with RC also greatly decreased chemotaxis in response to CXCL8. Strikingly, GRPR antagonist does not affect migration of human neutrophils toward the chemoattractants fMLP (where fMLP is *N*-formylmethionine-leucyl-phenylalanine peptide) and LTB4, indicating a possible interaction between a CXCL8 and its receptors and RC (Fig. 1B). To investigate whether RC inhibitory effect on CXCL8-induced neutrophil migration would be due to GPCR internalization [14], human neutrophils were treated with different concentrations of RC or CXCL8 and stained for CXCR2 on the surface of cells. As expected, CXCL8 induced CXCR2 internalization, but RC treatment maintained the receptor expression unaltered (Fig. 1C). Likewise, pretreatment with the GRPR antagonist was unable to change CXCL8-induced internalization (Fig. 1D). To exclude a possible direct cytotoxic effect of RC on neutrophils, percentage of viable and apoptotic neutrophils after 6 h treatment was tested and found similar to medium alone or vehicle (Fig. 1E). Nonstimulated neutrophils undergo spontaneous apoptosis after 20 h [13]. Viability was altered only when cells were stimulated with CXCL8 or a high concentration of RC at 24 h posttreatment (Fig. 1E). These data indicate that RC can modulate neutrophil migration toward an endogenous and essential chemoattractant, possibly through direct interaction with these cells. However, this effect does not involve the downregulation of membrane CXCR2 expression or cytotoxicity.

Acute liver injury is reduced by treatment with a GRPR antagonist

Based on the results above, we asked whether GRPR antagonist could reduce severity of disease along with CXCL8 production and neutrophil recruitment. We thus used a sterile inflammation model

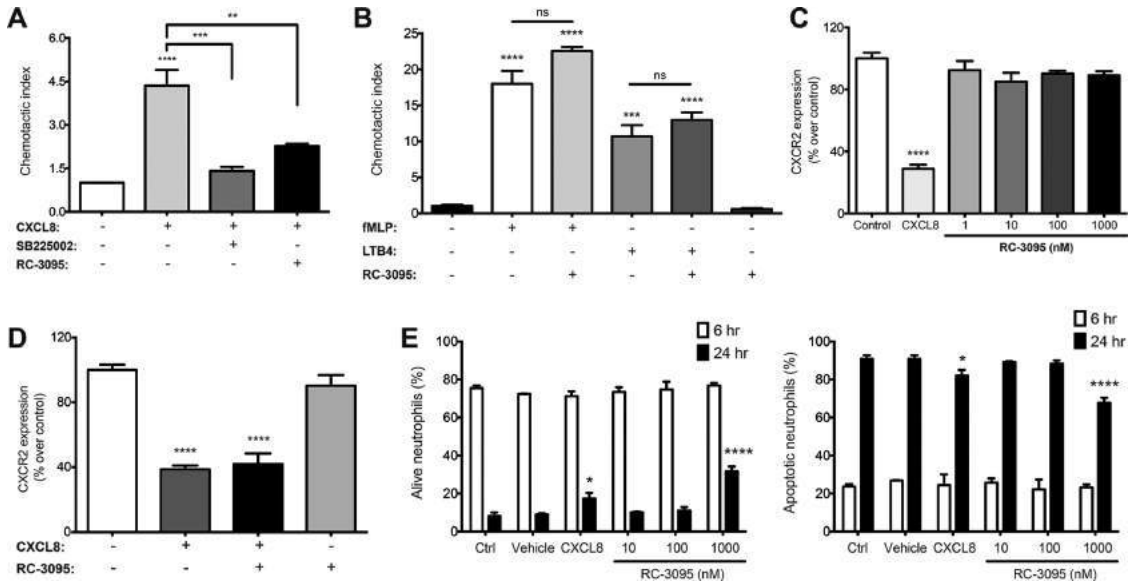

Figure 1. GRPR antagonist inhibits neutrophil chemotaxis toward CXCL8. (A, B) Human neutrophils were preincubated with CXCR2 antagonist, SB225002, or RC-3095 and allowed to migrate toward CXCL8, fMLP, or LTB4 using a Transwell System. Chemotactic index was determined as the ratio of the number of migrated neutrophils in chemoattractant-containing wells divided by negative control. (C, D) Surface expression of CXCR2 measured by FACS. CXCR2 internalization is expressed by percentage relative to negative control. (C) Neutrophils incubated with CXCL8 or RC-3095 and cells stained with anti-CXCR2 (CD182). (D) Neutrophils pretreated with RC-3095 or untreated and stimulated with CXCL8 for 2 h. (E) Viability of neutrophils was verified by AnnexinV/PI assay 6 h (black bars) or 24 h (white bars) after incubation with controls, CXCL8 or RC-3095. Data are presented as mean ± SE of a single experiment performed in triplicate for each sample group and is a representative of three independent experiments. $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{*}p < 0.05$; ns, not significant (one-way ANOVA).

of DILI triggered by overdose of APAP [1, 5]. The A-ALF model is characterized by acute liver damage up to 24 h after APAP intake, demonstrated by an increase in serum alanine aminotransferase (ALT) levels, release of CXCR2 ligands (CXCL1 and CXCL2), and accumulation of neutrophils within necrotic areas [4, 5]. To investigate whether RC would mitigate DILI, we orally overdosed mice with APAP. After 1 or 8 h of APAP administration, mice were i.v. treated with vehicle or RC [11, 15]. Pretreatment with RC significantly decreased transaminase levels in APAP-challenged mice in comparison to vehicle or RC-only controls by 24 h and this effect was sustained until 48 h after the overdose (Fig. 2A), a period where the liver is recovering and ALT levels decrease [4]. We also analyzed liver function 24 h after overdose by injecting indocyanine green (ICG) [16], which is rapidly cleared from the circulation by the liver. Interestingly, pretreatment with RC restores liver metabolic function after APAP overdose (Fig. 2B). Accordingly, histological examination of APAP-treated liver sections showed marked hemorrhagic congestion and extensive centrilobular necrosis, surrounded by viable hepatocytes presenting vesiculation (Fig. 2C) [4]. In line with our previous findings, a robust reduction in liver damage, congestion, and vesiculation was achieved when mice were pretreated with RC (Fig. 2C). Results indicate that RC effect is not a delay in injury progression, but rather a protection characteristic. To verify whether this reduction was due to inhibition of GRP released following liver damage, we measured GRP serum concentrations in mice at 24 h after APAP, the usual time point for peak serum concentrations of proinflammatory chemokines released in this model [4]. Remarkably, no serum GRP was detected in mice with A-ALF (not shown). Noteworthy, ALT and ICG measurement and histology of RC treated only show no major alterations, demonstrating minor influences in liver metabolism (Fig. 2A and B).

RC effect correlates with decreased neutrophil infiltration and motility in the liver

To further determine the effect of RC on liver damage reduction, we performed confocal intravital microscopy [17]. Twenty-four hours after APAP overdose, mice were anesthetized, injected with SYTOX Green, a viable cell-impermeable DNA dye and anti-Ly6G. We verified a decrease of necrotic liver areas in RC-treated mice when compared to APAP alone (Fig. 3A, left panels; Fig. 3B, top graph). In agreement with histological findings, intravital imaging revealed reduction of infiltrating neutrophils within the liver when mice received RC (Fig. 3A, middle panels; Fig. 3C). Moreover, most of neutrophils were found inside necrotic areas in the APAP group, whereas infiltrating neutrophils in RC-treated livers were dispersed throughout the tissue (Fig. 3A, right panels) [5]. Intravital recording of the liver revealed that GRPR antagonist markedly reduced neutrophil motility (Supporting Information Videos 1–3). Cell tracking analysis showed that although neutrophils migrated similar distances (track length), GRPR antagonist led to erratic neutrophil movement, with decreased directionality (meandering index). RC-treated infiltrating neutrophils also displayed lower crawling speed (velocity) and traveled smaller distances (displacement) (Fig. 3C and D). Taken together, these data show that neutrophil chemotaxis is altered in the liver upon RC treatment.

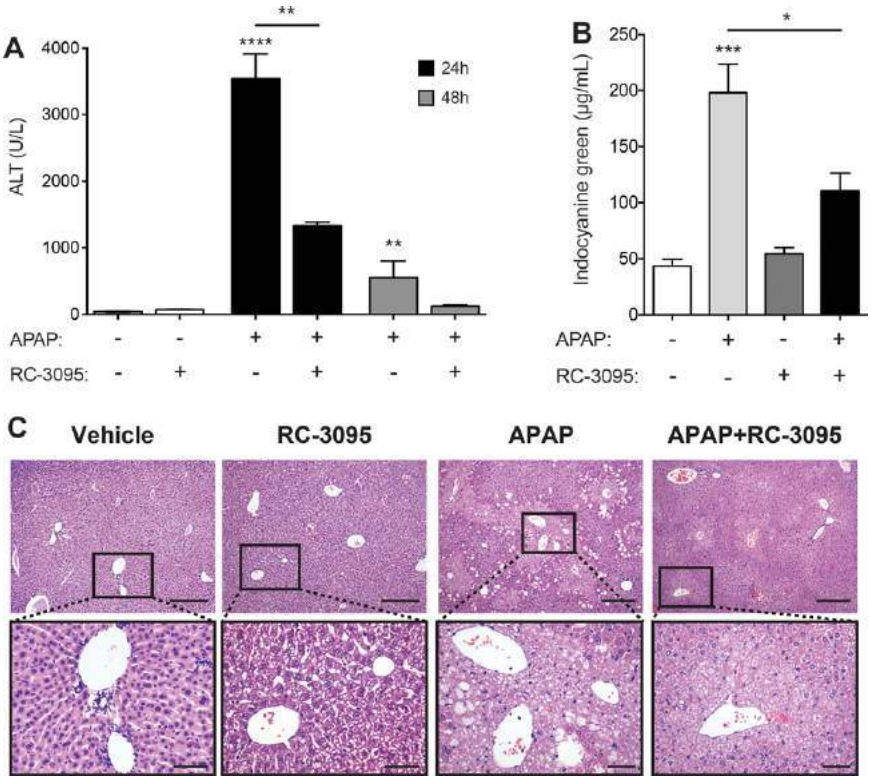

Figure 2. RC-3095 treatment reduces APAP-induced liver damage. DILI-induced mice were i.v. injected twice with vehicle or RC-3095. At 24 h or 48 h later, livers were harvested and serum collected. (A) Serum transaminase levels were determined by ELISA. (B) Mice received i.v. solution containing indocyanine green dye (ICG) and blood was collected 20 min after ICG administration. The absorbance was measured at 800 nm using a microplate reader. (C) Representative H&E liver sections of treated livers. Scale bars: 250 and 50 μm (top and bottom panels, respectively). Images are from a single experiment representative of three experiments with three to four mice per experiment. Data are presented as mean ± SE of a single experiment performed with $n = 3$–4 mice per group and is a representative of three independent experiments. $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{*}p < 0.05$; ns, not significant (Student's *t*-test).

GRPR antagonist activates MAPK signaling pathway and downregulates expression of adhesion molecules

To further investigate the mechanisms underlying the inhibitory effects of RC on neutrophil migration to CXCL8, we analyzed its effects on downstream signaling pathways involved in chemotaxis of the GRPR antagonist. We treated isolated human neutrophils with RC, stained for phosphorylated MAPKs (p38 and ERK 1/2), and analyzed them by FACS. We also stained for AKT, since it was one of the molecules involved in GRPR-mediated migration toward GRP [11]. Data revealed that RC treatment induces p38 (Fig. 4A) and ERK (Fig. 4B) activation, while AKT phosphorylation levels remained unaffected (Fig. 4C). To further investigate if MAPK activation promoted by GRPR antagonist could modulate expression of migration-relevant adhesion proteins, we challenged human neutrophils with CXCL8 and RC, and stained for Mac-1 (CD11b) and neutrophil activation maker CD66b. Accordingly, CXCL8 treatment resulted in upregulation of surface expression of CD11b and CD66b in neutrophils, but this was inhibited by pretreatment with RC (Fig. 4D). Collectively, these results indicate that RC has a direct effect on neutrophils, inhibiting migration toward CXCL8 gradients through a mechanism involving MAPK phosphorylation and modulation of adhesion molecules.

RC abrogates CXCL2-induced migration in vivo

In order to understand the direct effect of RC over CXCR2-driven migration, we investigated the direct effect of the compound on isolated mouse neutrophils. CXCL2 drives the activation of neutrophils observed by enhancing CD11b expression and removing L-selectin (CD62L) in the surface membrane [18–20]. Interestingly, pretreatment with RC prevents this activation process in vitro (Fig. 5A). Therefore, we used CXCL2-induced peritonitis model to evaluate GRPR antagonist effect in vivo [21, 22]. While i.p. injection of CXCL2 promotes strong neutrophil recruitment to the peritoneum within 4 h, an injection of RC 1 h prior the challenge with the chemokine diminishes neutrophil presence in the cavity (Fig. 5B and C). In addition, neutrophil-adhesion molecules CD11b and CD62L were altered in accordance with in vitro data (Fig. 5A and D). Altogether, these data show that RC can impair CXCL2-induced chemotaxis and limit its activation state.

In silico analysis suggests a RC–CXCR2 interaction

Our results from in vitro chemotaxis (Fig. 1A–D) opened a possibility to analyze an interaction with CXCR2 involving CXCL8 and RC. Therefore, we performed molecular docking to simulate the individual interaction of the protein CXCR2 with CXCL8 or with RC (Fig. 6A). Our in silico data show an interaction involving the ECL of CXCR2 and the N-loop residues of CXCL8, similar to previous findings [23]. Surprisingly, the best RC conformation appears to interact with the ECL of CXCR2 at the same site where CXCL8 is docking. In fact, both CXCL8 and RC seem to share binding residues in common, for example, serine in position 189 (Fig. 6B, side view), and are positioned in the same spot over CXCR2 (Fig. 6B, top view). Taken together, these results suggest that the

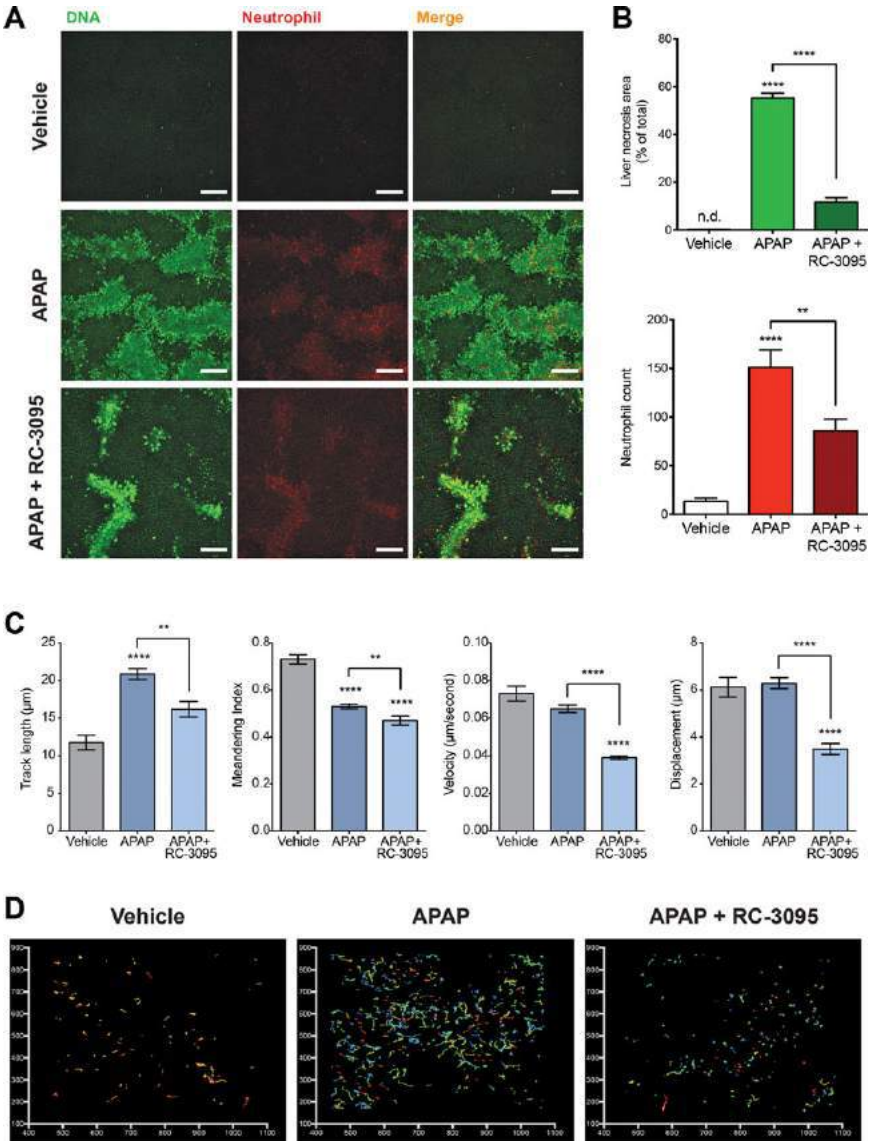

Figure 3. GRPR antagonist decreases hepatocyte necrosis and neutrophil infiltration by modulating neutrophil mobility. (A, B) Representative (A) and quantified (B) liver necrotic zones (green, left panels) and infiltrating neutrophils (red, middle panels) images were obtained by intravital microscopy. Scale bar = 200 μm. Images are from a single experiment with three to four mice per group sample and is representative of three independent experiments. (C, D) Digital cell tracking analysis of confocal intravital recordings (30 min duration, 1 frame/min) evaluating neutrophil motility (track length), meandering index (ratio track length/displacement), velocity, and displacement. Data are presented as mean ± SE of a single experiment performed in three to four mice per sample group and is a representative of two independent experiments. $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{*}p < 0.05$; ns, not significant (Student's *t*-test).

unappreciated effect of RC over neutrophils might be due to its direct interaction with CXCR2, expanding its anti-inflammatory potentialities.

Discussion

Current understanding of DILI is that it results from a combination of toxic metabolite byproducts and necrosis-driven acute inflammatory responses, promoting severe tissue damage [1]. Excessive APAP leads to accumulation of *N*-acetyl-*p*-benzoquinone imine (NAPQI), which interacts with mitochondria, inducing oxidative stress and hepatocyte necrosis [24]. Hence, DILI is a multifactorial scenario in which hepatocyte cell death, cytokines, and chemokines released, along with resident and peripheral immune cells, promote its outcome upon a drug stressor [25]. This environment attracts neutrophils, which significantly contribute to progression and severity of APAP-induced hepatocyte cell death [1]. Ab-mediated depletion [5, 26] and CXCR2-genetic ablation [27, 28] of neutrophils abrogates tissue injury, highlighting neutrophil migration impairment as a potential approach for the treatment of DILI [1].

The GRPR antagonist used in this study was initially developed to treat GRPR-expressing tumors and showed relevant results in preclinical, but not in clinical studies [29]. Nevertheless, RC ameliorates different inflammatory diseases [30]. Although these studies had shown decreased neutrophil infiltration within inflamed foci, a possible direct effect of this molecule on neutrophils was not considered until we showed that RC can prevent neutrophil migration in GRP-induced peritonitis [11]. GRP release in the serum correlates with rheumatoid arthritis [31], asthma [32], atopic dermatitis [33], and cancer [34]. Here, however, although no GRP was found in serum of mice with DILI, treatment with RC impaired neutrophil migration in vivo and in situ. Accordingly, septic rats treated with RC have been shown with decreased levels of ALT/aspartate aminotransferase

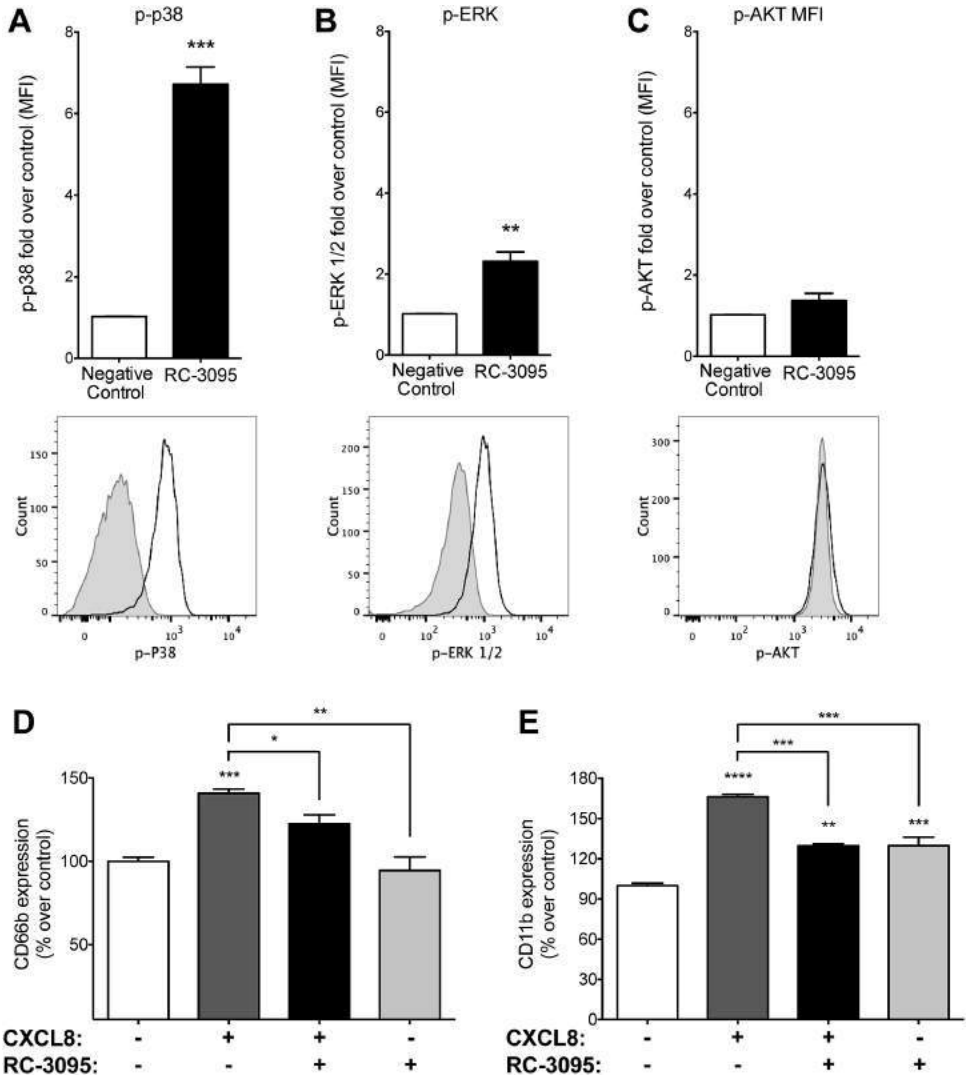

Figure 4. RC-3095 treatment effects MAPKs phosphorylation and adhesion molecules expression. (A–C) RC-3095-treated neutrophils stained for phosphorylated kinases: p38 (A), ERK 1/2 (B), and AKT (C). Phosphorylation of proteins was determined by flow cytometry and presented as fold increase relative to nonstimulated neutrophils (top graphs). Filled histograms, negative control neutrophils; solid black lines, RC-3095-stimulated neutrophils (bottom graphs). (D, E) Neutrophils pretreated or not with RC-3095 for 30 min and stimulated with CXCL8 for 1 h. Surface expression of CD66b and CD11b measured by FACS. Data are presented as mean ± SE of a single experiment performed in triplicate for each sample group and is a representative of three independent experiments. $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{*}p < 0.05$; ns, not significant (A–C: Student's *t*-test; D and E: one-way ANOVA).

(AST) [15]. Our results provide evidence of a new and unsuspected effect of this molecule, the interference with IL-8 induced neutrophil migration. Our in silico analysis reveal that both CXCL8 and RC could, and preferably would, interact with CXCR2 at equal positions, indicating competitive inhibitory potential, supporting our findings of RC impairing chemotaxis promoted by CXCL8, but not by other chemoattractants.

Further investigation on the mechanisms underlining such inhibition indicated that RC induces activation of MAPKs p38 and ERK, but not AKT. These kinases are ubiquitously involved in signaling pathways that regulate chemotaxis [6]. It is likely that they play multiple roles during chemokine receptor engagement, and only a few of these have been well characterized. DILI is marked by release of both CXCL8 and formyl-peptides, such as fMLP [4]. Migration via CXCR2 is governed by PI3K/AKT signaling, while chemotaxis via formyl peptide receptor 1 (fMLP receptor) depends on p38 [35]. In order to establish priority between the two inflammatory stimuli, neutrophils need phosphatase and tensin homolog, which dephosphorylates p38 products, allowing cells to migrate toward fMLP. Our observation that RC activates p38 and ERK simultaneously supports the idea that it interferes with fine tuning of a migratory route. Endotoxin was recently shown to impair neutrophil chemotaxis toward CXCL8 and hence the "prioritization" process of fMLP via a p38-dependent mechanism. Thus, the elevated levels of p38 phosphorylation found on RC-treated neutrophils could promote similar signaling inhibitory

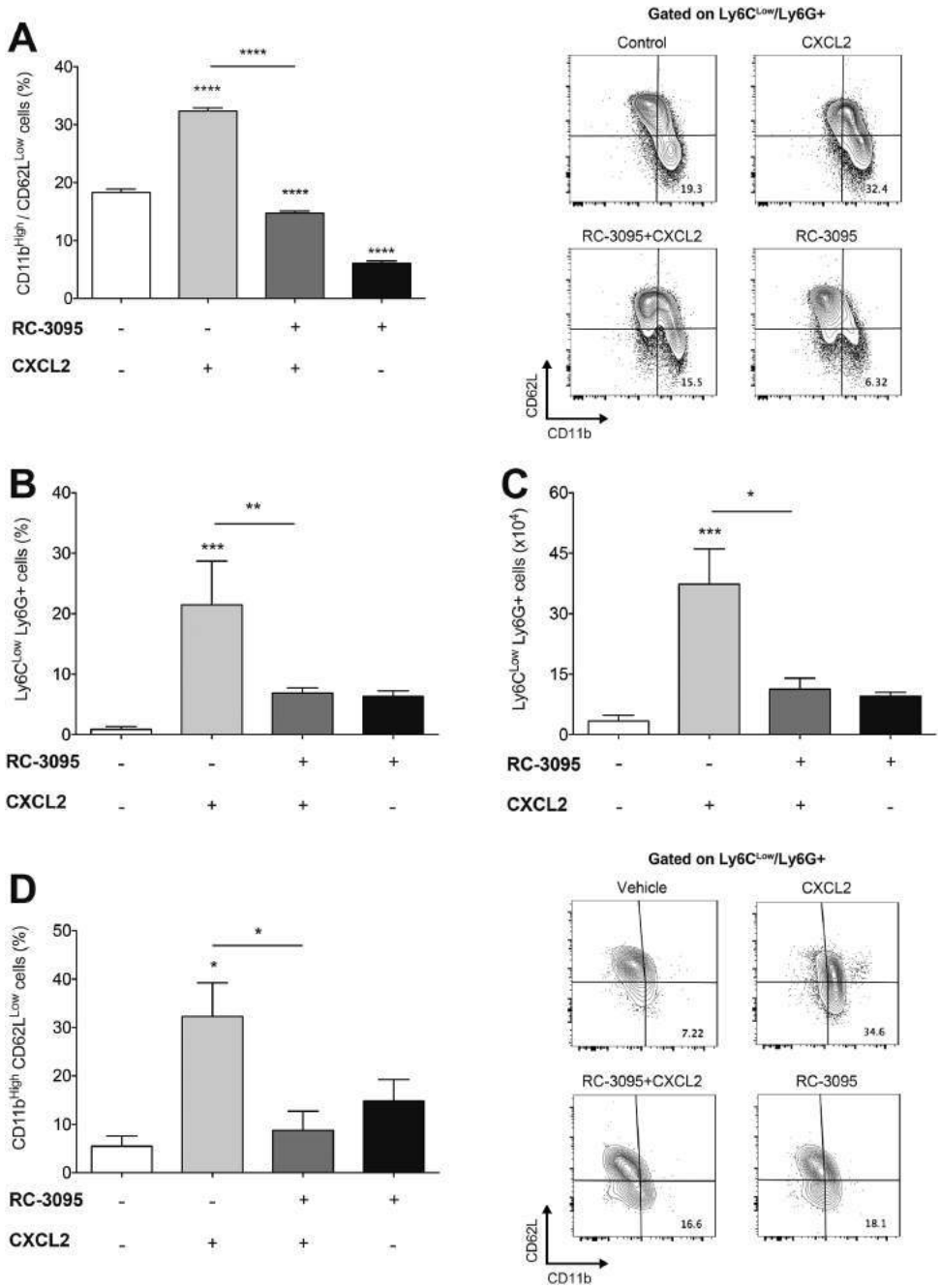

Figure 5. GRPR antagonist directly impairs neutrophils migration toward CXCR2 ligand in vivo. (A–D) Neutrophils were defined and gated based on Ly6C^{Low} Ly6G^{+} expression by FACS (Supporting Information Fig. 1). Percentage of CD11b^{High} CD62L^{Low} neutrophil-activated population was measured by flow cytometry. (A) Mouse neutrophils were pretreated with RC-3095 and/or stimulated with CXCL2 for 1 h in vitro. Percentage of activated neutrophils (left graph) and representative graph plots (right panel) are shown. Data are presented as mean ± SE of a single experiment performed in triplicate for each sample group and is a representative of four independent experiments. (B–D) Mice were i.v. treated with RC-3095 or vehicle. After 1 h i.p. injection of CXCL2 was performed. Four hours later, peritoneal wash was performed and stained for FACS. Percentage (B) and number (C) of migrated neutrophils to the peritoneum are shown. (D) Percentage of activated neutrophils (left graph) and representative graph plots (right panel) from mice shown in (B). Data are presented as mean ± SE of a single experiment performed in three to four mice per sample group and is a representative of four independent experiments. $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{*}p < 0.05$; ns, not significant (Student's t-test).

effects on CXCL8-induced migration [36]. Cell tracking data corroborates this hypothesis, as seen by changes in directionality to necrotic zones and distance traveled after RC. Additional studies need to be performed in order to evaluate phosphatase and tensin homolog, G-protein-coupled receptor kinases, and MAPKs status during CXCL8 and fMLP challenge in RC-treated neutrophils.

Finally, neutrophil activation is characterized by CD11b upregulation and loss of CD62L [18, 19, 37]. APAP-induced ALF

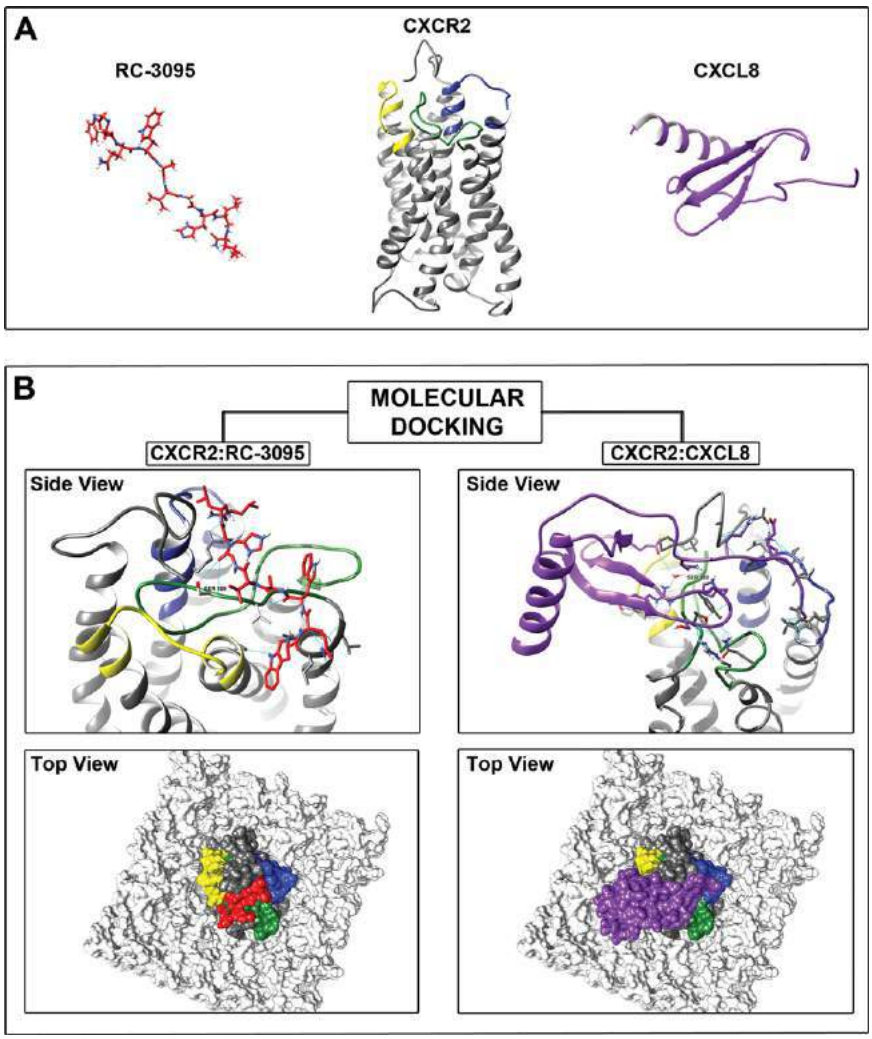

Figure 6. CXCL8 and RC-3095 are predicted to bind at the same site in CXCR2. (A) The molecular structures used as input to in silico assay: ligand RC-3095, chemokine receptor CXCR2 (with the extracellular loops (ECL) depicted in yellow (1ECL), green (2ECL), and blue (3ECL)), and chemokine CXCL8. (B) Molecular docking results. The side view shows hydrogen bonds (blue lines) between specific residues on CXCR2 and RC-3095 (left) or CXCL8 (right). The top view shows CXCR2 embedded into a membrane composed by phospholipids (white scaffold) interacting with RC-3095 (red) or CXCL8 (purple).

promotes upregulation of Mac-1 (CD11b/CD18) in murine and human neutrophils [24], and neutrophil migration toward fMLP relies on Mac-1 [38]. CD11b/CD62L perturbation induced here by RC could partially explain the reduction on neutrophil chemotaxis and motility depicted in situ. These alterations could be due to the inability of CXCR2 ligands to activate neutrophils when treated with RC. Another possibility is that such downregulation could occur via protein synthesis independent mechanisms, for example, Mac-1 membrane shedding. Integrin shedding is mainly driven by proteases [39], and is characteristic of transmigrated neutrophils [40]. Leukocyte shedding and release of soluble CD11/CD18 is increased in arthritis [41]. Our results also indicate that RC downregulates CD66b. This protein is uniquely expressed in human neutrophils and is a marker for activation and exocytosis of specific granules [42]. Downregulation of CD66b in RC-treated neutrophils could indicate that they might not be degranulating, agreeing with our findings.

Taken together, our results support the idea that RC exerts a potent suppressive effect, with potential use as an anti-inflammatory drug [11]. RC has been traditionally employed to investigate the role of GRPR/GRP axis in distinct biological systems [43–46], however our findings alert for an additional important effect of the compound besides its selective binding to GRPR. Additional studies are necessary to further characterize these responses and validate the direct inhibitory effect through CXCR2 binding. The optimization of RC use may thus provide a much-needed tool for acute liver injury treatment.

Material and methods

Reagents and mice

RC-3095 (RC) was synthesized by Ambiopharm (North Augusta, USA) and provided by Cristália Ltda (Itapira, Brazil). Human CXCL8 and mouse CXCL2 are from Peprotech (Rocky Hill, USA). PD98059 (ERK inhibitor), SB203580 (p38 inhibitor), and SB225002 (CXCR2 antagonist) from Cayman Chemical (Ann Arbor, USA). fMLP and LTB4 are from Sigma-Aldrich (St. Louis, USA). Mice (C57BL/6 females, 6–10 weeks old) were from CEBIO, UFMG (Brazil) or CeMBE, PUCRS (Brazil). All animal studies

were approved by the Animal Care and Use Committee at UFMG (CEUA no. 051/2011) and at PUCRS (CEUA no. 12/00321). Care and handling of the animals were in accordance with the NIH guidelines. All volunteers signed an informed consent term approved under Ethics protocol nr. 858/05-CEP.

Neutrophil isolation

Peripheral blood was collected from healthy volunteer donors. Neutrophils were purified as described [11]. Mouse BM neutrophils were isolated by discontinuous Percoll gradient (Sigma-Aldrich) as described [35].Neutrophil purity was confirmed by flow cytometry (FACS) using FACSCanto II (Becton Dickinson), resulting >90%. Cell viability was always higher than 97% (Trypan Blue assay).

Neutrophil chemotaxis assay

Neutrophil chemotaxis was assayed with Transwell System (Corning) [11] using 50 nM CXCL8 (IL-8), 100 nM fMLP, or 10 nM LTB4 added to the bottom wells in Roswell park memorial institute (RPMI) medium plus 2% FBS. Neutrophils in RPMI 2% FCS (2×10^5 cells/100 μL) were added to top wells and incubated for 2 h at 37°C 5% CO_2. Migrated neutrophils were counted in Neubauer chambers. Neutrophil migration toward RPMI medium alone was used as negative control. To analyze the effect of GRPR antagonist in CXCL8-driven neutrophil migration, cells were pretreated with RC (10 nM) or CXCR2 inhibitor SB225002 (300 nM) at culture conditions for 1 h. Chemotactic index is the ratio of the number of migrated neutrophils in chemoattractant-containing wells divided by negative control.

DILI model

APAP-induced ALF was performed as described [5, 17]. Briefly, mice were fasted before oral APAP administration (600 mg/kg; Sigma-Aldrich) or vehicle. After 24 h, blood (serum) and liver was collected. RC was injected i.v. twice (at 1 and 8 h after APAP challenge) 0.5 mg/kg as described previously [11, 15]. To observe direct effects of RC, mice were injected only with the GRPR antagonist (at 0 and 7 h) and analyzed together with other groups (at 23 h after injection of RC). Serum ALT activity was assayed using a kinetic test (Bioclin, Brazil).

Liver function test

Mice received i.v. solution containing 20 mg/kg of ICG dye (Cardiogreen, Sigma-Aldrich) diluted in sterile water for injection, as before [16, 47]. Briefly, mice were immediately anesthetized (i.v.) 20 min after ICG administration for blood collection. After plasma obtaining, samples were diluted in water for injection and plated (240 μL/well) in 96-wells polystyrene plate (Nunc, Denmark). The absorbance was measured at 800 nm using a microplate reader (Versa Max microplate reader). The results were compared to a standard curve and expressed in micrograms per milliliter.

Histopathological analysis

Tissues were fixed in 10% formalin, processed, dehydrated in increasing concentrations of ethanol (70–100 %), and embedded in paraffin. Sections of 5 μm were prepared and slides were stained and mounted in Entellan (Merck, SP, Brazil) at the Pathology Lab of São Lucas Hospital (PUCRS). Sections were submitted to H&E staining, performed according to standard protocol. Samples were examined by a pathologist blinded to the experimental groups on a fluorescence microscope (Zeiss Axioskop 40, Zeis Oberkochen, Germany) equipped with a Retiga 2000R CCD camera (QImaging, Surrey, Canada) and Image Pro Plus 6.0 image analysis software (Media Cybernetics, Rockville, USA).

Flow cytometry and measurement of MAPKs and AKT phosphorylation levels

Human and mouse neutrophils were stained with antibodies to CD182 (CXCR2) PE, CD66b FITC, and CD11b APC, Ly6G PE, Ly6C PerCP, and CD62L FITC (BD Biosciences, San Jose, USA). Expression levels were analyzed as MFI, normalized relative to negative control. Apoptosis and necrosis were measured with the Annexin-V Kit assay (BD Biosciences, USA). Phosphorylation of signaling proteins (phospho-AKT, phospho-P38, and phospho-ERK 1/2) was performed as described [11]. Neutrophils were incubated with RC (10 nM) in RPMI 2% FBS for 5 min, a time point sufficient to measure all three kinases together [9, 35]. Neutrophils were stained with Alexa 488 anti-phospho-p38, PE anti-phospho-AKT, and APC anti-phospho-ERK 1/2 antibodies for 30 min on ice. Data were acquired using a FACSCanto II (Beckton Dinckinson) analyzed by Flowjo® vX.

ELISA

Serum GRP concentrations of APAP-treated mice were estimated using the Enzyme Immunoassay kit (Phoenix Pharmaceuticals) in accordance with the supplier's assay instructions and as described before [11, 48].

In vivo mice imaging

Liver confocal intravital microscopy was performed as described [5, 17]. Briefly, mice were submitted to a laparotomy to expose the liver in an acrylic stage. Fluorophores SYTOX Green (100 μL/mouse, 50 μM, Invitrogen) and PE-conjugated anti-Ly6G (clone 1A8; 4 μg/mouse, eBioscience) were injected i.v. 10 min prior imaging. Extracellular DNA and liver necrosis quantification was performed using the ImageJ software (NIH, Bethesda, USA).

Neutrophil tracking was recorded in 30 min videos under the same conditions, one frame/minute. Neutrophil counts and digital tracking parameters were performed using Volocity software (PerkinElmer).

In vivo neutrophil migration assay

Single i.p. injection of CXCL2 (30 ng per cavity) and/or i.v. injection of RC (0.5 mg/kg) were performed. Control group received an i.p. injection of endotoxin-free saline vehicle solution. After 4 h, peritoneal wash was performed as described [11] and cells were stained for FACS with Ly6G, Ly6C, CD11b, and CD62L antibodies (BD Biosciences). Gating strategy at Supporting Information Fig. 1.

Molecular structures and molecular docking

CXCR2 protein was modeled with Modeller software [49] using the CXCR1 protein as template (PDB ID: 2LNL). Atomic composition of RC-3095 was obtained from PubChem (CID: 92043092), constructed and optimized through Automated Topology Builder (ATB) server [50]. CXCL8 monomer was obtained from PDB (PDB ID: 1IL8). The first residue of CXCL8 (Ser1) was modeled with PyMol [51] and energy minimized with GROMACS package [52]. CXCR2 and RC were submitted to molecular docking experiment using AutoDock Vina software [53]. To increase the accuracy of the process, we run the simulation 20 times, generating approximately 200 conformations [54] with an exhaustiveness of 100. The best conformation was chosen based on binding energy, frequency of explored conformations, and binding to CXCR2 extracellular residues. CXCR2 and CXCL8 monomer were submitted to Haddock 2.2 server [55] using default server parameters.

Statistical analysis

Data are presented as mean ± SE. Results were analyzed using GraphPad Prism 5. Statistical differences among the experimental groups were evaluated by ANOVA with Tukey correction or with Student's *t*-test. The level of significance was set at $p < 0.05$.

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Conflict of interest: Cristália Ltda. (Brazil) granted a fellowship to R.S.C. and supplied RC-3095. Cristália Ltda. holds a patent related to RC-3095. The other authors declare no commercial of financial conflicts of interest.

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Abbreviations: **A-ALF**: acetaminophen-induced acute liver failure · **ALF**: acute liver failure · **ALT**: alanine aminotransferase · **APAP**: acetaminophen · **CXCL1** : chemokine (C-X-C motif) ligand 1 · **CXCL2**: chemokine (C-X-C motif) ligand 2 · **CXCR1**: chemokine (C-X-C motif) receptor 1 · **CXCR2**: CXC chemokine receptor 2 · **DILI**: drug-induced liver injury · **fMLP**: *N*-formylmethionine-leucyl-phenylalanine peptide · **GPCRs**: G-protein-coupled receptors · **GRP**: gastrin-releasing peptide · **GRPR**: gastrin-releasing peptide receptor · **NAPQI**: *N*-acetyl-*p*-benzoquinone imine

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Supporting Information

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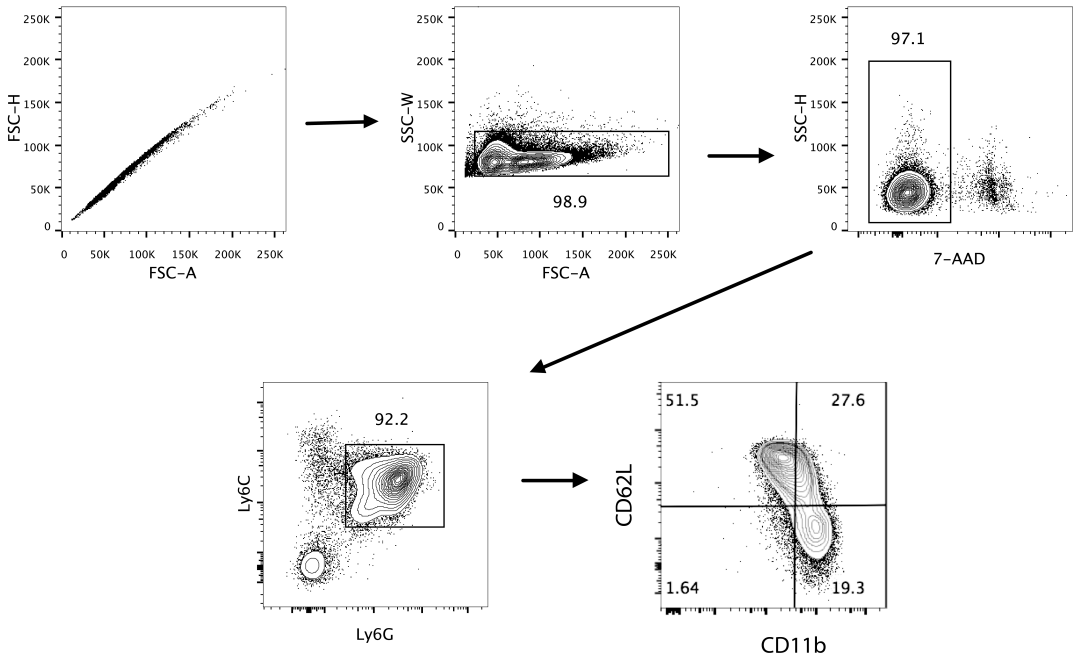
GRPR antagonist protects from drug-induced liver injury by impairing neutrophil chemotaxis and motility

SUPPORTING INFORMATION

SUPPORTING INFORMATION FIGURE 1

A



Supporting Information Figure 1: A. FACS gating strategy for mouse neutrophils. Isolated cells from bone marrow or peritoneum were acquired as singlets and single cells. Dead cells were excluded by 7-AAD staining and live cells were analyzed as neutrophils based on its Ly6C/Ly6G expression. Gating strategy was used in Figure 5 A and D.

SUPPORTING INFORMATION VIDEO LEGENDS

Supporting Information Video 1: Liver confocal intravital microscopy of 24 hours following ingestion vehicle. Liver necrotic cells can be seen by SYTOX green dye (green) and infiltrating neutrophils with anti-Ly6G PE (red). Scarce necrotic cells can be observed in this healthy liver. Neutrophils migration pattern is shown. Mice were imaged for the period of 30 minutes (at one framer per minute recording), and videos were accelerated to improve visualization of neutrophil chemotaxis (7 frames per second).

Supporting Information Video 2: Liver confocal intravital microscopy 24 hours following APAP overdose. Liver necrotic areas (SYTOX green dye, green) and infiltrating neutrophils (anti-Ly6G PE, red) are shown. Neutrophils migration pattern and patrolling behavior are shown. Mice were imaged for the period of 30 minutes (at one framer per minute recording), and videos were accelerated to improve visualization of neutrophil chemotaxis (7 frames per second).

Supporting Information Video 3: Liver confocal intravital microscopy of RC-3095-treated mice 24 hours following APAP overdose. Liver necrotic areas (SYTOX green dye, green) and infiltrating neutrophils (anti-Ly6G PE, red) are shown. Mice were imaged for the period of 30 minutes (at one framer per minute recording), and videos were accelerated to improve visualization of neutrophil chemotaxis (7 frames per second).

Capítulo 6

Considerações Finais

CONSIDERAÇÕES FINAIS

Neste trabalho procuramos investigar o papel do peptídeo liberador de gastrina (GRP) como uma molécula capaz de ativar respostas celulares. Levando em conta o envolvimento do GRP na proliferação tumoral bem como nosso achados anteriores na indução de migração de células imunes, propusemos correlacionar essas duas facetas. Assim, projetamos gerar um modelo *in vivo* onde a superexpressão do GRP em tumores seria a base para estudarmos os efeitos diretos do peptídeo sobre a proliferação, recrutamento de células imunes e promoção de agressividade tumoral e metástases. Todavia, dificuldades técnicas limitaram a execução de experimentos e a geração de dados para o cumprimento do objetivo principal da tese de doutoramento – objetivo específico 1. Mesmo assim, como os estudos sobre GRP como proteína moduladores de migração e respostas imunes é recente, identificamos diversas áreas onde nossos conhecimentos poderiam gerar informações relevantes sobre a biologia do GRP. Embora os projetos secundários que surgiram durante o período do doutorado não tenham centrado na investigação direta sobre microambiente tumoral e GRP, fomos capazes de realizar descobertas sobre mecanismos pelos quais o peptídeo age na indução de migração de tumores, ativação de neutrófilos induzindo produção de ROS e NETs. Finalmente, descobrimos e reportamos a promiscuidade de uma das moléculas tidas como um antagonista seletivo do GRPR, e mapeamos alguns de seus mecanismos de ação como um inibidor de dano inflamatório *in vivo*, usando um modelo de dano hepático induzido por droga.

Inicialmente hipotetizamos o papel do peptídeo liberador de gastrina no recrutamento de neutrófilos em direção à tumores produtores do peptídeo, sendo esse um novo mecanismo de indução de escape da vigilância tumoral imune, promovida através da formação de neutrófilos pró-inflamatórios N2, por exemplo. Entretanto, em nossos primeiros testes com B16-GRP, apenas conseguimos observar um aumento na presença de monócitos inflamatórios no microambiente tumoral. Essas células são conhecidamente envolvidas com a progressão tumoral, e são a fonte de reposição para macrófagos M2, considerados pró-tumorais (LEUSCHNER et al., 2011). Estudos sobre o efeito do GRP em monócitos são escassos, porém demonstram que o peptídeo aumenta a fagocitose e estimula a migração *in vitro* dessas células imunes (JIN et al., 1990; RUFF et al., 1985).

Infelizmente, esses estudos não receberam sequência, ou foram refutados pela literatura subsequente. Nossos próprios resultados publicados em 2012 mostravam que a migração de neutrófilos induzida por GRP era dependente de macrófagos e da produção de TNF-alfa. Assim, acreditamos que o GRP interaja diretamente com mais de uma subpopulação celular do compartimento mieloides, e continuaremos nossos estudos para clarificar tais interações.

O nosso modelo tumoral com superexpressão de GRP não alterou o crescimento tumoral, apesar do aumento de monócitos inflamatórios infiltrantes. Entretanto, novos experimentos de acompanhamento tumoral serão futuramente executados. Por exemplo, a injeção de um número menor de células tumorais irá permitir seguir por mais tempo a progressão e alteração do microambiente tumoral. Utilizamos o modelo padrão de número de células injetadas e tempo de acompanhamento (DAMSKY; BOSENBERG, 2010; KISHI et al., 2009). Todavia, o GRP está mais envolvido com tumores de evolução mais gradual e não extremamente agressiva como o melanoma metastático. Pretendemos finalizar estes estudos, incluindo no sistema o *knockdown* do receptor GRPR nas células tumorais, para tentar obter um sistema onde o efeito do GRP seja estudado separadamente no infiltrado, sem interferência do efeito que pode ter no tumor.

Os efeitos do GRP sobre a proliferação celular são amplamente conhecidos, entendidos como benéficos, para o reparo de danos teciduais, e de consequências graves, quando envolvidos em neoplasias (BARONI et al., 2008; PATEL; SHULKES; BALDWIN, 2006). Todavia, observamos nas células de adenocarcinoma humano de pulmão de não pequenas células (NSCLC, A549) que não houve um aumento da sua replicação após o tratamento com o peptídeo. Sendo assim, buscamos verificar se o GRP também promoveria migração tumoral. Vale salientar que a indução de migração por GRP em tumores já havia sido demonstrada em outros tipos tumorais, como cólon, próstata (GLOVER et al., 2005; NAGAKAWA et al., 2001; PATEL et al., 2014), e células endoteliais (MARTÍNEZ et al., 2005b), porém não nesse subtipo tumoral. Nossa caracterização buscou então comprovar de maneiras distintas que os efeitos do GRP sobre o A549 estavam restritos a quimiotaxia, excluindo efeitos proliferativos. Essa informação tem alta relevância, porque esse é o câncer que mais mata pacientes no mundo e apresenta, talvez não por coincidência, uma alta prevalência de expressão de GRPR e GRP, acima de 60% dos casos (MATTEI et al., 2014; TOI-SCOTT; JONES; KANE, 1996). Sendo assim, a visão do GRP como

fator migratório e por conseguinte um promotor de metástases pode auxiliar o diagnóstico e futuros tratamentos.

O entendimento do GRP atuando através de seu receptor GRPR na ativação de vias canônicas de proliferação, sobrevivência e migração (MAPK e PI3K/AKT), nos instigou a investigar efeitos gerais ainda não reportados para sua influência sobre neutrófilos (CZEPIELEWSKI et al., 2012). A reprogramação dos neutrófilos durante sua transformação de células circulatórias para fagócitos teciduais altamente eficientes requer a ativação e adaptações celulares para esse novo cenário após sua transmigração (AMULIC et al., 2012). Por isso, testamos se a estimulação induzida por GRP também acarretaria na produção de uma das marcas dos fagócitos, a produção de espécies reativas de oxigênio. Mecanismo esse, induzido por diversas moléculas quimiotáticas, como IL-8, fMLP, TNF (NAUSEEF, 2008). Encontramos que a migração estimulada por GRP depende de NADPH oxidase e que neutrófilos estimulados com o peptídeo produzem ROS. E mais interessante ainda, o peptídeo foi capaz de estimular os neutrófilos a ponto de promover NETosis. Este é uma resposta celular drástica dos neutrófilos. Soma-se a isso o fato desse tipo de reação contra moléculas endógenas estar relacionada com o desenvolvimento de doenças autoimunes (DARRAH; ANDRADE, 2012). Entretanto, nossos resultados sobre a liberação de NETs são preliminares. Novos ensaios devem ser conduzidos para afirmar mais contundentemente esse achados. Em outros trabalhos desenvolvidos durante o período dessa tese (Anexos **B** e **C**) iniciamos a estudar NETs na PUCRS. Investigamos mais profundamente a produção de NETs através da vizualização por microscopia confocal da co-localização das redes de DNA e enzimas neutrofílicas, como MPO e NE. Os próximos passos desse projeto serão a realização de experimentos similares, demonstrando a liberação de NETs e proteínas dos grânulos de neutrófilos. Planejamos também executar estudos *in vivo* em modelos de peritonite, e utilizando transferência adotiva de neutrófilos marcados e pré-tratados com diferentes inibidores para moléculas importantes na quimiotaxia induzida por GRP (por exemplo, NADPH oxidase, GRPR e FAK) como já realizado na literatura (SAKAI et al., 2012).

Nossos resultados também podem ser importantes para futuros estudos sobre a conexão de sistema imune e o sistema nervoso. A literatura sobre o GRP é principalmente dividida em dois: ações sobre tumores (como descrito acima); e ações no sistema nervoso central e periférico. GRP e GRPR são expressos em

neurônios em diversas localizações no cérebro e na medula espinhal, onde controlam processos fisiológicos diversos, como glicemia, liberação de gastrina, somatostatina, ácido gástrico e secreção pancreática, movimentos gastrointestinais, fome, ansiedade, aversão, aprendizagem e memória (KAMICHI et al., 2005; RAMOS-ÁLVAREZ et al., 2015; ROESLER et al., 2014). De maneira interessante, o GRP também participa da sinalização do prurido (coceira) atuando na medula (PEREIRA et al., 2015; SUN et al., 2009; SUN; CHEN, 2007), e conjuntamente com a degranulação de mastócitos (ANDOH et al., 2011). Essas conexões neuroimunes vêm sendo desvendadas e recentemente um trabalho demonstrou claramente como as terminações nervosas do intestino estão localizadas de maneira a interagir com células imunes residentes (nesta caso, macrófagos no intestino) para auxiliar o controle da homeostase local (GABANYI et al., 2016). É sabido também que condições de estresse emocional podem promover ou agravar doenças autoimunes (STOJANOVICH, 2010), asma (FRIERI, 2003) e outras condições inflamatórias (DHABHAR, 2014). Sendo assim, um neuropeptídeo de atuação sistêmica, modulado pelo sistema nervoso e que tem capacidade de ativação aguda de células imunes altamente móveis e numerosas, pode ter relação com a promoção de doenças inflamatórias crônicas e autoimunes.

Continuando os estudos sobre a migração celular estimulada por GRP *in vivo*, estabelecemos uma parceria com o Dr. Gustavo Menezes da UFMG a fim de visualizar através de microscopia intravital esse recrutamento. Observamos que o GRP promove rolamento e adesão de neutrófilos nos vasos mesentéricos quando injetamos GRP no peritônio (dados não publicados). No mesmo experimento utilizamos o antagonista do GRP, RC-3095, para verificar a especificidade dessa indução. Como controle, injetamos camundongos apenas com o antagonista. Para nossa surpresa, houve uma redução global da visualização da migração *in vivo* em níveis abaixo do controle negativo. Esse dado, somado às informações sobre a inibição da migração promovida por IL-8 levou-nos a procurar um modelo onde o decréscimo da migração dos neutrófilos fosse melhorar uma doença inflamatória aguda. Escolhemos o modelo de lesão hepática medicamentosa para verificar o potencial do RC-3095 no controle da quimiotaxia neutrofílica. Esse modelo é especialmente adequado, porque além do dano hepático, de depender da grande infiltração de neutrófilos, ele também consiste em um modelo de lesão estéril, ou seja, sem a presença de patógenos. Isso facilita o estudo das interações do sistema

imune e o dano tecidual, processo que acontece continuamente durante o crescimento de tumores sólidos.

Curiosamente, o antagonista do GRPR foi capaz de reduzir grandemente as lesões hepáticas normalmente promovidas pela overdose de acetaminofeno. *In vitro,* observamos alterações da migração de expressão de moléculas de adesão causados pelo antagonista. Esse resultados indicam que o antagonista RC-3095 não deve ser tão seletivo como a literatura vêm descrevendo e utilizando (PINSKI et al., 1992; RAMOS-ÁLVAREZ et al., 2015). Acreditamos que as sinalizações celulares geradas pelo RC-3095 promovem alterações nos processos normais de resposta à estímulos, limitando-os ou abolindo-os.

Um ângulo não explorado em nosso artigo é a influência do TLR4. Foi mostrado que o RC-3095 promove a diminuição de expressão de TLR4 no pulmão de ratos em sepse, sendo esse o possíveis mecanismos gerador do seu potencial protetor nesta doença inflamatória (PETRONILHO et al., 2012). O aumento da expressão de TLR4 no fígado após dano por APAP é bem conhecido (CAI et al., 2014; SHAH et al., 2013). E o TLR4 agrava o DILI através do reconhecimento em células mieloides do HMGB1, uma proteína nuclear liberada com a necrose dos hepatócitos. Sendo assim, uma possível redução de expressão de TLR4 induzido pelo RC-3095 nesse sistema poderia explicar os efeitos benéficos do antagonista.

Um trabalho intrigante mostrou que ao realizar um *screening* para ligantes do receptor quimiotático de neutrófilos para fMLP (FPR1 e FPR2), foi encontrado que antagonistas de GRPR (PD168368 e PD176252) foram capazes ativar neutrófilos, porém sem induzir produção de ROS (SCHEPETKIN et al., 2011). Entretanto, nossos resultados mostram um efeito inverso para o RC-3095. Todavia, o fato de antagonistas não peptídicos do GRPR aparentemente se ligarem ao FPR1/2 clama por uma melhor investigação sobre as interações químicas desse antagonista.

Um dos pontos fracos do manuscrito é a ausência do tratamento com RC-3095 em diferentes doses. Justificamos isso pela limitação experimental e a dose utilizada foi escolhida por apresentar efeitos benéficos em estudos anteriores (CZEPIELEWSKI et al., 2012; DAL-PIZZOL et al., 2006). Concentrações mais altas são um problema no modelo lesão hepática, porque o DMSO (utilizado como solvente para o RC-3095) apresenta efeitos protetores sobre o fígado (JEFFERY; HASCHEK, 1988).

Doutorado sanduíche

Com o intuito de aprender mais sobre modelos genéticos tumorais e com a vontade de produzir um camundongo GRPR *knockout*, fui realizar doutorado sanduíche no *Department of Immunology*, na *Icahn School of Medicina at Mount Sinai*, Nova Iorque, EUA. Apesar de não conseguir trabalhar diretamente com meu projeto de doutorado, esse foi o período mais intenso, esclarecedor, ampliador e de maior aprendizado de minha trajetória científica. Fazer parte do laboratório do Dr. Sérgio Lira foi uma oportunidade singular. Lá, pude acompanhar diversos projetos e acabei trabalhando com um modelo genético de Histiocitose de Langerhans (LCH, *langerhans cell histiocytosis*). Apresento no **Anexo A** desta Tese o manuscrito referente ao meu trabalho desenvolvido lá, que está em fase de finalização da parte escrita. Desenvolvemos um belíssimo trabalho, gerando um novo modelo genético para o estudo desta doença muito pouco compreendida, de fundo genético (provavelmente mutações no BRAF), com um forte envolvimento de células imunes mieloides. A LCH é uma doença que acomete em sua maioria crianças. Ela é marcada pela formação de lesões formadas por células mieloides, que apresentam marcadores celulares de células de Langerhans, como a langerina (CD207). As lesões são mais comuns na pele e ossos (casos menos graves), porém podem atingir em órgãos internos, tornando-se um risco a vida do paciente. E como a etiologia da doença não é compreendida, seu tratamento ainda é muito insipiente, mais voltado a remoção cirúrgica das lesões. Deste modo, um modelo para estudar essa doença é fundamental. No nosso modelo o gene mutado do BRAF(V600E) é ativado condicionalmente apenas nas células que expressam CX3CR1, o receptor da fractalquina (CX3CL1). Esse receptor está presente em diversas células mieloides e também em seus precursores. Imaginamos que a mutação nesses precursores ocasiona a formação dessa célula com marcadores de célula de Langerhans e macrófagos, que evolui na formação de lesões mieloides no fígado, pulmão e baço. Marcadores imunes, como achados histológicos, de expressão citocinas e quimiocinas, e a localização das lesões remetem à pacientes com LCH sistêmica. Sendo assim, nosso trabalho propõem um modelo murino para a compreensão e desenvolvimento de terapias para essa grave doença.

Por fim, entre os muitos trabalhos realizados durante esse doutorado, pude vislumbrar a influência das células mieloides em processos tumorais e inflamatórios. Com certeza muito será descoberto sobre os efeitos imunológicos do GRP e seus derivados, onde essas populações celulares serão atores importantes desta interação. Espero continuar acompanhando de perto esses avanços.

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ANEXOS

ANEXO A – Introdução do manuscrito do artigo produzido durante o Doutorado Sanduíche "*CX3CR1+ cells expressing BRAFV600E are the cell-of-origin of a systemic LCH-like disease in mice*"

ANEXO B – Artigo publicado "*Respiratory Syncytial Virus Fusion Protein Promotes TLR-4–Dependent Neutrophil Extracellular Trap Formation by Human Neutrophils*"

ANEXO C – Artigo publicado "*Gallic acid reduces the effect of LPS on apoptosis and inhibits the formation of neutrophil extracellular traps*"

ANEXO D – Carta de Aprovação do Comitê de Ética para o Uso de Animais

ANEXO A

"CX3CR1+ cells expressing BRAFV600E are the cell-of-origin of a systemic LCH-like disease in mice"

Rafael S Czepielewski, Monique F Beltrao, Lili Chen, Gerold Bongers, Alan Soto, Christopher Parkhurst, Juan JJ Lafaille, Martin McMahon, Maria Isabel Fiel, Miriam Merad, Glaucia C. Furtado, and Sergio A. Lira

Manuscrito do trabalho desenvolvido durante Doutorado Sanduíche

CX3CR1+ cells expressing *BRAF*^{V600E} are the cell-of-origin of a systemic LCH-like disease in mice

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Authorship contributions

RC, MFB, MEP, GCF, and LC performed the experiments. RC, MFB, MEP, GCF, JJ, LC and GB analyzed the data. CP, JJL and MM contributed reagents and materials. SAL designed the study. GF and SAL wrote the manuscript.

Abstract

Langerhans cell histiocytosis (LCH) is associated with activating mutations in *BRAF*, but the cell-of-origin of the disease remains unclear. Here we tested the hypothesis that CX3CR1-expressing cells are involved in LCH pathogenesis. To generate mice that express $BRAF^{V600E}$ in CX_3CR1^+ cells, we crossed $BRAF^{CA}$ mice with mice expressing a tamoxifen inducible cre-recombinase (creER) and the reporter gene YFP in the CX_3CR1 locus. After tamoxifen treatment, *FRBRAF* animals developed a syndrome that had neurological and hematological components. The neurological component was first observed at 12 weeks of treatment and consisted initially in mild hind limb weakness. By 20 weeks of treatment over 90% of the animals were affected and presented varying degrees of motor impairment and spasticity, which severely reduced their mobility. At this point, mice were hunched and had enlarged abdomens. Upon necropsy, the mice had marked hepatosplenomegaly, and inflammatory infiltrates were detected in the liver, lung, spleen, spinal cord and the brain. In most instances, granulomatous aggregates, rich in myeloid cells, multinucleated giant cells, and lymphocytes were detected in liver, spleen and the lungs. Remarkably, $CD11b^+MHCII^+$ Langerin $(CD207)^+$ cells, typically found within LCH lesions in humans, were also present in these infiltrates. Transfer of $CX_3CR1^+BRAF^{V600E}$ cells from the liver into a healthy host induced development of $CD207^+$ histiocytic clusters in the liver, lung and spleen, but not in the brain. Bone marrow transfer experiments confirmed that the disease-inducing cells originated from the bone marrow, rather than from a liver resident cell. Taken together these results indicate that CX_3CR1-expressing cells are the cells of origin of systemic LCH in mice.

Introduction

Langerhans cell histiocytosis (LCH) is a rare disease that affects primarily children, and occasionally adults (Dewan et al. 2008). In children, LCH appears most localized to the skin, and the bones (Geissmann 2001), but cases involving lungs (Suri et al. 2012), kidney (Segerer et al. 2008), liver (Hatemi et al. 2010), and less frequently, the central nervous system (Grois et al. 2005) have been reported (Badalian-Very et al. 2013). LCH is a localized disease in 80% of the patients, and it is easily treated. However, in 20% of the patients the disease shows multiorgan involvement and is refractory to treatment thus life threatening.

LCH is diagnosed based on its histopathological markers. Histologically, Langerhans cell histocytosis (LCH) is marked by the presence of $CD1a^+$, $CD68^+$ and $CD207(Langerin)^+$ large cells with abundant cytoplasm within the lesions (Abla et al. 2010, Berres et al. 2015). For many years it has been unclear if LCH is a reactive or neoplastic disorder, but the recent finding that approximately 60% of patients with LCH have an activating mutation of the *BRAF* gene (*BRAF*-^{V600E}) suggests that the disease may have a neoplastic nature (Badalian-Very et al. 2010). Recent work done by our laboratories further validate this notion by demonstrating that forced expression of the *BRAF*^{V600E} mutation in myeloid cells ($CD11c^+$ cells) promotes development of a LCH-like disease in mice (Berres et al. 2014). These animals developed hepatosplenomegaly, and lymphadenopathy with the presence of histiocytic clusters in the skin, liver, spleen, and lungs by 8 weeks of age.

In addition to dendritic cells (DC), $CD34^+$ progenitors and $CD14^+$ monocytes isolated from the bone marrow and peripheral blood of patients with high risk LCH express the mutated form of *BRAF* (Berres et al. 2014). Based on these results we asked wheter the

phenotype of LCH-like disease would be more severe if *BRAF-*^{V600E} expression was enforced in a monocyte, or monocyte progenitor cell (Yona et al. 2013). To test this hypothesis, we generated mice expressing *BRAF*^{V600E} in cells expressing the chemokine receptor CX_3CR1, which is also known as the fractalkine (CX_3CL1) receptor. CX_3CR1 is expressed by MDPs (Fogg et al. 2006, Yona et al. 2013), pre-DCs (Liu et al. 2009), as well as tissue-resident mononuclear phagocyte populations (Bar-On et al. 2010, Lewis et al. 2011, Niess et al. 2005, Varol et al. 2009), such as splenic and lung macrophages, Kupffer cells and brain microglia.

Our results show that expression of *BRAF*^{V600E} in CX_3CR1^+ cells promotes expansion of the myeloid compartment and development of a disease that resembles human LCH. Importantly, we show that transplantation of CX_3CR1^+*BRAF*^{V600E} cells into a normal host induces disease. These results directly implicate *BRAF*^{V600E} mutation in CX_3CR1^+ monocytes in the pathogenesis of LCH.

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ANEXO B

"Respiratory Syncytial Virus Fusion Protein Promotes TLR-4–Dependent Neutrophil Extracellular Trap Formation by Human Neutrophils"

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RESEARCH ARTICLE

Respiratory Syncytial Virus Fusion Protein Promotes TLR-4–Dependent Neutrophil Extracellular Trap Formation by Human Neutrophils

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Abstract

Acute viral bronchiolitis by Respiratory Syncytial Virus (RSV) is the most common respiratory illness in children in the first year of life. RSV bronchiolitis generates large numbers of hospitalizations and an important burden to health systems. Neutrophils and their products are present in the airways of RSV-infected patients who developed increased lung disease. Neutrophil Extracellular Traps (NETs) are formed by the release of granular and nuclear contents of neutrophils in the extracellular space in response to different stimuli and recent studies have proposed a role for NETs in viral infections. In this study, we show that RSV particles and RSV Fusion protein were both capable of inducing NET formation by human neutrophils. Moreover, we analyzed the mechanisms involved in RSV Fusion protein-induced NET formation. RSV F protein was able to induce NET release in a concentration-dependent fashion with both neutrophil elastase and myeloperoxidase expressed on DNA fibers and F protein-induced NETs was dismantled by DNase treatment, confirming that their backbone is chromatin. This viral protein caused the release of extracellular DNA dependent on TLR-4 activation, NADPH Oxidase-derived ROS production and ERK and p38 MAPK phosphorylation. Together, these results demonstrate a coordinated signaling pathway activated by F protein that led to NET production. The massive production of NETs in RSV infection could aggravate the inflammatory symptoms of the infection in young children and babies. We propose that targeting the binding of TLR-4 by F protein could potentially lead to novel therapeutic approaches to help control RSV-induced inflammatory consequences and pathology of viral bronchiolitis.

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Introduction

Respiratory Syncytial Virus (RSV)-induced acute bronchiolitis is the most prevalent respiratory disease in children under age 2 years, and its seasonal epidemics are associated with a significant number of hospital admissions, with a huge burden to communities worldwide [1]. Almost 70% of all children are infected with RSV during the first year of life, and by age 3, practically all children will have experienced at least one infection with this virus [2, 3]. RSV is a single stranded RNA virus, whose genome encodes up to 11 proteins [4]. The Fusion (F) protein, present at the virion surface, mediates fusion of the viral envelope with the target cell membrane during virus entry [5]. Only membrane-bound F protein is indispensable for virus replication *in vitro* and *in vivo* [6], and this protein is the primary target for both antiviral drug and vaccine developments [7, 8]. It has been demonstrated that RSV F protein activates pattern recognition receptors TLR-4 and CD14, inducing pro-inflammatory cytokine secretion [9]. In addition, it has been recently shown that RSV F protein directly interacts with the MD-2–TLR-4 complex, thus activating the transcription factor NF-κB [10]. These studies highlight the importance of specific signaling pathways activated by F protein to stimulate inflammation.

One of the characteristic features of RSV infection is the large amounts of neutrophils in the lower airways once infection is established [11]. It is also well recognized that neutrophils and their products are present in the airways of patients and animal models with RSV bronchiolitis [11–13], and also in virus-induced asthma [14, 15]. This body of evidence suggests that neutrophils play an important role in the pathogenesis observed in the airways of affected children [16,17].

Aside from the traditional mechanisms of phagocytosis, generation of reactive oxygen species (ROS), and degranulation, neutrophils can also produce neutrophil extracellular traps (NETs), an important strategy to immobilize and kill pathogens [18]. NETs are formed by decondensed chromatin fibers decorated with antimicrobial proteins, such as neutrophil elastase and myeloperoxidase [18]. NET-inducing stimuli include cell surface components of bacteria, such as LPS, whole bacteria, fungi, protozoan parasites, cytokines, and activated platelets, among others [18–22]. More recently, studies have demonstrated that viruses are also capable of inducing NET formation. *In vitro*, the production of NETs is modulated in neutrophils isolated from cats infected with feline immunodeficiency virus [23]. NETs activated after infection by Human Immunodeficiency Virus (HIV-1) are crucial for the elimination of virus [24]. NET release in the liver vasculature also protects host cells from poxvirus infection [25]. However, an excessive production of NETs contributes to the pathology of respiratory viral infections. NET formation is potently induced in lungs of mice infected with Influenza A virus, in areas of alveolar destruction [26], suggesting a putative role for NETs in lung damage.

We show that RSV virion was able to induce NET formation by human neutrophils and RSV F protein stimulated NET formation dependent on TLR-4 receptor activation. Moreover, F protein-induced NETs were decorated with neutrophil elastase and myeloperoxidase, granule proteins that can damage tissues. F protein potently induced NADPH Oxidase-derived ROS production and this was crucial for NET generation. Also, F protein induced NET production in an ERK and p38 MAPK phosphorylation-dependent manner. Together, these results provide compelling evidence to support a signaling mechanism activated by RSV F protein to induce NET formation. The massive production of NETs in the airways of children infected with RSV may worsen lung pathology and impair lung function.

Materials and Methods

Reagents

RSV A2 strain was provided by Dr. Fernando Polack (Vanderbilt University School of Medicine, USA). Human recombinant RSV Fusion protein was purchased from Sino Biological Inc. According to the manufacturer, the glycosylated protein purity is >95% and endotoxin level is <1.0 EU per 1 μg of the protein, as determined by the LAL method. PMA and Protease-free DNase 1 were from Promega. Dextran, LPS O111:B4 from *Escherichia coli*, Diphenyleneiodonium (DPI), N-acetyl-L-cysteine (NAC), and Histopaque-1077 were obtained from Sigma-Aldrich. *ECORI* and *HINDIII* were from Invitrogen. PD98059 and SB203580 were from Cayman Chemical. Polymyxin B and anti-RSV F protein (131-2A) were from Millipore. Blocking anti-TLR-4 (HTA125) and mouse IgG2a isotype control were from eBioscience. The 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diaceate, acetyl ester (CM-H_2DCFDA) was from Molecular Probes. RPMI 1640 was from Cultilab, and FCS was from Gibco.

Human neutrophil isolation

Whole blood (20 mL) was collected from healthy volunteer donors (with a mean age of 29 years, from both sexes) with documented verbal consent into heparin-treated tubes. Erythrocytes were removed using Dextran sedimentation followed by two rounds of hypotonic lysis. Neutrophils were isolated from the resulting cell pellet using Histopaque-1077 density centrifugation and then resuspended in RPMI 1640 medium. Neutrophil purity was evaluated by flow cytometry using FACSCanto II (Becton Dickinson), based on morphology and a granulocyte marker expression, resulting in around 97%. Only singlet cells were verified by gating on granulocytes size on the basis of forward scatter (FSC) and side scatter (SSC), followed by CD66b and CD3 expression discrimination. Cell viability was always higher than 99%, as examined by Trypan Blue exclusion assay.

RSV preparation and neutrophil stimulation

The RSV A2 strain was grown in Hep-2 cells. Virus was purified from cell culture supernatant and the viral titer was determined by infection of Hep-2 cell monolayers followed by a carboxymethylcellulose plaque assay. The virus aliquots were stored in -80°C. Human neutrophils (2 x 10^6/mL) were stimulated with RSV (10^2–10^4 PFU/mL) for 3 h at 37°C under 5% CO_2 atmosphere. These RSV concentrations were used because higher concentrations were cytotoxic to neutrophils (data not shown). After this period, culture supernatants were collected and NETs were quantified using Quant-iT dsDNA HS kit (Invitrogen), according to manufacturer's instructions.

Quantification of NET release

Neutrophils (2 x 10^6/mL) were stimulated with F protein (0.1–5 μg/mL), LPS (100 ng/mL), PMA (50 nM) or medium alone. After 1 h, 20 U/ml of each restriction enzyme (*ECORI* and *HINDIII*) was added to the cultures, and then kept for 2 h at 37°C, under 5% CO_2 atmosphere [19]. NETs were quantified in culture supernatants using Quant-iT dsDNA HS kit (Invitrogen), according to manufacturer's instructions. To evaluate the involvement of TLR-4, NADPHox-derived ROS, and MAPK (ERK and p38) on F protein-induced NET formation, neutrophils were pretreated with selective inhibitors at 37°C under 5% CO_2, as indicated in figure legends. The Trypan Blue exclusion assay was used to evaluate the viability of cells treated with these inhibitors, and at the end of incubation, the cellular viability was always higher than 97%.

Immunofluorescence

Neutrophils (2 x 10^5/300 μL) were incubated with F protein (1 μg/mL), LPS (100 ng/mL), PMA (50 nM) or medium alone for 3 h at 37°C under 5% CO_2 in 8-chamber culture slides (BD Falcon). After this period, cells were fixed with 4% paraformaldehyde (PFA) and stained with anti-elastase (1:1000; Abcam), followed by anti-rabbit Cy3 antibodies (1:500; Invitrogen) or anti-myeloperoxidase PE antibody (1:1000; BD Biosciences) and Hoechst 33342 (1:2000; Invitrogen). Confocal images were taken in a Zeiss LSM 5 Exciter microscope.

Assay of intracellular ROS generation

The determination of intracellular ROS generation was based on the oxidation of 0.5 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) to yield an intracellular fluorescent compound. Neutrophils (2 x 10^6 cells/microtube) were pretreated with NAC (1 mM) or DPI (10 μM) and stimulated with F protein for 60 minutes at 37°C under 5% CO_2. Afterwards, cells were incubated with CM-H_2DCFDA for 30 minutes at 37°C under 5% CO_2. Cytosolic ROS production was measured by flow cytometry using FACSCanto II flow cytometer (Becton Dickinson) with the BD FACSDiva software and analyzed with FlowJo v 7.5.

Expression of phospho-ERK and phospho-p38

The expression of phospho-ERK 1/2 and phospho-p38 in human neutrophils was measured by flow cytometry using BD Phosflow (BD Biosciences) protocol for human whole blood samples. Neutrophils were stimulated with F protein (1 μg/mL) for 5 minutes. Briefly, cells were fixed in Phosflow Buffer I for 10 minutes at 37°C. After washing, permeabilization was performed with Phosflow Perm Buffer II for 30 minutes on ice. Then, neutrophils were washed twice and stained with APC anti–phospho-ERK 1/2 and Alexa 488 anti–phospho-p38 antibodies for 30 minutes on ice. Also here, data were accessed by flow cytometry using FACSCanto II cytometer (Becton Dickinson) with BD FACSDiva software and analyzed with FlowJo v 7.5.

Statistical analyses

Data were presented as mean ± SEM. Results were analyzed using GraphPad Prim 5.0 statistical software package. Statistical differences among the experimental groups were evaluated by analysis of variance with Newman-Keuls correction or with Student's t Test. The level of significance was set at $p \leq 0.05$.

Ethics Statement

This study was reviewed and approved by the Research Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul (CEP/PUCRS) under protocol nr. CEP 310.623. CEP/PUCRS approved the use of verbal consent for this study and blood donors provided their verbal informed consent before blood collection. The authors have documented the verbal consent provided by the donors.

Results

RSV particles and RSV Fusion protein induce NET formation

It has been previously shown that neutrophils and their products are present in the airways of patients and animals infected with RSV [12–14]. Furthermore, recent studies demonstrated that viruses are able to induce NET formation [24, 25]. Therefore, we sought to investigate

whether RSV would be able to induce NET formation in human neutrophils by stimulating neutrophils with increasing concentrations of RSV and quantifying extracellular DNA after 3 h. Indeed, RSV was able to induce NET production in a concentration-dependent manner (Fig 1A). RSV Fusion protein is essential for viral replication [6] and it is known to activate human monocytes, inducing a pro-inflammatory response [9]. We hypothesized that RSV F protein could play a role on NET production. To test that, human neutrophils were stimulated with different concentrations of F protein *in vitro* and after 3 h of incubation extracellular DNA was quantified in culture supernatants. RSV F protein induced NET formation in a dose-dependent manner, with the concentration of 1 μg/mL inducing the strongest response (Fig 1B). In an alternative approach to demonstrate the production of extracellular DNA by F protein, we stimulated neutrophils with medium alone, LPS, PMA or F protein and performed confocal laser scanning microscopy analysis. All stimulants (F protein, PMA and LPS) were able to induce NET formation compared to medium alone (Fig 1C–1F). The expression of antimicrobial proteins on NETs is induced by different stimuli, including bacteria, fungi, and virus [18, 24, 27, 28]. We sought to characterize the composition of NETs induced by RSV F protein, analyzing it by immunostaining. F protein induced the formation of NETs containing the proteins from azurophilic granules, neutrophil elastase (NE) (Fig 1G–1I) and myeloperoxidase (MPO) (Fig 1J–1L), which co-localized with extracellular DNA.

Effect of different treatments on F protein-induced NETs generation

To ensure that the structures visualized and quantified were in fact NETs, we stimulated neutrophils with F protein or LPS, as a control, and treated the cells with protease-free DNase. DNase treatment was able to dismantle NETs induced by both LPS and F protein (Fig 2A), indicating that those structures were made of DNA, and consequently NETs. A major concern when characterizing any putative activator of TLR is the possible presence of microbial-derived contaminants. LPS is the prototype TLR-4 agonist and it is among the most potent proinflammatory stimuli both *in vivo* and *in vitro*. To test whether the effect of F protein could be due to LPS contamination, we stimulated neutrophils with F protein or LPS in the presence or absence of polymyxin B and quantified extracellular DNA in culture supernatants. As expected, LPS-induced NET release was inhibited by polymyxin B, which has been previously shown to bind and neutralize LPS [29]. In contrast, F protein was able to induce NET formation in the presence of polymyxin B (Fig 2B), indicating that the effect of F protein is not attributable to LPS contamination. Next, we treated F protein with proteinase K for 90 minutes, to digest the protein structure, or boiled F protein for 10 minutes at 100°C, to further exclude the possibility that the effect could be due to other heat-resistant contaminant. Both treatments profoundly inhibited F protein-induced NET formation (Fig 2C), confirming that only integral F protein is capable of inducing NET production. Finally, we treated the F protein solution with either a neutralizing antibody directed to RSV F protein or with an isotype-matched antibody and stimulated neutrophils with these preparations. The neutralized F protein was not able to induce NET release compared to the protein treated with the control antibody (Fig 2D), confirming its role on NET production.

F protein-induced NET formation is dependent on TLR-4 activation

RSV F protein activates the pattern recognition receptors TLR-4–CD14–MD-2 to induce the activation of the transcription factor NF-κB and proinflammatory cytokine secretion [9, 10]. We hypothesized that F protein could activate TLR-4 to induce NET production. A blocking antibody against TLR-4 was used to define the involvement of this receptor on F protein-induced NET formation. Pretreatment of neutrophils with anti-TLR4 significantly inhibited the

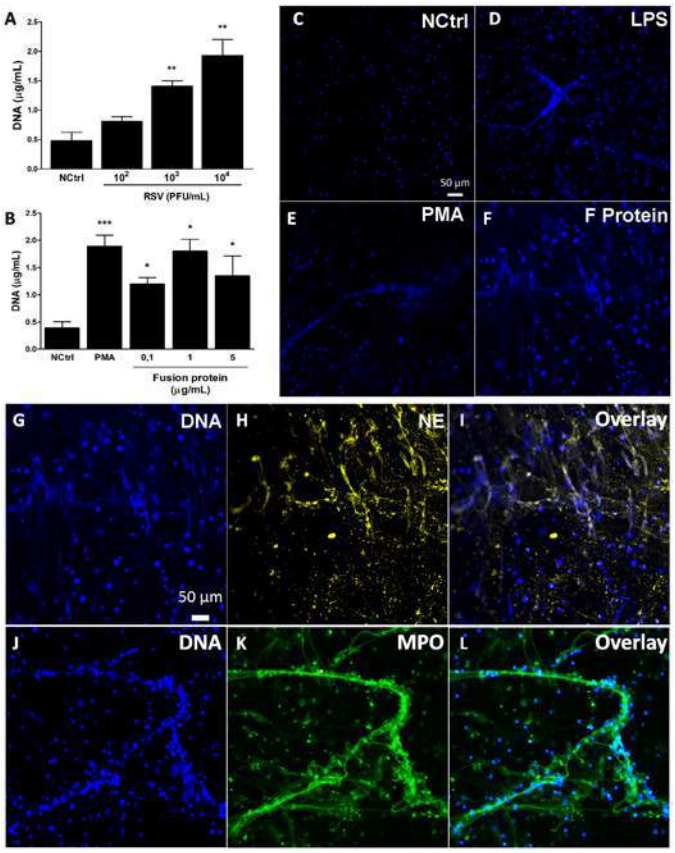

Fig 1. RSV particles and RSV Fusion protein induce NET formation. (A) Human neutrophils (2 x 10^6/mL) were stimulated with RSV (10^2–10^4 PFU/mL) or left unstimulated for 3 h at 37°C with 5% CO_2. (B) Neutrophils (2 x 10^6/mL) were stimulated with RSV F protein (0.1–5 µg/mL), PMA (100 nM) or medium alone for 3 h at 37°C with 5% CO_2. NETs were quantified in culture supernatants using Quant-iT dsDNA HS kit. Data are representative of at least 3 independent experiments performed in triplicates and represent mean ± SEM. *p<0.05; **p<0.01; ***p<0.001 when compared to negative control (NCtrl). (C-F) Neutrophils (2 x 10^5/300 µL) were stimulated with (C) medium, (D) LPS (100 ng/mL), (E) PMA (100 nM) or (F) F protein (1 µg/mL) for 3 h at 37°C with 5% CO_2. Cells were then fixed with 4% PFA and stained with Hoechst 33342 (1:2000). Images are representative of at least 4 independent experiments. (G-L) Neutrophils (2 x 10^5/300 µL) were stimulated with F protein (1 µg/mL) for 3 h at 37°C with 5% CO_2. Cells were fixed with 4% PFA and stained with: (G-I) Hoechst 33342 (1:2000), anti-elastase (1:1000), followed by anti-rabbit Cy3 (1:500) antibodies; (J-L) Hoechst 33342 (1:2000), anti-myeloperoxidase PE (1:1000) antibody. Overlay of the fluorescence images are shown in the last panels (I,L). Images are representative of 2 independent experiments. Images were taken in a Zeiss LSM 5 Exciter microscope. Scale bars = 50 µm.

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effect of F protein on NET release (Fig 3A). As an alternative approach to show the role of TLR-4 on NET formation by F protein, we visualized DNA fibers after pretreatment of cells with anti-TLR4. The release of DNA induced by F protein after pretreatment with the antibody is completely blocked (Fig 3B). These results indicate that RSV F protein induces NET formation via a TLR-4 activation pathway.

Essential role for NADPH Oxidase-derived ROS on F protein-induced NET generation

NET release induced by various agents has been previously shown to depend on ROS generation [27, 30, 31]. To characterize the involvement of ROS on F protein-induced NET production, neutrophils were treated with inhibitors of ROS generation. Treatment with NAC blocked

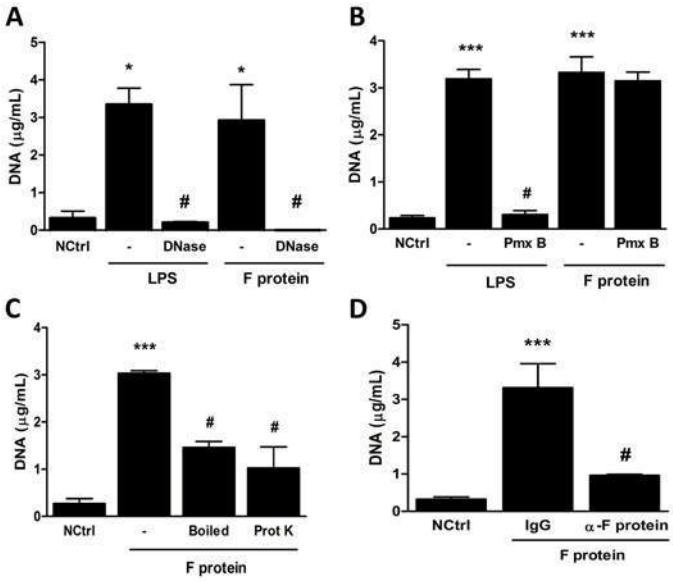

Fig 2. Effect of different treatments on F protein-induced NETs generation. Human neutrophils (2 x 10^6/mL) were stimulated with: (A) F protein (1 μg/mL) or LPS (100 ng/mL) in the presence or absence of DNase-1 (100U/mL); (B) F protein (1 μg/mL) or LPS (100 ng/mL) in the presence or absence of polymyxin B (Pmx B, 1 μg/mL); (C) F protein (1 μg/mL), boiled F protein (1 μg/mL, 10 min at 100°C) or F protein (1 μg/mL) treated with proteinase K (1 mg/mL for 90 min) for 3 h at 37°C with 5% CO_2. (D) F protein solution was treated with monoclonal anti-F protein (10 μg/mL) or isotype-matched (10 μg/mL) antibody and neutrophils (2 x 10^6/mL) were stimulated with these preparations for 3 h at 37°C with 5% CO_2. NETs were quantified in culture supernatants using Quant-iT dsDNA HS kit. Data are representative of at least 2 independent experiments performed in triplicates and represent mean ± SEM. *p<0.05; ***p<0.001 when compared to negative control (NCtrl); #p<0.05 when compared to LPS- or F protein-treated cells.

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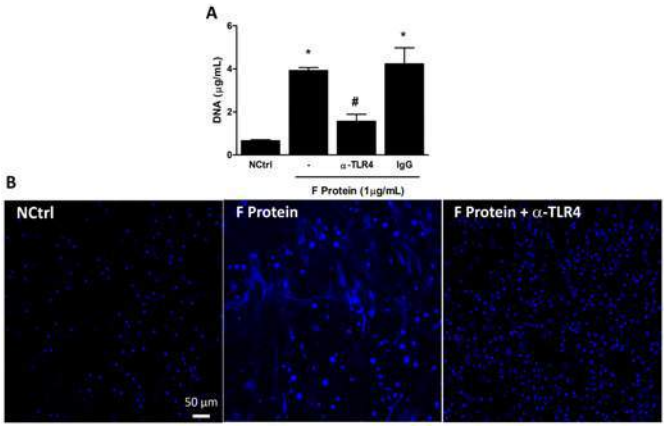

Fig 3. F protein-induced NET formation is dependent on TLR-4 activation. (A) Human neutrophils (2 x 10^6/mL) were pretreated with monoclonal anti-TLR4 (10 μg/mL) or isotype-matched (10 μg/mL) antibody for 1 h and stimulated with F protein (1 μg/mL) or medium for 3 h at 37°C with 5% CO_2. NETs were quantified in culture supernatants using Quant-iT dsDNA HS kit. Data are representative of at least 3 separate experiments performed in triplicates and represent mean ± SEM. *p<0.001 when compared to negative control (NCtrl); #p<0.05 when compared to F protein-treated cells. (B) Neutrophils (2 x 10^5/300 μL) were pretreated with anti-TLR4 (10 μg/mL) for 1 h at 37°C with 5% CO_2 and stimulated with F protein (1 μg/mL) or medium for 3 h at 37°C with 5% CO_2. Cells were fixed with 4% PFA and stained with Hoechst 33342 (1:2000). Confocal images were taken in a Zeiss LSM 5 Exciter microscope. Image is representative of 2 independent experiments. Scale bars = 50 μm.

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NET formation induced by F protein (Fig 4A) and abrogated F protein-induced ROS generation (Fig 4B). Similarly, treatment with DPI, a NADPH Oxidase inhibitor, significantly inhibited F protein-stimulated NET production (Fig 4C) and abolished ROS generation induced by F protein (Fig 4D). Together, these results indicate that F protein stimulates NET production dependent on NADPH Oxidase-derived ROS generation.

F protein activates ERK and p38 MAPK to induce NET formation

Recent studies have shown that ERK and p38 MAPK are indispensable for NET production [32,33]. To investigate the role of these MAPK on F protein-induced NET formation, we treated neutrophils with selective inhibitors of ERK and p38 MAPK. Pretreating neutrophils with PD98059 and SB203580, ERK and p38 inhibitors respectively, profoundly decreased DNA release induced by F protein (Fig 5A and 5B), pointing to a critical role for these MAPK on F protein-induced NET formation. We also evaluated whether treatment of neutrophils with F protein would activate these signaling pathways, analyzing phosphorylation of ERK 1/2 and p38. F protein rapidly activated phosphorylation of these signaling pathways (Fig 5C and 5D) leading to NET release, thus supporting the results obtained with the inhibitors.

Discussion

Neutrophils are key players in microbial containment due to their phagocytic properties, being able to deliver antimicrobial molecules in the phagolysosome and release neutrophil extracellular traps that entrap and kill a multitude of microorganisms [18, 34]. NETs are formed by a variety of stimuli, including bacteria, fungi, parasites, cytokines and endogenous proteins [18, 35–37].

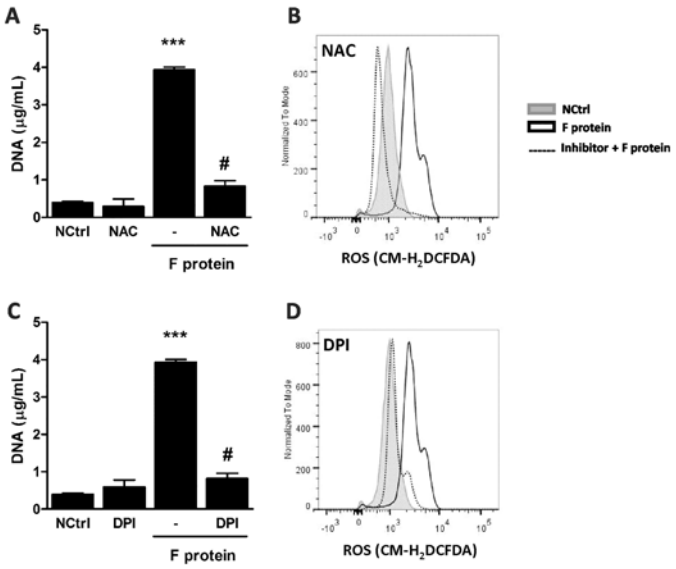

Fig 4. Essential role for NADPH Oxidase-derived ROS on F protein-induced NET generation. (A,C) Neutrophils (2 x 10^6/mL) were pretreated with NAC (1 mM) or DPI (10 µM) for 1 h and stimulated with F protein (1 µg/mL) for 3 h at 37°C with 5% CO_2. NETs were quantified in culture supernatants using Quant-iT dsDNA HS kit. Data are representative of 3 separate experiments performed in triplicates and represent mean ± SEM. ***p<0.001 when compared to negative control (NCtrl); #p<0.001 when compared to F protein-treated cells. (B,D) Neutrophils (2 x 10^6/microtube) were pretreated with NAC (1 mM) or DPI (10 µM) for 1 h, stimulated with F protein (1 µg/mL) for 1 h at 37°C with 5% CO_2 and incubated with 0.5 µM CM-H_2DCFDA for 30 min. ROS generation was analyzed by flow cytometry using FACSCanto II flow cytometer. Neutrophils gate was based on FSC x SSC distribution. Data are representative of 2 independent experiments performed in triplicates with similar results.

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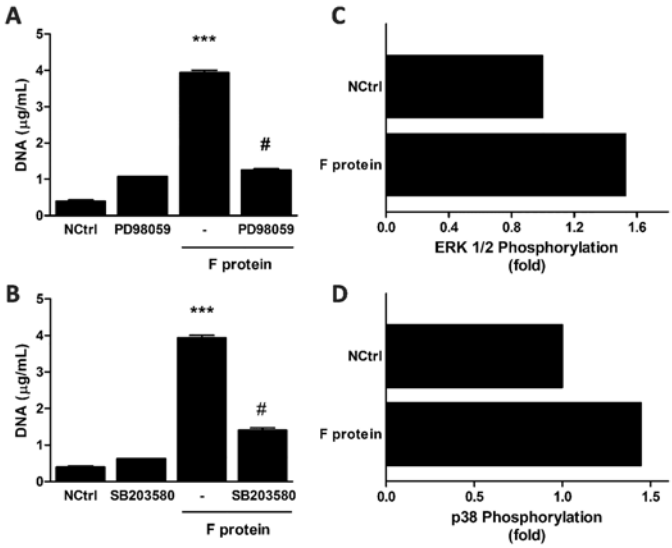

Fig 5. F protein activates ERK and p38 MAPK to induce NET formation. (A,B) Neutrophils (2 x 10^6/mL) were pretreated with PD98059 (30 μM) or SB203580 (10 μM) for 1 h and stimulated with F protein (1 μg/mL) for 3 h at 37°C with 5% CO_2. NETs were quantified in culture supernatants using Quant-iT dsDNA HS kit. Data are representative of 3 separate experiments performed in triplicates and represent mean ± SEM. ***p<0.001 when compared to negative control (NCtrl); #p<0.001 when compared to F protein-treated cells. (C,D) Neutrophils (1 x 10^6/mL) were stimulated with F protein (1 μg/mL) for 5 min at 37°C with 5% CO_2 and stained for phosphorylated proteins (ERK 1/2 and p38 MAPK), according to Materials and Methods. Proteins phosphorylation was analyzed by flow cytometry using FACSCanto II flow cytometer. Neutrophils gate was based on FSC x SSC distribution. Phosphorylation of protein pathways are presented as fold increase relative to unstimulated neutrophils (NCtrl). Data are representative of 2 separate experiments with similar results.

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Recent studies proposed a role for NETs in the control of viral infections [24, 25]. Neutrophil-derived NETs were able to capture HIV-1 particles and this effect was dependent on TLR-7 and TLR-8 activation [24]. Systemic injection of viral TLR ligands or poxvirus infection led to accumulation of neutrophils in liver sinusoids that formed aggregates with platelets and released NETs into the vessels [25]. These studies point out to a beneficial role for NETs in controlling and neutralizing viral infection. However, the excessive formation of NETs could be pathogenic to the host, mainly in respiratory viral infections, because NETs could expand more easily in the pulmonary alveoli, causing lung injury. It has been recently shown that Influenza A virus induced the formation of NETs, entangled with alveoli in areas of tissue injury, suggesting a potential link with lung damage [26].

In this study we were able to demonstrate that RSV particle and one of its membrane-expressed glycoproteins potently induced NET formation. RSV F protein caused the release of NETs coated with granular proteins NE and MPO. These proteins have been shown to be important for NET formation [38, 39] and to possess microbicidal activities [21, 24, 40]. MPO present in NETs provides the bactericidal activity against *S. aureus* [40] and promotes the elimination of HIV-1 [24]. NE expressed in NETs induced by the pathogenic mold *A. fumigatus* helps to inhibit its growth [21]. However, the antimicrobial proteins released with NETs are directly toxic to tissues and the massive production of NETs may damage host tissues [41], as is the case for elastase, which cleaves host proteins at the site of inflammation or infection [42]. Neutrophils actively producing NETs in the lung tissue disturb microcirculation and elicit pulmonary dysfunction [43]. Moreover, NETs directly induce epithelial and endothelial cell death [44]. NE and MPO expressed on DNA fibers stimulated by F protein could exacerbate lung pathology induced by RSV

infection, through the destruction of connective tissue, degradation of endothelial cell matrix heparan sulfate proteoglycan, resulting in post infection tissue injury [43].

The fibrous structure of NETs is essential for providing high local concentrations of antimicrobial proteins [45], but it can also be detrimental for host tissues, since it can impair lung function [46]. Furthermore, the characterization of NETs structure is a great concern when studying these DNA lattices and their function. With two different approaches, the quantification of extracellular DNA and fluorescence microscopy, we demonstrated that RSV F protein-induced NETs were dismantled by DNase treatment, confirming that their structural backbone is chromatin.

Together with G protein, F protein comprises the major glycoprotein on RSV surface and these proteins are the main targets of neutralizing antibodies against RSV. F protein mediates the fusion of virus with the target cell and it is essential for viral replication both *in vivo* and *in vitro* [6], being considered the primary target for vaccine and antiviral drug development. Monoclonal antibodies to F protein passively protect against RSV challenge in an animal model and reduce the severity of infection in premature and newborn babies [47, 48]. A major feature of RSV infection is the large numbers of neutrophils recruited to the airways of patients and animals [11–13, 49]. This phenomenon is more profound than in any other respiratory viral infection in childhood, in which mostly alveolar macrophages and T cells prevail.

Although neutrophils are essential effector cells of the innate immune system and have a crucial role in the clearance of microorganisms [50], it has been suggested that neutrophils may contribute to the pathology observed in the airways of patients and animals infected with RSV [51]. Moreover, it has been shown that RSV is able to activate neutrophils, inducing degranulation and IL-8 secretion [52] and also inhibit neutrophil spontaneous apoptosis [53]. It is plausible to reason that these effects could be mediated by F protein binding to TLR-4, once it has been demonstrated that F protein binds to TLR-4/CD14 and physically interacts with MD-2, an essential accessory molecule for TLR-4 activation [9, 10]. F protein induced NET formation in a TLR-4-dependent manner, since the treatment of neutrophils with a blocking antibody against TLR-4 profoundly inhibited extracellular DNA production. Our findings are in agreement with studies showing the activation of TLR-4 by different stimuli to induce NET generation [18, 20, 35]. Importantly, the activation of TLR-4 by F protein was not attributable to LPS contamination, since the treatment with polymyxin B did not inhibit NET formation induced by the protein, but did inhibit the effect of LPS on NET generation. Furthermore, the native conformation of F protein was required in order to stimulate NET formation, once the boiled or proteinase K-digested protein lost this effect, as well as the neutralized protein by a monoclonal antibody directed against RSV F protein.

Stimulation of TLR-4 initiates a signal transduction cascade that induces the assembly of NADPH Oxidase complex. Several studies indicate that ROS are required for NET formation [27, 30, 31]. Then, we sought to investigate whether F protein would be able to stimulate ROS production in neutrophils and whether this induction would be necessary for NET generation. Treatment with the ROS scavenger NAC abolished F protein-induced ROS and extracellular DNA production. Also, the oxidase inhibitor DPI, at the typical concentration needed to block the respiratory burst, completely blocked ROS production and NET formation induced by F protein. Thus, F protein-induced NET release is mediated by ROS generation. How ROS production contributes to DNA release is a question still open for debate. One possibility is that they promote the morphological changes seen in neutrophils secreting NETs [38]. In addition, it has been suggested that ROS can act as second messengers [54]. The requirement of ROS for NET generation induced by RSV F protein indicate that ROS act as second messengers for this stimulus, likely promoting downstream events that culminate in DNA release.

Recent evidence shows that NET formation needs additional signaling, with the involvement of ERK and p38 MAPK. Furthermore, activation of these MAP kinases is downstream of

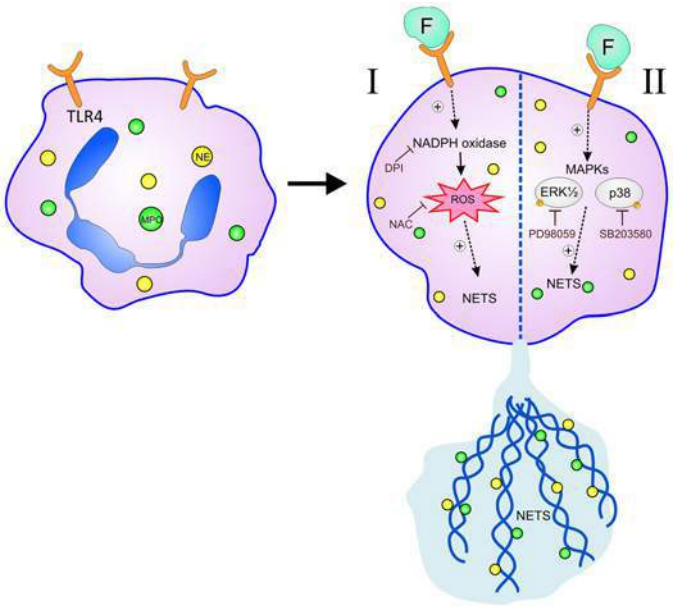

Fig 6. Mechanisms involved in RSV Fusion protein-induced NET formation in human neutrophils. (I) RSV F protein binds to and activates TLR-4, expressed by neutrophils, stimulating ROS production via NADPH Oxidase, which is essential for NET formation. (II) F protein is also able to activate ERK and p38 MAPK to induce NET release. RSV F protein stimulates the production of NETs decorated with the granular proteins NE and MPO.

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NADPH Oxidase-derived ROS production [32, 33]. We hypothesized that F protein would activate ERK and p38 MAPK to stimulate extracellular DNA release. Treatment of neutrophils with selective inhibitors of ERK and p38 MAPK almost abolished NET induction by F protein. Importantly, F protein was able to activate the phosphorylation of these MAP kinases. Taken together, these results indicate that RSV F protein-induced NET formation is mediated by the phosphorylation of p38 MAPK and ERK.

In conclusion, our study demonstrates that RSV particle stimulates NET formation by human neutrophils and RSV F protein is able to induce NET release through specific signaling pathways. This induction occurs through activation of TLR-4 and it is dependent on NADPH Oxidase-derived ROS generation, and on ERK and p38 MAPK phosphorylation (Fig 6). Neutrophils play an important role in the immunopathology during RSV infection and are continuously recruited from the bone marrow and blood stream to the lungs. The binding of RSV F protein to TLR-4 on neutrophils could induce the massive production of NETs, which can fill the lungs and impair lung function and consequently aggravate the inflammatory symptoms of the infection in young children and babies. We propose that targeting the binding of TLR-4 by F protein or even the associated use of DNase could potentially lead to novel therapeutic approaches to help control RSV-induced inflammatory consequences and pathology of viral bronchiolitis, which has a major disease burden among infants, worldwide.

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Author Contributions

Conceived and designed the experiments: BNP GAF. Performed the experiments: GAF NJ RSC MSM SPM. Analyzed the data: BNP GAF. Contributed reagents/materials/analysis tools: BNP RTS CB. Wrote the paper: BNP GAF.

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ANEXO C

“Gallic acid reduces the effect of LPS on apoptosis and inhibits the formation of neutrophil extracellular traps”

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Gallic acid reduces the effect of LPS on apoptosis and inhibits the formation of neutrophil extracellular traps

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ABSTRACT

Apoptosis and NETosis of neutrophils are two major mechanisms of programmed cell death that differ in their morphological characteristics and effects on the immune system. Apoptosis can be delayed by the presence of pathogens or chemical components such as lipopolysaccharide (LPS). Neutrophils have other antimicrobial strategy, called neutrophil extracellular traps (NETs), which contributes to the elimination and control of the pathogen. NETosis is induced by infection, inflammation or trauma and represents an innate immune activation mechanism. The objective of this study was to evaluate the effect of gallic acid (GA) in the modulation of apoptosis and NETs release. The results show that GA decreased the anti-apoptotic effect of LPS, blocked the induction of NETs and prevented the formation of free radicals induced by LPS. These findings demonstrate that the GA is a novel therapeutic agent for decreasing the exacerbated response of the body against an infectious agent.

1. Introduction

Sepsis is a complex syndrome that results in an exaggerated systemic inflammatory response against an infectious agent (Bone et al., 1997; Matot and Sprung, 2001). This reaction aims to destroy, dilute or immobilize the infectious agent. (Teixeira et al., 2003) Inflammations are divided into acute and chronic. Acute inflammation is characterized by the accumulation of fluid, fibrin, leukocytes (especially neutrophils) and red blood cells in the aggression area. Upon arrival in the inflamed site, neutrophils are already equipped with the necessary proteins to destroy infectious agents (Brinkmann et al., 2004). The encounter with the pathogen causes the activation of the cells with the immersion of the microorganism in a phagosome (Brinkmann et al., 2004; Guimarães-Costa et al., 2012; Fuchs et al., 2007). In the phagosome two events occur: first, there is great generation of reactive oxygen species (ROS), and second, the granules of neutrophils merge the phagosome, and unload antimicrobial peptides and enzymes. Together these two events lead to microbial death (Vaughan, 2013; Liu et al., 2000). The inflammatory reaction is mediated endogenously by active substances, called "inflammatory mediators" and excessive production of these mediators leads to an increase in host response, causing a metabolic imbalance that can propagate the inflammatory response (Teixeira et al., 2003; Fuchs et al., 2007; Esmann et al., 2010; Mello, 2012). Chronic inflammation is the sum of the reactions of the organism as consequence of the offending agent residence, which was not eliminated by the mechanisms of acute inflammation (Mello, 2012).

Septic shock is an example of the increase of uncontrolled inflammatory response that results in a metabolic imbalance (Radic, 2014). The shock and the complications are mainly related to the release of components of the bacterial wall. The endotoxin of gram-negative bacteria, lipopolysaccharide (LPS), and teichoic acid of gram-positive bacteria indirectly trigger the inflammatory cascade *via* induction of cytokine production by activated macrophages and monocytes, and sequentially, produce tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8). These cytokines interact with other cells and cellular elements (polymorphonuclear cells, endothelial cells, fibroblast cells, platelets and monocytes), inducing production and release of secondary mediators, which contribute to a delayed inflammatory response. (Sulowska et al., 2005) The

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overproduction or inappropriate expression of these factors can lead to a variety of pathological conditions, including septic shock and systemic toxicity (Mello, 2012; Radic, 2014; Kirchner et al., 2013).

NETosis and apoptosis of neutrophils are two major mechanisms of programmed cell death that differ in their morphological characteristics and their effects on the immune system (Brinkmann and Zychlinsky, 2012). Apoptosis is characterized by packaging of nuclear chromatin and nuclear fragments, subsequently occurring absorption of apoptotic cells by phagocytes, which generally suppress the immune response. Neutrophils under physiological conditions suffer apoptosis in 20 h. However, in infected tissues this can be delayed by microbial components such as LPS and pro-inflammatory stimuli (Teixeira et al., 2003; Esmann et al., 2010). Apoptosis of neutrophils is an important point in the physiological control of the immune response, playing an important role in the resolution of inflammation. In this context, apoptosis should be delayed until the essential functions of pathogen are completely phagocyted, then these cells must die to undo the inflammation and prevent tissue damage (Vaughan, 2013; Saffarzadeh and Preissner, 2013).

Recent studies have shown that neutrophils have another antimicrobial mechanism called NETosis, which can be induced by infection, inflammation or trauma and represents an innate immune activation mechanism (Brinkmann and Zychlinsky, 2012). When neutrophils are activated by phorbol myristate acetate (PMA), IL-8, LPS or fungi, they release means for the chromatin that are associated with different proteins, forming a complex called neutrophil extracellular traps (NETs), which capture and kill pathogens (Fuchs et al., 2007; Meng et al., 2012). NETs are abundant in inflamed sites, as found in patients with appendicitis, preeclampsia and infection by *Streptococcus pneumoniae* (Fuchs et al., 2007). Some studies suggest a pathophysiological role of NETs and their components in autoimmune diseases such as small vessel vasculitis, lupus nephritis, systemic lupus erythematosus (SLE), psoriasis, and rheumatoid arthritis (Meng et al., 2012; Bone, 1991; Thijs and Hack, 1995). Recent studies suggest that this action may cause tissue damage and the control of NETs release can result in beneficial effects in autoimmune diseases (Meng et al., 2012; Vilcek and Lee, 1991).

Gallic acid (GA) is a phenolic compound found in various plants, fruit and food, and it has antioxidant, anti-carcinogenic and anti-viral properties (You et al., 2011; Chandramohan Reddy et al., 2012). Other studies report that GA also has antibacterial, antifungal, anti-inflammatory, anti-malarial and anti-herpetic effects and is present in some of the most consumed beverages in the world, such as green tea (You et al., 2011; Chandramohan Reddy et al., 2012; Eslami et al., 2010). Hence, the objective of this study was to evaluate the effect of GA controlling apoptosis and formation of NETs in primary cultures of human neutrophils.

2. Materials & methods

2.1. Ethics statement

Study experimental protocol (443.648) was approved by the Ethics Research Committee of Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS).

2.2. Peripheral blood polymorphonuclear cells preparation

The peripheral blood polymorphonuclear cells (PMNs) were isolated from whole blood obtained from healthy human donors by Ficoll-Paque™ PLUS (GE Healthcare) density gradient centrifugation. Briefly, 12 mL of blood samples were collected by venipuncture in heparin-containing tubes. Plasma was discarded and blood cells were diluted 1:2 with saline solution. After, 4 mL Ficoll-Paque™ PLUS were added to cell solution and centrifuged at 720 ×*g* at room temperature for 20 min. After centrifugation, the supernatant was removed and the cells were washed with hypotonic lysis buffer, containing 0.83% NH_4Cl, to lyse red blood cells (erythrocytes). The solution was centrifuged at 200 ×*g* at 4 °C for 10 min and this procedure was repeated twice. The resultant pellet was washed with phosphate-buffered saline (PBS). Following isolation, neutrophils (2.0×10^5 / 200 μL) were maintained in RPMI 1640 medium supplemented with 10% autologous serum and 0.15% garamycin (Schering-Plough) in 96-well flat-bottom plates (Nunc™-Immuno Modules) at 37 °C in a humidified atmosphere containing 5% CO_2 (Morita et al., 2014; Czerwinska et al., 2013). Cell viability was assessed by trypan blue exclusion assay. Purity of this preparation was ≥95% of neutrophils. All reagents used were filtered through a disposable sterile filter unit 0.22 μM (Millex). All human subjects read and signed an informed consent.

2.3. Peripheral blood mononuclear cells (PBMCs) preparation

PBMCs were isolated from whole blood of healthy human donors (12 mL of heparinized blood) using Ficoll-Paque™ PLUS density gradient centrifugation. Briefly, plasma was discarded and the blood cells were diluted 1:2 with saline solution. After, 4 mL Ficoll-Paque™ PLUS were added to cell solution and centrifuged at 720 ×*g* at room temperature for 20 min. PBMCs were removed from the interface formed by centrifugation and washed with PBS. Cells were maintained in RPMI 1640 medium supplemented with 10% autologous serum and 0.15% garamycin (Schering-Plough) in 96-well flat bottom plates at 37 °C in a humidified atmosphere containing 5% CO_2. Cell viability was assessed by trypan blue exclusion assay. All reagents used were filtered through a disposable sterile filter unit 0.22 μM (Millex). All human subjects read and signed an informed consent.

2.4. Cytotoxicity assay

1.088 mg GA (MW 170.12) (Sigma Aldrich) was weighed and dissolved directly into 1 mL serum-free RPMI 1640 medium. After vortexing for a couple of minutes, GA is totally dissolved in the medium. From a stock solution of 6400 μM, the serial dilution was prepared as follows: 3200, 1600, 800, 400, 200, 100, 50, 25, and 12.5 μM. 100 μL of each concentration was added to wells containing 100 μL of RPMI 1640 medium + cells. Thus, the final concentrations in the wells were 1600, 800, 400, 200, 100, 50, 25, 12.5 and 6.25 μM, respectively. Neutrophils (2.0×10^5 cells/200 μL) were incubated for 16 h. Control group was composed of neutrophils in RPMI 1640 medium. LPS group (*Escherichia coli* 026: B6), the drug was diluted in RPMI 1640 medium and added directly to cell culture at the stated concentration (25, 50 and 100 ng/mL). GA group was composed by a GA serial dilution (6.25 to 1600 μM) and GA + LPS group was composed by the same serial dilution of GA + LPS (50 ng/mL), the drugs were diluted in medium and then added to the cell culture. All groups were made in triplicate and the viability was performed by trypan blue exclusion assay.

PBMCs (1.6×10^5 cells/200 μL) were incubated for 96 h. Control group was composed of PBMCs in RPMI 1640. GA group was composed by a GA serial dilution (6.25 to 1600 μM). All groups were made in triplicate and the viability was performed by trypan blue exclusion assay.

2.5. Apoptosis assay

Neutrophils were incubated in the presence of GA (25, 50 and 100 μM), LPS (50 ng/mL) or GA (25, 50 and 100 μM) + LPS (50 ng/mL) for 16 h. Untreated cells represented the control group. Apoptosis was evaluated by flow cytometry using Annexin-V Kit assay. Annexin-V and 7-AAD were added to 1×10^5 cell suspension according to the manufacturer's instructions (BD Biosciences) and then incubated for 15 min at room temperature in the dark. Subsequently 2×10^4 cells were analyzed by flow cytometry (FACS Canto

II, BD Bioscience) within 1 h. Early apoptotic cells were stained with Annexin V alone, whereas necrotic cells and late apoptotic cells were stained with both Annexin V and 7-AAD. Data were analyzed with FlowJo software v. 7.2.5 (Tree Star Inc., USA).

Morphological assessment of neutrophil apoptosis was evaluated by light microscopy analysis. Cells were spin down on a glass slide by a cytospin. Cells were fixed with methanol and stained with May–Grunwald–Giemsa staining solution.

2.6. Western blot

Neutrophils (1.0×10^6 cells/mL) were incubated in the presence of LPS (50 ng/mL) or GA (25, 50 and 100 μM) + LPS (50 ng/mL) for 16 h and re-suspended in a lysis buffer (10 mM pH 7.5 Tris-HCl, 100 mM NaCl, 0,3% CHAPS, 50 mM NaF, β-glicerol phosphate and protease inhibitors). The lysates (50 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with a blocking buffer (1 M Trizma pH 7.5, 5 M NaCl, 0.8% Tween 20 and 10% skim milk) for 2 h and stained with primary polyclonal antibodies – Human Anti-Caspase-3 – (1:500) in a blocking buffer overnight at 4 °C. The membranes were incubated with the secondary antibodies – Rabbit Anti-Mouse IgG – (1:7500) for 2 h at 4 °C. The bands were detected using Opti-4 CN revelator solution and the band intensities were obtained using the program ImageJ 3.0.

2.7. Induction and detection of NETs

NETs formation of neutrophils was quantitated in the supernatants (2.0×10^5 cells/200 μL). The cells were incubated in the presence of GA (25, 50, 100 μM), LPS (50 ng/mL) or GA (25, 50 and 100 μM) + LPS (50 ng/mL) for 16 h. Untreated cells represented the control group. To quantify levels of NETs (DNA), the Quant-iT™ PicoGreen® dsDNA Kit assay was used according to the manufacturer's instructions (Invitrogen). The fluorescence intensity of DNA was measured at excitation and emission wavelengths of 485 nm and 530 nm, respectively, in a microplate reader (Victor 3, PerkinElmer). A standard calibration curve was used. (Luo et al., 2014)

2.8. Immunofluorescence staining of NETs

The NETs formation was analyzed using the Kit Falcon™ Culture Slide (BD Biosciences). For immunofluorescence, freshly isolated PMNs were seeded on Poly-L-lysin coated cover slips, allowed to adhere (1 h), and stimulated with LPS (50 ng/mL) for NETs induction. To evaluate the effect of GA on the NETS formation, we incubated the cells with LPS (50 ng/mL) + GA (100 μM). After 16 h of incubation, cells were fixed with formaldehyde 4% for 2 h and permeabilized with PBS containing 0.03% Triton X-100 and 10% fetal bovine serum (Gibco — Life Technologies) for 30 min. To stain the NETs, samples were incubated with a primary monoclonal antibody – Mouse Anti-Myeloperoxidase – (1:200) for 30 min and then with a secondary

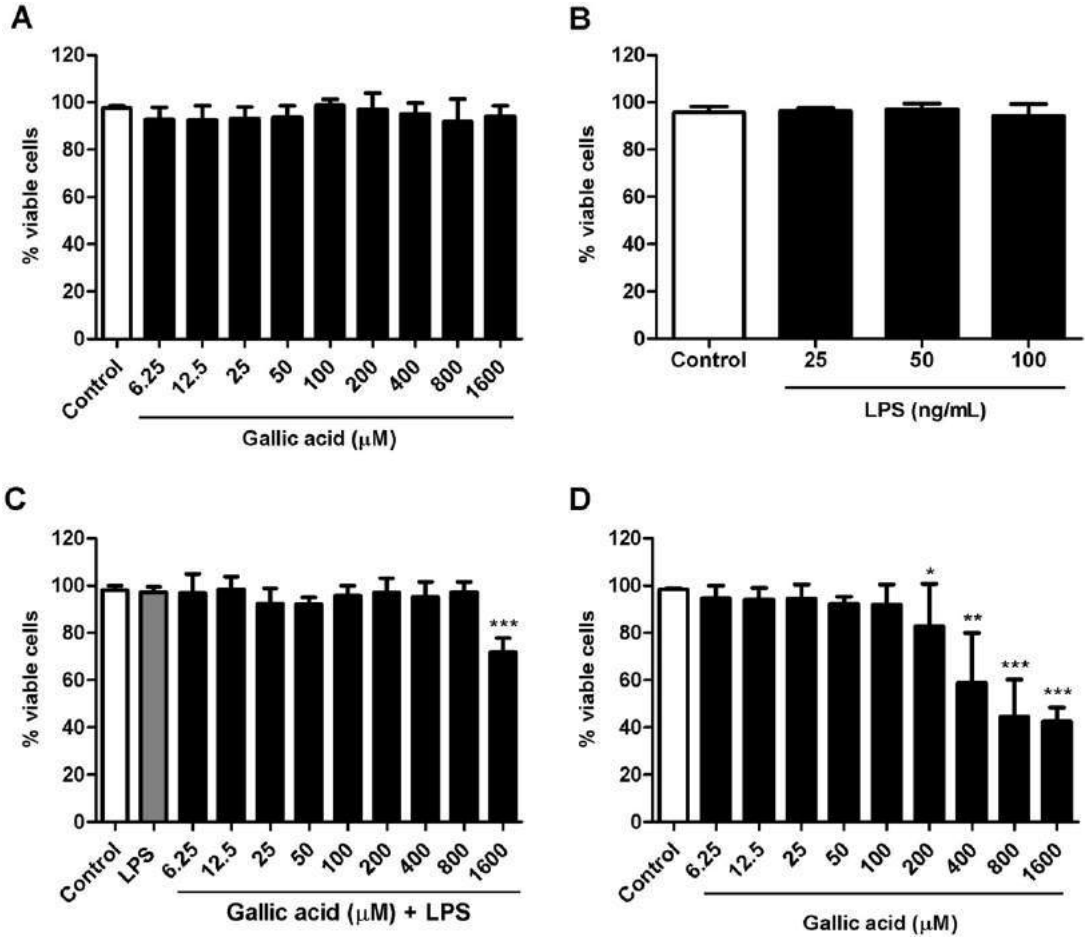

Fig. 1. Effect of different concentrations of GA on cell viability. Neutrophils were challenged with (A) GA (6.25 to 1600 μM), (B) LPS (50 ng/mL) or (C) GA (6.25 to 1600 μM) + LPS (50 ng/mL) for 16 h and cell viability was assessed by trypan blue exclusion assay. (D) PBMCs were challenged with GA (6.25 to 1600 μM) for 96 h and cell viability was assessed using trypan blue exclusion assay. Results were expressed as the percentage of viable cells. All data represent the mean ± SD (n = 5). ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ compared with control group.

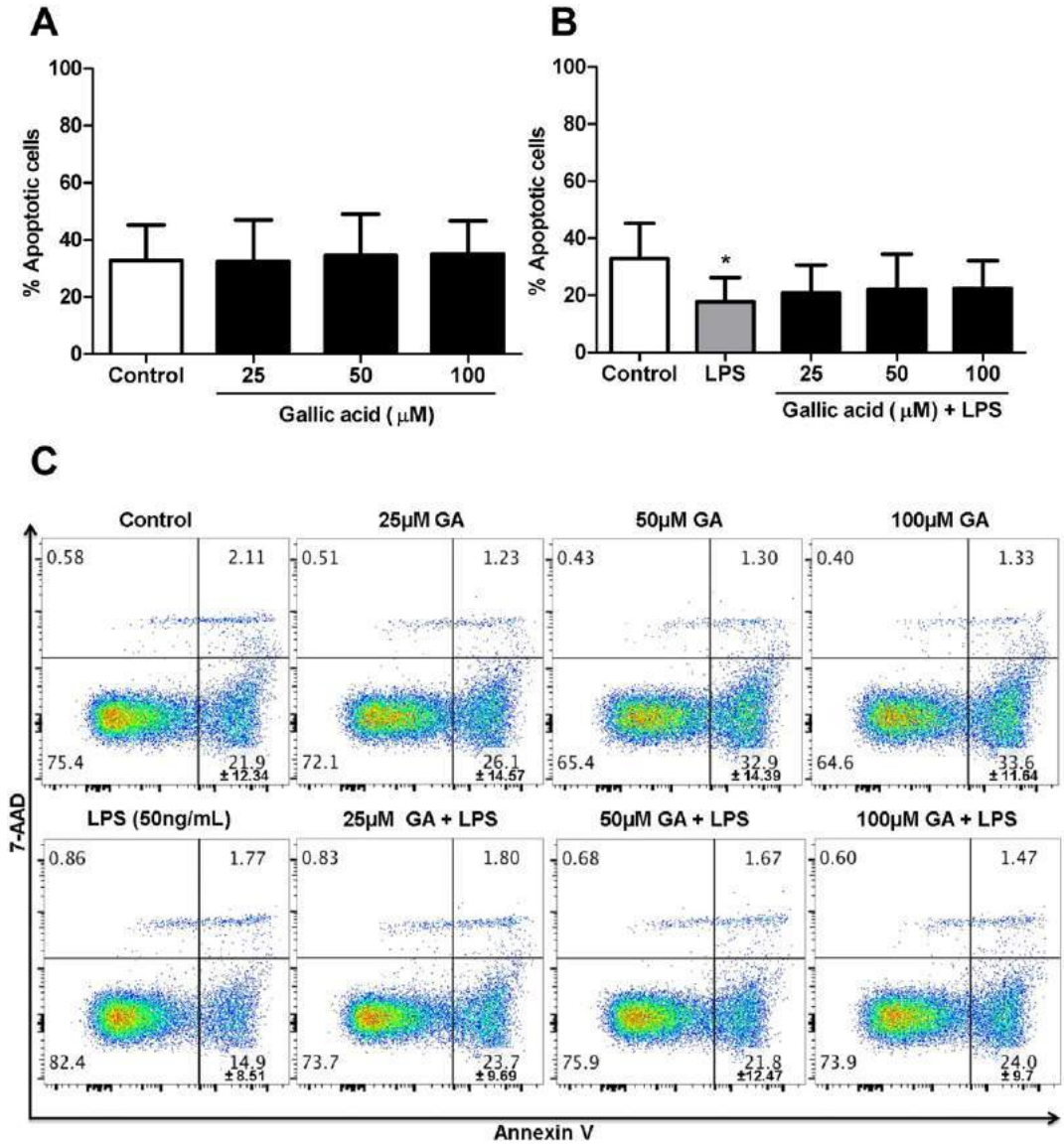

Fig. 2. Effects of GA, LPS and GA + LPS on apoptosis of neutrophils. (A) Cells were exposed to GA (25, 50 and 100 μM). (B) Cells were exposed to GA (25, 50 and 100 μM), LPS (50 ng/mL) and GA (25, 50 and 100 μM) + LPS (50 ng/mL). Results were expressed as percentage of apoptotic cells. Data represent the mean ± SD (n = 5). *$P < 0.05$ compared with control group. (C) Representative flow cytometric scatter plots of Annexin V (x axis)/7-AAD (y axis) stained control and GA- and/or LPS-treated cells for 16 h. The lower left quadrant shows viable cells, which are annexin V^- and 7-AAD^-. The lower right quadrant represents the apoptotic cells, annexin V^+ and 7-AAD^-. The upper right quadrant shows the late apoptotic or dead cells that are annexin V^+ and 7-AAD^+.

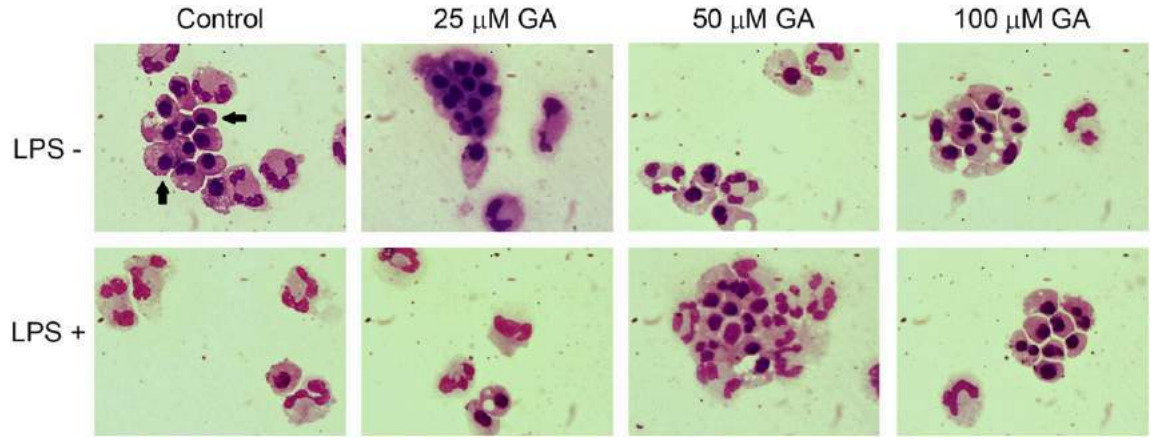

Fig. 3. Morphological changes of neutrophils after LPS stimulation visualized by optical microscopy. In this image, we could verify the morphological differences of cell in apoptosis. Arrows show morphological detail of an apoptotic cell which lost its original shape. 50 ng/mL LPS-treated cells show predominance of normal neutrophils, with fine granularity of chromatin and normal lobulated nucleus. Cells exposed to GA (25, 50 and 100 μM) and GA (50 and 100 μM) + LPS have the same pattern as control cells. Magnification ×400.

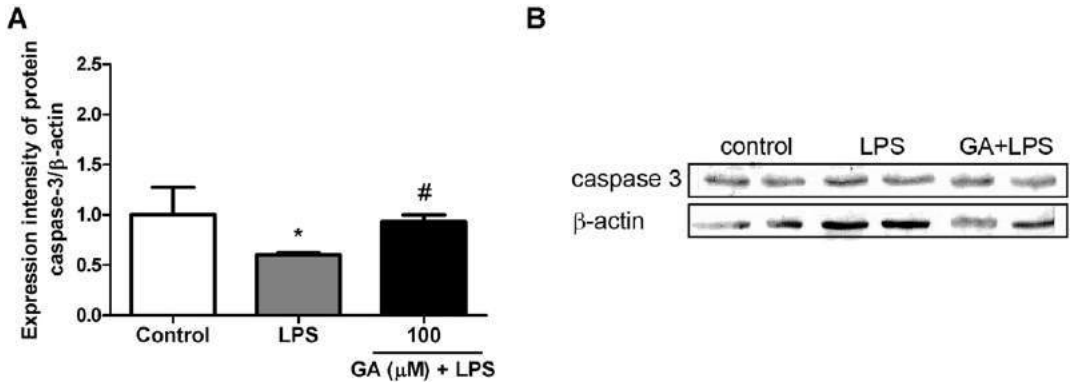

Fig. 4. Effect of GA and/or LPS on caspase-3 activation. Neutrophils were cultured in the presence of LPS (50 ng/mL) or GA (100 μM) + LPS (50 ng/mL) for 16 h. (A) Results were expressed as expression intensity of protein caspase-3/β-actin. Data represent the mean ± SD (n = 3). $^{*}P < 0.05$ compared with control group; $\#P < 0.05$ compared with LPS group. (B) Western blot analysis of extracts of neutrophils cultured for cleaved caspase-3 and β-actin proteins. Representative experiments are depicted (n = 3).

antibody – Rabbit Anti-Mouse IgG (H + L) Fluorescein (FITC) Conjugate – (1:500) for 30 min. After, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 2 min. Neutrophil-derived NETs formation was visualized by confocal immunofluorescence microscopy (Luo et al., 2014).

2.9. Antioxidant activity DPPH

The DPPH method is based on the capture of DPPH radical (2′,2′-diphenyl-1-picrylhydrazyl) by antioxidants, producing a decrease absorbance at 515 nm. The free radical scavenging activity was followed by preparing DPPH solution (60 μM) in methanol. Vitamin C (1 mg/mL) was taken as the reference standard. Different concentration of GA (25, 50 and 100 μM) and Vitamin C were diluted in methanol. 975 μL of DPPH solution (60 μM) was mixed with 25 μL of all GA concentrations. These mixtures were kept in dark about 5 min and measured the absorbance at 515 nm.

2.10. Measurement of production of reactive oxygen species

The generation of intracellular reactive oxygen species (ROS) of neutrophils (2.0×10^5 cells/200 μL) was evaluated based on the intracellular peroxide-dependent oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) which forms a fluorescent compound, 2′,7′-dichlorofluorescein (DCF). Briefly, the cells were incubated in the presence of GA (25, 50 and 100 μM), LPS (50 ng/mL) and GA (25, 50 and 100 μM) + LPS (50 ng/mL). After 16 h of incubation, cells were washed twice with PBS and then incubated with 200 μL/well of phosphate-buffered containing 10 μM of DCFH-DA at 37 °C for 30 min. The fluorescence intensity was measured at excitation and emission wavelengths of 485 nm and 530 nm, respectively, in a microplate reader (Victor 3, PerkinElmer).

2.11. IL-6, IL-8 and 1L-1β cytokines quantification

Cytokines production was evaluated in the supernatants of neutrophils (2.0×10^5 cells/200 μL). The cells were incubated in the presence of GA (25, 50 and 100 μM), LPS (50 ng/mL) or GA (25, 50 and 100 μM) + LPS (50 ng/mL) for 16 h. To quantify levels of IL-6, IL-8 and IL-1β, the Cytometric Bead Array (CBA) Kit assay was used according to the manufacturer's instructions (BD Biosciences) and analysis was performed by flow cytometry (FACS Canto II, BD Bioscience).

2.12. Statistical analysis

All experiments were done in triplicates. The normality of the data was analyzed by the Shapiro–Wilk test. The measures were parametric and then we calculated the mean and standard deviation of the mean for each of the variables analyzed. For comparison between groups analysis of variance (ANOVA) and post hoc LSD Test for multiple comparisons was applied. The differences were considered significant when the statistical analysis gives $P < 0.05$. SPSS (Statistical Package for Social Sciences) version 18.0 for Windows was used as a computational tool to analyze statistical data.

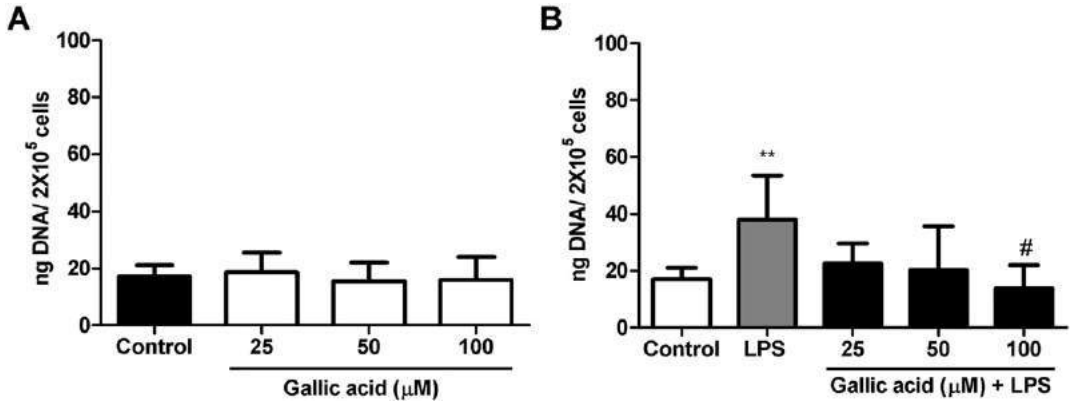

Fig. 5. Effect of GA and/or LPS on the NETs formation. (A) Cells were exposed to different concentrations of GA (25, 50 and 100 μM). (B) Cells were exposed to LPS (50 ng/mL) and different concentrations of GA (25, 50 and 100 μM) + LPS (50 ng/mL). Data represent the mean ± SD (n = 5). Results were expressed as ng DNA/2×10^5 cells. Data represent the mean ± SD (n = 5). $^{**}P < 0.01$ compared with control group; $\#P < 0.001$ compared with LPS group.

3. Results

3.1. Cytotoxic effect of GA, GA + LPS and LPS in human neutrophils and PBMCs

To evaluate cytotoxic effect of GA in human neutrophils, cells were exposed to different concentrations of GA (6.25 to 1600 μM). After 16 h of treatment, it was verified that GA did not decrease cell viability (Fig. 1A). We performed an apoptosis curve with LPS at concentrations of 25, 50 and 100 ng/mL (data not show). The concentration of 25 ng/mL had no significant anti-apoptotic effect, unlike the concentrations of 50 and 100 ng/mL. So, we tested the toxicity of LPS in neutrophils and verified that any concentration (25, 50 and 100 ng/mL) showed decrease in cell viability (Fig. 1B). Therefore, we chose the lowest concentration that possessed anti-apoptotic effect for the next experiments.

When we associate the LPS (50 ng/mL) with different concentrations of GA (6.25 to 1600 μM), only 1600 μM GA demonstrated toxicity (Fig. 1C). To evaluate cytotoxic effect of GA in other blood cells, PBMC cells were exposed to different concentrations of GA (6.25 to 1600 μM). The concentrations 200, 400, 800 and 1600 μM of GA decreased the cell viability (Fig. 1D).

3.2. Effect of GA, GA + LPS and LPS on apoptosis of human neutrophils

To analyze apoptosis induction, cells were exposed to different concentrations of GA (25, 50 and 100 μM) and LPS (50 ng/mL). GA alone did not induce apoptosis (Fig. 2A and C). However, LPS showed significant anti-apoptotic effect when compared with control, and GA inhibited this effect (Fig. 2B and C). Apoptosis was also evaluated by optical microscopy to verify morphological differences between apoptotic cells (the nucleus loses its original shape) and normal cells confirming the results by flow cytometry (Fig. 3).

3.3. Effect of LPS and GA + LPS on caspase 3 activation

As GA decreases anti-apoptotic effect of LPS, we decided to evaluate the activation of caspase-3. Cells treated with LPS (50 ng/mL) exhibited a significant reduction of caspase-3 when compared to control group and 100 μM GA + LPS (50 ng/mL) increased significantly the caspase-3 activation when compared to the cells treated with LPS (Fig. 4A and B).

3.4. Effect of GA, GA + LPS and LPS in the release of NETs of human neutrophils

The GA (25, 50 and 100 μM) alone did not induce the formation of NETs (Fig. 5A). LPS (50 ng/mL) increased the NETs formation and the GA decreased the LPS effect (Fig. 5B). This effect was visualized using immunofluorescence with confocal microscopy (Fig. 6).

3.1. Antioxidant effect of GA and Vitamin C

We observed that all concentrations of GA (25, 50 and 100 μM) decreased the free radical DPPH, showing a similar effect to Vitamin C (Fig. 7).

3.6. Effect of GA, GA + LPS and LPS on ROS generation

We observed that GA (25, 50 and 100 μM) alone did not induce the formation of ROS (Fig. 8A). 100 μM GA decreased significantly the ROS release when compared to LPS group (Fig. 8B).

3.7. Effect of GA + LPS and LPS on cytokine release

Cytokines have a key role in the resolution of inflammation, for this reason we decided to quantify the cytokines released by activated neutrophils. Cells stimulated with LPS increased IL-6, IL-8 and IL-1β levels when compared with control group. GA (50 and 100 μM) treatment decreased this effect only on IL-1β release (Fig. 9A, B and C).

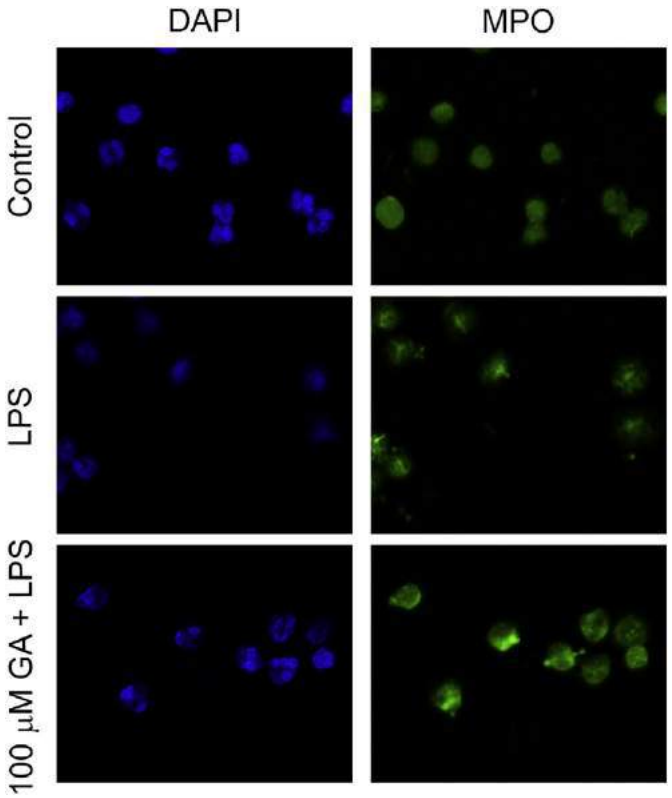

Fig. 6. NETs formation after LPS stimulation visualized by fluorescence. The image of control group shows the nuclear localization of DNA (blue fluorescence) and the granular patterns of myeloperoxidase (MPO) (green fluorescence). The LPS group shows the changes during NETs formation that can be determined with loss of granular integrity of MPO and nuclear lobules. GA + LPS group presents a similar morphology to the control group. Magnification ×630.

4. Discussion

Neutrophils are the first line of defense of our body and are the first cells to reach the focus of inflammation. In response to inflammatory stimuli, they migrate from the peripheral blood to infected tissues, where they efficiently bind, engulf, and inactivate bacteria (Brinkmann et al., 2004). These cells have a short half-life and die by apoptosis in a

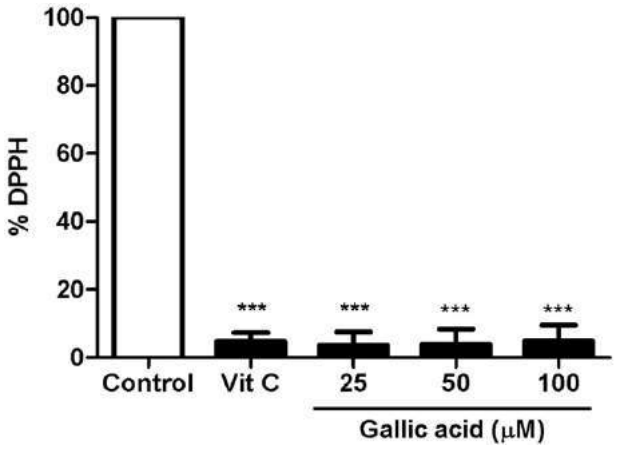

Fig. 7. Antioxidant effect of GA (25, 50 and 100 μM) and vitamin C (1 mg/mL). Data were expressed as percentage of control group. ***$P < 0.001$ compared with control group.

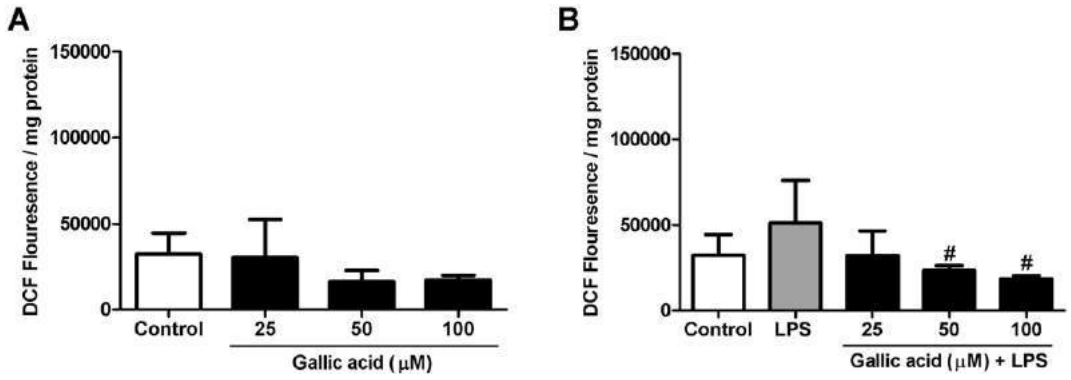

Fig. 8. Effect of GA, and/or LPS on neutrophils ROS release. (A) Cells were exposed to GA (25, 50 and 100 μM). (B) Cells were exposed to GA (25, 50 and 100 μM), LPS (50 ng/mL) and GA (25, 50 and 100 μM) + LPS (50 ng/mL). Data represent the mean ± SD (n = 3). Results were expressed as DCF Fluorescence/mg protein. #$P < 0.05$ compared with LPS group.

few hours. The presence of pathogens contributes to prolonging the life of neutrophils in the infected site. The permanence of these cells in the inflamed site, for a certain time, is beneficial to the host because it helps against the invasion, but on the other hand can lead to tissue damage by excessive release of toxic products. Neutrophils also have another antimicrobial strategy, called NETosis, which results in the death of these cells, and contributes to the elimination of the pathogens (Guimarães-Costa et al., 2012; Simon et al., 2000; Wallach-Dayan et al., 2006; Luo et al., 2014). Our study aimed to investigate the *in vitro* action of GA on LPS-induced apoptosis and NETosis of human neutrophils.

Our initial results showed that GA is not cytotoxic in neutrophils. In order to verify their possible cytotoxicity in other blood cells, we did experiments in primary cultures of human mononuclear cells. We found that the concentrations of 200, 400, 800 and 1600 μM of GA could decrease cell viability, thus they were not suitable for therapeutic purposes. For this reason, we chose 25, 50 and 100 μM concentrations to follow the study.

Our study showed that LPS decreased apoptosis in neutrophils. It is reported that LPS acts on TLR4 receptor and for consequence of this binding, occurs activation and increased lifetime of the cells. Neutrophil apoptosis is essential to regulate adult cell populations and in the resolution of inflammation. When the organism is under attack, these cells die slowly in order to control the infection, however they should die by apoptosis immediately after the combat against the pathogen. When LPS binds to TLR4 receptor, these cells release cytokines and produce ROS, and these inflammation mediators cause tissue damage (Brinkmann et al., 2004; Sabroe et al., 2005; Sabroe et al., 2002). In this study, GA decreased the anti-apoptotic effect of LPS, which indicates that GA has a protective role against infection-induced tissue damage.

The above-mentioned findings raised the question whether the caspase-3 was changed in GA-treated cells. Caspases play a key role of

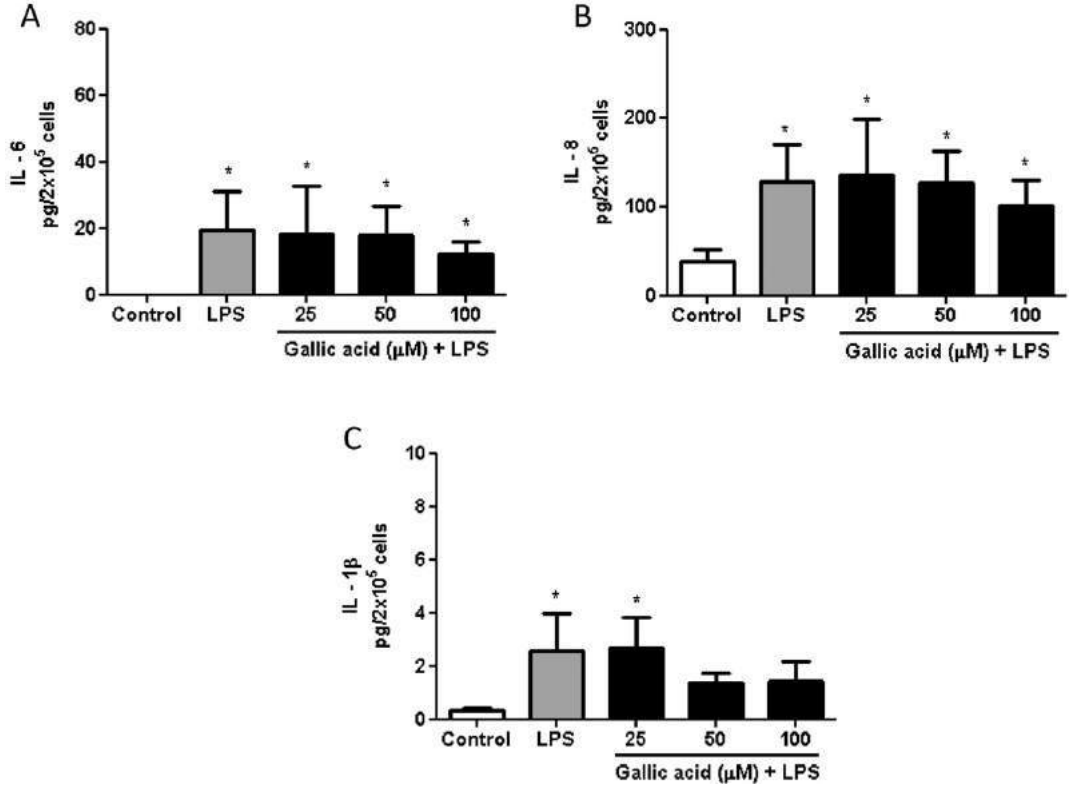

Fig. 9. Effect of GA on inflammatory cytokines release after 16 h treatment with GA (25, 50 and 100 μM) and/or LPS (50 ng/mL). Flow cytometric analyses of (A) IL-6, (B) IL-8 and (C) IL-1β in neutrophils supernatant. Cytokines IL-6 (A), IL-8 (B) and IL-1β (C) were analyzed. Data represent the mean ± SD (n = 5). Results were expressed as pg/2 × 10^5 cells. *$P < 0.05$ compared with control group.

apoptosis regulation and, in neutrophils, this programmed cell death is spontaneous (Gardai et al., 2004). Neutrophils apoptosis is inhibited during bacterial infection, because the inflammatory cytokines decrease caspase-3 activation, involving the NF-kβ (nuclear factor kappa B), ERK (extracellular-signal regulated kinases) and XIAP (X-chromosome linked apoptosis inhibitor) activation. XIAP inhibits the activity of caspase-3 (Gardai et al., 2004; Whitlock et al., 2000). Our results showed that LPS decreased caspase-3 expression and GA recovered this activation.

Studies describe that neutrophils, when activated by chemicals or pathogens, suffer NETosis and release NETs to the extracellular medium (Brinkmann et al., 2004). NETs are important to control and kill bacteria (Brinkmann et al., 2004) and represent a beneficial mechanism that is essential for the death of microorganism, preventing its spread in the body. However, the formation of NETs may have deleterious effects to the host due to the release of proteins, as proteases, which can injure the adjacent tissues (Meng et al., 2012). We were able to show that GA significantly decreased *in vitro* LPS-induced NETs release.

NETosis in response to chemical and biological stimuli is mediated by ROS production involving NADPH oxidase and MPO (Brinkmann et al., 2004; Guimarães-Costa et al., 2012). Preclinical studies have shown that GA possesses a variety of pharmacological activities, which include its action as an antioxidant and anti-inflammatory. In animal models, GA reduces oxidative stress and enhances the levels of glutathione (GSH), GSH peroxidase, GSH reductase, and GSH S-transferase in hepatic tissue, as well as catalase in the serum (Chen et al., 2013). In our study, GA demonstrated an antioxidant action, equivalent to the well-established antioxidant vitamin C. Moreover, GA also decreased ROS levels released by LPS-treated neutrophils, and, consequently, the NETs formation. Since ROS-dependent NETosis is also believed to play detrimental effects in autoimmune inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus, psoriasis, small-vessel vasculitis and lupus nephritis, pharmacological inhibition of ROS-dependent NETs formation could have a therapeutically effect on these disorders. Flavonoids are potentially effective in the treatment of these disorders (Kirchner et al., 2013) and, for that reason, GA can be potentially effective in the therapeutics of these diseases.

In response to pro-inflammatory stimuli, such as LPS, neutrophils are activated to reduce the action of pathogen on the tissue (Sabroe et al., 2002) and synthesize pro-inflammatory cytokines such as IL-6, IL-8 and IL-1β (Sabroe et al., 2002; Mitroulis et al., 2011). Our results corroborate with these studies demonstrating that LPS significantly increases the release of these cytokines. Even though, several pro-inflammatory cytokines have been associated with the early phase of acute inflammation, growing evidence derived from experimental and clinical studies indicates a pivotal role for IL-1β in the initiation of inflammatory process (Mitroulis et al., 2011). Experimental studies showed that IL-1β mediates the anti-apoptotic effect of LPS (Mitroulis et al., 2013) and it has protective effects against bacterial infection. Human studies have demonstrated that antagonists of IL-1β receptors are associated with increased susceptibility to bacterial infections. IL-1β exerts its protective effect against infection by the activation of many responses, which include rapid recruitment of neutrophils to the site of inflammation. However, when there is excessive inflammation and, consequently, an excessive release of IL-1β, severe mortality occurs. Excessive recruitment of neutrophils is also known to cause tissue damage, leading to multiple organ dysfunction and death. Our results demonstrated that GA decreased the LPS effect on IL-1β. However, it did not reverse the increase of IL-6 and IL-8. We showed that the GA modulates the release of IL-1β, and for this reason, exerts protective action against infections.

5. Conclusion

In this study, we demonstrated that GA significantly inhibits the release of ROS and formation of NETs in primary human neutrophils, indicating a correlation between these two phenomena. Our results also showed that GA reduces the anti-apoptotic effect of LPS in these cells. These actions suggest that GA may be used as a therapeutic strategy against diseases that are mediated by neutrophils activation.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

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ANEXO D

Carta de Aprovação do Comitê de Ética para o Uso de Animais

Ofício 037/13 – CEUA

Porto Alegre, 11 de junho de 2013.

Senhor Pesquisador,

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 12/00321, **"Mecanismos de Modulação de células imunes pelo peptídeo liberador de gastrina (GRP) e seu papel na progressão tumoral e metástases".**

Sua investigação está autorizada a partir da presente data.

Lembramos que é necessário o encaminhamento de relatório final quando finalizar esta investigação.

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

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