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REGULAÇÃO DA EXPRESSÃO DA ANEXINA A1 E SUA AÇÃO MODULATÓRIA EM PROCESSOS INFLAMATÓRIOS E INFECCIOSOS *IN VITRO* E *IN VIVO*

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Orientador: Luiz Ricardo Goulart

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Luiz Ricardo Goulart

"Importante é saber que tudo é um processo e não um acontecimento."

William P. Young

Ao meu esposo Rafael, também meu melhor amigo, por investir seus esforços e confiança, em todos os sentidos, na convicção da minha realização profissional. Sua admiração é parte do meu combustível! Muito obrigada!

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RESUMO

Introdução: As respostas inflamatórias exacerbadas ou prolongadas podem ser prejudiciais para o hospedeiro, então muitos mediadores antiinflamatórios atuam para regular as propriedades dos fatores pró-inflamatórios e garantir a homeostasia dos sistemas. **Objetivos:** Assim, o foco do presente trabalho foi estudar a regulação da expressão e as propriedades imunomodulatórias da anexina A1, uma proteína de 37 KDa, inicialmente conhecida como a segunda mensageira da ação dos glicocorticóides, em processos inflamatórios e infecciosos. **Metodologia:** Para tanto, por meio de técnicas moleculares (qPCR), bioquímicas (luminometria) e imunológicas (citometria de fluxo, imunofluorescência), foi investigado o papel endógeno da ANXA1 e exógeno do peptídeo N-terminal da ANXA1 em diferentes condições in vitro e in vivo: infecção fúngica, infecção viral por HIV-1 e SIV, doença inflamatória intestinal e injúria isquêmica renal. **Resultados:** O peptídeo N-terminal da ANXA1, Ac2-26, quando usado como tratamento prévio de PMN e PBMC do sangue periférico humano estimuladas por zymosan-opsonizado, inibe a produção de espécies reativas de oxigênio de modo dose-dependente. Paralelamente, a incubação dos fagócitos com o peptídeo demonstrou diminuir a expressão do receptor TLR2, responsável pelo reconhecimento de zymosan nos fagócitos, sugerindo um mecanismo de regulação de receptores de superfície pelo Ac2-26. Frente a este dado e a sinalização de ANXA1 via FPRs, a expressão do receptor de quimiocina e co-receptor do vírus HIV-1, CCR5, também foi investigada em tais condições in vitro. Os transcritos de CCR5 diminuíram em PBMCs de indivíduos HIV+ e saudáveis, parcialmente pela via ANXA1/FPR. Consistentemente, Ac2-26 também diminuiu a expressão do CCR5 na superfície de células T CD4+, os principais alvos do vírus. Em contraste, nos monócitos o peptídeo aumentou a expressão de CCR5, no entanto, induziu uma mudança na distribuição dos subfenótipos, diminuindo a quantidade dos monócitos com maior susceptibilidade a infecção por HIV-1 (CD14lowCD16+). A investigação da ANXA1 endógena em um modelo não humano de AIDS, realizado pela infecção de macacos por SIV, mostrou que a expressão transcricional da ANXA1 é regulada durante a progressão da doença nos principais compartimentos de infecção, o sangue e o intestino. Em comparação com as amostras de animais não-infectados, os transcritos de ANXA1 aumentaram gradualmente no sangue periférico entre as fases aguda e crônica da infecção por SIV, enquanto que na mucosa intestinal houve uma regulação negativa, atingindo os níveis basais apenas na infecção crônica. A análise de marcadores típicos da progressão da AIDS mostrou que a expressão de ANXA1 está relacionada à ativação de células T. No entanto, a expressão da ANXA1 tem uma correlação positiva com o número de transcritos de citocinas antiinflamatórias e uma correlação negativa com a carga viral e depleção de células T CD4+ no sangue e no intestino, sugerindo que a ativação da sua transcrição está relacionada a tentativa de diminuir a exaustão imunológica durante a infecção. Frente aos dados da modulação diferencial da ANXA1 no intestino de primatas, o próximo objetivo foi analisar a expressão da ANXA1 nos

portadores de IBD (doença inflamatória intestinal) tratados ou não com drogas imunossupressoras. Nos indivíduos com IBD, a expressão da ANXA1 é diminuída em nível de RNAm, no sangue periférico, e proteína, na mucosa colônica. Nestes pacientes, a imunoterapia com infliximab (anti-TNF-α) modulou sistemicamente a transcrição da ANXA1 e do TNF-α, e ainda a ativação linfocitária e a bacteremia. Em suma, a dinâmica da ANXA1 somada a outros elementos imunológicos e clínicos parecem estar associados a progressão do curso das IBDs. Finalmente, os processos inflamatórios agudo e crônico induzidos pelo procedimento de isquemia e reperfusão (I/R) revelou que o peptídeo mimético ANXA1 exógeno protegeu significativamente contra a lesão por I/R renal. Os animais pré-tratados com Ac2-26 mostraram menores taxas de filtração glomerular, osmolalidade urinária e desenvolvimento da necrose tubular aguda, possivelmente, por manter a integridade tecidual devido ao bloqueio total do extravasamento de neutrófilos, à atenuação de infiltração de macrófagos e à regulação da expressão protéica da ANXA1 endógena em células epiteliais renais. Estes resultados apontam um importante papel da ANXA1 na defesa das células epiteliais contra a lesão de I/R e indicam que os neutrófilos são mediadores-chave para o desenvolvimento da lesão do tecido renal após I/R. **Conclusões:** Em uma análise geral do conjunto de estudos apresentados, é possível concluir que a ANXA1 exógena e seus peptídeos derivados atuam como moléculas chave na modulação específica da ativação, imunofenótipo e distribuição dos fagócitos e linfócitos que participam como linha de defesa ou como alvo de agentes infecciosos, por meio da ligação com os FPRs ou por uma via ainda não elucidada. E, enfatizando a relevância da ANXA1 na defesa da homeostasia, ainda foi constatado que as vias relacionadas à regulação da ANXA1 endógena são ativadas diferencialmente em tecidos e fluidos sob inflamação aguda e crônica iniciadas quer seja por infecção viral, auto-imunidade ou hipóxia.

Palavras-chave: anexina A1, sangue, mucosa, AIDS, IBD, isquemia renal

ABSTRACT

Background: Exacerbated or prolonged inflammatory responses can be detrimental to the host, then any antiinflammatory mediators act to regulate the properties of pro-inflammatory factors and ensure the homeostasis of the organic systems. Objectives: **Aims:** This study has focused in the regulation of expression and the immunomodulatory properties of annexin A1, a protein of 37 KDa, originally known as the second messenger action of glucocorticoids, in inflammatory and infectious processes. **Methods:** Using molecular (qPCR), biochemical (luminometry) and immunological (flow cytometry, immunofluorescence) tools, we investigated the endogenous and exogenous of ANXA1 and its mimetic peptide (Ac2-26) in different conditions in vitro and in vivo: fungi infection, HIV/SIV infecion, bowel inflammatory disease and renal ischemic injury. **Results:** The Ac2-26 pre-treatment of PMN and PBMC of human peripheral blood stimulated with opsonized-zymosan inhibited the production of reactive oxygen species in a dose-dependent manner. In parallel, incubation of phagocytes with the peptide was shown to decrease expression of TLR2 receptor, responsible for the recognition of zymosan on phagocyte, suggesting a regulatory mechanism of surface receptor by ANXA1 peptide. Due to last data, the expression of CCR5, chemokine receptor and HIV co-receptor, was investigated in the same in vitro conditions. CCR5 transcription was down-regulated in PBMC from HIV-infected and healthy subjects, partially by ANXA1/FPR pathway. Consistently, both PBMC subject groups showed an impairment of percentage of CCR5+CD4+ T cells after Ac2-26 incubation. In contrast, Ac2-26 showed an up-regulation of CCR5 surface expression in monocytes, however the peptide switched the profile of monocyte subsets, increasing CD14highCD16- and decreasing CD14lowCD16+ populations. The last one is the most susceptible to HIV infection. In conclusion, the data suggest that the action of N-terminal ANXA1 is cell-dependent and may contribute indirectly modulating the HIV-1. Coherently, the study in vivo of ANXA1 expression in a non-human model of AIDS, showed that ANXA1 is regulated during the progression of the disease in the main compartments of infection, the blood and the gut. In comparison with samples of uninfected animals, the *ANXA1* transcripts in peripheral blood increased from acute to chronic SIV infection, whereas in the intestinal mucosa it was down-regulated, reaching baseline levels only in chronic infection. Analysis of typical markers of AIDS progression, showed that increased *ANXA1* transcripts was significantly correlated with the activation of T cells. However, the expression of *ANXA1* had a positive correlation with anti-inflammatory cytokines in the blood and in the intestine and a inverse dynamics of viral load and depletion of CD4 T cells, suggesting that activation of its transcription is related to an attempt to reduce the immune depletion during infection. In addition, based on the described differential modulation of the *ANXA1* in the gut of primates, another goal was to analyze the expression of *ANXA1* in

patients with IBD (inflammatory bowel disease) or not treated with immunosuppressive drugs. The correlation among those factors and clinical data were analyzed. In IBD patients, the expression of ANXA1 is reduced in level of mRNA in peripheral blood and protein in the colonic mucosa. In these patients, immunotherapy with infliximab (anti-TNF-alpha) modulates the transcription of *ANXA1* and *TNF-α,* and even systemic lymphocyte activation in accordance with the duration of treatment and the side effects of drugs, such as bacteremia. The dynamics of ANXA1 added to other clinical and immunological factors appear to be associated with the course of IBD. Finally, acute and chronic inflammatory process induced by ischemia/reperfusion (I/R) procedure, revealed that exogenous ANXA1 mimetic peptide granted a remarkable protection against kidney I/R injury, preventing glomerular filtration rate and urinary osmolality decreases and acute tubular necrosis development by affording striking structural protection due to the abortion of neutrophil extravasation, attenuation of macrophage infiltration and regulation of endogenous annexin A1 expression in renal epithelial cells. **Conclusions:** In a general analysis of all studies presented, we conclude that exogenous ANXA1 and its derived peptides act as key molecules in the modulation of specific activation, immunophenotype and distribution of phagocytes and lymphocytes participating as a line of defense or targeting of infectious agents, via FPRs or not. And, emphasizing the importance of ANXA1 in the defense of homeostasis, although it was found that the pathways related to the regulation of endogenous ANXA1 is differentially activated in tissues and fluids under acute and chronic inflammation caused by viral infection, autoimmunity or hypoxia.

Key words: annexin A1, blood, mucosa, AIDS, IBD, renal ischemic injury

LISTA DE FIGURAS

Capitulo III

Capitulo IV

LISTA DE TABELAS

Fundamentação Teórica

Capitulo V

LISTA DE ABREVIATURAS E SÍMBOLOS

A	Adenina
aa	Aminoácidos
ABC	Transportador do tipo ABC
Ac1-18	Peptídeo acetilado amino-terminal da ANXA1 (aa 1 ao 18)
Ac2-12	Peptídeo acetilado amino-terminal da ANXA1 (aa 1 ao 18)
Ac2-26	Peptídeo acetilado amino-terminal da ANXA1 (aa 2 ao 26)
Ac9-25	Peptídeo acetilado amino-terminal da ANXA1 (aa 9 ao 25)
ACTH	Hormônio Adrenocorticotrófico
AIDS	Síndrome da Imunodeficiência Adquirida
Akt	Proteína quinase B
ALXR	Receptor humano de peptídeos formilados-2 (nomenclatura prévia)
AMP	Adenosina mono-fosfato
ANXA	Proteínas anexinas encontradas em vertebrados
ANXA1	Proteína anexina A1
ANXA1	Gene da anexina A1
ANXA1 -/-	Nocaute para o gene *ANXA1*
ANXA2	Gene da anexina A2
ANXA3	Gene da anexina A3
ANXA5	Proteina anexina A5
ATP	Adenosina tri-fosfato
bHSV-1	Vírus Herpes bovino tipo 1
C	Citosina
Ca_{2+}	Ion cálcio
CAAT	Sequência promotora
CCR5	Receptor da quimiocina quimioatractante 5
CD	Doença de Crohn
CD3	Cluster de diferenciação 3 - População de células T
CD4	Cluster de diferenciação 4 - Subpopulação de células T auxiliadora
CD8	Cluster de diferenciação 8 - Subpopulação de células T citotóxica
CD11b	Cluster de diferenciação 11b
CD14	Cluster de diferenciação 14 - População de células apresentadoras de antígenos da linhagem monocítica/macrofágica
CD19	Cluster de diferenciação 19 - População de células B
CD38	Cluster de diferenciação 38
CD45	Cluster de diferenciação 45
CXCR4	Receptor da quimiocina quimioatractante X4
EBV	Vírus Epstein-Barr
EGF	Fator de Crescimento Epidérmico

EGFR	Receptor do Fator de Crescimento Epidérmico
ERK	Quinase regulada por sinais extracelulares
EROs	Espécies reativas de oxigênio
fMLF	Peptídeo formilado
FPR	Receptor de peptídeos formilados
FPR-1	Receptor humano de peptídeos formilados-1
FPR2/ALX	Receptor humano de peptídeos formilados-2
FPR-3	Receptor humano de peptídeos formilados-3
FSH	Hormônio Folículo-Estimulante
hCMV	Citomegalovírus humano
HLA-DR	Antígeno Leucocitário Humano tipo DR
HTLV	Vírus Linfotrópico de células T Humanas
G	Guanina
GAPDH	Gene da desidrogenase gliceraldeido-3-fosfato
GC	Glicocorticóide
gp120	Glicoproteina de 120 KDa do envelope do vírus HIV
gp41	Glicoproteina de 41 KDa do envelope do vírus HIV
GPCR	Receptor acoplado a proteína G
GR	Receptor de glicocorticóide
GRE	Elementos de resposta aos glicocorticóides
HGF	Fator de crescimento de hepatócitos
HGFR	Receptor do fator de crescimento de hepatócitos
HIV	Vírus humano da AIDS
HPA	Hipotálamo-Pituitária-Adrenal
HSP70	Proteína de choque térmico 70
HSP90	Proteína de choque térmico 90
I/R	Isquemia e reperfusão
IBD	Doença inflamatória intestinal
IKK	Complexo quinase IkB
IFN-γ	Interferon-gama
IL	Interleucina
IR	Isquemia-reperfusão
JEV	Vírus da encefalomielite japonesa
KDa	Quilodalton
LH	Hormônio Luteinizante
LPS	Lipopolissacarideo
MAPK-1	Proteína quinase ativada por mitogenos-1
MIP-1α	Proteína inflamatória de macrófago do tipo alfa
MIP-1ß	Proteína inflamatória de macrófago do tipo 1 beta

miR-196	Micro-acido ribonucléico -196
miRNA	Micro-acido ribonucléico
MSH	Hormônio estimulante de melanócitos
NEMO	Proteína essencial na regulação do NFkB
NFAT	Fator Nuclear Ativador de células T
NK	Células *Natural Killer*
OZ	Zymosan Opsonizado
PBMC	Células Mononucleares do Sangue Periférico
PGE2	Prostaglandina E2
PKC	Proteína quinase C
PLA_2	Fosfolipase A2
PMA	Acetato de forbol miristato
RANTES	quimiocina normalmente expressa e segregada por linfócitos T,
RIP-1	Proteína de Interação ao Receptor 1
RNAm/*mRNA*	Acido ribonucléico mensageiro
RPMI	Meio de cultura Roswell Park Institute
S100	Proteína de membrana ligada
SIV	Vírus símio da AIDS
SNP	Polimorfismo de nucleotídeo único
SOCS3	Supressor da sinalização das citocinas 3
STAT	Proteínas transdutoras de sinais e ativadoras de transcrição
T	Timina
TATA	Sequência gênica promotora
TCR	Receptor de células T
TGF-β	Fator de Crescimento Transformante beta
Th0	Célula T auxiliadora padrão)
Th1	Célula T CD4+ auxiliadora do tipo 1 ou padrão de resposta imune Th1
Th2	Célula T CD4+ auxiliadora do tipo 2 ou padrão de resposta imune Th2
Th17	Células T produtoras de IL-17 ou Padrão de resposta imune Th17
TLRs	Receptores *Toll-like*
TLR2	Receptor *Toll-like* do tipo 2
TRPM7	Transient receptor potential cation channel
TNF-α	Fator de Necrose Tumoral - alfa
TPA	12-O-tetradecanoil forbol acetato
Treg	Células T regulatórias
TSH	Hormônio estimulante da tireóide
UC	Colite Ulcerativa
UTR	Região não-traduzida

SUMÁRIO

APRESENTAÇÃO

O termo *saúde* significa, de acordo com os conceitos da Patologia, um estado de adaptação do organismo ao ambiente físico, psíquico ou social em que vive, no qual o indivíduo se sente bem e não apresenta sinais ou alterações orgânicas evidentes. Para que tal estado seja preservado ou recuperado é necessário que o conjunto de sistemas fisiológicos que compõem o organismo estejam em equilíbrio, ou seja, em homeostasia. Assim, inúmeros e complexos são os processos biológicos em atividade no corpo humano para a manutenção da homeostasia, sejam eles preventivos ou de resolução e reparo.

Um dos processos fisiopatológicos mais comuns na defesa da homeostasia de um organismo é a inflamação. Sucintamente, o processo inflamatório é a resposta da microcirculação e de eventos imunológicos subseqüentes na tentativa de conter e reparar alterações fisiológicas (por ex. o tecido uterino pós-gravídico) ou patológicas, causadas por agentes lesivos de origem química, física ou biológica (por ex., agentes infecciosos ou auto-imunidade). Na tentativa de reestabelecer o tecido lesado, células residentes do local, como macrófagos e mastócitos, são ativadas e, posteriormente, leucócitos circulantes são recrutados do sangue periférico, paralelamente às alterações do sistema microvascular e ao extravasamento de proteínas plasmáticas, estabelecendo os sinais cardinais da inflamação: dor, rubor, calor e edema. De acordo com a magnitude desta resposta, há a cronificação do processo, levando a exaustão do organismo e a perda da função tecidual resultante das inúmeras tentativas de reparo.

O cenário inflamatório também conta com a ação de diversas moléculas, sejam proteínas, lipídeos ou carboidratos, que agem como mediadores dos eventos acima descritos. O refinamento destes eventos ocorre através das múltiplas cascatas de sinalização celular, que se iniciam no meio extracelular, pela captação de sinais exógenos pelos receptores de superfície, ou no meio intracelular, pela ligação sincronizada de moléculas distribuídas no citosol ou nas organelas, de modo a atingir até mesmo o núcleo, levando a regulação gênica.

Dentre essa gama de mediadores, existem aqueles que podem ser considerados como moléculas chave, visto que estão envolvidos paralelamente

em níveis bioquímico e molecular do processo de resolução da alteração fisiopatológica e retorno à homeostasia. Neste ponto, as proteínas relacionadas aos efeitos farmacológicos dos glicocorticóides, drogas extremamente adotadas como terapia na maioria das condições inflamatórias, são alvos a serem extensivamente estudados.

A proteína anexina A1, membro da superfamília das anexinas, tem sido descrita como uma molécula chave, visto que não age somente como segunda mensageira das ações dos glicocorticóides, mas também atua em uma miríade de processos fisiológicos e patológicos, em nível molecular e/ou celular. Dentre as desordens nas quais esta proteína parece ser regulatória, estão os processos inflamatórios agudos frente a estímulos infecciosos, as doenças auto-imunes e os tumores malignos. No entanto, a partir de uma visão meta-analítica do que já foi demonstrado na literatura, incluindo artigos publicados de própria autoria ou co-autoria (Capítulo VI e Anexo I), é possível sugerir que a ação da anexina A1 é célula e tecido-específica.

Mediante o exposto, o presente estudo buscou investigar como a presença endógena e exógena da anexina A1 e de seu peptídeo N-terminal, quer seja em nível de RNAm ou proteína, se apresenta em processos inflamatórios associados a infecção ou hipóxia, como nas doenças inflamatórias intestinais (IBD), imunodeficiência causada por infecção pelos vírus HIV e SIV (AIDS), e lesão por isquemia e reperfusão renal.

FUNDAMENTAÇÃO TEÓRICA

1. Anexina A1: Uma Revisão

1.1 Descoberta da anexina A1 (ANXA1)

A busca por drogas antiinflamatórias tem estimulado esforços pelos cientistas há séculos, e em 1936 foi descrito o primeiro glicocorticóide (GC), uma molécula isolada e testada a partir de hormônios da glândula supra-renal com papel proposto na regulação da resposta inflamatória (Mason, Myers *et al.*, 1936). A partir disso, um dos mais importantes achados na área da biologia dos GCs foi a descoberta dos seu receptor (Schaumburg e Bojesen, 1968) e a descrição do mecanismo pelo qual a ocupação deste receptor intracelular (GR) levava ao aumento ou diminuição da transcrição de certos genes, estabelecendo assim a ação biológica dos esteróides (Buller, Schwartz *et al.*, 1976; Chan e O'malley, 1976; O'malley, 1976).

Dado que as prostaglandinas foram umas das primeiras moléculas descritas como elementos estimuladores da resposta inflamatória, e que a enzima revelada como responsável pela síntese deste mediador foi a fosfolipase A2 (PLA2), coube aos pesquisadores investigarem se os GCs apresentavam algum efeito inibitório sobre esta enzima. Por meio de um modelo murino de perfusão pulmonar, Flower e Blackwell (1979) não somente demonstraram que a dexametasona inibiu a ação da PLA2, assim como descreveram o mecanismo no qual o GC se ligou ao seu receptor e induziu a síntese de uma molécula com papel de “segunda mensageira dos GCs” nos macrófagos alveolares para bloquear a ação de tal enzima. Paralelamente, este trabalho foi suportado por um dado semelhante observado em macrófagos peritoneais por Di Rosa *et al.* (1984) e ainda pela evidência de que a “segunda mensageira” era uma proteína (Carnuccio, Di Rosa *et al.*, 1980). Então, neste mesmo ano, a proteína isolada por cromatografia de exclusão foi batizada de macrocortina, sendo que “macro” era referente a sua fonte celular, os macrófagos, e “cortina” referia-se a indução específica por GCs (Blackwell, Carnuccio *et al.*, 1980). Após a caracterização bioquímica correta, foi estabelecido que o nome da proteína anti-PLA2 seria lipocortina (Di Rosa, Flower *et al.*, 1984).

Posteriormente, diversos ensaios bioquímicos permitiram afirmar que a proteína, então conhecida como lipocortina, apresentava-se com 37KDa. Em

1986, a lipocortina foi seqüenciada e clonada, e esses avanços possibilitaram a descoberta de mais 12 lipocortinas, que também foram descritas e clonadas em mamíferos (Pepinsky, Tizard *et al.*, 1988; Raynal e Pollard, 1994). Atualmente estas proteínas compõem a Superfamília das Anexinas, assim nomeadas em razão do envolvimento destas no processo de agregação de vesículas fosfolipídicas e membranas naturais (Geisow, Walker *et al.*, 1987). De modo geral, as proteínas podem ser classificadas como anexinas se obedecerem a dois critérios estruturais e funcionais. Primeiro, deve conter uma estrutura conservada elementar de segmentos similares de 70 aminoácidos, e, além disso, apresentar a capacidade de se ligar a fosfolipídios negativamente carregados de modo Ca^{2+}-dependente. De acordo com estes critérios, como descrito na Figura 1, já são contabilizadas mais de 160 anexinas em 65 espécies, dentre elas fungos, plantas e vertebrados superiores (Smith e Moss, 1994; Gerke e Moss, 2002).

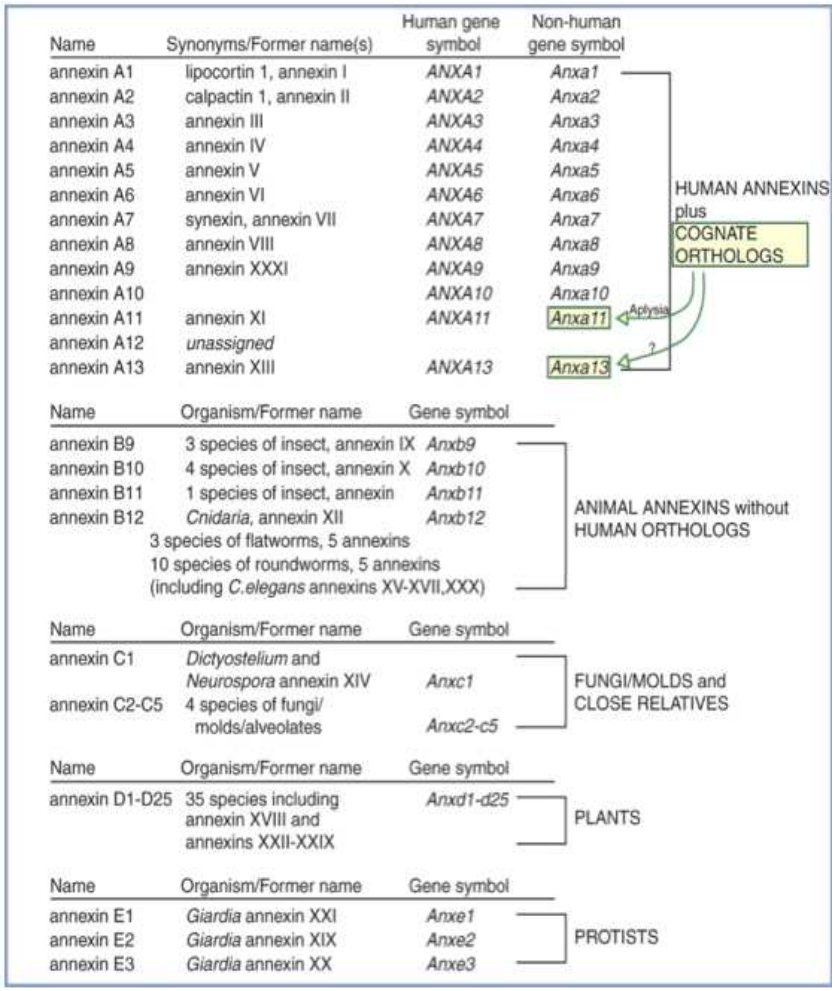

Figura 1. Nomenclatura das Anexinas. Os cinco grupos principais Anexina (A-E) são indicados, com detalhes das famílias mais estudadas. Os vertebrados são o grupo de A1-A13 (com exceção da A11 e A13, que podem ser encontrados em invertebrados). Dentro do grupo B, as anexinas do *C. elegans* ainda serão reclassificadas. Os fungos, plantas e protozoários estão classificados nos grupos das anexinas C, D e E, respectivamente. **Fonte:** modificado de Gerke e Moss (2002).

1.2 ANXA1: Do DNA à Proteína

1.2.1 Estrutura gênica e protéica

Os genes dos treze membros dessa família estão dispersos pelo genoma nos cromossomos 1, 2, 4, 5, 8, 9, 10 e 15. O gene *ANXA1* humano está localizado no cromossomo 9, mostrando estar localizado fisicamente próximo aos genes *ANXA2* e *ANXA3*, fato correlacionado com funções semelhantes em nível de proteína (Gerke e Moss, 2002).

A Figura 2 mostra que a estrutura gênica do *ANXA1* é dividida em um promotor mínimo (180 bp), 12 éxons, 25 pb de intróns flanquedores, e 341pb de região UTR. Cinco polimorfismos de nucleotídeo único (Dong, Li *et al.*, 2004) foram detectados, incluindo uma transição A3G silenciosa (Leu109Leu) no éxon 5 e quatro SNPs nas regiões não-codificantes do gene. Três transições A→G foram encontradas: uma no éxon não traduzido 1 (Hu, Flaig *et al.*, 2004); a segunda, no íntron 7, localizado a 28pb a partir da região 5' de exon 8 [seqüência de variâncias intrônicas 8-28A/G]; e uma terceira no intron 11, localizado a 31pb a partir do região 3' do éxon 11 (IVS11+31A/G). Além disso, foi encontrada uma transição T→G localizado a 11pb a partir da região 5' do exon 12 (IVS12-11T/G) (Lindgren, Nilsson *et al.*, 2001).

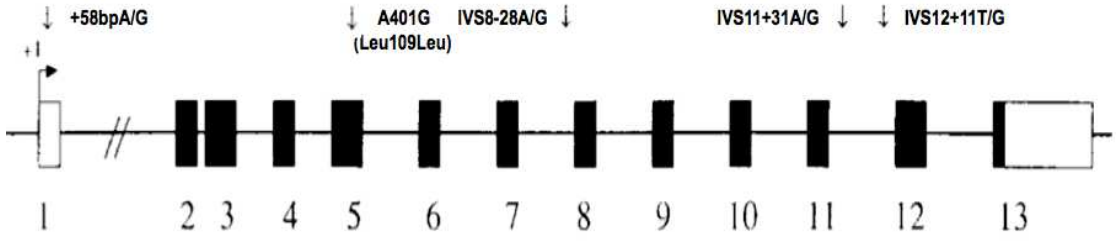

Figura 2. Estrutura esquemática do gene *ANXA1* humano. O início da transcrição é indicado com uma seta (+1). As áreas brancas representam as UTRs e as áreas pretas representam as regiões traduzidas. Os cinco SNPs identificados são vistos acima do local correspondente. A figura não está desenhada à escala. **Fonte:** Modificado de Lindgren, Nilsson *et al.* (2001).

O promotor do gene *ANXA1* contém regiões CAAT e TATA que, em estudos de deleção, foram mostradas serem essenciais para a atividade do promotor mínimo. A análise do promotor de *ANXA1* também levou à investigação da sensibilidade aos GCs, mostrando a indução desta droga no processo de transcrição da ANXA1 (Solito, De Coupade *et al.*, 1998b).

Estruturalmente, as proteínas anexinas compreendem dois domínios: uma pequena região N-terminal, variando em comprimento e composição (Pepinsky, Tizard *et al.*, 1988), e um domínio central, formado por quatro a oito dobras repetidas de uma seqüência conservada de 70 aminoácidos (Munn e Mues; Saris, Tack *et al.*, 1986). As anexinas diferem de outras famílias de proteínas ligantes de cálcio, como a calmodulina (ligação ao íon por meio de estrutura “E-F hand”), pois se ligam ao cálcio via uma seqüência formada por 70 aminoácidos denominada *annexin core* (Gerke e Moss, 2002). Cada anexina contém quatro sequências repetidas deste *core* na região C terminal, havendo forte homologia desta região entre as diferentes proteínas. Por outro lado, o N terminal (46 aa), contém resíduos tirosina e serina para fosforilação, bem como outros fatores de modificação pós-traducional, e ainda apresenta diferentes sítios de ligação para outras moléculas. Dessa forma, tem sido postulado que essa região confere as propriedades específicas de cada membro da superfamília (Rosengarth, Gerke *et al.*, 2001), inclusive da ANXA1.

Várias investigações têm caracterizado a região N-terminal da ANXA1 como a promotora da ação antiinflamatória e anti-proliferativa, sendo que os resultados experimentais com peptídeo-miméticos (sequências curtas de 1,5 a 3,5 KDa da região N-terminal), tais como o peptídeo Ac2-26, confirmam a presença desse sítio ativo anti-inflamatório (Perretti, 1998; Gavins, F., Yona, S. *et al.*, 2003). Uma representação esquemática da estrutura primária e o arranjo tridimensional da ANXA1 são mostrados na Figura 3.

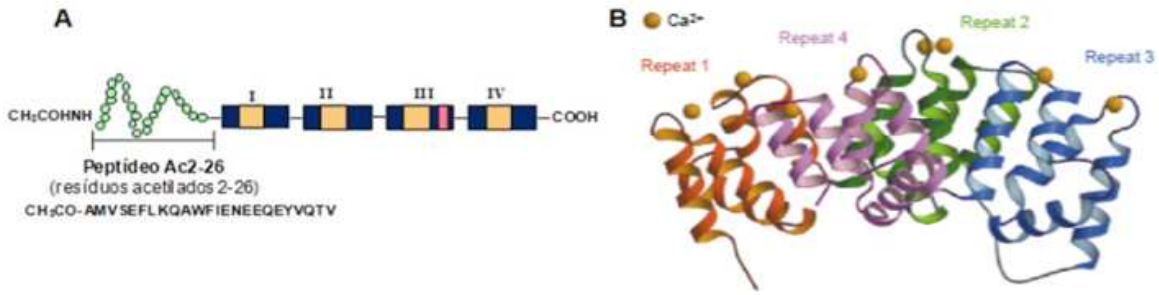

Figura 3. Representação esquemática da estrutura da proteína ANXA1, com destaque da região N-terminal em arranjo primário (peptídeo Ac2-26). B) Ilustração do arranjo tridimensional da proteína. **Fonte:** modificado de John, Christian *et al.* (2004).

Como já foi descrito, a região N-terminal da ANXA1 demonstrou ser a porção bioativa da proteína. Vários fragmentos N-terminais acetilados já foram testados, tais como Ac1-18 e Ac9-25, no entanto, foi o Ac2-26 que obteve resultados semelhantes aos da proteína inteira, sendo conhecido como um mimético funcional da ANXA1. Para tanto, estudos experimentais *in vivo,* como modelos de peritonite (Gastardelo, Damazo *et al.*, 2009), injúria da mucosa gástrica (Martin, Perretti *et al.*, 2008) e isquemia cerebral (Gavins, F. N., Dalli, J. *et al.*, 2007), e *in vitro,* tais como cultura de sinoviócitos (Tagoe, Marjanovic *et al.*, 2008), macrófagos (Reville, Crean *et al.*, 2006) e celulas tumorais da próstata (Carollo, Parente *et al.*, 1998), comprovaram a eficácia do tratamento com Ac2-26.

1.2.2 Regulação da expressão transcricional e traducional

Diversas evidências indicam que os GCs regulam a síntese e função da ANXA1, possivelmente através de uma combinação de processos genômicos e não-genômicos, dependendo do tipo celular e do tempo de indução (Perretti e D'acquisto, 2009). Dentre os processos genômicos, os GCs apresentam numerosos efeitos sobre a expressão gênica, de tal forma que tem sido proposto que cerca de 1% do genoma pode ser modulada por estas drogas. O GR é mantido no estado inativo por várias proteínas chaperonas, incluindo proteínas de choque térmico 70 (HSP70) e HSP90, porem, após a interação de um ligante, o complexo GC-receptor transloca-se para o núcleo, onde modula a expressão

gênica através da trans-repressão ou transativação. A transativação envolve a ligação direta deste complexo às seqüências específicas de nucleotídeos (denominado GRE, elementos de resposta aos GCs) para promover a transcrição de genes com propriedades antiinflamatórias, incluindo a MAPK-1 fosfatase (Clark, 2007), a qual esta envolvida com a diminuição de citocinas pró-inflamatórias. É possível sugerir que o mecanismo de regulação genômica da ANXA1 ocorra por esta mesma via, visto que células nocaute para MAPK-1 fosfatase também apresentam forte diminuição da expressão de *ANXA1* (Yang, Toh *et al.*, 2006).

Embora um estudo tenha revelado que o promotor de *ANXA1* não respondeu ao tratamento com dexametasona (Donnelly e Moss, 1998), outros trabalhos relataram algum nível de indução por GCs (Solito, De Coupade *et al.*, 1998b) e de inibição da transcrição em células tratadas com RU38486, um antagonista de GR (Solito, Mulla *et al.*, 2003). Os resultados diferentes podem corresponder ao uso de linhas de células diferentes em cada experimento ou pode refletir os tempos de exposição utilizados, que no primeiro caso foi estendido para 8h, e no segundo a 24h.

Assim como os GCs, outros hormônios que agem diretamente ou indiretamente no eixo hipotálamo-pituitário-adrenal (HPA) ou que sejam regulados por esta via, controlam positiva- ou negativamente a expressão de *ANXA1* em diferentes tipos celulares. Dentre os hormônios que contribuem para o aumento da ANXA1, estão: beta-estradiol, que aumenta a expressão em linhagem linfocítica, linhagem da neuroglia e na glândula pituitária (Castro-Caldas, Duarte *et al.*, 2001; Davies, Omer *et al.*, 2007); o cortisol, LH e FSH estão relacionados ao aumento em linfócitos (Mulla, Leroux *et al.*, 2005); e o neuropeptídeo hormonal alfa-MSH aumenta em células dendríticas (Min, Han *et al.*, 2011). Enquanto que o ACTH (Buckingham, John *et al.*, 2006) e o TSH diminuem os níveis de RNAm e proteína ANXA1, atuando nas células da tireóide e na glândula pituitária (El Btaouri, Claisse *et al.*, 1996; Mittag, Oehr *et al.*, 2007), e o hormônio enteropeptídico nas células L crípticas intestinais levam a diminuição da expressão de *ANXA1* (Nikoulina, Andon *et al.*, 2010).

Outras moléculas envolvidas na comunicação e manutenção das células, os fatores de crescimento, participam da via regulatória de ANXA1, geralmente aumentando sua expressão. Entre eles, vale citar o fator de crescimento epidérmico (EGF) (De Coupade, Gillet *et al.*, 2000; White, Bailey *et al.*, 2006) e o fator de crescimento de hepatócitos (HGF) (Lin, Jeng *et al.*, 2008), que atuam não somente induzindo os níveis de ANXA1, mas também modulam as modificações pós-traducionais.

Está claro que a regulação desta proteína é sutil, e que há muitos fatores que podem influenciar sua síntese e disposição. Além de esteróides e fatores de crescimento, a adição de citocinas, lipopolissacarídeo (De Coupade, Gillet *et al.*, 2000), e possivelmente outras condições que comprometem a integridade das células, tais como a adição de íons de metais pesados ou estresse térmico, levam ao aumento da expressão desta proteína (Rhee, Kim *et al.*, 2000). Estudos sobre a capacidade de resposta do promotor a estímulos pró-inflamatórios mostraram que o gene também pode ser induzido por PMA, por meio do fator nuclear de IL-6 (Solito, De Coupade *et al.*, 1998a).

Finalmente, os microRNAs (miRNAs), pequenos RNAs não-codificadores que regulam a expressão gênica, também parecem contribuir para a regulação da expressão de *ANXA1*. O miR-196b foi correlacionado inversamente com os níveis de RNAm de *ANXA1* em 12 linhas de células cancerígenas da mama, de esôfago e do endométrio. Em adição, células modificadas com uma super-expressão de miR-196 demonstraram a diminuição de ANXA1, em nível de RNAm e proteína, com evidências de que para tal efeito, o miRNA se liga a região promotora de *ANXA1* (Luthra, Singh *et al.*, 2008).

1.2.3 Modificações pós-traducionais

A fosforilação de proteínas regulatórias é um dos mecanismos primários pelos quais as células respondem a sinais extracelulares. Sabendo que a ANXA1 é estimulada por GCs e que ela atua em diferentes tipos celulares, tornou-se de suma importância a investigação do papel da fosforilação para esclarecer como esta proteína exerce suas atividades biológicas. Desde os primeiros trabalhos de

caracterização bioquímica, ficou confirmado que a fosforilação da ANXA1 ocorre nos sítios tirosina e serina (posições 5 e 27) e que inviabiliza sua capacidade de inibir a PLA2 e outras funções, especialmente aquelas envolvidas com a ação do N-terminal da proteína (Wang e Creutz, 1994; Porte, De Santa Barbara *et al.*, 1996; Kusumawati, Liautard *et al.*, 2001; John, Cover *et al.*, 2002). Por meio de ensaios com ATP marcado radioativamente, a primeira enzima descrita como responsável por este processo foi a PKA (proteína quinase A dependente de AMP-ciclico) (Hirata, 1983; Ohtsuki, Oh-Ishi *et al.*, 1992). Posteriormente, ficou claro que ANXA1 é um substrato para diversas tirosina-quinases (Geisow, Walker *et al.*, 1987), incluindo PKC intracitoplasmática (Johnstone, Hubaishy *et al.*, 1993; Farkas, Buday *et al.*, 1994), EGFR-quinase (Futter, Felder *et al.*, 1993), histidina quinase (Muimo, Hornickova *et al.*, 2000), HGFR-quinase (Skouteris e Schroder, 1996), TMPR7 com atividade quinase (Dorovkov e Ryazanov, 2004),

A regulação da fosforilação parece ser dependente de Ca^{+2} e pode ocorrer por diversos mecanismos positivos ou negativos, tais como: a interação com ANXA5, transglutaminase, melitina (Katoh, 2002) ou com S100, que levam a inibição do processo de fosforilação catalisado pela PKC (Raynal, Hullin *et al.*, 1993; Naka, Qing *et al.*, 1994; Mckanna, 1995); em contraste, a ação de angiotensina II (Salles, Gayral-Taminh *et al.*, 1993), ácido ocadáico e do TPA (Sato, Edashige *et al.*, 1995), gangliosídeos GT1b (Yamazaki, Nagatsuka *et al.*, 2006), hormônio de crescimento (Salles, Netelenbos *et al.*, 1996), proteína de membrana-1 codificada por EBV latente (Yan, Luo *et al.*, 2007), esfingosina (Katoh, 2004) e LPS (Solito, Christian *et al.*, 2006) induzem indiretamente à fosforilação dos sítios serina ou tirosina da ANXA1, dependente ou não de PKC, em diferentes tipos celulares.

A regulação da sinalização de ANXA1 parece não ser apenas condicionada à fosforilação, mas também a outros mecanismos de modificações pós-traducionais, como por exemplo, a carbonilação. Este processo parece ser dependente da sinalização redox e induz a degradação de ANXA1 via proteossoma (Wong, Cheema *et al.*, 2008). Outro mecanismo bem conhecido, a ubiquitinação, também tem sido descrito como uma modificação da ANXA1,

induzindo o seu direcionamento nuclear e o envolvimento com a transformação maligna em linhagens celulares de linfoma (Hirata, Thibodeau *et al.*, 2010).

1.2.4 Externalização e clivagem de ANXA1

As ações da ANXA1 parecem ser exercidas principalmente no meio extracelular, via ligação com receptores de membrana das células secretoras ou vizinhas, designando as funções autócrina, parácrina e justácrina da proteína. O acesso da ANXA1 a esses receptores é dependente da externalização da proteína a partir do citoplasma para a superfície celular de células ativadas. Após a ativação das células secretoras de ANXA1, o conteúdo intracelular pode ser externalizado e/ou secretado por meio de um de três mecanismos diferentes (Figura 4): através da ação do transportador ABC-ligante de ATP, formando um canal na membrana para a secreção da ANXA1 (Wein, Fauroux *et al.*, 2004); por meio da serino-fosforilação na região terminal, o que leva a sua ligação com determinados domínios lipídicos capazes de translocar o ligante para região externa da membrana celular e liberar para o meio a proteína fosforilada (Solito, Christian *et al.*, 2006) (Figura 4); ou ainda pela exocitose dos grânulos citoplasmáticos que estocam ANXA1 em determinados tipos celulares (Perretti e Flower, 2004).

Evidências substanciais suportam a visão de que a translocação de ANXA1 para a membrana é essencial para suas ações no sistema imunológico, como por exemplo, na modulação da migração de neutrófilos (Perretti, Chiang *et al.*, 2002). Além disso, a secreção dessa proteína parece ter um significado mais complexo, visto que é identificada em lavados peritoneais (Pepinsky, Sinclair *et al.*, 1986), lavados pulmonares de pacientes asmáticos (Ambrose e Hunninghake, 1990), fluido seminal (Christmas, Callaway *et al.*, 1991) e no sobrenadante de cultura primária, como ex. biópsia do colon de pacientes com colite ulcerativa (Vergnolle, Pages *et al.*, 2004), e de linhagens celulares (Wang, Wang *et al.*, 2007; Pupjalis, Goetsch *et al.*, 2011; Yang, Liu, Yao, Ping, Jiang, Liu, Xu, Huang, Mou, Gong, Chen, Bian e Ming Wang, 2011).

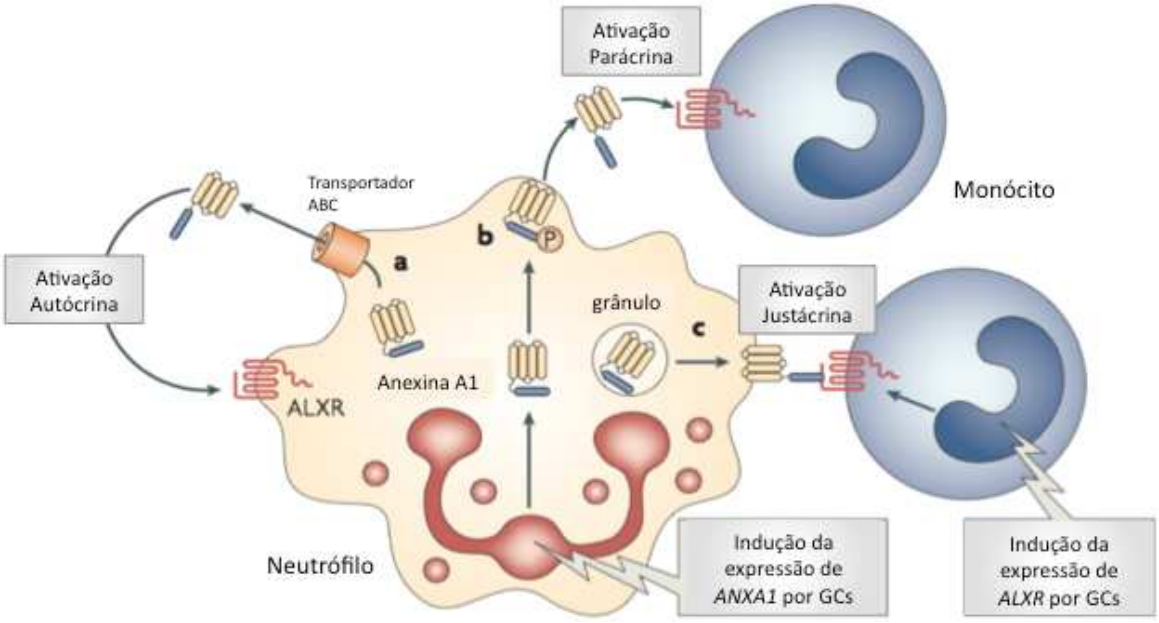

Figura 4. Mobilização de ANXA1 em células ativadas e seu modo de ação. Após a ativação celular, como na adesão ao endotélio, a ANXA1 intracelular é mobilizada para a membrana plasmática. Dependendo do tipo celular, a ANXA1 é, então, exteriorizada e/ou secretada por meio de três mecanismos descritos no texto (a, b, c). A ANXA1 pode funcionar de maneira autócrina, parácrina e justácrina (contato célula-célula) para ativar a sinalização do receptor ALXR (FPR2/ALX). O sistema ANXA1-FPR2/ALX pode ser manipulado por GCs, os quais induzem a expressão gênica de *ANXA1*, bem como *FPR2*, por células do sistema imune inato, aumentando assim os efeitos deste circuito antiinflamatório. **Fonte:** modificado de Perretti e D'acquisto (2009).

Dada a presença da ANXA1 no meio extracelular, é imprescindível comentar que a maior parte da proteína solúvel é clivada na região N-terminal (Oliani, S., Paul-Clark, M. *et al.*, 2001), e sabe-se que a proteólise na região N-terminal modifica fortemente as propriedades físicas e biológicas da ANXA1 (D'acquisto, Perretti *et al.*, 2008). Aparentemente, a enzima proteinase 3 (PR3) (Vong, D'acquisto *et al.*, 2007) e a catepsina D exercem o papel de catabolizar a ANXA1 secretada ou translocada para a superfície externa da membrana, clivando a região N-terminal e resultando em dois subprodutos: uma fração de proteína de 37KDa e outra de 33KDa. Esta fração de menor peso molecular parece ser inativa, o que poderia causar uma perda das funções homeostáticas da ANXA1, visto que somente as moléculas de ANXA1 resistentes a PR3 retém a função

antiinflamatória original da proteína (Pederzoli-Ribeil, Maione *et al.*, 2010). Em contraste, um estudo mais recente revelou que a enzima calpaína, além de clivar a proteína na região C-terminal, porção protéica sem ação previamente demonstrada, ainda induz a nova versão da ANXA1 a exercer funções pró-inflamatórias nos neutrófilos (Williams, Milne *et al.*, 2010).

1.2.5 Localização e função da ANXA1

A descrição adequada das funções da ANXA1 só é possível quando correlacionada a sua localização, dada a característica especifica de suas ações de acordo com o tipo celular, tecido ou condição fisiológica/patológica, em que esta proteína é expressa.

Inicialmente, uma varredura de vários tecidos, através do uso de um anticorpo monoclonal específico para western blotting e imunohistoquímica, revelou a distribuição da ANXA1 no cérebro, pulmão, fígado, rim, intestino, baço, timo, medula óssea (Fava, Mckanna *et al.*, 1989). Em adição, diversos estudos têm mostrado a presença desta proteína na maioria dos leucócitos e células do tecido conectivo que atuam no processo inflamatório, incluindo neutrófilos (Oliani, S. M., Paul-Clark, M. J. *et al.*, 2001; Oliani e Perretti, 2001), eosinófilos, mastócitos (Oliani, Damazo *et al.*, 2002; Sena, Provazzi *et al.*, 2006; Silistino-Souza, Rodrigues-Lisoni *et al.*, 2007), monócitos (De Caterina, Sicari *et al.*, 1993; Spurr, Nadkarni *et al.*, 2011), macrófagos (Diakonova, Gerke *et al.*, 1997), células dendriticas (Huggins, Paschalidis *et al.*, 2009; Min, Han *et al.*, 2011) e linfócitos T (Castro-Caldas, Duarte *et al.*, 2002; D'acquisto, Merghani *et al.*, 2007; D'acquisto, Paschalidis *et al.*, 2007; D'acquisto, Paschalidis *et al.*, 2008; Spurr, Nadkarni *et al.*, 2011).

A ANXA1 foi indicada como uma proteína bioativa em diversos mecanismos celulares e sistêmicos, incluindo: fusão de membrana (Oshry, Meers *et al.*, 1991; De La Fuente e Parra, 1995; Lambert, Gerke *et al.*, 1997; Bitto, Li *et al.*, 2000; Creutz e Snyder, 2005), fagocitose (Jitkaew, Witasp *et al.*, 2009; Patel, Ahmad *et al.*, 2011), exocitose (Mcarthur, Yazid *et al.*, 2009), diferenciação celular (Solito, De Coupade *et al.*, 1998a; Guzman-Aranguez, Olmo *et al.*, 2005; Huggins,

Paschalidis *et al.*, 2009; Bizzarro, Fontanella *et al.*, 2010; Paschalidis, Huggins *et al.*, 2010), regulação do crescimento celular e tumorigênese (Croxtall, Flower *et al.*, 1993; Croxtall, Waheed *et al.*, 1993; Kim, Yang *et al.*, 1996; Skouteris e Schroder, 1996; Alldridge e Bryant, 2003; Patton, Chen *et al.*, 2005; Ang, Nguyen *et al.*, 2009; Jackson e Evers, 2009), transdução de sinal (Renshaw, Montero-Melendez *et al.*, 2010), conformação do citoesqueleto (Ghitescu, Gugliucci *et al.*, 2001; Alldridge e Bryant, 2003; Patel, Ahmad *et al.*, 2011), anti-coagulação (Cirino e Cicala, 1993), regulação da secreção hormonal (Won, Kang *et al.*, 2003; Mcarthur, Yazid *et al.*, 2009), atividade chaperona (Kim, Lee *et al.*, 1997; Rhee, Kim *et al.*, 2000; Liu, Han *et al.*, 2011), apoptose (Perretti e Solito, 2004; Petrella, Festa *et al.*, 2005; Scannell e Maderna, 2006; D'acunto, Fontanella *et al.*, 2010; Pupjalis, Goetsch *et al.*, 2011) e modulação do processo inflamatório (Parente e Solito, 2004; Perretti e Flower, 2004; Kamal, Flower *et al.*, 2005; Scannell e Maderna, 2006; D'acquisto, Perretti *et al.*, 2008; Perretti e D'acquisto, 2009; Yazid, Ayoub *et al.*, 2010; Hutchinson, Rajagopal *et al.*, 2011).

1.2.6 Mapeamento da distribuição e função de ANXA1 em órgãos e sistemas

Nos últimos anos, ficou mais claro que, juntamente às vias iniciais da inflamação e antes do estabelecimento da imunidade adaptativa, alguns processos moleculares, bioquímicos e metabólicos são ativados. Estes processos não servem somente para limitar a extensão da inflamação por meio da supressão de mediadores inflamatórios clássicos, mas também para promover a transição de um estado de recuperação ativa caracterizada pelo *clearance* de células imunes e reparação regulada, processo denominado pró-resolução (Hutchinson, Rajagopal *et al.*, 2011). A ativação deste mecanismo conta com a regulação da expressão, conformação e interação de um diversificado conjunto de moléculas atuantes nos sistemas envolvidos em condições fisiopatológicas. Sendo assim, para verificar se a ANXA1 faz parte desse cenário, é fundamental que seja apresentada uma abordagem global das ações desta proteína, de modo seu papel seja descrito em cada um dos sistemas e seja possível sugerir como ela contribui para a atividade homeostática intrínseca do organismo (Tabela 1).

Tabela 1. Descrição geral da expressão e funções da ANXA1 nos diferentes órgãos e sistemas humanos.

Órgãos e Tecidos	*Condições Fisiológicas e Patológicas*	*Expressão e ações da ANXA1*	*Ref.*
Cérebro	Normal	Expressão constitutiva, especialmente na micróglia e astrócitos	(Eberhard, Brown *et al.*, 1994; Go, Zuiderveen *et al.*, 1994; Huitinga, Bauer *et al.*, 1998; Minghetti, Nicolini *et al.*, 1999; Knott, Stern *et al.*, 2000; Miyachi, Asai *et al.*, 2001; Lu, Tang *et al.*, 2006; Alexander, Minagar *et al.*, 2007; Schittenhelm, Trautmann *et al.*, 2009; Mcarthur, Cristante *et al.*, 2010)
	Injúria por IR Esclerose múltipla Encefalomielite Hemorragia cerebral Parkinsonismo	Aumento de expressão em todas as condições patológicas: Inibição do processo inflamatório, por regular a ação de citocinas, impedir a ativação de células endoteliais e induzir a fagocitose de neurônios e leucócitos apoptóticos pela micróglia	
	Astrocitoma, glioblastoma	Aumento da expressão envolvida na progressão tumoral	
Pulmão	Normal	Expressa em macrófagos alveolares, células epiteliais alveolares e ciliadas bronquiolares Envolvimento na secreção de surfactante	(Tsao, Chen *et al.*, 1994; Liu, Fisher *et al.*, 1995; Mayran, Traverso *et al.*, 1996; Katoh, Miyamoto *et al.*, 1999; Vishwanatha, Swinney *et al.*, 2003; Chung, Oh *et al.*, 2004; Bensalem,
	Infecção bact/viral Tabaco Alérgenos	Efeito protetor antiinflamatório nas infecções, asma, fibrose cística e efeitos do tabagismo	

	Câncer	Efeito pró-tumoral e envolvimento na agressividade do câncer de pulmão	Ventura *et al.*, 2005; Liu, Zhang *et al.*, 2011)
Coração e vasos sanguíneos	Normal	Expressão constitutiva no músculo estriado cardíaco	(Naka, Qing *et al.*, 1994; La, M., D'amico, M. *et al.*, 2001; Probst-Cousin, Berghoff *et al.*, 2004; Gavins, Kamal *et al.*, 2005; Cote, Lavoie *et al.*, 2010; Cheuk e Cheng, 2011)
	Injúria por IR	Efeito exógeno protetor antiinflamatório em condições de hipóxia	
	Miopatias e ateroma	Aumento da expressão em nível transcricional e traducional	
	Angiogênese	Inibição da migração de células endoteliais	
Intestino	Normal	Expressão no epitélio de revestimento, principalmente na região basolateral das células, e em varias linhagens celulares do intestino	(Beattie, Goulding *et al.*, 1995; Cuzzocrea, Tailor *et al.*, 1997; Massey-Harroche, Mayran *et al.*, 1998; Bensalem, Ventura *et al.*, 2005; Souza, Fagundes *et al.*, 2007; Babbin, Laukoetter *et al.*, 2008; Zhang, Huang *et al.*, 2010)
	Injúria por IR	Efeito protetor antiinflamatório em condições de hipóxia e em modelos de lesão por agentes químicos	
	Colite ulcerativa		
	Câncer	Efeito anti-tumoral in vitro e in vivo	
	Doença de Crohn	Auto-anticorpos anti-ANXA1 circulantes em doenças auto-imunes	

Rins e bexiga	Normal	Expressão no epitélio da maioria das estruturas renais e em linhagens celulares embrionários do rim	(Mckanna, Chuncharunee *et al.*, 1992; Sheu, Baum *et al.*, 1997; Touyz, He *et al.*, 2006; Zimmermann, Woenckhaus *et al.*, 2007; Araujo, Truzzi *et al.*, 2010; Yang, Yang *et al.*, 2010; Araujo, Truzzi *et al.*, 2011; Facio, Sena *et al.*, 2011)
	Hipertensão Nefrotoxicidade	Envolvimento na regulação da sinalização em vasos sanguíneos responsáveis pela hipertensão	
	Injúria por IR	Efeito protetor antiinflamatório e homeostático em condições de nefrotoxicidade e hipóxia	
	Câncer	Efeito pró-tumoral e envolvimento na agressividade do câncer dos rins e bexiga	
Fígado	Regeneração tecidual	Aumento de expressão em tecidos lesados, levando a regeneração hepática	(Masaki, Tokuda *et al.*, 1994; De Coupade, Gillet *et al.*, 2000; Seth, Leo *et al.*, 2003)
	Câncer	Envolvimento na transformação maligna e proliferação de células tumorais	
Pâncreas	Normal	Expressão constitutiva exclusiva das ilhotas de Langerhans	(Ohnishi, Tokuda *et al.*, 1994; Ohnishi, Tokuda *et al.*, 1995; Hong, Won *et al.*, 2002; Bai, Ni *et al.*, 2004)
	Secreção de insulina	Localização conjunta aos grânulos de insulina e secreção deste hormônio, após a fosforilação de ANXA1 em células beta	
	Câncer	Super-expressão relacionada à progressão tumoral	

Tireóide	Normal	Expressão regulada por TSH no tecido e linhagens celulares	(El Btaouri, Claisse *et al.*, 1996; Petrella, Festa *et al.*, 2005; 2006; Yamazaki, Tanigawa *et al.*, 2010)
	Câncer	Diminuição da expressão é relacionada a progressão tumoral *in vitro* e *in vivo* Iodoterapia aumenta os níveis de ANXA1 no tecido	
Mama	Normal	Envolvimento na indução da apoptose das células da glândula mamária, levando a regressão destas estruturas após a lactação.	(Mckanna, 1995; Ahn, Sawada *et al.*, 1997; Cicek, Samant *et al.*, 2004; Wang, Serfass *et al.*, 2004; Shen, Chang *et al.*, 2005; Shen, Nooraie *et al.*, 2006; Cao, Li *et al.*, 2008; Ang, Nguyen *et al.*, 2009; De Graauw, Van Miltenburg *et al.*, 2010; Maschler, Gebeshuber *et al.*, 2010)
	Câncer	A literatura é convergente com relação ao câncer, configurando duas possibiildades: - Perda da expressão de ANXA1 na fase precoce da tumorigênese, contribuindo para a transformação maligna. Em adição, o aumento da ANXA1 é induzido por estrógeno, mostrando ainda que a transfecção de ANXA1 inibe a metástase em células de tumores invasivos. - Aumento da expressão de ANXA1 de acordo com a progressão do câncer, colaborando para a metástase pulmonar e para a resistência a quimioterapia.	
Próstata	Normal	Presença constitutiva no fluido seminal	(Christmas, Callaway *et al.*, 1991; Paweletz, Ornstein *et al.*, 2000; Kang, Calvo *et al.*, 2002; Patton, Chen *et al.*, 2005; Hsiang, Tunoda *et al.*, 2006)
	Câncer	Perda da expressão contribui para o aumento da expressão de PLA2 e IL-6, que contribuem para a progressão do tumor Indução da expressão de ANXA1 leva a apoptose nas cél. tumorais	

Pele	Normal	Expressão relevante nos ductos glandulares e queratinócitos de todas as camadas da epiderme	(Ikai, Shimizu *et al.*, 1993; Ahluwalia, Mohamed *et al.*, 1994; Ahluwalia, 1998; Oliani, Ciocca *et al.*, 2008; Rondepierre, Bouchon *et al.*, 2009; Caputo, Maiorana *et al.*, 2011)
	Psoríase	Diminuição da expressão em todo o tecido	
	Melanoma	A perda da expressão está envolvida com a disseminação das células tumorais	
Articulações	Artrite aguda	Modelo murino: bloqueio da migração de neutrófilos via ação dos GCs/ANXA1 Inibe a ação pró-inflamatória dos sinoviócitos *in vitro* Ausência de expressão em animais deficientes geneticamente induz o aumento da severidade da doença	(Yang, Leech *et al.*, 1997; Yang, Hutchinson *et al.*, 1998; Yang, Y. H., Morand, E. F. *et al.*, 2004; D'acquisto, Merghani *et al.*, 2007; D'acquisto, Paschalidis *et al.*, 2008)
	Artrite reumatóide	Modelo murino e células humanas: estimulo *in vivo* e *in vitro* leva à ativação de linfócitos T CD4+ e piora o processo inflamatório crônico	
Nariz, ouvido e olhos	Mucosa nasal	Expressão intensa nas células ciliadas, ductos glandulares e células inflamatórias como mastócitos e eosinófilos (polipose) e perda da expressão em carcinomas nasofaríngeos	(Rodrigo, Garcia-Pedrero *et al.*, 2004; Rodrigo, Garcia-Pedrero *et al.*, 2005; Sena, Provazzi *et al.*, 2006; Kalinec, Webster *et al.*, 2009; Da Silva, Girol *et al.*, 2011)
	Ouvido interno	Baixa expressão nas células sensoriais, com aumento significativo nas células cocleares após tratamento com GCs	
	Uveíte	Ações anti-inflamatórias sobre mastócitos e neutrófilos em condição patológica no trato uveal dos olhos	

A compilação de todos estes dados referentes a atuação sistêmica da ANXA1, permitiu que algumas conclusões sejam sugeridas. A primeira delas é que a ANXA1 é constitutiva e não é uma proteína órgão-específica, todavia não são todos os tipos celulares que a expressam em abundância, tendo como principais fontes as células que constituem a defesa do organismo, seja física, como no caso das células epiteliais, ou imunológica, como os leucócitos e células inflamatórias do tecido conectivo. Em segundo lugar, fica claro que na maioria dos processos inflamatórios avaliados, a ANXA1 age como uma molécula antiinflamatória, valendo dizer que essa sugestão somente pode ser creditada se for considerada a fase temporal na qual estes processos foram observados, visto que existe uma gama de diferenças bioquímicas, moleculares e celulares entre as inflamações aguda e crônica. A terceira conclusão revela que a participação da ANXA1 na tumorigênese ainda é controversa, visto que, em órgãos vitais como cérebro, pulmão e rins, o aumento da expressão protéica está relacionado com a progressão ou agressividade do câncer, seja reflexo do estímulo direto nas células tumorais ou nos componentes teciduais que as mantém.

Todas as atividades da ANXA1 mostram que esta é uma proteína com poder de regulação e que auxilia em diversos processos fisiológicos ou patológicos em todo o organismo. Uma das possíveis explicações para este comportamento se dá pelas interações que a ANXA1 faz com uma ampla diversidade de moléculas, ativando vias de efeitos divergentes, como será descrito a seguir.

1.2.7 Interações moleculares

Como já descrito anteriormente, a proteína ANXA1 pode ser localizada no meio intracelular, associada a membrana, ou mesmo no meio extracelular, de acordo com a condição do microambiente. Portanto, para que suas funções e mecanismos sejam esclarecidos, os possíveis ligantes de ANXA1, há quase duas décadas, também são alvos de pesquisa.

Nos experimentos bioquímicos iniciais, foi observado que a proteína transglutaminase-2 se ligava à região N-terminal da ANXA1, auxiliando a

interação com o cálcio. Posteriormente, demonstraram a interação com as proteínas da família S100 (Mailliard, Haigler *et al.*, 1996) e com a proteína mu1 do complexo da clatrina (Creutz e Snyder, 2005), ambas relacionadas aos processos de exocitose/endocitose. Entre outras proteínas, já foram descritas afinidades bioquímicas entre ANXA1 e moléculas relacionadas ao citoesqueleto e mais propriamente na organização, fusão e permeabilidade da membrana, tais como as citoqueratinas 8 e 18 na linhagem celular A459 (Croxtall, Wu *et al.*, 1998) e profilina (envolvida com a organização da actina). Além disso, a sua interação físico-protéica com moléculas de sinalização da via de NF$\kappa\beta$, tal como as proteínas NEMO e RIP1 do complexo IKK, sugere o envolvimento de ANXA1 na metástase do câncer de mama.

A ANXA1 é também capaz de se ligar a moléculas de outras categorias bioquímicas, tais como os lipídeos, especialmente os negativamente carregados, dentre os quais estão a fosfatidilserina (Janshoff, Ross *et al.*, 2001) e a ceramida (Debret, El Btaouri *et al.*, 2003; Babiychuk, Monastyrskaya *et al.*, 2008; Babiychuk, Atanassoff *et al.*, 2011), ambas são moléculas sinalizadoras da apoptose; com os carboidratos, como foi identificada recentemente a associação cálcio-dependente com heparina e o heparan-sulfato (Horlacher, Noti *et al.*, 2011); e até mesmo com ácidos nucléicos, como ssDNA, estando envolvida na indução de mutações nesta molécula se associada a metais pesados ou exercendo papel de helicase (Hirata, Thibodeau *et al.*, 2010; Hirata, Corcoran *et al.*, 2011).

Por outro lado, muito do que já foi relatado sobre a interação entre ANXA1 e outras moléculas está vinculado aos receptores de membrana acoplados a proteína G e ligantes de peptídeos formilados, os FPRs.

1.2.8 Receptores de ANXA1 e de seus miméticos

Receptores de peptídeos formilados (FPRs) são um pequeno grupo de proteínas de sete domínios transmembrânicos acoplados a proteína G, que são expressos principalmente por células fagocitárias e epiteliais/endoteliais de mamíferos e são conhecidos por serem importantes na defesa do hospedeiro e inflamação. Os três FPRs humanos (FPR1, FPR2/ALX e FPR3) compartilham

homologia significativa e são codificadas por genes em cluster do cromossomo 19. Esses receptores se ligam um grupo extraordinariamente numeroso e estruturalmente composto por diferentes ligantes agonistas, incluindo peptídeos N-formilados e não formilados, que são quimioatractantes e ativam fagócitos. Peptídeos N-formilados, que são codificados na natureza apenas por genes de bactérias e mitocôndrias e resultam da obrigatoriedade da iniciação de síntese de proteína bacteriana e mitocondrial a partir de N-Formil-metionina, é a classe de ligantes comum a todos os três receptores humanos. Em comparação, foram identificados menos agonistas para FPR3, o terceiro membro desta família de receptores. Há quase de três décadas, uma grande quantidade de substâncias quimiotáticas tem sido identificada que inclui N-formil-metionil-leucil-fenilalanina (fMLF), C5a ativado, leucotrieno B4, fator ativador de plaquetas, e a superfamília de quimiocinas. Ambas as substâncias quimiotáticas ativam receptores transmembrânicas acoplados a proteína G expressos não somente em células de origem hematopoiéticas, mas também em outros tipos (Le, Li *et al.*, 2000). Estudos estruturais e funcionais do FPRs produziram informações importantes para a compreensão dos princípios gerais e farmacológicos que regem todos os receptores quimioatractantes de leucócitos.

Tabela 2. Lista dos diversos ligantes dos FPRs.

Receptor	Indução da expressão	Ligantes		
		Agonistas naturais	Agonistas sintéticos	Antagonistas
FPR1	TNF-γ IL-10 TLR4	Peptideos mitocondriais formilados Catepsina G ANXA1	fMLF (,1nM) AG-14 Ac2-26 Ac2-12 Ac9-25 T20/21 gp41 (HIV-1) WKYMVM MMWLL	Derivados de BOC Ciclosporina A Ciclosporina H Espinorfina CHIPS Acidos biliares piroxicam
FPR2/ ALX	GCs TNF-γ TLR2 TLR4 NOD2	Peptideos formilados SAA B-amilóide 1-42 ANXA1 Humanina Lipoxina A4 NADH-desidrogenase LL-37 CCL23 Hp(2-20)	fMLF (.10μM) AG-14 Peptideos derivados do HIV-1 Ac2-26 Ac2-12 Antiinflamina-2 Composto 43 CGEN-855ª Humanina formillada T21/N36 gp41 (HIV-1) Peptideo V3/F gp120 (HIV-1) Quin-C1 MMK-1 WKYMVM WKYMVm Temporina SHAAGtide	Derivados de BOC Peptideo WRW_4 PLIPr Quin-C7
FPR3	_	Peptideos formilados F2L Humanina Hp(2-20)	fMLF (.10μM) Ac2-26 WKYMVM WKYMVm	Peptideo WRW_4

Os agonistas humanos estão classificados como pró-inflamatório (vermelho), antiinflamatórios (azul) ou não-especificado (preto). O código de letras foi usado para sequências peptídicas. Abreviaturas: *BOC, butyloxycarbonyl moiety; CCL23, N-terminal truncated peptide of CCL23; CDCA/DCA, cheno/deoxycholic acid; CGEN-855A, Compugen lead peptide; CHIPS,chemotaxis inhibitory protein of S. aureus; F2L, peptide cleavage product of heme-binding protein; FLIPr, FPR2/ALX inhibitory protein; gG-2p20, Herpes simplex type 2 peptide; Hp (2–20), Helicobacter pylori; LL-37, enzymatic cleavage cathelicidin; MMK-1, sequence LESIFRSLLFRVM; SHAAGtide, processed peptide sequence of CCL23.* **Fonte:** Modificado de Dufton, Hannon *et al.* (2010).

O peptídeo Ac2-26 retém a maioria das ações antiinflamatórias de ANXA1, no entanto, usando um sistema de transfecção celular i*n vitro*, este fragmento mostrou ativar o FPR1, bem como o FPR2/ALX. Além disso, experimentos em células HEK293 transfectadas com esses dois receptores demonstraram que a proteína ANXA1 intacta apresenta mais afinidade pelo FPR2/ALX, enquanto que o Ac2-26 se liga e ativa ambos os receptores (Dufton, Hannon *et al.*, 2010).

Pela via do FPR2/ALX, o qual é também regulado pelos GCs (Sawmynaden e Perretti, 2006), a ANXA1 exerce suas ações contra-regulatórias em uma série de processos, tais como: extravasamento de neutrófilos (Walther, Riehemann *et al.*, 2000; Gavins, F. N., Yona, S. *et al.*, 2003; Gastardelo, Damazo *et al.*, 2009), produção de radicais livres em fagócitos (Karlsson, Fu *et al.*, 2005), migração e proliferação (Khau, Langenbach *et al.*, 2011; Yang, Liu, Yao, Ping, Jiang, Liu, Xu, Huang, Mou, Gong, Chen, Bian e Wang, 2011) de células tumorais, regulação da secreção de ACTH em roedores (John, Sahni *et al.*, 2007), cicatrização de úlceras gástricas (Martin, Perretti *et al.*, 2008), estimulação da secreção de metaloproteinases no processo de artrite reumatóide (Tagoe, Marjanovic *et al.*, 2008), sinalização da apoptose em neutrófilos (Pupjalis, Goetsch *et al.*, 2010).

Um estudo recente de genômica revelou que embora o tratamento com ANXA1 ou Ac2-26 pareça afetar predominantemente as mesmas funções, algumas diferenças podem ser observadas. Por exemplo, ANXA1 alterou um número maior de genes relacionados à proliferação e diferenciação celular, fatores de transcrição e transferases, enquanto o peptídeo Ac2-26 afetou mais genes envolvidos em imunidade e defesa, metabolismo de fosfolipídeos e quinases, para citar apenas alguns (Renshaw, Montero-Melendez *et al.*, 2010). Essa divergência no comportamento biológico destes elementos ainda sugere a possibilidade da existência de outros receptores envolvidos nas ações da ANXA1 e seus fragmentos.

1.3 Participação da ANXA1 nos mecanismos imunológicos e na patogênese de doenças inflamatórias e infecciosas

Neste tópico, iniciar a abordagem ressaltando o conceito geral dos processos inflamatórios e infecciosos dará ênfase e permitirá a compreensão das ações relevantes da ANXA1 na homeostasia. Logo, o processo de infecção é a colonização de um organismo hospedeiro por uma espécie estranha. Numa infecção, o organismo infectante utiliza os recursos do hospedeiro para se multiplicar, interferindo na fisiologia normal e resultando em evidentes prejuízos ao hospedeiro. Neste ponto, a inflamação é uma resposta biológica complexa de tecidos vasculares a estímulos nocivos, tais como agentes irritantes, patogênicos ou danos físicos. Este é um processo iniciado pelo organismo como resposta à lesão tecidual ou infecção, na tentativa de remover os patógenos invasores, bem como iniciar o processo de cicatrização. A ação combinada de um grupo de leucócitos e outros tipos celulares ativados, e a liberação de uma imensa gama de moléculas, tais como citocinas e quimiocinas, é a arma de defesa biológica, denominada como o sistema imunológico.

O estudo do papel da ANXA1 e do seu mimético no funcionamento do sistema imune tem demonstrado ações como a regulação de atividade enzimática, da expressão e secreção de metabólitos do acido araquidônico e de algumas citocinas, principalmente por células inflamatórias. Um progresso considerável foi feito na compreensão dos eventos celulares e moleculares que estão envolvidos na resposta inflamatória aguda à infecção e, em menor grau, a lesão tecidual. Além disso, os eventos que levam à inflamação crônica localizada, especialmente em infecções crônicas e doenças auto-imunes, são apenas parcialmente compreendidos. Propositalmente, as ações da ANXA1 sobre os elementos celulares atuantes no sistema imunológico serão descritas a seguir, visto que tal proteina parece desempenhar papéis muito divergentes quando a resposta imune inata e adaptativa são comparadas, como descrito na Tabela 3.

Tabela 3. Ações antagônicas da ANXA1 no sistema imunológico.

Sistema Imune	Inato	Adaptativo
In vitro	**Inibição de:** Ativação de PLA2 Produção de eicosanóides Geração de EROs Fagocitose **Indução de:** Ligação a L-selectina Apoptose de PMNs Fagocitose de PMNs apoptóticos	**Indução de:** Proliferação de células T Sinalização via TCR Resposta Th1 Inibição **de:** Resposta Th2
In vivo	**Inibição de:** Migração de PMNs Adesão de PMNs Liberação de histamina Síntese de citocinas Inflamação aguda (modelos) Inflamação crônica (modelos) Hiperalgesia Febre Lesão neuronal	**Indução de:** Respostas inflamatórias dependentes de células T

Fonte: Modificado de D'acquisto, Perretti *et al.* (2008).

1.3.1 Fagócitos: uma ação antiinflamatória

Há quase duas décadas, vários estudos têm descrito o papel da ANXA1 na função leucocitária, na fisiofarmacologia dos mediadores inflamatórios e no processo de ativação e recrutamento dos leucócitos (D'acquisto, Paschalidis *et al.*, 2008). Inicialmente, é essencial pontuar que uma das principais fontes de síntese de ANXA1 endógena é os neutrófilos (Perretti, Christian *et al.*, 2000; Movitz e Dahlgren, 2001; Oliani e Perretti, 2001), monócitos e macrófagos (Solito, De Caterina *et al.*, 1993; Comera e Russo-Marie, 1995), visto que mesmo em condições normais estes tipos celulares possuem altos níveis da proteína em seu citoplasma. Os neutrófilos humanos possuem uma grande proporção de ANXA1 citoplasmática estocada junto aos seus grânulos de gelatinase (Perretti, Christian *et al.*, 2000; Lominadze, Powell *et al.*, 2005), podendo ser rapidamente secretada se as células forem expostas baixas concentrações de quimioatractantes ou mesmo se houver adesão às células endoteliais ativadas (Vong, D'acquisto *et al.*, 2007). Vários trabalhos envolvendo lavado peritoneal ou linhagens celulares

também demonstraram que os monócitos/macrófagos contem uma abundante quantidade de ANXA1 e que esta pode ser secretada no meio (Blackwell, Carnuccio *et al.*, 1980; Peers, Smillie *et al.*, 1993). A ativação destes fagócitos estimula a mobilização da ANXA1 do citoplasma para a membrana de modo célula-específico, sendo que a realocação da proteína normalmente ocorre na superfície externa da membrana celular. Em adição, baixas concentrações extracelulares de cálcio induzem uma alteração conformação da ANXA1, tal qual a região N-terminal é então exposta, levando à forma ativa da proteína (Gerke, Creutz *et al.*, 2005).

A ANXA1 recombinante humana (hrANXA1) e os peptídeos derivados da região N-terminal da proteína, mantém as propriedades de inibição e de migração dos leucócitos em vários modelos de inflamação aguda (Perretti, Ahluwalia *et al.*, 1993; La, Tailor *et al.*, 2001; Brancaleone, Dalli *et al.*, 2011) e crônica (Gibbs, Carollo *et al.*, 2002; Yang, Y., Morand, E. *et al.*, 2004; D'acquisto, Merghani *et al.*, 2007). A atividade principal é exercida sobre a regulação da adesão dos neutrófilos (Perretti e Flower, 2004) e dos monócitos (Solito, De Coupade *et al.*, 2001) às células endoteliais ativadas, por meio da ligação da ANXA1 com os receptores FPR e da regulação das moléculas de adesão L-selectina e integrina (La, M, D'amico, M *et al.*, 2001). No entanto, a ação da proteína termina dentro de poucos minutos, após a sua clivagem realizada por enzimas (PR3, elastase) ligadas à membrana (Rescher, Goebeler *et al.*, 2006; Vong, D'acquisto *et al.*, 2007). Os modelos em ratos e camundongos demonstraram que a proteína ANXA1 e seus miméticos reduzem o dano induzido por leucócitos na lesão por isquemia/reperfusão no coração (Gavins, Leoni *et al.*, 2006), no cérebro (Gavins, F., Dalli, J. *et al.*, 2007), intestino (La, Tailor *et al.*, 2001). Recentemente, nosso grupo demonstrou que em um modelo de I/R nos rins, o tratamento prévio com o peptídeo Ac2-26 protegeu fortemente contra a migração de neutrófilos, impedindo a alteração funcional e mantendo a integridade tecidual das estruturas renais (Facio, Sena *et al.*, 2011) (vide Capítulo VI).

As atividades antiinflamatórias da ANXA1 ou dos seus miméticos é consistente com o fenótipo de neutrófilos *ANXA1*-/-, os quais mostram transmigração aumentada na microcirculação de camundongos, possivelmente

devido ao aumento da expressão de L-selectina e CD11b e conseqüente quimiotaxia, se comparados aos neutrófilos não-modificados geneticamente (Chatterjee, Yona *et al.*, 2005). Outras investigações com animais *ANXA1*-/- têm revelado o desenvolvimento de uma lesão articular mais severa, quando comparado aos animais selvagens, em um modelo de artrite monoarticular induzida por antígeno, como resultado do aumento exacerbado da expressão de IL-1 e IL-6 nesses tecidos (Yang, Y., Morand, E. *et al.*, 2004). Em um modelo de endotoxemia, ainda em animais deficientes versus selvagens, foi observado que há um aumento da sinalização intracelular e a aceleração da síntese de TNF-α, IL-1 e IL-6, contribuindo para a letalidade induzida pela administração de LPS (Damazo, Yona *et al.*, 2005).

A proteína ANXA1 não está só envolvida com a inibição do recrutamento de fagócitos, mas também participa da regulação de um dos mecanismos mais utilizados na resposta inicial a uma infecção, a liberação de espécies reativas de oxigênio (EROs). O pré-tratamento de neutrófilos e monócitos/macrófagos com peptídeos sintetizados a partir de diferentes regiões do N-terminal da proteína, tais como Ac2-26 e Ac9-25, parecem regular a produção anions superóxido e, de acordo com o receptor no qual eles se ligam (família dos FPRs), essas moléculas ativam ou inibem a liberação de EROs por um fagócito ativado (Karlsson, Fu *et al.*, 2005; Pickles, Brooks *et al.*, 2010).

Outros papéis relacionados à resolução da ação inflamatória causada pelos fagócitos têm sido atribuídos à ANXA1: a apoptose e a fagocitose (Lim e Pervaiz, 2007). O *clearance* dos leucócitos apoptóticos é um processo antiinflamatório, visto que previne a exposição dos tecidos lesados ao conteúdo potencialmente imunogênico das células necróticas. Nesse processo, a principal etapa é a fagocitose das células apoptóticas, evento que estimula a liberação de mediadores antiinflamatórios e inibe a secreção de citocinas pró-inflamatórias pelos fagócitos. Uma sequência de estudos demonstrou que a adição *in vitro* de sobrenadantes de macrófagos tratados com GCs, ou de neutrófilos apoptóticos ou do peptídeo Ac2-26, modula a fagocitose de PMN apoptóticos por macrófagos da medula, via FPR2/ALX (Dalli, Jones *et al.*, 2012). Este mecanismo leva ao rearranjo da actina, liberação de TGF-β1 e ativação de SOCS3, seguida de

inibição da síntese de IL-6, MIP-2 e IL-8 (Scannell, Flanagan *et al.*, 2007; Pupjalis, Goetsch *et al.*, 2011; Iwasa, Takahashi *et al.*, 2012). A ANXA1 ainda parece atuar na apoptose em um processo no qual a proteína é recrutada do citoplasma e exportada para a membrana externa, por um modo caspase-dependente, onde ela se co-localiza com a fosfatidilserina, auxiliando na sinalização do alvo apoptótico e contribuindo também para o *clearance* local (Arur, Uche *et al.*, 2003). O resumo das principais mecanismos da ANXA1 sobre os fagócitos está ilustrado na Figura 5.

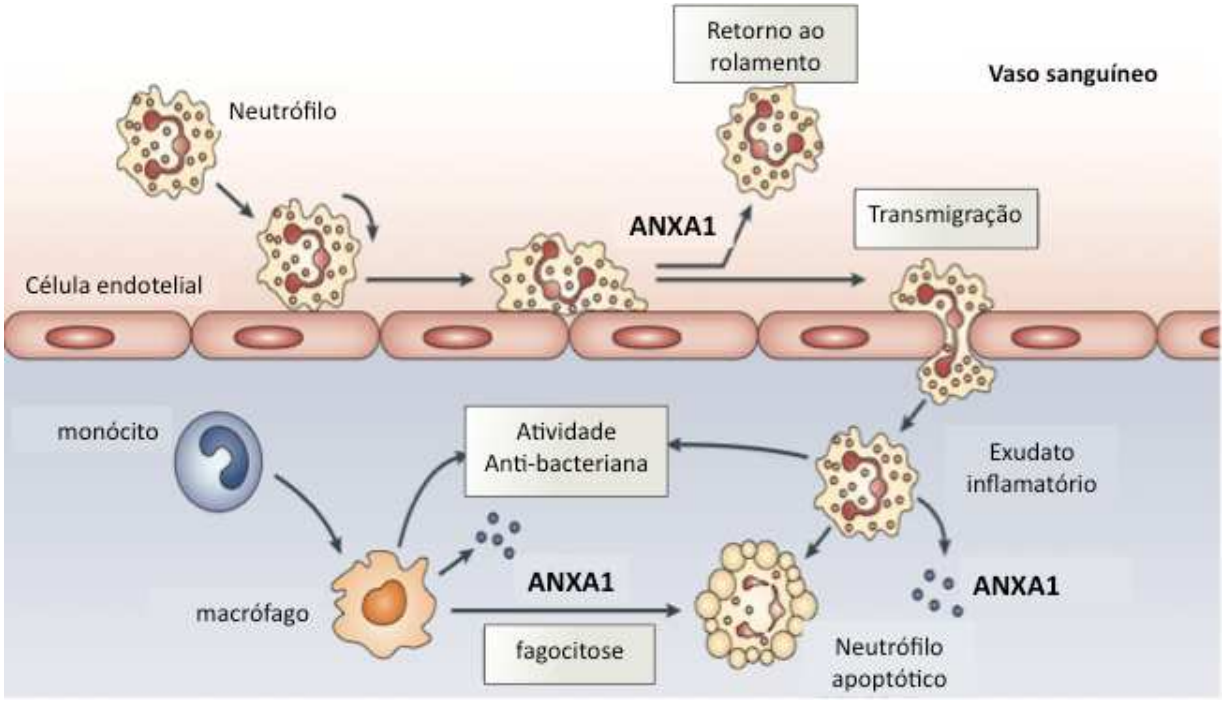

Figura 5. Resumo das principais atividades regulatórias da ANXA1 na resposta imune inata. Modificado de Perretti e D'acquisto (2009).

1.3.2 Linfócitos: uma ação pró-inflamatória

A ANXA1 tem uma distribuição e função totalmente diferente entre as células do sistema imune adaptativo. Estudos prévios e recentes demonstraram que, em contraste com as outras células do sangue periférico, os linfócitos totais parecem conter uma pequena quantidade da proteína tanto no meio intracelular, quanto na membrana celular (Morand, Hutchinson *et al.*, 1995; Spurr, Nadkarni *et*

al., 2011). Aparentemente, moléculas envolvidas com o receptor de GCs, tal como a Zap-70, é responsável pela regulação da expressão basal de ANXA1 nos linfócitos T (Ishaq, Degray *et al.*, 2007).

Apenas recentemente as investigações sobre os linfócitos, especialmente as células T, tem sido alvo de interesse dos pesquisadores, visto que atualmente há uma busca intensa pela resolução de inflamações crônicas, resultado de doenças com alta taxa de morbidade. Em um ensaio *in vitro*, células T foram tratadas com hrANXA1 após serem estimuladas com anti-CD3 (receptor de ativação) e anti-CD28 (co-receptor de ativação), para reproduzir um microambiente inflamatório com grande influxo de neutrófilos e macrófagos (D'acquisto, Merghani *et al.*). O resultado esperado seria a inibição ou diminuição da resposta mediada pelas células T, visto que a proteína exerce uma forte atividade antiinflamatória sobre os fagócitos. Contudo, a adição da proteína aumentou a ativação e a proliferação dos linfócitos pré-ativados, mas não daqueles em estado de repouso, sugerindo que a ativação sinalizada pelo TCR (CD3) contribui para a resposta das células T à ANXA1. Este estudo ainda revelou que houve um aumento da sinalização dos fatores NF$\kappa\beta$, NFAT e AP-1, e que esta atividade pró-inflamatória da ANXA1 foi realizada via FPR2/ALX, receptor mais mobilizado para a membrana em células T ativadas pelo TCR (D'acquisto, Merghani *et al.*, 2007) (Figura 6).

Considerando a importância das células T na inflamação crônica, a possibilidade da ANXA1 controlar várias vias de sinalização simultaneamente é um conceito atrativo. Neste ponto, vale ressaltar que os GCs demonstram ser novamente uma das moléculas responsáveis pela regulação da expressão da ANXA1, no entanto, na resposta adaptativa, os esteróides são capazes de diminuir a síntese de ANXA1 em células T CD4+ de pacientes com artrite reumatóide, tratados e monitorados. Estes mesmos pacientes antes do tratamento com GC expressavam mais ANXA1 nas células T CD4+ do sangue periférico, quando comparados a indivíduos saudáveis (D'acquisto, Paschalidis *et al.*, 2008).

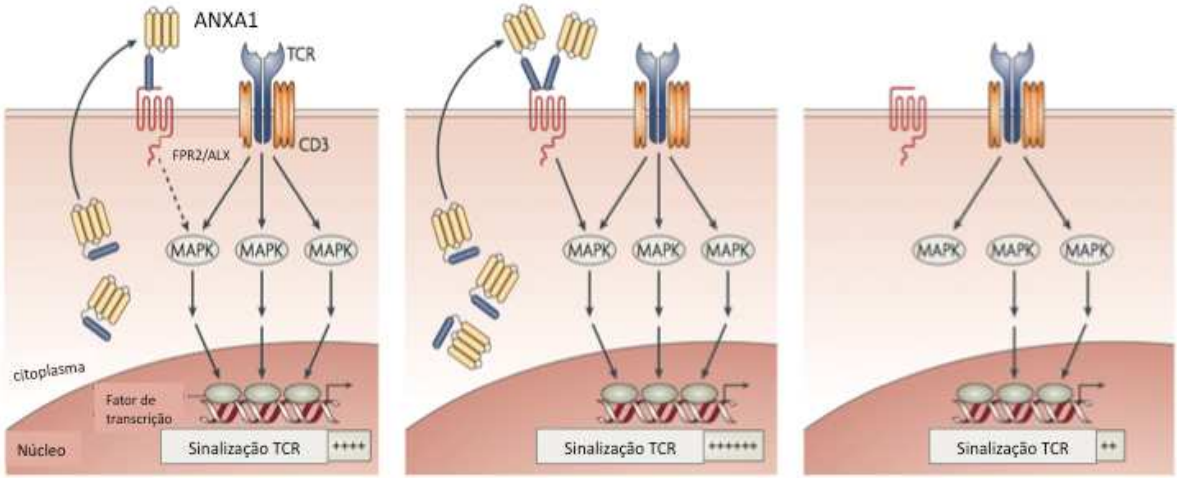

Figura 6. Via de sinalização da ANXA1 pelo FPR2/ALX e sua ação na ativação do TCR. A) condição fisiológica; B) hiperativação das células T, como nas doenças auto-imunes; C) ativação celular insuficiente, como nas imunodeficiências. **Fonte:** modificado de Perretti e D'acquisto (2009).

O fato de que a ANXA1 regula positivamente a sinalização do TCR, culminou na investigação do papel dessa proteína na diferenciação dos linfócitos. Dessa forma, quando células Th0 foram estimuladas com hrANXA1, elas adquiriram o fenótipo Th1, enquanto que a diferenciação de células T *ANXA1-/-* sob condições idênticas produziram altos níveis de citocinas de perfil Th2 (D'acquisto, Paschalidis *et al.*, 2007). A ANXA1 não só direciona a resposta, como exerce um papel primário e endógeno na seleção negativa e positiva dos timócitos, tendo como resultado um maior numero de células CD4+ na circulação, como foi demonstrado em um modelo de animais ANXA1-deficientes (Paschalidis, Huggins *et al.*, 2010).

Em contraste, quando os linfócitos são os protagonistas do cenário tumoral, e não especificamente inflamatório, o papel da ANXA1 é invertido. Estudos com linhagens celulares de leucemia e com linfoma tímico revelaram que a sua adição em condições *in vitro* ou a indução da expressão aumentada desta molécula no tecido leva a parada do ciclo celular e contribui para os mecanismos responsáveis pela apoptose de linfócitos T imaturos, respectivamente (Petrella, D'acunto *et al.*, 2008; Santos, Gonzalez-Sanchez *et al.*, 2009). Estes resultados deixam claro que a ANXA1 parece ter um papel homeostático que varia de acordo com a condição

patológica, mas instiga ainda mais a investigação dos mecanismos e vias pelos quais esta proteína alcança tal refinamento regulatório.

Em conclusão, os relevantes dados sobre ANXA1 na resposta imune adaptativa abrem uma infinidade de perguntas relativas a novos aspectos das células T e dos processos patológicos nos quais elas estão envolvidas. Isso inclui, por exemplo, o papel da ANXA1 em conjunto com os linfócitos na resposta imune das doenças infecciosas, especialmente, as virais.

1.3.3 Anexinopatias

"Anexinopatias" é um termo que tem sido usado para descrever doenças que, dentre suas características, apresentam uma síntese anormal, metabolismo ou ação de membros da superfamília das anexinas. Como já foi descrito anteriormente, tem havido várias sugestões de que a ANXA1 está envolvida em doenças inflamatórias e outras doenças crônicas humanas. Defeitos na função ou no metabolismo da ANXA1 em amostras de pacientes com doenças hereditárias têm sido relatados, tais como Febre Familial do Mediterrâneo (Shohat, Korenberg *et al.*, 1989; Garcia-Gonzalez e Weisman, 1992), síndrome do X frágil (Sun, Cohen et al., 2001) e doença de Weber-Christian (Akama, Tanaka *et al.*, 1995). O metabolismo anormal da ANXA1 também está envolvido na fibrose cística (Bensalem, Ventura *et al.*, 2005; Dalli, Rosignoli *et al.*, 2010) e outros distúrbios pulmonares relacionadas a alergia e a conseqüências do tabaco e quimioterapia (Van Hal, Overbeek *et al.*, 1996; Vishwanatha, Davis *et al.*, 1998; Fu, Cheng *et al.*, 2006; Damazo, Sampaio *et al.*, 2011; Quesada Calvo, Fillet *et al.*, 2011). A alteração da expressão ou da ação da ANXA1 ainda está ligada a desordens inflamatórias do sistema nervoso central, do sistema circulatório, da pele e das articulações (vide Tabela 1).

O fato da ANXA1 possuir uma ação sistêmica, com um papel crucial na maioria dos tecidos, instigou a investigação desta proteína ou de seus transcritos como possíveis biomarcadores de diversas patologias, mas principalmente nos cânceres. Na verdade, muitos dos trabalhos que revelam a ANXA1 como biomarcador, quer seja nos fluidos ou nos tecidos, alcançaram este resultado

abordando o problema com as ferramentas de pesquisa em larga escala, como os screenings proteômicos e transcriptômicos. Exemplos disso são os estudos que mostraram que a ANXA1 estava menos expressa nos carcinomas oral (Koike, Uzawa *et al.*, 2005), de esôfago (Huang, Xiong *et al.*, 2007), da nasofaringe (Cheng, Huang *et al.*, 2008), de cérvix uterino (Liu, Han *et al.*, 2011), em mieloma múltiplo (Xiao, Zhang et al., 2009), em melanoma (Caputo, Maiorana *et al.*, 2011); e em outros tumores, a ANXA1 foi demonstrada mais expressa, tais como tumores neurais e ependimomas (De Bont, Den Boer *et al.*, 2007; Wang, Piao *et al.*, 2011), e pulmonar (Brower, 2009). Em conseqüência dessas evidências, outras investigações com o foco especificamente na ANXA1 indicaram seu potencial diagnóstico nos cânceres gástrico (Yu, Wang *et al.*, 2008), oral (Zhang, Yang *et al.*, 2009; Faria, Sena *et al.*, 2010) e pulmonar (Wang, Piao *et al.*, 2011). Este novo biomarcador também foi descoberto ser útil na avaliação de doenças degenerativas, tal como o Alzheimer, condição na qual a ANXA1 pôde ser uma ferramenta de monitoramento da eficiência de terapias convencionais (Mhyre, Loy *et al.*, 2008). Vale dizer que é necessária a realização de investigações em escala clínico-epidemiológica, para definir com precisão em que condição a detecção de ANXA1 e de seus precursores pode ser realmente considerada um indicador clínico específico.

Em outra abordagem, os mecanismos pelos quais a ANXA1 atua nas doenças infecciosas ainda é pouco elucidado, com apenas alguns achados nas infecções bacteriana, fúngica e viral. Dadas as suas funções antiinflamatórias em modelos murinos induzidos por endotoxinas, inicialmente foi demonstrado que a proteína tem a capacidade de se ligar diretamente aos lipídeos A de bactérias gram-negativas, impedindo que essa endotoxina se ligue aos seus receptores celulares, como os receptores Toll-like, e desencadeie uma resposta exacerbada pelo hospedeiro (Eberhard e Vandenberg, 1998). Posteriormente, também foi demonstrado que a ANXA1 se liga no ácido lipoteicóico de *S. Aureus*, bactérias gram-positivas, prevenindo o reconhecimento destes agentes infecciosos e a fagocitose por macrófagos ativados (Gotoh, Takamoto *et al.*, 2005). Além disso, alguns estudos mostraram que a ANXA1 tem um papel benéfico ao hospedeiro, participando dos processos de maturação dos fagossomos e da indução de

apoptose dos fagócitos infectados por micobactérias (*M. avis* e *M. tuberculosis*) (Pittis, Muzzolin *et al.*, 2003; Gan, Lee *et al.*, 2008). No entanto, ambos agentes infecciosos modulam negativamente a ANXA1 para atingir o escape imunológico e manter a virulência. Em adição, a ANXA1 endógena, localizada na membrana das células epiteliais, parece ser a proteina efetora da inibição da proliferação do fungo pertencente à flora normal da mucosa oral, *Candida albicans,* em condições fisiológicas (Lilly, Yano *et al.*, 2010).

Do mesmo modo, nas infecções virais parece exercer uma função nos mecanismos antivirais, como por exemplo, na inibição parcial da infecção por citomegalovírus humano (hCMV) (Derry, Sutherland *et al.*, 2007) e vírus da encefalomielite japonesa (JEV) (Ding, Zhang *et al.*, 2011). A resposta da ANXA1 frente aos processos infecciosos tem sido descrita principalmente pela sua expressão, valendo citar que há uma aumento desta molécula em fibroblastos ou PBMCs em resposta a agentes patogênicos como o virus linfotrópico das células T humanas (HTLV), herpes vírus bovino – tipo 1 (bHSV-1) e vírus da febre suína (CSFV), enquanto a ANXA1 é inativada por meio da serino-fosforilação, pela proteína codificada pelo Epstein-Barr vírus (EBV).

Muito pouco se sabe, porém, sobre as causas e os mecanismos de inflamação crônica sistêmica, processo que ocorre em uma ampla variedade de enfermidades, como nas doenças auto-imunes e nas imunodeficiências. Neste ponto, frente a tudo que foi revisto sobre os papéis multifacetados da ANXA1 e sua regulação em inúmeros eventos fisiológicos e patológicos, cabe pontuar que é de extrema relevância investigá-la também nos tipos celulares e microambientes envolvidos nas doenças inflamatórias intestinais e na AIDS (Síndrome da Imunodeficiência Adquirida).

1.3.3.1 ANXA1 e AIDS

A AIDS ou Síndrome da Imunodeficiência Adquirida (SIDA) é causada pela infecção do vírus HIV-1. Em números, esta síndrome contabilizou aproximadamente 1.8 milhões de mortes somente em 2010, 2.7 milhões de novos casos e até então já tinha sido atingido 34 milhões de indivíduos infectados e

vivos em todo o mundo (WHO, 2011). Essas estatísticas epidemiológicas expõem a relevância de se engajar nas pesquisas que contribuam de alguma forma para este desafio que causa tanta vulnerabilidade a saúde da humanidade.

A partir da transmissão via contato sexual, exposição a fluidos corporais contaminados e transmissão vertical da gestante para o filho, os indivíduos infectados poderão ser acometidos com uma cascata de alterações imunológicas, acarretando na imunossupressão e falta de proteção ao desenvolvimento de doenças oportunistas. Assim, a patogênese da infecção pelo HIV-1 é caracterizada pela imunodeficiência linfocitária contextualizada ao descontrole da ativação imunológica generalizada, contando com intensa replicação viral e massiva depleção de células T CD4+ de memória durante a fase aguda, e com perda gradual das células T CD4+ remanescentes devido a persistência da hiper-ativação imune sistêmica, além da reposição insuficiente dos linfócitos depletados no sangue e nas mucosas, na fase crônica da infecção (Picker e Watkins, 2005; Simon, Ho *et al.*, 2006).

A entrada do HIV-1 nas células T e nos fagócitos requer interações seqüenciais entre o envelope viral, o receptor de membrana celular CD4 e um co-receptor (Caffrey, 2011). O receptor de β-quimiocionas CCR5 é essencial para o estabelecimento inicial da infecção pelo HIV-1 da cepa viral R5 (variante mais comum do vírus), visto que esta cepa possui uma sequência de aminoácidos que prediz o uso do CCR5 como co-receptor (Cocchi, Devico *et al.*, 1996; He, Chen *et al.*, 1997), e também pelo fato de que indivíduos geneticamente deficientes para a expressão de CCR5 são extremamente resistentes a infecção (Samson, Libert *et al.*, 1996). Dessa forma, muitos estudos têm sido realizados em busca de uma estratégia para bloquear ou inibir a expressão do CCR5.

Neste ponto, convém dizer que alguns agonistas dos receptores FPR1 e FPR2/ALX (por ex., fMLF, peptídeo W e proteína gp120 do envelope vírus HIV-1) são potentes inibidores do co-receptor de infecção do HIV-1, o CCR5 (Le, Li *et al.*, 2000). Experimentos *in vitro* mostraram que a ativação dos FPRs em monócitos leva a diminuição da expressão ou ao processo de fosforilação do co-receptor, desensibilizando o CCR5 e inibindo parcialmente a infecção (Shen, Proost *et al.*, 2000; Le, Oppenheim *et al.*, 2001; Li, Wetzel *et al.*, 2001). Em adição, foi revelado

recentemente que o FPR2/ALX também age com um co-receptor eficiente e alternativo dos vírus HIV-1, descrevendo-se uma nova via de entrada nas células CD4+ (Shimizu, Tanaka *et al.*, 2008; Nedellec, Coetzer *et al.*, 2009; Coetzer, Nedellec *et al.*, 2011). Interessantemente, acabam de ser descritas as primeiras associações entre ANXA1 e HIV/AIDS (Figura 7): 1) a possibilidade desta proteína e de seus peptídeos miméticos inibirem o CCR5, como os outros agonistas dos FPRs; 2) o compartilhamento do mesmo receptor e o viés que este fato pode acarretar, ou seja, a sugestão da competição entre a ANXA1 e o vírus.

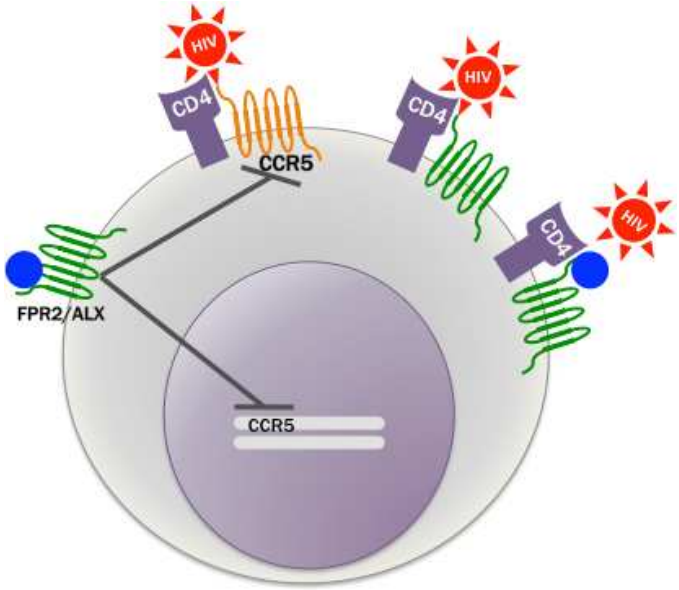

Figura 7. Proposta de atuação da ANXA1 e de seus miméticos na inibição da entrada do HIV-1 na célula alvo. Sinalização da ANXA1/peptídeos (esfera azul), via FPR2/ALX, induz a fosforilação ou regula a transcrição do co-receptor canônico do HIV-1, o CCR5; ANXA1/peptídeos competem pelo mesmo co-receptor não-canônico do vírus, o FPR2/ALX, e inibe parcialmente a infecção.

Por outro lado, desde os primeiros estudos, a imunoativação sistêmica foi reconhecida como um fator determinante para a progressão da imunodeficiência causada pela infecção por HIV-1 (Ascher e Sheppard, 1988), e agora este evento, resultante de uma inflamação crônica, é considerado crucial na patogênese da AIDS. Esta resposta é caracterizada pelo aumento da expressão de marcadores típicos do fenótipo ativado observado nas células T CD4+ e CD8+, B, NK e monócitos; níveis elevados de citocinas pró-inflamatórias (TNF-α, IL-6 and IL-1β),

quimiocinas (MIP-1α, MIP-1β e RANTES) (Appay e Sauce, 2008); a presença de hipergamaglobulinemia como resultado da ativação policlonal das células B (Moir e Fauci, 2008); um aumento do numero de células T em proliferação; hiperplasia dos linfonodos (Lederman e Margolis, 2008) e níveis séricos aumentados de marcadores de ativação, como a proteina C reativa e neopterina (Boasso e Shearer, 2008; Nixon e Landay, 2010; Redd, Eaton *et al.*, 2010). Todas essas características são resultantes da soma de fatores como a quantidade da carga viral, produtos virais antigênicos, e aumento do nível de LPS circulante associado à translocação microbiana proveniente da mucosa intestinal que é lesada durante a infecção aguda (Douek, 2007). Em contraposição à imunoativação sistêmica, muitos mediadores antiinflamatórios atuam para combater as propriedades dos fatores pró-inflamatórios, valendo citar que um potente mediador capaz de contribuir para a resolução da inflamação é a ANXA1 (Rothhut, Russo-Marie *et al.*, 1983; Flower e Rothwell, 1994; Perretti e D'acquisto, 2009; Perretti e Dalli, 2009).

Nesse sentido, a primeira questão a ser formulada é: como a ANXA1 é expressa no cenário de infecção por HIV-1? Existe, até o presente momento, um único estudo baseado na técnica de *microarray*, que mostrou um aumento na expressão transcricional da ANXA1 sistêmica em macacos sob infecção crônica por SIV, um modelo não-humano de AIDS (George, Reay *et al.*, 2005). A segunda questão é: se e como a ANXA1 participa dos mecanismos de resolução do organismo frente à infecção por HIV-1/SIV? A ANXA1 exerce papéis, em outras doenças, que seriam a colocariam em uma posição relevante na patogênese do HIV/AIDS, tais como imunomodulação da síntese de citocinas pró-inflamatórias, regulação da ativação de fagócitos, maturação de linfócitos e células dendríticas, indução de resposta Th1 e manutenção da integridade da mucosa intestinal (Babbin, Laukoetter *et al.*, 2008; D'acquisto, Perretti *et al.*, 2008; Huggins, Paschalidis *et al.*, 2009; Yang, Aeberli *et al.*, 2009; Paschalidis, Huggins *et al.*, 2010). A Figura 8 propõe as possibilidades de atuação da ANXA1 junto à fase crônica da infecção por HIV-1.

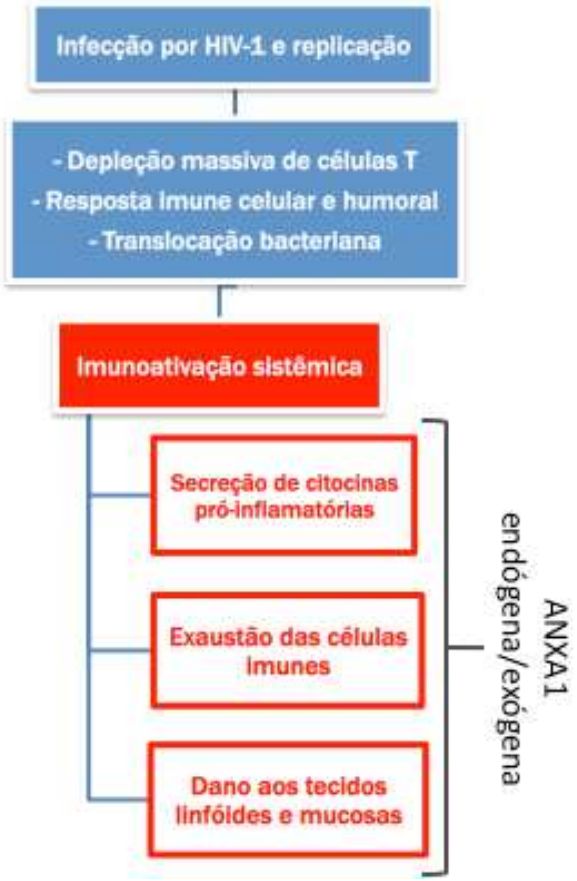

Figura 8. Proposta de atuação da ANXA1 e de seus miméticos, endógenos ou exógenos, nos eventos característicos da infecção crônica. Frente aos papéis já descritos, a proteína ANXA1 expressa pelo hospedeiro ou a administração de seus peptídeos funcionalmente miméticos elegem-se como potenciais candidatos a participar do mecanismo de resposta homeostática do indivíduo, contra progressão para a AIDS resultante da imunoativação sistêmica.

1.3.3.2 ANXA1 e doenças auto-imunes

As doenças auto-imunes constituem um grupo de mais de 80 desordens diferentes caracterizadas pelo ataque imune de componentes do próprio corpo, mediado por auto-anticorpos e células T auto-reativas (Cho e Gregersen, 2011). Estas condições de inflamação crônica não parecem ser causadas somente pela incitadores clássicos de inflamação: infecções e lesões. Em vez disso, elas parecem estar associadas à quebra da tolerância imunológica e ao mau funcionamento dos tecidos, isto é, com o desequilíbrio homeostático dos vários sistemas fisiológicos, resultando em inflamação e destruição tecidual (Medzhitov, 2008; Cho e Gregersen, 2011).

Há duas décadas, a ANXA1 vem sendo associada às doenças auto-imunes, devido a presença de auto-anticorpos circulantes contra a proteína em pacientes que sofrem de artrite reumatóide (Podgorski et al., 1992), lúpus eritematoso sistêmico (Pruzanski, Goulding *et al.*, 1994; Kretz, Norpo *et al.*, 2010), doença de Crohn e colite ulcerativa (Stevens, Smith et al., 1993; Beattie, Goulding et al., 1995). Considerando que a formação desses complexos proteína-anticorpo poderia neutralizar a ação da ANXA1, é possível sugerir que a quebra da ação endógena regulatória desta molécula bioativa contribua para o desenvolvimento e severidade das desordens auto-imunes.

Com base nesses achados, foi escolhido um modelo murino de desenvolvimento de colite ulcerativa, com a utilização de animais *ANXA1-/-*, e os resultados indicaram que a ANXA1 endógena diminui a infiltração de leucócitos no cólon e ainda apresenta propriedades reparadoras do epitélio da mucosa intestinal via FPR2/ALX (Babbin, Laukoetter *et al.*, 2008). Em contraste, a partir de um modelo de encefalomielite auto-imune com o mesmo tipo de animais foi sugerido que, neste caso, a ANXA1 endógena ativa os linfócitos Th1 e Th17, induzindo uma lesão mais severa, o que pode colaborar para o desenvolvimento da esclerose múltipla (Paschalidis, Iqbal *et al.*, 2009). Frente a estes dados, é importante enfatizar que há uma lacuna no que se diz respeito à expressão da ANXA1 endógena e de seus precursores nas doenças auto-imunes e na sua resposta às terapias atuais, o que possibilitaria uma maior compreensão do seu papel nestas condições crônico-inflamatórias.

2. Perspectivas

Diante da presente revisão, a abordagem sobre os efeitos da ANXA1 na sinalização e resposta dos fagócitos e linfócitos a diferentes antígenos ou agentes etiológicos, auxiliaria na compreensão geral do seu papel homeostático em doenças resultantes do desequilíbrio em múltiplos sistemas e no desenvolvimento de novas moléculas capazes de controlar as respostas imunológicas sistêmicas.

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OBJETIVOS

Objetivos Gerais

Investigar como a presença endógena e exógena da anexina A1 e de seu peptídeo N-terminal, quer seja em nível de RNAm ou proteína, se apresenta em processos inflamatórios associados a infecção ou hipóxia, como nas doenças inflamatórias intestinais (IBD), imunodeficiência causada por infecção pelos vírus HIV e SIV (AIDS), e lesão por isquemia e reperfusão renal.

Objetivos Específicos

Para compor a presente tese, foram cumpridos os seguintes objetivos:

1. Determinar *in vitro* o efeito do peptídeo N-terminal da ANXA1, Ac2-26, na produção de espécies reativas de oxigênio dos principais fagócitos do sangue periférico humano, estimulados por um componente da parede celular fúngica. Para complementar, o próximo passo foi investigar a ação deste peptídeo sobre a expressão de superfície do receptor TLR2, específco para o componente responsável pelo estímulo inflamatório nestes fagócitos;

2. Investigar *in vitro* o papel do Ac2-26 no imunofenótipo de leucócitos mononucleares do sangue periférico de indivíduos saudáveis e HIV-positivos. Simultaneamente, foi analisado o efeito e a via de sinalização deste peptídeo de ANXA1 sobre a expressão, em nível de RNAm e proteína de superfície, do co-receptor de infecção por HIV-1, o CCR5;

3. Investigar, em um modelo não-humano de AIDS, a cinética da expressão transcricional da ANXA1 no sangue periférico e na mucosa intestinal durante o período de infecção e progressão in vivo da doença, em macacos infectados por SIV. Em adição, analisar a correlação entre a presença de transcritos de ANXA1 e marcadores da infecção crônica por SIV/HIV, tais como ativação linfocitária, expressão de citocinas pró- e antiinflamatórias e carga viral;

4. Avaliar a expressão da ANXA1 em nível de RNAm e proteína, no sangue periférico e na mucosa intestinal, respectivamente, em indivíduos saudáveis e portadores de IBD (Doença de Crohn). Posteriormente, realizar a análise para identificar as associações entre a expressão de ANXA1 e os dados clínicos terapêuticos, a expressão transcricional de TNF-α, a presença de bacteremia e a ativação linfocitária;

5. Avaliar o papel do Ac2-26 como alternativa de tratamento in vivo na inflamação aguda e crônica causada por isquemia e reperfusão renal em modelo murino, analisando os parâmetros de função renal, a integridade tecidual microscópica e ultra-estrutural, a migração de células inflamatórias e a contribuição da expressão da ANXA1 endógena nesta condição.

CAPÍTULO I

A inibição da produção de ROS pela Anexina A1: regulação da produção de Eros através da diminuição da expressão de TLR2 via Receptores de Peptídeos Formilados*

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The ROS generation inhibition by the annexin A1 N-terminal peptide: a

possible link between TLR2 downregulation through Formyl Peptide Receptors

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Abstract

Toll-like receptor 2 (TLR2) mediates recognition of infectious agents, such as fungi and yeast, and signals to the production of inflammatory mediators, including reactive oxygen species (ROS). In additon to TLR2, other surface receptor has been proposed to participate in microbial recognition and ROS production, the FPR1. In this study we have examined whether ANXA1 peptide (Ac2-26), an anti-inflammatory agonist of FPRs, inhibits ROS production in human phagocytes (PMN and monocytes) stimulated with opsonized-zymosan particles, a yeast component wall recognized by TLR2. By chemiluminescence assay, we reported

that the Ac2–26 peptide inhibits, in a FPR1-dependent manner, human PMN/PBMC respiratory burst stimulated by these particles. In addition, using flow citometry tools, our data showed that Ac2–26 also inhibits the TLR2 surface expression in those cells. Our results propose a new role for ANXA1 peptide in the establishment of the crosstalk between FPR1 and TLR2 to control the innate immunity.

Introduction

Professional phagocytes, such as neutrophils and monocytes, recognize and kill invading microbes by releasing toxic molecules, which include ROS, cationic proteins and nitric oxide [1]. This reaction is linked to an important component of the host innate immune response: the recognition of PAMPs by the Toll-like (TLR) and formyl-peptide receptors (FPR) [2], which have established a crosstalk in mice's inflammatory cells [3].

It has been known that pro-inflammatory agonists of FPR1 and FPR2/ALX and specific TLR2 ligands have notable ability to induce the ROS production [4]. Given the potential toxicity of ROS to surrounding tissue, there is a need for regulatory mechanisms able to limit leukocyte activation. If a natural cell immune response cannot maintain homeostasis, a pharmacological approach may be necessary to avoid such a toxic response.

Here, our strategy to control ROS was based on the anti-inflammatory agonist of FPRs, the protein annexin A1 (ANXA1), aiming modulate both receptors simultaneouly. Firstly, the ANXA1 N-terminal peptides Ac1-18 and Ac9-25 have been shown acting as dose-dependent inhibitors of superoxide anion production in human neutrophils [5, 6]. Secondly, It has been demonstrated that some ANXA1 peptides bind to FPR1 and desensitizes cells towards subsequent stimulation with pro-inflammatory antigens [7], but the mechanism of desensitization has not been explained. On the other hand, in vitro studies showed that exogenous ANXA1 induced IL-10 and TGF-β release [8-10] by phagocytes, which could down-regulate the TLRs, since these anti-inflammatory cytokines are considered as negative regulators of TLR expression.

Taken together, given that ANXA1 actually modulates in a distinctive manner each signaling receptor involved in the free radical generation, we argued if the ANXA1 N-terminal domain could in fact inhibit ROS production via FPR1 binding and modulation of TLR2 expression. As a model for TLR2 activation, we have used zymosan, a component of cell walls of many fungi, which is able to stimulate phagocytosis, ROS production, inflammatory cytokines and chemokines secretion in myeloid cells [11] through its recognition by dectin-1 [12] and TLR2 [13], both expressed on phagocytes. Due to the non-modulation of endogenous

ANXA1 by dectin-1 expression in macrophages [14], we have focused our investigation on the effects of the Ac2-26 peptide on ROS generation and its possible involvement with TLR2 modulation during zymosan stimulation of phagocytes.

Material and Methods

Isolation of Human PMN and PBMC

The human PMNs and PBMC were purified from 10 mL of heparinized venous blood using Ficoll-Hypaqye gradient following the manucfature's procedure, with some modifications. Cells were counted using a Neubauer chamber and cellular viability of each sample was always greater than 95% as determined by the Trypan blue exclusion test. After isolation, cells were separately resuspended in 3 mL of RPMI 1640 without phenol red (LGC, Brazil) supplemented with 5% fetal calf serum (Hyclone, USA). Ethics Committee from UFTM, Brazil, approved this work.

Cell stimulation

Aliquots of PMN ($5x10^5$ cells) were incubated for 30min at 37°C with RPMI 1640 without phenol red (LGC, Brazil) containing different concentrations of Ac2-26 peptide (none, 15, 75, 150, 300μM). The N-terminal from annexin-A1 peptide (Ac2-26, Ac-AMVSEFLKQAWIFIENEEQEYVQTVK) was synthesized, HPLC-purified (GeneScript, USA), dissolved in sterile phosphate-buffered saline and stored at -20°C. In order to investigate the signaling pathway of Ac2-26, it was used a monoclonal neutralizing anti-FPR. The cells stimulation for ROS production was performed using opsonized-zymosan (13mg/mL) in all pre-treatment groups (PBS, Ac2-26, Ac2-26+anti-FPR, anti-FPR). Ac2-26 cellular cytotoxity was also assessed by MTT assay.

Determination of ROS production

The quantitative ROS determination was performed in a luminol-dependent

chemiluminescence assay. The principle of the method is based on luminal interaction with phagocyte-derived oxygen free radicals, which results in large amounts of light. Human PMNs (5 x 10^5 cells/100mL of RPMI without phenol red) were incubated for 30 minutes at 37^{o}C with different concentrations of Ac2-26 peptide or appropriated controls. After the incubation period, 500µL of luminol (10^{-4} M) was added to each luminescence tube and OZ used as a stimulus for ROS production. The final volume was adjusted for 700mL with RPMI-1640 without phenol red. The chemiluminescence measurements were performed in a luminometer (Lumat-LB 9501-EG&G Berthold, Germany). All experiments were performed from 3 different donors in duplicates and recorded during 35 min, which was sufficient to reach the peak of ROS production. The results were expressed as RLU/min (relative light units per minutes). Control experiments were performed simultaneously. The integral value (area under curve - AUC) of the chemiluminescence reaction represents the total ROS production by PMNs during the reaction. Individual responses were used to calculate mean ± SD of relative light units per minute (RLUs) after different stimulus.

Flow cytometry

After Ac2-26 pre-treatment, PMN and PBMC were stained with mouse anti-human TLR2-PE monoclonal antibodies (IMGENEX) in a 50µL staining buffer for 30min at room temperature in the dark. Control tubes included cells incubated with medium alone and cells incubated with PE-conjugated mouse IgG isotype antibodies. Cells were washed twice with PBS-FCS 1% and ressuspended in 500µL of PBS-FCS 1% + PFA 1%. Acquisition (20000 events/sample) was performed on a FACsCallibur cytometer (BD Biosciences, USA) and analysis was carried out using the FlowJo 7.6.1 (TreeStar Inc, USA). PMN analysis and gating were performed using forward-and-side-scatter parameters, while monocytes/PBMC were done using FITC-conjugated anti-CD14. The surface receptor expression was represented as percentage of gated positive cells.

Statistical Analysis

Data were analyzed by two-tailed Student's T test, considering significant $p<0.05$. Statistical analysis was carried out using GraphPad Prism 5.0.

Results and Discussion

The effects ANXA1 N-terminal peptides on PMN trafficking and activation have been tested using *in vitro* systems that resembles some physiologic events that occurred at inflamed sites [15, 17]. Our data showed that the Ac2-26 peptide exerted a potent inhibitory role on ROS production by human PMNs under opsonized-zymosan stimulus in dose-dependente manner (0-300uM; data not shown). Using both MTT assay and trypan blue exclusion test (data not shown), we were also able to demonstrate that the powerful concentrations (150 and 300μM) of Ac2-26 were not cytotoxic to human PMNs *in vitro*. Thus, data from the present work demonstrated that Ac2-26 peptide inhibits zymosan-induced ROS production (Figure 1 A-C) partially through FPR1 signaling. Similarly, it has been shown in an experimental peritonitis model that the Ac2-26 peptide inhibits PMN influx after zymosan stimulation [15]. However, data from other investigations with different sequences of N-terminal peptides revealed that at low concentrations these peptides inhibits PMN activation [5], in agreement with the idea of that it is unlikely that concentrations up to 50 μM of Ac2-26 could be synthesized *in vivo*.

The fact that zymosan particles are obtained from *S. cerevisae* cell walls, and binds to TLR2, an important innate PRR [22], may suggest that the annexin-A1 peptide could also affect the development of proper anti-oxidant responses against fungal infections. We tested the hypothesis that Ac2-26 bind to FPR1 and the downstream signaling inhibits ROS production in human PMNs by decreasing the expression of TLR2. PMN and PBMC treated with Ac2-26 peptide displayed a significant downregulation of TLR2 expression at cell surface as revealed by flow cytometry analysis (Figure 1 D-H). Interestingly, a previous work from our group revealed a dysregulation of TLR4 mRNA on ANXA-/- null murine macrophages, but not of TLR2 mRNA [16], suggesting that during our assays Ac2-26 leads only TLR2 internalization as inhibitory effect, as has been shown elsewhere [17]. In addition, early studies of our group also showed a simultaneous marked upregulation of ANXA1 synthesis in activated PMNs after zymosan stimulation [18]. Based on these evidences, we propose a new controlling mechanism for

ANXA1/FPR1 on the inflammatory response, since Ac2-26 may partially downregulate TLR2 expression on human blood phagocytes. This action could affect feedback and proresolving mechanisms, reducing the TLR2 recognition and activation by PAMPs, and consequently the ROS production in response to microbial agents.

Acknowledgments

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Figure legend ***(inserida individualmente junto à figura)***

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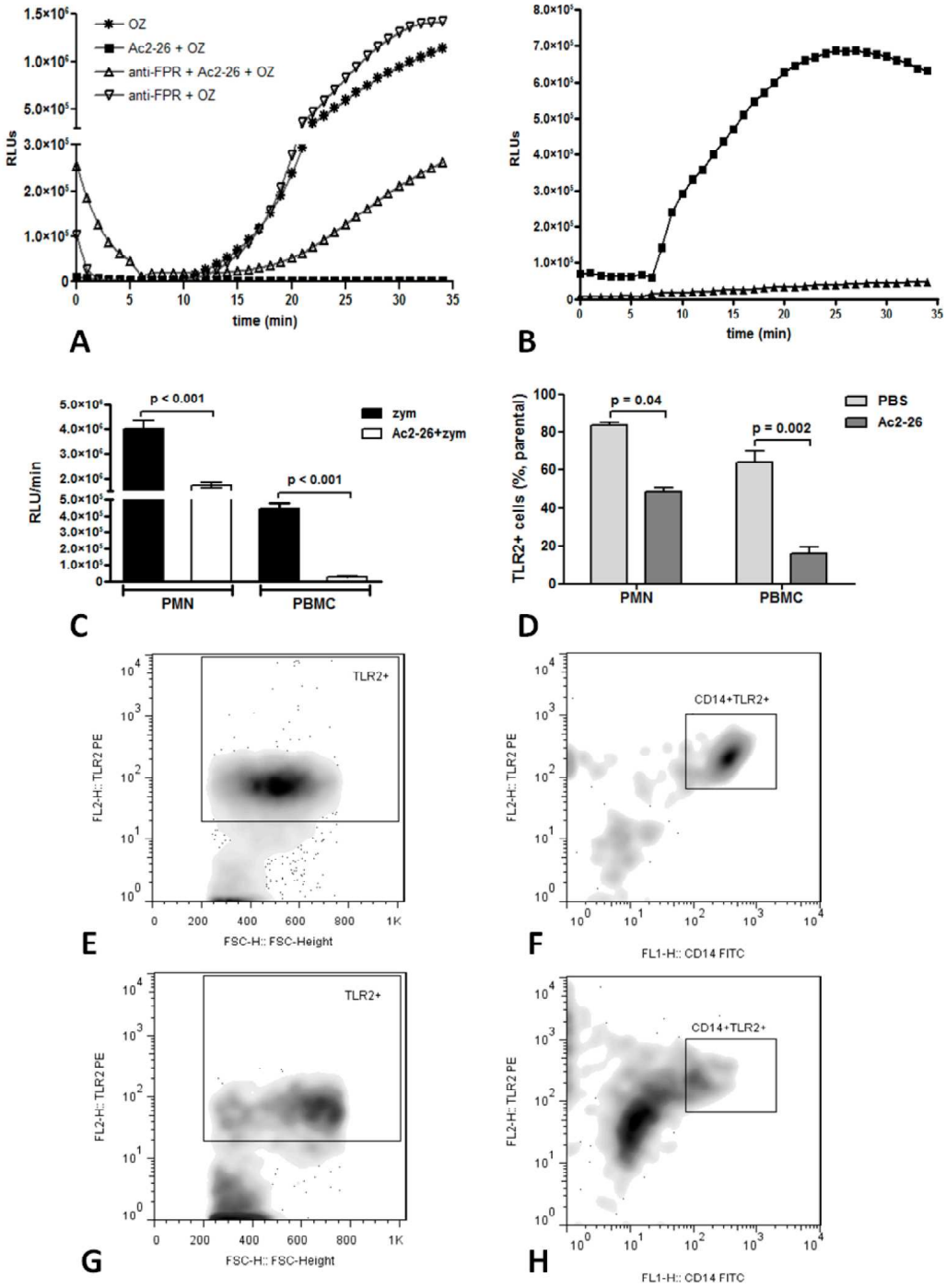

Figure 1. ***Effect of Ac2-26 on ROS production and TLR2 surface expression from PMN and PBMC.*** Representative chemiluminescence curves of ROS production vs time measured in zymosan-stimulated PMN (A) and PBMC (B) pre-treated or not with Ac2-26. (C) Data are expressed as mean ± SEM of integral values (area under the curve), representing total ROS production during the 35-minute reaction from three independent experiments in duplicate. (D) TLR2-positive cells after Ac2-26 pre-treatment. Density plots representing parental population of TLR2-positive neutrophils (E, G) and monocytes (CD14+) (F, H), without or with Ac2-26 peptide pre-treatment, respectively.

CAPÍTULO II

Efeito do peptídeo N-terminal da ANXA1 na infecção por HIV-1: regulação da expressão do CCR5 e conversão dos imunofenótipos de células T e monócitos*

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Immunophenotype switch of HIV-1-infected leukocyte subsets and modulation of CCR5 expression mediated by the Annexin A1 N-terminal peptide

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Abstract

Background: HIV most commonly uses CCR5, a seven-transmembrane G-protein-coupled receptor (GPCR), to enter target leukocytes. However, another important GPCR that is also target of the HIV gp120, the formyl peptide receptor (FPR) family, contrary to CCR5, is involved in host defense against pathogens, and also senses internal molecules that may constitute signals of cellular dysfunction. We hypothesize that the crosstalk interaction between FPRs and CCR5 is an essential cell signaling mechanism for a successful infection and disease progression. We have used a strong agonist of FPR2, the ANXA1 N-terminal peptide, that plays a homeostatic role in the activation of circulating monocytes and T cells, to test FPRs function on CCR5 expression, and on leuckocytes' profile under the disrupted balance caused by the HIV infection. **Methods:** Isolated PBMC of HIV-1-positive and healthy donors were *in vitro* treated with the Ac2-26 N-terminal peptide in combination or not with FPR pan-antagonist (BOC2). The CCR5 mRNA was measured by qPCR and its surface expression on subsets of T cells (CD4, CD8) and monocytes (CD14/CD16) were determined by flow cytometry. **Results:** CCR5 transcripts were downregulated by Ac2-26 treatment, as a consequence of decreased percentage of CD4+ T cells expressing CCR5, independently from the infection status, which was reverted by BOC2 in HIV-1-positive samples. Both infected and control groups showed reduction of CCR5+CD4+ T cells after the peptide stimulus. The Ac2-26 induced a monocyte subset switch with a selective expansion of CD14highCD16- and contraction of CD14lowCD16+ cells, predominating an anti-inflammatory and immature profile. However, all monocytes showed a trended up-regulation of CCR5 surface expression post-treatment, and even higher in the FPR antagonist plus Ac2-26-treated ones. **Conclusion:** The ANXA1 N-terminal peptide modulates CCR5 expression during HIV-1 infection in a cell-specific and infection status manner, suggesting that the crosstalk between these two receptors may have profound implications on the disease onset and progression.

Introduction

HIV-1 entry into T cells and phagocytes requires sequential interactions between envelope, CD4, and a co-receptor [1,2]. The β-chemokine receptor, CCR5, is critical in the initial establishment of chronic HIV-1 infection in vivo, since transmitted R5 strains (the variant of the virus that is common in earlier HIV infection) predominantly have sequences that predict the use of this co-receptor [3], and individuals in whom this receptor is genetically deficient are largely resistant to infection [4,5].

Due to its important co-action in the infection process, efforts have been made to inhibit its CCR5 expression, which led to the discovery of as a new class of antiretroviral drug, maraviroc and rapamycin [6]. On the other hand, CCR5 may also be subjected to heterologous desensitization when selective agonists of unrelated GPCRs activate the cells. An involved class of GPCR in this desensitization mechanism is the Formyl Peptide Receptor family, the FPRs [7]. The activation of FPR1 and FPR2/ALX by their agonists induces phosphorylation followed by inactivation of CCR5 in monocytes [8-10]. Additionally, the W peptide, a synthetic FPR2/ALX ligand, not only desensitized and phosphorilated CCR5 expressed in immature dendritic cells in vitro, but also partially inhibited HIV-1 infection in those cells [11].

These observations motivated us to investigate whether the annexin-A1 (ANXA1) N-terminal peptide, a natural FPR2/ALX ligand [12], is able to play any effect on the CCR5 expression in T cells and monocytes, at transcriptional and translational levels. ANXA1 is a member of a superfamily of annexin proteins that originally is characterized by binding to acidic phospholipids with high affinity in the presence of Ca^{2+}. This protein and its mimetic-peptides exert a myriad of roles in the physiological and pathological conditions, especially as a modulator of activated inflammatory cells [13]. It has been shown that the ANXA1 and its N-terminal peptides bind to FPRs and have interesting properties in activating this receptor by playing dual roles in inflammatory host responses [14].

On the other hand, ANXA1 has also been considered a modulator of TCR signaling and might represent a new target to pathologies with unbalanced

Th1/Th2 response or an aberrant T cell activation [15]. So, we hypothesized that ANXA1 may also have a role in controlling cell subsets expansion or contraction during HIV infection, and some of these may be involved in CCR5 expression, but may also present resistance to HIV infection, despite being CCR5 expression [16].

Therefore, because HIV-1 infection disrupts the homeostatic balance of T-cell and monocyte subsets, and that ANXA1 is probably a key molecule on both leukocytes activation status and CCR5 expression, we have characterized the cell immunphenotypes' distribution and CCR5 expression under stimulation of the N-terminal peptide Ac2-26 in the peripheral blood of HIV-1 patients and healthy controls, and implications to the HIV pathogenesis are discussed.

Material and Methods

Samples and stimulus

Peripheral blood samples (15ml) were collected from highly active antiretroviral therapy (HAART)-naïve patients that were enrolled at the CARES Clinic (Sacramento, CA) and through referring physicians. Written informed consent was obtained from the participants for this Institutional Review Board approved study (IRB no. 200311088-7). Control samples were collected from HIV-seronegative donors.

Then, PBMC were isolated using Ficoll-Paque gradient (GE Company, USA), according to manufacturer's instructions, and cultured in RPMI 1640 at 37^{o}C and CO_2 5%, which were separated in 4 groups of 1x10^{6} PBMCs. The first group of cells was treated with the PBS-dilluted recombinant ANXA1 N-terminal peptide, named Ac2-26, produced by GenScript USA Inc. (Piscataway, NJ). All tests were carried out using the pre-established dose (300μM) and incubation period (3 hours). To test whether FPRs mediated the Ac2-26 activity, a second group of cells were treated previously with BOC2 (1uM, GeneScript USA Inc.), a pan-antagonist of this receptor family, followed by described Ac2-26 treatment. Additionally, control PBMCs groups were treated with BOC2+PBS or only PBS. All of PBMC groups were submitted to RNA extraction and immunophenotyping procedures for further investigations.

Quantitative real-time RT-PCR

To quantify differences in mRNA levels for CCR5 between healthy controls and HIV-infected samples, total RNA was extracted utilizing the Qiagen RNeasy RNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The reaction was carried out using one tube of RT-PCR master mix (PE Applied Biosystems, CA) on the ABI Prism 7900 sequence detector (PE Applied Biosystems, CA). TaqMan real-time PCR validation of gene expression was performed using Assays on Demand systems (Applied Biosystems, CA). The level of gene transcription was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and expressed as a relative difference. The data were analyzed with Sequence Detector software and quantitated using the relative computational method [15].

Additionally, HIV RNA loads in plasma samples were determined by real-time reverse transcription-PCR (RT-PCR) as previously described [16]. Fluorescent signal was detected using an Applied Biosystems Prism 7900 sequence detection system, and data were analyzed with Sequence Detector software. Viral RNA copy numbers for experimental samples were determined by plotting CT values (the fractional cycle number, in which the fluorescence overcomes the fixed threshold detection value) against a regression curve derived from control transcript samples with known HIV copy numbers. This procedure was in order to normalize for the volume of the plasma samples.

Flow cytometry

Isolated PBMCs were then stained with Amcyan live amine dye (Invitrogen). We used antibodies to CD3 (APC)-Cy7–labeled; SP34.2), CD4 (Pacific Blue–labeled; OKT4), CD8 (APC-Cy5.5-labeled; 3B5), CD14 (phycoerythrin-labeled; 5A6.E9), CD16 (phycoerythrin-Cy5–labeled; DX2) and CCR5 (PE). After antibody cocktail incubation, cells were washed and fixed with 1% paraformaldehyde and analyzed by BD LSR II Flow Cytometer (BD Biosciences). A minimum of 1,000,000 events was collected for each sample, and data were analyzed using the FlowJo software version 7.3 (Tree Star Inc., Stanford, CA).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0 software. Data are expressed as mean ± SEM. Gene expression and flow cytometric data of total PBMC or leukocyte subsets from different PBMC groups were analyzed using unpaired Student's two-tailed t-test. For all comparisons, $p < 0.05$ was considered to be statistically significant.

Results

Ac2-26 peptide down-regulates CCR5 transcription in PBMC

Since ANXA1 is able to regulate the expression of some transcription factors [15,19], we investigated whether and how Ac2-26 affects CCR5 transcription. Firstly, we established the standard assays to be used hereafter. PBMCs of healthy donors were cultured in medium containing increasing concentrations (10-300μM) and time periods (1-3h), and transcript levels were measured by qPCR assays. Untreated cells from the same donors were used as controls. Additionally, after establishing the treatment parameters (300μM/3h) for the Ac2-26, toxicity analysis was performed by flow cytometry and no alteration in cell viability was observed (data not shown). It was found a consistent Ac2-26 effect on lowering CCR5 mRNA expression in PBMCs from healthy donors. Because FPRs are the receptors involved in ANXA1 downstream signaling, we have also added a pre-incubation with BOC2 (FPRs pan-antagonist) prior to Ac2-26 stimulus to counteract its effect. However, no change was observed when BOC2 was used, suggesting that the ANXA1 peptide signaling used an FPR-independent pathway or FPRs may have different affinities to BOC2 (Fig. 1A).

The expression of CCR5 transcripts had a light and non-significant decrease in PBMCs of HAART-naïve HIV-1-infected patients in the presence of Ac2-26 peptide (Fig. 1C). In contrast, PBMCs responded to the addition of BOC2 prior to the stimulation with Ac2-26, showing a total reversion of CCR5 expression impairment caused by the ANXA1 peptide, with increased in the transcript levels when compared to non-treated samples (control group).

Taken together, these results show that Ac2-26 peptide reduces CCR5 mRNA expression on PBMCs, suggesting that Ac2-26 interferes with CCR5 expression by reducing gene transcription using different pathways according to infection status.

Ac2-26 down-regulates CCR5 surface expression on CD4+ T cells

Based on the previous transcriptional data, we have proceeded to investigate the CCR5 surface expression in T cell subsets. We aimed to analyze whether Ac2-26 affected CCR5 cell surface expression on CD4+ T cells from HAART-naive HIV-infected and healthy donors.

A multicolor T cell marker panel included CD3, CD4, CD8 and CCR5. Importantly, Ac2-26 treatment did not interfer with the immunophenotype pattern of the typical T cell markers (data not shown). However, the Ac-26 peptide induced a statistical significant reduction in the CCR5 cell surface expression in the CD4+ T cells, the main target cells of HIV-1 (Figure 1 D). In addition, a modest decrease on CCR5 expression was evident on the CD8+ T cell subset from healthy donors (%), but not from HIV-1 infected patients (%) (data not shown).

ANXA1 peptide role on CCR5 modulation of CD4+ T cells is associated to resistance to HIV-1 infection

In order to understand if ANXA1 peptide and BOC2 exert a different action on modulation of CCR5 expression according to the viral load and CD4 counts, which confer resistance to HIV-1 infection, we analized individually 3 patients with distinctive resistance status to HIV-1 infection (Figure 2). Our data showed that the LTNP (long term non-progressor) HAART-naive patient presented none response to these molecules, once the LTNPs only have a minimal amount of CCR5-expressing CD4+ T cells. However, the intermediate resistant and HIV-1-susceptible patients showed a viral load-proportional decrease of CCR5+CD4+ T cells under Ac2-26 treatment. This Ac2-26 effect was partially reverted by the

BOC2 addition, suggesting the involvement of FPRs, particularly FPR1, in the modulation of CCR5 expression.

Immunophenotype switch of monocyte subsets from CD14lowCD16+ to CD14highCD16- after Ac2-26 stimulation with increased CCR5 surface expression

Human monocytes can be classified based on differential expression levels of CD14 and CD16 (FcγRIII). The CD14highCD16– cells are described as "classical" monocytes (90–95% in a healthy person), and the "nonclassical" or "proinflammatory" monocytes are associated with the FcγRIII-positive (CD14lowCD16+) subpopulation [20]. Despite representing the minority of circulating monocytes, CD14lowCD16+ monocytes are an important cellular target for HIV-1 entry [21]. The distribution of these monocyte's subsets is different according to infection status, showing that there are more CD14lowCD16+ in HIV-1-positive peripheral blood (Fig. 3A). However, our data also showed that Ac2-26 peptide stimulus led to a phenotypic switch, promoting an expansion of CD14highCD16- and a reduction of CD14lowCD16+ toward (Fig. 3A, B), but not through FPR signaling (Fig. 3A-F) in HIV-1 negative and positive samples. It suggests a role of the ANXA1 peptide in control of the maturation of monocytes during the infection processes, independently of FPR receptor.

In agreement with the literature, the CCR5 expression was higher in both monocyte subsets from infected samples (Fig. 3G, H). Therefore, besides the reduced CCR5 expression in T cells of HIV-1 infected samples, mainly the infected monocytes' subsets presented a trend to up-regulate the CCR5 surface expression following Ac2-26 treatment (Fig. 3G, H). This led to the even higher increase in the difference of CCR5 levels between HIV-1 negative and positive (Fig. 3G) in CD14lowCD16- subset. Also, in the CD14hiCD16- population, the addition of BOC2 plus Ac2-26 contributed to a statistically significant increase of CCR5 expression in the immature monocytes from both infected and non-infected samples (Fig. 3H). It suggests that the ANXA1 peptide functions according to the cellular type, infection status and FPR conformation.

Discussion

This pioneer investigation demonstrates the effect of a homeostatic molecule, Annexin A1, which exogenously targets the formyl peptide receptor 2, and signals to a series of cellular events that culminate with differential expression of the main co-receptor for HIV entry, CCR5, in different substes of immune cells, besides provoking expansion and contraction of specific T cell and monocyte subsets, which may have important implication to the HIV infection and disease progression.

The mechanisms of CCR5 regulation is critical for understanding AIDS pathogenesis [22], and until now the role of ANXA1 peptide as a CCR5 modulator at transcriptional and protein level in the HIV targeted subsets of leukocytes had not been explored. Previous analyses of ANXA1 and its derived-peptides revealed their involvement in a broad range of biological activities, including the control of several genes, such as transcription factors and signaling molecules [14]. In agreement with these findings, we provide evidences that the Ac2-26 peptide selectively regulates the expression of CCR5 in HIV targeted cells as T CD4+ cells and monocytes. Ac2-26-treated PBMCs down-regulates CCR5 transcription compared to non-treated cells. This result could be explained by the fact that ANXA1 can be physically associated with transcription factors involved in the regulation of CCR5 expression, such as NF-κB [23] and CCAAT enhancers [24], suppressing their transcriptional activity by preventing their binding to DNA. Furthermore, Ac2-26 treatment of cancer cell lines has also showed inhibition of NF-κB and N-FAT signaling as well as regulation of MAPK, p38, PKA/cAMP downstream pathways [15,23,25,26], which are implicated in the CCR5 expression [27].

As a proof-of-concept, an FPR antagonist was used to compete concomitantly with Ac2-26 for its FPR receptor; however, the expected ANXA1 peptide inhibition did not occur in PBMC of healthy individuals, and CCR5 expression was continuously low, similar to what has been observed with ANXA1 peptide alone. Interestingly, the opposite result happened in HIV positive samples, and the expected inhibitory effect of Ac2-26 was observed, reverting positively the CCR5 expression. This result suggests that the FPR of HIV-1-infected cells may present conformational changes or differential surface density, since it is

considered an unsual co-receptor for the HIV/SIV gp120 [28], which can only sense the ANXA1 peptide if previously modified by the virus. These results reinforce the role of ANXA1 as a key homeostatic molecule in cell signaling, which seems to depend on its own cell surface recognition and its endogenous signaling. This means that if endogenous ANXA1 becomes dysfunctional, a proper external signal through the secreted cleaved peptide may not occur, dysregulating the downstream signaling. However, HIV-1 infection has enabled Ac2-26 to signal through its FPR, triggering downstream effects, such as CCR5 gene repression in infected leukocytes.

The major target of HIV-1 is the CD4+CCR5+ T cells. Our data have shown for the first time that Ac2-26 treatment leads to a significant decrease of CCR5-expressing CD4+ T cells, independently of infection status. In contrast, it has been shown that exogenous ANXA1 stimulation of CD3/CD28-activated T cells augmented CD25 and CD69 expression, with paralleled increase of NFκβ, NFAT, and AP-1 expression [15]. Despite in vitro studies have proposed that human Th1 cells favor expression of CCR5 [29], a recent study demonstrated that TCR triggering leads to decreased CCR5 surface expression in circulating CD4+ T cells from SIV-infected monkeys [30], which is consistent with our data that showed an inhibition effect of ANXA1 peptide on human T cells. Analysis of treated group BOC2+Ac2-26 versus Ac2-26 alone did not showed any significant difference, supposing that ANXA1 decreases CCR5 by distinct pathway rather than through FPR signaling. Nevertheless, when each sample was plotted individually in order to check the effect of ANXA1 peptide according to HIV-1 infection resistance. It was revealed that Ac2-26 effect on CCR5 surface expression on CD4+ T cells was partially FPR1-dependent when is associated to higher susceptibility to infection.

On the other hand, our data indicated that ANXA1 peptide played divergently on monocytes. Firstly, the results showed that Ac2-26 treatment changed the CD14 and CD16 pattern of surface expression in the monocytes in all samples, diminishing the CD14lowCD16+ population and expanding the CD14highCD16-. The presence of phenotypically definable subpopulations of human monocytes has been recognized for over 20 years [31]. Refined analysis using flow cytometry and migration assays has been showed that CD14lowCD16+ subset has a pro-

inflammatory profile, since it moves on normal endothelium of blood vessels, invades tissues rapidly upon tissue damage, and becomes tissue macrophages, while CD14highCD16- subset has an anti-inflammatory and immature profile, remaining in the peripheral blood [32,33]. Since monocytes are susceptible targets for HIV infection [34], and macrophages, their tissue counterparts, constitute an important reservoir of virus [35], this immunophenotype switch may be one more advantage attribute of the ANXA1 peptide treatment over these target cells.

The only, but critical, concern about monocyte response is due to the Ac2-26 treatment and the up-regulation of CCR5 surface expression, in contrast to CD4+ T cells. Both monocytes subsets presented a trended increase of the CCR5 in their surface, especially the ones that had received BOC2 previously, suggesting that the up-regulation of the CCR5 provoked by Ac2-26 can be also enhaced by FPR pathway. Here, it is important considering that there is an epigenetic hypothesis to explain the cell type specific regulation of CCR5 expression, which is supported by the fact of CCR5-expressing CD14+ cells show much higher levels of acetylated histone H3 and methylation of the *CCR5* promoter, which means activation of transcription, when compared to the non–CCR5-expressing naïve T cells [36]. This can be secondary to the association of CREB-1 with *CCR5* promoter chromatin of T cells [36]. Consistently, there is a bridge linking ANXA1 and this epigenetic control of CCR5, since CREB-1 is involved in mouse AnxA1 regulation by cAMP and glucocorticoids [37]. Similarly, there is another evidence about differential regulation of CCR5 in HIV target cells, which reveals that ethanol can also decreases CCR5 expression in CD4+ T cells [38], however it leads to increase of the receptor in macrophages [39], corroborating our results.

Briefly, we provide evidences for a new function for exogenous ANXA1 N-terminal peptide, suggesting that it acts as a “Dr. Jekyll and Mr. Hyde” type-molecule on modulation of the immunophenotype of critical leukocytes subsets to HIV-1 infection. To better elucidate the ANXA1 role in the AIDS disease, the next step of is to investigate the involvement of exogenous and endogenous ANXA1 as inhibitors/activators of HIV-1 entry pathways.

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Figure Legends *(inseridas individualmente junto às figuras)*

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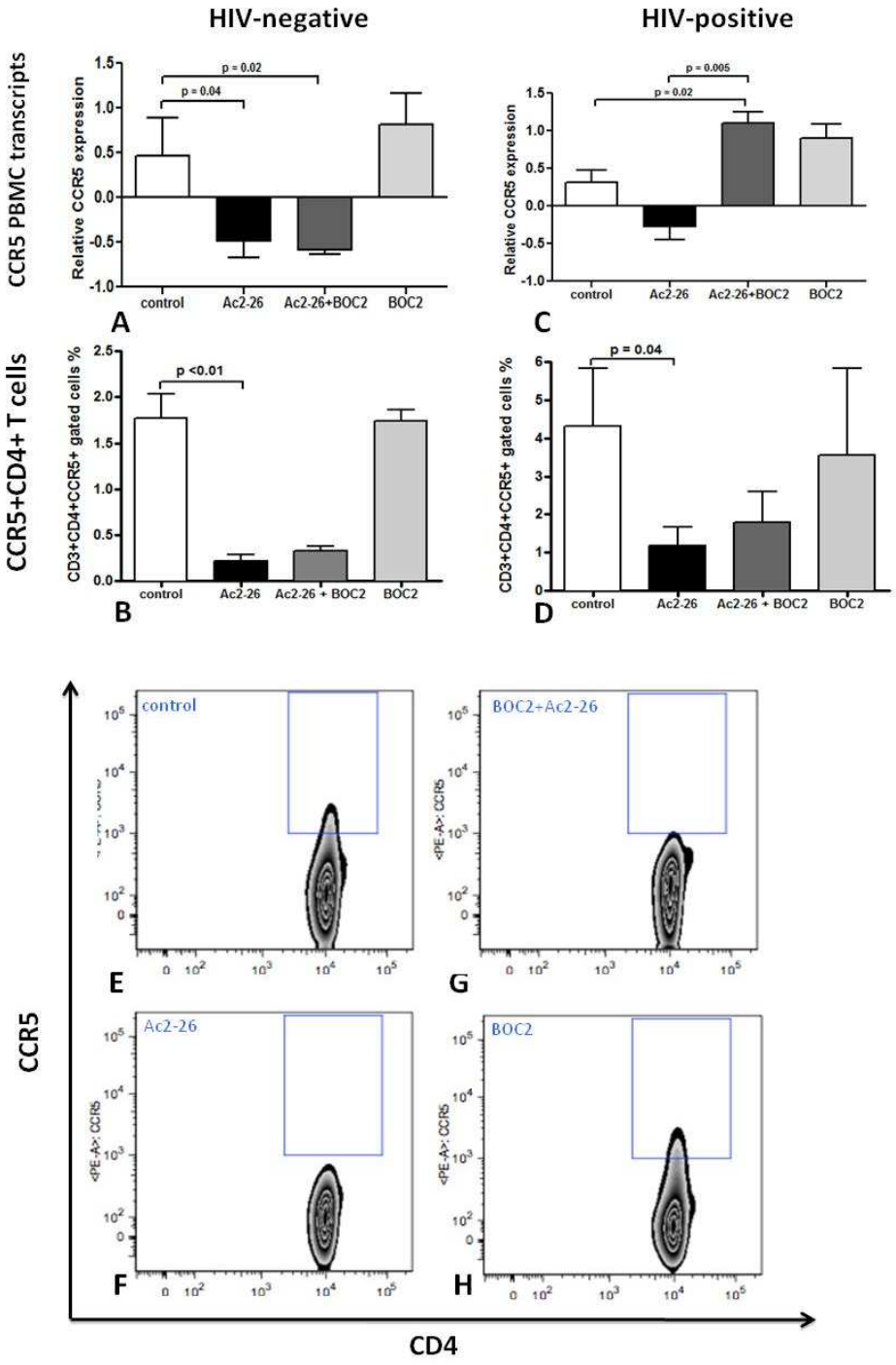

Figure 1. **Down-regulation of CCR5 expression in peripheral blood mononuclear cells by ANXA1 N-terminal peptide. A** and **C**: Relative expression of *CCR5* in PBMC from HIV-1-negative and HIV-1-positve samples, respectively. The transcription was assessed by real time PCR. **B** and **D**: Surface expression of CCR5 in peripheral blood CD3+CD4+ T cells expressed as percentage of positive cells, analyzed by flow cytometry. These data were represented by zebra plots in **E-H** figures. Cell groups were incubated for 3h with PBS (control), Ac2-26, BOC2 + Ac2-26 and BOC2. Results are expressed as means ± SEM. Values are significant at $p < 0.05$ (Student's t test).

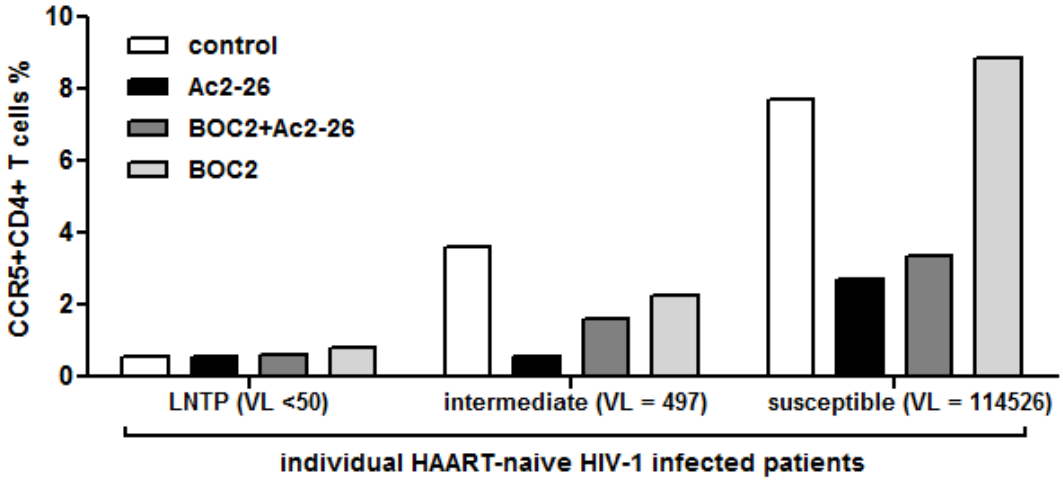

Figure 2. **Ac2-26 signaling and modulation of CCR5 expression in CD4+ T cells from HIV-1 infected peripheral blood according to viral load**. Three selected patients according to status of infection resistence represented as plasma viral load, showing different percentages of circulating CCR5+CD4+ T cells after treatment with PBS (control), Ac2-26, Ac2-26 + BOC2 and BOC2, as described. VL: number of copies/mL plasma.

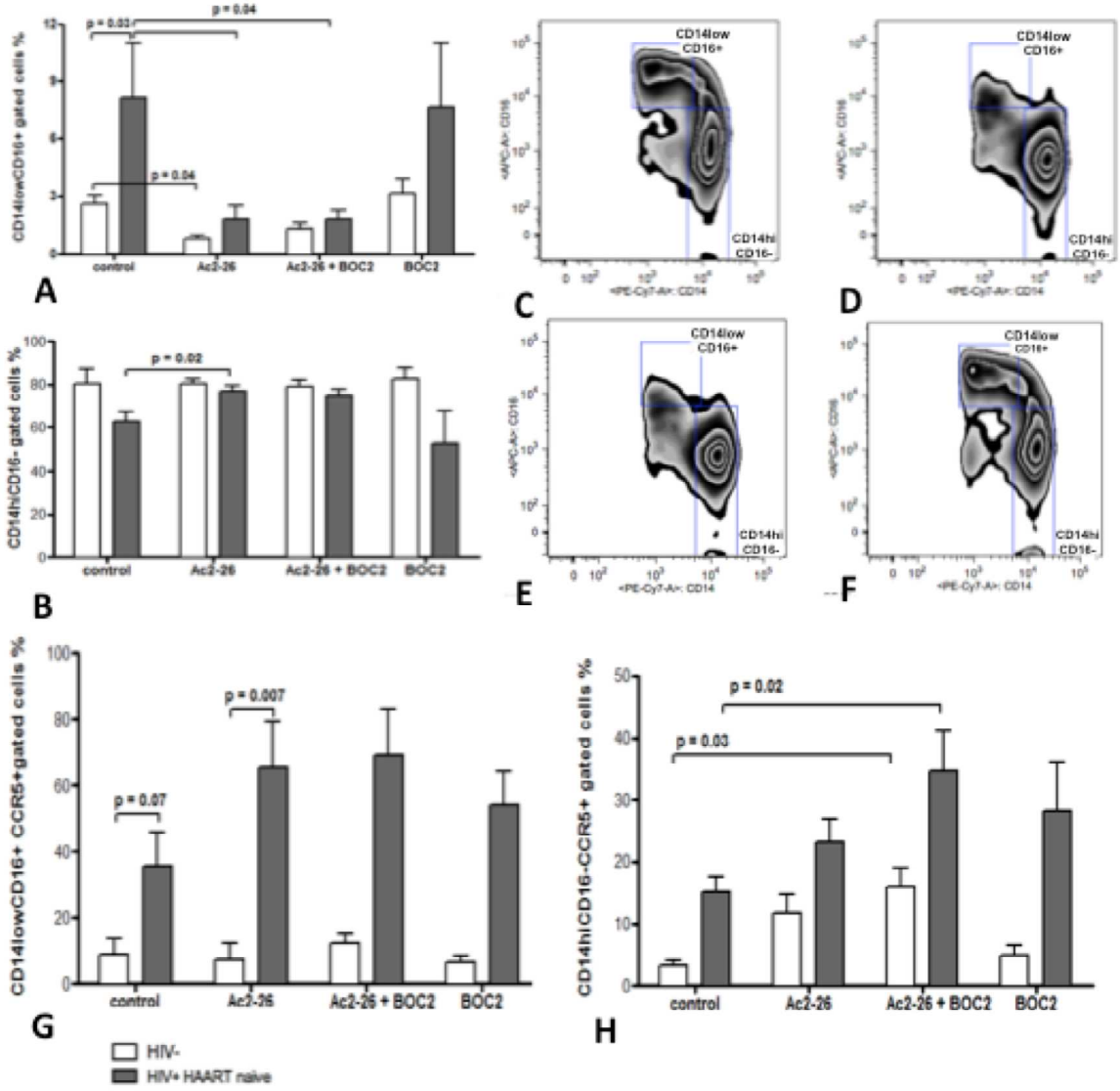

Figure 3. **immunophenotypic switch and up-regulation of CCR5 surface expression in monocyte subsets.** Changes in the distribution of the monocyte subsets (%) CD14lowCD16+ in **A,** and CD14highCD16- in **B** from HIV-1-negative and HIV-1-positve samples under treatment with PBS (control), Ac2-26, BOC2 + Ac2-26 and BOC2. These data were carried out by flow cytometry. **C – F:** Data are represented by zebra plots, indicating the regions of the monocyte subsets. Surface expression of CCR5 in of the monocyte subsets (%) CD14lowCD16+ in **G,** and CD14highCD16- in **H** expressed as percentage of positive cells, analyzed by flow cytometry. Results are expressed as means ± SEM. Values are significant at $p < 0.05$ (Student's t test).

CAPÍTULO III

Desequilíbrio da expressão de ANXA1 na infecção por SIV e progressão da doença: uma faca de dois gumes*

*Este capítulo está formatado como Artigo Original, de acordo com as normas do periódico *Plos Pathogens* (com algumas alterações para a Tese)

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Dysfunctional annexin A1 expression in SIV infection and disease progression: a double-edge sword

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Abstract

Two paradoxical hallmarks of HIV-1 infection are the systemic chronic immune activation and gut mucosal immune dysfunction, for which no single pathway has been associated with. The endogenous annexin A1 is considered a highly active mediator that counteracts systemic inflammation, but its role during HIV-1 infection has not been investigated, although its receptor has been implicated in the infection, once viral gp120 competes for the same binding site. Annexin A1 mRNA (ANXA1) expression was analyzed in both blood and gut of rhesus macaque under acute and chronic SIV infection, a model of AIDS. PBMC and jejunum samples from non-infected (12) and ART-naïve SIV-infected (53) colony-bred animals were collected, and ANXA1 expression was analyzed in association with other biomarkers by qPCR. Infected animals were i.v. inoculated with pathogenic SIVmac251 strain and euthanized during primary SIV infection (2wk; n=16), and chronic infection, at 10wk (n=22) and 27wk (n=15). The gut presented negative ANXA1 expression at 2wk and 10wk, while its expression in the blood during primary infection was kept in basal levels. However, both compartments had gradually higher expression levels during disease progression. The ANXA1 expression compared with plasma/gut viral loads and CD4 counts showed inverse dynamics during the infection course, and were negatively correlated with CCR5 in the blood. The immune activation, determined by HLA-DR in CD4+/CD8+ T cells of PBMC, revealed a positive correlation with ANXA1 expression. In contrast, the ANXA1 expression was negatively associated with TNF-α and IFNγ, and positively with IL-10 and TGFβ. Although ANXA1 over-expression in the blood seems to be associated with immune activation, its increase seems to confer resistance to the infection with an anti-inflammatory role, but does not protect CD4+ from apoptosis. However, it becomes deeply repressed at the gut mucosa, and may be a key modulator of both processes yet to be clarified.

Introduction

Despite progressive decline CD4+ T cells and plasma viral load [1], the virus inevitably establishes a chronic infection associated with a systemic immune activation [2]. However, the gastrointestinal tract is the major target site of HIV-1, and as virus replicates, a massive CD4+ T-cell depletion occurs [3]. Conversely to the systemic immune activation, the early loss of mucosal CD4+ T-cells induces a significant dysfunction of mucosal immunity, resulting in a critical damage to the gut tissue and consequently with impaired gut mucosa defenses, which is correlated with HIV progression [4]. Currently, due to the complexity of cellular events, no specific pathway has been able to establish a link between these two major hallmarks of HIV/SIV infection, the systemic immune activation and gut anergy.

There is no clear explanation for this immune discordance between compartments; however, we started looking for cell pathways that could be linked to the virus recognition and to inflammation as well, and surprisingly we identified a molecule that was a previously shown by our group that was differentially expressed in the gut of untreated SIV-infected macaques [5], the anti-inflammatory-related gene coding for Annexin A1 (ANXA1). ANXA1 is a 37 KDa protein known to be a downstream mediator of glucocorticoids, and a highly active mediator able to ensure a prompt resolution of inflammation [6,7,8,9].

The rationale that connects ANXA1 with both hallmarks, besides its differential modulation in the gut [5], is based on: i) the immune activation, characterized by an increased expression of typical inflammation markers (TNF-α, IL-6 and IL-1β, chemokines such as MIP-1α, MIP-1β and RANTES) in the activated phenotype observed in CD4+ and CD8+ T cells, B, NK and monocytes [2], and ANXA1 may function as an anti-inflammatory protein during the acute stage of inflammation and may also have an immediate inhibitory action on neutrophil emigration and macrophage and mast cell-mediator/cytokines production [10]; ii) the gut mucosal tissue disruption leads to immunological anergy as observed in the lamina propria of SIV-infected macaque tissues [11,12], and ANXA1 may play a protective role, as has been demonstrated in mucosa inflammation, including oral [13], nasal [14], lung [15] and gastrointestinal [16,17] damage, helping in the recovery of damaged tissues; and iii) the HIV-1 membrane

protein gp120 binds not only to CD4+ and CCR5 receptors for its cell entry [18], but also binds to formyl peptide receptors (FPRs) [19], competing for the same FPR binding site of ANXA1 [20].

We have used the simian immunodeficiency virus infection in rhesus macaques [21,22] a model of AIDS, in order to perform a thoroughly investigation in both peripheral blood and gut tissue, which would be more difficult to perform in humans. The transcription status of ANXA1 during the SIV infection and progression was investigated in both peripheral blood and gut tissue from SIV-infected and non-infected rhesus macaques at early, transitional and chronic stage of virus infection. In addition, we investigated ANXA1 association to viral load, CD4+ T cell counts, activated-T-cell subsets, and cytokines expression. We present evidences that link a dysfunctional ANXA1 expression to both microenvironments, which will be discussed.

Results

ANXA1 gene expression is differentially altered in peripheral blood and gut throughout of SIV infection

ANXA1 expression was assessed at both PBMC and gut (jejunum tissue) and samples were collected at similar time points: one day before SIV infection, 2, 10 and 27 weeks post-infection. PBMC represent an important sample for both initiating immune responses and as viral replication. Compared to uninfected control macaques, the systemic ANXA1 expression was up-regulated in the SIV-infected animals at all time points assessed, showing a significant difference at chronic stage of 27wk (fold change average [FC] = + 4.2, $p < 0.05$) as shown in Fig. 1A.

Whereas the ANXA1 mRNA levels in peripheral blood increases during SIV progression, in the jejunum tissue it was observed a striking reduction at early and acute-chronic transition stage of infection (FC = - 4.5) (Fig. 1B). These data was further contrasted by a previous microarray analysis [5] of ANXA1 expression in the gut from *Sooty Mangabey* macaques (natural SIV reservoirs), which showed no difference at 2 weeks post infection (fold change 1.1 ± 0.1 versus SIV negative animals) (data not published). At the later chronic stage in Rhesus group, ANXA1

transcripts are significantly returned to normal level of expression in the gut (27wk FC = + 0.5; p = 0.009 versus 2wk; p = 0.002 versus 10wk) (Fig. 1B). In summary, SIVMac25 infection leads to a differential systemic and mucosal modulation of ANXA1 expression.

Systemic ANXA1 transcripts present a similar expression profile to the immune activation biomarker HLA-DR in CD3+CD4+ and CD8+ blood cells from SIV-infected animals

It is well established that changes in HLA-DR expression on CD4+ T cells are correlated with changes on CD8+ T lymphocytes, with the presence of HIV antigen in the circulation, and with the disease stage [23]. We assessed the percentages of CD4+ and CD8+ T cells expressing HLA-DR through flow cytometry, and as expected, the number of HLA-DR+ T CD4+ and CD8+ cells increased at late chronic stage, when the immune activation is installed (Fig. 2A). Interestingly, ANXA1 expression in PBMC also presented a correlation with the cellular activation, revealing the same expression pattern of the HLA-DR in both cell types (Fig. 2B). However, the ANXA1 and HLA-DR positive correlation were discordant from the expected positive association with CD4 counts and viral loads in both compartments, which have shown contrary dynamics (Figures 4A, 4B).

The ANXA1 expression in the blood is increased during SIV infection, correlating positively with anti-inflammatory mediators and IL-6 signaling, and negatively with pro-inflammatory cytokines

Once increased ANXA1 expression can be considered as a biomarker of immune activation, the next step was the characterization of the immune response in the peripheral blood by analyzing mRNA expression of specific cytokines and signaling molecules by real-time PCR. Among those molecules, the pro-inflammatory cytokines IFN-γ, TNF-α, IL-17, IL-18, IL-22 (Th1 and Th17 responses); IL-6 with its signaling modulators STAT3 and SOCS3; and the anti-inflammatory cytokines IL-10 and TGF-β (Th2/Treg) were correlated to ANXA1

expression (Fig. 3) in order to verify if the ANXA1 could be playing a pro-inflammatory role.

Since ANXA1 mRNA expression tended to gradually increase from early (2 wk) to chronic stage of infection (10wk – 27wk), we were also expecting that the ANXA1 expression could be associated with high levels of pro-inflammatory cytokines. The pro-inflammatory cytokines, IL-18, IL-22 and IL-12, when associated to higher ANXA1 mRNA levels, presented a lower up-regulation when compared to uninfected animals (respectively, R = 0.49, p = 0.002; R = 0.46, p = 0.004; R = 0.40, p = 0.01). Changes in the expression of TNF-α, IFN-γ and IL-17 were less significant when associated with increased ANXA1 expression during SIV infection, although they have presented lower expression than in the acute stage, R = 0.36, p = 0.01; R = 0.36, p = 0.01; R = 0.26, p = 0.04; respectively) (Fig. 3A).

To further explore whether IL-6 signaling was also involved in this scenario, we assessed the expression of IL-6 and its regulatory molecules, STAT3 (activator) and SOCS3 (inhibitor), and compared to ANXA1 expression during SIV course. Coherently, the results showed strong positive correlations between ANXA1 mRNA and STAT3 (R = 0.60, p = 0.01), and IL-6 (R = 0.35, p = 0.02), while the expression of the SOCS3 (R = 0.60, p = 0.01) was negatively associated with ANXA1 transcripts (Fig. 3B).

We also investigated the anti-inflammatory cytokines expression, IL-10, and TGF-β, which presented increased expression throughout the disease progression, and a positive correlation with ANXA1 expression (R 0.44, p = 0.007; 0.38, p = 0.01; respectively) (Fig. 3C).

Despite the systemic anti-inflammatory profile, viral load and CD4+ T cell counts decreased as ANXA1 expression increased

Plasma viral load and CD4 counts presented opposite dynamics to the ANXA1 expression in PBMC, declining throughout the infection, while ANXA1, which is known to be anti-inflammatory, has gradually increased until the chronic stage (Fig. 4A). This results was further corroborated by a negative association between CCR5 and ANXA1 expression in PBMC, showing that the higher ANXA1

expression the lower the virus co-receptor (Fig. 4C), but not in the jejunum (data not shown). It was expected that the increased ANXA1 expression would counteract the systemic chronic activation, and could contribute to the CD4+T cells survival, but our results showed an inverse correlation, which means that the increased ANXA expression was assaociated with depletion of CD4+ T cells during disease progression (Fig. 4B).

ANXA1 expression in the gut also follows the anti-inflammatory cytokines' profile, but viral load and CD4+ T cell counts present an opposing dynamics to ANXA1 expression in both body compartments

Our previous studies have demonstrated that SIV infection can lead to impaired pro-inflammatory cytokine levels in the gut, as infection extends, leading to an immune dysfunction (data not published). Gene expression analysis were similar to that carried out for the peripheral blood; however, a parallel profile of the expression of IL-10, TGF-β, TNF-α and IFN-γ. ANXA1 expression decreased at the acute and transitional stages and presented a positive expression at the chronic stage. Divergently, pro-inflammatory cytokines presented higher levels in the acute stage, and diminished after 10 weeks of infection (Fig. 5A).

The viral load also decreased at 10wk and 27wk (Fig. 5B), consistently with the depletion of CD4 T cells in the gut mucosa (Fig. 5C), which were associated with increased ANXA1 expression in the jejunal cells (Fig. 5B) as disease progresses. Interestingly, when CD4 T cell counts was almost completely depleted at the chronic stage (around 10% of total CD3+ T cells), the ANXA1 expression returned to the basal levels (Fig. 5C).

The exogenous stimulation of PBMC and lamina propria lymphocytes from SIV-infected animal with ANXA1 N-terminal peptide induced both pro-inflammatory and anti-inflammatory responses

To investigate the role of the exogenous ANXA1 protein in the PBMC and lamina propria lymphocytes (LPL), we have stimulated cells with an N-terminal peptide of ANXA1 (Ac2-26), and cytokines' expression was assessed. The ANXA1 peptide induced IL-10, TGF-β, IL-6 and STAT3, but also led to increased TNF-α

and IFN-γ expression levels in PBMC from a SIV-infected animal at chronic stage of infection, when compared to an uninfected animal (Fig. 6A).

On the other hand, the LPLs stimulation by the ANXA1 peptide induced IFN-γ upregulation, suppressing all the others molecules, including IL-10, TGF-β, IL-6, STAT3 and TNF-α (Fig. 6B).

Discussion

The SIV infection and AIDS pathogenesis are intimately related to the activation state of the host immune system [24], and to the gut anergy [4], but these discordant events have not been explained. However, this is the first study that describes the ANXA1 expression in SIV infection in both compartments, which led us to propose that ANXA1 is dysfunctional in the peripheral blood and in the gut tissue, and is a probable link between systemic chronic activation and impaired gut responses.

Our study showed that ANXA1 expression the expression in the intestinal mucosa displays features that are distinct from those of the peripheral blood, suggesting the action of soluble HIV proteins, which are able to change the expression of glucocorticoid-regulated genes from monocytes/macrophages in the same direction as dexamethasone [25]. It also associated ANXA1 as an acute phase biomarker [26]. On the other hand, high throughput gene expression data indicated that mucosal CD4+ T-cell depletion is associated with downregulation of host genes involved in the mucosal growth and enterocyte function [5]. Thus, the deep repression of ANXA1 expression founded in the gut, mainly in initial and transitional stages of SIV infection, could be a consequence of the distinguishing rapid disruption of tissue integrity, since one of the major source of ANXA1 in the mucosa are the epithelial cells, especially the junctional epithelium, as seen in the oral mucosa [27]. Coherently, we have also observed a non-altered ANXA1 expression in the jejunum from natural SIV reservoirs (sooty mangabeys), which presented a mucosal CD4 depletion post-infection [28], but not tissue damage (data not published).

The constant expression of the systemic ANXA1 along the course of the SIV infection directed our efforts toward investigating its association with the typical

immune activation described during AIDS progression. HLA-DR+ T cells has been used as a common biomarker associated with the presence of systemic immune activation in HIV/AIDS [29,30,31], and surprisingly, our data have revealed a correlation between kinetics of HLA-DR+ T cell immunophenotype and higher ANXA1 transcription levels. We can argue that ANXA1 expression is upregulated in Th cells following activation and differentiation and [7], playing a homeostatic regulatory role in mature T cells by modulating the strength of TCR signaling via increasing levels of transcription factors such as AP-1, NF-kB and NFAT [32]. In pathological conditions, the increased expression of endogenous Anx-A1 might contribute to the basal hyperactivated state of these cells and to the increase of transcription factors that play a key role in the regulation of the expression of several inflammatory genes [10]. Consequently, at SIV/HIV infection, the augment of ANXA1 expression would provide the T cells with a positive modulator of TCR signaling and thus prevent immunosuppression, or could simply be triggered due to its potent anti-inflammatory role to counteract the active systemic inflammation. Paradoxically, since HIV replicates most efficiently in activated CD4 T lymphocytes [33], the chronic activation of the immune system seems to be important for maintaining viral replication by rendering the cells competent for viral replication, which is closely correlated to immune exhaustion, followed by a massive depletion of CD4+ T cells and disease progression [34,35]. Consistently, the present data also indicated a strong negative correlation between AnxA1 transcripts and CD4 counts during chronic stage of SIV infection (26 wks), showing less circulating CD4 T cells in animal with higher systemic AnxA1 expression. As a final and coherent point, it has been reported that ANXA1 and its peptides present in a conditioned medium from Jurkat T lymphocytes promote macrophage phagocytosis of apoptotic leukocytes [36], which suggests that ANXA1 may have a role in the CD4+ T cell apoptosis as a clearance modulator attempting to avoid further activation.

Although ANXA1 expression seems to be associated with T cell activation, we found that a significant negative correlation of ANXA1 and pro-inflammatory cytokines transcript levels in the PBMC from SIVmac25-infected animals. Contrarily to what was expected for T-cell activation, with presence of pro-inflammatory cytokines, our current data revealed that higher levels of ANXA1

expression may in fact contribute to lower transcription levels of those cytokines, promoting a conversion from Th1 to a Th2 response that leads to a defective T-cell activation, and consequently to disease progression, corroborated by an extensive literature reviewed elsewhere [37]. Also, in agreement with our results, the lack of ANXA1 expression in LPS-stimulated macrophages in knockout mice presented an over-secretion of TNF-α [38] and an in vitro assay showed that ANXA1-peptide Ac2-26 inhibited PPD-induced IFN-γ release from human PBMC in a concentration-dependent manner [39]. Also, another in vitro experiment demonstrated that glucocorticoid, the regulatory molecule of ANXA1 expression, partially suppressed the production of IL-18 in LPS/IL-2-stimulated human PBMC [40].

Unexpectedly, our data revealed a significant positive correlation between ANXA1 and IL-6 expression. To further support this result, the IL-6 signaling pathway was also investigated, and we could confirm that the transcription factor STAT3 was up-regulated and the suppressor of cytokine signaling 3 (SOCS3) was down-regulated in the blood of animals with a higher amount of ANXA1 transcripts. Nevertheless, these findings could be explained by the mechanism described in LPS-stimulated macrophages, in which the lack of SOCS3 expression converts IL-6 into induce an IL-10-like anti-inflammatory response [41].

Besides the negative correlation between ANXA1 and pro-inflammatory transcripts, we also showed that ANXA1 was also positively associated with systemic suppressors IL-10 and TGF-β expression. Previous study has shown that recombinant ANXA1 stimulated IL-10 release and inhibited of IL-12 mRNA in LPS-stimulated macrophages [42]. Our data on systemic IL-6, IL-10 and TGF-β together corroborate a previous study that demonstrated failure in pro-inflammatory chemokines up-regulation when T cells were stimulated with TGF-β and IL-6, which turned them as IL-10 producing cell with a potent anti-inflammatory activity [43], restraining an essential Th17 response to SIV infection. Other studies have also suggested that HIV-1 disease progression is associated with increasing secretion of IL-10 [44,45] and IL-10 mRNA levels are up-regulated in multiple PBMC subsets in HIV infected subjects compared to HIV-negative controls, particularly in T, B, and NK cells, although monocytes are a major source of this transcripts. These results indicate that multiple cell types contribute to IL-

10-mediated immune suppression in the presence of uncontrolled HIV viremia [46].

Our data also showed an opposite kinetics between plasma viral load and ANXA1 expression during SIV infection course and also this result was supported by a negative correlation with systemic CCR5 expression. However, while the viral fitness is similar in macaques and sooty mangabeys, the viral pathogenicity clearly differs between the two species. This difference appears to be mediated by reduced levels of immune activation and T cell turnover in the sooty mangabeys, as reviewed elsewhere [47].

Altogether, the peripheral blood data indicates that the typical anti-inflammatory function of ANXA1 observed in the SIV-infected animals might be due to specific transcriptional regulation on monocytes, the most abundant cell type. Interestingly, the presence of exogenous ANXA1 promotes the differentiation of IL-2- and IFN-γ-producing Th1 cells and suppresses the production of the Th2-type cytokines IL-4 and IL-13 [32], suggesting that the endogenous ANXA1 signaling pathway may be dysfunctional. In order to demonstrate this hypothesis, we have stimulated PBMC lymphocytes from SIV+ and SIV- rhesus macaques with an exogenous ANXA1 peptide. We demonstrated that an overload of ANXA1 in the blood may switch the immune response from anti-inflammatory to an inflammatory profile, characterized by elevated levels of TNF-α, IFN-γ and IL-6 expression. In these conditions, the presence of Tregs in a pro-inflammatory cytokine milieu may result in differentiation of naive CD4+ T cells to IL-17 production [Veldhoen M. et al. Immunity 24, 179–189, February 2006], which has an important role on SIV-infected CD4+ T IL-17 producing cells, found in very low frequency at mucosal and systemic sites [48].

On the other hand, at the jejunum compartment, a down-regulation of ANXA1 expression at acute and transitional stages was followed by high expression of TNF-α and IFN-γ, which may be linked to the tissue injury. The concurrent up-regulation of pro-inflammatory genes and down-regulation of ANXA1 may cause tissue damage due to excessive inflammation without a counteracting anti-inflammatory response, which also disrupts the gut regenerative capacity, resulting in loss of mucosal integrity. This injury may trigger an enhanced local inflammatory

reaction in the acute stage [5,49] and anergy in the chronic stage, due to the lack of sufficient CD4+ T cells [50].

As ANXA1 expression increased during SIV disease progression approaching basal levels at the chronic stage (27wk), a decreased transcript levels of the pro-inflammatory cytokines were followed by a gradual increase in IL-10 and TGF-β. The lack of ANXA1 expression at early stages of SIV infection may be a critical event that may have contributed to the lack of proper immune response leading to tissue injury; however, with its increase late in infection, with a predominant anti-inflammatory response the gut tissue advanced to a chronic and anergic phase, causing a progressive mucosal immune dysfunction, a key feature of the HIV/SIV-associated immune deficiency [4]. Again, looking back into our hypothesis of a dyfunctional endogenous ANXA1, we have also stimulated lamina propria lymphocytes with an exogenous ANXA1 peptide, and surprisingly, the anti-inflammatory cytokines (IL-10 and TGF-β) were inhibited followed by a significant increase in IFN-γ expression. The ability of exogenous ANXA1 peptide to shape an IFN-γ response in gut lymphocytes, and to restrain the acute phase mediators, IL-10 and TGF-β, support the notion of a possible recovery of the gut immune response, converting it from an anergic state to Th1-type response, but emphasizes even further that the endogenous ANXA1 may be dysfunctional, and the causative mechanism of this event must still be determined.

Although the exact cellular and molecular mechanisms underlying the complex interaction between HIV/SIV and the primate mucosal immune system are still poorly understood. Then, it is worth mentioning the biological outcomes reported for ANXA1 in epithelial cells, such as cell adhesion and actin remodeling [51,52,53,54,55], protective effect against tissue disruption in ischemia/reperfusion [56] and inflammatory bowel disease models [17], and respiratory and oral mucosal pathologies [14,51].

Our results pose a new cutting-edge challenge for the comprehension of the SIV/HIV infection and disease progression, and we are now focusing on pathways that are triggered by ANXA1 in order to demonstrate its pivotal role as a homeostatic molecule, linking the two major hallmarks of the disease; systemic chronic activation and gut immune dysfunction. Our data further reinforce the importance of understanding the crosstalk between systemic and mucosal

immunity, which are linked by ANXA1, a molecule that acts like a double-edged sword in both processes.

Material and Methods

Animals and Samples

Animals (65 colony-bred rhesus monkeys - Macaca mulatta) were housed in accordance with American Association for Accreditation of Laboratory Animal Care guidelines. Samples from 12 healthy, SIV-negative animals served as negative controls. Fifty-three animals were i.v. inoculated with animal infectious doses of pathogenic SIVmac251 and were euthanized during primary SIV infection (2wk) (n = 16), chronic infection (10wk) (n = 22), or advanced infection with simian AIDS (26wk) (SAIDS; n = 15). Longitudinal jejunal biopsy samples were collected by upper endoscopy and peripheral blood samples by venipuncture. Peripheral blood and intestinal tissue samples were isolated and evaluated as described previously.

Gene expression and Viral load

Total RNA from PBMC and jejunal biopsy samples were isolated using Qiagen RNeasy Plus Mini Kit in accordance with the manufacturer's protocol. Then, real-time PCR analysis was chosen to determine viral burden and changes in host gene expression. Briefly, primers and probes specific to the SIV RNA sequence or to human gene sequences for ANXA1, CCR5, STAT3, SOCS3, IL10, TGFβ, TNF-α, IFNγ, IL-6, IL-12, IL-17, IL-18, IL-22 were designed or ordered and used in real-time PCRs as previously described [59]. Fluorescent signal was detected with an ABI Prism 7700 sequence detector (PE Applied Biosystems). Data were captured and analyzed with Sequence Detector software (SDS). Viral copy number in PBMC was determined by plotting CT values (the fractional cycle number at which the fluorescence passes the fixed threshold for detection) against a standard curve derived from samples with known SIV copy numbers. In jejunal tissue samples, relative SIV viral RNA loads were calculated utilizing internal normalization of CT values to the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and/or CCR5 mRNA. For host gene expression studies, internal normalization of CT values was performed based on the housekeeping gene

GAPDH, and differential gene expression was calculated by comparison of mean CT values obtained from SIV-infected groups to the mean CT values from four healthy uninfected controls.

Flow cytometry

To immunophenotype cells, rhesus-compatible human antibodies to CD3 (BD Biosciences, clone SP-34-2, San Jose, CA), CD4 (Ebioscience, clone OKT4, San Diego, CA), CD8 (Invitrogen, clone RPA-T8) and HLA-DR (Biolegend, clone L243) were used. To test for viability, dead cells were stained with Amcyan LIVE amine dye (Invitrogen, Carlsbad, CA). Multicolor immunophenotyping of cells was performed on a modified BD LSRII with a minimum of 1,000,000 events collected. Flow cytometric analysis was performed using FlowJo version 7.3 (Treestar, Stanford, CA).

In vitro ANXA1 peptide exogenous stimulus

Annexin-1 mimetic peptide Ac2-26 (GenScript, USA) was used to stimulate primary isolate PBMCs and LPLs from SIV infected and uninfected Rhesus Macaques. Firstly, PBMC and LPL were isolated as described previously and rest at 37C for 1 hour in complete RPMI. Then, after spin down those cells separately at 1800rpm, they were aliquoted as $1x10^5$ cells and added per 100ul well (in a 100uM Ac2-26 solution in experimental wells or medium in control wells), in duplicate, and Incubated at 37C for 30 minutes. When the stimulus was done, cells were pipetted cells into 1.5ml tubes, spin down at 1800rpm, so the medium was discarded and the cells were cryo and stored at -80C until RNA extraction. Gene expression was carried out as described in this Material and Methods section.

Statistical Analysis

Comparison between two animal groups or different tissues was performed using two-tailed Mann-Whitney's U-test. Using the Pearson Test it was performed

correlation analysis among variables. For correlations including the PBMC and jejunum tissue viral load, we used the individual viral RNA copy numbers per ml plasma of each animal at the respective point in time when the assay was performed. For all tests were used the GraphPad Prism software, version 5 (Graph- Pad Software, San Diego, USA). Data was considered as statistically significant when $p < 0.05$.

Acknowledgements

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Figure Legends ***(inseridas individualmente junto às figuras)***

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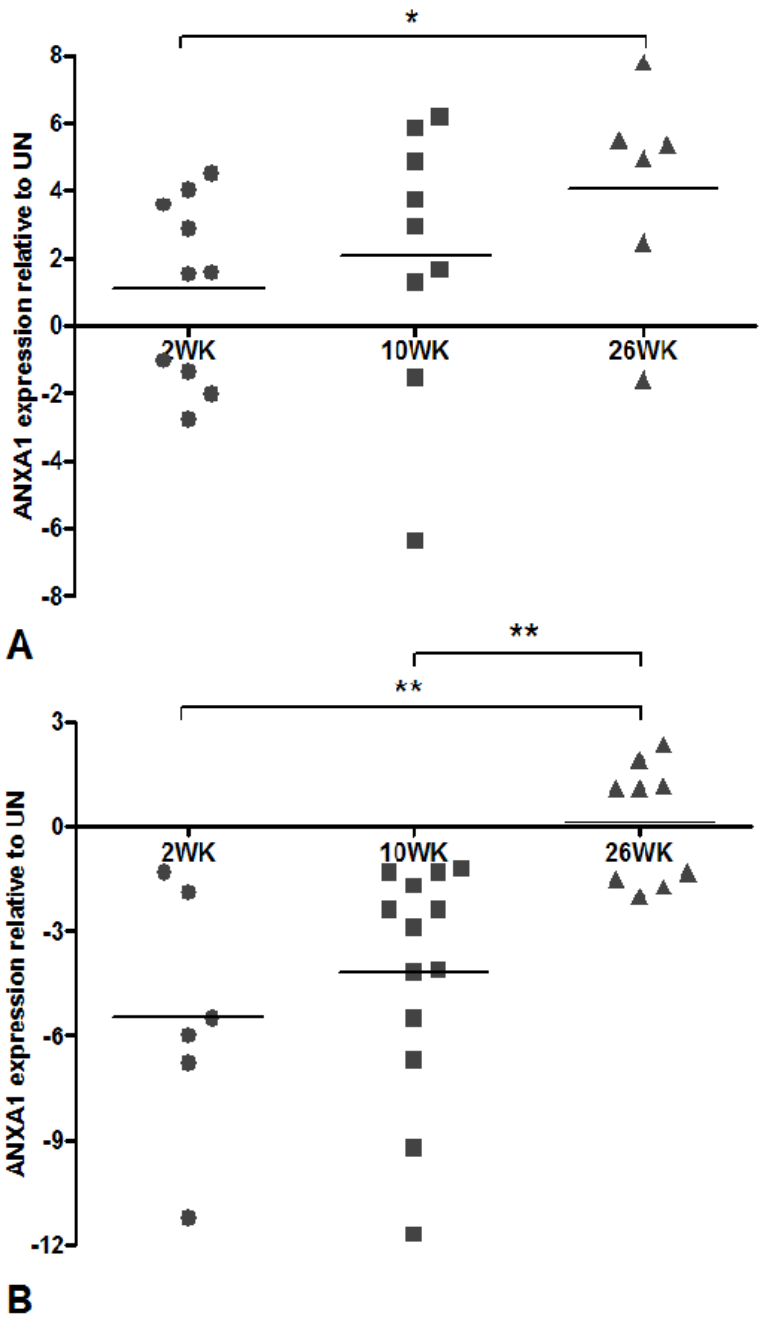

Figure 1. **Differential ANXA1 expression in blood and gut throughout SIV infection.** Relative expression of ANXA1 in PBMCs (A) and jejunum biopsy (B) after 2, 10 and 26 weeks of inoculation of Rhesus monkeys with pathogenic doses of SIVMac225. Data are presented as fold change of infected versus uninfected animal per dot, with a line representing the mean of the expression's group. Statistical analysis: P values were obtained on a per group basis (*) using the Mann-Whitney nonparametric test (when comparing different time points). *p value<0.05; ** p value<0.01.

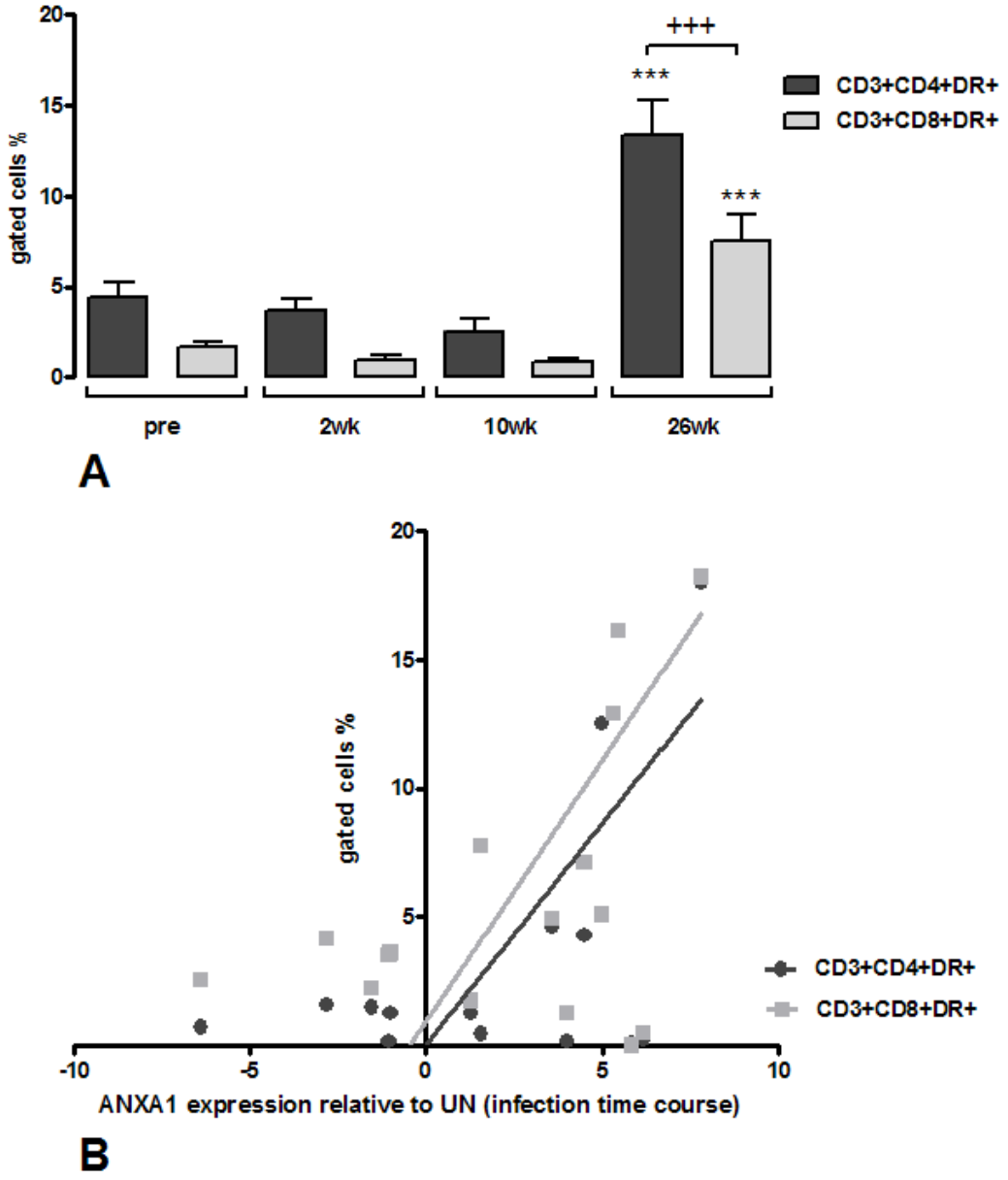

Figure 2. **Increasing systemic T cell activation correlate with ANXA1 expression.** (A) Percentual of cells positive for HLA-DR amongst total CD4+ and CD8+ T cells from peripheral blood (PBMCs) obtained at the indicated times before and after SIV infection (2, 10, 26 weeks). (B) Relationship of ANXA1 relative expression to the degree of systemic immune activation, as measured by percentage of the activated CD3+CD4+DR+ (R = 0.49) and CD3+CD8+DR+ (R = 0.47) T cells in the peripheral blood along the course of infection. In (A) data are represented as mean ± SEM. Statistical analysis: P values were obtained on a per group basis (*) using the Mann-Whitney nonparametric test (when comparing different time points).*** p value<0.001 vs early times before and after infection. +++ p value<0.001. In (B) Spearman's rank test was used to determine correlations between variables. Both correlations presented p value<0.05

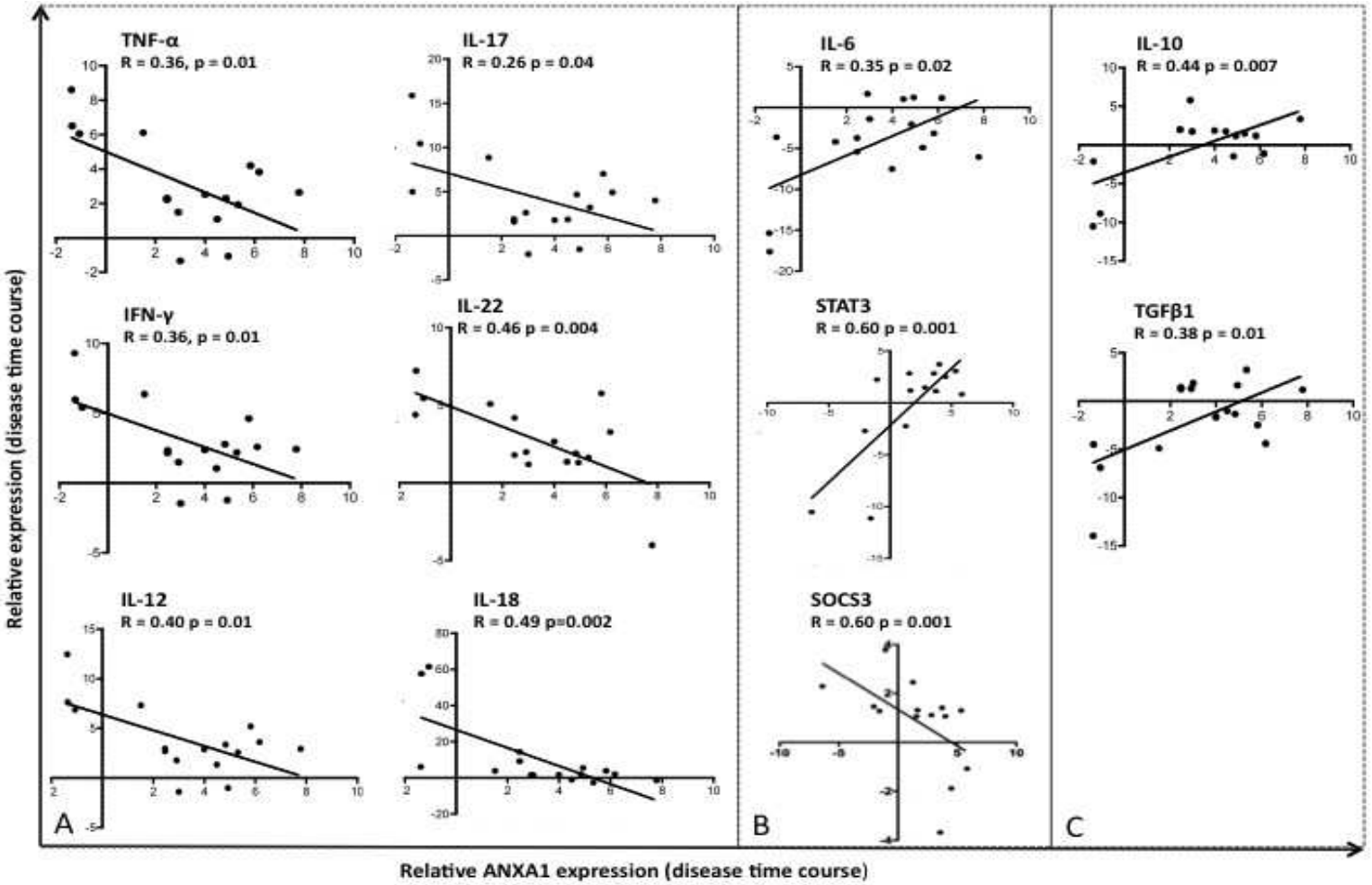

Figure 3. **Systemic ANXA1 expression is associated to anti-inflammatory profile of cytokines, but also with IL-6 signaling**. (B) Relationship of ANXA1 relative expression to the degree of systemic immune activation, as measured by percentage of the activated CD3+CD4+DR+ (R = 0.49) and CD3+CD8+DR+ (R = 0.47) T cells in the peripheral blood along the course of infection. In (A) data are represented as mean ± SEM. Statistical analysis: P values were obtained on a per group basis (*) using the Mann-Whitney nonparametric test (when comparing different time points).*** p value<0.001 versus early times before and after infection. +++ p value<0.001. In (B) Spearman's rank test was used to determine correlations between variables. Both correlations presented p value<0.05.

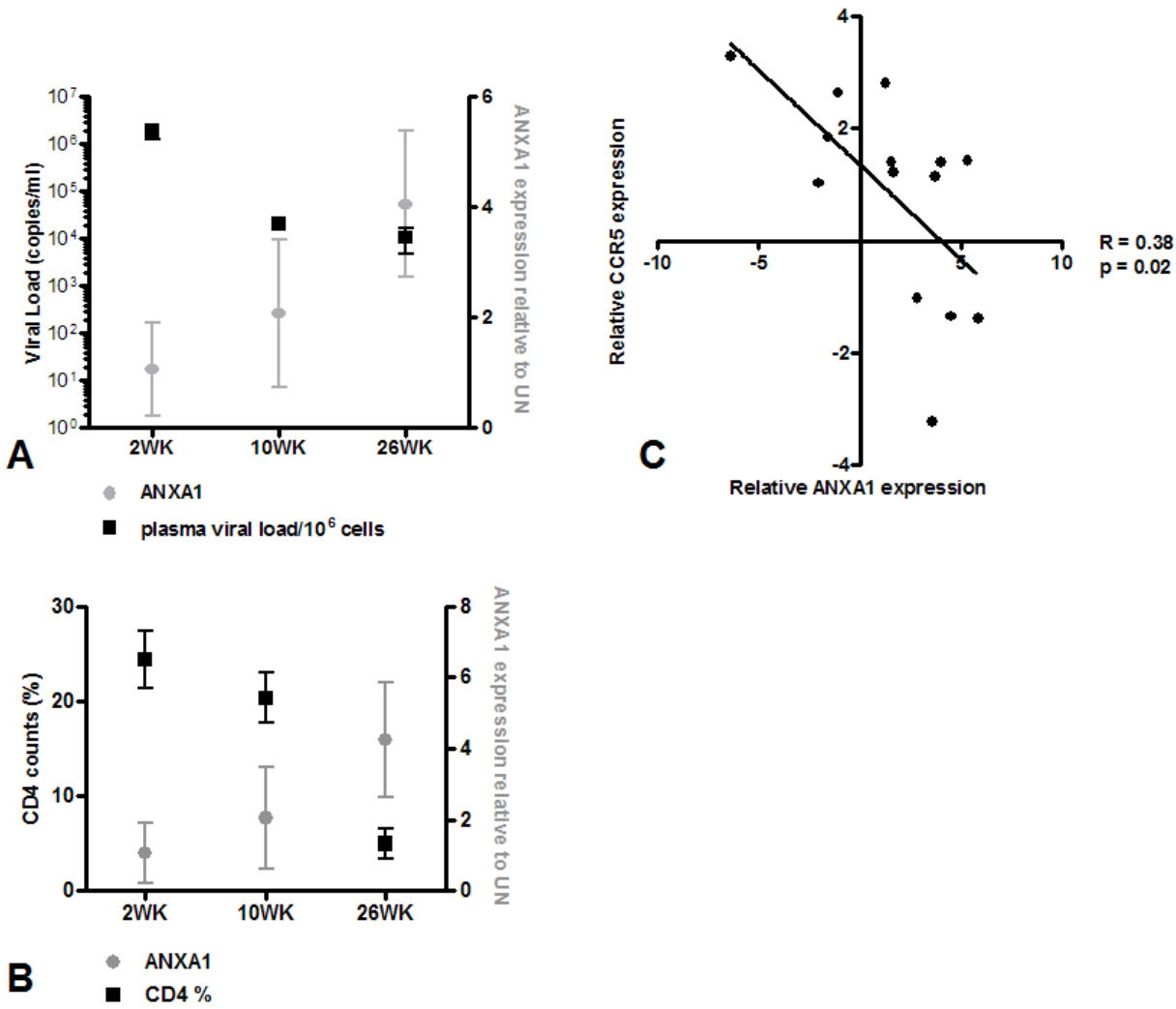

Figure 4. **Plasma viral load, CCR5 and CD4 T cell counts correlate to ANXA expression in the blood.** (A) Kinetics of plasma viral load and relative ANXA1 expression in PBMCs obtained at the indicated times after SIV infection (2, 10, 26 weeks). (B) Kinetics of CD4+ T cell counts (%) and relative ANXA1 expression in the peripheral blood as a function of time after infection. (C) Correlation between the relative CCR5 and ANXA1 expression in PBMCs. In (A; B) data are presented as mean ± SEM. Spearman's rank test was used to determine correlation between variables, which presented a moderate positive R = 0.38, p value<0.05.

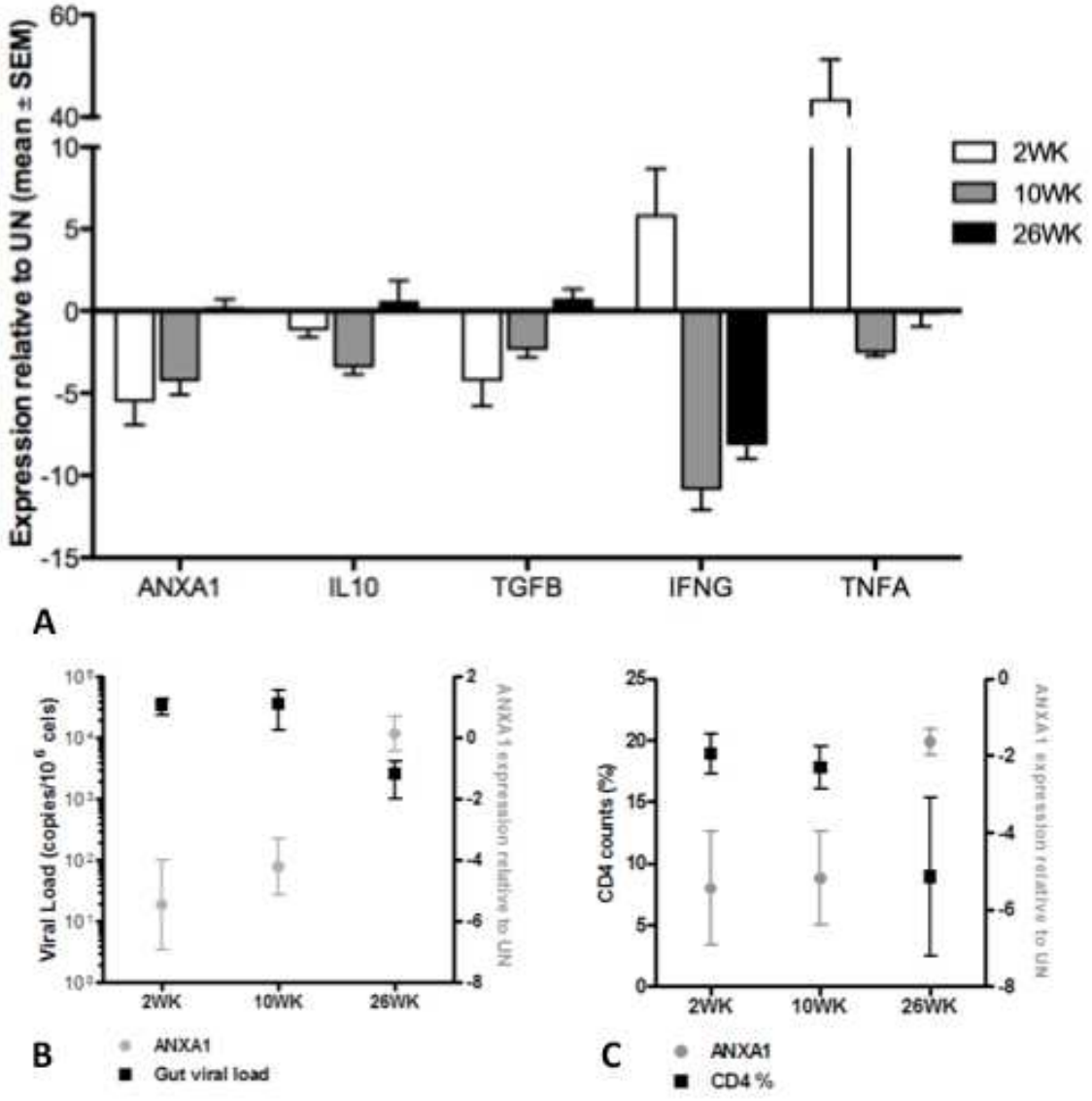

Figure 5. **Anti-inflammatory profile of ANXA1 expression in the gut is sustained, but is inversely correlated to viral load and CD4 T cell counts.** Profile of relative expression of the anti-inflammatory (IL-10, TGF-β) and pro-inflammatory (TNF-α, IFN-γ, IL-18) molecules compared to ANXA1 in the intestinal mucosa along the course of SIV infection. (B) Kinetics of gut viral load and relative ANXA1 expression in the jejunum tissue obtained at the indicated times after SIV infection (2, 10, 26 weeks). (C) Kinetics of CD4+ T cell counts (%) and relative ANXA1 expression in the jejunum tissue as a function of time after infection. Data are presented as mean ± SEM. Statistical analysis: P values were obtained on a per group basis (*) using the Mann-Whitney nonparametric test. *p value<0.05; ** p value<0.01; *** p value<0.001.

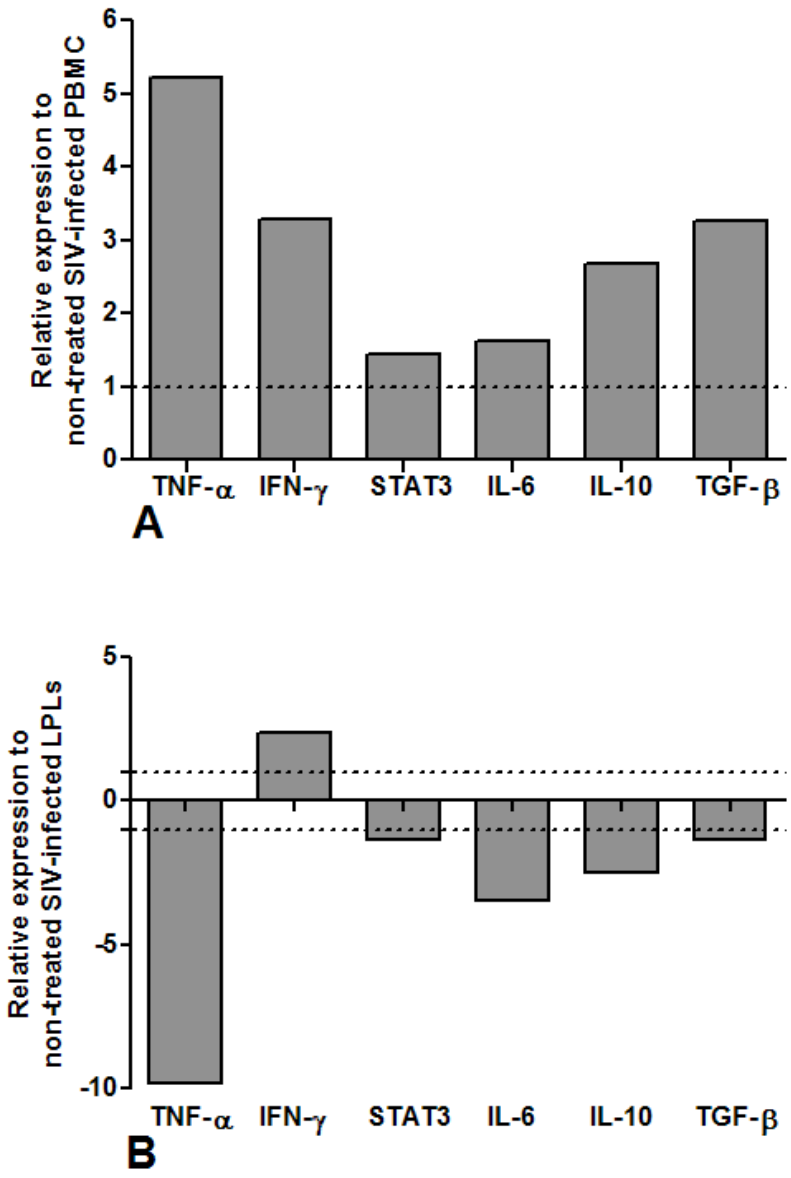

Figure 6. **ANXA1 N-terminal peptide addition switches the balance between pro and anti-inflammatory molecules in blood and gut lymphocytes.** Profile of relative expression of the anti-inflammatory (IL-10, TGF-β) and pro-inflammatory (TNF-α, IFN-γ, IL-6, STAT3) molecules in PBMCs (A) and LPLs (B) under exogenous stimulus of ANXA1 peptide (Ac2-26).

CAPÍTULO IV

A perda severa da expressão da ANXA1 é uma alteração molecular relacionada às IBDs e se reflete no processo de imunoativação sistêmica exacerbada pela terapia imunossupressora prolongada*

*Este capitulo está formatado como *Original Article*, de acordo com as normas do periódico

Inflammatory Bowel Diseases (com algumas alterações para a Tese).

Decreased expression of ANXA1 is an early dysfunctional outcome of IBD mirrored by a systemic immune activation that is exacerbated by continuous immunosuppressive therapy

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Abstract

There is no defined mechanism that elucidated the development of the inflammatory bowel disease (IBD), which prevents further improvement of appropriate therapeutic strategies. Currently, the neutralization of pro-inflammatory effectors is the treatment of choice for IBD, which is also a common procedure in autoimmune diseases. Multiple biomarkers have been used for IBD diagnosis, but none of them has successfully explained the disease pathogenesis. We are reporting for the first time the complete loss of the anti-inflammatory protein, annexin A1 (ANXA1), in the colonic mucosa of IBD patients, evidenced by confocal microscopy with an anti-ANXA1, an event that is mirrored by the ANXA1 mRNA expression in the peripheral blood, quantified by qPCR. Our data also showed that ANXA1 expression is upregulated at early infliximab (IFX) therapy, a TNF-α−specific antibody, reflecting a successful intervention. But, ANXA1 expression becomes downregulated with continuous therapy, which may be associated with its complete loss at the colonic mucosa. In parallel to the downregulation of ANXA1 expression, the TNF-α expression was increasingly higher after therapy, reflecting a dysfunctional mucosal barrier that led to increased plasma bacterial 16S levels and reduced ANXA1 expression. The IFX therapy also modified the cellular immune activation in the blood of IBD patients. In conclusion, ANXA1 seems to play an important role in IBD, which together with TNF-α, bacteremia and cell activation may explain the disease outcome and indicate the effectiveness of the therapy. However, it also suggests ANXA1 as a potential target for alternative therapies, aiming its recovery at the colonic mucosa.

Introduction

Inflammatory bowel disease [1] is a debilitating disease, characterized by severe inflammation of the gastrointestinal tract, often leading to physical symptoms, such as abdominal pain and recurrent diarrhea, adversely affecting an individual's quality of life [2, 3]. The natural history of IBD varies among patients, but if left untreated, may lead to significant complications, such intestinal hemorrhage, toxic megacolon, abscess formation, and fistula/stricture disease. One of the proposed mechanisms of IBD is the breakdown of the gut homeostasis, triggered as a consequence of dysregulated mucosal immunity [4, 5, 3]. Several hundred genetic defects are involved in the pathogenesis of IBD. Both human and murine studies support the critical roles genetic defects in innate immunity and aberrantly activated T cell subpopulations play in IBD [6, 7]. Additionally, an in vivo murine model study suggests that IBD symptoms may be due to TNF-α-induced intestinal T cell activation [8].

In the last decade, antibodies have been specifically used as tumor necrosis factor inhibitors (anti-TNF-α agents) have been demonstrated to be effective in IBD therapy. One of these agents, infliximab (IFX), is currently recommended by the National Institute for Health and Clinical Excellence (NICE; 2002) for patients with Crohn's disease who have severe disease activity, refractory to or intolerant of conventional treatment [9-13]. IFX, as well as other biologic therapy, has tremendously enriched IBD therapy as well as that of other immune mediated diseases [14-16]. Although the efficacy of IFX has undoubtedly been proven, there are numerous safety concerns with its use [17]. Among the side effects, increase in opportunistic infections has been observed in patients treated with TNF-α antagonists. This is consistent with current knowledge of the physiological actions of TNF-α and its role in combating infections [18]. IFX has been shown to produce early changes in certain blood proteins and epithelial (cell) expression profiles, which may assist with predicting clinical response to IFX treatment [19]. Currently, there is an increasing interest in the discovery of new IBD biomarkers that would aid in the diagnosis, treatment, and prognosis of IBD [20]. The establishment of clinical response predictors, however, would further help clinicians determine the benefits and risks of initiating IFX therapy on an individual basis [21, 19, 22]. Identification of factors that may play a significant role in IBD pathogenesis provides not only

potential biomarkers that can be monitored and assessed during treatment, but they may also be novel therapeutic targets for the treatment of IBD.

Annexin A1 (ANXA1) is an anti-inflammatory factor whose expression is decreased in the subcellular fraction of intestinal epithelial cells isolated from patients with Ulcerative colitis compared to patients with Crohn's disease and healthy controls [20]. ANXA1 is a 37kDa calcium-dependent phospholipid binding protein, originally reported as glucocorticoid-induced protein with anti-phospholipase activity [23-25]. ANXA1 has subsequently been shown to regulate diverse cellular functions in a variety of cell types. More importantly, ANXA1 exhibits profound inhibitory actions on leukocyte transmigration and activation, leading to the resolution of inflammation [26-29]. Its protective and anti-inflammatory role has been demonstrated in models of endotoxemia, peritonitis, arthritis, as well as cerebral and myocardial ischemia [30-37]. Studies have also demonstrated the role of ANXA1 in wound healing, especially in mucosal injury following intestinal inflammation [38, 39]. In agreement with the above data, other studies have identified a role for ANXA1 in promoting healing of indomethacin-induced gastric ulcers [39] and preventing the intestine mucosal injury in the murine model [40].

This study was designed to investigate the systemic effects of intestinal inflammation during the course of IBD treatment. The study focuses on the systemic and local ANXA1 expression as an indicator or predictor of response to infliximab therapy. An association was found between IBD, response to therapy, ANXA1, TNF-α expression, and lymphocyte activation in peripheral blood, and surprisingly we also report the complete loss of ANXA1 in colonic tissues from chronic IBD patients, demonstrating that ANXA1 may be a involved a potential mechanism of the immune dysfunction.

Material and Methods

Study Participants

Study participants were enrolled from the UCDHS at the University of California Medical Center, Sacramento. Participants with IBD (n = 33, 69 samples) and healthy controls with no prior history of IBD (n = 12, 12 samples) were enrolled in the study. The IBD patient group (consisting of 16 participants on longitudinal

follow-up of treatment with more than 1 time point and 17 participants with a single time point sample) was identified based on their clinical history and evaluation. Peripheral blood samples (20ml) were obtained from all participants. Concurrent peripheral blood and perioperative gut resection or colonic mucosal biopsy samples from selected IBD participants were obtained. Colonic biopsies were obtained from 5 healthy participants. DNA and RNA were extracted from PBMC (isolated using Ficoll-Hypaque density gradient centrifugation) and plasma using the Qiagen DNeasy and RNeasy extraction kits, respectively (Qiagen, Valencia) [41, 42]. Mucosal samples were stored in CryoPrep (American Master Tech Scientific, Lodi). The institutional review board at the University of California, Davis, approved this study protocol.

Real-Time PCR

Taqman$^{@}$ Real-time PCR assay was used to determine the ANXA1 and TNF-α mRNA levels in PBMC. Primer-probe pairs from ABI (*ANXA1, TNF-α and 16S*) were tested and validated to have an amplification efficiency of >95%, comparable to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative mRNA expression levels were calculated from normalized $\Delta \boldsymbol{C_T}$ (cycle threshold) values and are reported as the fold change expression. $\boldsymbol{C_T}$ values correspond to the cycle number at which the fluorescence signal exceeds the background fluorescence (threshold). In this analysis, the $\boldsymbol{C_T}$ value for the housekeeping gene (GAPDH) was subtracted from the $\boldsymbol{C_T}$ value of the target gene for each sample for normalization. For the detection of changes in gene expression in participant groups the RNA levels for each gene were compared with the levels in study group and are presented as the change in expression of each gene. ($\Delta\Delta \boldsymbol{C_T}$). The values were converted to a linear scale ($2^{\Delta\Delta \boldsymbol{CT}}$)(user bulletin 2; ABI Prism 7900 Sequence Detection System; Applied Biosystems) [43, 44]. Based on a previously determined standard curve, bacterial 16S rDNA levels in plasma samples was analyzed by real-time PCR assay using an ABI Prism 7900 sequence detector (Applied Biosystems [22].

Immunohistochemistry

Tissue sections were obtained from colon biopsies of five healthy controls and 5 IBD patients who were responsive to medical management and from colonic resections of three IBD patients who were not responsive to conventional therapy. Following standard immunohistochemical protocols, tissue sections were incubated overnight at 4°C with a 1:100 dilution of mouse anti–ANXA1 (AbCam, San Francisco, CA) and stained using a 1:100 dilution of rabbit FITC-conjugated anti-mouse (BioGenex, San Ramon, CA). DAPI was utilized to visualize nuclei [41, 43]. Tissue sections with no primary antibody and non-specific antibody containing serum were used as negative controls. Images were captured by confocal laser microscopy using LSM 5 and PASCAL software (Zeiss, New York). The images were processed using the ZEN 2009 software.

Immunophenotypic Analysis

Peripheral blood samples were processed and enriched for PBMC's as previously described [41, 42]. Isolated PBMCs were stained with Amcyan live amine dye (Invitrogen). PBMC's were then incubated with antibodies to CD3, CD4, CD8, CD19, CD38, and CD45 followed by PFA 1% fixation. We performed multicolor immunophenotyping on a modified LSRII with a minimum of 500,000 events collected. Flow cytometry data were analyzed using DIIVA software.

Statistical Analysis

The cell activation, ANXA1, TNF-α and 16S data were analyzed using unpaired t-test, Mann-Whitney and their correlations with another variables were performed with Pearson test. Statistical software included GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

Results

Study Participants Characteristics

Study participants were matched for age and sex and ethicity. About 50% were male in both the IBD as well as the control groups (Table 1). In the IBD group 28 of 33 were dignosed with Crohns Disease while 5 of 33 were diagnosed with Ulcerative Colitis. Most patients were either on Infliximab therapy or AZA/6MP/6TG therapy or both.

ANXA1 mRNA and protein levels are decreased in the blood and colonic mucosa from IBD patients

ANXA1 mRNA levels were measured in isolated PBMC. The level of expression of *ANXA1* gene was significantly lower in the PBMC from IBD patients in comparison to control subjects ($p < 0.05$) (Figure 1A). The level of ANXA1 mRNA in PBMCs obtained from IBD patients was between 2 and 500 fold lower than that of healthy controls. To corroborate the mRNA results in the blood and verify the histopathological profile of ANXA1 gene expression in inflamed colonic mucosa, we examined the protein expression in this tissue in IBD and control tissues by fluorescent immunohistochemistry (Figure 1B-F). ANXA1 protein was distinctively detected in the cytoplasm of surface and crypt epithelial cells and leukocytes dispersed in the connective tissue from normal mucosa (Figure 1B-D). In contrast, IBD mucosa demonstrated weaker immunolabeling in both cellular types from the villus structure (Figure 1E-F). The antibody negative control sections showed no staining (data not shown). The results show that the protein detected with anti-ANXA1 antibodies is specific and non-cross-reactive and may be present at lower levels in patients with IBD as compared to IBD negative controls.

PBMC ANXA1 mRNA levels are reflective of IFX therapy

ANXA1 expression over the course of IFX therapy is largely unknown. We focused on the ANXA1 mRNA levels in PBMC of patients on IFX therapy. Of the 33 IBD patients, longitudinal samples were obtained from 16. Four patients who were not on anti-TNF-α therapy were grouped as “no IFX” (Figure 2A) and revealed a

lower level of ANXA1 transcripts in the PBMC when compared to samples from patients initiating IFX therapy (Figure 2B, Table 2). Eighty-five percent of samples clustered together in IFX/ANXA1 negative producers, whom presented a down-regulation of ANXA1 expression in measurements from initial therapy to last sample at follow-up (Figure 2B, Table 2). On the other hand, 15% of patients were termed IFX/ANXA1 positive producers, since a modest up-regulation of ANXA1 was observed following IFX therapy (Figure 2C, Table 2). Interestingly, almost half (46%) of IFX/ANXA1 negative responders group presented an impairment of IBDQ score (mean = 126), while all of ANXA1 positive responders were associated to a better quality of life reaching a higher IBDQ average than the negative responders (mean = 173) (Figure 2D, Table 3).

Increase of PBMC TNF-α RNA expression is to IFX therapy

Correlation analysis demonstrated a trend towards a positive association between ANXA1 and TNF-α transcripts, approaching significance, showing a displacement of this correlation when compared blood samples from all patients under initial (Figure 3A) and continuous IFX therapy (Figure 3B). IFX is a monoclonal Anti TNF-α antibody resulting in the plasma TNF-α neutralization [38]. TNF-α transcription, assessed by real time-PCR, increases during IFX therapy as well as with increasing mucosal permeability as indicated by increasing bacterial 16S ribosomal DNA levels in plasma. TNF-α mRNA levels were significantly increased in the blood from IBD patients following IFX therapy (Figure 3C) ($p < 0.05$). Interestingly, a significant positive correlation was observed between plasma 16S rRNA levels and TNF-α transcripts ($R = 0.62$, $p = 0.02$) (Figure 3D). No correlation was found between TNF-α expression and lymphocyte activation.

Plasma bacterial 16S levels increases with IFX therapy and ANXA1 expression

Systemic cell activation was analyzed using immunophenotypic analysis [11, 17], and mucosal barrier function by quantitation of plasma 16S levels. Results indicated higher levels of plasma 16s levels in patients with IBD compared to healthy controls (Figure 4A). There was no significant difference between patients

initiating on IFX therapy and patients not ~~in~~ on IFX (Figure 4B). An increase in plasma 16S levels was observed in patients on IFX maintenance therapy. A significantly lower level of ANXA1 expression is associated with a higher bacterial load in IBD patients (R=.71, $p < 0.05$) (Figure 4C). The impact of bacterial load on T cell stimulation was assessed by linear regression and demonstrated a moderate correlation with memory CD8+ T cell numbers (R=.51, $p < 0.05$) (Figure 4D).

IFX therapy modifies cellular immune activation in blood from IBD patients

All patients with IBD had higher CD4+ T cell percentages and lower levels of CD8+ T cells in peripheral blood. Patients initiating IFX therapy and patients on continuing IFX therapy had significantly lower levels of CD4+ T cells compared to patients not on therapy (Figure5A). However, comparison of the pre (no IFX group) and post-IFX as initial (one single infusion) and continuous (at least 3 dose regimen) therapy values showed that the treatment was followed by a significant increase in the mean percentage of CD3+CD8+ T cells (from ≈ 10% to 29% and 26%, respectively, $p = 0.005$, Figure 5B) and a significant decrease in CD3+CD4+ (from ≈ 85% to 61% (initial), $p = 0.05$ Figure 5A). Following at least 3 months of IFX administration, the patients had a significantly increased percentage of CD4+CD38+ activated T cells (from ≈ 26% to 52%, $p = 0.03$, Figure 5C). In addition, there was a significantly increased concentrations of CD4+CD45+ not only during continuous treatment (from ≈ 24% to 47%, $p = 0.03$, Figure 5D), but also immediately after initial treatment (from ≈ 24% to 56%, $p = 0.04$). There was also non-significant increase in activated B cells, CD19+CD38 (from 18% to 42% and 45%, respectively, Figure 5E) during IFX therapy.

Discussion

Inflammatory bowel disease is characterized by chronic relapsing inflammation of parts of the gastrointestinal tract, but the origins of which are unknown. Our data indicated that ANXA1 protein distribution was reduced in the epithelial cells from activated IBD patients' colonic mucosa. This is consistent with current literature in which increased injury to the intestinal mucosa is observed in a model of induced

colitis performed in ANXA1-/- mice [39], but also by the fact of higher secretion of endogenous ANXA1 in the lumen from of UC patients [45]. Considering that ANXA1 mRNA might be a systemic biomarker for chronic disease such as cancer [46] and SIV/HIV infection (data not published), we report for the first time, the loss of ANXA1 protein in the microenvironment of the colonic mucosa of IBD patients, which is mirrored by the downregulation of ANXA1 expression in the peripheral blood. This data is supported by the role of ANXA1 protein in preventing neutrophil recruitment to tissue, so then this decrease in IBD development may lead to increased neutrophils, a common feature for IBD pathogenesis [47, 48]. Furthermore, early studies also reported ANXA1 imbalance in the blood, demonstrating that adults and children with Crohn's disease or ulcerative colitis (UC) presented elevated sera titration of anti-ANXA1, such as IgM [49], IgG and IgA [50].

Since IBD is treated with disease-modifying agents that down-regulate the immune response [17], we have also investigated how the IFX therapy influences ANXA1, a new biomarker of IBD, and TNF-α expression, which is the major target of this drug. The longitudinal analysis with patient's clinical follow-up data, allowed us to conclude that systemic ANXA1 transcription levels are affected by initial and continuous IFX therapy or the systemic immune response to the immunossupressive therapy. Besides the moderate recovery of ANXA1 transcripts at initial therapy, the majority of patients were IFX/ANXA1 negative producers, and continuous therapy may have led to a significant down-regulation of ANXA1 expression in the blood, resembling non-treated patients. Our data is corroborated by a recent study demonstrating that down-regulation of ANXA1 expression may have significantly contributed to the multidrug resistance in a lymphocyte lineage [51]. Interestingly, almost half of ANXA1 negative producers presented a decrease of IBDQ score, which is also evidenced by the fact that ANXA1 auto-antibody levels were directly related to disease activity scored clinically in patients with Crohn's disease [49], and suggesting that lack of ANXA1 at mRNA or protein levels might contribute to the pathogenic mechanisms underlying, a hypothesis that is strengthned through our confocal microscopy data showing a complete loss of the Annexin A1 protein at the colonic mucosa.

Several mechanisms have been proposed to explain the actions of IFX on IBD

patients. Besides the neutralization of TNF-α in its monomeric and trimeric forms, IFX causes apoptosis of activated T cells and lamina propria lymphocytes, inhibits neutrophil chemotaxis, stimulates production of reactive oxygen species, upregulates p38 MAP kinase activity, downregulates TNFR expression, and also alters cytokine secretion in the serum and lamina propria, decreasing IL-1, IL-6, IL-18 and IFN-γ production [52, 53]. Despite the aforementioned effects, our data showed that the numbers of TNF-α expression level was increased in PBMC of IBD patients under to continuous IFX therapy, suggesting a feedback regulation at the transcriptional level, a molecular mechanism yet to be determined. Because IFX treatment may also induce loss of immune surveillance [54, 55], we investigated the bacteremia in those patients, and we found an association among increased plasma bacterial load with continuous IFX therapy, with a concomitant increase of TNF-α expression.

Anti-TNF-α immunotherapy has indeed revolutionized the treatment of some inflammatory diseases, however, the major concern for patients receiving this therapy is the increased risk of fungal and bacterial infection [56]. Administration of LPS in a mouse model demonstrated that ANXA1 tissue protein and mRNA expression levels were increased [31], which are associated with reduced IL-6 and TNF-α levels in the sera [30]. Our data confirm these findings through a complementary result, showing that lower levels of ANXA1 mRNA were associated with higher bacterial load in the plasma from IBD patients, and did not significantly correlated with TNF-α mRNA augmentation. The ANXA1 deficiency in IBD patients as described here may induced an inadequate response to bacteria infection; previous studies have shown that this protein is essential for the formation of a stable apoptotic envelope in bacteria-infected macrophages [57] and phagocytosis signaling [58], besides exerting a fundamental role in the stimulation of Th1-driven response [59, 60]. This chronic inflammation is further aggravated by a continuous stimulus of TNF-α by bacterial components in circulation.

The description of a significant disequilibrium between the two signaling pathways, involving ANXA1 and TNF-α, clearly evidenced one of the most important causes of the disease pathogenesis, the loss of the immune homeostasis between two important pro-inflammatory and anti-inflammatory effectors. This may be a

critical event for the chronic inflammation found in IBD patients, despite a successful therapy outcome at the initial states of treatment.

Transcriptional regulation of many molecules involved in the immunity and the systemic infectious status of IBD patients may not completely explain the effects of IFX therapy. Because of that, we have also revisited the hypothesis that T cell activation could also be a factor related to the development of inflammatory condition in the gastrointestinal tract. Our results also indicated that T cell activation, particularly that of CD8+CD45+ T cells, was correlated with increased bacterial load in plasma of IBD patients. In terms of T cell response, TNF-α can also activate macrophages to induce anti-microbial activity, while cytolysis of the infected cells can kill the bacterium, a phenomenon observed in TB-like bacteria, a common microorganism in IFX-treated patients presenting side effects [56]. Most studies of the role of CD8 T cells in bacterial infection have focused in cytokine secretion and cytotoxicity and if these cells also express CD45 expression, a receptor correlated to activation or memory-type driven T cells [61], it makes sense suggesting these cell types are activated to defend the IBD paitents against bacteremia.

We have shown that continuous IFX therapy modified cellular immune activation in the blood from IBD patients, which was characterized by a significant disequilibrium in percentages of CD4+ and CD8+ T cells, CD38+ T and B cells, and also memory CD4+ cells (CD45RO+). Similar results have been partially reported elsewhere [62], in which increased numbers of circulating CD8 T cells in the peripheral blood of rheumatoid arthritis patients were observed three days after the IFX infusion and those effects were attributed to the IFX therapy. The same authors presented a hypothesis, suggesting that IFX blocks homing of Th1 cells, responsible for the secretion of pro-inflammatory cytokines, into the inflamed tissue, thus hese cells temporarily accumulate in the peripheral blood. Our results both from at the initial samples and after continuous IFX administration, for at least 3 months, indicated the accumulation of not only memory and activated T cells, but also activated B cells in the peripheral blood, supporting to some extent the above hypothesis.

There are several caveats to this study. The sample size was relatively small and the results may not be applicable to the general IBD positive population. However, this study indicates the need for larger studies testing this hypothesis. This study also provides clues to this novel mechanism of IBD associated pathology but further analysis into the molecular mechanisms and pathways involved will be needed. The characterization of a significant disequilibrium between ANXA1 and TNF-α expression in the blood, the complete loss of ANXA1 in the colonic mucosa, the increased levels of systemic TNF-α, the increased bacterial load demonstrating loss of the intestinal barrier function, and the lymphocyte activation, are some of the key factors which help further elucidate the mechanism behind IBD etiology/disease. Furthermore, the ANXA1 defect may also explain partially the increased bacteremia and consequently the increased levels of TNF-α without ANXA1 counteracting effect, which together may become the triggers of the abnormal inflammation. We believe that ANXA1 is a key molecule and may be a potential target for IBD treatment. Additional studies are needed to define the role of ANXA1 role in IBD, but convincing data is presented here, which expands the strategies for diagnosis and management of IBD.

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Figure Legends *(inseridas junto às figuras individualmente)*

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Table 1. **Patient characteristics.**

	Groups	
	IBD [n]	NC [n]
Total subjects	33	12
Sex		
Male	16	7
Female	17	5
Disease		
Crohn's disease	28	N/A
Ulcerative colitis	5	N/A
Medications		
Infliximab	29	N/A
AZA/6-MP/6-TG	22	N/A
Methotrexate	3	N/A
Prograf	5	N/A
Steroids	10	N/A
Mesalamines	13	N/A

Note: n = total subjects. Within parentheses: percentage of patients. Abbreviations: IBD (inflammatory bowel disease), NC (normal controls), N/A (not applicable).

Table 2. **Longitudinal follow-up of infliximab treatment vs ANXA1 expression in IBD patients.**

1-year longitudinal follow-up of patients	Infliximab (n=16)		No-infliximab (n=4)	p value[1]
ANXA1 expression	down-regulation (n=13)	up-regulation (n=3)		
first sample	-8.49 ± 1.32	-25.97 ± 14.33	-25.38 ± 1.27	0.004* ; 0.62
last sample	-34,95 ± 7.06	-4.92 ± 2.12		
p value[2]	0.0002*	0.10		

Note: n = total subjects.

Data are represented as mean ± SEM.

p values were obtained using Mann-Whitney test of comparison.

[1] p value vs non-IFX group; [2] p value vs first sample group.

* Statistically significant differences.

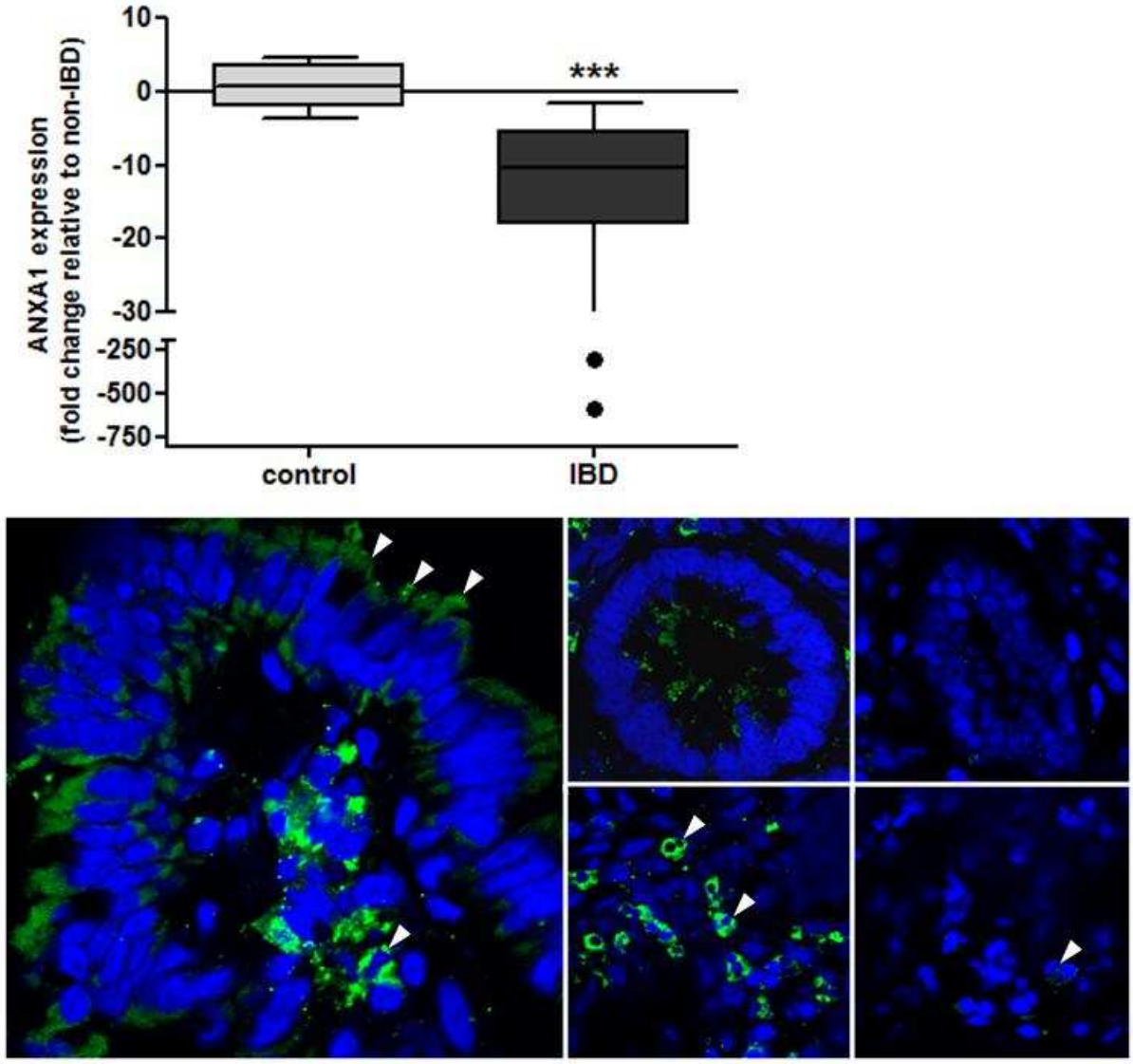

Figure 1. **ANXA1, a new biomarker, has its expression lower at mRNA and protein levels in blood and gut, respectively.** Level of ANXA1 mRNA (A) was determined by real-time RT-PCR analysis from peripheral blood. Values were normalized to endogenous housekeeping GAPDH mRNA and calibrated against the average of values from non-IBD controls. Indirect immunofluorescence using a mouse anti-human ANXA1 antibody is shown with cryostat sections of colon resections from non-IBD (B-D) and IBD patients (E-F) by confocal microscopy analysis. (B) ANXA1 protein is localized in cytoplasm of the epithelial cell layer (arrowheads) and dispersed leukocytes in the mucosa in the villus. (C) ANXA1 staining in the organized crypt from normal tissue is observed at apical portion of the cytoplasm toward to luminal surface. (D) Non-IBD submucosal PMN (arrowheads) are contrasting immunolabeled for ANXA1 in the cytoplasm. (E) ANXA1 protein is not detected in the crypt enterocytes and (F) weakly observed in some submucosal leukocytes (arrowhead) from IBD samples. (Magnification, 400×). ***$p < 0.001$.

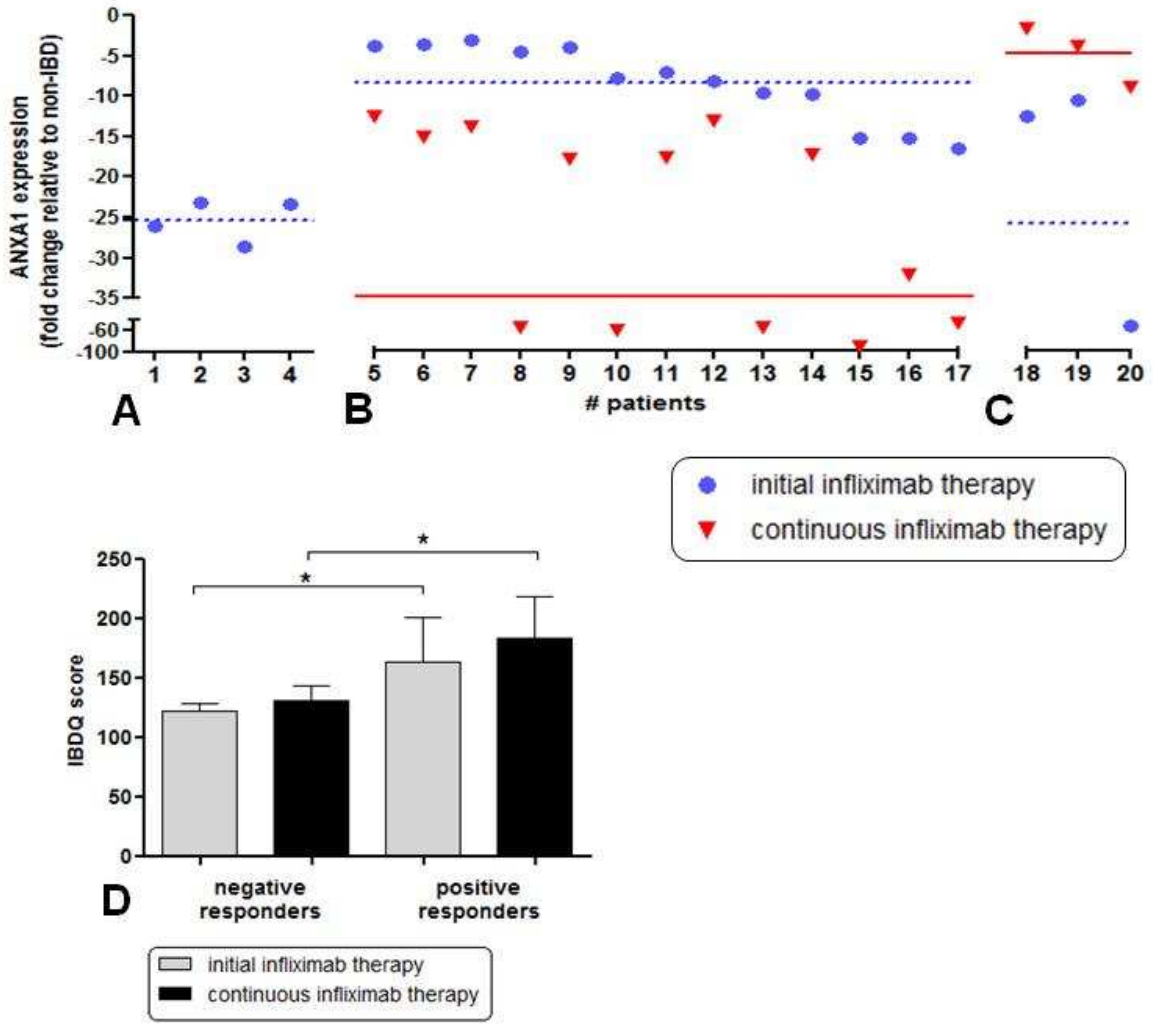

Figure 2. **Analysis of longitudinal follow-up of IFX therapy influence on systemic ANXA1 expression.** (A) Patients under none infliximab therapy; (B) Patient group (85%) showing down-regulation of ANXA1 expression after continuous infliximab therapy; (B) Patient group (15%) showing up-regulation of ANXA1 expression after continuous infliximab therapy; (D) Ratio of ANXA1 regulation between initial/none infliximab and continuous/initial infliximab on the groups of negative and positive therapy responders. The dashed lines are the mean of relative ANXA1 expression values at the first clinical attendance (blue) and at 3 months later under treatment (red) (longitudinal study of IBD patients = 16).

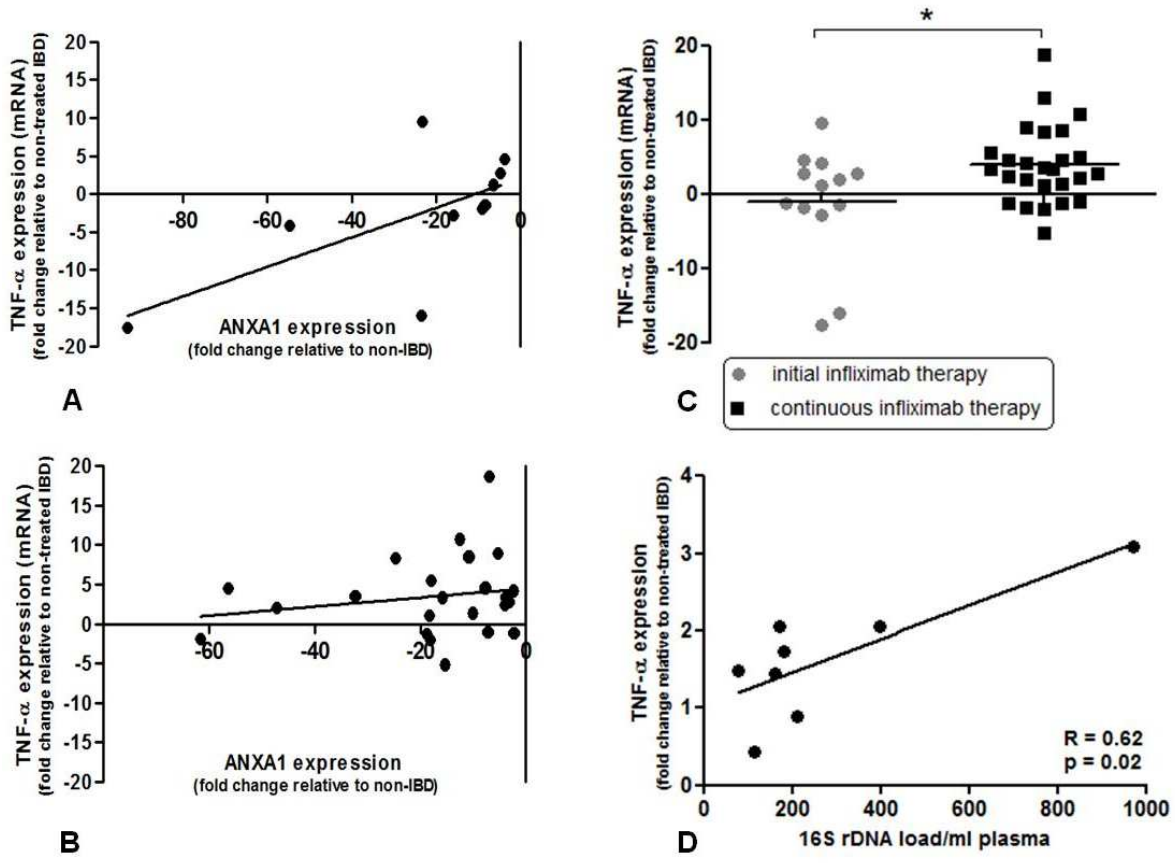

Figure 3. **Correlation of TNF-a vs ANXA1 transcripts revealed an Increase of TNF-a mRNA in response to IFX therapy, which is bacterial load-associated.** The linear regression graphs (A) and (B) showed the relation between ANXA1 and TNF-a expression in the blood from patients submitted to initial and continuous IFX therapy, respectively. As is possible to note that in (B) there is a crescent number of TNF-a transcripts, (C) indicated that blood cells from part of continuous IFX-treated IBD patients presented higher transcription of *TNFA*, which could be suggested is associated to significant correlation between (D) TNF-a expression and increased bacterial load (16S rDNA). *$p < 0.05$.

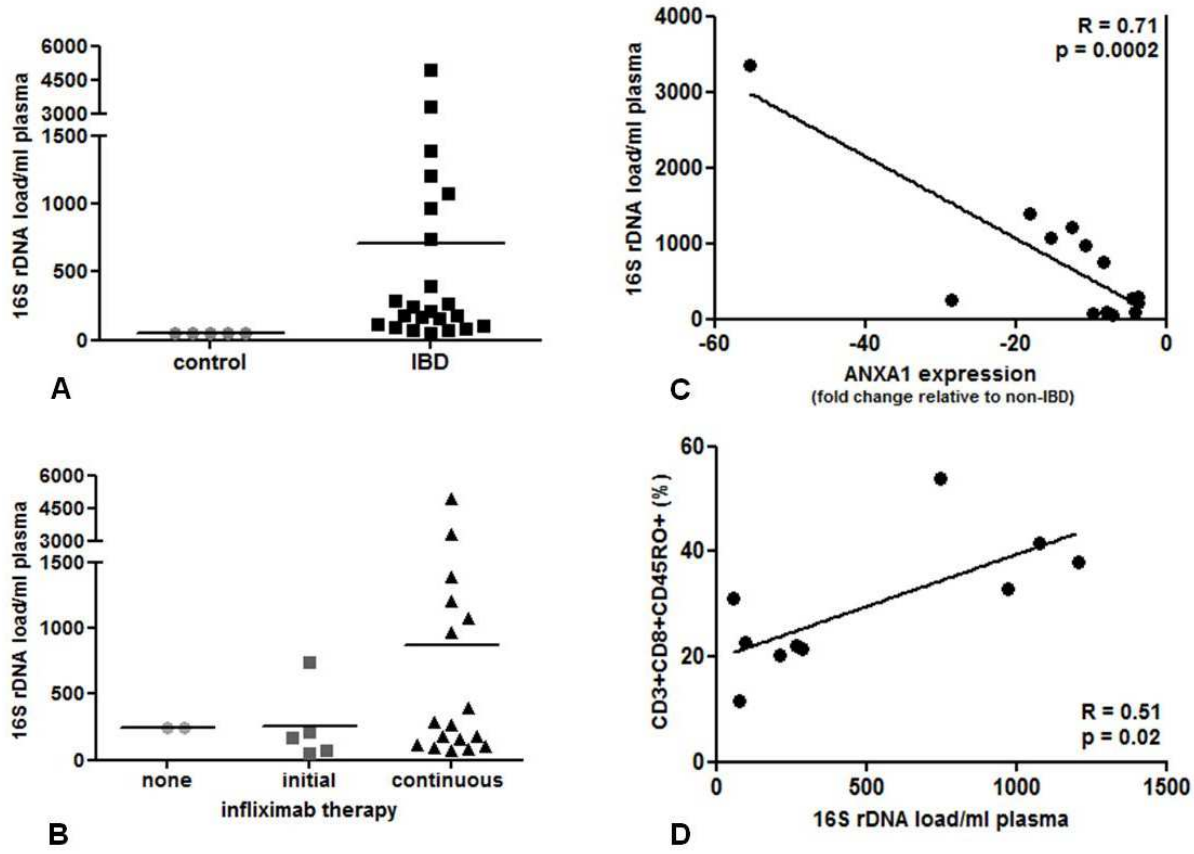

Figure 4. Correlation of bacterial load with IBD status, IFX therapy and ANXA1 expression. The abundance of bacteria in the blood from non-IBD and IBD patients (A) and under the IFX effect (B), as indicated by the number of 16S ribosomal DNA (rDNA) copies, was measured using quantitative PCR (qPCR). (C) Linear function was used to describe the inverse relationship between bacterial load and ANXA1 transcription level.

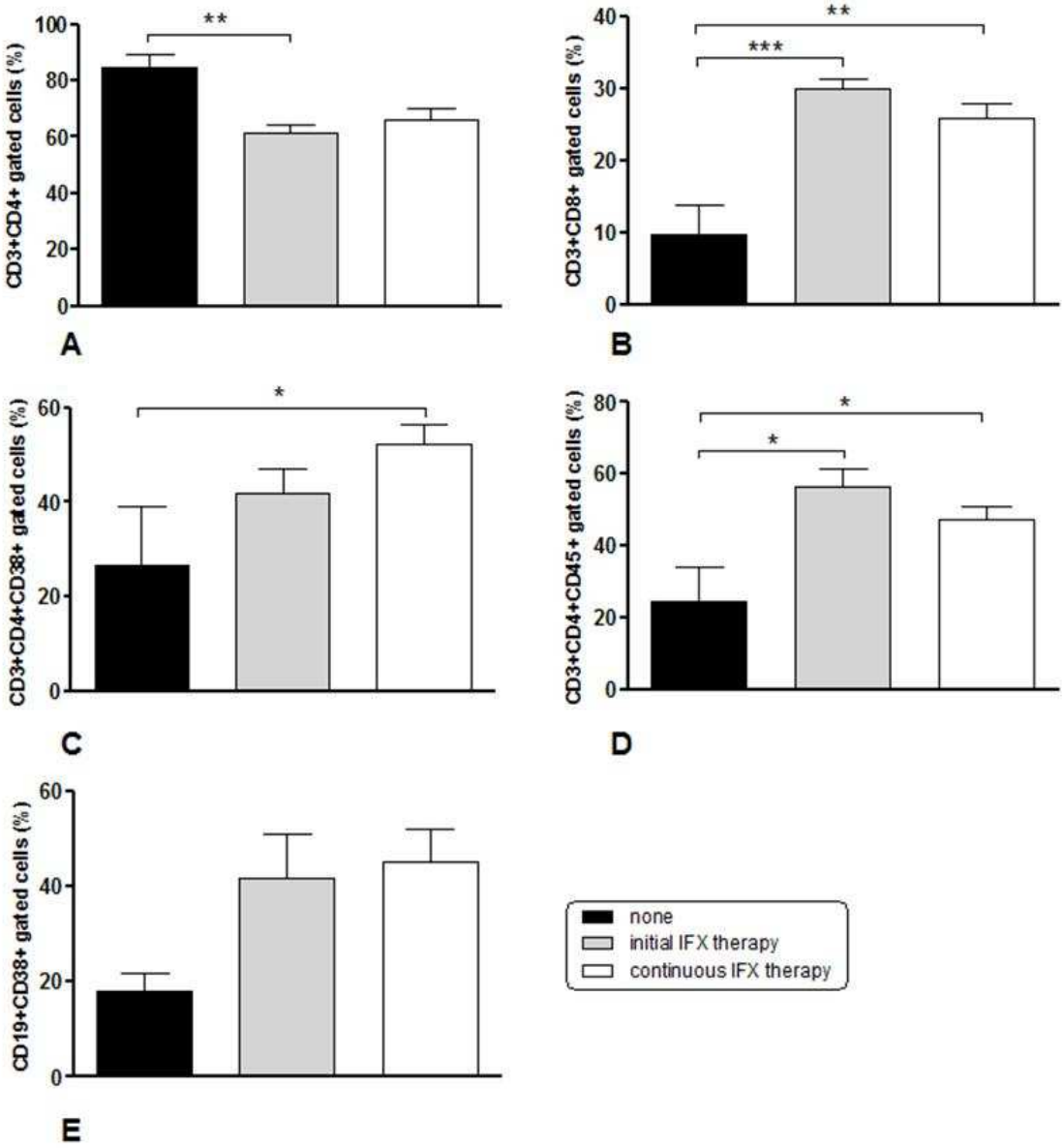

Figure 5. **Effect of IFX on lymphocyte counts and cell activation.** Flow cytometry analysis of peripheral blood samples from IBD patients with or without IFX therapy. A-E summarize the immunophenotypes (CD4+, CD8+, CD4+CD38+, CD4+CD45RO+, CD19+CD38+, respectively) have switched after using IFX at initial period or continuously for at least 3 months. Values are expressed as mean ± SEM of percentage of total cell number. *$p < 0.05$.

CAPÍTULO V

Annexin 1 mimetic peptide protects against renal ischemia/reperfusion injury in rats*

*Este capítulo está apresentado e editado na forma de *Original Article*, aceito e publicado no periódico *Journal of Molecular Medicine*.

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ORIGINAL ARTICLE

Annexin 1 mimetic peptide protects against renal ischemia/reperfusion injury in rats

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Abstract Inflammation is currently recognized as a key mechanism in the pathogenesis of renal ischemia–reperfusion (I/R) injury. The importance of infiltrating neutrophil, lymphocytes, and macrophage in this kind of injury has been assessed with conflicting results. Annexin 1 is a protein with potent neutrophil anti-migratory activity. In order to evaluate the effects of annexin A1 on renal I/R injury, uninephrectomized rats received annexin A1 mimetic peptide Ac2-26 (100 μg) or vehicle before 30 min of renal artery clamping and were compared to sham surgery animals. Annexin A1 mimetic peptide granted a remarkable protection against I/R injury, preventing glomerular filtration rate and urinary osmolality decreases and acute tubular necrosis development. Annexin A1 infusion aborted neutrophil extravasation and attenuated macrophage infiltration but did not prevent tissue lymphocyte traffic. I/R increased annexin A1 expression (assessed by transmission electron microscopy) in renal epithelial cells, which was attenuated by exogenous annexin A1 infusion. Additionally, annexin A1 reduced I/R injury in isolated proximal tubules suspension. Annexin A1 protein afforded striking functional and structural protection against renal I/R. These results point to an important role of annexin A1 in the epithelial cells defense against I/R injury and indicate that neutrophils are key mediators for the development of tissue injury after renal I/R. If these results were confirmed in clinical studies, annexin A1 might emerge as an important tool to protect against I/R injury in renal transplantation and in vascular surgery.

Keywords Annexin A1 · Acute kidney injury · Ischemia/reperfusion injury · Kidney · Neutrophils · Acute tubular necrosis

Part of this study was presented in abstract form at the Annual Meeting of the American Society of Nephrology, Philadelphia, Pennsylvania, 2005.

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Introduction

Ischemia is the most common cause of hospital-acquired acute kidney injury (AKI) [1] and acute tubular necrosis after renal transplantation [2], conditions that carry high mortality and morbidity. Inflammation and infiltrating leukocytes have been increasingly recognized as a key mechanism in the pathogenesis of renal ischemia–reperfusion (I/R) injury [3–10]. The role of neutrophils,

lymphocyte, and macrophage in this injury has been assessed with divergent results [11–16].

Annexin A1 protein is an important endogenous anti-inflammatory mediator, which is activated in response to cellular or tissue injury [17–20]. Annexin A1 treatment conferred protection in splanchnic, myocardial, and brain models of I/R injury [21–24], and diannexin protected against liver I/R injury [25, 26]. This protein is highly expressed in the cytosol of neutrophils, monocytes, eosinophils, and plasma cells [27]. Annexin A1 inhibits neutrophil adhesion and migration through inflamed postcapillary venule endothelium, decreases neutrophil recruitment in the inflamed tissue, detaches endothelium adhered leukocytes, accelerates polymorphonuclear cells apoptosis, induces L-selectin shedding tissue, and reduces phospholipase A_2 activity [17, 27–32]. Renal tissue annexin A1 expression changes dramatically after I/R [33].

The aim of this study was to assess the effects of exogenous annexin A1 mimetic peptide (Ac 2-26) administration in a rat model of renal I/R injury. This peptide has been previously shown to maintain the protectives anti-inflammatory effects of natural annexin A1, and as the parent compound, it has as main target the inhibition of neutrophil infiltration to inflamed tissue [23].

Material and methods

Animals

Adult male Wistar rats (breeding São José do Rio Preto Medical School colony) weighing 200 to 300 g were housed in a temperature- and light-controlled environment. They received standard salt and protein diet and were allowed free access to tap water. Experiments were done according to the Brazilian Law of Protection of Animals, and the experimental protocol was approved by the São José do Rio Preto Medical School Ethical Committee on Animal Experimentation.

In vivo ischemia/reperfusion model

Surgical procedure

Animals were anesthetized intraperitoneally with a solution of 20 mg/ml of xylazine and 50 mg/ml of ketamine (1 ml/100 g of rat). A polyethylene tube (PE-50) was placed in the left jugular vein for annexin 1 or vehicle (VH) infusion. The abdominal cavity was exposed by a ventral midline incision, and a right nephrectomy was performed. The left renal pedicle was identified, and the renal artery was carefully isolated from the renal vein. The left renal artery was occluded with a non-traumatic vascular clamp. Cessation of blood flow was documented by visual inspection. After 30 min, the clamp was removed and the left kidney was inspected for immediate and complete reperfusion. Only rats with adequate renal reperfusion (rapid and complete kidney reperfusion by visual inspection) were used in the study. Core body temperature was maintained stable (36.5±1°C) throughout all surgery by a thermostatically controlled heated table (Braile Biomedica, Sao Jose do Rio Preto, Brazil). At the end of the experiment, the abdominal wall was closed in two layers. Animals were allowed to recover and returned to their cage with food and water ad libitum. This model was selected because it causes moderate to severe, reversible renal injury in a consistent and reproducible way.

Sham surgical procedure

Animals in this group were submitted to exactly the same anesthesia and surgical procedures described above, but they did not have their renal artery clamped.

Experimental groups

Cohorts of eight rats were randomly allocated to one of the three following groups: sham-operated (control), vehicle-treated (phosphate-buffered saline (PBS), VH), and annexin 1 mimetic peptide Ac2-26-treated (ANXA1; 100 μg per rat). VH or ANXA1 were administered intravenously 30 min before renal artery occlusion. The dose of peptide Ac2-26 was selected according to previously published studies and after preliminary experiments at our laboratory. Animals were studied 2 and 7 days after reperfusion.

Renal function studies

Animals were housed into metabolic cages (Nalgene Co, Rochester, NY, USA) for 24 h urinary volume collection, and then glomerular filtration rate (GFR) was assessed by inulin clearance as previously described [34]. Briefly, on reperfusion days 2 and 7, animals were anesthetized and surgically prepared. A primer of inulin (Sigma Chemical Co, St. Louis, MO, USA) was given, followed by maintenance IV infusion of inulin solution (infusion pump, Harvard Apparatus, Holliston, MA, USA). After equilibration time, urine and blood samples were collected during three periods (20 min each). Inulin clearance results are expressed in milliliters per minute per 100 g, representing the mean of the three clearance periods.

Blood and urine analysis

Inulin was determined by chemical anthrone method (spectrophotometer, ByoSystems BTS 310, Barcelona,

Spain). Urinary and serum creatinine were determined by Jaffé colorimetric method (spectrophotometer, ByoSystems BTS 310, Barcelona, Spain). Urinary and serum sodium and potassium concentrations were determined by electrolyte analyzer (9180 Electrolyte Analyzer, AVL Scientific Co., Rosweel, GA, USA). Urinary osmolality was determined by freezing point (Osmette A, Precision Systems, Natick, MA, USA). The fractional excretion of sodium (FeNa) and potassium (FeK) were calculated by the usual formulas.

Histology and transmission electron microscopy

At the end of the experiment, animals were euthanized and the kidneys collected. Tissue preparation for histological and transmission electron microscopy (TEM) analysis was previously described [19]. Briefly, renal tissue fragments were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde, 0.1% sodium cacodylate buffer (pH 7.4) for 18 h at 4°C. They were then washed, dehydrated, and embedded in paraffin or LR Gold (London Resin Co., Reading, Berkshire, UK). Sections of renal tissue (1 μm thick) were stained with hematoxylin and eosin (HE) or Toluidine blue (TAAB Laboratories, UK), and histological analysis was done by light microscopy using an AXIOSKOP microscope (Zeiss, Germany). For TEM analysis, ultrathin sections (90 nm thick) were cut on an ultramicrotome (Reichert Ultracut; Leica, Vienna, Austria) and placed on nickel grids for immunogold labeling. All histological and immunocytochemical ultrastructural analyses were realized by observers blinded to rats treatment.

Score for acute tubular injury (counting of pyknotic nucleus)

Renal tissue fragments embedded in LR Gold were stained with HE and analyzed in a photonic microscope (NIKON, Japan) by an observer blinded to animal treatment. There were analyzed five fields from each fragment and four animals for each treatment group. Each field was randomly selected in the renal fragment, and the number of pyknotic tubule cell nucleus was counted.

Immunohistochemistry for mononuclear leukocytes detection

Paraffin-embedded sections (2 μm) were incubated for 1 h with sodium citrate at 96°C. Then, kidney fragments were treated with hydrogen peroxide (H_2O_2) at 3% diluted in methanol for 30 min and washed three times in PBS (pH 7.4) for 15 min. Sections were incubated overnight at 4°C with the primary antibody mouse anti-CD3 for lymphocytes or goat anti-MIP-1α for mononuclear phagocytic cells (Santa Cruz Biotechnology) working dilution of 1:100 and 1:200, respectively, with 1% bovine serum albumin in PBS. Then sections were washed in PBS (15 min) and incubated with a biotinylated secondary antibody, avidin-conjugated horseradish peroxidase, and enhanced with diaminobenzidine chromogenic substrate (detection kit LSAB) for 1 h at room temperature. At the end of the reaction, sections were washed thoroughly in distilled water, counterstained with hematoxylin, and mounted in BIOMOUNT (British BioCell International). Analysis was conducted with a microscope ZEISS AXIOSKOP 2, using the software AXIOVISION.

Post-embedding immunogold labeling

To detect ANXA1, an established immunogold staining procedure was used [19]. Tissue samples were prepared for electron microscopy by standard methods, and on sequence, a post-embedding immunogold labeling reaction was made. Ultrathin sections were incubated: (1) PBS containing 1% egg albumin (PBEA); (2) PBS containing 5% egg albumin (PBEA) for 30 min; (3) the sheep polyclonal antibody raised against the N-terminal peptide of human ANXA1 (peptide Ac2-26) for 2 h and used at a final dilution of 1:200 in PBEA; (4) normal sheep serum was used as control; (5) after washes in PBEA, in order to detect ANXA1, a donkey anti-sheep IgG antibody (1:50 in PBEA) conjugated to 15 nm colloidal gold (British Biocell, Cardiff, UK) was added; and (6) after 1 h, sections were washed in PBEA, then in distilled water. Ultrathin sections were stained with uranyl acetate and lead citrate before examination on a ZEISS LEO 906 electron microscope, EM Center, São José do Rio Preto, São Paulo, Brazil.

Western blotting

Renal tissue was sonicated in Tris–HCl solution (50 mM) containing phenylmethylsulfonilfluoride (1 mM, pH 7.4). Protein levels were determined by Bradford assay (Biorad, Hemel Hempsted, UK) and equalized prior to boiling in Laemmli buffer (4% sodium dodecyl sulfate, 20% glycerol, 1 mM DTT, 2 mM EDTA, and 1 mg/ml Coomassie brilliant blue). To detect AnxA1, protein extracts (25 μg per lane) were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis together with appropriate molecular weight markers (Amersham Pharmacia Biotech, Buckinghamshire, UK) and transferred to ECL Hybond nitrocellulose membrane. Membranes were incubated overnight in 5% nonfat dry milk diluted in TBS with 0.1% Tween 20 (TBST) before the addition of rabbit polyclonal ANXA1 (1:1,000, ZYMED, USA). This was followed by 30 min of washing with TBST and incubation for 60 min at room temperature with peroxidase-conjugated goat anti-rabbit IgG (1:2,000; Serotec, Oxford, UK) diluted

in TBST. Then, densitometry was performed by image analysis system (AxioVision Software, Zeiss, Germany).

Determination of tumor necrosis factor-α

Aliquots of blood (1 ml) from rats were centrifuged at 4,000×*g* for 10 min. Then, plasma concentration of tumor necrosis factor-α (TNF-α) was measured using a specific enzyme-linked immunosorbent assay kits (R&D System, Abingdon, UK).

In vitro model of I/R injury in isolated kidney tubules

Proximal tubules isolation

Proximal tubules (PT) were isolated according to methods previously described [35]. In brief, rats were anesthetized with sodium pentobarbital and underwent kidneys removal. Renal proximal tubules were isolated by collagenase digestion and separated by a Percoll gradient. Aliquots of 6 ml (containing 1.0–1.5 mg/ml of protein) were placed in ice-cold siliconized Erlenmeyer flasks for 5 min under 95% O_2/5% CO_2, followed by 10 min at room temperature.

Hypoxia/reoxygenation

After equilibration period, isolated tubules were divided into an experimental group (TP-H/R) and oxygenated group (TP-Oxygenated). Throughout the experiment, pO_2 was maintained within the 200–300-mmHg range in TP-Oxygenated. In TP-H/R, hypoxia (pO_2, 20–40 mmHg) was induced by superfusing with 95% N_2/5% CO_2 for 5 min. Duration of hypoxia period was 15 min. After hypoxia, tubules were reoxygenated superfusing with 95% O_2/5% CO_2 for 5 min (pO_2 returned to within the 200–300-mmHg range). Flasks were released and tubules were maintained under reoxygenation for 45 min. In the experimental groups, annexin A1 at dose of 4 μg/ml was added to tubule suspension in the beginning of the experiment. All samples were obtained at baseline, after hypoxia (at 15 min), and after reoxygenation (at 60 min).

Lactate dehydrogenase measurement

Cell injury was assessed by release of lactate dehydrogenase (LDH; percent). One milliliter of tubule suspension was centrifuged for 120 s in refrigerated centrifuge (1,500×*g*) to separate supernatant and pellet. Pellet was lysed with Triton X-100. LDH activity was measured in supernatant and pellet and converted to percentage release by dividing supernatant activity by total activity.

Statistical analysis

In vivo studies

Renal function data Results are mean ± standard error of mean (SEM). Comparisons were performed using one-way ANOVA followed by Student–Newman–Keuls multiple comparisons post-test or Kruskal–Wallis followed by Dunn's multiple comparisons test, as appropriate. p values<0.05 were considered significant.

Light and transmission electron microscopy data The density of intravascular and transmigrated neutrophils and total number of mononuclear leukocytes (macrophages and lymphocytes) and the histological and immunohistochemical analysis of these leukocytes in kidney were performed with the image analysis system (AxioVision Software, Zeiss, Germany).

Similarly, photographed sections of proximal tubular cells were used for the immunocytochemical ultrastructural analysis ($n=10$ per group). The area of proximal tubular cells was determined with AxioVision software (Zeiss, Germany). The density of immunogold (number of gold particles per cubic micrometer) was calculated and reported as mean ± SEM of number of the electron micrographs.

Comparisons were performed using one-way ANOVA followed by the Bonferroni post-test. p values<0.05 were considered significant.

In vitro tubule cell studies

Results are mean ± standard deviation. Comparisons were performed using one-way ANOVA followed by Student–Newman–Keuls multiple comparisons post-test. p values<0.05 were considered significant.

Results

In vivo model of renal injury

Renal function studies

At reperfusion day 2, vehicle-treated animals showed a significant decrease in GFR as compared to control and ANXA1-treated rats, whereas the GFR was similar in control and ANXA1 groups (Table 1). Diureses decreased significantly in VH and ANXA1 groups than in control animals. The FeNa and FeK of VH group were significantly higher as compared to control and ANXA1, whereas control and ANXA1 groups showed similar values. Urinary osmolality of VH group was significantly lower as

Table 1 Renal function 2 and 7 days after sham operation, reperfusion in vehicle and in annexin A1 mimetic peptide-treated rats (ANXA1)

	2 days			7 days		
	SHAM	VH	ANXA1	SHAM	VH	ANXA1
GFR (ml/min/100 g)	1.02±0.12	0.43±0.03*,**	0.84±0.06	1.10±0.09	0.49±0.06*,***	0.75±0.05*****
UV (ml/24 h)	15.7±1.4	9.3±0.3*	11.9±0.8*****	15.7±1.4	9.8±0.2***,*****	14.9±1.6
FeNa (%)	0.22±0.02	0.42±0.04*,****	0.18±0.02	0.29±0.02	0.53±0.04*,****	0.27±0.02
FeK (%)	34±2	60±3*,****	32±2	37±2	69±5*,****	30±2
*U*osmolality (mOsm/kg)	1,111±39	876±42****,*****	1,369±63*****	1,283±41	926±76***,*****	1,167±70

All groups of animals included eight rats. Data are mean ± SEM

GFR glomerular filtration rate, *UV* urinary volume, *Fe* fractional excretion, *Na* sodium, *K* potassium, $U_{osmolality}$ urinary osmolality

*$p<0.001$ vs. SHAM; **$p<0.01$ vs. ANXA1; ***$p<0.05$ vs. ANXA1; ****$p<0.001$ vs. ANXA1; *****$p<0.01$ vs. SHAM

compared to control and ANXA1. ANXA1-treated animals showed a significantly higher urinary osmolality than control animals (Table 1).

Reperfusion day 7 results were similar to those in day 2, except that GFR in ANXA1 group was now slightly, but significantly, lower than in control animals and that ANXA1 diuresis and urinary osmolality were similar to control and significantly higher than in VH group (Table 1).

Morphological assessment of kidney damage

The presence of I/R-induced renal damage was confirmed by light microscopy in the VH group (Fig. 1a, b), whereas ANXA1-treated rats presented renal histology similar to control animals (Fig. 1c, d). The number of pyknotic cells was significantly higher in VH animals, whereas control and ANXA1 animals showed similar number of cells (Fig. 1e).

Disruption of the renal integrity was seen post-reperfusion with extensive tubular necrosis and sloughing of epithelial cells into the tubular lumen in the VH group (Fig. 2c). Many tubules contained proteinaceous casts (Fig. 2c). This damage was absent in control and ANXA1 groups, in which brush border was clearly evident, together with a normal morphology of the basement membrane (Fig. 2a, e).

Kidney damage produced by I/R was confirmed by transmission electron microscope. Control animals showed normal renal tissue (Fig. 2b), whereas kidneys from VH group exhibited severe tubular injury characterized by loss of microvilli, sloughing of dead cells (casts) with luminal obstruction, tubular epithelial vacuolization, and mitochondrial cristolysis (Fig. 2d). In contrast, tubular morphology was preserved in ANXA1 group, as demonstrated by cell integrity and preservation of microvilli (Fig. 2f).

Recruitment of leukocytes in the kidney tissue

Histological assessment of the injured kidney showed the presence of leukocytes, mainly neutrophils. Cortical tissue from VH animals (Fig. 3a, b, e) disclosed a significantly higher neutrophil number interacting with endothelium, mainly at the glomerular capillaries and into the interstitial space, as compared to kidneys from control and ANXA1 rats. This neutrophil influx was more prominent at the medullary area (Fig. 3c, d). ANXA1 treatment decreased significantly I/R-induced renal neutrophil transmigration (Fig. 3f). There was no statistically significant difference between control and ANXA1 groups at any point.

Mononuclear phagocytic cells (monocytes/macrophages) were significantly increased in renal tissue of VH animals as compared to control or ANXA1 rats (Table 2). Likewise, VH animals presented a significant lymphocyte increase that was more marked at day 7 as compared to control or ANXA1 rats. Treatment with ANXA1-attenuated macrophages increase but did not prevent lymphocytes tissue influx (Table 2).

Expression of endogenous cellular AnxA1

AnxA1 expression detected by post-embedding immunogold labeling was analyzed to define the subcellular localization of the protein in the neutrophils (data not shown) and epithelial tubular cells. The localization of epithelial tubular cells endogenous AnxA1 was determined by immunocytochemical ultrastructural analysis (Fig. 4a–c). Epithelial tubular cells showed AnxA1 immunoreactivity throughout the nucleus and cytosol. Figure 4e illustrated these findings in a quantitative manner. Two days after reperfusion, AnxA1 expression was significantly increased in proximal tubule epithelial cells of VH (8.68±0.76) as compared to control (5.01±0.41, $p<0.01$) and to ANXA1 (4.22±0.25, $p<0.001$) groups. In addition, immunolabeling was higher on day 7 than on day 2 of reperfusion (VH day 7, 12.24±1.24 vs. day 2, $p<0.05$; ANXA1 day 7, 6.80±0.60 vs. day 2, $p<0.05$).

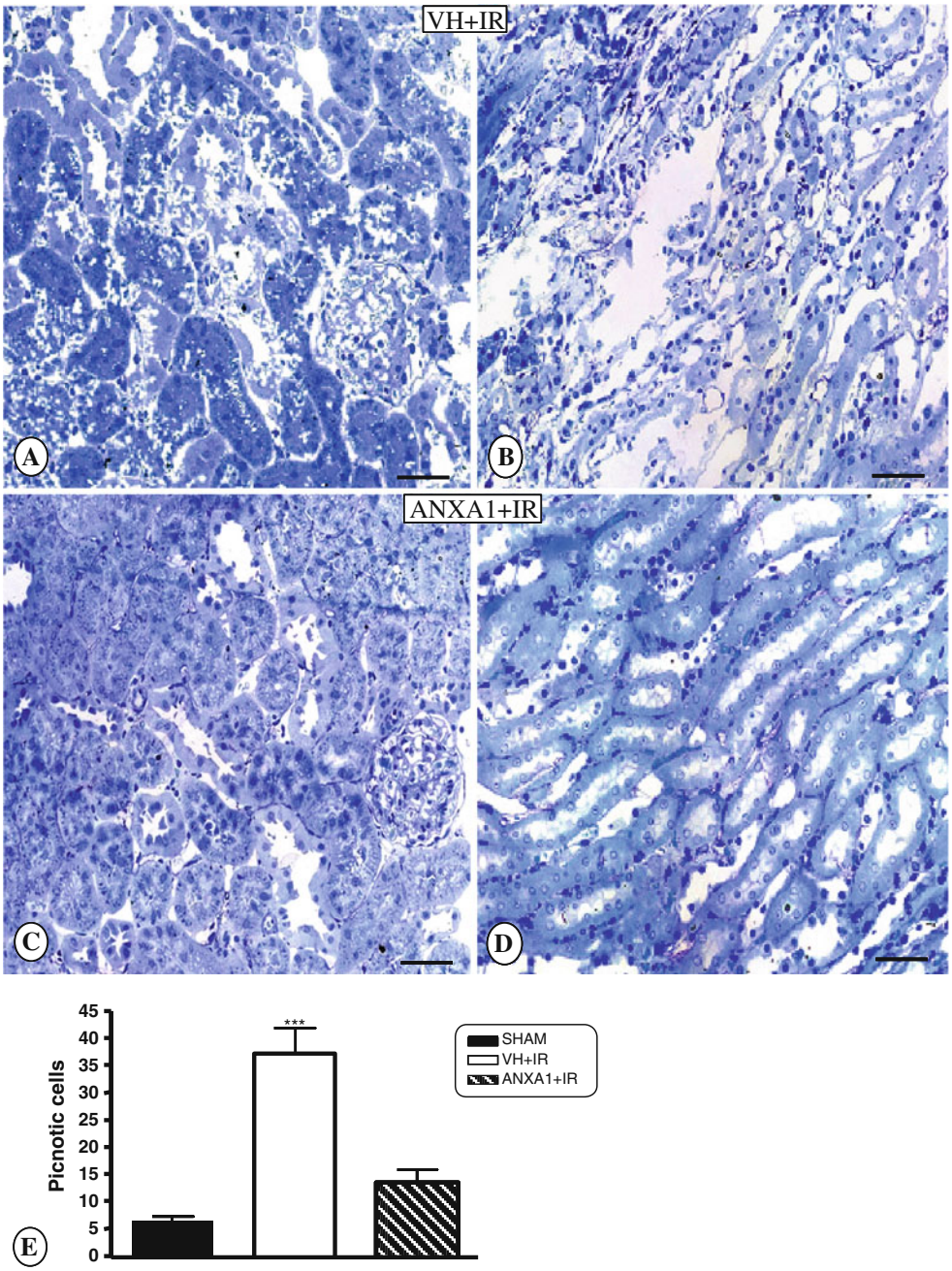

Fig 1 Annexin A1 (ANXA1) inhibits renal ischemia–reperfusion structural injury. Cortex (**a**) and medulla (**b**) from vehicle (VH + IR) group showing severe tubular damage by light microscopy. ANXA1 treatment (ANXA1 + IR) preserved renal structure at cortex (**c**) and medulla (**d**). Stain toluidine blue, *bars* 20 μm. **e** Quantitative analysis of pyknotic cells. Data are mean ± SEM. ***$p<0.001$ vs. SHAM-operated and ANXA1 + IR

Expression of endogenous AnxA1 in renal tissue

There was a significant AnxA1 expression increase in VH group, especially 2 days after I/R, when compared to control. Treatment with exogenous ANXA1 prevented protein expression increase in renal tissue, and so ANXA1 group results were similar to control as can be seen by semi-quantitative densitometry analysis of endogenous ANXA1 (Fig. 5a, b).

Determination of TNF-α in peripheral blood

TNF-α level in VH and ANXA1 animals were increased when compared to control group in both post-reperfusion periods. At reperfusion day 2, TNF-α was significantly ($p<0.01$) increased in VH and ANXA1 groups. At reperfusion day 7, TNF-α was elevated in VH and ANXA1 rats but the results did not show statistical significance (Fig. 6).

In vitro model of I/R injury in isolated proximal tubules

ANXA1 (4 μg/ml) had no effect on control PT or oxygenated PT up to 60 min incubation (Fig. 7). LDH release in control PT was 18±2.5% vs. 22±1.0% in control PT plus ANXA1 (NS). On the other hand, ANXA1 was partially protective against 15 min hypoxia followed

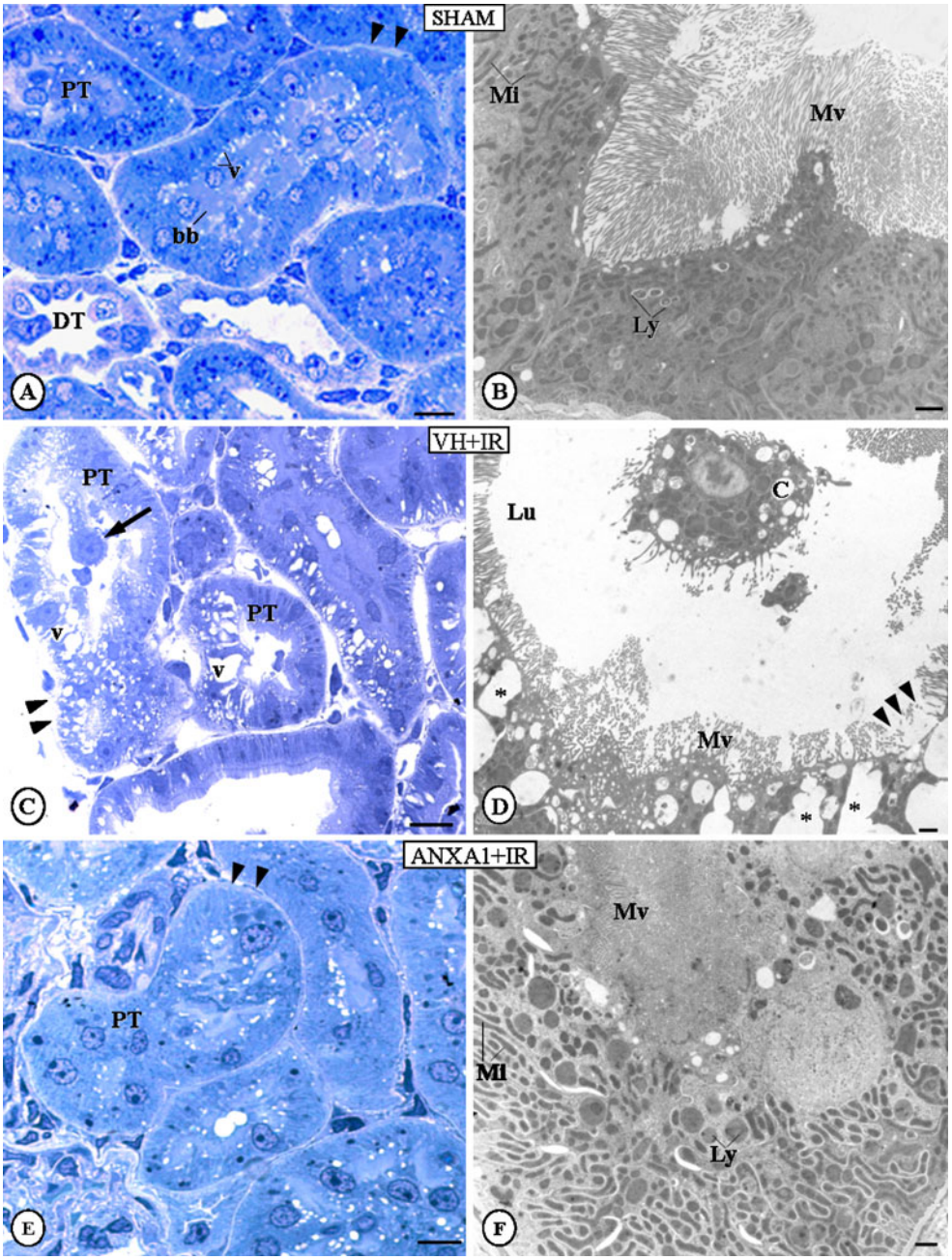

Fig 2 Assessment of renal cortex damage by light (**a**, **c**, **e**) and TEM (**b**, **d**, **f**). **a** Kidney of sham-operated rats with normal proximal (*PT*) and distal convoluted tubules set (*DT*). Note basement membrane (*arrowhead*) and brush border (*bb*) underlying endocytic vacuoles (*v*) in proximal tubule and **b** intact microvilli (*Mv*), mitochondria (*Mi*), and lysosomes (*Ly*). **c** The kidney of rats subjected to ischemia–reperfusion (VH + IR) showed focal lesion of PT expressed as acute tubular necrosis with basement membrane rupture (*arrowheads*), sloughing tubular cell (cast-c; *arrow*), and large vacuoles (*v*). **d** I/R injury can be seen clearly at TEM, evidenced by loss of microvilli (*arrowheads*), vacuolization (*asterisk*), and casts (*C*) into lumen (*Lu*) of tubule. **e** and **f** Treatment with ANXA1 afforded PT protection with preservation of cellular integrity, basement membrane (*arrowheads*), microvilli, lysosomes (*Ly*), and mitochondria. Stain toluidine blue, *bars* 10 μm and TEM 1 μm

by 45 min reoxygenation injury in PT. As shown in Fig. 7, at 60 min, LDH release in control PT was 18±2.5% compared with 60±6.6% in H/R PT alone ($p<0.001$ vs. control) and 50±5.4% in H/R PT plus ANXA1 ($p<0.05$ vs. H/R alone).

Discussion

The present study highlighted important and original aspects related to the role of annexin A1 and neutrophil in renal I/R injury.

Annexin A1

Infusion of annexin 1 mimetic peptide protected against renal I/R injury, preserving renal function and structure damage in a notable way. It is noteworthy that not only the GFR but also the tubular function, as indicated by the lower FeNa and higher urinary osmolality in annexin A1-treated animals, were protected. To the best of our knowledge, this is the first demonstration of a protecting effect of annexin A1 in renal I/R injury. There are previous evidences of annexin A1 protection in I/R injury in mesenteric, myocardial, and brain ischemia. In

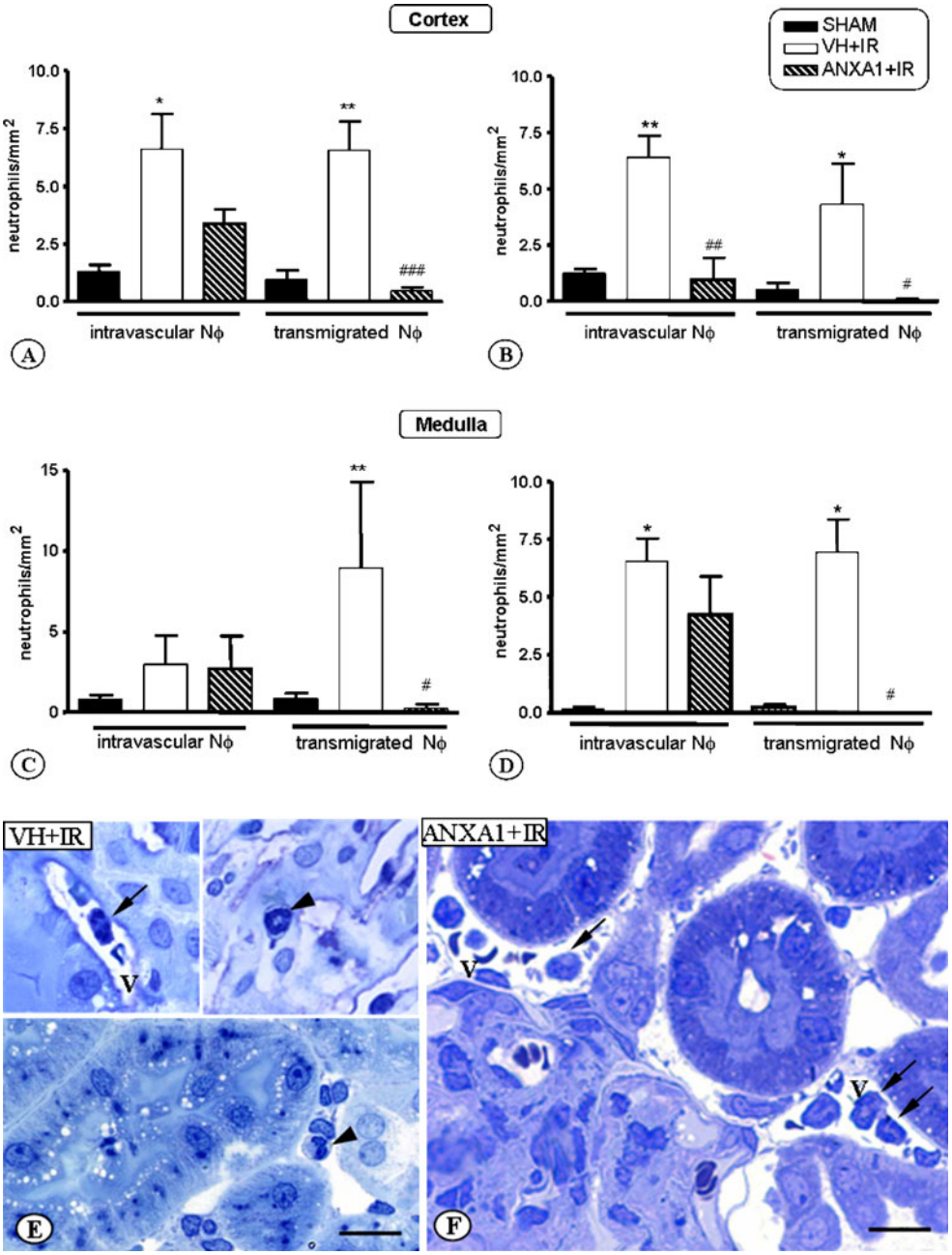

Fig 3 Quantitative analysis of intravascular and transmigrated neutrophils in renal cortex and medulla. Two days: cortex (**a**) and medulla (**c**) from sham-operated, saline-treated (VH+IR) and ANXA1-treated rats (ANXA1+IR, n=6 animals/group). Data are mean ± SEM. *p<0.05 vs. SHAM; **p<0.01 vs. SHAM; #p<0.05 vs. VH; ###p<0.001 vs. VH. Seven days: cortex (**b**) and medulla (**d**) from sham-operated, saline-treated (VH+IR) and ANXA1-treated rats (ANXA1+IR, n=6 animals/group). Data are mean ± SEM. *p<0.05 vs. SHAM; **p<0.01 vs. SHAM; #p<0.05 vs. VH; ##p<0.01 vs. VH. Histological assessment of kidney tissue showing **e** presence of intravascular (*arrows*) and transmigrated (*arrowheads*) neutrophils in VH + IR and **f** intravascular neutrophils in ANXA1 + IR. *v* vessels

all, annexin A1 limited structural and functional changes [21–24].

I/R caused a significant increase in renal annexin A1 expression, which was prevented by peptide Ac 2-26 administration. The increase in endogenous annexin A1 in vehicle-treated animals is probably a physiologic response trying to limit inflammation after reperfusion, which was clearly not successful as observed by the large number of leukocytes infiltrating renal tissue. McKanna et al. assessed rat kidney annexin A1 before and after renal ischemia [33]. In the normal kidney, annexin A1 was mainly found in Bowman's capsule epithelium, macula densa, and medullary and papillary collecting ducts, with light staining at the thick ascending limb. After ischemia, annexin A1 tissue expression increased significantly, in a similar way to the present results, and the thick ascending limb changed to heavy stain [33].

Transmigrated neutrophils are a possible source for the observed increase in annexin A1 expression, as previously observed [36]. An increased expression of annexin A1 associated with infiltrated leukocytes was already observed in injured myocardium after I/R, and consistently with the present results, it was prevented by administration of an annexin A1 peptide derived [23]. We also observed an increase in annexin A1 expression in epithelial cells, which was partially prevented by exogenous annexin A1 infusion. The biological outcomes reported for annexin A1 in

Table 2 Quantitative analysis of lymphocytes and mononuclear phagocytic cells 2 and 7 days after sham operation, reperfusion in VH and annexin A1 mimetic peptide-treated rats (ANXA1)

Cells/mm^2		2 days			7 days		
		SHAM	VH	ANXA1	SHAM	VH	ANXA1
Ly	Cortex	0.58±0.17	3.00±0.70	2.90±0.70	1.30±0.26	4.40±1.10**	4.30±2.00
	Medulla	0.52±0.08	3.10±0.60	3.20±0.69	1.50±0.61	3.80±0.76	4.10±0.69
MØ	Cortex	0.70±0.16	3.51±1.20	2.34±0.20*	0.69±0.18	3.49±0.98	2.23±0.24*
	Medulla	0.61±0.14	3.65±1.30	2.36±0.05*	0.64±0.17	3.44±1.10	2.49±0.12*

Data are mean of lymphocytes and mononuclear phagocytic cells. All groups of animals submitted to I/R procedure with vehicle or ANXA1 presented significant differences in relation to SHAM group with $p<0.01$

Ly lymphocytes, *MØ* mononuclear phagocytic cells

*$p<0.05$ ANXA1 vs. VH; **$p<0.05$ vs. VH at day 2

epithelial cells are anti-proliferative action, inhibition of cell activation, inhibition of phospholipase A_2 activity, and prostanoid generation [20, 37, 38], which are all linked to cell defense against I/R injury. The anti-inflammatory action of annexin A1, like lipoxin A4, is mediated by a formyl peptide receptor, which is expressed in epithelial [38] and mesangial cells [39]. In addition, annexin A1 attenuated I/R injury in isolated tubules suspension without inflammatory cells, indicating a protective effect directly related to the intracellular actions of this protein [28]. When all of these data are taken together, they point for an important role of annexin A1 in renal epithelial cells defense against I/R injury.

Neutrophils

In the present study, neutrophils were the most prominent cell infiltrating the kidneys 48 h after reperfusion. In fact,

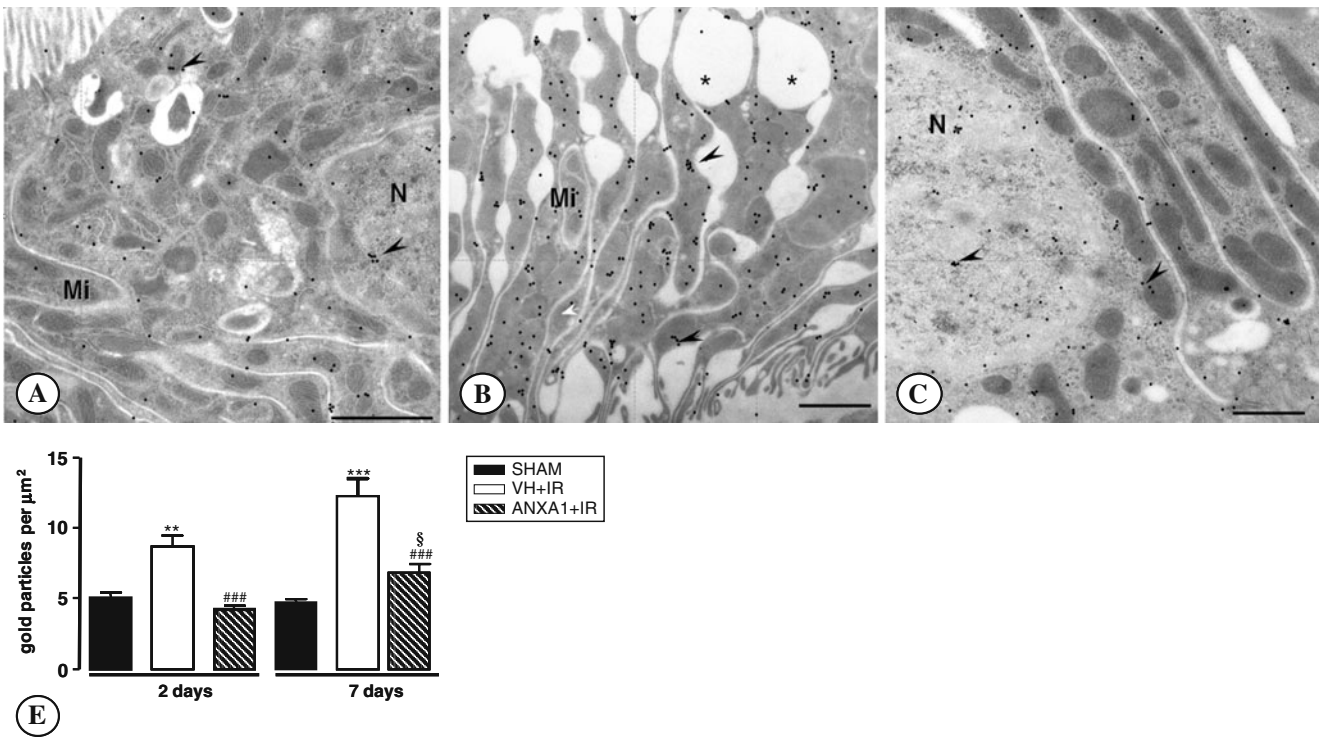

Fig 4 Annexin A1 (AnxA1) expression in epithelial tubular renal cells. **a** Epithelial tubular cell from SHAM-operated rats showed immunoreactivity throughout the cytosol (*arrowheads*) and nucleus (*N*). **b** Tubular cell from vehicle-treated animal presenting marked vacuolization (*asterisk*) and striking cytosol AnxA1 expression. **c** Similarly to SHAM, the ANXA1-treated group showed moderate AnxA1 expression in the tubular cells. **e** Quantitative analysis of AnxA1 in epithelial tubular cell by ultrastructural immunocytochemistry reaction. Data are mean ± SEM. **$p<0.01$ vs. SHAM (2 days); ***$p<0.001$ vs. SHAM (7 days); ###$p<0.001$ vs. VH+IR (2 and 7 days); +$p<0.05$ vs. VH+IR (2 days); §$p<0.05$ vs. ANXA1+IR (2 days)

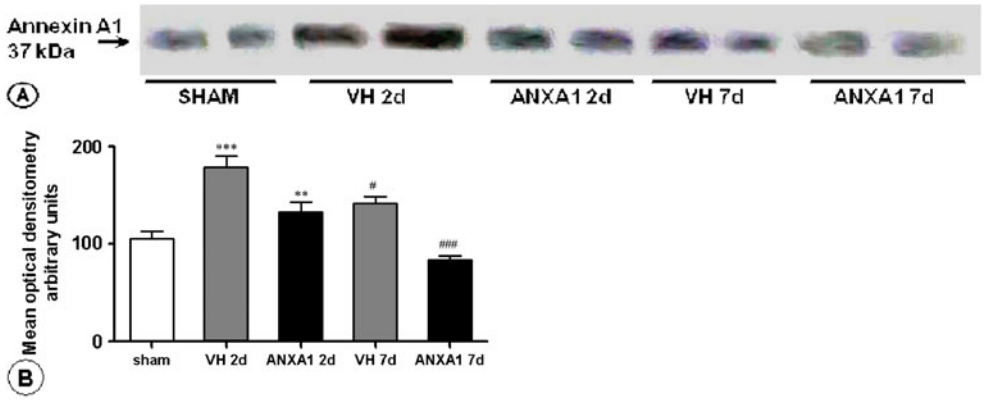

Fig 5 Endogenous annexin A1 (AnxA1) expression in renal tissue. Analysis of AnxA1 expression in renal tissue by Western blotting (**a**) and respective mean optical densitometry showing increase in the AnxA1 expression in vehicle-treated animals, which was prevented by exogenous ANXA1 treatment at 2 and 7 days post-treatment (**b**). Data are mean ± SEM. ***$p<0.001$ vs. SHAM; **$p<0.01$ vs. VH+IR 2 days; #$p<0.05$ vs. VH + IR 2 days; ###$p<0.001$ vs. VH + IR 7 days

on day 2 post-reperfusion, infiltrating neutrophils were approximately two times more frequent than monocytes and lymphocytes, and this influx was more prominent in the medullary area. Previous studies have also demonstrated an early renal neutrophil recruitment occurring predominantly in the medullar area [6, 10]. On day 7 post-reperfusion, neutrophil count decreased whereas lymphocytes number increased. In fact, many authors found that the first leukocytes infiltrating renal tissue after reperfusion are predominantly neutrophils, while later phases are characterized by lymphocytes and macrophages influx [5, 36]. Neutrophils can cause tissue injury and potentiate the inflammatory response by many ways such as plugging capillaries, producing cytotoxic oxygen free radicals, and liberating proteolytic enzymes [40, 41].

Neutrophils have been extensively studied in the renal I/R inflammatory lesion, but there is controversy about its importance as compared to other leukocytes in the injury genesis [3, 5–8, 10, 11, 42–45]. The present results indicate a major role for neutrophils in I/R injury. The modulation of blood-borne neutrophil tissue infiltration by annexin A1 mimetic peptide was certainly a key component in the observed protection against I/R injury, limiting the damage caused by these cells in the inflammatory site. In fact, neutrophil extravasation is a multistep process which is entirely sensitive to annexin A1. Analyses of inflamed vessels have restricted the annexin A1 site of action to adherent leukocytes, and annexin A1 administration reduced the number of neutrophils that adhere to and migrate through the inflamed post-capillary venule endothelium [30]. When blood-borne neutrophils adhere to inflamed vascular endothelium, up to 70% of total intracellular annexin A1 is rapid and actively mobilized from cytoplasm to cell surface. They interact with receptors of the G-protein-coupled receptors family, causing neutrophil detachment in an autocrine/juxtacrine way and ultimately inhibiting leukocytes transmigration [17, 46]. Annexin A1 might also act in a paracrine way, since endothelial cells have specific annexin A1 binding sites and acquire annexin A1 during the process of neutrophil diapedesis [17, 19, 46].

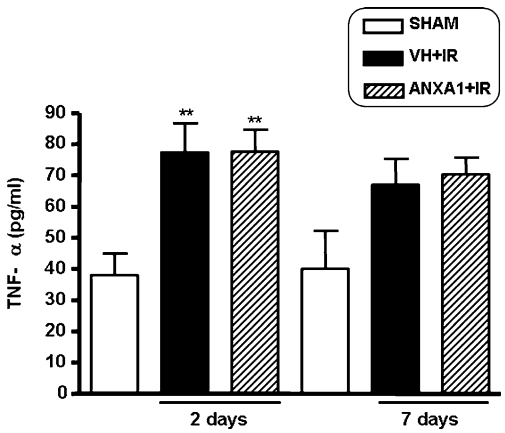

Fig 6 Determination of blood TNF-α. Sham-operated, vehicle group (VH + IR) and annexin A1-treated rats (ANXA1 + IR). Data are mean ± SEM. **$p<0.01$ vs. SHAM

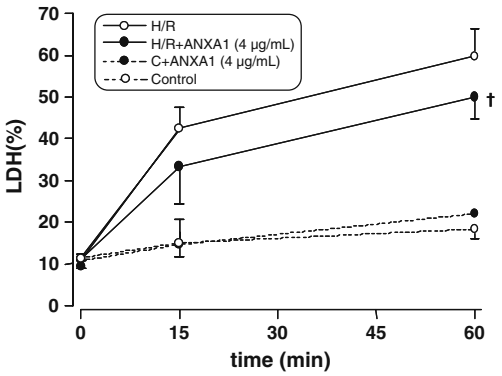

Fig 7 Effects of annexin-A1 (ANXA1) on control and hypoxic/reoxygenated (H/R) proximal tubules (PT). ANXA1 attenuated H/R injury in PT. †$p<0.05$ vs. H/R (60 min)

Finally, annexin A1 is associated with increased neutrophil apoptosis and L-selectin shedding from its surface [29].

Macrophage and lymphocyte

Some investigators have recently suggested that macrophage and lymphocyte infiltration has an early and significant role in I/R injury [13–16]. Indeed, I/R causes, as observed in the present study, a significant early and sustained macrophage infiltration, which has been associated to AKI development, long-term development of renal fibrosis, and renal tissue repair after renal I/R injury [14, 47–50]. Activated macrophages can damage renal tissue due to reactive oxygen species, nitric oxide, enzymes, and pro-inflammatory cytokines production. In the present study, annexin A1 treatment attenuated but not abolished macrophage extravasation. Our results are consistent with previous results showing that annexin A1 and diannexin inhibited macrophage trafficking in other models of I/R injury [20, 26]. It is possible that part of the annexin A1-induced protection observed was due to attenuation in macrophages renal influx.

The lymphocytes' role in I/R injury seems to be complex [5, 15]. In this study, a progressive renal tissue lymphocyte influx was observed after I/R. Several studies blocking T cell activation, depleting lymphocytes, or using genetically modified mice have pointed for these cells as important I/R injury mediators [13, 15, 16, 51], but others have not [12, 52]. Moreover, both protective and deleterious T cell subtypes have been described in renal I/R injury [53]. In contrast to neutrophils and macrophages, annexin A1 did not prevent lymphocyte infiltration after I/R, yet provided remarkable tissue protection.

Tumor necrosis factor

TNF-α is a pro-inflammatory cytokine upregulated in the early initiation phase of the ischemic injury and considered essential for neutrophil infiltration in inflamed tissue [43, 54]. TNF-α has been appointed as an important mediator of renal I/R injury through its cytotoxic and vasoconstrictive effects, its capacity to recruit neutrophils and macrophage and to induce renal epithelial cells apoptosis [52, 53]. Serum TNF was significantly increased in rats submitted to renal ischemia in this study. Although annexin A1 infusion prevented I/R-induced renal dysfunction, it did not affect TNF increase, pointing that this cytokine effects on renal function are dependent on tissue neutrophil extravasation.

Conclusions

This study indicates that annexin A1 has an important role in the renal defense against I/R injury. In addition, it showed that in this model of I/R injury, neutrophils are key components for the installation of renal structural and functional injury after reperfusion. Finally, these results may have a significant potential impact for the care and prevention of I/R injury in renal transplants and vascular surgery.

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Conflicts of interest The authors have no competing financial interests to disclose in relation to this manuscript.

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CONCLUSÕES

Diante dos dados obtidos a partir da realização dos objetivos iniciais, conclui-se que:

1. O peptídeo N-terminal da ANXA1, Ac2-26, demonstrou ter atividades antiinflamatórias, de modo dose-dependente, quando usado como tratamento prévio de células polimorfonucleares e mononucleares do sangue periférico humano estimuladas por zymosan-opsonizado (porção antigênica derivada da parece celular fúngica), inibindo a produção de espécies reativas de oxigênio, determinada por luminometria. Paralelamente, por meio de análise de citometria de fluxo, a incubação dos fagócitos com o peptídeo demonstrou diminuir a expressão dos receptores de superfície responsáveis pelo reconhecimento de zymosan-opsonizado, TLR2, nos neutrófilos e monócitos, respectivamente.

2. As células T CD4+ e monócitos humanos, envolvidos na infecção por HIV-1, tiveram seu imunofenótipo modulado por meio do tratamento in vitro com Ac2-26, de modo dose- e tempo-dependente. Nas células mononucleares do sangue periférico de indivíduos saudáveis ou HIV-positivos, o peptídeo modulou negativamente a transcrição de CCR5, avaliada por PCR em tempo real, sendo que em indivíduos HIV-positivos, estes resultados foram parcialmente revertidos pelo bloqueio de receptores FPR. Por meio de citometria de fluxo, os dados mostraram que o Ac2-26 também diminuiu a expressão do CCR5 na superfície de células T CD4+. No entanto, nos monócitos o peptídeo induziu uma mudança na distribuição dos subfenótipos, diminuindo aqueles com perfil pró-inflamatório (CD14lowCD16+), e, em contraste, aumentou a expressão de CCR5 em todos os monócitos. Em conclusão, os dados sugerem que a ação do N-terminal da ANXA1 é célula-dependente e que pode participar indiretamente da modulação da infecção por HIV-1.

3. Em um modelo não-humano de AIDS, realizado pela infecção por SIV em macacos, a análise da expressão transcricional demonstrou que a ANXA1 é regulada durante a progressão da doença nos principais compartimentos de infecção. Em comparação com as amostras de animais não-infectados, os transcritos de ANXA1 aumentaram no sangue periférico nas fases aguda, transicional e crônica da infecção por SIV, enquanto que na mucosa intestinal houve uma regulação negativa, atingindo os níveis basais apenas na infecção

crônica. A análise de marcadores típicos da progressão da AIDS, mostraram que os transcritos de ANXA1 aumentam significativamente em associação com a ativação de células T. No entanto, a expressão da ANXA1 tem uma correlação positiva com o número de transcritos de citocinas antiinflamatórias no sangue e no intestino, sugerindo que a ativação da sua transcrição está relacionada a tentativa de diminuir a exaustão imunológica durante a infecção. Em adição, a dinâmica da transcrição da ANXA1 é contrária a da replicação viral, representada pela carga viral plasmática.

4. Nos indivíduos com doenças inflamatórias intestinais (IBD), especialmente a Doença de Crohn, a expressão da ANXA1 é diminuída em nível de RNAm, no sangue periférico, e proteína, na mucosa colônica. Nestes pacientes, a imunoterapia com infliximab (anti-TNF-α) modulou a transcrição da ANXA1 e do TNF-a, e ainda a ativação linfocitária sistêmica, de acordo com a duração do tratamento e com os efeitos colaterais desta droga, tal como a bacteremia. Em suma, a dinâmica da ANXA1 somada a outros elementos imunológicos e clínicos parecem estar associados ao curso das IBDs e da qualidade de vida dos pacientes.

5. O último estudo indicou que a ANXA1 tem um papel importante na resposta antiinflamatória aguda contra lesão renal por isquemia e reperfusão. Além disso, mostrou que no modelo de lesão por I/R renal em ratos, os neutrófilos são elementos essenciais para a instalação da lesão renal estrutural e funcional após a reperfusão.

Em uma análise geral do conjunto de estudos apresentados, é possível concluir que a ANXA1 exógena e seus peptídeos derivados atuam como moléculas chave na modulação específica da ativação, imunofenótipo e distribuição dos fagócitos e linfócitos que participam como linha de defesa ou como alvo de agentes infecciosos, por meio da ligação com os FPRs ou por uma via ainda não elucidada. E, enfatizando a relevância da ANXA1 na defesa da homeostasia, ainda foi constatado que as vias relacionadas à regulação da ANXA1 endógena são ativadas diferencialmente em tecidos e fluidos sob inflamação aguda e crônica iniciadas por infecção viral, auto-imunidade ou hipóxia.

ANEXO

Ver o link do arquivo **Tese Angela ASS Priuli – Anexo**

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Expression of annexin A1 mRNA in peripheral blood from oral squamous cell carcinoma patients

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SUMMARY

Several studies have been suggesting annexin A1 protein as an active player in tumorigenesis of many organs. Nevertheless, its tumor biomarker role has been mainly studied in tissues by immunohistochemistry or cell culture. Hence, in this investigation, the peripheral blood from 27 oral squamous cell carcinoma (OSCC) patients and 25 negative control individuals were examined by quantitative real-time PCR. Down-regulated *ANXA1* expression at mRNA level was observed in OSCC samples ($p = 0.026$). Significantly diminished mRNA levels correlated to age, sex and the anatomical site of the tumor lesion were observed. Moreover, the ROC curve analysis revealed the performance of *ANXA1* expression as a suitable biomarker for patients with oral cavity cancer, especially those with 60 years of age or older and/or women. For the first time, *ANXA1* mRNA is revealed as blood-based biomarker, and its adoption for complementary non-invasive diagnosis of OSCC is suggested. These results suggest that, beyond the anti-inflammatory function, annexin A1 may also play a tumor suppressor role in peripheral blood cells, such as leukocytes.

Introduction

Oral squamous cell carcinoma (OSCC), the sixth most common cancer worldwide, continues to be the most prevalent cancer related to the consumption of tobacco, alcohol and other carcinogenic products[1] affecting about 500,000 patients worldwide each year.[2,3] Every year more than 300,000 new cases are reported in America of which approximately 9000 die of the disease, with a 5-year survival rate around 50%, despite several advancements in therapeutic regimens.[4]

The progression and metastasis of oral cancer involve elements detected in sera such as copper, iron and selenium, and also present tissue up-regulation of oncogenes (Ras, c-Myc, c-erb2),[5] mutation, deletion or down-regulation of tumor suppressor genes (p53),[6] cell cycle associated kinases and their inhibitors, growth factor receptors (EGFR, IGFR) and nuclear receptors such as RARalpha.[7] In addition, the immunohistochemistry has been a significant tool to identify altered expression levels of other cellular markers such as PCNA, cytokeratins, enzymes (cyclooxygenase-2), anti-apoptotic genes (Bcl family), pro-angiogenic genes (VEGF family) and immunomodulators (IL-10 and IL-12), which have been implicated in squamous cell carcinoma progression.[7] Early detection of oral cancer has remained elusive for appropriate clinical management of the disease. Unfortunately, specific biomarkers that distinguish different tumor conditions and behaviors needed for the early diagnosis and efficient treatment are still lacking.

Annexin A1 (*ANXA1*), an anti-inflammatory and calcium-dependent protein[8] of the superfamily of annexins, may have important

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regulatory roles in tumor development and progression. Recent interest in the biological activity of this intriguing molecule has unraveled important functional attributes of *ANXA1* in a variety of inflammatory pathways, on cell proliferation machinery, in the regulation of cell death signaling, in phagocytic clearance of apoptosing cells, and most importantly in the process of carcinogenesis.[9–11] The differential expression in various tissue cancers from prostate, ovary, breast, bladder, esophagus, larynx, pancreas, B cell, lymphoma and head and neck shows that this protein play a biomarker role when is up or down-regulated in distinct tumor stages.[9]

Recent study shows that annexin A1 has lower expression at protein and mRNA level in tissue and oral squamous cancer cells culture.[2] Previously, all of investigations associating cancer and *ANXA1* were performed with tissues or cells.[12–16] Recent advances in the field of biological sciences have sparked new interest in the area of identifying cancer biomarkers in bodily fluids.[17] It has been shown that identical mutation present in the primary tumor can be identified in the bodily fluids of the affected patients. Advantages of using bodily fluid as a diagnostic tool also resulted from its relatively non-invasive manner. mRNA in blood, semen, urine, and saliva has been proved as a novel resource to supplant conventional tools for disease identification.[18]

In the present study, the *ANXA1* gene expression was investigated by real-time PCR, for the first time, in peripheral blood samples of patients with oral squamous cell carcinoma and control subjects. Correlations with clinical parameters and disease status were then estimated, suggesting its significance as important protein expressed in blood and so as a new biomarker to oral cancer patients.

Patients and methods

Sample collection and preparation

Peripheral blood from 27 OSCC patients was collected at the time of surgery from April to December 2008 at the Clinical Hospital of Uberlândia. As non-cancer control subjects, blood samples of 25 volunteers without history of cancer were collected at the Stomatology Diagnosis Unit of the Federal University of Uberlândia. All the primary tumor sections were evaluated by a histopathological analyses and clinical information (age, sex, smoking, drinking, clinical history, treatment, recurrence) was collected. The clinical stage was determined postoperatively according to the tumor-node (TNM) staging system[19] and the histopathological grading according to the World Health Organization grading scheme.[20] All patient identifiers were coded to protect confidentiality. The study was approved by the University Ethics Committee under the number 249/09, and all of the patients gave written informed consent.

RNA isolation and reverse transcription

Total RNA was isolated by the guanidine isothiocyanate extraction method[21] with minor modifications. RNA concentration was obtained through spectrophotometric readings at 260 and 280 nm, and quality was evaluated by gel electrophoresis. Validated samples were stored at −80 °C. Two micrograms of total RNA from blood were reverse transcribed at 37 °C for 1 h in a 20 μL solution containing 40 U of Murine Moloney Leukemia Virus Reverse Transcriptase (MMLV-RT) (Amersham Biosciences, São Paulo, Brazil), 1× MMLV-RT Buffer, 10 U RNAse inhibitor (Invitrogen), 0.2 mM of each dNTP, and 126 pmol of hexamer random primers (Invitrogen). The final volume of each reaction was completed with DEPC (diethylpyrocarbonate)-treated water.

Quantitative real-time polymerase chain reaction (qPCR)

The qPCR assay was performed by using the 7300 Real-time PCR System (PE Applied Biosystems, Foster City, California, USA). Initially, the quality of cDNA was evaluated through PCR of the housekeeping gene β-2-microglobulin (*B2M*). The primers' set were: 5′–3′ CCT GCC GTG TGA ACC ATG T and GCG GCA TCT TCA AAC CTC C. Quantitative PCRs were carried out according to the instructions for SybrGreen PCR Core Reagent (PE Applied Biosystems, Foster City, California, USA).

The *ANXA1* forward and reverse primers were respectively: 5′-GAT TCA GAT GCC AGG GCC T-3′ and 5′-CAC TCT GCG AAG TTG TGG AT-3′. The relative expression of each specific product was calculated by $2^{-\Delta\Delta C_T}$ (CT = fluorescence threshold value; $\Delta C_T = C_T$ of the target gene $- C_T$ of the reference gene (*B2M*); $\Delta\Delta C_T = \Delta C_T$ of the oral cancer sample $- \Delta C_T$ of the calibrator sample). All samples were run in duplicates and the relative quantification of each target gene expression was performed twice.

Statistical analysis

The GraphPad Prism 4.0 software was used to perform statistical analysis and create diagrams. Analysis of the association between *ANXA1* mRNA expression and clinicopathological parameters are described and grouped in Table 1. Mann–Whitney

Table 1
Correlation between the status of *ANXA1* blood expression and the clinicopathologic characteristics of oral cancer patients.

Classification	Case number %	Relative mRNA expression	*p*-Value
Anatomical site			
Lip	26	2.09 ± 0.38	
Oral cavity	74	1.06 ± 0.11	0.02[a]
Age			
Under 60	52	1.33 ± 0.24	
Over 60	48	1.05 ± 0.14	0.04[a]
Sex			
Male	52	1.64 ± 0.27	
Female	48	1.10 ± 0.10	0.04[a]
Smoking			
Yes	89	1.26 ± 0.18	
No	11	1.17 ± 0.17	0.29
Drinking			
Yes	70	1.28 ± 0.22	
No	30	1.18 ± 0.13	0.17
T stage			
T1	29.6	1.189 ± 0.24	
T2	26	1.755 ± 0.32	
T3	18.4	1.309 ± 0.26	
T4	26	1.426 ± 0.38	0.51
N			
N0	78	1.332 ± 0.21	
N+	22	1.339 ± 0.24	0.44
Histopathological grade			
Well	48	1.395 ± 0.22	
Moderately	52	1.364 ± 0.23	
Poorly	0	–	0.46
Chemotherapy			
Yes	33	1.505 ± 0.52	0.33
No	67	1.32 ± 0.16	
Radiotherapy			
Yes	37	1.636 ± 0.48	0.20
No	73	1.291 ± 0.17	
Recurrence			
Yes	37	0.89 ± 0.18	0.10
No	73	1.31 ± 0.18	

[a] *p*-Value significant at 0.05 probability level.

U-tests were performed to compare gene expression levels between the independent groups of cancerous and non-cancerous blood samples. Kruskal–Wallis test was used to compare the relative quantitative gene expression in tumor patients' blood stratified into two independent patient groups, considering the parameters age, sex, anatomical sites, TNM grading system, recurrence and therapy, due to the lack of normality of the original expression data. Significance was considered with probability levels below 5%.

To visualize the efficacy of the biomarker to discriminate tumor versus control in blood individuals (in the absence of an arbitrary cutoff value), we summarized the data in a ROC (receiver operating characteristic) curve. This curve plots the sensitivity (true positives) on the *Y* axis against the 1 − specificity (false positives) on the *X* axis, considering each observed value as a possible cutoff value. The AUC (area under curve) was calculated as a single measure for the discriminative efficacy of a marker. When a marker has no discriminative value, the ROC curve will lie close to the diagonal and the AUC is close to 0.5. When a test has strong discriminative value, the ROC curve will move up to the upper left-hand corner and the AUC will be close to 1.0. The Statistical Package for Social Sciences (SPSS) was used for analyses.

Results

Decreased expression of annexin A1 mRNA in peripheral blood patients

Amplicons obtained with *ANXA1* and *B2M* primers were detected by agarose gel electrophoresis to verify the expected size and the quality of the samples (Fig. 1). *ANXA1* mRNA was expressed in all of OSCC samples and healthy samples. Comparative analysis of OSCC blood patients showed significantly lower *ANXA1* expression when compared to blood sample of control individuals ($p = 0.026$). The results of relative gene expression for *ANXA1* of peripheral blood from OSCC patients are presented in Fig. 2A. Real-time PCR revealed that a low expression level of *ANXA1* transcripts was detected in the group with OSCC, conferring 1.5-fold decrease compared to the control subjects, and odds ratio of 0.33 for cancer event ($p = 0.06$). Diagnostic efficiency was tested by the ROC curve analysis and showed AUC = 0.65 (95% CI; 0.51–0.78, $p = 0.05$) (Fig. 2A).

Differential detection of annexin A1 at mRNA in agreement with clinical data

Clinical profile of cancer and control group is summarized in Table 1. The majority of OSCC patients presented lesions in intraoral regions, mainly tongue ($n = 8$), followed by gum ($n = 7$). Generally, the OSCC was found in individuals under 60 years of age ($n = 14$) and in male patients ($n = 14$). Moreover, the smoking ($n = 24$) and drinking ($n = 19$) habits were remarkable characteristics of cancer patients. TN stage system and histopathological grade presented no difference among cancer individuals. Combined chemotherapy and radiotherapy was applied in the majority of the investigated OSCC patients.

Expression data and their correlation with clinicopathological findings are displayed in Table 1 and significant results are illustrated in Fig. 2B–D. Comparative analysis detected significant down-regulation of annexin A1 in the tumor group with lesions in the oral cavity (tongue, gum, floor and palate) versus those with inferior and superior lips' cancers ($p = 0.043$). mRNA expression was also sex-related and showed decreased *ANXA1* mRNA levels in the peripheral blood of the female patient group ($p = 0.009$). In addition, patients over 60 years old presented diminished expression than younger individuals ($p = 0.04$). We have also compared the areas under the ROC curve (AUCs), for the *ANXA1* expression in the tumor site (oral cavity) of the OSCC and in female and/or elderly (>60 years old) patients, which were significantly different from the AUCs obtained for the control individuals (Fig. 2B, C, and D; $p = 0.05$, $p = 0.03$, $p = 0.005$, respectively, at 95% CI).

There were no significant differences between patients' subgroups in relation to smoking, drinking, recurrence, TNM staging, histopathological grading or therapies. No statistical differences about age and sex characteristics were observed in negative controls.

Discussion

The global increase in frequency and mortality of oral cancer has intensified current research efforts in the field of prevention and early detection of this disease. Therefore, the search for alternative approaches to biopsy and a number of molecular-based diagnostic markers have been attempted to detect the presence of oral cancer.[4] For the first time, the *ANXA1* was identified in the peripheral blood by real-time PCR and was suggested as a potential diagnostic biomarker. The present study revealed the *ANXA1* mRNA as new transcript biomarker for early detection of OSCC in the peripheral blood of patients, which is a very different approach used by other investigators that have only reported differential annexin A1 protein expression by immunohistochemistry in OSCC tissues in agreement with the histopathological grade.[2]

At mRNA level, real-time PCR revealed a down-regulation of *ANXA1* in the peripheral blood from OSCC patients in any clinical stage (histopathological grading and TN system) when compared to control individuals without cancer history. Previous studies have indicated that annexin A1 protein is present intracellularly in a variety of tissues including human peripheral blood leukocytes.[9] Annexin A1 is present in all leukocyte subsets, except B lymphocytes, presenting maximal content in monocytes and polymorphonuclear neutrophils and least in lymphocytes,[22] exerting diverse and antagonistic immune functions.[23] Indeed, it is thought that major cell types in the peripheral blood from cancer patients are lymphocytes, so may be *ANXA1* mRNA down-regulation in cancerous blood cells is proportional to T cell quantities during tumoral immunological surveillance.

An important finding was the anatomical site-dependent transcriptional regulation of *ANXA1*, exhibiting higher expression in the peripheral blood of patients bearing lip tumor, whereas the mRNA levels in the peripheral blood of patients with tumors in the oral cavity were lower. In this way, it is known that tumors

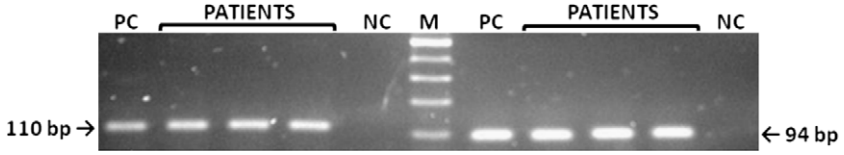

Figure 1 Expression of annexin A1 mRNA and clinical data of OSCC patients. Detection of PCR products by ethidium bromide staining in agarose gel electrophoresis. Primers for *ANXA1* (110 bp) and *B2M* (94 bp) were initially tested and amplicons were detected in three OSCC blood samples. Lane M: 100 bp size marker. Lanes PC: positive controls. Lanes NC: negative controls.

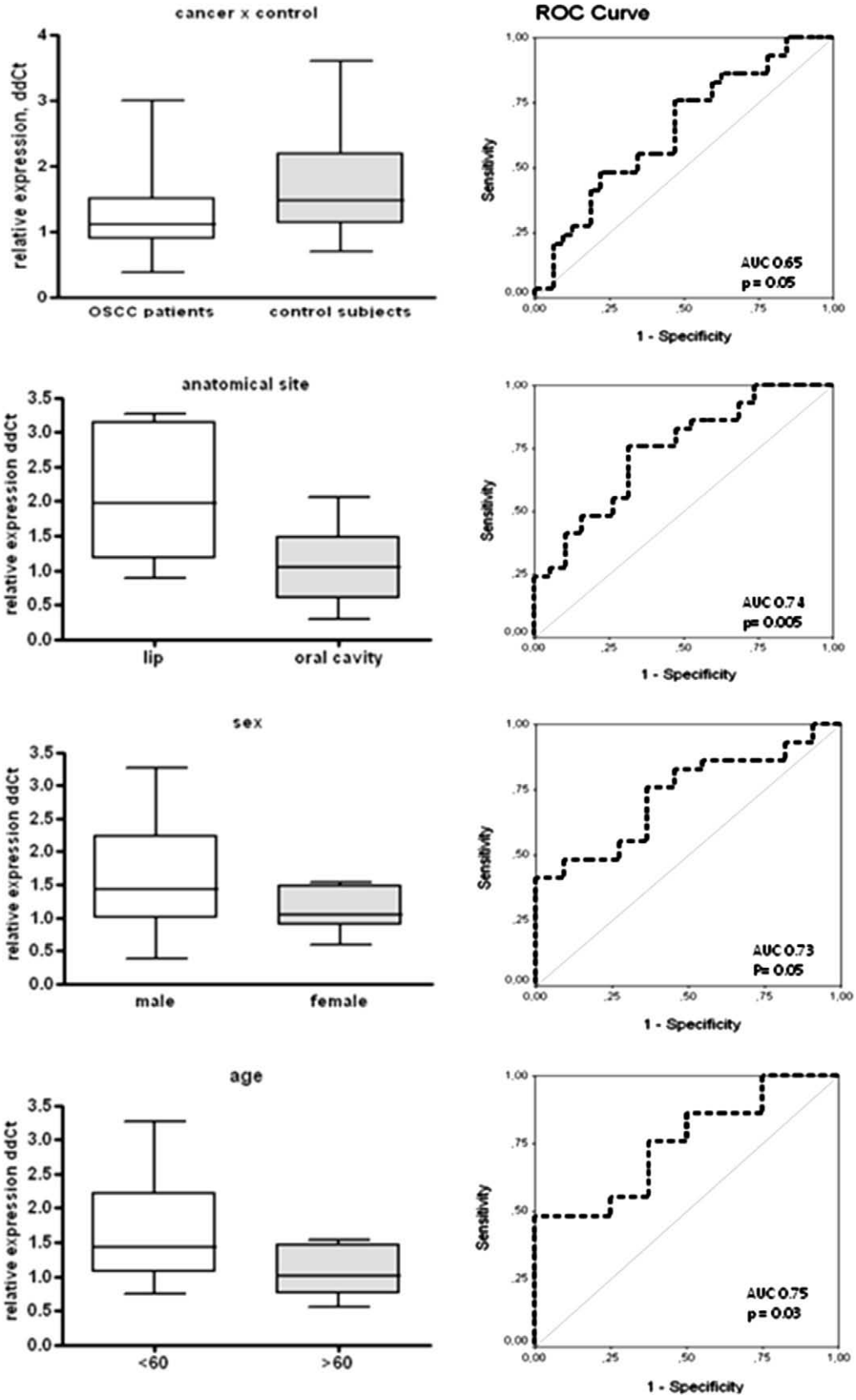

Figure 2 *ANXA1* expression in the peripheral blood as a biomarkers for OSCC. (A) The box-plot diagram shows significantly down-regulated *ANXA1* mRNA expression in the peripheral blood of OSCC patients compared to healthy samples. (B, C and D) Box-plot diagrams showing gene expression in cancer subsets according to the clinical data. The line within the boxes indicates the median. The top edge of the boxes represents the 75th percentile, the bottom edge the 25th percentile. The range is shown as a vertical line, extreme values were excluded. (A–D) ROC curves were combined with representative box-plots of qPCR, indicating the diagnostic efficiency of AUC and their *p*-values. Broken line illustrates the *ANXA1* expression and solid one represents the reference line.

originated from different sites in the mouth, like the tongue, mouth floor, and buccal mucosa, have different cancer development characteristics.[17] Consistent with this data, a recent study revealed the involvement of increased annexin A1 expression in melanoma dissemination, which may explain the higher expression profile of this blood marker in patients with lip tumors.[24] Elderly patients (>60 years old) also indicated striking down-regulation of *ANXA1* and this observation fits with an epidemiology work that showed worse overall survival in older patients.[25] In addition, the lower *ANXA1* expression in female patients might be involved not only with gender differences, but also with increased incidence of OSCC in female patients.[26] ROC curves demonstrated the discriminating power of the diagnostic test of *ANXA1* mRNA levels in the peripheral blood, and were in agreement with clinical data, indicating that this gene may be involved in cancer progression in specific OSCC patients and represents a good marker for previously diagnosing cancer in people presenting these clinical characteristics.

The development of oral cancer, a subgroup of head and neck cancers, has been linked to long term use of tobacco and alcohol[27]; however, our results do not show differences in *ANXA1* expression in the peripheral blood samples of smoking and drinking habits within the OSCC groups. This data is consistent with previous investigation performed in cancerous tissue at protein and mRNA levels.[2] Limited by the small overall sample size ($N = 27$), we were not able to generate additional important data to see if the real-time PCR data can segregate clinical characteristics as TNM staging, histopathological grade, therapy and recurrence.

In this way, although the precise mechanism is still not clear, the described pro-apoptotic[28] and anti-proliferative[29] roles of annexin A1 suggest that there is a regulation pathway for its expression in tumor conditions, especially in tissues and body fluids, which lead to a critical decrease in *ANXA1* mRNA levels that are associated with cancerous phenotype. We propose that *ANXA1* play an important role as a tumor suppressor gene. On the other hand, a phagocytosis study showed that down-regulation of annexin A1 homolog protein prevented efficient engulfment of determined cells from *Caenorhabditis elegans*.[30] This data suggests a "eat me" signal role for annexin A1 in tumor cells, as showed previously in neutrophils,[31] allying its diminished expression and known immune evasion described in tumor biology.

Non-invasive fluid biomarkers are the most widely used alternative to biopsy and have recently emerged as innovative diagnostic tests and advanced healthcare technology solutions that help improve patient care. Compared to tissue biopsies, blood is an easily accessible fluid and our findings demonstrated that *ANXA1* can be used as a non-invasive blood-based biomarker for oral squamous cell carcinoma. Therefore, the purpose of investigating the absence or presence of any peripheral blood mRNA marker, including *ANXA1*, yet is recognized as a powerful tool of diagnosis and biology comprehension due to the localization of mRNA,[32] which is disposed in cells as standby transcripts that may be translated or not under specific stimulus.

Another potentially profitable area for future investigation may be to examine the *ANXA1* expression in saliva of these patients. The application of the salivary proteomics may be explored for early detection of human cancers, predicting aggressiveness and prognosis, and surveillance for cancer recurrence, which may eventually lead to simple clinical tools for early detection and monitoring of cancers such as OSCC. In addition, saliva represents a readily accessible body fluid that may be repeatedly sampled for in vivo assessment of efficacy and toxicity of anticancer drugs.

We believe that novel discoveries and technological advances in conjunction with the ability to diagnose a disease by a non-invasive biofluid screening, such as the peripheral blood, will foment a revolutionary change in medicine. The use of *ANXA1* as a biomarker in OSCC opens a new perspective for cancer diagnosis, by demonstrating its important association with specific tumor characteristics, like mucosal cancers, and may become of great significance when appropriately employed for differential diagnosis.

Conflict of interest statement

None declared.

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