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Análise da proteína Anexina A1 na diferenciação morfofenotípica e funcional dos mastócitos no câncer colorretal

Tese apresentada para obtenção do título de Doutor em Biociências, Área de Concentração Genética.

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***Adorina** e **Marcelo**,*
pessoas admiráveis,
que me dedicaram suas vidas
e me ensinaram o valor do saber.
Sou eternamente grato a eles
por terem me dado raízes
e asas.

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um pilar em minha vida.
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me permitiram chegar ao final desse trabalho.

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"[...] O que procuraste em ti ou fora de

teu ser restrito e nunca se mostrou,
mesmo afetando dar-se ou se rendendo,
e a cada instante mais se retraindo,

olha, repara, ausculta: essa riqueza
sobrante a toda pérola, essa ciência
sublime e formidável, mas hermética,

essa total explicação da vida,
esse nexo primeiro e singular,
que nem concebes mais, pois tão esquivo

se revelou ante a pesquisa ardente
em que te consumiste... vê, contempla,
abre teu peito para agasalhá-lo."

[Carlos Drummond de Andrade em "A máquina do mundo"]

RESUMO

O câncer colorretal (CCR) é o terceiro mais comum no mundo e a quarta causa de morte por câncer no ocidente. A origem e desenvolvimento dessa patologia têm sido amplamente associados com alterações no estado inflamatório do intestino. A Anexina A1 (AnxA1) é uma proteína anti-inflamatória envolvida na manutenção da homeostase intestinal e na regulação do desenvolvimento tumoral. Os mastócitos (MCs), que também expressam AnxA1, são amplamente envolvidos nos danos associados no trato gastrointestinal promovido por agentes carcinógenos. No presente estudo foi avaliado o papel da AnxA1 na resposta funcional de MCs na carcinogênese colorretal induzida por N-metil-N'-nitro-N-nitrosoguanidina (MNNG). Para esse objetivo, animais deficientes de AnxA1 ($AnxA1^{-/-}$) receberam instilações de MNNG e o efeito agudo do composto foi avaliado após 8, 24 e 48h. Nossos resultados mostram que o MNNG induz, no epitélio do cólon, aumento da expressão da AnxA1, que limita a lesão tecidual e inflamação local, regulando a produção exacerbada de IL-1, IL-6, IL-12, TNF- α, INF- γ na condição controle e, principalmente, após 24h da exposição. A AnxA1 também reduz a migração e desgranulação de MCs, polarizando a diferenciação dessas células para maior expressão de quimase após o dano carcinogênico. Em seguida, a contribuição dos MCs à tumorigênese induzida pelo MNNG foi avaliada 24 semanas após a exposição crônica dos camundongos deficientes de mastócitos ($Kit^{-/-}$) ao composto. Nessas condições foi observado aumento do número de MCs totais, desgranulados, triptase e quimase positivos e o desenvolvimento de lesões tumorais nos animais selvagens ($Kit^{+/+}$) e reconstituídos de mastócitos ($Kit^{-/-}$+MC). Diferentemente, foi verificada menor susceptibilidade aos tumores induzidos por MNNG nos $Kit^{-/-}$. Na condição crônica, o MNNG promoveu a formação de um microambiente imunorregulado rico em AnxA1, com baixa expressão das citocinas estudadas e aumento de IL-4. Esse cenário imunossupressor sugere dependência dos MCs, células que também foram associadas com a maior proliferação de células epiteliais colônicas. Em conjunto, os resultados obtidos indicam a AnxA1 como um possível alvo terapêutico no CCR, pois regula os danos iniciais induzidos pelo MNNG e a atividade e diferenciação dos MCs que, cronicamente, contribuem para o desenvolvimento dos tumores.

Palavras-chave: microambiente tumoral, carcinogênese, inflamação, imunidade inata, intestino

ABSTRACT

Colorectal cancer (CRC) is the third most common cancer in the world, the forth cancer-related death cause in the Western counties, and its origin and development has been wildly related to alterations in bowel inflammatory state. Annexin A1 (AnxA1) is a protein involved in the maintenance of intestinal homeostasis and the regulation of tumoral development through the inhibition of inflammatory pathways. Mast cells (MCs), which also expressed AnxA1, were extensively involved in carcinogens-associated damage in gastrointestinal tract. The present study was conducted to evaluate the role of AnxA1 in the functional response of MCs in colorectal carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Thus, we measured MCs recruitment, activation, proteases (tryptase and chymase) expression and the levels of cytokines (IFN-γ, IL-1, IL-4, IL-6, IL12 and TNF-α) in the colon of AnxA1-deficient mice (AnxA1-/-) during the acute response to MNNG. Mast cells-deficient mice (Kit-/-) were also exposed to MNNG carcinogenic activity in order to check, after 24 weeks, the contribution of MCs in tumor growth. Our results showed that MNNG damage increased the expression of AnxA1 which promoted tissue protection and regulated the migration and activation of MCs. The carcinogen induced local immune deregulation with increased levels of pro inflammatory cytokines found in AnxA1-/-. Chymase positive MCs were found at higher number in acute exposure to MNNG and also in the established tumor environment. Interestingly, a greater number of chymase-positive cells were observed in the presence of AnxA1. At chronic condition, MNNG promoted an immunoregulated AnxA1-rich microenvironment dependent on the MCs, which stimulates the proliferation of colonic epithelial cells. In conclusion, our results indicate that AnxA1 regulates MNNG-induced damage and the activity of MCs which, chronically, seems to contribute to tumor development.

Keywords: Anexin A1, mast cells, colorectal cancer, tumor macroenvironment

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°C	graus Celsius
g	grama
<	menor
%	porcentagem
kg	quilograma
µg	micrograma
µL	microlitro
µm	micrômetro
mg	miligrama
mL	mililitro
mm	milímetro
ng	nanograma
nm	nanômetro
pg	picograma
ANOVA	análise de variância
AnxA1	Anexina A1
$AnxA1^{-/-}$	camundongos BALC/c deficientes para AnxA1
$AnxA1^{+/+}$	camundongos BALC/c selvagens
$AnxA1_{2-26}$	peptídeo derivado da porção n-terminal da Anexina A1
BALB/c	linhagem de camundongos albinos
BSA	albumina do soro bovino
C57/BL6	linhagem de camundongos de pelagem escura
Caspase-3	marcador de apoptose celular
CCR	câncer colorretal
CD	cluster de diferenciação
CTMCs	mastócitos do tecido conjuntivo
DAB	3-3 tetrahidrocloreto diaminobenzidin
DAMPs	padrões moleculares associados ao dano celular
DMEM	meio de cultivo celular Dulbecco
DMH	1,2-dimetil-hidrazina
DNA	ácido desoxirribonucleico
DOM	densidade óptica média
DSS	sulfato sódico de dextrana

EERP	Escola de Enfermagem de Ribeirão Preto
FCF	Faculdade de Ciências Farmaceutícas
FGF-2	fator de crescimento dos fibroblastos 2
FPRs	receptores para peptídeos formilados
hCMA1	gene da quimase de mastócitos humanos
HCT116	linhagem de células tumorais colônicas
Hep-2	linhagem celular de câncer de laringe
i.r.	via intrarretal
IBILCE	Instituto de Biociências, Letras e Ciências Exatas da UNESP
IFN-γ	interferon γ
IL-	interleucina
INCA	Instituto Nacional do Câncer
$Kit^{-/-}$	animais deficientes de mastócitos (KIT knock-out)
$Kit^{-/-}$+MC	camundongos deficientes de mastócitos reconstituídos
$Kit^{+/+}$	camundongos selvagens WB/B6
MC_C	mastócitos somente quimase positivos
MC_T	mastócitos somente triptase positivos
MC_{TC}	mastócitos triptase quimase positivos
mMCP	protease de mastócitos murinos
MMCs	mastócitos de mucosa
MNNG	Metil-N´-Nitro-N-Nitroso-Guanidina
NF-κB	fator de transcrição nuclear κB
NLRs	receptores semelhantes ao NOD
p53	gene supressor de tumor
PAMPs	padrões moleculares associados à patógenos
PBS	solução tampão fosfato
PCNA	antígeno nuclear de proliferação celular
RNA	ácido ribonucleico
RNAi	RNA de interferência
SCF	fator de crescimento de célula-tronco
SOCS3	fator de transcrição celular
Stat3	fator de transcrição celular
TBS	solução Tris base
TLRs	receptores semelhantes ao Toll
TNBS	ácido 2,4,6-trinitrobenzenosulfônico

TNF-α	fator de necrose tumoral
Tyk2	fator de transcrição celular
UNESP	Universidade Estadual Paulista “Júlio de Mesquita Filho”
USP	Universidade de São Paulo
VEGF	fator de crescimento do endotélio vascular
WBB6F1-Kit$^{W/W-v}$	camundongos Kit$^{-/-}$

APRESENTAÇÃO

Nesse trabalho, dois modelos experimentais diferentes que se complementam foram utilizados na busca pela influência da proteína Anexina A1 (AnxA1) sobre a atividade dos mastócitos na carcinogênese de cólon, induzida pelo carcinógeno MNNG. No primeiro modelo, os animais deficientes de AnxA1 foram expostos à atividade aguda do MNNG. No segundo, os animais deficientes de mastócitos ($Kit^{-/-}$) foram submetidos à tumorigênese crônica, com o objetivo de avaliar o papel dessas células no ambiente tumoral estabelecido, bem como o envolvimento da AnxA1 nesse cenário.

Os resultados dessa tese de Doutorado originaram o manuscrito intitulado *"Secreted annexin A1 modulates mast cell pro-tumoral actions in N-methyl-N'-nitro-N-nitrosoguanidine-induced colorectal cancer"* (APÊNDICE 1) submetido para a revista *British Jounal of Cancer* (ANEXO 4). Paralelamente, outras investigações foram desenvolvidas em colaboração e resultaram na publicação de dois artigos científicos, anexados na íntegra à tese. O artigo intitulado "*Humoral imune responses against the malaria vaccine candidate antigen Plasmodium vivax AMA-1 and IL-4 polymorphisms in individuals living in an endemic área of the Brazilian Amazon"* foi publicado na *Cytokine*, cujo assunto está relacionado com a interferência de polimorfismos no gene da IL-4 no perfil de resposta Th2 e na produção de anticorpos contra a malária (APÊNDICE 3). O outro artigo intitulado *"Heterogeneity of mast cells and expression. of Annexin A1 protein in a seconde degree burn model with silver sulfadiazine treatment"* foi publicado na *Plos One* e explora o envolvimento dos mastócitos na regeneração tecidual de queimaduras tratadas com sulfadiazina (APÊNDICE 2).

SUMÁRIO

1. INTRODUÇÃO

1.1. Câncer colorretal

O câncer colorretal (CCR) é o terceiro tipo de câncer mais comum no mundo e a quarta causa de morte por câncer no ocidente (FAVORITI et al., 2016). Com o aumento da expectativa de vida da população brasileira, as neoplasias vêm ganhando cada vez mais importância no perfil de morbidade e mortalidade do país. A última estimativa do Instituto Nacional do Câncer (INCA) projetou que, em 2016, o CCR seria o segundo mais comum entre as mulheres com 17.620 casos, ocupando, por sua vez, a terceira posição entre os homens, com 16.660 casos novos, sendo a maior prevalência nas regiões sul e sudeste.

A nomenclatura "câncer colorretal" se refere a todas as neoplasias malignas originadas no epitélio do intestino grosso (cólon) e do reto (BELOV; ZHOU; CHRISTOPHERSON, 2010). De acordo com os diferentes sítios anatômicos de origem do tumor, esses podem ser divididos em três entidades (câncer de cólon proximal, distal e retal) (LI; LAI, 2009). O adenocarcinoma (ADC) é a manifestação histológica mais frequente, correspondendo a mais de 90% dos casos (HAMILTON et al., 2010) enquanto os demais tipos histológicos incluem ADC mucinoso, carcinoma de células escamosas e carcinomas indiferenciados (ZAMPINO et al., 2009).

A história natural dessa neoplasia apresenta uma transição de criptas normais a adenoma e, posteriormente, a adenocarcinoma durante um período de 10 a 20 anos (INCA – MINISTÉRIO DA SAÚDE, 2012). Os pólipos adenomatosos (ou ADs) são protuberâncias que se projetam no lúmen intestinal, classicamente considerados lesões pré-malignas que fazem parte das etapas da carcinogênese colorretal. Evidências demonstram redução da morbimortalidade associada à detecção precoce de focos de criptas aberrantes (FCAs), lesões invasivas e precursoras de pólipos adenomatosos (PAs). Nenhuma outra malignidade apresenta tão acentuada abundância de lesões pré-neoplásicas e/ou pré-cancerosas como o CCR (TANAKA; ISHIKAWA, 2013a). O período de tempo necessário para o desenvolvimento do ADC a partir do AD é longo, mesmo com estimativas conservadoras indicando um intervalo de 5 a 10 anos. O surgimento do AD e sua progressão para ADC é resultado do acúmulo de alterações genéticas e epigenéticas que, dentre outras causas, promovem o desequilíbrio entre a apoptose e a proliferação das células epiteliais da mucosa intestinal (DAVIES; MILLER; COLEMAN, 2005).

As modalidades terapêuticas disponíveis para o tratamento do CCR incluem a ressecção cirúrgica da área do intestino afetada, seguida de quimioterapias e radioterapias, combinada

com anticorpos monoclonais ou proteínas contra o fator de crescimento do endotélio vascular (VEGF) e o receptor do fator de crescimento epidermal (EGRF) (BUTLER et al., 2016; CHAN et al., 2017; PINTER et al., 2016). Sem tratamento adicional, aproximadamente 50% dos pacientes submetidos à ressecção cirúrgica apresentam recidivas e progressão da doença metastática (MEDEIROS; OSHIMA; FORONES, 2010). Entretanto, o uso de terapias combinadas também não representa a cura total do câncer, sendo que a recorrência em 5 anos é de cerca de 40% dos pacientes (AUGESTAD et al., 2015). Embora aprimoradas ao longo do tempo, estas técnicas de controle e erradicação do tumor, que despontaram a partir da década de 70 e atualmente parecem ter atingido seus índices ótimos, ainda apresentam percentuais de sucesso insatisfatórios na garantia de sobrevida de cinco anos para o paciente. Tais percentuais estão em torno de 50% no caso de tumores detectados precocemente e de 35% para tumores avançados (SINHA; HONEY, 2012). Contudo, os sintomas clínicos podem não ser aparentes até o final do curso da doença e, por essa razão, o CCR é frequentemente diagnosticado em uma fase avançada (TANAKA, 2009).

A história pessoal de adenoma (AD) e familiar de CCR e de condições hereditárias, como Polipose Adenomatosa Familiar (Familial Adenomatous Polyposis - FAP) e Câncer Colorretal Hereditário Não-Polipoide (Hereditary Non-Polyposis Colorectal Cancer - HNPCC) ou Síndrome de Lynch são fatores que predispõem ao aparecimento dessa neoplasia (ITZKOWITZ; YIO, 2004). Tradicionalmente, o CCR pode ser dividido em casos esporádicos, familiares e hereditários, sendo que aproximadamente 80% são esporádicos (ZAMPINO et al., 2009). Dentre os fatores genéticos envolvidos na etiologia do CCR esporádico destaca-se, como evento inicial, uma mutação no gene supressor de tumor APC encontrada em 80% dos casos, sugerindo que a tumorigênese colorretal esporádica siga a sequência "adenoma-carcinoma", assim como na FAP (CHEAH, 2009). A progressão da mucosa normal para AD, e deste para ADC, também está associada ao acúmulo de mutações em genes como o TP53, KRAS e SMAD2/4, considerados de grande importância para esse tipo de progressão tumoral, apesar de não explicarem com êxito o surgimento de todos os CCRs (DAVIES; MILLER; COLEMAN, 2005), entre outras alterações genéticas.

Duas vias de desenvolvimento do CCR esporádico foram identificadas de acordo com os eventos moleculares que lhe dão origem. Uma delas é denominada via "canônica" (sequência de adenoma a carcinoma) ou "supressora" por envolver a perda da expressão de genes supressores de tumor. Esta via é a mais comum e caracteriza-se por mutação ou por deleção de K-ras, APC, DCC, e p53, entre outros genes, como c-MYC e c-ERBB2 (HAMILTON, 1993; KAPITANOVÍC et al., 1997; CALVERT; FRUCHT, 2002). A segunda via, menos frequente, é a "mutante" e envolve a instabilidade de microssatélites. A sua

característica molecular principal é um grande acúmulo de mutações em sequências de microssatélites ao longo do genoma, causadas por alterações primárias nos genes do sistema de reparo de erros do DNA (MMR) (MORÁN et al., 2010). Estas duas vias têm distintas características clínicas e histopatológicas (LEA; JACKSON; DUNNICK, 2009), sendo que e os tumores originados pela via supressora têm melhor resolução clínica (COLUSSI et al., 2013). A frequência de alterações e alguma das associações clínicas para alguns desses genes são apresentados na Tabela 1.

Tabela 1 - Oncogenes e Genes supressores de tumor frequentemente associados ao Câncer Colorretal

Gene	Frequência	Associação
APC	85%	Associado a Polipose Familiar Hereditária e sua inativação é encontrada em grande parte dos cânceres colorretais esporádicos.
TP53	35-55%	Associado a transição de pólipo para câncer invasivo, enquadrando-se como última chave deste processo.
KRAS	35-45%	Pacientes com câncer colorretal estádio IV e mutação de KRAS não respondem a terapia com inibidores de EGFR.
TGFBR2	25-30%	Mutação presente em 90% dos tumores com instabilidade microssatélite e 15% dos cânceres colorretais com estabilidade microssatélite
MLH1, MSH2, MSH6	15-25%	Associado a câncer colorretal hereditário sem polipose HNPCC.
SMAD4	10-35%	Mutação encontrada na polipose familiar juvenil com risco aumentado de câncer colorretal em mais de 60% dos casos após a terceira ou quarta décadas de vida.

Fonte: Adaptado de PARREIRAS et al., 2013.

Algumas características importantes do estilo de vida como o consumo excessivo de carne vermelha (HOGG, 2007), alimentos gordurosos (LIU et al., 2011a) ou álcool (etilismo) (FEDIRKO et al., 2011) contribuem para o aparecimento esporádico (não hereditário) da doença. Outros fatores de risco incluem a idade acima dos 50 anos, a obesidade, o sedentarismo (BEYDOUN; BEYDOUN, 2008) e o diabetes (LARSSON; ORSINI; WOLK, 2005). Intervenções sobre esses hábitos reduzem o risco a que os pacientes estão sujeitos, e nos casos de recorrência familiar o acompanhamento possibilita a detecção precoce (ANAND et al.,

2008). Em comum, todos esses fatores tem a capacidade de modificar o estado inflamatório do intestino (ANAND et al., 2008). Por isso, o uso de aspirina tem sido proposto em ensaios clínicos na terapia preventica do CCR pela sua atividade de inibição da síntese da prostaglandina E2 (PGE_2) (DREW; CAO; CHAN, 2016). As evidências da intrínseca associação entre inflamação e o estabelecimento de neoplasias (GRIVENNIKOV; GRETEN; KARIN, 2010) reforçam a necessidade do estudo do papel das células inflamatórias na carcinogênese colorretal, um tema que vem ganhando destaque nos últimos anos (DI CARO et al., 2013).

1.2. Mastócitos no câncer colorretal

Os mastócitos são células imunes residentes, ricas em grânulos citoplasmáticos metacromáticos contendo diversos moduladores inflamatórios como citocinas, proteases, histamina, seratonina e eicosanóides. Os mastócitos são derivados de células progenitoras pluripotentes da medula óssea, CD34+/CD117+ em humanos (KENNELLY et al., 2011). A partir daí, o desenvolvimento de mastócitos maduros nos tecidos é dependente da ativação de KIT (CD117) pela associação com o seu principal ligante, o fator de crescimento de células-tronco (SCF) (HALOVA; DRABEROVA; DRABER, 2012). Entretanto, o estímulo somente com IL-3, independente de SCF, também promove a manutenção de mastócitos murinos *in vitro*, uma vez que ambos induzem a ativação do fator de transcrição STAT5, importante regulador da proliferação e sobrevivência dessas células (SHELBURNE et al., 2003).

Mastócitos diferenciados no tecido são células residentes de longa vida, contudo dependentes do estímulo contínuo de SCF. A suspensão da atividade catalítica do KIT resulta na apoptose de mastócitos, que também é promovida pela privação de SCF, danos do DNA e outros estímulos intrínsecos que resultam na ativação de caspases (WOIDACKI et al., 2013). Assim, o silenciamento do gene codificante de KIT resulta na deficiência de mastócitos e constitui um modelo de estudo importante no entendimento da atividade dessas células em diversas condições fisiopatológicas. No cólon normal, os mastócitos são localizados no mesênquima adjacente ao epitélio intestinal e sua função é diretamente associada à organização da membrana basal (CHICHLOWSKI et al., 2010), uma vez que os animais deficientes de mastócitos ($Kit^{-/-}$) apresentam alterações da arquitetura intestinal (GROSCHWITZ et al., 2009).

Os mastócitos expressam uma grande diversidade de receptores de superfície capazes de regular sua proliferação migração e ativação. A ativação dos mastócitos desencadeia uma série de eventos bioquímicos que culmina em uma cascata de sinalização intracelular, que resulta na liberação do conteúdo dos grânulos citoplasmáticos no espaço extracelular por exocitose, processo denominado desgranulação. O reconhecimento de antígenos específicos via IgE ligada

a receptores de superfície nos mastócitos é o principal estímulo conhecido para a ativação dos mastócitos. Contudo, a presença de receptores semelhantes ao Toll (TLRs) e NOD (NLRs) é amplamente aceita nessas células, fornecendo a capacidade de responder também à padrões moleculares associados aos patógenos (PAMPs) e dano celular (DAMPs) (DIETRICH et al., 2010).

A ativação dos mastócitos resulta na rápida produção de mediadores inflamatórios derivados de lipídeos da cascata do ácido aracdônico, eicosanóides, prostaglandinas e leucotrienos, a partir de fosfolipídios de membrana e esfingolipídios. Nesse momento, também ocorre a liberação de mediadores pré-formados armazenados nos grânulos dos mastócitos como aminas bioativas, principalmente a histamina, proteoglicanos e proteases, sobretudo triptases e quimases (MOON; DEAN BEFUS; KULKA, 2014). Após esses eventos iniciais, ocorre aumento da expressão gênica de citocinas, como o fator de necrose tumoral (TNF-α), interleucinas 1, 4 e 6 (IL-1, IL-4 e IL-6), além de quimiocinas e fatores de crescimento como o fator de crescimento dos fibroblastos 2 (FGF-2) e o fator de crescimento do endotéliol vascular (VEGF). Esses mediadores influenciam profundamente os tecidos ao redor, sendo relacionados recentemente à indução de respostas imunes, hiperplasia celular, angiogênese e, também, tumorigênese (AZOUZ et al., 2015).

Os mastócitos têm sido implicados no desenvolvimento dos efeitos deletérios induzidos no intestino por carcinógenos, como a radiação gama (BLIRANDO et al., 2011), 1,2-dimetil-hidrazina (DMH) (WEDEMEYER; GALLI, 2005a) e o sulfato sódico de dextrana somado ao ácido 2,4,6-trinitrobenzenosulfônico (DSS/TNBS) (HAMILTON et al., 2011). Essas células também são encontradas em todos os estágios do desenvolvimento tumoral, sendo consideradas uma das principais populações celulares do estroma neoplásico (VARRICCHI et al., 2017). No entanto, no microambiente tumoral estabelecido, a descrição dos mediadores produzidos pelos mastócitos e, consequentemente, a atuação dessas células em relação às tumorais é altamente heterogênea (HEIJMANS et al., 2012). Alguns desses mediadores, como TNF-α, IL-1, IL-4 e IL-6 e o condroitin sulfato, são associados à inibição do desenvolvimento tumoral, além de induzir a apoptose das células tumorais e quimiotaxia dos leucócitos, favorecendo o controle da progressão tumoral (CONTI et al., 2007). No entanto, a secreção de histamina, VEGF, FGF-2, triptase, quimase, IL-8 e IL10 sugere que os mastócitos podem favorecer o desenvolvimento tumoral, estimulando a vasodilatação, angiogênese, proliferação, morfogênese e degradação da matriz extracelular, promovendo um ambiente propício para o processo neoplásico e metastático (MALTBY; KHAZAIE; MCNAGNY, 2009; WASIUK et al., 2009).

A diversidade funcional dos mastócitos pode ser, ao menos em parte, devida a limitação das análises que avaliam os mastócitos como uma população celular homogênea. No entanto,

diferenças moleculares e morfológicas condicionam a existência de subpopulações de mastócitos, derivadas de um progenitor comum circulante (MAANINKA; LAPPALAINEN; KOVANEN, 2013). A diversidade fenotípica entre mastócitos do tecido conjuntivo (CTMCs) e de mucosa (MMCs) foi descrita pela primeira vez em ratos por Enerback em 1986, em função das diferentes propriedades histológicas no jejuno. Décadas mais tarde, foi demonstrado que os CTMCs, provenientes do intestino delgado, peritôneo e pele dos camundongos, são capazes de expressar a quimase mMCP-4, a elastase mMCP-5, e duas triptases tetraméricas, mMCP-6 e -7, enquanto os MMCs do intestino delgado expressam apenas dois tipos de quimases mMCP-1 e -2. Desse modo, os mastócitos murinos exibem variação anatômica na expressão de proteases, sendo que a expressão quimase no intestino é verificada mesmo em condições controle.

A expressão variável das enzimas triptase, quimase e catepsina G também tem levado ao reconhecimento de diferentes subpopulações de mastócitos humanos (XING et al., 2011). Mastócitos que expressam triptase e quimase (MC_{TC}) são abundantes no tecido conjuntivo da pele (derme) e na submucosa do trato gastrointestinal, enquanto aqueles que expressam triptase, mas pouca ou nenhuma quimase (MC_T) são localizados, geralmente, na mucosa dos tratos respiratório e gastrointestinal. Uma terceira e menor população de mastócitos, que expressa somente quimase e catepsina G (MC_C), também foi identificada na mucosa e submucosa intestinal (WEIDNER; AUSTEN, 1993). No entanto, alguns estudos, têm mostrado que tais classificações não são rígidas, podendo ser modificadas dentro do microambiente tumoral (GURISH et al., 1995; MALTBY; KHAZAIE; MCNAGNY, 2009).

1.3. Diferenciação dos mastócitos e implicações no desenvolvimento tumoral

A capacidade dos mastócitos para se transdiferenciarem entre as subpopulações, modificando sua síntese de mediadores em resposta às citocinas, hormônios e espécies reativas de oxigênio, foi demonstrada inicialmente *in vitro* (KITAMURA, 1986). Essa condição, adquirida em cultura, não é mantida quando as células são transferidas novamente para os tecidos de origem. Em humanos, poucos dados da literatura descrevem a possibilidade de transdiferenciação de mastócitos. No entanto, a alteração do perfil de mastócito, observado no mesmo tecido em condições normais e patológicas, tem sido relatada (MOON et al., 2010; WALLS et al., 1990). Embora o recrutamento de mastócitos maduros, de tecidos adjacentes, seja plausível para explicar este fenômeno, os achados sobre plasticidade de diferentes células, como linfócitos e macrófagos, ressaltam que a expressão de marcadores e mediadores não é estável nas células do sistema imune, estando diretamente associada às condições do microambiente em que essas células estão inseridas (XING et al., 2011), conforme ilustrado na figura 1.

Ainda que sutis, as diferenças entre os subtipos de mastócitos podem ocasionar alterações notáveis no quadro clínico de doenças respiratórias (KOSANOVIC et al., 2013), cutâneas (BABINA et al., 2006) e intestinais (BEIL et al., 2002). Em todos os casos é importante destacar que, em vista do seu potencial pleitrópico, qualquer efeito desempenhado por essas proteases em benefício ou detrimento do hospedeiro é necessariamente contexto-específico (CAUGHEY, 2007). A expressão de quimases e tripstases dos mastócitos está correlacionada com a maturação dessas células nos tecidos e ao processo de angiogênese durante a progressão de tumor quimicamente induzido em camundongos BALB/c (ANTONIO et al., 2012). Dessa forma, a caracterização dos subtipos de mastócitos, infiltrados no processo neoplásico, pode ser útil na descrição da ação anti ou pró-tumoral dessas células.

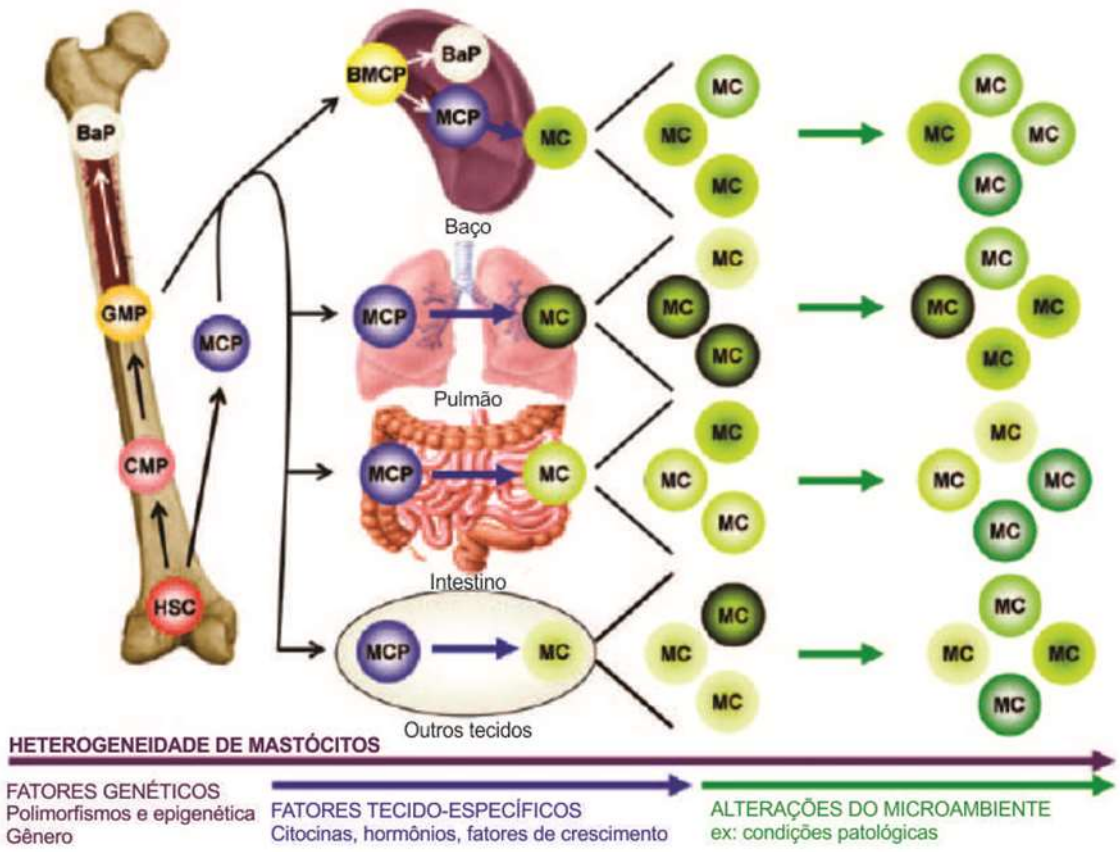


Figura 1. Desenvolvimento e heterogeneidade dos mastócitos. As células progenitoras de mastócitos (MCP) podem se desenvolver a partir de células tronco-hematopoiéticas (HSC) mas, também, de células progenitoras de granulócitos/macrófagos (GMP), diferenciadas de um progenitor mielóide comum (CMP). No baço de camundongos C57BL/6 é descrita, ainda, a diferenciação de MCPs de células progenitoras comuns de mastócitos e basófilos (BMCP), que também originam células progenitoras de basófilos (BAP). A maturação dos mastócitos (MC) é influenciada por fatores do microambiente e resulta no desenvolvimento de diferentes fenótipos. Variações no microambiente em condições fisiológicas (como mudanças hormonais no ciclo menstrual) e patológicas podem causar alterações no fenótipo dos mastócitos. Além disso, fatores genéticos como polimorfismos e a regulação epigenética da expressão gênica pode afetar a heterogeneidade dos mastócitos. Adaptado de Moon, 2010.

O aumento do número de mastócitos quimase-positivos está relacionado, por exemplo, ao mal prognóstico no câncer pulmonar (NAGATA et al., 2003). No trato intestinal normal, a expressão de quimase não é observada (ALDENBORG; ENERBÄCK, 1994). Contudo, o número de mastócitos quimase-positivos é aumentado na doença inflamatória intestinal (ANDOH et al., 2006) e ocorre predomínio destas células nos pacientes com CCR (TAN et al., 2005). Entre seus efeitos conhecidos, a quimase interfere na resistência transepitelial e na permeabilidade paracelular *in vitro*, podendo estar relacionada ao desenvolvimento da irritação intestinal (GROSCHWITZ; HOGAN, 2009). Paralelamente, a quimase pode estar intimamente relacionada com a formação de novos vasos e progressão do câncer gástrico (KONDO et al., 2006). Ainda, a capacidade da quimase e catepsina G de converterem a angiotensina I em angiotensina II estimulando a angiogênese (SUEKANE et al., 2010) possivelmente contribui para a progressão do tumor. Na maioria dos tumores humanos, a alta densidade de mastócitos está associada com intensa vascularização e diminuição da sobrevida do paciente (MALTBY; KHAZAIE; MCNAGNY, 2009).

Modelos experimentais murinos mostram que a protease 4 de mastócitos murinos, similar a quimase humana, é capaz de degradar o TNF em suas formas solúvel e transmembrânica (PILIPONSKY et al., 2012), propiciando a evasão tumoral, além de atuar na ativação de MMP-9 e patogênese de cólon induzida por DSS (ISHIDA et al., 2008). É importante ressaltar que o surgimento desses subtipos é estritamente relacionado aos mediadores presentes no microambiente (GALLI; TSAI, 2008).

1.4. Proteína anexina 1 e seu papel no microambiente tumoral

A Anexina A1 (AnxA1), originalmente descrita como uma proteína induzida por glicocorticóide com ações inibitórias da fosfolipase A_2 (FLOWER; BLACKWELL, 1979; GOULDING et al., 1990), é uma proteína anti-inflamatória de 37 kDa, formada por 346 aminoácidos. Ela é o primeiro membro de uma família de 13 anexinas descritas em mamíferos (RAYNAL; POLLARD, 1994). As anexinas são agrupadas em função das suas características estruturais, incluindo a presença de um núcleo, que é constituído por quatro repetições de 60 a 70 aminoácidos cada, com alta afinidade ao cálcio (GERKE; CREUTZ; MOSS, 2005), ligado a uma sequência N-terminal, que confere especificidade de ação para cada membro da superfamília anexina (GERKE; MOSS, 2002). Uma representação esquemática da estrutura primária e o arranjo tridimensional da ANXA1 são mostrados na figura 2.

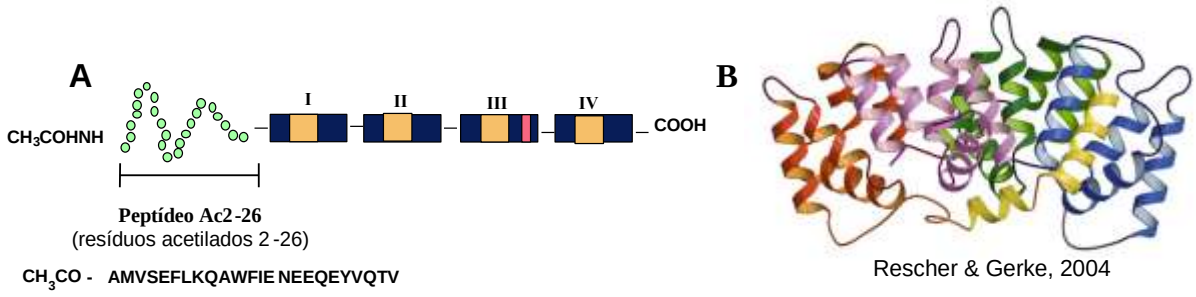


Figura 2. A proteína anti-inflamatória Anexina A1. (A) Representação esquemática da estrutura primária, com destaque do sítio ativo anti-inflamatório (peptídeo $ANXA_{2-26}$). (B) Ilustração do arranjo tridimensional desta proteína. Retirado de Rescher & Gerke, 2004.

A AnxA1 é amplamente distribuída no organismo, sendo encontrada nos leucócitos, células do estroma e fluidos biológicos (PERRETTI; D'ACQUISTO, 2009). Várias células expressam a AnxA1 e há diferenças na localização intracelular da proteína em mastócitos (OLIANI et al., 2000; SILISTINO-SOUZA et al., 2007), neutrófilos (OLIANI et al., 2001) e eosinófilos (OLIANI; DAMAZO; PERRETTI, 2002). A diferenciação celular e, em alguns casos a ativação, é um grande estímulo para a síntese de AnxA1, embora os processos moleculares não estejam totalmente esclarecidos. Exemplos dessa regulação têm sido relatados nos fibroblastos estimulados pelo TNF-α (TAGOE et al., 2008) e nas células epiteliais (CROXTALL; FLOWER, 1992).

Os glicocorticoides regulam a síntese e função da ANXA1 (PARENTE; SOLITO, 2004; RHEN; CIDLOWSKI, 2005), possivelmente por meio de uma combinação de processos não-genômicos e genômicos, dependendo do tipo celular (PERRETTI; D'ACQUISTO, 2009). Dexametasona e outros esteroides aumentam a localização da ANXA1 na superfície das células (SOLITO et al., 2006). Este processo não exige síntese proteica *de novo* e está associado com mudanças rápidas na localização celular da proteína (processo não genômico). O aumento tardio de expressão da ANXA1 na superfície celular é consequente da ativação do gene (processo genômico) (PERRETTI; DALLI, 2009).

A *ANXA1*, localizada na região cromossômica 9q12-9q21.2 (HUEBNER et al., 1988), está envolvida em algumas das ações benéficas dos glicocorticoides, incluindo a inibição da proliferação celular e diapedese leucocitária (PERRETTI; D'ACQUISTO, 2009), a regulação da diferenciação celular (FLOWER; ROTHWELL, 1994) e o tráfico de membranas (DIAKONOVA et al., 1997). Além disso, essa proteína tem sido associada com a progressão em alguns tumores invasivos, sugerindo um papel na regulação da proliferação, migração e invasão das células epiteliais (BAI et al., 2004; CICEK et al., 2004; LIN et al., 2008). No

entanto, os mecanismos moleculares pelos quais a ANXA1 modula essas respostas celulares não são completamente entendidos (KHAU et al., 2011). Uma interessante função da ANXA1, na proliferação celular, é o seu papel como um substrato do domínio tirosina quinase, pertencente ao receptor do fator de crescimento epitelial (EGFR) (DE et al., 1986; RADKE et al., 2004), que inibe a proliferação epitelial (CROXTALL et al., 1996). Algumas das ações biológicas da AnxA1 são apresentadas na figura 3.

Várias investigações indicam que a ação reguladora da AnxA1 sobre a migração transendotelial leucocitária, pode ser mediada pelo FPR (PERRETTI et al., 2001; GAVINS et al., 2003; ERNST et al., 2004; DUFTON et al., 2010). A família FPR apresenta divergência evolutiva significante em espécies de mamíferos, com notável expansão diferencial de genes em murinos (GAO et al., 1998).

Em camundongos, a família de genes *Fpr* é complexa e está localizada no cromossomo 17, compreendendo sete membros. Enquanto, em humanos, apenas três tipos de receptores foram encontrados e incluem, FPR1, FPR2/ALX (também conhecido como FPRL-1) e FPR3 (antigamente designado com FPRL-2) (YE et al., 2009). Ambos FPR2/ALX e FPR3 estão colocalizados com o FPR1 na região cromossômica 19q13.3 (BAO et al., 1992). Os receptores fpr1 e fpr2 de roedores (anteriormente designados Fpr-rs1 e Fprs-rs2) correspondem, respectivamente, ao FPR1 e FPR2/ALX em humanos, com os quais compartilham homologias de 77 e 76% (DUFTON; PERRETTI, 2010; GAO et al., 1998; YE et al., 2009).

Embora os FPRs sejam classicamente conhecidos por agirem como receptores quimiotáticos, regulando a migração leucocitária, eles são expressos em populações celulares diversas e ativados por uma variedade de ligantes endógenos e exógenos que provocam respostas biológicas diferentes, tais como lipoxina A4 (LXA4), amiloide sérica A (SAA) e AnxA1 (LE et al., 2001); (DUFTON; PERRETTI, 2010). A AnxA1 ativa somente o receptor FPR2/ALX (HAYHOE et al., 2006), já o peptídeo Ac2-26, *in vitro,* ativa todos os três receptores da família FPR (RESCHER et al., 2002; ERNST et al., 2004). No entanto, tais respostas são bloqueadas por antagonistas não seletivos dos receptores FPRs, os antagonistas Boc1 e Boc2 (STENFELDT et al., 2007), por meio da inibição do fluxo de cálcio induzido pela AnxA1 em PMNs (LA et al., 2001a).

A função do receptor FPR nas ações antimigratórias da AnxA1 e dos seus peptídeos derivados também foi demonstrada com o uso de camundongos deficientes para o FPR na peritonite (PERRETTI et al., 2001), na microcirculação mesentérica após isquemia-reperfusão e no edema de pata induzido por carragenina (DUFTON; PERRETTI, 2010). Investigações realizadas em nosso laboratório mostraram, pela primeira vez na literatura, a colocalização ultraestrutural da AnxA1 com o receptor Fpr-rs2 (fpr2) em neutrófilos de camundongos

induzidos à peritonite aguda, indicando que a ação anti-inflamatória da AnxA1 pode ser mediada pelo receptor fpr2 (GASTARDELO et al., 2009).

A expressão do receptor FPR2 pode estar intimamente relacionada com a expressão da AnxA1. O estudo realizado por Sawmynaden e Perretti (2006) mostrou que o tratamento com dexametasona aumenta a expressão proteica da AnxA1 e do FPR2/ALX em monócitos. A hipótese proposta por Perretti e D'Acquisto (2009) é que os GCs, inicialmente, promovem rápida mobilização da AnxA1 na superfície celular onde o receptor está localizado, por mecanismos não-genômicos. Em seguida, levam ao aumento da expressão dos genes *AnxA1* e *FPR2* por mecanismos genômicos.

Os mecanismos moleculares responsáveis pela mobilização da AnxA1 para a membrana plasmática são dependentes do tipo celular. A proteína pode ser externalizada pela exocitose dos grânulos de gelatinase dos neutrófilos ou após ativação do sistema transportador cassete ligante do ATP (*ABC-transporter*) nos macrófagos (PERRETTI; DALLI, 2009).

O acesso da AnxA1 endógena aos receptores de membrana pode, ainda, depender de processos de fosforilação que possibilitam a translocação da proteína do citoplasma para a superficie celular (SOLITO et al., 2003; SOLITO et al., 2006; SOLITO et al., 2008). Mecanismos não genômicos, mas que requerem as vias proteina quinase C (PKC), fosfatidilinositol 3-quinase (PI3K) e proteina quinase ativadora de mitógeno (MAPK) foram observados em células pituitárias folículoestelares humanas, nas quais, os GCs e o LPS induziram a fosforilação da AnxA1 em serina e translocação da proteína recém-fosforilada para a superfície celular (SOLITO et al., 2003, 2006). Curiosamente, a dexametasona não teve nenhum efeito sobre a fosforilação da AnxA1 em tirosina (SOLITO et al., 2003)(SOLITO et al., 2003).

Poucos pesquisadores têm estudado a possível expressão do receptor FPR e sua interação com ANXA1 nos tumores. Em uma recente investigação, foi mostrado que a ANXA1 e seu peptídeo N-terminal $ANXA1_{2-26}$ podem promover a invasão das células SKCO-15 (linhagem celular derivada de adenocarcinoma coloretal) por meio da ativação do receptor FPR2 em uma maneira autócrina/parácrina (BABBIN et al., 2006). A associação entre a expressão da AnxA1 em biópsias de tumores de laringe, bem como experimentos *in vitro* com a linhagem tumoral Hep-2 sugere o envolvimento desses mediadores no crescimento tumoral e mestástase (GASTARDELO et al., 2014).

A expressão alterada da ANXA1 tem sido associada com a transformação celular, progressão tumoral e metástase (LIU et al., 2011b). Estudos têm demonstrado que o padrão da expressão da ANXA1 no câncer humano não é bem definido. O aumento da expressão dessa proteína foi previamente observado no glioblastoma (LOGING et al., 2000), adenocarcinoma

mamário (AHN et al., 1997), carcinoma hepatocelular (MASAKI et al., 1996), câncer de pâncreas (BAI et al., 2004) e adenocarcinoma de pulmão (LIU et al., 2011b). Enquanto, outras investigações relataram redução ou perda da expressão da ANXA1 no câncer de cabeça e pescoço (GARCIA PEDRERO et al., 2004; PAWELETZ et al., 2000), esôfago (PAWELETZ et al., 2000), próstata (PAWELETZ et al., 2000; XIN et al., 2003), linfoma não-Hodgkin (VISHWANATHA; SALAZAR; GOPALAKRISHNAN, 2004) e adenocarcinoma sinonasal (RODRIGO et al., 2011) e no carcinoma invasivo de mama (SHEN et al., 2006), embora a expressão aumentada seja verificada no tecido hiperplásico. A maioria dos dados correlaciona as alterações na expressão de AnxA1 com pior prognóstico (BOUDHRAA et al., 2016). Em tumores gastrointestinais, a expressão de AnxA1 regula a invasividade das célualas tumorais, resultando na metástase para a cavidade peritonial e baixa sobrevida (KANDA; KODERA, 2016).

Várias investigações têm caracterizado a região N-terminal como promotora da ação anti-inflamatória da AnxA1, de modo que os resultados experimentais com o peptídeo sintético $AnxA1_{2-26}$, contendo o mesmo sequenciamento de aminoácidos, confirmam a presença desse sítio ativo anti-inflamatório (GAVINS et al., 2003; PERRETTI, 1998). Em nosso grupo de pesquisa, demonstramos que o tratamento com o peptídeo $AnxA1_{2-26}$, na linhagem celular de câncer de laringe (Hep-2), resultou no aumento da expressão da AnxA1 e na inibição do crescimento tumoral, sugerindo um papel regulatório dessa proteína no desenvolvimento tumoral (GASTARDELO et al., 2014). A AnxA1 também interfere na ocorrência de metástases em alguns tumores, regulando o processo de migração/invasão celular (ALLDRIDGE; BRYANT, 2003; WANG et al., 2004). Similarmente, a falta de AnxA1 no tumor, torna as células tumorais resistentes a apoptose após indução por agentes quimioterápicos (LIM; PERVAIZ, 2007), sugerindo essa proteína como um biomarcador negativo no desenvolvimento e na progressão do câncer.

No trato gastrointestinal, a AnxA1 foi revelada como um fator diferencial entre portadores de DC e RU. Enquanto pacientes com DC apresentam regulação negativa da AnxA1 nas células epiteliais e imunes transmigradas para o tecido (SENA et al., 2013), portadores de RU têm alta expressão dessa proteína nos mesmos tipos celulares (VONG et al., 2012). Essa diferença demonstra que, independentemente dos mecanismos específicos de cada DII, a AnxA1 é um importante mediador no contexto da inflamação intestinal. Ainda, outros estudos têm demonstrado o papel da AnxA1 na prevenção de lesão da mucosa intestinal em modelo murino (MARTIN et al., 2008) e, também, na cicatrização de úlceras induzidas por indometacina no estômago ou em lesões consequentes de DII (BABBIN et al., 2006, BABBIN et al., 2008; LEONI et al., 2013).

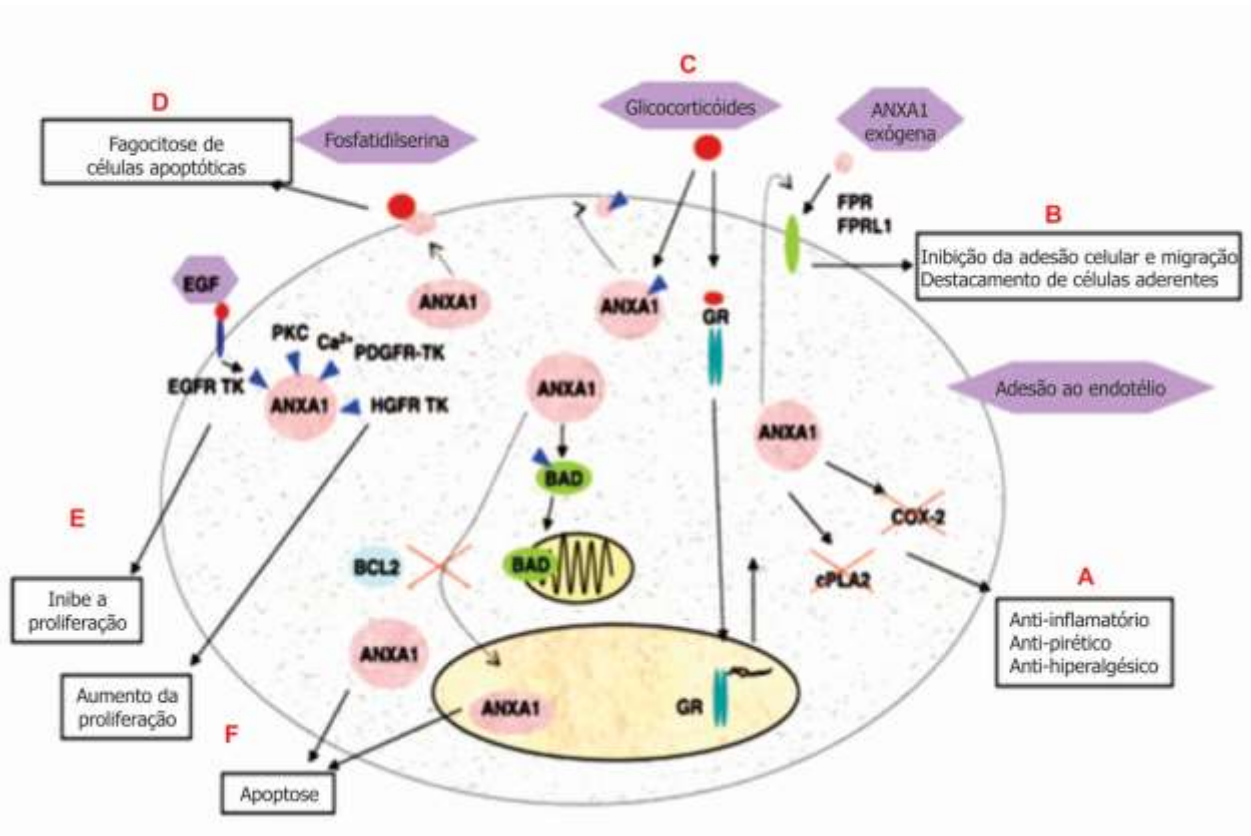


Figura 3. Algumas das ações biológicas da ANXA1. A) Inibição da fosfolipase A2 e ciclo-oxigenase-2. B) Inibição da diapedese leucocitária mediada pelo receptor FPR. C) Expressão aumentada da ANXA1 após tratamento com glicocorticoide. D) Estimulação de fagocitose de células apoptóticas. E) Inibição da proliferação por meio da fosforilação pela tirosina quinase EGFR. F) Indução da apoptose pela superexpressão e translocação para o núcleo. Retirado de Lim e Pervaiz, 2007.

No sistema imune, a AnxA1 tem um papel contraditório, exibindo ações inibitórias sobre a ativação e transmigração de neutrófilos e monócitos e conduzindo à resolução da inflamação aguda (PERRETTI et al., 1996; OLIANI et al., 2001; CHATTERJEE et al., 2005; GIL et al., 2006) e, por outro lado, podendo levar à proliferação e diferenciação de células T à resposta Th1 (Figura 3) (D'ACQUISTO et al., 2007).

Um estudo realizado pelo nosso grupo demonstrou que a indução de peritonite com carragenina, promove a reorganização dos grânulos citoplasmáticos e a síntese *de novo* da AnxA1, que desempenha um papel importante na regulação do infiltrado inflamatório e citocinas produzidas no intestino (OLIANI et al., 2000, 2008). Deste modo, a AnxA1 é descrita como capaz de inibir a desgranulação de mastócitos (BANDEIRA-MELO et al., 2005), atuando na atividade de estabilizadores farmacológicos via FPR (YAZID et al., 2013; SINNIAH et al., 2016). Além disso, foi demonstrado recentemente que a ativação da via FPR2/ALX nos mastócitos, reduz a migração de neutrófilos na resposta imune intata (HUGHES et al., 2017). No entanto, pouco é conhecido sobre a atividade da AnxA1 sobre a regulação da síntese de mediadores pelos mastócitos, sobretudo no microambiente tumoral.

Com essas considerações, um melhor entendimento dos mecanismos moleculares responsáveis pelos efeitos do tumor na expressão de mediadores pelos mastócitos e, consequentemente, o efeito sobre o processo oncogênico é de grande importância para o delineamento de novas estratégias terapêuticas anti-câncer. Além disso, essa investigação contribui para o melhor entendimento do papel dos subtipos de mastócitos e da proteína AnxA1 no adenocarcinoma colorretal, pois poucos estudos têm investigado essas ações e, possivelmente, poderão resultar em dados efetivos para prevenção, diagnóstico e/ou tratamento desse tipo de câncer.

2. OBJETIVOS

2.1. Objetivo geral

No presente projeto avaliamos a influência da AnxA1 sobre a atividade e o fenótipo dos mastócitos nos estágios iniciais da carcinogênese e no microambiente tumoral colônico estabelecido, assim como a produção dos mediadores farmacológicos e seu envolvimento na neoplasia colorretal induzida quimicamente.

2.2. Objetivos específicos

Camundongos BALB/c selvagens e deficientes de AnxA1 foram utilizados para avaliar o efeito agudo de instilações intrarretais com MNNG sobre:

i. a expressão endógena da AnxA1 nos animais selvagens;
ii. as alterações estruturais induzidas pelo carcinógeno no epitélio colônico;
iii. o número e o estado de ativação dos mastócitos;
iv. a presença de subpopulações de mastócitos no microambiente tumoral, pela detecção de triptase e quimase;
v. a produção de IL-1β, IL-4, IL-6, IL-12, TNFα, IFN-γ no tecido colônico.

Camundongos C57/BL6 selvagens e deficientes de mastócitos, foram avaliados após o desenvolvimento crônico de tumores colorretais:

i. a interferência dos mastócitos no número de lesões tumorais formadas, na condição clínica dos animais e na arquitetura das criptas colônicas;
ii. o número e estado de ativação dos mastócitos;
iii. a presença de subpopulações de mastócitos no microambiente tumoral, pela detecção de triptase e quimase;
iv. a expressão de AnxA1 e a produção das citocinas (IL-1β, IL-4, IL-6, IL-12, TNFα, IFN-γ) no tecido colônico;
v. o número de células proliferativas e apoptóticas no cólon pela expressão dos marcadores PCNA e Caspase-3.

3. MATERIAIS E MÉTODOS

3.1. Animais

Camundongos BALC/c ($AnxA1^{+/+}$) selvagens e deficientes para a proteína ($AnxA1^{-/-}$) foram gentilmente cedidos pela Profa. Dra. Sandra Helena Poliselli Farsky (FCF-USP, São Paulo – SP). Camundongos selvagens C57/BL6 ($Kit^{+/+}$) e geneticamente deficientes de mastócitos WBB6F1-$Kit^{W/W-v}$ ($Kit^{-/-}$) foram adquiridos junto ao Biotério de Animais Especiais da Faculdade de Medicina de Ribeirão Preto (FMRP USP), por intermédio da Profa. Dra. Gabriela Silva Bisson (EERP-USP, Ribeirão Preto – SP). Os experimentos foram realizados no Laboratório de Imunomorfologia do Instituto de Biociências, Letras e Ciências Exatas da UNESP de São José do Rio Preto (IBILCE – UNESP) após indução tumoral realizada no Laboratório de Genômica e Imunobiologia da Escola de Enfermagem de Ribeirão Preto (EERP – USP Ribeirão Preto). Animais machos, com idade entre 5 e 7 semanas, pesando aproximadamente 25g foram utilizados. Estes foram mantidos em gaiolas com grupos de 5 animais, ciclo de claro-escuro de 12h e água e comida *ad libitum*. Os procedimentos adotados foram aprovados pelos Comitês de Ética em Pesquisa Animal do IBILCE/UNESP (73/2014), EERP/USP (13.1.124.20.2) e FCF/USP (01200.003570/2015-17) (ANEXOS 1, 2 e 3 respectivamente).

3.2. Administração do carcinógeno MNNG

Os camundongos receberam instilações intrarretais a 3 cm da margem anal de 0,1 mL do composto carcinogênico Metil-N´-Nitro-N-Nitroso-Guanidina (MNNG) (Sigma-Aldrich, Milwaukee, WO, USA) (20 mg/kg peso corporal). Os animais do grupo controle receberam 0,1 mL de PBS 1 mM (pH 7,0) pela mesma via (CHE et al., 2010). Em virtude do alto potencial carcinogênico do composto, sua manipulação em forma sólida foi realizada por um técnico especializado no Departamento de Patologia, Faculdade de Medicina de Ribeirão Preto (FMRP/USP). Após, no IBILCE, os animais receberam instilações intrarretais de 0,1 ml da solução de MNNG (5 mg/ml), a 3 cm da margem anal, utilizando uma sonda metálica de 5 cm. Para evitar exposição desnecessária, os pesquisadores envolvidos foram paramentados com jaleco, máscara e luvas de nitrila.

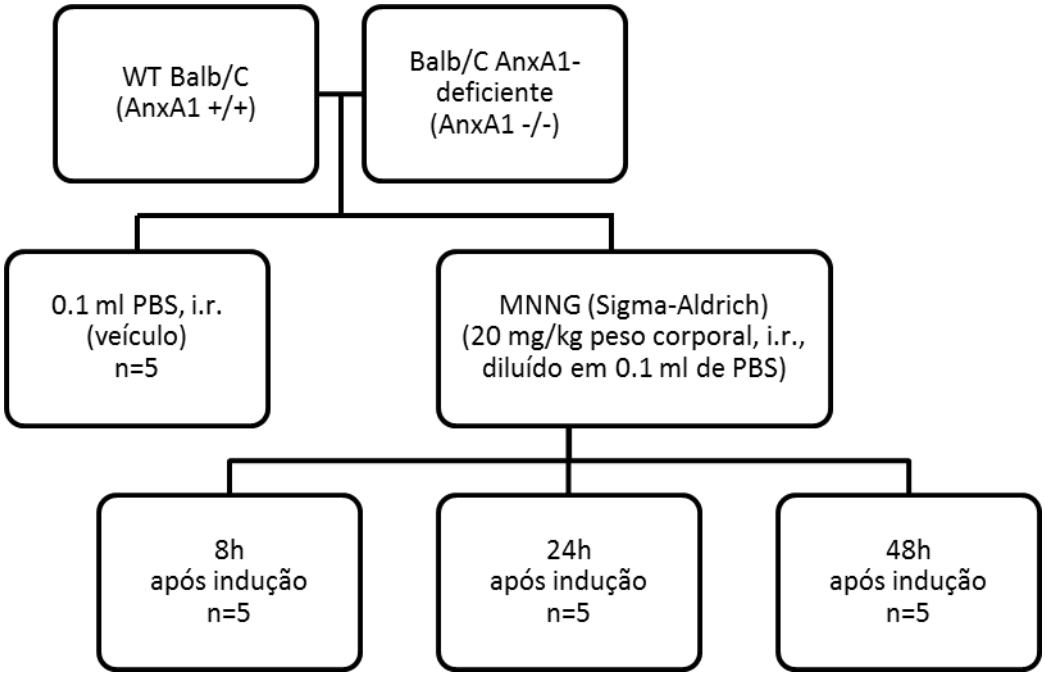
3.3. Transplante de células da medula óssea para os camundongos $Kit^{-/-}$

Para a reconstituição de mastócitos, camundongos $Kit^{-/-}$ (n=5/grupos) receberam injeções i.v. de $2x10^7$ células isoladas a partir da medula de camundongos normais C57/BL6 ($Kit^{+/+}$) (GRIMBALDESTON et al., 2005). Resumidamente, células da medula óssea foram retiradas do fêmur de camundongos $Kit^{+/+}$ com meio Dulbecco (DMEM) (Sigma Chemical Co.,

St. Louis, MO, USA) e cultivadas com o mesmo meio suplementado com 10 ng/ml de IL-3 (PeproTech, Rocky Hill, NJ, USA) até que mais de 95% das células fossem identificadas como mastócitos derivados de cultivo celular (BMCMCs). Esse fenótipo celular foi identificado pela coloração com May Grunwald-Giemsa (Bio-OpticaSpA, Milan, Italy) e expressão positiva de FcεRI (anti-mouse conjugado com PE, 1:200, clone MAR-1, Biolegend San Diego, California, USA) e c-Kit (Anti-mouse CD117 conjugado com FITC, 1:200, 2B8, Biolegend, San Diego, California, USA) por citometria de fluxo no FACSCanto I® (Becton Dickinson, San Jose, CA). Células viáveis foram contadas com azul de tripan 0,4% (Thermo Fisher Scientific Inc, Waltham, MA,USA) e $2x10^7$ células diluídas em 200 mL de DMEM foram injetadas pela veia caudal em camundongos $Kit^{-/-}$ com 4 semanas de idade. Animais controle receberam somente 200 mL de DMEM.

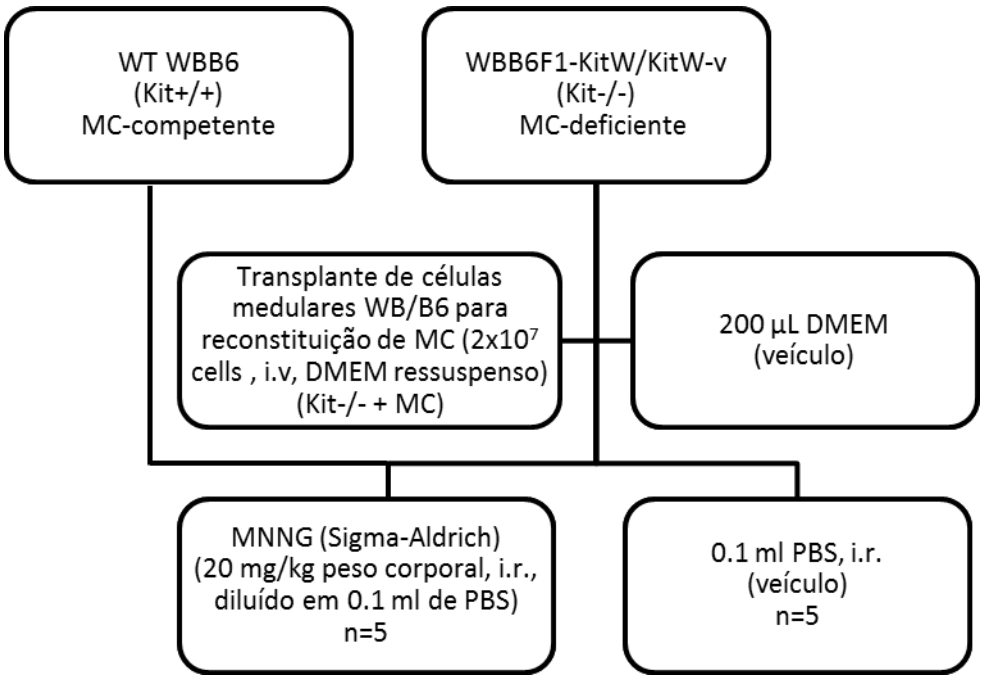
3.4. Grupos experimentais

A resposta inicial dos mastócitos, ao estímulo do MNNG, foi avaliada em camundongos Balb/C $AnxA1^{+/+}$ e $AnxA1^{-/-}$ (n=5/grupos). Esses animais receberam o carcinógeno e foram submetidos à eutanásia para análises específicas após 8, 24 e 48 horas. O esquema a seguir ilustra a descrição dos grupos de estudo:



O envolvimento dos mastócitos no desenvolvimento de tumores colorretais foi observado nos camundongos C57BL/6 $Kit^{+/+}$, $Kit^{-/-}$ e $Kit^{-/-}$+MC (n=5/grupos). Estes receberam instilações intrarretais de 0,1 mL de solução de MNNG (5mg/mL), 2 vezes por semana, durante 2 semanas, totalizando 4 instilações, a 3 cm da margem anal, utilizando uma sonda metálica de

5 cm. Os animais controle receberam instilações intrarretais de PBS. Após 24 semanas do término das instilações, os animais foram submetidos à eutanásia para realização das análises.



3.5. Análises histopatológicas

Fragmentos colônicos foram abertos longitudinalmente, o mais próximo possível da borda mesentérica, em toda sua extensão. Após macrodissecção, o cólon distal foi fixado em formol tamponado a 10%, por 24horas, lavado em água corrente, desidratado em etanol e incluído em parafina. Cortes de 4 µm foram obtidos e os fragmentos corados com Hematoxilina-Eosina para avaliação histopatológica e Azul de Toluidina a 0,5 % para análise dos mastócitos. Cortes longitudinais seriados dos segmentos colônicos foram utilizados para identificação e quantificação dos mastócitos, com base em suas características morfológicas (íntactos e desgranulados). A análise foi realizada no microscópio ZEISS, AXIOSKOP 2, do Laboratório de Imunomorfologia, IBILCE-UNESP, São José do Rio Preto, SP. As áreas de cada tecido foram obtidas com o auxílio do software analisador de imagem Axiovision (ZEISS). Os dados foram expressos como média ± desvio padrão (D.P.) do número de mastócitos por mm^2.

3.6. Análises imuno-histoquímicas

Outros segmentos colônicos, também incluídos em parafina, cortados serialmente, mantendo a orientação transversal original para fornecer secções circulares, foram usados para as reações de imuno-histoquimica. As secções foram processadas conforme descrito por Femia e colaboradores (2008). Para a análise da expressão de AnxA1, PCNA (proliferação celular),

caspase-3 (apoptose), triptase e quimase o método biotina estreptoavidina foi realizado, utilizando anticorpos primários como descrito previamente. Para os controles negativos, o anticorpo primário foi excluído. A coloração imuno-histoquímica foi realizada utilizando o método de detecção avidina-biotina do Kit de Detecção Universal Novostain (Novocastra Laboratories, Newcastle, UK) com algumas modificações, seguido de reação com 3-3 tetrahidrocloreto diaminobenzidina (DAB) (Sigma-Aldrich Inc.). Os fragmentos foram contracorados com hematoxilina. A reação positiva foi mostrada como um precipitado marrom claro-marrom-escuro no núcleo, membrana, citoplasma e/ou perinúcleo para células. As análises densitométricas da AnxA1 foram realizadas com auxílio do software Axiovision. Para isso, foram mensurados 15 pontos de 3 lâminas diferentes do cólon de cada animal para obtenção de uma média relacionada com a intensidade da imunomarcação. Os valores foram obtidos como unidades arbitrárias O índice de marcação de PCNA (iPCNA) e caspase-3 (iCaspase-3) foi expresso como razão de células corados por núcleos totais contados em 100 criptas. Para triptase e quimase, as células positivas foram quantificadas e os resultados expressos como número de células positivas por área observada no cólon.

3.7. Ensaios Multiplex para análise de citocinas e outros mediadores farmacológicos

A fim de quantificar as citocinas produzidas no cólon dos animais utilizamos o kit Millipore MILLIPLEX MAP MCYTOMAG 70K no instrumento LUMINEX xMAP MAGPIX (Millipore Corporation, Billerica, MA, USA) para a detecção multiplex quantitativa de múltiplos analitos, por meio de esferas magnéticas. As amostras foram homogeneizadas em solução salina tamponada (10% peso/volume) na presença de inibidores de proteases (Pierce) e centrifugadas a 4°C, por 15 min, a 12000g. O sobrenadante foi retirado, centrifugado, aliquotado e mantido a -20ºC até o momento do uso. As esferas magnéticas, soluções controles, tampão de lavagem, soro matriz e padrões foram preparados e homogeneizados conforme as instruções descritas nos kits. Inicialmente, foram adicionados 25 µL dos padrões, soluções controles e amostras na placa magnética de 96 poços, lavada previamente com o tampão de lavagem. Em seguida, 25 µL de tampão de ensaio foram adicionados às amostras, 25 µL do soro matriz aos padrões e 25 µL de beads magnéticas revestidas com anticorpos específicos em todos os poços (controles, padrões e amostras). A placa foi revestida com um selante próprio e incubada *overnight* a 4˚C, sob agitação. No dia seguinte, sempre sob agitação, a placa foi lavada duas vezes com 200 µL de tampão de lavagem e, incubada com 25 µL de anticorpo de detecção ligado a biotina à temperatura ambiente, por uma hora. Para completar a reação, 25 µL de ficoeritrina conjugada à estreptavidina foi adicionada e incubada por 30 minutos, protegida da luz à temperatura ambiente. A placa foi lavada duas vezes e incubada com 150 µL

do fluido do dispositivo por cinco minutos à temperatura ambiente. Em seguida, a leitura da placa foi realizada pelo equipamento LUMINEX xMAP MAGPIX.

A concentração dos analitos foi determinada pelo software MAGPIX xPONENT (Millipore Corporation, Billerica, MA, USA). Os ensaios foram realizados em triplicata para os seguintes analitos: interleucinas 1β (IL-1β), 4 (IL-4), 6 (IL-6), 10 (IL-10), fator de necrose tumoral (TNFα) e interferon gama (IFN-γ).

3.8. Análises estatísticas

Os dados foram demonstrados como média ± desvio padrão das médias. Quando atestada a distribuição normal dos dados pelo teste de normalidade de Shapiro-Wilk as diferenças estatísticas foram comparadas pelo teste de Análise de Variância (ANOVA), e, se significativas, seguidas pelo teste de Bonferroni. Quando a distribuição normal esteve ausente em algum dos grupos, o teste não paramétrico de Kruskal-Wallis, seguido pelo pós-teste de Dunns foi empregado. Os valores de P menores que 0,05 (5%) foram considerados estatisticamente significantes. As análises estatísticas e os gráficos foram feitas usando o programa GraphPad Prism, versão 5.0 (GraphPad Software, San Diego, CA, USA).

4. RESULTADOS

4.1. MNNG induz a expressão da AnxA1 endógena nas células epiteliais colônicas

Inicialmente, em condições normais, a AnxA1 foi fracamente detectada na membrana apical das células epiteliais colônicas, que compõe a porção secretora das glândulas tubulares (Figura 4A). Entretanto, logo após 8 horas da instilação com MNNG, o aumento significante da expressão de AnxA1 foi observado (Figura 4B). Após 24 horas, a imunorreatividade para AnxA1 foi mais evidente (Figura 4C) e sua intensidade foi reduzida 48 horas depois (Figura 4D). Não foi observada imunorreatividade nas secções incubadas com 10% TBS-BSA (Figura 4E). Diferenças entre os grupos foram quantificadas pela medida de densidade óptica média (DOM) (Figura 4F).

4.2. A AnxA1 endógena reduz os danos do cólon induzidos por MNNG e evita a produção de citocinas pró-inflamatórias

No cólon dos camundongos AnxA1$^{-/-}$ foi observada leve inflamação transmural, mesmo em secções de cólon do grupo veículo (Figura 5B), quando comparado ao grupo AnxA1$^{+/+}$ (Figura 5A). A deficiência de AnxA1 também foi associada com a produção aumentada das citocinas pró-inflamatórias IL-1, IL-6 e IL-12 e com níveis reduzidos de IL-4 (Figura 5I-N). Apesar do forte desequilíbrio promovido pelo MNNG no microambiente do cólon, não foram observadas diferenças nos níveis de citocinas entre as linhagens em 8 horas após a instilação do carcinógeno. O aumento da morte de células epiteliais e influxo de células inflamatórias foram também observados neste tempo experimental (Figura 5C e D).

Após 24 horas da exposição ao carcinógeno, o edema e a hiperemia do cólon foram evidentes (Figura 5E), com maior gravidade nos animais AnxA1$^{-/-}$ (Figura 5F) e maior concentração de IL-1, IL-6, IL-12, TNF-α e IFN-γ, em comparação com os AnxA1$^{+/+}$ (Figura 5I-M). Finalmente, 48 horas após a instilação do MNNG (Figura 5G-H), foi verificada intensa degeneração hidrópica vacuolar, perda de criptas inteiras e do epitélio superficial, além da diminuição significativa das células caliciformes, edema submucoso e infiltrado maciço de leucócitos na mucosa, com expressão aumentada de IL-1, TNF-α e IFN-γ (Figura 5I, L e M).

4.3. Modulação do recrutamento de mastócitos, ativação e diferenciação pela AnxA1 em resposta ao MNNG

Os mastócitos totais e desgranulados foram identificados pela coloração com azul de toluidina (Figura 6A-D). Em todas as condições avaliadas, o número de mastócitos foi aumentado nos camundongos AnxA1$^{-/-}$ em comparação aos AnxA1$^{+/+}$ (Figura 6E). Aumento

significativo no número de mastócitos desgranulados foi observado nos animais AnxA1$^{-/-}$ após 8 horas da instilação de MNNG (Figura 6F). No grupo veículo de ambas as linhagens (AnxA1$^{+/+}$ e AnxA1$^{-/-}$), a imunomarcação para triptase foi observada, diferentemente da baixa marcação para quimase (Figura 6F). Em 24 e 48 horas após a indução por MNNG, intensidade elevada destas proteases foi verificada, permitindo a quantificação de mastócitos triptase e quimase positivos em ambas as linhagens de camundongos (Figura 6P e Q, respectivamente). Por outro lado, foi encontrado menor número de mastócitos quimase positivos em animais AnxA1$^{-/-}$.

4.4. Susceptibilidade reduzida à formação de pólipos adenomatosos induzidos por MNNG nos camundongos deficientes de mastócitos (Kit$^{-/-}$)

A administração intrarretal de MNNG resultou, após 24 semanas, em número aumentado de pólipos tumorais no cólon dos camundongos selvagens (Kit$^{+/+}$) e menor tumores nos camundongos deficientes de mastócitos (Kit$^{-/-}$) (Figura 7A e B). Os camundongos reconstituídos com mastócitos (Kit$^{-/-}$+MC) exibiram um número semelhante de tumores em relação ao fenótipo selvagem (Figura 7A-B). O peso corporal foi reduzido em Kit$^{+/+}$ MNNG, em comparação com o respectivo grupo veículo ($p < 0,0001$, Figura 7C).

As secções de cólon do grupo controle, Kit$^{+/+}$ Veículo, apresentaram arquitetura normal das criptas (Figura 7D) e não foram observadas diferenças histológicas significativas em comparação com veículo do grupo Kit$^{-/-}$ Veículo (Figura 7E) ou Kit$^{-/-}$+MC Veículo (Figura 7F). Adenomas pedunculados benignos foram verificados na análise histopatológica dos tumores. A maioria deles era composta de estruturas tubulares revestidas por epitélio displásico com uma drástica redução no número de células caliciformes. No entanto, alguns pólipos adenomatosos exibiram alto grau de displasia e adenocarcinomas intra-epiteliais, conforme observado na figura 7G e I.

O número de mastócitos foi observado aumentado nos animais que desenvolveram tumores, Kit$^{+/+}$ e Kit$^{-/-}$+MC quando comparados com os respectivos grupos veículos (Figura 8E). Como esperado, os mastócitos estavam ausentes nos camundongos Kit$^{-/-}$. Secções histológicas sequenciais obtidas para imuno-histoquímica foram coradas com azul de toluidina para confirmar que as células imunomarcadas eram mastócitos. Nos grupos Veículo (Figura 8F-H e L-N), verificou-se mastócitos triptase positivos, contudo com baixa expressão de quimase. Por outro lado, a maioria dos mastócitos encontrados nos adenomas expressavam fortemente a triptase e a quimase (Figura 8I-K e O-P).

4.5. Aumento da proliferação de células epiteliais do cólon após exposição MNNG e imunorregulação do microambiente tumoral associada aos mastócitos

A expressão epitelial reduzida de AnxA1 foi encontrada em $Kit^{-/-}$ quando comparada com $Kit^{+/+}$ e $Kit^{-/-}$+MC (Figura 9A-F). Nestes camundongos, que apresentam mastócitos e que desenvolvem tumores, também foi verificada uma produção reduzida de citocinas pró-inflamatórias (IL-1, IL-6, IL-12, IFN-y e TNF-α) (Figura 9I-M) e a expressão aumentada de IL-4 (Figura 5F).

A proliferação e apoptose das células epiteliais do cólon foram analisadas por imuno-histoquímica para PCNA e Caspase-3, respectivamente (Figura 10A-C e F-H). Aumento significativo do número de células epiteliais positivas para PCNA em camundongos $Kit^{+/+}$ e $Kit^{-/-}$+MC expostos ao MNNG foi observado em comparação aos respectivos grupos Veículos ($p < 0,001$) (Figura 10E). No entanto, essa diferença não foi observada entre os grupos $Kit^{-/-}$ MNNG e Veículo (Figura 10E). Não houve, também, diferenças significativas para as células Caspase-3 positivas entre os camundongos $Kit^{+/+}$, $Kit^{-/-}$ e $Kit^{-/-}$+MC após a administração de MNNG, em relação aos respectivos grupos Veículo ($p < 0,001$) (Figura 10J). A especificidade de imunomarcação foi confirmada pelo controle da reação (Figura 10D e I).

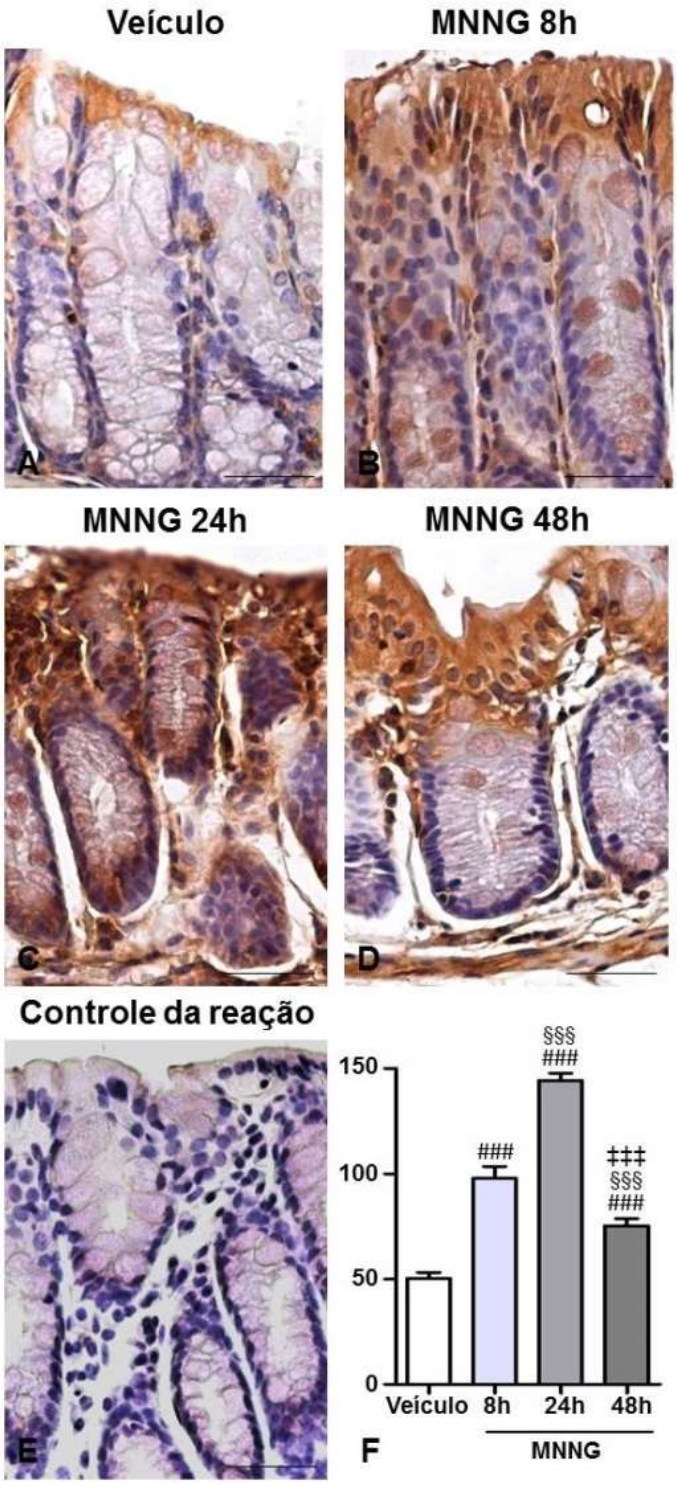


Figura 4. MNNG induz a expressão de AnxA1 no cólon murino. Imunomarcação para AnxA1 no epitélio do cólon do grupo Balb/C Veículo (A) e 8 (B), 24 (C) e 48 (D) horas após a exposição ao MNNG. Controle negativo da reação (E). Intensa imunorreatividade para AnxA1 foi observada em 24 horas após a exposição ao MNNG. Secções: 2 μm. Barras: 10 μm. Contracoloração: Hematoxilina. F) Densitometria óptica média (DOM): AnxA1. Média ± DP. ### p <0,001 *vs* grupo Veículo, §§§ p < 0.001 *vs* 8 h; ## p < 0.001 *vs* 24 h.

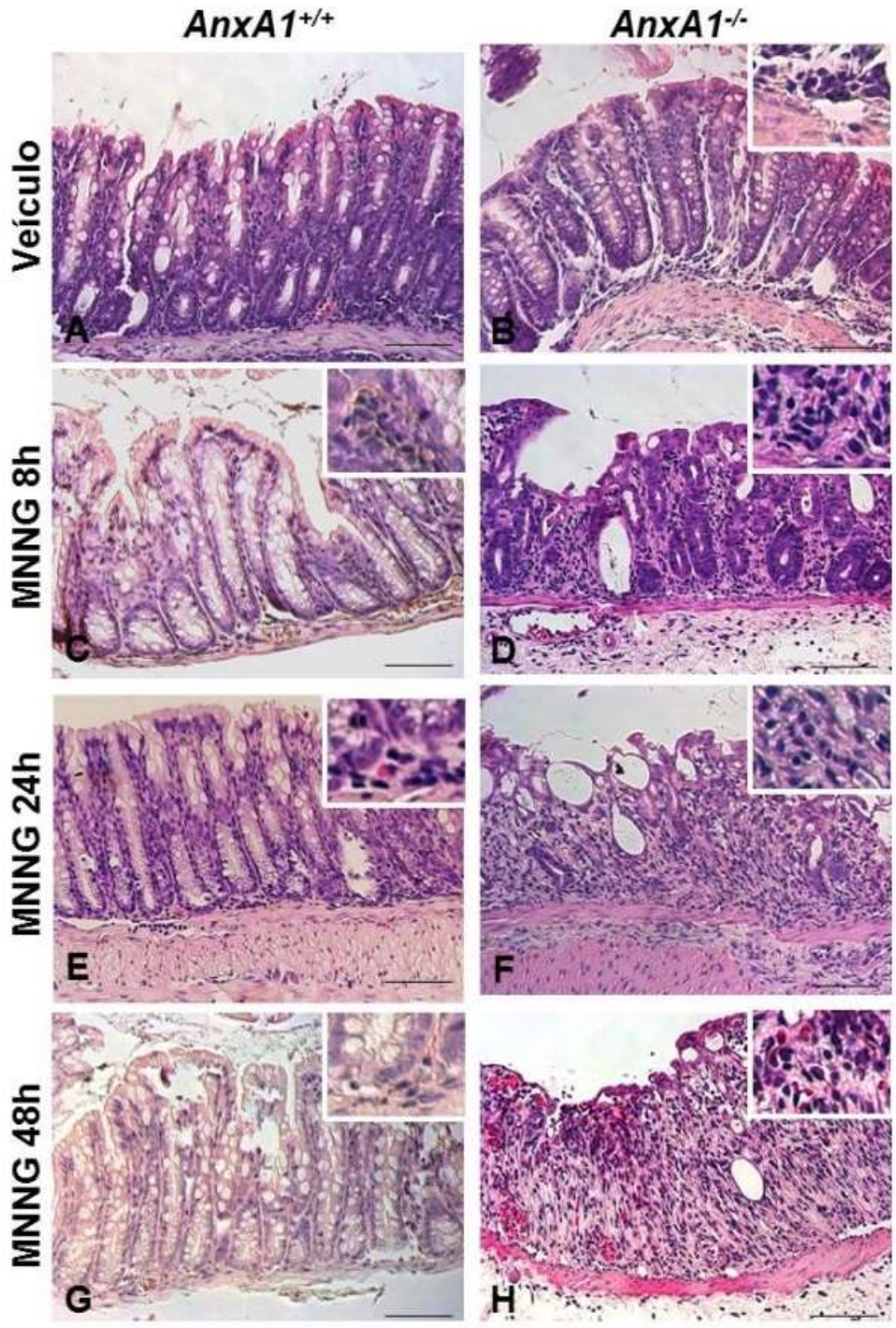


Figura 5. Histopatologia do cólon distal e análise de citocinas em camundongos deficientes de AnxA1 (AnxA1$^{-/-}$) após indução aguda ao MNNG. (A e B) Morfologia intestinal controle (C-H) Histoarquitetura alterada do cólon após a instilação do MNNG. Presença de infiltrado inflamatório na mucosa intestinal, células necróticas, criptas displásicas e degeneração hidrópica vacuolar. As lesões teciduais foram observadas após 24h em camundongos AnxA1$^{-/-}$ (F) e 48h em AnxA1$^{+/+}$ (G). Secções: 2 µm. Coloração: Hematoxilina-Eosina. Barras: 50 µm.

Figura 5 (continuação)

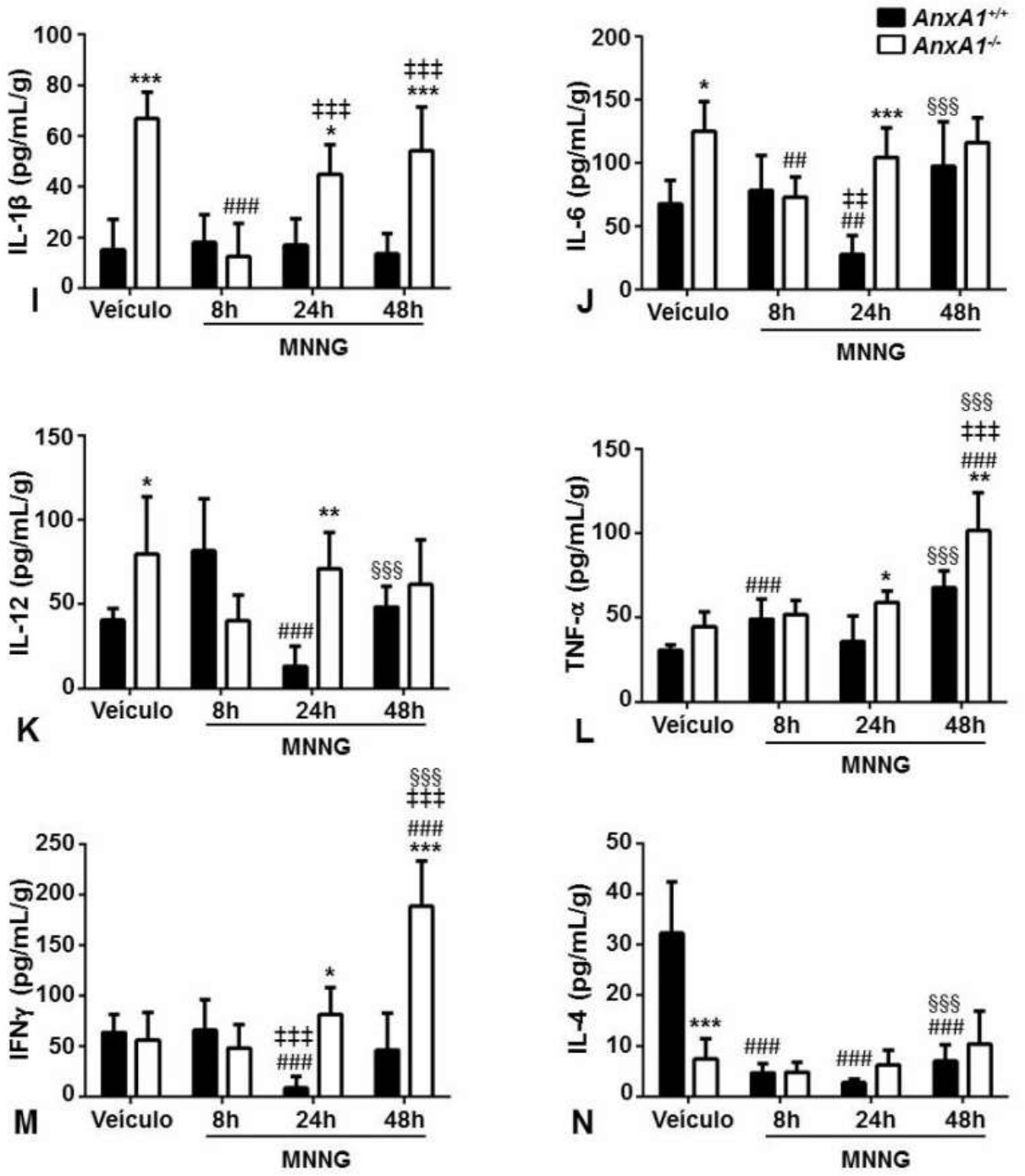


Figura 5 (continuação). Expressão das citocinas IL-1, (J) IL-6, (K) IL-12p40, (L) TNF-a, (M) IFN-y e (N) IL- 4 no cólon proximal de camundongos AnxA1$^{+/+}$ e AnxA1$^{-/-}$ após 8, 24 e 48 horas da exposição ao MNNG. Média ± DP. n = 5 animais/grupo. *** p <0,001 *vs* grupo AnxA1$^{+/+}$ respectivo, ### p <0,001 *vs* grupo Veículo, §§§ p < 0.001 *vs* 8 h; ‡‡‡ p < 0.001 *vs* 24 h.

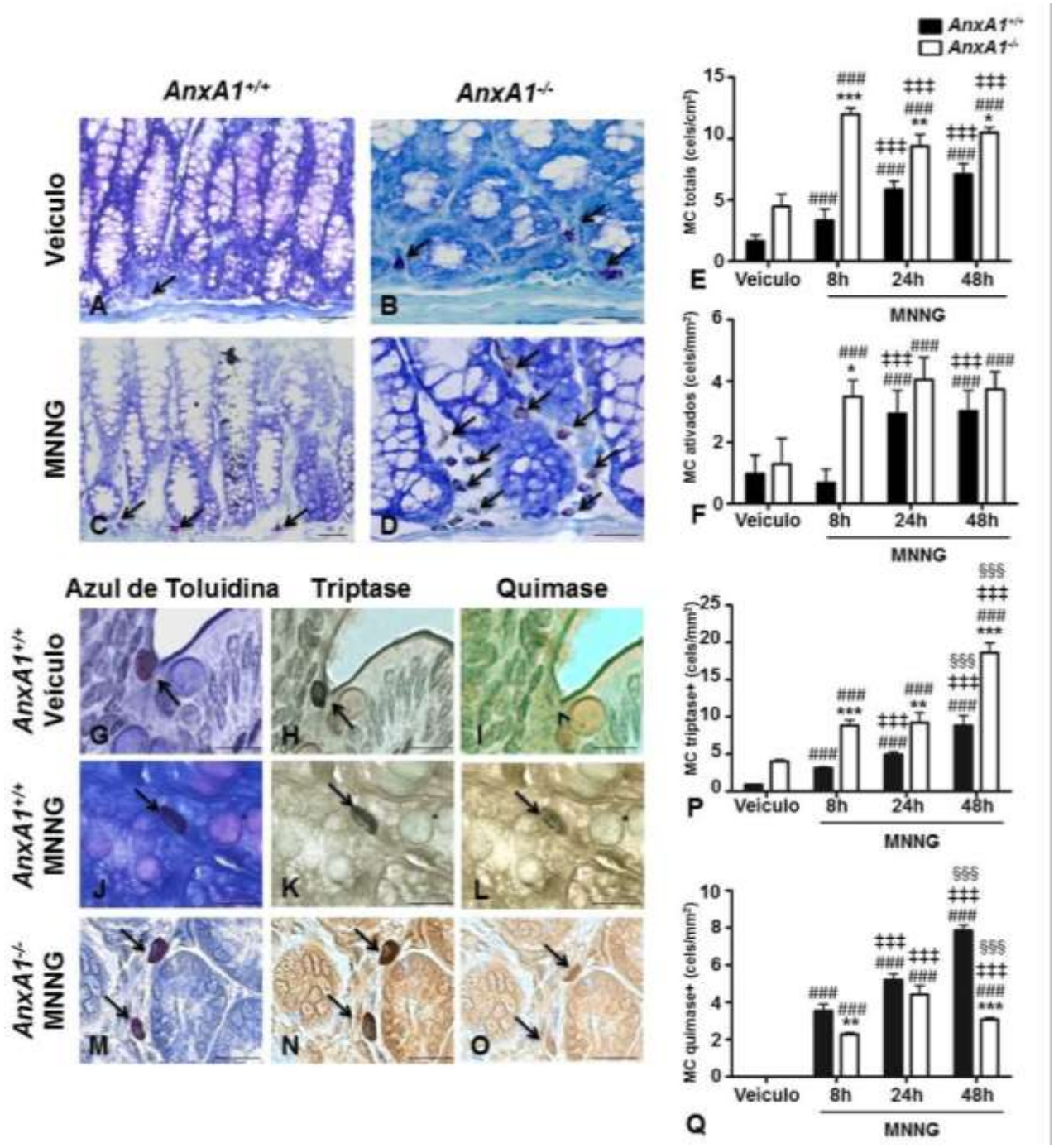


Figura 6. AnxA1 modula os efeitos agudos do MNNG sobre o recrutamento, diferenciação e a ativação de mastócitos. Mastócitos metacromáticos (setas) no cólon distal do grupos Veículo (A-B) e grupos induzidos por MNNG (C-D). (E e F) Quantificação de mastócitos (MC) totais e desgranulados em AnxA1$^{+/+}$ e AnxA1$^{-/-}$. Média ± DP. *** p <0,001 *vs* respectivo grupo Veículo. Avaliação imuno-histoquímica das proteases dos mastócitos nos camundongos AnxA1$^{+/+}$ (G-L) e AnxA1$^{-/-}$ (M-O). Mastócitos triptase positivos (setas) com baixa expressão de quimase (cabeças de seta) no grupo Veículo (H-I). Mastócitos positivos para triptase/quimase (setas) 8 horas após a indução com MNNG (K-L e N-O). Secções: 2 µm. Coloração: Azul de Toluidina (A-D, G, J e M); Contra-coloração: Hematoxilina (H, I, K, L, N e O). Barras: 50 µm (A-D); 20 µm (G-O). Quantificação de mastócitos (MC) triptase (P) e quimase (Q) positivos no cólon distal nos animais veículo e em 8, 24 e 48 horas após a indução com MNNG. Média ± SD. *** p <0,001 *vs* respectivo grupo veículo, ### p <0,001 *vs* grupo Veículo, §§§ p < 0.001 *vs* 8 h; ## p < 0.001 *vs* 24 h.

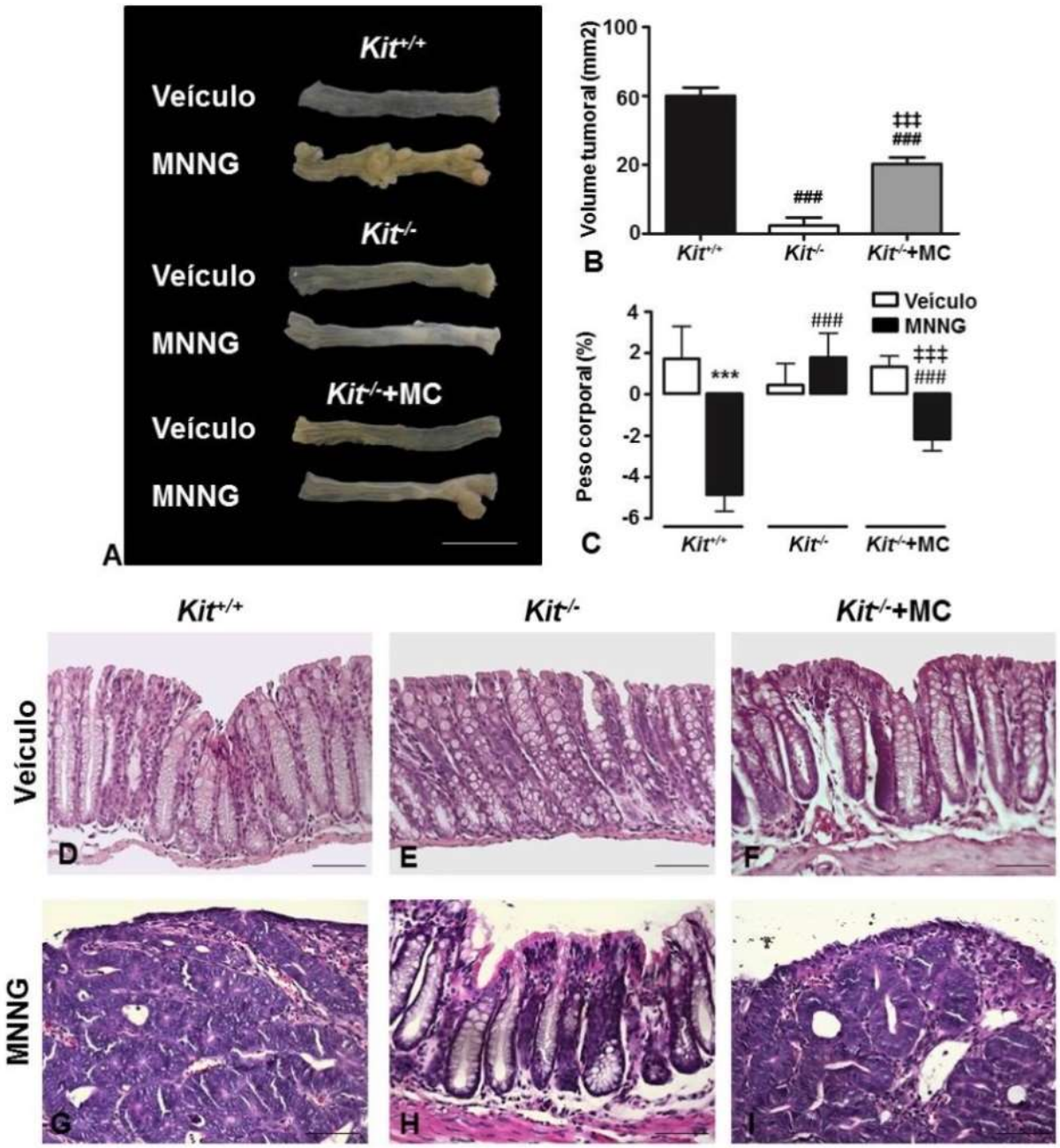


Figura 7. Alterações morfofisiológicas induzidas por MNNG em camundongos deficientes em mastócitos (Kit$^{-/-}$). (A) Cólon distal representativo de Kit$^{+/+}$, Kit$^{-/-}$ e Kit$^{-/-}$+MC após 24 semanas de indução com PBS (Veículo) e carcinógeno (MNNG); (B) volume tumoral e (C) diferença de peso corporal de camundongos após o mesmo período. Média ± DP de n = 5 animais/grupo. ### p <0,001 *vs* Kit$^{+/+}$ grupo MNNG; P <0,001 *vs* Kit$^{-/-}$ grupo MNNG; *** p <0,001 *vs* respectivo grupo veículo. Fotomicrografias representativas do cólon de camundongos veículos (D-F), formação de adenomas em Kit$^{+/+}$ e Kit$^{-/-}$+MC (G e I) e infiltrado inflamatório na mucosa colônica de Kit$^{-/-}$ camundongos (H). Células caliciformes praticamente ausentes das criptas que formam os adenomas (G e I). Secções: 2 µm. Coloração: Hematoxilina-Eosina. Barra: 50 µm (D-I).

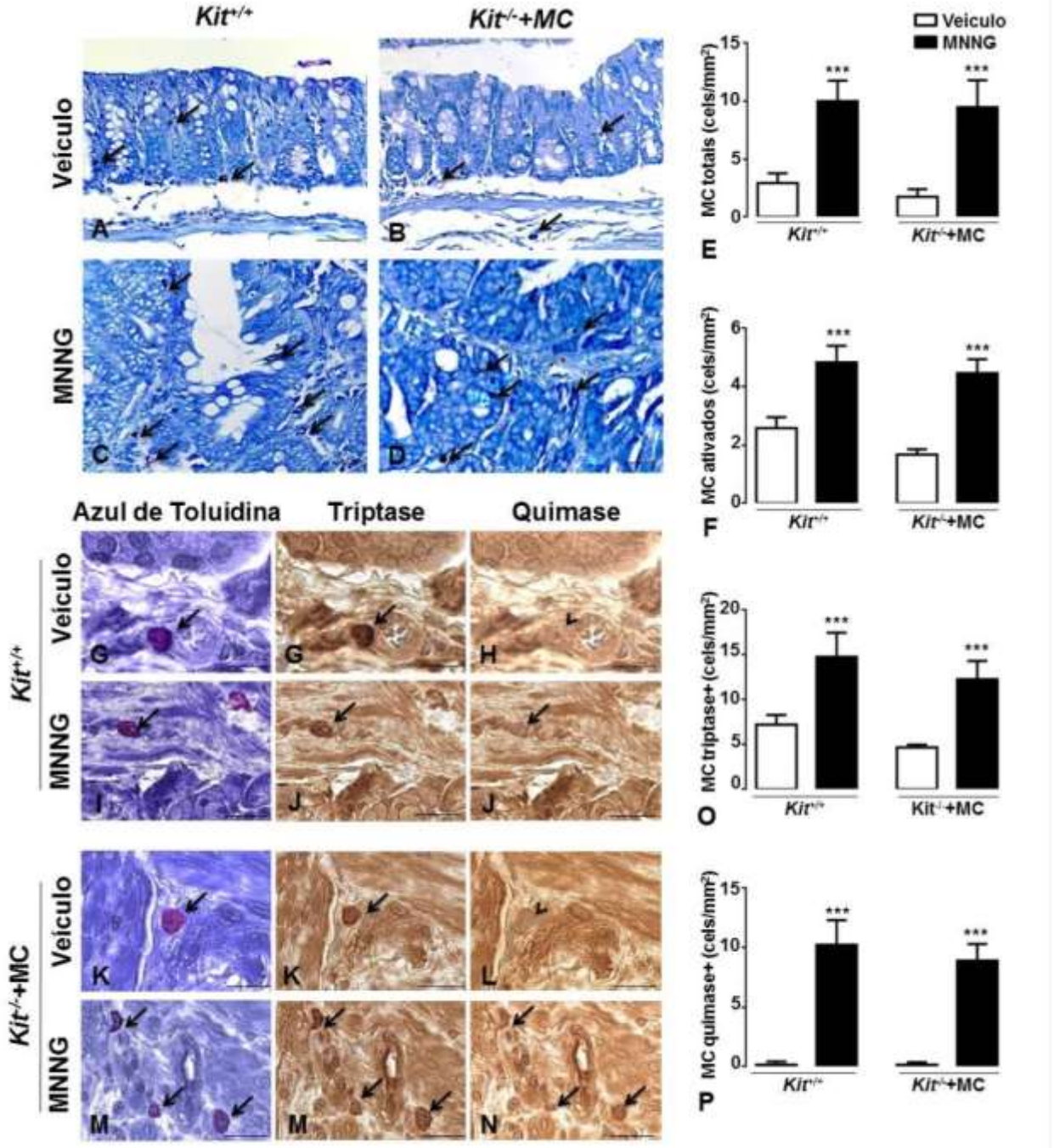


Figura 8. Avaliação quantitativa e fenotípica de mastócitos após indução tumoral por MNNG. Mastócitos metacromáticos (setas) no cólon distal dos animais induzidos por Veículo (A-B) e MNNG (C-D). Quantificação de mastócitos totais (E) e ativados (F) (Média ± DP. *** p <0,001 em relação ao respectivo grupo Veículo). Imunohistoquímica para proteases no cólon dos camundongos $Kit^{+/+}$ (G, H, K e L) e $Kit^{-/-}$ MC (I, J, M e N). A baixa expressão de quimase (cabeças de seta) foi observada em mastócitos na condição Veículo (H e L). Mastócitos quimase positivos (setas) observados após instilações com MNNG (J e N). Para confirmar as mesmas células observadas no tecido imunomarcado, secções seriadas foram coradas com Azul de Toluidina (G, I, K e M). Secções: 2 µm. Barras: 50 µm (A-D) e 20 µm (G-N). Quantificação de mastócitos triptase (O) e quimase (P) positivos no cólon distal nas condições Veículo e após indução com MNNG. Média ± DP. *** p <0,001 *vs* respectivo grupo Veículo.

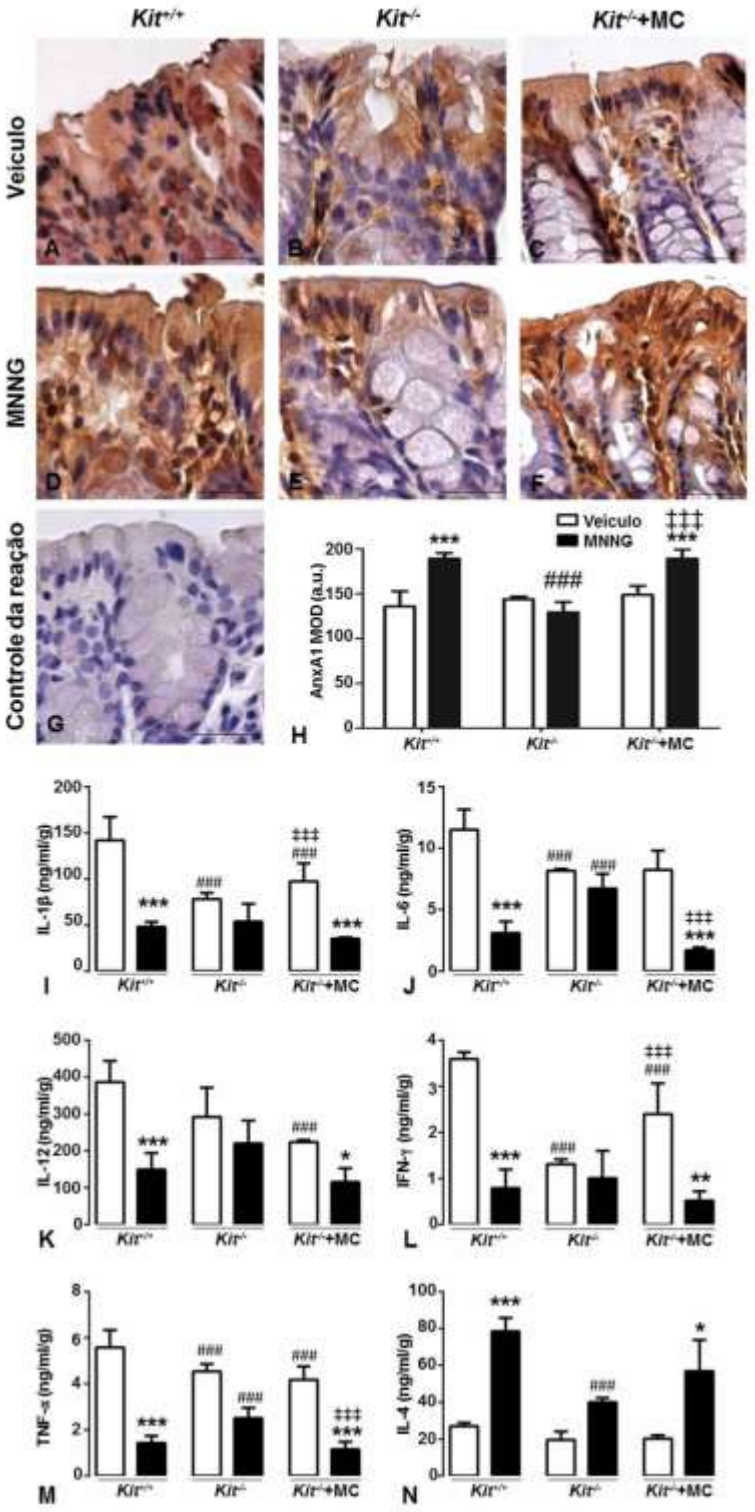


Figura 9. Expressão de AnxA1 e citocinas no cólon de camundongos deficientes em mastócitos após indução por MNNG. Imunomarcação de AnxA1 no epitélio no grupo Veículo (A-C) e após 24 semanas da exposição ao MNNG (D-E). Camundongos com mastócitos ($Kit^{+/+}$), deficientes ($Kit^{-/-}$) e reconstituídos ($Kit^{-/-}$+MC) foram avaliados em ambas as condições. Controle negativo da reação (E). Expressão intensa de AnxA1 observada no tecido afetado por MNNG nos animais $Kit^{+/+}$ e $Kit^{-/-}$+MC. Secções: 2 µm. Barras: 30 µm. Contracoloração: Hematoxilina. H) Densitometria óptica média (DOM): AnxA1. Média ± DP. *** p <0,001 *vs* grupo Veículo. ### p <0,001 *vs* respectivo grupo $Kit^{+/+}$. ‡‡‡ p <0,001 *vs* respectivo grupo $Kit^{-/-}$. Expressão de IL-1 (I), IL-6 (J), IL-12 (K), IFN-γ (L) TNF-α (M) e IL-4 (N) no cólon proximal de $Kit^{+/+}$, $Kit^{-/-}$ e $Kit^{-/-}$+MC após a exposição ao MNNG. Média ± DP de n = 5 animais/grupo. *** p <0,001 *vs* respectivo grupo Veículo.

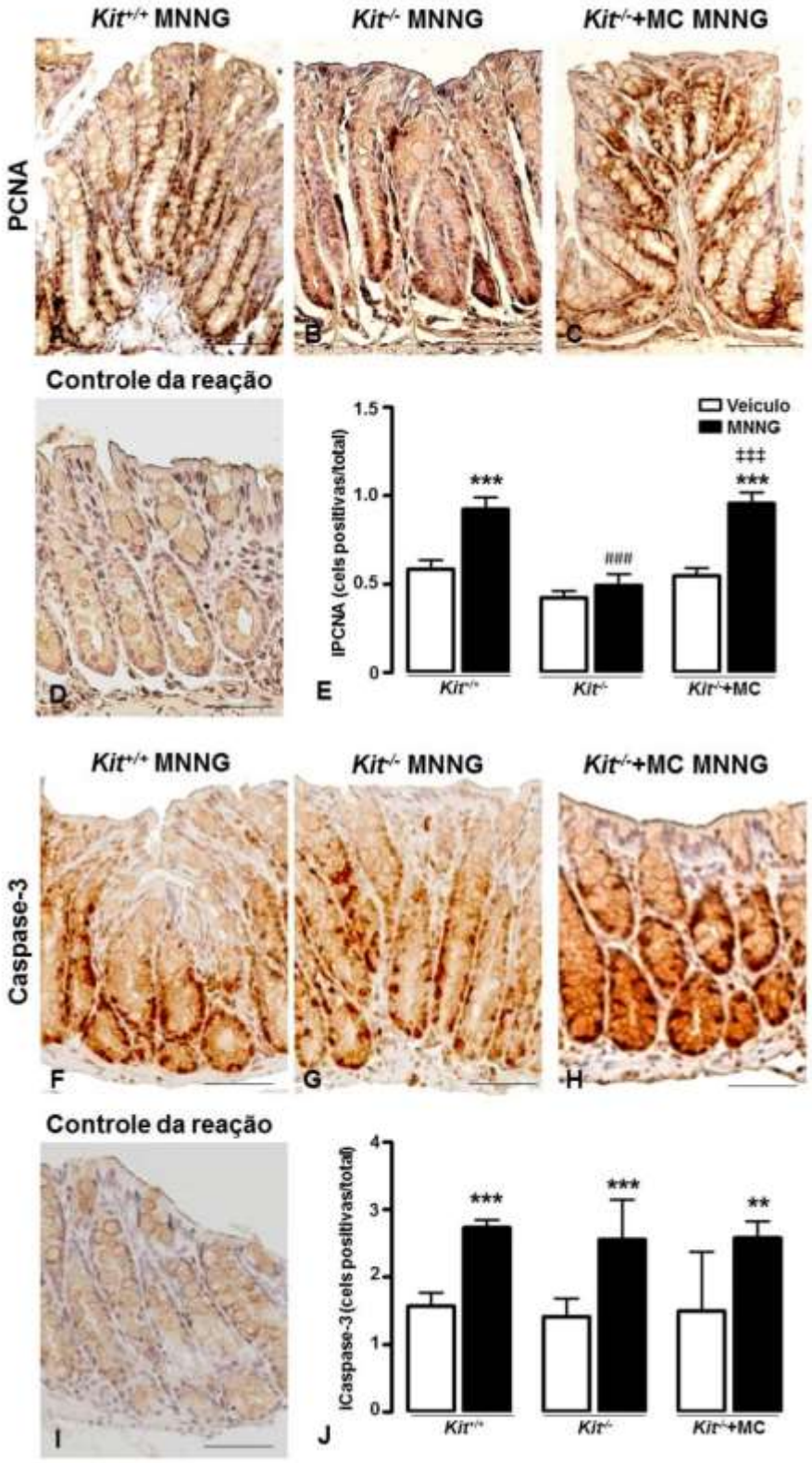


Figura 10. Proliferação e apoptose de células epiteliais colônicas induzida por MNNG na presença de mastócitos. Imunorreatividade do antígeno nuclear de proliferação celular (PCNA) e Caspase-3 no cólon distal dos $Kit^{+/+}$ (A e F), $Kit^{-/-}$ (B e G) e $Kit^{-/-}$+MC (C e H), respectivamente. Controle negativo do ensaio (D e I). Secções: 2 µm. Contra-coloração: Hematoxilina. Barras: 50 µm. (E e J) Índice relativo de células PCNA e Caspase-3 positivas em criptas colônicas, respectivamente. Média ± DP. *** p <0,001 *vs* respectivo grupo Veículo.

5. DISCUSSÃO

Nas últimas décadas emergiu novamente a ênfase sobre a associação entre inflamação e câncer (COLOTTA et al., 2009). Os papéis opostos da inflamação, nas fases inicias em contraposição às fases tardias do desenvolvimento tumoral, são suportados por evidências clínicas e experimentais (MANTOVANI et al., 2008). De fato, a tumorigênese é modulada pelas células estromais, sobretudo células imunes como os mastócitos, que participam das alterações no microambiente em formação e que podem propiciar ou comprometer o desenvolvimento tumoral (QUAIL; JOYCE, 2013). No presente trabalho, utilizamos o modelo murino de carcinogênese de cólon, induzida pelo carcinógeno MNNG, para investigar as relações entre a proteína AnxA1 e a atividade dos mastócitos nos estágios iniciais e avançados do processo oncogênico. Nossos resultados indicam que a AnxA1 participa da manutenção da organização do epitélio em resposta ao dano decorrente da exposição ao MNNG. Além disso, essa proteína regula o recrutamento, a ativação e a diferenciação dos mastócitos, evidenciando um papel importante na regulação da progressão tumoral.

A participação da AnxA1 nos processos tumorigênicos é controversa. Embora alguns estudos mostrem que ela modula a proliferação celular (BABBIN et al., 2008), sua associação no desenvolvimento da metástase, em alguns tumores, sugere que essa proteína regula o processo de migração/invasão celular (ALLDRIDGE; BRYANT, 2003; WANG et al., 2004). Similarmente, a baixa expressão de AnxA1 em linhagens de células tumorais as torna resistentes a apoptose após indução por agentes quimioterápicos (WANG et al., 2004; YU et al., 2014), sugerindo essa proteína como um biomarcador no prognóstico do câncer.

No CCR, a AnxA1 é aumentada, com expressão mais intensa nos estágios pouco diferenciados em comparação com tumores em avançado estágio de diferenciação (XUE et al., 2007). Além disso, a AnxA1 é altamente expressa nas doenças gastrointestinais, como úlceras gástricas e colites, e favorece a resolução da inflamação local e da regeneração tecidual (DE PAULA-SILVA et al., 2016; MARTIN et al., 2008).

Nesse sentido, nossos dados mostram que ocorre a expressão aumentada da AnxA1 no microambiente intestinal após a exposição aguda ao carcinógeno e, também, nos camundongos $Kit^{+/+}$ e $Kit^{-/-}$+*MC* que desenvolveram tumores. Uma vez que o gene *ANXA1* é induzido por estímulos inflamatórios (VO; FLOWER; D'ACQUISTO, 2006) e, também, por p53 em resposta à hipóxia no microambiente tumoral (LIU et al., 2007), o aumento da expressão da AnxA1 no cólon pode estar relacionado aos mecanismos de reparo e eliminação de células aberrantes.

Precocemente na carcinogênese, a ativação do fator de transcrição nuclear NF-κB dos leucócitos inflamatórios residentes em sítios pré-neoplásicos, assim como os mastócitos, exacerba a inflamação local com o aumento da expressão de TNF-α, IL-1, IL-12 e expressão reduzida de IL-4. Assim, foi observado que a instilação do carcinógeno MNNG promove o dano do epitélio intestinal e recrutamento de células inflamatórias, ação exacerbada na ausência de AnxA1. Também foi verificado que, mesmo em condições controle, que a deficiência dessa proteína anti-inflamatória resulta num cenário imunológico desbalanceado, com expressão aumentada de citocinas pró-inflamatórias e redução de IL-4. A expressão reduzida dessa citocina pode estar associada ao desenvolvimento de doenças intestinais crônicas (BAMIAS; ARSENEAU; COMINELLI, 2014).

A AnxA1 é conhecida como um indutor da expressão de IL-4 em condições inflamatórias (GIMENES et al., 2015). Essa citocina tem um papel importante na regulação da tolerância periférica intestinal e sua deficiência é diretamente associada à Doença de Chron e Doença Inflamatória Intestinal (CONNELLY et al., 2014; TOPTYGINA et al., 2014). Na literatura, há relatos de que a proteína AnxA1 tem sua expressão induzida por citocinas pró-inflamatórias de fase aguda, especialmente, IL-6 e TNF-α (YANG et al., 2009), amplamente associadas a retocolite ulcerativa (WANG et al., 2009). Além disso, a deficiência de AnxA1 condiciona o número reduzido de células inflamatórias, principalmente neutrófilos e macrófagos, capazes de expressar IL-1, IL-2 e IL-12 em condições de inflamação intestinal (GAO et al., 2015; LU et al., 2014). Desse modo a AnxA1, é apontada como importante regulador da homeostase intestinal.

A exposição ao carcinógeno promoveu um distúrbio intenso do microambiente intestinal, de maneira que horas depois do estímulo lesivo não foram observadas diferenças na expressão de citocinas nas linhagens animais $AnxA1^{+/+}$ e $AnxA1^{-/-}$. Em concentrações acima de 100µM, o MNNG é tóxico, compromete as funções celulares e é capaz de provocar lesão da membrana celular e, consequentemente, necrose (WĘSIERSKA-GĄDEK; GUEORGUIEVA; WOJCIECHOWSKI, 2003). As células necróticas fornecem forte estimulação ao sistema imune, promovendo o recrutamento de novas células, ativação das mesmas e das células residentes (BARTHOLOMAE et al., 2004; KOTERA; SHIMIZU; MULÉ, 2001).

A presença de mastócitos e seu estado de ativação foi verificada nas criptas colônicas dos animais expostos ao MNNG, com aumento do número de mastócitos totais e desgranulados, sobretudo nos animais com deficiência de AnxA1. Nosso grupo de pesquisa foi o responsável por identificar, pela primeira vez na literatura, a expressão de AnxA1 nos mastócitos (OLIANI et al., 2000), inclusive demonstrando sua co-localização com o receptor FPR2 na membrana dessas células (GASTARDELO et al., 2014). A associação AnxA1-FPR2 é

sugerida como capaz de mediar as funções reguladoras da AnxA1 sobre os mastócitos, como o controle da ativação celular (KAMAL; FLOWER; PERRETTI, 2005).

Os mastócitos são células imunes fundamentais para o desenvolvimento do CCR (BLIRANDO et al., 2011; HAMILTON et al., 2011; TANAKA; ISHIKAWA, 2013b; WEDEMEYER; GALLI, 2005b) que também expressam grandes quantidades de AnxA1 (DA SILVA; GIROL; OLIANI, 2011; KAMAL; FLOWER; PERRETTI, 2005). Os mastócitos são derivados de progenitores derivados da medula óssea que transmigram para a circulação para alcançarem, respectivamente a circulação sanguínea e os tecidos, onde completam a sua maturação ((DAHLIN; HALLGREN, 2015). Progenitores de mastócitos expressam as integrinas $\alpha4/\beta1$ e $\alpha4/\beta7$, que se ligam aos receptores no endotélio ativado ou células da mucosa na transmigração no intestino (RIBATTI, 2015). O fator de crescimento de células-tronco (SCF) produzido por fibroblastos, células estromais e endoteliais é o principal mediador na sobrevivência, ativação e circulação de progenitores de mastócitos, mas IL-4, IL-9, IL-10, fator de crescimento transformante (TGF-β) e o fator de crescimento de nervos (NGF) atuam em sinergia com o SCF (RIBATTI, 2015). Nós supomos que a AnxA1 pode controlar diretamente as vias de transmigração e locomoção dos progenitores de mastócitos, uma vez que a AnxA1 endógena modula o tráfego de granulócitos da medula óssea para o sangue e os tecidos inflamados (MACHADO et al., 2016; PERRETTI; D'ACQUISTO, 2009) ou, indiretamente, reduzindo a liberação de mediadores envolvidos na transmigração e quimiotaxia (SUGIMOTO et al., 2016). De fato, observamos níveis reduzidos de interleucinas envolvidas na adesão celular nos vasos, como a IL-1β and IL-6, na inflamação provocada pelo MNNG.

Embora não se conheça o mecanismo pelo qual a AnxA1 possa modular a migração e desgranulação dos mastócitos, algumas vias especialmente ativas nessas células são relacionadas a essa proteína. Por exemplo, a AnxA1 estimula *in vivo* a via Tyk2/Stat3 (MASCHLER et al., 2010), importante para a transcrição para o gene da quimase em mastócitos humanos (hCMA1). Nesse sentido, foi observado um número aumentado de mastócitos quimase positivos no cólon dos animais deficientes de AnxA1 expostos ao MNNG e no microambiente tumoral rico em AnxA1, sugerindo um papel regulador dessa protease. Este efeito pode ser mediado por receptores para peptídeos formilados (FPRs), que pertencem à família de receptores acoplados à proteína G, resultando na ativação de STAT3 (LI et al., 2011). Nesse sentido, um número aumentado de mastócitos quimase-positivos foi também observado em biópsias de endometriose ectópica, correlacionado com a alta expressão de AnxA1 co-localizada com o receptor FPR1 em células glandulares (PAULA-JÚNIOR et al., 2015). Estas funções são relevantes, sobretudo no cenário inflamatório inicial após exposição

ao carcinógeno onde os mastócitos parecem desempenhar um papel-chave na promoção tumoral.

Nossos dados mostram que os mastócitos estão relacionados ao aumento da susceptibilidade do desenvolvimento tumoral induzido pelo MNNG quando comparado aos *Kit*$^{-/-}$. Como esperado, a reconstituição dos *Kit*$^{-/-}$ com mastócitos derivados da medula óssea resultou na incidência aumentada de tumores. Resultados semelhantes foram observados quando foi utilizado 1,2-dimetilhidrazina (DMH) or azoximetano (AOM) para induzir tumores colorretais (RIGONI et al., 2015; WEDEMEYER; GALLI, 2005a). Consequentemente, nossos resultados suportam a hipótese de que os mastócitos contribuem para o desenvolvimento/crescimento tumoral.

O número de mastócitos totais e ativados também foi verificado aumentado no cólon dos animais que desenvolveram tumores. Essas células apresentam importantes funções tanto na fase aguda quanto na fase de remodelação de lesões (LEE et al., 2013). No processo de desgranulação, a liberação de triptase e/ou quimase para o meio extracelular contribui na degradação da matriz, promoção da angiogênese e remodelação tecidual por meio de proteólises seletivas na matriz e ativação de metaloproteinases (PANSRIKAEW et al., 2010; SIMIONESCU et al., 2013). Estes processos estão intimamente ligados a tumorigênese e a quimase, especificamente, tem sido apontada como um dos mais potentes indutores da produção de angiotensina 2 no organismo (MIYAZAKI et al., 2006), que somada à produção intensa de VEGF, pode contribuir para a angiogênese associada às células pró-tumorais originadas pelo dano carcinogênico.

O microambiente estabelecido após a indução crônica com MNNG demonstra que os mastócitos contribuem para a formação dos tumores, pela imunorregulação da resposta anti-tumoral e possivelmente associada à expressão aumentada de AnxA1. O MNNG é alquilante direto do DNA (WĘSIERSKA-GĄDEK; GUEORGUIEVA; WOJCIECHOWSKI, 2003) que resulta em transições G:C para A:T após a replicação do DNA que não podem ser corrigidas pelo sistema de reparo de bases mal pareadas (MMR - do inglês mismatch repair) (YORK; MODRICH, 2006). Desse modo, o MNNG induz quebras no DNA, o arraste do ciclo celular, e focos nucleares persistentes nos sítios de dano no DNA (STOJIC et al., 2004). A instilação intrarretal de MNNG é um método confiável e eficiente de induzir tumores seletivamente na área do cólon exposta ao carcinógeno (CHE et al., 2010). Assim, nós demonstramos que esse efeito depende dos mastócitos constitutivos, sendo que o desenvolvimento de tumores foi acentuadamente reduzido nos camundongos deficientes para ambas as cópias do Kit (*Kit*$^{-/-}$), em que resulta na ausência de mastócitos (KITAMURA; GO; HATANAKA, 1978).

Também destacamos que a expressão da AnxA1 no microambiente durante a fase tardia do crescimento tumoral é modulada pelos mastócitos, pois a maior expressão dessa proteína foi detectada nos camundongos $Kit^{+/+}$ e nos $Kit^{-/-}$ reconstituídos ($Kit^{-/-}$+MC), em comparação com os animais $Kit^{-/-}$. Juntos, esses dados mostram a interação entre os mastócitos e as células epiteliais durante o desenvolvimento do CCR para promover a expressão da AnxA1, principalmente, pelas células epiteliais. Como mencionado, os níveis da AnxA1 são um indicados de mal prognóstico nos tumores (ONOZAWA et al., 2017; YI; SCHNITZER, 2009)

Além disso, foi verificada a maior proporção de células PCNA positivas e, também, a expressão aumentada de AnxA1 no cólon dos animais $Kit^{+/+}$ e $Kit^{-/-}$+*MC*. Assim, a ação dos mastócitos nesse modelo parece ser fundamental para a proliferação de células epiteliais que resulta na formação de tumores. Nesse mesmo sentido, outros autores demonstram que a proteína AnxA1 é capaz de regular a proliferação das células epiteliais intestinais (BABBIN et al., 2008). Modelos experimentais e estudos *in vitro* indicam que a ANXA1 imobiliza o NF-κB impedindo sua translocação para o núcleo, a ativação da transcrição subseqüente (ZHANG et al., 2010) e pode retardar a malignização dos tumores de cólon (CALON et al., 2012). Esses efeitos anti-tumorais diretos, bem como sua influência sobre a atividade e fenótipo dos mastócitos, apontam para a AnxA1 como um importante alvo terapêutico no CCR.

Considerando as possibilidades já existentes de impedir a ativação dos mastócitos (SOUCEK et al., 2011) e a inibição seletiva de alguns mediadores importantes, como a quimase (SCHLATTER et al., 2012), a caracterização da AnxA1 como um possível mediador envolvido na regulação da diferenciação das subpopulações de mastócitos pode aprimorar a intervenção terapêutica anti-tumoral para o CCR.

6. CONCLUSÕES

Os resultados obtidos, nas condições de indução pelo carcinógeno MNNG no cólon, permitem concluir que:

1. O dano tecidual agudo induz o aumento da produção de AnxA1 endógena no cólon;

2. A AnxA1 contribui para a manutenção da morfologia e homeostasia intestinal, regulando a produção de IL-4 e o infiltrado celular, inibindo a produção das citocinas pró-inflamatórias IL-1, IL-6, IL-12, TNF-α e IFN-γ e o dano tecidual, sobretudo 24 horas após a exposição ao composto;

3. O carcinógeno promove recrutamento, ativação e diferenciação dos mastócitos triptase e quimase positivos na exposição inicial ao carcinógeno e no estabelecimento dos tumores em 24 semanas;

4. Os efeitos do MNNG sobre os mastócitos são modulados pela AnxA1, que inibe o recrutamento e a desgranulação dessas células nos estágios iniciais da carcinogênese e diferenciam os mastócitos para expressão aumentada de quimase;

5. Os mastócitos contribuem para a expressão de AnxA1 no microambiente tumoral estabelecido após 24 semanas da indução do composto;

6. Os mastócitos afetam a susceptibilidade à carcinogênese, dados confirmados pelo menor volume tumoral e morfofisiologia preservada nos animais deficientes de mastócitos ($Kit^{-/-}$);

7. Os mastócitos, conforme observado na condição crônica, estimulam o estabelecimento de um ambiente imunossupressor, rico em AnxA1, com aumento de IL-4 e baixa expressão das citocinas pró-inflamatórias. Ainda, contribuem para a proliferação aumentada de células epiteliais colônicas, resultando na tumorigênese colorretal;

Associadas, nossas conclusões fornecem subsídios para o entendimento de que a proteína AnxA1 endógena está envolvida nas alterações do cólon após a exposição ao MNNG, modulando o recrutamento, a ativação e a diferenciação dos mastócitos, eventos que estimulam a tumorigênese no cólon. No entanto, estudos adicionais são necessários para elucidar o papel específico destas células e seus mediadores na progressão do câncer colorretal.

7. REFERÊNCIAS BIBLIOGRÁFICAS

AHN, S. H. et al. Differential expression of annexin I in human mammary ductal epithelial cells in normal and benign and malignant breast tissues. **Clin Exp Metastasis**, v. 15, n. 2, p. 151–156, 1997.

ALDENBORG, F.; ENERBÄCK, L. The immunohistochemical demonstration of chymase and tryptase in human intestinal mast cells. **Histochem J**, v. 26, p. 587–596, 1994.

ALLDRIDGE, L. C.; BRYANT, C. E. Annexin 1 regulates cell proliferation by disruption of cell morphology and inhibition of cyclin D1 expression through sustained activation of the ERK1/2 MAPK signal. **Experimental cell research**, v. 290, n. 1, p. 93–107, 15 out. 2003.

ANAND, P. et al. Cancer is a preventable disease that requires major lifestyle changes. **Pharm Res**, v. 25, n. 9, p. 2097–2116, set. 2008.

ANDOH, A. et al. Immunohistochemical study of chymase-positive mast cells in inflammatory bowel disease. **Oncol Rep**, v. 16, p. 103–107, 2006.

ANTONIO, D. et al. Expression of mast cell proteases correlates with mast cell maturation and angiogenesis during tumor progression. **PLoS One**, v. 7, n. 7, p. e40790, 2012.

AUGESTAD, K. M. et al. Metastatic spread pattern after curative colorectal cancer surgery . A retrospective , longitudinal analysis. **Cancer Epidemiology**, v. 39, n. 5, p. 734–744, 2015.

AZOUZ, N. P. et al. Investigating Mast Cell Secretory Granules; from Biosynthesis to Exocytosis. **Journal of Visualized Experiments**, n. 95, p. 1–7, 2015.

BABBIN, B. A. et al. Annexin I regulates SKCO-15 cell invasion by signaling through formyl peptide receptors. **Journal of Biological Chemistry**, v. 281, n. 28, p. 19588–19599, 2006.

BABBIN, B. A. et al. Annexin A1 regulates intestinal mucosal injury, inflammation, and repair. **Journal of immunology (Baltimore, Md. : 1950)**, v. 181, n. 7, p. 5035–44, 1 out. 2008.

BABINA, M. et al. Baseline and stimulated turnover of cell surface c-Kit expression in different types of human mast cells. **Exp Dermatol**, v. 15, p. 530–537, 2006.

BAI, X. F. et al. Overexpression of annexin 1 in pancreatic cancer and its clinical significance. **World Journal of Gastroenterology**, v. 10, n. 10, p. 1466–1470, 2004.

BAMIAS, G.; ARSENEAU, K. O.; COMINELLI, F. Cytokines and mucosal immunity. **Current opinion in gastroenterology**, v. 30, n. 6, p. 547–52, nov. 2014.

BANDEIRA-MELO, C. et al. A novel effect for annexin 1-derived peptide ac2-26: reduction of allergic inflammation in the rat. **J Pharmacol Exp Ther**, v. 313, p. 1416–1422, 2005.

BARTHOLOMAE, W. C. et al. T cell immunity induced by live, necrotic, and apoptotic tumor cells. **Journal of immunology (Baltimore, Md. : 1950)**, v. 173, n. 2, p. 1012–22, 15 jul. 2004.

BEIL, W. J. et al. Selective alterations in mast cell subsets and eosinophil infiltration in two complementary types of intestinal inflammation: ascariasis and Crohn's disease. **Pathobiology**, v. 70, p. 303–313, 2002.

BELOV, L.; ZHOU, J.; CHRISTOPHERSON, R. I. **Cell Surface Markers in Colorectal Cancer Prognosis**. [s.l: s.n.]. v. 12

BEYDOUN, H. A.; BEYDOUN, M. A. **Predictors of colorectal cancer screening behaviors among average-risk older adults in the United StatesCancer Causes and Control**, 2008.

BLIRANDO, K. et al. Mast cells are an essential component of human radiation proctitis and contribute to experimental colorectal damage in mice. **Am J Pathol**, v. 178, n. 2, p. 640–651, 2011.

BOUDHRAA, Z. et al. Annexin A1 localization and its relevance to cancer. **Clinical science (London, England : 1979)**, v. 130, n. 4, p. 205–20, 2016.

BUTLER, C. T. et al. A Quininib Analogue and Cysteinyl Leukotreine Receptor Antagonist inhibits

VEGF-Independent Angiogenesis and Exerts an Additive Anti-angiogenic Response with Bevacizumab. 2016.

CALON, A. et al. Dependency of colorectal cancer on a TGF-β-driven program in stromal cells for metastasis initiation. **Cancer cell**, v. 22, n. 5, p. 571–584, 13 nov. 2012.

CAUGHEY, G. H. Mast cell tryptases and chymases in inflammation and host defense. **Immunology Review**, p. 141–154, 2007.

CHAN, E. et al. Phase II study of the Multikinase inhibitor of angiogenesis , Linifanib , in patients with metastatic and refractory colorectal cancer expressing mutated KRAS. 2017.

CHATTERJEE, B. E. et al. Annexin 1-deficient neutrophils exhibit enhanced transmigration in vivo and increased responsiveness in vitro. **Journal of leukocyte biology**, v. 78, n. 3, p. 639–646, 2005.

CHE, T. C. H. et al. Early lesions induced in rat colon epithelium by N-methyl-N'-nitro-N-nitrosoguanidine. **Tissue and Cell**, v. 42, p. 190–194, 2010.

CHICHLOWSKI, M. et al. Role of mast cells in inflammatory bowel disease and inflammation-associated colorectal neoplasia in IL-10-deficient mice. **PLoS One**, v. 5, p. e12220, 2010.

CICEK, M. et al. Identification of metastasis-associated proteins through protein analysis of metastatic MDA-MB-435 and metastasis-suppressed BRMS1 transfected-MDA-MB-435 cells. **Clinical and Experimental Metastasis**, v. 21, n. 2, p. 149–157, 2004.

COLOTTA, F. et al. **Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instabilityCarcinogenesis**, 2009.

COLUSSI, D. et al. Molecular pathways involved in colorectal cancer: implications for disease behavior and prevention. **International journal of molecular sciences**, v. 14, n. 8, p. 16365–85, 7 ago. 2013.

CONNELLY, T. M. et al. An interleukin-4 polymorphism is associated with susceptibility to Clostridium difficile infection in patients with inflammatory bowel disease: results of a retrospective cohort study. **Surgery**, v. 156, n. 4, p. 769–74, out. 2014.

CONTI, P. et al. Role of mast cells in tumor growth. **Ann Clin Lab Sci**, v. 37, p. 315–322, 2007.

CROXTALL, J. D. et al. Lipocortin 1 and the control of cPLA2 activity in A549 cells. Glucocorticoids block EGF stimulation of cPLA2 phosphorylation. **Biochemical Pharmacology**, v. 52, n. 2, p. 351–356, 1996.

CROXTALL, J. D.; FLOWER, R. J. Lipocortin 1 mediates dexamethasone-induced growth arrest of the A549 lung adenocarcinoma cell line. **Proc Natl Acad Sci U S A**, v. 89, p. 3571–3575, 1992.

D'ACQUISTO, F. et al. Annexin-1 modulates T-cell activation and differentiation. **Blood**, v. 109, n. 3, p. 1095–1102, 2007.

DA SILVA, P. S.; GIROL, A. P.; OLIANI, S. M. Mast cells modulate the inflammatory process in endotoxin-induced uveitis. **Mol Vis**, v. 17, p. 1310–1319, 2011.

DAHLIN, J. S.; HALLGREN, J. Mast cell progenitors: Origin, development and migration to tissues. **Molecular Immunology**, v. 63, n. 1, p. 9–17, 2015.

DAVIES, R. J.; MILLER, R.; COLEMAN, N. Colorectal cancer screening: prospects for molecular stool analysis. **Nature reviews. Cancer**, v. 5, n. 3, p. 199–209, 2005.

DE, B. K. et al. A calcium-dependent 35-kilodalton substrate for epidermal growth factor receptor/kinase isolated from normal tissue. **Journal of Biological Chemistry**, v. 261, n. 29, p. 13784–13792, 1986.

DE PAULA-SILVA, M. et al. Role of the protein annexin A1 on the efficacy of anti-TNF treatment in a murine model of acute colitis. **Biochemical Pharmacology**, v. 115, p. 104–113, 2016.

DI CARO, G. et al. Immune cells: plastic players along colorectal cancer progression. **J Cell Mol Med**,

v. 17, p. 1088–1095, 2013.

DIAKONOVA, M. et al. Localization of five annexins in J774 macrophages and on isolated phagosomes. **Journal of cell science**, v. 110 (Pt 1, p. 1199–1213, 1997.

DIETRICH, N. et al. Mast cells elicit proinflammatory but not type I interferon responses upon activation of TLRs by bacteria. **Proceedings of the National Academy of Sciences**, v. 107, n. 19, p. 8748–8753, 2010.

DREW, D. A.; CAO, Y.; CHAN, A. T. Aspirin and colorectal cancer: the promise of precision chemoprevention. **Nature Publishing Group**, n. Ci, p. 8–11, 2016.

DUFTON, N.; PERRETTI, M. **Therapeutic anti-inflammatory potential of formyl-peptide receptor agonistsPharmacology and Therapeutics**, 2010.

FAVORITI, P. et al. Worldwide burden of colorectal cancer: a review. **Updates in Surgery**, v. 68, n. 1, p. 7–11, 11 mar. 2016.

FEDIRKO, V. et al. Alcohol drinking and colorectal cancer risk: an overall and dose-response meta-analysis of published studies. **Ann Oncol**, v. 22, p. 1958–1972, 2011.

FEMIA, A. P. et al. Identification of mucin depleted foci in the human colon. **Cancer Prev Res (Phila)**, v. 1, p. 562–567, 2008.

FLOWER, R. J.; BLACKWELL, G. J. Anti-inflammatory steroids induce biosynthesis of a phospholipase A2 inhibitor which prevents prostaglandin generation. **Nature**, v. 278, n. 5703, p. 456–459, 29 mar. 1979.

FLOWER, R. J.; ROTHWELL, N. J. Lipocortin-1: cellular mechanisms and clinical relevance. **Trends in pharmacological sciences**, v. 15, n. 3, p. 71–6, 1994.

GALLI, S. J.; TSAI, M. Mast cells: versatile regulators of inflammation, tissue remodeling, host defense and homeostasis. **J Dermatol Sci**, v. 49, p. 7–19, 2008.

GAO, J.-L. et al. Differential expansion of the N-formylpeptide receptor gene cluster in human and mouse. **Genomics**, v. 51, n. 2, p. 270–6, 1998.

GAO, X. et al. Neutrophil extracellular traps contribute to the intestine damage in endotoxemic rats. **The Journal of surgical research**, v. 195, n. 1, p. 211–8, 1 maio 2015.

GARCIA PEDRERO, J. M. et al. Annexin A1 down-regulation in head and neck cancer is associated with epithelial differentiation status. **The American journal of pathology**, v. 164, n. 1, p. 73–9, 2004.

GASTARDELO, T. S. et al. Inflammation and Cancer: Role of Annexin A1 and FPR2/ALX in Proliferation and Metastasis in Human Laryngeal Squamous Cell Carcinoma. **PloS one**, v. 9, n. 12, p. e111317, jan. 2014.

GAVINS, F. N. et al. Leukocyte antiadhesive actions of annexin 1: ALXR- and FPR-related anti-inflammatory mechanisms. **Blood**, v. 101, p. 4140–4147, 2003.

GERKE, V.; CREUTZ, C. E.; MOSS, S. E. Annexins: linking Ca2+ signalling to membrane dynamics. **Nature Reviews Molecular Cell Biology**, v. 6, n. 6, p. 449–461, 2005.

GERKE, V.; MOSS, S. E. Annexins: From Structure to Function. **Physiological Reviews**, v. 82, n. 2, p. 331–371, 2002.

GIL, C. D. et al. Interaction of human neutrophils with endothelial cells regulates the expression of endogenous proteins annexin 1, galectin-1 and galectin-3. **Cell Biology International**, v. 30, n. 4, p. 338–344, 2006.

GIMENES, A. D. et al. Beneficial effect of annexin A1 in a model of experimental allergic conjunctivitis. **Experimental eye research**, v. 134, p. 24–32, maio 2015.

GOULDING, N. J. et al. Anti-inflammatory lipocortin 1 production by peripheral blood leucocytes in

response to hydrocortisone. **The Lancet**, v. 335, n. 8703, p. 1416–1418, 1990.

GRIMBALDESTON, M. A. et al. Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. **The American journal of pathology**, v. 167, n. 3, p. 835–48, set. 2005.

GRIVENNIKOV, S. I.; GRETEN, F. R.; KARIN, M. Immunity, inflammation, and cancer. **Cell**, v. 140, p. 883–899, 2010.

GROSCHWITZ, K. R. et al. Mast cells regulate homeostatic intestinal epithelial migration and barrier function by a chymase/Mcpt4-dependent mechanism. **Proc Natl Acad Sci U S A**, v. 106, p. 22381–22386, 2009.

GROSCHWITZ, K. R.; HOGAN, S. P. Intestinal barrier function: molecular regulation and disease pathogenesis. **The Journal of allergy and clinical immunology**, v. 124, n. 1, p. 3-20–2, jul. 2009.

GURISH, M. F. et al. Tissue-regulated differentiation and maturation of a v-abl-immortalized mast cell-committed progenitor. **Immunity**, v. 3, p. 175–186, 1995.

HALOVA, I.; DRABEROVA, L.; DRABER, P. Mast cell chemotaxis chemoattractants and signaling pathways. **Frontiers in Immunology**, v. 3, n. MAY, p. 1–19, 2012.

HAMILTON, M. J. et al. Essential role for mast cell tryptase in acute experimental colitis. **Proc Natl Acad Sci U S A**, v. 108, p. 290–295, 2011.

HAMILTON, S. R. et al. Carcinoma of the colon and rectum. In: BOSMAN, F. T. et al. (Eds.). . **WHO Classification of Tumours of the Digestive System**. 4. ed. [s.l.] Lyon: IARC, 2010. p. 134–146.

HEIJMANS, J. et al. Role of mast cells in colorectal cancer development, the jury is still out. **Biochim Biophys Acta**, v. 1822, n. 1, p. 9–13, 2012.

HOGG, N. Red meat and colon cancer: heme proteins and nitrite in the gut. A commentary on "diet-induced endogenous formation of nitroso compounds in the GI tract". **Free radical biology & medicine**, v. 43, n. 7, p. 1037–9, 1 out. 2007.

HUEBNER, K. et al. Chromosomal localization of the human genes for lipocortin I and lipocortin II. **Oncogene Res**, v. 2, n. 4, p. 299–310, 1988.

HUGHES, E. et al. Mast Cells Mediate Early Neutrophil Recruitment and Exhibit Anti-Inflammatory Properties via FPR2/ALX. **British Journalournal**, 2017.

INCA, M. DA S.-. **Estimativas da incidência e mortalidade por câncer no Brasil**, 2012.

INSTITUTO NACIONAL DE CANCER JOSÉ ALENCAR GOMES DA SILVA. **INCA - Instituto Nacional de Câncer - Estimativa 2016**. [s.l: s.n.].

ISHIDA, K. et al. Role of chymase-dependent matrix metalloproteinase-9 activation in mice with dextran sodium sulfate-induced colitis. **J Pharmacol Exp Ther**, v. 324, p. 422–426, 2008.

KAMAL, A. M.; FLOWER, R. J.; PERRETTI, M. An overview of the effects of annexin 1 on cells involved in the inflammatory process. **Memórias do Instituto Oswaldo Cruz**, v. 100 Suppl, p. 39–47, mar. 2005.

KANDA, M.; KODERA, Y. Molecular mechanisms of peritoneal dissemination in gastric cancer. v. 22, n. 30, p. 6829–6840, 2016.

KENNELLY, R. et al. Mast Cells in Tissue Healing: From Skin to the Gastrointestinal Tract. **Current Pharmaceutical Design**, v. 17, n. 34, p. 3772–3775, 2011.

KHAU, T. et al. Annexin-1 signals mitogen-stimulated breast tumor cell proliferation by activation of the formyl peptide receptors (FPRs) 1 and 2. **The FASEB Journal**, v. 25, n. 2, p. 483–496, 2011.

KITAMURA, B. Y.; GO, S.; HATANAKA, K. Decrease of Mast Cells in W/ W" Mice and Their Increase by Bone Marrow Transplantation. **Blood**, v. 52, n. 2, 1978.

KITAMURA, Y. **Transdifferentiation between mast cell subpopulations** (T. Nakano, Ed.)Development Growth and Differentiation, 1986.

KONDO, K. et al. Expression of chymase-positive cells in gastric cancer and its correlation with the angiogenesis. **J Surg Oncol**, v. 93, p. 33–36, 2006.

KOSANOVIC, D. et al. Mast cell chymase: an indispensable instrument in the pathological symphony of idiopathic pulmonary fibrosis? **Histol Histopathol**, v. 28, n. 6, p. 691–699, 2013.

KOTERA, Y.; SHIMIZU, K.; MULÉ, J. J. Comparative analysis of necrotic and apoptotic tumor cells as a source of antigen(s) in dendritic cell-based immunization. **Cancer research**, v. 61, n. 22, p. 8105–9, 15 nov. 2001.

LARSSON, S. C.; ORSINI, N.; WOLK, A. Diabetes mellitus and risk of colorectal cancer: A meta-analysis. **Journal of the National Cancer Institute**, v. 97, n. 22, p. 1679–1687, 2005.

LEA, I. A.; JACKSON, M. A.; DUNNICK, J. K. Genetic pathways to colorectal cancer. **Mutation research**, v. 670, n. 1–2, p. 96–8, 2009.

LEE, H. et al. Mucosal mast cell count is associated with intestinal permeability in patients with diarrhea predominant irritable bowel syndrome. **Journal of neurogastroenterology and motility**, v. 19, n. 2, p. 244–50, 1 abr. 2013.

LEONI, G. et al. Annexin A1, formyl peptide receptor, and NOX1 orchestrate epithelial repair. **The Journal of clinical investigation**, v. 123, n. 1, p. 443–54, jan. 2012.

LI, F.; LAI, M. Colorectal cancer, one entity or three. **Journal of Zhejiang University SCIENCE B**, v. 10, n. 3, p. 219–229, 2009.

LI, Y. et al. Pleiotropic regulation of macrophage polarization and tumorigenesis by formyl peptide receptor-2. **Oncogene**, v. 30, p. 3887–3899, 2011.

LIM, L. H. K.; PERVAIZ, S. Annexin 1: the new face of an old molecule. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology**, v. 21, n. 4, p. 968–75, abr. 2007.

LIN, C.-Y. et al. Nuclear localization of annexin A1 is a prognostic factor in oral squamous cell carcinoma. **Journal of surgical oncology**, v. 97, n. 6, p. 544–50, 2008.

LIU, L. et al. Is dietary fat associated with the risk of colorectal cancer? A meta-analysis of 13 prospective cohort studies. **Eur J Nutr**, v. 50, p. 173–184, 2011a.

LIU, T. et al. Hypoxia induces p53-dependent transactivation and Fas/CD95-dependent apoptosis. **Cell death and differentiation**, v. 14, p. 411–421, 2007.

LIU, Y. F. et al. Identification of annexin A1 as a proinvasive and prognostic factor for lung adenocarcinoma. **Clinical and Experimental Metastasis**, v. 28, n. 5, p. 413–425, 2011b.

LOGING, W. T. et al. **Identifying potential tumor markers and antigens by database mining and rapid expression screening [5]Genome Research**, 2000.

LU, N. et al. Activation of the epidermal growth factor receptor in macrophages regulates cytokine production and experimental colitis. **Journal of immunology (Baltimore, Md. : 1950)**, v. 192, n. 3, p. 1013–23, 1 fev. 2014.

MAANINKA, K.; LAPPALAINEN, J.; KOVANEN, P. T. Human mast cells arise from a common circulating progenitor. **J Allergy Clin Immunol**, v. 132, p. 463–9.e3, 2013.

MACHADO, I. D. et al. Annexin A1 Is a Physiological Modulator of Neutrophil Maturation and Recirculation Acting on the CXCR4 / CXCL12 Pathway. n. February, 2016.

MALTBY, S.; KHAZAIE, K.; MCNAGNY, K. M. Mast cells in tumor growth: angiogenesis, tissue remodelling and immune-modulation. **Biochim Biophys Acta**, v. 1796, p. 19–26, 2009.

MANTOVANI, A. et al. Cancer-related inflammation. **Nature**, v. 454, n. 7203, p. 436–44, 24 jul. 2008.

MARTIN, G. R. et al. Annexin-1 modulates repair of gastric mucosal injury. **American journal of physiology. Gastrointestinal and liver physiology**, v. 294, n. 3, p. G764–G769, 2008.

MASAKI, T. et al. Enhanced expression of the protein kinase substrate annexin in human hepatocellular carcinoma. **Hepatology (Baltimore, Md.)**, v. 24, n. 1, p. 72–81, 1996.

MASCHLER, S. et al. Annexin A1 attenuates EMT and metastatic potential in breast cancer. **EMBO Mol Med**, v. 2, p. 401–414, 2010.

MEDEIROS, M.; OSHIMA, C. T. F.; FORONES, N. M. Depression and anxiety in colorectal cancer patients. **Journal of gastrointestinal cancer**, v. 41, n. 3, p. 179–84, set. 2010.

MIYAZAKI, M. et al. Pathological roles of angiotensin II produced by mast cell chymase and the effects of chymase inhibition in animal models. **Pharmacology & therapeutics**, v. 112, n. 3, p. 668–76, dez. 2006.

MOON, T. C. et al. Advances in mast cell biology: new understanding of heterogeneity and function. **Mucosal Immunol**, v. 3, n. 2, p. 111–128, 2010.

MOON, T. C.; DEAN BEFUS, A.; KULKA, M. Mast cell mediators: Their differential release and the secretory pathways involved. **Frontiers in Immunology**, v. 5, n. NOV, p. 1–18, 2014.

MORÁN, A. et al. Differential colorectal carcinogenesis: Molecular basis and clinical relevance. **World J Gastrointest Oncol**, v. 2, n. 3, p. 151–158, 15 mar. 2010.

NAGATA, M. et al. Chymase-positive mast cells in small sized adenocarcinoma of the lung. **Virchows Arch**, v. 443, p. 565–573, 2003.

OLIANI, S. M. et al. An immunocytochemical and in situ hybridization analysis of annexin 1 expression in rat mast cells: modulation by inflammation and dexamethasone. **Laboratory investigation; a journal of technical methods and pathology**, v. 80, p. 1429–1438, 2000.

OLIANI, S. M. et al. Neutrophil interaction with inflamed postcapillary venule endothelium alters annexin 1 expression. **Am J Pathol**, v. 158, p. 603–615, 2001.

OLIANI, S. M. et al. Fluctuation of annexin-A1 positive mast cells in chronic granulomatous inflammation. **Inflammation research : official journal of the European Histamine Research Society ... [et al.]**, v. 57, n. 10, p. 450–6, out. 2008.

OLIANI, S. M.; DAMAZO, A. S.; PERRETTI, M. Annexin 1 localisation in tissue eosinophils as detected by electron microscopy. **Mediators Inflamm**, v. 11, p. 287–292, 2002.

ONOZAWA, H. et al. Annexin A1 is involved in resistance to 5-FU in colon cancer cells. p. 235–240, 2017.

PANSRIKAEW, P. et al. Correlation of mast cell density, tumor angiogenesis, and clinical outcomes in patients with endometrioid endometrial cancer. **Asian Pacific journal of cancer prevention : APJCP**, v. 11, n. 3, p. 623–6, jan. 2010.

PARENTE, L.; SOLITO, E. **Annexin 1: More than an anti-phospholipase proteinInflammation Research**, 2004.

PARREIRAS, F. C. et al. Genetic aspects of colorectal cancer and its impact on disease management. **Revista Médica de Minas Gerais**, v. 23, n. 2, p. 221–227, 2013.

PAULA-JÚNIOR, R. et al. The intricate role of mast cell proteases and the annexin A1-FPR1 system in abdominal wall endometriosis. **Journal of molecular histology**, v. 46, n. 1, p. 33–43, fev. 2015.

PAWELETZ, C. P. et al. Loss of annexin 1 correlates with early onset of tumorigenesis in esophageal and prostate carcinoma. **Cancer Research**, v. 60, n. 22, p. 6293–6297, 2000.

PERRETTI, M. et al. Mobilizing lipocortin 1 in adherent human leukocytes downregulates their

transmigration. **Nature medicine**, v. 2, n. 11, p. 1259–1262, 1996.

PERRETTI, M. Lipocortin 1 and chemokine modulation of granulocyte and monocyte accumulation in experimental inflammation. **Gen Pharmacol**, v. 31, p. 545–552, 1998.

PERRETTI, M. et al. Involvement of the receptor for formylated peptides in the in vivo anti-migratory actions of annexin 1 and its mimetics. **The American journal of pathology**, v. 158, n. 6, p. 1969–73, 2001.

PERRETTI, M.; D'ACQUISTO, F. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. **Nat Rev Immunol**, v. 9, n. 1, p. 62–70, 2009.

PERRETTI, M.; DALLI, J. **Exploiting the Annexin A1 pathway for the development of novel anti-inflammatory therapeuticsBritish Journal of Pharmacology**, 2009.

PILIPONSKY, A. M. et al. The chymase mouse mast cell protease 4 degrades TNF, limits inflammation, and promotes survival in a model of sepsis. **Am J Pathol**, v. 181, p. 875–886, 2012.

PINTER, T. et al. Original Study Placebo-Controlled Trial of Peg fi lgrastim in Patients Receiving First-Line FOLFOX / Bevacizumab or FOLFIRI / Bevacizumab for Locally Advanced or Metastatic Colorectal Cancer : Final Results of the Peg fi lgrastim and Anti-VEGF Evaluation . **Clinical Colorectal Cancer**, 2016.

QUAIL, D.; JOYCE, J. Microenvironmental regulation of tumor progression and metastasis. **Nature medicine**, v. 19, n. 11, p. 1423–1437, 2013.

RADKE, S. et al. Specific association of annexin 1 with plasma membrane-resident and internalized EGF receptors mediated through the protein core domain. **FEBS Letters**, v. 578, n. 1–2, p. 95–98, 2004.

RAYNAL, P.; POLLARD, H. B. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. **Biochimica et biophysica acta**, v. 1197, n. 1, p. 63–93, 5 abr. 1994.

RESCHER, U.; GERKE, V. Annexins--unique membrane binding proteins with diverse functions. **Journal of cell science**, v. 117, n. Pt 13, p. 2631–2639, 2004.

RHEN, T.; CIDLOWSKI, J. A. Antiinflammatory Action of Glucocorticoids — New Mechanisms for Old Drugs. **New England Journal of Medicine**, v. 353, n. 16, p. 1711–1723, 2005.

RIBATTI, D. Mast cells as therapeutic target in cancer. **European Journal of Pharmacology**, v. 778, p. 1–6, 2015.

RIGONI, A. et al. Mast cells infiltrating inflamed or transformed gut alternatively sustain mucosal healing or tumor growth. **Cancer Research**, v. 75, n. 18, p. 3760–3770, 23 jul. 2015.

RODRIGO, J. P. et al. Down-regulation of annexin A1 and A2 protein expression in intestinal-type sinonasal adenocarcinomas. **Human Pathology**, v. 42, n. 1, p. 88–94, 2011.

SCHLATTER, D. et al. Generation, characterization and structural data of chymase binding proteins based on the human Fyn kinase SH3 domain. **MAbs**, v. 4, p. 497–508, 2012.

SENA, A. et al. Dysregulation of Anti-Inflammatory Annexin A1 Expression in Progressive Crohns Disease. **PLoS ONE**, v. 8, n. 10, p. 1–13, 2013.

SHELBURNE, C. P. et al. Stat5 expression is critical for mast cell development and survival. **Blood**, v. 102, n. 4, 2003.

SHEN, D. et al. Decreased expression of annexin A1 is correlated with breast cancer development and progression as determined by a tissue microarray analysis. **Human Pathology**, v. 37, n. 12, p. 1583–1591, 2006.

SILISTINO-SOUZA, R. et al. Annexin 1: differential expression in tumor and mast cells in human larynx cancer. **Int J Cancer**, v. 120, p. 2582–2589, 2007.

SIMIONESCU, C. et al. Tumor angiogenesis, macrophages and mast cell microdensities in endometrioid endometrial carcinoma. **Oncology letters**, v. 6, n. 2, p. 415–420, ago. 2013.

SINHA, V. R.; HONEY. Critical aspects in rationale design of fluorouracil-based adjuvant therapies for the management of colon cancer. **Critical Reviews in Therapeutic Drug Carrier Systems**, v. 29, n. 1, p. 89–148, 2012.

SINNIAH, A. et al. The role of the Annexin-A1/FPR2 system in the regulation of mast cell degranulation provoked by compound 48/80 and in the inhibitory action of nedocromil. **International Immunopharmacology**, v. 32, p. 87–95, 2016.

SOLITO, E. et al. A novel calcium-dependent proapoptotic effect of annexin 1 on human neutrophils. **The FASEB journal : official publication of the Federation of American Societies for Experimental Biology**, v. 17, n. 11, p. 1544–1546, 2003.

SOLITO, E. et al. Post-translational modification plays an essential role in the translocation of annexin A1 from the cytoplasm to the cell surface. **The FASEB journal : official publication of the Federation of American Societies for Experimental Biology**, v. 20, n. 9, p. 1498–1500, 2006.

SOUCEK, L. et al. Modeling pharmacological inhibition of mast cell degranulation as a therapy for insulinoma. **Neoplasia**, v. 13, p. 1093–1100, 2011.

STOJIC, L. et al. Mismatch repair-dependent G2 checjpoint induced by low doses of SN1 type methylating agents requires the ATR kinase. **Genes and Development**, v. 18, n. 11, p. 1331–1344, 2004.

SUEKANE, T. et al. Phenotypic change and accumulation of smooth muscle cells in strictures in Crohn's disease: relevance to local angiotensin II system. **J Gastroenterol**, v. 45, p. 821–830, 2010.

SUGIMOTO, M. A. et al. Annexin A1 and the Resolution of Inflammation: Modulation of Neutrophil Recruitment, Apoptosis, and Clearance. **Journal of immunology research**, v. 2016, p. 8239258, 2016.

TAGOE, C. E. et al. Annexin-1 mediates TNF-alpha-stimulated matrix metalloproteinase secretion from rheumatoid arthritis synovial fibroblasts. **J Immunol**, v. 181, p. 2813–2820, 2008.

TAN, S. Y. et al. Prognostic significance of cell infiltrations of immunosurveillance in colorectal cancer. **World J Gastroenterol**, v. 11, p. 1210–1214, 2005.

TANAKA, T. Colorectal carcinogenesis: Review of human and experimental animal studies. **Journal of Carcinogenesis**, v. 8, n. 1, p. 5, 2009.

TANAKA, T.; ISHIKAWA, H. Mast cells and inflammation-associated colorectal carcinogenesis. **Semin Immunopathol**, v. 35, p. 245–254, 2013a.

TANAKA, T.; ISHIKAWA, H. Mast cells and inflammation-associated colorectal carcinogenesis. **Semin Immunopathol**, v. 35, n. 2, p. 245–254, 20 mar. 2013b.

TOPTYGINA, A. P. et al. Cytokine profile in children with inflammatory bowel disease. **Biochemistry. Biokhimiia**, v. 79, n. 12, p. 1371–5, dez. 2014.

VARRICCHI, G. et al. Are Mast Cells MASTers in Cancer ? v. 8, n. April, p. 1–13, 2017.

VISHWANATHA, J. K.; SALAZAR, E.; GOPALAKRISHNAN, V. K. Absence of annexin I expression in B-cell non-Hodgkin's lymphomas and cell lines. **BMC cancer**, v. 4, p. 8, 2004.

VO, P.; FLOWER, R.; D'ACQUISTO, F. Inflammatory stimuli modulate the Annexin-A1 promoter by in human monocytesitle. **FASEB JOURNAL**, v. 20, p. A1376–A1376, 2006.

VONG, L. et al. Up-regulation of Annexin-A1 and lipoxin A4 in individuals with ulcerative colitis may promote mucosal homeostasis. **PLoS ONE**, v. 7, n. 6, p. 1–9, 2012.

WALLS, A. F. et al. Histochemical heterogeneity of human mast cells: disease-related differences in mast cell subsets recovered by bronchoalveolar lavage. **Int Arch Allergy Appl Immunol**, v. 92, p. 233–241, 1990.

WANG, S. et al. NF-kappaB signaling pathway, inflammation and colorectal cancer. **Cellular & molecular immunology**, v. 6, n. 5, p. 327–34, out. 2009.

WANG, Y. et al. Annexin-I expression modulates drug resistance in tumor cells. **Biochemical and biophysical research communications**, v. 314, n. 2, p. 565–70, 6 fev. 2004.

WASIUK, A. et al. Mast cells as regulators of adaptive immunity to tumours. **Clin Exp Immunol**, v. 155, p. 140–146, 2009.

WEDEMEYER, J.; GALLI, S. J. Decreased susceptibility of mast cell-deficient Kit(W)/Kit(W-v) mice to the development of 1, 2-dimethylhydrazine-induced intestinal tumors. **Lab Invest**, v. 85, p. 388–396, 2005a.

WEDEMEYER, J.; GALLI, S. J. Decreased susceptibility of mast cell-deficient Kit(W)/Kit(W-v) mice to the development of 1, 2-dimethylhydrazine-induced intestinal tumors. **Laboratory investigation; a journal of technical methods and pathology**, v. 85, n. 3, p. 388–96, mar. 2005b.

WEIDNER, N.; AUSTEN, K. F. Heterogeneity of mast cells at multiple body sites. Fluorescent determination of avidin binding and immunofluorescent determination of chymase, tryptase, and carboxypeptidase content. **Pathol Res Pract**, v. 189, p. 156–162, 1993.

WĘSIERSKA-GĄDEK, J.; GUEORGUIEVA, M.; WOJCIECHOWSKI, J. MNNG Induces Dramatic DNA Damage and Non-Apoptotic Changes in Cervical Carcinoma HeLa Cells. **Annals of the New York Academy of Sciences**, v. 1010, n. 1, p. 278–282, 24 dez. 2003.

WOIDACKI, K. et al. Mast cells rescue implantation defects caused by c-kit deficiency. **Cell death & disease**, v. 4, n. 1, p. e462, 2013.

XIN, W. et al. Dysregulation of the Annexin Family Protein Family Is Associated with Prostate Cancer Progression. **The American Journal of Pathology**, v. 162, n. 1, p. 255–261, 2003.

XING, W. et al. Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. **Proc Natl Acad Sci U S A**, v. 108, p. 14210–14215, 2011.

XUE, L.-Y. et al. [Expression of annexin I in different histological types of carcinomas]. **Zhonghua zhong liu za zhi [Chinese journal of oncology]**, v. 29, n. 6, p. 444–8, jun. 2007.

YANG, Y. H. et al. Annexin-1 regulates macrophage IL-6 and TNF via glucocorticoid-induced leucine zipper. **Journal of immunology (Baltimore, Md. : 1950)**, v. 183, n. 2, p. 1435–45, 15 jul. 2009.

YAZID, S. et al. Anti-Allergic Cromones Inhibit Histamine and Eicosanoid Release from Activated Human and Murine Mast Cells by Releasing Annexin A1. v. 8, n. 3, p. 1–10, 2013.

YE, R. D. et al. Nomenclature for the Formyl Peptide Receptor (FPR) Family. **Pharmacological Reviews**, v. 61, n. 2, p. 119–161, 2009.

YI, M.; SCHNITZER, J. E. Impaired tumor growth , metastasis , angiogenesis and wound healing in annexin A1-null mice. 2009.

YORK, S. J.; MODRICH, P. Mismatch repair-dependent iterative excision at irreparable O 6-methylguanine lesions in human nuclear extracts. **Journal of Biological Chemistry**, v. 281, n. 32, p. 22674–22683, 2006.

YU, S. et al. Correlation of ANXA1 expression with drug resistance and relapse in bladder cancer. **International journal of clinical and experimental pathology**, v. 7, n. 9, p. 5538–48, 2014.

ZAMPINO, M. G. et al. K-ras status in squamous cell anal carcinoma (SCC): It's time for target-oriented treatment? **Cancer Chemotherapy and Pharmacology**, v. 65, n. 1, p. 197–199, 2009.

ZHANG, Z. et al. Annexin 1 induced by anti-inflammatory drugs binds to NF-kappaB and inhibits its activation: anticancer effects in vitro and in vivo. **Cancer Res**, v. 70, n. 6, p. 2379–2388, 15 mar. 2010.

7. APÊNDICES

Apêndice 1: Artigo científico submetido para publicação contendo os resultados relacionados à tese de doutorado

Secreted annexin A1 modulates mast cell pro-tumoural actions in N-methyl-N'-nitro-N-nitrosoguanidine-induced colorectal cancer

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ABSTRACT

Background: Colorectal cancer (CRC) is a frequently occuring disease in western countries with poor prognosis. Mast cells (MCs) are involved in carcinogens-associated damage in the gastrointestinal tract; therefore, knowledge of MCs recruitment and functions is pivotal to comprehension of CRC genesis. Annexin A1 (AnxA1) is a protein expressed by MCs that modulates gastrointestinal tumoural development. Here we evaluated the role of AnxA1 in the functional response of MCs in CRC carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

Methods: Wild type (*AnxA1*$^{+/+}$) and AnxA1-deficient (*AnxA1*$^{-/-}$) male Balb-c mice received intra-rectal instillation of MNNG (4μgμl^{-1}) or PBS (100μL), and intestinal tissue was recovered 4, 24 or 48 hours later. MCs-sufficient WB/B6 (*Kit*$^{+/+}$), genetically MCs-deficient WBB6F1-KitW/KitW-v (*Kit*$^{-/-}$) and *Kit*$^{-/-}$ reconstituted with *Kit*$^{+/+}$ MCs received intra-rectal instillation of MNNG or PBS (4μgμl^{-1}) twice a week for four consecutive weeks, and intestinal tissue was collected 24 weeks after the first instillations.

Results: MNNG damaged the tissue and increased the expression of AnxA1 in epithelial cells. The lesion and MCs numbers were higher in *AnxA1*$^{-/-}$ mice. Increased levels of pro-inflammatory cytokines were found in the CRC tissue of *AnxA1*$^{-/-}$. Higher and lower numbers of chymase and tryptase-positive cells, respectively, were observed in the presence of AnxA1. At the late phase of CRC, immunossupression was detected in CRC tissue with higher recruitment of MCs, AnxA1 expression and epithelial proliferation, which were not detected in *Kit*$^{-/-}$ strain. Furthermore, the MNNG-induced lesion was reduced in *Kit*$^{-/-}$ mice. Reconstitution of MCs to *Kit*$^{-/-}$ mice induced epithelial lesion and proliferation and immunossupression.

Conclusions: Our results show the pivotal participation of MCs in the initial and late phase of CRC and highlight the interplay of MCs and AnxA1 on both the initial and late phase of the carcinogenesis.

Keywords: cytokines, MCs-deficient WBB6F1-KitW/KitW-v mice, immunossupression, AnxA1 deficient mice, chymase mast cells, tryptase mast cells

Colorectal cancer (CRC) is the third most common type of cancer worldwide and the second leading cause of cancer death in the western world (Siegel *et al.*, 2012). The treatment is mainly based on surgical resection, followed by chemotherapy combined with monoclonal antibodies or proteins against vascular endothelial growth factor (VEGF) and epidermal growth receptor (EGFR) (Butler *et al.*, 2016; Pinter *et al.*, 2016; Chan *et al.*, 2017; Lee *et al.*, 2017). Nevertheless, they do not represent total cure since cancer recurrence within 5 years occurs in about 40% of cured patients (Augestad *et al.*, 2015). The risk factors of CRC include genetic profile, environmental agents, and chronic inflammation in the gastrointestinal tract. In the latter context, aspirin intake has been proposed in clinical trials as a chemopreventive drug to CRC because it inhibits prostaglandin E_2 (PGE_2) secretion (Drew *et al.*, 2016).

Mast cells (MCs) are a heterogeneous population of immune cells with differences in their morphology, ultra-structure, mediator content, and surface receptors. They are found in solid tumours and are considered one of the major cell

populations of the tumour stroma (Varricchi *et al.*, 2017). Tumour-derived stem cell factor (SCF) binds to its receptor c-Kit on MCs, driving and activating MCs in the tumour microenvironment, where they are involved in inflammation and immunosuppression (Huang *et al.*, 2008). Indeed, several reports have shown the participation of MCs in the development of CRC induced by different carcinogens. An increased number of MCs was detected in the CRC induced by gamma radiation, 1,2-dimethylhydrazine (DMH) or sodium dextran sulfate added to 2,4,6-trinitrobenzene sulfonic acid (DSS/TNBS). Furthermore, the genetic deficiency of MCs or c-Kit receptors in mice reduced the incidence and number of tumours, attenuating the acute and chronic damage caused by these carcinogens in the gastrointestinal tract (Wedemeyer & Galli, 2005; Blirando *et al.*, 2011; Hamilton *et al.*, 2011; Tanaka & Ishikawa, 2013). Therefore, blocking MCs recruitment and activation may be a potential strategy on CRC therapy.

Activated MCs produce a wide spectrum of mediators, including cytokines, proteases (mainly tryptases and chymases), amines, nitric oxide and products from arachidonic acid metabolism, which may contribute to inflammation in the tumour microenvironment (Varricchi *et al.*, 2017). On the other hand, MCs also secrete anti-inflammatory agents such as the agonists of Annexin A1 formyl peptide receptor-2/ALX (FPR2/ALX), lipoxin A4 and annexin A1 (AnxA1) (da Silva *et al.*, 2011). Moreover, it was recently shown that activation of FPR2/ALX pathway in MCs impairs the neutrophil migration in innate immune response (Hughes *et al.*, 2017).

AnxA1 is a 37-KDa protein member of calcium and phospholipid-binding proteins of the annexin superfamily widely distributed in the body, found in biological fluids and expressed in stromal cells, differentiated haematopoietic cells, tumour cells, fibroblasts, epithelial cells and MCs (Oliani *et al.*, 2000, 2008; Perretti & D'Acquisto, 2009). Secreted AnxA1 modulates different physiological phenomena, such as granulopoiesis and neutrophil mobilization into blood (Machado *et al.*, 2016), and acts as an anti-inflammatory mediator by limiting pro-inflammatory responses and inducing

pro-resolutive effects (Perretti & D'Acquisto, 2009). Furthermore, AnxA1 expression is observed in diverse tumour microenvironments, and the majority of data show its correlation with poor prognosis, although the pro-tumoural mechanisms have not been described (Boudhraa *et al.*, 2016). AnxA1 activates the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) pathway in diverse cells, and it may be a possible mechanism (Perretti & D'Acquisto, 2009; Boudhraa *et al.*, 2016). In gastointestinal tumours, AnxA1 expression regulates cell invasiveness, leading to invasion and metastasis to the peritoneal cavity and poor survival (Kanda & Kodera, 2016).

MCs are important producers of AnxA1, which, in turn, performs inhibitory actions over this cell, being an important mediator of MC-stabilizing drug response (Yazid *et al.*, 2010, 2013). Although both AnxA1 and MCs are pivotal to the development of gastrointestinal cancer, the interplay of AnxA1 and MCs on the genesis of disease has not been investigated. Therefore, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced CRC was carried out in AnxA1 or WBB6F1-KitW/KitW-v null mice. Our data show the pivotal participation of MCs in the initial and late phase of CRC and highlight the connection of MCs and AnxA1 in both phases of the carcinogenesis process. AnxA1 modulates the MCs influx in the early inflammatory phase of CRC, and MCs in the microenvironment of tumour is fundamental to AnxA1 expression by epithelial cells.

MATERIALS AND METHODS

Mice. Wild type Balb/C (*AnxA1*$^{+/+}$) and Anx1 deficient (*AnxA1*$^{-/-}$) mice were provided from the Animal House at the School of Pharmaceutical Sciences, University of São Paulo (Brazil) and wild type MC-sufficient WB/B6 (*Kit*$^{+/+}$) and genetically MC-deficient WBB6F1-KitW/KitW-v (*Kit*$^{-/-}$) mice were provided from the animal facilities of the School of Medicine of Ribeirão Preto, University of São Paulo (Brazil). All experiments were performed in male animals that were 5 to 7 weeks old, age and weight-matched. Mice

were kept in community cages at 12h light–dark cycle and allowed food and water *ad libitum*. The experiments were performed in strict accordance with the Brazilian laws of protection, and this study was approved by the Animal Research Ethics Committee of the IBILCE/UNESP (73/2014), EERP/USP (191/2013) and FCF/USP (01200.003570/1998-08).

Bone Marrow Transplantation into *Kit*$^{-/-}$ mice. For MCs reconstitution, *Kit*$^{-/-}$ mice received i.v. injection of $2x10^7$ cells isolated from the bone marrow of *Kit*$^{+/+}$ (Grimbaldeston *et al.*, 2005). Briefly, bone marrow cells were flushed from the femurs of *Kit*$^{+/+}$ mice with cold Dulbecco's minimal essential medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) and cultured in the same medium supplemented with IL-3 ($10ngml^{-1}$; PeproTech, Rocky Hill, NJ, USA) until >95% of the cells were identified as bone marrow progenitor cells (MCps). Cell phenotype was indicated by May Grunwald-Giemsa (Bio-OpticaSpA, Milan, Italy) staining and positive expression of both FcεRI (PE conjugated anti-mouse, 1:200, clone MAR-1, Biolegend, San Diego, California, USA) and c-Kit (FITC Anti-mouse CD117, 1:200, 2B8, Biolegend, San Diego, California, USA) by flow cytometry using a FACSCanto I® (Becton Dickinson, San Jose, CA). Viable cells were counted with 0.4% trypan blue (Thermo Fisher Scientific Inc., Waltham, MA, USA), and $2x10^7$ cells suspended in 200mL of DMEM were injected via tail vein into 4-week-old *Kit*$^{-/-}$ mice. Control animals received 200mL of DMEM.

Induction of Colonic Tumours with MNNG. Mice received intra-rectal instillation of $4\mu g\mu l^{-1}$ (equivalent to $20mgkg^{-1}$ body weight) of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; Sigma-Aldrich, Aldrich Chemical Co., Inc., Milwaukee, WI) at 3cm from the anal margin. Control groups received 100µl of PBS 1mM (pH 7.0) by the same method (Che *et al.*, 2010). To evaluate the initial response of MCs cells on tissue damage evoked by MNNG, AnxA1$^{+/+}$ or AnxA1$^{-/-}$ mice were sacrificed at 8, 24 and 48 hours after

carcinogenic induction. The late response of MCs was investigated in *Kit*$^{-/-}$ *and Kit*$^{+/+}$ mice treated twice a week for 4 consecutive weeks with 4μgμl^{-1} of MNNG or 100μL of PBS 1mM. Mice were sacrificed 24 weeks after the first treatments. All animals were weighed weekly and evaluated after 24 weeks to colon analysis.

Colon sampling and tumour analysis. Mice were euthanized by isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) inhalation. The laparotomy was performed to remove the colon, and the colonic mucosa was exposed with a longitudinal incision to be observed under a stereomicroscope at x 5–30 magnification. Number, size and location of tumours along the colon were documented. Subsequently, large intestine fragments were processed for histopathological and immunohistochemical analysis (distal colon), as well as cytokine measurement (proximal colon).

Histopathological analysis. Samples of the distal portion of the colon were fixed 4% buffered paraformaldehyde for 24h, dehydrated in graded ethanol and embedded in paraffin. Sections of 2μm were stained with Hematoxylin and Eosin (HE) for histopathological assessment. Tissue samples were well oriented with longitudinally cut crypts to precisely assess alterations in the colon tissue architecture. The number and activation state of mast cells were measured by 0.1% Toluidine Blue staining. Analysis was conducted using a high-power objective (40X) on an Axioskop 2-Mot Plus Zeiss microscope (Carl Zeiss, Jena, Germany).

Immunohistochemical analysis. Sections of the colon (2μm) were incubated with sodium citrate buffer at 96°C for 30min. The endogenous peroxide activity was blocked with 3% hydrogen peroxide for 30min, followed by incubation with either polyclonal rabbit anti-AnxA1 (Zymed Laboratories, Cod. 713400, Cambridge, UK) at 1:500, polyclonal rabbit anti-caspase-3 (Abcam, Cod. 52293, Cambridge, UK) at 1:50, Proliferating Cells Nuclear Antigen (PCNA) (Abcam, Cod. 2426, Cambridge, UK) at

1:2000, tryptase (Abcam, Cod. 2378, Cambridge, UK) at 1:200 or chymase (Abcam, Cod. 13136, Cambridge, UK) at 1:500, in 10% TBS-BSA (Sigma-Aldrich, San Luis, USA). The slides were incubated overnight at 4°C. Additionally, negative control sections were incubated with 10% TBS-BSA instead of the primary antibody. Following the wash step, sections were incubated with a secondary Ab conjugated with HRP (Abcam, Cambridge, UK). Positive staining was detected using 3,3'-diaminobenzidine (DAB substrate; Invitrogen, USA). Finally, sections were counterstained with Hematoxylin and mounted. Analysis was conducted on Axioskop 2-Mot Plus Microscope (Carl Zeiss, Jena, Germany), using AxioVision software for densitometric and quantitative analysis. Densitometric analysis was used to determine AnxA1 intensity in colonic epithelial (40x) on an arbitrary scale from 0 to 255. Caspase-3 and PCNA positive epithelial cells (iCaspase-3 and iPCNA) were quantified as a ratio of immunoreactive cells per total cells counted in 100 crypts. Tryptase and chymase positive mast cells were identified in consecutive serial sections with Toluidine blue.

Cytokine levels evaluation. To quantify the levels of interleukins (IL)-1β, IL-4, IL-6, IL-12, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) in the proximal colon samples, we used the multiplex instrument LUMINEX xMAP MAGPIX (Millipore Corporation, Billerica, MA, USA). Antibody beads, controls, wash buffer and standards were prepared following the manufacturer's instructions (MILLIPLEX MCYTOMAG-70K kit). The plate was analysed using MAGPIX with xPONENT software.

Statistical Analysis. All results are expressed as Mean ± S.D. Data obtained from the different experiments were analysed using the software GraphPad Prism 5.0. The incidence of tumour development was evaluated by one-way ANOVA. The mast cell numbers/mm^2 of tissue and others analysis in various groups were compared by two-way ANOVA. When differences among groups were observed, Bonferroni (parametric)

or Dunn's test (non-parametric) pot-tests were applied. A p-value below 0.05 was considered significant. All data are presented as mean±S.D.

RESULTS

MNNG rectal instillation increases endogenous expression of AnxA1 in colonic epithelial cells. AnxA1 is weakly detected in the apical membrane of colon epithelial cells of mice (Figure 1A). MNNG instillation enhanced AnxA1 expression, mainly visualized at 8 and 24h after instillation with a reduction 48h later. Sections were incubated with 10% TBS-BSA instead of the primary antibody to provide a negative control for the immunohistochemistry reaction. Differences of AnxA1 expression among the groups were quantified by Mean Optical Density (MOD) measurement (Figure 1B).

Expression of endogenous AnxA1 reduces MNNG-induced colon damage and prevents pro-inflammatory cytokine production. In vehicle groups, the absence of AnxA1 induced enhanced expression of the pro-inflammatory cytokines IL-1β, IL-6, and IL-12 (Figure 1D) and reduced IL-4 levels, with a slight inflammatory infiltrate in the mucosal layer (Figure 1C). After 8h of MNNG instillation, death of crypt epithelial cells and inflammatory cell influx were observed with higher severity in *AnxA1*$^{-/-}$ mice (Figure 1C), and no differences in cytokines levels were observed between the strains. Nevertheless, levels of IL-1β were markedly reduced in *AnxA1*$^{-/-}$ mice compared to WT (Figure 1D).

At 24h after exposure, edema and hyperemia of the colon became evident in *AnxA1*$^{-/-}$ mice (Figure 1C). Moreover, IL-1β, IL-6, IL-12, TNF-α, and IFN-γ were highly expressed in these animals compared to *AnxA1*$^{+/+}$ (Figure 1D). We next verified the effects of MNNG after 48h of instillation. The damages were more prominent in *AnxA1*$^{-/-}$ mice, represented by vacuolar hydropic degeneration, structural damages in the epithelial layer with decrease of goblet cells, submucosal edema and massive

infiltration of leukocytes into the mucosa (Figure 1C), simultaneous to higher expression of IL-1β, TNF-α, and IFN-γ (Figure 1D).

Modulation of AnxA1 protein in the recruitment, activation and differentiation of Mcs in response to MNNG. Total and activated MCs were identified by Toluidine blue staining (Figure 2A). The number of MCs was enhanced 24 and 48h after MNNG instillation in *AnxA1*$^{+/+}$ mice, while an elevated number of MCs was detected since the first 8h after MNNG instillation in *AnxA1*$^{-/-}$ mice (Figure 2B). However, the MCs' migration was higher in *AnxA1*$^{-/-}$ mice compared to *AnxA1*$^{+/+}$ in all periods of times after MNNG instillation (Figure 2B).

Analysis of tryptase and chymase positive MCs showed that the number of tryptase label cells was augmented 24 and 48h after MNNG instillation, and the number of chymase labeled was markedly enhanced since the first 8h after MNNG instillation in *AnxA1*$^{+/+}$ mice (Figure 2C). Conversely, the numbers of both MCs labeled proteases were enhanced in *AnxA1*$^{-/-}$ mice after 8h of MNNG instillation (Figure 2C). The comparative analyses between the strains showed higher numbers of tryptase positive mast cells in *AnxA1*$^{-/-}$ mice in all experimental periods and reduced chymase positive cells 48h after MNNG instillation (Figure 2D).

Reduced susceptibility to MNNG-induced adenomatous polyps in MCs deficient mice. Intrarectal administration of MNNG induced, after 24 weeks, the development of tumoural polyps in the *Kit*$^{+/+}$ colon, which was significantly smaller in MCs deficient mice (*Kit*$^{-/-}$) (Figure 3A). The reconstitution of MCs in *Kit*$^{-/-}$ mice (*Kit*$^{-/-}$*+MC*) partially rescued the volume of tumours (Figure 3B). In addition, the body weight was reduced in *Kit*$^{+/+}$ MNNG compared to the respective vehicle group, and the reconstitution of MCs from *Kit*$^{+/+}$ mice reduced the loss of weight (Figure 3B).

Colon sections from *Kit*$^{+/+}$ mice instillated with vehicle (Figure 3C) presented normal architecture, and no significant histological differences were observed in

comparison with *Kit*$^{-/-}$ or *Kit*$^{-/-}$*+MC* instillated with vehicle. Benign pedunculated adenomas were verified in histopathological analysis of tumours in *Kit*$^{+/+}$ and *Kit*$^{-/-}$*+MC* groups. Most of them were composed of tubular structures lined by dysplastic epithelium with a drastic reduction in the number of goblet cells. However, some adenomatous polyps exhibited a high grade of dysplasia and intraepithelial adenocarcinomas. These drastic changes were not present in *Kit*$^{-/-}$ MNNG group (Figure 3C).

MCs number increased in the sites of tumour development in *Kit*$^{+/+}$ and *Kit*$^{-/-}$*+MC* compared to the respective vehicle instillated groups (Figure 4A and B). As expected, MCs were absent in *Kit*$^{-/-}$ mice (data not shown). Sequential histological sections of those obtained for immunohistochemistry were stained with Toluidine blue to confirm that immunostained cells were MCs. In vehicle groups (Figure 4C), tryptase expression was verified, but chymase was found at low levels in MCs. On the other hand, most of the MCs found in the adenomas strongly expressed tryptase and chymase (Figure 4C). Quantification of protease-positive MCs is presented in Figure 5D.

Microenvironmental immunoregulation and enhanced proliferation of epithelial colonic cells after MNNG exposure. Expression of AnxA1 was equivalent in all groups of Vehicle mice (Figure 5A). Higher expression of the protein was detected in *Kit*$^{+/+}$ mice and *Kit*$^{-/-}$*+MC* mice instillated with MNNG compared to *Kit*$^{-/-}$ mice. Also, reduced epithelial expression of AnxA1 was found in *Kit*$^{-/-}$ instillated with MNNG compared to *Kit*$^{+/+}$ MNNG and *Kit*$^{-/-}$*+MC* MNNG. AnxA1 Mean Optical Density (MOD) measurement are presented in Figure 5B.

Reduced levels of pro-inflammatory cytokine IL-1β, IL-6, IL-12, IFN-γ and TNF-α and enhanced levels of IL-4 were found in *Kit*$^{+/+}$ mice with tumours (Figure 5C). The proliferation and apoptosis of epithelial colonic cells were analysed by immunohistochemistry to PCNA and Caspase-3, respectively (Figure 6A and C). Significantly increased epithelial PCNA-positive cell numbers were detected in MNNG-

exposed *Kit*$^{+/+}$ and *Kit*$^{-/-}$*+MC* mice compared to the respective vehicles ($p<0.001$) (Figure 6B). In addition, this difference was not observed between *Kit*$^{-/-}$ MNNG and vehicle groups. Furthermore, there were no statistically significant differences for Caspase-3-positive cells among *Kit*$^{+/+}$, *Kit*$^{-/-}$ and *Kit*$^{-/-}$*+MC* mice after MNNG administration compared to the respective vehicle groups ($p<0.001$) (Figure 6D).

DISCUSSION

To our knowledge we show, for the first time, the interplay of MCs and AnxA1 expression on epithelial cells on the early and late phases of gastrointestinal tumour development evoked by MNNG in mice. Expression of AnxA1 by epithelial cells protects the early gastrointestinal inflammatory damage caused by MNNG, depending on inhibition of MCs infiltration into the site of the lesion. Furthermore, infiltration of MCs is fundamental to the AnxA1 super expression by epithelial cells in later phases of tumour development, to epithelial cell proliferation and to secretion of immunossupressive cytokines in the tumour microenvironment. Therefore, AnxA1 expressed by epithelial cells in CRC is connected to MCs' infiltration and displays anti- and pro-tumoural actions depending on the phase of the process.

AnxA1 is expressed in the gastric and intestinal tissues and exerts functional and morphological properties to maintain the tissue homeostasis (Babbin *et al.*, 2008; Leoni *et al.*, 2013). Moreover, AnxA1 is highly expressed on gastrointestinal diseases, as gastric ulcera and colitis, and favours the resolution of local inflammation and tissue regeneration (Martin *et al.*, 2008; de Paula-Silva *et al.*, 2016). AnxA1 is also expressed in gastrointestinal cancers, and its expression in advanced stages of the disease is associated to metastasis and poor prognosis (Su *et al.*, 2010; Boudhraa *et al.*, 2016). Here we show that in the early stages of CRC, AnxA1 is expressed by epithelial cells and protects the tissue damage caused by MNNG. The protective effect of AnxA1 may be due to anti-inflammatory properties since higher lesion in *AnxA1*$^{-/-}$ mice was correlated to unbalanced levels of pro- and anti-inflammatory cytokines, with higher

amounts of pro-inflammatory mediators. Indeed, the genesis and initial development of CRC is associated with inflammation in the gut (Neurath, 2014; Wessler *et al.*, 2017), as aspirin intake has been proposed to prevent CRC drug and the mechanism involved seems to be the inhibition of PGE2 (Drew *et al.*, 2016).

Nowadays, it is strongly evident that the tumoural microenvironment is pivotal to the regression or development of the tumour. The tumour microenvironment is composed of a diversity of stromal and immune cells that secrete pro- or anti-tumoural mediators resulting in a complex cascade of cells activations (Tahmasebi Birgani & Carloni, 2017; Wang *et al.*, 2017). MCs are immune cells pivotal to CRC development (Wedemeyer & Galli, 2005; Blirando *et al.*, 2011; Hamilton *et al.*, 2011; Tanaka & Ishikawa, 2013) that also express high amounts of AnxA1 (Kamal *et al.*, 2005; da Silva *et al.*, 2011). MCs are derived from committed MCs progenitor cells in the bone marrow (MCps) that transmigrate in the bone marrow's and peripheral tissues' microvasculature to be delivered, respectively, into blood and recruited into tissues, where they undergo complete maturation (Dahlin & Hallgren, 2015). MCps express $\alpha4/\beta1$ and $\alpha4/\beta7$ integrins, which bind to counter receptors in the activated endothelium or mucosal cells in the gut to transmigration (Ribatti, 2015). Stem cell factor (SCF) secreted by fibroblasts, stromal and endothelial cells is the main mediator of MCps survival, activation and circulation in the blood, but IL-3, IL-4, IL-9, IL-10, transforming growth factor beta (TGF-beta), and nerve growth factors (NGF) act in synergism with SCF (Ribatti, 2015). We suppose that AnxA1 can directly control the transmigration and locomotion pathways of MCps because endogenous AnxA1 modulates the traffic of granulocytes from the bone marrow and into the blood, and the homing to bone marrow and inflammatory tissues (Perretti & D'Acquisto, 2009; Machado *et al.*, 2016), or indirectly by reducing the secretion of inflammatory mediators involved in transmigration and chemotaxis (Sugimoto *et al.*, 2016). Indeed, we here have shown reduced levels of interleukins involved on cell adhesion into vessels, such as IL-1β and IL-6, in the inflammation caused by MNNG.

AnxA1 also inhibits MCs degranulation in the brochoalveolar fluid in allergic inflammation (Bandeira-melo *et al.*, 2005) or after compound 48/80 challenge (Sinniah *et al.*, 2016). Here we show that endogenous AnxA1 is protective in early phases of MNNG-induced carcinogenesis by blocking MCs migration and degranulation, and it may represent a mechanism to impair tumour development.

MCs homing into tissue maturates into two distinct populations according to their enzimatic profiles, such as tryptase or chymase subtypes (Caughey, 2007). Both proteases have been related to induction of angiogenesis, which supports cancer feeding and growth (Ribatti *et al.*, 2011; Douaiher *et al.*, 2014). Although both enzymes produce similar effects, literature correlates the higher density of tryptase-positive MCs to a poor prognosis in some types of cancer, with an especial role in gastrointestinal primary cancers and metastasis (Malfettone *et al.*, 2013; Suzuki *et al.*, 2015; Ammendola *et al.*, 2016). In the context of MCs heterogeneity, our data bring novelty in AnxA1 functions, since we show, for the first time, that the higher total number of tryptase-positive MCs is observed in the absence of AnxA1 in the CRC experimental model. Indeed, de Paula-Junior *et al.* (2015) demonstrated higher density of chymase-positive MCs in the ectopic endometria from human biopsies of endometriosis, correlated with higher expression of AnxA1 co-localized with the receptor FPR1 in the glandular cells.

MNNG is a direct alkylator of DNA (WĘSIERSKA-GĄDEK *et al.*, 2003) that forms adducts at the O^6 position of guanine, which results in G:C to A:T transitions after DNA replication that cannot be repaired by the DNA mismatch repair system (York & Modrich, 2006). Thus, MNNG induces DNA breaks/gaps, cell cycle arrest, and persistent nuclear foci at sites of DNA damage (Stojic *et al.*, 2004). Intrarectal instillation of MNNG is a reliable and efficient method to induce tumours selectively in the area exposed to the carcinogen in the colon (Che *et al.*, 2010). Indeed, we here show carcinogenesis after 24 weeks of the initial exposure to MNNG, and we also demonstrate that this effect depends on constitutive MCs, because the tumour

development was markedly reduced in mice lacking both copies of Kit (*Kit*$^{-/-}$), which results in a virtual absence of tissue MCs (Kitamura *et al.*, 1978). These data corroborate the literature about using other tumour-inducer compounds (Tanaka & Ishikawa, 2013; Rigoni *et al.*, 2015). Moreover, the contribution of MCs to CRC development was corroborated by the appearance of tumours in *Kit*$^{-/-}$ mice reconstituted with normal MCps before the beginning of exposures.

We also here highlight that AnxA1 expression in the microenvironment during the late phase of tumour growth is modulated by MCs cells because higher expression of the protein was detected in *Kit*$^{+/+}$ mice and in *Kit*$^{-/-}$ mice reconstituted with MCs, but not in *Kit*$^{-/-}$ animals. Together, these data show the interplay of MCs and epithelial cells during CRC development to enhance AnxA1 expression, mainly by the epithelial cells. As mentioned, AnxA1 levels are indicator of poor prognosis in CRC tumours (Yi & Schnitzer, 2009; Onozawa *et al.*, 2017). Indeed, our data show an immunosuppressive profile of cytokines mediators in CRC infiltrated with MCs and expressing AnxA1 that are involved in the immune escape of tumour cells. It has shown that reduced and elevated levels of pro-inflammatory and anti-inflammatory chemical mediators, respectively, are involved in the anergy of the immune cells during cancer initiation and progression (Neurath, 2014). Similarly, we detected enhanced epithelial cell proliferation in CRC infiltrated with MCs cells. Together, our data corroborate the pro-tumoural activity of MCs in CRC in experimental models and in patients (Rigoni *et al.*, 2015; Ammendola *et al.*, 2016), and we believe that this activity may be related to immune suppression in the microenvironment and cell proliferation. The contribution of secreted AnxA1 on CRC growth may be supposed because AnxA1 causes cancer cell proliferation and metastasis (Sato *et al.*, 2011) and induces the secretion of immunossupressive cytokines in inflammatory sites (C'hng *et al.*, 2006; Perretti & D'Acquisto, 2009).

In conclusion, our data show the complexity of cell interactions in tumour development by showing the relevance and distinct interplay of AnxA1 and MCs during

the initial and late phase of CRC growth. The connection of MCs and AnxA1 in the late phase of tumour development represents a goal to be addressed in the near future.

Competing interests: the authors declare that no competing interests exist.

References

Ammendola M, Patruno R, Sacco R, Marech I, Zuccalà V, Luposella M, Zizzo N, Gadaleta C, Porcelli M, Gadaleta CD, Ribatti D (2016) Mast Cells Positive to Tryptase and Tumour- Associated Macrophages Correlate with Angiogenesis in Locally Advanced Colorectal Cancer Patients Undergone to Surgery. **8222**: doi:10.1517/14728222.2016.1158811.

Augestad KM, Bakaki PM, Rose J, Crawshaw BP, Lindsetmo RO, Dørum LM, Koroukian SM, Delaney CP (2015) Metastatic spread pattern after curative colorectal cancer surgery . A retrospective , longitudinal analysis. *Cancer Epidemiol* **39**: 734–744, doi:10.1016/j.canep.2015.07.009.

Babbin BA, Laukoetter MG, Nava P, Koch S, Lee WY, Capaldo CT, Peatman E, Severson EA, Flower RJ, Perretti M, Parkos CA, Nusrat A (2008) Annexin A1 regulates intestinal mucosal injury, inflammation, and repair. *J Immunol* **181**: 5035–5044.

Bandeira-melo C, Bonavita GC, Diaz BL, Silva PMR, Carvalho F, Jose PJ, Flower RJ, Perretti M, Martins MA (2005) A Novel Effect for Annexin 1-Derived Peptide Ac2-26 : Reduction of Allergic Inflammation in the Rat. **313**: 1416–1422, doi:10.1124/jpet.104.080473.1997.

Blirando K, Milliat F, Martelly I, Sabourin JC, Benderitter M, François A (2011) Mast cells are an essential component of human radiation proctitis and contribute to experimental colorectal damage in mice. *Am J Pathol* **178**: 640–651, doi:10.1016/j.ajpath.2010.10.003.

Boudhraa Z, Bouchon B, Viallard C, D'Incan M, Degoul F (2016) Annexin A1 localization and its relevance to cancer. *Clin Sci (Lond)* **130**: 205–220,

doi:10.1042/CS20150415.

Butler CT, Reynolds AL, Tosetto M, Dillon ET, Guiry P, Cagney G, Sullivan JO, Kennedy BN (2016) A Quininib Analogue and Cysteinyl Leukotreine Receptor Antagonist inhibits VEGF-Independent Angiogenesis and Exerts an Additive Anti-angiogenic Response with Bevacizumab. doi:10.1074/jbc.M116.747766.

C'hng S, Wallis RA, Yuan L, Davis PF, Tan ST (2006) Mast cells and cutaneous malignancies. 149–159, doi:10.1038/modpathol.3800474.

Caughey GH (2007) Mast cell tryptases and chymases in inflammation and host defense. *Immunol Rev* 141–154.

Chan E, Goff LW, Cardin DB, Ancell K, Smith SJ, Whisenant JG, Ye F, Berlin JD, Chan E (2017) Phase II study of the Multikinase inhibitor of angiogenesis , Linifanib , in patients with metastatic and refractory colorectal cancer expressing mutated KRAS. doi:10.1007/s10637-017-0458-8.

Che TCH, François S, Bouchet S, Chapel A, Forgue-Lafitte ME (2010) Early lesions induced in rat colon epithelium by N-methyl-N'-nitro-N-nitrosoguanidine. *Tissue Cell* **42**: 190–194, doi:10.1016/j.tice.2010.04.002.

Dahlin JS, Hallgren J (2015) Mast cell progenitors: Origin, development and migration to tissues. *Mol Immunol* **63**: 9–17, doi:10.1016/j.molimm.2014.01.018.

Douaiher J, Succar J, Lancerotto L, Gurish MF, Orgill DP, Qi X, Hong J, Chaves L, Zhuang Y, Chen Y, Chabon J, Graham B, Ohmori K, Li Y, Huang H (2014) NIH Public Access. **39**: 1–37, doi:10.1016/B978-0-12-800267-4.00006-7.Development.

Drew DA, Cao Y, Chan AT (2016) Aspirin and colorectal cancer: the promise of precision chemoprevention. *Nat Publ Gr* 8–11, doi:10.1038/nrc.2016.4.

Grimbaldeston MA, Chen C-C, Piliponsky AM, Tsai M, Tam S-Y, Galli SJ (2005) Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. *Am J Pathol* **167**: 835–848, doi:10.1016/S0002-9440(10)62055-X.

Hamilton MJ, Sinnamon MJ, Lyng GD, Glickman JN, Wang X, Xing W, Krilis SA, Blumberg RS, Adachi R, Lee DM, Stevens RL (2011) Essential role for mast cell

tryptase in acute experimental colitis. *Proc Natl Acad Sci U S A* **108**: 290–295, doi:10.1073/pnas.1005758108.

Huang B, Lei Z, Zhang GM, Li D, Song C, Li B, Liu Y, Yuan Y, Unkeless J, Xiong H, Feng ZH (2008) SCF-mediated mast cell infiltration and activation exacerbate the inflammation and immunosuppression in tumor microenvironment. *Blood* **112**: 1269–1279, doi:10.1182/blood-2008-03-147033.

Hughes E, Becker F, Flower R, Buckingham J, Gavins F (2017) Mast Cells Mediate Early Neutrophil Recruitment and Exhibit Anti-Inflammatory Properties via FPR2/ALX. *Br Journalournal* doi:10.1111/bph.13847.

Kamal AM, Flower RJ, Perretti M (2005) An overview of the effects of annexin 1 on cells involved in the inflammatory process. *Mem Inst Oswaldo Cruz* **100 Suppl**: 39–47, doi:/S0074-02762005000900008.

Kanda M, Kodera Y (2016) Molecular mechanisms of peritoneal dissemination in gastric cancer. **22**: 6829–6840, doi:10.3748/wjg.v22.i30.6829.

Kitamura BY, Go S, Hatanaka K (1978) Decrease of Mast Cells in W/ W" Mice and Their Increase by Bone Marrow Transplantation. *Blood* **52**:.

Lee SJ, Lee SY, Lee WS, Yoo JS, Sun J, Lee J, Park SH, Park JO, Ahn M, Lim HY (2017) Phase I trial and pharmacokinetic study of tanibirumab , a fully human monoclonal antibody to vascular endothelial growth factor receptor 2 , in patients with refractory solid tumors. doi:10.1007/s10637-017-0463-y.

Leoni G, Alam A, Neumann P-A, Lambeth JD, Cheng G, McCoy J, Hilgarth RS, Kundu K, Murthy N, Kusters D, Reutelingsperger C, Perretti M, Parkos CA, Neish AS, Nusrat A (2012) Annexin A1, formyl peptide receptor, and NOX1 orchestrate epithelial repair. *J Clin Invest* **123**: 443–454, doi:10.1172/JCI65831DS1.

Machado ID, Spatti M, Hastreiter A, Santin ER, Osio RA, Gil CD, Oliani SM, Perretti M, Helena S, Farsky P (2016) Annexin A1 Is a Physiological Modulator of Neutrophil Maturation and Recirculation Acting on the CXCR4 / CXCL12 Pathway. doi:10.1002/jcp.25346.

Malfettone A, Silvestris N, Saponaro C, Ranieri G, Russo A, Caruso S, Popescu O, Simone G, Paradiso A, Mangia A (2013) High density of tryptase-positive mast cells in human colorectal cancer: a poor prognostic factor related to protease-activated receptor 2 expression. *J Cell Mol Med* **17**: 1025–1037, doi:10.1111/jcmm.12073.

Martin GR, Perretti M, Flower RJ, Wallace JL (2008) Annexin-1 modulates repair of gastric mucosal injury. *Am J Physiol Gastrointest Liver Physiol* **294**: G764–G769, doi:10.1152/ajpgi.00531.2007.

Neurath MF (2014) Cytokines in inflammatory bowel disease. *Nat Rev Immunol* **14**: 329–342, doi:10.1038/nri3661.

Oliani SM, Christian HC, Manston J, Flower RJ, Perretti M (2000) An immunocytochemical and in situ hybridization analysis of annexin 1 expression in rat mast cells: modulation by inflammation and dexamethasone. *Lab Invest* **80**: 1429–1438.

Oliani SM, Ciocca GAP, Pimentel TA, Damazo AS, Gibbs L, Perretti M (2008) Fluctuation of annexin-A1 positive mast cells in chronic granulomatous inflammation. *Inflamm Res* **57**: 450–456, doi:10.1007/s00011-008-7222-7.

Onozawa H, Saito M, Saito K, Kanke Y, Watanabe Y (2017) Annexin A1 is involved in resistance to 5-FU in colon cancer cells. 235–240, doi:10.3892/or.2016.5234.

de Paula-Silva M, Barrios BE, Macció-Maretto L, Sena AA, Farsky SHP, Correa SG, Oliani SM (2016) Role of the protein annexin A1 on the efficacy of anti-TNF treatment in a murine model of acute colitis. *Biochem Pharmacol* **115**: 104–113, doi:10.1016/j.bcp.2016.06.012.

Perretti M, D'Acquisto F (2009) Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat Rev Immunol* **9**: 62–70, doi:10.1038/nri2470.

Pinter T, Klippel Z, Cesas A, Croitoru A, Decaestecker J, Gibbs P, Hotko Y, Jassem J, Kurteva G, Novotny J, Reilly SO, Salek T, Reiner M, Morrow PK, Choi MR, Whittaker S (2016) Original Study Placebo-Controlled Trial of Peg fi lgrastim in Patients Receiving First-Line FOLFOX / Bevacizumab or FOLFIRI / Bevacizumab for Locally Advanced or

Metastatic Colorectal Cancer : Final Results of the Peg fi lgrastim and Anti-VEGF Evaluation . *Clin Colorectal Cancer* doi:10.1016/j.clcc.2016.08.008.

Ribatti D (2015) Mast cells as therapeutic target in cancer. *Eur J Pharmacol* **778**: 1–6, doi:10.1016/j.ejphar.2015.02.056.

Ribatti D, Ranieri G, Nico B, Benagiano V, Crivellato E (2011) Tryptase and chymase are angiogenic in vivo in the chorioallantoic membrane assay. **102**: 99–102, doi:10.1387/ijdb.103138dr.

Rigoni A, Bongiovanni L, Burocchi A, Sangaletti S, Danelli L, Guarnotta C, Lewis A, Rizzo A, Silver AR, Tripodo C, Colombo MP (2015) Mast Cells In fi ltrating In fl amed or Transformed Gut Alternatively Sustain Mucosal Healing or Tumor Growth. **75**: 3760–3771, doi:10.1158/0008-5472.CAN-14-3767.

Sato Y, Kumamoto K, Saito K, Okayama H (2011) Up-regulated Annexin A1 expression in gastrointestinal cancer is associated with cancer invasion and lymph node metastasis. 239–243, doi:10.3892/etm.2011.210.

Siegel RL, Ward EM, Jemal A (2012) Trends in Colorectal Cancer Incidence Rates in the United States by Tumor Location and Stage , 1992 – 2008. 411–417, doi:10.1158/1055-9965.EPI-11-1020.

da Silva PS, Girol AP, Oliani SM (2011) Mast cells modulate the inflammatory process in endotoxin- induced uveitis. 1310–1319.

Sinniah A, Yazid S, Perretti M, Solito E, Flower RJ (2016) International Immunopharmacology The role of the Annexin-A1 / FPR2 system in the regulation of mast cell degranulation provoked by compound 48 / 80 and in the inhibitory action of nedocromil. *Int Immunopharmacol* **32**: 87–95, doi:10.1016/j.intimp.2016.01.003.

Stojic L, Mojas N, Cejka P, Di Pietro M, Ferrari S, Marra G, Jiricny J (2004) Mismatch repair-dependent G2 checjpoint induced by low doses of SN1 type methylating agents requires the ATR kinase. *Genes Dev* **18**: 1331–1344, doi:10.1101/gad.294404.

Su N, Xu X, Chen H, Gao W, Ruan C, Wang Q, Sun Y (2010) Increased Expression of Annexin A1 Is Correlated with K-Ras Mutation in Colorectal Cancer. 243–250,

doi:10.1620/tjem.222.243.

Sugimoto MA, Vago JP, Teixeira MM, Sousa LP (2016) Annexin A1 and the Resolution of Inflammation: Modulation of Neutrophil Recruitment, Apoptosis, and Clearance. *J Immunol Res* **2016**: 8239258, doi:10.1155/2016/8239258.

Suzuki S, Ichikawa Y, Nakagawa K, Kumamoto T, Mori R, Matsuyama R, Takeda K, Ota M, Tanaka K, Tamura T, Endo I (2015) High infiltration of mast cells positive to tryptase predicts worse outcome following resection of colorectal liver metastases. *BMC Cancer* 1–8, doi:10.1186/s12885-015-1863-z.

Tahmasebi Birgani M, Carloni V (2017) Tumor Microenvironment , a Paradigm in Hepatocellular Carcinoma Progression and Therapy. 7–10, doi:10.3390/ijms18020405.

Tanaka T, Ishikawa H (2013) Mast cells and inflammation-associated colorectal carcinogenesis. *Semin Immunopathol* **35**: 245–254, doi:10.1007/s00281-012-0343-7.

Varricchi G, Galdiero, Maria Rosaria Loffredo S, Marone G, Iannone R, Marone G, Granata F (2017) Are Mast Cells MASTers in Cancer? **8**: 1–13, doi:10.3389/fimmu.2017.00424.

Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, Gong Z (2017) Role of tumor microenvironment in tumorigenesis. *J Cancer* **8**: doi:10.7150/jca.17648.

Wedemeyer J, Galli SJ (2005) Decreased susceptibility of mast cell-deficient Kit(W)/Kit(W-v) mice to the development of 1, 2-dimethylhydrazine-induced intestinal tumors. *Lab Invest* **85**: 388–396, doi:10.1038/labinvest.3700232.

WĘSIERSKA-GĄDEK J, GUEORGUIEVA M, WOJCIECHOWSKI J (2003) MNNG Induces Dramatic DNA Damage and Non-Apoptotic Changes in Cervical Carcinoma HeLa Cells. *Ann N Y Acad Sci* **1010**: 278–282, doi:10.1196/annals.1299.048.

Wessler S, Krisch LM, Elmer DP, Aberger F (2017) From inflammation to gastric cancer – the importance of Hedgehog / GLI signaling in Helicobacter pylori -induced chronic inflammatory and neoplastic diseases. 1–13, doi:10.1186/s12964-017-0171-4.

Yazid S, Leoni G, Getting SJ, Cooper D, Solito E, Flower RJ (2010) Anti-allergic cromones inhibit neutrophil recruitment onto vascular endothelium via annexin-A1

mobilization. *Arterioscler Thromb Vasc Dis* **30**: 1718–1724, doi:10.1161/ATVBAHA.110.209536.Anti-allergic.

Yazid S, Sinniah A, Solito E, Calder V, Flower RJ (2013) Anti-Allergic Cromones Inhibit Histamine and Eicosanoid Release from Activated Human and Murine Mast Cells by Releasing Annexin A1. **8**: 1–10, doi:10.1371/journal.pone.0058963.

Yi M, Schnitzer JE (2009) Impaired tumor growth , metastasis , angiogenesis and wound healing in annexin A1-null mice.

York SJ, Modrich P (2006) Mismatch repair-dependent iterative excision at irreparable O 6-methylguanine lesions in human nuclear extracts. *J Biol Chem* **281**: 22674–22683, doi:10.1074/jbc.M603667200.

Figure Legends

Figure 1. Distal colon AnxA1 expression, histopathology, and cytokine analysis in WT and AnxA1-deficient mice (*AnxA1*$^{-/-}$) after acute MNNG induction. (A) Representative immunostaining of AnxA1 in colon epithelium of *AnxA1*$^{+/+}$ Balb/c. **(B)** Mean Optical Densitometry (MOD): AnxA1. **(C)** Altered histoarchitecture of colon after MNNG instillation with the presence of dysplasic crypts and mucosal inflammatory infiltrate (details). Tissue injuries become evident after 8 h in AnxA1$^{-/-}$ mice and 48h in *AnxA1*$^{+/+}$. **(D)** Cytokines expression (IL-1β, IL-6, IL-12p40, TNF-α, IFN-γ, and IL-4). Scale bars: (A) 30 μm; (C) 50 μm. ### $p < 0.001$ vs Vehicle group, §§§ $p < 0.001$ vs 8 h; ǂǂǂ $p < 0.001$ vs 24 h.

Figure 2. AnxA1 modulates MNNG acute effects over mast cells recruitment, differentiation and activation. Metachromatic mast cells (arrows) in distal colon of vehicle and MNNG-induced groups. (B) Total and degranulated mast cells quantification. (C) Immunohistochemical mast cell proteases evaluation on serial 2 μm sections of *AnxA1*$^{+/+}$ and *AnxA1*$^{-/-}$ mice. High tryptase and low chymase expression in mast cells at Vehicle group (arrowhead). Tryptase/chymase positive mast cells

(arrows) 8 h after MNNG induction. (D) Tryptase and chymase positive cells quantification. Scale bars: (A) 50 µm; (C) 20 µm. * p < 0.05, ** p < 0.01, *** *p* < 0.001 vs Vehicle *AnxA1*$^{+/+}$; ### p < 0.001 vs Vehicle *AnxA1*$^{-/-}$; ‡‡‡ p < 0.001 vs 8 h; §§§ p < 0.001 vs 24 h.

Figure 3. MNNG-induced morphophysiological alterations in mast cells-deficient mice (*Kit*$^{-/-}$) colon. (A) Representative distal colon of C57BL/6 *Kit*$^{+/+}$, *Kit*$^{-/-}$ and *Kit*$^{-/-}$*+MC* after 24 weeks of the induction with PBS (Vehicle) and carcinogen (MNNG); (B) tumoral volume and mice corporal weight difference after the same period. ### *p* < 0.001 vs *Kit*$^{+/+}$ MNNG group; ‡‡‡ *p* < 0.001 vs *Kit*$^{-/-}$ MNNG group; *** *p* < 0.001 vs respective vehicle group. (C) Histopathological sections from the distal colon of vehicle groups, adenomas formation in *Kit*$^{+/+}$ and *Kit*$^{-/-}$+MC and, inflammatory infiltrate in the colonic mucosa from *Kit*$^{-/-}$ mice. Goblet cells are virtually absent of the crypts forming the adenomas. Scale bars: (A) 1cm; (C) 50 µm.

Figure 4. Quantitative and phenotypic assessment of mast cells after MNNG tumoral induction. (A) Metachromatic mast cells (arrows) in distal colon. (B) Quantification of total and activated mast cells. (C) Low intensity chymase expression (arrowhead) was observed in mast cells at Vehicle condition. Chymase positive cells with higher expression (arrows) are shown after MNNG instillations. Scale bars: (A) 50 µm; (C) 20 µm. (D) Tryptase and chymase positive cells quantification. *** *p* < 0.001 *vs* respective Vehicle group.

Figure 5. AnxA1 and cytokines expression in mast cells-deficient mice after MNNG induction. (A) Representative immunostaining of AnxA1 in the colon epithelium. More intense AnxA1 expression was found in MNNG-affected tissue in the presence of mast cells. Scale bars: 20 µm. (B) Mean Optical Densitometry (MOD): AnxA1. (D) Cytokines expression (IL-1β, IL-6, IL-12p40, IFN-γ, TNF-α, and IL-4). *** *p*

< 0.001 *vs* respective Vehicle group. ### $p < 0.001$ *vs* respective *Kit*$^{+/+}$ group. ‡‡‡ $p <$ 0.001 *vs* respective *Kit*$^{-/-}$ group.

Figure 6. MNNG-induced proliferation and apoptose of colonic epithelial cells in the presence of mast cells. (A and C) Immunoreactivity of Proliferating Cell Nuclear Antigen (PCNA) and Caspase-3 in distal colon. Scale bars: 50 µm. (B and D) Relative PCNA and Caspase-3 positive cells in colonic crypts, respectively. *** $p < 0.001$ *vs* respective Vehicle group. ### $p < 0.001$ *vs* respective *Kit*$^{+/+}$ group. ‡‡‡ $p < 0.001$ *vs* respective *Kit*$^{-/-}$ group.

Figure 1

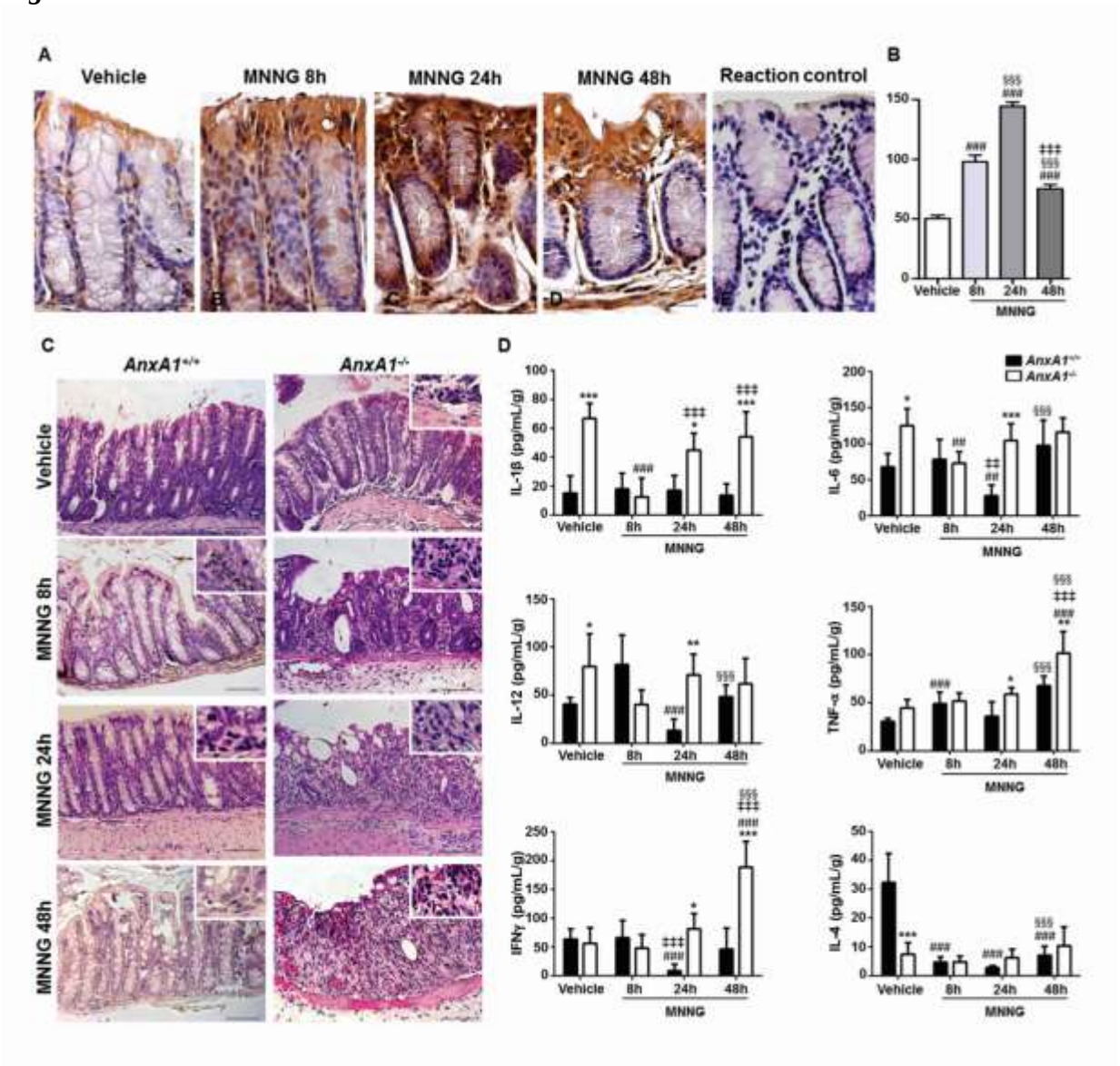

A
Vehicle
MNNG 8h
MNNG 24h
MNNG 48h
Reaction control
B
C
AnxA1+/+
AnxA1-/-
Vehicle
MNNG 8h
MNNG 24h
MNNG 48h
D
IL-1β (pg/mL/g)
IL-6 (pg/mL/g)
IL-12 (pg/mL/g)
TNF-α (pg/mL/g)
IFNγ (pg/mL/g)
IL-4 (pg/mL/g)
Vehicle
8h
24h
48h
MNNG

Figure 2

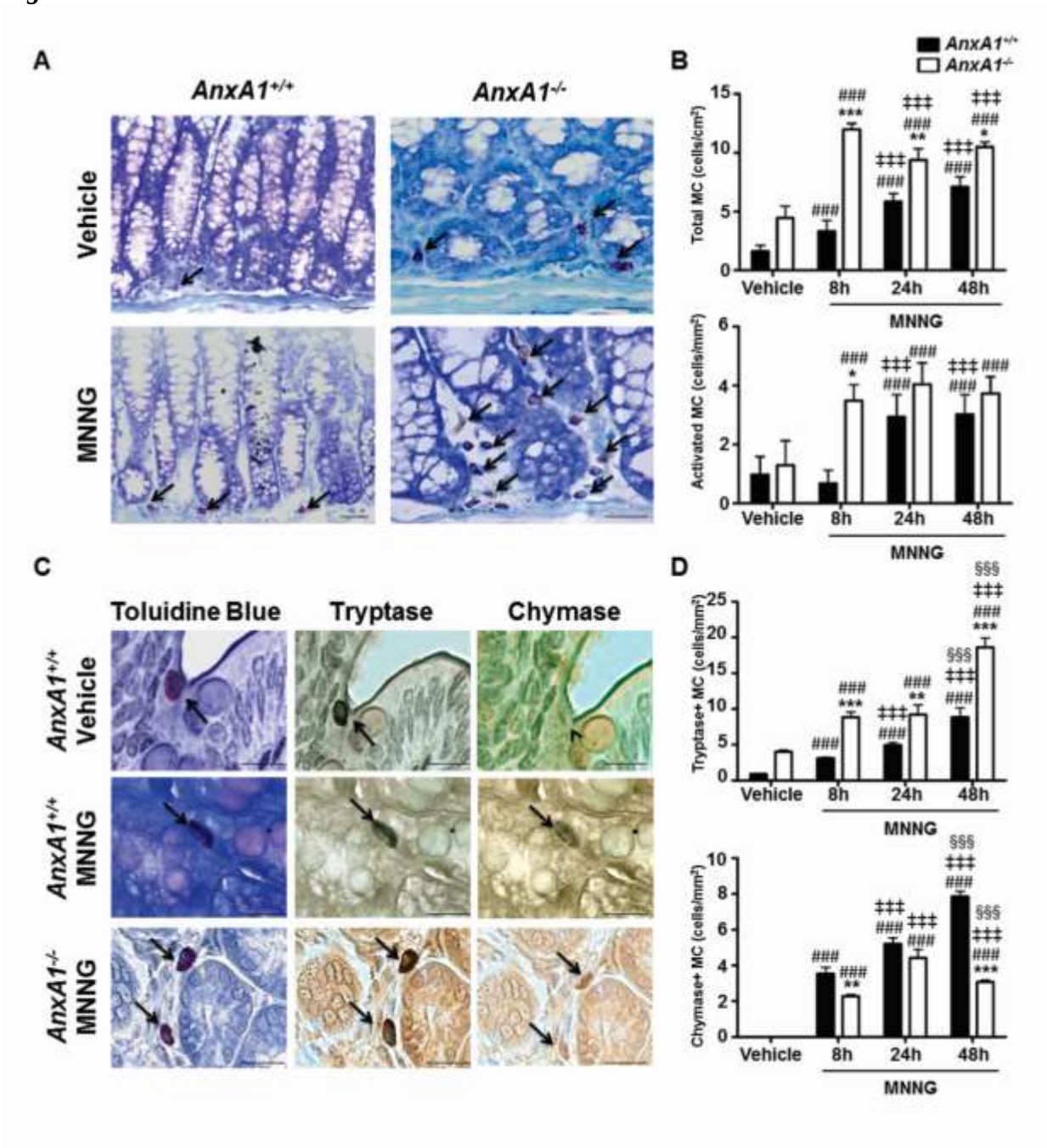

A
AnxA1+/+
AnxA1-/-
Vehicle
MNNG
B
AnxA1+/+
AnxA1-/-
Total MC (cells/cm²)
Activated MC (cells/mm²)
Vehicle
8h
24h
48h
MNNG
C
Toluidine Blue
Tryptase
Chymase
AnxA1+/+ Vehicle
AnxA1+/+ MNNG
AnxA1-/- MNNG
D
Tryptase+ MC (cells/mm²)
Chymase+ MC (cells/mm²)

Figure 3

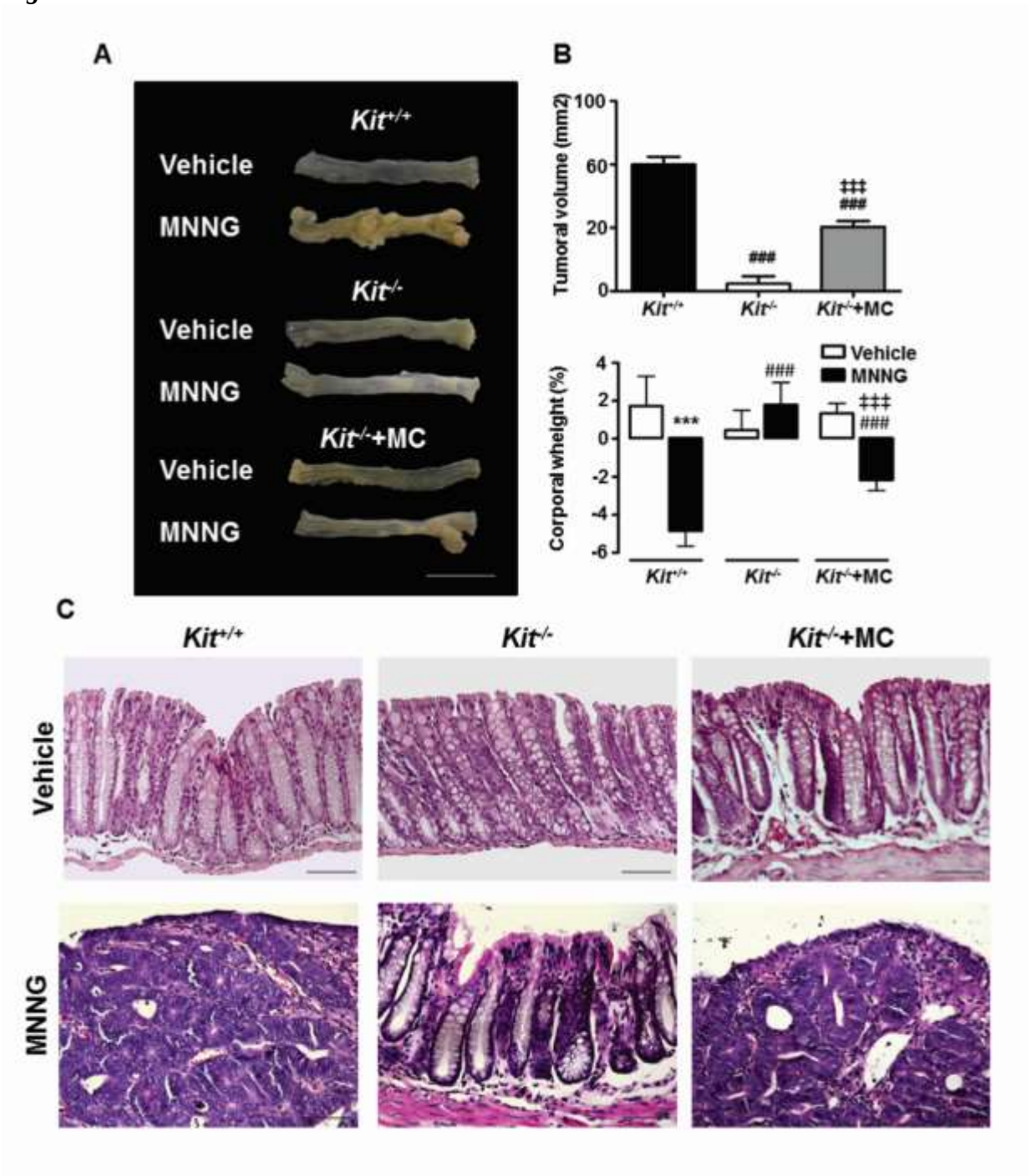

A
Kit+/+
Vehicle
MNNG
Kit-/-
Vehicle
MNNG
Kit-/-+MC
Vehicle
MNNG
B
Tumoral volume (mm2)
100
60
20
0
###
‡‡‡
###
Kit+/+
Kit-/-
Kit-/-+MC
Corporal whelght (%)
4
2
0
-2
-4
-6
***
###
‡‡‡
###
Vehicle
MNNG
Kit+/+
Kit-/-
Kit-/-+MC
C
Kit+/+
Kit-/-
Kit-/-+MC
Vehicle
MNNG

Figure 4

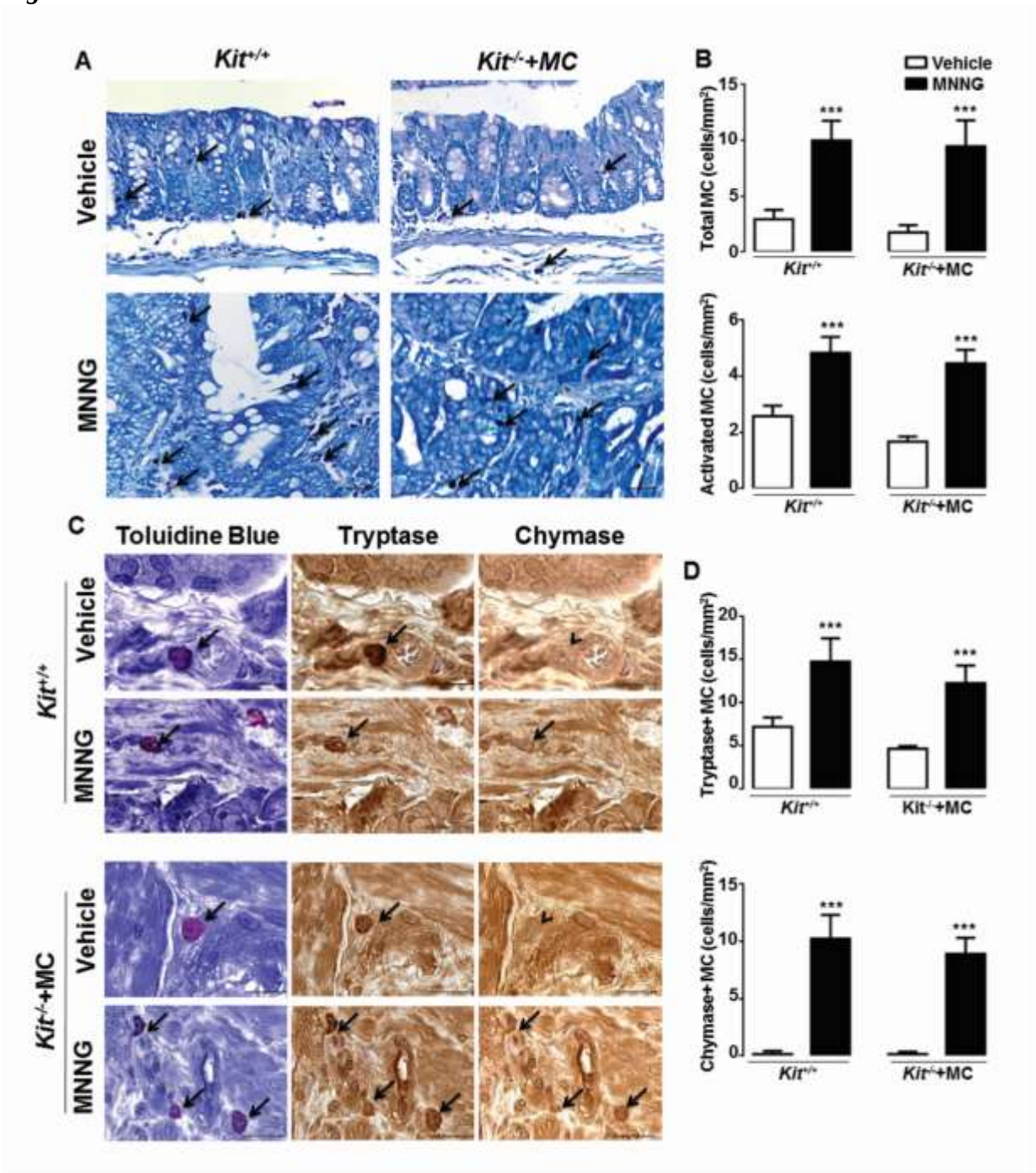

A
Kit+/+
Kit-/-+MC
Vehicle
MNNG
B
Vehicle
MNNG
Total MC (cells/mm²)
Activated MC (cells/mm²)
Kit+/+
Kit-/-+MC
C
Toluidine Blue
Tryptase
Chymase
Kit+/+
Kit-/-+MC
D
Tryptase+ MC (cells/mm²)
Chymase+ MC (cells/mm²)
***

Figure 5

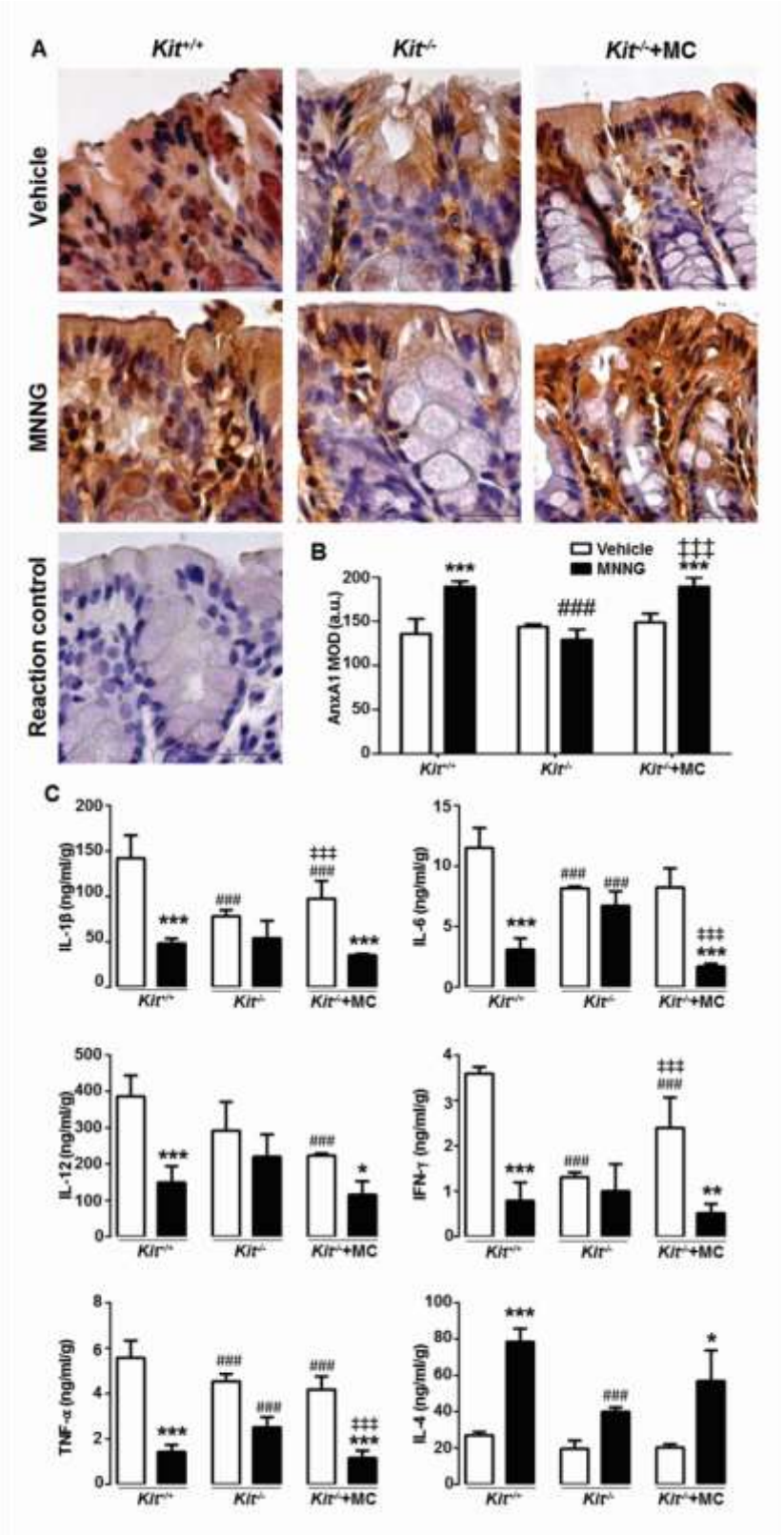
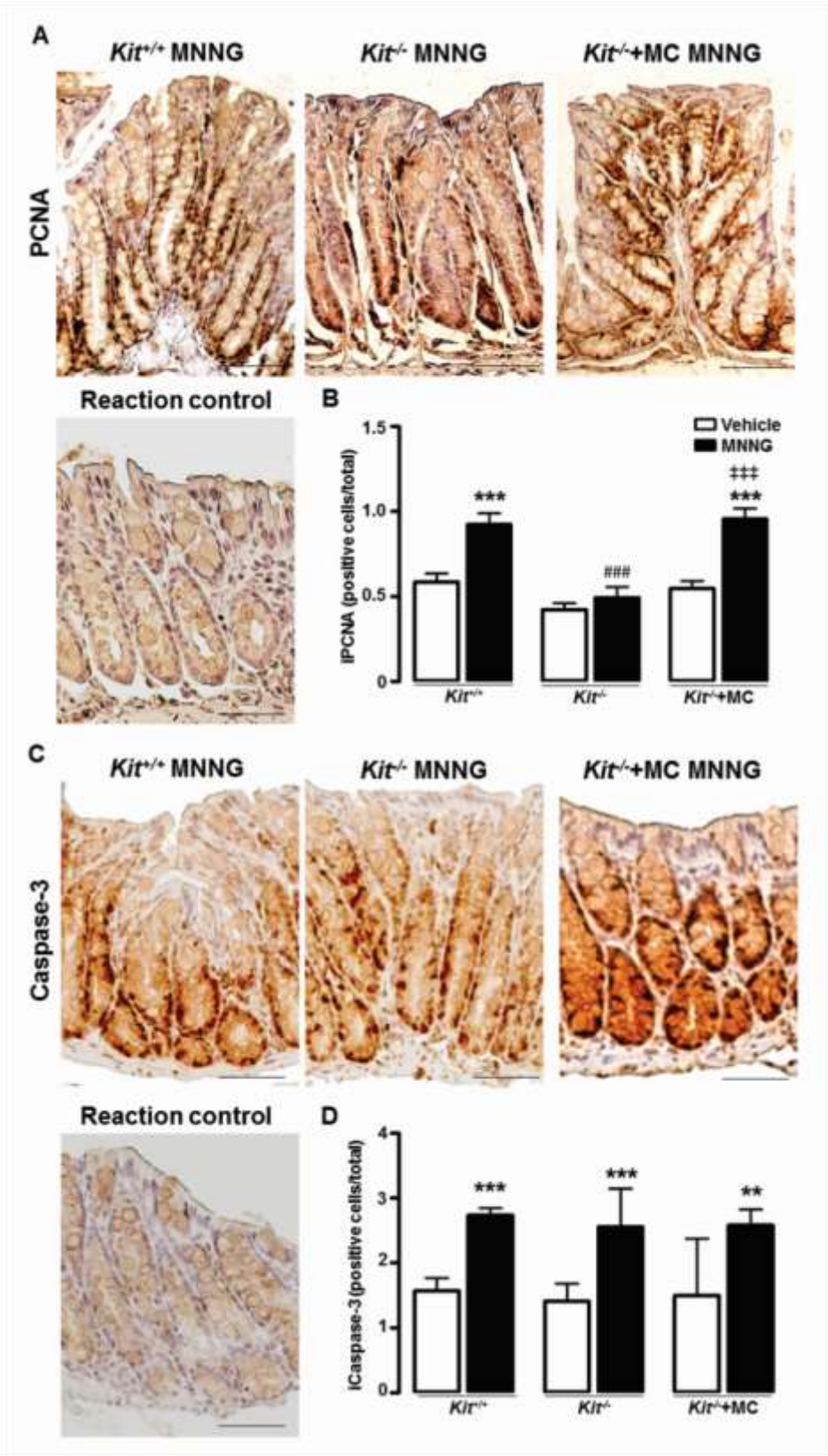
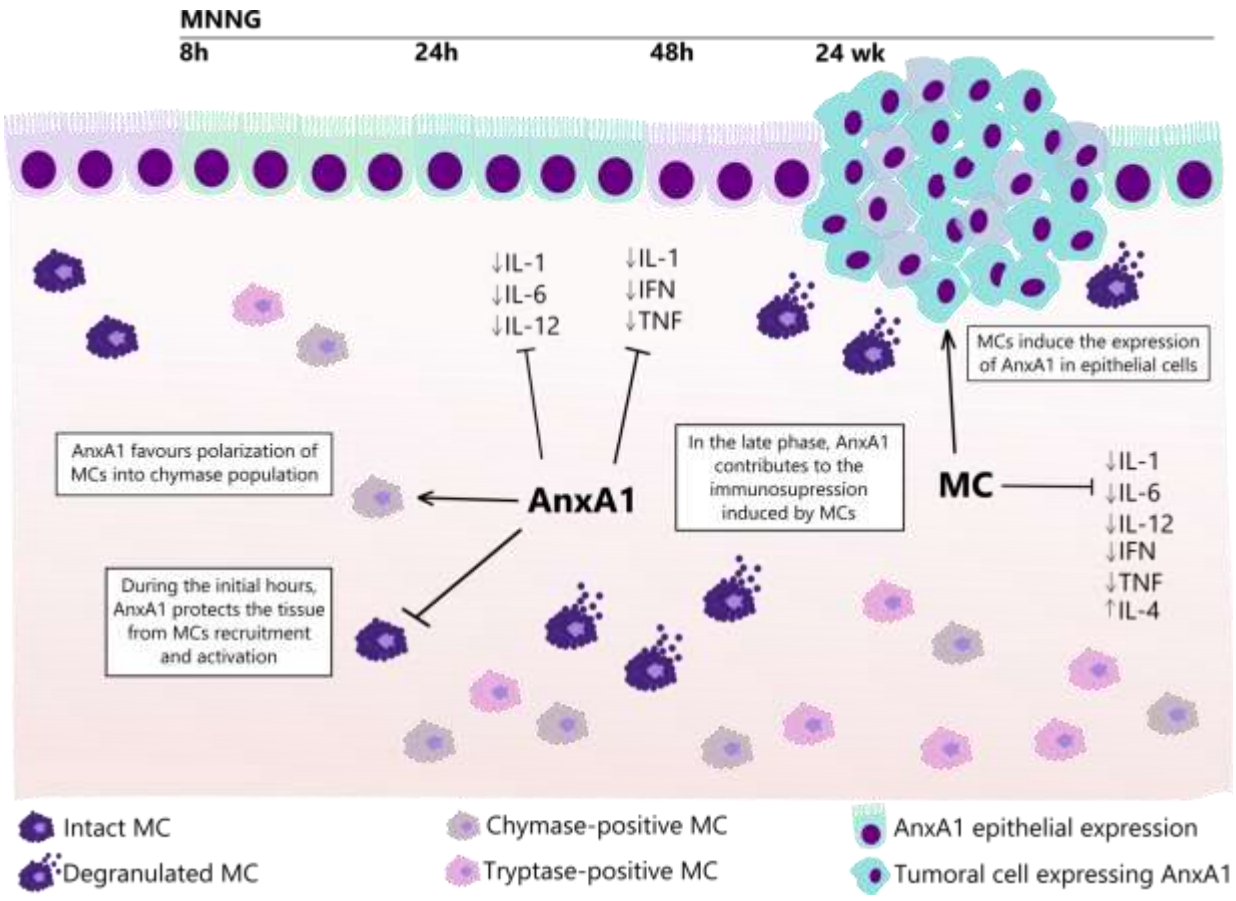

A
Kit+/+
Kit-/-
Kit-/-+MC
Vehicle
MNNG
Reaction control
B
Vehicle
MNNG
AnxA1 MOD (a.u.)
C
IL-1β (ng/ml/g)
IL-6 (ng/ml/g)
IL-12 (ng/ml/g)
IFN-γ (ng/ml/g)
TNF-α (ng/ml/g)
IL-4 (ng/ml/g)

Figure 6


A
Kit+/+ MNNG
Kit-/- MNNG
Kit-/-+MC MNNG
PCNA
Reaction control
B
Vehicle
MNNG
IPCNA (positive cells/total)
1.5
1.0
0.5
0
***
###
‡‡‡
***
Kit+/+
Kit-/-
Kit-/-+MC
C
Kit+/+ MNNG
Kit-/- MNNG
Kit-/-+MC MNNG
Caspase-3
Reaction control
D
ICaspase-3 (positive cells/total)
4
3
2
1
0
***
***
**
Kit+/+
Kit-/-
Kit-/-+MC

Supplementary figure



Proposed schema to ilustrate the interactions of annexin A1 (AnxA1) and mast cells (MCs) in the colorectal cancer (CRC) development. During the initial phase, AnxA1 is highly expressed by epithelial cells, mediating the protection of colonic histoarchitecture, downregulating the expression of pro-inflammatory cytokines and favouring the incidence of chymase-positive MCs into the tissue. In the late phase, the presence of MCs induces the expression of AnxA1 by epithelial cells, which correlates with the tumor formation and an immunosupressive profile.

Apêndice 2: Artigo científico publicado na revista Cytokine

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Humoral immune responses against the malaria vaccine candidate antigen *Plasmodium vivax* AMA-1 and *IL-4* gene polymorphisms in individuals living in an endemic area of the Brazilian Amazon

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ABSTRACT

Background: Several studies have recently demonstrated that the immune responses against malaria is governed by different factors, including the genetic components of the host. The *IL-4* gene appears to be a strong candidate factor because of its role in the regulation of the Th2 response. The present study investigated the role of *IL-4* polymorphisms in the development of IgG antibodies against PvAMA-1 and the IL-4 levels in individuals infected with *Plasmodium vivax* in a malaria endemic area in the Brazilian Amazon.

Methods: The study sample included 83 patients who were diagnosed with *P. vivax* infection using thick smear and confirmed by nested-PCR. The *IL-4* −590 C>T and *IL-4* −33 C>T polymorphisms were genotyped by PCR–RFLP, and the intron 3 VNTR was genotyped by PCR. A standardised ELISA protocol was used to measure the total IgG against PvAMA-1. The cytokine/chemokine levels were measured using a Milliplex multiplex assay (Millipore). All of the subjects were genotyped with 48 ancestry informative markers to determine the proportions of African, European and Amerindian ancestry using STRUCTURE software.

Results: Of the 83 patients, 60 (73%) produced IgG antibodies against PvAMA-1. A significant decrease in the percentage of respondents was observed among the primo-infected individuals. No significant differences were observed in the frequencies of genotypes and haplotypes among individuals who were positive or negative for IgG antibodies against PvAMA-1. Furthermore, no significant correlation was observed between the *IL-4* polymorphisms, antibody levels, IL-4 levels, and parasitemia.

Conclusions: This study indicated that the polymorphisms identified in the *IL-4* gene are not likely to play a role in the regulation of the antibody response against PvAMA-1 and IL-4 production in vivax malaria.

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1043-4666/

1. Background

Malaria is a parasitic disease of major importance worldwide. It is estimated that over 200 million cases occurred in 2012 alone. *Plasmodium vivax* is a widely distributed species, with high incidence in Central and South America, Asia, and Oceania. Despite being less lethal than *P. falciparum*, *P. vivax* is responsible for the high morbidity in these regions [1].

Vaccine development is considered one of the most promising strategies to control malaria, and the proteins involved in the

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recognition, adhesion, and internalisation of erythrocytes are considered to be the main target candidates for vaccine development. Apical membrane antigen-1 (AMA-1) appears to be a strong candidate because of its role in the invasion of erythrocytes by *Plasmodium* [2]. Monoclonal antibodies against AMA-1 were able to inhibit the *in vitro* invasion of erythrocytes by merozoites of different parasite species, including those of *P. knowlesi*, *P. falciparum*, and *P. reichenowi* [3–5]. Recently, antibodies obtained from mice immunised with *P. vivax* AMA-1 (PvAMA-1) inhibited erythrocyte invasion by *P. vivax* merozoites [6]. In addition, several seroepidemiological studies have indicated that AMA-1 is immunogenic in natural infections. In Brazil, the antibody response against PvAMA-1 has been evaluated in naturally exposed individuals from different endemic regions in the Amazon [7–10].

It is known that the heterogeneity of the immune responses can be attributed to several factors, including genetic components of the host [11]. Moreover, it has been reported that the production of antibodies against various *P. falciparum* proteins is controlled by immune-related genes, including those of the HLA system such as *IL-1*, *IL-3*, *IL-4*, *IL-5*, *IL-10*, *IL-13*, *TNF*, *FCGR2A*, *FCGR3B*, and *CD36* [12–23]. However, few studies have evaluated the immune responses against *P. vivax* proteins, and the genetic mechanisms involved in the humoral immune responses against this species remain poorly understood [10,12,24–27].

Because of its role in the regulation of different stages of the immune responses, the IL-4 molecule seems to be an excellent candidate for modulating the immune responses directed against *P. vivax*. This Th2 cytokine induces the exchange of IgM/IgG to IgE isotypes and enhances the antigen presentation ability of B lymphocytes [28]. In falciparum malaria, higher IL-4 levels were detected in children with severe malaria compared with children with mild malaria [29,30], and IL-4 levels have been correlated with parasitemia [29,31]. The *in vitro* production of IL-4 by $CD4^+$ T cells in individuals living in malaria endemic areas has been associated with elevated antibody levels [32].

Polymorphisms in the promoter region of the *IL-4* gene more specifically, the single nucleotide polymorphism (SNP) −590 C>T have been associated with the production of antibodies against *P. falciparum* proteins, including MSP-1, MSP-2, CSP, and Pf332 [19,21,33]. However, the role of *IL-4* gene polymorphisms in the immune responses against *P. vivax* has not been investigated. Therefore, the present study aimed to evaluate the effect of *IL-4* gene polymorphisms (*−590 C>T*, *−33 C>T* and one intron 3 variable-number tandem repeat (VNTR) on the production of IgG antibodies against PvAMA-1 and on IL-4 levels in individuals naturally infected with *P. vivax* in the Brazilian Amazon.

2. Methods

2.1. Study participants

This study was conducted in the city of Goianésia do Pará, located in Pará, one of the states encompassed by the Brazilian Amazon. Malaria transmission in this region occurs throughout the year; in 2012, this region had an annual parasite index of 46.74, with approximately 80% of the cases caused by *P. vivax* [34]. The study sample was composed of 83 individuals recruited between February and August 2012. The subjects were aged >14 years, exhibited classic symptoms of malaria, and sought medical assistance in the city. Only those diagnosed with *P. vivax* by microscopic examination were included in the study. A control group consisted of 40 individuals living in a non-endemic area in São José do Rio Preto, southeastern Brazil they complied with the following criteria: they were over 18 years old, of both genders and who reported never having visited malaria transmission areas were used as controls. The control samples were only used for the determination of serological response.

2.2. Sample collection and malaria diagnosis

After the participants answered a questionnaire on epidemiological data, approximately 10 mL of peripheral blood was collected by venipuncture, and the serum samples were separated by centrifugation. Malaria was diagnosed using thick blood smears stained with Giemsa and subsequently confirmed by nested-PCR [35]. Parasitemia was defined as the number of parasites per microliter of blood after examination of 100 microscopic fields. All of the participants or their guardians signed an informed consent form. The project was approved by the health authorities of the city of Goianésia do Pará and by the Research Ethics Committee of the Department of Medicine of São José do Rio Preto (CEP/FAMERP No. 4599/2011).

2.3. Genotyping

DNA was extracted from the white blood cells using an EasyDNA™ extraction kit (Invitrogen, California, USA). All of the individuals were genotyped for a set of 48 bi-allelic insertion/deletion (INDELS) polymorphisms validated as ancestry informative markers, performed by three multiplex reactions as described elsewhere [36].

For the genotyping of the *IL-4* gene, the VNTR, a 70-bp short tandem repeat in intron 3 (rs8179190), was detected by PCR, and the 183- and 253-bp alleles were identified as the B1 and B2 alleles, respectively. The SNPs −590 C>T (rs2243250) and −33 C>T (rs2070874) were genotyped by PCR, followed by enzymatic digestion. The genotyping of the *IL-4* gene followed a previously described protocol [37], except for the cycling conditions of the 3 polymorphisms, which were changed to 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; and 1 cycle of 72 °C for 5 min. The PCR products were visualised on a 2% agarose gel stained with Gel Red (Biotium, CA, USA).

2.4. Antigen and antibody measurements

A recombinant protein containing the ectodomain (amino acids 43-487) of the AMA-1 protein of *P. vivax* (Belém strain) was expressed in *Pichia pastoris* and purified as described previously [6]. An ELISA test was performed to determine the levels of naturally acquired IgG antibodies against PvAMA-1, following a previously described protocol [6]. To classify the sample as positive or negative, the reactivity index (RI) was calculated by dividing the sample OD by the cutoff value. Samples with RI ⩾ 1 were considered positive, and samples with RI < 1 were considered negative. The cutoff value was established by considering the mean OD value from 40 control samples plus 3 standard deviations.

2.5. Cytokine assay

The concentration of IL-4 in the serum samples was quantified using a customised Milliplex MAP Human Cytokine/Chemokine Panel (# HCYTOMAG-60K, Millipore, Boston, MA, USA). The assays were performed according to the manufacturer's instructions. The samples were analysed in duplicate on a Luminex 200 system (Luminex Corporation), and the data were collected by Luminex xPONENT software.

2.6. Statistical analysis

The individual proportions of African, European, and Native American genetic ancestry were estimated using the software

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STRUCTURE version 2.3.1. The remaining statistical analyses were performed using the program R version 2.11.1 (http://www.r-project.org). The genotypic and allelic frequencies for each variant were obtained using the package *genetics* [38]. Using this package, deviations from the Hardy–Weinberg equilibrium were evaluated using the chi-squared test, and the linkage disequilibrium between pairs of loci was analysed with the parameter D'. The haplotype frequencies were estimated using the maximum likelihood method with the expectation-maximisation (EM) algorithm, which is part of the *haplo.stats* package [39]. The proportion differences were assessed using the chi-squared test. The nonparametric Kruskal–Wallis test was used to determine the differences in the antibody and IL-4 levels in relation to the genotypes. The correlation between the haplotypes, antibody levels, and IL-4 levels was evaluated by logistic regression, using the haplo.glm function in the *haplo.stats* package. The *p* value was adjusted for covariables using a logistic regression model. The graphs were generated using the *ggplot* package, and $p < 0.05$ was considered significant.

3. Results

3.1. Study population

The population profile is shown in Table 1. Among the 83 patients infected with *P. vivax* included in the study, 60 (73%) produced IgG antibodies against PvAMA-1 (RI ⩾ 1), whereas 20 (27%) did not (RI < 1). No significant differences were observed between these 2 groups regarding the gender, age, number of previous episodes of malaria, exposure time, or genetic ancestry. However, a higher proportion of primo-infected individuals was observed among the subjects without PvAMA-1 antibodies compared with those positive for PvAMA-1 antibodies (33.0% and 3.7%, respectively, $p < 0.00001$). No significant differences were detected between PvAMA-1 antibody positive and negative individuals with respect to parasitemia (medians of 2000 and 1000 parasites/μL, respectively, $p = 0.15$) or IL-4 levels (medians of 1.04 and 1.18 pg/mL, respectively, $p = 0.26$).

3.2. Association between IL-4 polymorphism and AMA-1 antibody response

The *IL-4 −590 C>T*, *IL-4 −33 C>T*, and VNTR polymorphisms were genotyped successfully in all 83 patients. No significant deviations from the Hardy–Weinberg equilibrium were observed for any polymorphism ($p > 0.23$ for all). For the *IL-4 −590 C>T* SNP, the *T* allele was the most frequent (51.8%). For the *IL-4 −33 C>T* SNP, the *C* allele was the most frequent (64.5%), and for the VNTR, the *B2* allele was the most frequent (66.3%). No significant differences were observed in the genotype frequency between individuals with and without IgG antibodies against PvAMA-1 (Table 2), even when these genotypes were grouped according to genetic models (recessive, dominant, or additive) (data not shown). A multivariate analysis adjusting for the primo-infected status did not change this result. Similar to the results for the rate of the respondents, none of the polymorphism genotypes significantly affected the levels of IgG antibodies against PvAMA-1 (Fig. 1A).

Table 2
Distribution of IL-4 polymorphisms among malaria patient AMA-1 responders or non-responders.

IL-4 Polymorphism	Frequency (%)		p-value
	AMA-1 positive	AMA-1 negative	
−590 C>T (rs2243250)			0.48
CC	19.6	19.0	
CT	53.6	66.7	
TT	26.8	14.3	
−33 C>T (rs2070874)			0.91
CC	37.5	42.8	
CT	51.8	52.4	
TT	10.7	4.8	
VNTR (rs81790190)			0.88
B1B1	7.1	9.5	
B1B2	53.6	47.6	
B2B2	39.3	42.9	

3.3. Cytokine quantification and association with IL-4 polymorphisms

The IL-4 levels among the patients with vivax malaria ranged between 0.61 and 40.36 pg/mL, with a median of 1.05 pg/mL. No significant differences were observed between the IL-4 levels in the genotypes evaluated (Fig. 1B). Furthermore, no correlation was observed between the IL-4 levels and parasitemia (*rho* = −0.08, $p = 0.48$, Spearman correlation) and the antibody levels against PvAMA-1 (*rho* = 0.02, $p = 0.85$, Spearman correlation).

3.4. Linkage disequilibrium and haplotype association

Because of the proximity of polymorphisms on chromosome 5, a high linkage disequilibrium was observed between the *IL-4 −590 C>T* and *IL-4 −33 C>T* ($D' = 0.96$, $r^2 = 0.47$) SNPs, between the *IL-4 −590 C>T* SNP and VNTR ($D' = 0.95$, $r^2 = 0.43$), and between the *IL-4 −33 C>T* SNP and VNTR ($D' = 0.79$, $r^2 = 0.58$). Five haplotypes with a frequency >4% were observed and were evaluated to determine the PvAMA-1 IgG antibody levels and IL-4 levels. Of these, the most frequent haplotype was *IL-4*$_{-590C-T/-33C-T/VNTR}$ *C/C/B2* (47.5%). No significant differences were observed in the

Table 1
Characteristics of malaria patients according to AMA-1 antibody response.

Characteristics	All malaria patients (*n* = 83)	AMA-1 positive (*n* = 60)	AMA-1 negative (*n* = 23)	p-value
Gender (male)[a]	73.5	75.0	71.4	0.88
Age (years)[b]	33 (15–68)	31 (15–68)	34 (18–65)	0.54
Exposure period (years)[b]	7 (0.1–30)	7 (0.1–30)	11 (0.1–21)	0.90
Previous malaria episodes[c]	5.3 ± 3.3	5.4 ± 2.9	4.9 ± 4.3	0.72
Primo-infected[a]	11.0	3.7	33.3	<0.0001
Genetic ancestry[c]				
African	0.324 ± 0.119	0.325 ± 0.119	0.322 ± 0.122	0.92
European	0.435 ± 0.131	0.435 ± 0.130	0.434 ± 0.137	0.99
Native Amerindian	0.240 ± 0.096	0.239 ± 0.099	0.245 ± 0.087	0.66
Parasite density (parasites/μL)[b]	1500 (5–10,000)	2000 (10–10,000)	1000 (5–7000)	0.15
IL-4 concentration (pg/mL)[b]	1.05 (0.615–40.365)	1.04 (0.725–9.325)	1.18 (0.615–40.365)	0.26

[a] Percentages.
[b] Median (range).
[c] Mean ± standard deviation.

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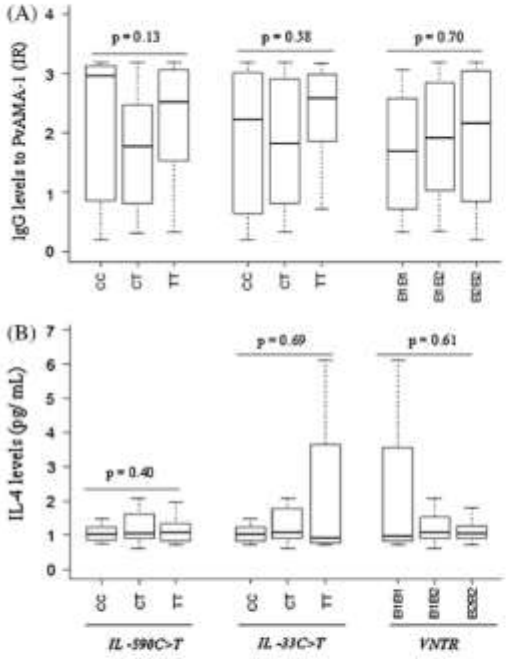

Fig. 1. (A) The AMA-1 levels (IR) and; (B) IL-4 levels according to the genotype. The boxes delimit the median values, and the bars denote the 25th and 75th percentiles. The differences between the genotypes were examined with the Mann–Whitney U test. A single individual whose concentration of IL-4 was 40.36 pg/mL was omitted from the graph.

Table 3
Association between the *IL-4* haplotype frequencies and PvAMA-1 antibody response.

Haplotype[a]	PvAMA-1			Global score statistic[c]
	Frequency		*p*	
IL-4 $_{-590/-33/VNTR}$	Pos[b]	Neg[b]		
C/C/B2	0.458	0.524	0.42	χ^2 = 2.09941
T/T/B1	0.285	0.285	0.99	df = 5
T/C/B2	0.127	0.118	0.83	*p*-value = 0.83
T/T/B2	0.076	0.024	0.23	
T/C/B1	0.044	0.048	0.97	

[a] Haplotypes with frequencies <4% were omitted.
[b] Pos = Positive and Neg = Negative.
[c] The overall association between the haplotypes and the response.

haplotype frequency between individuals who were positive and negative for PvAMA-1 antibodies (Table 3). Using a regression model for the quantitative traits in the *haplo.stats* package, the variation in the antibody and IL-4 levels was determined for each haplotype in relation to a reference haplotype (in this case, the most frequent haplotype). No significant differences were observed in these levels (Fig. 2).

4. Discussion

Although the genes at the HLA class I and class II loci are the best known immunogenetic determinants of the humoral immune responses to malaria [10,11,15,25,27,40–42], recent immunogenetic studies have demonstrated that polymorphisms at many loci in several genes are associated with the humoral antibody response against different Plasmodium proteins [12,14,15,17,19–21,23]. In the present study, we investigated 3 polymorphisms in the *IL-4* gene (*IL-4* −*590 C>T*, *IL-4* −*33 C>T*, and a 70-bp VNTR in intron 3) that are associated with falciparum malaria. To our knowledge, this is the first study that investigates the influence of *IL-4* polymorphisms on the IgG antibody response against *P. vivax*. The present study was conducted in a mixed population in a malaria endemic area in the Brazilian Amazon, and possible biases owing to the population heterogeneity were carefully considered by employing informative markers of ancestry to characterise the study population.

The potential involvement of *IL-4* gene polymorphisms in immunity against malaria has been demonstrated in a previous study [33], wherein differences in allele frequencies at the *IL-4* −*590 C>T* SNP (previously designated *IL-4* −*524 C>T*) were detected among sympatric African tribes. The *T* allele was more frequent in the Fulani tribe, whose members admittedly have increased antibody levels and decreased susceptibility against malaria compared with those in the neighbouring tribes of Rimaibé and Mossi. In the Fulani tribe, the *T* allele was associated with significantly higher levels of IgG antibodies against CSP and PF332 *P. falciparum* antigens. Since then, other studies have investigated this SNP but with contradictory results [20,21,23,37].

In the present study, we found no role for *IL-4* polymorphisms in the production of IgG antibodies against PvAMA-1. Recently, a genome-wide association study (GWAS) investigated the effect of 6 *IL-4* SNPs, including SNP −*590 C>T*, on the acquisition of IgE antibodies against PvAMA-1 in individuals exposed to malaria in Sri Lanka, and no significant association was found [12]. A previous study conducted in Thailand [20] demonstrated that the acquisition of specific IgG1 and IgG3 antibodies against *P. falciparum* in patients with complicated and uncomplicated malaria was dependent on the *IL-4* −*590 C>T* genotype, but this finding was only apparent after separating the patients who had been previously infected with malaria from those who never had the disease. In fact, it is well known that exposure to malaria can affect the acquisition of antibodies against malaria. In the present study, a strong association was observed between individuals who never had malaria and a low IgG antibody response against AMA-1. However, the low proportion of primo-infected individuals in the study sample did not allow us to perform an analysis similar to that from a previous study [20]. It is important to point that the sample size of our study is small and that a panel of genetic variations in humoral immune responses in larger sample size should be further investigated.

We investigated the influence of *IL-4* polymorphisms on the IL-4 cytokine levels. The *IL-4* −*590 C>T* SNP affects the binding of *NFAT*, a key transcriptional activator of the *IL-4* gene [43], whereas the *IL-4* −*33 C>T* SNP has been associated with an increased promoter activity of the gene [44]. Moreover, it has been proposed that distinct numbers of VNTR copies in intron 3 may affect its transcriptional activity [45]. In the present study, no significant differences were observed in the serum IL-4 levels among the different genotypes and haplotypes. This result is in agreement with that found in a previous study [19], which concluded that there was no correlation between the genotypes and haplotypes of *IL-4* −*590 C>T* and *IL-4* −*33 C>T* with the IL-4 plasma levels in mothers with placental malaria from a cohort study. However, 2 other studies of patients infected with *P. falciparum* found increased IL-4 levels in the plasma of patients with the genotypes *IL-4* −*590TT* [29] and VNTR B1B2 [30]. This discrepancy may be related to the lack of function of these polymorphisms. Alternatively, the production of IL-4 may be regulated by polymorphisms only in the presence of other genetic determinants.

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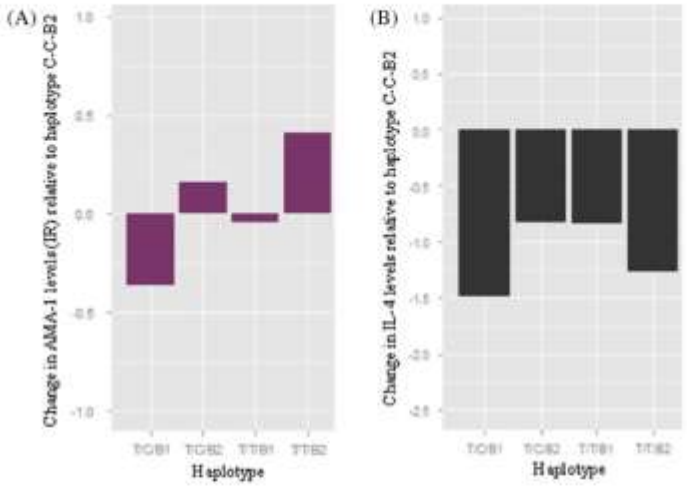

Fig. 2. Relative effects of the haplotypes on (A) the AMA-1 levels (IR) and (B) IL-4 levels. The regression coefficients from the haplo.glm analysis were estimated for each haplotype. The coefficients reflect the difference in the mean per copy relative to the haplotype *IL-4*$_{-590/-33/mVNTR}$ C/C/B2, the most frequent haplotype. There were no significant differences.

The IL-4 levels found in the present study were low (median = 1.5 pg/mL). Similar results were observed in 2 other studies in the Brazilian Amazon involving patients with vivax malaria [46,47]. In contrast, higher plasma IL-4 levels appear to be associated with the disease severity in individuals infected with *P. falciparum* [29,30]. In fact, IL-4 levels appear to vary substantially during the course of infection, and higher concentrations are observed in the intermediate and late stages of the disease [29,48]. Therefore, the fact that malaria patients from the studied area have easy access to medical services and seek diagnosis and treatment a few days after the onset of symptoms may explain the low IL-4 levels observed in the present study. To better understand the role of this cytokine in the development of the humoral immune responses against malaria, we investigated whether the IL-4 concentration is correlated with the IgG antibody response against PvAMA-1, but no correlation was observed. In an experimental mouse model, the protection induced by immunisation with AMA was not strictly dependent on IL-4, suggesting that an antibody response can be induced even in the absence of this cytokine [49]. In fact, it is much more likely that the balance between different cytokines may be more important than the absolute levels of a particular cytokine to the result of the immune responses to malaria [29].

5. Conclusions

This study demonstrated that the *IL-4* polymorphisms studied are not likely to play a role in the regulation of the antibody response against PvAMA-1 and IL-4 production in vivax malaria. However, the delicate balance of various cytokines may be more important than any specific cytokine in regulating the immune responses to malaria. In this respect, future studies evaluating a range of cytokines may help elucidate this aspect. In addition, other genes may be involved, considering the variations observed in antibody acquisition. Additional studies being conducted by our group will explore these issues in detail and will include an investigation of the acquisition of antibodies against various candidate *P. vivax* vaccine antigens.

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References

[1] World Health Organization (WHO). World Malaria Report 2013. Geneva (Switzerland): World Health Organization; 2013.
[2] Remarque EJ, Faber BW, Kocken CH, Thomas AW. Apical membrane antigen 1: a malaria vaccine candidate in review. Trends Parasitol 2008;24:74–84.
[3] Thomas AW, Deans JA, Mitchell GH, Alderson T, Cohen S. The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit *Plasmodium knowlesi* invasion of erythrocytes. Mol Biochem Parasitol 1984;13:187–99.
[4] Kocken CH, Narum DL, Massougbodji A, Ayivi B, Dubbeld MA, Vanderwel A, et al. Molecular characterisation of *Plasmodium reichenowi* apical membrane antigen-1 (AMA-1), comparison with *P. falciparum* AMA-1, and antibody-mediated inhibition of red cell invasion. Mol Biochem Parasitol 2000;109:147–56.
[5] Kocken CH, Withers-Martinez C, Dubbeld MA, Vanderwel A, Hackett F, Valderrama A, et al. High-level expression of the malaria blood-stage vaccine candidate *Plasmodium falciparum* apical membrane antigen 1 and induction of antibodies that inhibit erythrocyte invasion. Infect Immun 2002;70:4471–6.
[6] Vicentin EC, Françoso KS, Rocha MV, Ioustov D, Dos Santos FL, Kalhously PS, et al. Invasion-inhibitory antibodies elicited by immunization with *Plasmodium vivax* apical membrane antigen-1 expressed in Pichia pastoris yeast. Infect Immun 2014;82:1296–307.
[7] Rodrigues MH, Rodrigues RM, Oliveira TR, Cônsolo NA, Rodrigues MM, Kocken CH, et al. Antibody response of naturally infected individuals to recombinant *Plasmodium vivax* apical membrane antigen-1. Int J Parasitol 2005;35:185–92.
[8] Miñalo BC, Gentil F, Bargieri DY, Costa FT, Rodrigues MM, Soares IS. *Plasmodium vivax* apical membrane antigen-1: comparative recognition of different domains by antibodies induced during natural human infection. Microbes Infect 2008;10:1266–73.
[9] Bueno LL, Lobo FP, Morais CG, Mourão LC, De Ávila RA, Soares IS, et al. Identification of a highly antigenic linear B cell epitope within *Plasmodium vivax* apical membrane antigen 1 (AMA-1). PLoS ONE 2011;6:e21289.
[10] Storti-Melo LM, da Costa DR, Souza-Neiras WC, Cassiano GC, Couto VS, Póvoa MM, et al. Influence of HLA-DRB-1 alleles on the production of antibody against CSP, MSP-1, AMA-1, and DBP in Brazilian individuals naturally infected with Plasmodium vivax. Acta Trop 2012;121:152–5.

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[11] Duah NO, Weiss HA, Jepson A, Tetteh KK, Whittle HC, Conway DJ. Heritability of antibody isotype and subclass responses to *Plasmodium falciparum* antigens. PLoS ONE 2009;4:e7381.
[12] Dewasurendra RL, Suriyaphol P, Fernando SD, Carter R, Rockett K, Corran P, et al. Genetic polymorphisms associated with anti-malarial antibody levels in a low and unstable malaria transmission area in southern Sri Lanka. Malar J 2012;11:281.
[13] Maiga B, Dolo A, Touré O, Daca V, Tapily A, Campino S, et al. Human candidate polymorphisms in sympatric ethnic groups differing in malaria susceptibility in Mali. PLoS ONE 2011;8:e75675.
[14] Kajegukha D, Mwanziva C, Daou M, Ndaro A, Mutombo S, Mbugi E, et al. CD36 c.1264 T>G null mutation impairs acquisition of IgG antibodies to Plasmodium falciparum $MSP1_{19}$ antigen and is associated with higher malaria incidences in Tanzanian children. Scand J Immunol 2012;75:355–60.
[15] Sabbagh A, Courtin D, Milet J, Massaro JD, Castelli EC, Migot-Nabias F, et al. Association of HLA-G 3′ untranslated region polymorphisms with antibody response against Plasmodium falciparum antigens: preliminary results. Tissue Antigens 2013;82:53–8.
[16] Afridi S, Atkinson A, Garnier S, Fumoux F, Rihet P. Malaria resistance genes are associated with the levels of IgG subclasses directed against Plasmodium falciparum blood-stage antigens in Burkina Faso. Malar J 2012;11:308.
[17] Adu B, Jepsen MP, Gerds TA, Kyei-Baafour E, Christiansen M, Dodoo D, et al. Fc gamma receptor 3B (FCGR3B-c.233C>A-rs5030738) polymorphism modifies the protective effect of malaria specific antibodies in Ghanaian children. J Infect Dis 2014;209:285–9.
[18] Carpenter D, Abushama H, Bereczky S, Färnert A, Rooth I, Troye-Blomberg M, et al. Immunogenetic control of antibody responsiveness in a malaria endemic area. Hum Immunol 2007;68:165–9.
[19] Lokossou AG, Dechavanne C, Bouraïma A, Courtin D, Le Port A, Ladékpo R, et al. Association of IL-4 and IL-10 maternal haplotypes with immune responses to P. falciparum in mothers and newborns. BMC Infect Dis 2013;13:215.
[20] Tangteerawatana P, Perlmann H, Hayano M, Kalambaheti T, Troye-Blomberg M, Khusmith S. IL4 gene polymorphism and previous malaria experiences manipulate anti-Plasmodium falciparum antibody isotype profiles in complicated and uncomplicated malaria. Malar J 2009;8:286.
[21] Vafa M, Maiga B, Israelsson E, Dolo A, Doumbo OK, Troye-Blomberg M. Impact of the IL-4 – 590 C/T transition on the levels of Plasmodium falciparum specific IgE, IgG, IgG subclasses and total IgE in two sympatric ethnic groups living in Mali. Microbes Infect 2009;11:779–84.
[22] Johnson A, Leke R, Harun L, Ginsberg C, Ngugang J, Stowers A, et al. Interaction of HLA and age on levels of antibody to Plasmodium falciparum rhoptry-associated proteins 1 and 2. Infect Immun 2000;68:2231–6.
[23] Veera F, Luoni G, Calissano C, Troye-Blomberg M, Perlmann P, Perlmann H, et al. IL4-589C/T polymorphism and IgE levels in severe malaria. Acta Trop 2004;90:205–9.
[24] Capobianco MP, Cassiano GC, Fantui AA, Storti-Melo LM, Pavanno EC, Galbiatti AL, et al. No evidence for association of the CD40, CD40L and BLYS polymorphisms, B-cell co-stimulatory molecules, with Brazilian endemic Plasmodium vivax malaria. Trans R Soc Trop Med Hyg 2013;107:377–83.
[25] Lima-Junior JC, Rodrigues-da-Silva RN, Banic DM, Jiang J, Singh B, Fabricio-Silva GM, et al. Influence of HLA-DRB1 and HLA-DQB1 alleles on IgG antibody response to the P. vivax MSP-1, MSP-3α and MSP-9 in individuals from Brazilian endemic area. PLoS ONE 2012;7:e36419.
[26] Pandey JP, Morais CG, Fontes CJ, Braga EM. Immunoglobulin GM 3 23 5,13,14 phenotype is strongly associated with IgG3 antibody responses to Plasmodium vivax vaccine candidate antigens PvMSP1-19 and PvAMA-1. Malar J 2010;9:229.
[27] Oliveira-Ferreira J, Pratt-Riccio LR, Arruda M, Santos F, Ribeiro CT, Goldberg AC, et al. HLA class II and antibody responses to circumsporozoite protein repeats of *P. vivax* (VK210, VK247 and *P. vivax*-like) in individuals naturally exposed to malaria. Acta Trop 2004;92:63–8.
[28] Mosmann TR, Coffman RL. Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv Immunol 1989;46:111–47.
[29] Tangteerawatana P, Pichyangkul S, Hayano M, Kalambaheti T, Looareesuwan S, Troye-Blomberg M, et al. Relative levels of IL-4 and IFN-gamma in complicated malaria: association with IL-4 polymorphism and peripheral parasitemia. Acta Trop 2007;101:258–65.
[30] Cabantous S, Poudiougou B, Oumar AA, Traore A, Barry A, Vitte J, et al. Genetic evidence for the aggravation of Plasmodium falciparum malaria by interleukin 4. J Infect Dis 2009;200:1530–9.
[31] Mshana RN, Boulandi J, Mshana NM, Mayumbo J, Mendome G. Cytokines in the pathogenesis of malaria: levels of IL-1 beta, IL-4, IL-6, TNF-alpha and IFN-gamma in plasma of healthy individuals and malaria patients in a holoendemic area. J Clin Lab Immunol 1991;34:131–9.
[32] Troye-Blomberg M, Riley EM, Kabilan L, Holmberg M, Perlmann H, Andersson U, et al. Production by activated human T cells of interleukin 4 but not interferon-gamma is associated with elevated levels of serum antibodies to activating malaria antigens. Proc Natl Acad Sci USA 1990;87:5484–8.
[33] Luoni G, Verra F, Arca B, Sirima BS, Troye-Blomberg M, Coluzzi M, et al. Antimalarial antibody levels and IL-4 polymorphism in the Fulani of West Africa. Genes Immun 2001;2:411–4.
[34] **Ministério da saúde: Informações de saúde: Indicadores municipais do col de diretrizes, objetivos, metas e indicadores 2013–2015 – Pará. (Municipal indicators of the list of guidelines, objectives, targets, and indicators, 2013–2015, Pará.)** <http://tabnet.datasus.gov.br/cgi/tabnet.exe?pacto/2013/cnv/coapmunpa.def>.
[35] Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infection. Mol Biochem Parasitol 1993;58:283–92.
[36] Santos NP, Ribeiro-Rodrigues EM, Ribeiro-dos-Santos AK, Pereira R, Gusmão L, Amorim A, et al. Assessing individual interethnic admixture and population substructure using a 48-insertion-deletion (INDEL) ancestry-informative marker (AIM) panel. Hum Mutat 2010;31:184–90.
[37] Gyan BA, Goka B, Cvetkovic JT, Kurtzhals JL, Adabayeri V, Perlmann H, et al. Allelic polymorphisms in the repeat and promoter regions of the interleukin-4 gene and malaria severity in Ghanaian children. Clin Exp Immunol 2004;138:145–50.
[38] **Warnes G, Gorjanc G, Leisch F, Man M. Genetics: population genetics. R package version 1.3.6.** <http://CRAN.R-project.org/package=genetics>.
[39] **Sinnwell JP, Schaid DJ. Haplo.stats. Statistical analysis of haplotypes with traits and covariates when linkage phase is ambiguous. R package version 1.4.4.** <http://CRAN.R-project.org/package=haplo.stats>.
[40] Zhang Q, Xue K, Xu X, Wang C, Chang W, Pan W. Influence of HLA-DRB1 alleles on antibody responses to PfCP-2.9-immunized and naturally infected individuals. J Clin Immunol 2009;29:454–60.
[41] Pratt-Riccio LR, Lima-Junior JC, Carvalho LJ, Thetsen M, Espindola-Mendes EC, Santos F, et al. Antibody response profiles induced by Plasmodium falciparum glutamate-rich protein in naturally exposed individuals from a Brazilian area endemic for malaria. Am J Trop Med Hyg 2005;73:1096–103.
[42] Johnson AH, Leke RG, Mendell NR, Sibon D, Suh YJ, Bomba-Nkolo D, et al. Human leukocyte antigen class II alleles influence levels of antibodies to the *Plasmodium falciparum* asexual-stage apical membrane antigen 1 but not to merozoite surface antigen 2 and merozoite surface protein 1. Infect Immun 2004;72:2762–71.
[43] Rockman MV, Hahn MW, Soranzo N, Goldstein DB, Wray GA. Positive selection on a human-specific transcription factor binding site regulating IL4 expression. Curr Biol 2003;13:2118–23.
[44] Kim BS, Park SM, Uhm TG, Kang JH, Park JS, Jang AS, et al. Effect of single nucleotide polymorphisms within the interleukin-4 promoter on aspirin intolerance in asthmatics and interleukin-4 promoter activity. Pharmacogenet Genomics 2010;20:748–58.
[45] Vandenbroeck K, Martinez G, Marrosu M, Consiglio A, Zaffaroni M, Vaccargiu S, et al. Occurrence and clinical relevance of an interleukin-4 gene polymorphism in patients with multiple sclerosis. J Neuroimmunol 1997;76:189–92.
[46] Medina TS, Costa SPT, Oliveira MD, Ventura NA, Souza JM, Gomes TF, et al. Increased interleukin-10 and interferon-g levels in Plasmodium vivax malaria suggest a reciprocal regulation which is not altered by IL-10 gene promoter polymorphism. Malar J 2011;10:264.
[47] Gonçalves RM, Scopel KK, Bastos MS, Ferreira MU. Cytokine balance in human malaria: does Plasmodium vivax elicit more inflammatory responses than Plasmodium falciparum? PLoS ONE 2012;7:e44394.
[48] Torre D, Speranza F, Giola M, Matteelli A, Tambini R, Biondi G. Role of Th1 and Th2 cytokines in immune response to uncomplicated Plasmodium falciparum malaria. Clin Diagn Lab Immunol 2002;9:348–51.
[49] Burns Jr JM, Flaherty PR, Nanavati P, Weidanz WP. Protection against Plasmodium chabaudi malaria induced by immunization with apical membrane antigen1 and merozoite surface protein 1 in the absence of gamma interferon or interleukin-4. Infect Immun 2004;72:5603–12.

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Apêndice 2: Artigo científico publicado na revista Plos One

PLOS ONE

RESEARCH ARTICLE

Heterogeneity of mast cells and expression of Annexin A1 protein in a second degree burn model with silver sulfadiazine treatment

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Abstract

Mast cells (MCs) participate in all stages of skin healing and one of their mediators is the Annexin A1 protein (AnxA1), linked to inflammation, proliferation, migration and apoptosis processes, but not studied in thermal burns yet. Therefore, our objectives were to evaluate the behavior of MCs and AnxA1 in a second degree burn model, treated or not with silver sulfadiazine 1% (SDP 1%) and associated to macrophages quantification and cytokines dosages. MCs counts showed few cells in the early stages of repair but increased MCs in the final phases in the untreated group. The normal skin presented numerous tryptase-positive MCs that were reduced after burning in all analyzed periods. Differently, few chymase-positive MCs were observed in the early stages of healing, however, increased chymase-positive MCs were found at the final phase in the untreated group. MCs also showed high immunoreactivity for AnxA1 on day 3 in both groups. In the tissue there was a strong protein expression in the early stages of healing, but in the final phases only in the SDP treated animals. TNF-α, IL-1β, IL-6, IL-10 and MCP-1 levels and macrophages quantification were increased in inflammation and reepithelialization phases. Reduced IL-1β, IL-6 and IL-10 levels and numerous macrophages occurred in the treated animals during tissue repair. Our results indicate modulation in the profile of MCs and AnxA1expression during healing by the treatment with SDP 1%, pointing them as targets for therapeutic interventions on skin burns.

Introduction

The tissue repair process in the healing of burns can be divided into phases of inflammation, proliferation and maturation, where each stage orchestrates the beginning of the next phase [1–4]. The maturation phase is characterized by neogenesis of the epithelial appendages and the extracellular matrix (ECM) remodeling, but pathological scars may be formed due to excessive collagen synthesis [1].

Among the mediators involved in inflammation, there are the pro-inflammatory cytokines interleukin-1 beta (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α) and the anti-inflammatory

Competing interests: The authors have declared that no competing interests exist.

cytokine IL-10, which differ in their expression during wound healing [5–7]. The initial phase also involves the release of monocytes chemoattractant protein-1 (MCP-1), that attracts macrophages and mast cells (MCs) [8,9].

The MCs have important functions in the different wound repair phases [10]. In the process of degranulation, MCs release chemotactic factors and specific proteases, such as tryptase and chymase, to the ECM contributing to the degradation of ECM, promotion of angiogenesis and tissue remodeling through selective proteolysis in the matrix and activation of matrix metalloproteinases (MMPs) [11–13]. Previous investigations have also indicated the importance of chymase in the healing process of burn in rats, related to the density of capillaries and accumulation of collagen [12,14]. The variable expression of proteases has led to the recognition of MCs subpopulations in humans and murines [15,16].

Moreover, studies have indicated that the granules of MCs store the protein annexin A1 (AnxA1) [17,18], the first cloned member of the superfamily of annexins proteins. The annexins are grouped according to their structural characteristics and display a central domain consisting of four replicates of 60 to 70 amino acids each, with affinity for Ca^{++} and connected to a N-terminal sequence, which confers specificity of action for each member of the superfamily [19]. The AnxA1 protein presents various functions related to inflammation [20,21] growth [22], migration, cell proliferation and differentiation, besides membrane transport and apoptosis [21,23].

In the skin under normal conditions, the expression of AnxA1 is reduced [24], however, abnormal distribution and expression of the protein have been observed in inflammations and skin tumors [24–27] and mucosal injury [28–30]. The AnxA1 is strongly expressed in skin lesions in lupus [27], in differentiated squamous cell carcinoma [24] and melanoma [26]. In patients infected with *Leishmania braziliensis*, the expression of AnxA1 was stronger in macrophages CD163+ and lymphocytes CD4+ and CD8+ on infected skin compared to normal skin [31]. Immunohistochemical analyzes, in a model of granulomatous inflammation, showed strong expression of AnxA1 in MCs in the initial (7 days) and late (28 days) phases of the inflammatory reaction [25].

The AnxA1 was also indicated as the key mediator in the death of keratinocytes in Stevens-Johnson syndrome and in toxic epidermal necrolysis, important cutaneous drug reactions [32]. The pharmacological treatment with the mimetic peptide of the N-terminal region of AnxA1 (Ac2-26) was used in different models of studies. In vitro investigations indicated that the peptide was able to stimulate the migration of human fibroblast WS1 lineage [33]. In *in vivo* studies, they also showed protective peptide effects with increased skin transplant survival in allograft model in rats [34] and improvement in the healing process of excisions in mice skins, observed in a dose-dependent manner [35]. However, there are no known reports on the expression and function of AnxA1 in the repair of burns.

As MCs and AnxA1 have been little explored in burns, the aim of this study was to analyze the profile of these cells by the assessment of their number and heterogeneity for tryptase and chymase, and also to evaluate the expression of AnxA1 in MCs and in skin flaps in a second degree burn model using the silver sulfadiazine at 1% (SDP 1%), considered the standard treatment for partial thickness burns due to its antimicrobial properties [36,37]. The results of these analyses may be useful for a better understanding of t he role of MCs and the protein AnxA1 in the wound healing process.

Materials and methods

Animals

Wistar rats (n = 40) weighing approximately 250g were obtained from the Integrated College Padre Albino Foundation (FIPA). The animals were kept in cages in a controlled environment

(24 to 25˚C, 12h light/dark cycle) with water and food ad libitum. The experiments were conducted after approval and in accordance with the rules of the Ethics Committee for Animal Use of FIPA (Protocol 12/14).

Experimental model of burn and treatment protocols

The animals were anesthetized intraperitoneally with 0,2mL/100g of ketamine and 0,05mL/100g of xylazine and submitted to trichotomy of the dorsal region, after a metal block with dimensions of 2x2cm^2 and water heated to 100˚C was applied for 10 seconds to characterize a second degree burn [38]. Immediately after the trauma, the lesions were covered with gauze moistened in cold saline solution. The animals were given analgesic codeine (1mL/kg) right after the injury induction by gavage and it was offered diluted in drinking water on the following days. The control of water intake and weight of the animals was daily performed.

The topical treatments were begun 24h after the induction of burns. The wounds were cleansed with saline daily. Regarding the treatment, two groups were established. One group was the control group and it received no treatment (C Groups), the other group was treated with SDP 1% ointment (SDP Groups) once a day. Each group, control and treated, was subdivided into 4 groups according to the time the lesion was collected, (n = 5/group) so that different phases of the tissue repair could be analyzed. Thus, the animals were euthanized by overdose of isoflurane for the lesions removal after 3, 7, 14 and 21 days of injury. For comparison of the physiological state of the tissue in relation to fragments of regenerated skin, normal skin flaps (n = 5) were also taken from control animals, from not burned regions of skin. The animals' water and food intake and their behavior in the cages were monitored daily. A veterinarian followed the wounds to check for signs of infection. No animal died as a result of the injuries.

Quantitative analysis of cytokine levels

Fragments from all groups were macerated in liquid nitrogen and added 650μL of a solution containing protease and phosphatase inhibitors (Merck, Millipore Corporation, USA) following the manufacturer's instructions. The material was incubated for 20 minutes at 4˚C under constant agitation and then centrifuged at 14.000 RPM for 10 minutes at 4˚C. The supernatants were collected and frozen at -80˚C.

TNF-α, IL-1β, IL-6, IL-10 and MCP-1 were quantified in the supernatant using the MILLIPLEX MAP Kit (RECYTMAG-65K; Merck Millipore Corporation, USA) and analyzed on the Luminex xMAP MAGPIX device (Merck Millipore Corporation, USA). The concentration of the analytes was determined by the MAGPIX xPONENT Software and expressed in pg/mL.

Histopathological analysis and quantification of cells

Fragments of normal skin and lesions were fixed in 4% formalin, processed for inclusion in paraffin and sectioned at 5μm for histopathologic, quantitative and immunohistochemical analysis. The repair process was evaluated histologically by Hematoxylin-Eosin (HE) and the organization of the collagen fibers was evidenced by Picrosirius Polarization method.

MCs were stained with 0.1% toluidine blue (TB+ MCs) and evaluated according to their morphological characteristics in intact or degranulated. The histamine accumulation by MCs was evidenced after staining with 2.5% Safranin-O (S-O+ MCs) [16,39,40]. The quantification of MCs in the skin fragments was performed in 10 images per slide obtained by 40X objective in a Leica microscope (DM500). The areas of each tissue were obtained using the Leica Image Analysis Software.

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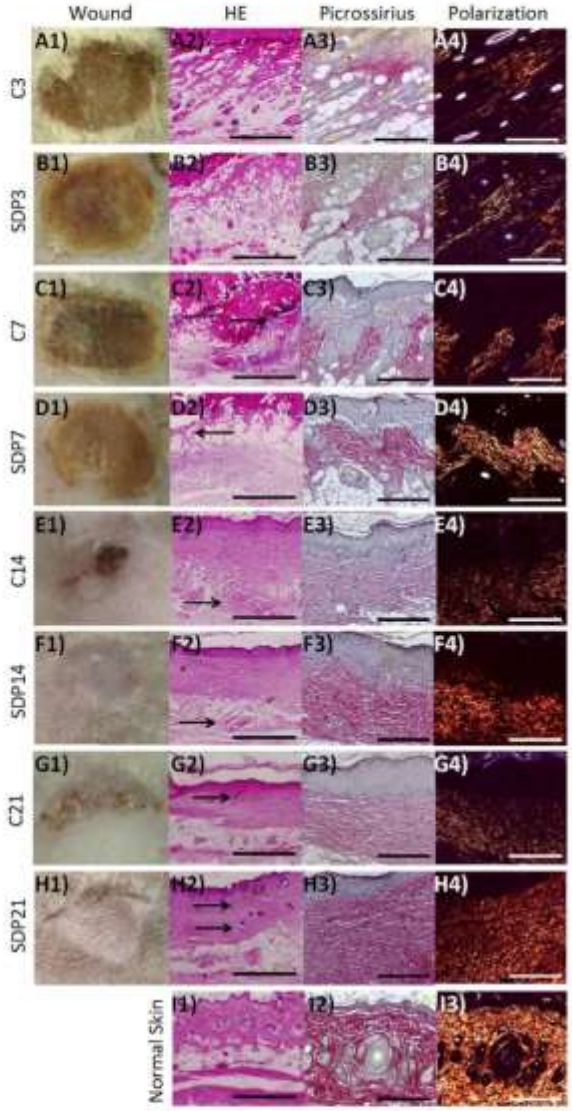

Fig 1. Macroscopic and histopathologic analysis of the healing process in a second degree burn. (a) C3 and (b) SDP3, inflammation phases, with leukocytes influx and presence of adipocytes in both groups. (c) C7 and (d) SDP7, proliferation phase with re-epithelialization (arrows). Up to 7 days weakly stained collagen fibers (a3, b3, c3 and d3) and low birefringent after polarization (a4, b4, c4 and d4) in the dermis of both groups. (e) C14 and (f) SDP14, complete reepithelialization and fast healing in SDP 1% group (e1 and f1). Dermis and hypodermis (e2, f2—arrows) are better organized and collagen fibers more strongly stained in the group treated with SDP 1% (e3, f3). (g) C21 and (h) SDP21, remodeling phase, the epithelial attachments (g1, h1, g2, h2—arrows) may be observed in larger amount and with increased birefringence under polarized light

in the groups treated with SDP 1% (g4 and h4). Normal Skin (i). (a1, b1, c1, d1, e1, f1, g1, h1 and i1) Macroscopic analisys. (a2, b2, c2, d2, e2, f2, g2, h2 and i2) Staining: HE. Bars 500 μm. Picrossirius staining without (a3, b3, c3, d3, e3, f3, g3, h3 and i3) and after (a4, b4, c4, d4, e4, f4, g4, h4 and i4) polarization. Bars 200μm.

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TNF-α levels were found in both groups compared to N ($p<0.001$) and also to previous phase (C7: 154.18 ± 27.14, $p<0.001$ *vs* C3; SDP7: 143.98 ± 31.91, $p<0.001$ *vs* SDP3; Fig 2A).

The IL-1β dosages indicated a significant increase in both groups, on days 3 and 7 compared to N group (N: 224.22 ± 21.21; C3: 530.64 ± 94.37; $p<0.05$; SDP3: 582.94 ± 99.01; $p<0.01$; C7: 641.79 ± 122.76; $p<0.01$; SDP7: 684.68 ± 140.04; $p<0.001$; Fig 2B). Interestingly, in the next period, there was a higher amount of this cytokine in the C14 group in relation to N ($p<0.001$) and reduction of these levels in SDP14 group compared to the previous phase ($p<0.05$) and the control group (C14: 901.29 ± 70.61; SDP14: 314.39 ± 79.83, $p<0.001$ *vs* C14; Fig 2B).

Regarding the IL-6, overexpression ($p<0.001$) of this cytokine was observed in the lesions collected on day 7 compared to N (N: 0.49 ± 0.02; C7: 9192.64 ± 2743.42; SDP7: 5100.97 ± 1600.30; Fig 2C), with lower dosage in SDP7 ($p<0.001$) compared to C7 group.

The quantification of IL-10 showed higher levels in the inflammation (N: 15.67 ± 12.76, C3: 157.19 ± 21.29, $p<0.05$; SDP3: 262.90 ± 68.59; $p<0.001$; Fig 2D) and cell proliferation phases (C7: 347.06 ± 63.59; $p<0.001$; SDP7: 202.02 ± 66.58; $p<0.01$; Fig 2D), in relation to N. Again, the treatment with SDP 1% reduced the cytokine levels on day 7 compared to control ($p<0.01$).

Finally, the dosages of the MCP-1 chemokine showed elevated levels ($p<0.001$) on days 3 and 7 of the healing process in relation to N (N: 100.27 ± 50.95; C3: 6525.44 ± 1569.07; SDP3: 8051.84 ± 1520.58; C7: 6774.98 ± 467.06; SDP7: 6796.24 ± 845.05; Fig 2E). Similarly, in the quantification of macrophages (Fig 2G), there was an increase ($p<0.001$) in the number of these cells in both groups 3 days after the injury compared to N (N: 16.15 ± 4.43; C3: 60 ± 19.41; SDP3: 75 ± 22.68; Fig 2F). However, in the next phase, only the SDP7 group presented numerous macrophages compared to N (SDP7 70 ± 5.77; $p<0.001$; Fig 2F).

The number and morphology of the MCs are modulated by treatment with SDP 1%

After confirming the lesion model, we quantified the MCs in the region of the lesion as evidenced by the Toluidine Blue dye and morphologically differentiate these cells between intact mast cells, that is, cells with well-defined contour and that are not in the clear process of releasing the contents of their cytoplasmic granules (Fig 3A and 3F), and degranulated MCs with irregular contours and dispersed granules (Fig 3B and 3C). Quantification of MCs showed a large number of intact cells in N (N: 74.91 ± 5.84; Fig 3A and 3G). On days 3 and 7, in both groups, there was a significant decrease in the total number of MCs compared to N (C3: 30.94 ± 4.89; SDP3: 18.28 ± 3.08; $p<0.001$: 80.01 ± 6.01; C7: 18.51 ± 8.12; SDP7: 39.55 ± 13.29 $p<0.01$; Fig 3B, 3C and 3G). In the SDP group, the MCs were observed mostly degranulated 7 days post injury (SDP7: 32.73 ± 22.55, $p<0.05$ *vs* N: 5.09 ± 1.97; Fig 3H).

In the final phases of repair, the amount of MCs increased only in C group. These cells were observed mostly degranulated 14 days post injury (C14: 59.88 ± 12.87 $p<0.05$ *vs* SDP14: 38.31 ± 9.55; Fig 3D and 3H) but intact after 21 days (C21: 53.75 ± 6.81 $p<0.001$ *vs* SDP21: 15.66 ± 5.66; Fig 3F and 3H).

Histamine storage by mast cells during healing

After observing the modulation promoted by SDP 1% in MCs, reducing their amount in the final stages of repair, as well as the number of degranulated MCs, we analyzed the histamine

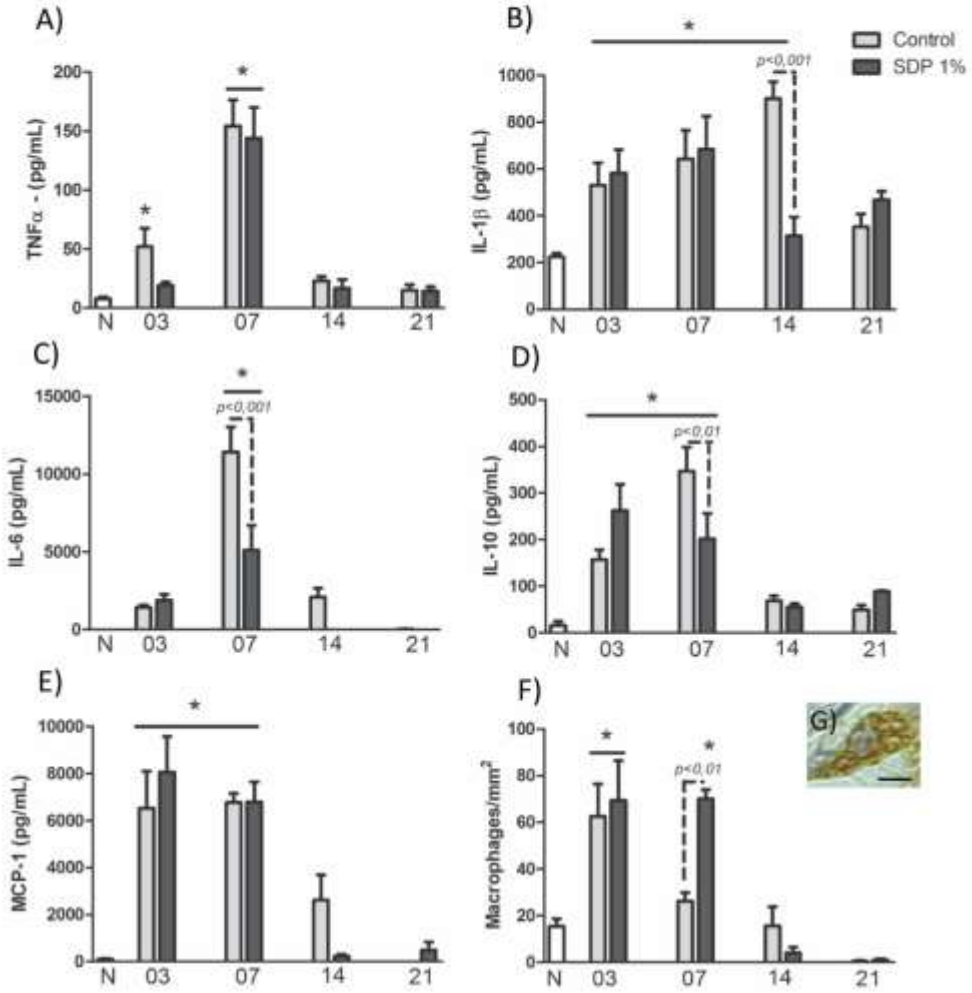

Fig 2. Cytokines and macrophages in wound healing in a second degree burn. (a) TNF-α: high dosages in C3 and in both groups after 7 days. (b) IL-1β: high levels in both groups on days 3 and 7 and also in C14. (c) IL-6: overexpression on day 7, especially in the control group. (d) IL-10: Increased levels on days 3 and 7. (e) MCP-1: overexpression on days 3 and 7. (f) Macrophages: numerous macrophages on day 3 and SDP7. (g): Macrophage. Counter-staining: Hematoxilin. Bar 2μm. Values are presented as mean ± S.E.M. (n = 5/group). * *p<0.05 vs* Normal Skin (N).

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accumulation in the citoplasmatic granules of MCs, during the tissue repair by reaction to S-O (Fig 3J) [(40)]. Then, the quantification of the TB+ MCs (Total MCs) was compared to the S-O + MCs (MCs with accumulation of histamine) (Fig 3I).

Numerous S-O+ MCs were observed in N (S-O+: 70 ± 7.01; Fig 3J). In the lesions, a marked difference between TB+ MCs and S-O+ MCs occurred on day 7 only in the SDP group (SDP7

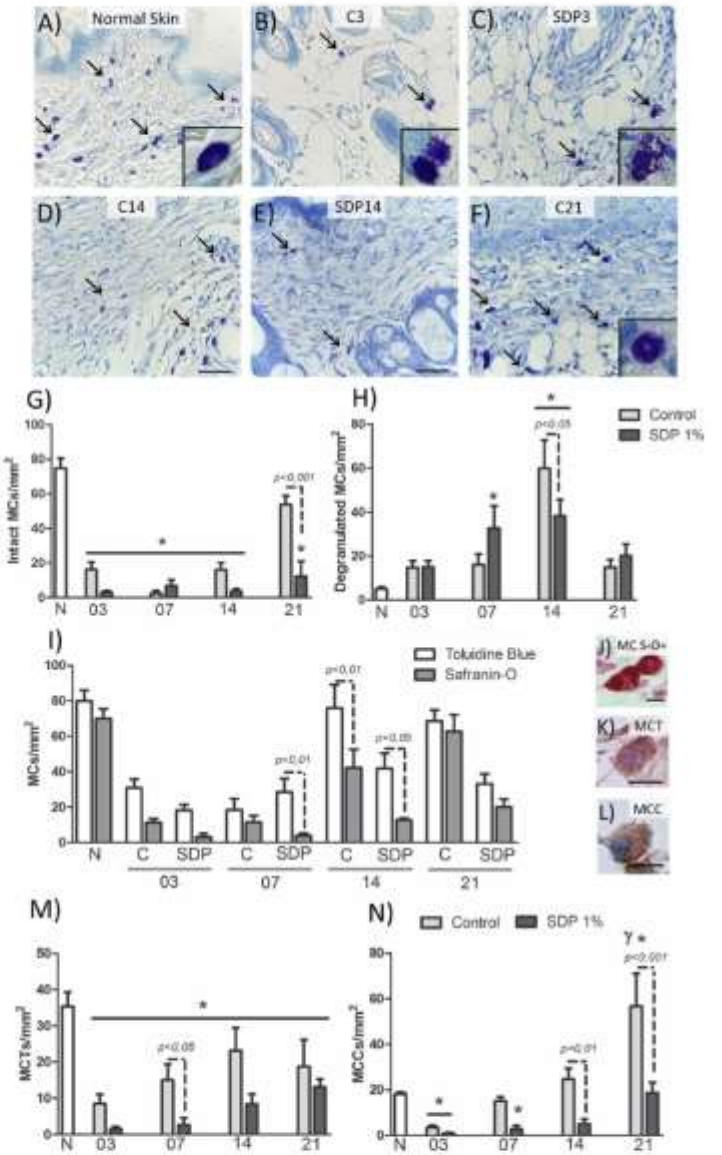

Fig 3. Morphology of MCs in burn healing process. (a) MCs (arrows), most intact in Normal Skin (N). (b) C3 and (c) SDP3 with few MCs and mostly degranulated. (d) C14 (e) SDP14, MCs degranulated, mainly in control group. (f) C21, numerous intact MCs. Details of intact (a and f) and degranulated MCs (b and c). Staining: Toluidine Blue. Bars 50 μm. Quantification of MCs: (g) numerous intact MCs in N and C21 and (h) degranulated MCs in SDP7 and in both groups on day 14. (i) Differences between quantification of MCs

evidenced by Toluidine blue or Safranin-O, with few MCs S-O+ (MCs histamine storage) in SDP7, C14 and SDP14 compared to the MC TB+ (total MCs in the tissue). (j) MC stained with Safranin-O (MC S-O+); (k) reactive MC for tryptase (MCT) and (l) chymase (MCC). Bars 50 μm. Heterogeneity of MCs, showing many MCTs (m) in N and numerous MCCs (n) in C21. Values are presented as mean ± S.E.M. ($n = 5$/group). * $p<0.05$ *vs* N and γ $p<0.001$ *vs* C14.

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TB+: 39.55 ± 29.73 $p<0.01$ *vs* SDP7 S-O+: 3.94 ± 1.28; Fig 3I) and on day 14 in both groups (C14 TB+: 76 ± 13.14 $p<0.01$ *vs* C14 S-O+: 42.17 ± 10.51; SDP14 TB+: 41.9 ± 11.01 $p<0.05$ *vs* SDP14 S-O+: 20.83 ± 5; Fig 3I). This difference between TB+ MCs and S-O+ MCs occurred in the same periods in which these cells were in degranulation process. It indicates little accumulation of histamine within MCs in these periods, possibly due to degranulation.

Treatment with the SDP 1% influences the heterogeneity of MCs

To analyze the heterogeneity of MCs during the healing process these cells were quantified after immunohistochemistry for the tryptase (MCT; Fig 2K) and chymase (MCC; Fig 2L). In the N group, the MCs were mostly tryptase + (MCT: 33.3 ± 4.79; Fig 3M MCC: 18.7 ± 2.1; Fig 3N).

At all stages of tissue repair a reduction of MCTs was observed in both groups compared to N, with greater differences ($p<0.001$) in the early stages (C3: 8.5 ± 2.57; SDP3: 1.5 ± 0.61; C7: 15 ± 5.68; SDP7: 2.5 ± 1.93; Fig 3M) than in the final stages of repair (C14: 18.65 ± 7.81; $p<0.05$ *vs* N; SDP14: 10.5 ± 4.56; $p<0.01$ *vs* N; C21: 18.75 ± 9.60; $p<0,05$ *vs* N; SDP21: 13.12 ± 2.77; $p<0.01$ *vs* N; Fig 3M).

On day 3 of the healing, there was a reduction in the number of MCCs ($p<0.001$), compared to N group (C3: 4 ± 1.27; SDP3: 0.87 ± 0.52; Fig 3N), however, after 7 days of injury, the amount of MCCs was reduced ($p<0.05$) only in SDP7 compared to N group (C7: 12.5 ± 4.67; SDP7: 2.8 ± 1.64; Fig 3N). On day 14, the treated group showed fewer MCCs ($p<0.01$) compared to C14 (C14: 24.75 ± 5.37; SDP14: 5 ± 2.23; Fig 3N). Finally, after 21 days of burn lesion, increased MCCs were found in C21 group in relation to the other groups (C21: 60 ± 21.21, $p<0.001$ *vs* N and $p<0.01$ *vs* C14; SDP21: 18.75 ± 5.90, $p<0.001$ *vs* C21; Fig 3N).

The expression of AnxA1 in the cytoplasm of MCs, epidermis and dermis varies during the wound healing

A reactivity for the AnxA1 protein was observed in the cytoplasm of MCs (Fig 4L and 4M). The densitometric analysis showed increased immunostaining for AnxA1 ($p<0.001$) in the phase of inflammation, in C3 and SDP3 groups compared to the others (Fig 4K). The specificity of the immunolabeling was checked by control of the reaction and the counterstaining with TB in serial sections.

In the tissues, there was reduced AnxA1 protein expression in the normal skin (Fig 4G, 4I and 4J). After burn, on day 3 (Fig 4A and 4B), the expression of AnxA1 was increased ($p<0.001$) in both groups in the epithelium, especially in keratinocytes (Fig 4I) and stroma (Fig 4J) compared to N, especially in the C3 group ($p<0.05$; Fig 4I and 4J).

On day 7, the AnxA1 expression remained increased with respect to N (Fig 4C and 4D), in regions of reepithelization (C7: $p<0.05$; SDP7: $p<0.001$; Fig 4I) and in the dermis ($p<0.001$; Fig 4J). However, the SDP7 group showed higher immunostaining for AnxA1 compared to C7 group ($p<0.05$; Fig 4I and 4J).

After 14 days of injury, the immunoreaction for AnxA1 was high only in the epithelium in C ($p<0.01$) and SDP groups ($p<0.05$) compared to N (Fig 4I). Thereafter, further increase of the protein expression occurred in SDP21 group (Fig 4F) compared to N and C21 groups

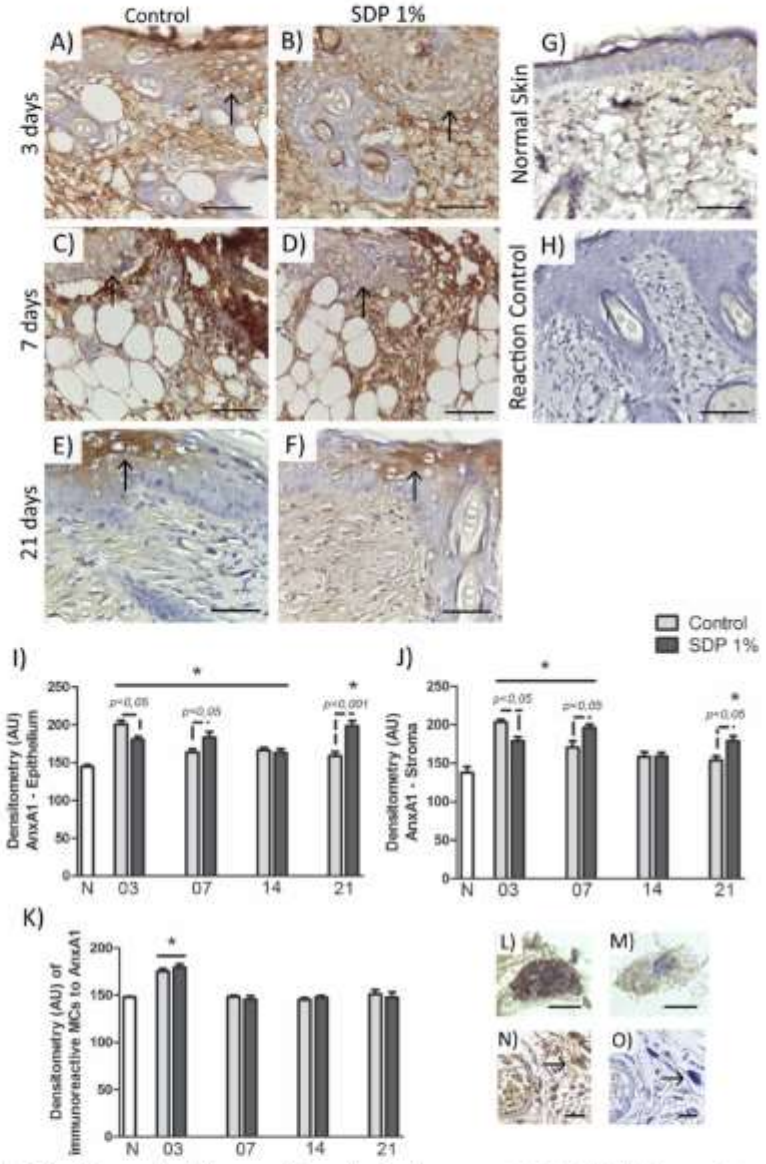

Fig 4. AnxA1 expression in the second degree burn healing process. (a) C3 and (b) SDP3, increased expression of the protein in the inflammation phase, 3 days post injury, in the surface epithelium (arrows) and dermis. (c) C7 and (d) SDP7, increased expression in the stroma and epithelium (arrows), 7 days post injury. (e) C21 and (f) SDP21 higher expression of AnxA1 in the group treated with SDP 1%, surface epithelium (arrows). (g) Low expression of AnxA1 in normal skin. (i) No immunoreactivity in the reaction control. Counter-staining:

hematoxylin. Bars 50μm. Optical densitometry of immunostained AnxA1 in (i) Epithelium and (j) Stroma. (k) Densitometric analysis of AnxA1 in the cytoplasm of MCs. Values are presented as mean ± S.E.M. (n = 5/group). Values $p<0.05$ *vs* Normal Skin (N). (l) strongly AnxA1 immunoreactive MC in the inflammatory phase. (m) weakly AnxA1 immunoreactive MC, remodeling phase. Bars 20 μm. (n) MC immunoreactive for AnxA1 and (o) the same cell in serial seccion with counterstaining of hematoxylin and toluidine blue, arrows indicate MCs. Bars 50 μm.

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(Fig 4E) in the dermis (SDP21: $p<0.001$ *vs* N; $p<0.05$ *vs* C21; Fig 4J) and epidermis ($p<0.001$ *vs* N and C21; Fig 4I) especially observed near the epithelial appendages development.

Discussion

Burns are complex traumas, with high morbidity and mortality rates [41,42] and generate strong economic and psychosocial impact due to treatment time [43].

In burn lesions, the interactions between inflammatory cells, chemical mediators, growth factors and ECM, coordinate the healing process, and angiogenesis is essential for tissue remodeling [4,44]. The MCs and the anti-inflammatory protein AnxA1 are important in the wound repair and angiogenesis processes [10,34,35,45]. However, the biological roles of MCs and AnxA1 are still poorly explored in burns. For these reasons, in the present study we investigated the heterogeneity of MCs and the expression of AnxA1 during the healing process of second degree lesions in control animals and animals treated with SDP 1%.

Histopathological findings confirmed the depth of the injury as a superficial second degree burn [38] and also the best regeneration in the treated group [36,46]. Furthermore, on day 21 of the repair process, the analysis of the lesions showed formation of epithelial appendages, especially in the SDP group. The appendages are functional parts of the skin, therefore, a fast regeneration is important to the restoration of normal anatomy, structure and function [4].

Next, knowing the importance of the inflammatory mediators, levels of TNF-α, IL-1β, IL-6, IL-10 and MCP-1 were quantified in the supernatant of the macerated fragments. On days 3 and 7, the dosages of IL-1β, IL-10 and MCP-1 were increased compared to normal skin. Differently, the higher levels of IL-6 were observed only 7 days post injury. The TNF-α dosages were elevated in both burned groups on day 7, but only in the control group on day 3. Increased levels of IL-1β were also found in the control group 14 days after injury. The results of our measurements are consistent with other studies in skin burns [5–9,47].

The pro-inflammatory cytokines TNF-α, IL-1β and IL-6 and the chemokine MCP-1 are important for leukocyte recruitment and reepithelialization [8]; nevertheless, the prolonged inflammation can be harmful [36]. However, our evaluations showed that the treatment with SDP 1% decreased levels of IL-6 and IL-1β and increased the dosage of MCP-1 on day 7. Besides, the macrophage quantification followed the cytokines levels. In an *in vitro* study, the dosage of MCP-1 was decreased in fibroblasts derived from keloids, emphasizing the protective role of MCP-1 in the healing process [48]. This chemokine is released primarily by keratinocytes near the injury and recruits monocytes, macrophages, lymphocytes and also MCs [8,9].

After verifying that the model used in this study was suitable for the induction of second degree burns, we proceed to the analysis of MCs. The MCs were observed in large quantities and mainly intact in the dermis of the normal skin, with a significant reduction of total number on days 3 and 7 after injury, as in other investigations [3,12,14]. In addition, mice deficient in MCs presented an impaired skin wound closure, indicating the importance of the mediators of these cells to this process [49].

Later, in the following phases, there was an increase of MCs number in the untreated group, as also observed in other studies [12,14]. The MCs were found especially degranulated

on day 14, but intact 21 days after injury in the control group. The reduction of these cells in the treated group may be related to the leukopenic property of SDP, due to cytotoxicity promoted by the drug components to stem cells from bone marrow [36,50].

Several *in vivo* and *in vitro* studies have linked the increased presence of MCs with the proliferation of fibroblasts and formation of keloids [51–55]. Thus, the control of MCs proliferation in the repair of the skin lesion is an important therapeutic strategy in the treatment of burn patients. Still, in the later stages of healing, a decrease in the number of macrophages and an increase in the number of MCs [3] were observed, characteristics which were also found in our cell quantifications.

With that in mind, following the investigation, we studied the histamine accumulation and heterogeneity of MCs. The process of histamine accumulation was verified by S-O+ MCs, comparing these cells to TB+ MCs. Our results showed differences in the number of TB+ and S-O+ MCs in the same periods in which these cells were found to be active, corroborating that the MCs were still releasing the content of their granules in these periods.

Regarding the heterogeneity of MCs for tryptase and chymase, after 3 days of burn, decreased immunoreactive cells were observed for both proteases. The reduction in the number of MCTs remained in all examined periods of wound healing. These results are in agreement to another study [56] that showed the reduction of MCTs and MCCs in human skin recent wounds. The same study found increased tryptase activity in older scars when compared to normal human skin. In addition, tryptase is considered a biomarker for mastocytosis [57]. Given the MCCs, our analysis showed reduced cells in the initial phases, but numerous MCCs were observed in the lesions from day 14. In the lesions of the untreated groups, the increased MCCs in the late phases of repair match with the elevated IL-1β levels found in our quantifications and are in line with studies that indicate the involvement of chymase in the cleavage of pro-IL-1β to IL-1β, activating it [58]. Furthermore, these results indicate the relation between the MC histamine storage and chymase accumulation and are in agreement with a study that demonstrated the importance of histamine to the maturation of MCs and accumulation of proteases [40]. Additionally, other *in vitro* study demonstrated that during MC maturation process, the chymase is expressed by most cells and in larger quantities than tryptase [59], indicating again that, in our model, these cells are in the process of infiltration and maturation in the newly regenerated tissue.

Besides that, several studies associate the chymase to the formation of fibrosis and keloids [10,52,53,60,61]. While other investigations indicate that both tryptase and chymase are important for collagen degradation [13,49,56,62]. Thus, our results reinforce that the heterogeneity of MCs, in different periods and in response to treatment with SDP 1%, is related to their function in the cellular microenvironment, indicating that the MCs actively participate in the remodeling phase of the repair process.

Continuing our study, we evaluated the expression of AnxA1 in the MCs cytoplasm, during tissue healing and normal skin. Our evaluations showed strong MC immunoreactivity for the protein, mainly on day 3 in the inflammatory phase in both groups. These results are in accordance to different investigations that also showed the presence of AnxA1 in the granules of MCs [17–19,63] with increased synthesis during the inflammatory processes [63].

Due to the importance of AnxA1, demonstrated by the pharmacological treatment with the mimetic peptide Ac2-26 in a skin allograft model in rats that increased the survival of the transplantation related to inhibition of neutrophil transmigration and induction of apoptosis, reducing tissue damage, [34] and also by the application of hydrogels containing the AnxA1 peptide that provided rapid healing of dorsal wounds in mice [35], we analyzed the expression of Anxa1 in the epidermis, especially in keratinocytes, and in the dermis during the healing process on burns. In the tissue evaluations, a weak immunostaining was found in normal skin,

in accordance with previous studies [24,64]. However, an increased AnxA1 expression was observed in both groups 3 and 7 days after injury, which was observed strongly expressed in the dermis and in reepithelialization. These results relate the expression of AnxA1 to the inflammatory process, corroborating with other researches in different models of inflammation [20,65], and are also in agreement with studies that have shown the participation of AnxA1 in the process of cellular proliferation [21] and epidermal proliferation [24]. Other studies have indicated the importance of AnxA1 in different pathological processes, such as in inflammations and neoplasias [26,64], in the intestinal mucosa during the closure of intestinal epithelium lesions [28–30], migration and proliferation of endothelial cells [22], differentiation of keratinocytes [24] and their stratification and keratinization [66] and also in the motility of fibroblasts [67].

In the remodeling phase, on day 21, higher immunostaining for AnxA1 was observed in the SDP group, both in the stroma and epithelial regions, especially close to the neogenesis of hair follicles and glands. The presence of AnxA1 was detected in the duct of sweat glands, demonstrated by in situ hybridization, in another research [64]. In addition, studies have contributed to the understanding of the AnxA1 antifibrotic properties in the matrix of different organs, by decreasing inflammation and subsequent development of fibrosis [68] or by acting directly on the phenotype of fibroblasts, reducing the expression of collagen, α-smooth muscle actin (α-SMA) and the growth transformation factor (TGF-β1) [69,70]. Therefore, our results indicate the role of AnxA1 in tissue repair in burns, both in epithelial regeneration as in the protection against keloid formation.

Moreover, IL-6 and TNF-α can be linked to AnxA1 expression [71]. Although IL-6 can induce the expression of AnxA1 [19], in turn, the AnxA1 can equally reduce TNF-α and IL-6 expressions [71], which explains the modulation of these cytokines during the repair process after 7 days of injury and in the SDP group, when the AnxA1 expression was increased. Our observations also indicated increase in IL-10 levels in the phases of inflammation and cell proliferation, that is again coincident with the phases in which the AnxA1 protein expression is higher in the lesions. While pro-inflammatory cytokines perpetuate inflammation, other mechanisms operate to regulate this activity, such as IL-10 [8] and AnxA1 protein [21,23]. Thus, our results suggest that AnxA1 protein and IL-10 act together in the burn healing process.

In general, our investigation revealed that degranulation of MCs in the early phases of wound healing is important for modulation of the microenvironment with the secretion of proteases and the AnxA1 protein, which may stimulate the release of chemical mediators such as TNF-α, IL-1β, IL-6, IL-10 and MCP-1. Furthermore, our analysis showed that treatment with SDP 1% promotes slower repopulation of the tissue by MCs, controlling mainly MCCs, which may exert a protective effect against the formation of keloids. In addition, our studies revealed increased expression of the protein AnxA1 especially in the treated group, concomitant with keratinocytes proliferation and differentiation and also stromal remodeling, with the possibility of anti-fibrotic action.

Altogether, our data show modulation in the number, degranulation state, maturity and heterogeneity of MCs, and the relation of these cells with the AnxA1 and cytokines expression during the tissue repair process in a model of second degree burn. However, additional studies are needed to better understand the biological role of MCs and their mediators, the AnxA1 protein among them, during the phases of skin repair in burns in the search for new therapeutic tools.

Author Contributions

Conceptualization: HRS SMO APG.

Data curation: HRS LA SMO APG.

Formal analysis: HRS APG.

Funding acquisition: SMO APG.

Investigation: HRS APG.

Methodology: HRS LA LP SSC MMIP.

Project administration: HRS SMO APG.

Resources: SMO APG.

Supervision: SMO APG.

Validation: HRS SMO APG.

Visualization: HRS SMO APG.

Writing – original draft: HRS APG.

Writing – review & editing: HRS SMO APG.

References

1. Stroncek JD, Reichert WM. Overview of Wound Healing in Different Tissue Types. Indwelling Neural Implant Strateg Contend with Vivo Environ. 2007; pp. 3–33. http://www.ncbi.nlm.nih.gov/books/NBK3938/
2. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. Nature. 2008; 453: 314–21. doi: 10.1038/nature07039 PMID: 18480812
3. Ehrlich HP. A Snapshot of Direct Cell-Cell Communications in Wound Healing and Scarring. Adv wound care. 2013; 2: 113–21.
4. Takeo M, Lee W, Ito W. Wound Healing and Skin Regeneration. Cold Spring Harb Perspect Med. 2015; 5 1–13.
5. Kubo H, Hayashi T, Ago K, Ago M, Kanekura T, Ogata M. Temporal expression of wound healing-related genes in skin burn injury. Leg Med. 2014; 16: 8–13.
6. Oppeltz RF, Rani M, Zhang Q, Schwacha MG. Gamma delta (γδ) T-cells are critical in the up-regulation of inducible nitric oxide synthase at the burn wound site. Cytokine. 2012; 60: 528–34. doi: 10.1016/j.cyto.2012.07.003 PMID: 22831879
7. Gauglitz GG, Song J, Herndon DN, Finnerty CC, Boehning D, Barral JM, et al. Characterization of the Inflammatory Response During Acute and Post-Acute Phases After Severe Burn. Shock. 2008; 30: 503–7. doi: 10.1097/SHK.0b013e31816e3373 PMID: 18391855
8. Gillitzer R, Goebeler M. Chemokines in cutaneous wound healing. J Leukoc Biol. 2001; 69: 513–21. PMID: 11310836
9. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen. 2008; 16: 585–601. doi: 10.1111/j.1524-475X.2008.00410.x PMID: 19128254
10. Dong X, Geng Z, Zhao Y, Chen J, Cen Y. Involvement of mast cell chymase in burn wound healing in hamsters. Exp Ther Med. 2013; 5: 643–7. doi: 10.3892/etm.2012.836 PMID: 23408248
11. Douaiher J, Succar J, Lancerotto L, Gurish MF, Orgill DP, Hamilton MJ, et al. Development of mast cells and importance of their tryptase and chymase serine proteases in inflammation and wound healing. Adv Immunol. 2014; 122: 211–52. doi: 10.1016/B978-0-12-800267-4.00006-7 PMID: 24507159
12. Nishikori Y, Kakizoe E, Kobayashi Y, Shimoura K, Okunishi H, Dekio S. Skin mast cell promotion of matrix remodeling in burn wound healing in mice: relevance of chymase. Arch Dermatol Res. 1998; 290: 553–60. PMID: 9836506
13. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. Nat Rev Immunol. Nat Rev Immunol. 2014; 14: 478–94. doi: 10.1038/nri3690 PMID: 24903914
14. Bayat M, Vasheghani MM, Razavie N, Jalili MR. Effects of low-level laser therapy on mast cell number and degranulation in third-degree burns of rats. J Rehabil Res Dev. 2008; 45: 931–8. PMID: 19009479
15. Bischoff SC. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. Nat Rev Immunol. 2007; 7: 93–104. doi: 10.1038/nri2018 PMID: 17259966

16. Welle M. Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. J Leukoc Biol. 1997; 61: 233–45. PMID: 9060446
17. Oliani SM, Christian HC, Manston J, Flower RJ, Perretti M. An immunocytochemical and in situ hybridization analysis of annexin 1 expression in rat mast cells: modulation by inflammation and dexamethasone. Lab Invest. 2000; 80:1429–38. PMID: 11005211
18. Paula R, Oliani AH, Vaz-Oliani DCM, D'Ávila SCGP, Oliani SM, Gil CD. The intricate role of mast cell proteases and the annexin A1-FPR1 system in abdominal wall endometriosis. J Mol Histol. 2015; 46: 33–43. doi: 10.1007/s10735-014-9595-y PMID: 25201101
19. D'Acquisto F, Perretti M, Flower RJ. Annexin-A1: a pivotal regulator of the innate and adaptive immune systems. Br J Pharmacol. 2008; 155: 152–69. doi: 10.1038/bjp.2008.252 PMID: 18641677
20. Girol AP, Mimura KKO, Drewes CC, Bolonheis SM, Solito E, Farsky SHP, et al. Anti-inflammatory mechanisms of the annexin A1 protein and its mimetic peptide Ac2-26 in models of ocular inflammation in vivo and in vitro. J Immunol. 2013; 190: 5689–701. doi: 10.4049/jimmunol.1202030 PMID: 23645879
21. Lim LHK, Pervaiz S. Annexin 1: the new face of an old molecule. FASEB J. 2007; 21: 968–75. doi: 10.1096/fj.06-7464rev PMID: 17215481
22. Côté MC, Lavoie JR, Houle F, Poirier A, Rousseau S, Huot J. Regulation of vascular endothelial growth factor-induced endothelial cell migration by LIM kinase 1-mediated phosphorylation of annexin 1. J Biol Chem. 2010; 285: 8013–21. doi: 10.1074/jbc.M109.098665 PMID: 20061392
23. Gavins FNE, Hickey MJ. Annexin A1 and the regulation of innate and adaptive immunity. Front Immunol. 2012; 3: 1–11.
24. Bastian BC, van der Piepen U, Römisch J, Pâques EP, Bröcker EB. Localization of annexins in normal and diseased human skin. J Dermatol Sci. 1993; 6: 225–34. PMID: 8136320
25. Oliani SM, Ciocca GAP, Pimentel TA, Damazo AS, Gibbs L, Perretti M. Fluctuation of annexin-A1 positive mast cells in chronic granulomatous inflammation. Inflamm Res. 2008; 57: 450–6. doi: 10.1007/s00011-008-7222-7 PMID: 18827967
26. Boudhraa Z, Rondepierre F, Ouchchane L, Kintossou R, Trzeciakiewicz A, Franck F, et al. Annexin A1 in primary tumors promotes melanoma dissemination. Clin Exp Metastasis. 2014; 31: 749–60. doi: 10.1007/s10585-014-9665-2 PMID: 24997993
27. Meng Z, Shi Z-R, Tan G-Z, Yin J, Wu J, Mi X-B, et al. The association of anti-annexin1 antibodies with the occurrence of skin lesions in systemic lupus erythematosus. Lupus. 2014; 23: 183–7. doi: 10.1177/0961203313513820 PMID: 24300781
28. Leoni G, Alam A, Neumann P, Lambeth JD, Cheng G, Mccoy J, et al. Annexin A1, formyl peptide receptor, and NOX1 orchestrate epithelial repair. J Clin Invest. 2013; 123: 1–12.
29. Leoni G, Neumann P, Kamaly N, Quiros M, Nishio H, Jones HR, et al. Annexin A1 –containing extracellular vesicles and polymeric nanoparticles promote epithelial wound repair. J Clin Invest. 2015; 125: 1–13.
30. Leoni G, Neumann P-A, Sumagin R, Denning TL, Nusrat A. Wound repair: role of immune-epithelial interactions. Mucosal Immunol. 2015; 8: 959–68. doi: 10.1038/mi.2015.63 PMID: 26174765
31. Silva HAL, De Lima GS, Boité MC, Porrozzi R, Hueb M, Damazo AS. Expression of annexin A1 in leishmania-infected skin and its correlation with histopathological features. Rev Soc Bras Med Trop. 2015; 48: 560–7. doi: 10.1590/0037-8682-0183-2015 PMID: 26516965
32. Saito N, Qiao H, Yanagi T, Shinkuma S, Nishimura K, Suto A, et al. An annexin A1-FPR1 interaction contributes to necroptosis of keratinocytes in severe cutaneous adverse drug reactions. Sci Transl Med. 2014; 6: 245–95.
33. Bizzarro V, Fontanella B, Carratù A, Belvedere R, Marfella R, Parente L, et al. Annexin A1 N-Terminal Derived Peptide Ac2-26 Stimulates Fibroblast Migration in High Glucose Conditions. PLoS One. 2012; 7: 1–9.
34. Teixeira RAP, Mimura KKO, Araujo LP, Greco KV, Oliani SM. The essential role of annexin A1 mimetic peptide in the skin allograft survival. J Tissue Eng Regen Med. 2013; 10: 524–31.
35. Del Gaudio P, De Cicco F, Aquino RP, Picerno P, Russo P, Dal Piaz F, et al. Evaluation of in situ injectable hydrogels as controlled release device for ANXA1 derived peptide in wound healing. Carbohydr Polym. 2015; 115: 629–33. doi: 10.1016/j.carbpol.2014.09.040 PMID: 25439941
36. Atiyeh BS, Costagliola M, Hayek SN, Dibo S a. Effect of silver on burn wound infection control and healing: Review of the literature. Burns. 2007; 33: 139–48. doi: 10.1016/j.burns.2006.06.010 PMID: 17137719
37. Marx DE, Barillo DJ. Silver in medicine: The basic science. Burns. 2014; 40: S9–18. doi: 10.1016/j.burns.2014.09.010 PMID: 25418438

38. Meyerholz DK, Piester TL, Sokolich JC, Zamba GKD, Light TD, David K. Morphological parameters for assessment of burn severity in an acute burn injury rat model. Int J Exp Path. 2009; 90:26–33.
39. Feyerabend TB, Hausser H, Tietz A, Blum C, Hellman L, Straus AH, et al. Loss of Histochemical Identity in Mast Cells Lacking Carboxypeptidase A Loss of Histochemical Identity in Mast Cells Lacking Carboxypeptidase A. Society. 2005; 25: 6199–210.
40. Nakazawa S, Sakanaka M, Furuta K, Natsuhara M, Takano H, Tsuchiya S, et al. Histamine synthesis is required for granule maturation in murine mast cells. Eur J Immunol. 2014; 44: 204–14. doi: 10.1002/eji.201343838 PMID: 24002822
41. Summer GJ, Puntillo KA, Miaskowski C, Green PG, Levine JD. Burn Injury Pain: The Continuing Challenge. J Pain. 2007; 8: 533–48. doi: 10.1016/j.jpain.2007.02.426 PMID: 17434800
42. Parihar A, Parihar MS, Milner S, Bhat S. Oxidative stress and anti-oxidative mobilization in burn injury. Burns. 2008; 34: 6–17. doi: 10.1016/j.burns.2007.04.009 PMID: 17905515
43. Richardson P, Mustard L. The management of pain in the burns unit. Burns. 2009; 35:921–36. doi: 10.1016/j.burns.2009.03.003 PMID: 19505764
44. Evers LH, Bhavsar D, Mailänder P. The biology of burn injury. Exp Dermatol. 2010; 19: 777–83. doi: 10.1111/j.1600-0625.2010.01105.x PMID: 20629737
45. Guo C, Liu S, Sun M-Z. Potential role of Anxa1 in cancer. Future Oncol. 2013; 9: 1773–93. doi: 10.2217/fon.13.114 PMID: 24156336
46. Bayat M, Vasheghani MM, Razavi N, Taheri S, Rakhshan M. Effect of low-level laser therapy on the healing of second-degree burns in rats: A histological and microbiological study. J Photochem Photobiol B Biol. 2005; 78: 171–7.
47. Babcock GF, Hernandez L, Yadav E, Schwemberger S, Dugan A. The burn wound inflammatory response is influenced by midazolam. Inflammation. 2012; 35: 259–70. doi: 10.1007/s10753-011-9313-9 PMID: 21400122
48. Yeh F-L, Shen H-D, Tai H-Y. Decreased production of MCP-1 and MMP-2 by keloid-derived fibroblasts. Burns. 2009; 35: 348–51. doi: 10.1016/j.burns.2008.06.018 PMID: 18950946
49. Weller K, Foitzik K, Paus R, Syska W, Maurer M. Mast cells are required for normal healing of skin wounds in mice. FASEB J. 2006; 20: 2366–8. doi: 10.1096/fj.06-5837fje PMID: 16966487
50. Gamelli RL, Paxton TP, O'Reilly M. Bone marrow toxicity by silver sulfadiazine. Surg Gynecol Obstet. 1993; 177: 115–20. PMID: 8342089
51. Levi-Schaffer F, Rubinchik E. Activated mast cells are fibrogenic for 3T3 fibroblasts. J Invest Dermatol. 1995; 104: 999–1003. PMID: 7769271
52. Dong X, Zhang C, Ma S, Wen H. High concentrations of mast cell chymase facilitate the transduction of the transforming growth factor-β1/Smads signaling pathway in skin fibroblasts. Exp Ther Med. 2015; 9: 955–60. doi: 10.3892/etm.2015.2216 PMID: 25667659
53. Dong X, Zhang C, Ma S, Wen H. Mast cell chymase in keloid induces profibrotic response via transforming growth factor-β1/Smad activation in keloid fibroblasts. Int J Clin Exp Pathol. 2014; 7: 3596–607. PMID: 25120737
54. Harunari N, Zhu KQ, Armendariz RT, Deubner H, Muangman P, Carrougher GJ, et al. Histology of the thick scar on the female, red Duroc pig: final similarities to human hypertrophic scar. Burns. 2006; 32: 669–77. doi: 10.1016/j.burns.2006.03.015 PMID: 16905264
55. Huttunen M, Aalto ML, Harvima RJ, Horsmanheimo M, Harvima IT. Alterations in mast cells showing tryptase and chymase activity in epithelializating and chronic wounds. Exp Dermatol. 2000; 9: 258–65. PMID: 10949547
56. Hermes B, Feldmann-Böddeker I, Welker P, Algermissen B, Steckelings MU, Grabbe J, et al. Altered expression of mast cell chymase and tryptase and of c-Kit in human cutaneous scar tissue. J Invest Dermatol. 2000; 114: 51–5. doi: 10.1046/j.1523-1747.2000.00837.x PMID: 10620115
57. Pejler G, Rönnberg E, Waern I, Wernersson S, Dc W, Ro E. Mast cell proteases: multifaceted regulators of inflammatory disease. Blood. 2011; 115: 4981–90.
58. Gabay C, Lamacchia C, Palmer G. IL-1 pathways in inflammation and human diseases. Nat Rev Rheumatol. 2010; 6: 232–41. doi: 10.1038/nrrheum.2010.4 PMID: 20177398
59. Ma F, Kambe N, Wang D, Shinoda G, Fujino H, Umeda K, et al. Direct development of functionally mature tryptase/chymase double-positive connective tissue-type mast cells from primate embryonic stem cells. Stem Cells. 2008; 26: 706–14. doi: 10.1634/stemcells.2007-0348 PMID: 17991916
60. Artuc M, Steckelings UM, Grützkau A, Smorodchenko a, Henz BM. A long-term coculture model for the study of mast cell-keratinocyte interactions. J Invest Dermatol. 2002; 119: 411–5.

61. Dong X, Chen J, Zhang Y, Cen Y. Mast cell chymase promotes cell proliferation and expression of certain cytokines in a dose-dependent manner. Mol Med Rep. 2012; 5: 1487–90. doi: 10.3892/mmr.2012.851 PMID: 22470087

62. Saarinen J, Kalkkinen N, Welgus HG, Kovanen PT. Activation of human interstitial procollagenase through direct cleavage of the Leu83-Thr84 bond by mast cell chymase. J Biol Chem. 1994; 269: 18134–40. PMID: 8027075

63. Kamal AM, Flower RJ, Perretti M. An overview of the effects of annexin 1 on cells involved in the inflammatory process. Mem Inst Oswaldo Cruz. 2005; 100(SUPPL. 1): 39–48.

64. Sato-Matsumura KC, Koizumi H, Matsumura T, Ohkawara A, Takasu T, Furuta Y, et al. Localization of annexin I (lipocortin I, p35) mRNA in normal and diseased human skin by in situ hybridization. ArchDermatolRes. 1996; 288: 565–9.

65. Patel N. Fat Injection in Severe Burn Outcomes: A New Perspective of Scar Remodeling and Reduction. Aesthetic Plast Surg. 2008; 32: 470–2. doi: 10.1007/s00266-008-9120-3 PMID: 18351415

66. Sato-Matsumura K, Matsumura T, Nakamura H, Sawa H, Nagashina K, Koizumi H. Membrane expression of annexin I is enhanced by calcium and TPA in cultured human keratinocytes. Arch Dermatol Res. 2000; 292: 496–9. PMID: 11142771

67. Bizzarro V, Fontanella B, Carratù A, Belvedere R, Marfella R, Parente L, et al. Annexin A1 N-Terminal Derived Peptide Ac2-26 Stimulates Fibroblast Migration in High Glucose Conditions. PLoS One. 2012; 7(9):1–9.

68. amazo AS, Sampaio AL, Nakata CM, Flower RJ, Perretti M, Oliani SM. Endogenous annexin A1 counter-regulates bleomycin-induced lung fibrosis. BMC Immunol. 2011; 12: 1–13.

69. Neymeyer H, Labes R, Reverte V, Saez F, Stroh T, Dathe C, et al. Activation of annexin A1 signalling in renal fibroblasts exerts antifibrotic effects. Acta Physiol. 2015; 215:144–58.

70. Trentin PGT Ferreira TP, Arantes aCS, Ciambarella BT, Cordeiro RSB, Flower RJ, et al. Annexin A1-mimetic peptide controls the inflammatory and fibrotic effects of silica particles in mice. Br J Pharmacol. 2015; 172: 3058–71. doi: 10.1111/bph.13109 PMID: 25659822

71. Jia Y, Morand EF, Song W, Cheng Q, Stewart A, Yang YH. Regulation of lung fibroblast activation by annexin A1. J Cell Physiol. 2013; 228: 476–84. doi: 10.1002/jcp.24156 PMID: 22777765

8. ANEXOS

Anexo 1. Formulário de apreciação do Comitê de Ética em Pesquisa FCF/USP

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Comissão de Ética no Uso de Animais - CEUA

Ofício CEUA/FCF **44.2015-P500**

CERTIFICADO

A Comissão de Ética no Uso de Animais, da Faculdade de Ciências Farmacêuticas, da Universidade de São Paulo, **CERTIFICA** que o Projeto de Pesquisa "**Modulação da proteína anexina A1 em modelos experimentais de inflamação intestinal e angiogênese**" (Protocolo CEUA/FCF/500), de responsabilidade do(a) pesquisador(a) **Profa. Dra. Sandra Helena Poliselli Farsky**, está de acordo com as normas do Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi APROVADO em reunião de **03 de julho de 2015**. Conforme a legislação vigente, deverá ser apresentado, no encerramento deste Projeto de Pesquisa, o respectivo **relatório final**.

São Paulo, 6 de julho de 2015.

Prof. Dr. Joilson de Oliveira Martins
Coordenador da CEUA/FCF/USP

Av. Prof. Lineu Prestes, 580, Bloco 13 A, Cidade Universitária, CEP 05508-900, São Paulo, SP
Telefone: (11) 3091 3622 - e-mail: ceuafcf@usp.br

Anexo 2. Formulário de apreciação do Comitê de Ética em Pesquisa EERP/USP

UNIVERSIDADE DE SÃO PAULO
ESCOLA DE ENFERMAGEM DE RIBEIRÃO PRETO

Centro Colaborador da OPAS/OMS para o Desenvolvimento da Pesquisa em Enfermagem

Avenida Bandeirantes, 3900 - Ribeirão Preto - São Paulo - Brasil - CEP 14040-902
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www.eerp.usp.br - eerp@eerp.br

CERTIFICADO

Certificamos que o projeto intitulado "Atividade da fosfoetanolamina sintética em modelo de carcinogênese experimental", protocolo nº 12.1.124.20.2, sob responsabilidade de Lucas Ribeiro de Azevedo e Gabriela Silva Bisson que envolve a manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica encontra-se de acordo com os preceitosa da Lei nº 11,794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Prefeitura do Campus de Ribeirão Preto (CEUA PUSP-RP) na Reunião Ordinária da CEUA de 10/08/2012.

Vigência do Projeto	01/08/2012 a 31/07/2013
Espécie/Linhagem e Nº de animais	Camundongo heterogênico C57/BL6
Nº de animais	120
Idade/Peso	6 a 8 semanas /25 gramas
Sexo	Macho
Origem	Biotério Central PUSP-RP

Ribeirão Preto, 10 de agosto de 2012.

Profª Drª Angelita Maria Stabile
Vice Presidente da CEUA EERP

Anexo 3. Formulário de apreciação do Comitê de Ética em Pesquisa IBILCE/UNESP

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Câmpus de São José do Rio Preto

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – IBILCE/UNESP-CSJRP

CERTIFICADO

Certificamos que o projeto/disciplina de pesquisa intitulado "**Análise morfofenotípica e funcional dos mastócitos no câncer colorretal**", protocolo nº. 118/2015 – CEUA, sob a responsabilidade da Professora Doutora Sonia Maria Oliani, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata para fins de pesquisa científica (ou de ensino), encontra-se de acordo com os Preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6899, de 15 de julho de 2009, e com as normas editadas pelo Conselho de Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), do IBILCE/UNESP, em reunião de 01 de setembro de 2015.

Vigência do Projeto	01 de setembro de 2013 a 03 de março de 2017
Espécie/linhagem	Camundongo heterogênico (Balb/c) Camundongo *Knockout* (Balb/c)
Nº de animais	24 camundongos heterogênicos (Balb/c) 24 camundongos *Knockout* (Balb/c)
Peso/idade	De 20 a 25 gramas De 8 a 10 semanas
Sexo	48 fêmeas
Origem	Biotério USP/FCF

São José do Rio Preto, 13 de setembro de 2013.

Prof. Dr. Luiz Henrique Florindo
Presidente da CEUA

Instituto de Biociências, Letras e Ciências Exatas – Comissão de Ética no Uso de Animais
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Anexo 4. Comprovante de submissão do manuscrito

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Detailed Status Information

Manuscript #	-2017-0XXXX
Current Revision #	0
Current Stage	Under consideration
Title	Secreted annexin A1 modulates mast cell pro-tumoural actions in N-methyl-N'-nitro-N-nitrosoguanidine-induced colorectal cancer
Running Title	MCs and AnxA1 in MNNG-induced carcinogenesis
Manuscript Type	Full Paper
Corresponding Author	Prof. Sonia Oliani (smoliani@ibilce.unesp.br) (IBILCE/UNESP)
Contributing Authors	Dr. Lucas Azevedo (IBILCE/UNESP) , Mrs. Marina Silva (USP) , Dr. Diego Miranda (EERP/USP) , Dr. Gabriela Pereira-da-Silva (EERP/USP) , Prof. Sandra Farsky (USP)
Abstract	Background: Colorectal cancer (CRC) is a frequently occuring disease in western countries with poor prognosis. Mast cells (MCs) are involved in carcinogens-associated damage in the gastrointestinal tract; therefore, knowledge of MCs recruitment and functions is pivotal to comprehension of CRC genesis. Annexin A1 (AnxA1) is a protein expressed by MCs that modulates gastrointestinal tumoural development. Here we evaluated the role of AnxA1 in the functional response of MCs in CRC carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Methods: Wild type (AnxA1+/+) and AnxA1-deficient (AnxA1-/-) male Balb-c mice received intra-rectal instillation of MNNG (4µgµl-1) or PBS (100µL), and intestinal tissue was recovered 4, 24 or 48 hours later. MCs-sufficient WB/B6 (Kit+/+), genetically MCs-deficient WBB6F1-KitW/KitW-v (Kit-/-) and Kit-/- reconstituted with Kit+/+ MCs received intra-rectal instillation of MNNG or PBS (4µgµl-1) twice a week for four consecutive weeks, and intestinal tissue was collected 24 weeks after the first instillations. Results: MNNG damaged the tissue and increased the expression of AnxA1 in epithelial cells. The lesion and MCs numbers were higher in AnxA1-/- mice. Increased levels of pro-inflammatory cytokines were found in the CRC tissue of AnxA1-/-. Higher and lower numbers of chymase and tryptase-positive cells, respectively, were observed in the presence of AnxA1. At the late phase of CRC, immunossupression was detected in CRC tissue with higher recruitment of MCs, AnxA1 expression and epithelial proliferation, which were not detected in Kit-/- strain. Furthermore, the MNNG-induced lesion was reduced in Kit-/- mice. Reconstitution of MCs to Kit-/- mice induced epithelial lesion and proliferation and immunossupression. Conclusions: Our results show the pivotal participation of MCs in the initial and late phase of CRC and highlight the interplay of MCs and AnxA1 on both the initial and late phase of the carcinogenesis.
Subject Editor	Not Assigned
Keywords	cytokines, MCs-deficient WBB6F1-KitW/KitW-v mice, immunossupression, AnxA1 deficient mice, chymase mast cells, tryptase mast cells
Subject Terms	Biological sciences/Immunology/Innate immune cells Biological sciences/Cancer/Cancer microenvironment
Conflict of Interest	There is no conflict of interest
Applicable Funding Source	This work was supported by Grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (2016/02012-4 to S.M.O.); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Grants 308144/2014-7 to S.M.O.). [Oliani]

Stage	Start Date
Under consideration	14th Jun 17
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