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EFEITOS DO ESTRESSE NEONATAL POR LIPOPOLISSACARÍDEO SOBRE A RESPOSTA A UM ESTIMULO INFLAMATÓRIO EM CAMUNDONGOS

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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular, como requisito parcial para a obtenção do grau de Mestre em Biologia Celular e Molecular.

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LISTA DE ABREVIATURAS

ACTH	Hormônio adrenocorticotrófico
HPA	Hipotálamo-Hipófise Adrenal
LPS	Lipopolissacarídeo
OMS	Organização Mundial da Saúde
TLR-4	*Toll-like receptor 4*
PGE2	Prostaglandina E2
NF-kB	Fator nuclear kappa B
SNC	Sistema Nervoso Central
IL-8	Interleucina-8
TNF-α	Fator de necrose tumoral alfa
NETs	Armadilhas extracelulares dos neutrófilos
DNA	Ácido desoxirribonucleico
IFN-α / γ	Interferon alfa e gama α / γ
ROS	Espécies reativas de oxigênio
PMA	Phorbol-12-miristato-13-acetato
DPP	Dia Pós-Parto

RESUMO

A exposição neonatal ao lipopolissacarídeo (LPS) gera um estimulo estressor capaz de afetar o desenvolvimento de importantes sistemas, dando origem a alterações comportamentais e neuroendócrinas duradoras. O objetivo desta dissertação é avaliar a resposta a um estímulo inflamatório em camundongos Balb/c submetidos à estresse neonatal por LPS em diferentes idades e sexos. Os camundongos receberam, no 3º e 10º dias de vida, injeção i.p. de LPS (100µg /Kg) ou solução salina. Após 21 ou 60 dias de vida, os mesmos foram submetidos à injeção de solução salina ou a um estímulo inflamatório através da injeção de carragenina 1%. Foram mensurados no lavado peritoneal citocinas inflamatórias, espécies reativas de oxigênio e produção de Redes Extracelulares de Neutrófilos (NETs). Os resultados encontrados demonstram que o estresse neonatal por LPS diminui a liberação de citocinas inflamatórias nos animais jovens machos e fêmeas. Já nos adultos, somente as fêmeas apresentaram a mesma resposta. Foi encontrado um aumento na produção de NETs nos animais do grupo nLPS com 60 dias quando comparado com animais nLPS com 21 dias, em ambos os sexos, indicando um efeito do estresse neonatal sobre a ontogenia da formação de NETs. A produção de ROS não sofreu influência do estresse neonatal, porém foi encontrada uma maior produção na idade adulta, independente da intervenção neonatal. Os resultados do presente estudo indicam que o estresse neonatal por LPS altera a produção de citocinas em resposta a um estímulo inflamatório em diferentes idades, em um efeito dependente do sexo. Além disso, parece que a formação de NETs ao longo da vida também é influenciada pelas alterações no período neonatal.

Palavras-chaves : Estresse Neonatal, LPS, Inflamação, NETs

ABSTRACT

Neonatal exposure to lipopolysaccharides (LPS) generates a stressful stimulus capable of affecting the development of important systems, which causes lasting behavioral and neuroendocrine alterations. The aim of this study is to evaluate the response to an inflammatory stimulus in Balb/c mice exposed to LPS-induced neonatal stress at different ages and genders. Mice underwent intraperitonial injections on postnatal days 3 and 10 with either LPS (100ug/Kg) or saline solution. After 21 or 60 days of age, either saline solution was injected or an inflammatory stimulus was induced by the injection of 1% carrageenan. Inflammatory cytokines, reactive oxygen species and NETs production were measured in the peritoneal lavage. Our findings indicate that LPS-induced neonatal stress can reduce inflammatory cytokines in young animals, males and females. In adults, however, only females showed the same response pattern. In nLPS group, an increase in NETs production was observed in animals that were 60-days-old when compared to 21-days-old in both genders, which indicates an effect of neonatal stress on the ontogeny of NETs formation. The production of ROS was not affected by neonatal stress, even though an increased production was found among adults regardless of neonatal intervention. The results shown here indicate that LPS-induced neonatal stress can alter cytokine production in response to inflammatory stimuli at different ages, in a gender dependent way. Moreover, it seems like the formation of NETs throughout life is also influenced by alterations undergone during the neonatal period.

Keywords: Neonatal stress, LPS, Inflammation, NETs

SUMÁRIO

Capítulo 1 – Apresentação do Tema

1. Introdução

1.1 Estresse

Em 1936 o fisiologista canadense Hans Selye introduziu o termo "*stress*" no campo da saúde para designar a resposta geral e inespecífica do organismo a um estressor ou a uma situação estressante. O conceito de estresse baseia-se na observação de que diferentes tipos de condições físicas ou psicológicas que ameaçam a homeostase do organismo eliciam o mesmo conjunto de alterações corporais – a chamada "síndrome de adaptação geral" (1).

A resposta mais característica ao estresse é a liberação do hormônio adrenocorticotrófico (ACTH) e corticóides (cortisol em humanos e corticosterona em roedores) na corrente sanguínea como resultado da ativação do eixo HPA (hipotálamo-hipófise-adrenal) (2,3). Além do eixo HPA, o estresse agudo também ativa o componente simpático do sistema nervoso neurovegetativo como parte da reação de luta ou fuga, ou "resposta de emergência". Como resultado disso, a noradrenalina das fibras nervosas simpáticas periféricas é liberada em diferentes tecidos, bem como a adrenalina da medula adrenal na corrente sanguínea (4, 5).

Diferenças sexuais também podem ter interferência na resposta ao estresse. Sabe-se que fêmeas de muitas espécies geralmente apresentam aumento da resposta imune e da resistência à infecção em comparação com machos. Já os machos, por sua vez, com a liberação da testosterona deprimem a imunidade humoral e mediada por células, aumentando a susceptibilidade a infecções bacterianas e virais (6).

1.1.1 Estresse neonatal

O estresse neonatal pode ser desencadeado por qualquer estressor de natureza física ou psicológica aplicado ao recém-nascido. Nos seres humanos, a negligência emocional

aplicada no inicio da vida pode levar a um aumento da probabilidade de o indivíduo, na idade adulta, desenvolver ansiedade, depressão crônica e até mesmo vício em drogas (7). Processos importantes como divisão, diferenciação, crescimento, migração e morte celular ocorrem neste período, sendo uma fase crítica para o desenvolvimento neural. Estudos demonstram que o estresse neonatal aplicado em modelos experimentais pode alterar significativamente a resposta do animal ao estresse na idade adulta. Ainda, diferenças relativamente sutis no cuidado materno podem alterar a resposta ao estresse de um animal para toda a sua vida (8).

As alterações comportamentais e fisiológicas provocadas em indivíduos que sofreram algum tipo de estresse no período neonatal são inúmeras e parecem bem estabelecidas. Estudos apontam que o estresse neonatal pode provocar efeitos sobre o desenvolvimento de diversos sistemas, incluindo o sistema imune (7, 9), neural (10) e neuroendócrino (11-13).

1.1.2 Estresse neonatal por LPS

Sabemos que infecções na fase inicial da vida são frequentes e que em algumas situações podem causar uma resposta imunológica ao estresse com grandes proporções. De acordo com a Organização Mundial da Saúde (OMS), nascem em média 130 milhões de recém-nascidos anualmente e destes, cerca de 4 milhões morrem, sendo infecção a causa de 36% desses óbitos (14). O estresse neonatal por LPS (Lipopolissacarídeo) consiste em um modelo experimental para avaliar as repercussões de variações imunológicas no início da vida. Esse estímulo ativa no hospedeiro uma série de mecanismos, como o recrutamento de células fagocitárias, ativação do complemento, liberação de imunoglobulinas, entre outros. Os componentes da parede bacteriana, as endotoxinas (lipopolissacarideos) dos microorganismos gram-negativos, que ativam resposta no hospedeiro podem desencadear o que chamamos de endotoxemia sistêmica, que resulta em um aumento de expressão e síntese de numerosos mediadores como citocinas, moléculas de adesão, enzimas proteolíticas e radicas livres de

oxigênio (15). O LPS liga-se ao receptor de Toll-like 4 (TLR4), expressos nas células de defesa, que quando ativado inicia uma cascata que culmina na liberação local de prostaglandina E2 (PGE2), que atua no nervo vago causando elevação inicial de glicocorticoides (11). Um das ações da corticosterona é sua capacidade para inibir a atividade da transcrição fator NF-kB (Fator Nuclear Kappa B), pela redução da fosforilação do IκB-α, refletindo em uma diminuição da transcrição de mediadores pró-inflamatórios (16). Além disso, alguns estudos vem demonstrando que animais que são expostos a essas endotoxinas no inicio da vida tem uma maior liberação de ACTH e corticosterona durante toda sua vida, podendo levar a uma predisposição a doenças que possuem um componente regulatório do eixo HPA significante, como doenças inflamatórias (12, 17).

A resposta ao estresse imunológico durante os períodos pré-natal e neonatal é conhecida por ter uma influência duradoura na resposta fisiológica a estímulos estressores na idade adulta. Estudos demonstram que algumas respostas do Sistema Nervoso Central (SNC) mediadas pelo sistema imune podem ser influenciadas pela experiência estressante neonatal precoce. Por exemplo, o estresse fisiológico por LPS no período neonatal pode afetar profundamente a resposta do eixo HPA em adultos (17, 18). Ellis et al. (16) demonstraram que um único desafio imune durante o período neonatal resulta em alterações duradouras na resposta imune inata a infecção em adultos. Ratos submetidos à injeção de LPS no período neonatal apresentavam uma redução na resposta febril na idade adulta em resposta a uma nova aplicação de LPS, assim como uma redução na produção de citocinas pró-inflamatórias, como interleucina-8 (IL-8) e fator de necrose tumoral alfa (TNF-α) (16).

Shanks et al. (12) demonstraram que ratos que sofreram exposição a LPS no período neonatal apresentavam alterações no desenvolvimento de sistemas neurais que comandam as respostas endócrinas ao estresse e isso pode predispor a doenças relacionadas ao estresse. Pesquisas mais recentes como a de Iwasa et al. (2010), correlacionam a exposição neonatal ao LPS com a regulação do peso corporal na idade adulta, demonstrando que pode haver um

aumento do desenvolvimento do peso corporal como também um aumento na ingestão de alimentos na idade adulta. Isso se explicaria pelas mudanças no sistema endócrino, neuropeptídeos, e sistemas de regulação de citocinas (19).

Em um modelo de tolerância ao LPS em camundongos no período neonatal, Landoni et al. (2012), mostraram que os mesmos apresentaram uma maior produção de NETS (*neutrophil extracellular traps)* em comparação ao grupo controle. Os camundongos receberam doses de LPS do 1º ao 4º dia de vida e, após chegar à idade adulta, foram submetidos a um desafio polimicrobiano. Avaliando a formação dos NETS, observou-se que os animais tolerantes apresentavam uma maior formação e, consequentemente, uma "limpeza" mais eficiente da infecção causada pelo desafio polimicrobiano (20).

1.2 Carragenina com agente inflamatório

A carragenina é um polissacarídeo sulfatado extraído de uma alga marinha, *Chondrus crisp*. É comum seu uso na indústria alimentícia com aditivo alimentar. Entretanto, ela vem sendo usada há décadas na pesquisa, devido seu potencial para indução de inflamação (21). A carragenina é frequentemente utilizada em modelos experimentais de pleurisia (22, 23), peritonite (24, 25) e edema (25, 26).

Quando injetada no peritônio, em um período de 4h, a mesma promove uma inflamação aguda, com migração leucocitária, liberação de enzimas proteolíticas, espécies reativas de oxigênio e citocinas. A migração celular é preferencialmente de neutrófilos, que são atraídos para o local inflamado devido à ação de prostaglandinas e agentes quimiotáticos como interleucinas (IL-8) e complemento (C5) (27). Entretanto, ainda não está estabelecido se a carragenina é capaz de ativar diretamente os neutrófilos. Bhattacharyya et al. (28), demonstraram que a inflamação induzida por carragenina em colonócitos humanos ocorre por meio da ativação do receptor TLR4 e produção de espécies reativas de oxigênio nestas células

(29). Considerando que os neutrófilos também expressam TLR4, é possível que a carragenina possa induzir a sua ativação por essa mesma via.

1.3 Neutrophil Extracellular Traps

Os neutrófilos são as células mais abundantes do sistema imunológico inato e possuem papel fundamental na luta do organismo contra as infecções. Estas células são as primeiras a serem recrutadas para o sítio da infecção, sendo assim, constituem a primeira linha de defesa do organismo (30, 31).

Na infecção, os neutrófilos são atraídos por quimiocinas e mediadores inflamatórios como IL-8 e interferon γ (IFN- γ) liberados pelas células endoteliais ativadas, macrófagos e mastócitos, e pelos produtos C3a e C5a do complemento. Durante a fase aguda da inflamação, principalmente em casos de infecção bacteriana, além do rápido recrutamento de neutrófilos para o sítio inflamatório há também grande aumento na sua produção. Atualmente, conhecemos três mecanismos utilizados pelos neutrófilos para eliminar os microorganismos invasores: fagocitose; degranulação(32); e a recentemente descrita liberação de NETs (33).

Os NETs são estruturalmente uma malha de fibras de ácido desoxirribonucleico (DNA) que constituem de histonas e proteínas antimicrobianas liberadas pelos neutrófilos no espaço extracelular (33, 34). Neste fenômeno biológico o núcleo dos neutrófilos perdem a sua forma característica de lóbulos, eucromatina e heterocromatina se homogeneízam, ocorre ruptura da membrana nuclear e os grânulos citoplasmáticos se dissolvem permitindo que os componentes celulares e nucleares se misturem para finalmente produzir a ruptura da membrana celular e posterior liberação do conteúdo para o meio extracelular. Desde que foi considerado que o resultado final deste processo é a morte dos neutrófilos, vários autores referem-se a este mecanismo como morte por "netose" (34, 35). Entretanto, em 2009, Yousefi et al. (35, 36)., demonstrou que os NETs produzidos pelos neutrófilos pode conter DNA mitocondrial e nuclear, porém não implica na morte dos neutrófilos. Um ano depois, Pilsczek

et al. (37, 38) descreveu a formação de NETs, como um processo não ligado com a lise da membrana plasmática como um evento primário e sim por brotamento de vesículas, que seriam preenchidas com DNA nuclear liberadas para o exterior da célula .

A base molecular da formação dos NETs ainda é pouco compreendida. No entanto, as ROS (espécies reativas de oxigênio) desempenham um papel central para iniciar o programa. Fuchs et al.(39), demonstraram que os neutrófilos derivados de pacientes com deficiência no sistema imunitário causado por mutações em genes que codificam a NADPH oxidase apresentam defeito na formação dos NETs, devido a incapacidade do complexo para gerar ROS. Portanto, pode-se afirmar que a NADPH oxidase é necessária para desencadear um sinal que culmina na formação destas redes (35, 39). Sugere-se que a maior parte dos neutrófilos em circulação é capaz de produzir estas redes. Uma vez que se verificou que a formação dos NETs não exigem caspases e não é acompanhado por fragmentação do DNA, é sabido que este processo é claramente independente da apoptose. Além disso, a morte celular passiva (necrose), por exemplo, induzida por toxinas ou complemento não é relacionada com a formação dos NETs. De fato, a formação dos NETs ocorre muito mais rapidamente do que a apoptose, como também, os estímulos conhecidos para "atrasar" a apoptose podem induzir a formação dos NETs (39, 40).

A descoberta dos NETs gerou, nestes últimos anos, muitos estudos para esclarecer e avaliar esse novo mecanismo de defesa apresentado pelos neutrófilos. Yost et al. (41), utilizando neutrófilos de recém-nascidos estimulados 1 h com LPS, fator de ativação de plaquetas, PMA (Phorbol-12-miristato-13-acetato) e algumas bactérias, concluíram que estes não foram capazes de gerar NETs. Deste modo, levantou-se a possibilidade de que a não formação dos NETs pudesse contribuir para a predisposição dos lactentes para alguns tipos de infecções precoces. Este grupo atribui esta falha como uma nova deficiência imune em recém-nascidos. Ao contrário do estudo pubicado por Yost, Marcos et al. (42) relataram que os neutrófilos de recém-nascidos poderiam sim gerar NETs, como em adultos, porém com um

tempo de resposta muito maior. Esses autores observam que, enquanto os adultos respondem dentro de 1 hora na presença de indutor, a formação das redes dos neutrófilos de neonatos começam em 2 horas após a indução, sendo que adquirem a quantidade de NETs igual a formada por adultos em aproximadamente 3 horas. O que continua a se estabelecer é se este atraso na geração NETs tem implicações para a resposta inicial à infecção em lactentes (34, 43).

2. JUSTIFICATIVA

A resposta ao estresse imunológico durante o período neonatal é conhecida por ter uma influência de longa duração na resposta fisiológica equivalente ao estresse na idade adulta. Vários estudos relatam os efeitos sobre o desenvolvimento físico, neuroquímico e comportamental em animais que sofreram algum tipo de estresse neste período. Sendo assim, torna-se relevante um melhor entendimento de como eventos no início da vida, como o estresse neonatal, podem ter influência, em curto e longo prazo, na resposta inflamatória e imune, através da liberação de citocinas, ROS e formação dos NETs. Estes achados podem representar a possibilidade de desenvolvimento de estratégias de prevenção e tratamento para esses eventos tão prevalentes em recém-nascidos no nosso meio.

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar o efeito do estresse neonatal por LPS sobre a resposta a um estímulo inflamatório induzido por carragenina em camundongos Balb/c.

3.2 Objetivos Específicos

1. Avaliar o efeito do estresse neonatal sobre a resposta a um estímulo inflamatório em diferentes idades e sexos.
2. Quantificar citocinas inflamatórias (IL-6, IL-10 e TNF) no lavado peritoneal.
3. Avaliar a produção de ROS no lavado peritoneal.
4. Avaliar a formação dos NETs no lavado peritoneal.

Capítulo 2 – Artigo Científico

2.1 Artigo Científico 1: LPS-induced neonatal stress in mice affects the response profile to an inflammatory stimulus in an age and sex-dependent manner.

Artigo submetido ao Clinical and Experimental Immunology

LPS-induced neonatal stress in mice affects the response profile to an inflammatory stimulus in an age and sex-dependent manner

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Abstract:

Neonatal exposure to lipopolysaccharides (LPS) generates a stressful immunologic stimulus capable of affecting the development of important systems, which causes lasting behavioral and neuroendocrine alterations. The aim of this study is to evaluate the response to an inflammatory stimulus in Balb/c mice exposed to LPS-induced neonatal stress at different ages and sexes. Mice were submitted to intraperitoneal injections on postnatal days 3 and 10 with either LPS (nLPS), 100µg/Kg, or saline solution (nSal). At 21 or 60 days of age, either saline solution was injected or an inflammatory stimulus was induced by the injection of 1% carrageenan. Inflammatory cytokines, reactive oxygen species and neutrophil extracellular traps (NETs) production were measured in peritoneal fluid. Our findings indicate that LPS-induced neonatal stress can reduce inflammatory cytokines in young animals, males and females. In adults, however, only females showed the same response pattern. An increase in NETs production was observed in nLPS animals that were 60-days-old when compared to 21-days-old in both sexes, which indicates an effect of neonatal stress on the ontogeny of NETs formation. The production of ROS was not affected by neonatal stress, even though an increased production was found among adults regardless of neonatal intervention. The results shown here indicate that LPS-induced neonatal stress can alter cytokine production in response to inflammatory stimuli at different ages, in a sex-dependent effect. Moreover, data also indicate that the formation of NETs throughout life is also influenced by early life adversities.

Keywords: Neonatal stress, LPS, Inflammation, NETs

Introduction

Infections are frequent early in life and can cause long-lasting effects in the physiological response to stressor stimuli in adulthood, a mechanism known as *programming* (1,2,3). Several studies have shown that neonatal exposure to lipopolysaccharides (LPS) affects the development of important systems by causing long-lasting behavioral and neuroendocrine changes (4, 5). LPS binds to toll-like receptor 4 (TLR4), which is expressed in immune cells and, when activated, can initiate a signaling cascade that culminates in local release of prostaglandin E2 (PGE2), which then acts in the vagus nerve by triggering an initial rise in glucocorticoids (6). One of the main activities of corticosterone is its capability to reduce the activity of NF-kB leading to a decrease in the transcription of proinflammatory mediators (7).

Although the effects of neonatal stress on the response of several systems in adulthood are well-established (3, 7-9), the ontogenetic differences of these changes are still poorly known. A previous study (10) showed that the effect of neonatal stress on neurogenesis is different in young and in adult mice, which points to different consequences throughout development. Considering the fact that the immune system begins its development before birth, studies have demonstrated the connection between its activation early in life and the consequences in the regulation of immune responses in adulthood that influence stress susceptibility (3, 11).

Furthermore, the effect of stress may also present differences in response to infections in different sexes. Studies have shown that females from many species usually present an increase in immune response and infection resistance when compared to males (12). In males, on the other hand, testosterone release depresses the humoral and cell-mediated immunity, which increases susceptibility to viral and bacterial infections (12).

Regardless of ontogeny or sex differences, an inflammatory process can activate several mechanisms in the organism, such as the recruitment of immune cells, complement

activation, immunoglobulin production and release of reactive oxygen species (ROS) (13). Neutrophil migration towards the infection site aims to eliminate the invading pathogen through well-known mechanisms, such as phagocytosis and degranulation, but also through a recently described mechanism of neutrophil extracellular traps (NETs) release. NETs are, structurally, a net of DNA (deoxyribonucleic acid) fibers composed of histones and antimicrobial proteins that are released by neutrophils in the extracellular space in order to immobilize and kill pathogens and thus stop the infection (14, 15).

Considering the high prevalence of infection during neonatal period and its long-lasting consequences, it is relevant to further study the impact of neonatal stress in both short and long-term basis. The influences of neonatal stress on the response profile to an inflammatory stimulus at different stages of life, as well as possible sex-dependent effects, are still poorly known. Therefore, the purpose of this study is to evaluate the response to an inflammatory stimulus in Balb/c mice undergoing neonatal stress by LPS at different ages and in different sexes. Towards that goal, inflammatory cytokines, reactive oxygen species and NETs formation were measured.

Materials and Methods

Animals

Primiparous Balb/C female mice (20-30g) from the vivarium of the Institute of Biomedical Research (IPB) at PUC/RS University, were mated. After birth, each litter was standardized in 5-7 pups and after weaning they were kept in colonies of 4-5 animals per cage, in a controlled temperature environment (21 ± 1 ºC) with light-dark cycles of 12:12 hours and free access to food and water. The experimental protocol was approved by the Ethics Committee for the Use of Animals from PUC/RS University under number 13/00345, and all experiments followed the National Institute of Health Guide for The Care and Use of Laboratory Animals (16).

Experimental design

After the birth and standardization of the litters, all animals were weighed in days post-partum (DPP) 1, 3, 10, 21 and 60. On the 3rd DPP, the animals were divided into two groups: stressed (nLPS) and control (nSal). The stressed group was subjected to intraperitoneal injection (i.p.) on the 3rd and 10th DPP with 100 µg/Kg of LPS (*E. coli* serotype *026B6*; Sigma-Aldrich, St Louis, MO, dissolved in saline solution). In the control group, animals received an injection with sterile saline solution (NaCl 0.9%) in the same days. Newborns remained with the mothers until weaning, on the 21st DPP. Experiments were performed on the 21st or 60th DPP. Mice were divided again into two groups, one that received an intraperitoneal injection with carrageenan (1%) and the other that received an injection with saline solution. Four hours after administration, the animals were euthanized with isoflurane followed by decapitation. A peritoneal wash was performed and samples stored to measure inflammatory cytokines, ROS and NETs. In all experiments performed on both 21st and 60th DPP, the experimental groups were composed of male and female mice.

Carrageenan-induced peritonitis and peritoneal wash

In order to induce the migration of neutrophils into the peritoneal cavity of mice, 0.25 mL of 1% carrageenan (Sigma Aldrich) was administered intraperitoneally. After 4 hours, the cavity was washed with 2 mL of cold RPMI 1640 (Cultilab). The contents of peritoneal lavage were separated for DNA quantification and visualization (NETs), ROS quantification and cytokines measurements.

NETs quantification

For the quantification of NETs, 300 µL of washing supernatant were precipitated with 750 µL of absolute ethanol and 105 µL of Sodium Acetate 3 M. Samples were incubated overnight at -20 °C, then centrifuged for 15 minutes at 13000 rpm and the pellet was ressuspended in 100 µL of 70% ethanol and centrifuged again. Pellet was then ressuspended in 40 µL of nuclease free water and DNA quantification was performed by using Quant-iT

dsDNA HS kit (Invitrogen) in the Qubit fluorometer (Invitrogen), according to manufacturer's instructions.

Immunofluorescence

For visualization of DNA, 300 µL of the peritoneal lavage were incubated in 8-chambe culture slides (BD Falcon) for 2 hours at 37 °C with 5% CO_2. After this period, cells were fixed with 4% paraformaldehyde (PFA) and stained with Hoechst 33342 (1:2000; Invitrogen). A Zeiss LSM 5 Exciter confocal microscope was used for imaging.

ROS quantification

For ROS quantification, 60 µL of peritoneal lavage were incubated with 240 µL of dichlorofluorescein diacetate - DCFH - 100 µM (Sigma- Aldrich) at 37 °C for 30 minutes in a dark plate proper for fluorescein. After incubation, samples were analyzed in the fluorescence reader Victor-X3 (PerkinElmer) with wavelengths for excitation and emission of 480 and 530 nm, respectively. To calculate ROS concentration in the samples, a concentration curve was performed by using 1 mM 2',7'dichlorofluorescein (Sigma- Aldrich).

Determination of cell death

Granulocytes in the peritoneal fluid were analyzed for apoptosis and necrosis with Annexin-V and Propidium Iodide, respectively, according to manufacturer's instructions (BD Pharmingen^{TM}). Briefly, cells were washed twice, stained with Annexin V-FITC and Propidium Iodide for 15 minutes at room temperature and analyzed by flow cytometry (FACS Canto II, BD Bioscience) within 1 h. Cells were initially selected by granulocytes size on the basis of forward scatter (FSC) and side scatter (SSC). Cells were then gated on both SSC and FSC singlets, ensuring individual cell staining. Data were analyzed with FlowJo software v. 7.2.5 (Tree Star Inc., USA).

Cytokine measurement

Samples obtained from peritoneal lavage were used in the determination of cytokine levels. Interleukin 6 (IL6), tumor necrosis factor (TNF) and interleukin 10 (IL10) were

simultaneously measured by flow cytometry with the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, USA). Sample acquisition was performed in a FACSCanto II flow cytometer (BD Biosciences, USA). Data analysis was performed in FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary).

Statistical Analysis

Data normality was evaluated by Shapiro-Wilk test and due to its normal distribution was presented as mean and standard error of the mean. Differences between experimental groups were analyzed by Student's t-test for independent samples. The significance level was established as 5% in all cases. All analyses were performed using the GraphPad Prism 5.02 software.

Results

LPS-induced neonatal stress does not alter body weight

Regarding body weight analysis, no significant differences were observed between groups of the same sex at any different ages (data not shown).

LPS-induced neonatal stress alters the release of inflammatory cytokines in a sex-dependent manner

Significant differences were found in IL-6, IL-10 and TNF levels from peritoneal washings. On the 21st DPP, after the carrageenan inflammatory stimulus, a significant decrease in those markers was observed in LPS-induced neonatal stress groups, both in males (Fig. 1A, C, E) and females (Fig. 1B, D, F). In adult females (inflammatory stimulus performed on the 60th DPP), there was a significant decrease in the concentration of IL-6 and TNF (Fig. 2B and D). However, no significant difference was observed for any of the inflammatory markers in males (Fig. 2 A, C and E), indicating sex-dependent differences in the regulation of this response. When the inflammatory markers at different ages (21 and 60 days) were compared, we found a significant difference in all three markers, which

demonstrates an increased production in adulthood regardless of neonatal stress in males (Fig. 3A, C, E) and females (Fig. 3B, D and F).

Neonatal stress has no influence in ROS production

No significant differences were found in ROS measurements between nSal and nLPS groups in both males and females at different ages (data not shown). Nevertheless, when we compared ROS concentration at different ages (21 and 60 days), there was a significant difference both for males (Fig. 4A) and females (Fig. 4B), which indicates an increase in ROS concentration in adulthood regardless of neonatal stress.

Increase in NETs formation in adulthood is influenced by neonatal stress

NETs formation after carrageenan-induced inflammatory stimulus was evaluated through quantification of DNA in peritoneal washing and imaging after DNA staining, as shown in figure 5. Although an increase in NETs formation after carrageenan stimulus can be observed in all experimental groups, there was no significant difference between nSal and nLPS groups, for males (Fig. 5 A, C) and females (Fig. 5B, D) at different ages. When DNA concentration was compared at different ages (21 and 60 days), a significant increase in NETs production was observed in adult males (Fig. 6A) and females (Fig. 6B) in the nLPS group. Moreover, no differences were found in the nSal group, indicating an effect of neonatal stress on the ontogeny of NETs formation.

Carrageenan does not induce apoptosis or necrosis in peritoneal granulocytes from stressed and non-stressed animals

In order to rule out that DNA release could be occurring as a consequence of cell death, cells from the peritoneal washing were stained for Annexin-V and PI. In fact, DNA release induced by carrageenan was not due to apoptosis or necrosis, since cell viability remained higher than 80% in all groups (Fig. 7).

Discussion

Data presented here shows that LPS administration in the neonatal period can influence the response of inflammatory cytokine release later in life. Four hours after carrageenan administration (i.p.), we found a decrease in two pro-inflammatory cytokines (TNF and IL-6) and in one anti-inflammatory cytokine (IL-10) in the nLPS group. Several studies have demonstrated the effect of LPS-induced stress in the regulation of the HPA axis (1, 6, 17). Shanks and collaborators have shown an increase in plasma concentration of corticosterone in adult rats that were exposed to LPS neonatally, as well as long-lasting effects on the immune regulation, revealing an alteration in the inflammatory response (4). In our study, it is possible that the reduction in inflammatory cytokines observed on animals from the nLPS group as a response to carrageenan application could be related to the regulation of the HPA axis. Therefore, neonatal stress might have induced an increase in the release of corticosterone, leading to a decrease in cytokine production through regulation of the immune system, since glucocorticoids inhibit the activity of NF-κB transcription factor (18). Ellis and collaborators showed that mice that underwent LPS stress neonatally had a decline in the production of pro-inflammatory cytokines (TNF and IL-6) after a second immunologic challenge. This finding is attributed to a reduction in IκB-α phosphorylation, which reflects a decrease in NF-κB activation, ultimately leading to a decrease in the transcription of these mediators (5, 7). On the other hand, the decrease in IL-10 concentration (anti-inflammatory) seen in the peritoneal washing from the nLPS group could be a consequence of the reduced inflammatory response (reduction of TNF), since IL-10 has the function of inhibiting the production of TNF- α (19, 20).

Furthermore, data presented here reveal that there is a difference in the production of TNF and IL-6 pro-inflammatory cytokines in adult females in response to stress. Considering that this response was unchanged in adult males when compared to the control group, there seems to be a hormonal influence on these effects. Sex differences in neonatal stress response are well known(21) and different mechanisms involved in immune system regulation have

been widely studied regarding such differences (22, 23). It is known that females are more prone to inflammatory and autoimmune diseases, effects that comprise female hormones (estradiol and progesterone) (24) and glia function (25) as important factors in the regulation of the response.

The inflammatory process induced by carrageenan activates ROS production (26), which results in an antimicrobial action that facilitates the death of invasive microorganisms through the degradation of the plasma membrane by lipid oxidation (26, 27). Our data show that there is ROS production in response to carrageenan, however no difference in this response was observed between the groups that received LPS or saline in the neonatal period. These findings indicate that neonatal stress had no influence in the secondary oxidative response to an inflammatory stimulus. Nonetheless, we observed an increased production of ROS in adulthood (21x60 days), which corresponds to the same response found to inflammatory cytokines, indicating that young mice present an inflammatory response profile that is different when compared to adults, regardless of neonatal intervention.

Like ROS, NETs play a major role in the defense process against microbial infections (28, 29). To our knowledge, this is the first study to investigate the effects of neonatal stress on NETs formation. To perform this analysis, in addition to quantifying DNA directly, neutrophils were stained with a DNA specific dye for the visualization of the nets. In order to confirm that the released DNA was in fact netosis, assays for the evaluation of apoptosis and necrosis were performed and results showed that most cells remained alive. Besides, data found indicates that neonatal stress did not alter NETs release. However, there was an increase in the production of DNA nets in animals from the nLPS group at 60 days of age when compared to animals from the same group at 21 days of age for both sexes, which indicates that there is an effect of neonatal stress on the ontogeny of NETs formation. Yost and collaborators have shown that activated neutrophils from newborns cannot generate NETs, raising the possibility that this inability could contribute to the predisposition of infants

to some types of infection early in life (30). Marcos and collaborators, though, have described that neutrophils from newborns could indeed generate NETs, as well as the adults, although with a much longer response period (31). These evidences point to the existence of differences in response to NETs formation at different stages of life, and our data shows that stress during a critical period for the immune development can have an important role in the regulation of this response.

ROS (30, 32) and TNF (33) are described in the literature as activators of NETs release. Our data shows an increase in ROS and TNF release in animals at 60 days when compared to 21 days in both nSal and nLPS groups. This could be related to the increase in NETs production in adult animals, although this result was found only for the nLPS group. The nSal adult group did not show a significant difference in NETs production, indicating an interaction between the effect of neonatal stress and the ontogeny of this response. Moreover, when nSal and nLPS groups were compared, both at 21 and 60 days, we did not observe a significant difference in ROS production. Also, there was a decrease in TNF production for the group that underwent stress during neonatal period. Considering the fact that NETs formation was similar in both nSal and nLPS groups, it seems unlikely that ROS and TNF release have a direct action in the effects found on the activation of NETs in the present study.

In the course of time, we create an immunologic memory that is capable of defense in a faster and more efficient way against known infectious agents, given that older people usually show good response to antigens they have had contact with in their youth, but they can't respond well to new antigens (34), (35) (36). Another hypothesis to explain the increase in DNA nets in adult life could be through an immunologic memory mechanism, since LPS (37, 38) administered in the neonatal period, as well as carrageenan (26, 39), make use of the TLR-4 pathway, that culminates in the activation of the innate immune system, leading to a different response that is mediated by neutrophils. Landoni and collaborators have revealed that mice that received higher doses of LPS from the 1st to the 4th days of life followed by a

polymicrobial challenge in adulthood presented an increased NETs production in comparison to the control group, which implies that tolerant animals presented a more efficient infection "cleanness" (40). Thus, considering that our data point to an increase in NETs production in adult mice from the nLPS group, it is possible that immunologic memory mechanisms are involved.

In conclusion, data in the present study suggests that LPS-induced neonatal stress alters cytokine production in response to an inflammatory stimulus at different ages, in a sex-dependent effect. Also, it seems that NETs formation throughout life can be influenced by early-life adversities, such as neonatal stress.

References

1. Xiong F, Zhang L. Role of the hypothalamic-pituitary-adrenal axis in developmental programming of health and disease. Front Neuroendocrinol. 2013;34(1):27-46.

2. Chintamaneni K, Bruder ED, Raff H. Programming of the hypothalamic-pituitary-adrenal axis by neonatal intermittent hypoxia: effects on adult male ACTH and corticosterone responses are stress specific. Endocrinology. 2014;155(5):1763-70.

3. Bilbo SD, Levkoff LH, Mahoney JH, Watkins LR, Rudy JW, Maier SF. Neonatal infection induces memory impairments following an immune challenge in adulthood. Behav Neurosci. 2005;119(1):293-301.

4. Shanks N, Windle RJ, Perks PA, Harbuz MS, Jessop DS, Ingram CD, et al. Early-life exposure to endotoxin alters hypothalamic-pituitary-adrenal function and predisposition to inflammation. Proc Natl Acad Sci U S A. 2000;97(10):5645-50.

5. Boissé L, Mouihate A, Ellis S, Pittman QJ. Long-term alterations in neuroimmune responses after neonatal exposure to lipopolysaccharide. J Neurosci. 2004;24(21):4928-34.

6. Walker AK, Nakamura T, Byrne RJ, Naicker S, Tynan RJ, Hunter M, et al. Neonatal lipopolysaccharide and adult stress exposure predisposes rats to anxiety-like behaviour and blunted corticosterone responses: implications for the double-hit hypothesis. Psychoneuroendocrinology. 2009;34(10):1515-25.

7. Ellis S, Mouihate A, Pittman QJ. Early life immune challenge alters innate immune responses to lipopolysaccharide: implications for host defense as adults. FASEB J. 2005;19(11):1519-21.

8. Caldji C, Diorio J, Meaney MJ. Variations in maternal care in infancy regulate the development of stress reactivity. Biol Psychiatry. 2000;48(12):1164-74.

9. Iwasa T, Matsuzaki T, Kinouchi R, Fujisawa S, Murakami M, Kiyokawa M, et al. Neonatal LPS injection alters the body weight regulation systems of rats under non-stress and immune stress conditions. Int J Dev Neurosci. 2010;28(1):119-24.

10. Herpfer I, Hezel H, Reichardt W, Clark K, Geiger J, Gross CM, et al. Early life stress differentially modulates distinct forms of brain plasticity in young and adult mice. PLoS One. 2012;7(10):e46004.

11. Bilbo SD, Newsum NJ, Sprunger DB, Watkins LR, Rudy JW, Maier SF. Differential effects of neonatal handling on early life infection-induced alterations in cognition in adulthood. Brain Behav Immun. 2007;21(3):332-42.

12. Schwarz JM, Bilbo SD. Sex, glia, and development: interactions in health and disease. Horm Behav. 2012;62(3):243-53.

13. Smith JA. Neutrophils, host defense, and inflammation: a double-edged sword. J Leukoc Biol. 1994;56(6):672-86.

14. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004;303(5663):1532-5.

15. Yam-Puc JC, García-Marín L, Sánchez-Torres LE. [Neutrophil extracellular traps (NET), consequence of a cellular suicide]. Gac Med Mex. 2012;148(1):68-75.

16. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals. 8th edition. Washington (DC): National Academies Press (US); 2011. Available from: http://www.ncbi.nlm.nih.gov/books/NBK54050/.

17. Shanks N, Larocque S, Meaney MJ. Neonatal endotoxin exposure alters the development of the hypothalamic-pituitary-adrenal axis: early illness and later responsivity to stress. J Neurosci. 1995;15(1 Pt 1):376-84.

18. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. Science. 1995;270(5234):286-90.

19. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol. 1991;147(11):3815-22.

20. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med. 1991;174(5):1209-20.

21. Davis M, Emory E. Sex differences in neonatal stress reactivity. Child Dev. 1995;66(1):14-27.

22. Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. Hum Reprod Update. 2005;11(4):411-23.

23. Olsen NJ, Kovacs WJ. Gonadal steroids and immunity. Endocr Rev. 1996;17(4):369-84.

24. Baker AE, Brautigam VM, Watters JJ. Estrogen modulates microglial inflammatory mediator production via interactions with estrogen receptor beta. Endocrinology. 2004;145(11):5021-32.

25. Schwarz JM, Sholar PW, Bilbo SD. Sex differences in microglial colonization of the developing rat brain. J Neurochem. 2012;120(6):948-63.

26. Bhattacharyya S, Dudeja PK, Tobacman JK. Carrageenan-induced NFkappaB activation depends on distinct pathways mediated by reactive oxygen species and Hsp27 or by Bcl10. Biochim Biophys Acta. 2008;1780(7-8):973-82.

27. Hakim J. [Reactive oxygen species and inflammation]. C R Seances Soc Biol Fil. 1993;187(3):286-95.

28. Ermert D, Urban CF, Laube B, Goosmann C, Zychlinsky A, Brinkmann V. Mouse neutrophil extracellular traps in microbial infections. J Innate Immun. 2009;1(3):181-93.

29. Arazna M, Pruchniak MP, Demkow U. Neutrophil extracellular traps in bacterial infections: Strategies for escaping from killing. Respir Physiol Neurobiol. 2013.

30. Yost CC, Cody MJ, Harris ES, Thornton NL, McInturff AM, Martinez ML, et al. Impaired neutrophil extracellular trap (NET) formation: a novel innate immune deficiency of human neonates. Blood. 2009;113(25):6419-27.

31. Marcos V, Nussbaum C, Vitkov L, Hector A, Wiedenbauer EM, Roos D, et al. Delayed but functional neutrophil extracellular trap formation in neonates. Blood. 2009;114(23):4908-11; author reply 11-2.

32. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol. 2007;176(2):231-41.

33. Neeli I, Khan SN, Radic M. Histone deimination as a response to inflammatory stimuli in neutrophils. J Immunol. 2008;180(3):1895-902.

34. Marcos, Miguel AR, Toribio, María Luisa, and Gaspar, María Luisa(Jul 2007) Immune System: Early Ontogeny. In: eLS. John Wiley & Sons Ltd, Chichester. http://www.els.net [doi: 10.1002/9780470015902.a0001132].

35. Vetro SW, Bellanti JA. Fetal and neonatal immunoincompetence. Fetal Ther. 1989;4 Suppl 1:82-91.

36. Metcalf D, Brumby M. The role of the thymus in the ontogeny of the immune system. J Cell Physiol. 1966;67(3):Suppl 1:149-68.

37. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J Biol Chem. 1999;274(16):10689-92.

38. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol. 1999;162(7):3749-52.

39. Bhattacharyya S, Gill R, Chen ML, Zhang F, Linhardt RJ, Dudeja PK, et al. Toll-like receptor 4 mediates induction of the Bcl10-NFkappaB-interleukin-8 inflammatory pathway by carrageenan in human intestinal epithelial cells. J Biol Chem. 2008;283(16):10550-8.

40. Landoni VI, Chiarella P, Martire-Greco D, Schierloh P, van-Rooijen N, Rearte B, et al. Tolerance to lipopolysaccharide promotes an enhanced neutrophil extracellular traps

formation leading to a more efficient bacterial clearance in mice. Clin Exp Immunol. 2012;168(1):153-63.

Legends to the Figures:

Fig. 1. Inflammatory cytokine release 4 hours after the injection of carrageenan (CGN) or saline (Sal) solution in young male and female mice that underwent neonatal stress induced by LPS. TNF (A: males - p=0.0008 and B: females - p=0.01), IL-6 (C: males - p=0.04 and D: females - p=0.003) and IL-10 (E: males - p=0.02 and F: females - p=0.001). Data shown represent 21 days old mice from nSal (saline solution administered in the 3rd and 10th days of life) and nLPS (LPS administered in the 3rd and 10th days of life) groups. n = 5-8 animals per group.

Fig. 2. Inflammatory cytokine release 4 hours after the injection of carrageenan (CGN) or saline (Sal) solution in adult male and female mice that underwent neonatal stress induced by LPS. TNF (A: males - p= 0.07 and B: females - p= 0.002), IL-6 (C: males - p= 0.98 and D: females - p= 0.04) and IL-10 (E: males - p= 0.23 and F: females - p= 0.20). Data shown represent 60 days old mice from nSal (saline solution administered in the 3rd and 10th days of life) and nLPS (LPS administered in the 3rd and 10th days of life) groups. n = 5-8 animals per group.

Fig. 3. Comparison of the cytokines release between young and adult mice (21 and 60 days old) after the inflammatory stimulus by carrageenan (CGN) injection. TNF (A: males and B: females; p<0.0001 in all cases), IL-6 (C: males and D: females, p<0.0001 in all cases) and IL-10 (E: males - nSal p=0.04 and nLPS p= 0.0003; F: females - nSal p=0.04 and nLPS p=0.0009). Data shown corresponds to experimental groups nSal (saline solution given on the 3rd and 10th days of life) and nLPS (LPS given on the 3rd and 10th days of life). n = 5-8 per group.

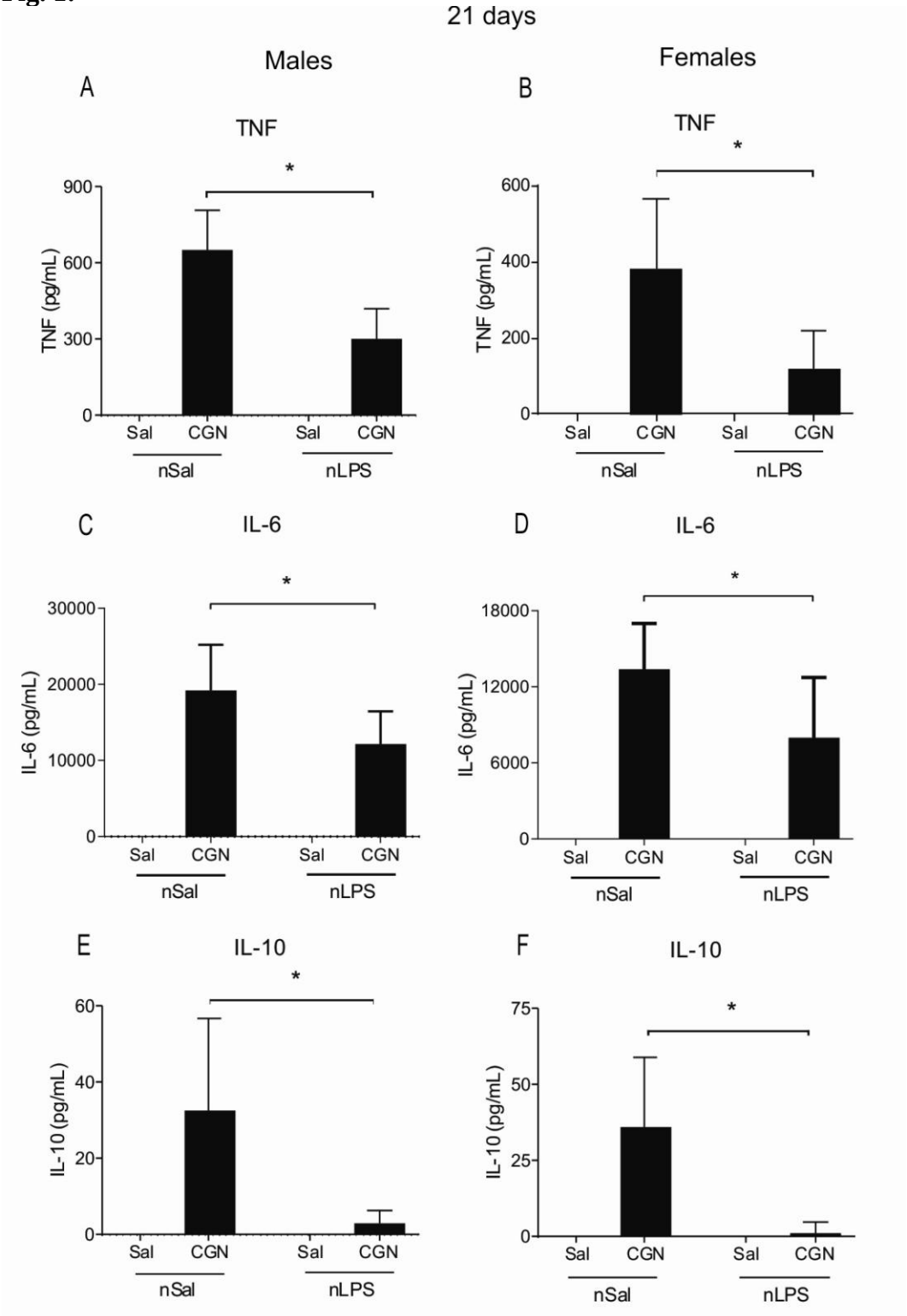
Fig. 4. Comparison of ROS production between animals with 21 and 60 days of age after inflammatory stimulus by carrageenan (CGN) injection. In (A) males (nSal p=0.001 and nLPS p=0.001) and (B) females (nSal p=0.005 and nLPS p=0.009). Data shown corresponds to experimental groups nSal (saline solution given on the 3rd and 10th days of life) and nLPS (LPS given on the 3rd and 10th days of life). n = 4 per group.

Fig. 5. Response of NETs release 4 hours after carrageenan (CGN) or Saline (Sal) injection in animals that underwent neonatal stress induced by LPS. Data shown here correspond to mice with 21 (A: males and B: females) and 60 days of age (C: males and D: females) in experimental groups nSal (saline solution given on the 3rd and 10th days of life) and nLPS (LPS given on the 3rd and 10th days of life). n = 7-11 per group. Visualization of DNA nets stained with Hoechst in cells obtained from the peritoneal washing 4 hours after the injection of (F) Carrageenan and (E) Saline. Images were taken in a Zeiss (LSM 5 Excite) confocal microscope. Scale bars: 20 um.

Fig. 6. Comparison of NETs release 4 hours after carrageenan (CGN) injection in animals with 21 or 60 days of age. In (A) males (nLPS p=0.02) and (B) females (nLPS p=0.01) on experimental groups nSal (saline solution on third and tenth days of life) and nLPS (LPS o third and tenth says of life). n = 7-11 per group.

Fig. 7. Carrageenan does not induce apoptosis or necrosis in peritoneal granulocytes from stressed and non-stressed animals. Flow cytometric analysis of Annexin-V binding and Propidium Iodide uptake in peritoneal fluid cells from: males at 21days from nSal group (A) and nLPS group (B); females at 21days from nSal group (C) and nLPS group (D); males at 60 days from nSal group (E) and nLPS group (F); females at 60 days from nSal group (G) and nLPS group (H).

Fig. 1:


21 days
Males
Females
A
TNF
TNF (pg/mL)
900
600
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0
*
Sal
CGN
Sal
CGN
nSal
nLPS
B
TNF
TNF (pg/mL)
600
400
200
0
*
Sal
CGN
Sal
CGN
nSal
nLPS
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IL-6
IL-6 (pg/mL)
30000
20000
10000
0
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Sal
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Sal
CGN
nSal
nLPS
D
IL-6
IL-6 (pg/mL)
18000
12000
6000
0
*
Sal
CGN
Sal
CGN
nSal
nLPS
E
IL-10
IL-10 (pg/mL)
60
40
20
0
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Sal
CGN
Sal
CGN
nSal
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IL-10 (pg/mL)
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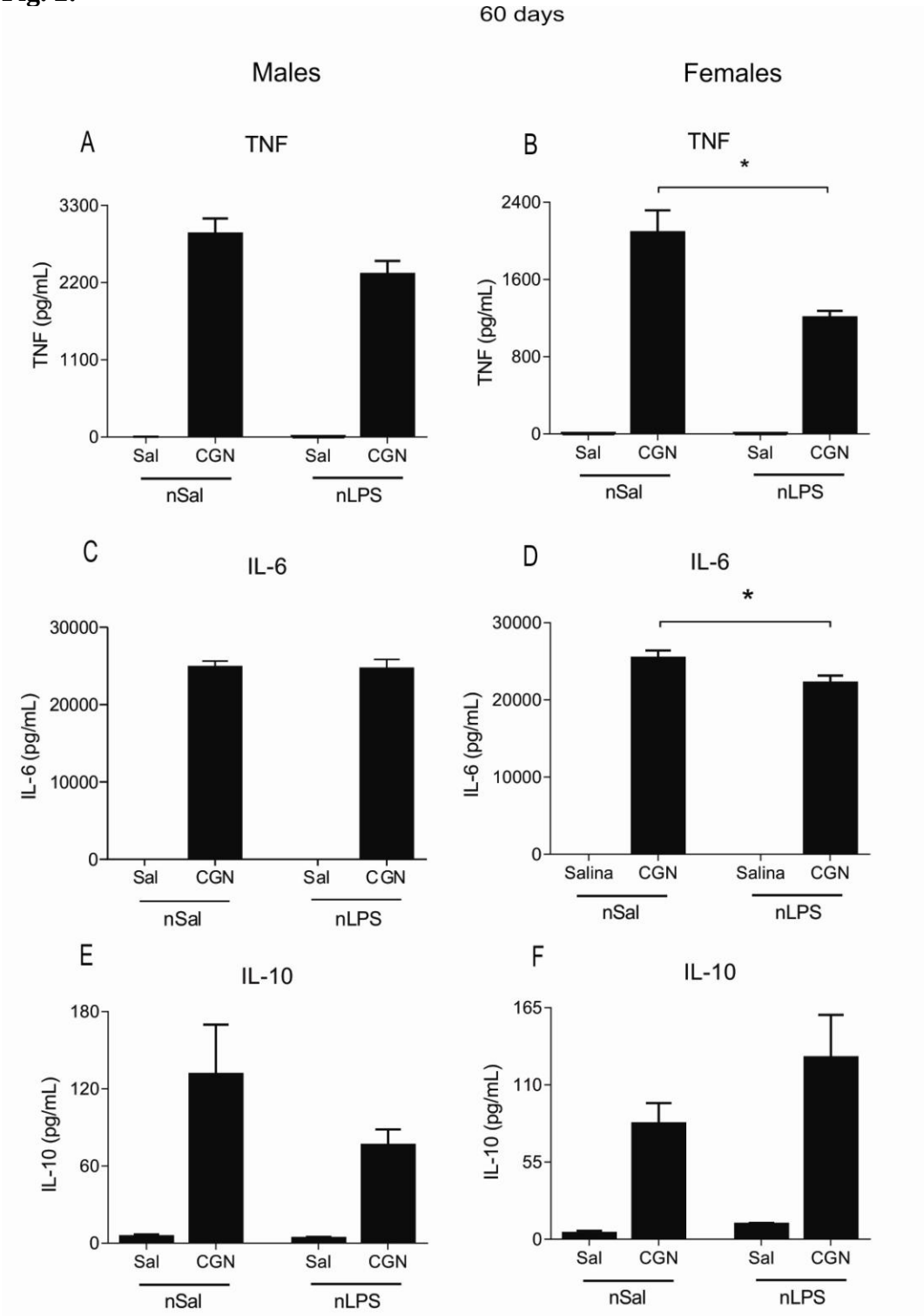
Fig. 2:


60 days
Males
Females
A
TNF
B
TNF
*
TNF (pg/mL)
3300
2200
1100
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2400
1600
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Sal
CGN
nSal
nLPS
C
IL-6
D
IL-6
*
IL-6 (pg/mL)
30000
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IL-10
IL-10 (pg/mL)
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110
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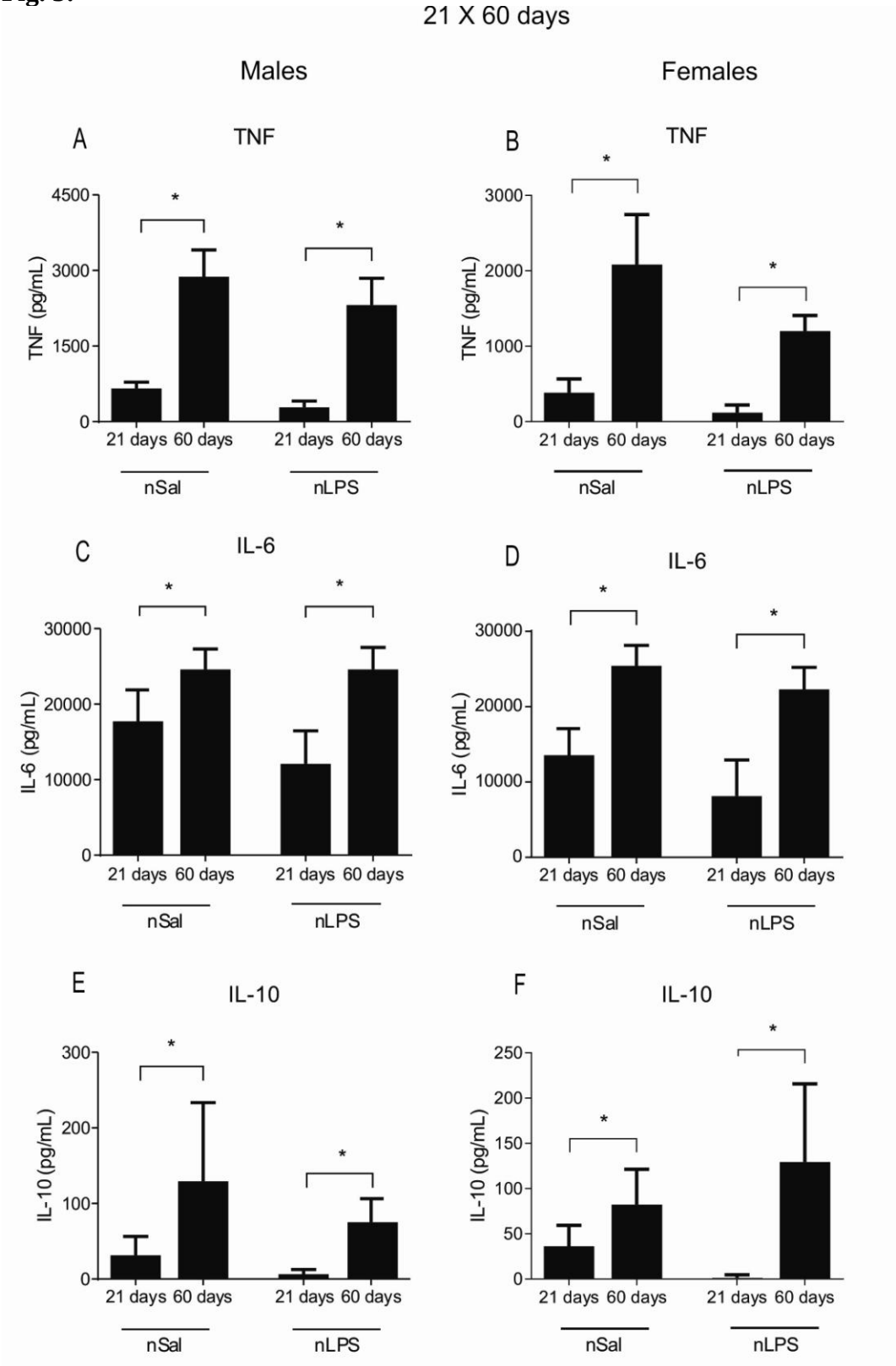
Fig. 3:


21 X 60 days
Males
Females
A
TNF
B
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TNF (pg/mL)
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1500
0
3000
2000
1000
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*
21 days 60 days
21 days 60 days
nSal
nLPS
C
IL-6
D
IL-6
IL-6 (pg/mL)
30000
20000
10000
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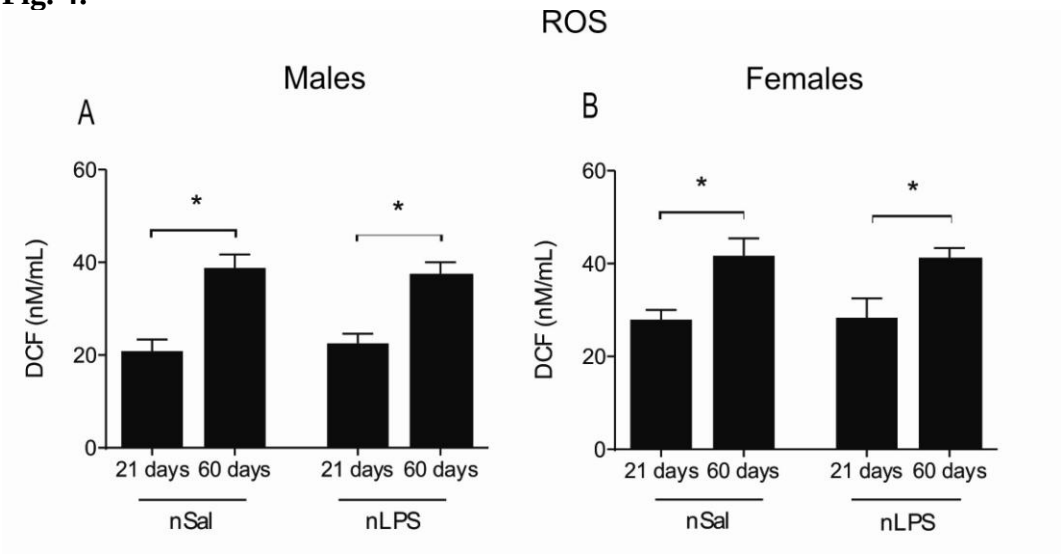
Fig. 4:


ROS
Males
A
Females
B
DCF (nM/mL)
60
40
20
0
*
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21 days
60 days
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21 days
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60 days
nSal
nLPS
nSal
nLPS

Fig. 5:

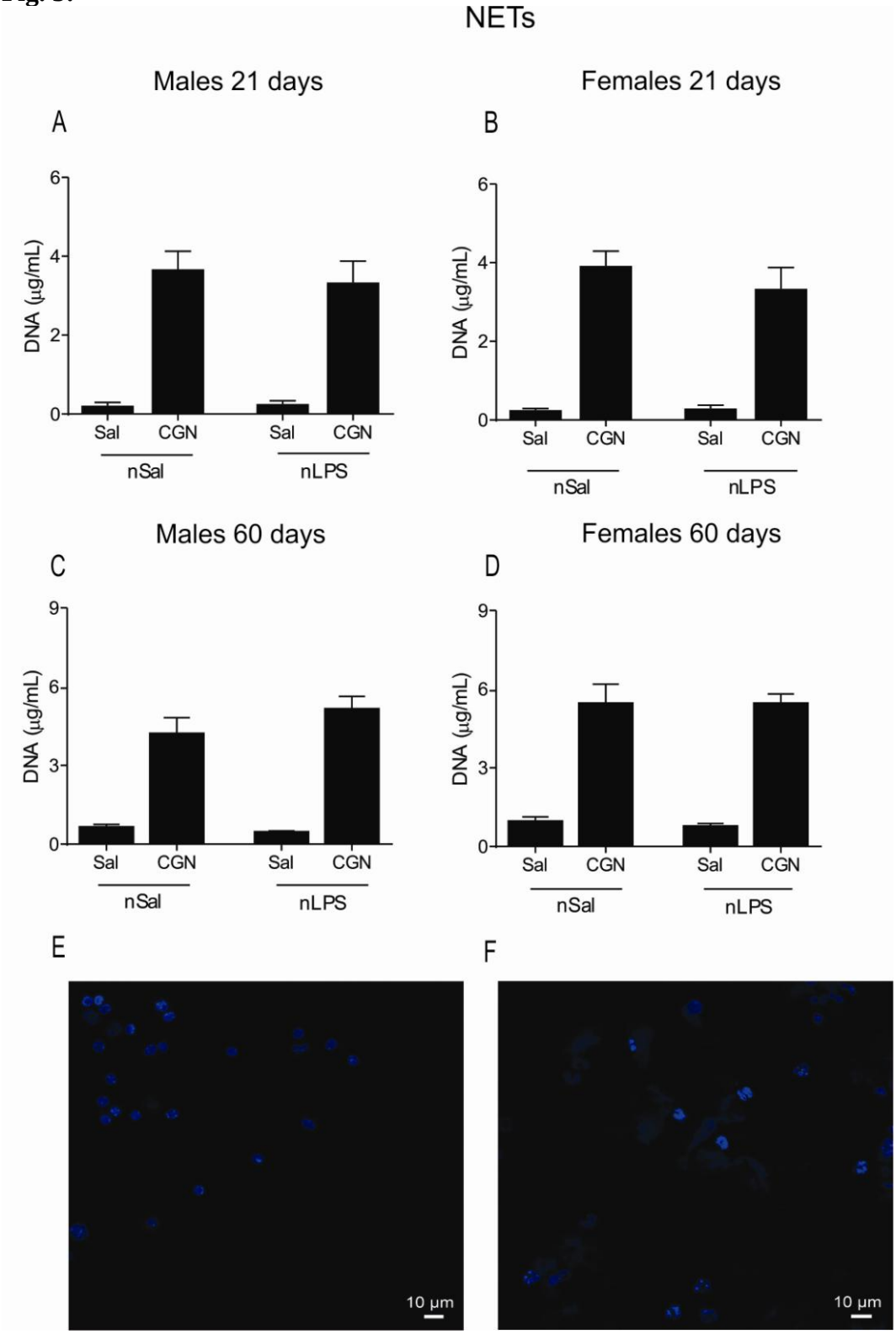

NETs
Males 21 days
A
DNA (µg/mL)
0
2
4
6
Sal
CGN
Sal
CGN
nSal
nLPS
Females 21 days
B
DNA (µg/mL)
0
2
4
6
Sal
CGN
Sal
CGN
nSal
nLPS
Males 60 days
C
DNA (µg/mL)
0
3
6
9
Sal
CGN
Sal
CGN
nSal
nLPS
Females 60 days
D
DNA (µg/mL)
0
3
6
9
Sal
CGN
Sal
CGN
nSal
nLPS
E
10 µm
F
10 µm

Fig. 6:

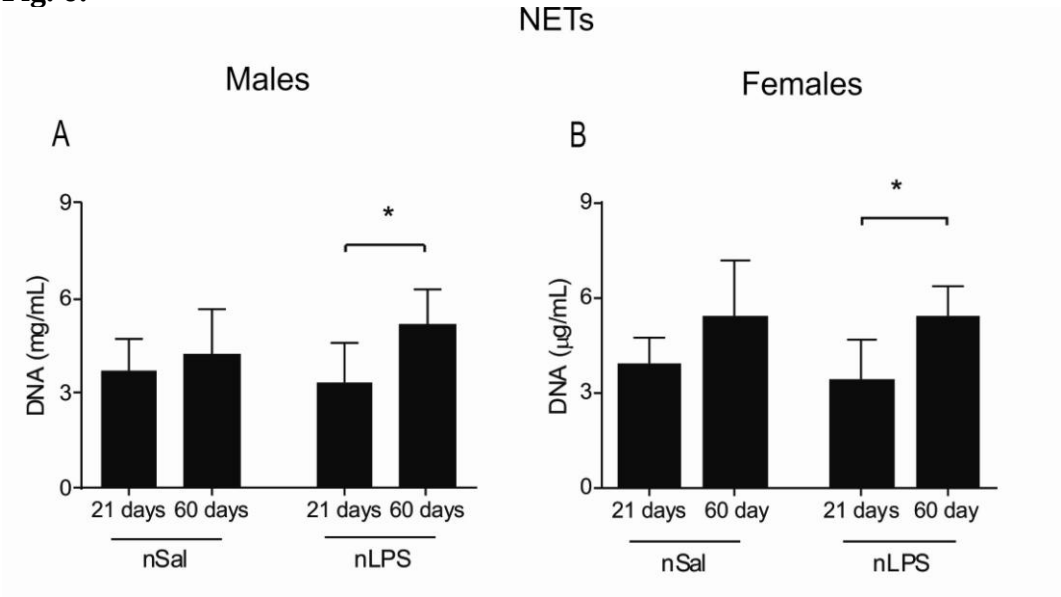
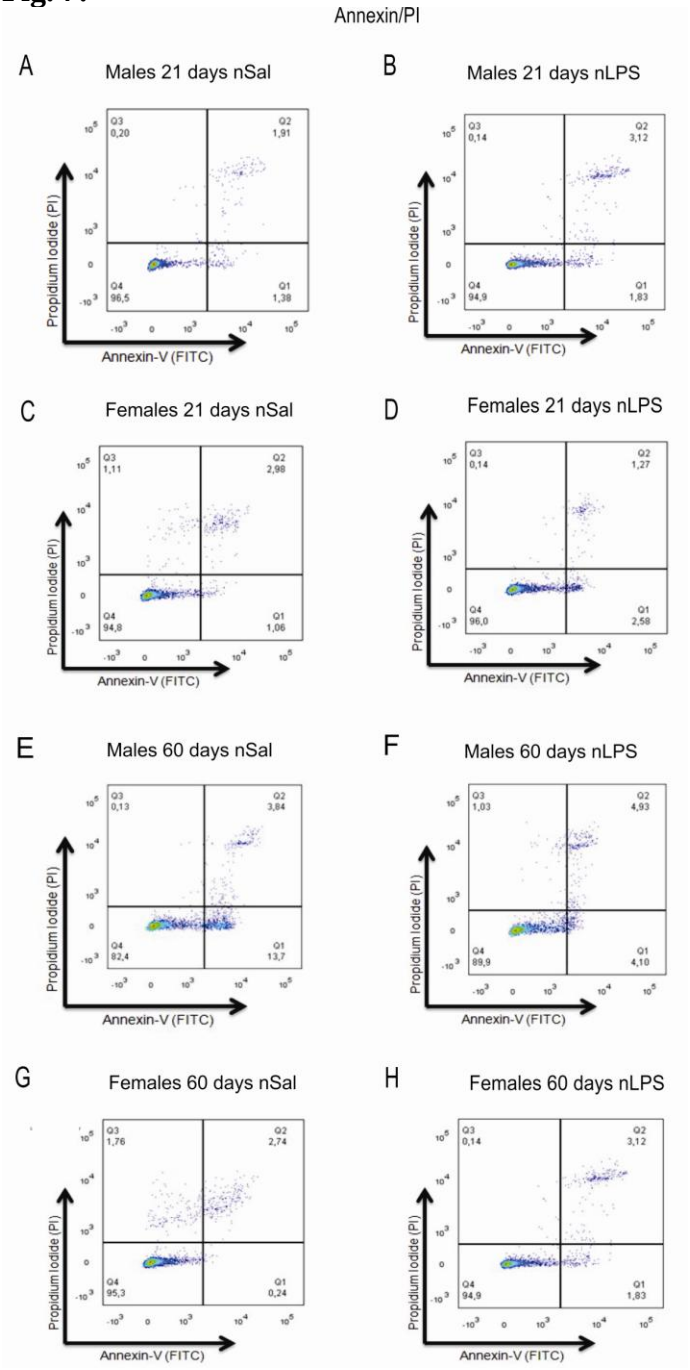

NETs
Males
Females
A
B
DNA (mg/mL)
DNA (μg/mL)
9
6
3
0
*
*
21 days
60 days
21 days
60 days
21 days
60 day
21 days
60 day
nSal
nLPS
nSal
nLPS

Fig. 7:


Annexin/PI
A
Males 21 days nSal
Q3 0,20
Q2 1,91
Q4 96,5
Q1 1,38
B
Males 21 days nLPS
Q3 0,14
Q2 3,12
Q4 94,9
Q1 1,83
C
Females 21 days nSal
Q3 1,11
Q2 2,98
Q4 94,8
Q1 1,06
D
Females 21 days nLPS
Q3 0,14
Q2 1,27
Q4 96,0
Q1 2,58
E
Males 60 days nSal
Q3 0,13
Q2 3,84
Q4 82,4
Q1 13,7
F
Males 60 days nLPS
Q3 1,03
Q2 4,93
Q4 89,9
Q1 4,10
G
Females 60 days nSal
Q3 1,76
Q2 2,74
Q4 95,3
Q1 0,24
H
Females 60 days nLPS
Q3 0,14
Q2 3,12
Q4 94,9
Q1 1,83
Propidium Iodide (PI)
Annexin-V (FITC)

2.2. Artigo Científico 2: Carrageenan-induced inflammation promotes neutrophil extracellular trap formation in a mouse model.

Artigo submetido ao European Journal of Immunology.

Carrageenan-induced inflammation promotes neutrophil extracellular trap formation in a mouse model

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Abstract

Neutrophil Extracellular Traps (NETs) are the release of the inner content of neutrophils in response to different stimuli to combat microorganisms. Carrageenan is usually used due to its potential to induce inflammation. Here we demonstrate that carrageenan injection can induce NETs release in a mice model of acute peritonitis.

Key Words: NETs, Neutrophils, Carrageenan

Introduction

Neutrophils are the first cells involved in defense against pathogens and present a mechanism, called Neutrophil Extracellular Traps (NETs), which are extracellular structures able to trap and kill pathogens [1, 2]. These NETs are fibers made of chromatin (histones and DNA) decorated with antimicrobial proteolytic enzymes present in the granules of neutrophils (such as neutrophil elastase and myeloperoxidase) [3] and other nuclear components, all apparently involved in its mechanism of action [4]. There are a variety of stimuli yet described in the literature including proteins, bacteria, fungi, viruses and cytokines capable of inducing NETs release [1, 5-7]. NETs are present in a plenty of *in vivo* inflammatory conditions, such as endometriosis [8], sepsis [9], thrombosis [10], autoimmunity [11, 12]. These studies point out to the importance of exploring inflammatory stimuli involved in NETs formation.

Carrageenan is a sulfated polysaccharide extracted from *Chondrus crispus*, a marine algae, used for decades in research for its potential to induce inflammation [13]. Also, carrageenan is widely used by the food industry as a gelling agent [13]. Due to the potential induction of inflammation, carrageenan is often used as a model of pleurisy [14, 15], peritonitis [16, 17] and edema [17, 18] in experimental animals. The injection of carrageenan

triggers an acute inflammation associated with hyperalgesy, which is characterized by edema and an exacerbated response to thermal and mechanical stimuli [13]. Bhattacharyya and colleagues found that carrageenan-induced inflammation in human colonocytes occurs through activation of Toll-like receptor-4 (TLR4) (19) and reactive oxygen species production [20].

Experimental models to further study the role and mechanisms of NET formation may be an important tool for future research. Thus, present study evaluated the use of carrageenan to induce inflammation in a mouse model of acute peritonitis and its effect on neutrophil extracellular trap formation.

Methods

Animals

Balb/C mice weighing 20-30g were used. Animals were housed in the vivarium of the Institute of Biomedical Research (PUCRS) with 12:12 h light/dark cycle, free access to water and food, and controlled temperature (21 ± 1^{o}C). All experimental protocols were approved by the Ethics Committee on Animal Use at Pontifícia Universidade Católica do Rio Grande do Sul (CEUA/PUCRS), under protocol number 13/00345, and all experiments followed the National Institute of Health Guide for The Care and Use of Laboratory Animals [21].

Induction of Acute Peritonitis and Peritoneal Lavage

Acute peritonitis was induced by an intraperitoneal injection of 1% carrageenan in a volume of 250 µL. The control group received an intraperitoneal injection of endotoxin-free PBS. After 4 h of injection, animals were euthanized and their peritoneal cavities were rinsed with 2 mL of cold RPMI 1640 medium. Total leukocytes in peritoneal fluid were determined in Neubauer chambers after dilution in Trypan solution. Differential counting of leukocytes was carried out in Diff-Quick (Laborclin)-stained slides. Images were obtained with an Olympus BX43 microscope.

Quantification of NETs release

Neutrophil extracellular traps were quantified in peritoneal fluid supernatants using Quant-iT dsDNA HS kit (Invitrogen), according to manufacturer's instructions.

Immunofluorescence

Peritoneal cells from carrageenan-treated mice were incubated in 8-chamber culture slides (BD Falcon) for 2 hours at 37 °C under 5% CO_2. After this period, cells were fixed in 4% paraformaldehyde (PFA), and stained with anti-neutrophil elastase (1:1000; Abcam), followed by anti-rabbit Cy3 antibody (1:500; Invitrogen) and Hoechst 33342 (1:2000; Invitrogen). Images were obtained with a Zeiss LSM 5 Exciter confocal microscope.

Annexin-V assay

Granulocytes in the peritoneal fluid were analyzed for apoptosis and necrosis with Annexin-V and Propidium Iodide respectively, according to manufacturer's instructions (BD Pharmingen^{TM}). Briefly, cells were washed twice, stained with Annexin V-FITC and Propidium Iodide for 15 minutes at room temperature and analyzed by flow cytometry (FACS Canto II, BD Bioscience) within 1 h. Cells were initially selected by granulocytes size on the basis of forward scatter (FSC) and side scatter (SSC). Cells were then gated on both SSC and FSC singlets, ensuring individual cell staining. Data were analyzed with FlowJo software v. 7.2.5 (Tree Star Inc., USA).

Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). Results were analyzed using a statistical software package (GraphPad Prism 5). Statistical differences between experimental groups were evaluated by analysis of variance or with Student's t Test. The level of significance was set at $p \leq 0.05$.

Results and Discussion

Neutrophil migration induced by Carrageenan

Carrageenan-induced peritonitis promoted the migration of neutrophils when compared to saline-treated group (Fig. 1A). More than 60% of cells found in peritoneal fluids after carrageenan injection were neutrophils, as illustrated in Fig. 1E and F. Previous studies have demonstrated that carrageenan is able to trigger neutrophil migration to the site of injection (14-17). Although the percentage of other leukocytes, such as macrophages, lymphocytes and mast cells (Fig. 1B-D) has changed as a consequence of the carrageenan-induced neutrophils migration, the absolute number was not altered.

Carrageenan injection induces the release of Neutrophil Extracellular Traps

Many inflammatory stimuli were shown to be capable of inducing neutrophil extracellular trap formation by human neutrophils [1, 5, 8, 22, 23]. To date, only few reports have demonstrated the production of NETs by mouse neutrophils [10, 24, 25]. Here, we were able to demonstrate that intraperitoneal injection of the polysaccharide from *Chondrus crispus*, Carrageenan, induces NETs formation *in vivo* (Fig. 2A). NETs release induced by carrageenan was not due to apoptosis or necrosis, since cell viability remained high in both saline- and carrageenan-treated mice (Fig. 2B). Also, peritoneal cells from carrageenan-treated mice showed a negative staining for annexin-V and propidium iodide (Fig. 2C). These results indicate that carrageenan-induced NETs formation is an active process. The major components of NETs are decondensed DNA and antimicrobial proteins, such as neutrophil elastase (NE) [3]. In addition, NE together with myeloperoxidase, was shown to be important for NETs formation [3]. To characterize the constituents of the extracellular DNA found after intraperitoneal injection of carrageenan, we analyzed it by immunofluorescence. Hoescht 33342 staining showed that these extracellular structures consist of extracellular DNA fibers (Fig 2D-F), which co-localized with NE (Fig. 2G-I), confirming that carrageenan induced the formation of NETs in the peritoneum of mice.

In conclusion, the results show for the first time that carrageenan-induced inflammation in the peritoneum of mice is able to induce NETs formation. These findings

may contribute to future studies using *in vivo* experimental models to further investigate NETs. The mechanisms by which carrageenan induces NETs formation in mice should yet be addressed in future research.

Acknowledgments

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References

1. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004;303(5663):1532-5.

2. Urban CF, Reichard U, Brinkmann V, Zychlinsky A. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. Cell Microbiol. 2006;8(4):668-76.

3. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol. 2010;191(3):677-91.

4. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog. 2009;5(10):e1000639.

5. Neeli I, Khan SN, Radic M. Histone deimination as a response to inflammatory stimuli in neutrophils. J Immunol. 2008;180(3):1895-902.

6. Saitoh T, Komano J, Saitoh Y, Misawa T, Takahama M, Kozaki T, et al. Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. Cell Host Microbe. 2012;12(1):109-16.

7. Jenne CN, Wong CH, Zemp FJ, McDonald B, Rahman MM, Forsyth PA, et al. Neutrophils recruited to sites of infection protect from virus challenge by releasing neutrophil extracellular traps. Cell Host Microbe. 2013;13(2):169-80.

8. Berkes E, Oehmke F, Tinneberg HR, Preissner KT, Saffarzadeh M. Association of neutrophil extracellular traps with endometriosis-related chronic inflammation. Eur J Obstet Gynecol Reprod Biol. 2014;183:193-200.

9. Luo L, Zhang S, Wang Y, Rahman M, Syk I, Zhang E, et al. Proinflammatory role of neutrophil extracellular traps in abdominal sepsis. Am J Physiol Lung Cell Mol Physiol. 2014;307(7):L586-96.

10. Brill A, Fuchs TA, Savchenko AS, Thomas GM, Martinod K, De Meyer SF, et al. Neutrophil extracellular traps promote deep vein thrombosis in mice. J Thromb Haemost. 2012;10(1):136-44.

11. Hakkim A, Fürnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A. 2010;107(21):9813-8.

12. Dwivedi N, Radic M. Citrullination of autoantigens implicates NETosis in the induction of autoimmunity. Ann Rheum Dis. 2014;73(3):483-91.

13. Di Rosa M. Biological properties of carrageenan. J Pharm Pharmacol. 1972;24(2):89-102.

14. Cuzzocrea S, Santagati S, Sautebin L, Mazzon E, Calabrò G, Serraino I, et al. 17beta-estradiol antiinflammatory activity in carrageenan-induced pleurisy. Endocrinology. 2000;141(4):1455-63.

15. Vinegar R, Truax JF, Selph JL. Some quantitative temporal characteristics of carrageenin-induced pleurisy in the rat. Proc Soc Exp Biol Med. 1973;143(3):711-4.

16. Syam S, Bustamam A, Abdullah R, Sukari MA, Hashim NM, Mohan S, et al. β Mangostin suppress LPS-induced inflammatory response in RAW 264.7 macrophages in vitro and carrageenan-induced peritonitis in vivo. J Ethnopharmacol. 2014;153(2):435-45.

17. Gonçalves DO, Calou IB, Siqueira RP, Lopes AA, Leal LK, Brito GA, et al. In vivo and in vitro anti-inflammatory and anti-nociceptive activities of lovastatin in rodents. Braz J Med Biol Res. 2011;44(2):173-81.

18. Lucetti DL, Lucetti EC, Bandeira MA, Veras HN, Silva AH, Leal LK, et al. Anti-inflammatory effects and possible mechanism of action of lupeol acetate isolated from Himatanthus drasticus (Mart.) Plumel. J Inflamm (Lond). 2010;7:60.

19. Bhattacharyya S, Gill R, Chen ML, Zhang F, Linhardt RJ, Dudeja PK, et al. Toll-like receptor 4 mediates induction of the Bcl10-NFkappaB-interleukin-8 inflammatory pathway by carrageenan in human intestinal epithelial cells. J Biol Chem. 2008;283(16):10550-8.

20. Bhattacharyya S, Dudeja PK, Tobacman JK. Carrageenan-induced NFkappaB activation depends on distinct pathways mediated by reactive oxygen species and Hsp27 or by Bcl10. Biochim Biophys Acta. 2008;1780(7-8):973-82.

21. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals. 8th edition. Washington (DC): National Academies Press (US); 2011. Available from: http://www.ncbi.nlm.nih.gov/books/NBK54050/.

22. Martinelli S, Urosevic M, Daryadel A, Oberholzer PA, Baumann C, Fey MF, et al. Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. J Biol Chem. 2004;279(42):44123-32.

23. Oehmcke S, Mörgelin M, Herwald H. Activation of the human contact system on neutrophil extracellular traps. J Innate Immun. 2009;1(3):225-30.

24. Ermert D, Urban CF, Laube B, Goosmann C, Zychlinsky A, Brinkmann V. Mouse neutrophil extracellular traps in microbial infections. J Innate Immun. 2009;1(3):181-93.

25. Meng W, Paunel-Görgülü A, Flohé S, Hoffmann A, Witte I, Mackenzie C, et al. Depletion of neutrophil extracellular traps in vivo results in hypersusceptibility to polymicrobial sepsis in mice. Crit Care. 2012;16(4):R137.

Figures Legends

Figure 1. Cellular profile from saline (SAL) and carrageenan-injected (CGN) mice. Carrageenan induced peritonitis with neutrophil migration (A) and mast cells (D) increase. Peritoneal fluids from saline-injected mice had predominance of macrophages (B) and lymphocytes (C). Cytospin of leukocytes in slides and Diff-Quick (Laborclin)-stained cells from peritoneal fluids in both saline (E) and carrageenan (F) groups. The images were obtained by light microscopy (400x). *indicates significant difference ($p<0.05$) when saline and carrageenan groups were compared. n=12 per group.

Figure 2. Carrageenan injection induces the release of Neutrophil Extracellular Traps. Peritoneal NETs formation was quantified using a Quant-iT dsDNA kit (A). Cell viability was evaluated using Trypan Blue (B). Apoptosis/necrosis was measured using Annexin-V and Propidium Iodide (C). Cells from peritoneal fluids were fixed with paraformaldehyde 4% and stained with Hoechst 33342. Saline (D), carrageenan – 20 µm (E) and carrageenan – 10 µm (F) images are presented. Co-localization of DNA and neutrophil elastase was performed in carrageenan-induced NETs. DNA stained with Hoechst (G), neutrophil elastase stained with anti-neutrophil elastase (H) and overlay (I). All images were obtained by Confocal

Microscopy. *indicates significant difference ($p<0.05$) when saline and carrageenan groups were compared. n=8-9 per group.

Fig. 1:

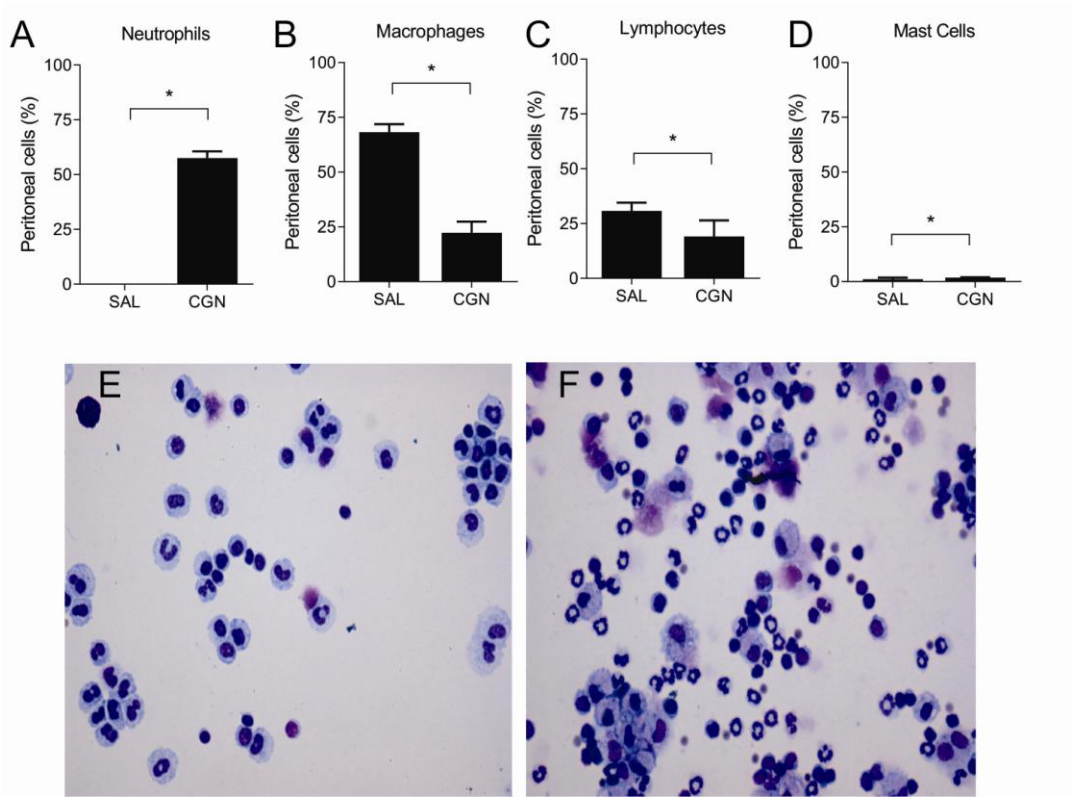
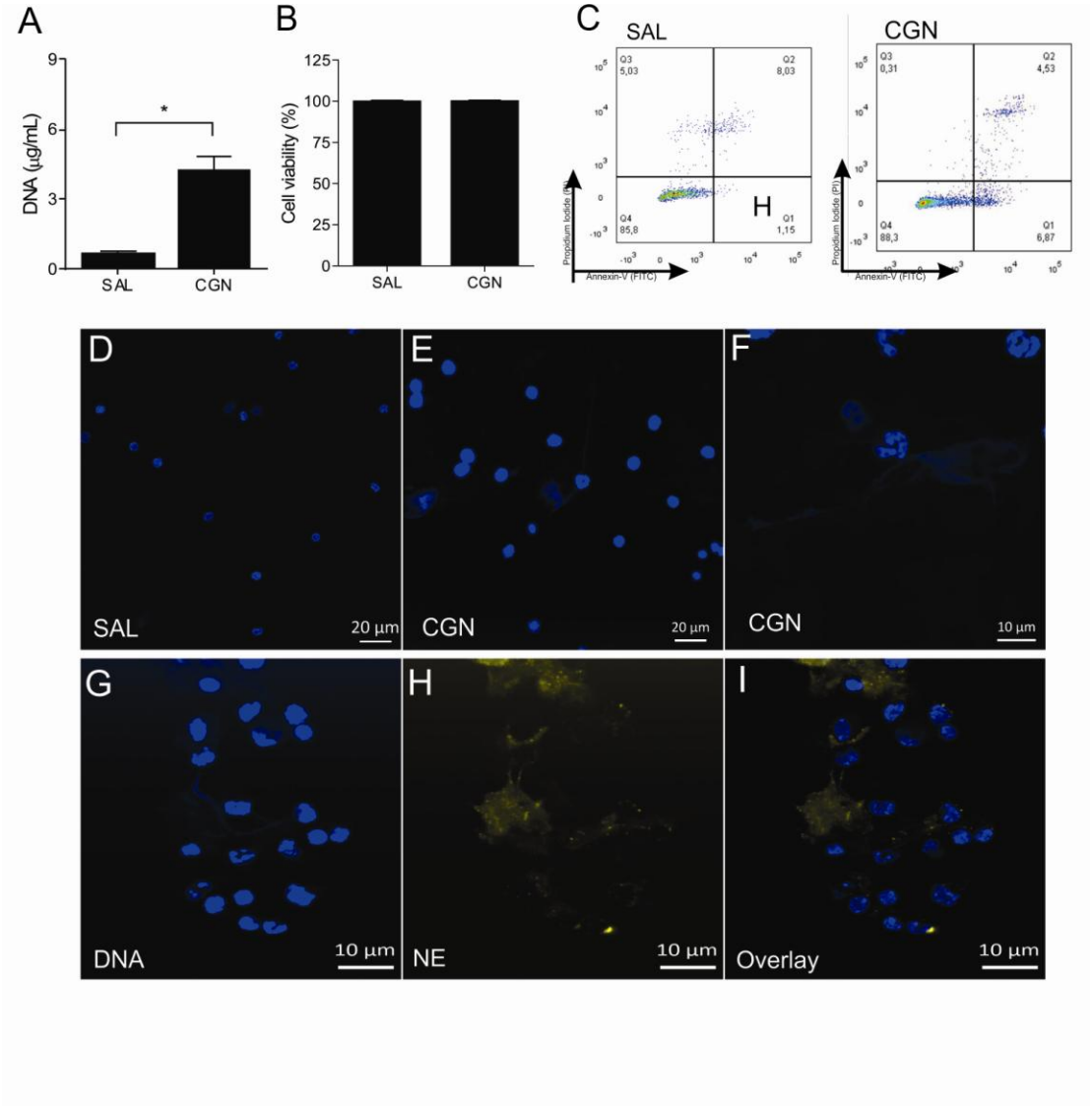


Fig. 2:


A
DNA (µg/mL)
9
6
3
0
*
SAL
CGN
B
Cell viability (%)
125
100
75
50
25
0
SAL
CGN
C
SAL
CGN
Q3
5,03
Q2
8,03
Q4
85,8
Q1
1,15
H
Q3
0,31
Q2
4,53
Q4
88,3
Q1
6,87
Propidium Iodide (PI)
Annexin-V (FITC)
D
SAL
20 µm
E
CGN
20 µm
F
CGN
10 µm
G
DNA
10 µm
H
NE
10 µm
I
Overlay
10 µm

Capítulo 3 – Considerações Finais

Neste estudo demonstramos que o estresse neonatal por LPS em camundongos Balb/c tem interferência na resposta inflamatória através da liberação de citocinas. Esse efeito parece ser influenciado pela idade e pelo sexo dos animais. Apesar da liberação de ROS e NETs não ter sido alterada pelo estresse no início da vida, os resultados demonstram um efeito ontogenético sobre esses parâmetros e sobre a liberação de citocinas.

Também demonstramos neste estudo que um estímulo inflamatório causado por carragenina, além de ativar diversos mecanismos inflamatórios como produção de citocinas e ROS, é capaz de ativar o sistema imune inato com a migração de células de defesa, preferencialmente neutrófilos. Desta forma, demonstramos pela primeira vez que a carragenina é capaz de ativar a liberação de NETs pelos neutrófilos.

4. Referências Bibliográficas

1. Selye H. A syndrome produced by diverse nocuous agents. 1936. J Neuropsychiatry Clin Neurosci. 1998;10(2):230-1.

2. Levine S. The psychoendocrinology of stress. Ann N Y Acad Sci. 1993;697:61-9.

3. Hostinar CE, Sullivan RM, Gunnar MR. Psychobiological mechanisms underlying the social buffering of the hypothalamic-pituitary-adrenocortical axis: a review of animal models and human studies across development. Psychol Bull. 2014;140(1):256-82.

4. Khansari DN, Murgo AJ, Faith RE. Effects of stress on the immune system. Immunol Today. 1990;11(5):170-5.

5. Wright RJ. Stress and atopic disorders. J Allergy Clin Immunol. 2005;116(6):1301-6.

6. Schwarz JM, Bilbo SD. Sex, glia, and development: interactions in health and disease. Horm Behav. 2012;62(3):243-53.

7. Spencer SJ, Galic MA, Pittman QJ. Neonatal programming of innate immune function. Am J Physiol Endocrinol Metab. 2011;300(1):E11-8.

8. Caldji C, Diorio J, Meaney MJ. Variations in maternal care in infancy regulate the development of stress reactivity. Biol Psychiatry. 2000;48(12):1164-74.

9. Bilbo SD, Levkoff LH, Mahoney JH, Watkins LR, Rudy JW, Maier SF. Neonatal infection induces memory impairments following an immune challenge in adulthood. Behav Neurosci. 2005;119(1):293-301.

10. Adams-Chapman I, Stoll BJ. Neonatal infection and long-term neurodevelopmental outcome in the preterm infant. Curr Opin Infect Dis. 2006;19(3):290-7.

11. Walker AK, Nakamura T, Byrne RJ, Naicker S, Tynan RJ, Hunter M, et al. Neonatal lipopolysaccharide and adult stress exposure predisposes rats to anxiety-like behaviour and blunted corticosterone responses: implications for the double-hit hypothesis. Psychoneuroendocrinology. 2009;34(10):1515-25.

12. Shanks N, Larocque S, Meaney MJ. Neonatal endotoxin exposure alters the development of the hypothalamic-pituitary-adrenal axis: early illness and later responsivity to stress. J Neurosci. 1995;15(1 Pt 1):376-84.

13. Gomes CM, Donadio MV, Franskoviaki I, Anselmo-Franci JA, Franci CR, Lucion AB, et al. Neonatal handling reduces angiotensin II receptor density in the medial preoptic area and paraventricular nucleus but not in arcuate nucleus and locus coeruleus of female rats. Brain Res. 2006;1067(1):177-80.

14. Lawn JE, Cousens S, Zupan J, Team LNSS. 4 million neonatal deaths: when? Where? Why? Lancet. 2005;365(9462):891-900.

15. van Deventer SJ, Büller HR, ten Cate JW, Aarden LA, Hack CE, Sturk A. Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. Blood. 1990;76(12):2520-6.

16. Ellis S, Mouihate A, Pittman QJ. Early life immune challenge alters innate immune responses to lipopolysaccharide: implications for host defense as adults. FASEB J. 2005;19(11):1519-21.

17. Shanks N, Windle RJ, Perks PA, Harbuz MS, Jessop DS, Ingram CD, et al. Early-life exposure to endotoxin alters hypothalamic-pituitary-adrenal function and predisposition to inflammation. Proc Natl Acad Sci U S A. 2000;97(10):5645-50.

18. Boissé L, Mouihate A, Ellis S, Pittman QJ. Long-term alterations in neuroimmune responses after neonatal exposure to lipopolysaccharide. J Neurosci. 2004;24(21):4928-34.

19. Iwasa T, Matsuzaki T, Kinouchi R, Fujisawa S, Murakami M, Kiyokawa M, et al. Neonatal LPS injection alters the body weight regulation systems of rats under non-stress and immune stress conditions. Int J Dev Neurosci. 2010;28(1):119-24.

20. Landoni VI, Chiarella P, Martire-Greco D, Schierloh P, van-Rooijen N, Rearte B, et al. Tolerance to lipopolysaccharide promotes an enhanced neutrophil extracellular traps formation leading to a more efficient bacterial clearance in mice. Clin Exp Immunol. 2012;168(1):153-63.

21. Di Rosa M. Biological properties of carrageenan. J Pharm Pharmacol. 1972;24(2):89-102.

22. Cuzzocrea S, Santagati S, Sautebin L, Mazzon E, Calabrò G, Serraino I, et al. 17beta-estradiol antiinflammatory activity in carrageenan-induced pleurisy. Endocrinology. 2000;141(4):1455-63.

23. Vinegar R, Truax JF, Selph JL. Some quantitative temporal characteristics of carrageenin-induced pleurisy in the rat. Proc Soc Exp Biol Med. 1973;143(3):711-4.

24. Syam S, Bustamam A, Abdullah R, Sukari MA, Hashim NM, Mohan S, et al. β Mangostin suppress LPS-induced inflammatory response in RAW 264.7 macrophages in vitro and carrageenan-induced peritonitis in vivo. J Ethnopharmacol. 2014;153(2):435-45.

25. Gonçalves DO, Calou IB, Siqueira RP, Lopes AA, Leal LK, Brito GA, et al. In vivo and in vitro anti-inflammatory and anti-nociceptive activities of lovastatin in rodents. Braz J Med Biol Res. 2011;44(2):173-81.

26. Lucetti DL, Lucetti EC, Bandeira MA, Veras HN, Silva AH, Leal LK, et al. Anti-inflammatory effects and possible mechanism of action of lupeol acetate isolated from Himatanthus drasticus (Mart.) Plumel. J Inflamm (Lond). 2010;7:60.

27. Alves CF, Alves VB, de Assis IP, Clemente-Napimoga JT, Uber-Bucek E, Dal-Secco D, et al. Anti-inflammatory activity and possible mechanism of extract from Mikania laevigata in carrageenan-induced peritonitis. J Pharm Pharmacol. 2009;61(8):1097-104.

28. Bhattacharyya S, Gill R, Chen ML, Zhang F, Linhardt RJ, Dudeja PK, et al. Toll-like receptor 4 mediates induction of the Bcl10-NFkappaB-interleukin-8 inflammatory pathway by carrageenan in human intestinal epithelial cells. J Biol Chem. 2008;283(16):10550-8.

29. Bhattacharyya S, Dudeja PK, Tobacman JK. Carrageenan-induced NFkappaB activation depends on distinct pathways mediated by reactive oxygen species and Hsp27 or by Bcl10. Biochim Biophys Acta. 2008;1780(7-8):973-82.

30. Kennedy AD, DeLeo FR. Neutrophil apoptosis and the resolution of infection. Immunol Res. 2009;43(1-3):25-61.

31. Fox S, Leitch AE, Duffin R, Haslett C, Rossi AG. Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. J Innate Immun. 2010;2(3):216-27.

32. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest. 2000;80(5):617-53.

33. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004;303(5663):1532-5.

34. Yam-Puc JC, García-Marín L, Sánchez-Torres LE. [Neutrophil extracellular traps (NET), consequence of a cellular suicide]. Gac Med Mex. 2012;148(1):68-75.

35. Brinkmann V, Zychlinsky A. Neutrophil extracellular traps: is immunity the second function of chromatin? J Cell Biol. 2012;198(5):773-83.

36. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ. 2009;16(11):1438-44.

37. Pilsczek FH, Salina D, Poon KK, Fahey C, Yipp BG, Sibley CD, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. J Immunol. 2010;185(12):7413-25.

38. Arazna M, Pruchniak MP, Demkow U. Neutrophil extracellular traps in bacterial infections: Strategies for escaping from killing. Respir Physiol Neurobiol. 2013.

39. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol. 2007;176(2):231-41.

40. Lögters T, Margraf S, Altrichter J, Cinatl J, Mitzner S, Windolf J, et al. The clinical value of neutrophil extracellular traps. Med Microbiol Immunol. 2009;198(4):211-9.

41. Yost CC, Cody MJ, Harris ES, Thornton NL, McInturff AM, Martinez ML, et al. Impaired neutrophil extracellular trap (NET) formation: a novel innate immune deficiency of human neonates. Blood. 2009;113(25):6419-27.

42. Marcos V, Nussbaum C, Vitkov L, Hector A, Wiedenbauer EM, Roos D, et al. Delayed but functional neutrophil extracellular trap formation in neonates. Blood. 2009;114(23):4908-11; author reply 11-2.

43. Sharma AA, Jen R, Butler A, Lavoie PM. The developing human preterm neonatal immune system: a case for more research in this area. Clin Immunol. 2012;145(1):61-8.