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GABRIELA CALIXTO RIBEIRO DE HOLANDA

EFEITO DA INFECÇÃO ESQUISTOSSOMÓTICA MATERNA: EXPRESSÃO DAS HISTONAS DESACETILASES EM DESCENDENTES ADULTOS E ANÁLISE PROTEÔMICA DO LEITE

RECIFE – PE
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Tese apresentada ao Programa de Pós-Graduação em Biologia Aplicada à Saúde do Centro de Biociências, Laboratório de Imunopatologia Keizo Asami da Universidade Federal de Pernambuco como parte dos requisitos para obtenção do título de Doutora em Biologia Aplicada à Saúde.

Orientador: Prof. Dr. José Luiz de Lima Filho

Coorientador: Profª. Drª. Valdenia Maria Oliveira de Souza

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BANCA EXAMINADORA

_____

Prof. Dr. José Luiz de Lima Filho (Orientador)

Universidade Federal de Pernambuco

_____

Profª. Dra. Danyelly Bruneska Gondim Martins (Examinador interno)

Universidade Federal de Pernambuco

_____

Profª. Dra. Maria Tereza Cartaxo Muniz (Examinador externo)

Universidade de Pernambuco

_____

Profª. Dra. Vlaudia Maria Assis Costa (Examinador externo)

Universidade Federal de Pernambuco

_____

Profª. Dra. Virgínia Maria Barros de Lorena (Examinador externo)

Universidade Federal de Pernambuco

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Aos meus pais, por todo amor, compreensão, apoio e incentivo. Vocês me ensinam todos os dias sobre perseverança, superação e dedicação.

Dedico

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(THEODORE ROOSEVELT, 1899)

RESUMO

A gestação e a amamentação em mães esquistossomóticas pode alterar a resposta imune da prole a longo prazo. Assim, o objetivo deste estudo foi avaliar a expressão de HDACs, produção de citocinas por linfócitos T e B e macrófagos e frequência de células CD4+CD25+FoxP3+ de descendentes adultos nascidos e/ou amamentados por mães esquistossomóticas, além de avaliar as proteínas do soro do leite de camundongos infectadas pelo *Schistosoma mansoni*. Para isto, fêmeas *Swiss webster* foram submetidas a infecção com *S. mansoni* (20 cercárias), sincronização do estro e acasalamento. Logo após nascimento, a amamentação adotiva foi realizada em que descendentes de mães infectadas mamaram nas mães não infectadas (MI) e filhotes de mães não infectadas foram amamentados pelas mães infectadas (AI). Um grupo de animais nascidos de mães esquistossomóticas permaneceu amamentando nas próprias mães (MIAI). Para grupo Controle, utilizou-se animais nascidos e amamentados em mães não infectadas. Os leites das mães infectadas (LMI) e não infectadas (LMNI) foram coletados entre o 10º e 12º dias de amamentação e a gordura e caseína foi removida. Foi realizada nanoUPLC-MS^E para caracterizar as proteínas dos leites LMI e LMNI. Os descendentes (sete semanas de idade) tiveram seus esplenócitos cultivados com/sem Concanavalina A (5 μg/mL). Após 24h, parte das células foi utilizada na qPCR e a outra parte foi utilizada para imunofenotipagem, com anticorpos monoclonais ligados a fluorocromo para a avaliação de CD4+IL-4+, CD4+IL-10+, CD4+IL-2+, CD4+IFN-γ+, CD45R/B220+IL-10+, CD14+IL-10+, CD4+CD25+FoxP3+. Comparado ao Controle, o grupo MI apresentou aumento da expressão de HDAC9 e frequência de células CD4+IL-10+. O grupo AI teve um aumento da expressão de HDAC1, HDAC2, HDAC6, HDAC7, HDAC10, Sirt2, Sirt5, Sirt6 e Sirt7 enquanto o grupo MIAI apresentou um aumento apenas de HDAC10. Os grupos AI e MIAI apresentaram frequências diminuídas de células CD4+IL-4+ e CD4+CD25+FoxP3+, seguidas de uma maior frequência de células CD14+IL-10+ e CD45R/B220+IL-10+. O grupo MIAI também apresentou alta frequência de células CD4+IL-10+. A análise proteômica do leite identificou 29 proteínas diferencialmente expressas, das quais 15 foram encontradas apenas no LMI, 4 encontravam-se reguladas negativamente e 10 proteínas encontradas apenas no LMNI. As análises de ontologia gênica, enriquecimento de vias e interação proteína-proteína indicaram proteínas diferencialmente expressas ligadas a vias e processos biológicos, tais como: processos metabólicos e glicolíticos da frutose 1,6-bifosfato, metabolismo da glicose e degranulação de neutrófilos. As proteínas reguladas negativamente encontram-se envolvidas na regulação positiva da ativação das células

B e via de sinalização do receptor, resposta imune inata, ativação do complemento e fagocitose. A amamentação induziu a expressão de HDACs de diferentes classes, envolvidas na redução da resposta inflamatória, enquanto a gestação levou a alta expressão de uma única HDAC. A amamentação e gestação parecem favorecer diferentes vias IL-10-dependentes, mas não células com fenótipo regulatório. Além disso, a proteômica do leite revelou um perfil proteico que pode estar envolvido na ativação/regulação do sistema imune da prole, conferindo um caráter protetor devido ao contato prévio com o leite de mães infectadas.

Palavras-chave: Epigenômica. Espectrometria de massa. Esquistossomose. Proteômica. Relação materno-fetal.

ABSTRACT

Breastfeeding or gestation in schistosomotic mothers can alter the immune response of the offspring in the long term. Thus, the aim of this study was to evaluate the expression of HDACs, production of cytokines by T and B lymphocytes and macrophages, and frequency of CD4+CD25+FoxP3+-cells from adult offspring born and/or suckled by schistosomotic mothers and evaluate whey proteins from the milk of *Schistosoma mansoni*-infected mice in order to identify the fractions which can act as potential immunomodulatory tools. For this, *Swiss webster* females were submitted to infection with *S. mansoni* (20 cercariae), estrus synchronization and mating. After birth, adoptive breastfeeding was performed, in which offspring of infected mothers suckled on non-infected mothers (BIM) and offspring of non-infected mothers suckled on infected mothers (SIM). Another group of animals born from schistosomotic mothers remained breastfeeding on their own mothers (BSIM). For the CONTROL group, animals born and suckled on non-infected mothers were used. Milk from infected (MIM) and non-infected (MNIM) mothers was collected between the 10th and 12th days of breastfeeding and fat and casein was removed. It was conducted a nanoUPLC-MSE analysis to characterize the proteomic profile of milk from infected (MIM) and non-infected mice (MNIM). Seven-week-old offspring had their splenocytes cultured with/without Concanavalin A (5 μg/mL). After 24h part of the cells was used for immunophenotyping, with fluorochrome-linked monoclonal antibodies for evaluation of CD4+IL-4+, CD4+IL-10+, CD4+IL-2+, CD4+IFN-γ+, CD45R/B220+IL-10+, CD14+IL-10+, CD4+CD25+FoxP3+. The other part of the cells was used in qPCR. Compared to Control, BIM mice showed increased expression of HDAC9 and frequency of CD4+IL-10+-cells. SIM group had increased expression of HDAC1, HDAC2, HDAC6, HDAC7, HDAC10, Sirt2, Sirt5, Sirt6 and Sirt7. BSIM group had only increased HDAC10. SIM and BSIM groups presented decreased frequencies of CD4+IL-4+-cells and CD4+CD25+FoxP3+-cells followed by higher frequency of CD14+IL-10+-cells and an increase of CD45R/B220+IL-10+-cells. BSIM group has also shown high frequency of CD4+IL10+-cells. Milk proteomics analysis identified 29 differentially expressed proteins, of which, 15 were only found in MIM, 4 were down-regulated and 10 only found in MNIM. Gene Ontology (GO), pathways enrichment analysis and protein-protein interaction (PPI) analyses indicated differentially expressed proteins linked to biological processes and pathways such as: fructose 1,6-biphosphate metabolic and glycolytic processes, glucose metabolism and neutrophil degranulation pathways. Those which were

down-regulated were involved in the positive regulation of B cell activation and receptor signaling pathway, in the innate immune response, complement activation and phagocytosis. Breastfeeding induced the expression of HDACs from different classes, which are involved in the reduction of inflammatory response. However, gestation enhanced the expression of a single HDAC. Breastfeeding or gestation appear to favor different IL-10-dependent pathways, but not cells with regulatory phenotype. Besides that, milk proteomics revealed a protein profile that may be involved in the activation and regulation of the offspring's immune system in the long term, conferring a protective character due to the previous contact with milk from infected mothers.

Keywords: Epigenomics. Maternal-fetal relationship. Mass spectrometry. Proteomics. Schistosomiasis.

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LISTA DE ABREVIATURAS

APC	*Antigen-presenting cell* (Células apresentadoras de antígenos)
CD	*Cluster of differentiation* (Grupo de diferenciação)
Cepa BH	Cepa Belo Horizonte
ConA	Concanavalina A
CTLA-4	*Cytotoxic T-lymphocyte–associated antigen 4*
DNA	Ácido desoxirribonucleico
FoxP3	*Forkhead box P3*
GITR	*Glucocorticoid-induced TNFR family related gene*
HAT	*Histone acetyltransferases*
HDAC	*Histone deacetylases*
IFN-γ	Interferon-γ
IFN-α	Interferon-α
IFN-β	Interferon-β
IgE	Imunoglobulina E
IgG	Imunoglobulina G
IL-1	Interleucina 1
IL-2	Interleucina 2
IL-4	Interleucina 4
IL-5	Interleucina 5
IL-6	Interleucina 6
IL-10	Interleucina 10
IL-12	Interleucina 12
JNK	*c-Jun N-terminal kinases*
LC-MSE	Cromatografia líquida com espectrômetro de massa
LXRα	*Liver X receptor alpha*
MAPK	*Mitogen activated protein kinases*
MCP-1	*Monocyte chemoattractant protein-1*
NF-κβ	Fator nuclear kappa beta
OA	Ovalbumina
PCR	*Polymerase Chain Reaction*

RNA Ácido ribonucleico

ROS Espécies reativas de oxigênio

SEA *Soluble eggs antigen from Schistoma mansoni* (Antígeno solúvel do ovo do *S.mansoni*)

Sirt *Sirtuin*

STAT *Signal transducers and activators of transcription*

SWAP *Soluble worm antigen preparation* (Antígeno do verme adulto do *S. mansoni*)

TGF-β *Transforming growth factor beta* (Fator de transformação do crescimento beta)

Th1 Linfócitos T *helper* tipo 1

Th2 Linfócitos T *helper* tipo 2

TNF-α *Tumoral necrose factor alfa* (Fator de necrose tumoral alfa)

Treg Linfócitos T regulatórios

TLR *Toll-like receptor*

SUMÁRIO

1 INTRODUÇÃO

A alta prevalência de esquistossomose crônica em gestantes, bem como mulheres em idade fértil, tem sido amplamente relatada (SALAWU; ODAIBO, 2013). O status imunológico das mães esquistossomóticas é capaz de alterar resposta imune do descendente ao longo prazo (OTHMAN et al., 2010; SANTOS et al., 2010, 2014). Foi observado, em um estudo experimental, que os camundongos adultos, previamente amamentados em mães infectadas, apresentaram uma potencialização da produção de anticorpos anti-ovalbumina (OA) (SANTOS et al., 2010) e uma melhora na habilidade de apresentação antigênica dos Linfócitos B na fase indutora da resposta imune anti-OA (SANTOS et al., 2014). Descendentes adultos que apenas nasceram de mães infectadas e não foram amamentados por estas mães apresentaram alta produção de IL-10 e baixa produção de anticorpos anti-OA (SANTOS et al., 2010) e na fase indutora da resposta imune anti-OA tiveram menor frequência de linfócitos B e a capacidade de apresentação antigênica de células CD11+ parcialmente prejudicada (SANTOS et al., 2014). Entretanto, foi observado que quando os camundongos nascidos de mães infectadas quando também são amamentados em mães infectadas ocorre uma reversão da supressão de anticorpos anti-OA, acompanhada do aumento da produção de IL-2 e diminuição de IL-10 e recuperação da produção de anticorpos (SANTOS et al., 2010).

Estudos vem relacionando alterações pós-transcricionais na cromatina, através do processo de acetilação/desacetilação de histonas, com a resposta imune (CHENG et al., 2014; SINGH et al., 2010; VISHWAKARMA et al., 2013; ZOETEN et al., 2011). Além disso, existem estudos que demonstram o papel de marcadores epigenéticos, que podem ser remodelados durante o período perinatal e podem desencadear influências duradouras sobre o epigenoma dos descendentes (ATTIG et al., 2010; LOKE et al., 2013). Camundongos que tiveram administração pré-natal de *Acinetobacter lwoffii* F78 tiveram na sua prole uma maior acetilação da histona H4 no gene de IFN-γ e isso conferiu proteção contra asma após desafio com OA, associada a uma regulação positiva na produção de IFN-γ (BRAND et al., 2011). Song et al. (2014) viram que animais descendentes de mães alérgicas a amendoim tiveram elevados níveis de IgE-específico após sensibilização, além de níveis elevados de histamina seguidos de maior produção de citocinas Th2 e redução da metilação do DNA em sítios CpG do promotor do gene de IL-4.

Em relação ao leite materno, é visto que o mesmo possui compostos bioativos que são importantes como precursores da imunidade do recém-nascido e auxiliam na maturação do

sistema imune (BALLARD; MORROW, 2013). Attallah et al. (2003) verificaram que os recém-nascidos amamentados em mães infectadas apresentam o antígeno 63-kD *Schistosoma* por até 24 meses pós-parto, enquanto em crianças não amamentadas, o antígeno foi identificado na urina até 28 dias pós-parto. Sales et al. (2014) verificaram em leite de camundongos infectados, através de separação de proteínas por gel unidimensional (1D), proteínas como Albumina, Serotransferrina, Alfafetoproteína, Alfa-Actina 3 e 4 e Interleucina 17F, proteínas estas predominantemente relacionadas ao transporte celular, processos metabólicos, estruturais e modulação da resposta imune. Ao analisar o leite de camundongos não infectados e em conjunto com o SEA (antígenos do ovo), foram observadas proteínas que estavam associadas a poucas características biológicas. Neste estudo, não foi feita uma análise proteômica do leite de camundongos mais apurada.

Para os estudos com mamíferos, a herança epigenética transgeneracional é amplamente usada para descrever os efeitos de base que pode ser herdada de uma geração para a seguinte. Embora sejam encontrados estudos que relacionam alterações epigenéticas e a relação materno-fetal, não foram encontrados estudos que relatam os efeitos da gestação e amamentação, de forma separada, e nem suas implicações no sistema imune desses indivíduos quando descendentes de mães esquistossomóticas.

Para isso, o presente estudo teve por objetivo avaliar a expressão de enzimas que atuam na remodelação da cromatina através de desacetilação de histonas (HDACs) em células esplênicas de camundongos adultos descendentes de mães esquistossomóticas, além de descrever os componentes do soro do leite de camundongos fêmeas infectadas pelo *S. mansoni*. Os resultados advindos deste trabalho contribuem para melhor entendimento a respeito do caráter imunomodulatório da ação de HDACs e do leite materno como possíveis ferramentas imunomodulatórias para indivíduos que tenham contato no início da vida com antígenos parasitários e anticorpos anti-parasita, seja in utero e/ou via leite, na resposta imune à longo prazo.

1.2 OBJETIVO GERAL

Comparar a expressão gênica das histonas desacetilases (HDACs) dos esplenócitos e associar com a produção de citocinas em linfócitos T e B e macrófagos de descendentes adultos nascidos e/ou amamentados em mães infectadas pelo *S. mansoni* e descrever a proteômica do soro do leite destas mães.

1.2.1 Objetivos específicos

- Comparar a expressão de HDACs das classes I, IIa, IIb, III e IV em leucócitos esplênicos em descendentes adultos nascidos e/ou amamentados em mães infectadas pelo *S. mansoni* e descendentes nascidos e amamentados em mães não infectadas.
- Comparar em cultura de leucócitos esplênicos a frequência de linfócitos T (CD4+) produtores de IL-2, IL-4, INF-γ e IL-10, linfócitos B (CD45+/B220) e macrófagos (CD14+) produtores de IL-10, e células T regulatórias (CD4+CD25+FoxP3+) em resposta ao mitógeno (Concanavalina A) em descendentes adultos nascidos e/ou amamentados em mães infectadas pelo *S. mansoni* e descendentes nascidos e amamentados em mães não infectadas.
- Comparar a proteômica do leite de mães infectadas pelo *S. mansoni* e não infectadas.

2 REVISÃO DE LITERATURA

2.1 A ESQUISTOSSOMOSE E RELAÇÃO MATERNO-FETAL

Com mais de 700 milhões de pessoas vivendo em áreas de risco, distribuídas em 78 países, a esquistossomose é uma doença tropical, com alta morbidade e mortalidade que afeta atualmente cerca de 240 milhões de pessoas em todo o mundo e, por isso, é considerada uma das mais importantes doenças helmínticas (WHO et al., 2014).

Segundo o Ministério da Saúde (BRASIL, 2009), no Brasil encontra-se distribuída em 19 unidades federativas se apresentando de forma endêmica em uma vasta área que vai do estado do Maranhão até Minas Gerais. Pernambuco é um dos estados que exibe uma das mais altas taxas de prevalência, ocupando o terceiro lugar na Região Nordeste (SILVA; DOMINGUES, 2011). Além da infecção persistente em municípios rurais da zona da mata, a transmissão e os casos de infecção aguda têm sido verificados na zona costeira, especialmente em localidade de praias turísticas (GOMES et al., 2014).

A alta prevalência de esquistossomose crônica em gestantes, bem como mulheres em idade fértil, tem sido amplamente relatada (SALAWU; ODAIBO, 2013). O *status* imunológico das mães esquistossomoticas foi capaz de alterar resposta imune do descendente ao longo prazo (ATTALAH et al., 2006; OTHMAN et al., 2010; SANTOS et al., 2010, 2014). Dentre estes

estudos, experimentalmente, foi relacionado o efeito da gestação, separadamente da amamentação, na reposta imune para o antígeno heterólogo - ovalbumina (OA) (SANTOS et al., 2010, 2014). Com relação ao efeito da amamentação, foi observado que os camundongos adultos, previamente amamentados em mães esquistossomóticas (amamentação adotiva) apresentaram uma potencialização da produção de anticorpos anti-OA (SANTOS et al., 2010) e uma melhora na habilidade de apresentação antigênica dos Linfócitos B, na fase indutora da resposta imune anti-OA através de um aumento na frequência de células B CD40+/CD80+ (SANTOS et al., 2014).

Componentes do leite de mães infectadas parecem interferir na produção de anticorpos anti-OA, visto que descendentes que tiveram contato com leite de mães não-infectadas, não produziram níveis de anticorpos anti-OA tão elevados quando comparado aos que foram amamentados em mães infectadas.

Do contrário, descendentes adultos que nasceram de mães infectadas e não foram amamentados por estas mães apresentaram um perfil imunológico mais supressor, com alta produção de IL-10 e baixa produção de anticorpos anti-OA (SANTOS et al., 2010). Além disso, nestes descendentes, na fase indutora da resposta imune anti-OA tiveram menor frequência de linfócitos B e a capacidade de apresentação antigênica de células CD11+ parcialmente prejudicada na (SANTOS et al., 2014).

Entretanto, foi observado que quando os camundongos nascidos de mães infectadas são também amamentados em mães infectadas ocorre uma reversão da supressão de anticorpos anti-OA, acompanhada do aumento da produção de IL-2 e diminuição de IL-10 e recuperação da produção de anticorpos (SANTOS et al., 2010).

Com relação à modulação para os antígenos homólogos, do parasita, foi demonstrado, frente à infecção pós-natal, que animais nascidos de mães infectadas amamentados por mães controle mostraram maior quantidade de granulomas hepáticos e deposição de colágeno, ao passo que os animais amamentados apresentaram granulomas menores. Os animais nascidos e amamentados por mães infectadas exibiram os menores níveis de anticorpos anti-SEA e anti-SWAP. Dessa forma, a gestação em mães infectadas intensificou a fibrose hepática, enquanto a amamentação diminuiu intensamente os granulomas nos descendentes (SANTOS et al., 2016).

Sendo assim, estes achados mostram que mães infectadas por *S. mansoni* afetam a resposta imune pós-natal para antígenos não relacionados e antígenos parasitários e realçam o papel do leite de mães infectadas como um estimulador da imunidade que é mantida a longo

prazo.

2.2 O LEITE MATERNO E A ESQUISTOSSOMOSE

O leite materno humano apresenta fatores solúveis como proteínas, gorduras, carboidratos, minerais, eletrólitos, vitaminas e água. Além disso, possui compostos bioativos que são importantes como precursores da imunidade do recém-nascido e auxiliam na maturação das células do sistema imune (BALLARD; MORROW, 2013). Nesse contexto, foi demonstrado um total de 285 peptídios não redundantes encontrados em banco de dados, incluindo um "core" de 106 proteínas que eram compartilhadas com o proteoma do leite bovino (ALESSANDRO et al., 2010). Mais recentemente, Picarielloa et al. (2013) identificaram proteínas no leite materno com diversas funções como transporte e sinalização celular, metabolismo da gordura, processos metabólicos, síntese de proteínas e modulação da resposta imune. Com relação às características imunológicas, a amamentação confere proteção ao recém-nascido, visto que o leite contém substâncias antimicrobianas e fatores imunomodulatórios, por exemplo, citocinas, oligossacarídeos, anticorpos, fatores de crescimento, bem como macrófagos, neutrófilos, linfócitos e células epiteliais. Dentre os fatores solúveis, como as citocinas, destacam-se a IL-1, TNF-α, IFN-γ, IL-6, IL-10 e TGF-β (BLEWETT et al., 2008). Diante do exposto, a proteômica tem se tornado uma ferramenta fundamental para caracterização das proteínas e descobertas de biomarcadores. Essa técnica vem sendo definida como a análise do conjunto de proteínas presentes em tecidos, células ou compartimentos subcelulares, usada para identificar proteínas diferencialmente expressas e potencialmente relacionadas a processos biológicos.

Em relação ao leite de mães esquistossomóticas, é sabido do seu potencial em alterar o grau de competência imune dos seus descendentes devido à presença de antígenos parasitários ou de anticorpos anti-parasito (BHARGAVA et al., 2012). Nesse contexto, Attallah et al. (2003) verificaram que os recém-nascidos amamentados em mães infectadas apresentam o antígeno 63 Kd por até 24 meses após o parto. Contudo, nas crianças não amamentadas, o antígeno foi identificado na urina até 28 dias após o parto. Em camundongos, Sales et al. (2014) verificou em leite de camundongos infectados, através de separação de proteínas por gel unidimensional (1D), proteínas como Albumina, Serotransferrina, Alfafetoproteína, Alfa-Actina 3 e 4 e Interleucina 17F, proteínas estas predominantemente relacionadas ao transporte celular, processos metabólicos, estruturais e modulação da resposta imune. Ao analisar o leite de

camundongos não infectados e adicionado de SEA, foi observado proteínas que estavam associadas a poucas características biológicas. Mostrando que o leite de mães infectadas foi capaz de modular a resposta imune dos descendentes, independente dos antígenos do SEA. Estudos tem demonstrado que PIII, fração solúvel do SWAP (Antígeno do verme adulto), é capaz de diminuir a reação granulomatosa e a proliferação celular, induz baixos níveis de IFN-γ e altos níveis de IL-10. Outro antígeno oriundo do PIII, é o P24 que é uma fração do PIII, o mesmo desempenha funções semelhantes ao PIII, tendo como possíveis mecanismos para a modulação do granuloma, os altos níveis de IL-10 e IgG1 (ZOUAIN et al., 2004). Apesar de componentes do leite de mães infectadas terem sido anteriormente descritos, existem constituintes proteicos presentes neste leite que permanecem não elucidados. Sabendo das vantagens de se utilizar uma técnica de identificação de proteína mais refinada, o presente projeto se propõe a verificar os componentes do leite de camundongos infectadas por LC-MS^E.

2.3 EPIGENÉTICA E A RESPOSTA IMUNE

Modificações epigenéticas são mudanças reversíveis baseadas na organização da cromatina celular e que permite a modulação das atividades de expressão gênica em resposta a sinais externos sem alterar a sequência dos nucleotídeos. Incluem metilação do ácido desoxiribonucleico (DNA), modificações pós-traducionais das histonas e dos ácidos ribonucleicos (RNAs) não codificantes, sendo cruciais para estabelecer a programação correta da expressão dos genes.

Os nucleossomos e as histonas são estruturas associadas ao DNA com a função de organização da cromatina. Tal organização está intimamente associada à expressão gênica. O nucleossomo é a unidade fundamental da cromatina e consiste em aproximadamente 146 pb de DNA enroladas ao redor de um octâmero central formado por quatro tipos de histonas: um tetrâmero H3-H4 e dois dímeros H2A e H2B. As histonas são proteínas básicas que consistem em um domínio globular C-terminal, onde o filamento de DNA se enrola e de uma cauda N-terminal flexível (BANNISTER; KOUZARIDES, 2011). Do ponto de vista bioquímico, as principais modificações das histonas são acetilação, metilação, fosforilação, ubiquitinação, e sumoilação. Os efeitos de tais modificações variam de ativação a silenciamento gênico e reparo do DNA (SEUTER et al., 2012; CHATUVERDI et al., 2014).

A acetilação é a alteração mais comumente estudada, considerada como um dos mecanismos de estímulo à transcrição, é controlada por duas famílias de enzimas: as histonas-

acetiltransferases (HATs) e as histonas-desacetilases (HDACs). As HATs atuam como co-ativadores da transcrição através da adição de um grupo acetil às histonas e as HDACs por sua vez, removem um grupo acetil, causando repressão da transcrição (VILLAR-GAREA et al., 2004; PETERSON et al., 2005). A acetilação das histonas é regulada de modo reversível por um balanço entre as atividades das HATs e HDACs (ELSHEIKH et al., 2009). A hiperacetilação das histonas por agentes inibidores específicos das HDACs pode afetar processos de apoptose, proliferação celular e diferenciação (MINUCCI et al., 2006), e o grau de modificação da histona se correlaciona com o nível de transcrição (SCHUBELER et al., 2004). As histonas-desacetilases (HDACs) têm sido categorizadas como classe I (HDAC1, HDAC2, HDAC3, e HDAC8), classe IIa (HDAC4, HDAC5, HDAC7, e HDAC9), classe IIb (HDAC6 e HDAC10), classe III (SIRT1 a SIRT7), e classe IV (HDAC11) (HABERLAND et al., 2009) e estão sendo estudadas devido a interferência na rota de mecanismos relacionados à patogênese de diversos tipos de cânceres e doenças inflamatórias como apresentado na tabela 1.

TABELA 1 Ação das Histonas desacetilases (HDACs)

	Histona Deacetilase	**Ação**	**Autores**
Classe I	HDAC1	Inibição de NF-kβ.	Dahllöf et al., 2015
	HDAC2	Regula negativamente TNF-α	Kim et al., 2018
	HDAC3	Essencial à maturação de CD4+ e células B (B220+).	Stengel et al., 2017
	HDAC8	Inibição de HDAC8 aumenta Treg induzida com produção de IL-10.	Kim et al., 2018
Classe IIa	HDAC4	Indução da diferenciação miofibroblástica por elevação de TGF-β1.	Glenisson et al., 2007
	HDAC5	Depleção de HDAC5 prejudica função supressora da Treg.	Xiao et al., 2016
	HDAC7	Redução da expressão de TNF, IL-1 e IL-6.	Barneda- Zahonero et al., 2013
	HDAC9	Aumento de IFN do tipo I.	Li et al., 2016
Classe IIb	HDAC6	Suporte do perfil regulatório com aumento da expressão de Treg (FoxP3), IL-10 e CTLA-4 e menor produção de TNF-α e IL-6 por macrófagos.	Zoeten et al., 2011 Vishwakarma et al., 2013
	HDAC10	Expressão de IL-6 e IL-8 e ativação de NF-kβ.	Liao et al., 2019
Classe III	Sirt1	Produção de IL-12 e TGF-β1 por DCs. Sua deficiência leva à	Schug et al., 2010

		produção de citocinas pró-inflamatórias por macrófagos	
	Sirt2	Inibe a expressão de IL-1β e IL-6 e ativação de NF-kβ. Reduz espécies reativas de oxigênio (ROS).	Lo Sasso et al., 2014
	Sirt3	Redução de ROS através do aumento de SOD e catalase.	Sundaresan et al., 2009
	Sirt4	Depleção gera aumento de IL-1β, IL-6, IL-8, COX-2, MMP-9, ICAM-1.	Tao et al, 2015
	Sirt5	Antagoniza Sirt2 e leva à ativação de NF-kβ.	Qin et al., 2017
	Sirt6	Diminuição dos níveis de TNF-α.	Lee et al., 2013
	Sirt7	Estabilização da proteína receptora do TGF-β tipo I.	Yamagata et al., 2018
Classe IV	HDAC11	Repressor transcricional de IL-10.	Cheng et al., 2014

As histonas das células T naive, tanto no locus de IFN-γ e IL-4 são hipoacetiladas (FIELDS et al., 2002), enquanto a metilação do DNA é considerado o principal mecanismo epigenético que controla negativamente a expressão de citocinas Th1 (WHITE et al., 2002). Além disso, a metilação do DNA em genes de citocinas do tipo Th1 apoia o desenvolvimento de células Th2 (MAKAR et al., 2004), que é reforçado por acetilação das histonas, que fornece acessibilidade no loci tanto de IFN-γ quanto IL-4, para células Th1 e Th2 (SINGH et al., 2010).

Além dos genes de Th1 e Th2, evidências sugerem que a metilação do DNA e acetilação das histonas também ocorrem no locus da IL-10 durante a diferenciação de células T em ambos perfis Th2 e Treg (SARAIVA et al., 2005). Com relação à acetilação e expressão de IL-10 e Treg, um estudo experimental, Lian et al. (2012) mostraram que a supressão de HDAC11 foi capaz de promover a expressão de IL-10 em células de Kupfer. Em estudo recente, foi relatada a possibilidade da HDAC11 em regular negativamente a proliferação e função de células MDSC (Myeloid derived suppressor cell) (SAHAKIAN et al., 2014). Em estudo com colite em animais *knockout* para HDAC6 foi verificado que a ausência desta enzima gerou maior expressão de Foxp3, CTLA-4, IL-10 e GITR (ZOETEN et al., 2011). Quando avaliada células T regulatórias em relação a neuroinflamação secundária após lesão cerebral aguda, foi visto que a Tubastatina (inibidor da HDAC6) serviu como potente indutor de expressão de Foxp3 e melhorou a função anti-inflamatória cerebral de Tregs (LIESZ et al., 2013). A tubastatina também é capaz de inibir a produção de TFN-alfa e IL-6 em macrófagos estimulados com LPS (VISHWAKARMA et al., 2013). Quando estudado um novo inibidor da HDAC6 (Quinazolin),

foi visto que ele foi capaz de inibir de forma mais eficaz o crescimento tumoral na leucemia mieloide aguda, mesmo quando a dose utilizada foi quatro vezes menor do que o SAHA (HDACi) (YANG et al., 2015). Em estudo experimental, utilizando células apresentadoras de antígeno (APCs) demonstrou papéis diferentes entre a HDAC6 e HDAC11 na regulação transcricional da IL-10 por estas células, sendo a HDAC6 necessária para ativação transcricional da expressão do gene de IL-10 em macrófagos e células dendríticas através da ativação de STAT3 e a HDAC11 atua como repressor transcricional de IL-10. Além disso, eles observaram que estas HDACs interagem entre si nas APCs (CHENG et al., 2014). É possível que HDAC6/HDAC11 possam participar na regulação de outros genes relacionados ao sistema imunológico além da sua atuação no gene da IL-10.

2.4 HERANÇA EPIGENÉTICA TRANSGENERACIONAL

O período perinatal, que envolve tanto o desenvolvimento *in utero* quanto a amamentação no recém-nascido, é um momento crucial para a maturação e indução de tolerância imunológica. É bem aceito que ambos os fatores endógenos e exógenos conduzem esta fase de maturação tardia do sistema imunitário. Exposições durante este período podem primar o risco de doença na vida adulta (FALL, 2013).

Estudos têm demonstrado o papel de marcadores epigenéticos, que podem ser remodelados durante o período de desenvolvimento crítico, portanto a exposição a ambientes adversos pode desencadear influências duradouras sobre o epigenoma da célula em diferenciação (ATTIG et al., 2010). Para os estudos com mamíferos, a herança epigenética transgeneracional é amplamente usada para descrever os efeitos de base que pode ser herdada de uma geração para a seguinte.

Foi mostrado em camundongos que a administração pré-natal de bactérias Gram-negativas resultou em acetilação da histona H4 no gene de IFN-γ e conferiu proteção contra asma após desafio com OVA, associada a uma regulação positiva na produção de IFN-γ no descendente (BRAND et al., 2011). Além disso, a nutrição materna foi identificada como fator capaz de alterar perfil epigenético do descendente, como por exemplo, através da redução de acetilação na região do promotor de histona H3 (K9, 14), silenciando a expressão de LXRα e levando ao aparecimento de diabetes na prole (VO et al., 2013). Estes mecanismos corroboram as diferenças observadas na função imune presente no nascimento em crianças predispostas a doenças alérgicas na infância (TULIC et al., 2011). Embora ainda não foi totalmente explorada,

os estudos sugerem que a exposição materna pode potencialmente alterar a epigenética fetal em genes chave do sistema imunológico.

Yu et al. (2015) observaram que animais nascidos e amamentados em fêmeas submetidas à dieta com alta quantidade de lipídio induziu hipermetilação global do DNA, incluindo ácidos graxos e genes relacionados com o metabolismo do colesterol. Além disso, em ovelhas obesas, foi visto que a ativação do *Toll-like receptor-4* (TRL-4) por meio de ácidos graxos livres maternos poderia ativar o NF-kB e JNK, vias de sinalização inflamatória (ZHU et al., 2010). Outras vias inflamatórias foram ativadas devido ao índice de massa corporal materno, como p38 MAPK e STAT-3 (AYE et al., 2014).

Um estudo experimental que avaliou a formação do Lábio leporino/Fenda palatina em camundogos descendentes C57BL/6J, foi observado que quando administrado 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) nas fêmeas durante o período gestacional, os descendentes tiveram em conjunto com a formação do lábio leporino, menor acetilação na histona H3 no 15° dia de gestação em comparação ao grupo controle. Levando a crer que a acetilação desta histona esteja relacionada com um dos mecanismos pelos quais o TCDD induz lábio leporino (CUIPING et al., 2014). Quando utilizado embriões precoces advindos de fêmeas com dieta de baixa proteína, os embriões tiveram redução de acetilação nas histonas H3 e H4 no promotor de Gata6, levando a baixa expressão a nível de mRNA e proteína. Além disso, foi verificado um estímulo na expressão de HDAC1 (SUN et al., 2015). Em estudo com camundongos, os ovócitos *knockout* para HDAC2, através de cruzamento com animais Zp3-Cre, foi observado que essa enzima parece ser largamente responsável pela desacetilação da H4K16 durante a maturação destes ovócitos (MA; SCHULTZ, 2013). Song et al. (2014) viram que animais descendentes de mães alérgicas a amendoim tiveram elevados níveis de IgE-específico após sensibilização, além de níveis elevados de histamina e apresentaram mais sintomas anafiláticos em comparação com descendentes de mães controle. Estes resultados foram seguidos de maior produção de citocinas Th2 em cultura de espenócitos e linfonodos mesentéricos de descendentes de mães alérgicas e redução da metilação do DNA em sítios CpG do promotor do gene de IL-4.

Embora sejam encontrados estudos que relacionam alterações epigenéticas e a relação materno-fetal, não foram encontrados estudos que relatam os efeitos da gestação e amamentação de forma separada e nem suas implicações no sistema imune desses indivíduos quando descendentes de mães esquistossomóticas. Para isso, o presente estudo teve por objetivo avaliar a expressão de enzimas que atuam na remodelação da cromatina através de desacetilação

de histonas (HDACs) em células esplênicas de camundongos adultos descendentes de mães esquistossomóticas, além de descrever os componentes do soro do leite de camundongos fêmeas infectadas pelo *S. mansoni*. Os resultados advindos deste trabalho contribuem para melhor entendimento a respeito do caráter imunomodulatório da ação de HDACs e do leite materno como possíveis ferramentas imunomodulatórias para indivíduos que tenham contato no início da vida com antígenos parasitários e anticorpos anti-parasita, seja *in utero* e/ou via leite, na resposta imune à longo prazo.

3 METODOLOGIA

3.1 ANIMAIS E INFECÇÃO MATERNA

Camundongos fêmeas *Swiss Webster* com 4 semanas de idade foram infectadas, via percutânea, com 20 cercárias da cepa São Lourenço da Mata (SLM) de *S. mansoni*. Após 45 dias de infecção, o Método de Kato-Katz (KATZ et al., 1972) foi utilizado para confirmação da infecção e mensuração do número de ovos de *S. mansoni* por grama de fezes. Sessenta dias pós-infecção, as fêmeas tiveram seus estros sincronizados (WANG et al., 2001) através da administração de hormônios. No primeiro dia foi administrado 5 UI (100 µL) de Gonadotrofina Coriônica e no terceiro dia 5 UI de Gonadotrofina Coriônica Humana (hCG). Posteriormente, as fêmeas foram acasaladas (duas fêmeas para um macho) e foi observada a formação do "plug" vaginal para confirmação do acasalamento. O mesmo procedimento foi realizado para as fêmeas não infectadas. Os animais foram fornecidos e mantidos em boas condições de saúde e nutrição no Biotério do Instituto Aggeu Magalhães (IAM/FIOCRUZ).

3.2 FORMAÇÃO DOS GRUPOS DE ESTUDO

Imediatamente após o nascimento, parte dos descendentes machos foi submetida à amamentação adotiva (SANTOS et al., 2010): parte dos animais nascidos de mães infectadas foram amamentados por mães não infectadas (Grupo MI). Parte dos filhotes de mães não infectadas foram amamentados em mães infectadas (Grupo AI). Além disso, a outra parte dos animais permaneceram sendo amamentados em suas próprias mães: animais nascidos e amamentados em mães infectadas (Grupo MIAI) e um grupo de animais nascidos e amamentados em mães não infectadas (Grupo CONTROLE).

3.3 OBTENÇÃO DO LEITE MATERNO

Entre 10 e 12 dias após o parto, as mães foram separadas dos seus filhotes por ~2 horas, anestesiadas e receberam ocitocina (100 µl -1 UI), minutos antes da ordenha. Sobre as tetas, foi feita utilização de gaze estéril umedecida com água aquecida e posteriormente realizada uma massagem manual. O leite foi coletado com auxílio de uma pipeta (200 µl/animal) e acondicionado em tubos de vidro esterilizados e o inibidor de protease (Protease Inhibitor Mix - GE Healthcare) foi adicionado aos leites obtidos em uma proporção de leite:inibidor = 1: 100 (w/w). Posteriormente, os leites foram centrifugados em centrífuga refrigerada (4000 *g* por 10 minutos a 4 °C) para remoção de gordura. Em seguida, as proteínas do soro foram obtidas a partir do leite desnatado ajustando o pH a 4,6 com ácido acético a 10% (v/v) e centrifugando a 3000 *g* por 10 min para obter um sobrenadante das proteínas do soro e as caseínas isoeletricamente precipitadas (POLIDORI; VINCENZETTI, 2012). As amostras foram armazenadas a -80 °C para uso posterior.

3.4 CULTURA DE CÉLULAS ESPLÊNICAS

Foram retirados os baços dos descendentes dos diferentes grupos de estudo com sete semanas. As suspensões de células esplênicas foram obtidas assepticamente por maceração do órgão em meio RPMI 1640. Após centrifugação (400 *g* por 10 min./4 °C), as hemácias provenientes do órgão foram lisadas pela adição de água estéril ao precipitado por 20 segundos. As células foram ressuspendidas em meio RPMI suplementado com 5% de Soro Bovino Fetal - SBF (Sigma-Aldrich® Chemical, St. Louis, Mo., USA), mantidas no gelo por 5 min. para deposição do estroma e, após esse período, o sobrenadante foi transferido para outro tubo, sendo a contagem das células e o teste de viabilidade realizados com o auxílio de câmara de Neubauer e Azul de Trypan a 10% (90 µL de Azul de Trypan a 10% + 10 µL de suspensão de células). As células foram cultivadas no intervalo de 24 h (2_x10^7 células/poço) para posterior imunofenotipagem e qPCR. Parte das células foram armazenadas em RNALater™ Solution (Invitrogen). Os cultivos foram realizados em placas de 24 poços e as células submetidas ou não a estímulo mitogênico com Concanavalina A - ConA (5 µg/mL). As placas foram incubadas a 37 °C em estufa umidificada de CO_2 a 5%.

3.5 ENSAIO DE CITOMETRIA DE FLUXO PARA IMUNOFENOTIPAGEM

Após 24 horas de cultivo foram adicionados a cada poço, contendo as suspensões de células esplênicas, sob os diferentes estímulos acima descritos, 5 μL de Golgi Stop (para cada 2_x10^7 células), agitados em vórtex e devolvidas a estufa de CO_2 a 37 ºC por um intervalo de quatro horas. Passado este período de incubação, as células foram lavadas com 6,0 mL de PBS 0,01M + 5% de SBF estéril gelado, em seguida foram agitadas em vórtex e centrifugadas a 400 g por 10 minutos sob a temperatura de 4 ºC. Posteriormente, o sobrenadante foi descartado e as células ressuspendidas em PBS 0,01M + 5% de SBF estéril, no mesmo volume da cultura inicial e distribuídas nos tubos de citometria, sendo 100 μL por tubo com concentração final de 1x106 células por tubo. Realizou-se a marcação celular com os anticorpos de superfície (anti-CD4; anti-CD25; anti-CD14; anti-CD45R/B220), sendo acrescentado ao tubo 0,5 μL de cada anticorpo em ambiente escuro, seguido de agitação em vórtex e incubação por 30 minutos a 4 ºC no escuro. Passado o tempo de incubação, as células foram lavadas uma vez com 1 mL de PBS 0,01M + 5% de SBF estéril gelado com agitação em vórtex e posterior centrifugação a 400 *g* por cinco minutos. Depois disso, o sobrenadante foi descartado e acrescentado 200 μL de tampão de fixação e as células foram incubadas por 15 minutos a 4 ºC no escuro. Após este tempo, foi realizada nova lavagem com 1 mL de PBS 0,01M + 5% de SBF estéril gelado. O sobrenadante foi descartado e foi adicionado aos tubos 500 μL da solução de permeabilização, diluída quatro vezes e incubados por 30 minutos a 4ºC no escuro. Posteriormente as células foram lavadas com 1 mL de PBS 0,01M + 5% de SBF estéril gelado. Após a centrifugação o sobrenadante foi descartado e adicionou-se a cada tubo 1μl dos anticorpos para marcação intracelular (anti-IL-2, anti-IL-4, anti-IFN-γ, anti-IL-10 e anti-FoxP3) e incubados por 30 minutos a temperatura ambiente em ambiente escuro. Uma nova lavagem foi realizada com 1 mL por tubo da solução de permeabilização diluída 10x em água destilada estéril, seguida de agitação no vótex e centrifugação em 400 *g* por cinco minutos a 4 ºC. Depois o sobrenadante foi descartado e acrescentou-se a cada tubo 300 μL da solução de fixação para posterior leitura em citômetro de fluxo. Os eventos (50.000 para linfócitos e 5.000 para macrófagos) foram adquiridos através do equipamento FACSCalibur™ e analisados com o programa FlowJo (BD-Pharmingen®, San Diego, CA, USA).

3.6 ANÁLISE DE PCR QUANTITATIVA EM TEMPO REAL (qPCR)

O RNA total de céulas esplênicas foi extraído utilizando ReliaPrepTM RNA Cell Miniprep System Kit (Promega, Madison, WI) de acordo com as instruções do fabricante. O DNA complementar (cDNA) foi gerado com QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Alemanha). A PCR quantitativa foi realizada utilizando SYBR Green master mix (Applied Biosystems, Foster City, CA) no equipamento 7500 Real Time System (Applied Biosystems). Os resultados foram normalizados para o gene endógeno β-Actina. Os níveis de expressão relativos foram calculados como $2(^{Ct}(\beta\text{-Actina})-^{Ct}(\text{gene}))$. Os primers foram desenhados usando o software Primer3Plus e as sequências foram descritas na Tabela 1.

TABELA 2 Sequência de primers utilizados na qPCR (*forward e reverse*)

Target gene (Accession number)	**Sequence 5'-3' (Forward and reverse)**	**Tm**	**CG %**	**Amplicon length (bp)**
Hdac1 (NM_008228.2)	CCGGTTAGGTTGCTTCAATC	59.6	50	118
	AACATTCCGGATGGTGTAGC	59.8	50	
Hdac2 (NM_008229.2)	TATTATGGCCAGGGTCATCC	59.6	50	119
	TCAGCAGTGGCTTTATGAGG	59	50	
Hdac3 (NM_010411.2)	ATGCAGGGTTTCACCAAGAG	60.1	50	117
	TGTTGCTCCTTGCAGAGATG	60.1	50	
Hdac4 (NM_207225.2)	CCGCCAGCAGTTTTAAAGTC	59.9	50	92
	ACCGAATGGAGATGCTCAAC	60.1	50	
Hdac5 (NM_001077696.1)	ACTTTCCCCTCCGTAAAACG	60.3	50	116
	AACAGTGCCATCCTTTCGAC	60.1	50	
Hdac6 (NM_010413.3)	ATCTCAGCTGGCTTTGATGC	60.5	50	116
	ATAATACGGCCACCAGCAAG	60.0	50	
Hdac7 (NM_001204275.1)	ATGATGGCCTGGAACATAGG	59.8	50	75
	GATGCTGCTGCAGAGAAATG	59.7	50	
Hdac8 (NM_027382.4)	AGGGAATCTGAAGCATGTGG	60.1	50	131
	CAAATTTCCCCTGCAGTCAC	60.5	50	
Hdac9 (NM_001271386.1)	TTTGAGGTGGCAGAATCCTC	60.2	50	106
	GAGCTGAAGCCTCATTTTCG	60.1	50	
Hdac10 (NM_199198.2)	AACAGGAGCTGTGCACAATG	59.9	50	143
	TCCTCTGCAGCCCATATTTC	60.2	50	
Hdac11 (NM_144919.2)	TGATGGGGTTGAACACTGAG	59.5	50	128
	AGCAGCCCCTTAAAAACTCC	59.7	50	
Sirt1 (NM_019812.3)	GCCCTCAATTTCTGTTCTGC	59.8	50	150
	TTTTGAGTGCTCCAGACACG	60.0	50	
Sirt2 (NM_019812.3)	ACGGCTGCTCATTAACAAGG	60.3	50	88
	GTCAAAATCCATGCCACCTC	60.3	50	

Sirt3 (NM_001177804.1)	CATATGGGCTGATGTGATGG	59.8	50	141
	AGATCTGCCAAAGCGAAGTC	59.6	50	
Sirt4 (NM_001167691.1)	CGAGCAAAAGCTCCCAATAG	60.0	50	145
	TTCCAGCCTTTGGACATCAG	61.2	50	
Sirt5 (NM_178848.3)	CCAGCTTTAGCAGGAAAAGG	59.1	50	139
	CCAGGTTTTCTCCAAACCAC	59.4	50	
Sirt6 (NM_181586.3)	TGTCCAACACAGCTCCTTTC	58.9	50	97
	CTTCCACATGTGTGGGATTC	58.8	50	
Sirt7 (NM_001363439.1)	AGCTTCGGGATACCATTGTG	60	50	104
	CAGGATTGTGTCTGCTTTGC	59.4	50	
β-Actin (NM_007393.5)	TTGCTGACAGGATGCAGAAG	61.1	50	147
	TGATCCACATCTGCTGGAAG	59.8	50	

HDAC: Histona deacetilase 1-11; Sirt: Sirtuína 1-7. bp: pares de bases. Tm (temperature de melting) foi calculado em configurações padrão de concentração de oligo 0,25 mM e Na 50 mM. Os primers foram desenhados utilizando o Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

3.7 QUANTIFICAÇÃO DE PROTEÍNAS

As proteínas do soro resultantes da depleção das caseínas foram submetidas à quantificação de proteínas através do 2-D Quant Kit™ (80-6483-56 GE Healthcare®). Através desse kit, foi possível precipitar quantitativamente apenas as proteínas presentes nos pools, deixando os outros componentes plasmáticos interferentes em solução. Isso foi feito através da adição de 500 µL do "agente precipitante" e "co-precipitante" em cada tubo contendo 10 µL dos pools Leite de Mães Infectadas (MIM) e Leite de Mães não Infectadas (MNIM). Os eppendorfs foram então centrifugados a 10.000 *g* e o sobrenadante descartado. Foi adicionado então 100 µL da solução de cobre e 400 µL de água ultra-pura (Milli-Q®, 18.2 MΩ•cm). Os tubos foram novamente agitados em vórtex e então foi adicionado 1 mL de reagente de cor a cada eppendorf. Após 20 minutos, foi feita a medida das amostras e da curva analítica em 480 nm, em Espectrofotômetro (GE HEALTHCARE, 2010). Nesse método, o cobre livre é quantificado, portanto os valores das absorbâncias são inversamente proporcionais à concentração de proteínas. A curva analítica padrão foi preparada em uma faixa linear de 0-50 µg de uma solução de Albumina Bovina Sérica (BSA - Albumina Sérica Bovina, GE Healthcare). Todo experimento foi realizado em triplicata.

3.8 PRECIPITAÇÃO DE PROTEÍNAS

Em seguida, as proteínas foram precipitadas por um método adaptado de Williams; Stone

(1997). Para cada 150 µg/mL de proteínas foi adicionado 278 µL de 50% Ácido Tricloroacético e 139 µL de 0,1% Triton. As amostras foram incubadas em gelo por 60 minutos e depois submetidas à centrifugação a 10.000 *g* por 10 minutos à 4 ºC. O sobrenadante foi removido e descartado e o *pellet* foi lavado duas vezes com acetona a 90% (WILLIAMS; STONE, 1997). Após a lavagem a acetona foi removida e o *pellet* foi ressolubilizado em Água Milli-Q (Milli-Q®, 18.2 MΩ•cm) e a solução foi submetida a uma nova quantificação de proteínas.

3.9 DIGESTÃO TRÍPTICA E PREPARAÇÃO DE AMOSTRAS PARA LC-MSE

Aproximadamente 50 µg de proteína do grupo experimental e controle foram diluídos em 60 µL de NH_4HCO_3 50 mM. Em seguida, foram adicionados 25 µL de *RapiGest SF* (Waters) a 0,2%, um surfactante utilizado para desnaturar proteínas e melhorar a digestão enzimática, por amostra. A reação ocorreu a 80 ºC até a completa solubilização das proteínas. Posteriormente, as proteínas foram reduzidas pela adição de 2,5 µL de ditiotreirol (100 mM) a 60 °C por 30 minutos, depois foram resfriadas à temperatura ambiente e alquiladas com 2,5 µL de iodoacetamida (300 mM) por 30 minutos ao abrigo da luz. Para a digestão de proteínas, adicionou-se tripsina*Gold* (Promega) a uma razão de proteína: proteína de 1:100 (p/p) e as amostras foram incubadas durante 18h a 37 °C. Após a digestão, o *RapiGest SF* foi hidrolisado com a adição de ácido trifluoroacético a 5% durante 90 minutos a 37 ºC. As amostras foram centrifugadas e o sobrenadante foi transferido para um recipiente *Waters Total Recovering Vial* (Waters). Para a quantificação peptídica absoluta, adicionaram-se 5 pmol de Álcool desidrogenase da *Saccharomyces cerevisiae* (ADH, Uniprot P00330, Waters) a cada amostra como padrão interno. Em seguida, foram adicionados 85 µL de acetonitrila a 3% contendo ácido fórmico a 0,1%, de modo que as concentrações finais de 250 ng/µL e 25 µmol/µL para proteína e ADH, respectivamente, foram atingidas. As amostras foram mantidas a 6 °C para a análise nanoUPLC-MS^E.

3.10 NANOUPLC E AQUISIÇÃO INDEPENDENTE DE DADOS (MS^E) LIVRE DE MARCAÇÃO (*LABEL-FREE*) POR ESPECTROMETRIA DE MASSAS

Os produtos da digestão tríptica foram separados pelo sistema nanoACQUITY UPLC (Waters) numa coluna de fase reversa HSS T3 C18 (1,8 µm x 100 µm x 100 mm) em conjunto com uma coluna Symmetry C18, 5 µm, usando os seguintes gradientes em uma taxa de fluxo de 450 nL/min: 7 a 40% B em 115 minutos; um gradiente de limpeza de 40 a 85% B em 4

minutos e mantido em 85% de B durante 4 minutos; em seguida, reequilíbrio da coluna para 7% B em 2 minutos. A temperatura da coluna foi mantida a 55 °C e as fases móveis A e B consistiram, respectivamente, de água ultrapura e acetonitrila, ambas contendo ácido fórmico a 0,1%. Utilizou-se um espectrômetro de massa Synapt HDMS (Waters) para análise independente de dados (MS^E) dos peptídeos trípticos. O instrumento foi operado no modo resolução e todas as análises foram realizadas no modo “V”, utilizando-se ionização por nano-eletropulverização no modo íon positivo (nanoESI $^+$). Os dados do espectrômetro de massa foram adquiridos usando uma infusão de canal de sonda NanoLockSpray de Glu-Fib (Glu1) derivado de fibrinopeptídeo B humano (M + 2H) 2+ = 785.2486, e os fragmentos MS/MS Glu-Fib foram usados para calibração final do instrumento. Todas as análises foram realizadas usando um canal de massa Glu-Fib em intervalos de 30 segundos. Os dados exatos de tempo de retenção de massa (EMRT) nanoLC-MSE foram coletados com o padrão alternativo MS (3 eV) e elevada energia de colisão (MSE 15–55 eV) aplicadas à armadilha 'T-wave' CID (colisão-dissociação induzida) com gás argônio, usando um tempo de varredura de 0,8 segundos e interseção automática para cada varredura MS de m/z 50 a 2.000. O *offset* de RF (perfis MS) foi ajustado de tal forma que os dados LC/MS foram efetivamente adquiridos de 350 a 2.000 m/z, o que garantiu que qualquer massa observada nos dados LC/MS^E < 350 m/z surgiu de dissociações na célula de colisão.

3.11 PROCESSAMENTO DE DADOS, IDENTIFICAÇÃO DE PROTEÍNA E QUANTIFICAÇÃO

Os dados obtidos da análise de $nanoUPLC/MS^E$ foram processados para a identificação de proteínas pelo software *Protein Lynx Global Server v. 2.4* (PLGS) com os bancos de dados *Mus musculus* UniProtKB (UniProt mouse downloaded em 03 de dezembro de 2018) e NCBI (NCBI mouse downloaded em 04 de dezembro de 2018), ao qual a sequência de aminoácidos da proteína padrão interna (ADH, UniprotP00330) foi anexada. Para acessar a taxa de identificação de falso positivo, foi utilizada a sequência reversa dos bancos de dados. Os parâmetros padrão do PLGS para o processamento de espectros e busca de banco de dados foram: uma perda de clivagem por tripsina, uma modificação fixa de carbamidometil para Cisteína (Cys) e uma modificação variável de oxidação para Metionina (M). Os limiares de busca utilizados foram: fragmentos mínimos por peptídeo (3), peptídeos mínimos por proteína (1), fragmentos mínimos por proteína (7), e taxa de descoberta falsa para identificação de proteínas (2). Os três peptídeos mais abundantes (método de Hi3 livre de marcadores) de todas

as proteínas detectadas foram comparados com os três peptídeos mais intensos da proteína de referência cravada (ADH) de concentração conhecida e usados para calcular as quantidades molares carregadas na coluna (quantificação absoluta) para cada proteína. As proteínas identificadas foram organizadas usando o PLGS em uma lista detalhando a proteína única para cada condição e uma relação logarítmica entre os diferentes grupos. Apenas as proteínas com scores de frequência e intervalos de confiança superiores a 99% de acordo com o algoritmo, respectivamente foram consideradas aceitáveis nessas buscas na base de dados, e quando a mesma proteína foi identificada para diferentes íons do fragmento, aqueles que apresentaram a maior pontuação foram considerados para comparações adicionais e apresentação de dados (CHAMBERY et al., 2009; GEROMANOS et al., 2009; LI et al., 2009).

3.12 ANÁLISE ESTATÍSTICA

Os resultados foram submetidos ao teste de Barlett para verificar se a distribuição dos dados era normal. Após verificar-se que os resultados não seguem a distribuição normal, foi utilizado o método de Kruskal-Wallis. Quando esta análise exibiu diferença significativa foi realizada a comparação múltipla de Dunn. Nos testes estatísticos foram considerados significativos valores de $P<0,05$. O programa utilizado para a obtenção dos cálculos estatísticos foi o Graph Pad Prism 5.0. Além disso, foi realizada análise descritiva dos componentes do leite materno. Todos os procedimentos foram repetidos três vezes para avaliar a reprodutibilidade dos resultados e foi apresentado um estudo representativo dos três estudos independentes.

4 RESULTADOS

Os resultados da pesquisa são apresentados em forma de artigos.

4.1 ARTIGO 1 - GESTATION AND BREASTFEEDING IN SCHISTOSOMOTIC MICE DIFFERENTIALLY ALTERS THE EXPRESSION OF HISTONE DEACETYLASES (HDACS) IN ADULT OFFSPRING

Gestation and breastfeeding in schistosomotic mice differentially alters the expression of histone deacetylases (hdacs) in adult offspring

Gabriela Calixto Ribeiro de Holanda[1], Fabrício Oliveira Souto[1], Maria da Conceição Silva[1], Virgínia Maria Barros de Lorena[2], Vlaudia Maria Assis Costa[1], Monica Camelo Pessôa de Azevedo Albuquerque[1], Valdênia Maria Oliveira de Souza[1]/+, José Luiz de Lima Filho[1]

[1]Universidade Federal de Pernambuco, Laboratório de Imunopatologia Keizo Asami, Recife, PE, Brasil
[2]Fundação Oswaldo Cruz-Fiocruz, Instituto de Pesquisas Aggeu Magalhães, Recife, PE, Brasil

+ Corresponding author
Valdenia Maria Oliveira de Souza E-mail address: valdenia.souza@gmail.com
Laboratory of Immunopathology Keizo Asami (LIKA)
ORCID: 0000-0002-4976-009x
Av. Moraes Rego, 1235 - Cidade Universitária, 50670-901 Recife, Pernambuco, Brazil.
Zip code: 50670-901 Phone/Fax: +55 81 2126 8485

Abstract

BACKGROUND Breastfeeding or gestation in schistosomotic mothers can cause long-term alterations in the immune response of offspring.
OBJECTIVES Evaluate the expression of histone deacetylases (HDACs) (all classes), the production of cytokines by T and B lymphocytes and macrophages, and the frequency of

CD4+CD25+FoxP3+-cells in adult offspring born and/or suckled by schistosomotic mothers.
METHODS We harvested splenocytes from offspring born to (BIM), suckled by (SIM), or born to/suckled by (BSIM) schistosomotic mothers and animals from noninfected mothers (Control) at seven-weeks old and cultured them with/without Concanavalin A. HDAC expression was evaluated by real-time quantitative polymerase chain reaction (qPCR), and cytokines and membrane markers were evaluated by fluorescence-activated cell sorting (FACS).
FINDINGS Compared to Control, BIM mice showed increased expression of HDAC9 and frequency of CD4+IL-10+-cells. The SIM group had increased expression of HDAC1, HDAC2, HDAC6, HDAC7, HDAC10, Sirt2, Sirt5, Sirt6, and Sirt7. The BSIM group only had increased HDAC10 expression. The SIM and BSIM groups exhibited decreased frequencies of CD4+IL-4+-cells and CD4+CD25+FoxP3+-cells, along with a higher frequency of CD14+IL-10+-cells and an increase in CD45R/B220+IL-10+-cells. The BSIM group also showed a high frequency of CD4+IL10+-cells.
MAIN CONCLUSIONS Breastfeeding induced the expression of HDACs from various classes involved in reducing inflammatory responses. However, gestation enhanced the expression of a single HDAC and breastfeeding or gestation appears to favour multiple IL-10-dependent pathways, but not cells with a regulatory phenotype.

Key words: breastfeeding - epigenomics - histone deacetylases - pregnancy - schistosomiasis

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Introduction

A high prevalence of chronic schistosomiasis in pregnant women and women of childbearing age has been reported,[1] and the effects of maternal infection have raised questions regarding the immunity of the offspring.

It is known that the immunological status of schistosomotic mothers can induce long-term alterations in the immune response of the offspring.[2,3,4,5] An experimental study on the effects of gestation and breastfeeding in infected mothers, separately, showed that gestation in these mothers led to potential immunosuppression in adult offspring, with elevated production of IL-10 and lower levels of anti-ovalbumin (OA) antibodies.[2] In addition, offspring born to

infected mothers had a lower frequency of B lymphocytes, and the capacity for antigen presentation by CD11c+ cells was partially impaired.[3] In contrast, it has also been observed that adult mice previously breastfed by schistosomotic mothers exhibited improvement in the production of anti-OA antibodies[2] and in the antigen presentation ability of B lymphocytes through an increase in surface frequency of CD40+/CD80+ in these cells.[3] However, whether these alterations in the immune response of adult offspring from infected mothers are due to epigenetic changes from the perinatal period remains unclear.

Studies have correlated post-transcriptional changes in the chromatin, through histone acetylation/deacetylation, with the immune response.[6,7,8] In an experimental study on antigen presenting cells (APCs), it was demonstrated that histone deacetylase (HDAC)6 is required for transcriptional activation of IL-10 gene expression in macrophages and dendritic cells through activation of STAT3.[6] Another study using pancreatic beta cell lines showed that knockdown of HDAC1 increased IFN-γ-induced STAT1 phosphorylation.[7] Kosciuczuk et al.[8] showed that deacetylation of cyclin-dependent kinase 9 induced by Sirtuin 2 promotes STAT1 phosphorylation during type I interferon responses.

In addition, it has been demonstrated that the role of epigenetic markers can be remodelled during the perinatal period, and may trigger lasting influences on the epigenome of the offspring.[9] Mice prenatally administered with *Acinetobacter lwoffii* F78 displayed increased acetylation of histone H4 in the interferon (IFN)-γ gene in their offspring, and conferred protection against asthma after challenge with OA, which is associated with positive regulation of IFN-γ production.[10] Song et al.[11] found that offspring from mothers with peanut allergy had elevated IgE-specific levels, high levels of histamine and resultant increased production of Th2 cytokines, and reduction of DNA methylation at CpG sites of the IL-4 gene promoter after sensitisation.

Histone acetylation is the most commonly studied epigenomic alteration, for stimulation of transcription, and in turn, is reversibly regulated by the balance between the activity of histone acetyltransferases (HATs) and HDACs.[12] HDACs have been classified as class I (HDAC1, HDAC2, HDAC3 and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), class III (SIRT1 to SIRT7), and class IV (HDAC11)[13] and are increasingly studied due to their interference in the pathways of mechanisms associated with the pathogenesis of various cancers and other inflammatory diseases.[14,15,16]

Although research that relates epigenetic alterations to the maternal-foetal relationship can be found, there are no studies that report the effects of gestation and/or breastfeeding on the

expression of HDACs, and the implications for the immune system of offspring from schistosomotic mothers. To investigate, we have evaluated whether the expression of enzymes involved in chromatin remodeling through histone deacetylation can be altered due to gestation or breastfeeding from *Schistosoma mansoni*-infected mothers. Our results could aid in the discovery of therapeutic targets that improve the immunity of individuals who previously contacted immunological factors resulting from infection during perinatal period.

Materials and Methods

Animals and maternal infection – Four-week-old Swiss Webster female mice were infected subcutaneously (s.c.) with 20 *S. mansoni* cercariae, strain São Lourenço da Mata (SLM). On the 45th day, infection was confirmed by the Kato-Katz method.[17] On the 60th day post-infection (dpi), oestrus was synchronised among mice via the administration of 5 i.u. (100 μL) of equine chorionic gonadotrophin hormone, followed by an additional injection with 5 i.u. (100 μL) of human chorionic gonadotrophin 48 h later. Females were housed with male mice at a 1:1 ratio, and successful mating was confirmed by presence of a vaginal plug. The same procedure was performed in noninfected females, and sevenweek-old male offspring were taken for the experimental and control groups. The mice were housed in the animal care facility at the Aggeu Magalhães Institute (IAM), Oswaldo Cruz Foundation (Fiocruz), municipality of Recife, State of Pernambuco, Brazil.

Adoptive nursing and study groups – Immediately after birth, new-born mice from *S. mansoni*-infected or noninfected mothers were rehoused with mothers from the opposite group. After adoptive nursing, offspring born from infected mothers (BIM) were suckled by noninfected mothers, and offspring from noninfected mothers were suckled by infected mothers (SIM). A separate group was born from and suckled by schistosomotic mothers (BSIM). Animals born from noninfected females were suckled by their mothers (Control).

Cell culture – Spleens from each animal (sevenweeks-old) were harvested after euthanasia by cervical dislocation. Cell suspensions were prepared in RPMI-1640 (Sigma-Aldrich, St. Louis, USA) supplemented with HEPES (10 μM), 2-mercaptoethanol (0.05 μM), 216 mg of L-glutamine/L, gentamicin (50 mg/L), and 5% of foetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, USA). Cells from each group (n = 8-10) were cultivated at a final concentration of 2×10^7 cells/mL in tissue culture plates (Costar Culture Plates, City, USA) and stimulated with

concanavalin-A (Con-A) (5 μg/mL), or without antigenic stimulus (Basal), at 37ºC in 5% CO2. Cultured cells were harvested after 24 h and assayed for immunophenotyping and real-time quantitative polymerase chain reaction (qPCR).

Flow cytometry analyses – 5 μL of Golgi Stop (per 2×10^7 cells) were added to each well containing splenic cells under different stimuli, then the cells were vortexed and returned to the CO2 incubator at 37ºC for four additional hours. Spleen cells were subjected to double-labelling with fluorochrome-labelled antibody solutions at a concentration of 0.5 mg/10^6 cells: FITC anti-mouse CD4, and PE anti-mouse IL-4, APC anti-mouse IFN-γ, PE anti-mouse IL-10, or PerCP-Cy-5.5 anti-mouse IL-2; FITC anti-mouse CD4, PE anti-mouse CD25, and APC anti-mouse FoxP3; FITC anti-mouse CD45R (B220) or FITC anti-mouse CD14, and PE anti-mouse IL-10 (BD Biosciences Pharmingen). After staining, preparations were washed with phosphate-buffered saline (PBS) containing azide (0.1%) and FBS (3%). After centrifugation, the cell pellet was resuspended in PBS with paraformaldehyde (0.5%) and maintained at 4ºC until data acquisition, which was performed using a FACSCalibur (BD-Pharmingen, New Jersey, USA) flow cytometer and acquisition of a minimum 50,000 lymphocytes or 5,000 monocytes. The frequency of positive cells was analysed using FlowJo software, with quadrant gating set based on negative populations and isotype controls. A descriptive analysis of the frequency of cells in the upper right quadrant (double-positive cells) was performed. Distinct gating strategies were used to analyse each subpopulation of cells (Fig. 1). T lymphocyte subpopulations were first selected as CD4 high cells on FL1/anti-CD4-FITC versus laser side-scatter (SSC) dot plots (Fig. 1A). Following this gating procedure, a second gate was set using FL1/anti-CD4-FITC versus FL2/anti-CD25-PE; then, a third gate was established to generate representative 2-dimensional graphics using FL1/anti-CD4-FITC versus FL4/anti-FoxP3-APC to identify triple staining for CD4+CD25+FoxP3+ (Fig. 1B). The frequency of cytokine-expressing cells was further determined on FL1/anti-CD4-FITC versus FL2/anti-IL10-PE or anti-IL4-PE, FL3/anti-IL2-Percp-Cy-5.5 or FL4/anti-IFN-γ-APC dot plots by quadrant statistic measurements, and expressed as percentage of cytokine T CD4+ lymphocyte (Fig. 1C). B Cells and monocytes were first selected on CD45-high or CD14-high cells using FL1/anti-CD45 or CD14-FITC versus SSC dot plots, and the frequency of IL-10 producing cells was subsequently determined using FL1/CD45-FITC or FL1/CD14-FITC versus anti-IL10-PE dot plots and quadrant statistic measurements (Fig. 1D-E). The results are expressed as the median frequency of cells from each group ± standard error.

qPCR analysis – Total RNA from splenic cells was extracted using the ReliaPrepTM RNA Cell Miniprep System Kit (Promega, Madison, WI) according to the manufacturer's instructions. Complementary DNA (cDNA) was generated with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative PCR was performed using SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) on the 7500 Real Time System (Applied Biosystems Foster City, CA, USA) machine. Results were normalised to the housekeeping gene β-Actin. Relative expression levels were calculated using $2^{\Delta\Delta Ct}$. Primers were designed using Primer3Plus software, and the sequences are described in Table.

Statistical analysis – Results were subjected to Barlett's test to verify whether the distribution of the data was normal. After verifying that the results did not follow a normal distribution, the Kruskal-Wallis test was used, followed by Dunn's multiple comparison test when statistical significance was shown. For statistical analysis, we used GraphPad Prism v.5.0 (GraphPad Software, San Diego, CA, USA) and findings were considered significant at $p < 0.05$. All procedures were performed in triplicate to evaluate reproducibility, and images refer to one representative of at least three independent studies.

Ethics – The animal protocol was approved by the Ethical Commission on Animal Use of the Fiocruz (113/2017) and is in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation.

Results

Relative expression of HDAC in animals born and/or breastfed from schistosomotic mothers – To verify the epigenetic profile of the animals born and/or breastfed from schistosomotic mothers, real time qPCR was performed on spleen cells cultured for 24 h in absence (basal) or presence of mitogenic stimulus (ConA). For class I HDAC, the basal relative expressions of HDAC1 and HDAC2 were not altered, but with mitogenic stimulus, the group of animals which were breastfed only (SIM) exhibited increased expression compared to the Control (Fig. 2A-B). The groups BIM, SIM, and BSIM had decreased basal relative expression of HDAC3, but

with mitogenic stimulus the relative expression was similar to Control (Fig. 2C). There was no difference in the relative expression of HDAC8 either at baseline or with mitogenic stimulation

(Fig. 2D).

Class IIa HDACs were analysed, and we saw that there was no difference in HDAC4 expression (Fig. 3A). The relative expression of HDAC5 in the SIM group was lower compared to Control, BIM and BSIM at baseline, but there was no difference among the groups under mitogenic stimulus (Fig. 3B). For HDAC7 the SIM group had lower expression than Control at baseline, while under mitogenic stimulus the SIM group had increased expression compared to the BIM and BSIM groups (Fig. 3C). Regarding HDAC9, there was no difference at baseline, but the BIM group showed a higher relative expression compared to Control under mitogenic stimulus (Fig. 3D).

Regarding HDACs 6 and 10 (class IIb), there was similar expression among all groups, and did not differ significantly from Control. However, in response to mitogenic stimulus, the expression of HDAC6 and HDAC10 was increased in the SIM group, and HDAC10 was increased in the BSIM group (Fig. 4A-B).

Among sirtuins (class III), the expressions of Sirt1, Sirt3, and Sirt4 were found to be similar among all groups under the culture conditions used (Fig. 5A-C). Sirt2 and Sirt5 did not show any differences in the basal groups, but increased expression was observed in the SIM group compared to Control under mitogenic stimulation (Fig. 5D-E). Fig. 5F shows that compared to Control, the expression of Sirt6 in the SIM group was lower at baseline. However, under mitogenic stimulus, there was a significant increase in the expression of Sirt6 in all experimental groups (BIM, SIM, and BSIM). Although there was no baseline difference for Sirt7, the SIM group had increased expression with mitogenic stimulus compared to the Control, BIM, and BSIM groups (Fig. 5G).

Class IV is composed only of HDAC11 which in this study showed no differences compared to Control, but the SIM group had higher relative expression compared to BIM under mitogenic stimulus (Fig. 6).

Intracellular cytokines in T and B lymphocytes, and monocytes, and the frequency of regulatory T lymphocytes in animals born and/or breastfed – Cytokine producing T lymphocytes were observed by labelling with CD4+/IL-4+, CD4+/IFN-γ+, CD4+/IL-10+, or CD4+/IL-2+, while B lymphocytes and monocytes were labelled with anti-CD45R/B220+ and anti-CD14+ antibodies, respectively, together with anti-IL-10+. T lymphocytes with a regulatory profile were evaluated by triple labelling CD4+CD25+FoxP3+. Frequencies were evaluated with mitogenic stimulus (ConA) and without (basal).

Compared to the Control group, the frequency of CD4+/IL-4+ cells under basal conditions and mitogenic stimulation was lower in the SIM and BSIM groups (Fig. 7A). IL-10 production by CD4+ cells was higher in the BIM and BSIM groups under mitogenic stimulation (Fig. 7B). There were no differences among groups when the frequencies of CD4+IL-2+ and CD4+/IFN-γ+ cells (Fig. 7C-D) were analysed.

Regarding IL-10 production by B lymphocytes (Fig.7E), it was slightly higher in the SIM and BSIM groups in response to mitogen. The SIM and BSIM groups also had an increased frequency of CD14+IL-10+ in comparison to the Control and BIM groups both at baseline and in response to mitogen (Fig. 7F).

For cells expressing CD4+CD25+FoxP3+, the SIM and BSIM groups exhibited decreased frequency relative to the Control and BIM groups, with and without mitogenic stimulus (Fig. 7G).

Discussion

Maternal infection by *S. mansoni* can alter the degree of immune competence of the offspring in the long term, either through in-utero contact or breastfeeding.[2,3,4,5] In this study, the effects of gestation and breastfeeding were evaluated separately in *S. mansoni*-infected mothers. Therefore, the expression of HDACs, and cytokine production by lymphocytes and macrophages was assessed. These experiments were conducted using an in vitro system with broadly activated splenic cells, achieved using mitogenic stimulation.

Our findings show that breastfeeding from infected mothers induced the expression of HDACs from different classes which are involved in reducing the inflammatory response; however, gestation enhanced the expression of a single HDAC. These enzymatic changes induced by breastfeeding or gestation appear to inhibit cells with a regulatory phenotype (CD4+CD25+FoxP3+), but favour an IL-10-dependent pathway.

Only offspring generated from infected mothers showed increased expression of HDAC9. According to Tao et al.[18], HDAC9 is linked to decreased generation and performance of regulatory T cells. It has been observed that animals genetically deficient in HDAC9 had increased mRNA expression of Foxp3, CTLA-4, and GITR and increased CD4+FoxP3+ cells in lymphoid tissues. Regarding the cell phenotypes studied here, we observed that the generated offspring had a higher frequency of IL-10 producing CD4+ T cells. Together, these data suggest that immunomodulation in utero induced by maternal infection does not favour the generation

of cells with a regulatory phenotype but does favour IL-10 production via HDAC9 in offspring. In studies using immunization with ovalbumin as adjuvant, gestation in infected mothers was correlated with increased production of IL-10.[2,5] Furthermore, Li et al.[19] reported the positive regulation of HDAC9 in immature peritoneal macrophages through the action of acetyltransferase Dnmt3, leading to increased production of IFN-α and IFN-β via deacetylation of kinase TBK1. Mice deficient for Dnmt3a showed greater susceptibility to infection by vesicular stomatitis virus (VSV). Thus, it is possible that gestation in schistosomotic mothers could alter the innate immune response against viral infections in offspring.

Prior exposure to the breast milk of schistosomotic mothers positively altered the expression of HDACs from different classes linked to reduction and/or resolution of the inflammatory response.[8,20,21] There was an increase in the relative expression of HDACs 1, 2 and 7, all of which show anti-inflammatory activity by inhibiting the transcription of NF-κβ and decreasing the production of inflammatory cytokines (TNF-α, IL-1, IL-6).[7,22,23] There was a decrease in HDAC3 expression in all experimental groups (at baseline), but this recovered to levels similar to Control in response to mitogen.

HDAC6 and HDAC11 were also increased in animals that only received milk from infected mothers. Wang et al.[24] when using an HDAC6 inhibitor, observed a reduction in HDCA6 recruitment to the IL-10 promoter along with enhanced TNF-α, IL-12p40, and IFN-γ production, as well as increased influx of macrophages, dendritic cells (DCs), and neutrophils to the lungs in response to Mycobacterium tuberculosis infection. In fact, Cheng et al.[6] reported that HDAC6 is required for transcriptional activation of IL-10 gene expression in macrophages and dendritic cells through STAT3 activation, while HDAC11 acts as an IL-10 transcriptional repressor. Here, the expression of HDAC6 seemed to overlap with the repressive effect of HDAC11. These data are corroborated by the increased IL-10 production in macrophages (CD14+IL-10+) in the SIM group. It is worth noting that the increase in HDAC6 expression, together with the baseline HDAC5 decrease in breastfed animals, may be related to the decrease in Treg cell activity and markers (FoxP3, CTLA-4 and GITR).[25,26] These data are corroborated by the lower frequency of CD4+CD25+-FoxP3+ cells in animals which were breastfed under mitogenic stimulus. Regarding the production of cytokines by T lymphocytes, decreased production of IL-4 and IFN-γ was observed (although there was no statistical difference for the latter). There was a subtle increase in the frequency of IL-10 producing B lymphocytes under mitogenic stimulation, and the baseline frequency was high in all study groups. These data corroborate the dependence of IL-10 on phagocytic cells in producing the

suppressive and epigenetic effects of breast milk from schistosomotic mothers.

The high expression of HDAC10 was related to activation of the IL-1β-mediated NF-κβ signalling pathway in a study with mesenchymal stem cells derived from the synovial membrane in temporo-mandibular joint repair.[27] However, HDAC10 was shown to be important in regulating the production of reactive oxygen species (ROS) in gastric cancer, and its inhibition led to the accumulation of ROS, triggering the intrinsic apoptotic pathway.[28] It is known that ROS acts both in the innate and adaptive immune systems by attracting polymorpho-nuclear leukocytes, monocytes, and macrophages through Toll like receptors (TLR).[29,30] Here, there were increases in the expression of this enzyme in the group that received only milk (SIM) as well as in those that were born and breastfed (BSIM). This finding may be related to the activity of antioxidant enzymes in breast milk.[31]

Milk from infected mothers also led to increased expression of class III HDACs, Sirt2, and Sirt7. It is known that Sirt2 acts on the anti-inflammatory pathway in M2 macrophages through expression of Arginase 1 (Arg1) and Cd11c,[21] and reduces ROS by increasing superoxide dismutase 2 (SOD2), catalase, and glutathione peroxidase.[8] The increase in Sirt2 was related to inhibition of the NF-κβ pathway and reduced expression of IL-1β, IL-6, and TNF-α.[21] Sirt7 acts on the stabilisation of TGF-β receptor type 1, allowing for efficient signaling of TGF-β.[32] This targeting to an anti-inflammatory response profile is consistent with the tolerogenic effect of breast milk.[33] According to our data, offspring who were only breastfed by schistosomotic mothers and who had undergone postnatal infection by *S. mansoni* also had elevated levels of TGF-β, in addition to decreased nitric oxide production (Unpublished observations).

High expression of Sirt6 was observed in all experimental groups (born and/or breastfed animals). According to Lee et al.,[20] high expression of Sirt6 acts to inhibit the NF-κβ pathway and blocks the effect of TNF-α. Li et al.[34] showed that overexpression of Sirt6 inhibits RIGI-like receptor (RLR) and Toll-like receptor 3 (TLR3) in Dengue virus (DENV) infection, and the sirtuin core domain of SIRT6 is required for the inhibition of NF-κB p65 function, negatively regulating DENV-induced inflammatory responses via the RLR and TLR3 signalling pathways. These observations support the inhibition of inflammation we observed in the offspring of schistosomotic mothers.

The high expression of Sirt5 was also observed in the SIM group. It has been shown that Sirt5 is responsible for deacetylating STAT3, disrupting its activity at the mitochondrial level.[35] Qin et al.[36] demonstrated that deficiency of this enzyme led to a decrease in the innate

inflammatory response, with lowered production of IL-6, TNF-α and, MCP-1 both in the hyper inflammatory and hypo inflammatory stages of sepsis in an animal model. In addition, it was observed that high expression of Sirt5 leads to an increased pro-inflammatory response by decreasing the interaction between Sirt2 and NF-κβp56.[36] Here, there was no decrease in Sirt2, and the increase in Sirt6 and Sirt7 may have overlapped with the effect of Sirt5.

Thus, HDACs were epigenetically altered by maternal infection and were involved in the suppression of pro-inflammatory mediators and increased IL-10 production but did not favour cells with a regulatory phenotype. Even knowing the importance of cell-cell interactions in both in vivo and in vitro systems, it would be important to culture purified cells to understand if this pattern of expression of HDACs, indicating a predisposition to IL-10 dependent anti-inflammatory effects, was due to previous programming of T lymphocytes and macrophages in the BIM and SIM groups, respectively.

In conclusion, gestation in infected mice led to increased expression of HDAC9 alone, while breastfeeding from schistosomotic mothers led to increased expression of HDACs of all classes. It is true that the extrapolation of these data from mice to humans should be carefully evaluated, but our findings highlight the importance of experimental and clinical approaches to investigate the efficacy of therapeutic targets in allergy, autoimmunity, and cancer models after long-term epigenetic changes result in offspring from areas endemic for schistosomiasis. Therefore, our results shed light on immunological factors resulting from infection during early age, caused by modifications in epigenetic profile. In view of this, it will be important to evaluate factors in breast milk of infected mothers and how these components may modify the epigenetic and immunological profile of offspring.

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Authors' contribution

GCRH, VMOS and JLLF - Conceived and designed the proposal; GCRH, MCS and FOS - performed lab experiments and processed the data; VMBL, FOS and MCPAA – contributed reagents/materials/analysis/tools; GCRH - writing – original draft; GCRH, VMOS, FOS, VMAC and JLLF - writing, review and editing; JLLF and VMOS - supervision. The authors declare no conflicts of interest.

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TABLE

Primers used in quantitative real-time polymerase chain reaction (PCR)

Target gene (Accession number)	Sequence 5'-3' (Forward and reverse)	Tm	CG %	Amplicon length (bp)
Hdac1 (NM_008228.2)	CCGGTTAGGTTGCTTCAATC	59.6	50	118
	AACATTCCGGATGGTGTAGC	59.8	50	
Hdac2 (NM_008229.2)	TATTATGGCCAGGGTCATCC	59.6	50	119
	TCAGCAGTGGCTTTATGAGG	59	50	
Hdac3 (NM_010411.2)	ATGCAGGGTTTCACCAAGAG	60.1	50	117
	TGTTGCTCCTTGCAGAGATG	60.1	50	
Hdac4 (NM_207225.2)	CCGCCAGCAGTTTTAAAGTC	59.9	50	92
	ACCGAATGGAGATGCTCAAC	60.1	50	
Hdac5 (NM_001077696.1)	ACTTTCCCCTCCGTAAAACG	60.3	50	116
	AACAGTGCCATCCTTTCGAC	60.1	50	
Hdac6 (NM_010413.3)	ATCTCAGCTGGCTTTGATGC	60.5	50	116
	ATAATACGGCCACCAGCAAG	60.0	50	
Hdac7 (NM_001204275.1)	ATGATGGCCTGGAACATAGG	59.8	50	75
	GATGCTGCTGCAGAGAAATG	59.7	50	
Hdac8 (NM_027382.4)	AGGGAATCTGAAGCATGTGG	60.1	50	131
	CAAATTTCCCCTGCAGTCAC	60.5	50	
Hdac9 (NM_001271386.1)	TTTGAGGTGGCAGAATCCTC	60.2	50	106
	GAGCTGAAGCCTCATTTTCG	60.1	50	
Hdac10 (NM_199198.2)	AACAGGAGCTGTGCACAATG	59.9	50	143
	TCCTCTGCAGCCCATATTTC	60.2	50	
Hdac11 (NM_144919.2)	TGATGGGGTTGAACACTGAG	59.5	50	128
	AGCAGCCCCTTAAAAACTCC	59.7	50	
Sirt1 (NM_019812.3)	GCCCTCAATTTCTGTTCTGC	59.8	50	150
	TTTTGAGTGCTCCAGACACG	60.0	50	
Sirt2 (NM_019812.3)	ACGGCTGCTCATTAACAAGG	60.3	50	88
	GTCAAAATCCATGCCACCTC	60.3	50	
Sirt3 (NM_001177804.1)	CATATGGGCTGATGTGATGG	59.8	50	141
	AGATCTGCCAAAGCGAAGTC	59.6	50	
Sirt4 (NM_001167691.1)	CGAGCAAAAGCTCCCAATAG	60.0	50	145
	TTCCAGCCTTTGGACATCAG	61.2	50	
Sirt5 (NM_178848.3)	CCAGCTTTAGCAGGAAAAGG	59.1	50	139
	CCAGGTTTTCTCCAAACCAC	59.4	50	
Sirt6 (NM_181586.3)	TGTCCAACACAGCTCCTTTC	58.9	50	97
	CTTCCACATGTGTGGGATTC	58.8	50	
Sirt7 (NM_001363439.1)	AGCTTCGGGATACCATTGTG	60	50	104
	CAGGATTGTGTCTGCTTTGC	59.4	50	
β-Actin (NM_007393.5)	TTGCTGACAGGATGCAGAAG	61.1	50	147
	TGATCCACATCTGCTGGAAG	59.8	50	

bp: base pair; CG: cytosine and guanine content; HDAC: histone deacetylase 1-11; Sirt: Sirtuin 1-7; Tm (melting temperature) was calculated at default settings of 0.25 μM oligo concentration and 50 μM Na. Primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

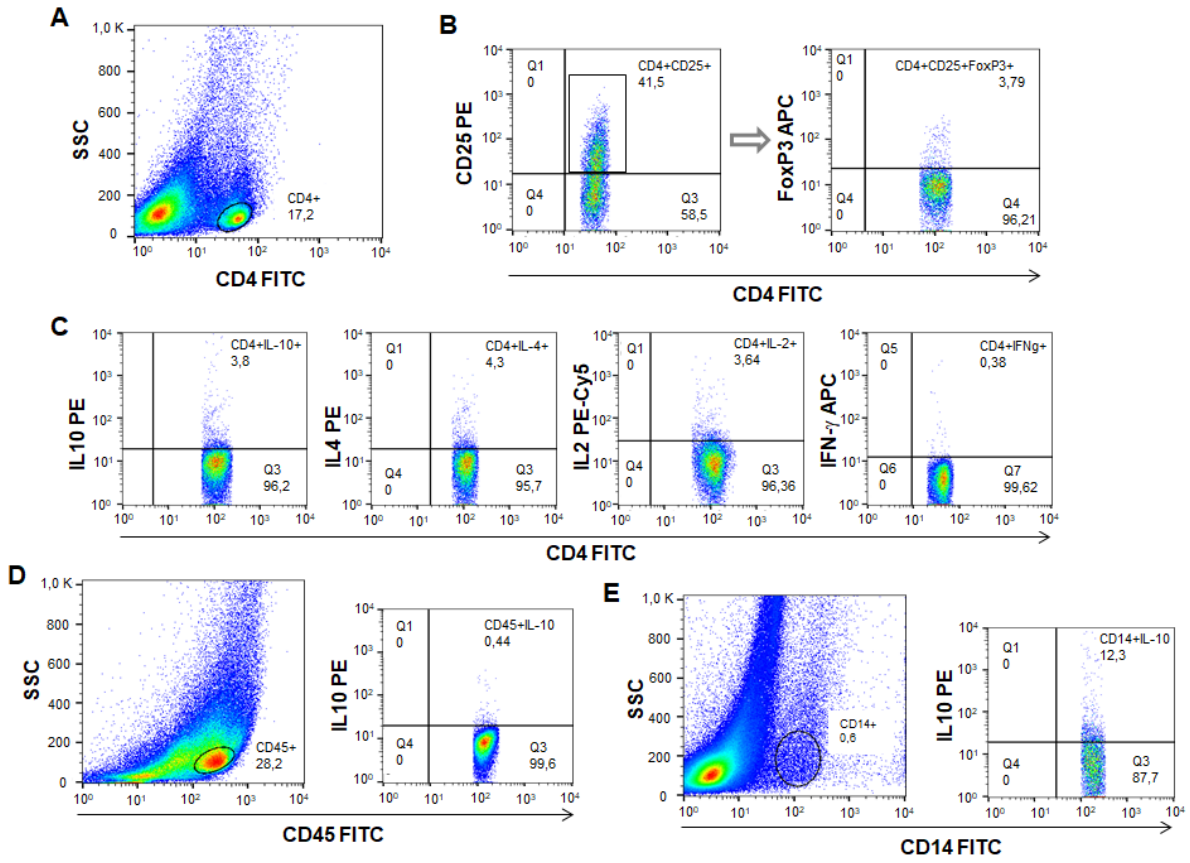

Fig. 1: representation of the gating strategy used to analyse the different subpopulations of cells. FL1/anti-CD4-FITC versus laser side-scatter (SSC) dot plot (A), CD4+CD25+FoxP3+ cells dot plots (B), cytokine producing T CD4+ lymphocytes dot plots (C), IL-10 producing CD45R/B220+ cells dot plot (D) and IL-10 producing CD14+ cells dot plot (E).

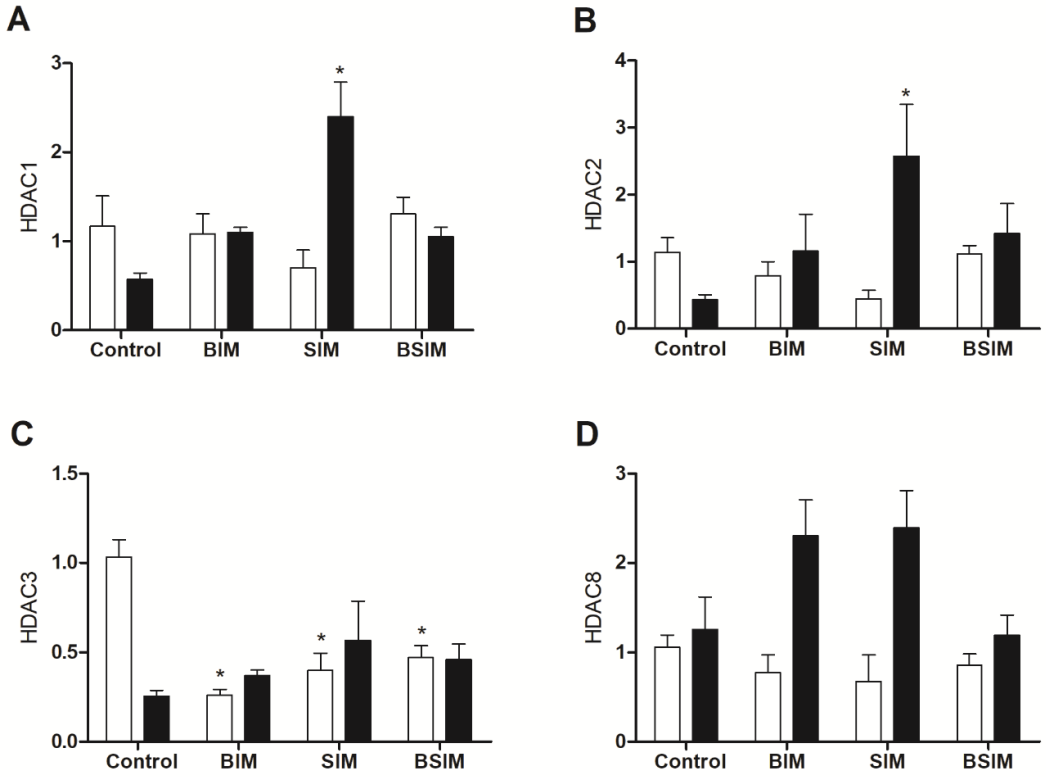

Fig. 2: relative expression of class I histone deacetylases (HDACs). HDAC1 (A), HDAC2 (B), HDAC3 (C) and HDAC8 (D) in splenocytes from Swiss Webster mice (seven weeks) born (BIM), breastfed (SIM), or born and breastfed (BSIM) in infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 μg/mL, black bar) or with culture medium (BASAL, white bar). The relative expression was verified by real-time quantitative polymerase chain reaction (qPCR). The results represent the median and standard error for 8-10 animals/group. *$p < 0.05$ compared to control group.

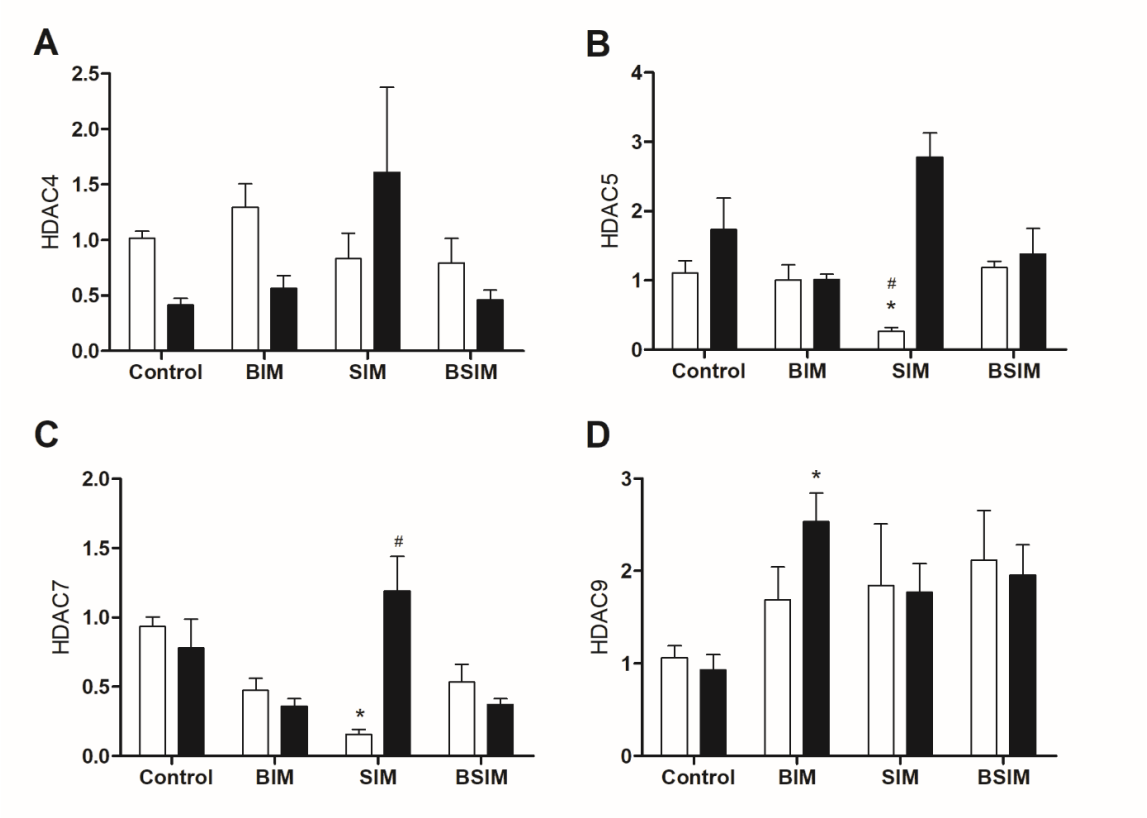

Fig. 3: relative expression of class IIa histone deacetylases (HDACs). HDAC4 (A), HDAC5 (B), HDAC7 (C) and HDAC9 (D) in splenocytes from Swiss Webster mice (seven weeks) born (BIM), breastfed (SIM), or born and breastfed (BSIM) in infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 μg/mL, black bar) or with culture medium (BASAL, white bar). The relative expression was verified by real-time quantitative polymerase chain reaction (qPCR). The results represent the median and standard error for 8-10 animals/group. *$p < 0.05$ compared to control group. #$p < 0.05$ compared to BIM and BSIM groups.

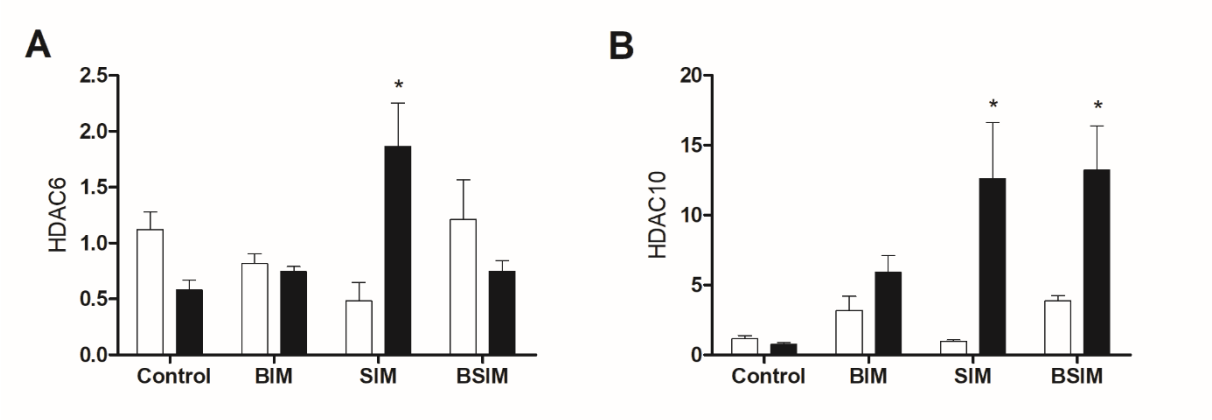

Fig. 4: relative expression of class IIb histone deacetylases (HDACs). HDAC6 (A) and HDAC10 (B) in splenocytes from Swiss Webster mice (seven weeks) born (BIM), breastfed (SIM), or born and breastfed in (BSIM) infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 μg/mL, black bar) or with culture medium (BASAL, white bar). The relative expression was verified by real-time quantitative polymerase chain reaction (qPCR). The results represent the median and standard error for 8-10 animals/group. *$p < 0.05$ compared to control group.

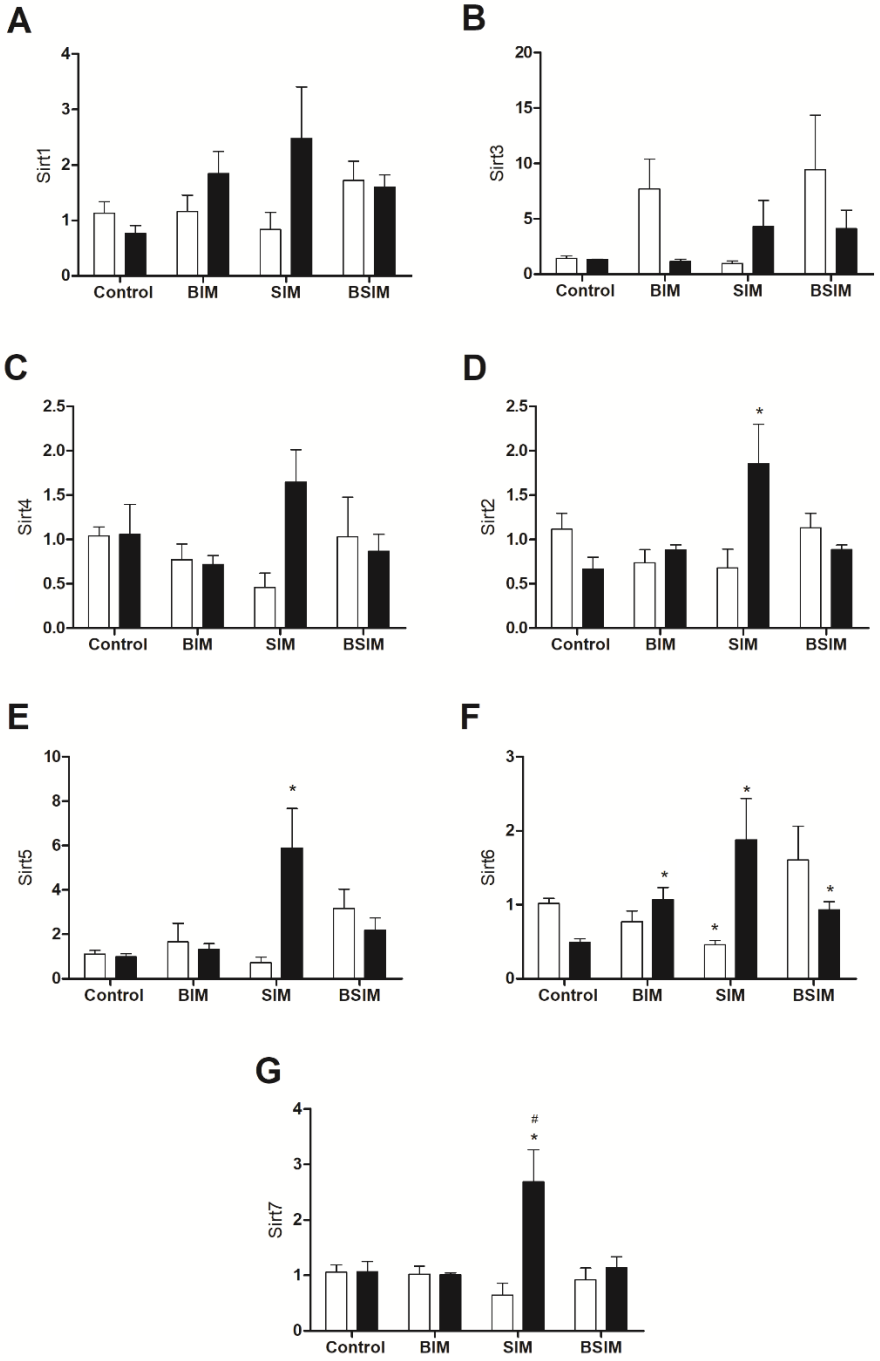

Fig. 5: relative expression of class III histone deacetylases (HDACs). Sirt1 (A), Sirt3 (B), Sirt4 (C), Sirt2 (D), Sirt5 (E), Sirt6 (F) and Sirt7 (G) in splenocytes from Swiss Webster mice (seven weeks) born (BIM), breastfed (SIM) or born and breastfed (BSIM) in infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 μg/mL, black bar) or with culture medium (BASAL, white bar). The relative expression was verified by real-time quantitative polymerase chain reaction (qPCR). The results represent the median and standard error for 8-10 animals/group. *p < 0.05 compared to control group. #p <0.05 compared to BIM and BSIM groups.

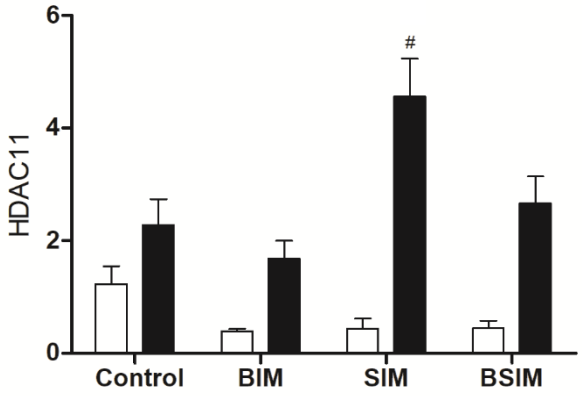

Fig. 6: relative expression of class IV histone deacetylases (HDACs). HDAC11 in splenocytes from Swiss Webster mice (seven weeks) born (BIM), breastfed (SIM) or born and breastfed(BSIM) in infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 μg/mL, black bar) or with culture medium (BASAL, white bar). The relative expression was verified by real-time quantitative polymerase chain reaction (qPCR). The results represent the median and standard error for 8-10 animals/group.

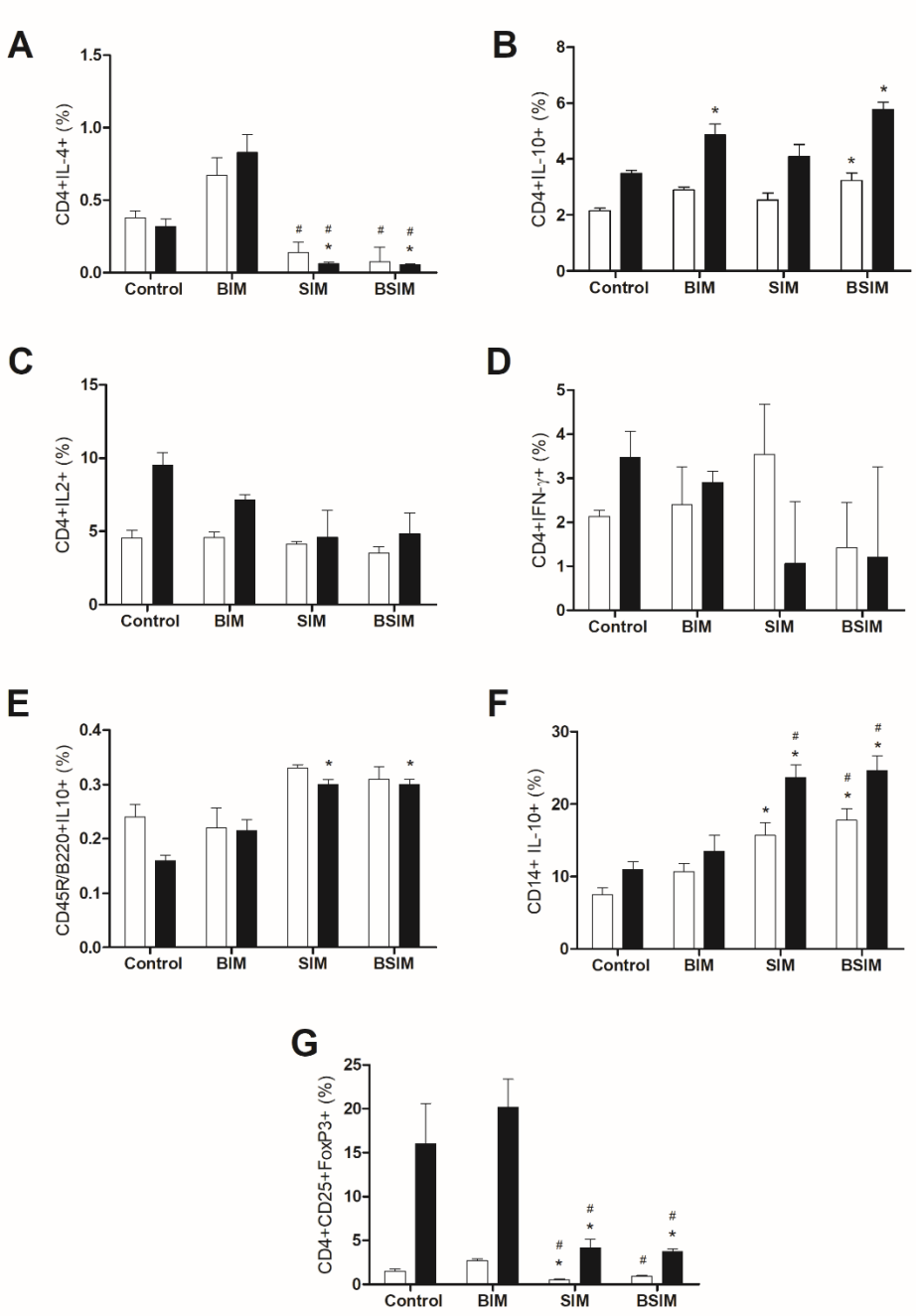

Fig. 7: cytokine production by T and B lymphocytes and monocytes, and splenocytes expressing CD4+ CD25+ FoxP3+. Frequency of CD4+IL-4+(A), CD4+IL-10+ (B), CD4+IL-2+ (C), CD4+IFN-γ+ (D), CD45R/B220+IL-10+ (E), CD14+IL-10+ (F) and CD4+CD25+FoxP3+ (G) in splenocytes from Swiss Webster mice (seven weeks) born (BIM), breastfed (SIM) or born and breastfed (BSIM) in infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 μg/mL, black bar) or with culture medium (BASAL, white bar). The frequencies were verified by flow cytometry. The results represent the median and standard error for 8-10 animals/group. *$p < 0.05$ compared to control group. #$p < 0.05$ compared to BIM and BSIM groups.

4.2 ARTIGO 2 - WHEY MILK PROTEOMICS FROM *Schistosoma mansoni* INFECTED MICE REVEALS PROTEINS INVOLVED IN IMMUNOMODULATION OF THE OFFSPRING

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Whey milk proteomics from *Schistosoma mansoni* infected mice reveals proteins involved in immunomodulation of the offspring

Gabriela Calixto Ribeiro de Holanda[1], Iasmim Lopes de Lima[1], Sandra Nara Naressi Scapin[2], Maria da Conceição Silva[1], Iana Rafaela Fernandes Sales[3], José Mauro Granjeiro[2], Roberto Afonso da Silva[1], Valdenia Maria Oliveira de Souza[1], José Luiz de Lima Filho[1]

[1]Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Recife, PE, Brasil

[2] Instituto Nacional de Metrologia, Qualidade e Tecnologia – INMETRO, Duque de Caxias, RJ, Brasil

[3] Centro Universitário Maurício de Nassau, Recife, PE, Brasil

*Corresponding author

Valdenia Maria Oliveira de Souza E-mail address: valdenia.souza@gmail.com

Laboratory of Immunopathology Keizo Asami (LIKA)

ORCID: 0000-0002-4976-009x

Av. Moraes Rego, 1235 - Cidade Universitária, 50670-901 Recife, Pernambuco, Brazil. Zip code: 50670-901 Phone/Fax: +55 81 2126 8485

Author contributions

Gabriela Calixto Ribeiro de Holanda, Valdenia Maria Oliveira de Souza and José Luiz de Lima Filho - conceived and designed the proposal; Gabriela Calixto Ribeiro de Holanda, Iasmim Lopes de Lima and Maria da Conceição Silva - performed lab experiments and processed the data; Sandra Nara Naressi Scapin, Iana Rafaela Fernandes Sales, José Mauro Granjeiro- contributed reagents/materials/analysis/tools; Gabriela Calixto Ribeiro de Holanda - writing - original draft; Gabriela Calixto Ribeiro de Holanda, Valdenia Maria Oliveira de

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Abstract

Milk from schistosomotic mothers can modulate the immune response of their offspring. However, its characterization and potential of modulating immunity has not yet been fully elucidated. Thus, the aim of this study was to evaluate whey proteins from the milk of *Schistosoma mansoni*-infected mice in order to identify the fractions which can act as potential immunomodulatory tools. For this, we did a mass spectrometry (nanoUPLC-MSE) analysis to characterize the proteomic profile of milk from infected (MIM) and non-infected mice (MNIM). It was possible to identify 29 differentially expressed proteins: 15 were only found in MIM, 10 only found in MNIM and 4 were down-regulated in MIM group. Gene Ontology (GO), pathways enrichment analysis and protein-protein interaction (PPI) analyses indicated differentially expressed proteins linked to biological processes and pathways in MIM group such as: fructose 1,6-biphosphate metabolic and glycolytic processes, glucose metabolism and neutrophil degranulation pathways. The down-regulated and unique proteins identified in MNIM group were involved in the positive regulation of B cell activation and receptor signaling pathway, in the innate immune response, complement activation and phagocytosis. The present findings revealed a protein profile that may be involved in the activation and deactivation of the offspring's immune system in the long term, conferring a protective character due to the previous contact with milk from infected mothers.

Keywords: Breastfeeding; Immune response; Mass spectrometry; Offspring; Proteomics; Schistosomiasis.

Introduction

Schistosomiasis is considered one of the most important helminthic diseases due to its high morbidity and mortality. It affects around 240 million people and is distributed in 78 countries (WHO 2015). Among the afflicted individuals, there is a high prevalence of chronic schistosomiasis in pregnant women and in women of childbearing age (Salawu and Odaibo

2013) which causes concerns regarding breastfeeding in infected mothers due to the modulation of the offspring's immune response.

Milk from schistosomotic mothers can alter the immune competence of their offspring due to the presence of parasite antigens and/or anti-parasite antibodies (Thomas and Harn 2005; Bhargava et al. 2012). Attallah et al. (2003) have demonstrated that newborns which were suckled by infected mothers presented the 63-kD *S. mansoni* antigen for up to 24 months after delivery. However, in non-suckled children, the antigen was identified in their urine up to 28 days postpartum. Regard to anti-Schistosoma antibodies, Colley et al. (1999) observed that mice, which received the antibody against *Schistosoma mansoni* soluble egg antigens (SEA) during breastfeeding, were infected during adulthood and had higher production of anti-SEA antibodies.

An experimental study on the effect of breast milk from infected mothers, separated from gestation, enhanced the production of anti-ovalbumin (OA) in adult mice (Santos et al. 2010) and improved the ability of antigen presentation by B lymphocytes in the inducing phase of the anti-OA immune response (Santos et al. 2014). For homologous antigens, it was demonstrated that breastfeeding on infected mothers decreased granulomas in adult offspring which were infected postpartum (Santos et al. 2016). Thus, these findings highlight that the milk from *S. mansoni* infected mothers is capable of improving the long-term immunity. Another experimental study showed that milk from *S. mansoni* infected mothers induced the expression of HDACs from various classes which were involved in reducing inflammatory responses along with a higher frequency of CD14+IL-10+-cells and an increase in CD45R/B220+IL-10+-cells in the offspring (Holanda, et al., 2019).

It has already been described that the breast milk has important bioactive compounds which can act as precursors of the newborn's immunity and assist on the maturation of their immune system (Ballard and Morrow 2013). Some examples of these compounds are: oligosaccharides, antibodies, growth factors, macrophages, neutrophils, lymphocytes and epithelial cells. There are also mediators like the cytokines IL-1, TNF-α, IFN-γ, IL-6, IL-10 and TGF-β (Blewett et al. 2008; Brenmoehl et al., 2018). However, the potential of proteins from milk of infected mothers on modulating the offspring's immunity has not been fully elucidated.

Proteomics analysis has become indispensable for protein characterization and biomarkers discovery. The characterization of proteins from tissues, cells, subcellular compartments and fluids can be used to identify differentially expressed proteins which are potentially related to biological processes. Thus, the aim of the present study was to evaluate

whey proteins from the milk of *Schistosoma mansoni* infected mice in order to identify proteins which can act as potential immunomodulatory tools.

Materials and methods

Animals and infection

Four-week-old Swiss Webster female mice were infected subcutaneously (s.c.) with 20 *S. mansoni* cercariae, São Lourenço da Mata strain (SLM). On the 45th day, the infection was confirmed by the Kato-Katz method (Katz; Chaves; Pellegrino 1972). On the 60th day post-infection (dpi), estruses were synchronized by the administration of 5 i.u. (100 µL) of equine chorionic gonadotrophin hormone and, after 48h, an additional injection of 5 i.u. (100 µL) of human chorionic gonadotrophin. The females were caged with male mice at a 1:1 ratio and successful mating was checked by the presence of a vaginal plug. The same procedure was performed in noninfected females. The mice were housed in the animal care facility at the Aggeu Magalhães Institute (IAM), Oswaldo Cruz Foundation (Fiocruz), Recife, Pernambuco, Brazil.

Milk harvesting

Milk was obtained from 20 Swiss Webster female mice infected with *Schistosoma mansoni*, and from 20 healthy female mice (control group). Between 10 and 12 days after delivery, mothers were separated from their offspring for approximately 2 hours, then, they were anesthetized (Xilazine-0.1 µL and Ketamine-0.2 µL) and received oxytocin (100 µL -1 UI) minutes before milking. Sterile gauze moistened with warm water (~30 °C) was used on their teats and then a manual massage was applied. The milk was collected with a pipette (200 µL/animal) in sterile glass tubes, pooled in two groups (Milk from Infected Mothers - MIM and Milk from Non-Infected Mothers - MNIM) and the Protease Inhibitor Mix (GE Healthcare) was added in a ratio of 1:100 - milk:inhibitor (w/w). Subsequently, for fat removal, the milk was centrifuged in a refrigerated centrifuge (4,000 x g for 10 minutes at 4 °C). After that, whey proteins were obtained from skimmed milk by adjusting the pH to 4.6 with 10% (v/v) acetic acid. After a centrifugation of 3,000 x g for 10 min, it was possible to obtain a supernatant containing whey proteins and isoelectrically precipitated caseins (Polidori and Vincenzetti 2012). The samples were stored at -80 °C until further use.

Protein Quantification and Clarification

The whey proteins obtained from the depletion of caseins were quantified using the 2-D Quant Kit™ (80-6483-5-GE Healthcare) following the manufacturer's instructions in triplicate. The calibration curve was made using the bovine serum albumin as the standard. Then, the proteins were precipitated by Williams and Stone (1997) with modifications. For every 150 µg/mL of proteins, it was added 278 µL 50 % Trichloroacetic Acid and 139 µL 0.1 % Triton. The samples were incubated on ice for 60 minutes and centrifugated to 13,000 x g for 10 minutes at 4 ºC. The supernatant was discarded and the pellet was washed twice in 90% Acetone. After that, the pellet was resuspended in Ultrapure water (Milli-Q®, 18.2 MΩ•cm) and submitted to a new quantification. The protein solution was concentrated in the vacuum concentrator 5301 (Eppendorf) and stored protected from moisture until further use.

Tryptic Digestion and Sample Preparation for LC- MSE

Approximately 50 µg of proteins from control and experimental pools were diluted in 60 µL of 50-mM NH4HCO3. Then, 25 µL of 0.2% RapiGest SF (Waters), a surfactant used to denature proteins and enhance enzymatic digestion, were added per sample. The reaction occurred at 80 ºC on a dry bath incubator until complete solubilization of the proteins. Afterwards, the proteins were reduced by adding 2.5 µL of 100-mM dithiothreitol at 60 °C for 30 minutes, then, they were cooled down to room temperature and alkylated with 2.5 µL of 300-mM iodoacetamide for 30 minutes in the dark. For protein digestion, Trypsin (Promega) was added at a 1:100 (w/w) enzyme:protein ratio and the samples were incubated overnight at 37 °C. Following the digestion, the RapiGest SF was hydrolyzed with the addition of 5% TFA for 90 min at 37 ºC. The samples were centrifuged, and the supernatant was transferred to a Waters Total Recovering Vial (Waters). For absolute peptide quantification, 5 pmol of yeast alcohol dehydrogenase (ADH, UniProt entry P00330, Waters) was added to each sample as an intern standard. Then, 85 µL of 3% acetonitrile containing 0.1% formic acid was added up to final concentrations of 250 ng/µL and 25 fmol/µL for protein and ADH, respectively, were reached. The samples were kept at 6 °C for the nanoUPLC-MSE analysis. The products obtained from tryptic digestion were separated by nanoACQUITY UPLC system (Waters) on an HSS T3 C18 reverse-phase column (1.8 µm x 100 µm x 100 mm) in conjunction with a Symmetry C18, 5 µm trap column, using the following gradient at a flow rate of 450 nL/min: 7 to 40% B in 115 min; a cleaning gradient of 40 to 85% B in 4 min and maintained in 85% B

for 4 min; then column re-equilibration to 7% B in 2 min. The column oven temperature was kept at 55 °C and mobile phases A and B consisted, respectively, of ultrapure water and acetonitrile, both containing 0.1 % formic acid. A Synapt HDMS mass spectrometer (Waters) was used for data-independent analysis (MSE) of the tryptic peptides. The instrument was operated in the resolution mode, and all analyses were performed in the "V" mode, using nano-electrospray ionization in the positive-ion mode (nanoESI+). The mass spectrometer data were acquired using a NanoLockSpray probe channel infusion of Glu–Fib (Glu1) derived from fibrinopeptide B human (M + 2H)2+ = 785.2486, and the MS/MS Glu–Fib fragments were used for final calibration of the instrument. All analyses were performed using a Glu–Fib mass channel at 30-s intervals. The exact mass retention time (EMRT) nanoLC-MSE data were collected with alternative standard MS (3 eV) and elevated collision ramp energies (MSE 15–55 eV) applied to the trap 'T-wave' CID (collision-induced dissociation) cell with argon gas, using a scan time of 0.8 s and automatic interscan for each MS scan from m/z 50 to 2,000. The RF offset (MS profiles) was adjusted such that LC/MS data were effectively acquired from m/z 350 to 2,000, which ensured that any mass observed in the LC/MSE data <m/z 350 arose from dissociations in the collision cell.

Data Processing, Protein identification and Quantification

The raw data obtained from the analysis of nanoUPLC/MSE were processed by ProteinLynx Global SERVERTM v. 2.4 software (PLGS, Waters). Processed files were searched against UniProtKB (UniProt mouse downloaded in December 03, 2018) and NCBI (NCBI mouse downloaded in December 04, 2018) Mus musculus annotated databases, using IdentityE (PLGS, Waters) database algorithm. Amino acid sequence from the standard internal protein (ADH, UniProt entry P00330) was appended. To access the false positive identification rate, we used the reverse sequence of the databases. The PLGS default parameters for spectra processing and database search were: peptide tolerance (10 ppm), fragment tolerance (20 ppm), one missed trypsin-cleavage, a fixed carbamidomethyl modification for Cysteine and a variable oxidation modification for Methionine. The search thresholds used were: minimum fragment ion matches per peptide (3), minimum fragment ion matches per protein (7), minimum peptides per protein (1), and False discovery rate for protein identification (2). The three most abundant peptides (label-free Hi3 method) of all detected proteins were compared to the three most powerful peptides of the spiked-in reference protein (ADH) of known concentration and used to calculate the molar amounts loaded onto the column (absolute quantification) for each

protein. The identified proteins were organized using PLGS into a list detailing unique proteins to each condition and a logarithm ratio between the different groups. The average quantitative values of all proteins were calculated, and the p value ($p < 0.05$) calculated using ExpressionE software (PLGS, Waters) to refer to the differences between biological replicates. Only proteins with attendance scores (>100) and at least 2 unique peptides were maintained in the expression analysis. For proteins identified by different fragment ions, those with the highest score were considered for comparisons and data presentation. (Chambery et al. 2009; Li et al. 2009).

Functional Annotation and construction of protein-protein interaction networks

Gene Ontology analysis (GO) related to Biological Process, Molecular Function and Cellular Component was carried out through the Database for Annotation, Visualization and Integrated Discovery (DAVID) V6.8 (https://david.ncifcrf.gov). The signaling pathways classification was performed by the Reactome (https://reactome.org). The set of differentially expressed proteins were screened and inputted in the online Search Tool for the Retrieval of Interacting Genes database (STRING) (http://string-db.org) to visualize the protein-protein interaction (PPI) network.

Ethics statement

The animal protocol was approved by the Ethics Committee on Animal Use of Fiocruz (113/2017) and it is in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation.

Results

Differentially expressed proteins (DEPs)

During the analysis by the LC-MSE, 78 proteins from infected mice (MIM) and 75 proteins in the milk from non-infected mice (MNIM) were both identified with scores ≥100 and FDR rates ≤ 2%. The assays of differentially expressed proteins (DEPs) by PLGS ExpressionE detected 15 proteins identified only in the MIM group and 10 in MNIM group. It was detected 4 down-regulated proteins (Ig alpha chain C region, Serine protease inhibitor A3M, Serine protease inhibitor A3N, Serine protease inhibitor A3K) in MIM (Fold-chance < 0.5) compared to the healthy milk (MNIM). The unique proteins identified in MIM and MNIM samples were considered as Up and Down regulated in MIM group, respectively (Table 1 and Table 2). For

both cases, the exclusiveness of a protein can be interpreted as the detection of its concentration by LC-MSE in a single sample or group.

Gene Ontology (GO) analysis

According to gene ontology analysis (GO) by DAVID software, DEPs have been associated with 35 terms on the category of "Biological Process," 29 in "Molecular Function" category and 71 in "Cellular Component" (Figures 1 and 2).

Regard to biological processes, the up-regulated proteins were grouped in: Fructose 1,6-bisphosphate metabolic process (2 proteins, p= 0.0055); Glycolytic process (2 proteins, p= 0.022); Heat response (2 proteins, p= 0.034); Epithelial cell differentiation (2 proteins, p= 0.04) and protein homotetramerization (2 proteins, p= 0.044) (Fig. 1a). Down-regulated proteins, on the other hand, were mainly grouped in Negative regulation of peptidase activity (4 proteins, p= 0.000056); Response to peptide hormone (3 proteins, p= 0.001); Response to cytokine (3 proteins, p= 0.0013); Positive regulation of B cell activation (2 proteins, p= 0.022); Acute-phase response (2 proteins, p= 0.023); Phagocytosis, recognition (2 proteins, p= 0.025); Innate immune response (3 proteins, p= 0.028); Phagocytosis, engulfment (2 proteins, p= 0.029); B cell receptor signaling pathway (2 proteins, p= 0.036); Complement activation classical pathway (2 proteins, p= 0.036) (Fig. 2a).

In the GO category of cellular components the overrepresented terms to up-regulated proteins included Extracellular exosome (9 proteins, p= 0.000079); Cytoplasm (12 proteins, p= 0.00024); Myelin sheath (3 proteins, p= 0.0069); Extracellular space (5 proteins, p= 0.014) and mitochondrion (5 proteins, p= 0.022) (Fig. 1b). From down-regulated proteins the main terms were: Blood microparticle (7 proteins, p= 0.000000000076); Extracellular space (9 proteins, p= 0.00000043); Extracellular exosome (9 proteins, p= 0.000034); Extracellular region (7 proteins, p= 0.00029); External side of plasma membrane (3 proteins, p= 0.015) and immunoglobulin circulating complex (2 proteins, p= 0.018) (Fig. 2b).

The most enriched GO terms on the molecular function category in up-regulated proteins were Cytoskeletal protein binding (3 proteins, p= 0.0008); Fructose-bisphosphate aldolase activity (2 proteins, p= 0.0031); Nucleotide binding (5 proteins, p= 0.026) and Poly(A) RNA binding (4 proteins, p= 0.029) (Fig. 1c). Regarding down-regulated proteins, the terms were Serine-type endopeptidase inhibitor activity (5 proteins, p= 0.0000011); Peptidase inhibitor activity (4 proteins, p= 0.000072); Protein homodimerization activity (4 proteins, p= 0.015) and Immunoglobulin receptor binding (2 proteins, p= 0.021) (Fig. 2c).

Reactome pathway analysis

The reactome pathway enrichment analysis revealed the implication of DEPs in 25 statistically significant pathways. The main pathways related to up-regulated proteins were: Gluconeogenesis (2 protein, p= 0.003); Glycolysis (2 proteins, p= 0.005); Tyrosine catabolism (1 protein, p= 0.007); Glucose metabolism (2 proteins, p= 0.007); Phenylalanine and tyrosine metabolism (1 protein, p= 0.015); Josephin domain DUBs (1 protein, p= 0.016); Metabolism of Angiotensinogen to Angiotensins (1 protein, p= 0.024); Neutrophil degranulation (3 proteins, p= 0.024); RHO GTPases Activate WASPs and WAVEs (1 protein, p= 0.047) and Regulation of TP53 Degradation (1 protein, p= 0.047) (Fig. 3a). From down-regulated proteins, the main pathways were: Glucocorticoid biosynthesis (1 protein, p= (1 protein, p= 0.013); DNA unwinding (1 protein, p= 0.016); Trafficking and processing of endosomal TLR (1 protein, p= 0.017); Dissolution of Fibrin Clot (1 protein, p= 0.017); Pentose phosphate pathway (1 protein, 0.02); DNA strand elongation (1 protein, p= 0.003); Activation of the pre-replicative complex (1 protein, p= 0.003); Gluconeogenesis (1 protein, p= 0.003); Metabolism of steroid hormones (1 protein, p= 0.003) and Activation of ATR in response to replication stress (1 protein, p= 0.003) (Fig. 3b).

Protein-protein interactions analysis

The STRING database was used to evaluate the interactions of 26 differentially expressed proteins (Fig. 4). The network was constituted of 26 nodes and 19 edges with an average node degree of 1.46 and a clustering coefficient of 0.273. The expected number of edges was 4, which was significantly lower than the actual number of edges, showing that there are statistically relevant interactions (score > 0.800) in the network with a PPI enrichment p-value of 3.03e-08. The analysis showed interactions between up- and down-regulated proteins. The up-regulated protein Fructose-biphosphate aldolase A interacts with Fructose-biphosphate aldolase C and Protease serine 1 and also interacts with the down-regulated proteins Glucose-6-phosphate 1-dehydrogenase, Gamma-enolase, Alpha-2-antiplasmin and Serine protease inhibitors A3M and A3N. Fructose-biphosphate aldolase C interacts with Protease serine 1, Glucose-6-phosphate 1-dehydrogenase and Gamma-enolase. Another up-regulated protein, Actin-related protein 2, interacts with Protease serine 1 and the down-regulated proteins Serine protease inhibitors A3M and A3N. The down-regulated proteins whose presents PPI were: Alpha-2-antiplasmin and Serine protease inhibitors A3M and A3N, which were co-expressed.

Discussion

Milk from schistosomotic mothers can modulate the immune response of the offspring against homologous and heterologous antigens (Santos et al. 2010, 2014, 2016; Straubinger et al. 2014; Fernandes et al. 2018). Milk composition is dynamic, and it can be influenced by maternal and environmental factors (Andreas et al., 2015). Also *S. mansoni* infection changes the expression of proteins present in plasma (Barsoum et al., 2013) which can possibly affect the milk composition because part of the milk proteins is derived from plasma (Wall et al., 2015). However, we have a poor understanding about what it is in the milk from infected mothers that could cause the immunomodulation in these descendants. In the present study, using LC-MSE, we have identified a total of 29 proteins that are differentially expressed between milk from infected mice (MIM) and non-infected mice (MNIM), where 15 of them were up-regulated and 14 were down-regulated.

The dichotomous character of breast milk in stimulating the immune system has already been described, as well as its ability of containing factors linked to immunological tolerance (Blewett et al. 2008; Castellote et al. 2011; Brenmoehl et al., 2018). In the milk of infected mice (MIM) were found proteins that seem to favor this dichotomy. For better understand the biological function of differentially expressed proteins present in milk from infected mice (MIM), we have performed GO analysis. The results revealed that differentially expressed proteins are mainly associated with fructose 1,6-biphosphate metabolic, glycolytic processes, heat response and cell differentiation. Notably, Fructose-bisphosphate aldolase A and C (proteins most linked to biological process in MIM) are part of neutrophil degranulation and glucose metabolism pathways. It is known that activated neutrophils can secrete cytokines and present antigens via MHC Class II allowing the activation of T cells (Wright et al. 2010). Interestingly, studies have been using recombinant fructose-1, 6-bisphosphate aldolase (FBPA) as a vaccine candidate antigen against *Trichinella spiralis* and *Streptococcus pneumoniae* since this protein can increase Th1, Th2 and Th17 cytokines in mice (Elhaik et al., 2016; Yang et al., 2019). Furthermore, Michalek et al. (2011) demonstrated that the differentiation of CD4 effector T cells requires energy through the glucose metabolism with a high expression of Glut1, a glucose transporter protein on the surface of these cells. Besides that, osteopontin (another up-regulated protein) plays a role in the survival of dendritic cells since the blockage of this protein results in a reduced expression of both costimulatory and MHC class II molecules

(CD86/HLA-DR), which favors apoptosis (Kawamura et al. 2005). These data corroborate with studies from Santos et al. (2010, 2014) in which offspring suckled by infected mothers demonstrated an increase in the production of IL-2, suggesting the activation of effector T cells, and a higher frequency of CD86+ dendritic cells.

The protein-protein interaction network suggested that Fructose-bisphosphate aldolases A and C interact with the down regulated protein glucose-6-phosphate 1-dehydrogenase (G6PD). Which is a protein linked to the oxidative pentose-phosphate pathway that represents a route for the dissimilation of carbohydrates other than glycolysis. Deficiency in this protein provides protection against malaria (Ruwende and Hill 1998; Mbanefo et al. 2017) and in other study with G6PD-deficient peritoneal macrophages, an increase of IL-10 production was observed by these cells (Wilmanski et al. 2005). Another up-regulated protein in MIM is carboxylesterase 1D (Ces1d). Ces1d deficiency leads to reduction of hepatic lipogenesis through fatty acid oxidation increase (Lian et al. 2016). Michalek et al. (2011) demonstrated that the differentiation of CD4+ cells into induced regulatory T cells depends on lipid oxidation. Together, these data suggest a higher production of IL-10 by macrophages and a lower capacity of Treg generation by MIM. The offspring from infected mothers showed a bigger predisposition in the frequency of CD4+CD25+FoxP3+ cells, while, against mitogenic/antigenic stimulus, this higher frequency was not sustained (Santos et al. 2016; Fernandes et al. 2018). A new work about the effects of gestation and breastfeeding of offspring from schistosomotic mothers showed that breastfeeding in these mothers led to lower CD4+CD25+FoxP3+-cells frequency along with a higher frequency of CD14+IL-10+-cells and higher HDAC6 expression in the offspring (Holanda et al., 2019). It is known that HDAC6 is required for the transcriptional activation of IL-10 gene expression in macrophages (Cheng et al., 2014). If this fact is linked to early contact with this protein, it deserves to be investigated.

Regarding the 40S ribosomal protein 7 and the ATP-dependent RNA helicase DDX5 protein, it is known that they act increasing p53 activity, inducing apoptosis and inhibiting cellular proliferation (Bates et al. 2005; Zhu et al. 2009). In addition to these proteins, phosphatidylethanolamine-binding protein 1 was also up-regulated. It is known that the latter protein acts inhibiting Raf-1 by negatively regulating the Raf-MEK-ERK pathway (Yeung et al. 2000; Rath et al. 2008), being also able to inhibit the activation of the transcription factor NF-κβ (Yeung et al. 2001). Holanda et al. (2019) showed that the milk of infected mothers was responsible for altering the expression of HDAC1, HDAC2, HDAC7, Sirt2 and Sirt6 in the offspring, which are linked to inhibition of NF-κβ pathway. It is possible that these proteins are

involved in a predisposition to minimize inflammatory processes in offspring suckled by infected mothers (Attallah et al. 2006; Othman et al. 2010; Straubinger et al. 2014; Santos et al. 2016).

Concerning down-regulated proteins, among them 10 proteins found only in MIMN, the GO analysis revealed proteins which were linked to several biological processes. Immunoglobulin alpha chain C region and immunoglobulin kappa constant participate in the positive regulation of B cell activation; recognition and engulfment in phagocytosis; B cell receptor signaling pathway; complement activation (classical pathway) and innate immune response with the Complement factor B. Moreover, GO analysis showed that these two proteins have as molecular function immunoglobulin receptor binding. Innate immune defensive proteins are commonly found in breast milk (Stein et al., 2004; 2007). It is known that the passive transmission of antibodies through breastfeeding is better and lasts longer than through the uterus (Colley et al. 1999; Attallah et al. 2003; Fusaro et al. 2007; Nóbrega et al. 2012). However, studies on the participation of these antibodies in the modulation of the offspring immunity were performed at an early stage of birth, meaning it does not rule out the interference from the gestational period. Here, complement factor B and immunoglobulin fractions were down-regulated, suggesting a lower participation of the classical pathway of the complement system and of the antibodies on the milk modulation in infected animals.

The up-regulated protein Protease serine 1 precursor and the down-regulated proteins serine protease inhibitors A3M and A3N interact with each other as demonstrated in the protein-protein interaction network. Besides them, serine protease inhibitor A3K and alpha-2-antiplamin are also down-regulated and the last one also shows a co-expression with serine protease inhibitor A3N. The protease serine 1 precursor acts on serine cleavage and these down-regulated proteins act in the partial or total inhibition of biosynthesis and of the action of serine proteases (Laskowski and Qasim 2000). Hara et al. (2014) have demonstrated in patients with hepatic fibrosis that latent TGF-β is activated in a manner which is dependent upon proteolytic cleavage by plasma kallikrein serine protease (PLK), therefore, in an inhibition system of serine proteases, there is a shift towards a bigger activation of TGF- β with an immunosuppressive activity. Cathepsin B was also found down-regulated in MIM, and its high expression was observed in diseases such as rheumatoid arthritis, osteoarthritis and cancer (Lai et al. 2004; Tong et al. 2014; Yang et al. 2016). In an animal model of meningitis caused by Streptococcus pneumoniae, treatment with the cathepsin B inhibitor improved the course of the disease, decreased brain inflammation and reduced IL-1β (Hoegen et al. 2011). Thus, it is possible that

an enhancement of TGF-β gene expression after postnatal schistosomiasis infection in animals (Othman et al. 2010), which were born and suckled by infected mothers, together with the reduction of the granulomatous inflammatory reaction (Santos et al. 2016) and allergy (Straubinger et al. 2014) are involved in the downregulation of these proteins in the infected mother's milk.

In conclusion, our findings suggest that milk from *S. mansoni* infected mice has differentially expressed proteins which are involved in biological processes and pathways linked to the immune system. This protein profile may be involved with the activation and/or deactivation of the offsprings immune system in the long term, conferring a protective character due to the previous contact with milk from infected mothers. The proteins described here act primarily through the cellular metabolism, thus, they could be used as potential therapeutic targets which would enhance the immune response against heterologous antigens such as those within vaccines, tumors, autoimmune diseases and allergies.

Compliance with Ethical Standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animal protocol was approved by the Ethics Committee on Animal Use of Fiocruz (113/2017) and it is in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation.

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Conflict of interest The authors declare that they have no conflict of interest.

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Table 1. Up-regulated milk proteins in mice infected with *Schistosoma mansoni*

ID UniProt/ NCBI	Gene	Proteins	1:0_ Ratio	Ave Score	Ave Seq. Cover (%)	Ave ngram	SD
P62082	Rps7	40S ribosomal protein S7[a,b]	-	266.66	17.01	880	-
NP_666355.1	Actr2	actin-related protein 2[a,b]	-	236.23	11.41	-	-
Q8BFZ3	Actbl2	Beta-actin-like protein 2[a,b]	-	133.81	7.18	-	-
Q8VCT4	Ces1d	Carboxylesterase 1D[a]	-	400.16	12.21	16783.66	571.71
P05064	Aldoa	Fructose-bisphosphate aldolase A[a,b]	-	185.89	23.08	5673	-
P05063	Aldoc	Fructose-bisphosphate aldolase C[a]	-	286.76	19.28	3645	877
P35505	Fah	Fumarylacetoacetase[a,b]	-	241.53	13.13	1436	-
P10923	Spp1	Osteopontin[a]	-	852.77	14.97	7431	1328.64
NP_061346,2	Pebp1	phosphatidylethanolamine-binding protein 1[a,b]	-	245.62	26.20	-	-
P0CG49	Ubb	Polyubiquitin-B[a,b]	-	172.57	5.25	4540	-
XP_017172700.1	Dxo	PREDICTED: decapping and exoribonuclease protein isoform X1[a,b]	-	301.53	9.51	-	-
XP_006532765.1	Bzrap1	PREDICTED: peripheral-type benzodiazepine receptor-associated protein 1 isoform X1[a,b]	-	126.58	4.93	-	-
NP_031866.2	Ddx5	probable ATP-dependent RNA helicase DDX5[a,b]	-	128	7.64	-	-
NP_444473.1	Prss1	protease, serine, 1 precursor[a,b]	-	224.64	12.20	-	-
NP_001106798.1	Fam131b	protein FAM131B isoform b[a,b]	-	186.39	11.21	-	-

[a] Unique proteins in milk from infected mice with *Schistosoma mansoni* (MIM).

[b] Values of Ave ngram and/or standard deviation (SD) not calculated due to the appearance of the protein concentration in only one replicate or none.

Table 2. Down-regulated milk proteins in mice infected with *Schistosoma mansoni*

ID UniProt/ NCBI	Gene	Proteins	1:0_ Ratio	Ave Score	Ave Seq. Cover (%)	Ave ngram	SD
P01878	Igh-VJ558	Ig alpha chain C region	0.49	2138.76	33.14	12394	397.0214
Q03734	Serpina3m	Serine protease inhibitor A3M	0.49	624.2	27.59	1841.5	529.5
Q91WP6	Serpina3n	Serine protease inhibitor A3N[b]	0.49	359.91	10.77	548	-
P07759	Serpina3k	Serine protease inhibitor A3K	0.48	3266.82	49.76	41534.66	196.0482
Q61247	Serpinf2	Alpha-2-antiplasmin [a,b]	-	186.28	11.81	5635	-
P10605	Ctsb	Cathepsin B [a,b]	-	150.52	7.96	2804	-
P04186	Cfb	Complement factor B [a,b]	-	190.94	11.56	31495	-
Q06770	Serpina6	Corticosteroid-binding globulin [a]	-	303.32	8.18	5652.5	98.5
P97310	Mcm2	DNA replication licensing factor MCM2 [a,b]	-	111.45	4.42	21821	-
P17183	Eno2	Gamma-enolase [a,b]	-	209.05	11.06	878	-
NP_032088.1	G6pdx	glucose-6-phosphate 1-dehydrogenase X [a,b]	-	161.66	6.21	-	-
P01756		Ig heavy chain V region MOPC 104E [a]	-	793.71	40.74	4599	87.9355
P01837	Igkc	Immunoglobulin kappa constant [a]	-	1234.42	49.84	3600.33	1120.4303
NP_001182194.1	Pdzd7	PDZ domain-containing protein 7[a,b]	-	171,28	12.05	-	-

[a] Unique proteins in milk from non-infected mice (MNIM).

[b] Values of Ave ngram and/or standard deviation (SD) not calculated due to the appearance of the protein concentration in only one replicate or none.

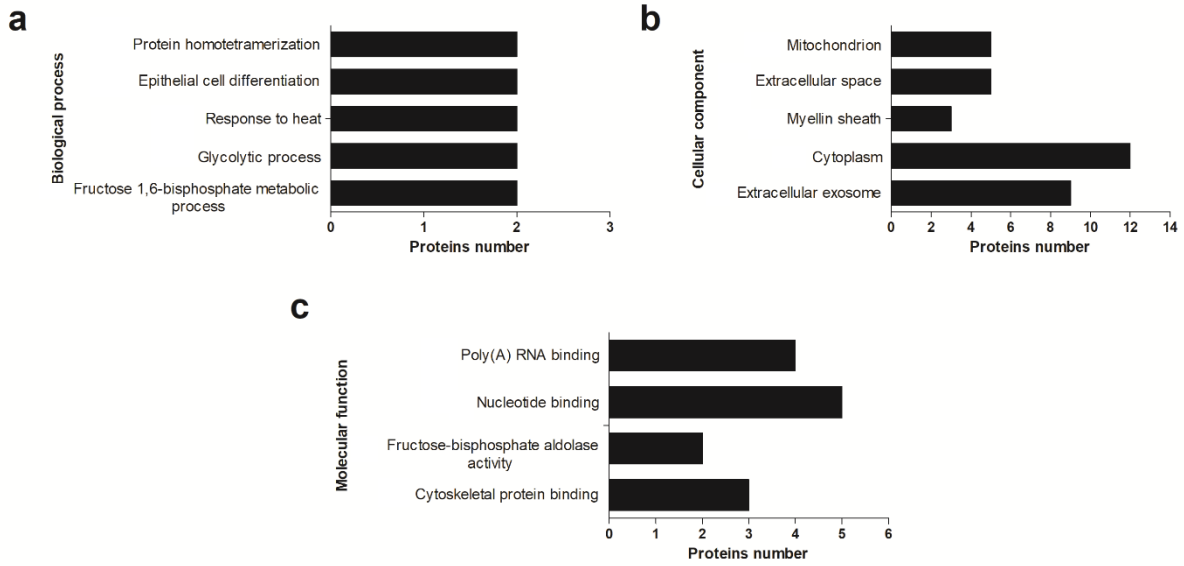

Fig. 1 Top Gene Ontology (GO) terms obtained from GO functional analysis of up-regulated milk proteins from *Schistosoma mansoni* infected mice by DAVID. a: TOP 5 Biological process; b: TOP 5 Cellular components; c: Top 4 Molecular functions

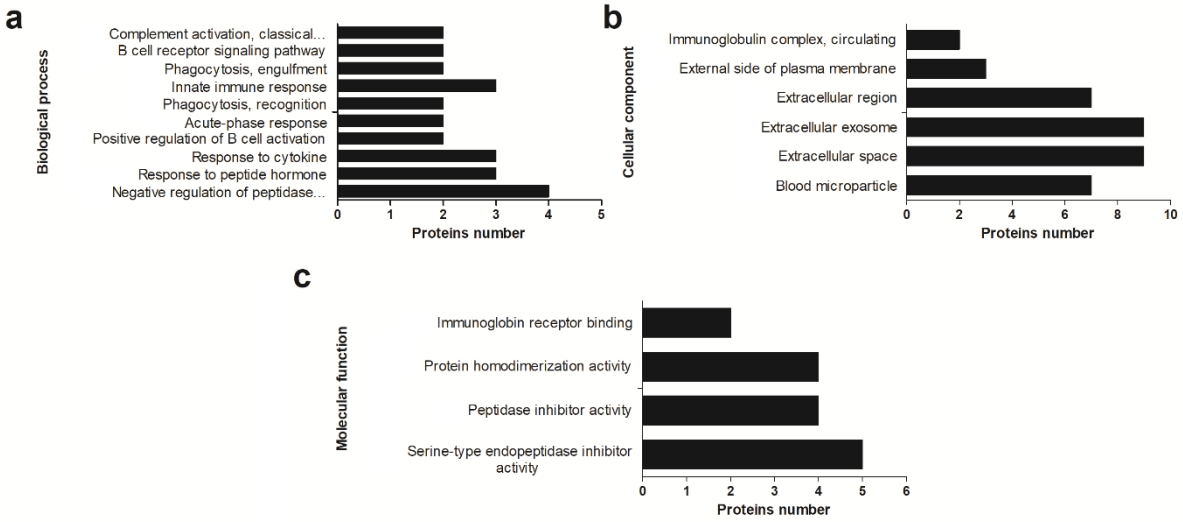

Fig. 2 Top Gene Ontology (GO) terms obtained from GO functional analysis of down-regulated milk proteins from *Schistosoma mansoni* infected mice by DAVID. a: TOP 10 Biological process; b: TOP 6 Cellular components; c: Top 4 Molecular functions

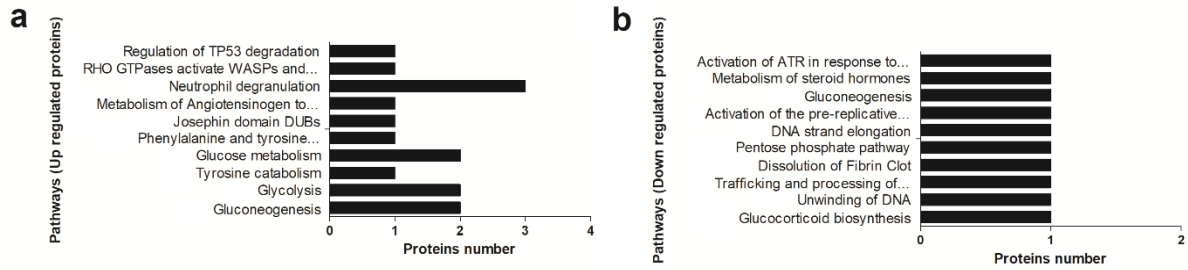

Fig. 3 Pathways enrichment analysis of differentially expressed proteins in milk from *Schistosoma mansoni* infected mice by Reactome. a: TOP 10 pathways from up-regulated proteins; b: TOP 10 pathways from down-regulated proteins

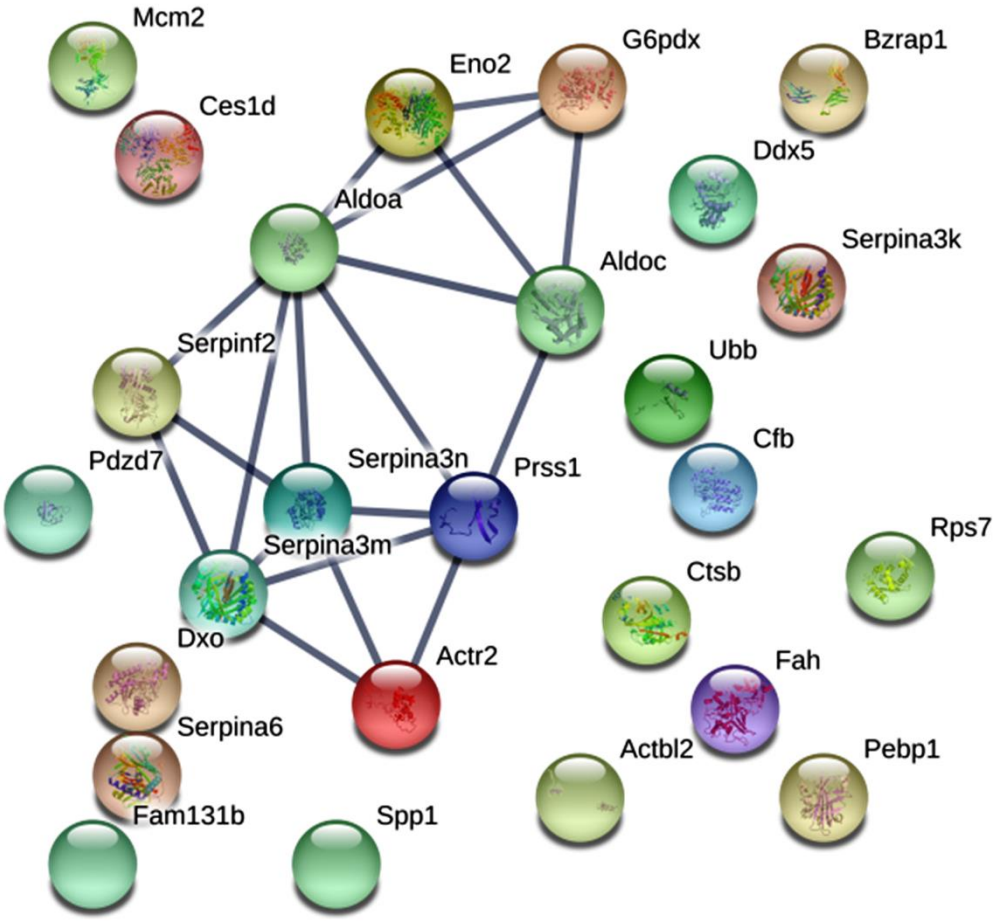

Fig. 4 The protein-protein interaction network of milk from *Schistosoma mansoni* infected mice that were differentially expressed. The network was analyzed by STRING. The PPI network was constructed using the cut-off value of a confidence score > 0.70

5 CONCLUSÕES

A gestação e a amamentação em camundongos infectadas pelo *S. mansoni* foram capazes de modificar de maneira diferente a expressão das histonas desacetilases, podendo servir como potenciais alvos terapêuticos para melhoramento da imunidade dos descendentes.

A gestação em mães infectadas modificou a expressão apenas da HDAC9, envolvida na produção de interferon do tipo I, podendo modular a resposta do descendente contra infecções virais. Por outro lado, o leite de mães infectadas levou a maior expressão de HDAC1, HDAC2, HDAC6, HDAC7, HDAC10 e Sirt2, Sirt5, Sirt6 e Sirt7. Todas essas enzimas, menos a Sirt5, possuem ação anti-inflamatória reduzindo citocinas inflamatórias, espécies reativas de oxigênio e inibição de NF-κβ. A Sirt5 atua antagonizando a Sirt2, o que neste estudo não foi evidenciado.

Em conjunto com estes dados, foi observado que a gestação em mães infectadas levou ao aumento de IL-10 por linfócitos T e a amamentação levou a maior frequência de células B e monócitos produtores de IL-10 e menor frequência de células Treg.

A análise proteômica do soro do leite de mães infectadas pelo *S. mansoni* revelou proteínas reguladas diferencialmente (alta e baixa expressão) que estão envolvidas em processos biológicos e vias ligadas ao sistema imunológico da prole a longo prazo. Esse perfil proteico pode estar envolvido com a ativação e/ou desativação do sistema imunológico da prole a longo prazo, conferindo um caráter protetor devido ao contato prévio com o leite das mães infectadas. As proteínas descritas aqui atuam primariamente através do metabolismo celular, portanto, podem ser usadas como alvos terapêuticos potenciais que melhorariam a resposta imune contra antígenos heterólogos, como aqueles dentro de vacinas, tumores, doenças autoimunes e alergias.

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APÊNDICE A – ARTIGO 1 PUBLICADO NA REVISTA MEMÓRIAS DO INSTITUTO OSWALDO CRUZ

ORIGINAL ARTICLE Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. 114: e190366, 2019 1|10

Gestation and breastfeeding in schistosomotic mice differentially alters the expression of histone deacetylases (HDACs) in adult offspring

Gabriela Calixto Ribeiro de Holanda[1], Fabrício Oliveira Souto[1], Maria da Conceição Silva[1], Virgínia Maria Barros de Lorena[2], Vlaudia Maria Assis Costa[1], Monica Camelo Pessôa de Azevedo Albuquerque[1], Valdênia Maria Oliveira de Souza[1]/+, José Luiz de Lima Filho[1]

[1]Universidade Federal de Pernambuco, Laboratório de Imunopatologia Keizo Asami, Recife, PE, Brasil
[2]Fundação Oswaldo Cruz-Fiocruz, Instituto de Pesquisas Aggeu Magalhães, Recife, PE, Brasil

BACKGROUND Breastfeeding or gestation in schistosomotic mothers can cause long-term alterations in the immune response of offspring.

OBJECTIVES Evaluate the expression of histone deacetylases (HDACs) (all classes), the production of cytokines by T and B lymphocytes and macrophages, and the frequency of $CD4^+CD25^+FoxP3^+$-cells in adult offspring born and/or suckled by schistosomotic mothers.

METHODS We harvested splenocytes from offspring born to (BIM), suckled by (SIM), or born to/suckled by (BSIM) schistosomotic mothers and animals from noninfected mothers (Control) at seven-weeks old and cultured them with/without Concanavalin A. HDAC expression was evaluated by real-time quantitative polymerase chain reaction (qPCR), and cytokines and membrane markers were evaluated by fluorescence-activated cell sorting (FACS).

FINDINGS Compared to Control, BIM mice showed increased expression of HDAC9 and frequency of $CD4^+IL\text{-}10^+$-cells. The SIM group had increased expression of HDAC1, HDAC2, HDAC6, HDAC7, HDAC10, Sirt2, Sirt5, Sirt6, and Sirt7. The BSIM group only had increased HDAC10 expression. The SIM and BSIM groups exhibited decreased frequencies of $CD4^+IL\text{-}4^+$-cells and $CD4^+CD25^+FoxP3^+$-cells, along with a higher frequency of $CD14^+IL\text{-}10^+$-cells and an increase in $CD45R/B220^+IL\text{-}10^+$-cells. The BSIM group also showed a high frequency of $CD4^+IL10^+$-cells.

MAIN CONCLUSIONS Breastfeeding induced the expression of HDACs from various classes involved in reducing inflammatory responses. However, gestation enhanced the expression of a single HDAC and breastfeeding or gestation appears to favour multiple IL-10-dependent pathways, but not cells with a regulatory phenotype.

Key words: breastfeeding - epigenomics - histone deacetylases - pregnancy - schistosomiasis

A high prevalence of chronic schistosomiasis in pregnant women and women of childbearing age has been reported,[1] and the effects of maternal infection have raised questions regarding the immunity of the offspring.

It is known that the immunological status of schistosomotic mothers can induce long-term alterations in the immune response of the offspring.[2,3,4,5] An experimental study on the effects of gestation and breastfeeding in infected mothers, separately, showed that gestation in these mothers led to potential immunosuppression in adult offspring, with elevated production of IL-10 and lower levels of anti-ovalbumin (OA) antibodies.[2] In addition, offspring born to infected mothers had a lower frequency of B lymphocytes, and the capacity for antigen presentation by CD11c+ cells was partially impaired.[3] In contrast, it has also been observed that adult mice previously breastfed by schistosomotic mothers exhibited improvement in the production of anti-OA antibodies[2] and in the antigen presentation ability of B lymphocytes through an increase in surface frequency of CD40+/CD80+ in these cells.[3] However, whether these alterations in the immune response of adult offspring from infected mothers are due to epigenetic changes from the perinatal period remains unclear.

Studies have correlated post-transcriptional changes in the chromatin, through histone acetylation/deacetylation, with the immune response.[6,7,8] In an experimental study on antigen presenting cells (APCs), it was demonstrated that histone deacetylase (HDAC)6 is required for transcriptional activation of IL-10 gene expression in macrophages and dendritic cells through activation of STAT3.[6] Another study using pancreatic beta cell lines showed that knockdown of HDAC1 increased IFN-γ-induced STAT1 phosphorylation.[7] Kosciuczuk et al.[8] showed that deacetylation of cyclin-dependent kinase 9 induced by Sirtuin 2 promotes STAT1 phosphorylation during type I interferon responses.

In addition, it has been demonstrated that the role of epigenetic markers can be remodelled during the perinatal period, and may trigger lasting influences on the epigenome of the offspring.[9] Mice prenatally adminis-

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Financial support: FACEPE, CNPq.
+ Corresponding author: valdenia.souza@gmail.com
https://orcid.org/ 0000-0002-4976-009x
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tered with *Acinetobacter lwoffii* F78 displayed increased acetylation of histone H4 in the interferon (IFN)-γ gene in their offspring, and conferred protection against asthma after challenge with OA, which is associated with positive regulation of IFN-γ production.[10] Song et al.[11] found that offspring from mothers with peanut allergy had elevated IgE-specific levels, high levels of histamine and resultant increased production of Th2 cytokines, and reduction of DNA methylation at CpG sites of the IL-4 gene promoter after sensitisation.

Histone acetylation is the most commonly studied epigenomic alteration, for stimulation of transcription, and in turn, is reversibly regulated by the balance between the activity of histone acetyltransferases (HATs) and HDACs.[12] HDACs have been classified as class I (HDAC1, HDAC2, HDAC3 and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), class III (SIRT1 to SIRT7), and class IV (HDAC11)[13] and are increasingly studied due to their interference in the pathways of mechanisms associated with the pathogenesis of various cancers and other inflammatory diseases.[14,15,16]

Although research that relates epigenetic alterations to the maternal-foetal relationship can be found, there are no studies that report the effects of gestation and/or breastfeeding on the expression of HDACs, and the implications for the immune system of offspring from schistosomotic mothers. To investigate, we have evaluated whether the expression of enzymes involved in chromatin remodelling through histone deacetylation can be altered due to gestation or breastfeeding from *Schistosoma mansoni*-infected mothers. Our results could aid in the discovery of therapeutic targets that improve the immunity of individuals who previously contacted immunological factors resulting from infection during perinatal period.

MATERIALS AND METHODS

Animals and maternal infection - Four-week-old Swiss Webster female mice were infected subcutaneously (s.c.) with 20 *S. mansoni* cercariae, strain São Lourenço da Mata (SLM). On the 45th day, infection was confirmed by the Kato-Katz method.[17] On the 60th day post-infection (dpi), oestrus was synchronised among mice via the administration of 5 i.u. (100 µL) of equine chorionic gonadotrophin hormone, followed by an additional injection with 5 i.u. (100 µL) of human chorionic gonadotrophin 48 h later. Females were housed with male mice at a 1:1 ratio, and successful mating was confirmed by presence of a vaginal plug. The same procedure was performed in noninfected females, and seven-week-old male offspring were taken for the experimental and control groups. The mice were housed in the animal care facility at the Aggeu Magalhães Institute (IAM), Oswaldo Cruz Foundation (Fiocruz), municipality of Recife, State of Pernambuco, Brazil.

Adoptive nursing and study groups - Immediately after birth, new-born mice from *S. mansoni*-infected or noninfected mothers were rehoused with mothers from the opposite group. After adoptive nursing, offspring born from infected mothers (BIM) were suckled by noninfected mothers, and offspring from noninfected mothers were suckled by infected mothers (SIM). A separate group was born from and suckled by schistosomotic mothers (BSIM). Animals born from noninfected females were suckled by their mothers (Control).

Cell culture - Spleens from each animal (seven-weeks-old) were harvested after euthanasia by cervical dislocation. Cell suspensions were prepared in RPMI-1640 (Sigma-Aldrich, St. Louis, USA) supplemented with HEPES (10 µM), 2-mercaptoethanol (0.05 µM), 216 mg of L-glutamine/L, gentamicin (50 mg/L), and 5% of foetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, USA). Cells from each group (n = 8-10) were cultivated at a final concentration of 2×10^7 cells/mL in tissue culture plates (Costar Culture Plates, City, USA) and stimulated with concanavalin-A (Con-A) (5 µg/mL), or without antigenic stimulus (Basal), at 37°C in 5% CO_2. Cultured cells were harvested after 24 h and assayed for immunophenotyping and real-time quantitative polymerase chain reaction (qPCR).

Flow cytometry analyses - 5 µL of Golgi Stop (per 2×10^7 cells) were added to each well containing splenic cells under different stimuli, then the cells were vortexed and returned to the CO_2 incubator at 37°C for four additional hours. Spleen cells were subjected to double-labelling with fluorochrome-labelled antibody solutions at a concentration of 0.5 mg/10^6 cells: FITC anti-mouse CD4, and PE anti-mouse IL-4, APC anti-mouse IFN-γ, PE anti-mouse IL-10, or PerCP-Cy-5.5 anti-mouse IL-2; FITC anti-mouse CD4, PE anti-mouse CD25, and APC anti-mouse FoxP3; FITC anti-mouse CD45R (B220) or FITC anti-mouse CD14, and PE anti-mouse IL-10 (BD Biosciences Pharmingen). After staining, preparations were washed with phosphate-buffered saline (PBS) containing azide (0.1%) and FBS (3%). After centrifugation, the cell pellet was resuspended in PBS with paraformaldehyde (0.5%) and maintained at 4°C until data acquisition, which was performed using a FACSCalibur (BD-Pharmingen, New Jersey, USA) flow cytometer and acquisition of a minimum 50,000 lymphocytes or 5,000 monocytes. The frequency of positive cells was analysed using FlowJo software, with quadrant gating set based on negative populations and isotype controls. A descriptive analysis of the frequency of cells in the upper right quadrant (double-positive cells) was performed. Distinct gating strategies were used to analyse each subpopulation of cells (Fig. 1). T lymphocyte subpopulations were first selected as CD4 high cells on FL1/anti-CD4-FITC versus laser side-scatter (SSC) dot plots (Fig. 1A). Following this gating procedure, a second gate was set using FL1/anti-CD4-FITC versus FL2/anti-CD25-PE; then, a third gate was established to generate representative 2-dimensional graphics using FL1/anti-CD4-FITC versus FL4/anti-FoxP3-APC to identify triple staining for CD4+CD25+FoxP3+ (Fig. 1B). The frequency of cytokine-expressing cells was further determined on FL1/anti-CD4-FITC versus FL2/anti-IL10-PE or anti-IL4-PE, FL3/anti-IL2-Percp-Cy-5.5 or FL4/anti-IFN-γ-APC dot plots by quadrant statistic measurements, and expressed as percentage of cytokine T CD4+ lymphocyte (Fig. 1C). B Cells and monocytes were first

selected on CD45-high or CD14-high cells using FL1/anti-CD45 or CD14-FITC versus SSC dot plots, and the frequency of IL-10 producing cells was subsequently determined using FL1/CD45-FITC or FL1/CD14-FITC versus anti-IL10-PE dot plots and quadrant statistic measurements (Fig. 1D-E).

The results are expressed as the median frequency of cells from each group ± standard error.

qPCR analysis - Total RNA from splenic cells was extracted using the ReliaPrep™ RNA Cell Miniprep System Kit (Promega, Madison, WI) according to the manufacturer's instructions. Complementary DNA (cDNA) was generated with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative PCR was performed using SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) on the 7500 Real Time System (Applied Biosystems Foster City, CA, USA) machine. Results were normalised to the housekeeping gene β-Actin. Relative expression levels were calculated using $2^{-\Delta\Delta Ct}$. Primers were designed using Primer3Plus software, and the sequences are described in Table.

Statistical analysis - Results were subjected to Barlett's test to verify whether the distribution of the data was normal. After verifying that the results did not follow a normal distribution, the Kruskal-Wallis test was used, followed by Dunn's multiple comparison test when statistical significance was shown. For statistical analysis, we used GraphPad Prism v.5.0 (GraphPad Software, San Diego, CA, USA) and findings were considered significant at $p < 0.05$. All procedures were performed in triplicate to evaluate reproducibility, and images refer to one representative of at least three independent studies.

Ethics - The animal protocol was approved by the Ethical Commission on Animal Use of the Fiocruz (113/2017) and is in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation.

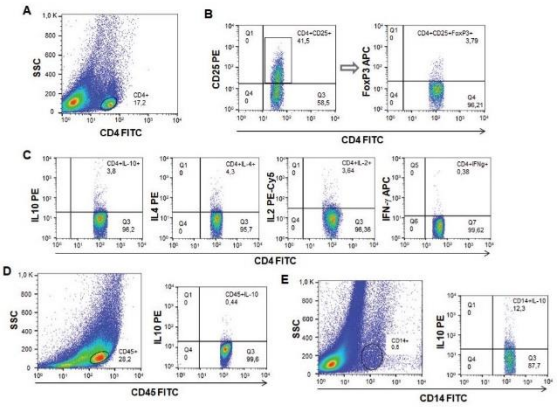

Fig. 1: representation of the gating strategy used to analyse the different subpopulations of cells. FL1/anti-CD4-FITC versus laser side-scatter (SSC) dot plot (A), CD4+CD25+FoxP3+ cells dot plots (B), cytokine producing T CD4+ lymphocytes dot plots (C), IL-10 producing CD45R/B220+ cells dot plot (D) and IL-10 producing CD14+ cells dot plot (E).

RESULTS

Relative expression of HDAC in animals born and/or breastfed from schistosomotic mothers - To verify the epigenetic profile of the animals born and/or breastfed from schistosomotic mothers, real time qPCR was performed on spleen cells cultured for 24 h in absence

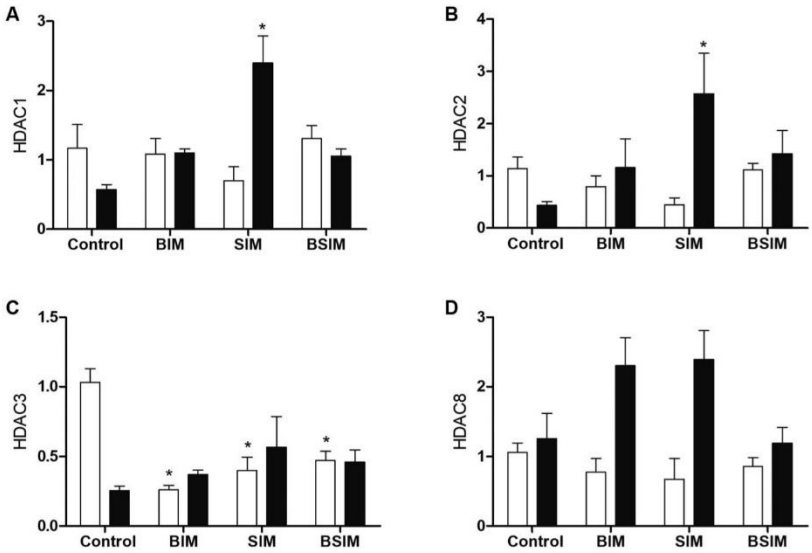

Fig. 2: relative expression of class I histone deacetylases (HDACs). HDAC1 (A), HDAC2 (B), HDAC3 (C) and HDAC8 (D) in splenocytes from *Swiss Webster* mice (seven weeks) born (BIM), breastfed (SIM), or born and breastfed (BSIM) in infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 µg/mL, black bar) or with culture medium (BASAL, white bar). The relative expression was verified by real-time quantitative polymerase chain reaction (qPCR). The results represent the median and standard error for 8-10 animals/group. *$p < 0.05$ compared to control group.

TABLE
Primers used in quantitative real-time polymerase chain reaction (qPCR)

Target gene (Accession number)	Sequence 5'-3' (Forward and reverse)	Tm	CG %	Amplicon length (bp)
HDAC1 (NM_008228.2)	CCGGTTAGGTTGCTTCAATC	59.6	50	118
	AACATTCCGGATGGTGTAGC	59.8	50	
HDAC2 (NM_008229.2)	TATTATGGCCAGGGTCATCC	59.6	50	119
	TCAGCAGTGGCTTTATGAGG	59	50	
HDAC3 (NM_010411.2)	ATGCAGGGTTTCACCAAGAG	60.1	50	117
	TGTTGCTCCTTGCAGAGATG	60.1	50	
HDAC4 (NM_207225.2)	CCGCCAGCAGTTTTAAAGTC	59.9	50	92
	ACCGAATGGAGATGCTCAAC	60.1	50	
HDAC5 (NM_001077696.1)	ACTTTCCCCTCCGTAAAACG	60.3	50	116
	AACAGTGCCATCCTTTCGAC	60.1	50	
HDAC6 (NM_010413.3)	ATCTCAGCTGGCTTTGATGC	60.5	50	116
	ATAATACGGCCACCAGCAAG	60.0	50	
HDAC7 (NM_001204275.1)	ATGATGGCCTGGAACATAGG	59.8	50	75
	GATGCTGCTGCAGAGAAATG	59.7	50	
HDAC8 (NM_027382.4)	AGGGAATCTGAAGCATGTGG	60.1	50	131
	CAAATTTCCCCTGCAGTCAC	60.5	50	
HDAC9 (NM_001271386.1)	TTTGAGGTGGCAGAATCCTC	60.2	50	106
	GAGCTGAAGCCTCATTTTCG	60.1	50	
HDAC10 (NM_199198.2)	AACAGGAGCTGTGCACAATG	59.9	50	143
	TCCTCTGCAGCCCATATTTC	60.2	50	
HDAC11 (NM_144919.2)	TGATGGGGTTGAACACTGAG	59.5	50	128
	AGCAGCCCCTTAAAAACTCC	59.7	50	
Sirt1 (NM_019812.3)	GCCCTCAATTTCTGTTCTGC	59.8	50	150
	TTTTGAGTGCTCCAGACACG	60.0	50	
Sirt2 (NM_019812.3)	ACGGCTGCTCATTAACAAGG	60.3	50	88
	GTCAAAATCCATGCCACCTC	60.3	50	
Sirt3 (NM_001177804.1)	CATATGGGCTGATGTGATGG	59.8	50	141
	AGATCTGCCAAAGCGAAGTC	59.6	50	
Sirt4 (NM_001167691.1)	CGAGCAAAAGCTCCCAATAG	60.0	50	145
	TTCCAGCCTTTGGACATCAG	61.2	50	
Sirt5 (NM_178848.3)	CCAGCTTTAGCAGGAAAAGG	59.1	50	139
	CCAGGTTTTCTCCAAACCAC	59.4	50	
Sirt6 (NM_181586.3)	TGTCCAACACAGCTCCTTTC	58.9	50	97
	CTTCCACATGTGTGGGATTC	58.8	50	
Sirt7 (NM_001363439.1)	AGCTTCGGGATACCATTGTG	60	50	104
	CAGGATTGTGTCTGCTTTGC	59.4	50	
β-Actin (NM_007393.5)	TTGCTGACAGGATGCAGAAG	61.1	50	147
	TGATCCACATCTGCTGGAAG	59.8	50	

bp: base pair; CG: cytosine and guanine content; HDAC: histone deacetylase 1-11; Sirt: Sirtuin 1-7; Tm (melting temperature) was calculated at default settings of 0.25 µM oligo concentration and 50 µM Na. Primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

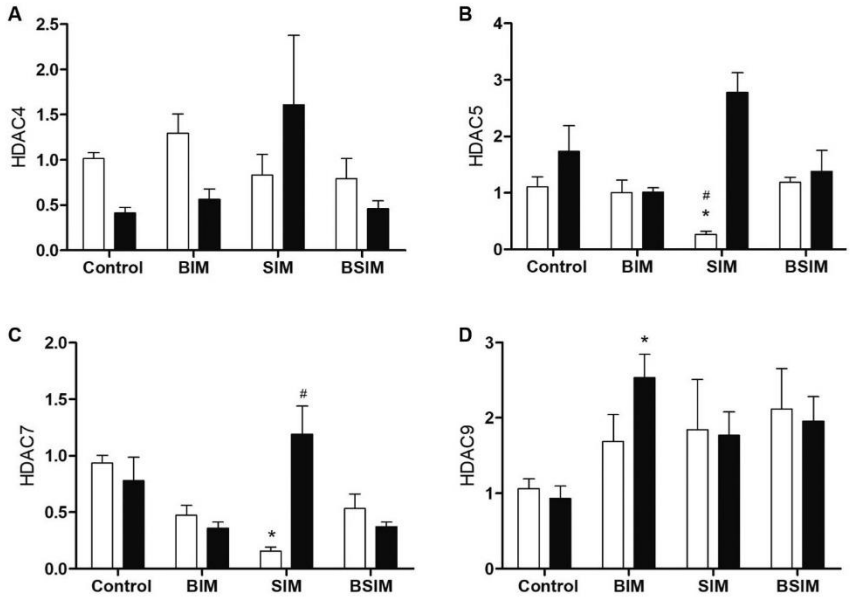

Fig. 3: relative expression of class IIa histone deacetylases (HDACs). HDAC4 (A), HDAC5 (B), HDAC7 (C) and HDAC9 (D) in splenocytes from *Swiss Webster* mice (seven weeks) born (BIM), breastfed (SIM), or born and breastfed (BSIM) in infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 µg/mL, black bar) or with culture medium (BASAL, white bar). The relative expression was verified by real-time quantitative polymerase chain reaction (qPCR). The results represent the median and standard error for 8-10 animals/group. $^{*}p < 0.05$ compared to control group. $^{\#}p < 0.05$ compared to BIM and BSIM groups.

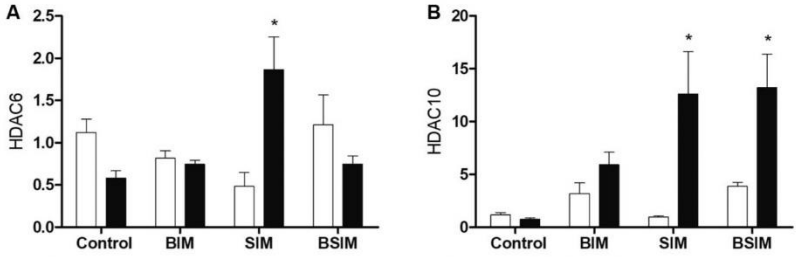

Fig. 4: relative expression of class IIb histone deacetylases (HDACs). HDAC6 (A) and HDAC10 (B) in splenocytes from *Swiss Webster* mice (seven weeks) born (BIM), breastfed (SIM), or born and breastfed in (BSIM) infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 µg/mL, black bar) or with culture medium (BASAL, white bar). The relative expression was verified by real-time quantitative polymerase chain reaction (qPCR). The results represent the median and standard error for 8-10 animals/group. $^{*}p < 0.05$ compared to control group.

(basal) or presence of mitogenic stimulus (ConA). For class I HDAC, the basal relative expressions of HDAC1 and HDAC2 were not altered, but with mitogenic stimulus, the group of animals which were breastfed only (SIM) exhibited increased expression compared to the Control (Fig. 2A-B). The groups BIM, SIM, and BSIM had decreased basal relative expression of HDAC3, but with mitogenic stimulus the relative expression was similar to Control (Fig. 2C). There was no difference in the relative expression of HDAC8 either at baseline or with mitogenic stimulation (Fig. 2D).

Class IIa HDACs were analysed, and we saw that there was no difference in HDAC4 expression (Fig. 3A). The relative expression of HDAC5 in the SIM group was lower compared to Control, BIM and BSIM at baseline, but there was no difference among the groups under mitogenic stimulus (Fig. 3B). For HDAC7 the SIM group had lower expression than Control at baseline, while under mitogenic stimulus the SIM group had increased expression compared to the BIM and BSIM groups (Fig. 3C). Regarding HDAC9, there was no difference at baseline, but the BIM group showed a higher relative expression compared to Control under mitogenic stimulus (Fig. 3D).

Regarding HDACs 6 and 10 (class IIb), there was similar expression among all groups, and did not differ significantly from Control. However, in response to mitogenic stimulus, the expression of HDAC6 and HDAC10 was increased in the SIM group, and HDAC10 was increased in the BSIM group (Fig. 4A-B).

Among sirtuins (class III), the expressions of Sirt1, Sirt3, and Sirt4 were found to be similar among all groups under the culture conditions used (Fig. 5A-C).

Sirt2 and Sirt5 did not show any differences in the basal groups, but increased expression was observed in the SIM group compared to Control under mitogenic stimulation (Fig. 5D-E). Fig. 5F shows that compared to Control, the expression of Sirt6 in the SIM group was lower at baseline. However, under mitogenic stimulus, there was a significant increase in the expression of Sirt6 in all experimental groups (BIM, SIM, and BSIM). Although there was no baseline difference for Sirt7, the SIM group had increased expression with mitogenic stimulus compared to the Control, BIM, and BSIM groups (Fig. 5G).

Class IV is composed only of HDAC11 which in this study showed no differences compared to Control, but the SIM group had higher relative expression compared to BIM under mitogenic stimulus (Fig. 6).

Intracellular cytokines in T and B lymphocytes, and monocytes, and the frequency of regulatory T lymphocytes in animals born and/or breastfed - Cytokine producing T lymphocytes were observed by labelling with CD4+/IL-4+, CD4+/IFN-γ+, CD4+/IL-10+, or CD4+/IL-2+, while B lymphocytes and monocytes were labelled with anti-CD45R/B220+ and anti-CD14+ antibodies, respectively, together with anti-IL-10+. T lymphocytes with a regulatory profile were evaluated by triple labelling CD4+CD25+FoxP3+. Frequencies were evaluated with mitogenic stimulus (ConA) and without (basal). Compared to the Control group, the frequency of CD4+/IL-4+ cells under basal conditions and mitogenic stimulation was lower in the SIM and BSIM groups (Fig. 7A). IL-10 production by CD4+ cells was higher in the BIM and BSIM groups under mitogenic stimulation (Fig. 7B). There were no differences among groups when the frequencies of CD4+IL-2+ and CD4+/IFN-γ+ cells (Fig. 7C-D) were analysed.

Regarding IL-10 production by B lymphocytes (Fig. 7E), it was slightly higher in the SIM and BSIM groups in response to mitogen. The SIM and BSIM groups also had an increased frequency of CD14+IL-10+ in comparison to the Control and BIM groups both at baseline and in response to mitogen (Fig. 7F).

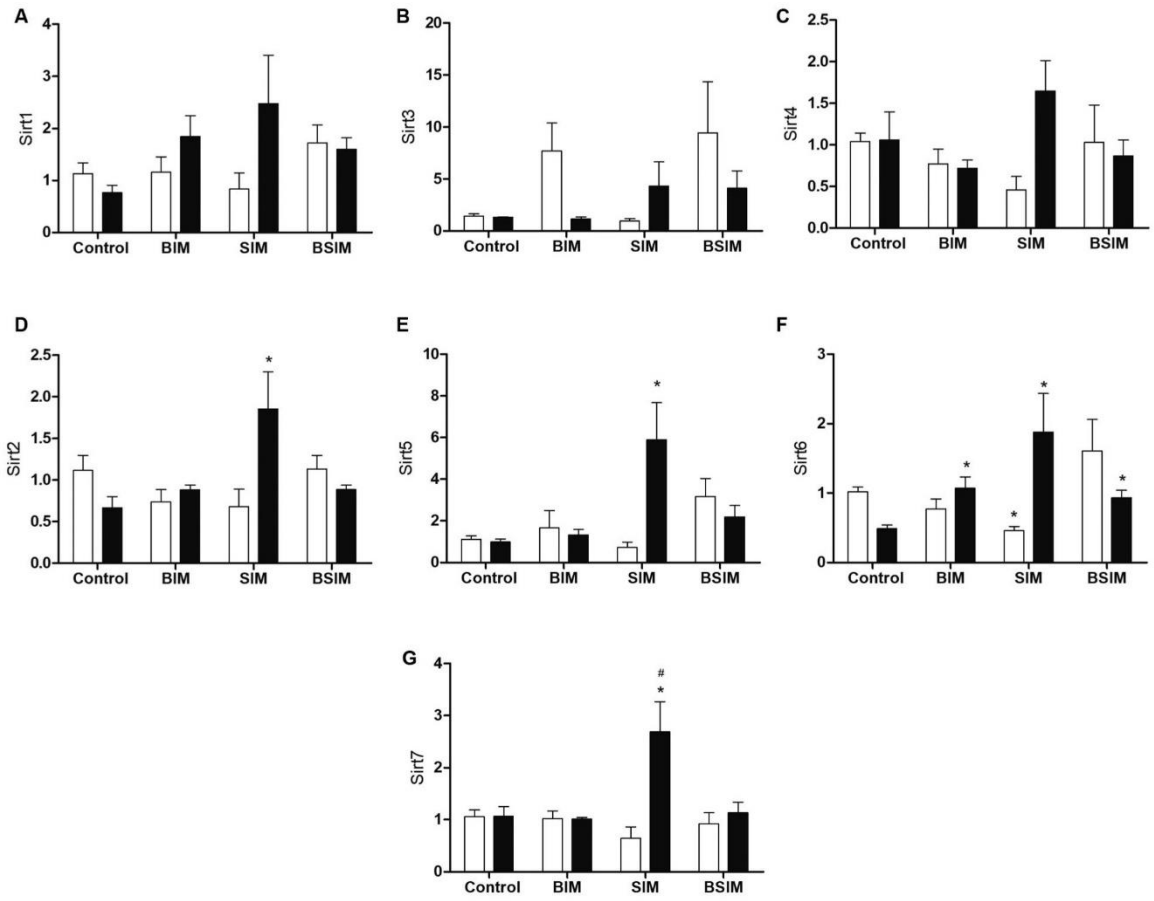

Fig. 5: relative expression of class III histone deacetylases (HDACs). Sirt1 (A), Sirt3 (B), Sirt4 (C), Sirt2 (D), Sirt5 (E), Sirt6 (F) and Sirt7 (G) in splenocytes from *Swiss Webster* mice (seven weeks) born (BIM), breastfed (SIM) or born and breastfed (BSIM) in infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 μg/mL, black bar) or with culture medium (BASAL, white bar). The relative expression was verified by real-time quantitative polymerase chain reaction (qPCR). The results represent the median and standard error for 8-10 animals/group. $^{*}p < 0.05$ compared to control group. $^{\#}p < 0.05$ compared to BIM and BSIM groups.

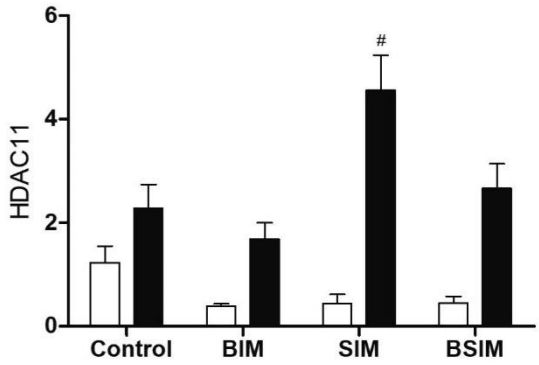

Fig. 6: relative expression of class IV histone deacetylases (HDACs). HDAC11 in splenocytes from *Swiss Webster* mice (seven weeks) born (BIM), breastfed (SIM) or born and breastfed(BSIM) in infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 µg/mL, black bar) or with culture medium (BASAL, white bar). The relative expression was verified by real-time quantitative polymerase chain reaction (qPCR). The results represent the median and standard error for 8-10 animals/group.

For cells expressing CD4+CD25+FoxP3+, the SIM and BSIM groups exhibited decreased frequency relative to the Control and BIM groups, with and without mitogenic stimulus (Fig. 7G).

DISCUSSION

Maternal infection by *S. mansoni* can alter the degree of immune competence of the offspring in the long term, either through in-utero contact or breastfeeding.[2,3,4,5] In this study, the effects of gestation and breastfeeding were evaluated separately in *S. mansoni*-infected mothers. Therefore, the expression of HDACs, and cytokine production by lymphocytes and macrophages was assessed. These experiments were conducted using an *in vitro* system with broadly activated splenic cells, achieved using mitogenic stimulation.

Our findings show that breastfeeding from infected mothers induced the expression of HDACs from different classes which are involved in reducing the inflammatory response; however, gestation enhanced the expression of a single HDAC. These enzymatic changes induced by

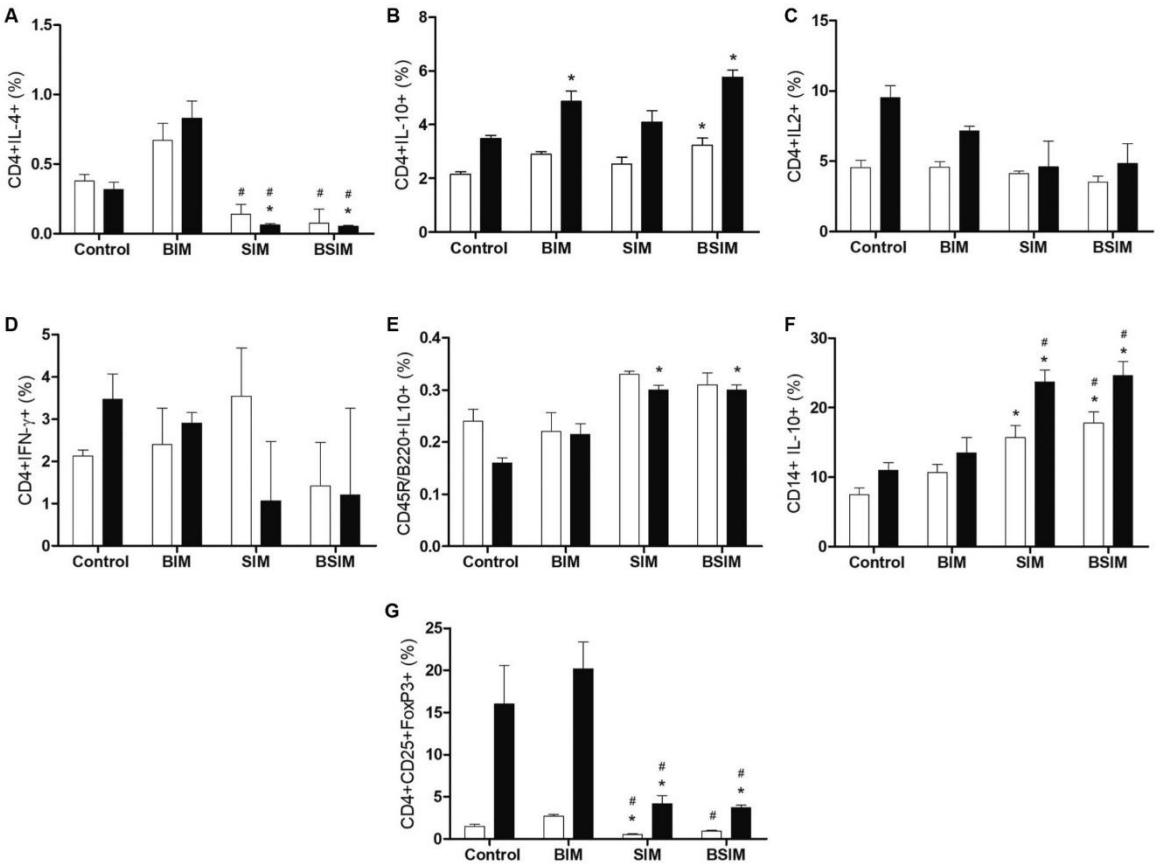

Fig. 7: cytokine production by T and B lymphocytes and monocytes, and splenocytes expressing CD4+ CD25+ FoxP3+. Frequency of CD4+IL-4+ (A), CD4+IL-10+ (B), CD4+IL-2+ (C), CD4+IFN-γ+ (D), CD45R/B220+IL-10+ (E), CD14+IL-10+ (F) and CD4+CD25+FoxP3+ (G) in splenocytes from *Swiss Webster* mice (seven weeks) born (BIM), breastfed (SIM) or born and breastfed (BSIM) in infected mothers, or uninfected mothers (Control), cultured for 24 h in the presence of mitogen (Concanavalin A) (5 µg/mL, black bar) or with culture medium (BASAL, white bar). The frequencies were verified by flow cytometry. The results represent the median and standard error for 8-10 animals/group. $^{*}p < 0.05$ compared to control group. $^{\#}p < 0.05$ compared to BIM and BSIM groups.

breastfeeding or gestation appear to inhibit cells with a regulatory phenotype (CD4+CD25+FoxP3+), but favour an IL-10-dependent pathway.

Only offspring generated from infected mothers showed increased expression of HDAC9. According to Tao et al.[18], HDAC9 is linked to decreased generation and performance of regulatory T cells. It has been observed that animals genetically deficient in HDAC9 had increased mRNA expression of Foxp3, CTLA-4, and GITR and increased CD4+FoxP3+ cells in lymphoid tissues. Regarding the cell phenotypes studied here, we observed that the generated offspring had a higher frequency of IL-10 producing CD4+ T cells. Together, these data suggest that immunomodulation in utero induced by maternal infection does not favour the generation of cells with a regulatory phenotype, but does favour IL-10 production via HDAC9 in offspring. In studies using immunisation with ovalbumin as adjuvant, gestation in infected mothers was correlated with increased production of IL-10.[2,5]Furthermore, Li et al.[19] reported the positive regulation of HDAC9 in immature peritoneal macrophages through the action of acetyltransferase Dnmt3, leading to increased production of IFN-α and IFN-β via deacetylation of kinase TBK1. Mice deficient for Dnmt3a showed greater susceptibility to infection by vesicular stomatitis virus (VSV). Thus, it is possible that gestation in schistosomotic mothers could alter the innate immune response against viral infections in offspring.

Prior exposure to the breast milk of schistosomotic mothers positively altered the expression of HDACs from different classes linked to reduction and/or resolution of the inflammatory response.[8,20,21] There was an increase in the relative expression of HDACs 1, 2 and 7, all of which show anti-inflammatory activity by inhibiting the transcription of NF-κβ and decreasing the production of inflammatory cytokines (TNF-α, IL-1, IL-6).[7,22,23] There was a decrease in HDAC3 expression in all experimental groups (at baseline), but this recovered to levels similar to Control in response to mitogen.

HDAC6 and HDAC11 were also increased in animals that only received milk from infected mothers. Wang et al.[24] when using an HDAC6 inhibitor, observed a reduction in HDCA6 recruitment to the IL-10 promoter along with enhanced TNF-α, IL-12p40, and IFN-γ production, as well as increased influx of macrophages, dendritic cells (DCs), and neutrophils to the lungs in response to *Mycobacterium tuberculosis* infection. In fact, Cheng et al.[6] reported that HDAC6 is required for transcriptional activation of IL-10 gene expression in macrophages and dendritic cells through STAT3 activation, while HDAC11 acts as an IL-10 transcriptional repressor. Here, the expression of HDAC6 seemed to overlap with the repressive effect of HDAC11. These data are corroborated by the increased IL-10 production in macrophages (CD14+IL-10+) in the SIM group. It is worth noting that the increase in HDAC6 expression, together with the baseline HDAC5 decrease in breastfed animals, may be related to the decrease in Treg cell activity and markers (FoxP3, CTLA-4 and GITR).[25,26] These data are corroborated by the lower frequency of CD4+CD25+-FoxP3+ cells in animals which were breastfed under mitogenic stimulus. Regarding the production of cytokines by T lymphocytes, decreased production of IL-4 and IFN-γ was observed (although there was no statistical difference for the latter). There was a subtle increase in the frequency of IL-10 producing B lymphocytes under mitogenic stimulation, and the baseline frequency was high in all study groups. These data corroborate the dependence of IL-10 on phagocytic cells in producing the suppressive and epigenetic effects of breast milk from schistosomotic mothers.

The high expression of HDAC10 was related to activation of the IL-1β-mediated NF-κβ signalling pathway in a study with mesenchymal stem cells derived from the synovial membrane in temporo-mandibular joint repair.[27] However, HDAC10 was shown to be important in regulating the production of reactive oxygen species (ROS) in gastric cancer, and its inhibition led to the accumulation of ROS, triggering the intrinsic apoptotic pathway.[28] It is known that ROS acts both in the innate and adaptive immune systems by attracting polymorpho-nuclear leukocytes, monocytes, and macrophages through Toll-like receptors (TLR).[29,30] Here, there were increases in the expression of this enzyme in the group that received only milk (SIM) as well as in those that were born and breastfed (BSIM). This finding may be related to the activity of antioxidant enzymes in breast milk.[31]

Milk from infected mothers also led to increased expression of class III HDACs, Sirt2, and Sirt7. It is known that Sirt2 acts on the anti-inflammatory pathway in M2 macrophages through expression of *Arginase 1 (Arg1)* and *Cd11c*,[21] and reduces ROS by increasing superoxide dismutase 2 (SOD2), catalase, and glutathione peroxidase.[8] The increase in Sirt2 was related to inhibition of the NF-κβ pathway and reduced expression of IL-1β, IL-6, and TNF-α.[21] Sirt7 acts on the stabilisation of TGF-β receptor type 1, allowing for efficient signalling of TGF-β.[32] This targeting to an anti-inflammatory response profile is consistent with the tolerogenic effect of breast milk.[33] According to our data, offspring who were only breastfed by schistosomotic mothers and who had undergone postnatal infection by *S. mansoni* also had elevated levels of TGF-β, in addition to decreased nitric oxide production (Unpublished observations).

High expression of Sirt6 was observed in all experimental groups (born and/or breastfed animals). According to Lee et al.,[20] high expression of Sirt6 acts to inhibit the NF-κβ pathway and blocks the effect of TNF-α. Li et al.[34] showed that overexpression of Sirt6 inhibits RIG-I-like receptor (RLR) and Toll-like receptor 3 (TLR3) in Dengue virus (DENV) infection, and the sirtuin core domain of SIRT6 is required for the inhibition of NF-κB p65 function, negatively regulating DENV-induced inflammatory responses via the RLR and TLR3 signalling pathways. These observations support the inhibition of inflammation we observed in the offspring of schistosomotic mothers.

The high expression of Sirt5 was also observed in the SIM group. It has been shown that Sirt5 is responsible for deacetylating STAT3, disrupting its activity at the mitochondrial level.[35] Qin et al.[36] demonstrated that deficiencyof this enzyme led to a decrease in the

innate inflammatory response, with lowered production of IL-6, TNF-α and, MCP-1 both in the hyper inflammatory and hypo inflammatory stages of sepsis in an animal model. In addition, it was observed that high expression of Sirt5 leads to an increased pro-inflammatory response by decreasing the interaction between Sirt2 and NF-κβp56.[36] Here, there was no decrease in Sirt2, and the increase in Sirt6 and Sirt7 may have overlapped with the effect of Sirt5.

Thus, HDACs were epigenetically altered by maternal infection and were involved in the suppression of pro-inflammatory mediators and increased IL-10 production, but did not favour cells with a regulatory phenotype. Even knowing the importance of cell-cell interactions in both *in vivo* and *in vitro* systems, it would be important to culture purified cells to understand if this pattern of expression of HDACs, indicating a predisposition to IL-10 dependent anti-inflammatory effects, was due to previous programming of T lymphocytes and macrophages in the BIM and SIM groups, respectively.

In conclusion, gestation in infected mice led to increased expression of HDAC9 alone, while breastfeeding from schistosomotic mothers led to increased expression of HDACs of all classes. It is true that the extrapolation of these data from mice to humans should be carefully evaluated, but our findings highlight the importance of experimental and clinical approaches to investigate the efficacy of therapeutic targets in allergy, autoimmunity, and cancer models after long-term epigenetic changes result in offspring from areas endemic for schistosomiasis. Therefore, our results shed light on immunological factors resulting from infection during early age, caused by modifications in epigenetic profile. In view of this, it will be important to evaluate factors in breast milk of infected mothers and how these components may modify the epigenetic and immunological profile of offspring.

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AUTHORS' CONTRIBUTION

GCRH, VMOS and JLLF - Conceived and designed the proposal; GCRH, MCS and FOS - performed lab experiments and processed the data; VMBL, FOS and MCPAA - contributed reagents/materials/analysis/tools; GCRH - writing - original draft; GCRH, VMOS, FOS, VMAC and JLLF - writing, review and editing; JLLF and VMOS - supervision. The authors declare no conflicts of interest.

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ANEXO A - PARECER DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA - IAM/FIOCRUZ)

Ministério da Saúde
FIOCRUZ
Fundação Oswaldo Cruz
Instituto Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o projeto intitulado **"AVALIAÇÃO *IN VITRO* DOS LINFÓCITOS B E FATORES EPIGENÉTICOS, EM CAMUNDONGOS ADULTOS DESCENDENTES DE MÃES ESQUISTOSSOMÓTICAS, EM RESPOSTA A ANTÍGENOS TUMORAIS E PROTÉICOS"** protocolado sob nº **113/2017** pelo (a) pesquisador (a) **Virginia Maria Barros de Lorena** está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Instituto de Pesquisas Aggeu Magalhães / Fundação Oswaldo Cruz (CEUA/IAM) em reunião 22/11/2017. Na presente versão, este projeto está licenciado e tem validade até 01 de dezembro de 2021 com a finalidade de pesquisa científica. Esses animais são advindos do Biotério de criação do Instituto de Pesquisas Aggeu Magalhães/Fiocruz-PE. É responsabilidade do coordenador do projeto notificar à CEUA de quaisquer alterações em relação ao projeto. O coordenador concorda que nenhuma dessas mudanças serão implementadas antes de serem aprovadas pela CEUA/IAM.

Quantitativo de Animais Aprovados	
Espécie/Linhagem/Raça	**Nº de Animais/Peso/Idade/Sexo**
Camundongo heterogênico (Swiss)	70 (25-30gr/ 4 semanas/ machos) e 140 (25-45gr/ 4 semanas/fêmeas)
Camundongo heterogênico (Swiss) - Descendentes dos animais acima citados	448 (peso variados/0-120 dias/machos) e 672 (peso variado/0-120 dias/fêmeas)
Total	**1.332**

Recife (PE, Brasil), 01 de dezembro de 2017

Dr Lindomar José Pena
Coordenador CEUA/IAM

Lindomar José Pena
Coordenador da Comissão de Ética no Uso de Animais - CEUA
Mat. Siape: 1971435
e-mail: Lindomar.pena@cpqam.fiocruz.br
IAM / Fiocruz

Av. Professor Moraes Rego, s/n - Cidade Universitária – Campus da UFPE
Recife - PE - CEP: 50.670-420
Telefone: (81) 2101-2500/2101-2600 Fax: (81) 2453-1911
www.cpqam.fiocruz.br