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Expressão de microRNAs no hipocampo de ratos submetidos a meningite pneumocócica e tratados com vitamina B12

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Dissertação apresentada à Faculdade de Medicina Veterinária de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para obtenção do título de Mestre em Ciência Animal (Medicina Veterinária Preventiva e Produção Animal)

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RESUMO

A meningite se caracteriza pela inflamação da aracnóide, pia-máter e líquor; causando danos às estruturas corticais e subcorticais. As meningites bacterianas estão intimamente relacionadas ao nível de desenvolvimento socioeconômico do país e são consideradas endêmicas no Brasil. Apesar dos esforços para o desenvolvimento de medicamentos e vacinas, a doença ainda possui altos índices de morbidade. Ela ocorre quando bactérias atravessam barreiras de proteção do corpo e alcançam o sistema nervoso central, desencadeando uma resposta imunológica. Sabe-se que durante o curso da doença os níveis de homocisteína do líquor aumentam, promovendo desmielinização e danos neuronais, e que a vitamina B12 é um tratamento utilizado para diminuir danos. Os microRNAs (miRNAs) são instrumentos de resposta fisiológica, tendo sua expressão modificada em diferentes tecidos, em decorrência de diferentes estímulos fisiológicos e patológicos. Estão associados ao controle da expressão de diferentes mediadores inflamatórios e sua ausência é capaz de causar danos severos a resposta imune. Considerando a importância dos miRNAs para regulação de processos imunes o presente estudo visou elucidar os padrões de expressão dos miRNAs durante o processo inflamatório da meningite pneumocócica (MP), bem como observar esses padrões em resposta ao tratamento adjuvante da doença com vitamina B12. Observamos um total de 37 miRNAs diferencialmente expressos, a infecção regulou positivamente 22 deles, e outros 7 negativamente, o tratamento adjuvante com vitamina B12 em animais não-infectados aumentou a expressão de 2, e diminuiu a expressão de 1 miRNA; o tratamento adjuvante com vitamina B12 em animais infectados amentou a expressão de 2 e diminui a de 6 miRNAs. Vias de sinalização, intimamente relacionadas à meningite pneumocócica foram encontradas reguladas, tanto pela MP, quanto pelo tratamento adjuvante com vitamina B12 em animais infectados. O uso da vitamina B12 não regulou nenhuma via em animais não infectados. Pode-se concluir que o presente estudo corrobora com achados recorrentes durante as meningites bacterianas, e serviu de início para a investigação dos efeitos da terapia adjuvante com vitamina B12 durante a MP, entretanto são necessários estudos futuros sobre os miRNAs regulados durante o tratamento adjuvante e seus alvos experimentais.

Palavras-chave: Meningite. MicroRNAs. Vitamina B12. Hipocampo. Epigenômica.

SCARAMELE, N. F. **Hippocampal expression of microRNAs in rats with pneumococcal meningitis treated with vitamin B12.** 2019. 65 f. Dissertação (Mestrado) – Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2019.

ABSTRACT

Meningitis is characterized by an arachnoid, pia mater and cerebrospinal fluid inflammation; causing damage to the cortical and subcortical structures. Bacterial meningitis is closely related to the level of socioeconomic development of country and is considered endemic in Brazil. Despite efforts to develop drugs and vaccines, the disease still has high rates of morbidity and mortality. Occurring when bacteria cross body protection barriers and reach the central nervous system, triggering immune response. It is known that during disease course the the levels of homocysteine increase in cerebrospinal fluid, leading to demyelination and neuronal damage, and that vitamin B12 is a treatment used to reduce those damages. MicroRNAs (miRNAs) are instruments of physiological response, having their expression modified in different tissues, due to different physiological and pathological stimuli. They are associated with expression control of different inflammatory mediators and their absence is capable of causing severe damage to the immune response. Considering the importance of miRNAs in regulation of immune processes, the present study aimed to elucidate miRNA expression patterns during the inflammatory process resulting from pneumococcal meningitis (PM), and to observe these patterns in response to adjuvant treatment of vitamin B12 in infected rats. We observed a total of 37 differentially expressed miRNAs; the infection positively regulated 22 and negatively regulated 7 of them, while the adjuvant treatment with vitamin B12 in non-infected animals increased the expression of 2, and decreased the expression of 1 miRNA; the adjuvant treatment with vitamin B12 in infected animals upregulated 2 and downregulated 6 miRNAs. Signaling pathways, closely related to PM, were found to be regulated by both, PM and adjuvant treatment with vitamin B12 in infected animals. The adjuvant therapy with vitamin B12 did not regulate any pathways in non-infected rats. We can concluded that, the present study corroborates with recurrent findings during bacterial meningitis course, and served as a start for the investigation of vitamin B12 adjuvant therapy effects during PM. However, future studies about the adjuvant treatment regulated miRNAs, and their experimental targets are needed.

Keywords: Meningitis. MicroRNAs. Vitamin B12. Hippocampus. Epigenomics.

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1 INTRODUÇÃO GERAL

A inflamação na aracnóide, na pia-máter e no líquor, é capaz de se disseminar por todo espaço subaracnóideo, caracterizando a meningite, que pode ser resultante tanto de processos não infecciosos, por exemplo, substâncias químicas exógenas, neoplasias e traumas (1); como decorrente de agentes infecciosos: bactérias, vírus, fungos e outros (2,3). Em ambos os casos a inflamação causa danos às estruturas corticais e subcorticais (4), afetando não só as membranas de proteção do sistema nervoso central (SNC), como a barreira hemato-encefálica (BHE), barreira hemato-liquórica (BHL), mas também o hipocampo, córtex e outras estruturas (5).

Haemophilus influenzae, Neisseria meningitidis e *Streptococcus pneumoniae* (pneumococo) são as principais bactérias causadoras de meningite (6). A incidência de cada uma está intimamente relacionada à variação de idade, nível de desenvolvimento socioeconômico do país (6,7), e disponibilidade de medicamentos e vacinas (8). Dados do Ministério da Saúde (2) apontam que 5.848 dos casos de meningite notificados no país correspondem a meningites bacterianas, e desse total 16% são causadas pelo *Streptococcus pneumoniae,* atingindo, sobretudo, crianças menores de um ano.

O pneumococo é uma bactéria gram-positiva e encapsulada geralmente disposta aos pares (9); sua cápsula polissacarídica varia estruturalmente, fazendo com que existam, atualmente, 98 sorotipos desta bactéria (10). Para alguns destes sorotipos estão disponíveis vacinas, a vacina pneumocócica polissacarídica (VPP) 23-valente, que confere prevenção aos sorotipos 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F e 33F e as vacinas pneumocócicas conjugadas (VPG) 10-valente e 13-valente (11). A vacina conjugada 10 valente oferece proteção contra os sorotipos 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F e 23F, enquanto a vacina 13-valente adiciona proteção aos tipos 3, 6A e 19A (12).

A introdução de medicamentos tornou as meningites bacterianas curáveis, contudo os índices de morbidade da doença ainda são altos (6,13–15). Inicialmente o antibiótico de escolha para tratamento das meningites bacterianas era a penicilina, e devido à ocorrência de cepas de pneumococo penicilina-resistente descritas em várias regiões do mundo optou-se pelo uso das cefalosporinas, antibióticos do tipo

β-lactâmicos de terceira geração, sendo o mais comum a ceftriaxona, utilizada tanto para tratamento empirico, quanto como primeira escolha após diagnóstico diferencial para *S. pneumoniae* (16–18).

O pneumococo coloniza a nasofaringe humana, fazendo de lactentes e crianças jovens seu principal reservatório (2). Receptores presentes na nasofaringe tal como GalNAc(β1-3)Gal, GalNAc(β1-4)Gal, e o ácido siálico são alvos para algumas das mais de 500 proteínas de superfície do pneumococo, como a fosforilcolina e as proteínas ligantes de colinas (Cbp), que participam da adesão do patógeno ao epitélio (6,19,20). A fosforilcolina se liga aos receptores do fator ativador de plaquetas (PAF) (21), enquanto a CbpA é capaz de se ligar ao receptor polimérico de imunoglobulina (RPIg) (22) que normalmente reconhece anticorpos do tipo IgA (23), transportando-os através da mucosa; isso facilita a transcitose pelo epitélio que, em conjunto com a presença da hialuronato liase, um dos fatores de virulência do *Streptococcus pneumoniae,* é capaz de catalisar a degradação da matriz extracelular (24), permitindo que o micro-organismo atinja a corrente sanguínea causando assim uma bacteremia.

Uma vez que o sistema imune do hospedeiro reconhece o estado de bacteremia, inicia-se a resposta inata com ativação do complemento. O pneumococo entra na chamada fase de variação, aumentando a espessura de sua cápsula e usando de sua variação antigênica para evitar a fagocitose e inibir a ativação do complemento (21). Os imunocomplexos pneumocócicos se ligam as hemácias, que os transferem para macrófagos. Quando os imunocomplexos são fagocitados, ocorre liberação de pneumolisina, e esta aumenta a virulência do *S. pneumoniae*, pois se liga ao complemento, limitando a fagocitose da bactéria (25).

Para que ocorra infecção do SNC pneumococo precisa ultrapassar dois obstáculos, a BHL e BHE. A BHL localiza-se no plexo coroide, que é revestido por um epitélio ependimal; essa barreira é formada por capilares fenestrados, permissivos a passagem de substâncias específicas (26). Embora o estudo de Quagliarello e colaboradores (27) tenha revelado que durante o curso da doença ocorra o rompimento de junções endoteliais, o mecanismo preciso de entrada do pneumococo no líquor ainda não foi completamente elucidado (6). A BHE possui células endoteliais diferenciadas com presença de junções oclusivas, e um sistema de carreamento específico que visam limitar e regular a troca de substâncias no local (28), sendo fisiologicamente controlada pelos fatores derivados de astrócitos

(6,29). Iovino et al. (30), observaram claramente a adesão do pneumococo ao endotélio cerebral, sugerindo que esta seja a principal rota de alcance do patógeno no SNC, onde a liberação de compostos bacterianos como peptideoglicanos e fragmentos da parede celular, desencadeiam a resposta inata (21), e consequente produção de citocinas e outros mediadores inflamatórios (31).

Estudos demonstraram que na fase aguda da meningite bacteriana os níveis liquóricos de homocisteína em crianças estão elevados (32); sabe-se que a homocisteína, um aminoácido sulfurado, induz a apoptose neuronal (33). A hiper-homocisteinemia é comumente tratada com vitamina B12 (34,35), substância que pertence à família dos compostos de cobalamina e executa diversas funções metabólicas no SNC e periférico, auxiliando no processo de metilação da metionina, impedindo que a homocisteína seja formada (35). Ademais a deficiência desta vitamina é capaz de causar desmielinização, e outros danos neuronais (36).

Mecanismos fisiológicos precisam estar em equilíbrio para que um indivíduo mantenha seu estado de saúde, e o principal centro de regulação destes mecanismos é o nosso DNA, responsável pela codificação de genes que, por sua vez, é intensamente regulada por processos epigenéticos, garantindo assim a regulação temporal e tecidual da expressão gênica. O nível de condensação da cromatina, mecanismos de metilação de DNA e RNAs não-codificadores são exemplos de mecanismos epigenéticos. Tais processos são dinâmicos, podendo agir concomitante ou separadamente, permitindo a rápida repressão ou ativação de um gene específico, de um conjunto de genes ou mesmo de proteínas, e desenvolve papel essencial em processos de modulação imune (37).

Entre os RNAs não-codificadores está a família dos microRNAs (miRNAs); formados por uma pequena sequência endógena de nucleotídeos que na sua fase madura possui cerca de 22 nucleotídeos e tem função reguladora pós-transcricional (38–40). A biogênese dos miRNAs tem inicio no núcleo celular com a transcrição de miRNAs primários (pri-miRNAS) pela RNA polimerase II (41) e III (42), que ainda dentro do núcleo são processados por proteínas como a Drosha e seu cofator DGCR8 (43), originando os pré-miRNA, estruturas de aproximadamente 70 nucleotídeos em forma de haste-alça, ou "hairpin" (44). Os pré-miRNAs são levados do núcleo ao citoplasma pelas exportinas, e então fragmentados por uma proteína de nome Dicer, que fragmenta a parte da alça, originando um RNA complementar em dupla fita, em torno de 20 pares de base, nos mamíferos a Dicer pode ser

auxiliada pelas proteínas Argonautas 2 (AGO2). O miRNA maduro tem origem na separação desse RNA complementar em duas fitas simples (45,46).

Um miRNA maduro precisa se associar a um complexo efetor, conhecido como Complexo de Silenciamento Induzido por RNA (RISC, sigla do inglês "RNA Induced Silencing Complex") (47,48). Quando o RISC está ativo ele é capaz de inutilizar moléculas de RNA mensageiro (mRNA), impedindo que essas sejam traduzidas para proteínas (49). Isso porque o RISC é formado por uma endonuclease da família Argonauta, por enzimas como a Dicer e as helicases, e também por uma proteína TRBP de ligação ao RNA. Sendo assim, ele é capaz não só de se ligar ao mRNA pela extremidade 3' UTR, bloqueando sua tradução, como também pode quebrar a sequência do mRNA complementar ao miRNA presente no complexo (40).

Sabe-se que a expressão de miRNAs ocorre de forma temporal, e é variada em diferentes tecidos (40). O miRNA miR-155, por exemplo, está associado a uma variedade de mediadores inflamatórios (50) e sua ausência é capaz de causar danos severos a resposta imune (51), sugerindo a possibilidade da expressão alterada destas moléculas durante a meningite pneumocócica (MP).

Sabendo da importância dos miRNAs para regulação de processos imunes, e da agressividade e importância da meningite, o presente estudo visa elucidar como os miRNAs se comportam durante o processo inflamatório da meningite pneumocócica, bem como observar possíveis efeitos do tratamento adjuvante da doença com vitamina B12.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

Analisar o microtranscriptoma (expressão global de miRNAs) do hipocampo de ratos com MP, bem como os efeitos do tratamento adjuvante com vitamina B12 nestes animais.

1.2.2 Objetivos Específicos

1) Determinar a expressão dos miRNAs no hipocampo de ratos com MP, tratados ou não com vitamina B12;

2) Predizer quais são os mRNA alvos dos miRNAs diferencialmente expressos;

3) Predizer em quais vias os alvos estão envolvidos, desvendando deste modo os mecanismos de regulação da inflamação e do metabolismo, observando a atuação da vitamina B12 como tratamento adjuvante para esta doença.

2 CAPÍTULO 1 - Patterns of hippocampal miRNome during pneumococcal meningitis infection and adjunctive vitamin B12 therapy

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Abstract

Acute bacterial meningitis occurs when bacteria reach the central nervous system, triggering an exacerbate innate immune response culminating in necrotic lesions in the cortex and apoptosis of progenitor cells and postmitotic neurons of the hippocampus gyrus. One of the main bacteria to cause meningitis is *Streptococcus pneumoniae* (pneumococcus). After bacteremia, pneumococus causes blood-brain barrier (BBB) disruption, reach the cerebral endothelium and infect the cerebrospinal fluid triggering host inflammatory response and hyperhomocysteinemia, usually treated with vitamin B12. Thus, we hypothesized the influence of a post-transcriptional epigenetic mechanism, microRNA expression profile (miRNome), in regulate the host response to pneumococcal meningitis and adjunctive therapy with vitamin B12. Infant rats (11 days) were intracysternally infected with *S. pneumoniae* (~$2x10^6$ c.f.u.) and treated with vitamin B12 (6.25 mg/ kg) 3h and 18 h post infection. After that all rats received antibiotic therapy. Twenty-four hours p.i., the rats were deeply anesthetized, perfused and total RNA extract from left hippocampi was used to perform microarray (miRNA 4.1 Array strips from Affymetrix, Santa Clara, California, EUA) and qRT-PCR (miScript miRNA PCR System from Qiagen-Valencia, CA, USA) analysis. A total of 37 miRNAs were modulated, 22 and 7 were, respectively up and downregulated by infection, 6 were downregulated and 2 were upregulated by adjunctive B12 therapy in infected rats and 3 were modulated by adjunctive therapy in sham group, being 2 positively and 1 negatively regulated. Ingenuity Pathway Analysis software was employed to determine the experimental mRNA targets and pathways enrichment analysis, that unveiled critical canonical pathways strongly related to the pathophisiology of pneumococcal meningitis, during infection. In conclusion, our study has contributed to the understanding of hippocampal miRNome modulation during bacterial meningitis and in response to adjunctive therapy with vitamin B12.

Keywords: meningitis, *Streptococcus pneumoniae*, vitamin B12, neuroprotection, microRNA, miRNome.

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2.1 Introduction

Streptococcus pneumoniae (pneumococcus) is an infectious agent capable of initiating an inflammatory process that can lead to damage to cortical and subcortical structures (1) that characterizaes meningitis (2). Pneumococcus are responsible for more than 45% of bacterial meningitis (BM) cases (3) and, their ability to colonize the human nasopharynx, makes infants and young children their main target (4,5).

Despite the development of vaccines and other drugs, BM morbidity rates are still high (4,6), primarily due to capsule diversity in distinct serotypes (3,7). Survival rates of BM caused by pneumococcus vary between 30 and 35% and, among those, 50% of survivors (8) develop cognitive (9) , sensory-motor and hearing/cochlear deficiencies, impairing their quality of life (10,11). These sequelae are closely related to hippocampal damage (granular cells of the dentate gyrus apoptosis), as demonstrated by Queiroz et al, 2018 (12), since the hippocampus, a cortical structure, is responsible for neuronal plasticity and production of basal dendrites, important synaptic cells (13–15).

Hippocampal damage occurs after pneumococus causes bacteremia, blood-brain barrier (BBB) disruption, reach the cerebral endothelium and infect the cerebrospinal fluid (CSF) (2,16,17) causing central nervous system (CNS) infection, which is characterized by the release of bacterial compounds such as peptideoglycans and cell fragments (18). Released compounds stimulate pattern recognition receptors (PRR), present in immune cells that act as sentinels in the CSF and trigger host inflammatory response. White blood cells are chemo attracted (19,20), and infiltrate the tissue, starting production of inflammatory mediators through MyD88, an essential signal transducer in the interleukin-1 (IL-1) and Toll-like receptor (TLR) signaling pathways, and activating G coupled protein complex (21) , reactive oxygen species (ROS) (22), among other substances (23). In addition, studies have demonstrated an increase in CSF levels of homocysteine, a sulfurized amino acid that can induce neuronal apoptosis (24,25) during the acute fase of BM in children (26).

Increase of homocysteine occurs due to transmethylation, methionine loses an ATP and transforms into S-adenosyl methionine (SAM), which is transmethylated, that is, it releases a methyl group in S-adenosyl homocysteine (SAH); when SAH releases its adenosine, it originates homocysteine, which can be transformed into

glutathione or recycled into methionine (27,28). Hyperhomocysteinemia, is usually treated with vitamin B12 (B12) (29,30), given that B12 acts as a cofactor for recycling homocysteine into methionine, preventing its increase (30).

Availability of methyl groups is essential for the occurrence of the epigenetic processes of DNA and histone methylation (31,32). DNA methylation, along with histone modifications and non-coding RNAs (ncRNA) are responsible for transcriptional and post-transcriptional control of gene expression (reviewed by (33)). Small ncRNAs (~22 nucleotides), called microRNAs (miRNA), are major players in post-transcriptional regulation of gene expression. These miRNAs are able to associate and activate a complex of proteins, called RNA Induced Silencing Complex (RISC) (34) and, by sequence complementarity, disable messenger RNA (mRNA), preventing their translation into proteins (35).

MicroRNAs are involved in regulating a plethora of biological processes, previously reviewed (36,37), such as angiogenesis, cancer, gastrointestinal and cardiac diseases, immune host response against viral (38,39) and bacterial infections (36,37). Interestingly, Wang et al. (40) also reviewed the mutual control between miRNAs and DNA methylation, describing that miRNAs can regulate methyltransferases and other proteins, and can, in turn, be regulated by hyper and hypo methylation. Moreover, each miRNA can target and control hundreds of genes (41,42) in a temporally and tissue-specific manner (43), making them potential diagnostic and therapeutic targets (44).

In a previous study (12), our group investigated the expression of miRNAs in response to adjuvant treatment with resveratrol in rats with meningitis, using the Open Array methodology. To our knowledge, no investigation has been conducted to investigate miRNA expression associated with adjuvant vitamin B12 treatment in the pneumococcal meningitis model, therefore in this present study, we aim to investigate the effects of BM and adjunctive therapy with B12 on global miRNA expression (miRNome) in the hippocampus of infant rats, in a model of pneumococcal meningitis.

2.2 Methods

2.2.1 Animal model and experimental design

All animal procedures were approved by the Ethics Committee of Care and Use of Laboratory Animals (CEUA-FIOCRUZ, protocol LW-22/13).

Experiments were conducted according to an established experimental model of pneumococcal acute meningitis in infant rats (45). At postnatal day 11, Wistar rats (20 ± 2 g) were infected by intracisternal injection of 10 µL saline containing ~2 × 10^6 c.f.u. / mL of *S. pneumoniae* (serotype 3) (46). Rats in the SHAM group were intracisternally injected with 10 µL of sterile saline. Infected (INF) and SHAM-infected (SHAM) infant rats (N = 16) were subdivided; individuals were treated at 3 and 18 hours post-infection with 10 µL of intramuscular B12 (Merck, Naucalpan de Juárez, Mexico; 6.25 mg/kg), or 10 µL of intramuscular saline (placebo), resulting in the following 4 experimental groups: SHAM+placebo, SHAM+B12, INF+placebo and INF+B12. Following 18 h of infection, CSF (10 to 30 µL) was obtained by puncture of the cisterna magna, and 10 µL was cultured to confirm meningitis (12), briefly, after 24 hours of CSF incubation at 37°C, rats with bacterial titters in the CSF ≥ 1 × 10^8 c.f.u./mL were diagnosed positive to pneumococcal acute meningitis, and only animals with titers ~1 × 10^8 c.f.u./mL were included in the study, in order to control for infection intensity. All animals received antibiotic therapy (ceftriaxone at 100 mg/kg) (EMS Sigma Pharma Ltda., São Paulo, Brazil) at 18h p.i. Twenty-four hours p.i., rats were deeply anesthetized by an intraperitoneal injection of Ketamine (80 mg/kg) + Xylazine (10 mg/kg) (Syntec, São Paulo, Brazil). Immediately after, animals were perfused via the left cardiac ventricle with 7.5 mL of RNAse-free ice-cold phosphate buffered saline (PBS). Hippocampi were removed from the left hemispheres. Samples were dissected and stored on RNA later until use.

2.2.2 Total RNA extraction and quantification

Total RNA was obtained from post-treatment hippocampi samples using a miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All samples were stored at -80ºC after quantification by NanoDrop (ND-2000 spectrophotometer, Thermo Scientific, Wilmington, DE, USA). Quality of samples was assessed using capillary electrophoresis (Bioanalyzer 2100 Agilent, Santa Clara, CA, USA), and the minimum of RNA Integrity Number, an indicator for quality, required was 7.

2.2.3 Microarray

Total RNA (250ng/sample) was labelled using the FlashTag™ Biotin HSR RNA Labeling Kit, according to the manufacturer's instructions, including an ELOSA quality control assay. Hybridization to the Affymetrix® miRNA 4.1 Array strips (Affymetrix, Santa Clara, California, EUA) was carried at 48ºC for 20h. Subsequently, strips were processed and scanned using the GeneAtlas® System (Affymetrix). Raw intensity values were background corrected, log2 transformed and then quantile normalized by the software Transcriptome Analysis Console (TAC) 4.0 (Thermo Fisher Scientifc) using the Robust Multi-array Average (RMA) algorithm. Statistical analysis was also performed in the TAC software 4.0 (Affymetrix) by two-way ANOVA (fold-change ± 1.5, $p<0.01$), comparing groups according to their biological relevance. To observe the effect of infection, we compared INF+placebo vs. SHAM+placebo; to observe the effect of adjuvant treatment in non-infected animals the contrast SHAM+B12 vs. SHAM+placebo was performed, and for the analysis of adjuvant treatment effect on infected animals, INF+B12 vs. INF+placebo comparison was applied. Data files were deposited at Gene Expression Omnibus with the access number GSE131262.

2.2.4 Target, pathways and functional enrichment analysis

In a way to indentify target genes for the miRNAs with an altered expression in the hippocampus, following infection and/or adjuvant therapy, we employed the miRNA Target Filter Analysis from the Ingenuity Pathway Analysis (IPA) software (Qiagen). We opted to use conservative filters, allowing only experimentally observed targets to be identified. For the identification of canonical pathways potentially regulated by the differentially expressed miRNAs, the Fisher test –log (p-value)>2was employed in the IPA software.

Further, we used the software miRPath v3.0 (47) to further investigate the effect of adjuvant treatment in infected animals (INF+B12 vs. INF+placebo). Targets of miRNAs annotated in Tarbase 7.0 were used, considering only experimentally validated interactions, and, for pathway analysis, the following parameters were considered: Pathways Union, FDR corrected p-value<0.01; Enrichment Analysis Method chosen was Fisher's Exact Test.

2.2.5 Validation by qRT-PCR

For validation of miRNA expression, miScript miRNA PCR System (Qiagen-Valencia, CA, USA) were employed, according to manufacturer´s instructions. Validated inventoried primers employed were purchased from Qiagen. Stratagene QPCR System Mx3005P (Agilent Technologies, Santa Clara, CA, USA), was used to perform PCR following instructions on the miScript miRNA PCR System´s manual. Expression levels were determined using standard curves for all miRNAs at each individual run; standard curves were constructed based on a pool of the samples, in serial dilution. Expression of each candidate miRNA is demonstrated as a ratio to the endogenous control miRNA SNORD96A.

2.2.6 Statistical analysis

Statistical analysis of miRNA differential expression was performed in the TAC software 4.0 (Affymetrix) by two-way ANOVA (fold change ± 1.5, $p<0.01$). Real time PCR data was analyzed using least-squares analysis of variance and the general linear model procedures of SAS (SAS Institute, Cary, NC, USA). Comparison of means was done using Duncan's multiple range test and p-values<0.05 were considered to be statistically significant.

2.3 Results

2.3.1 Expression profile of miRNAs is modulated during BM and by B12 adjuvant treatment in infected animals

Of the total 1177 miRNAs representing the rat species in Affymetrix® miRNA 4.1 Array strips (Affymetrix, Santa Clara, California, EUA), 37 miRNAs were modulated (fold change ± 1.5; $p<0.01$) (Figure 1) between comparisons of interest, which represents 3.14% of the total array content for the species. Pneumococcal meningitis (PM) (when comparing INF+placebo vs. SHAM+placebo) downregulated 7 (Figure 2A). Further, 6 miRNAs were downregulated, and 2 were upregulated by the interaction between pneumococcal acute BM and adjunctive therapy with B12 (when comparing INF+B12 vs. INF+placebo) (Figure 2B). Three miRNAs were regulated by B12 in the SHAM individuals (when comparing SHAM+B12 vs. SHAM+placebo), two

of them were upregulated and 1 was downregulated in B12 treated animals (Figure 2C).

2.3.2 Target genes and pathways identification

All differentially expressed miRNAs, upregulated and downregulated by pneumococcal acute BM and by adjunctive therapy in sick or healthy animals were analyzed in the IPA software. Using the tool miRNA Target Filter, a total of 240 targets that have been experimentally observed previously in the literature, were identified for the list of regulated miRNAs (Table 1). The list of target genes is available as Online resource 1. In order to indentify relevant pathways in each comparison we proceeded with the Core Analysis tool, within IPA, using significance –log (p-value)>2. Our target gene list generated 151 canonical pathways represented following BM infection (Online resource 2, the top 10 was show in Table 2), 8 canonical pathways were identified following adjuvant treatment with B12 in infected rats (Online resource 3). No canonical pathways were identified in the vitamin B12 treatment in SHAM rats. Notwithstanding, upon further analysis using miRPath 3.0 software, 3 significant ($p<0.01$) signaling pathways were identified for the adjuvant treatment with B12 in infected rats (Table 3).

2.3.3 qRT-PCR Validation

Two differentially expressed miRNAs were validated by real-time PCR, using Agilent´s Stratagene Mx 3005P cycler and commercial primers. Briefly, RNA samples were transformed into cDNA, and from the cDNA samples real-time amplification was performed, and quantified by means of a specific standard curve for each amplicon. Results are presented as relative to the endogenous SNORD96A control (Figure 3). Strong correlation coefficients (Pearson test), 0.95 for miR-223-3p and 0.96 for miR-155-5p, were observed between methods.

2.4 Discussion

Functional enrichment analysis unveiled critical canonical pathways strongly related to the pathophisiology of pneumococcal meningitis (PM), that can be regulated by changes in the hippocampal miRNome during the acute phase of the disease.

We have found up (miR-155-5p, miR-146a-5p, miR-451a, miR-223-3p, miR-200b-3p and miR-193a-5p) and downregulated (miR-328-3p) miRNAs that have already been described as regulators of several inflammatory molecules. For instance, Interleukin-1 Receptor (IL-1R) is a cytokine receptor gene cluster comprised by eight receptors, containing an immunoglobulin domain (48), and four of those molecules (IL1R1, IL1RAP, IL1RAPL2, IL1RL2) were identified as targets of miR-146a-5p, upregulated by infection in our experiment. IL1RL2 acts as a negative regulator of inflammation, precluding the IL-1 interaction with IL-1R (48), which would suggest increased binding of IL-1 to IL-1R1, in our infected animals. Yet the receptor reduction could be detrimental once this receptor deficiency decreased survival in mice (49). It's know that the levels of cytokines and others mediators are elevated during bacterial meningitis (50). Our results have shown that, not only interleukine-1-receptors (ILR-1), but also interleukin-1 receptor-associated kinase 1/2 (IRAK1/2) are downstream molecules, targets of an infection associated overexpressed miRNA (miR-146a-5p). This same miRNA targets interleukin 36 receptor antagonist (IL-36RN), an activation inhibitor of NF-kB, and TNF receptor associated factor 6 (TRAF6), an enzyme responsible for mediation of signal transduction in response to inflammatory cytokines (51,52). These two molecules are essential for the IL-1 Signaling Pathway (53). Taken together, these results suggest that the IL-1 Pathway is indirectly impaired in BM animals, following overexpression of miR-146a-5p.

In our study, TLR4 and TLR9 are candidate targets for downregulation by miR-146a-5p, which is overexpressed during infection, the same way as MYD88 is a target of miR-155-5p, also upregulated following infection. Toll-like receptors (TLR) are a type of pattern-recognition receptors, that are responsible to recognize microbial compounds and initiate the inflammatory cascade (54). TLR2, TLR4 and TLR9 are responsible for the recognition of lipoproteins, pneumolysin and DNA, respectively (18,55), and evidences show that the loss of these receptors lead to pathogen survival, through downregulation of their downstream member MYD88 and consequent cytokine production levels (55). In light of the literature cited above, our results could suggest an attempt to reduce cytokine output and, consequently, hamper an appropriate immune response.

In line with the complexity of immune responses, and considering the duality of host;parasite communication, our results also point to an attempt to rescue cytokine production, given that miR-155-5p targets the mRNA for suppressor of cytokine

signaling 1 (SOCS1), a protein capable of directly binding tyrosine-kinase activity of Janus kinase (JAK) family to prevent activation of the STAT family. Interestingly, STAT1 is a target to miR-146a-5p, upregulated by infection in our study and has characteristic functions in immune response assisting T cell differentiation, controlling chemokine production (56) and microglial and macrophage activation (57,58), and is also related with cell death and apoptosis (21,58). Further, possible decreased levels of SOCS, could be involved in destabilizing the interendothelial cell junctions in BBB (59). Notwithstanding, host organism is capable to recognize the pathogen and their released products, such as LPS. A example for this recognition are C-reactive protein (CRP), target of upregulated miR-146a-5p, a protein with capacity to bind to the surface of pathogens, leading to complement system activation, and also binds to receptors as macrophages and dendritic cells (60–62). Also, CRP, is potentially reduced by upregulation of miR-146a-5p during infection, and is a phosphorylcholine (Pcho) ligand which, under normal conditions, has the capacity to decrease Pch binding with PAF receptors, decreasing vacuole internalization of bacteria (63). Thus, in our experiment, Pcho would be free to bind PAF, facilitating bacterial passage from bloodstream to brain, suggesting that this decrease may play a role in disease progression. Other example is LPS binding protein (LBP) primarily known as responsive to gram-negative bacteria, was later shown to be essential for immune response to pneumococci (gram-positive bacteria) (64), LPS is a target of upregulated miR-146a-5p, and in LBP knockout mice, leukocyte levels in CSF remain decreased throughout the acute phase of PM (65), which contributes to a decrease in inflammation. However, the control of inflammatory signs can be considered beneficial only to a certain extent (65), since low concentrations of leukocytes and cytokines are generally synonymous with an increase of circulating bacteria and, consequently, poor disease outcome.

In the Neuroinflammation Signaling Pathway, downregulation of miR-328-3p, is capable of positively regulating beta-secretase 1 (BACE1), a peptidase involved with neuronal migration (66). When BACE1 isn't present, neuroblast maturation and migration to dentate gyrus are impaired (66), besides BACE1 controls proliferation and differentiation of precursor neurons (67). As demonstrated by Queiroz et al. (12), the apoptotic scores are high in rats with PM, thus downregulation of miR-328-3p, as an attempt to increased BACE1 and consequently neurogenesis, seems inefficient to reestablish granular layer population cells. In this same pathway, Colony

Stimulating Factor 1 Receptor (CSF1R) seems to be affected by the overexpression of miR-155-5p, it's known that in normal CNS conditions, this receptor is only expressed in microgial cells and is essential for normal brain anatomy (68). CSFR1 inhibition decreases microglia proliferation (69) and, given that microgia cells are the specialized macrophages of the CNS (50,70), this could suggest a potential influence in immune response deficiency during PM.

MiR-155-5p regulates, among others, the CCAAT/enhancer binding protein beta (CEBPβ), also known as NF-IL6 (71), a transcription factor found in a variety of tissues and in immune granulocytic cells and monocytes (72), wich is able to act in a plethora of differentiated cell types, including neuronal cells (72) and other cells associated with inflammation (73,74). It is also capable of mediating PTG activation. PTG is a protein responsible for regulating glycogen concentrations (75), which is the primary energy reserve in the brain (76). Glycogen and glucose levels are affected during hypoxia (77,78), hypoxia is extensively related to excess nitric oxide and reactive oxygen species (79), but is also related to an increase of intracellular pathogens (80). During PM, nitric oxide (NO) and reactive oxygen species (ROS) seems to be elevated in response to LPS (81), and this increase is capable of causing DNA damage (82), this stimuli could be a trigger for the regulation of transcriptional related pathways, p53 Signaling Pathway, p38 MAPK Signaling and NF-κB Signaling that are affected during PM in our experiment, a reasonable amount of molecules in those pathways are involved with cell apoptosis. TNF receptor superfamily member 10a (TNFRSF10A), tumor protein p53 inducible nuclear protein 1 (TP53INP1), phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1) and Fas associated via death domain (FADD) have been previously reported to be proapoptotic poteins (83–86), and are candidate targets for downregulation by the upregulated miR-155-5p during infection, suggesting a likely host response to an infection-induced apoptotic process.

During PM, injury in BBB starts with pathogen LPS release (87), which can stimulate a target of miR-146a-5p (upregulated after infection): CXCL8, a vasogenic substance, that acts by increasing paracellular permeability (88), contributing to local pleocytosis (20,89). Other important component of BBB are the transporters, specially ABCB1, also known as P-glycoprotein, responsible for efflux transport mechanisms, mainly of lipophilic substances (90). In infected rats, we observed an upregulation of miR-451a, pointing to a decrease of its target, ABCB1. This gene

plays a role in the elimination of xenobiotics (91), and its reduction could lead to CNS accumulation of cytokines and other substances, such as glutamate, which can cause neuronal cell death during bacterial meningitis (12,92). Curiously, miR-451a is downregulated when infected rats are treated with B12 adjunctive therapy, suggesting a potential reestablishment of ABCB1 molecules, aiming at a consequent cytokine and xenobiotics homeostasis in the CNS.

The adjunctive vitamin B12 therapy upregulated miR-764-3p and miR-3552, and downregulated miR-18a-3p, miR-92a-2-5p, miR-21-3p, miR-147, miR-451-5p and mir-1956. From those, three (miR-18a-3p, miR-92a-2-5p and miR-451-5p) downregulated miRNAs had experimentally observed targets: ABCB1, AKTIP, AR, FBXO33, KRAS, MIF, according to the IPA Target Filter tool. Four of them (ABCB1, AR, KRAS, MIF) are involved in significant pathways (Fisher test -log (p-value)>2). The ABCB1, as previously mentioned, is a target for miR-451-5p. This miRNA is regulated in opposite directions when we compare the influence of the infection and the effect of adjuvant therapy on infected animals, thus suggesting a reestablishment of miRNA expression with the use of vitamin B12 as adjuvant therapy. The ABCB1 gene, along with the KRAS gene (target of downregulated miR-18a-3p) are present in the IL-6 signaling pathway, the IL-6 is a proinflammatory cytokine, which can be associated with neuroprotection and with growth and neuronal differentiation (93), whilst KRAS, an oncogenic GTPase, under normal conditions controls cell growth, proliferation and motility (94–96), and it has also been demonstrated to be expressed during meningococcal meningitis (97).

Using the miRPath 3.0 tool, we observed that the downregulation of miR-451a and miR-18a-3p, in response to adjunctive therapy, could positively regulate molecules of Parkinson Disease Pathway (hsa05012), especially the ubiquitin (UB) in the ubiquitin pathway in dopaminergic neurons. UB is used by ubiquitin proteassome system (UPS) to mark and degrade abnormal proteins (98), failures in this system have already been related to *in vitro* apoptosis of dopaminergic cells (99) and accumulation of cytotoxic molecules in Parkinson's disease (100), demonstrating its importance in biomolecule control and repair. The association between our vitamin B12 treatment and a possible positive regulation on UB expression indicates positive effects of B12 treatment during PM, which yields further investigation on its potential to decrease apoptosis events.

In addition, negative regulation of miR-92a-2-5p during adjuvant treatment using vitamin B12 in infected rats, suggests an increase in collagen type I α1 (COL1A1), collagen type V α1 (COL5A), fibronectin I (FN1), and integrin α5 (ITGA5) expression. COL5A1, generally less abundant, and COL1A1, the most abundant in all tissue types (101), are fibrillar collagens, which interact by, forming heterotypic fibers (102); a greater pesence of collagen type 5, event observed during the tissue regeneration process (103), lead to less rigid fibers. Type I collagen is also widely associated with fibronectin, and together they are able to stabilize tensile forces and increase cell adhesion (104,105). Integrins are important receptors for adhesion between cells and extracellular matrix proteins (106), ITGA5 specifically, is involved with vascular and neural development, as reviewed by Hynes (106), as ITGA5 knockout causes vascularization defects and apoptosis in the neural crest. Thus, our experiment corroborates the results previously found by Queiroz et al (12), where rats with pneumococcal meningitis receiveing resveratrol as an adjuvant treatment, also had a potential increase of focal adhesion molecules in ECM-Receptor Interaction Pathway, which may contribute to BBB integrity.

Taken together, our data suggests that pneumococcal-regulated miRNAs act in an attempt to control the exacerbated inflammation characteristic of BM. Further, regulation of miR-451a and miR-147 in an opposite direction, compared to PM, suggests possible effects of adjuvant therapy with B12 on the regulation of miRNAs during PM infection, warranting further investigation on the use B12 as an adjuvant treatment.

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

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Figures List

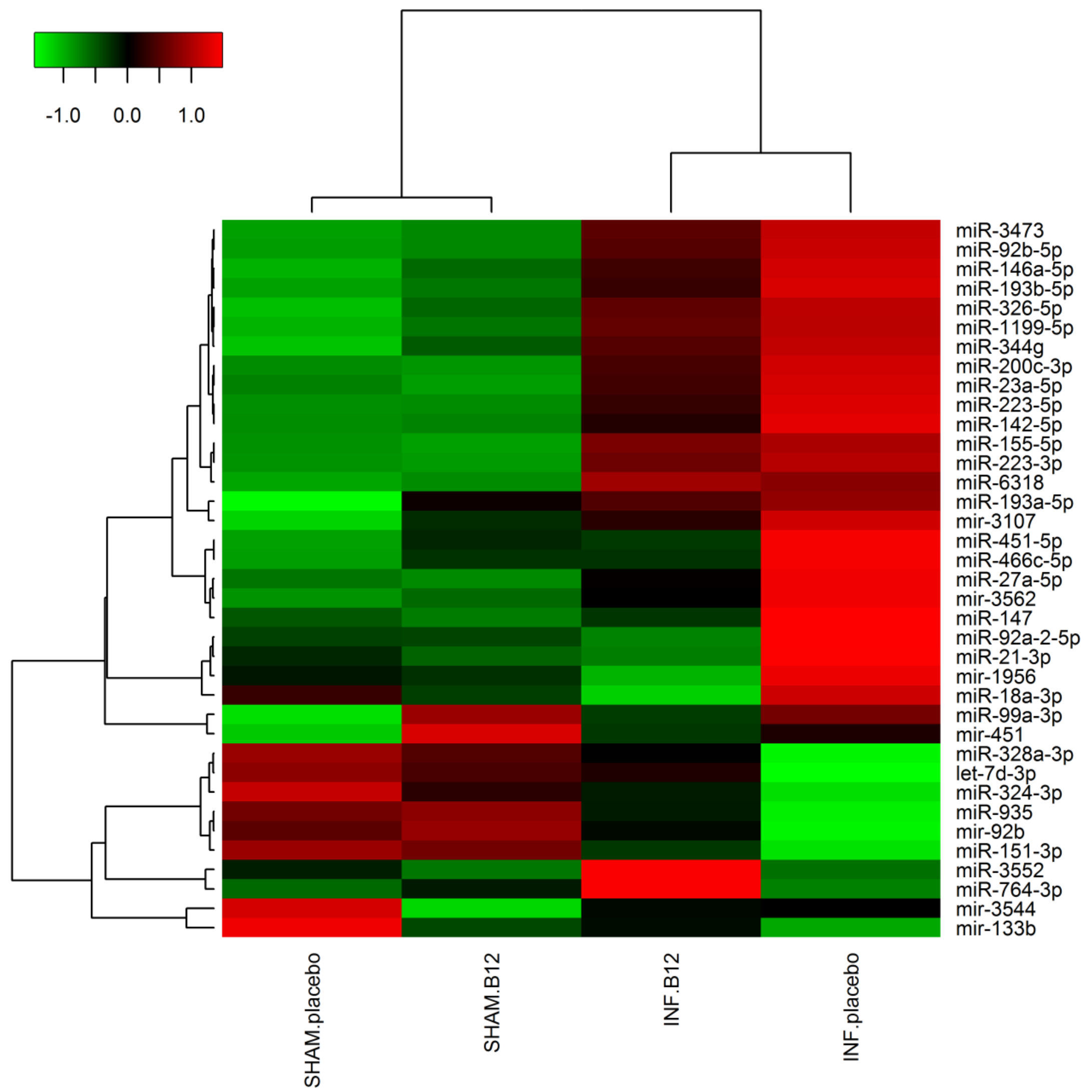

Fig.1 Heatmap of miRNAs modulated by infection, and/or adjuvant treatment in SHAM and infected rats. MiRNAs were grouped by hierarchical clustering based on the correlations between their expression profiles. Heatmap shows the average signal for 37 differentially expressed miRNAs (FC±1.5; *P*<0.01). This analysis was performed using RStudio.

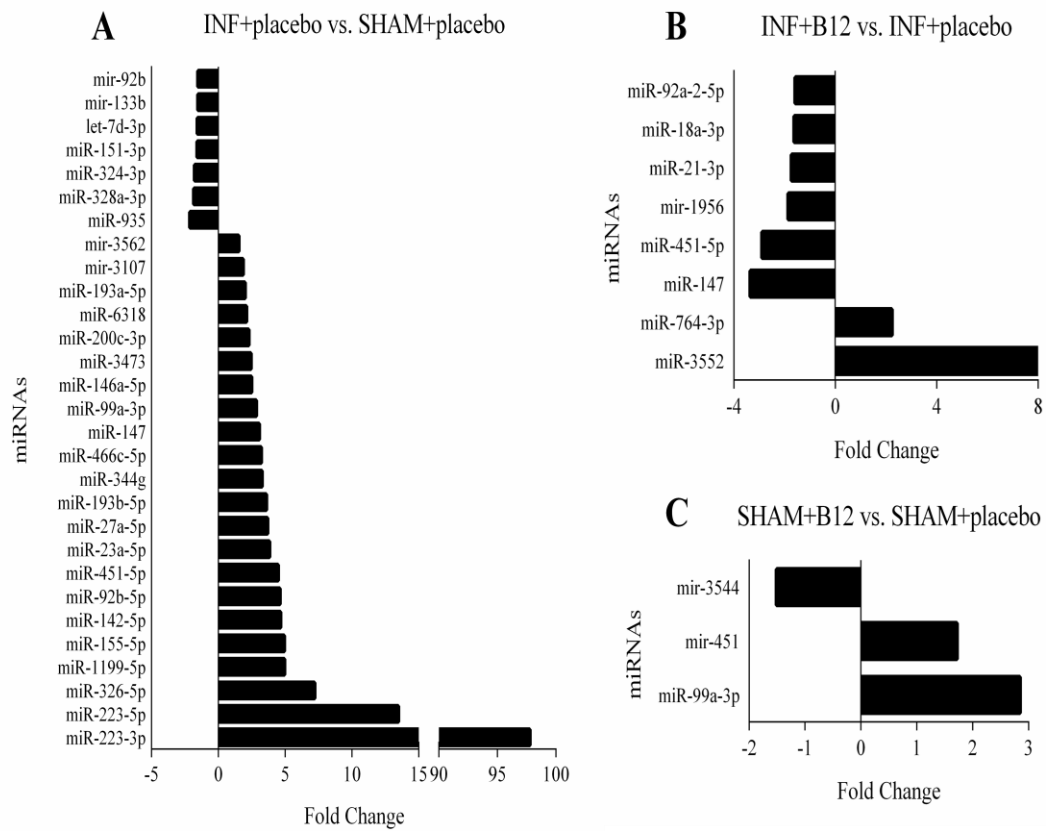

Fig.2 Fold Change of miRNAs modulated by infection, and/or adjuvant treatment in SHAM and infected rats. Columns represent the number of up regulated and down regulated miRNAs (FC±1.5; p-value<0.01) in the comparisons of interest: A) INF+placebo vs. SHAM+placebo; B) INF+B12 vs. INF+placebo and C) SHAM+B12 vs. SHAM+placebo.

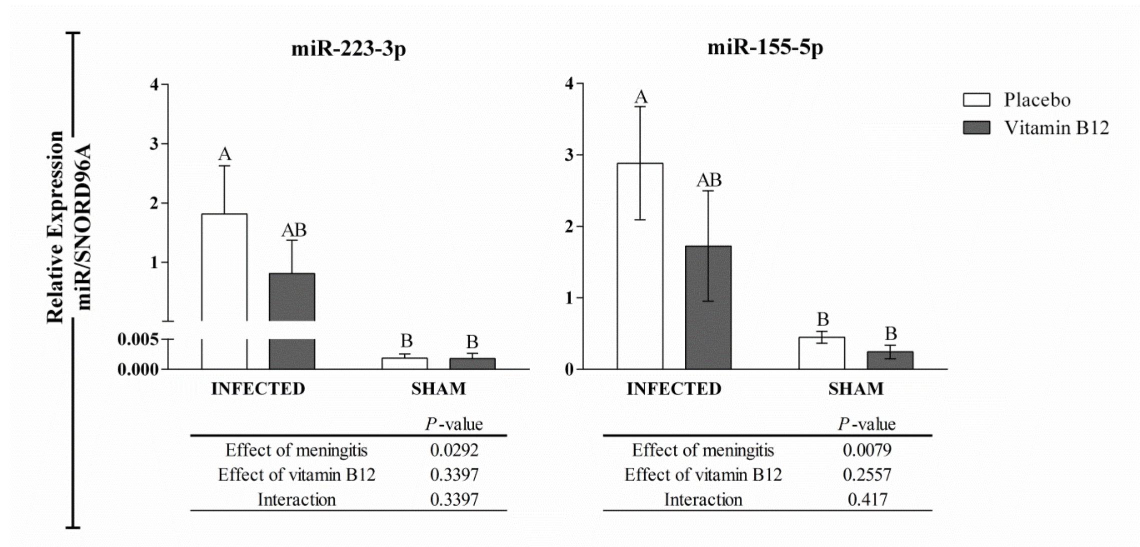

Fig.3 qRT-PCR of miRNA expression levels in hippocampi of SHAM and infected rats, treated or no with vitamin B12. A) Relative expression of miR-223-3 and Relative expression of miR-155-5p. Data is presented as mean±SEM. Different superscript letters differ significantly (p<0.05).

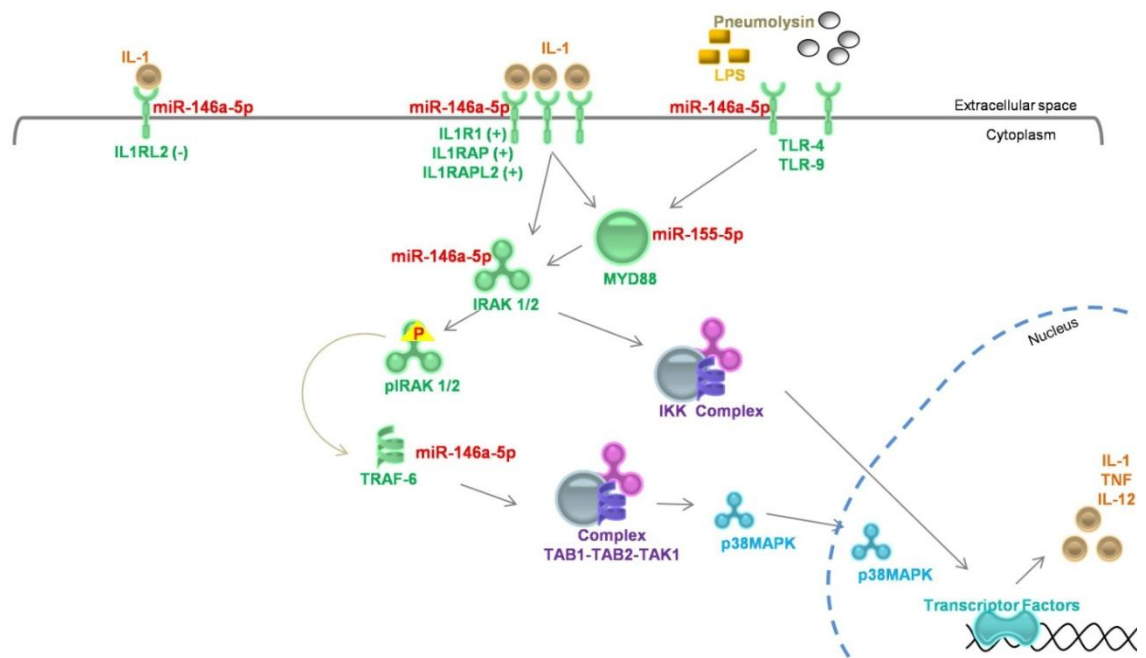

Fig.4 PM upregulates miRNAs targeting components of IL-1 and TLR signaling pathway. Upregulation of miR-146a-5p and miR-155-5p could reduce target expression, and indirectly lead to a decrase in proinflammatory cytokines levels.

Tables List

Table 1: Number of miRNA experimentally observed targets to the differentially expressed miRNAs.

miRNA	SHAM+B12 x SHAM+placebo		INF+placebo x SHAM+placebo		INF+B12 x INF+placebo		Number of Targets
	Fold Change	*p*-value	Fold Change	*p*-value	Fold Change	*p*-value	
rno-miR-935	-1.09	0.7846	**-2.13**	**0.0041**	1.81	0.0599	
rno-miR-328a-3p	1.01	0.5595	**-1.83**	**0.0036**	1.75	0.0527	3
rno-miR-324-3p	-1.17	0.2689	**-1.77**	**0.0073**	1.47	0.1689	
rno-miR-151-3p	-1.12	0.7199	**-1.58**	**0.0043**	1.31	0.1533	
rno-let-7d-3p	-1.01	0.5353	**-1.57**	**0.0026**	1.49	0.0203	
rno-mir-133b	-1.23	0.0234	**-1.53**	**0.0048**	1.11	0.2182	
rno-mir-92b	1.08	0.5796	**-1.52**	**0.0065**	1.21	0.0405	
rno-mir-3544	**-1.51**	**0.0029**	-1.24	0.0981	1.04	0.9462	
rno-miR-764-3p	1.03	0.5206	-1.24	0.8463	**2.24**	**0.0073**	
rno-miR-3552	-1.01	0.4046	1.04	0.4477	**8.15**	**0.0039**	
rno-miR-18a-3p	-1.2	0.2909	1.23	0.1714	**-1.62**	**0.0015**	1
rno-mir-451	**1.72**	**0.0037**	1.44	0.0791	-1.21	0.5109	
rno-miR-92a-2-5p	1.04	0.9914	1.44	0.0216	**-1.58**	**0.0073**	1
rno-miR-21-3p	-1.05	0.596	1.5	0.0233	**-1.73**	**0.0049**	
mmu-mir-1956	-1.06	0.8001	1.53	0.023	**-1.86**	**0.0009**	
rno-mir-3562	1.11	0.7495	**1.54**	**0.0061**	-1.41	0.0662	
mmu-mir-3107	1.36	0.1959	**1.86**	**0.0041**	-1.27	0.1975	
mmu-miR-193a-5p	1.6	0.0363	**2.01**	**0.0032**	-1.07	0.5353	3
rno-miR-6318	1.04	0.7884	**2.11**	**0.0054**	1.08	0.81	
rno-miR-200c-3p	-1.13	0.863	**2.30**	**7.80E-06**	-1.39	0.0184	21
rno-miR-3473	-1.06	0.8003	**2.46**	**0.0013**	-1.30	0.2621	
rno-miR-146a-5p	1.14	0.324	**2.50**	**7.21E-05**	-1.60	0.059	77
rno-miR-99a-3p	**2.85**	**0.0012**	**2.86**	**0.0028**	-1.64	0.0859	
rno-miR-147	-1.17	0.6602	**3.08**	**0.0009**	**-3.35**	**0.0022**	
rno-miR-466c-5p	-1.08	0.4275	**3.23**	**0.0078**	-2.09	0.0403	
rno-miR-344g	1.55	0.4221	**3.28**	**0.0079**	-1.54	0.4007	
hsa-miR-193b-5p	1.04	0.6657	**3.61**	**0.001**	-2.17	0.1055	
rno-miR-27a-5p	-1.14	0.8412	**3.69**	**0.0032**	-2.31	0.036	
rno-miR-23a-5p	1.09	0.7724	**3.84**	**0.0035**	-1.88	0.1523	
rno-miR-451-5p	1.59	0.2446	**4.49**	**0.0012**	**-2.89**	**0.0099**	4
rno-miR-92b-5p	-1.20	0.8542	**4.62**	**0.0041**	-2.20	0.287	
rno-miR-142-5p	1.01	0.9332	**4.67**	**0.0085**	-2.3	0.1353	
rno-miR-155-5p	-1.19	0.7469	**4.95**	**3.91E-06**	-1.55	0.2906	124
rno-miR-1199-5p	1.58	0.5515	**4.96**	**0.0027**	-1.10	0.4049	
rno-miR-326-5p	1.61	0.35	**7.22**	**0.0008**	-1.16	0.316	1
rno-miR-223-5p	1.01	0.9637	**13.49**	**0.0002**	-6.54	0.0391	1
rno-miR-223-3p	-1.16	0.849	**97.79**	**2.49E-06**	-3.10	0.1219	9

Table 2: Top 10 canonical pathways, and respectively targets, regulated by pneumococcal meningites, when compare INF+palcebo vs. SHAM+placebo.

Ingenuity Canonical Pathways	-log(p-value)	Regulated Molecules in Pathway
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	23.60	SOCS1,LTB,IL1F10,CCND1,IRAK1,TLR10, IL36G,IL1RL2,TLR1,CHUK,CTNNB1,NOS2, IL1RAP,CXCL8,MIF,IL1RAPL2,IL10,MYD88, IL36A,PLCG1,IKBKE,CEBPB,IL1R1,IL37, TLR9,IL36B,TRAF6,TLR4,PRKCI,RIPK1, IRS1,RHOA,IL36RN,TCF7L2,IRAK2
IL-6 Signaling	17.50	SOCS1,CXCL8,ABCB1,IL1RAPL2,IL36A, IKBKE,CEBPB,IL1R1,IL37,IL1F10,TLR9, IL36B,TRAF6,IL36G,IL1RL2,IRS1,CRP, IL36RN,CHUK,LBP,IL1RAP
Toll-like Receptor Signaling	17.10	MYD88,TAB2,IL36A,IL37,IL1F10,TLR9, IL36B,IRAK1,TRAF6,TLR4,IL36G,TLR10, TLR1,IL36RN,CHUK,LBP,IRAK2
Hepatic Cholestasis	16.00	CXCL8,ABCB1,IL1RAPL2,MYD88,IL36A, IKBKE,IL1R1,IL37,IL1F10,IL36B,IRAK1, TRAF6,TLR4,IL36G,PRKCI,IL1RL2,IL36RN, CHUK,LBP,IL1RAP,IRAK2
Dendritic Cell Maturation	15.30	IL10,MYD88,IFNB1,IL36A,LTB,PLCG1, IKBKE,IL37,IL1F10,TLR9,IL36B,TRAF6, TLR4,CD1D,IL36G,CD40,IL1RL2,IRS1, IL36RN,CHUK,STAT1,IFNA1/IFNA13
IL-10 Signaling	14.90	IL1RAPL2,IL10,IL36A,IKBKE,IL1R1,IL37, IL1F10,IL36B,TRAF6,IL36G,IL1RL2,IL36RN, CHUK,LBP,IL1RAP
Neuroinflammation Signaling Pathway	13.80	CXCL8,IL10,MYD88,IFNB1,PLCG1,BACE1, IKBKE,IL1R1,TLR9,CSF1R,IRAK1,TRAF6, TLR4,TLR10,RIPK1,CD40,IRS1,TLR1,CRP, CHUK,STAT1,CTNNB1,NOS2, IFNA1/IFNA13,IRAK2
p38 MAPK Signaling	13.70	IL1RAPL2,TAB2,IL36A,IL1R1,IL37,IL1F10, IL36B,IRAK1,TRAF6,FADD,IL36G,IL1RL2, IL36RN,MEF2C,STAT1,IL1RAP,IRAK2
NF-κB Signaling	13.50	MYD88,TAB2,IL36A,IL1R1,IL37,IL1F10, TLR9,IL36B,IRAK1,TRAF6,FADD,TLR4, TLR10,IL36G,RIPK1,CD40,IRS1,TLR1, IL36RN,CHUK
Acute Phase Response Signaling	12.90	SOCS1,MYD88,IL36A,IKBKE,CEBPB,IL1R1, IL37,IL1F10,F2,IL36B,IRAK1,TRAF6,IL36G, RIPK1,CRP,IL36RN,CHUK,LBP,IL1RAP

Table 3: Significant signaling pathways were identified for the adjuvant treatment with B12 in infected rats, when miRPath 3.0 was employed.

KEGG pathway	p-value	#genes	#miRNAs
ECM-receptor interaction	1.19E-14	4	1
Parkinson's disease	4.76E-06	6	2
Fatty acid elongation	2.89E-04	2	1

Online Resource

Online resource 1

ID	miRNA	Number of Targets	Targets
20500950	rno-miR-328a-3p	3	ABCG2, BACE1, CD44
20501345	rno-miR-18a-3p	1	KRAS
20501406	rno-miR-92a-2-5p	1	AR
20500387	mmu-miR-193a-5p	3	Ccl6, IL10, IL2RG
20501519	rno-miR-200c-3p	21	BAP1, ELMO2, ERBIN, ERRFI1, FHOD1, FOXF2, GEMIN2, KLHL20, MARCKS, PLCG1, PPM1F, PTEN, PTPN12, PTPN13, PTPRD, RERE, WASF3, WDR37, ZEB1, ZEB2, ZFPM2
20501476	rno-miR-146a-5p	77	ATOH8, BLMH, BRCA1, C8A, CAMP, CCL8, CCNA2, CCR3, CD1D, CD40, CDKN3, CFH, CHUK, COL13A1, CRP, CXCL8, CXCR4, DMBT1, FADD, IFNA1/IFNA13, IFNB1, IL10, IL12RB2, IL1F10, IL1R1, IL1RAP, IL1RAPL2, IL1RL2, IL36A, IL36B, IL36G, IL36RN, IL37, IRAK1, IRAK2, IRF5, KIF22, LALBA, LBP, LTB, LTF, MCM10, MCPH1, METTL7A, MMP16, MR1, NFIX, NLGN1, NOS2, NOVA1, PA2G4, PBLD, PDIK1L, PEX11G, PGLYRP1, PGLYRP2, PLEKHA4, POLE2, PRR15, PTAFR, PTGES2, RAD54L, S100A12, SDCBP2, SFTPD, STAT1, SYT1, TIMELESS, TLR1, TLR10, TLR4, TLR9, TMSB15A, TRAF6, TRIM14, UHRF1, VWCE
20502448	rno-miR-451-5p	4	ABCB1, AKTIP, FBXO33, MIF
20529126	rno-miR-155-5p	124	ABHD16A, AGTR1, AICDA, AMIGO2, ANKFY1, ARFIP1, ARFIP2, ARID2, ARL10, ARL5B, ATG3, ATP6V1C1, BACH1, BET1, BRPF3, CBFB, CCND1, CD47, CDK5RAP3, CEBPB, CHAF1A, CLDN1, CSF,1R, CTLA4, CTNNB1, CUL4B, CUX1, CYP51A1, CYR61, DCAF7, DHX40, DNAJB1, DNAJC19, DPP7, DSG2, ETS1, F2, FADD, FADS1, FAR1, FGF7, FMNL2, GNA13, HSD17B12, HSDL1, IKBKE, IL13RA1, INPP5D, JARID2, LCLAT1, LDOC1, LPL, LY6K, MAF, MARC1, MATR3, MEIS1, MET, METTL7A, MOSPD2, MPZL1, MSI2, MYB, MYD88, MYO10, MYO1E, NARS,NT5E, PDE3A, PDLIM5, PHC2, PICALM, PKN2, PLXND1, PMAIP1, PODXL, POLE3, POLE4, PPL, PPP5C, PRAF2, PRKCI, PTPRJ, RAB23, RAB27B, RAB34, RAB5C, RAB6A, RAI14, RCN2, RCOR1, RHEB, RHOA, RIPK1, SATB1, SCAMP1, SDCBP, SH3BP4, SLA, SLC30A1, SMAD1, SMAD2, SNAP29, SOCS1, SPI1, SYNE2, SYPL1, TAB2, TACSTD2, TBCA, TCF7L2, TM6SF1,TNFRSF10A, TP53INP1, TRAM1, TRIM32, TRIP13, TXNDC12, TXNRD1, UBE2J1, UFL1, VAMP3, WDFY1, WEE1
20500944	rno-miR-326-5p	1	AR
20501556	rno-miR-223-5p	1	AR
20501557	rno-miR-223-3p	9	CNTN4, IRS1, LMO2, MEF2C, NFIA, RHOB, SCN3A, STMN1, VIM

Online resource 2

Ingenuity Canonical Pathways	-log(p-value)	Regulated Molecules in Pathway
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	23.60	SOCS1,LTB,IL1F10,CCND1,IRAK1,TLR10,IL36G, IL1RL2,TLR1,CHUK,CTNNB1,NOS2,IL1RAP,CXCL8, MIF,IL1RAPL2,IL10,MYD88,IL36A,PLCG1,IKBKE, CEBPB,IL1R1,IL37,TLR9,IL36B,TRAF6,TLR4,PRKCI, RIPK1,IRS1,RHOA,IL36RN,TCF7L2,IRAK2
IL-6 Signaling	17.50	SOCS1,CXCL8,ABCB1,IL1RAPL2,IL36A,IKBKE,CEBP, IL1R1,IL37,IL1F10,TLR9,IL36B,TRAF6,IL36G,IL1RL2, IRS1,CRP,IL36RN,CHUK,LBP,IL1RAP
Toll-like Receptor Signaling	17.10	MYD88,TAB2,IL36A,IL37,IL1F10,TLR9,IL36B,IRAK1, TRAF6,TLR4,IL36G,TLR10,TLR1,IL36RN,CHUK,LBP, IRAK2
Hepatic Cholestasis	16.00	CXCL8,ABCB1,IL1RAPL2,MYD88,IL36A,IKBKE,IL1R1, IL37,IL1F10,IL36B,IRAK1,TRAF6,TLR4,IL36G,PRKCI, IL1RL2, IL36RN,CHUK,LBP,IL1RAP,IRAK2
Dendritic Cell Maturation	15.30	IL10,MYD88,IFNB1,IL36A,LTB,PLCG1,IKBKE,IL37, IL1F10,TLR9,IL36B,TRAF6,TLR4,CD1D,IL36G,CD40, IL1RL2,IRS1,IL36RN,CHUK,STAT1,IFNA1/IFNA13
IL-10 Signaling	14.90	IL1RAPL2,IL10,IL36A,IKBKE,IL1R1,IL37,IL1F10,IL36B, TRAF6,IL36G,IL1RL2,IL36RN,CHUK,LBP,IL1RAP
Neuroinflammation Signaling Pathway	13.80	CXCL8,IL10,MYD88,IFNB1,PLCG1,BACE1,IKBKE, IL1R1,TLR9,CSF1R,IRAK1,TRAF6,TLR4,TLR10,RIPK1, CD40,IRS1,TLR1,CRP,CHUK,STAT1,CTNNB1,NOS2, IFNA1/IFNA13,IRAK2
p38 MAPK Signaling	13.70	IL1RAPL2,TAB2,IL36A,IL1R1,IL37,IL1F10,IL36B, IRAK1,TRAF6,FADD,IL36G,IL1RL2,IL36RN,MEF2C, STAT1,IL1RAP,IRAK2
NF-κB Signaling	13.50	MYD88,TAB2,IL36A,IL1R1,IL37,IL1F10,TLR9,IL36B, IRAK1,TRAF6,FADD,TLR4,TLR10,IL36G,RIPK1,CD40, IRS1,TLR1,IL36RN,CHUK
Acute Phase Response Signaling	12.90	SOCS1,MYD88,IL36A,IKBKE,CEBPB,IL1R1,IL37, IL1F10,F2,IL36B,IRAK1,TRAF6,IL36G,RIPK1,CRP, IL36RN,CHUK,LBP,IL1RAP
Communication between Innate and Adaptive Immune Cells	12.70	CXCL8,IL10,IFNB1,IL36A,IL1F10,IL37,TLR9,IL36B, TLR4,IL36G,TLR10,CD40,IL36RN,TLR1,IFNA1/IFNA13
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	12.60	IL1RAPL2,IL10,TAB2,IL36A,IKBKE,IL1R1,IL37,IL1F10, TLR9,CSF1R,IL36B,TRAF6,IL36G,IL1RL2,IRS1, IL36RN,CHUK,CTNNB1,SMAD1,IL1RAP,TCF7L2
IL-12 Signaling and Production in Macrophages	12.10	IL10,MYD88,MAF,IKBKE,IL12RB2,CEBPB,TLR9,SPI1, TRAF6,TLR4,PRKCI,CD40,IRS1,CHUK,NOS2,STAT1, IFNA1/IFNA13
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	11.90	IL10,IL36A,LTB,IL1F10,IL37,TLR9,IL36B,TLR4,IL36G, TLR10,CD40,IL36RN,TLR1,CHUK
LXR/RXR Activation	11.20	TLR4,IL36G,IL1RAPL2,IL1RL2,IL36RN,LPL,IL36A,LBP, IL1F10,IL37,IL1R1,NOS2,IL1RAP,CYP51A1,IL36B
iNOS Signaling	10.20	TRAF6,TLR4,MYD88,IKBKE,CHUK,LBP,NOS2,STAT1, IRAK1,IRAK2
PPAR Signaling	9.98	TRAF6,IL36G,IL1RAPL2,IL1RL2,IL36RN,IL36A,IKBKE, CHUK,IL1F10,IL37,IL1R1,IL1RAP,IL36B
Granulocyte Adhesion and Diapedesis	9.69	CXCL8,IL1RAPL2,CXCR4,MMP16,IL36A,IL1F10,IL37, IL1R1,IL36B,IL36G,IL1RL2,CCL8,CLDN1,IL36RN,Ccl6,I L1RAP

Role of Cytokines in Mediating Communication between Immune Cells	9.41	CXCL8,IL36G,IL10,IL36RN,IFNB1,IL36A,IL1F10,IL37, IFNA1/IFNA13,IL36B
Role of Hypercytokinemia/hyp erchemokinemia in the Pathogenesis of Influenza	9.02	CXCL8,IL36G,IL36RN,IFNB1,IL36A,IL1F10,IL37, IFNA1/IFNA13,IL36B
Activation of IRF by Cytosolic Pattern Recognition Receptors	8.72	FADD,TRAF6,RIPK1,CD40,IL10,IFNB1,IKBKE,CHUK, STAT1,IFNA1/IFNA13
Hepatic Fibrosis / Hepatic Stellate Cell Activation	8.53	SMAD2,CXCL8,IL1RAPL2,IL10,IL1R1,MET,TLR4, COL13A1,CD40,IL1RL2,LBP,STAT1,IL1RAP, IFNA1/IFNA13,AGTR1
Type I Diabetes Mellitus Signaling	8.37	FADD,TRAF6,SOCS1,RIPK1,MYD88,IKBKE,CHUK, IL1R1,NOS2,STAT1,IL1RAP,IRAK1
LPS/IL-1 Mediated Inhibition of RXR Function	8.32	ABCB1,IL1RAPL2,MYD88,IL36A,IL1F10,IL37,IL1R1, IRAK1,IL36B,TRAF6,TLR4,IL36G,IL1RL2,IL36RN,LBP, IL1RAP
TREM1 Signaling	7.96	TLR4,CXCL8,TLR10,CD40,MYD88,IL10, TLR1,PLCG1,TLR9,IRAK1
Osteoarthritis Pathway	7.78	SMAD2,CXCL8,IL1RAPL2,IL1R1,CEBPB,FADD,TLR4, IL1RL2,MEF2C,CHUK,CTNNB1,NOS2,SMAD1,IL1RAP, TCF7L2
Cholecystokinin/Gastri n-mediated Signaling	7.49	IL36G,PRKCI,RHOB,RHOA,IL36RN,IL36A,MEF2C, GNA13,IL1F10,IL37,IL36B
Agranulocyte Adhesion and Diapedesis	7.44	CXCL8,IL36G,CCL8,CLDN1,CXCR4,MMP16,IL36RN, IL36A,PODXL,IL1F10,IL37,IL1R1,Ccl6,IL36B
IL-1 Signaling	7.14	TRAF6,MYD88,TAB2,IKBKE,CHUK,GNA13,IL1R1, IL1RAP,IRAK1,IRAK2
Th1 and Th2 Activation Pathway	6.71	SOCS1,CCR3,IL2RG,CD40,IL10,CXCR4,IRS1,MAF, IL12RB2,STAT1,TLR9,SPI1,IFNA1/IFNA13
Atherosclerosis Signaling	6.68	CCR3,CXCL8,IL36G,CD40,CXCR4,IL36RN,LPL, IL36A,IL1F10,IL37,IL36B
Role of PKR in Interferon Induction and Antiviral Response	6.49	FADD,TRAF6,TAB2,IFNB1,IKBKE,CHUK,STAT1
Systemic Lupus Erythematosus Signaling	6.33	IL10,IL36A,PLCG1,IL37,IL1F10,C8A,TLR9,INPP5D, IL36B,IL36G,CD40,IRS1,IL36RN,IFNA1/IFNA13
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	6.32	TRAF6,TLR4,CXCL8,PRKCI,MYD88,IL10,IRS1,TLR1, IFNB1,TLR9,IFNA1/IFNA13
IL-8 Signaling	6.27	CXCL8,IKBKE,TLR9,CCND1,IRAK1,TRAF6,PRKCI, RHOB,RHOA,IRS1,GNA13,CHUK,IRAK2
Role of RIG1-like Receptors in Antiviral Innate Immunity	6.27	FADD,TRAF6,RIPK1,IFNB1,IKBKE, CHUK,IFNA1/IFNA13
Macropinocytosis Signaling	6.17	MET,PRKCI,IRS1,RHOA,ANKFY1,PLCG1, RAB34,TLR9,CSF1R
Colorectal Cancer Metastasis Signaling	5.93	SMAD2,MMP16,TLR9,CCND1,TLR4,TLR10,RHOB,IRS 1,RHOA,TLR1,STAT1,CTNNB1,NOS2,TCF7L2
PPARα/RXRα Activation	5.89	TRAF6,SMAD2,IL1RAPL2,IL1RL2,IRS1,LPL,PLCG1, MEF2C,IKBKE,CHUK,IL1R1,IL1RAP
iCOS-iCOSL Signaling	5.84	IL2RG,CD40,IRS1,PLCG1,IKBKE,CHUK,PLEKHA4,

in T Helper Cells		TLR9,INPP5D,PTEN
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	5.65	TLR4,PRKCI,RHOB,IRS1,RHOA,PLCG1,IKBKE,CHUK, NOS2,STAT1,TLR9,SPI1
Phagosome Formation	5.62	TLR4,MARCKS,TLR10,PRKCI,RHOB,IRS1,RHOA, TLR1,PLCG1,TLR9
PI3K Signaling in B Lymphocytes	5.53	TLR4,PRKCI,CD40,IRS1,PLCG1,IKBKE,CHUK, PLEKHA4,INPP5D,PTEN
Tec Kinase Signaling	5.43	FADD,TLR4,PRKCI,RHOB,IRS1,RHOA,PLCG1,GNA13, STAT1,TLR9,TNFRSF10A
p53 Signaling	5.29	TP53INP1,PMAIP1,IRS1,BRCA1,CTNNB1,TLR9, TNFRSF10A,CCND1,PTEN
Small Cell Lung Cancer Signaling	5.23	TRAF6,PA2G4,IRS1,IKBKE,CHUK,TLR9,CCND1,PTEN
Th2 Pathway	5.07	CCR3,IL2RG,CD40,CXCR4,IL10,IRS1,MAF, IL12RB2,TLR9,SPI1
Regulation of the Epithelial-Mesenchymal Transition Pathway	4.85	MET,ETS1,SMAD2,IRS1,RHOA,ZEB2,ZEB1, CTNNB1,TLR9,FGF7,TCF7L2
Graft-versus-Host Disease Signaling	4.82	IL36G,IL36RN,IL36A,IL1F10,IL37,IL36B
Prostate Cancer Signaling	4.69	AR,PA2G4,IRS1,CHUK,TLR9,CTNNB1,CCND1,PTEN
Adrenomedullin signaling pathway	4.65	IL36G,IRS1,IL36RN,IL36A,PLCG1,CEBPB,CFH,IL1F10, IL37,TLR9,IL36B
Gα12/13 Signaling	4.47	IRS1,RHOA,MEF2C,IKBKE,CHUK,GNA13, CTNNB1,TLR9,F2
Molecular Mechanisms of Cancer	4.40	SMAD2,PMAIP1,PA2G4,TAB2,TLR9,CCND1,FADD, PRKCI,RHOB,IRS1,RHOA,GNA13,CTNNB1,BRCA1, SMAD1
Ovarian Cancer Signaling	4.28	PA2G4,IRS1,CD44,BRCA1,CTNNB1,TLR9,CCND1, TCF7L2,PTEN
Crosstalk between Dendritic Cells and Natural Killer Cells	4.20	TLR4,IL2RG,CD40,IFNB1,LTB,TLR9,IFNA1/IFNA13
Prolactin Signaling	4.14	SOCS1,PRKCI,IRS1,PLCG1,CEBPB,TLR9,STAT1
Th17 Activation Pathway	4.14	TRAF6,MYD88,IL10,IL12RB2,IL1R1,IRAK1,IRAK2
Endocannabinoid Cancer Inhibition Pathway	4.13	IRS1,RHOA,VIM,GNA13,NOS2,CTNNB1,TLR9,CCND1, TCF7L2
IL-17 Signaling	4.08	TRAF6,CXCL8,IRS1,CRP,CEBPB,NOS2,TLR9
IL-7 Signaling Pathway	4.08	MET,SOCS1,IL2RG,IRS1,STAT1,TLR9,CCND1
Gαq Signaling	4.05	PRKCI,RHOB,IRS1,RHOA,PLCG1,IKBKE,CHUK,TLR9, AGTR1
LPS-stimulated MAPK Signaling	4.02	TLR4,PRKCI,IRS1,IKBKE,CHUK,LBP,TLR9
PEDF Signaling	4.02	IRS1,RHOA,IKBKE,ZEB1,CHUK,TLR9,TCF7L2
Sumoylation Pathway	3.99	ETS1,AR,RHOB,RHOA,MYB,RCOR1,ZEB1
Lymphotoxin β Receptor Signaling	3.92	TRAF6,IRS1,LTB,IKBKE,CHUK,TLR9
Role of JAK1, JAK2 and TYK2 in Interferon Signaling	3.87	SOCS1,IFNB1,STAT1,IFNA1/IFNA13
Leukocyte Extravasation	3.83	PRKCI,CXCR4,CLDN1,IRS1,MMP16,RHOA,CD44, PLCG1,CTNNB1,TLR9

Signaling		
Glucocorticoid Receptor Signaling	3.81	SMAD2,CXCL8,IL10,IKBKE,ARID2,CEBPB,TLR9, TRAF6,AR,IRS1,CHUK,STAT1,NOS2
Th1 Pathway	3.81	SOCS1,CD40,IL10,IRS1,IL12RB2,TLR9,STAT1, IFNA1/IFNA13
FAT10 Cancer Signaling Pathway	3.80	SMAD2,CXCR4,IKBKE,CHUK,CTNNB1
Protein Kinase A Signaling	3.77	CDKN3,PRKCI,PTPRJ,PTPRD,PTPN13,RHOA,PDE3A, PLCG1,CHUK,GNA13,CTNNB1,PTPN12,TCF7L2, PTEN
T Cell Exhaustion Signaling Pathway	3.67	SMAD2,IL10,IRS1,PLCG1,IL12RB2,STAT1,TLR9, CTLA4,IFNA1/IFNA13
IL-15 Production	3.60	PRKCI,IFNB1,STAT1,IFNA1/IFNA13
IL-17A Signaling in Airway Cells	3.56	TRAF6,IRS1,IKBKE,CHUK,TLR9,PTEN
CD40 Signaling	3.53	TRAF6,CD40,IRS1,IKBKE,CHUK,TLR9
Superpathway of Inositol Phosphate Compounds	3.46	MET,PTPRJ,PTPN13,IRS1,PLCG1,TLR9,PTPN12, PPP5C,INPP5D,PTEN
ILK Signaling	3.41	RHOB,IRS1,RHOA,VIM,NOS2,CTNNB1,TLR9,CCND1, PTEN
Growth Hormone Signaling	3.39	SOCS1,PRKCI,IRS1,PLCG1,TLR9,STAT1
HGF Signaling	3.37	MET,ETS1,PRKCI,IRS1,PLCG1,TLR9,CCND1
Glioma Signaling	3.35	PRKCI,PA2G4,IRS1,PLCG1,TLR9,CCND1,PTEN
NGF Signaling	3.29	TRAF6,IRS1,RHOA,PLCG1,IKBKE,CHUK,TLR9
FXR/RXR Activation	3.26	IL36G,LPL,IL36RN,IL36A,IL1F10,IL37,IL36B
Signaling by Rho Family GTPases	3.25	STMN1,ARFIP2,PRKCI,RHOB,IRS1,RHOA,WASF3, VIM,GNA13,TLR9
Factors Promoting Cardiogenesis in Vertebrates	3.23	SMAD2,PRKCI,MEF2C,CTNNB1,SMAD1,TCF7L2
Clathrin-mediated Endocytosis Signaling	3.23	MET,SH3BP4,RAB5C,PICALM,IRS1,TLR9,FGF7, MYO1E,F2
Induction of Apoptosis by HIV1	3.22	FADD,RIPK1,CXCR4,IKBKE,CHUK
TWEAK Signaling	3.22	FADD,RIPK1,IKBKE,CHUK
IL-17A Signaling in Fibroblasts	3.22	TRAF6,IKBKE,CHUK,CEBPB
PI3K/AKT Signaling	3.20	RHEB,IKBKE,CHUK,CTNNB1,CCND1,INPP5D,PTEN
Glioblastoma Multiforme Signaling	3.17	RHOB,IRS1,RHOA,PLCG1,TLR9,CTNNB1, CCND1,PTEN
Interferon Signaling	3.17	SOCS1,IFNB1,STAT1,IFNA1/IFNA13
NF-κB Activation by Viruses	3.16	PRKCI,RIPK1,IRS1,IKBKE,CHUK,TLR9
Role of Tissue Factor in Cancer	3.14	CXCL8,IRS1,GNA13,CYR61,TLR9,F2,PTEN
CXCR4 Signaling	3.14	PRKCI,RHOB,CXCR4,IRS1,RHOA,GNA13, TLR9,ELMO2
IL-4 Signaling	3.14	SOCS1,IL2RG,IL13RA1,IRS1,TLR9,INPP5D
Acute Myeloid Leukemia Signaling	3.07	IRS1,TLR9,SPI1,CCND1,CSF1R,TCF7L2
p70S6K Signaling	3.00	IL2RG,PRKCI,IRS1,PLCG1,TLR9,AGTR1,F2
HMGB1 Signaling	2.97	CXCL8,TLR4,RHOB,IRS1,RHOA,IL1R1,TLR9
RANK Signaling in Osteoclasts	2.95	TRAF6,IRS1,TAB2,IKBKE,CHUK,TLR9
Thrombopoietin	2.90	PRKCI,IRS1,PLCG1,TLR9,STAT1

Signaling		
Endometrial Cancer Signaling	2.90	IRS1,TLR9,CTNNB1,CCND1,PTEN
T Helper Cell Differentiation	2.87	IL2RG,CD40,IL10,IL12RB2,STAT1
B Cell Receptor Signaling	2.81	ETS1,IRS1,MEF2C,IKBKE,CHUK,TLR9,INPP5D,PTEN
Hereditary Breast Cancer Signaling	2.79	IRS1,WEE1,ARID2,TLR9,BRCA1,CCND1,PTEN
Chronic Myeloid Leukemia Signaling	2.77	PA2G4,IRS1,IKBKE,CHUK,TLR9,CCND1
Thyroid Cancer Signaling	2.77	CXCL8,CTNNB1,CCND1,TCF7L2
Glioma Invasiveness Signaling	2.74	RHOB,IRS1,RHOA,CD44,TLR9
Role of JAK1 and JAK3 in γc Cytokine Signaling	2.74	SOCS1,IL2RG,IRS1,TLR9,STAT1
Type II Diabetes Mellitus Signaling	2.74	SOCS1,PRKCI,IRS1,IKBKE,CHUK,CEBPB,TLR9
IL-9 Signaling	2.73	IL2RG,IRS1,STAT1,TLR9
NRF2-mediated Oxidative Stress Response	2.69	PRKCI,IRS1,DNAJC19,MAF,DNAJB1,TLR9,TXNRD1,BACH1
3-phosphoinositide Biosynthesis	2.69	MET,PTPRJ,PTPN13,IRS1,TLR9,PTPN12,PPP5C,PTEN
T Cell Receptor Signaling	2.69	IRS1,PLCG1,IKBKE,CHUK,TLR9,CTLA4
GM-CSF Signaling	2.67	ETS1,IRS1,TLR9,STAT1,CCND1
TNFR1 Signaling	2.63	FADD,RIPK1,IKBKE,CHUK
IL-15 Signaling	2.60	CXCL8,IL2RG,IRS1,PLCG1,TLR9
Pancreatic Adenocarcinoma Signaling	2.60	SMAD2,PA2G4,IRS1,TLR9,STAT1,CCND1
Thrombin Signaling	2.58	PRKCI,RHOB,IRS1,RHOA,PLCG1,GNA13,TLR9,F2
Non-Small Cell Lung Cancer Signaling	2.58	PA2G4,IRS1,PLCG1,TLR9,CCND1
Rac Signaling	2.57	PRKCI,ARFIP2,IRS1,RHOA,CD44,TLR9
Sphingosine-1-phosphate Signaling	2.53	RHOB,IRS1,RHOA,PLCG1,GNA13,TLR9
Erythropoietin Signaling	2.51	SOCS1,PRKCI,IRS1,PLCG1,TLR9
Endocannabinoid Developing Neuron Pathway	2.50	IRS1,RHOA,BRCA1,TLR9,CTNNB1,CCND1
IL-3 Signaling	2.47	PRKCI,IRS1,TLR9,STAT1,INPP5D
Renin-Angiotensin Signaling	2.46	PRKCI,IRS1,PLCG1,TLR9,STAT1,AGTR1
JAK/Stat Signaling	2.45	SOCS1,IRS1,CEBPB,TLR9,STAT1
STAT3 Pathway	2.43	SOCS1,IL2RG,IL1RL2,IL13RA1,IL12RB2,IL1R1
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	2.43	PRKCI,VAMP3,PLCG1,INPP5D,PTEN
FGF Signaling	2.43	MET,IRS1,PLCG1,TLR9,FGF7
Neuregulin Signaling	2.41	PRKCI,ERBIN,PLCG1,ERRFI1,PTEN
Death Receptor	2.41	FADD,RIPK1,IKBKE,CHUK,TNFRSF10A

CD28 Signaling in T Helper Cells	2.40	IRS1,PLCG1,IKBKE,CHUK,TLR9,CTLA4
Bladder Cancer Signaling	2.39	CXCL8,PA2G4,MMP16,FGF7,CCND1
HER-2 Signaling in Breast Cancer	2.35	PRKCI,IRS1,PLCG1,TLR9,CCND1
PDGF Signaling	2.31	IRS1,PLCG1,TLR9,STAT1,INPP5D
Retinoic acid Mediated Apoptosis Signaling	2.29	FADD,IFNB1,TNFRSF10A,IFNA1/IFNA13
Melanoma Signaling	2.27	IRS1,TLR9,CCND1,PTEN
Human Embryonic Stem Cell Pluripotency	2.23	SMAD2,IRS1,TLR9,CTNNB1,SMAD1,TCF7L2
Role of NFAT in Regulation of the Immune Response	2.21	IRS1,PLCG1,MEF2C,IKBKE,GNA13,CHUK,TLR9
Role of JAK2 in Hormone-like Cytokine Signaling	2.20	SOCS1,IRS1,STAT1
Role of IL-17A in Arthritis	2.11	CXCL8,IRS1,TLR9,NOS2
EGF Signaling	2.11	IRS1,PLCG1,TLR9,STAT1
SAPK/JNK Signaling	2.07	FADD,RIPK1,IRS1,GNA13,TLR9
Ephrin B Signaling	2.06	CXCR4,RHOA,GNA13,CTNNB1
IL-2 Signaling	2.06	SOCS1,IL2RG,IRS1,TLR9
3-phosphoinositide Degradation	2.06	PTPRJ,PTPN13,PTPN12,PPP5C,INPP5D,PTEN
IGF-1 Signaling	2.05	SOCS1,PRKCI,IRS1,TLR9,CYR61
Mouse Embryonic Stem Cell Pluripotency	2.04	IRS1,TLR9,CTNNB1,SMAD1,TCF7L2
April Mediated Signaling	2.03	TRAF6,IKBKE,CHUK
MSP-RON Signaling Pathway	2.02	TLR4,IRS1,TLR9,NOS2
D-myo-inositol-5-phosphate Metabolism	2.01	PTPRJ,PTPN13,PLCG1,PTPN12,PPP5C,PTEN

Online resource 3

Ingenuity Canonical Pathways	-log(p-value)	Regulated Molecules in Pathway
Cancer Drug Resistance By Drug Efflux	3.77	ABCB1,KRAS
Prostate Cancer Signaling	3.23	AR,KRAS
IL-6 Signaling	2.99	ABCB1,KRAS
Eumelanin Biosynthesis	2.74	MIF
Xenobiotic Metabolism Signaling	2.31	ABCB1,KRAS
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.25	MIF,KRAS
Glucocorticoid Receptor Signaling	2.19	AR,KRAS
Polyamine Regulation in Colon Cancer	2.1	KRAS

APÊNDICE A – Referências da Introdução Geral

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Anexo A – Normas da Revista Molecular Neurobiology

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Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

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Manuscripts should be submitted in Word.

Use a normal, plain font (e.g., 10-point Times Roman) for text.

Use italics for emphasis.

Use the automatic page numbering function to number the pages.

Do not use field functions.

Use tab stops or other commands for indents, not the space bar.

Use the table function, not spreadsheets, to make tables.

Use the equation editor or MathType for equations.

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Please use no more than three levels of displayed headings.

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Abbreviations should be defined at first mention and used consistently thereafter.

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Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

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Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

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Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.

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