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INVESTIGAÇÃO DA VIA DA QUINURENINA NO MODELO DE ENCEFALOMIELITE AUTOIMUNE EXPERIMENTAL EM CAMUNDONGOS.

TESE DE DOUTORADO

MICHELI STÉFANI ZARZECKI

Itaqui, RS, Brasil.

2020

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Por

MICHELI STÉFANI ZARZECKI

Tese apresentada ao Programa de Pós Graduação em Bioquímica, da Universidade Federal do Pampa (UNIPAMPA), como requisito parcial para obtenção do grau de Doutora em Bioquímica

Orientadora: Profª. Dra. Silvana Peterini Boeira
Coorientador: Prof. Dr. Leandro Cattelan Souza

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Tese defendida e aprovada em 21 de dezembro de 2020.

Banca Examinadora:

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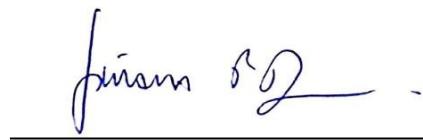
Profª. Drª. Silvana Peterini Boeira (Orientadora)

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Profª. Drª. Shanda de Feitas Couto (UNIPAMPA)

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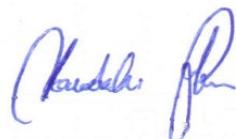
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Dr. Carlos Borges Filho (UNIPAMPA)

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Prof. Dr. Lucian Del Fabbro (Universidade do Contestato)

DEDICATÓRIA (S)

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1 PARTE I

2 RESUMO

A Esclerose Multipla (EM) é uma doença neurológica, crônica, autoimune, de caráter progressivo e não tem cura. Combinação de predisposição genética juntamente com fatores tóxicos ambientais podem ser determinantes no desencadeamento dessa doença. Os sintomas variam entre motores, sensoriais e cognitivos. As manifestações clínicas variam muito de um individuo para outro, sendo um dos problemas na hora do tratamento dessa doença. A encefalomielite autoimune experimental (EAE) é um modelo que simula a esclerose múltipla do tipo Remitente-Recorrente (EMRR) em roedores. Evidências sugerem que a ativação da indoleamina-2,3-dioxigenase (IDO), a enzima limitadora da velocidade da via da quinurenina (VQ), desempenha um papel crucial nas doenças relacionadas à inflamação. O presente estudo teve como objetivo investigar o envolvimento do processo inflamatório e dos componentes do VQ em um modelo de EAE em camundongos. A compreensão da influencia dessa via poderá possibilitar a delimitação de alvos terapêuticos, bem como estudar a participação da vida da quinurenina nessa doença. Para isso a EAE foi induzida por imunização subcutânea (s.c.) com 200 μl de emulsão contendo 200 μg do peptídeo da proteína de oligodendrócito de mielina (MOG35-55) e 500 μg de extrato de Mycobacterium tuberculosis. Para identificar o papel do VQ na patogênese da EAE, os camundongos receberam o inibidor IDO (INCB024360) na dose de 200 mg/kg (por via oral) por 25 dias. Demonstramos que o inibidor de IDO mitigou os sinais clínicos da EAE, em paralelo com a redução dos níveis de citocinas (cérebro: córtex pré-frontal e hipocampo, medula espinhal, baço e linfonodo) e expressão do gene da proteína adaptadora de ligação de cálcio ionizada-1 (Iba-1) na região central sistema nervoso de camundongos com EAE. Além disso, o inibidor IDO causa uma diminuição significativa nos níveis de triptofano, quinurenina e metabólitos neurotóxicos de VQ, como 3-hidroxiquinurenina (3-HK) e ácido quinolínico (QUIN) no córtex pré-frontal, hipocampo, medula espinhal, baço e linfonodo em camundongos com EAE. A expressão de mRNA e a atividade enzimática de IDO e quinurenina 3-monooxigenase (KMO) também foram reduzidas pelo inibidor de IDO. Esses achados indicam que o processo inflamatório concomitante à ativação de IDO/VQ está envolvido nos mecanismos patogênicos da EAE. A modulação do VQ é um alvo promissor para um tratamento coadjuvante em paciente com EM.

3   ABSTRACT

Multiple sclerosis (MS) is a neurological, chronic, autoimmune disease, progressive in nature and has no cure. Combination of genetic predisposition together with toxic environmental factors can be decisive in triggering this disease. Symptoms vary between motor, sensory and cognitive. Clinical manifestations vary widely from one individual to another, being one of the problems when treating this disease. Experimental autoimmune encephalomyelitis (EAE) is a model that simulates remitting-recurrent multiple sclerosis (EMRR) in rodents. Active evidence that the activation of indoleamine-2,3-dioxigenase (IDO), an enzyme that limits the speed of the kynurenine pathway (KP), plays a crucial role in diseases related to inflammation. The present study aimed to investigate the involvement of the inflammatory process and the components of the KP in a model of EAE in mice. Understanding the influence of this pathway can enable the delimitation of therapeutic targets, as well as studying the participation of the life of quinurenine in this disease. For this, an EAE was induced by subcutaneous immunization (s.c.) with 200 µl of emulsion containing 200 µg of myelin oligodendrocyte protein peptide (MOG35-55) and 500 µg of Mycobacterium tuberculosis extract. To identify the role of VQ in the pathogenesis of EAE, the mice received the inhibitor IDO (INCB024360) at a dose of 200 mg/kg (orally) for 25 days. We demonstrated that the IDO inhibitor mitigated the clinical signs of EAE, in parallel with the reduction of cytokine levels (brain: prefrontal cortex and hippocampus, spinal cord, spleen and lymph node) and expression of the ionized calcium binding adapter gene (Iba-1) in the central nervous system of mice with EAE. In addition, the inhibitor IDO causes a decrease in levels of tryptophan, quinurenine and KP neurotoxic metabolites such as 3-hydroxyquinurenine (3-HK) and quinolinic acid (QUIN) in the prefrontal cortex, hippocampus, spinal cord, spleen and lymph node in mice with EAE. An mRNA expression and an enzyme activity of IDO and quinurenine 3-monooxygenase (KMO) were also reduced by the IDO inhibitor. These findings indicate that the inflammatory process concomitant with the activation of IDO/VQ is related to the pathogenic mechanisms of EAE. A modulation of KP is a promising target for an adjunctive treatment in patients with MS.

Keywords: multiple sclerosis, autoimmune disease, microglia, astrocytes, indoleamine-2,3-dioxigenase, kynurenine pathway, IDO inhibitor, INCB024360

4 LISTA DE ILUSTRAÇÕES

5 LISTA DE TABELAS

6 LISTA DE ABREVIATURAS E SIGLAS

5-HT – serotonina ou 5-hidroxitriptamina

ATP – Adenosina Trifosfato

BHE – barreira hematoencefalica

DATASUS - Departamento de Informática do Sistema Único de Saúde

EAE – encefalomielite autoimune experimental

EM – esclerose multipla

HAA - Ácido 3-hidroxiantranílico

IDO – Indoleamina 2,3-dioxigenase

IFN – interferon

IL – interleucina

KAT - quinurenina aminotransferase

KMO - quinurenina-3-monooxigenase

KYN - quinurenina

KYNA - ácido quinurênico

NAD - Nicotinamide adenine dinucleotide

NADP - nicotina adenina dinucleótido fosfato

3-OHK - 3-hidroxiquinurenina

QUIN ou QA– ácido quinolínico

SNC – sistema nervoso central

TDO – triptofano 2,3-dioxigenase

TRP - triptofano

VQ – via da quinurenina

SUMÁRIO

APRESENTAÇÃO

A presente tese foi dividida em três partes principais. Na parte I encontram-se a INTRODUÇÃO e OBJETIVOS. Os resultados que fazem parte desta tese estão apresentados sob a forma de 1 artigo publicado em periódico científico e 1 manuscrito, os quais se encontram nos itens ARTIGO e MANUSCRITO I. As seções materiais e métodos, resultados e referências bibliográficas, encontram-se no próprio artigo e representa a íntegra deste estudo.

A CONCLUSÃO, encontra-se na parte III desta tese. O item REFERÊNCIAS refere-se somente às citações que aparecem nos itens introdução e conclusão desta tese. No item PERSPECTIVAS, estão expostos os possíveis sugestões de estudos que podem dar continuidade a este trabalho.

1 INTRODUÇÃO

A Encéfalo Mielite Autoimune Experimental é o modelo animal que mimetiza a Esclerose Múltipla (EM), a EM é uma doença autoimune do SNC, onde as células de defesa do nosso organismo se voltam contra a mielina dos neurônios (VAMOS et al., 2009; SUNDARAM et al., 2014.) A Bainha de Mielina é uma membrana lipídica rica em glicofosfolipídeos e colesterol ao qual protege a parte do neurônio chamada de axônio. A mielina tem a função de isolamento, de não deixar o impulso nervoso sair do axônio enquanto é conduzido de um local para outro. Quando há esse ataque a mielina, os neurônios não conseguem desempenhar o seu papel tanto na transmissão do impulso nervoso entre o próprio neurônio nem a comunicação com os demais (na sinapse) (MENDES e MELO 2011).

EM afeta cerca de dois milhões de pessoas no mundo, na sua grande maioria jovens adultos, nas faixas etárias entre 20 a 40 anos, prevalentemente do sexo feminino (GOLD & VOSKUHL, 2016). Dados do DATASUS (Departamento de informática do SUS) de 2014 revelam que no Brasil, há 35 mil portadores e cerca de 13 mil em tratamento.

Está bem estabelecido que as células-T ativadas e as células gliais cerebrais secretam citocinas/quimiocinas pró-inflamatórias juntamente com a geração de mediadores inflamatórios incluindo as espécies reativas do oxigênio que levam à perda axonal (HAFLER, 2004; GOVERMAN, 2009). Portanto, a patogênese da EM é um processo complexo, envolvendo células TH1 e TH17, macrófagos, células dendríticas, astrócitos e microglia, bem como uma gama de mediadores inflamatórios produzidos por essas células (DONG & BENVENISTE, 2001; HAFLER, 2004; FARINA et al., 2007; GOVERMAN, 2009; FELDMANN & STEINMAN, 2005; FLETCHER et al., 2010; JACK et al., 2005). Outras características dos danos causados pela EM incluem também a perda neuronal, lesão axonal e atrofia do SNC, devido a uma reação inflamatória progressiva que envolve tanto o sistema imune adaptativo quanto o inato (HAFLER, 2004; KAWAKAMI et al., 2005; GOVERMAN, 2009).

A via da quinurenina, derivada do triptofano, que tem como produto final o NAD, também tem dois braços, onde se produz dois metabolitos e esses tem sido alvos de investigação em diversas patologias do SNC (MYINT et al., 2012; SOUZA et al. 2016a.)

Embora fatores genéticos e ambientais também estejam implicados na patogênese da EM (BARANZINI et al., 2010; SELLNER et al., 2011), há uma incerteza sobre a fisiopatologia subjacente da EM e, ainda que a pesquisa indique que a EM possui uma patogênese imuno mediada, os alvos de resposta imunológica não estão bem definidos (SRIVASTAVA et al., 2012). Portanto, é fundamental a investigação de novos alvos terapêuticos, uma vez que essa doença não tem cura e apresenta diversas manifestações clínicas e patológicas. O estudo dos mecanismos envolvidos na

doença possibilitará uma constante e significativa evolução na qualidade de vida dos pacientes, uma vez que o tratamento é realizado por meio de medicamentos que visam coibir a progressão da doença, ou seja, estabilizar a escala de estado de incapacidade desses indivíduos.

2   REFERENCIAL TEÓRICO

Esclerose Múltipla

A EM é uma doença neurológica, acomete o sistema nervoso central (SNC), crônica, autoimune, de caráter progressivo e não tem cura. Chama-se Esclerose por que como resultado da doença forma-se um tecido parecido a uma cicatriz, chama-se Múltipla porque pode atingir várias áreas do cérebro e da medula espinhal (CHANADAY & ROTH, 2016; DE PAULA-SILVA et al., 2017). É uma doença neurológica pois ocorre uma reação autoimune contra a mielina dos neurônios. A perda de mielina em diferentes locais é justamente o fator determinante para tais respostas e os sintomas dessa doença, como: deficiências motoras, cognitivas e comportamentais, incluindo dormência ou dor nas extremidades, perda da visão e demência ((FROHMAN et al, 2006; MCFARLAND & MARTIN, 2007; BHARGAVA & CALABRESI, 2016, YANDAMURI & LANE, 2016).

Não existe um padrão definido, cada paciente com EM possui seu tipo peculiar de manifestação da doença, recidiva ou não, portanto terá o seu conjunto de sinais e sintomas, o que dificulta, sobremaneira, o controle e evolução da doença e assim traçar um possível prognóstico. A doença pode ser classificada pelo conjunto de manifestações dos sintomas, bem como os sintomas isolados, em três subtipos: EM recorrente e remitente (EMRR), EM progressiva secundária (EMPS) e EM progressiva primária (EMPP). EMRR é a forma clínica mais comum e é responsável por 85% dos casos (HUISMAN et al., 2017), e caracteriza-se pela ocorrência de surtos e remissões sucessivas, em geral, com boa recuperações neurológicas. A EMPS A doença é inicialmente recorrente-remitente, mas após algum tempo, torna-se progressiva. EMPP a doença evolui, desde o início, de maneira lenta e progressiva. Os Critérios que são utilizados para o diagnóstico da esclerose múltipla são denominados de Critérios de McDonald (McDonald et al., 2001).

Podemos salientar que a imprevisibilidade da doença é a sua marcante característica e isto interfere nos sintomas e na reação do paciente frente aos tratamentos oferecidos, além da forma como reage com relação à família, trabalho e sociedade. Um ponto importante é o surgimento da doença que ocorre principalmente na fase socialmente produtiva. Não podendo assim deixar de se verificar o impacto social, pessoal e econômico que pode ocorrer na vida do indivíduo e da sociedade, pois a medida que a doença progride, sintomas motores debilitantes se desenvolvem, podendo levar eventualmente a paralisia completa e até a morte (SORCE et al., 2017).

A causa exata da EM ainda é desconhecida, sobre os fatores envolvidos na patogênese, os fatores genéticos e ambientais podem ser responsáveis pelo desenvolvimento e progressão da doença (GHASEMI et al., 2017). Sobre os fatores genéticos, já foram identificados polimorfismos de genes de citocinas que participem como reguladores na cascata do sistema imunológico (FORTE et al., 2006; ZHANG et al., 2019).

Entre os fatores ambientais capazes de desencadear a patogênese da EM em um contexto de suscetibilidade genética, as infecções virais são de particular relevância. O herpesvírus humano (HHV-6), vírus Varicella-Zoster (VZV) e principalmente vírus Epstein-Barr (EBV), e a expressão de Retrovírus Endógenos Humanos (HERVs) (MORANDI et al., 2017), além de infecções virais temos a exposição ao sol e consequente níveis baixos de vitamina D crônicos (ISMAILOVA et al., 2019), exposição ao tabagismo (HEYDARPOUR et al., 2018), obesidade e exposição a solventes orgânicos e ao cobre (MCKAY et al., 2017; GERHARDSSON et al., 2020; SARMADI et al., 2020). Além disso, menor ingestão de ferro (mas não outros fatores dietéticos) foram associados ao aumento risco de EM pediátrica, enquanto maior gordura saturada e ingestão de vegetais foi associada com aumento e diminuição do risco de recaída, respectivamente (NOURBAKHSH et al., 2018).

Embora fatores genéticos e ambientais também estejam implicados na patogênese da EM (BARANZINI et al., 2010; SELLNER et al., 2011), há uma incerteza sobre a fisiopatologia subjacente da EM e, ainda que a pesquisa indique que a EM possui uma patogênese imuno mediada, os alvos de resposta imunológica não estão bem definidos (SRIVASTAVA et al., 2012). Portanto, é fundamental a investigação de novos alvos terapêuticos, uma vez que essa doença não tem cura e apresenta diversas manifestações clínicas e patológicas. O estudo dos mecanismos envolvidos na doença possibilitará uma constante e significativa evolução na qualidade de vida dos pacientes, uma vez que o tratamento é realizado por meio de medicamentos que visam coibir a progressão da doença, ou seja, estabilizar a escala de estado de incapacidade desses indivíduos.

Sintomas da EM e a relação dos tecidos estudados

A EM é uma doença do SNC, as partes afetadas por essa doença compreendem o cérebro, a medula espinhal e as partes neurais do olho e tem uma relação única com o sistema imunológico (ENGELHARDT et al., 2016). A EM difere no padrão de distribuição das lesões dentro do SNC. A maioria dos pacientes com EM exibe suas lesões principalmente no cérebro, já em modelos animais com EM, apresentam a maioria das placas inflamatórias na medula espinhal e no nervo óptico (SIMMONS et al., 2013). As lesões e placas inflamatórias influenciam nos sintomas, visto que os sintomas da EM variam entre motores, sensoriais e cognitivos isso se dá aos locais afetados pelos processos de neuroinflamação, desmielinização e neurodegeneração.

Um dos sintomas mais comuns e que se torna incapacitante da EM é a fadiga cognitiva, que afeta cerca de 80% dos pacientes. É uma sensação avassaladora de exaustão mental que está relacionada a mudanças funcionais no córtex frontal e/ou nos gânglios da base, e perda de conectividade entre eles. Outro déficit cognitivo comum na EM presente em 50-70% dos pacientes, é a disfunção executiva (capacidades de organização, planejamento, abstração, conceituação, execução, monitoramento, entre outra), que também está relacionada a alterações em várias áreas do córtex frontal (CHANADAY & ROTH, 2016).

Neuroinflamação, desmielinização e neurodegeneração em áreas cerebrais profundas como o estriado e o hipocampo, junto com suas correlações comportamentais, são amplamente estudadas na EM (CHANADAY & ROTH, 2016). Algumas das manifestações clínicas da EM, como comprometimento da memória e depressão, estão, pelo menos em parte, relacionadas ao envolvimento do hipocampo. Estudos patológicos mostraram extensa desmielinização, dano neuronal e anormalidades sinápticas no hipocampo de pacientes com esclerose múltipla, e os avanços na tecnologia de ressonância magnética forneceram novas maneiras de avaliar o envolvimento do hipocampo in vivo. O hipocampo também tem papéis importantes na plasticidade e neurogênese, os quais contribuem potencialmente para a preservação e restauração funcional (ROCCA et al., 2018).

O SNC há muito tempo é considerado um órgão imunologicamente único, e pelo fato de algumas doenças terem origem nesse local, é de se esperar que seja investigado, porém não podemos deixar de lado os outros órgãos que servem de apoio e assim contribuem no processo inflamatório, que podem representar uma extensão do meio imunológico do cérebro (ENGELHARDT et al., 2016). Em humanos, há cerca de 500 -700 nódulos linfáticos no total, e muitos deles potencialmente recebendo drenagem da medula espinhal. Os gânglios linfáticos cervicais são as primeiras estações de drenagem do cérebro e, portanto, desempenham um papel fundamental nas doenças neuroinflamatórias, bem como na EM (DI et al., 2017). Os estudos realizados durante a última década revelaram um sistema linfático meníngeo funcional que drena líquido, acromoléculas e células imunes do líquido cefalorraquidiano (LCR) para os linfonodos regionais. Várias anormalidades neuropatológicas, incluindo edema do cérebro, axônios inchados, degeneração dos gânglios e proliferação da glia, foram relatadas quando todos os linfonodos cervicais foram ressecados e os vasos linfáticos cervicais profundos foram ligados em modelos experimentais de EM (SCHNEIDER et al., 2006). Além disso, com a remoção cirúrgica dos linfonodos cervicais e lombares em modelo animais de EM, observou-se uma redução da inflamação cerebral e também a carga de recidiva, indicando que os linfonodos podem contribuir para a patologia dessa doença (DI et al., 2017).

Além do estudo das diferentes regiões do cérebro, medula e os linfonodos, o estudo sobre outros órgãos responsáveis por papéis na inflamação são de suma importância. O baço é o maior órgão imunológico secundário no corpo e é responsável por iniciar reações imunológicas a antígenos transmitidos pelo sangue e por filtrar o sangue de materiais estranhos e glóbulos vermelhos velhos ou danificados (BRENDOLAN et al., 2007).

Uma das vantagens da utilização de modelos animais, além de poder replicar a doença, é a possibilidade de poder avaliar diferentes orgãos ao mesmo tempo e a interação deles diante de uma doença em especifico. Os achados da interação entre os orgãos sublinham a importância da avaliação do corpo como um todo, não apenas o SNC, para assim melhorar a compreensão das manifestações clínicas da EM bem como os mecanismos e os demais orgãos envolvidos nessa patologia.

Função das células presentes no SNC

A função das células não neuronais do SNC, células da glia, geralmente chamadas neuroglia (em grego, γλία: "cola"), é proporcionar suporte e nutrição aos neurônios. A neuroglia pode ser classificada como dois grandes grupos distintos entre morfologia e função, de acordo com a origem embriológica: a microglia, responsável pela função de defesa imune do SNC e a macroglia, compreende síntese de mielina, revestimento e crescimento.

A Microglia monitora o estado funcional de sinapses, influencia mudanças neuroplásticas por remodelar espaços extracelulares e eliminar elementos sinápticos por fagocitose. Em resposta a estímulos nocivos, a microglia sofre uma série de mudanças, um aumento em número devido à proliferação e através do recrutamento de monócitos do sangue periférico, a produção de citocinas pró-inflamatórias e a expressão de vários antígenos de superfície celular são também características de resposta inflamatória microglial. A molécula adaptadora de ligação de cálcio ionizado 1 (Iba-1) é expressa pela microglia independente do contexto celular, já foi mostrado em estudos a utilização desse medidor, Iba-1, como um marcador da atividade da microglial (JACK et al., 2005; CALCIA et al., 2016).

Já os astrócitos são importantes por fornecerem suporte nutricional e estrutural para neurônios, por participarem funcionalmente da barreira hematoencefálica e por participarem de funções como: detoxificação, fagocitose, funções imunes e entre outras. Estudos sobre o desenvolvimento embrionário, morfologia, funções e participação de astrócitos em processos patológicos têm sido frequentemente investigados, bem como estudos que investigaram a expressão de marcadores específicos de células para astrócitos (ENACHE et al., 2019). Em processos

patológicos, os astrócitos respondem prontamente, e, por outro lado, alterações celulares em astrócitos são indicadores confiáveis de lesão do SNC. A proteína glial fibrilar ácida (GFAP) é a principal proteíína como filamento intermediário dos astrócitos, desempenha um papel essencial na manutenção da forma e motilidade dos processos astrocíticos e contribui para a arquitetura da matéria branca, mielinização e integridade da barreira hematoencefálica (MAYER et al., 2013). O GFAP é um marcador clássico de astrocitoma, tanto em ambientes clínicos quanto experimentais (VAN et al., 2019).

A patogênese da EM e o modelo experimental

A EM é caracterizada pela ativação de células mononucleares, predominantemente de células TCD4+ e CD8+, que migram através da barreira hematoencefálica para o SNC e são reativados pela apresentação das células gliais do cérebro e da medula espinhal. Este tráfico de células é um processo fortemente regulado que está associado com a ativação das células-T pela barreira hematoencefálica (STEINMAN, 2007; GOVERMAN, 2009), bem como a secreção de várias quimiocinas (PRAT et al, 2000).

Durante o curso da EM, os antígenos virais ou infecciosos ativam as células-T auto-reativas na periferia, que apresentam similaridade molecular com o antígeno do SNC, diferenciam-se em células-T *helper* tipo 1 (TH1) ou T *helper* tipo 17 (TH17), migram através da barreira hematoencefálica (BHE) e induzem lesões distribuídas ao longo do SNC (SOSPEDRA & MARTIN, 2005, MCFARLAND & MARTIN, 2007, ORSINI et al., 2014, LANZ et al., 2017) (Figura 1).

Está bem estabelecido que as células-T ativadas e as células gliais cerebrais secretam citocinas/quimiocinas pró-inflamatórias juntamente com a geração de mediadores inflamatórios incluindo os radicais livres altamente reativos (espécies reativas do oxigênio) que levam à perda axonal (HAFLER, 2004; GOVERMAN, 2009). Portanto, a patogênese da EM é um processo complexo, envolvendo células TH1 e TH17, macrófagos, células dendríticas, astrócitos e microglia, bem como uma gama de mediadores inflamatórios produzidos por essas células (DONG & BENVENISTE, 2001; HAFLER, 2004; FARINA et al., 2007; GOVERMAN, 2009; FELDMANN & STEINMAN, 2005; FLETCHER et al., 2010; JACK et al., 2005). Outras características dos danos causados pela EM incluem também a perda neuronal, lesão axonal e atrofia do SNC, devido a uma reação inflamatória progressiva que envolve tanto o sistema imune adaptativo quanto o inato (HAFLER, 2004; KAWAKAMI et al., 2005; GOVERMAN, 2009).

Figura 1. Células T ativadas na EM
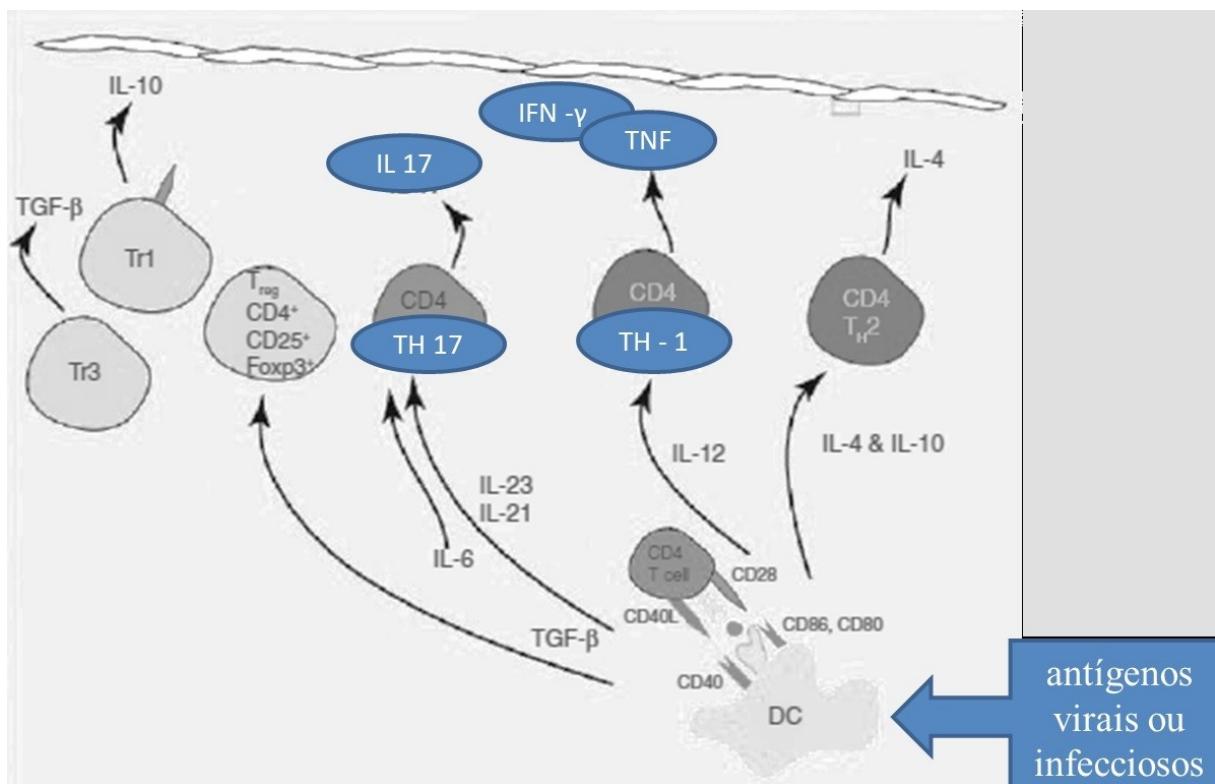
Fonte: Adaptado de MCFARLAND & MARTIN, 2007

A natureza devastadora e complexa da EM, bem como os eventos etiológicos e patológicos que regem as doenças inflamatórias e desmielinizantes do SNC, combinada com a falta de uma cura e dificuldades na recapitulação de uma doença humana, levou ao emprego de tais modelos animais para ajudar a elucidar os mecanismos de progressão da EM (GOLD & VOSKUHL, 2016). A encefalomielite autoimune experimental (EAE) é o modelo animal mais conhecido da EM, este modelo reproduz muitos dos sintomas patológicos e clínicos da EM e pode ser induzida em roedores susceptíveis e outros animais por imunização (ORISINI, 2014; ROSSI & CONSTANTIN 2016; DE PAULA-SILVA et al., 2017).

O modelo de EAE requer a administração da toxina adjuvante imunológica pertussis microbiana (MOG), sua função é no aumento da permeabilidade da barreira hematoencefálica (BHE), expandindo as células mieloides e os linfócitos T específicos do antígeno, reduzindo linfócitos T reguladores e modulando a expressão de citocinas inflamatórias (HOFSTETTER et al., 2002; CHEN et al., 2006; YANDAMURI & LANE, 2016). Já se sabe que as células-TCD4 diferenciadas desempenham um papel essencial na defesa contra patógenos e são atuantes em doenças autoimunes, como podemos mostrar na figura 2 na indução da EAE (MUFAZALOV et al., 2017), a maioria dos pacientes com EM exibe suas lesões principalmente no cérebro, já em modelos animais com EAE, apresentam a maioria das placas inflamatórias na medula espinhal e no nervo óptico (SIMMONS et al., 2013). Como na EM o curso clínico da EAE pode ser altamente variável, os pesquisadores podem obter um curso clínico semelhante ao da EM com base na cepa murina, antígeno injetado e adjuvante (KRISHNAMOORTHY & WEKERLE 2009).

O modelo EAE é caracterizado por uma resposta auto-imune contra as proteínas do sistema nervoso central (SNC), que culmina em infiltrado inflamatório, gliose, lesão da bainha de mielina e morte neuronal (HAFLER, 2004; FROHMAN et al, 2006; MCFARLAND & MARTIN, 2007; GOVERMAN, 2009). Quando analisados em modelos animais, podemos distinguir as fases da progressão da doença, conforme o tempo de imunização realizada e as apresentações relacionadas a manifestação e caracterizada por pontuações clínicas da doença, sendo divididas entres as fases: aguda, remissão e de recaída (KWIDZINSKI et al, 2005). Portanto, foi definido que nos dias 0 a 7 após a imunização os animais estariam na fase de indução da doença, já nos dias 7-15 vai de encontro com a fase aguda da doença e nos dias 15-25 após a imunização, caracterizado como a fase crônica da doença (DUTRA et al, 2011).

Figura 2. Papel das células T CD4 no modelo de EAE
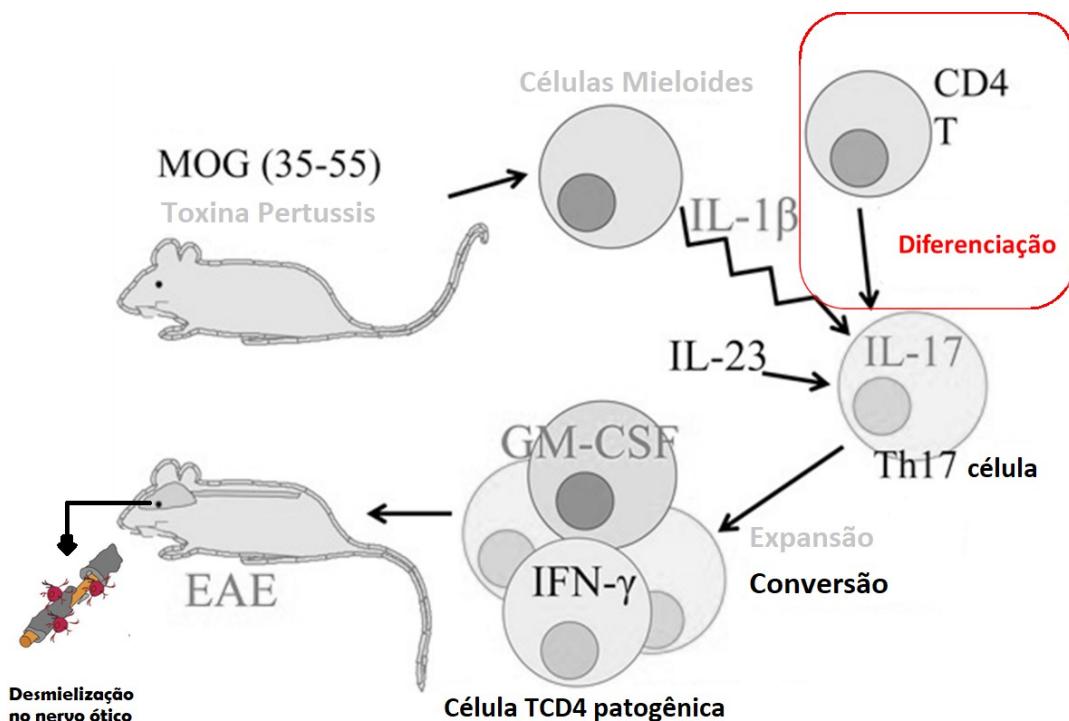
Fonte: Adaptado de MUFAZALOV et al., 2017.

Via da Quinurenina

Na busca de novos alvos terapêuticos, tem se a necessidade do estudo de vias alternativas que possam estar relacionadas a cascata inflamatória e pode desencadear ou não um efeito neuroprotetor dentro das doenças autoimunes. A via da quinurenina (VQ) é uma das vias oriundas do aminoácido essencial triptofano (Trp), e os seus metabolitos estão relacionados com o sistema vascular, sistema imune, imunotolerância e infecções (GAETANI et al., 2020). Já o Trp participa da síntese de proteínas, na liberação de catabólitos imunomoduladores, é precursor na síntese do neurotransmissor aminérgico serotonina (5-hidroxitriptamina ou 5-HT), na síntese do neuro-hormônio melatonina, participa da síntese de vários metabólitos quinuramínicos neuroativos da

melatonina e traços de amina triptamina e também participa daVQ. Os níveis do Trp e a função dos seus derivados há muito tempo são assuntos de interesse de pesquisa em autoimunidade. Nesse contexto tem-se dedicado o estudo aos metabolitos da quinurenina e seu papel diante dos processos inflamatórios que ocorrem nas células do cérebro.

A VQ em seu estado fisiológico normal é metabolizada no fígado e age sobre processos celulares básicos, como na formação de nicotina adenina dinucleotídeo (NAD). Somente 1% do Trp disponível no corpo vai para a síntese de 5-HT, em condições fisiológicas normais, 99% do Trp é metabolizado no fígado com a enzima limitante no primeiro processo da via, a 2,3-dioxigenase de triptofano (TDO) (GAETANI et al., 2020), já em estados de inflamação, infecção ou estresse oxidativo, esse mecanismos ativam a enzima indoleamina-2;3-dioxigensase (IDO) nos tecidos extra-hepáticos, como os pulmões, placenta, rins, baço, sangue e o cérebro (GUILLEMIN et al., 2003). No cérebro a VQ está presente nos macrófagos e nas células microgliais (GUILLEMIN et al., 2003) e, em parte, nos astrócitos (GUILLEMIN et al., 2001a).

Após o Trp ser catabolizado pela IDO ou TDO em quinurenina (KYN), é adicionalmente catabolizado em 3-hidroxiquinurenina (OHK) pela enzima kynurenina-3-monoooxigenase (KMO ou K3MO). Após OHK, a degradação adicional continua a ácido 3-hidroxiantranilico (HAA) através da ação da quinunerinase. Depois disso, o catabolismo prossegue no fígado para a via de oxidação completa e forma ATP ou em ácido quinolínico (QUIN ou QA) que finalmente é degradado em NAD. Pelo outro braço da VQ o metabolito KYN também pode ser catabolizado pelas aminotransferases de quinurenina (KATs) em ácido quinurênico (KYNA) (MYINT et al., 2012).

No cérebro, o catabolismo do Trp ocorre principalmente nos astrócitos e na micróglia (GRANT et al., 2000). Enquanto os astrócitos produzem principalmente KYNA por falta de enzima KMO, microglia e macrófagos produzem principalmente QUIN (GUILLEMIN et al., 2005; GUILLEMIN et al., 2001b; GUILLEMIN et al., 2000). Os astrócitos metabolizam o QUIN produzido pela microglia vizinha (GUILLEMIN et al., 2001b). A via KYN também desempenha um papel no metabolismo da glicose, podendo ativar a via da glicólise ou inibir a gliconeogênese. Portando, sob condições fisiológicas normais, no cérebro, a VQ tem como principal função servir, principalmente para armazenamento de glicogênio e síntese de pequenas quantidades de NAD necessárias para o sistema nervoso central (MYINT et al., 2012).

Durante a inflamação, a formação de 3-hidroxiquinurenina (OHK) aumenta muito mais rapidamente do que a formação de ácido quinurênico (KYNA) e o equilíbrio entre a forma de OHK e KYNA é deslocado para o lado OHK. Na presença de inflamação, os monócitos ativados são os fortes produtores de QUIN (CHIARUGI et al., 2001a), a produção de QUIN persiste até que o

processo inflamatório seja concluído. Uma vez que alguns dos metabolitos KYN ativam uma reação inflamatória, isso poderia prolongar a síntese de QUIN, enquanto alguns dos metabólitos inibem a proliferação de células-T e células naturais killer (FRUMENTO et al., 2002) inibem o processo inflamatório adicional e impedem a formação de QUIN.

A VQ é altamente ativada no cérebro a partir de sua própria fonte central de KYN e de fontes periféricas. O ácido quinurênico (KYNA) provou ser neuroprotetor em vários cenários experimentais (KLEIN et al., 2013). Por outro lado, o ácido quinolínico (QUIN) é uma potente neurotoxina com uma propriedade adicional pela produção de radicais livres (KLIVENYI et al, 2004; SUNDARAM et al., 2014). Resultados de Guillemin et al., (2001a) sugerem que os astrócitos isolados são neuroprotetores, minimizando a produção de QUIN e maximizando a síntese do ácido quinurênico. No entanto, é provável que, na presença de macrófagos e/ou microglia, os astrócitos se tornem indiretamente neurotóxicos pela produção de grandes concentrações de quinurenina que podem ser secundariamente metabolizadas pela vizinhança ou pela infiltração de células monocíticas para formar a neurotoxina QUIN. Os metabólitos KYN contribuem diretamente para as alterações neuroprotetoras e neurodegenerativas no cérebro através de seus efeitos atingirem diretamente em várias rotas que envolvem o processo inflamatório e a via dos neurotransmissores (VAMOS et al., 2009).

No cérebro, o aumento da degradação de Trp induz uma baixa disponibilidade para síntese de 5-HT. Além disso, com inflamação e ativação de IDO, a serotonina é degradada não apenas pela monoamina oxidase em ácido 5-hidroxi-indol acético, mas também pela enzima IDO em formil-5-hidroxi quinurenina e, portanto, menos serotonina estará disponível para uma neurotransmissão serotoninérgica ideal (MYINT et al., 2012; Figura 3).

Figura 3. Metabolismo do triptofano pela Via da quinurenina.
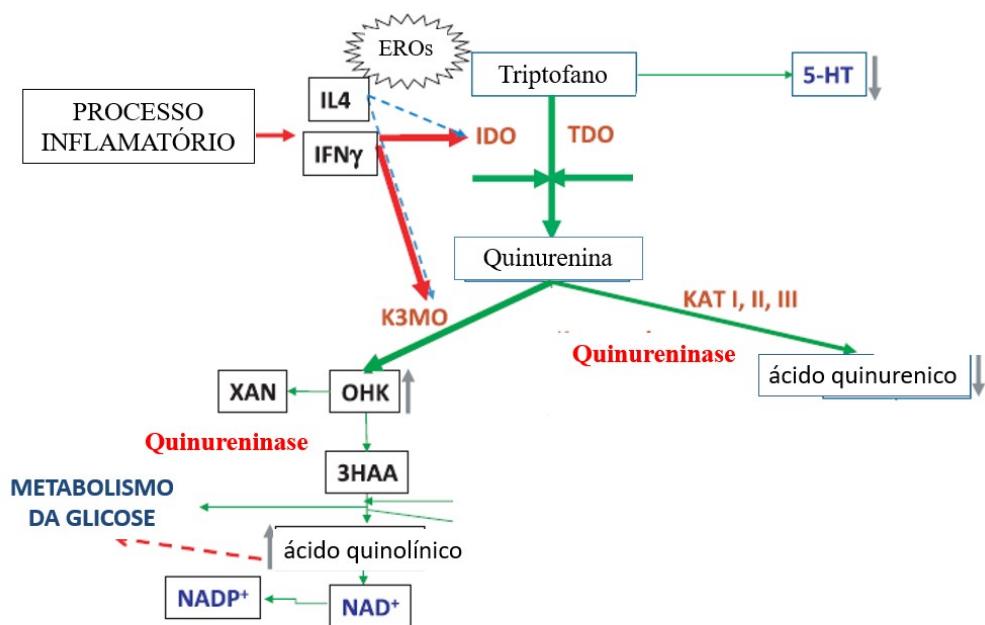
FONTE: Adaptado de Myint et al., 2012.

Inibidores da IDO

A IDO, a enzima chave na via da quinurenina, é acentuada por citocinas pró-inflamatórias, como o interferon-γ (IFN- γ) e inibidas pela citocinas anti-inflamatórias, como a interleucina-4 (IL-4) (MYINT et al., 2012; DE GOMES et al. 2018b; SOUZA et al. 2017b)(Figura 3). A atividade do KMO também é reforçada por citocinas pró-inflamatórias (MYINT et al., 2012). Portanto, sabendo que citocinas pró-inflamatórias estão relacionadas com o aumento da atividade da enzima IDO, para verificar a influência e o papel dos metabolitos da via sobre as diferentes doenças, além do uso de animais knockout, usa-se inibidores dessa enzima. A família de genes da IDO incluem a IDO1 e a IDO2. Comumente é mais utilizada a IDO1, pois já se sabe que esse gene tem maior eficiência catalítica e é mais abundante nos tecidos do que a IDO2 (WANG et al., 2020).

Dos inibidores da IDO os mais utilizados na literatura são o INCB024360 e o 1 -MT, mas também são encontrados outros inibidores da IDO na literatura, o que difere é que são divididos pelo tipo de inibição (competitiva ou não) e pelo solvente utilizado para os ensaios cinéticos (TOURINO, 2012; Tabela 1).

Tabela 1. inibidores da ido já descritos na literatura, divididos pelo tipo de inibição (competitiva ou não) e solvente utilizado para os ensaios cinéticos.

Solvente	Nome Inibidores Não Competitivas
MeOH	Adociaquinona B
	Adociaquinona A
	Annulin B
	Annulin C
	Naftoquinona
	Annulin A
	Warfarina
	Cumarina
	Dihidroquinona
Tampão	DMT
	4-fenilimidazol
	2-mercaptol-benzotiazol
	2-mercapto-feniltiazol
	Feniltiazol
	Norharman
DMSO	TRY
Solvente	Nome Inibidores Competitivas
Tampão	1-MT
DMSO	Brassinina
	5-br-brassinina

Fonte: Adaptado de Tourino, Melissa Cavalheiro (2012); Cady, S. G. (1991); Banerjee, T. (2007); Sono, M. (1989), Rohring, U.F. (2010), Andersen, R. J. (Patente WO 2006/005185,2006)

O 1-metil-triptofano (1-MT) é um inibidor competitivo da IDO-1 e alvo de estudos clínicos. O 1-MT é encontrado nas formas enantioméricas D- e L- e embora o enantiômero 1-L-MT seja mais eficiente na inibição da IDO-1, é o 1-D-MT o enantiômero mais eficiente na redução experimental de tumores. Esse dado sugere que o 1-MT pode ter ações adicionais à inibição da IDO (RAJDA et al., 2015).

O INCB024360, de nome comercial Epacadostat, é um inibidor seletivo da enzima IDO-1, que foi desenvolvido e está atualmente sob experimentação clínica em vários tipos de doenças, alguns tipos de câncer, como o de ovário (BEATTY et al., 2017; KRISTELEIT et al., 2017). A estrutura molecular do INCB24360 contém vários grupos funcionais anteriormente desconhecidos ou subutilizados em substâncias medicamentosas, incluindo hidroxiamidina, furazano, brometo e sulfamida (Figura 4). Esse conjunto de substâncias in vitro são consistentes com a boa permeabilidade celular e também com a biodisponibilidade oral observada em todas as espécies (rato, cão, macaco) analisadas (YUE et al., 2017).

Figura 4. Estrutura química do INCB024360
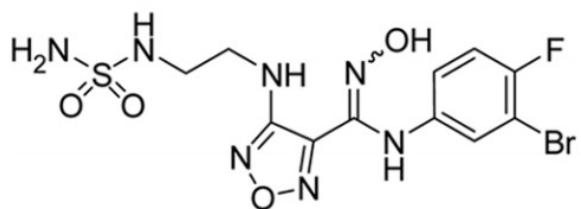
Epacadostat (INCB24360) **4f**

Fonte: Adaptado de YUE et al., 2017.

3   JUSTIFICATIVA

Podemos salientar que a imprevisibilidade da doença é a sua marcante característica e isto interfere nos sintomas e na reação do paciente frente aos mesmos e aos tratamentos oferecidos, além da forma como reage consigo mesmo, com relação à família, trabalho e sociedade. Um ponto importante é o surgimento da doença que ocorre principalmente na fase socialmente produtiva. Não podendo assim deixar de se verificar o impacto social, pessoal e econômico que pode ocorrer na vida do indivíduo e da sociedade, uma vez que a medida que a doença progride, sintomas motores debilitantes se desenvolvem, podendo levar eventualmente a paralisia completa e até a morte. Além disso, investigar novos alvos e mecanismos envolvidos nas doenças autoimunes do SNC, faz com que possamos chegar mais próximo de um possível tratamento ou a compreensão do desenvolvimento dessa doença, uma vez que essa doença não tem cura e apresenta diversas manifestações clínicas.

4   OBJETIVOS

Objetivo Geral

O objetivo desse estudo foi investigar a participação dos metabolitos e das enzimas da via da quinurenina em um modelo de Encefalomielite Autoimune Experimental (EAE) em camundongos.

Objetivos Específicos

- Investigar o efeito do inibidor da IDO na EAE em camundongos.

- Averiguar a participação dos metabolitos e das enzimas da via da quinurenina na EAE em camundongos.

- Analisar a influência da via da quinurenina sobre os parâmetros inflamatórios na EAE.

- Verificar a participação de IBA-1 e GFAP na EAE.

- Revisar as evidências sobre o papel da via da quinurenina como um possível biomarcador para e esclerose múltipla, pontuando sobre as principais vias de diagnóstico em humanos.

5   PARTE II

ARTIGO: Involvement of Indoleamine-2,3-Dioxygenase and Kynurenine Pathway in Experimental Autoimmune Encephalomyelitis in Mice

ORIGINAL PAPER

Involvement of Indoleamine-2,3-Dioxygenase and Kynurenine Pathway in Experimental Autoimmune Encephalomyelitis in Mice

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Abstract

The experimental autoimmune encephalomyelitis (EAE) is a model that mimics multiple sclerosis in rodents. Evidence has suggested that the activation of indoleamine-2,3-dioxygenase (IDO), the rate-limiting enzyme in the kynurenine pathway (KP), plays a crucial role in inflammation-related diseases. The present study aimed to investigate the involvement of the inflammatory process and KP components in a model of EAE in mice. To identify the role of KP in EAE pathogenesis, mice received IDO inhibitor (INCB024360) at a dose of 200 mg/kg (per oral) for 25 days. We demonstrated that IDO inhibitor mitigated the clinical signs of EAE, in parallel with the reduction of cytokine levels (brain, spinal cord, spleen and lymph node) and ionized calcium-binding adaptor protein-1 (Iba-1) gene expression in the central nervous system of EAE mice. Besides, IDO inhibitor causes a significant decrease in the levels of tryptophan, kynurenine and neurotoxic metabolites of KP, such as 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN) in the prefrontal cortex, hippocampus, spinal cord, spleen and lymph node of EAE mice. The mRNA expression and enzyme activity of IDO and kynurenine 3-monooxygenase (KMO) were also reduced by IDO inhibitor. These findings indicate that the inflammatory process concomitant with the activation of IDO/KP is involved in the pathogenic mechanisms of EAE. The modulation of KP is a promising target for novel pharmacological treatment of MS.

Keywords Autoimmune disease · Central nervous system · Glial activation · Inflammation · Tryptophan metabolism · Multiple sclerosis

Introduction

Multiple sclerosis (MS) is an inflammatory neurodegenerative disease characterized by neuronal demyelination in the central nervous system (CNS), which is related to an autoimmune response against components of myelin [1]. MS is marked by neurological disability, including sensorial, motor, autonomic and neurocognitive deficits. Affecting around 2.5 million individuals worldwide, the prevalence and incidence of MS are higher for women than men (ranging female to male ratio of 2:1) [2].

Although genetic and environmental factors are implicated in the pathogenesis of MS, the molecular mechanisms underlying the pathophysiology of this disease is not fully elucidated. It is believed that autoreactive T cell responses have a crucial role in the pathogenesis of MS [1–3]. Indeed, it has been proposed that MS occurs in genetically predisposed individuals following exposure to an environmental trigger that activates myelin-specific T cells. Upon activation, the myelin-specific CD4+ and CD8+ T cells cross the blood–brain barrier (BBB) and are reactivated by CNS-resident antigen-presenting cells (APCs), e.g. perivascular macrophages and dendritic cells. The reactivation of T cells by APSc in the CNS triggers the recruitment of innate immune system that has a crucial role in mediating demyelination and axonal damage [1].

Previous studies have reported that CNS autoimmunity can be mediated by two distinct lineages of autoreactive T cells that result in T cell differentiation, such as T-helper type 1 (Th-1) or T-helper type 17 (Th-17). These T cells subtypes orchestrate an immune response against myelin, causing inflammatory lesions distributed throughout the CNS [1,

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4]. In this context, the experimental autoimmune encephalomyelitis (EAE) is a rodent model that mimics MS. This model is characterized by an autoimmune response against proteins of the CNS, which culminates in the inflammatory infiltrate, gliosis, deterioration of myelin sheath and neuronal death [1, 2, 5, 6].

A cure for MS is not currently known and the available treatment does not effectively change the course of the disease, as well as often associated with adverse effects. Thus, it is necessary to identify new molecular targets involved in the pathogenesis of MS, to provide new insights for the development of novel pharmacological treatments for this disease.

The kynurenine pathway (KP) is the main metabolic route for tryptophan (TRP) degradation in most mammalian tissues, including the brain [7–9]. The rate-limiting step of KP is the conversion of TRP to kynurenine (KYN) by indoleamine 2,3-dioxygenase (IDO) in extrahepatic tissues, occurring mainly in the blood and lymphoid tissues [10, 11]. Although some neurons also contain IDO in the brain, TRP catabolism occurs mainly in astrocytes and microglia [12–14].

The IDO induction is stimulated by proinflammatory cytokines such as interferon-gamma (IFN-γ) [15, 16] and this enzyme is inhibited by anti-inflammatory cytokines such as interleukin-4 (IL-4) [8, 9]. In neurological diseases, microglia and macrophages are highly responsive to IFN-γ, which upregulates the expression of KP enzymes, including IDO-1 and kynurenine 3-monooxygenase (KMO), which culminates in the production of neurotoxic metabolites such as quinolinic acid (QUIN). These neurotoxic metabolites circulate systemically or are released locally in the brain and can contribute to the excitotoxic death of oligodendrocytes and neurons [17]. It has been already known that some metabolites of KP possess neuroprotective or neurotoxic role; the 3-hydroxykynurenine (3-HK) and QUIN derived from microglia are neurotoxic whereas kynurenic acid (KYNA) generated by astrocytes is neuroprotective [7, 14, 18, 19].

Emerging studies from the past decade have indicated that KP exerts important immunomodulatory roles in the pathology of neurodegenerative disorders, anxiety-depression states, pain syndromes and autoimmune diseases [8, 9, 20, 21]. For instance, experimental and indirect studies have been demonstrated that IDO is overactivated in various autoimmune disorders, including MS [20, 22–24]. It is presumed that this activation is an endogenous immunosuppressive response to counteract the autoreactive process; however, the toxic levels reached by known neurotoxic catabolites of KP in EAE and MS are currently under-investigated [20]. It has been reported that the inhibition of IDO with 1-methyltryptophan (1-MT) and IDO knockout (IDO$^{-/-}$) mice increased the severity of clinical symptoms of EAE [25–27]. Conversely, studies evaluating the effect of Epacadostat

(INCB024360) (a potent and selective IDO-1 inhibitor with immunomodulatory action and good cell permeability and oral bioavailability) [28] were not found in the literature to assess the involvement of the KP in modulating the clinical symptoms of EAE.

Therefore, we sought to investigate the relationship between the levels of neuroactive metabolites of KP in the CNS with the clinical course of EAE in mice treated with MOG peptide. For this aim, the IDO inhibitor (INCB024360) was administered to MOG-treated mice and the body weight, clinical signs and parameters of inflammation and KP dysregulation were analyzed in the inguinal lymph nodes, brain and spinal cord.

Materials and Methods

Animals

Experiments were conducted using adult C57BL/6 female mice (20–25 g, 90 days old). Animals were housed, divided into groups of 6 in Plexiglas cages (41 cm × 34 cm × 16 cm) with the floor covered with sawdust. They were kept in a room with a light–dark cycle of 12 h with the lights on between 7:00 and 19:00 h and temperature controlled (20–25 ℃) and received water and food ad libitum. All protocols were previously approved by the Animal Ethics Committee of the Federal University of Pampa (CEUA protocol number: 034/2017).

EAE Induction and IDO Inhibitor Administration

EAE is a useful model for the investigation of immunological mechanisms responsible for the inflammatory autoimmune process in MS [29]. The animals were randomly allocated into 4 groups (n = 6 per group; total number = 32): (A) vehicle/naïve (sham control); (B) INCB024360/naïve; (C) vehicle/EAE; and (D) INCB024360/EAE. EAE was induced by subcutaneous (s.c.) immunization into the flanks with 200 μl of emulsion containing 200 μg of myelin oligodendrocyte glycoprotein 35–55 peptide (MOG35–55) and 500 μg of *Mycobacterium tuberculosis* extract H37RA (Difco Laboratories, Detroit, MI, USA) in incomplete adjuvant Freund (Sigma Chemical Co., St. Louis, MO, USA) as previously described [30]. MOG35–55 is a potent encephalitogenic peptide in C57BL/6 mice, and immunization with this peptide leads to chronic progressive disease [29]. This procedure was repeated after 7 days to increase the incidence of EAE [31]. In addition, the animals received 300 ng of Pertussis toxin (PT) (Sigma Chemical Co., St. Louis, MO, USA) i.p. on day 0 and day 2 post-immunization. PT as adjuvant, which is thought to enhance EAE induction by increasing BBB permeability, expanding myeloid cells

and antigen-specific T lymphocytes, reducing regulatory T lymphocytes, and modulating expression of inflammatory cytokines [32]. The vehicle/naïve mice received PBS instead MOG35–55 peptide.

The clinical signs of EAE were evaluated according to the following scores: 0, with no signs of disease; 1, loss of tone in the tail; 2, paresis of the hind limbs; 3, paralysis of the hind limbs; 4, tetraplegia; and 5, dying and/or death [30, 31, 33].

The IDO inhibitor Epacadostat (INCB024360) (Incyte Corporation) was dissolved in 3% N,N-dimethylacetamide and 10% (2- hydroxypropyl) β-cyclodextrin and administered by oral gavage at 200 mg/kg every day for 25 days [34]. INCB024360/vehicle group received PBS (per oral). Mice were weighed and observed daily for clinical signs of EAE starting on day 0 until day 25 post-immunization. Day 7 was the day when the peculiar clinical signs became apparent. Mice that did not develop the disease were excluded from the study. The experimental procedure is explained in Fig. 1.

Tissue Preparation for Ex-Vivo Assays

After the mean clinical score evaluation, animals were euthanized with barbiturate overdose (pentobarbital sodium 150 mg/kg; i.p. route) and the prefrontal cortex, hippocampus, spinal cord, spleen and inguinal lymph nodes were

dissected, removed, weighed and homogenized in 50 mM Tris–HCl, pH 7.4. The homogenate was centrifuged at $2400 \times g$ for 15 min at 4 °C, and a low-speed, supernatant fraction (S1) was used for assays.

Measurement of IFN-γ and Interleukin-17 (IL-17) Levels

Levels of IFN-γ and IL-17 in the prefrontal cortex (PFC), hippocampus (HC), spinal cord (SC), spleen and lymph node (LN) were measured using sample aliquots of 100 µl and mouse cytokine ELISA DuoSet Kits from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's instructions (protein range of 31.25–2000 pg). The level of cytokine was estimated by interpolation from a standard curve by colorimetric measurements at 450 nm (correction wavelength 540 nm) on an ELISA plate reader (Berthold Technologies-Apollo 8-LB 912, KG, Germany). Results are shown as pg/mg of protein.

RNA Extraction and Quantitative Real-Time-Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted using the Trizol protocol. The reverse transcription assay was carried out as described in the M-MLV Reverse Transcriptase (Invitrogen, Carlsbad,
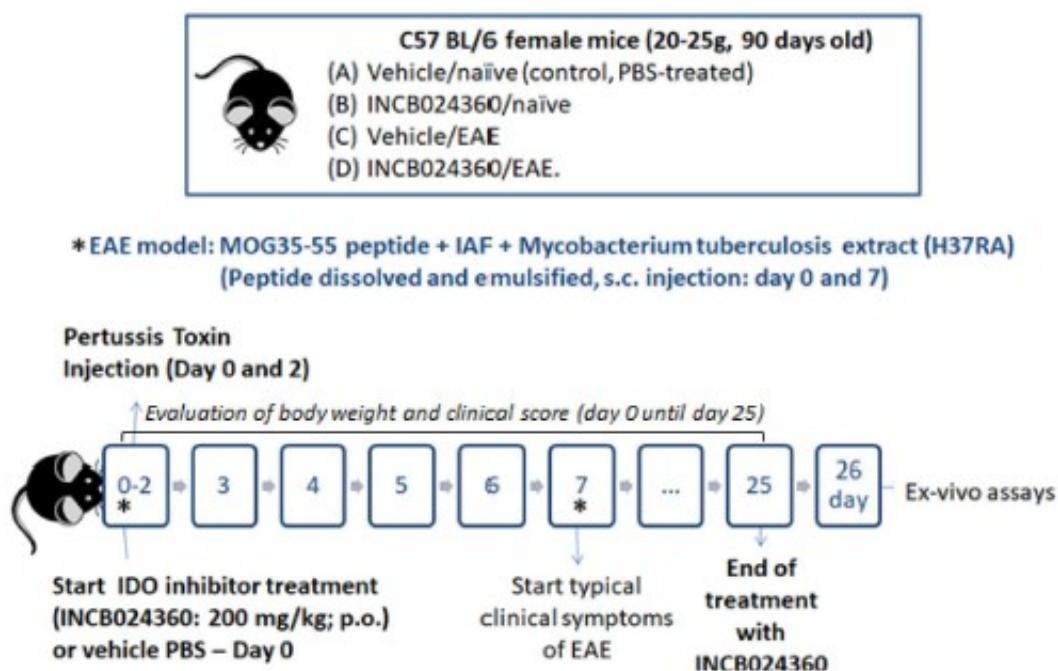

Fig. 1 Overview of study design. *EAE* experimental autoimmune encephalomyelitis; *MS* multiple sclerosis; *MOG35–55* Myelin oligodendrocyte glycoprotein; *INCB024360* indoleamine-2,3-dioxygenase inhibitor

CA, USA), according to the manufacturer's instructions. Real-time quantitative PCR analysis of mRNA was performed as described previously [33], in StepOnePlusTM using the TaqManH Universal PCR Master Mix Kit (Applied Biosystems, Foster City, CA, USA) for quantification of the amplicons, and 100 ng of cDNA were used in each reaction. The cDNA was amplified in triplicate using specific TaqMan Gene Expression target genes, the 39 quencher MGB and FAM-labelled probes used for detect Iba1 (Mm00479862_g1), GFAP (Mm01253033_m1), IDO-1 (Mm00492586_m1), KMO (Mm00505511_m1), KAT-II (Mm00496169_m1) and GAPDH (Mm99999915_g1), which were obtained from Applied Biosystems (Foster City, CA, USA). The housekeeping gene GAPDH was used as an endogenous control to normalize gene expression data.

IDO, KMO and KAT Activities

IDO activity in the PFC, HC, SC, spleen and LN was determined as previously described [35], with minor modifications. The amount of the enzyme as expressed in terms of its heme content based on the absorbance at 406 nm. The S1 (0.2 ml) was added to 0.8 ml of the reaction mixture containing 400 µM L-TRP, 20 mM ascorbate, 10 µM methylene blue, and 100 µg catalase in 50 mM potassium phosphate buffer pH 6.5. The reaction was carried out at 37 °C under agitation for 60 min. Then, it was blocked by adding 0.2 ml of 30% trichloroacetic acid and further incubated at 50 °C for 30 min to convert the N-formylkynurenine to L-KYN. Samples were centrifuged at 13,000g for 10 min at 4 °C. The supernatants were filtered through microspin ultrafiltrates with a cut-off of 10,000M, before being taken for measurement of IDO. The amount of L-KYN formed from TRP was determined by reversed phase high pressure liquid chromatography (HPLC). One hundred µl of the reaction product was injected onto a Merck LiChrospher column (150 mm long, 4.6 mm diameter, packed with 5 lm silica beads holding 18C long carbon chains). A cartridge guard column containing the same material as the analytical column was used. The mobile phase consisted of 0.1 M ammonium acetate buffer (pH 4.65) with 5% acetonitrile. Flow rate was 1 ml/min. KYN was detected using a spectrometer measuring absorbency at a wavelength of 365 nm and was quantified using known amounts of L-kynurenine. The retention time of KYN was around 5.35 min. All determinations were performed in duplicate. One unit of the activity was defined as 1 nmol KYN/h/mg protein at 37 °C.

For the determination of KMO activity in the PFC, HC, SC, spleen and LN, tissues were homogenized 1:5 (wt/vol) in ultrapure water and further diluted 1:5 (vol/vol) in 100 mM Tris–HCl buffer (pH 8.1) containing 10 mM KCl and 1 mM ethylenediamine tetraacetic acid (EDTA). Eighty µl of the tissue preparation were incubated for 40 min at 37 °C in a solution containing 1 mM nicotinamide adenine dinucleotide phosphate (NADPH), 3 mM glucose-6-phosphate, 1 U/ml glucose-6 phosphate dehydrogenase, 100 µM KYN, 10 mM KCl and 1 mM EDTA in a total volume of 200 µl. The reaction was stopped by the addition of 50 µl of 6% perchloric acid. Blanks were obtained by adding the specific enzyme inhibitor Ro 61-8048 (100 µM) in the incubation solution. After centrifugation (16,000×g, 15 min), 20 µl of the supernatant were applied to HPLC to measure 3-HK.

The KAT activity was performed according to the method of Guidetti et al. [36]. Briefly, the PFC, HC, SC, spleen and LN were harvested and homogenized in distilled water. After centrifugation (12,000g, 10 min), KAT activity was measured in a total volume of 200 µl, containing 80 µl of supernatant fluid, 150 mm Tris–acetate buffer, pH 7.4, 2 lm KYN, 1 mm pyruvate and 80 lm pyridoxal-5-phosphate. Samples were incubated for 24 h at 37 °C, and the reaction was terminated by adding 50% (w/v) trichloroacetic acid. After successive washes with 0.1 m HCl and distilled water, KYNA was eluted from the column with 2 × 1 ml of distilled water and quantified by HPLC.

Analysis of TRP and KYN Levels

The levels of TRP and its metabolite KYN in the PFC, HC, SC, spleen and LN were performed in a Shimadzu LC-10A liquid chromatograph, according to Cooper et al. [37]. The chromatographic separation was achieved using a 250- by 4.6-mm (inner diameter) C18 reverse-phase column (particle size, 4 lm; Aquapore RP-300 C-18). For Trp measurement, the column was eluted isocratically at flow rate of 1.0 ml/min with 0.015 M sodium acetate (pH 4.5) containing 15% methanol. For KYN determination, the column was eluted with acetonitrile at a 1:47 dilution in 0.1 M acetic acid–0.1 M ammonium acetate (pH 4.65). The peaks of TRP or KYN were identified by comparison with the retention times of standard compounds (Sigma), and quantification was based on the ratios of the peak areas of compound to the internal standard. The mobile phase contained 50 nM glacial acetic acid, 100 mM zinc acetate and 3% acetonitrile dissolved in double-distilled NANO pure water HPLC grade H_2O. The pH was adjusted to 4.9 using 5 M NaOH. Supernatant of prefrontal cortex, hippocampus, spinal cord, spleen and lymph node were sonicated in 1 ml of mobile phase containing 7% perchloric acid spiked with 50 ng/20 µl of N-methyl 5-HT as an internal standard. The resultant solution was centrifuged at 44,800g for 20 min and the supernatants were placed into new Eppendorf tubes using a syringe fitted with a 0.45-µm filter (Phenomenex). Twenty µl of the filtered supernatant was injected using a Waters auto sampler and a Reverse Phase analytical column (Kinetex™ Core Shell Technology column with specific area of 4.6 mm and particle size of 2.6 µl, Phenomenex) was used for the separation

of metabolites. A PDA-UV detector (Shimadzu SPD-M10A VP), calibrated to integrate at 230 and 250 nm, as well as a fluorescent detector (Shimadzu RF- 20 A XS prominence fluorescence detector), set to excitation wavelength 254 nm and emission wavelength 404 nm, were used to detect the metabolites. Chromatographs were generated by CLASS-VP software (Shimadzu). The results are expressed as pg/mg protein.

Analysis of KYNA, 3-HK and QUIN Levels

KYNA, 3-HK and QUIN levels were measured in the PFC, HC, SC, spleen and LN samples using HPLC [16]. The mobile phase contained 50 nM glacial acetic acid, 100 mM zinc acetate and 3% acetonitrile dissolved in double-distilled NANO pure water HPLC grade H_2O. The pH was adjusted to 4.9 using 5 M NaOH. S1 of PFC and HP were sonicated in 1 ml of mobile phase containing 7% perchloric acid spiked with 50 ng/20 µl of N-methyl-5-HT as an internal standard. The resultant solution was centrifuged at 20,000 rpm for 20 min and the supernatants were placed into new Eppendorf tubes using a syringe fitted with a 0.45-µm filter (Phenomenex). Approximately 20 µl of the filtered supernatant was injected using a Waters auto sampler and a Reverse Phase analytical column (Kinetex™ Core Shell Technology column with specific area of 4.6 mm and particle size of 2.6 µl, Phenomenex) was used for the separation of metabolites. A PDA-UV detector (Shimadzu SPD-M10A VP), calibrated to integrate at 230 and 250 nm, as well as a fluorescent detector (Shimadzu RF-20A XS prominence fluorescence detector), set to excitation wavelength 254 nm and emission wavelength 404 nm, were used to detect the metabolites. Chromatographs were generated by CLASS-VP software (Shimadzu). The results are expressed as ng/g tissue.

Protein Determination

Protein concentration was measured by the method Bradford [38], using bovine serum albumin as the standard.

Statistical Analysis

The data distribution was verified by applying the Kolmogorov–Smirnov test. Comparisons between the experimental and control group were performed by two-way analysis of variance (ANOVA), followed by Bonferroni post hoc test when appropriate. Nonparametric comparisons were performed by the Kruskal–Wallis test, with Dunn's post-hoc test comparisons to analyze clinical score data. A value of $p < 0.05$ was considered to be significant. All tests were carried out using the GraphPad software 6.0 (San Diego, CA, USA).

Results

IDO Inhibitor, INCB024360, Alleviates Clinical Signs and Body Weight Loss Induced by EAE

Peculiar clinical signs, e.g. tail atony and clumsy gait, appeared on day 7 post-MOG-injections and continue elevated until 25 on the vehicle/EAE group, as demonstrated by mean clinical score (Fig. 2a). Statistical analysis revealed that the control group differed from EAE in day 10 until day 25. In days 10–12 and 15–25 EAE differed from IDO inhibitor-treated EAE (Figs. 1a, 2a). Treatment with IDO inhibitor afforded significant improvement of clinical signs in EAE mice, as measured by the area under the curve (AUC) (Fig. 2b). Moreover, IDO inhibitor administration prevented the marked loss of body weight elicited by EAE (Fig. 2c). These results suggest that IDO/KP activation contributes to the appearance of clinical signs in EAE model.

Cytokine Levels in the PFC, HC, SC, Spleen and LN

EAE increased IFN-γ and IL-17 levels in the PFC, HC, SC, spleen and LN and IDO inhibitor (200 mg/kg) partially reverted this increase, indicating that IDO is implicated in the proinflammatory response of EAE (Table 1).

Effect of EAE and INCB024360 on GFAP and Iba-1 mRNA Levels

The results of RT-PCR analyses are presented as fold-changes relative to the naïve group received vehicle. The analysis of GFAP (astrocytes) and Iba-1 (microglia) mRNA levels showed that MOG35–55/CFA peptide and PT significantly increased the expression of Iba-1 gene in the PFC, HC and SC of EAE animals. The expression of Iba-1 gene was attenuated by IDO inhibitor (INCB024360) at the dose of 200 mg/kg (p.o.). In contrast, no significant modification was observed in GFAP (Fig. 3a–f). These findings suggest that IDO plays a role in the increase of Iba-1 gene expression in EAE mice.

Effect of INCB024360 on KP Metabolites in EAE Mice

In response to EAE model, a significant increase in TRP levels was found in the PFC, HC, and SC when compared to the naive/vehicle group. In contrast, TRP levels in IDO inhibitor/EAE group showed a significant decrease when compared to the control mice (naïve/group). A per se effect of IDO inhibitor was observed in the PFC, HC and SC, as revealed by the significant decrease in TRP levels in IDO inhibitor/vehicle group. In addition, EAE-treated
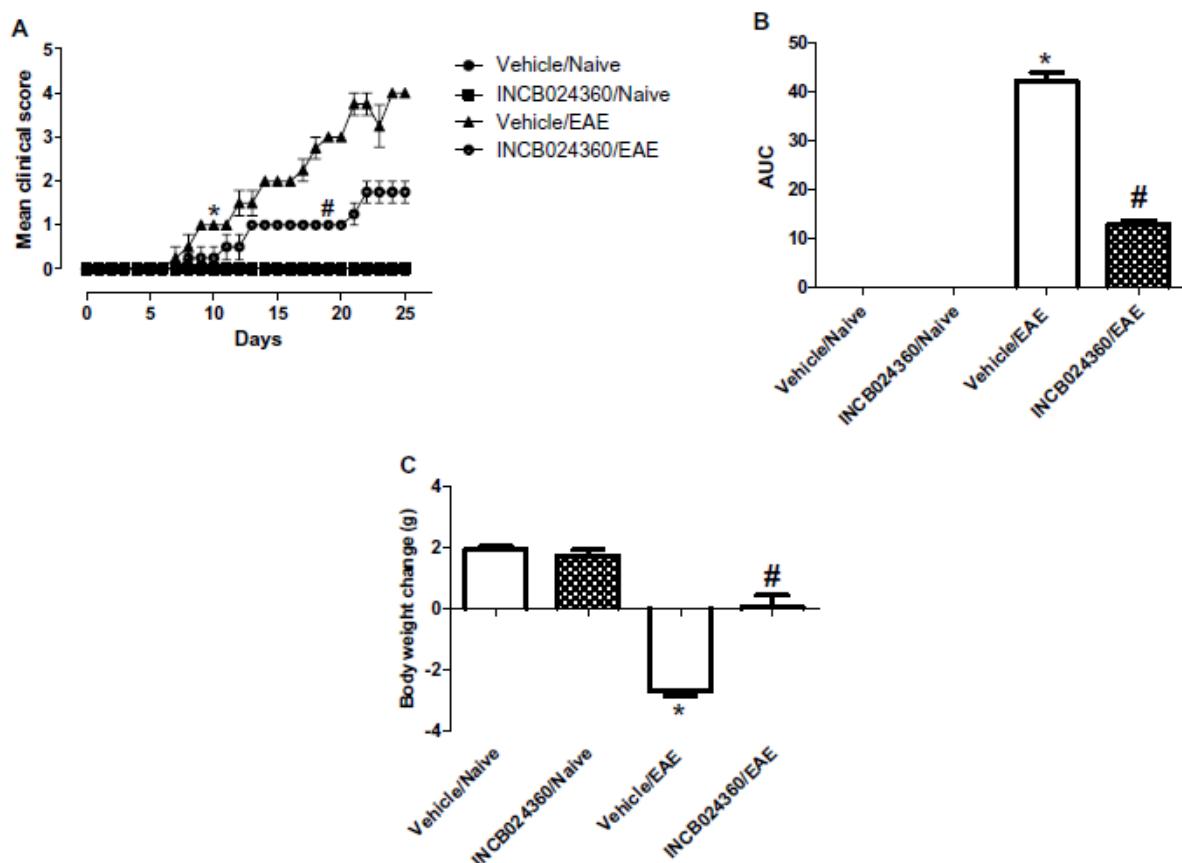
Fig. 2 Effect of IDO inhibitor (INCB024360) in the clinical signs induced by EAE in mice. Mean clinical score (**a**), area under the curve (AUC) (**b**) and body weight change (**c**) in an EAE model induced by immunization with MOG35–55/CFA peptide and pertussis toxin. The 100% incidence of clinical score 4 (tetraplegia) was observed in day 25 in EAE mice. Mice treated with IDO inhibitor displayed 83% of incidence of clinical score 2 (paresis of the hind limbs) and 17% of incidence of clinical score 1 (loss of tone in the tail) 25 days post-MOG injection (**a**). Statistical analysis revealed that IDO inhibitor significantly mitigates the severity of mean clinical score in EAE mice. Values of mean clinical score were analyzed by Kruskal–Wallis test, with Dunn's post-hoc test. Values of AUC and body weight were analyzed by 2-way ANOVA and Bonferroni multiple comparison test. Each value is expressed as mean ± S.E.M. (n = 6). *p < 0.05 when compared to vehicle/naive and #p < 0.05 when compared to Vehicle/EAE

mice showed a significant increase in KYN levels and KYN/TRP ratio in the PFC, HC, SC, spleen and LN when compared to control counterparts. Moreover, IDO inhibitor normalized the elevated KYN levels in the PFC, HC, SC and spleen and mitigated KYN levels in the LN. IDO inhibitor also restored KYN/TRP ratio in these organs. (Table 2).

EAE/vehicle demonstrated a significant increase in the levels of the neurotoxic metabolites 3-HK and QUIN in the PFC, HC, SC, spleen and LN when compared to control group. In contrast, IDO inhibitor abolished this effect. We did not found any significantly changes in the levels of neuroprotective kynurenic acid (KYNA) in the all tissues analyzed (Figs. 4a–f, 5a–f, 6a–c).

These findings indicate that KP metabolites are increased in the CNS and lymphoid organs of EAE mice, and IDO inhibition prevents this alteration.

EAE Increased the Gene Expression and Activity of IDO, KMO Without Affecting KAT

The IDO, KMO and KAT gene expression (mRNA levels) and activity were analyzed in the PFC, HC, SC, spleen and LN. EAE/vehicle group increased the IDO-1 and KMO mRNA levels in the PFC, HC, SC, spleen and LN compared to the control group. IDO-1 inhibitor partially prevented these increases in the gene expression of IDO-1 and KMO in all tissues of EAE mice. No significant differences were

Table 1 Effect of experimental autoimmune encephalomyelitis (EAE) and indoleamine-2,3-dioxygenase inhibitor (INCB024360) on the cytokine levels interferon-gamma (IFN-γ) and interleukin-17 (IL-17)

(pg/mg protein)	Vehicle		INCB024360	
	Naive	EAE	Naive	EAE
Prefrontal cortex				
IFN-γ	21.1±1.02	91.8±5.11[a]	19.7±0.95	28.9±2.01[b]
IL-17	9.17±0.56	48.8±1.01[a]	12.3±1.22	17.8±0.96[b]
Hippocampus				
IFN-γ	28.9±1.44	124.8±6.89[a]	32.8±1.49	44.2±2.31[b]
IL-17	14.8±1.71	60.2±3.29[a]	18.7±1.20	21.8±1.35[b]
Spinal cord				
IFN-γ	34.8±2.10	178.8±8.10[a]	37.8±2.11	48.9±2.88[b]
IL-17	24.4±1.37	59.7±1.99[a]	30.7±3.17	34.4±2.91[b]
Spleen				
IFN-γ	78.5±6.01	247.8±5.74[a]	89.7±5.41	102.8±9.11[b]
IL-17	44.1±2.38	127.9±3.01[a]	50.8±4.19	55.9±4.91[b]
Lymph node				
IFN-γ	67.9±5.10	177.8±8.54[a]	80.1±7.49	85.7±6.71[b]
IL-17	37.8±2.10	97.7±5.67[a]	42.8±2.63	47.7±2.31[b]

The values were analyzed by two-way ANOVA and Bonferroni multiple comparison test. Each value is expressed as the mean±S.E.M. (n=6)

[a] $p < 0.05$ when compared with vehicle/naive

[b] $p < 0.05$ when compared with vehicle/EAE

found in KAT-II mRNA levels between the experimental groups (Figs. 7a–f, 8a–f, 9a–c). Besides, the increase in the IDO and KMO activity was prevented by IDO inhibitor in the PFC, HC, SC, spleen and LN (Table 3). These results suggest that both activation and gene expression of IDO and KMO are increased in the CNS and lymphoid tissues of EAE mice.

Discussion

Despite the advances obtained in recent years, much remains to be clarified about the etiological and pathological events governing inflammatory and demyelinating diseases of the CNS, such as MS. Due to the limitation found in the development of more extensive ante-mortem studies with humans, most of the information available is derived from animal models, such as murine EAE [39]. In this study, we investigated the effects of treatment with oral administration of a potent IDO inhibitor Epacadostat (INCB024360, 200 mg/kg), a competitive tryptophan inhibitor, in EAE induced by immunization with MOG35–55/CFA peptide and PT in female mice. We aimed to identify where the induction of clinical signs is related to the activation of IDO and neurotoxic metabolites of KP.

IDO Inhibitor (INCB024360) Ameliorates Clinical Signs of EAE

It has been reported that the first hallmarks of illness in the MOG-induced EAE model is the loss of body weight and the clinical signs as tail tonus and hand limb paralysis [40]. Here, we observed that EAE/vehicle group significantly lost weight and exhibited the highest score of disease (about four points in the grading scale of disease). This result is in accordance with a previous report [40] and a recent study of our laboratory [30]. The administration of IDO inhibitor significantly prevented the EAE symptomology, alleviating clinical scores when compared to control counterparts.

These results are in contrast with previous studies where the pharmacological blockage of IDO with 1-MT [25–27] and IDO-deficient mice [27] enhanced the severity of EAE, exacerbating the clinical parameters. Although the IDO activation is thought to be an endogenous self-protective response in MS [20], it is of note that increased IDO activation during CNS inflammation leads to a higher elevation in neurotoxic QUIN than neuroprotective KYNA [41]. This hypothesis was proposed by Kwidzinski and Bechmann [42]. The authors have suggested that the anti-inflammatory efforts of IDO activation might act as a double-edged sword and contribute to the neurodegenerative aspect of MS. Another important explanation for the discrepancy of our findings is the highly variable course of the disease, and the relapsing and remitting nature of neuroinflammation and clinical signs in MS [20]. Hence, we provide evidence that IDO inhibition improves the clinical signs of EAE in mice.

IDO Inhibitor (INCB024360) Blunted the Inflammatory Response in EAE Mice

We have recently shown that EAE-treated mice showed an increase in the proinflammatory cytokines IFN-γ and IL-17 in the spleen, PFC, HC and SC [30]. Here, we demonstrate that IDO inhibitor administration (200 mg/kg; p.o) during 25 days blunted the increase of IFN-γ and IL-17 level in the PFC, HC, SC, spleen and LN of EAE mice. Th1 and Th17 cells are the two major pathogenic mechanisms in CNS autoimmunity [27, 43]. IFN-γ, the hallmark cytokine of Th1 cells and IL-17, the hallmark cytokine of Th17 cells, plays an important role in EAE pathogenesis [43, 44]. IFN-γ promotes oligodendrocyte death, thus inducing demyelination [45]. In addition, transgenic mice overexpressing IFN-γ develop spontaneous inflammatory demyelinating disease [46]. Therefore, our study supports the notion that these cytokines may cause the mobilization of precursor cells in the brain during acute inflammatory processes in the CNS, initiating repair mechanisms. These findings indicate an important role for IFN-γ signaling in autoimmune CNS inflammation.

Fig. 3 Effect of EAE and IDO inhibitor (INCB024360) on the mRNA levels of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor protein-1 (Iba-1) in the prefrontal cortex (**a, b**), hippocampus (**c, d**) and spinal cord (**e, f**). Values were analyzed by 2-way ANOVA and Bonferroni multiple comparison test. Each value is expressed as mean ± S.E.M. (n = 6). *p < 0.05 when compared to vehicle/naive and #p < 0.05 when compared to vehicle/EAE
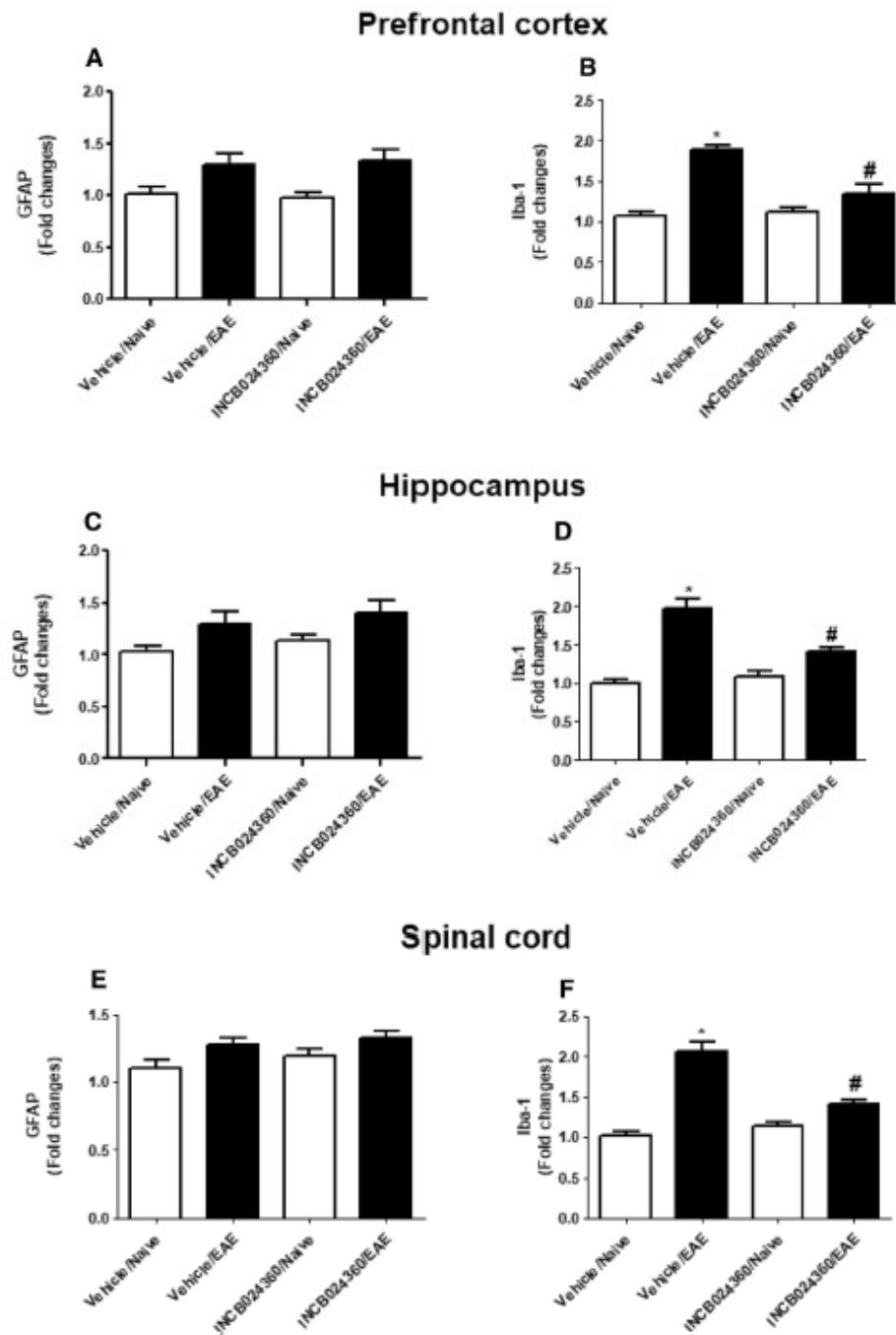
GFAP positive astrocytes analysis (a hallmark of astrocyte activation) and Iba-1 positive microglia (hallmark of microglia activation) were evenly distributed in the brain with normal morphology [47]. Our analysis revealed an increase of Iba-1 mRNA levels in the PFC, HC, SC, spleen and LN of EAE mice, a result that is in agreement with previous studies [48–50]. On the other hand, administration of 200 mg/kg of IDO inhibitor suppressed the increase in gene expression of Iba-1, but not significantly modulated GFAP genes in mice with EAE. Our findings are corroborated by a previous study of Mayer et al. [51], which showed that GFAP plasma levels were low in the vast majority of MS patients. Thus, in the current study, GFAP was not significantly modified in EAE mice, while Iba-1 was expressively increased in lymphoid tissues and CNS. Taken together, our findings support the notion that proinflammatory cytokines

Table 2 Effect of experimental autoimmune encephalomyelitis (EAE) and indoleamine-2,3-dioxygenase inhibitor (INCB024360) on the levels of kynurenine pathway metabolites in the organs of mice

(pg/mg protein)	Vehicle		INCB024360	
	Naive	EAE	Naive	EAE
Prefrontal cortex				
TRP	256.8 ± 12.1	412.2 ± 20.5[a]	214.5 ± 15.8[a]	311.8 ± 17.9[b]
KYN	84.4 ± 6.74	146.1 ± 9.77[a]	65.4 ± 5.40	76.7 ± 4.89[b]
KYN/TRP	32.86 ± 1.93	35.44 ± 0.61[a]	30.48 ± 0.27	24.59 ± 0.16[b]
Hippocampus				
TRP	298.5 ± 11.6	401.1 ± 23.6[a]	253.4 ± 8.7[a]	292.4 ± 12.7[b]
KYN	75.8 ± 4.10	132.4 ± 8.47[a]	61.7 ± 11.1	84.8 ± 6.29[b]
KYN/TRP	25.39 ± 0.39	33.01 ± 0.17[a]	24.35 ± 3.55	29.00 ± 0.89[b]
Spinal cord				
TRP	145.7 ± 6.78	231.1 ± 14.8[a]	126.4 ± 9.84[a]	155.4 ± 13.0[b]
KYN	32.7 ± 2.11	67.4 ± 1.77[a]	26.7 ± 1.49	36.1 ± 0.97[b]
KYN/TRP	22.4 ± 0.40	29.2 ± 1.10[a]	21.1 ± 0.47	23.2 ± 1.33[b]
Spleen				
TRP	748.4 ± 32.1	812.1 ± 27.5	779.5 ± 31.2	798.1 ± 30.9
KYN	178.4 ± 16.4	385.9 ± 19.8[a]	149.2 ± 17.4	177.8 ± 12.0[b]
KYN/TRP	23.8 ± 1.2	47.5 ± 0.8[a]	19.1 ± 1.5	22.2 ± 0.6
Lymph node				
TRP	674.7 ± 19.4	594.1 ± 20.1	633.1 ± 13.5	644.1 ± 21.8
KYN	156.4 ± 10.7	279.5 ± 9.8[a]	134.4 ± 7.7	177.4 ± 5.8[b]
KYN/TRP	23.2 ± 0.9	47.0 ± 0.1[a]	21.2 ± 0.7	27.5 ± 0.1[b]

Tryptophan (TRP), kynurenine (KYN) and KYN/TRP ratio. The values were analyzed by two-way ANOVA and Bonferroni multiple comparison test. Each value is expressed as the mean ± S.E.M. (n = 6)

[a] $p < 0.05$ when compared with vehicle/naive

[b] $p < 0.05$ when compared with vehicle/EAE

concomitant with increased Iba-1 gene expression may be involved in the severity of the disease or the vulnerability to EAE.

The Neurotoxic Metabolites of KP are Involved in the Pathophysiology of EAE

Previous studies of our laboratory have shown that proinflammatory cytokines were able to activate the KP in the CNS [8, 21, 52]. The KP is modulated when cells are activated or exposed to a variety of cytokines, especially IFN-γ [14]. In the brain, IDO activation plays a key role in the development of CNS diseases [53].

Evidence has suggested that IDO activation is associated with potent immunosuppressive effects, participating in the promotion of immunotolerance [20, 24]. The concept

of IDO immunomodulation in MS is supported by studies wherein reported that IDO counteracts the effects of autoreactive lymphocytes via TRP depletion, which is essential for T lymphocyte survival, reducing the severity of EAE [24]. Nevertheless, in the present study we observed an increase of TRP levels in the PFC, HC and SC in EAE-induced mice. Although it seems counterintuitive, this result could be explained as a compensatory mechanism of the brain in response to inflammation. This finding has already been demonstrated by previous studies from our research group [8, 21, 52]. Of note, the administration of IDO inhibitor resulted in the reduction of TRP levels of EAE-treated mice. These findings suggest that the pharmacological blockade of IDO ameliorates TRP alterations in EAE mice.

The metabolic route of TRP is the conversion into a variety of neuroactive substances, including the well-known neurotransmitters serotonin and melatonin, as well as the formation of KYN, the central molecule of KP, that is catalyzed by IDO [54]. Once synthetized, KYN can be further metabolized through two distinct branches, which are considered neurotoxic or neuroprotective, depending on the metabolites produced. In brief, KYNA is a part of a neuroprotective branch of KP produced by astrocytes and catalyzed by KAT enzyme. In contrast, 3-HK and QUIN belongs to neurotoxic branch, that is derived from microglial cells and is be initially catalyzed by KMO activity [18, 54, 55]. In addition to the combined effect of TRP depletion, the presence of downstream KYN metabolites 3-HK and QUIN have been reported to inhibit T cell proliferation and the reduction of Th1 cells, developing a immunotolerogenic phenotype [25–27]. Likewise, the neurotoxic 3-HK has been found to be increased in the spinal cords of EAE-induced rats [23] and QUIN levels were found elevated in the spinal cords of rats with EAE [22].

In the present study, EAE mice showed increased levels of KYN, 3-HK and QUIN in the brain, SC, spleen and LN. In contrast, our study demonstrated that the treatment with IDO inhibitor blocked these effects. QUIN is a *N*-methyl-D-aspartate (NMDA) receptor agonist that, when it is overproduced, can lead to acute neuronal death or chronic and progressive neuronal dysfunction by multiple mechanisms, mainly by glutamatergic excitotoxicity and reactive species production [14]. At human level, our results are in accordance with previous data, which reported that QUIN is found in pathophysiological levels in MS patients [56]. Regarding 3-HK, it has demonstrated that it is neurotoxic and induces cell death, and has a synergistic neurotoxicity effect with QUIN [24].

In our study, the neuroprotective KYNA, an NMDA receptor antagonist, was not altered by either EAE or IDO inhibitor treatment in all tissues studied, suggesting that this neuroprotective metabolite is not altered in MOG-induced EAE. In contrast with previous evidence which

Fig. 4 Effect of EAE and IDO inhibitor (INCB024360) on the levels of kynurenine pathway metabolites 3-hydroxykynurenine (3-HK), quinolinic acid (QUIN) and kynurenic acid (KYNA) in the prefrontal cortex (**a, c** and **e**) and hippocampus (**b, d** and **f**). Values were analyzed by 2-way ANOVA and Bonferroni multiple comparison test. Each value is expressed as mean ± S.E.M. (n = 6). *p < 0.05 when compared to vehicle/naive and #p < 0.05 when compared to vehicle/EAE

Prefrontal cortex

Hippocampus
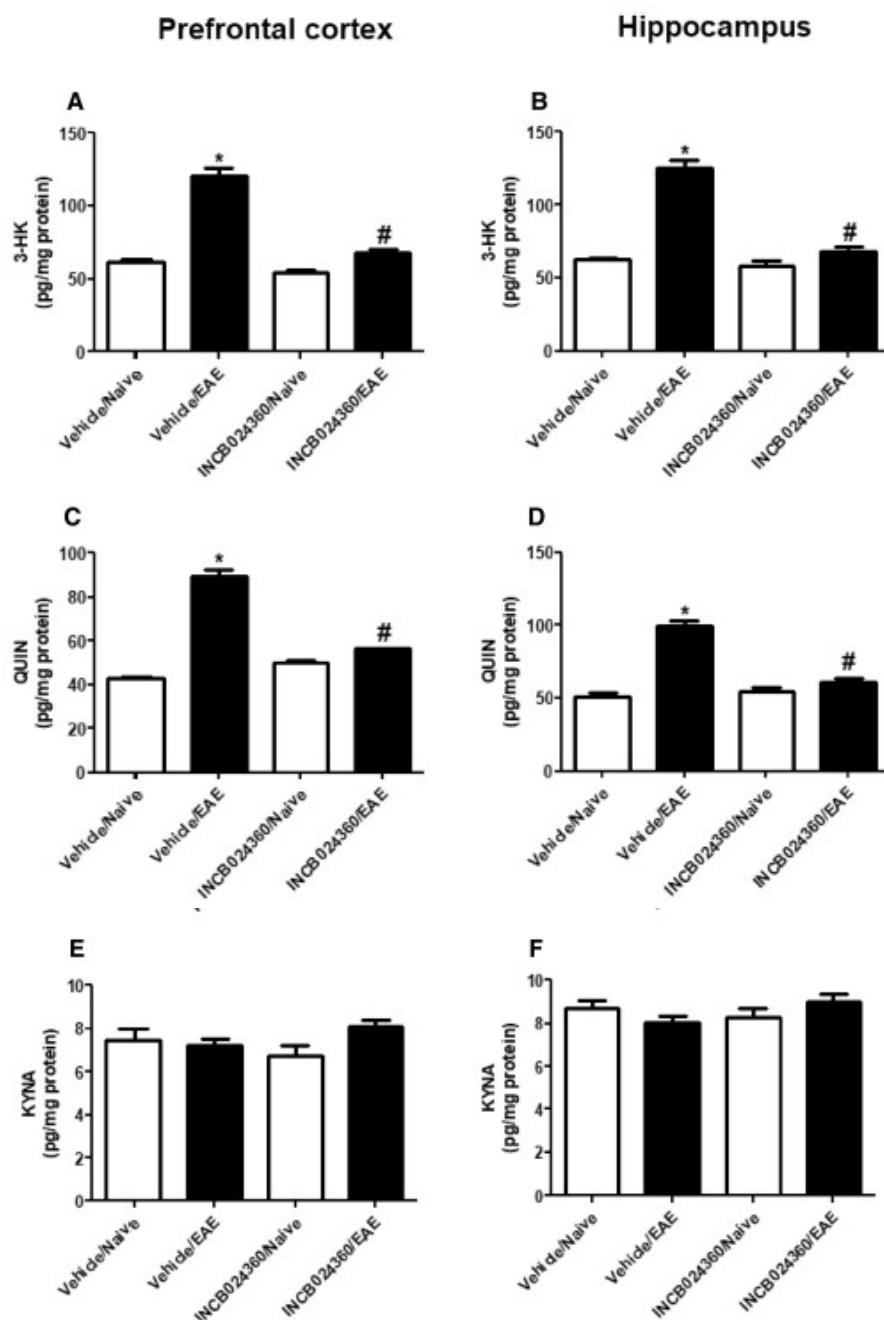
reported that KYN and downstream metabolites, such as 3-HK and QUIN, acts as immunosuppressive metabolites in autoimmunity diseases, here we supported the idea that these metabolites in toxic levels contribute to EAE/MS pathology. In other words, our results indicate that the imbalance between neurotoxic and neuroprotective metabolites of the KP favoring the neurotoxic ones might contribute to neurodegeneration in EAE, in part through

NMDA receptor-mediated neurotoxicity, results that are in line with Lim et al. [57].

The Activation and Overexpression of IDO and KMO Contribute to Clinical Signs of EAE

Our present study also demonstrates that EAE-induced mice had a substantial increase in the IDO-1 and KMO mRNA

Fig. 5 Effect of EAE and INCB024360 on the levels of kynurenine pathway metabolites 3-hydroxykynurenine (3-HK), quinolinic acid (QUIN) and kynurenic acid (KYNA) in the spinal cord (**a**, **c** and **e**) and spleen (**b**, **d** and **f**). Values were analyzed by 2-way ANOVA and Bonferroni multiple comparison test. Each value is expressed as mean ± S.E.M. (n = 6). *$p < 0.05$ when compared to vehicle/naive and #$p < 0.05$ when compared to vehicle/EAE
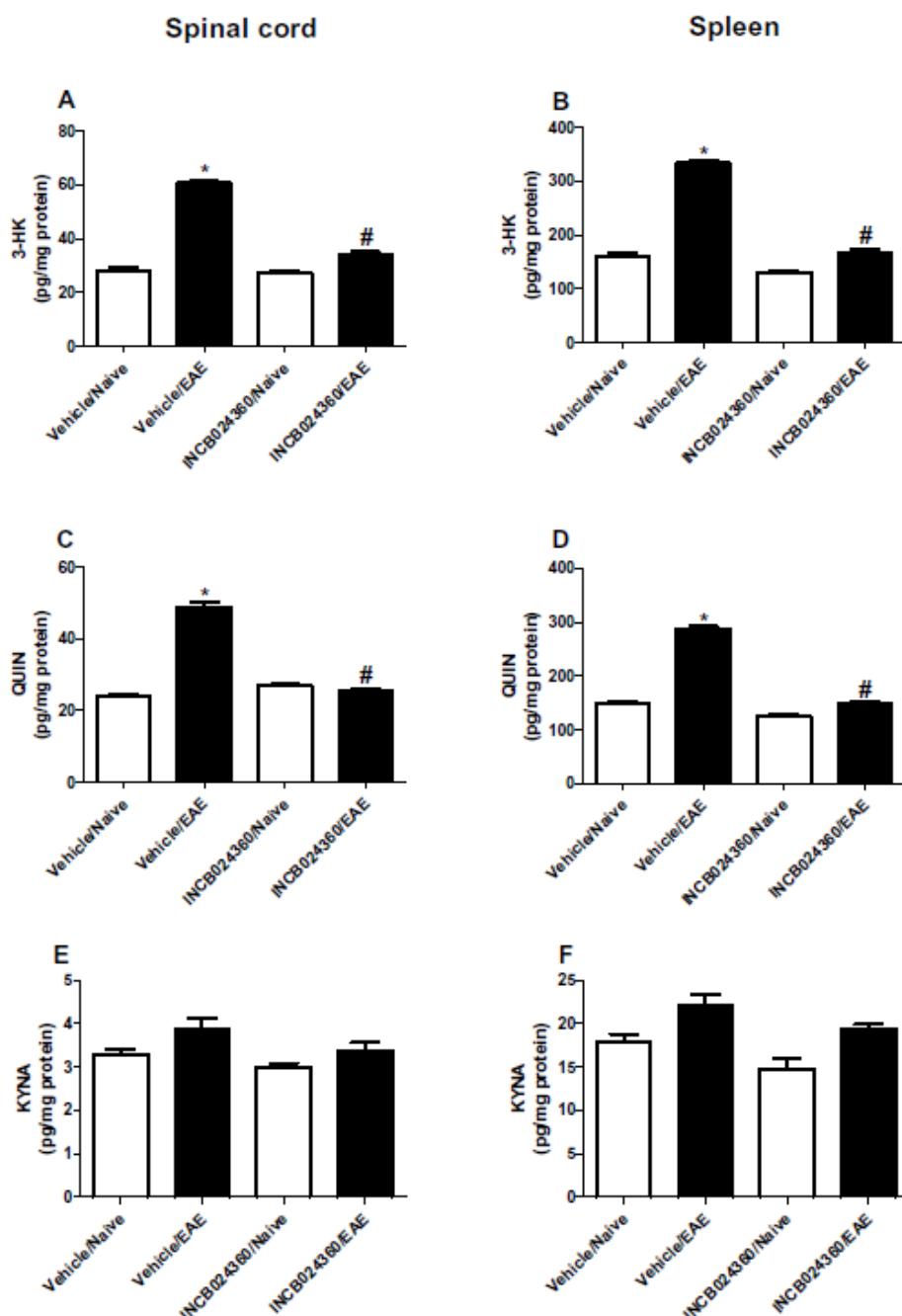

expression in the PFC, HC, SC, spleen and LN. The central role of overactivated IDO in the CNS of brain diseases, such as depression and Alzheimer's disease is already being demonstrated by our previous researches [8, 9, 16, 21, 52] and other laboratories [39, 58]. However, it has been reported that IDO activation in autoimmune disorders acts as an endogenous negative regulator of the immune system to counteract autoimmunity [24].

In the present study, the treatment with IDO inhibitor blunted the increase in activity and gene expression of IDO-1 and KMO in the CNS and lymphoid tissues, effect that occurred in parallel with the amelioration in clinical signs of EAE mice. No changes in KAT enzyme (mRNA levels and enzyme activity) were observed in all groups of the present studied. Hence, our results are in contradistinction to reports that IDO inhibition exacerbates EAE symptoms. Our findings

Fig. 6 Effect of EAE and INCB024360 on the levels of kynurenine pathway metabolites 3-hydroxykynurenine (3-HK) (a), quinolinic acid (QUIN) (b) and kynurenic acid (KYNA) (c) in the lymph node. Values were analyzed by 2-way ANOVA and Bonferroni multiple comparison test. Each value is expressed as mean $\pm$ S.E.M. (n= 6). $*p < 0.05$ when compared to vehicle/naive and $\#p < 0.05$ when compared to vehicle/EAE
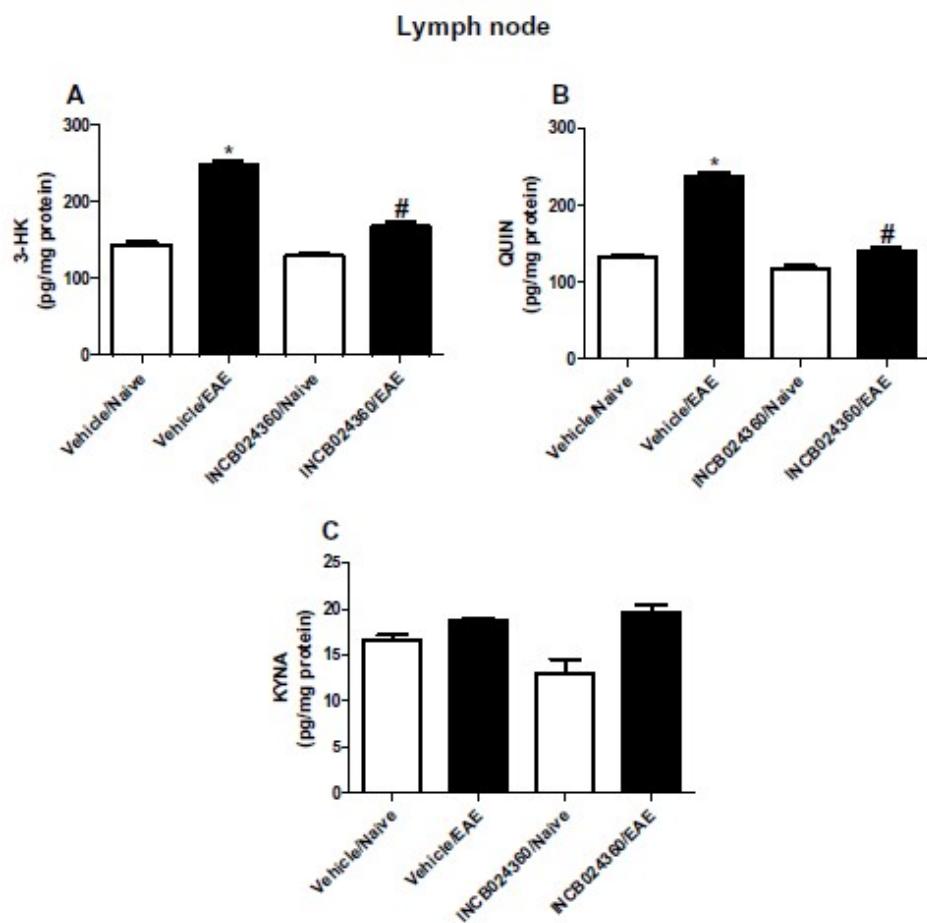
indicate that IDO-1 and KMO participate in the regulation of EAE, supporting the notion that the immunomodulatory effects of IDO in MS/EAE are dichotomous, acting in an immunosuppressive or neurotoxic manner (when exacerbated levels of 3-HK and QUIN are produced). Additional investigations are needed to assess the role of tryptophan-2,3-dioxygenase-2 (TDO-2) in the EAE model.

These data together demonstrated that there is a dialogue between the immune system and KP in the MOG-induced EAE model, and the pharmacological modulation of KP might be of therapeutic value for MS patients. Further investigations are necessary to elucidate the exact immunomodulatory mechanism of IDO inhibitor (INCB024360) in EAE model, assessing the degree of insult in the brain and spinal cord. A summary of our major findings can be seen in Fig. 10.

Conclusions

Our data suggest that KP dysregulation in EAE mice occurs in response to the inflammatory process and is marked by the actions of neurotoxic metabolites 3-HK and QUIN in the brain, SC, spleen and LN of mice. The administration of IDO inhibitor (INCB024360) was able to alleviate weight loss, clinical signs and blunting inflammatory process, suggesting that the increase in IDO/KMO activity and mRNA levels are involved in the severity of the clinical course of EAE. The above alterations in IDO and neurotoxic metabolites of KP indicate their potential utility as biomarkers of disease course and support molecular targets for the treatment of MS. More studies are needed to identify the unequivocal link between KP and MS.

Fig. 7 Effect of EAE and INCB024360 on the gene expression (mRNA levels) of the kynurenine pathway enzymes indoleamine-2,3-di-oxygenase (IDO-1), kynurenine 3-monooxygenase (KMO) and kynurenine aminotransferase (KAT-II) in the prefrontal cortex (**a**, **c** and **e**), hippocampus (**b**, **d** and **f**). Values were analyzed by 2-way ANOVA and Bonferroni multiple comparison test. Each value is expressed as mean ± S.E.M. (n = 6). *p < 0.05 when compared to vehicle/naive and #p < 0.05 when compared to vehicle/EAE

Prefrontal cortex

Hippocampus

Spinal cord

Spleen
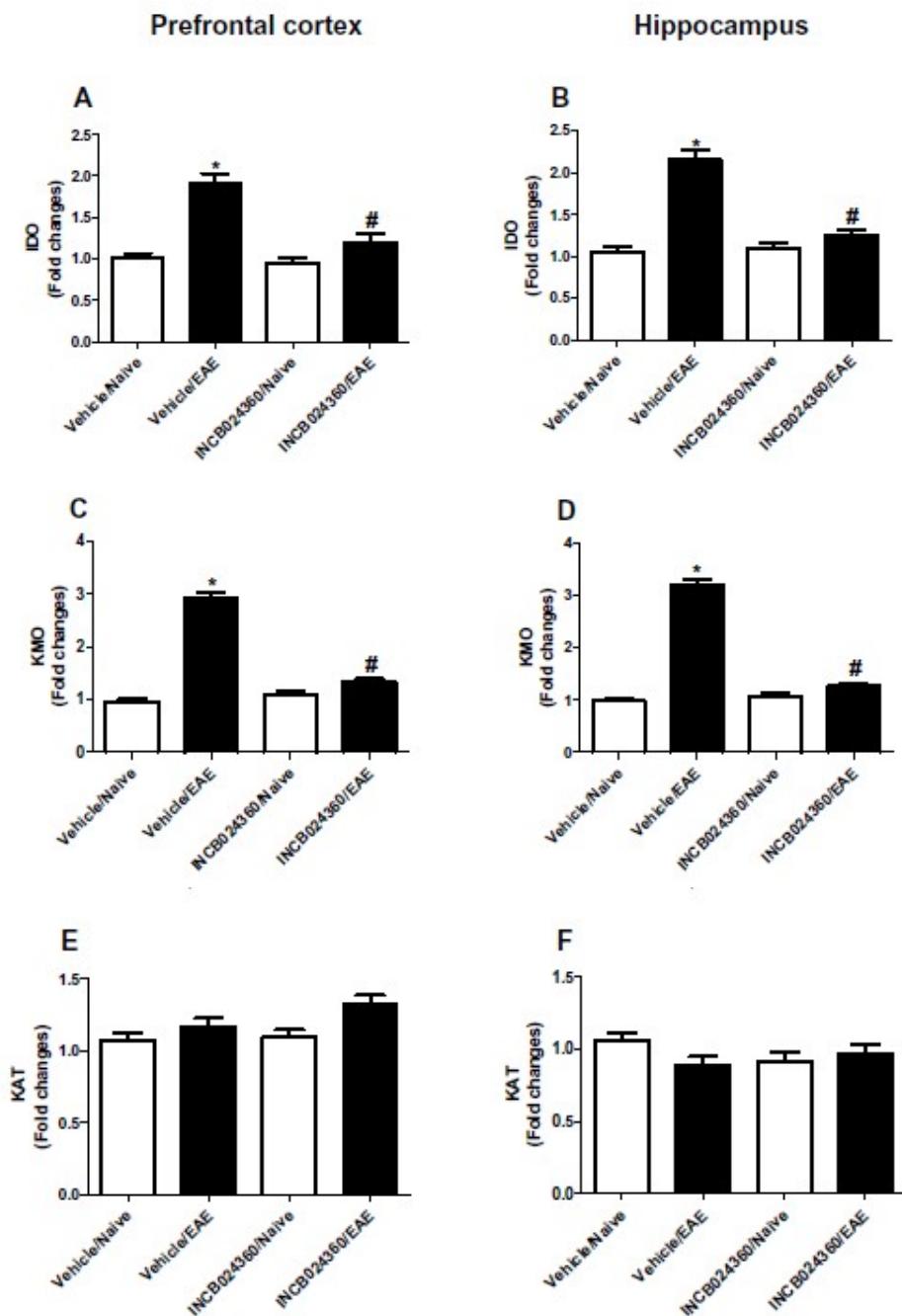
Fig. 8 Effect of EAE and INCB024360 on the gene expression (mRNA levels) of the kynurenine pathway enzymes indoleamine-2,3-di-oxygenase (IDO-1), kynurenine 3-monooxygenase (KMO) and kynurenine aminotransferase (KAT-II) in the spinal cord (**a**, **c** and **e**) and spleen (**b**, **d** and **f**). Values were analyzed by 2-way ANOVA and Bonfer-roni multiple comparison test. Each value is expressed as mean ± S.E.M. (n= 6). *p < 0.05 when compared to vehicle/naive and #p < 0.05 when compared to vehicle/EAE
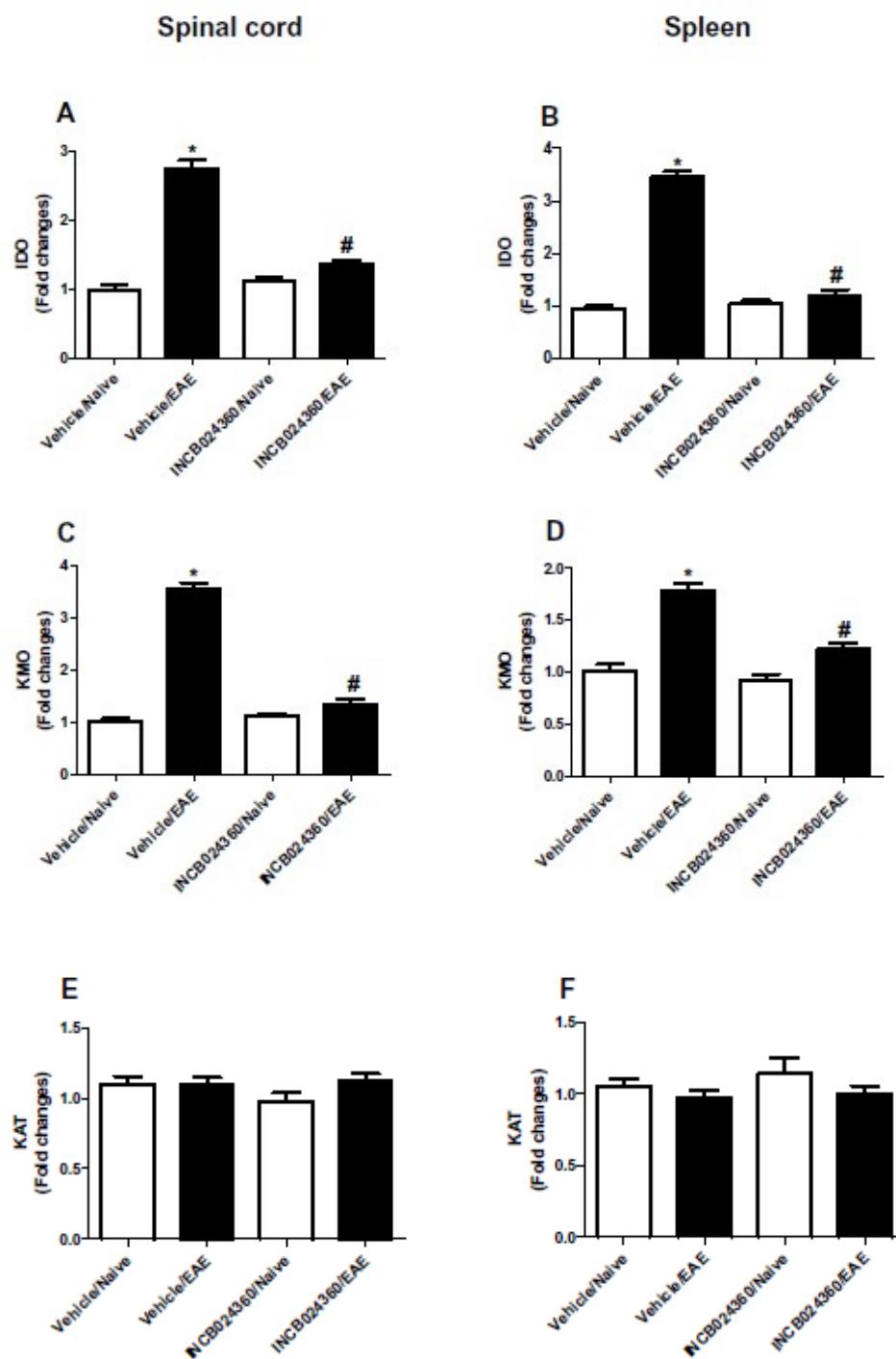
Fig. 9 Effect of EAE and INCB024360 on the gene expression (mRNA levels) of the kynurenine pathway enzymes indoleamine-2,3-dioxygenase (IDO-1) (**a**), kynurenine 3-monooxygenase (KMO) (**b**) and kynurenine aminotransferase (KAT-II) (**c**) in the lymph node. Values were analyzed by 2-way ANOVA and Bonferroni multiple comparison test. Each value is expressed as mean ± S.E.M. (n= 6). *p < 0.05 when compared to vehicle/naive and #p < 0.05 when compared to vehicle/EAE


Lymph node
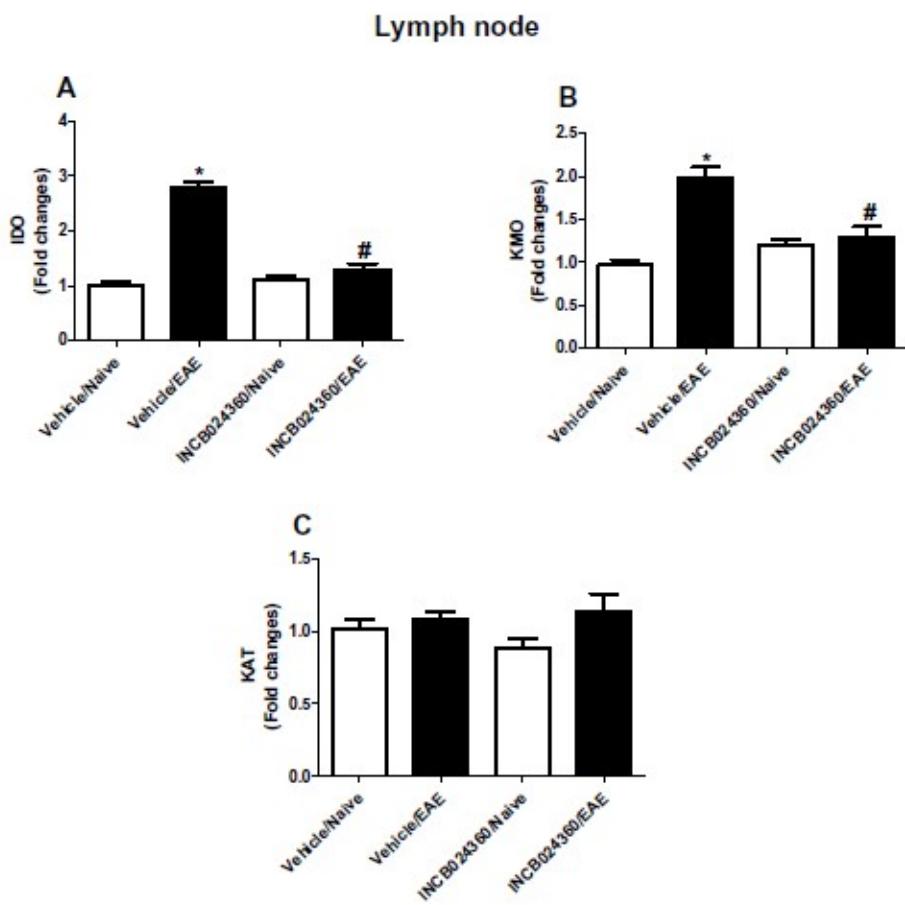
Table 3 Effect of experimental autoimmune encephalomyelitis (EAE) and indoleamine-2,3-dioxygenase inhibitor (INCB024360) on the enzyme activities along the kynurenine pathway in the organs of mice

(pg/h/mg protein)	Vehicle		INCB024360	
	Naive	EAE	Naive	EAE
Prefrontal cortex				
IDO	87.7 ± 2.41	187.7 ± 8.11[a]	67.7 ± 4.11[a]	90.1 ± 5.13[b]
KMO	57.7 ± 3.11	121.4 ± 11.8[a]	66.7 ± 6.12	72.7 ± 5.14[b]
KAT	24.8 ± 1.94	21.2 ± 2.12	28.7 ± 3.27	25.8 ± 1.49
Hippocampus				
IDO	98.4 ± 4.27	167.0 ± 5.62[a]	81.4 ± 2.77[a]	105.7 ± 5.77[b]
KMO	68.9 ± 4.02	151.1 ± 6.31[a]	77.4 ± 5.77	80.4 ± 7.19[b]
KAT	32.1 ± 3.44	36.7 ± 2.79	30.9 ± 3.11	39.7 ± 2.10
Spinal cord				
IDO	54.7 ± 2.31	79.7 ± 3.03[a]	44.7 ± 2.10[a]	58.4 ± 3.11[b]
KMO	40.2 ± 2.83	67.8 ± 5.11[a]	37.8 ± 2.09	45.4 ± 2.31[b]
KAT	18.7 ± 1.05	21.4 ± 0.88	17.8 ± 0.73	20.1 ± 0.89
Splenn				
IDO	278.7 ± 17.4	412.7 ± 17.4[a]	221.7 ± 10.2[a]	293.5 ± 11.2[b]
KMO	154.7 ± 18.7	321.8 ± 15.7[a]	167.4 ± 10.2	172.8 ± 14.1[b]
KAT	61.4 ± 3.77	70.8 ± 4.78	68.8 ± 4.01	60.8 ± 3.46
LN				
IDO	312.8 ± 15.6	541.7 ± 15.1[a]	335.7 ± 17.1	341.2 ± 13.2[b]
KMO	143.8 ± 13.8	287.9 ± 10.3[a]	168.7 ± 12.1	167.7 ± 10.8[b]
KAT	58.4 ± 5.12	54.7 ± 4.21	61.1 ± 3.55	67.7 ± 4.88

Indoleamine 2,3-dioxygenase (IDO), kynurenine 3-monooxygenase (KMO) and kynurenine aminotransferase (KAT). The values were analyzed by two-way ANOVA and Bonferroni multiple comparison test. Each value is expressed as the mean ± S.E.M. (n=6)

[a] $p < 0.05$ when compared with vehicle/naive

[b] $p < 0.05$ when compared with vehicle/EAE

Fig. 10 Diagram showing the mechanisms of EAE-induced inflammation and KP dysregulation. In this study, the inflammatory process, reflected by the increased Iba-1 gene expression and elevated interferon-gamma (IFN-γ) and interleukin-17 (IL-17) levels, causes an increase in indoleamine-2,3-dioxygenase (IDO) activity and mRNA levels in the prefrontal cortex (PFC), hippocampus (HC), spinal cord (SC), spleen and lymph node (LN). The overactivation of IDO resulted in increasing kynurenine (KYN), 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN) levels. The administration of IDO inhibitor INCB024360 (200 mg/kg; p.o) during 25 days abrogated these alterations in MOG-induced EAE in mice. These findings suggest a key role for IDO induction and neurotoxic downstream metabolites of KP in the development of weight loss and clinical signs of experimental autoimmune encephalomyelitis (EAE)
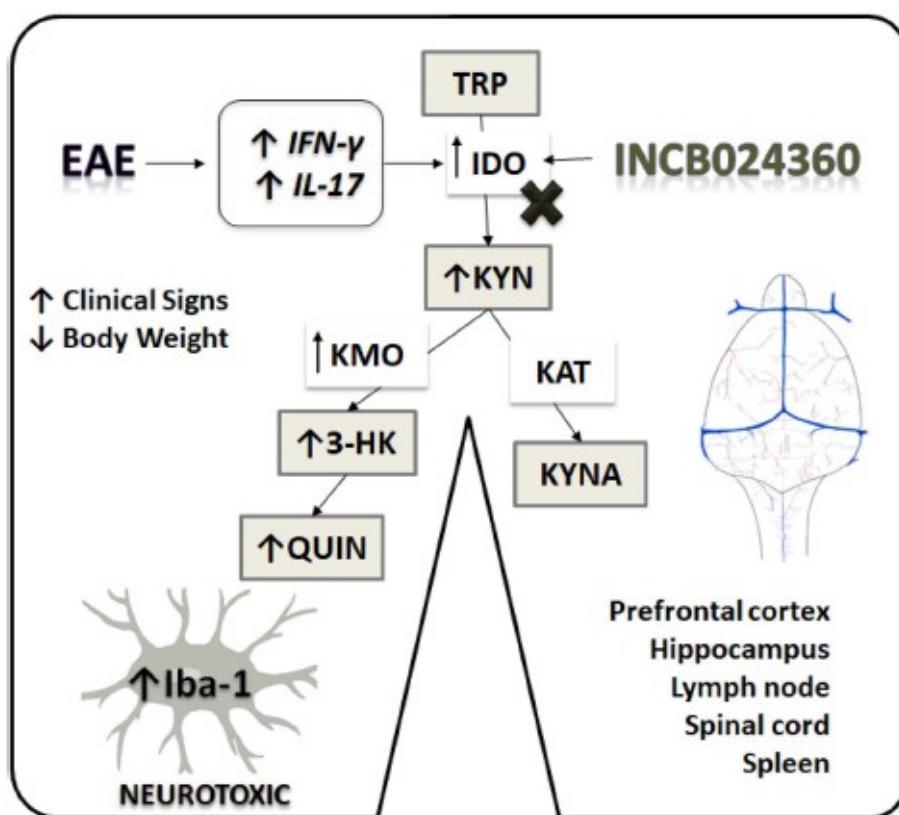
Compliance with Ethical Standards

Conflict of Interest The authors have no personal conflicts of interest related to this study.

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6   PARTE III

MANUSCRITO: MULTIPLE SCLEROSIS: KYNURENINE PATHWAY AS A POSSIBLE BIOMARKER

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Purpose of review

Multiple sclerosis is it is a neurological, chronic, autoimmune disease, progressive in nature and without cure. This review will discuss the role of the quinurenine pathway as a possible biomarker for this autoimmune disease and the main diagnostic pathways in humans affected by this disease.

Recent findings

The prognosis of multiple sclerosis varies substantially across individual patients, and a combination of clinical, imaging, and laboratory markers can be useful in predicting clinical course and optimizing treatment in individual patients.

Summary

The studies demonstrated in this review used blood, urine, feces and cerebrospinal fluid (CSF) samples to assess inflammatory parameters in patients with multiple sclerosis, in the recurrent remitting multiple sclerosis (RRMS) and secondary progressive multiple sclerosis (SPMS) subtypes. The inhibitors used to study the Kynurenine pathway were from, enzymes present in the pathway (IDO, TDO, KAT and KMO) and the metabolites derived from tryptophan are (KYN, KYNA, QUIN and serotonin) in the course of the disease. As well as studies to characterize the metabolites of the quinurenine pathway as biomarkers. There are studies associating the quinurenine pathway as tolerogenic signals.A number of recent advances have been made in the clinical diagnosis and prognostication of multiple sclerosis patients. Future research will enable the development of more accurate biomarkers of disease categorization and prognosis, which will enable timely personalized treatment in individual multiple sclerosis patients.

Keywords

tryptophan metabolism; kynurenine, Multiple Sclerosis, prognosis, subtypes

1 INTRODUCTION

Multiple sclerosis (MS) is an autoimmune and inflammatory disease of the central nervous system, but the symptoms go beyond the affected site, extending to the optic nerve and the spinal cord. The disease is characterized by damage to myelin, a substance responsible for protecting neurons, isolating and making the passage of information from one neuron to another, more effective and faster. The damage also extends to neuronal death and consequent irreparable damage, such as paralysis of limbs and organs. It is not easy to distinguish since the symptoms vary from episodes related to blurred vision, and difficulty in movement, being very confused with other diseases and often, therefore more difficult to be diagnosed and when diagnosed it may be that the stage of the disease is already be more advanced. Furthermore, MS can be classified into 3 subtypes by the level of characteristic episodes of the disease, that is, by the levels of manifestations of the characteristic symptoms of this disease, as well as the symptoms emitted, are Relapsingremitting multiple sclerosis (RRMS); primary progressive multiple sclerosis (PPMS) and secondary progressive multiple sclerosis (SPMS) (KATZ 2015; BISHOP and RUMRILL, 2015; HUISMAN et al., 2017).

Much is said that genetic and environmental factors are present in the occurrence of this disease, what is already known is that this is an autoimmune disease at the neuronal level, but what triggers this reaction is still being investigated. When we know what are the mechanisms that lead to the occurrence of this type of disease, we can find out what the means and preventive measures were. MS is a major cause of neurological disability worldwide. The importance of discovering new therapeutic targets is in line with the fact that if we study the mechanisms involved in the origin of the pathogenesis of diseases, we can better understand their path, the path of the disease, being able to propose alternatives to the damage caused by the disease and that improve the quality of life of these patients. The existing clinical treatments are not curable, and better treatments are urgently needed (ZHANG et al., 2019; ISMAILOVA et al., 2019; LI et al., 2019).

Epidemiologically speaking, MS manifests itself in the age group of young adults, questioning the level of productivity and the stage in which this individual is, an important point is the emergence of the disease that occurs mainly in the socially productive phase. Thus, the social, personal and economic impact that can occur in the life of the individual and society cannot be neglected. If there is a need for a more accessible diagnosis, we look for biomarker studies capable of being analyzed via blood or urine and according to the other means of diagnosis, we are able to start treatment early, thus avoiding major disorders (NOURBAKHSH et al., 2018, GAETANI et al., 2020).

Comprehensive understanding of such mechanistic subtleties will benefit future attempts in the rational design of salient therapeutic agents, including next generation anti- anti-inflammatory and anti-neurodegenerative drug targets with amplified effectivity. Several studies report that Kynurenine pathway modulation reverses the experimental autoimmune encephalomyelitis mouse disease progression. Therefore, this review aimed to analyze and compile the latest studies focused on the Kynurenine Pathway both in animal models and in patients with multiple sclerosisin the last ten years in a database.

METHODOLOGY

For this narrative review, the PubMed database was searched with the searches were performed using keywords such as the following: "kynurenine pathway", "IDO inhibitor" OR "kynurenine" AND "experimental autoimmune encephalomyelitis", "EAE" OR "multiple sclerosis", search concentrated in publications filtered for the last 10 (2010 through 2020.) years and articles that were not reviews. A total of 176 articles were found, 52 articles were related to the quinurenine pathway with the EAE model and 124 articles were related to the quinurerine pathway with multiple sclerosis. After independent analysis by the reviewers, 24 articles for this study were included in this review, of these 11 articles in the animal model of EAE and 13 studies in samples of patients with multiple sclerosis.

KYNURENINE PATHWAY IN EAE ANIMALS MODEL

Evidences of the kynurenine pathway in EAE a model of Multiple Sclerosis is summarized in table 1. Most studies use some inhibitor of some enzyme that limits the pathway, such as IDO, TDO and KMO and Kat II inhibitors have also been studied. As we can see in the results obtained by administration of IDO inhibitor (INCB024360) was able to alleviate weight loss, clinical signs and blunting inflammatory process, suggesting that the increase in IDO/KMO activity and mRNA levels are involved in the severity of the clinical course of EAE and probably in MS. (ZARZECKI et al., 2020). The synergistic immune suppressive effects of rat fetal NSCs expressing IDO (rfNSCs-IDO) were validated by mixed leukocyte reaction (MLR). Systemic rfNSCs-IDO injections resulted in significant local immune suppression in the cervical lymph nodes and CNS, evidenced by a reduction in the number of activated T lymphocytes and an increase in regulatory T cell numbers, which induced significantly fewer clinical symptoms and faster recovery (LEE et al., 2015).

Stimulator of Interferon Genes (STING - signaling adaptor to stimulate interferon type 1 (IFN-I) production) agonists to boost IDO activity and anipulating the Kyn pathway downstream of IDO is an effective strategy to enhance tolerogenic responses (LEMOS et al., 2020) KP was steadily upregulated correlating with disease severity and associated with a shift towards increasing

concentrations of the KP metabolite quinolinic acid, a neuro- and gliotoxin. KP modulation at the KMO level to preserve immune tolerance and limit neurodegeneration in EAE. (SUNDARAM et al., 2020).

In the steady state, Smad7 regulates the expression of the transcription factors Batf3 and IRF8 that promote the development of CD8+ CD103+ DCs in the spleen and Smad7 directs DC function by regulating the expression of indoleamine 2,3-dioxygenase in response to IFN-γ signaling. During inflammation, Smad7 governs the tolerogenic function of conventional DCs by regulating IDO-mediated Treg induction. (LUKAS et al., 2017) Infusion of Mesenchymal stromal cells exhibited a infiltration of mononuclear cells and demyelination of the spinal cords were both reduced in CNS of the mice, the frequency of CD5+ IL-10+ B cells in the mice was significantly increased. Moreover, those effects could be eliminated while the indoleamine 2,3-dioxygenase (IDO) inhibitor, D/L-1MT, was added to the co-cultured cells (LI et al., 2019).

Some studies try to investigate the levels of tryptophan both in its consumption and metabolism, as well as the concentration factor related to the pathways that it will be metabolized, since the tryptophan participates in pathways: protein synthesis, release of immunomodulating catabolics, synthesis of the aminergic neurotransmitter - serotonin, Neurohormone melatonin, various neuroactive cinuranic metabolites of melatonin, traces of amine tryptamine (GAETANI et al., 2020). Let's look at the work in which theTryptophan 10 mg/mouse/day was safe and increased serum kynurenine levels, at subtoxic concentrations suppresses antigen-specific Th1 responses. This treatment resulted in suppression of myelin-specific Th1 responses, there was no relevant impact on clinical disease activity (LANZ et al., 2017)

Or the supplementation of some quinurerine metabolite, such as systemic administration of CA (0.1-10 mg/kg, i.p.; Cinnabarinic acid (CA) is an endogenous metabolite of the kynurenine pathway) was highly protective against EAE. CA fully prevents neuroinflammation in mice by enhancing regulatory T-cell activity and decreases neuroinflammation by suppressing Th17 cells (FAZIO et al., 2014)

The rate-limiting step is controlled by indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO). IDO is expressed in antigen presenting cells during immune reactions, hepatic TDO regulates blood homeostasis of tryptophan and neuronal TDO influences neurogenesis.TDO deficiency did not influence myelin-specific T cells, leukocyte infiltration into the CNS, demyelination and disease activity. TDO-deficiency protected from neuronal loss in the spinal cord but not in the optic nerves (LANZ TV, Williams 2017)

The kynurenine pathway is a paramount source of several immunoregulatory metabolites, including L-kynurenine (Kyn), the main product of indoleamine 2,3-dioxygenase 1 (IDO1) that catalyzes the rate-limiting step of the pathway.

The other two pathways provide the transamination and decarboxylation of Trp. The hydroxylation in serotonin occurs for only 1% of dietary Trp (GAETANI et al., 2020). In the serotonin pathway, the metabolite Nacetylserotonin (NAS) has been shown to possess antioxidant, antiinflammatory, and neuroprotective properties. Study shows that N-acetylserotonin directly binds IDO1 and acts as a Positive Allosteric Modulator of the IDO1 enzyme in vitro and in vivo. N-acetylserotonin confers immunosuppressive effects on dendritic cells via increased catalytic activity but not expression of IDO1 (MONDANELLI et al.,2020).

Prostaglandins can mediate as bone marrow mesenchymal stem cells to differentiate into their multiple cell lines, as well as glial cells and neurons, not only being part of cell differentiation but also participating in the recruitment and proliferation of immune responses influenced by T cells. , lymphocytes and other cells present in the inflammatory process. Prostaglandin E2 (PGE2) is generated from arachidonic acid by cyclooxygenases (COXs) and prostaglandin synthases, and the synthesis of PGE2 can be efficiently blocked by nonsteroidal anti-inflammatory drugs, in particular cyclooxygenase-2 (COX-2) inhibitors (prostaglandin E2-induction of IDO-dependent immunoregulation (MATYSIAK et al., 2011).

KYNURENINE PATHWAY IN PATIENT SAMPLES WITH MULTIPLE SCLEROSIS

Metabolism of the essential amino acid tryptophan (TRP), catabolism via the kynurenine pathway is considered to represent a major link between inflammation and various diseases, including neurodegenerative disorders, depression, schizophrenia, multiple sclerosis, cardiovascular disease, and cancer. Therefore, it is related to is a key endogenous immunosuppressive pathway restricting inflammatory responses. Interestingly, the metabolic products of the kynurenine pathway are known to have several effects on vascular system, immune system, immunotolerance, and infections. (LANZ et al., 2017; ARNHARD et al., 2018; GAETANI et al., 2020). Evidences of the kynurenine pathway in EAE a model of Multiple Sclerosis is summarized in table 2.

The enzyme catalyzing the rate-limiting step of tryptophan catabolism along the kynurenine pathway, (indoleamine 2,3-dioxygenase 1- IDO1), belongs to the class of inhibitory immune checkpoint molecules. Such regulators of the immune system are crucial for maintaining self-tolerance and thus, when properly working, preventing autoimmunity, A dysfunctional IDO1 has recently been associated with a specific single nucleotide polymorphism (SNP) and with the occurrence of autoimmune diabetes and multiple sclerosis. (MONDANELI et al., 2020)

The kynurenine pathway and levels of TRP and its metabolites kynurenine (KYN), kynurenic acid (KYNA) and quinolinic acid (QUIN) are well regulated under physiological conditions but may be altered as part of the activated immune response (ARNHARD et al., 2018). Tryptophan metabolites promote regulatory T cell (Treg) differentiation and suppress proinflammatory T helper cell (Th)1 and Th17 phenotypes (LANZ et al., 2017)

In addition to tryptophan, the most studied metabolites are KYN, QUIN, KYNA associated with the disease subtype which metabolite is most present. The raise QUIN levels of Relapsingremitting multiple sclerosis (RRMS) patients in relapse and generally decreased levels of TRP in secondary progressive MS (SPMS) may report to neurotoxicity and failure of remyelination, respectively (AEINEHBAND et al., 2016). The pattern of KP metabolites in RRMS patients could not predict neurocognitive symptoms. Tryptophan- and phenylalanine metabolism were found to be commonly altered between SPMS and both RRMS patients and controls.

Metabolomics is a comprehensive profiling of the dynamic molecular networks composed of low-weight molecules or metabolites. These metabolites essentially correspond to the intermediate and end products of ongoing pathophysiological processes. Different metabolomic technologies have been used to study pathophysiology in MS. In this sense, the study of metabolites that are associated with MS is of fundamental value, in this study 117 metabolites were identified, of which 21 have some relationship with the subtypes of patients with SPMS and RRMS. In addition to being related to several routes. The pathway analysis revealed eight biochemical pathways that were affected in SPMS compared with RRMS patients: aminoacyl-tRNA biosynthesis; phenylalanine metabolism; tryptophan metabolism; valine, leucine and isoleucine biosynthesis; pyrimidine metabolism; nitrogen metabolism; valine, leucine and isoleucine degradation and purine metabolism Tryptophan an metabolism demonstrated strong relevance as it achieved the highest impact in the comparison between SPMS and controls and the second highest in the comparison with RRMS patients (HERMAN et al., 2019)

Tryptophan is catabolized 95% towards kynurenine metabolites by the rate limiting enzyme indoleamine-2,3-dioxygenase (IDO). Its metabolite, quinolinic acid (QUIN), is produced by activated microglia and resident macrophages in the central nervous system (CNS), but not by neurons and astrocytes. QUIN is involved in neuronal death; it acts as an agonist of N-methyl-D-aspartate (NMDA) receptors. It has been studied that the metabolites of the quinurerine pathway have two metabolites participating in the neurotoxic pathway, as present in moments that were being a neuroproteror metabolite, in the case of both the QUIN and neurotoxic metabolite and the Kyna metabolite as increased in times of neuroprotective. In most studies we can observe the measurement of both pathway metabolites (TRP, KYN, QUIN and KYNA).

The importance of analyzing human samples of multiple sclerosis is in line with the different subtypes of the disease and their respective inflammatory responses, one of the investigated parameters being some inflammatory biomarkers and their association with the most present symptoms and also with coadjuvant treatments, such as physical exercise. Correlation between exercise-induced changes in KYN/TRP, evaluate 30 minutes of moderate-intensity exercise, inflammatory responses (IL-6, IFN-γ, IL-1RA and TNF-α) and TRP, KYN (DONIA et al., 2019) Study of Bansi et al., (2018) metabolites were evaluated Trp, Kyn and serotonin (5HT) in RRMS and SPMS and the modification occurred by acute aerobic exercise. Serotonin increased after training, whereas the kynurenine pathway was only activated in persons with RRMS. Further research is warranted to investigate whether these changes are associated with clinical measures (e.g. depressions and immune function). KP parameters have a strong association with MS subtype, correlating with disease severity scores. The changing levels of KP metabolites we observed also provides a mechanistic insight that may explain the transition from the milder RRMS form to the more debilitating SPMS disease form. (LIM et al., 2017)

Neurofilament light (NFL) has proved to be a good prognostic factor in multiple sclerosis (MS), as its level is proportionally elevated with extended neuraxonal damage. Neopterin is a nonspecific marker of inflammation present during viral as well as immunological inflammatory processes significant correlations were found between NFL, neopterin, and QUIN, and between kynurenine and neopterin. Normalized NFL, QUIN, and neopterin were the best independent predictors of neurological disability in pwMS (RAJDA et al., 2020).

In 220 patient with autoimmune neuroinflammation include MS, Kyn concentrations correlated strongly with CSF markers of neuroinflammation in bacterial and viral CNS infections (SÜHS et al., 2019) The concentration of QUIN dramatically increased, whereas that of KYNA slightly decreased in the RRMS group, resulting in a significantly increased QUIN/KYNA ratio and significantly decreased PICA/QUIN ratio (TÖMÖSI et al., 2020)

MS can manifest in different forms, mainly represented by relapsing-remitting MS (RRMS; affecting about 85% of MS patients and marked by flare-ups of symptoms followed by periods of remission) and primary progressive MS (PPMS; a disease that affects ~10% of MS patients and continues to worsen gradually from the beginning). In the serotonin pathway, the metabolite Nacetylserotonin increases IDO1 activity in peripheral blood mononuclear cells (PBMCs) from MS patients (MONDANELLI et al.,2020)

When environmental and genetic factors are involved in the etiology of the disease under study, it is fundamental to important data for studies that take into account both, and our microbiota and as related to the development of autoimmune diseases. Trp metabolism by the gut microbiota

and the kynurenine pathway may be relevant to the risk of MS in children as well as MS activity and severity. Specific urinary signature of host/microbiome Trp metabolism can be potentially identified so as to select novel biomarkers (GAETANI et al., 2020). While higher serum levels of Trp and relative abundance of índole lactate, a Trp metabolite (produced by gut microbiota and an AHR ligand) were associated with a lower risk of pediatric MS, they were not associated with MS phenotype (relapse, EDSS and SDMT). Elevated kynurenine levels were associated with higher relapse rate. Reduced serum Trp and indole lactate in patients with MS compared to controls were the strongest and the most consistent findings in that study (NOURBAKHSH et al., 2018)

MS patients and found that the patients exhibited an increase in the frequency of B cells, but a markedly decrease in frequency of CD5+ and IL-10+ B cells compared to healthy controls. PBMCs or B cells from MS patients were co-cultured with MSCs, the frequency of CD5+ IL-10+ B cells also increased, the proliferative and immunosuppressive capacity of CD5+ B cells were significantly enhanced while the apoptosis ratio of this cellular subset significantly decreased (LI et al., 2019)

METABOLOMICS AND BIOMARKERS

Investigating its regulation in CNS infections would improve our understanding of pathophysiology and end-organ damage, and, furthermore, open doors to its evaluation as a source of diagnostic and/or prognostic biomarkers (SÜHS et al., 2019).

Tryptophan (TRP) is the least-abundant essential amino acid. TRP is metabolized in mammalians via two important pathways. The first pathway involves degradation towards serotonin, and the second pathway, which is called the kynurenine pathway, is leading to the production of nicotinamide adenine dinucleotides (NAD and NADH). The kynurenine pathway accounts for catabolism of more than 90% of ingested tryptophan not used for protein synthesis. Due to its importance in linking different kinds of disease with inflammation, metabolite profiling of intermediates of the kynurenine pathway has gained a lot of interest in medical research.

There remains a need for sensitive and reliable biomarkers that can be used longitudinally in patients with multiple sclerosis. Metabolomics has also been done on urine samples, which are readily available for analysis, and this has been used as a potential source of biomarkers in MS. It's already gone identified eight metabolites characterizing EAE mice that are commonly found in plasma and urine and are potential biomarkers. (GAETANI et al., 2020) Thus, a combined metabolomics analysis in urine, where both changes in host inflammatory/metabolic responses and during MS may be highlighted, might help identifying novel biomarkers. The combination of the neurodegenerative biomarkers together with biomarkers of neuroinflammation could provide

additional information on the underlying pathomechanism of disease activity, which is essential for the identification of patients at risk of developing cumulative disabilities (RAJDA et al., 2020)

Metabolomics Several Trp metabolites downregulate CNS imune responses through activation of the aryl hydrocarbonreceptor (AHR) on glial cells. interestingly, these Trp metabolites are produced by intestinal bacteria and this may affect the risk of MS (NOURBAKHSH et al., 2018)

Here, we sought to discuss whether, in MS patients, a specific urinary signature of host/microbiome Trp metabolism can be potentially identified so as to select novel biomarkers and guide toward the identification of specific metabolic pathways as drug targets in MS. (GAETANI et al., 2020)

The concentration of QUIN dramatically increased, whereas that of KYNA slightly decreased in the multiple sclerosis group, resulting in a significantly increased QUIN/KYNA ratio and significantly decreased PICA/QUIN ratio (TÖMÖSI et al., 2020).

Thus, tissue injury and regeneration are not restricted to MS; neurofilaments are not specific for the disease, but they can give a picture of its activity and severity (RAJDA et al., 2020). The combination of the neurodegenerative biomarkers together with biomarkers of neuroinflammation could provide additional information on the underlying pathomechanism of disease activity, which is essential for the identification of patients at risk of developing cumulative disabilities (RAJDA et al., 2020)

The balance between these metabolites is important as it determines overall excitotoxic activity at the N-methyl-D-Aspartate (NMDA) serum KP metabolic signatures in patients can discriminate clinical MS subtypes with high sensitivity and specificity. analysis of KP metabolites in MS patient serum may have application as MS disease biomarkers.The changing levels of KP metabolites we observed also provides a mechanistic insight that may explain the transition from the milder RRMS form to the more debilitating SPMS disease form. KP profiling is likely to be relevant to the pathogenesis of other diseases characterized by inflammation and neurodegeneration, like Alzheimer's disease, Parkinson's disease and ALS, where aberrant KP metabolism has been reported (LIM et al., 2017).

Ease of diagnosis and thus aiming at a prognosis and therapeutic possibilities in order to delay the demyelination and/or death of neurons and improve the quality of life of these individuals. Not only is the discovery of new biomarkers important, but new and rapid methodologies in tissues that are easier to be analyzed such as: Blood, urine, cerebrospinal fluid (CSF), or the combination of more than one evaluation, as well as the use McDonald criteria, combination of clinical, imaging,

and laboratory markers can be useful it's very importante in predicting clinical course and optimizing treatment in individual patients.

TOLEROGENIC

Highest IDO1 expression and catalytic activity occur in dendritic cells (DCs), the most potent antigen-presenting cells that, upon IDO1 up-regulation, acquire tolerogenic functions; IDO1 may represent a major therapeutic target in MS. (MONDANELLI et al.,2020)

An imbalance in tryptophan (TRP) metabolites is associated with several neurological and inflammatory disorders. A change in the relative ratios of these metabolites can provide important insights in predicting the presence and progression of neuroinflammation in disorders such multiple sclerosis, among other diseases (LESNIAK et al., 2013)

Reinforcing defective tolerogenic processes slows progression of autoimmune (AI) diseases and has potential to promote drug-free disease remission (FAZIO et al., 2014; LUKAS et al., 2017; LI et al., 2019; LEMOS et al., 2020; MONDANELLI et al., 2020)

CONCLUSIONS

The prognosis of multiple sclerosis varies substantially across individual patients, and a combination of clinical, imaging, and laboratory markers can be useful in predicting clinical course and optimizing treatment in individual patients. The metabolites of the participation of the Kynurenine pathway in neuroinflammation are well defined in the literature. However, what should be delimited are the treatment routes according to the diagnosis and mainly the subtype of multiple sclerosis. In animals, we cannot yet define these disease subtypes, but we can evaluate more tissue treated in neurodegenerative diseases. In patients, the possibility would be to evaluate the largest number of metabolites to be watching, both in the development and in the progression of the disease. The studies demonstrated in this review used blood, urine, feces and cerebrospinal fluid (CSF) samples to assess inflammatory parameters in patients with multiple sclerosis, in the RRMS and PRMS subtypes. The inhibitors used to study the Kynurenine pathway were from IDO, TDO, KAT and KMO and the metabolites derived from tryptophan are KYN, KYNA, QUIN and serotonin in the course of the disease. Further studies are carried out to carry out biomarkers compatible with the target audience and the specific disease. In addition to the scope of the discovery of metabolites related to tolerogenic function.

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TABLES

Table 2. Evidences of the kynurenine pathway in EAE a model of Multiple Sclerosis.

Author, year	Sample/biomarkers	Inhibit or knockout	Results
Matysiak et al., 2011	Spleen – IDO, PGE2	-	PGE2 induction of IDO-dependent immunoregulation
Fazio et al., 2014	Splenocytes and BILs, cervical lymph nodes – Cytokine Th1, Th2, Th17, IL-17 and IFN-g TGF-b , IL-10	-	Cinnabaric acid (0.1-10 mg / kg, i.p.) was highly protective against EAE. Exogenous CA amplified endogenous production inducing IDO1 and quinureninase
Lee et al., 2015	Spleen, lymph node, and brain - CD3+ T, CD25+, FoxP3+	1-MT	Combination of neuronal stem cells (NSCs) and IDO expression protects from damage caused by EAE
Lanz TV, Becker, 2017	Sérum - TRP and KYN Lymph Node - T Cells	TDO-deficient mice	Combination of neuronal stem cells (NSCs) and IDO expression protects from damage caused by EAE
Lanz TV, Williams 2017	Lymph Nodes -T Cells Spinal Cord – Iba-1 and GFAP, Demyelination Score	-	TDO deficiency did not influence myelin-specific T cells, leukocyte infiltration into the CNS, demyelination and disease activity.
Lukas et al., 2017	spleen and LN -Th1 and Th17 Cell Differentiation Smad7, IRF8, Batf3, IDO1, and IDO2 TGF-β	1-MT	Smad7 is a negative regulator of growth transforming factor-β signaling, Smad7 directs the function of dendritic cells by regulating the expression of IDO in response to IFN-γ signaling.
Li et al., 2019	spinal cords, peripheral blood,	D/L-1MT	suggest that mesenchymal stromal cells may promote CD5 + B cell proliferation, survival and function via the IDO pathway
Lemos et al., 2020	brain/spleen tissue; sérum, spleen – IDO	PF-04859989 4Cl-HAA	increased IDO activity and manipulate pathway metabolites as a tolerogenic form
Mondanelli et al.,2020	IL-6, IL-10, IL-12 p70, IL-27, and transforming growth factor β	-	N-acetylserotonin acts as a positive allosteric modulator of IDO1

	(TGF-β) spinal cord		
Sundaram et al., 2020	Spleen - IDO e KMO TRP, KYN, KYNA, QUIN and PIC, NAD+ and NADH, GFAP, Iba, NeuN, GFAP, IBA1, MBP	1-MT Ro 61-8048	Significant improvement in disease severity was seen treated with 1-MT but was better with KMO inhibitor
Zarzecki et al., 2020	Brain, spinal cord, spleen and lymph node – IDO, KMO, KAT, TRP, KYN, 3-HK, QUIN, GFAP and IBA-1; INF- and IL-17	INCB024360	administration of INCB024360 was able to alleviate weight loss, clinical signs and blunting inflammatory process, suggesting that the increase in IDO/KMO activity and mRNA levels are involved in the severity of the clinical course of EAE.
Legend: N-acetylserotonin (NAS); Positive Allosteric Modulator (PAM); dendritic cells (DCs); ionized calcium-binding adaptor protein-1 (Iba-1), glial fibrillary acidic protein (GFAP), tryptophan-2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase (IDO), Cinnabarinic acid (CA) is an endogenous metabolite of the kynurenine pathway; Experimental Autoimmune Encephalomyelitis (EAE); brain-infiltrating leukocytes (BILs); Kat II and HAAO; tryptophan (TRP), kynurenine (KYN), kynurenic acid (KYNA) and quinolinic acid (QUIN), 3-hydroxykynurenine (3-HK); relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive (PPMS); kynurenine/Trp (K/T) ratio; kynurenine/anthranilate (K/A) ratio; 3-hydroxykynurenine; (3-HK) 3-hydroxyanthranilate; (5-) serotonin; NeuN (neuron), QUIN/IBA1 (macrophage/microglia), and QUIN/MBP (oligodendrocytes), prostaglandin E2 (PGE2), IDO inhibitor (1-MT), IDO inhibitor (INCB024360), KMO (inhibito Ro 61-8048 r), Kat II inhibitor (PF-04859989 or PF), HAAO inhibitor (4-chloro-3-hydroxyanthranilic acid or 4Cl-HAA).			

Table 3. Evidences of the kynurenine pathway in patients with Multiple Sclerosis (MS)

Author, year	Patients/ treatment	Sample - metabolite	Results
Aeinehband et al., 2016	MS n=38	TRP, KYN, QUIN and KYNA	↑ QUIN in patients with recurrent RRMS and ↓ TRP in SPMS may report neurotoxicity and remyelination failure
Lim et al., 2017	n =136 (50 RRMS) (20 SPMS, (17 PPMS) and (49 HC) Cohort 1,2 and 3	Sérum and CSF - TRP, KYN; 3-HK, 3-HAA, and AA, KA, QA, PA, FGF, TNF ALFA, IL-2, IL-7, MIP-1α, MIP-1β	KP have a strong association with the MS subtype, strategies aim to rebalance KP, mainly in terms of QA/KA
Nourbakhsh et al., 2018	Global metabolomics 69/67 cases/controls. Targeted Trp 82 cases, 50 controls and a validation group (92 cases, 50 controls), functional gut microbiome - 17 cases.	Sérum and Stool samples - tryptophan, kynurenine, kynurenate, N-formyl anthranilic acid, xanthurenate, picolinate, serotonin, Nformyl anthranilic acid, indole lactate, indole acetate, indole propionate and 3-indoxyl sulfate	↑ kynurenine were associated with a higher relapse rate. The reduction in serum Trp and indole lactate (microbiota) in patients with MS
Arnhard et al., 2018	100 patients	Plasma-TRP, KYN, QUIN and KYNA	validation of the method to evaluate the metabolites in the plasma of a patient with MS.
Bansi et al., 2018	RRMS n = 33 and SPMS n = 24/ acute aerobic **exercise**	Plasma -Trp, Kyn and 5HT	↑ serotonin after acute resistance training, while the kynurenine pathway was activated only in people with RRMS.
Donia et al., 2019	Thirteen individuals (3 male, 10 female)/30 minutes of moderate-intensity exercise	Sérum -IL-6, TNF alfa and IFN-, IL-1RA, TRP, KYN,	exercise-induced changes in inflammation may have exerted its effects via kynurenine-dependent

Herman et al., 2019	CSF - SPMS n = 16 and RRMS n = 30, control n = 10	117 metabolites	KP and serotonin changed in SPMS/RRMS, as well as a possible connection with the microbiota through the serotonergic system.
Sühs et al., 2019	220 patient with autoimmune neuroinflammation include MS	CSF - TRP, KYN	Kyn CSF low or undetectable MS, but ↑ in bacterial and viral infections of the CNS
Li et al., 2019	10 MS	Heparinized peripheral blood - CD5+ and IL-10+ B cells	mesenchymal stromal cells attenuate MS via IDO-dependent, increasing the suppressor ratio of CD5 + IL-10 B cells. RRMS ↓ Kyn urine but not related to other studies that used other samples (blood, CSF ..)
Gaetani et al., 2020	Urine	Urinary -Trp; kyn; anthranilate; 3-hydroxykynurenine; 3-hydroxyanthranilate; serotonin; tryptamine; indole-3-acetic acid; indole-3-acetamide; indole-3-lactic acid; indole-3-propionic acid.	RRMS ↓ Kyn urine but not related to other studies that used other samples (blood, CSF)
Mondanelli et al.,2020*	RRMS n = 59/NAS (10 mg/kg i.p.)	Supernatants - IFN-γ	reinstallation of the physiological activity of IDO1 in peripheral mononuclear cells of the blood of patients with RRMS. N-acetylserotonin acts as a positive allosteric modulator of IDO1
Rajda et al., 2020	pwMS; n = 37	CSF - NFL, QUIN, neopterin	Neopterin (is a catabolic product of guanosine triphosphate) and QUIN, Neurofilament light (NFL) has been shown to be a good prognostic factor for MS, as its level is proportionally high with extended neuraxonal damage.
Tömösi et al.,	RRMS n = 20	Serum e CSF-KYNA, 3-HK,	validation of UHPLC-MS method serum samples and CSF. ↑ QUIN and

2020		and quinolinic acid QUIN, SER	↓ KYNA pwMS

Legends: * study with the animal model and patients with MS. central nervous system (CNS), patients with Multiple Sclerosis (pwMS); Relapsingremitting multiple sclerosis (RRMS); secondary progressive multiple sclerosis (SPMS); primary progressive; multiple sclerosis (PPMS); healthy controls (HC), N-acetylserotonin (NAS); cerebrospinal fluid (CSF); peripheral blood mononuclear cells (PBMCs); Neurofilament light (NFL); kynurenic acid (KA) and quinolinic acid (QA) tryptophan, kynurenine, TRP, KYN, 3-hydroxykynrenine (3-HK), 3-hydroxyanthranilic acid; (3-HAA), and anthranilic acid (AA) kynurenic acid (KA) quinolinic acid (QA) pilonic acid (PA) fibroblast growth fator (FGF) Tumor necrosis fator (TNF).

CONCLUSÃO

O presente trabalho demonstrou que a VQ é altamente influenciada pela inibição da sua principal enzima, a IDO, visto que essa enzima influencia na cascata da VQ e consequentemente nos níveis de citocinas inflamatórias. Nossos resultados demonstraram, ainda, a associação de metabólitos da VQ e seu papel neuroprotetor (produzidas nos astrócitos) ou neurotóxico (oriundas da microglia) (Figura 5).

Alterações na VQ foram descritas em investigações pré-clínicas e clínicas no modelo de EAE. A via da quinurenina é um novo alvo promissor através do qual se pode influenciar o sistema imunológico e alcançar a neuroproteção, e mais pesquisas são necessárias com o objetivo de desenvolver novas drogas para o tratamento da EM e outras doenças autoimunes. Além disso, a possibilidade de influenciar a VQ para reduzir o ácido quinolínico e aumentar o nível de ácido quinurênico no cérebro oferece um novo alvo para a ação de drogas projetada para alterar o equilíbrio, diminuindo as excitotoxinas e aumentando os neuroprotetores.

Portanto, interferir experimentalmente com o catabolismo do triptofano pode ajudar a elucidar previamente os efeitos estimados dos metabólitos da quinurenina e relacionar essa via a novos alvos terapêuticos para proteger do dano secundário durante a neuroinflamação. Portanto, nossos resultados sugerem que o tratamento com inibidor IDO pode ser usado como um adjuvante no tratamento de doenças neurodegenerativas.

Figura 5. Principais resultados desse estudo
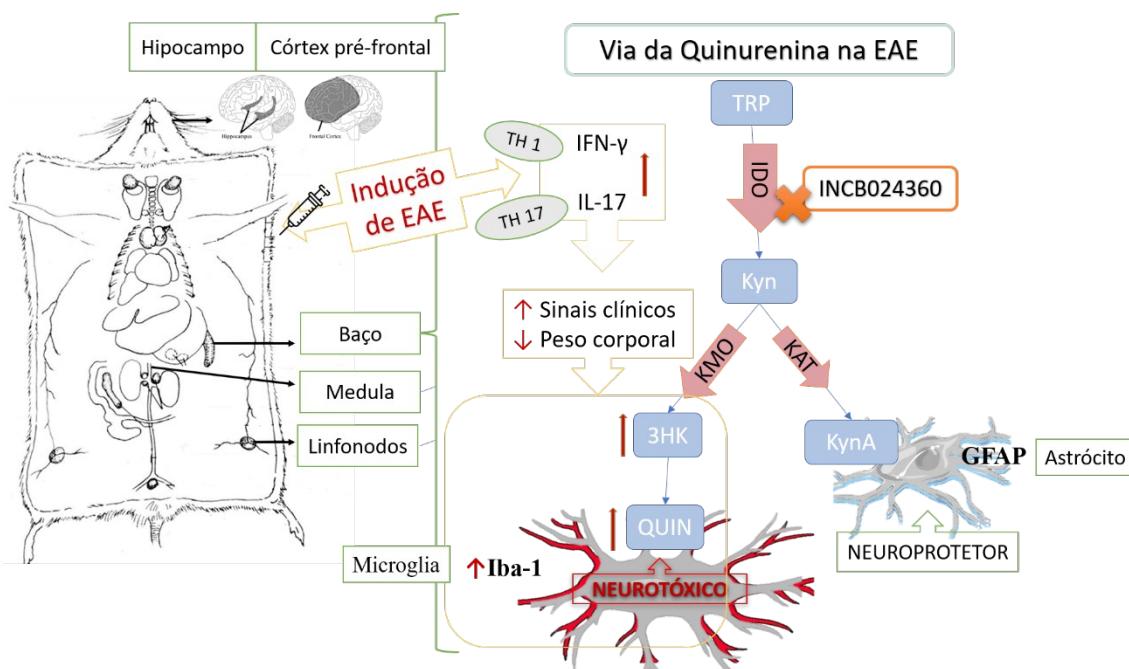
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8   PERSPECTIVAS

Em nosso entendimento, para quem pretender dar continuidade a esse trabalho, será necessário que outros biomarcadores sejam investigados no intuito de se compreender melhor como a VQ está envolvida na EM e como poderá melhorar a progressão da doença. Entendemos a importância da utilização de outros inibidores da IDO, como o 1-MT, bem como algum inibidor da IDO-2. A utilização de animais knockout para os genes da IDO e das demais enzimas da via da quinurenina também se faz importante. Em nosso estudo, investigamos apenas a expressão do KAT-II, no entanto, existem três KATs adicionais expressos que podem gerar KynA (KAT-I, KAT-III, KAT-IV), que podem ser investigados para podermos tirar conclusões sobre os KATs e o ramo neuroprotetor da VQ. Adicionalmente, poderíamos avaliar o efeito sistêmico dos metabólitos da VQ, bem como o seu papel como biomarcadores.

Além de modelos animais, uma sugestão do Professor Cristiano (idealizador da pesquisa), seria coletar amostras de sangue e avaliar o nível dos metabolitos em pacientes acometidos com doenças neurodegenerativas na região da fronteira oeste, visto que há uma grande preocupação e empenho por parte dos órgãos públicos ao atendimento a esses pacientes, acometidos com doenças neurodegenerativas e demência, através da colaboração dos Centros de Atenção Psicossocial (CAPS), que são instituições brasileiras que tratam indivíduos com transtornos mentais graves e persistentes. A utilização e combinação de diferentes ferramentas para o prognostico de doenças autoimunes como o uso de exames clínicos, laboratórias e de imagem, ressonância magnética como determina as diretrizes para o diagnostico da EM, é de fundamental importância para a compreensão do desfecho dessas doenças.

9 Anexo

Parecer de aprovação do Comitê de ética no Uso de Animais da UNIPAMPA

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação (PROPII)

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

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CEUA
Comissão de Ética em Uso de Animais

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: 034/2017 - ADENDO

Título: Investigação da via das quinureninas em um modelo de Encefalomielite autoimune experimental em camundongos.

Data da aprovação: 09/08/2017

Período de vigência do projeto: 09/08/2019

Pesquisadores(a): Cristiano Ricardo Jesse

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Finalidade	( ) Ensino (X) Pesquisa
Espécie/Linhagem/Raça	Camundongos C67BL
Nº de animais	40 + 72 (acréscimo)
Peso/Idade	20 - 22 g / 18 meses
Sexo	Machos
Origem	Biotério da Universidade Federal de Pelotas

Profª. Drª. Vanusa Manfredini
Coordenadora CEUA/UNIPAMPA

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