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USO DO SARS-CoV-2 INATIVADO PARA ESTUDOS PRÉ-CLÍNICOS DE INFLAMAÇÃO PULMONAR E DE EFEITO NA ENCEFALOMIELITE AUTOIMUNE EXPERIMENTAL

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de Botucatu, para obtenção do título de Mestre em Doenças Tropicais.

Orientadora: Profa. Dra. Alexandrina Sartori
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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Souza, William Danilo Fernandes de.
Uso do SARS-CoV-2 inativado para estudos pré-clínicos de inflamação pulmonar e de efeito na encefalomielite autoimune experimental / William Danilo Fernandes de Souza. - Botucatu, 2022

Dissertação (mestrado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina de Botucatu
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Capes: 21104000

1. Encefalomielite autoimune experimental. 2. Pulmões - Doenças. 3. Resposta imune. 4. COVID-19 (Doença). 5. Pneumonia.

Palavras-chave: C57BL/6; Inflamação; Pulmão; Resposta imune; SARS-CoV-2.

AGRADECIMENTOS

A minha família pelo auxílio e apoio em todos os momentos e por acreditar e apoiar todas as minhas escolhas. Agradeço em especial as mulheres da minha vida, minha mãe e minha avó, que sempre me deram o amor necessário para continuar acreditando que a vida é linda e vai muito além de qualquer dificuldade que, por eventualidade, nos confronte em nossa jornada.

Ao meu vovô, que mesmo sem entender nada do que eu executava em laboratório, sempre fazia questão de perguntar como as coisas estavam indo no “trabalho”. Ele se foi no ano passado, mas sei que ele estaria orgulhoso pela minha eventual obtenção de título, não que isso significasse algo para ele, mas eu sei que ele estaria feliz pelo simples fato de seu neto estar feliz.

Ao meu namorado que sempre esteve ao meu lado sem julgar meus momentos de estresse e que sempre me incentivou e torceu pelas minhas conquistas.

A minha orientadora Profa. Dra. Alexandrina Sartori, por me acolher em seu laboratório. Agradeço pela oportunidade, paciência, ensinamentos, dedicação e confiança. Hoje ela encerra sua brilhante carreira na pesquisa e eu tenho a honra de fazer parte da sua história, espero fazer jus ao seu nome ao longo da minha jornada. Passamos juntos por um momento caótico, a Pandemia COVID-19, e isso dificultou muito nosso trabalho, mas jutos vencemos o desafio. Devo tudo ao seu comprometimento e dedicação.

A minha coorientadora Profa. Dra. Dra. Sofia Fernanda Gonçalves Zorzella-Pezavento pela maestria e leveza que conduziu nosso projeto. Devo a ela todo o conhecimento adquirido ao longo desses anos. Além de ser uma excelente profissional, sempre teve a delicadeza de ser um ombro amigo nas horas difíceis.

A Profa. Dra. Denise Moraes da Fonseca pela receptividade em seu laboratório e entusiasmo em nosso trabalho, sua colaboração intelectual e

prática é imensurável, e é por isso que hoje podemos apresentar sua conclusão. Agradeço também a todos os seus alunos, em especial a Marina e Caio, que me auxiliaram com perfeição na execução de técnicas que eu pouco compreendia no início dessa jornada.

Ao professor Dr. José Luiz Proença-Módena e colaboradores pelo auxílio no desenvolvimento do projeto.

Ao professor Dr. João Pessoa Araújo Jr. que confiou em nosso trabalho e aceitou ser o responsável pelo projeto, o que nos permitiu dar o início aos experimentos e concluir nosso estudo.

A Dra. Maria Luiza Cotrim Sartor de Oliveira, que nos permitiu utilizar os equipamentos do laboratório de patologia.

A coordenação e administração da pós-graduação em Doenças Tropicais, Empresa JBS, CAPES e CNPq pelo auxílio financeiro.

E por último, mas não menos importante, aos animais que utilizamos ao longo desse projeto. Agradeço por suas vidas em prol da ciência, no decorrer de cada experimento me comprometi a lhes o oferecer o máximo de respeito e cuidado como tentativa de retribuir qualquer desconforto que tenhamos lhes causado.

A Deus sobre todas as coisas.

“Quando a vida parece difícil, os corajosos não se deitam e aceitam a derrota; em vez disso, estão ainda mais determinados a lutar por um futuro melhor.”

- Rainha Elizabeth II

RESUMO

A COVID-19 é uma doença infecciosa causada pelo coronavírus SARS-CoV-2 que se disseminou para a maioria dos países constituindo uma pandemia. O principal órgão-alvo dessa doença costuma ser o pulmão cujo comprometimento varia de leve a grave. Parte significativa do envolvimento pulmonar tem sido atribuída a um processo inflamatório exacerbado envolvendo tanto a imunidade inata quanto a específica. Na primeira fase desta investigação caracterizamos um modelo de inflamação pulmonar desencadeado pela inoculação intranasal do SARS-CoV-2 inativado em camundongos e avaliamos a capacidade da vitamina D de controlar ou modificar este processo. A instilação nasal de SARS-CoV2 desencadeou um processo inflamatório associado com diferentes tipos celulares e mediadores o qual foi controlado pela administração nasal de vitD. Na segunda fase desta investigação utilizamos o SARS-CoV-2 inativado como uma preparação vacinal para analisar seu efeito na evolução clínica e imunológica da encefalomielite autoimune experimental (EAE) que é o modelo mais utilizado para estudos de esclerose múltipla. Para isto, camundongos C57BL/6 foram, inicialmente, imunizados com o vírus inativado associado ao alúmen e avaliados quanto à indução da resposta imune humoral e celular específicas. Posteriormente, animais assim imunizados foram submetidos à indução da EAE por imunização com MOG na presença de Adjuvante Completo de Freund e toxina pertussis. Até o momento foram avaliados variação de peso corporal e escores clínicos. A imunização com o vírus inativado não agravou o desenvolvimento da EAE.

Palavras-chave: SARS-CoV-2, encefalomielite autoimune experimental, C57BL/6, esclerose múltipla, inflamação, resposta imune, vacinação, pulmão, sistema nervoso central.

ABSTRACT

COVID-19 is an ongoing pandemic triggered by the coronavírus SARS-CoV-2. The main targets of this infectious disease are the lungs and a significant part of its involvement is associated to an accentuated inflammatory process involving innate and specific immunity. Initially, we established and characterized a pulmonary inflammatory process triggered by intranasal instillation of inactivated SARS-CoV-2 in C57BL/6 mice and then evaluated the ability of vitamin D to control or modify this process. Intranasal instillation of inactivated SARS-CoV-2 triggered an inflammatory process constituted of various cells and mediators which was significantly controlled by intranasal vitD administration. We then employed the inactivated SARS-CoV-2 as a vaccine antigen to analyze its effect on the clinical and immunological evolution of experimental autoimmune encephalomyelitis (EAE) which is the most used animal model for multiple sclerosis studies**.** C57BL/6 mice were then immunized with the virus associated to alúmen to characterize the humoral and cellular specific immune responses. Similarly immunized mice were then submitted to EAE induction by inoculation of MOG emulsified with Complete Freund Adjuvant in the presence of pertussis toxin. The following parameters were evaluated up to now: body weight variation and clinical scores. Immunization with the inactivated SARS-CoV-2 did not aggravate EAE development.

Keywords: SARS-CoV-2, experimental autoimmune encephalomyelitis, C57BL/6, multiple sclerosis, inflammation, immune response, vaccination, lung, central nervous system.

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CAPÍTULO 1

1 Revisão de literatura

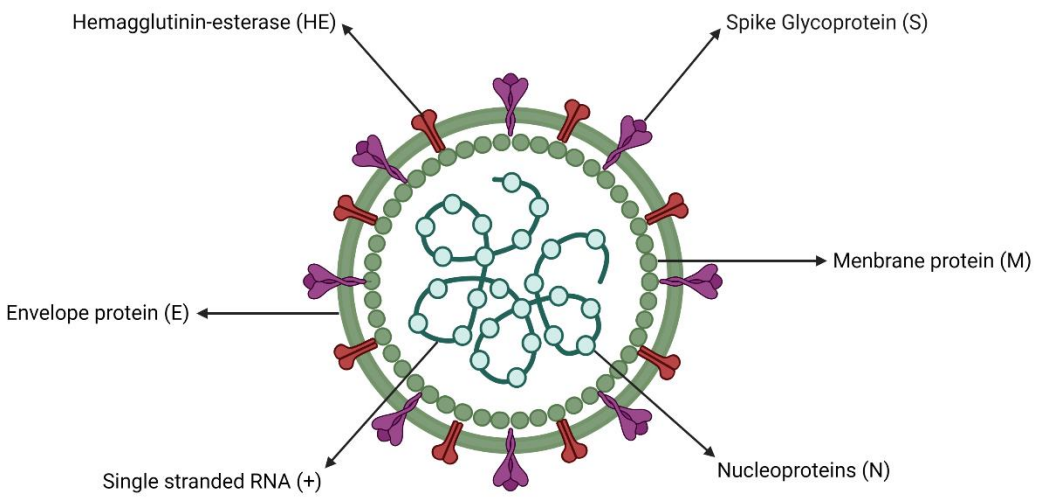
1.1 COVID-19: aspectos epidemiológicos, agente etiológico e formas clínicas

O SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2) é um novo coronavírus, inicialmente identificado em Wuhan, província chinesa de Hubei e que desencadeou uma pandemia denominada COVID-19. A família *Coronaviridae* é grande e inclui 7 espécies capazes de causar infecções em seres humanos que podem ser brandas (resfriados comuns) ou mais graves como é o caso das associadas às pandemias: SARS-CoV, causadora da Severe Respiratory Syndrome Coronavirus entre 2002-2003; MERS-CoV, causadora da Middle East Respiratory Syndrome Coronavirus em 2012 e a espécie SARS-CoV-2, causadora da atual pandemia. Até esta data foram registrados mais de 608 milhões de casos confirmados de Covid-19 e mais de 6 milhões de mortes associadas a este vírus WHO (2020).

A origem deste novo coronavírus permanece obscura. Segundo ANDERSEN et al., (2020), estudos de estrutura genética sugerem que é bastante improvável que o vírus tenha surgido por manipulação laboratorial. Estes pesquisadores sugerem 3 hipóteses para explicar sua origem: 1. seleção natural em um hospedeiro animal seguida de transferência zoonótica, 2. transferência zoonótica para humano seguida de seleção natural no hospedeiro humano, 3. seleção ocorrida in vitro ou in vivo em culturas celulares ou modelo animal, respectivamente, seguida de transferência para ser humano. Estes processos de seleção envolveriam especialmente mutações na região RBD (receptor binding domain) que é responsável pela interação com a molécula receptora nas células humanas que é a angiotensin-converting enzyme 2 (ACE2). Morcegos e pangolins são sugeridos como os prováveis reservatórios do vírus progenitor.

A estrutura viral e o mecanismo de infecção das células-alvo já são bem descritos na literatura. O SARS-CoV-2, que pertence ao grupo dos beta-coronavírus juntamente com o SARS-CoV e o MERS-CoV, é um vírus

envelopado contendo uma única molécula de RNA+ de fita simples associada com várias cópias de nucleoproteínas. Este nucleocapsídeo é envolto por uma bicamada lipídica na qual estão ancoradas 4 glicoproteínas estruturais: spike, hemaglutinina-acetil transferase, glicoproteína de membrana, glicoproteína do envelope e uma fosfo-proteína também associada ao envelope, como ilustrado na **figura 1**.

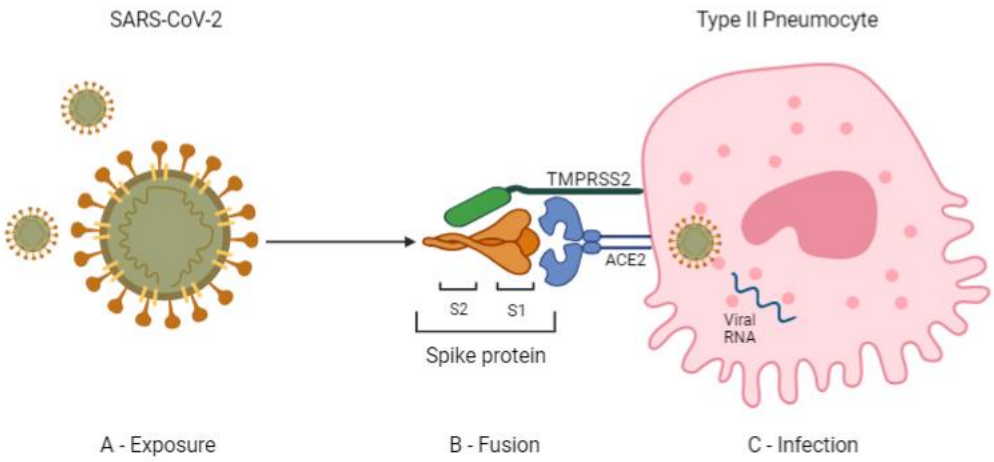


Fonte: Created with Biorender.com

Figura 1. Representação da estrutura do SARS-CoV-2. A partícula viral é constituída pelas glicoproteínas estruturais: Spike (S), Hemaglutinina-acetil Transferase (HE), Envelope (E) e Membrana (M) e contém em seu núcleo RNA (+) de fita simples associado a nucleoproteínas (N), que juntos formam o nucleocapsídeo.

O processo de infeção envolve a proteína S (spike) que é exposta sob a forma trimérica e cuja distribuição na superfície da partícula viral forma estruturas similares a espículas conferindo a aparência de uma coroa no microscópio eletrônico; esta conformação resultou no nome coronavírus (HELMY et al., 2020). A Spike é composta pelos domínios S1 e S2, sendo que a região RBD, que é responsável pela ligação na célula-alvo, está

localizado no S1 enquanto a região responsável pela fusão entre o vírus e a célula-alvo está situada no domínio S2 (BORGES, 2020). A ACE2, como referido acima, funciona como receptora do vírus enquanto a serina protease transmembrana 2 (TMPRSS2), também presente na superfície da célula-alvo, catalisa a clivagem proteolítica facilitando a fusão viral com a célula-alvo. O processo de infecção está ilustrado na **figura 2**. Vários receptores alternativos e proteases celulares adicionais estão envolvidos neste processo de infecção (MASRE et al., 2021). Apesar do pulmão e da traqueia serem descritos como os alvos primários do vírus, já se sabe que o vírus também pode infectar rins, intestino delgado, pâncreas, vasos sanguíneos, glândulas sudoríparas e células endoteliais vasculares (LIU et al., 2021).



Fonte: Created with Biorender.com

Figura 2. Processo de infecção de célula humana pelo SARS-CoV-2. (A) Os indivíduos são expostos ao SARS-CoV-2 principalmente através das vias aéreas superiores e por esse motivo as células da traqueia e do pulmão são consideradas alvos primários de infecção. (B) Após a exposição ao vírus, a proteína denominada Spike (subdividida em domínio S1 e S2), expressa na superfície da partícula viral, interage com as proteínas de membrana do hospedeiro (ACE2 e TRPMSS2) respectivamente, resultando na fusão do vírus/célula. (C) Uma vez concluídos os processos de fusão e passagem do material genético do vírus para o interior da célula, a replicação tem início.

Diferentes graus de gravidade podem ser observados nos indivíduos com COVID-19. Segundo (BAJ et al., 2020), estas manifestações podem ser agrupadas em 5 graus de gravidade crescente cujos sintomas mais típicos são listados no quadro abaixo.

Quadro 1. Níveis de gravidade e seus sintomas na infecção por SARS-CoV-2. Indivíduos com COVID-19 têm sido classificados em 5 níveis distintos de acordo com os sintomas.

QUADRO CLÍNICO DE PACIENTES COM COVID-19 – GRAVIDADE x SINTOMAS	
GRAVIDADE DA DOENÇA	**SINTOMAS**
Assintomática	- Ausência de manifestações clínicas - Sem alterações na tomografia e raio X
Leve	- Febre, tosse seca, dor de garganta, espirros, cansaço, dor muscular, dor de cabeça e perda do olfato - Usualmente sem alterações nos exames de imagem pulmonar
Moderada	- Sintomas de quadro pneumônico, febre, tosse seca - Exames de imagem do pulmão mostram opacidade pulmonar lembrando vidro fosco além de áreas de consolidação
Severa	- Dispnéia, hipóxia e mais de 50% de comprometimento pulmonar, diarréia, vômitos e náusea - Exames de imagem mostram opacidades típicas, consolidação, efusão pleural e linfadenopatia
Crítica	- Dificuldade respiratória, dor torácica, limitação da mobilidade e fala, evolução para síndrome do estresse respiratório, arritmia, insuficiência cardíaca e hepática, encefalopatia, coagulação intravascular disseminada e choque séptico

Fonte: (BAJ et al., 2020)

1.2 Resposta imune, “cytokine storm” e patogênese pulmonar

Segundo AHMADPOOR & ROSTAING (2020), na maioria dos pacientes, os quais desenvolvem a forma benigna da doença, ocorre ativação da resposta imune inata seguida de resposta imune específica que juntas controlam a replicação viral e determinam recuperação clínica. De acordo com estes autores, em indivíduos saudáveis e não idosos, a interação do vírus com o TLR-7, localizado nos endossomas, desencadeia uma produção inicial de IFN-γ, IL-12 e IL-6, seguida da expansão de células Th, ativação de linfócitos B com subsequente produção de anticorpos e diferenciação de linfócitos Tc. Entretanto, quando o sistema imunológico do indivíduo afetado é incapaz, por razões diversas (comorbidades e imunossenescência, por exemplo), de montar uma resposta imune eficiente, ocorrerem várias alterações imunológicas envolvendo tanto a imunidade inata quanto a específica. As alterações mais comuns incluem queda na quantidade de linfócitos, incluindo na subpopulação de T reguladores e exaustão funcional de linfócitos citotóxicos (Tc e NK) (YE; WANG; MAO, 2020; ZHENG et al., 2020). A diminuição de linfócitos inclui tanto os TCD4+ quanto os TCD8+ e é acompanhada de queda na produção de IFN-γ (WU et al., 2020). Os casos mais graves também são caracterizados por elevada quantidade de polimorfonucleares e aumento nos níveis de proteína C reativa (OUYANG et al., [s.d.]).

Um dos aspectos que vem sendo enfatizado na patogênese da COVID-19 é a ocorrência de uma “cytokine storm” derivada principalmente de células da imunidade inata. Segundo vários relatos, esse fenômeno está associado com doença mais grave e determina a síndrome do desconforto respiratório que pode progredir para a síndrome de falência de múltiplos órgãos e finalmente para óbito do paciente (YE; WANG; MAO, 2020). Evidências clínicas confirmam um estado hiperinflamatório nestes pacientes caracterizado por níveis elevados de diversas citocinas e quimiocinas, incluindo IL-1β, TNF-α, IL-2, IL-6, IL-7, GM-CSF, IL-12, IL-17,

IP-10, MIP-1α, MCP-1 (MEHTA et al., 2020). Exames post-mortem mostraram necrose tecidual e infiltração intersticial de macrófagos no pulmão, coração e mucosa gastrointestinal em pacientes com elevada concentração de citocinas inflamatórias (XU et al., 2020b).

A COVID-19 atinge de forma mais brutal a estrutura pulmonar. Segundo MASON (2020), considerando uma perspectiva biológica, a gravidade do envolvimento pulmonar está associada à região do pulmão na qual o vírus está em processo de multiplicação. Resumidamente, este comprometimento envolve 3 estágios. No estágio inicial, que seria assintomático, o vírus inalado se liga em células epiteliais presentes na cavidade nasal e inicia um processo de replicação que é acompanhado de uma discreta ativação da resposta imune inata. No estágio 2, caracterizado pelo aparecimento de sintomas clínicos brandos, o vírus se propaga para a parte inferior do sistema respiratório e a resposta imune inata se torna mais acentuada. Algumas quimiocinas, como por exemplo a CXCL10, têm sido sugeridas como marcadores preditores de síndrome respiratória aguda grave (TANG et al., 2005). No estágio 3 o vírus atinge as unidades de troca gasosa, infecta células alveolares e, possivelmente, causa apoptose e dano alveolar difuso.

No contexto acima queremos investigar se o processo inflamatório que ocorre no pulmão e a produção elevada de citocinas podem ser mimetizados pela instilação nasal do vírus morto em modelo murino. Uma vez confirmada esta possibilidade, avaliaremos se estas alterações podem ser controladas por vitamina D (vitD). A escolha dessa vitamina se baseia fundamentalmente em nossa linha de pesquisa. Já demonstramos anteriormente que a vitD controla de forma eficaz o processo inflamatório que ocorre no sistema nervoso central (SNC) durante o desenvolvimento da EAE (CHIUSO-MINICUCCI et al., 2015; DE OLIVEIRA et al., 2020; ZORZELLA-PEZAVENTO et al., 2017). Aliado a isto, a literatura indica que a vitD aplicada por via intranasal (IN) determina tanto efeito anti-inflamatório local quanto no SNC (ENKHJARGAL et al., 2017; KIM et al., 2019). Além

destas propriedades anti-inflamatórias, a vitD também vem sendo apontada na literatura recente como uma das substâncias com potencial terapêutico/imunorregulador para a COVID-19. Tem sido sugerido, por exemplo, que a deficiência de vitD em países do hemisfério norte seria um fator adicional para a maior gravidade da infecção nestas regiões em comparação com o hemisfério sul (RHODES et al., 2020). Em revisão recente, GRANT et al., (2020), discutem alguns aspectos que justificariam a suplementação com esta vitamina para reduzir o risco de infecção por vírus influenza e SARS-CoV-2. De acordo com estes autores, os principais efeitos protetores da vitD seriam através de indução de catelicidinas e defensinas as quais possuem efeito antiviral, e de inibição sobre a produção de citocinas pró-inflamatórias.

A possibilidade de que a vitD possa controlar, de forma concomitante, processos inflamatórios no SNC e no pulmão, nos parece muito promissora no contexto atual da COVID-19, tanto para os portadores de EM como de outras patologias neurológicas.

1.3 Esclerose múltipla: modelo experimental, imunopatogênese e fatores ambientais

A EM é uma doença inflamatória e desmielinizante do SNC que é descrita como a principal causa de incapacidade neurológica em adultos jovens (HOHLFELD, 2009). A doença afeta 2,3 milhões de pessoas em todo o mundo, tendo maior prevalência na Europa e na América do Norte (AARLI et al., 2014). No continente americano a incidência é de 8,3 casos a cada 100.000 habitantes (WHO, 2008) e a Associação Brasileira de EM estima que 40 mil brasileiros estão atualmente afetados.

A maioria dos pacientes apresenta um quadro transitório de sintomas com períodos de exacerbação e remissão da doença, seguido por uma fase secundária progressiva, caracterizada por perdas irreversíveis e neurodegeneração (HOHLFELD, 2009). As manifestações clínicas da EM

incluem fraqueza de um ou mais membros, perda da visão, falta de coordenação motora, depressão e fadiga (GELFAND, 2014).

O modelo experimental mais utilizado para estudar diferentes aspectos da EM tais como imunopatogênese e alternativas terapêuticas é conhecido como encefalomielite autoimune experimental (EAE) a qual é induzida em animais de diferentes espécies, principalmente em distintas cepas de camundongos. A EAE é desencadeada por imunização com antígenos derivados da bainha de mielina (doença ativa) ou por transferência adotiva de linfócitos T isolados de animal previamente imunizado com estes antígenos (doença passiva). As manifestações clínicas e as alterações imunopatogênicas observadas na EAE variam um pouco entre os diferentes animais e as distintas cepas de camundongos, mas parecem refletir justamente a heterogeneidade encontrada na doença humana correspondente. As duas cepas de camundongos utilizadas com mais frequência para indução de EAE são a C57BL/6 e a SJL (SIMMONS et al., 2013) as quais mimetizam a EM de caráter crônico e remitente-recorrente, respectivamente. Nestes dois modelos ocorre um processo inflamatório no SNC, em especial na medula espinal, seguido de desmielinização e manifestações de paralisia flácida ascendente os quais permitem investigar o efeito deletério ou protetor de vários agentes infecciosos, drogas e procedimentos vacinais.

Estudos em amostras derivadas de pacientes e de animais com EAE vêm permitindo revelar detalhes da imunopatogênese. Está demonstrado que o processo inflamatório no SNC resulta em desmielinização, morte dos oligodendrócitos, dano axonal, gliose e neurodegeneração (HEMMER; KERSCHENSTEINER; KORN, 2015; KAWACHI; LASSMANN, 2017). Linfócitos Th1, Th17 e Tc específicos para antígenos do SNC são ativados nos órgãos linfoides secundários e então migram para o SNC onde são reativados por reconhecimento local de antígenos próprios, iniciando assim o processo de lesão inflamatória autoimune (DENDROU; FUGGER; FRIESE, 2015; HARTUNG et al., 2014). As células Th17 também

desempenham um papel importante na patogênese da EM contribuindo para a ruptura da barreira hematoencefálica e para o processo inflamatório (WOJKOWSKA et al., 2014). Ênfase também tem sido dada à participação de células T reguladoras (Tregs), visto que deficiências numéricas e funcionais destas células têm sido descritas em pacientes com EM (KOUCHAKI et al., 2014). Células da imunidade inata tais como microglia e macrófagos também participam ativamente da patogênese da EM/EAE. Estudos mostram que estes fagócitos internalizam e degradam a bainha de mielina, dando início aos processos inflamatório e desmielinizante (FISCHER; ZELINKA; MILANI-NEJAD, 2015). A contribuição dos astrócitos para a progressão da doença também vem sendo investigada (CORREALE, 2014). Esse processo inflamatório é significativamente mediado pelas espécies reativas de oxigênio (EROs), ou seja, radicais livres derivados do oxigênio molecular, os quais podem ser formados nas células durante os processos oxidativos, principalmente na cadeia respiratória através do transporte mitocondrial de elétrons (OHL; TENBROCK; KIPP, 2016). Microglia ativada e macrófagos infiltrantes liberam vários mediadores pró-inflamatórios e radicais livres como superóxido, hidroxil, peróxido de hidrogênio e óxido nítrico (COLTON and GILBERT, 1993). As EROs oxidam a maioria dos componentes celulares como lipídios, proteínas, carboidratos e ácidos nucléicos, provocando alterações estruturais e, consequentemente funcionais, através do processo de peroxidação. Para impedir os efeitos deletérios das EROS são ativados mecanismos de defesa antioxidantes. Existem basicamente dois grupos de antioxidantes: enzimas com atividade antioxidante (superóxido dismutase, glutationa peroxidase e catalase) e antioxidantes não enzimáticos (vitamina C, α-tocoferol, glutationa reduzida, vitamina D e selênio) (Halliwell and Gutteridge, 2007; RAYMAN, 2012). Entretanto, a produção excessiva de EROs e/ou redução no sistema de defesa antioxidante endógeno determina estresse oxidativo (Lopes, Oliveira, and Soares Fortunato, 2008) que é considerado um evento primordial na

patogênese da EM (MATUTE-BLANCH; MONTALBAN; COMABELLA, 2017). O estresse oxidativo está diretamente associado com um sistema denominado inflamassoma que amplifica os danos determinados pelas EROs. O inflamassoma é um complexo proteico macromolecular composto por um sensor molecular, a proteína adaptadora ASC e a protease caspase-1. O inflamassoma é ativado em resposta a infecções, dano tecidual ou desequilíbrios metabólicos, iniciando programas celulares responsáveis pela maturação e liberação de citocinas pró-inflamatórias como IL-1β e IL-18 e também por um tipo de morte celular denominado piroptose (MIAO; RAJAN; ADEREM, 2011). O inflamassoma NLRP3 é o que se encontra descrito com maiores detalhes até o momento e está amplamente associado às doenças inflamatórias crônicas e autoimunes (WEN; TING; O'NEILL, 2012). Para ativação de NLRP3 e formação do inflamassoma são necessários dois sinais: o sinal 1 ou priming, desencadeado pela ativação do NF-κB por TLRs, NLRs e receptores de citocinas, o que resulta na transcrição de NLRP3 e pró-IL-1β (BAUERNFEIND et al., 2009). O segundo sinal é determinado por agonistas que ativam o complexo inflamassoma e consequentemente a caspase-1. Esse segundo sinal pode ser por efluxo de potássio (K+), translocação mitocondrial, produção de EROs e liberação de DNA mitocondrial. Está comprovado que as EROs ativam o inflamassoma NLRP3 no decurso do diabetes tipo II experimental em camundongos (ZHOU et al., 2010). As EROs também têm sido descritas como ativadoras do inflamassoma NLRP3 no SNC em camundongos infectados com vírus. Aspectos referentes à participação do inflamassoma em diferentes doenças inflamatórias que afetam o SNC vêm sendo alvo de muita investigação e discussão tanto para que os mecanismos patogênicos destas doenças sejam conhecidos como também para se avaliar a possibilidade de utilizar esta plataforma como alvo terapêutico (VOET et al., 2019). A literatura sugere fortemente que a ativação do inflamassoma esteja relacionada com a progressão da EM visto que as citocinas sintetizadas e liberadas através deste complexo proteico, como é o caso

das IL-1β e IL-18, atuam na diferenciação e manutenção das células Th1 e Th17 que são subpopulações celulares altamente envolvidas na patogênese da doença (CHUNG et al., 2009). Além disso, há uma elevada expressão de caspase-1 nas lesões localizadas no SNC e nas células mononucleares do sangue periférico de pacientes com EM (MING et al., 2002). Em consonância com estes achados, camundongos com EAE exibem elevada expressão de NLRP3 na medula espinhal, enquanto camundongos NLRP3-/- desenvolvem doença mais branda, decorrente da redução do infiltrado inflamatório, menor produção de citocinas encefalitogênicas e preservação da bainha de mielina (GRIS et al., 2010).

O desenvolvimento da EM depende de predisposição genética, estilo de vida e fatores ambientais. Existem fortes evidências da associação entre EM e baixos níveis de vitamina D, obesidade durante a adolescência, tabagismo e infecções virais (ALFREDSSON; OLSSON, 2019). A possibilidade de que o vírus Epstein-Barr (EBV) seja um gatilho importantíssimo para o desenvolvimento da EM foi recentemente reforçada pelo trabalho de (BJORNEVIK et al., 2022). Estes autores estudaram uma coorte de mais de 10 mil jovens adultos (militares) e encontraram 955 indivíduos com EM. Constataram que o risco de desenvolver esta patologia aumentou 32 vezes após a infecção com o EBV mas não aumentou após a infecção com outros vírus. Um achado bastante interessante foi o aumento dos níveis de neurofilamento de cadeia leve que é um biomarcador de degeneração neuroaxonal, após a soroconversão anti-EBV.

Nosso laboratório dispõe de bastante experiência com modelos experimentais de EM, especialmente com o modelo de doença crônica pois este vem sendo utilizados no estudo de fatores ambientais que podem afetar o desenvolvimento da EAE e na busca de terapias alternativas ou adjuntas para a EM (FRAGA-SILVA et al., 2015; PINKE et al., 2020; ZORZELLA-PEZAVENTO et al., 2017). Neste trabalho vamos explorar o modelo desenvolvido por animais C57BL/6 imunizados com um peptídeo derivado da mielina do oligodendrócito (MOG35-55). Este modelo de

doença inflamatória crônica, que mimetiza a imunopatogênese observada na EM, será utilizado para investigar se a resposta imune induzida por imunização subcutânea com vírus inativado determina agravamento desta patologia desmielinizante.

1.4 Esclerose múltipla & COVID-19 & vacinação

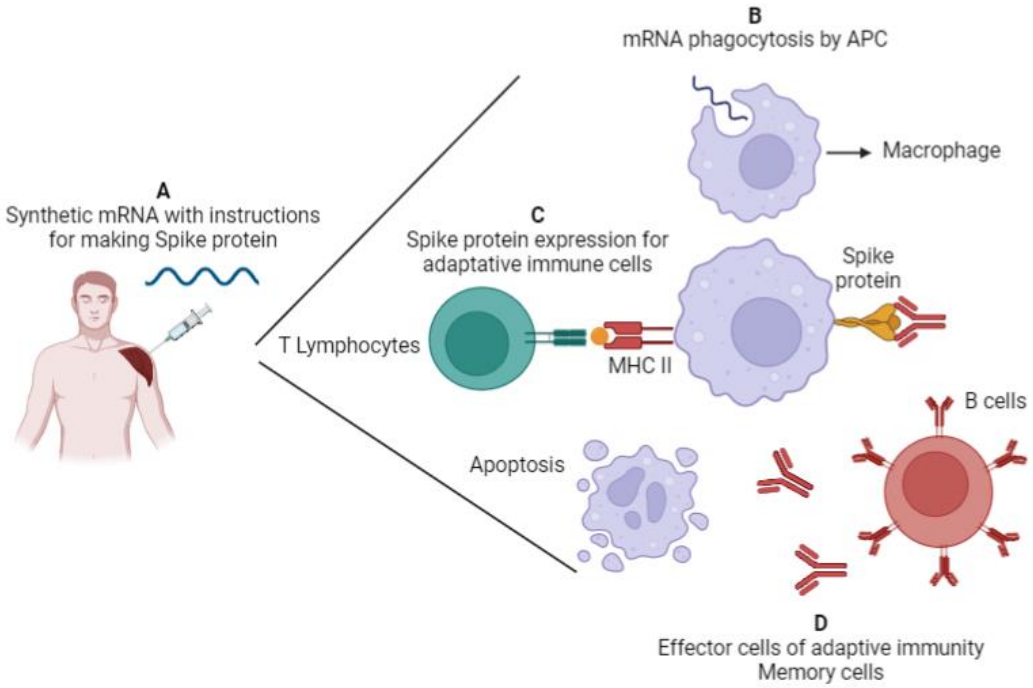
A relação entre COVID-19 e EM é bastante complexa e deve ser analisada sob diferentes ângulos. Uma das primeiras indagações nesta área foi se os pacientes com EM, por estarem sob terapia imunossupressora ou imunomoduladora, teriam maior risco de adquirir esta infecção. Algumas publicações têm indicado que a susceptibilidade ao SARS-CoV-2 é afetada pelo tipo de droga em questão; IFNs e acetato de glatiramer foram associados com risco reduzido enquanto o uso de anticorpos monoclonais anti-CD20 determinou aumento do risco (REDER et al., 2021). Fatores como comorbidades, obesidade e ancestralidade negra também parecem contribuir para aumentar o risco de infecção nestes pacientes (REDER et al., 2021). Outros relatos não confirmaram este risco aumentado de COVID-19 em pacientes com EM mas sim um maior risco de hospitalização nestes indivíduos (EVANGELOU et al., 2021; ZABALZA et al., 2021).

Outra questão importante refere-se ao possível efeito desta infecção na evolução da EM. Investigações indicam que o SARS-CoV-2 pode desencadear tanto pseudo surtos quanto surtos verdadeiros (BELLUCCI et al., 2021; GARJANI et al., 2021). Um aspecto muito interessante que vem sendo também discutido é a possibilidade de que a infecção pelo SARS-CoV-2 atue como um fator ambiental que contribui para a indução de autoimunidade envolvendo o SNC, incluindo a EM. A entrada do SARS-CoV-2 no SNC ativa a microglia, desencadeando neuroinflamação crônica e possivelmente neurodegeneração. A análise transcriptométrica do SARS-CoV-2 revela similaridade com vários epítopos do SNC o que pode,

juntamente com a cytokine storm e vários autoanticorpos, culminar em uma condição autoimune (GUPTA; WEAVER, 2021).

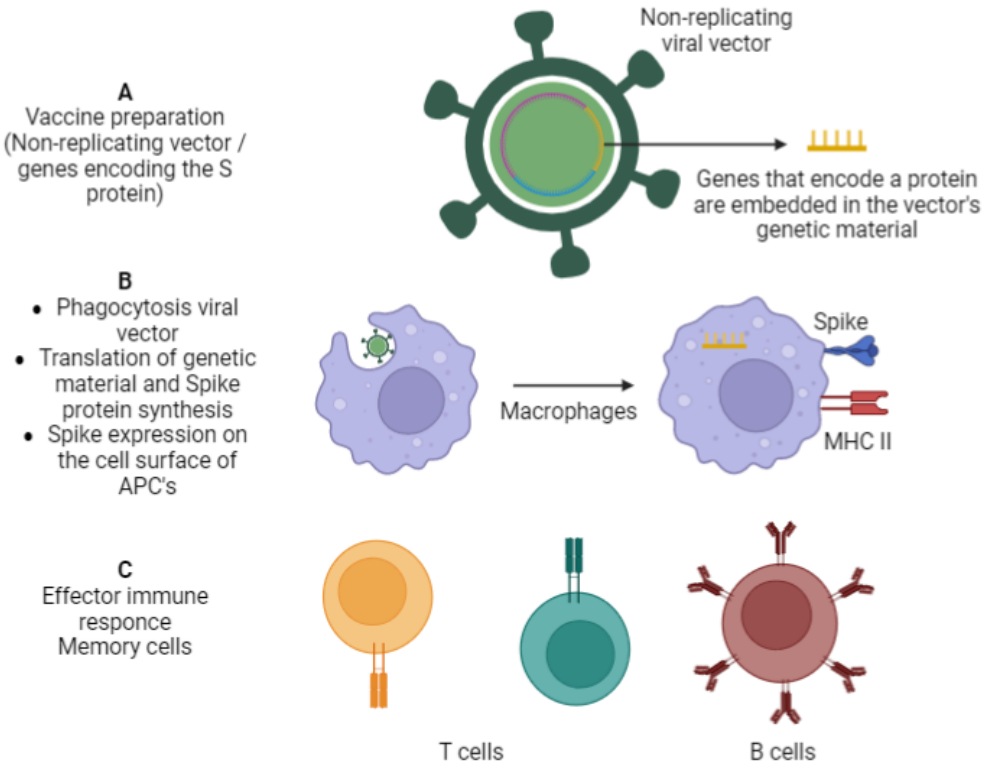
Existe uma concordância geral de que pacientes com EM não devem suspender a terapia mas que devem receber a imunização anti-COVID-19. Recomenda-se, entretanto, uma análise acurada do tipo de terapia uma vez que algumas delas são imunodepletoras o que pode diminuir consideravelmente a eficácia das vacinas. Por exemplo, pacientes em tratamento com ocreluzimab, rituximab e alemtuzimab devem discutir com seus médicos a estratégia de imunização para otimizar a eficácia da vacina.

Várias vacinas, baseadas em distintos mecanismos de ação, vêm sendo aprovadas para controle da COVID-19, incluindo principalmente as inativadas, as baseadas em vetores virais não replicantes e aquelas contendo subunidades tais como proteínas e mRNA (FRANCIS et al., 2021). Os programas vacinais adotados priorizaram, inicialmente, os indivíduos mais vulneráveis e os pacientes com EM têm sido vistos como de risco mais elevado. Todas as vacinas aprovadas são capazes de induzir anticorpos neutralizantes contra a proteína Spike mas só as baseadas em vetor viral e em ácidos nucléicos (mRNA neste caso), desencadeiam imunidade celular do tipo Tc (JEYANATHAN et al., 2020). Segundo a National Multiple Sclerosis Society, as três vacinas mais recomendadas para os pacientes que convivem com a EM são a Pfizer BioNTech, a Moderna e a Janssen/J&J. As 2 vacinas de mRNA são constituídas por mRNA codificando a Spike e envolto por nanopartículas.



Fonte: Created with Biorender.com

Figura 3. Mecanismo de ação das vacinas de mRNA. O mRNA mensageiro sintético contendo as informações para síntese da proteína (S) é produzido em laboratório e introduzido no indivíduo através da vacinação. (B) Uma vez localizado pelas células apresentadoras de antígeno APCs), ocorre o processo de fagocitose desse material genético. (C) As APC's então sintetizam a proteína codificada, processam a mesma e apresentam os epítopos, associados às moléculas de histocompatibilidade de classe, aos linfócitos Th. (D)Essa apresentação inicia a imunidade efetora e a geração de memória imunológica.



Fonte: Created with Biorender.com

Figura 4 Mecanismo de ação da vacina baseada em vetor viral não replicante. (A) A vacina é elaborada com a introdução dos genes responsáveis pela síntese da proteína Spike no material genético de um vírus sem capacidade de causar doença e replicação. (B) O vírus é fagocitado pelas APCs e os genes são copiados em mRNA sendo a Spike resultante da tradução do mRNA expressa na superfície das células do hospedeiro, além de também serem processadas e apresentadas através de moléculas de MHC II. (C) A células da imunidade adaptativa são especificamente ativadas ocorrendo a montagem da resposta imune efetora e memória imunológica.

Dois aspectos vêm recebendo atenção especial no contexto da vacinação dos pacientes com EM. O primeiro deles se refere à eficácia dessas vacinas considerando que estes indivíduos estão sob terapia imunossupressora ou que podem apresentar alterações imunológicas. O uso de DMTs (disease modifying therapies) pode determinar decréscimo da eficácia vacinal e isto foi observado nos pacientes fazendo uso de ocrelizumab, rituximab, cladribine e esteróides. O grau de segurança das vacinas anti-COVID-19 vem sendo investigado e tem mostrado resultados

divergentes. Evidências preliminares realmente não apontaram para aumento de risco de surtos nestes pacientes (ACHIRON et al., 2021). Entretanto, alguns achados têm sugerido o aparecimento de surtos após vacinação. Inicialmente foram relatados 2 casos isolado de surtos agudos após vacinação anti-COVID-19 (ETEMADIFAR et al., 2021; MANISCALCO et al., 2021). Mais recentemente, NISTRI et al., (2021) relataram o aparecimento de surtos em 16 pacientes convivendo com EM os quais foram acompanhados entre os meses de março e junho de 2021, após terem sido vacinados. Três destes 16 pacientes desenvolveram o primeiro episódio de EM após terem sido vacinados e os outros 13 já tinham diagnóstico prévio de EM. As vacinas empregadas nestes indivíduos foram Pfizer (10 pacientes), Moderna (2 pacientes) e AstraZeneca (4 pacientes). Todos os surtos ocorreram entre 3 dias e 3 semanas após a primeira dose ou após o reforço. Os 16 pacientes descritos apresentaram evidências de comprometimento na MRI condizente com o aparecimento dos surtos. Os autores alertam para a necessidade de esclarecer se esta associação temporal foi causal ou incidental.

2 Justificativa

Um dos aspectos mais importantes desta infecção é o comprometimento pulmonar que é o órgão alvo inicial e que dependendo da gravidade, pode resultar em doença muito prolongada ou morte do paciente. A linha de pesquisa do nosso laboratório está voltada para a imunopatogênese dos processos inflamatórios e seu controle por diferentes produtos, como por exemplo a vitamina D. A primeira abordagem dessa investigação foi estabelecer e caracterizar um modelo de inflamação pulmonar induzido por instilação nasal de SARS-CoV-2 inativado e, posteriormente, avaliar a eficácia terapêutica ou imunomoduladora da administração de vitamina D. A relevância desta análise reside no fato deste processo inflamatório ser provavelmente o aspecto mais acentuado da patogênese desta infecção e

por ter a vitamina D características que facilitariam sua adoção como terapia. Neste sentido, ressaltamos que esta vitamina é produzida de forma endógena após exposição solar, é dotada de reconhecida capacidade imunomoduladora e já vem sendo sugerida ou adotada terapeuticamente em doenças inflamatórias (AO; KIKUTA; ISHII, 2021). Inclusive LAKKIREDDY et al., (2021) mostraram recentemente que elevadas doses de vitamina D diminuíram vários marcadores inflamatórios em pacientes com COVID-19 sem desencadear efeitos colaterais.

Outro processo inflamatório no qual temos interesse ocorre no sistema nervoso central (SNC) no decurso da esclerose múltipla (EM) que é uma doença associada a uma resposta autoimune contra componentes da mielina e descrita como a principal causa de incapacidade neurológica em adultos jovens (HOHLFELD, 2009). Para estudá-la utilizamos um modelo murino desta doença denominado encefalomielite autoimune experimental (EAE). Neste contexto, nos interessamos pelos possíveis efeitos da resposta imune induzida pelo vírus, incluindo por preparações vacinais, na evolução da EAE. Avaliamos então o efeito da imunização com o SARS-CoV-2 inativado e associado ao alúmen, o que é semelhante ao processo vacinal com a Coronavac, amplamente empregada em nosso país, no desenvolvimento clínico e imunológico da EAE.

3 Objetivos

1. Induzir um modelo murino de inflamação pulmonar por instilação nasal de SARS-CoV-2 Inativado.
2. Caracterizar as populações celulares envolvidas na inflamação.
3. Investigar o efeito do tratamento com vitamina D.
4. Investigar se a resposta immune induzida por antígeno vacinal (virus morto associado ao alúmen afeta as características clínicas e imunológicas da EAE.

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CAPÍTULO 2

Artigo submetido à Frontiers in Pharmacology em 22.09.2022

Experimental lung inflammation induced by inactivated SARS-CoV-2 is controlled by intranasal instillation of vitamin D

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Keywords: SARS-CoV-2, COVID-19, lung, inflammation, mice, vitamin D

Abstract

COVID-19 is a pandemic triggered by the coronavírus SARS-CoV-2 whose peak occurred in the years 2020 and 2021. The main target of this virus is the lung, and the disease is associated to an accentuated inflammatory process involving mainly the innate arm of the immune system. Here, we described the induction of a pulmonary inflammatory process triggered by the intranasal (IN) instillation of UV inactivated SARS-CoV-2 in C57BL/6 mice and then the evaluation of vitamin D (VitD) ability to control or modify this process. The assays used to assess the severity of lung involvement included total and differential number of cells in the BALF, histopathological

analysis, quantification of T cell subsets and inflammatory mediators by RT-PCR, cytokine quantification in lung homogenates and flow cytometric analysis of cells recovered from lung parenchyma. IN instillation of inactivated SARS-CoV-2 triggered a pulmonary inflammatory process, consisting of various cell types and mediators, resembling the typical inflammation found in COVID-19 patients. This inflammatory process was significantly downmodulated by IN VitD administration, suggesting that this hormone has the potential to be an adjunct therapy for COVID-19.

1 Introdução

The SARS-CoV-2, a newly identified β-coronavirus, is the causative agent of the pandemic respiratory pathology known as COVID-19 whose peak occurred in 2020 and 2021. Even though most of the affected individuals are asymptomatic or develop mild symptoms, a minor proportion evolve towards a severe pathology. A plethora of factors related to the host, the environment, and the virus itself can affect disease outcome (SAMADIZADEH et al., 2021). Even though the lung is considered the primary target of this infection, the virus can spread to many other organs as kidneys, liver, spleen, muscles, and the nervous system (MACHHI et al., 2020). Pulmonary manifestations vary from asymptomatic or mild pneumonia to a severe disease accompanied by hypoxia, shock, respiratory failure, and multiorgan deterioration or death (GAVRIATOPOULOU et al., 2020). The complexity of this infection includes its aggravation by other comorbidities as hypertension, diabetes and cardiovascular diseases (ZHOU et al., 2020) and by the adverse outcomes that may manifest after acute illness. According to NALBANDIAN et al., (2021), most of the more prevalent post-COVID-19 manifestations, commonly known as long COVID, include fatigue, joint pain, dyspnea, chest pain, sleep disturbances, lost of taste or smell, and headache.

It is well established that the innate immune system functions as the

first line of defense against the pathogens, including the SARS-CoV-2. This initial response is intended to limit viral infection and to promote the development of the adaptive immunity. The sense of danger is detected by pattern-recognition receptors (PRRs) present in the surface, cytosol or nucleus of macrophages, monocytes, dendritic cells (DCs), neutrophils and innate lymphoid cells (ILCs) which recognize PAMPs (pathogen-associated molecular patterns). Several PRRs are able to mediate signaling pathways in response to interaction with SARS-CoV-2 including Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). A detailed description of this interaction was recently published by (DIAMOND; KANNEGANTI, 2022). A growing body of clinical data has been suggested that COVID-19 severity is mostly determined by inflammation and the associated cytokine storm (HU; HUANG; YIN, 2021; KARKI et al., 2021). The use of appropriated animal models is allowing a better understanding of infection and pathogenesis triggered by SARS-CoV-2. Most of the experimental *in vivo* studies have been done using macaques, cats, ferrets, hamsters and mice, being hamsters and genetically modified mice widely employed. Recently, (BEDNASH et al., 2022) demonstrated that hamsters intranasally (IN) inoculated with SARS-CoV-2 developed a viral pneumonia and systemic illness showing histological evidence of lung injury, increased pulmonary permeability, acute inflammation, and hypoxemia. They also demonstrated upregulation of inflammatory mediators that persisted after infection clearance.

Many of the findings described in mice are consistent with severe COVID-19 in patients. For example, the IN inoculation of SARS-CoV-2 in transgenic mice expressing the ACE2 receptor driven by the cytokeratin-18 resulted in high virus levels in the lungs. An accentuated deterioration in pulmonary function was identified a few days later which coincided with a local infiltration of monocytes, neutrophils and activated T cells, and with an impressive up-regulation of innate immunity characterized by signatures of

type I and II IFN signaling and leukocyte activation pathways (WINKLER et al., 2020). Standard laboratory mice strains and non-infectious virus components have also been used to establish models of lung inflammation. XIA et al., (2021), for example, described the induction of acute lung injury associated with inflammation by SARS-CoV-2 N protein intratracheal inoculation in C57BL/6, C3H/HeJ, and C3H/HeN through the NF-kB activation. PUTHIA et al., (2022) described a model of pulmonary inflammation induced by lung coadministration of aerosolized S protein together with LPS in C57BL/6 mice. This procedure significantly increased the NF-kB activation, the amount of inflammatory macrophages and polimorphonuclear cells (PMNs) in the brochoalveolar lavage fluid (BALF) and also triggered pathognomonic changes in the lungs. BALF analysis revealed an increased level of inflammatory cytokines and chemokines resembling a cytokine storm.

Most of the therapeutic strategies consist in clinical trials of repurposing existing drugs already used for other infectious or inflammatory pathologies. Anti-viral drugs, monoclonal antibodies, high titer convalescent plasma, and immunomodulators are being investigated (ALUNNO et al., 2021; MAJUMDER; MINKO, 2021; ŞİMŞEK YAVUZ; KOMŞUOĞLU ÇELİKYURT, 2021). Observational studies have shown that serum Vitamin D (VitD) levels were inversely correlated with COVID-19 incidence and severity suggesting that supplementation with this hormone could be explored to prevent or treat COVID-19 patients (MERCOLA; GRANT; WAGNER, 2020). Since then, VitD has been tested, alone or associated with other pharmaceuticals, as a potential prophylactic, immunoregulatory and even neuroprotective measure for this infection (ALEXANDER et al., 2020; XU et al., 2020a). According to the ClinicalTrials.gov, there are 31 completed studies involving tests with VitD in COVID-19 patients. Some of these trials aimed to assess its effect on the lungs and they indicated that one single dose did not prevent the respiratory worsening of hospitalized patient (MARIANI et al., 2022) neither reduced hospital length in moderate

to severe COVID-19 (MURAI et al., 2021). On the other hand, other reports, mainly by using multiple doses of this vitamin, have been more promising. (OHAEGBULAM et al., 2020), described shorter lengths of stay, lower oxigen requirements and a reduction in inflammatory markers status. As reported by (SABICO et al., 2021), a 5000 IU daily supplementation during 15 days in VitD deficient patients reduced the time to recovery for cough and gustatory sensory loss.

To the best of our knowledge, most of these trials were done by administration of VitD by oral route. In this context, we initially evaluated if the IN instillation of UV inactivated SARS-CoV-2 in C57BL/6 mice was able to trigger an inflammatory pulmonary process and then we investigated if this process could be modulated by VitD administered by systemic (intraperitoneal) or local (IN) routes.

2 Material and methods

2.1 General experimental design

In this investigation we characterized a model of pulmonary inflammation induced by IN administration of 3 doses of inactivated SARS-CoV-2 in C57BL/6 mice and then evaluated the ability of vitamin D, administered by intraperitoneal (4 doses) or IN (3 doses) routes to control or modify this process. The following methodologies were used: total and differential count of cells present in the BALF, histopathological analysis of the target tissue, determination of lymphocyte subpopulations and inflammatory mediators by RT-PCR, flow cytometric analysis of cells recovered from the lung and cytokine quantification in pulmonary homogenates. These analyses were performed on the seventh day after administration of the first virus dose. Induction of lung inflammation and evaluation of VitD therapeutic potential are outlined in Scheme 1- General Experimental design, A and B, respectively, provided at the supplementary

data section.

2.2 Animals

Female C57BL/6 mice were acquired from the Animal Facility of the Animal Research and Production Center (ARPC/IBTECH), UNESP, Botucatu or from the Animal Facility of the University of Sao Paulo. Animals were housed in polypropylene cages with a maximum capacity for 4-5 animals, in a rack with individual ventilation (Alesco). The temperature was controlled by air conditioning and maintained at about 22ºC. The animals received water and commercial feed ad libitum and were handled according to the standards of the ethics committee in animal experimentation of IB, UNESP, Botucatu (CEUA Protocol No. 1959140820, ID: 000129) and the ethics committee in animal experimentation of the ICB, USP (CEUA Protocol 3147240820).

2.3 SARS-CoV-2 propagation and inactivation

In this study was used a B lineage isolate of SARS-CoV-2 (SARS.CoV2/SP02.2020, GenBank accession number MT126808) kindly provided by Edison Luiz Durigon (PhD, ICB-USP), recovered from a sample collected on Feb 28, 2020, in Brazil. The virus was propagated in Vero cells (CCL-81; ATCC, Manassas, VA, USA) according to the previously described protocol (WÖLFEL et al., 2020) in a biosafety level 3 laboratory (BSL-3) located in the University of Campinas. All viral stocks used in the study were titrated by plaque forming assay according to previously published studies (COIMBRA et al., 2022). Briefly, decimal serially diluted samples were incubated with Vero cells into 24-wells plates for 1h at 37°C and 5% CO_2. After adsortion, cells were overlaid and maintained with semi-solid medium (1% w/v carboxymethylcellulose in DMEM supplemented with 5% FBS) for

4 days. After fixation with 8% formaldehyde solution and staining with 1% methylene blue (Sigma-Aldrich), the viral titer was determined by dividing the average number of plates by the value obtained from the multiplication between the dilution factor and the volume of the viral suspension added to the plate. The results were expressed as viral plaque forming units (PFU)/mL of sample. The virus used in this study, with a titer of $8x10^6$ PFU/mL, was inactivated by exposure to 7,560 mJ/cm2 of UVC (30 min) according described previously (BISPO-DOS-SANTOS et al., 2021). The supernatant of non-infected Vero cells, inactivated by UVC, was used as a negative control. Inactivation efficacy was determined inoculating the UVC inactivation product into Vero cells. The Vero cells infected with UVC inactivated SARS-CoV-2 showed no cytopathic effect. In addition, no virus were detected in the supernatant of these cells by plaque forming assay or quantitative RT-PCR.

2.4 Induction and characterization of the pulmonary inflammation induced by SARS-CoV-2

We adopted the protocol described by Roberts et al., 2007. Briefly, the animals received 3 doses of 4.10^5 PFU/50 ul, administered on days 1, 3 and 5 which were dispensed with a tip connected to a pipette. The pulmonary inflammatory response was analyzed on the seventh day by using 5 methodologies: total and differential cell counts performed in the BALF, RT-PCR for quantification of transcription factors, cytokines and inflammasome genes, flow cytometry for identification of cells present in the parenchyma, histopathological analysis and cytokine quantification in lung homogenates.

2.5 Bronchoalveolar lavage procedure

The bronchoalveolar washes were obtained from mice previously euthanized with ketamine and xylazine. The animal´s trachea was exposed with the help of scissors and tweezers and a catheter was introduced through which 1ml of sterile PBS was injected and then aspirated. This PBS injection/aspiration process was repeated 3 consecutive times and the samples were centrifuged at 4ºC for 10 min, 1500 rpm. The pellets were pooled and resuspended in 300 ul and the total cell concentration determined by using a Neubauer chamber. Smears for differential cell counts were prepared by cytocentrifugation at 600 rpm for 5 min and then stained with the Rapid Pannotic Kit (Laborclin, Paraná, Brazil).

2.6 Quantitative RT-qPCR analysis

Total RNA from lung samples was extracted with the reagent TRIZOL (Invitrogen, Carlsbad, CA, USA) and the synthesis of cDNA (High Capacity RNA-to-cDNA Converter Kit Applied Biosystems, Foster city, CA, USA), according to manufacture recommendations. The quantitative expression of mRNA for the transcription factors *Tbx21* (Mm00450960_m1), *GATA3* (Mm00484683_m1), *RORc* (Mm01261022_m1) and *Foxp3* (Mm00475162_m1), cytokines *IL-6* (Mm00446190_m1), *TNF-α* (Mm0043258_m1), *IFN-y* (Mm01168134_m1), *IL-12* (Mm00434169_m1), *IL-17* (Mm00439618_m1), inflammasome components as *NLRP3* (Mm09840904_m1), *IL-1β* (Mm00434228_m1) and *Caspase-1* (Mm00438023_m1), and other inflammatory markers as *iNOS* (Mm00440502_m1), *CPA3* (Mm00483940_m1) and *Arginase* (Mm00475988_m1) were analyzed by real-time PCR, using the TaqMansystem with primers and probes sold by Life Technologies (Applied Biosystems) according to the manufacturer´s recommendations. Gene expression was based on GAPDH (Mm99999915_g1), a reference gene, and presented as a relative change in the fold (2-ΔΔct), using the control group as a calibrator.

2.7 Lung histopathological analysis

Left lung samples were collected on the seventh day after the beginning of IN instillations and then washed with PBS, fixed in 10% buffered formalin for 24 hours, washed and stored in 70% ethanol until inclusion. Five um thick sections from Control (Saline), Culture medium and SARS-CoV-2 groups, were obtained using a Leica RM2245 microtome and stained with H&E. Histopathological alterations were evaluated in a Carl Zeiss microscope GmbH, Oberkochen, Germany, attached to a digital camera (AxioCamHRc, Carl Zeiss).

2.8 Isolation of lung cells and cytometry analysis

In order to differentiate the parenchyma-infiltrating leukocytes from the vascular-associated fraction, mice were intravenously injected with 3ug of FITC-labeled anti-CD45 antibody (Biolegend) in 200 ul of sterile saline solution. After 3 min, mice were euthanized and lungs were perfused and collected for tissue processing. The vascular fraction of leukocytes were identified based on the anti-CD45 FITC staining.

The right lungs, which were removed soon after euthanasia, were shredded, processed in digestion buffer (incomplete RPMI medium – (Sigma, St Louis MO) containing 0,5 mg/ml DNAse I – (Sigma Alddrich) and 1 mg/ml Colagenase IV – (Sigma Alddrich) and incubated at 37ºC, 30 min, 180 rpm. Once homogenized, the digested samples were passed through 70 µm cell strainers, transferred to conical centrifuge tubes containing 8 ml of complete RPMI (3% Fetal bovine serum – (LGC Biotechnology), 10mg/ml penicillin + 10000 units/ml streptomycin – (Hyclone), 0,3 g/ml L-glutamine – (Sigma-Aldrich), 0,0040 g/ml betamercaptoethanol – (Sigma Aldrich), 0,0089 g/mL non-essential amino acids – (Sigma-Aldrich), 0,0089 g/mL sodium pyruvate – (Sigma Aldrich)) and then centrifuged at 4ºC, 8 min, 1600 rpm. Supernatants were discarded, the cells resuspended in 500µL of

ACK erythrocyte lysis buffer and incubated on ice. After 2 min, were added 10 ml of complete RPMI and centrifuged again at 4ºC, 8 min, 1600 rpm, being the supernatant discarded and the cells resuspended in 1ml of complete RPMI, counted and prepared for cytometry analysis. Two million of lung cells were stained for surface markers or for transcription factors, according to the **Table 1**. All the antibodies and intranuclear staining were conduced according to the manufacture instructions, by using eBioscience Transcription Factor Buffer set.

Alternatively, 2 million cells were used for intracellular cytokine detection. For labelling of cytokine-producing cells, the cells were incubated for 4 hours with 100µL of stimulus solution (50ng/ml of Phorbol myristate acetate (PMA) (Sigma Aldrich) + 500ng/ml ionomycin (Sigma Aldrich) + 1µl/ml of stop Golgi (BD Biosciences). Cytokines, transcription factors and cells from innate and specific immunity were then labeled with fluorochrome-conjugated antibodies. Prior to addition of the antibody mix, as specified in **Table 1** (available at the supplemmentary data section), all samples from all panels were incubated by 20 min, 4ºC with 30µL of Live Dead (LD, Thermo Fisher Scientific), followed by surface staining and intracellular staining (BD-Citofix-Citoperm kit). The data were acquired in the BD LSRFFortessa X-20 flow cytometer (BD Biosciences) and the compensation and data analyses were performed using the FlowJo software. Gate strategies are described in **Supplementary Figures 2, 3 and 4**.

Table 1: Clones of antibodies used in flow cytometry for immunophenotyping of lung cells

Antibody	Manufacturer	Clone	Antibody	Manufacturer	Clone
CD45	Biolegend	103151	F4/80	eBiosciences	BM8
LD	Invitrogen	N/A	IFN-y	Biolegend	XMG1.2
CD103	eBiosciences	46-1031-82	IL-17	eBiosciences	Ebio 17B7
CD19	BD Biosciences	HIB19	TNF-α	eBiosciences	MP6-XT22
CD11b	Biolegend	101257	IL-6	Biolegend	MP5-20F3
Ly6G	Biolegend	127626	Thy 1.2	BD Biosciences	105331
CD11c	Biolegend	117336	TCR-β	BD Biosciences	47-5961-82
MHC II	Biolegend	M5/114.15.2	FC Block (anti CD16/32)	Bioxcell	2.4G2
Ly6C	Biolegend	HK1.4	SinglecF	BD Biosciences	562757
CD64	Biolegend	X54-5/7-1			

2.9 Vitamin D administration by IP and IN routes

1α,25-dihydroxyvitamin D3 (1,25-VitD3, Sigma-Aldrich) was administered intranasally (IN) or intraperitoneally (IP). The 2 therapeutic protocols with 1α,25-dihydroxyvitamin D3 (VitD) were carried out through different strategies. In the IN protocol, each animal was treated with 3 doses of VitD (0.1ug/dose) which were administered simultaneously with the SARS-CoV-2 inoculum (4.10^5 PFU/each inoculum) in a final volume of 57 µl. This volume was divided between the two nostrils on days 1, 3 and 5. In the IP protocol, each animal was treated with 4 doses of VitD (0.1 ug/100 ul/dose) that were delivered on days 0, 2, 4 and 6 to mice that were instilled with 50 ul of SARS-CoV-2 on days 1, 3 and 5. In both cases, euthanasia was performed at the 7th day after the beginning of the protocol.

2.10 Measurement of serum calcium levels

Blood samples collected after anesthesia were centrifuged, and the sera stored at −20 °C until further analyses. Serum levels of calcium were

measured according to the instructions of the manufacturer (Cálcio Arsenazo III, Bioclin-Quibasa Química Básica Ltda, Belo Horizonte, MG, Brazil). In this technique calcium quantification is based on a colorimetric reaction in which calcium reacts with arsenazo III, in an acidic medium, generating a blue complex whose intensity is proportional to calcium concentration in the sample.

2.11 Statistical analysis

In the case of parametric variables, the values were presented in mean and standard error of the mean (SEM) and the comparison between two groups was performed by unpaired t-Test and among three or more groups was performed by ANOVA followed by Tukey test. When the variables were non-parametric, the results were presented in median and interquartile intervals and the comparison between the groups was performed by Mann-Whitney test or Kruskal-Wallis test followed by Dunn's test. The level of significance adopted was 5%. The data were analyzed by the SigmaPlot for Windows version 2.0 statistical package (1995, Jandel Corporation, California, USA). For t-distributed stochastic neighbor embedding (t-SNE) algorithm analysis, 100,000 (**Figure 2**) or 50.000 (**Figure 5**) events per sample were downsampled from the live parenchymal leukocytes gate (**Supplementary Figure 2**) and concatenated. The t-SNE algorithm was applied in the concatenated samples using 2000 interaction and perplexity 80. After that, the cell clusters were identified based on the main cell subsets gated according to Supplementary Figure 2, and the percentage of each cell subset was calculated after segregating the groups based on the sample IDs.

3 Results

3.1 Cell infiltration in the BALF point out to a pulmonary inflammatory process in mice intranasally instilled with inactivated SARS-CoV-2

The ability of inactivated SARS-CoV-2 to trigger a lung inflammatory process was initially investigated by analyzing the amount and identity of white blood cells (WBC) obtained from the BALF of mice. Two control groups were included in all initial experimental procedures and were identified as saline and culture medium which corresponded to animals that were anesthetized and instilled with saline 0.9% or with the culture medium used for VERO cell culture and virus propagation, respectively. The total number of WBC and specific cell populations, which were identified in cytospin smears, are shown in **Figure 1A** and indicate a significant increase in total cell number, as well as in lymphocytes and neutrophils in animals that received SARS-CoV-2 in comparison to the control groups. The percentage of each specific cell population, as illustrated in **Figure 1B**, was also affected by SARS-CoV-2 and characterized by higher percentuals of lymphocytes and neutrophils, and lower percentual of macrophages. Animals injected with saline or culture medium displayed a similar profile characterized by a much smaller number of all cell types, indicating that the culture medium present in SARS-CoV-2 preparation was not triggering a significant pulmonary airway inflammation.

3.2 RT-qPCR from lung homogenates shows alterations in T cell subsets, cytokines and other inflammatory mediators

Next we measured the relative expression of several genes by RT-PCR which revealed differences between culture medium and SARS-CoV-2 groups. In the SARS-CoV-2 group there was a significantly higher expression of *Foxp3* **(Figure 1F**), IL-6 (**Figure 1G**) and *GM-CSF* (**Figure 1L**) transcripts and a higher, even though not statistically significant,

expression of *TNF-α* (**Figure 1H**), *IL-17* (**Figure 1K**), *IL-1β* (**Figure 1O**), and *NLRP3* (**Figure 1P**) transcripts. On the other hand, we found a significant decrease in the expression of *RORc* (**Figure 1E**) and *iNOS* (**Figure 1M**) in the lungs of SARS-CoV-2 group compared to the culture medium controls. Other genes as *T-bet*, *GATA-3*, *IFN-g,* IL-12 and *CPA3* (**Figures 1C, D, I, J** and **N**, respectively) were similarly expressed in culture medium and SARS-CoV-2 groups.

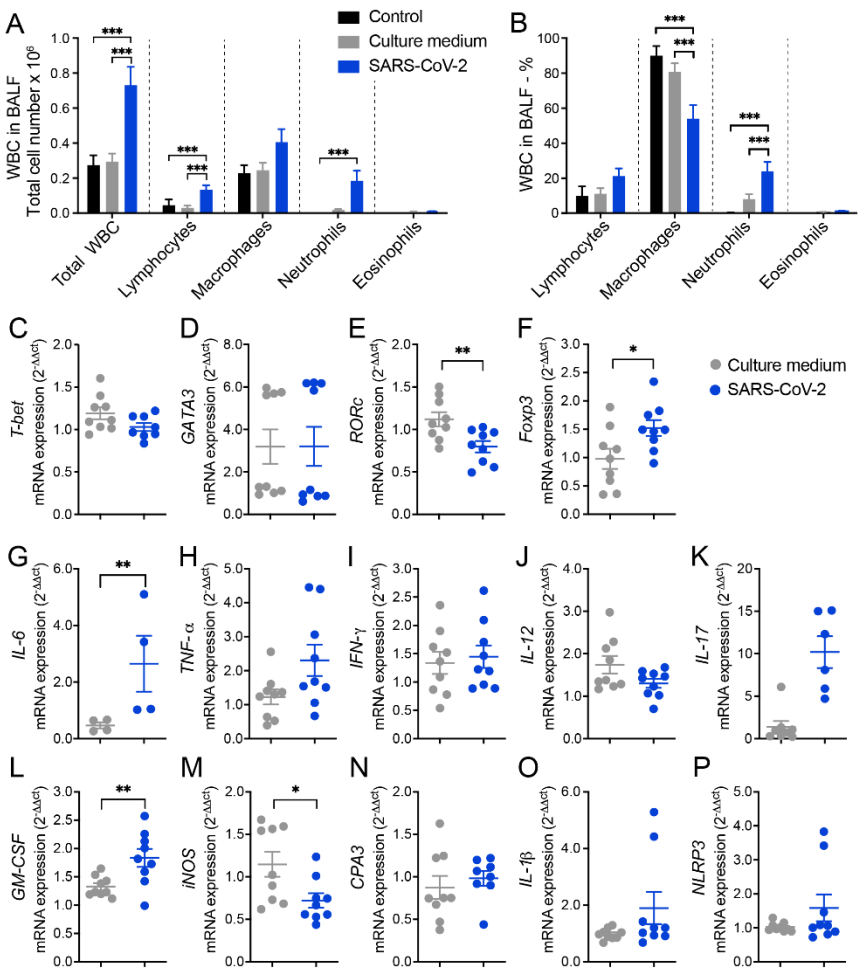


Figure 1. Cell counts in the BALF and lung mRNA transcripts for T cell subsets, cytokines, and other indicators of inflammation in mice intranasally instilled with UV inactivated SARS-CoV-2. C57BL/6 mice were instilled with the virus (3 doses of 4.10^5 PFU/each) on days 1, 3 and 5. At the 7th day, the BALF and the left lower lobe were

collected for WBC differential count and mRNA transcipts determinations, respectively. Total number (**A**) and percentage (**B**) of WBC; *T-bet* (**C**), *GATA3* (**D**), *RORc* (**E**), *Foxp3* (**F**), *IL-6* (**G**), *TNF-α* (**H**), *IFN-γ* (**I**), *IL-12* (**J**), *IL-17* (**K**), *GM-CSF* (**L**), *iNOS* (**M**), *CPA3* (**N**), *IL-1β* (**O**), *NLRP3* (**P**). In A and B figures the results are expressed as mean ± SEM and the statistical significance of the differences was analyzed by ANOVA test followed by Tukey´s test. In C-P figures the results were expressed in median and interquartile intervals and the comparison between the groups was performed by the t-Test. Data showed in A, B and C-P is derived from two experiments with similar results which were combined (n=9 mice/experimental group, except *IL-6* determination that included 4 animals. * p <0.05; ** p<0.01; ***p<0,001.

3.3 Histopathology and cytometric analysis reveal an impressive infiltration of inflammatory cells into the pulmonary parenchyma

The histopathological evaluation, which is illustrated in **Figure 2A**, was done by analyzing serial sections obtained from the left lung lobe stained with hematoxylin and eosin (H&E). As expected, lung architecture was totally preserved in the animals of the control group, allowing the visualization of alveoli and longitudinally and transversally sectioned blood vessels. Culture medium and SARS-CoV-2 groups displayed inflammatory focci, however, the ones found in the virus instilled animals were clearly more numerous and intense. These inflammatory focci were, in both cases, located around the vessels and bronchi as indicated by black and green arrows, respectively. The presence of neutrophilic infiltrates (green arrow head), macrophage infiltrates (blue arrow head), and lymphocytic infiltrates (yellow arrow head) are indicated in the microphotographs. Numerous consolidation areas were present in the lungs of SARS-CoV-2 instilled animals but were rare and absent in culture medium and saline control groups, respectively. To confirm these findings, we evaluated the leukocytes infiltrating the lung parenchyma by using cells isolated from mice previously injected with FITC-labeled anti-CD45 antibodies to distinguish circulating cells from the tissue infiltrate. Indeed, confirming the histopathological

findings, the quantification of total and parenchymal infiltrating $CD45^+$ leukocytes revealed a significant increase in cell number in the group of mice that received UV-inactivated SARS-CoV-2 compared to both control groups.

Next, we characterized the tissue infiltrating leukocytes based on the surface molecules expression by flow cytometry and used t-SNE algorithm for dimension reduction (**Figure 2C** and **Supplementary Figure 2**). In the lungs of mice exposed to the inactivated vírus, we found an enrichment in the clusters of cells that indicate the presence of neutrophils, eosinophils, macrophages (tissue-resident), monocytes and in the $CD103^-CD11b^+$ DC subset (**Figure 2C**). On the other hand, the frequency of other cells subsets, such as $CD103^+CD11b^-$ DCs, alveolar macrophages and B cells as reduced (**Figure 2C**). Furthermore, the frequency of proinflammatory cytokine-producing cells was also increased in the SARS-CoV-2 group, in particular, the frequency of IFN-*g* and TNF-α-producing $TCR\beta^+$ T cells and IL-6 and TNF-α-producing CD11b+ myeloid cells (**Figure 2D**). The quantification of each cell subset number is shown in the **Figure 2F-Q**). The predominant profile of all tested cells was characterized by a significantly higher amount of cells enriched in the t-SNE analysis in the SARS-CoV-2 group in comparison to the control group. This was the case for the total number of neutrophils (**Figure 2F**), eosinophils (**Figure 2G**), monocytes (**Figure 2H**), inflammatory monocytes (**Figure 2I**), monocytes-derived macrophages (**Figure 2J**), resident macrophages (**Figure 2K**), dendritic cells (DCs) (**Figure 2L**), CD11b+CD103- DCs (**Figure 2M**), IFN-g^+- and TNF-α^+-producing T cells (**Figure 2N-O** and **Supplementary Figure 3**) and TNF-α^+- and IL-6-producing myeloid cells (**Figure 2P-Q** and **Supplementary Figure 3**). Contrastingly, the total number of each cell population in the culture medium instilled animals was intermediate between control and SARS-CoV-2 groups and did not present statistical differences when compared with the two other experimental groups. Therefore, this data shows that the IN instillation of the inactivated virus is sufficient to promote a proinflammatory lung milieu that resembles most of the markers of the

infection with SARS-CoV-2.

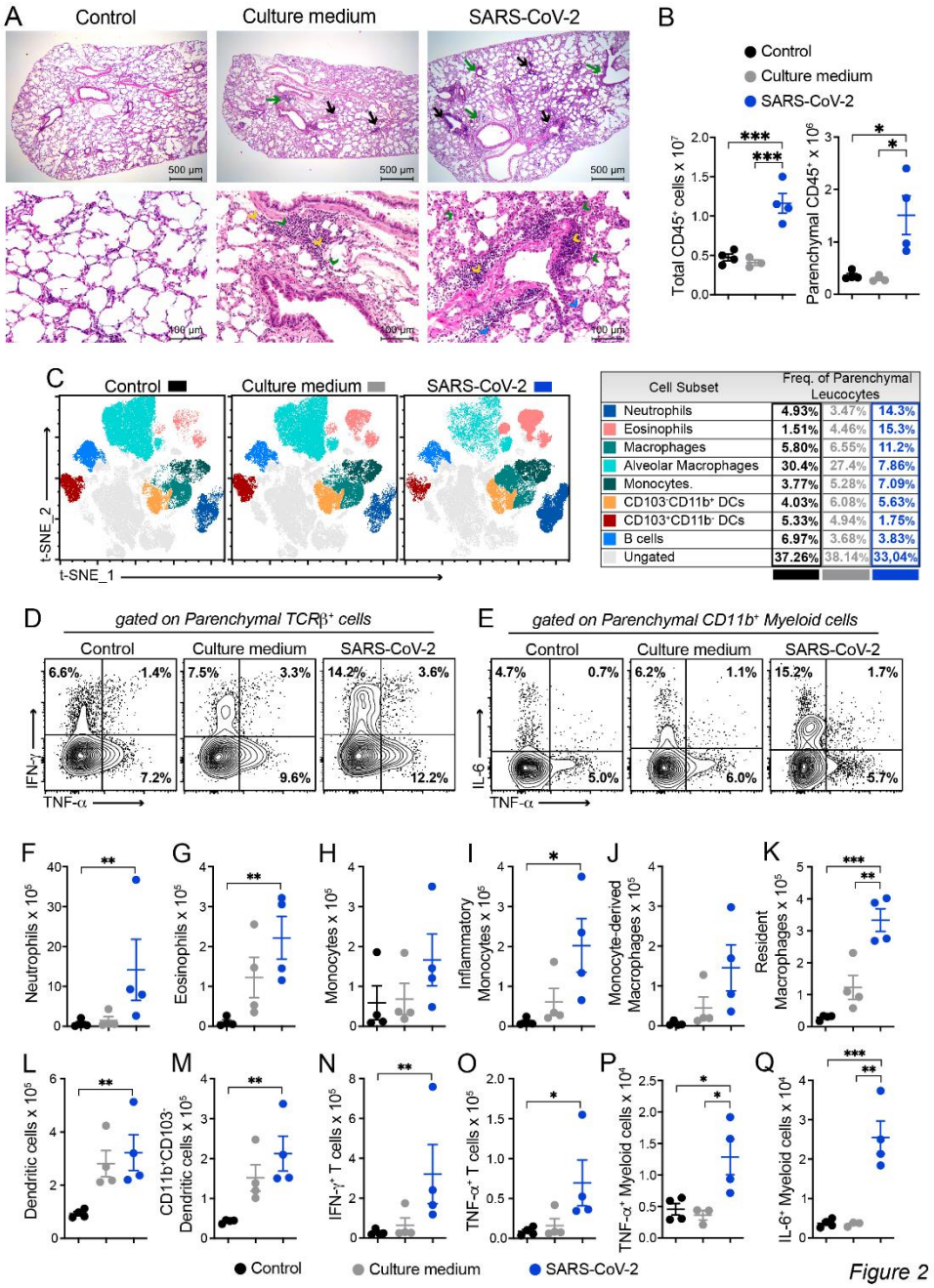


Figure 2. Characterization of the inflammatory process induced by IN instillation of inactivated SARS-CoV-2 by histopathological and cytometric analyses. C57BL/6 mice were instilled with the virus (3 doses of 4.10^5 PFU/each) on days 1, 3 and 5. At the 7th day, the upper left lobe and the whole right lung were collected for histopathological and flow cytometry analyses, respectively. Five um thick sections from Control (Saline), Culture

medium and SARS-CoV-2 groups were stained with H&E and representative images are shown in (**A**). Inflammatory focci around the vessels (black arrows) and around the bronchi (green arrows), neutrophilic infiltrates (green arrow head), macrophage infiltrates (blue arrow head), and lymphocytic infiltrates (yellow arrow head). (**B**) Cells were isolated from the lung tissue and total $CD45^+$ leukocytes or the parenchymal infiltrating leukocyte fraction (identified based on anti-CD45 intravenous injection) were quantified by flow cytometry and specific cells subsets were evaluated according to the gating strategy described in **Supplemmentary Figure 2**. (**C**) t-distributed stochastic neighbor embedding (t-SNE) analysis illustrating the distribution of cell clusters in each experimental group according to gate strategy described in the **Supplementary Figure 2**. The table on the right side indicates the frequency of each cell cluster relative to the $CD45^+$ parenchyma infiltrating leukocyte in the Control (black), Culture medium (grey) and SARS-CoV-2 (blue) groups. Representative contour plots of IL-6, TNF-α or IFN-γ staining in parenchymal $TCR\beta^+$ T cells (**D**) or $CD11b^+$ myeloid cells (**E**), according to gate strategy described in the **Supplementary Figure 3**. The total cell numbers of each parenchymal-infiltrating cell subsets are expressed in mean ± SEM, including Neutrophils (**F**), Eosinophils (**G**), Monocytes (**H**), Inflammatory monocytes (**I**), Monocyte-derived macrophages (**J**), Resident macrophages (**K**), Dendritic cells (**L**), CD11b+CD103- Dendritic cells (**M**), IFN-γ^+ $TCR\beta^+$ cells (**N**), TNF-α^+ $TCR\beta^+$ cells (**O**), TNF-α^+ $CD11b^+$ myeloid cells (**P**) and IL-6^+CD11b^+ myeloid cells (**Q**). Data shown in **A** is derived from one experiment between two showing similar findings (n=4); **B-Q** data are derived from two experiments with similar results which were combined (n=6-8). The comparison between the groups in C-P was performed by the Kruskal-Wallis test followed by the Duun test. * $p<0.05$; ** $p<0.01$; ***$p<0,001$.

3.4 BALF and cytokine levels in lung homogenates suggest that VitD modulates pulmonary inflammation induced by SARS-CoV-2

Considering that VitD has a strong effect over the immune system and its given consideration for prophylactic or therapeutic application in COVID-19 patients, we tested its IP and IN immunomodulatory efficiency towards lung inflammation. As already observed in previous studies, the IP treatment with VitD decreased body weight (**Figure 3A**) and increased serum calcium levels (**Figure 3B**). Contrary to our expectation, VitD applied via IN similarly affected body weight and calcium levels, as illustrated in

(**Figures 3A** and **B**), respectively. BALF findings suggested a modulatory action of VitD administered by both routes and characterized by reduction, although not statistically significant, of lymphocytes, macrophages, neutrophils and eosinophils as illustrated in **Figures 3C** and **D**.

To analyze if SARS-CoV-2 induced lung inflammation model was also mimicking the cytokine storm-like phenomenon and to reinforce the presumed downmodulatory effect of VitD, we tested the presence of pro-inflammatory cytokines in lung homogenates. As can be observed in **Figure 3E**, SARS-CoV-2 group produced higher levels of IL-17A, TNF, and IL-6. The level of these pro-inflammatory cytokines was reduced by both VitD administration routes, being this effect more accentuated by IN pathway.

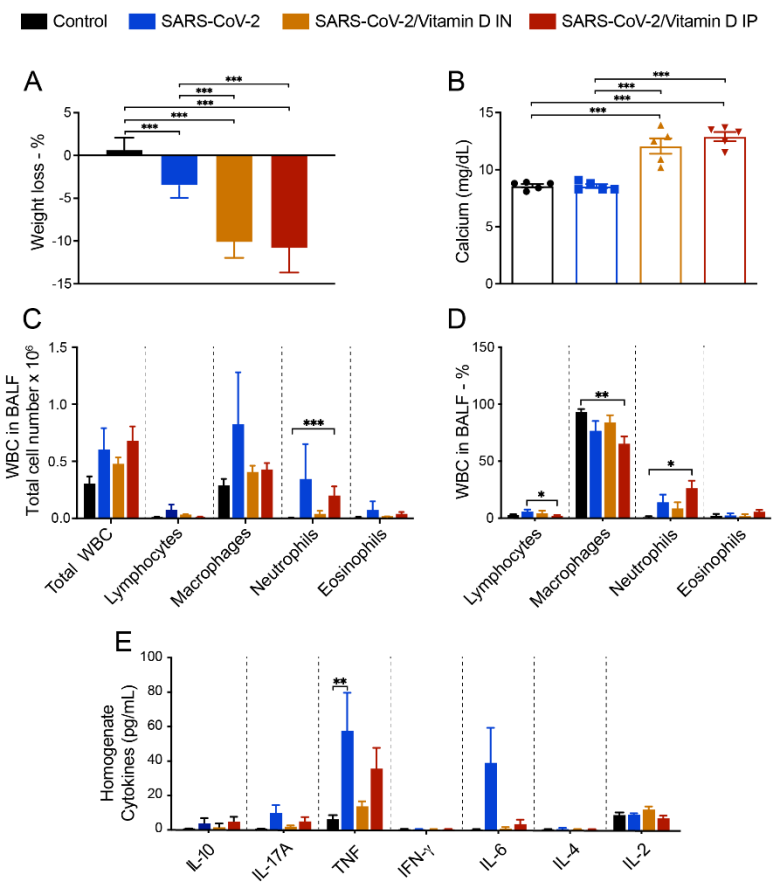


Figure 3. Evaluation of VitD effects on body weight, serum calcium levels, BALF cell counts and cytokine levels in the lungs of mice instilled with inactivated SARS-CoV-

2. C57BL/6 mice were instilled with the virus (3 doses of 4.10^5 PFU/each) on days 1, 3 and 5. Body weight was checked daily and, at the 7th day, were collected blood samples for calcium measurement, BALF for WBC analysis and lower left lobe for cytokine quantification. Body weight loss (**A**), serum calcium levels (**B**), total WBC and WBC subsets in BALF (**C**), percentage of WBC in BALF (**D**), and cytokines in lung homogenates (**E**). Data showed in A, B and D derive from one experiment (n=5 mice/ experimental group). Data showed in C and D derive from 3 experiments with similar results which were combined (n= 16 mice/experimental group). The results are expressed as mean ± SEM and the comparison between the 4 groups was performed by the ANOVA test followed by Tukey. * p<0.05; ** p<0.01; p<0,001.

3.5 Differential effects of VitD delivered by IN and IP routes on *RORc* and inflammasome genes expression

The expression of various genes was similar in the three compared groups as was the case of *T-bet*, *GATA3*, *Foxp3*, *TNF-α, IFN-g*, *IL-12*, *GM-CSF*, and *INOs* (**Figures 4A, B, D, E, F, G, H, I** and **J**). However, the expression of *ARG*, *IL-1β*, and *NLRP3* was significantly higher in the IP VitD-treated group in comparison to the IN VitD-treated one (**Figures 4C, K** and **L**).

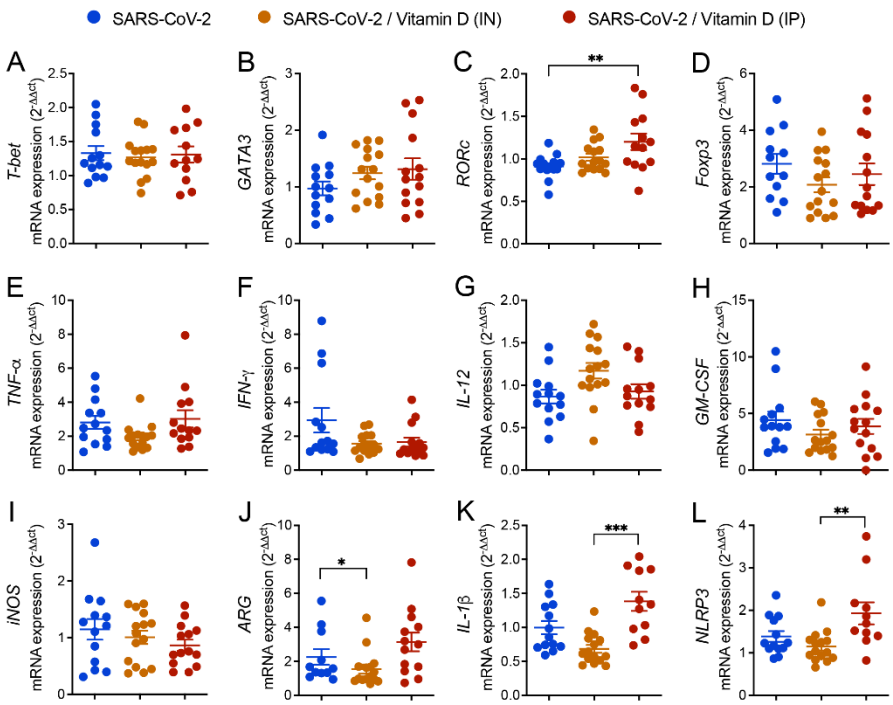


Figure 4. Effects of IN and IP vitamin D delivery on T cell transcription factors, cytokines and inflammasome gene transcripts in lungs of mice IN challenged with UV inactivated SARS-CoV-2. C57BL/6 mice were instilled with the virus (3 doses of 4.10^5 PFU/each) on days 1, 3 and 5. In the IN protocol, mice was treatment with 3 VitD doses (0,1 µg/dose) simultaneously with the SARS-CoV-2 inoculum. In the IP protocol, each animal was treated with 4 VitD doses delivered on days 0,2,4 and 6. At the 7th day, the lower left lobe was removed and RNA extracted and submitted to RT-PCR. Tested genes included *T-bet* (**A**), *GATA3* (**B**), *RORc* (**C**), *Foxp3* (**D**), *TNF-α* (**E**), *IFN-γ* (**F**), *IL-12* (**G**), *GM-CSF* (**H**), *iNOS* (**I**), *ARG* (**J**), *IL-1β* (**K**), *NLRP3* (**L**). Data derives from three experiments with similar results which were combined (n=13-15 mice/experimental group). The results are presented in median and interquartile intervals and the comparison between the groups was performed by the Kruskal-Wallis test followed by the Dunn test. * p<0.05; ** p<0.01; *** p<0.001.

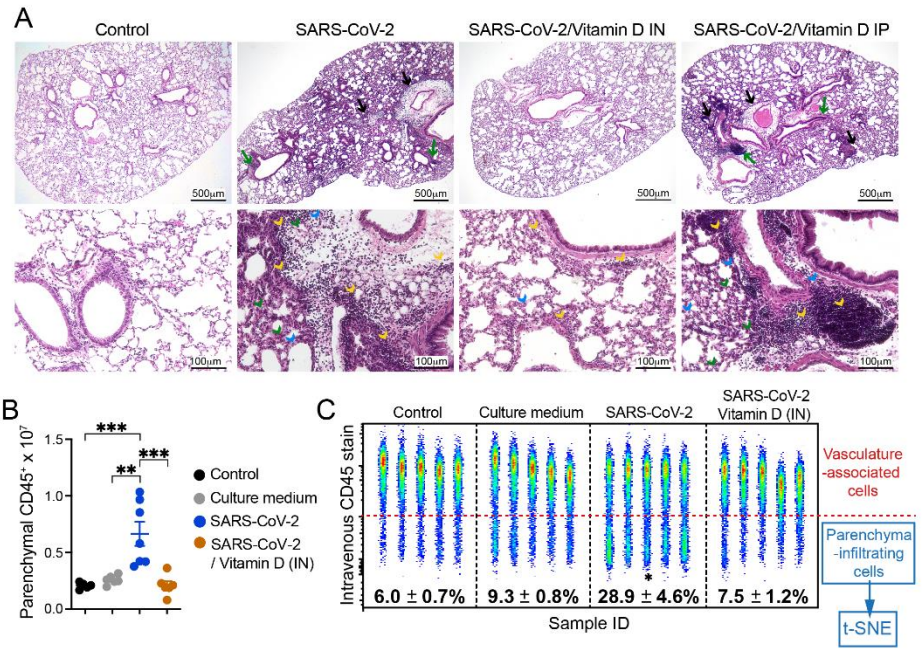
3.6 IN VitD treatment efficiently controls pulmonary inflammation

The analysis of the histological sections clearly indicated the strong ability of IN VitD to control lung inflammation (**Figure 5A**). In this case, there

was a convincing interruption of the accumulation of inflammatory cells in the lung parenchyma of IN VitD treated mice that were exposed to the inactivated virus. Even though BALF and cytokines in lung homogenates have suggested a possible downmodulatory effect of IP VitD, this modulatory activity was not confirmed by histopathological analysis of the lungs. These findings can be clearly observed in **Figure 5A**. Inflammatory focci are easily observed around the vessels (black arrows) and bronchi (green arrows) in SARS-CoV-2 and SARS-CoV-2/Vitamin D IP groups. The presence of neutrophilic infiltrates (green arrow head), macrophage infiltrates (blue arrow head), and lymphocytic infiltrates (yellow arrow head) are also numerous in these two experimental groups but rare in animals treated by IN route.

To clarify whether this anti-inflammatory effect of IN VitD involved the modulation of specific cell types, including myeloid, DCs, ILCs and lymphocytes, we used flow cytometry to identify the cells infiltrating in the lung parenchyma, as described above. The total number and the frequency of $CD45^+$ cells infiltrating the lung parenchyma of mice instilled with inactivated SARS-CoV-2 was significantly increased compared to both control groups (Control and Culture medium) (**Figures 5B** and **C**). Notably, the IN VitD treatment significantly reduced the number and frequency of leukocytes infiltrating the lung parenchyma of mice receiving the inactivated virus (**Figures 5B** and **C**).

Next, to better understand the modulatory effects of VitD in the virus-induced parenchymal lung inflammation, we analyzed the frequency of distinct cell subsets by t-SNE and found that the VitD treatment reverted the recruitment of neutrophils, eosinophils, monocytes and CD103-CD11b+ DCs induced by the inoculation of inactivated SARS-CoV-2 (Figures 5D, 5E, 6C). In addition. the VitD treatment slightly increased the percentage of B cells and CD103+CD11b- DCs in the lung parenchyma in comparison to the SARS-Cov-2 group (Figures 5E, 6C), suggesting that the treatment might be selectively controlling the inflammatory immune tone in the lung.



E

Cell Subset	Frequency of Parenchymal Leucocytes			
	Control	Culture medium	SARS-CoV-2	SARS-CoV-2 Vitamin D (IN)
Neutrophils	1.92 %	2.72 %	14.5 %	4.58 %
Eosinophils	0.45 %	6.17 %	8.53 %	1.50 %
Macrophages	5.75 %	4.51 %	4.76 %	4.41 %
Alveolar Macrophages	1.58 %	1.84 %	1.01 %	2.14 %
Patrolling Monocytes	0.58 %	2.10 %	3.42 %	1.67 %
Inflammatory Monocytes	6.04 %	5.56 %	6.99 %	6.86 %
CD103$^-$CD11b$^+$ DCs	8.05 %	8.73 %	8.81 %	5.67 %
CD103$^+$CD11b$^-$ DCs	9.52 %	6.99 %	3.87 %	6.99 %
B cells	10.8 %	8.63 %	10.6 %	13.1 %
T cells	22.3 %	32.6 %	24.8 %	22.3 %
Ungated	33.01 %	20.15 %	12.71 %	30.78 %

Figure 5

Figure 5. Effect of IN vitamin D on lung histopathology and infiltration of cytokine-producing cells triggered by IN instillation of UV inactivated SARS-CoV-2. C57BL/6 mice were instilled with the virus (3 doses of 4.10^5 PFU/each) on days 1, 3 and 5. In the IN protocol, mice was treatment with 3 VitD doses (0,1 µg/dose) simultaneously with the SARS-CoV-2 inoculum. In the IP protocol, each animal was treated with 4 VitD doses delivered on days 0,2,4 and 6. At the 7th day, the upper left lobe and the right lung were collected for histopathological and flow cytometry analyses, respectively. The upper left

lobe was washed, fixed and stained with H&E, and then evaluated concerning the presence of inflammatory focci (A). Cells from lung parenchyma were eluted and analyzed after labeling with an array of specific antibodies (B). Data showed in A is derived from one experiment (n=5 animals /experimental group) and data shown in B is derived from one experiment (n= 7-8 animals /group). Results are presented in median and interquartile intervals and the comparison between the groups was performed by the Kruskal-Wallis test followed by the Dunn´s test. * $p<0.05$; ** $p<0.01$.

In addition, the functional impact of VitD treatment on inflammatory cytokine production was also analyzed by flow cytometry in myeloid cells, B cells, Tgd, TCD4+ cells and ILCs (**Figure 6**). Concerning cytokine production by myeloid cells, were analysed the ones producing TNF-α, IL-6 or both cytokines. While the inactivated virus promoted the production of TNF-α and IL-6 by lung myeloid cells, the VitD treatment controlled the frequency of cytokine-producing cells (**Figure 6A**). When we quantified the number of cytokine-producing myeloid cells, the only population that displayed a significant difference was the one producing both cytokines. Even though the % of TNF-α^+IL-6^+ $CD11b^+$ cells was similar in SARS-CoV-2 and SARS-CoV-2/VitD groups (**Figure 6E**), the total number of these cells was significantly lower in the VitD-treated animals as shown in **Figure 6D**. This data could be explained by the consistent reduction in total cell recruitment to the lung parenchyma in the VitD-treated mice.

The % of DCs was only slightly reduced by VitD (not shown). As already observed during the characterization phase of the lung inflammatory process, the amount of DCs was very similar in SARS-CoV-2 and its respective control group (culture medium). In spite of this, VitD therapy was able to significantly reduce the total number of DCs (**Figure 6F**) and also of the two checked subsets, that is, $CD103^-CD11b^+$ (**Figure 6G**) and $CD103^+CD11b^-$ (**Figure 6H**), but not the frequency of the $CD103^+CD11b^-$ (**Figure 5B** and **D**). Once again, this inconsistency in the modulation of cell number but no cell frequency could be attributed to the considerable reduction of leukocyte recruitment to the lung parenchyma of VitD-treated

animals (**Figure 5B**).

The total number of IFN-g, IL-17 and IL-6 producing ILCs were usually increased in SARS-CoV-2 group in comparison to the control that received the culture medium and VitD therapy triggered a clear tendency to decrease the total number of theses cytokine-producing cells as shown in **Figures 6A, I, J** and **K.** Concerning the Tgd lymphocytes, the most relevant alterations were detected in the cells that were producing IL-17 or IFN-g. As shown in figures **Figures 6A, L** and **N**, VitD significantly downregulated the total cell number of IL-17 and IFN-g producing cells. VitD also downmodulated, although not significantly, their percentages as illustrated in **Figures 6M** and **O** for IL-17 and IFN-g, respectively. In regard to $TCD4^+$ lymphocytes, the most pronounced differences were also observed in IL-17- and IL-17/IFN-g-producing cells. The % of these cells was reduced by treatment with VitD being this reduction statistically significant in the case of $TCD4^+IL17^+$ (**Figures 6A** and **Q**). The total amount of these two cell subsets was also decreased by VitD therapy, being this reduction statistically significant regarding $TCD4^+IL\text{-}17^+IFN\text{-}g^+$ (**Figures 6P, R** and **S**). The % and the total number of B cells were similar in culture medium and SARS-CoV-2 groups (**Figures 6T** and **U**). However, a significant increase in these two parameters was triggered by local VitD administration as shown in figures T and U. Interestingly, the number of $IL\text{-}6^+TNF\text{-}\alpha^+$ B cells, which was increased in the SARS-CoV-2 group, was significantly downregulated by VitD (**Figure 6V**).

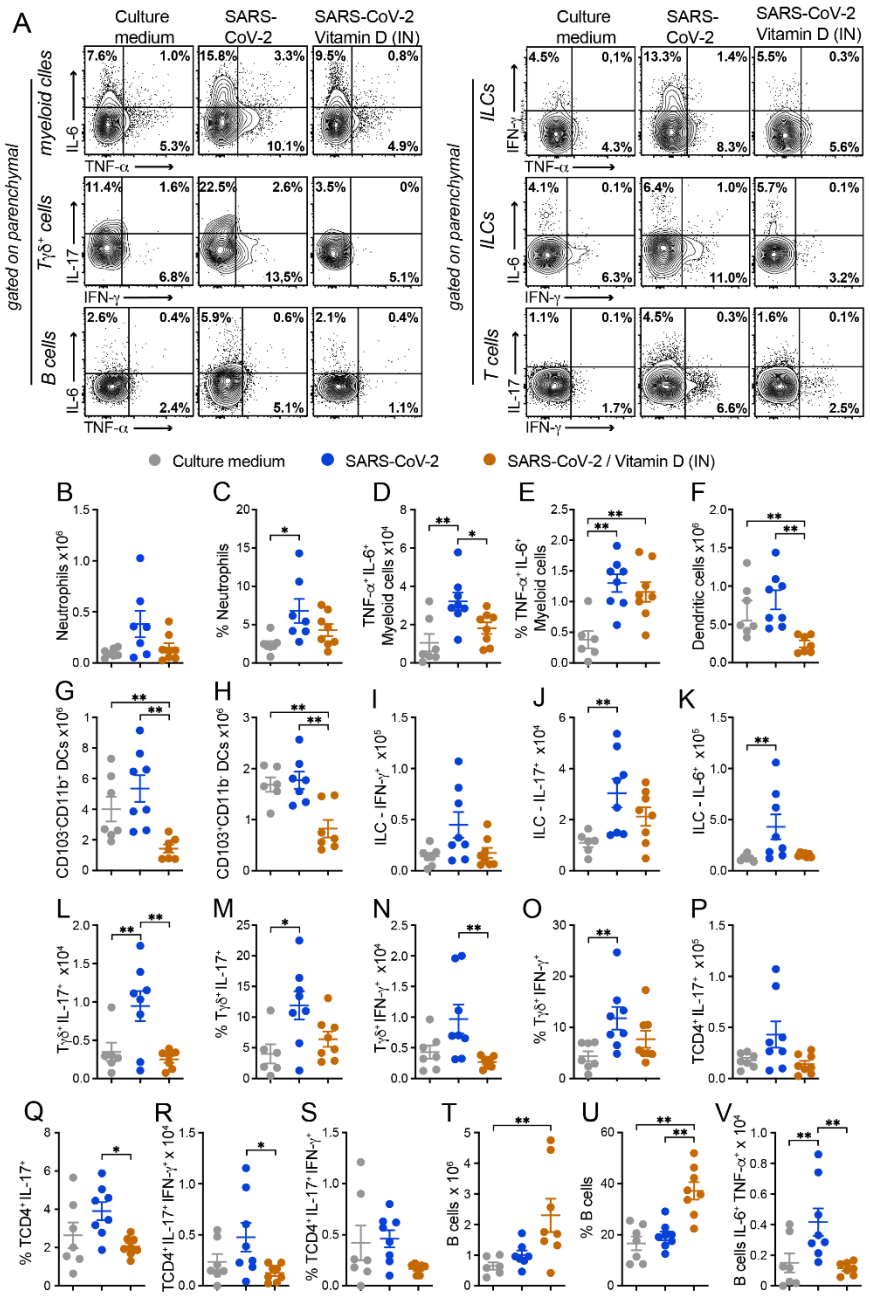


Figure 6

Figure 6. Effect of IN vitamin D on lung histopathology and cell infiltration triggered by IN instillation of UV-inactivated SARS-CoV-2. C57BL/6 mice were instilled with the virus (3 doses of 4.10^5 PFU/each) on days 1, 3 and 5. In the IN protocol, mice was treatment with 3 VitD doses (0,1 µg/dose) simultaneously with the SARS-CoV-2 inoculum. In the IP protocol, each animal was treated with 4 VitD doses delivered on days 0,2,4 and 6. At the 7th day, the upper left lobe and the right lung were collected for flow cytometry analyses. In order to differentiate the parenchyma-infiltrating leukocytes from the vasculature-

associated fraction, mice were intravenously injected with FITC-labeled anti-CD45 antibody 3 min before euthanasia. Specific cell subsets infiltrating the lungs of mice were analyzed according to the gate strategy described in **Supplementary Figures 2, 3** and **4**. Intracellular cytokine production was detected by flow cytometry in PMA/Ionomycin/brefeldin *in vitro* stimulated cells. (**A**) Representative contour plots of each experimental group indicating the frequency of cytokine production by each parenchymal cell subset, as indicated in the Y axis of the figure. (**B-V**) Absolute numbers and/or percentage of each cell subset or cytokine producing cell as indicated in each graph Y axis. Results are presented in median and interquartile intervals and the comparison between the groups was performed by the Kruskal-Wallis test followed by the Dunn´s test. * p<0.05; ** p<0.01; ***p<0,001

Taken together, our data suggest that local administration of VitD was sufficient to suppress the recruitment of inflammatory cells to the lung parenchyma induced by the exposure to inactivated SARS-CoV-2**.**

4 Discussion

This investigation was conducted considering that COVID-19 can be a lethal pathology whose treatment is not well established nowadays. In this study we initially used C57BL/6 mice, instilled with UV-inactivated SARS-CoV-2, to characterize a working model of inflammation in the lung that is the initial and main target of COVID-19 infection (BÖSMÜLLER et al., 2021). We then employed this model to investigate the potential of VitD to control local inflammation. The choice of C57BL/6 mice strain and inactivated virus would, in our view, make the model more accessible to a greater number of researchers and laboratory facilities.

The initial results, obtained by analyzing the BALF, suggested the ability of inactivated SARS-CoV-2 to trigger a local inflammatory process characterized by a clear increase in WBC, including in lymphocytes and neutrophils. As the BALF obtained from the culture medium group presented a profile very similar to the other control group (saline), most of the inflammatory process can be attributed to the virus itself and not to the

content of the medium used to grow the virus. Even though the subsequent analyses provided much more enlightening information about this model, this preliminary data was considered relevant because BALF procedures have been largelly employed as a tool to study a plethora of experimental and human diseases (MEYER; RAGHU, 2011; VAN HOECKE et al., 2017). In addition, this technique has been explored in experimental and clinical investigations involving the SARS-CoV-2 virus itself (LIAO et al., 2020).

Data obtained from RT-PCR assays performed with RNA extracted from lungs reinforced the initial findings showing increased expression of *GM-CSF* and *Foxp3* and a tendency towards increased values for *IL-17*, *IL-1β* and *NLRP3* mRNA expression. The possible contribution of inflammasome activation to COVID-19 immunopathogenesis is highly supported by the literature. It has been reported that inflammasome activation is triggered by SARS-CoV-2 components (PAN et al., 2021); that its higher activation is possibly involved in COVID-19 severity (RODRIGUES et al., 2021), and that specific inhibition of the NLRP3 inflammasome was able to decrease the intensity of a COVID-19-like pathology in mice (ZENG et al., 2022).

Histopathological analysis together with the flow cytometric evaluation of the cells obtained from the lung parenchyma allowed a more clear evaluation of the intensity and the quality of this inflammatory process. H&E stained sections clearly showed that IN instillation of inactivated SARS-CoV-2 induced a multifocal and interstitial pneumonia characterized by perivascular and perialveolar inflammation.The flow cytometric analysis performed with cells isolated from the lung parenchyma allowed a more precise identification of the cells involved in local inflammation. The presence of PMNs, eosinophils, macrophages, and lymphocytes was confirmed and the use of a combination of certain monoclonal antibodies allowed the differentiation of, for example, monocyte-derived macrophages and parenchyma-resident macrophages as described by (HOU et al., 2021). All these cell types have been associated with COVID-19 and their

presumed contribution to disease immunopathogenesis has been apprised by clinical and SARS-CoV-2 animal model investigations. The detection of mononuclear cells, including macrophages and lymphocytes, in our study is entirely supported by literature data showing that all COVID-19 patients presented a pulmonary inflammatory reaction predominantly composed of lymphocytes and macrophages (XU et al., 2020c). These cell populations have also been characterized in COVID-19 experimental disease model (YINDA et al., 2021). Our findings indicated the presence of a significant amount of inflammatory macrophages in the lungs of SARS-CoV-2 instilled mice, including an increase in monocyte-derived macrophages. Interestingly, the hyper inflammation observed in COVID-19 has been partially attributed to this cell type which is recruited from the blood by a plethora of chemoattractants (MERAD; MARTIN, 2020). An increased amount of PMNs is also described in the bloodstream and in the lungs of COVID-19 patients and strong evidences indicate that they play a paramount role on disease pathophysiology (REUSCH et al., 2021). A neutrophilic mucositis involving the entire lower respiratory tract has been described in lung autopsies from COVID-19 deceased patients (BARNES et al., 2020). Moreover, a neutrophil activation signature predicted critical illness and mortality in COVID-19 (MEIZLISH et al., 2021). Most of the damage triggered by PMNs has been attributed to their extensive and prolonged activation which leads to an excessive ROS release composed of superoxide radicals and H_2O_2 (CAVALCANTE-SILVA et al., 2021). In addition, according to PARACKOVA et al., (2020), PMNs have been seen as drivers of hyperinflammation by enhanced degranulation and pro-inflammatory cytokine production. The release of neutrophil extracellular traps (NETs) by PMNs is also pointed as a major promotor of damage in COVID-19 by causing endothelial injury and necroinflammation via complement activation, and by promoting the venous thrombus formation (TOMAR et al., 2020). This exuberant contribution of PMNs to the interstitial pneumonia that occurs in COVID-19 was, in many aspects, reproduced in

hACE2 mouse model infected with SARS-CoV-2.

The contribution of eosinophils to this pathology is still a matter of debate but their quantification in the blood indicates that eosinophilia and eosinopenia are associated with better and worst disease outcomes, respectively (XIE et al., 2021). Considering these and other findings, some authors believe that they could play a protective role. Nevertheless, (KIM et al., 2022), by analyzing BALF of COVID-19 patients, found an eosinophil-mediated lung inflammation associated with elevated natural killer T cell response. In accordance with these findings, we also detected eosinophils in BALF what was further confirmed by cytometry. To the best of our knowledge, there are no published studies concerning the involvement of eosinophils in the murine lung inflammation triggered by SARS-CoV-2.

The presence of DCs in the pulmonary parenchyma also deserves attention considering that they are fundamental for both, innate and specific anti-viral immune response but can also contribute to viral dissemination and immunopathogenesis during COVID-19 (MARONGIU et al., 2021). In this regard, by analyzing circulating DCs and monocyte subsets from hospitalized COVID-19 patients, (WINHEIM et al., 2021) described their impaired function and delayed regeneration. Flow cytometry also allowed the identification of lymphoid and myeloid cells producing cytokines as TNF-α, IFN-γ, and IL-17 which are among the most important mediators of COVID-19 immunopathogenesis (DARIF et al., 2021).

Having confirmed that SARS-CoV-2 IN instillation triggered a pulmonary inflammation similar to that developed by COVID-19 patients, we used this model to test VitD ability to modulate this inflammatory process. The option for VitD was based on the extensive literature attesting the powerfull immunomodulatory property of this hormone (SASSI; TAMONE; D'AMELIO, 2018), the robust evidences linking its low levels with poor COVID-19 outcomes (KARONOVA et al., 2021) and our own previous experience indicating its ability to counteract the inflammatory process that damages the central nervous system in a multiple sclerosis murine model

(DE OLIVEIRA et al., 2020; MIMURA et al., 2021). As indicated by our results, VitD administered by both routes, was capable to partially control pulmonary inflammation by downmodulating both, the amount of infiltrating cells and the local expression of pro-inflammatory cytokines. Interestingly, its IN application was way more effective which was confirmed by histopathological and flow cytometric analyses. H&E sections from animals that received VitD by the IN route revealed very well preserved lung structures, similar to those observed in the animals from the control group injected with saline. The flow cytometry protocol indicated that VitD was able to impair the recruitment of several cell types, as PMNs, DCs, and lymphocytes as $CD4^+$ and Tγδ in the lungs of mice challenged with SARS-CoV-2. This approach also allowed the identification of various cell subsets whose cytokine production was being decreased by VitD as myeloid, ILCs, Tγδ, $TCD4^+$ and B cells. These findings were considered specially relevant because the main detected cytokines, as TNF-α, IL-6, IL-17 and IFN-γ, have been identified as some of the major villains of the cytokine storm associated with COVID-19 and were significantly downmodulated by VitD. The possible association between this disease and VitD levels has been investigated from different perspectives, including the possible role of its deficiency and worst disease outcomes (RADUJKOVIC et al., 2020) and its prophylactic, immunorregulatory and protective role in COVID-19 (XU et al., 2020a). Its therapeutic benefit is also being widely pursued but a final conclusion is not possible yet due to the discordant results reported so far (ENTRENAS CASTILLO et al., 2020; MARCINKOWSKA; BROWN, 2022). As far as we know, there are no publications concerning the administration of IN VitD to control lung inflammation triggered by SARS-CoV-2 in animal models neither in patients up to now. In this context and considering the efficacy of its local instillation, we believe that once this effect has been proven in SARS-CoV-2-infected animals too, that it would be worth going to clinical trials.

Our initial hypothesis already predicted a superior efficacy of IN VitD

because other lung inflammatory pathologies, as asthma and rhinitis, are efficiently controlled by local drug delivery (FENG et al., 2021; KIM et al., 2019). Theoretically, a higher local concentration of vitamin D could be more efficient and, possibly, less toxic. The higher efficacy of local delivery was confirmed by our findings, however, we still detected increased calcium levels and body weight loss associated with this route. Hypercalcemia could be related to VitD systemic diffusion, subsequent increased calcium absorption from the intestine and increased bone mobilization (TEBBEN; SINGH; KUMAR, 2016). Body weight loss is possibly related to the effect of VitD in the brain considering that IN administration can provide a route for easier delivery of substances to the CNS (RHEA et al., 2020). Also, the brain is the master regulator of weight through the hypothalamus that controls both weight and express vitamin D receptors. According to SISLEY et al., (2016), administration of 1,25-D3 into the third ventricle of the brain dramatically decreased body weight by lowering food intake in obese rodents through action within the arcuate nucleus. These authors also found that vitamin D receptor colocalized with and activated key appetite-regulating neurons in the arcuate, namely proopiomelanocortin neurons.

Considering that IN VitD efficacy was much more impressive than IP VitD, we believe that most of its effect is locally occurring and is being mediated by some of its well-established immunomodulatory mechanisms. The significant reduction in many cytokine-producing cells and also in the level of these mediators in lung homogenates supports this possibility. In addition, the total levels of many proinflammatory cells in both, the pulmonary parenchyma, showed by cytometry, and in the alveolar space, demonstrated by BALF analysis, were also clearly downmodulated by VitD. Different mechanisms could be involved in this anti-inflammatory VitD effect as, for example, the reduction in TLR expression in lung-resident cells what could decrease the intensity of the initial interaction of SARS-CoV-2 with these cells (BILEZIKIAN et al., 2020). This could decrease the release of chemokines and therefore control the subsequent movement of leukocytes

towards the lung. Classical immunomodulatory mechanisms involving the innate immunity as inhibition of DCs maturation and blockage of antigen presentation to T helper cells could also occur. Inhibition of Th1 and Th17 effector cells and induction of Th2 lymphocytes could also contribute to VitD efficacy. In addition, VitD suppresses the release of a plethora of pro-inflammatory cytokines (XU et al., 2020b), which seem, considering our results, to play a major role in its therapeutic effect when delivered intranasally. The model of inflammation limited to the lung, used in this work, does not allow us to predict whether IN vitD would control extrapulmonary inflammatory processes triggered by SARS-CoV-2 infection. As IN VitD was able to attenuate LPS-induced acute lung inflammation (SERRÉ et al., 2022), it is expected that its application by this route would also be effective to control pulmonary inflammatory processes triggered by other infectious agents or substances.

It is important to highlight that this anti-inflammatory effect of IN vitD is very distinc from the activity that has been attributed to a series of IN substances recommended for COVID-19 prophylaxis as, for example, PVP-I, H_2O_2, chlordextrin (TELLES-ARAUJO et al., 2020), sprayable acid-oxidizing solution (GIARRATANA et al., 2021) and povidone-iodine solution (NAQVI et al., 2020). These compounds are virucidal and have been indicated as a hygiene measure to reduce viral load in the upper respiratory system whereas IN VitD, as implied by our results, would be indicated to control inflammation induced by SARS-CoV-2 in the lung. An alternative procedure to IN virucidal products is mechanical viral load reduction as described by (LYNN BAXTER et al., [s.d.]). These authors demonstrated that isotonic saline IN irrigation significantly reduced patients hospitalization. This strategy, which is based on virus removal, is also different from our preceeding in which a small volume of VitD, not able to displace the virus, is instilled concomitantly with the inactivated SARS-COV-2.

Even though the focus of our work had been the control of lung inflammation, we conceive that the possible adoption of IN VitD could bring

additional advantages to COVID-19 patients. In this sense, we highlight the stabilizing activity towards BBB disruption and the anti-fibrotic property of VitD considering that increased BBB (KRASEMANN et al., 2022) and lung fibrosis had been associated to more severe COVID-19 cases.

Our study is mainly limited by the fact that we did not show that this anti-inflammatory effect of VitD also happens during experimental SARS-CoV-2 infection. However, considering its adjunct therapeutic potential for COVID-19, we understand that this anti-inflammatory activity determined by IN VitD deserves to be further and fully investigated in preclinical and clinical assays.

Considering the results provided by this investigation and the fact that most of the clinical trials done with vitamin D employed other supplementation routes, mainly the oral one, it is temptative to suppose that the IN one could be more efficient. This clarification depends upon clinical trials in which vit D ability to control pulmonary inflammation is compared after its administration by various routes.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements: Dr. Edison Luiz Durigon (IBS-USP) for the donation of SARS-CoV-2, Dr Maria Luisa Cotrim Sartor de Oliveira (Pathology laboratory UNESP/SP) for the use of the equipment, and Flow Cytometry and Imaging Research FLUIR-CEFAP/USP core facility for the support on the flow cytometry experiments.

Funding: This study was supported by JBS S.A. and with the support of the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) - 88882.495054/2020-01, São Paulo Research Support Foundation (FAPESP, scholarship no. 2020/04558-0). J.L.P.-M. is

supported by CNPq scholarship no. 305628/2020-8 and A.S. is supported by the CNPq no. 307269/2017-5 grant. DMF is supported by CNPq scholarship 313429/2020-0 and FAPESP 2021/06881-5. MCA, CSL, BCO, GWS were supported by FAPESP scholarships 2019/12691-4, 2019/13916-0, 2019/07771-9 and 2021/12768-7, respectively.

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CAPÍTULO 3

COVID-19 & Multiple Sclerosis relationship: a two-way road
A narrative review

Abstract

COVID-19 is an infectious disease triggered by the virus SARS-CoV-2 which is responsible for a pandemic whose morbidity and mortality peaked between 2020 and 2021. To date, nearly 180 million cases have been diagnosed globally, with 3.8 million deaths (https://coronavirus.jhu.edu/map.html). One of the initial concerns, among many, was that this disease could trigger or worsen multiple sclerosis (MS) or other autoimmune diseases. The main objective of this review is to show the existence of a complex relationship between COVID-19 and Multiple Sclerosis. In this sense will be highlighted their association with inflammation, autoimmunity and central nervous system dysfunction. The association of their risk of development and severity with low vitamin D levels and the possible inclusion of vitamin D supplementation as an adjunct therapy will also be addressed. Lastly, will be suggested some experimental animal models that may be used to further clarify this challenging interplay.

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1.Introduction

COVID-19 is a pandemic infection caused by the virus SARS-CoV-2 whose severity will vary depending on host conditions and characteristic of the virus itself. Even though the lungs are predominantly affected, other organs and tissues as, for example, kidneys, heart, and the nervous system can be injured. Most of the pathophysiological findings in this disease have been attributed to an hyperinflammatory syndrome resulting from a dysregulated innate immune response. However, specific autoimmune response plays an additional role in its patognomonic effects. This review is focused on the possible interrelationship between COVID-19 and multiple sclerosis (MS) that is an autoimmune pathology that damages the central nervous system (CNS). In this scenario, we will first provide general informations about COVID-19 clinical manifestations, its ethiological agent and immunopathogenesis, and the nervous system involvement that can occur in some patients. The occurrence of autoimmune phenomena involving antibodies and cellular immunity, in the course of the disease, will also be addressed. MS clinical manifestations, the most relevant stages of its immunopathogenesis and the recognized relevance of viral infection to its development will then be described. The relevance of low vitamin D levels for development and severity of both diseases and the possibility to supplement patients to improve their modulatory potential towards inflammatory processes will also be reported. The final section of this review will be dedicated to briefly describe and disccuss some animal disease models that could be employed to investigate both disease at the same time.

2.COVID-19: clinical manifestations, etiology, and immunopathogenesis

A novel coronavirus named as 2019-nCoV or SARS-CoV-2 was

originally detected in Wuham, China, in 2019, but in a few months, it spread out to most countries causing a pandemic. This viral agent causes COVID-19 which affects predominantly the respiratory system causing flu-like symptoms as fever, cough, sore throat, dyspnea and fatigue (SINGHAL, 2020). However, this infection is characterized by a variety of clinical manifestations. Around 80% of the affected persons display very mild symptoms or can be even asymptomatic while about 15% can develop more severes symptoms. The remaining 5% of patients can advance to severe pathological conditions characterized by acute respiratory distress syndrome (ARDS), septic shock, and multiorgan failures associated to an elevated risk of death (DA ROSA MESQUITA et al., 2021). Evolution to more severe conditions has been specially favored by advanced age, coexistence of comorbidities as hypertension, diabetes and heart diseases, and genetic and epigenetic factors (ESPINOSA et al., 2020; SHAHID et al., 2020; YILDIRIM et al., 2021). Even though this infection is most well characterized by a significant respiratory impairment, it can also trigger several extrapulmonary manifestations including thrombotic complications, myocardial dysfunction and arrhythmia, acute coronary syndromes, acute kidney injury, gastrointestinal symptoms, hepatocellular lesions, hyperglycemia and ketosis, neurologic alterations, visual disturbances, and dermatologic complications (ELROBAA; NEW, 2021; JOHNSON et al., 2020). More recently, there is also growing concern about the existence and severity of post-covid sequelae, that is, those that appear after the predicted virus elimination as the ones affecting the respiratory and the neurological system (WANG; KREAM; STEFANO, 2020). Immediate and long-term consequences of COVID-19 infections for the development of neurological disease, with special focus in multiple sclerosis will be addressed later.

At least in part, these pulmonary and extra-pulmonary manifestations have been attributed to a direct virus damage, given that ACE2 (angiotensin converting enzyme), which is the entry receptor for the SARS-CoV-2, is expressed in the lungs and in these other extrapulmonary. Analogously to

other Coronavirus, SARS-CoV-2 consists of four structural proteins: Spike (S), membrane (M), envelope (E) and nucleocapsid (N). Spike comprises two functional subunits: S1, which binds to target cells and S2, that triggers the fusion between the viral and the target cell membranes (YUKI; FUJIOGI; KOUTSOGIANNAKI, 2020). The Sars-CoV-2 uses two host proteins to enter the target cell. The ACE2 is used for attachement to S1 whereas the transmembrane serine protease 2 (TMPRSS2) is used to activate the protease activity of S2. Detailed informations about the molecular mechanisms of this initial virus-target cell interaction are already available (SHANG et al., 2020).

The first line of defense against pathogens, including SARS-CoV-2, is the innate immunity. In the case of SARS-CoV-2, the recognition by tissue-resident immune cells within the lung provides a local immune response resulting in the recruitment of further innate immune cells from the blood. Innate immune cells, including monocytes, macrophages, PMNs and innate lymphoid cells (ILCs) express pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). SARS-CoV-2 is able to interact with various PRRs, especially Toll-like receptors (TLRs), retinoic acid-inducible gene 1(NLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and inflammasomas, initiating innate immunity activation (DIAMOND; KANNEGANTI, 2022). PRRs signaling engaged by SARS-CoV-2 induces concurrent release of both IFNs and other pro-inflammatory cytokines (DIAMOND; KANNEGANTI, 2022). Type I and III interferon (IFN) responses act as the first line of defense against viral infection and are activated by the recognition of viruses by infected cells and innate immune cells. This interaction initiates a series of signaling pathways to eliminate the virus. The most relevant mediators at this initial phase are type I and III interferons (IFNs). In the most favorable scenarios, these IFNs will directly control infection or will contribute to the induction of the specific immune response.

What is exactly happening with this early IFN response is not well

deciphered. Evidences have been suggested impaired and robust type I IFN responses KIM & SHIN (2021), and delayed and impaired IFN response BORDALLO et al., (2020), in patients with severe COVID-19. Clinical studies have evaluated recombinant type I and III IFNs as therapeutic options considering that they have been successfully used for the treatment of SARS-CoV-1 and Middle East respiratory syndrome (MERS)-CoV patients (CHOI; SHIN, 2021). Boosting the innate immunity with BCG, LPS and b-glucan, a process named trained immunity, is another strategy that has been proposed for protection against COVID-19.

However, due to virus-triggered immune evasion strategies or to pre-existing medical or genetic conditions, the infection can escalate and trigger an exaggerated secretion of cytokines and chemokines and to recruit and activate a plethora of immune cells establishing a state known as cytokine storm characterized by elevated levels of several pro-inflammatory cytokines (ZANZA et al., 2022). As will be discussed later, this cytokine storm could interfere in multiple sclerosis (MS) evolution. In the context of cytokine storm, recent studies suggest blocking of certain cytokines, such as interleukin (IL)-1 and IL-6 or Januskinase (JAK) pathway and traditional anti-inflammatory drugs for cOVID-19 treatment (JONES; HUNTER, 2021).

3.Neurological involvement associated to COVID-19

A growing body of evidence indicates that SARS-CoV-2, similarly to other coronaviruses, can reach and to establish itself at the nervous system triggering a variety of clinical and pathological disturbances which can vary a lot in severity. According to ABBOUD et al., (2020), the following manifestations have been found in some COVID-19 patients: headache, myalgia, dizziness and fatigue, described as mild; hyposmia, hypogeusia, visual disturbances, encephalopathy, epilepsy, paralysis and consciouness disorder, identified linked to moderate severity; and cerebrovascular events, acute necrotizing encephalopathy, meningitis, encephalitis and Guillain-

Barré Syndrome, considered as severe symptoms. The prevalence of these manifestations seems to be specially elevated among hospitalized patients as described by (CHOU et al., 2021). By analyzing 3744 patients they found around 80% of prevalence, including headache (37%), anosmia or ageusia (26%), encephalopathy (49%), coma (17%), and stroke (6%). However, the prevalence of neurological manifestations associated with COVID–19 varies widely among the different studies. The pathogenesis of CNS infection by SARS-CoV-2 and the neurological complications are still poorly understood.

Most of these symptoms had been attributed to the arrival of the virus to the nervous system. Laboratory findings have supported the neuroinvasion by SARS-CoV-2, as for example, the study by ARAÚJO et al., (2021), that detected the virus in the cerebrospinal fluid of a patient suffering from Guillain-Barré Syndrome. Also SONG et al., (2021), demonstrated neuroinvasion by using human brain organoids, mice expressing human ACE2 and autopsies from deceased patients. Two major routes had been associated to neuroinvasion by this virus according to REZA-ZALDÍVAR et al., (2021): through peripheral neurons and throught hematogeneous dissemination. It is believed that the most common route used by SARS-CoV-2 to reach the CNS is by using the peripheral nerve terminals. The olfactory nerve is considered the major candidate because it is located very near to the olfactory epithelium which, by expressing ACE2 and TMPRSS2, allows virus initial replication (GASMI et al., 2021). This process has been described as transcribrial route because it occurs across the cribriform plate of the ethmoid bone, followed by virus spread retrograde via transsynaptic transfer using an endocytosis or exocytosis mechanism and a fast axonal transport. According to LIU et al., (2021), and ESPOSITO et al., (2020), the virus could also gain access to the CNS via the vagal afferents from the upper airways and the enteric nervous system, respectively. Another possible route is through the virus presence in the bloodstream from where it can reach the nervous system by a direct interaction with brain capillary cells or by means of an infected leukocyte.

The detection of viral particles in capillary endothelial cells in the front lobe tissue obtained at a postmortem analysis from an infected patient gives some support to this interaction with endothelial cells, PANIZ-MONDOLFI et al., (2020). Infected leukocytes could also pass through the brain blood barrier (BBB), acting as a Trojan Horse. More recently, this hypothesis that infected cells can cross the BBB as a Trojan Horse has been extended to also include exossomes and high-density lipoproteins associated with SARS-CoV-2 (LAM; HUANG; SHUI, 2022).

Different experimental models have been used to study the presence and the consequences of SARS-CoV-2 in the CNS: neural cell lines, animal models and brain organoids. Published studies have shown that SARS-CoV-2 can infect and replicate in induced pluripotent stem cell (iPSCs)-derived human neural progenitor cells (hNPCs) and in neurospheres or brain organoids produced from these cells (ZHANG et al., 2020a). The neuropathology associated to COVID-19 is a complex condition related to the presence of the virus, the induced local and peripheral immune response and to altered coagulation and vascular systems. The most recurrent neuropathological findings in COVID-19 patients include microglial activation, lymphoid inflammation with a clear predominance of TCD8+ cells, astrogliosis, myelin loss, hypoxia-ischemic changes, brain infarcts and hemorrhage and microtrombi (PRIORI, 2021). Part of these findings are due to SARS-CoV-2 infection of microglia and neurons which express different receptors for spike as ACE2, ephrin ligands and Eph receptors, neuropilin 1 (NRP-1), P2X7, and CD147 (ZALPOOR et al., 2022). Similarly, to peripheral infection, this CNS infection also triggers a cytokine and chemokine avalanche causing neurotoxicity, dysruption of the neuro-glia homeostasis and neuronal death (SAVELIEFF; FELDMAN; STINO, 2022).

4.Connection between Covid-19 and autoimmunity

A possible relationship between COVID-19 and and autoimmunity

brought another challenge to a disease already very complex. Some of the aspects that had been more emphasized by the experts in this field include the potential of the virus to trigger autoimmunity, to underlying brain autoimmunity and other neurodegenerative diseases, autoimmune response against ACE2, contribution of autoimmune phenomena to post-acute disease sequelae and effects of pre-existing autoimmunity in COVID-19 severity.

The possible induction of autoimmunity by SARS-CoV-2 is not unexpected as viral infections had been classically associated with multiple autoimmune pathologies (SMATTI et al., 2019). Case reports of arthritis GASPAROTTO et al., (2021) and possibly of Type 1 diabetes in children AMBATI et al., (2022) after COVID-19 have been described. More recently, JUANES-VELASCO et al., (2022), by using functional proteomics approaches, characterized the auto-immune profile of COVID-19 patients presenting ARDS. They found several auto-antibodies in ARDS patients targeting cytokines, chemokines, glycoproteins, and phospholipoproteins and they also suggested the possibility to identify differential profiles in patients presenting distinct levels of lung damage. Other reports endorse the appearance of autoimmune diseases after SARS-CoV-2 infection as cold agglutinin syndrome, autoimmune haemolytic anemia PATIL; HERC; GIRGIS, (2020) and Guillain-Barré syndrome (MEHTA; SUNDER, 2021). Several mechanisms have been blamed for autoimmune phenomena detected in patients with COVID, including stimulation of inflammatory signaling, superantigenic properties, molecular and functional mimicry, bystander activation of T cells, transient immunosuppression and pro-inflammatory cytokines (KNIGHT et al., 2021; MOBASHERI et al., 2022). The postulate that there is a molecular mimicry between the virus and human epitopes has received a lot of attention. On the basis of analysis in silico, KOVAČIĆ; JOTANOVIĆ; LAKOVIĆ, (2021), identified several homologous regions between SARS-CoV-2 proteome and the human proteins PARP14, PARP9 and MACROD1, suggesting therefore that they

could be involved in autoimmune responses.

Another protein that has gained attention is ACE2 that allows the binding to human target cells. Autoimmunity to ACE2 was initiall hypothesized by KNIGHT et al., (2021); TOWNSEND (2020), as a possible cause of tissue inflammation in COVID-19. Investigations carried out at that time confirmed the presence and association of IgM anti-ACE2 in the sera of patients with severe COVID-19 (CASCIOLA-ROSEN et al., 2022). Later on, ARTHUR et al., (2021), confirmed this finding showing a high prevalence (81%) of anti-ACE2 antibodies in a disease-convalescent group and in 93% of acutely hospitalized patients. On the other hand, there was absence or only 5% prevalence of this antibody in control or outpatients, respectively.

The brain has been viewed as a possible target of self-reactivity. According to the viewpoint of GUPTA & WEAVER (2021), based on similarities between SARS-CoV-2 and diverse human CNS protein epitopes, which could trigger a myriad of autoantibodies and also a local cytokine storm, COVIDD-19 is a trigger of brain autoimmunity. More than 2 years after the beginning of the pandemic, the condition called “Long COVID” or Post-COVID-19 syndrome” can be clearly appreciated. This term is employed to refer to a diversified group of symptoms that remain during months after disease onset. More common examples of these manifestations include shortness of breathe, fatigue, cough, chest pain, rashes and neurological and cognitive deficits (TARIBAGIL; CREER; TAHIR, 2021). GUPTA & WEAVER (2021), especulated that SARS-CoV-2 infection could, in the context of this long COVID, play a role as a long-term risk factor for neurodegenerative diseases as Alzheimer and Parkinson.

5.Connection between COVID-19 and vitamin D

Numerous questions have been raised concerning the relationship between COVID and vitamin D. In our opinion some of the most relevant ones, for practical purposes are:1. Is there vitD deficiency in COVID-19

patients and is this a risk factor to get the infection and to develop a more severe pathology? 2. Is vitD supplementation indicated for Covid-19 patients? 3. How is the vitD presumed to control COVID-19 pathogenesis?

An analysis between vitD levels and the number of COVID-19 cases in 20 european countries showed a significant negative correlation suggesting that higher levels of this hormone could afford some protection against SARS-CoV-2 infection (ALI, 2020). These findings were reinforced by the observation that many hospitalized COVID-19 patients have vitD serum levels considered below the normal expected ones (DEMIR; DEMIR; AYGUN, 2021; HERNÁNDEZ et al., 2021; SZARPAK et al., 2021). The majority of the findings also support an inverse correlation between vitD deficiency and a worst prognosis for COVID-19. According to CAMPI et al., (2021), low vitD levels at hospital admission were associated with increased IL-6 levels and predicted both the severity of respiratory distress and mortality during the course of hospitalization. VANEGAS-CEDILLO et al., (2022), also found this association between lower vitD levels and mortality. The authors believe that these low levels could contribute to the pro-inflammatory and pro-thrombotic state, increasing therefore the risk for adverse COVID-19 outcomes. Increased risk of COVID-19 severity and fatal outcome have been confirmed by other similar studies (CARPAGNANO et al., 2021; INFANTE et al., 2022; KARONOVA et al., 2021). From a theoretical point of view, based mainly on vitD immunomodulatory properties, its adoption as an adjunct therapy for COVID-19 seems consistent and is, at some extension, sustained by clinical assays. SABICO et al., (2021), for example, investigated the effect of vitD oral supplementation in mild to moderate ill patients that had low levels of this vitamin. They observed that 5000 IU of vitD reduced the time to recovery for cough and gustatory sensory loss. Göner et al., (2021) conducted a prospective study in which supplementation of hospitalized patients presenting vitD deficiency shortened hospital stay and decreased mortality rate by 2,14 times. Contrasting with these findings, CHEN et al., (2021), by

employing a meta-analysis and GRADE assessment of coort studies and RCTs, arrived to opposite conclusions. They infered that low vitD levels do not play a role in disease severity and that supplementation does not improve outcomes in hospitalized patients with COVID-19. Considering that this is a very complex pathology, the explanation for these conflicting results could be partially related to intra- and inter-coorts variability. Other parameters as supplementation protocols (doses, duration of supplementation), pacient´s age, presence of comorbidities and even the risk of bias, could contribute to this variability. This ambiguous scenario has prevented an official recommendation concerning prophylactic or therapeutic use of vitD for COVID-19 control (FARID et al., 2021; MARTINEAU; CANTORNA, 2022). The ability of vitD to presumably control COVID-19 infectivity and severity is mediated by different mechanisms. This vitamin is able to increase the production of an antimicrobial peptide called cathelicidin LL-37 by macrophages and respiratory epithelial cells (BARLOW et al., 2011). A preprint posted online by Roth et al., (2020), described the ability of this peptide to inhibit, in vitro, the virus binding to its receptor by the binding of this peptide to SARS-CoV-2 spike protein. One of the hallmarks of severely affected COVID-19 patients is the presence of a cytokine storm triggered by the activation of cells from both, the innate and specific immunity. The very well-established ability of vitamin D to control cytokines and chemokines production could provide another mechanism for vitD usefulness as an adjunct thepy in COVID-19. This effect derives mostly from the downmodulatory of vitD over Th1 and Th17 cells differentiation and cytokine production (CHAUSS et al., 2022; ZHANG; SHIH; ZHANG, 2013). This mechanism is already suggested by clinical findings in COVID-19 patients. BİLİR et al., (2022), described that vitD supplementation to geriatric intensive care patients suffering from COVID-19 reduced many inflammatory parameters, including IL-16, C-reactive protein, procalcitonin, D-Dimer, ferritin and lactate dehydrogenase. Adicional possible mechanisms could be mediated by its effects against endothelial disfunction

KIM et al., (2020) and vascular thrombosis (MOHD et al., 2021).

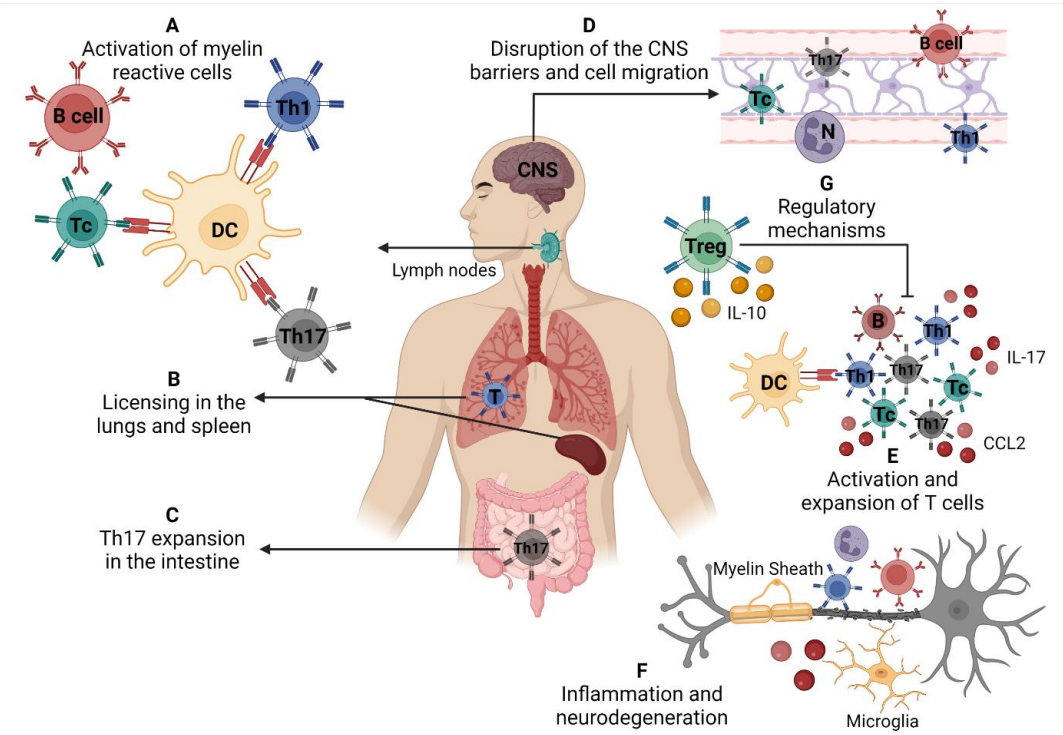
6. Multiple Sclerosis: clinical manifestations and immunopathogenesis

Multiple sclerosis (MS) is classicaly described as an inflammatory and demyelinating disease originated from an autoimmune disturbance. It is characterized by the presence of multifocal and scattered lesions through the grey and white matter from brain and spinal cord. Damaged myelin sheath commonly results in vision and coordination loss, muscle weakness, muscle stiffness and spasms, pain and changes in bladder and bowel function. MS diagnosis is usually based on the clinical presentation, supported by neuroimaging and in some cases by CSF analysis to search for inflammatory markers and oligoclonal antibody bands (GARG; SMITH, 2015). Even though MS can follow very different patterns of evolution, according to CONFAVREUX & VUKUSIC (2006), this pathology is considered one disease that presents different fenotypes and this could be related to the fact that it is a multifactorial etiology affected by the genetic background and a plethora of environmental elements as infectious agents, mainly virus, and vitD levels as will be commented later. Understanding how these factors affect this disease is fundamental because this is a handicapping pathology whose incidence and prevalence is increasing in the world. Four basic disease courses, which can also be refered as types or phenotypes, are recognized: clinically isolated syndrome, relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS). RRMS is considered the most common phenotype. Affecting around 85% of the patients. As indicated by its designation, it is characterized by alternating relapses and remmissions which are periods of neurological dysfunction or absence of neurological symptoms, respectively (KLINEOVA; LUBLIN, 2018). A noteworthy finding is the strong association of relapses with infections, postpartum period,

genetic risk factors, stress, and vitamin D levels (XIE et al., 2020). An increased relative risk for relapses have been associated with infections located at the upper respiratory system, and urinary and gastrointestinal tracts (VOLLMER, 2007).

MS immunopathogenesis has been unrevealed by using both, patient´s samples and informations and animal models, specially experimental autoimmune encephalomyelitis (EAE). This disease is induced, mainly in mice and rats, by immunization with myelin-derived proteins and peptides in the presence of complete Freund's adjuvant and pertussis toxin (ROBINSON et al., 2014).

The supposed main stages that lead to MS development, which are characterized below, are outlined in **Figure 1**.



Fonte: Created with Biorender.com

Figure 1. Immunopathogenic scheme of multiple sclerosis/ experimental autoimmune encephalomyelitis. (A) Selfreactive T cells against myelin antigens are activated in secondary lymphoid organs. (B) The licensing of selfreactive cells occurs in the lungs and spleen (C) and differentiation for the Th17 subpopulation occurs in the intestine. (D) These cells migrate to the central nervous system and pass through the Blood Brain

Barrier, (E) are reactivated and ince the inflamtory process that leads to (F) demyelinization and neurodegeneration. There is action of regulatory T cells and anti-inflamtory cytokines, but they are insufficient to inhibit the progress of the disease.

1.Peripheral activation of myelin-specific lymphocytes

It is accepted that activation of myelin-specific T cells could occur in peripheral lymphoid organs by recognition of microbial epitopes that share homology with self-antigens (molecular mimicry) CUSICK; LIBBEY; FUJINAMI, (2012), release of myelin from the CNS by a local insult STOHLMAN & HINTON (2006) or by bystander activation (WALDNER; COLLINS; KUCHROO, 2004). Other mechanisms including induction of costimulation, polyclonal activation, altered processing and expression of cryptic antigens could be also get started by an infectious agent (KHAN; GHAZANFAR, 2018).

2.Alleged T cell licensing

A fairly new concept has emerged from EAE studies in the last few years and is called “licensing” or more clearly “licensing for pathogenicity”. This stage, which seems to occur in the lungs, spleen or maybe both, allows the T cells to become pathogenic, to reach the CNS and to orchestrate a local inflammatory process (ODOARDI et al., 2012; TAN et al., 2017). This licensing process is accomplished by a change in the pattern of gene expression in which there is downregulation of genes related to proliferation/activation and upregulation of migration-promoting genes (RANSOHOFF, 2012). Th17 cells play a major encephalitogenic role by opening the blood-brain barrier (BBB) KEBIR et al., (2007) and by acting as a highly pro-inflammatory source triggering neurodegeneration (MOSER et al., 2020).

3.Expansion of Th17 in the intestine

The immune system associated with the intestinal mucosa has recently been recognized as pivotal to MS and EAE development. This crucial role in the pathogenesis occurs mainly by promoting activation and acquisition of the Th17 phenotype (IVANOV et al., 2009); Cosorich et al., 2017). It is assumed that activation of effector Th17 cells occurs mostly in the murine small intestine, independent of their ensuing function (GABORIAU-ROUTHIAU et al., 2009; IVANOV et al., 2009). Interestingly, the development of steady-state or pathogenic Th17 cells is critically determined by microbiota composition. Segmented filamentous bacteria, for example, induce brain autoimmunity in mice by selectively promoting Th17 differentiation (LEE et al., 2011).

4.Breakdown of the blood-barriers and cell migration to the CNS

There are two barriers that protect the CNS integrity and functionality which are termed blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier. These two barriers are located in distinct CNS compartments, and their dysfunction can allow leukocyte access and ensuing neurodegeneration in MS and EAE (ORTIZ et al., 2014). Different conditions can affect the integrity of these barriers including peripheral inflammation, infections and pro-inflammatory cytokines. It is well established that excessive inflammation is detrimental to the host. There is a series of mechanisms by which an exaggerated inflammatory process can induce dysruption of these barriers, including changes in tight junctions, damage to endothelial cells, activation of astrocytes and microglia, and effects of peripheral immune cells (HUANG; HUSSAIN; CHANG, 2021). The invasion of the CNS by neurotropic viruses, by hematogenous or non-hematogeneous routes, can be associated to structural and functional BBB alterations that leads to its breakdown (CHEN; LI, 2021). Certain cytokines

have been more often linked to alterations in these barriers as shown by (FURUTAMA et al., 2020). They found that periodontal inflammation-induced IL-6 is associated to neuroinflammation and BBB dysruption in the hippocampus.

5.Local inflammation, neurodegeneration, possible reactivation

A plethora of infiltrating cells, consisting mainly of γδT, Th1 and Th17 cells, are locally expanded and release cytokines that will activate microglia and oligodendrocytes. These activated cells, together with the ones mobilized from the periphery, will release inflammatory mediators such as IL-8, IL-17, GM-CSF, CCL2 and enzymes that will, alltogether, trigger neurodegeneration (KARPUS, 2020; MCGINLEY et al., 2018). A great deal of contribution to this damaging process has been imputed to B cells, mitochondrial dysfunction, oxidative stress and inflammasome activation (GHARIBI et al., 2020; LANG et al., 2018). This process will eventually be controlled by the activation of regulatory mechanisms involving different cellular types as Treg CD25+Foxp3+, Tr1, Qa-1 restricted CD8, Bregs, NK, and CNS-derived MDSCS (RUIZ; VIGNE; POT, 2019). However, results described by PANDIT et al., (2021), suggest that viruses, namely EBV and HHV6, could have a role in triggering relapses through a peripheral mechanism, rather than a direct role through intrathecal multiplication.

7.Connection between MS and virus (EBV and SARS-CoV-2)

Epstein-Barr virus (EBV) establishes lifelong infection, usually subclinical, in more than 90% of the adult population worldwide. However, it is also the causal agent of infectious mononucleosis, some cancer types, and some severe lymphoproliferative diseases. Epidemiological, serological and virological evidence support its role also in MS development

(BJORNEVIK et al., 2022). Primary EBV infection occurs in the squamous epithelial cells where it replicates and from where it reaches and infects the B lymphocytes of Waldeyer´s tonsilar ring. This infection triggers a process by which these naive B cells undergo a germinal center-like activation and differentiation program affecting more than 11,000 genes. This process culminates in proliferating immortalized B cells resembling plasmablasts and early plasma cells (MROZEK-GORSKA et al., 2019). In the context of MS, EBV is understood as a disease trigger. BJORNEVIK et al., (2022), tested this possibility in a very large cohort constituted by more than 10 million US army personnel. They observed a 32-fold increase of MS diagnosis in individuals who became seropositive compared with those that remained seronegative. This role of EBV has been mainly attributed to a cross-reactivity between self-antigens and EBV antigens, involving both, cellular and humoral immunity (SOLDAN; LIEBERMAN, 2022). Additionally, as EBV-infected B cells and plasma cells have been identified in the brain of deceased MS patients, but not in controls SERAFINI et al., (2010), it is believed that induction of B cell trafficking to the CNS is also involved in this mechanism (ROBINSON; STEINMAN, 2022). The recognized efficacy of monoclonal antibodies as rituximab for example, that targets the B cell surface marker CD20, adds more credibility to this contribution of EBV to MS development (MILO, 2019).

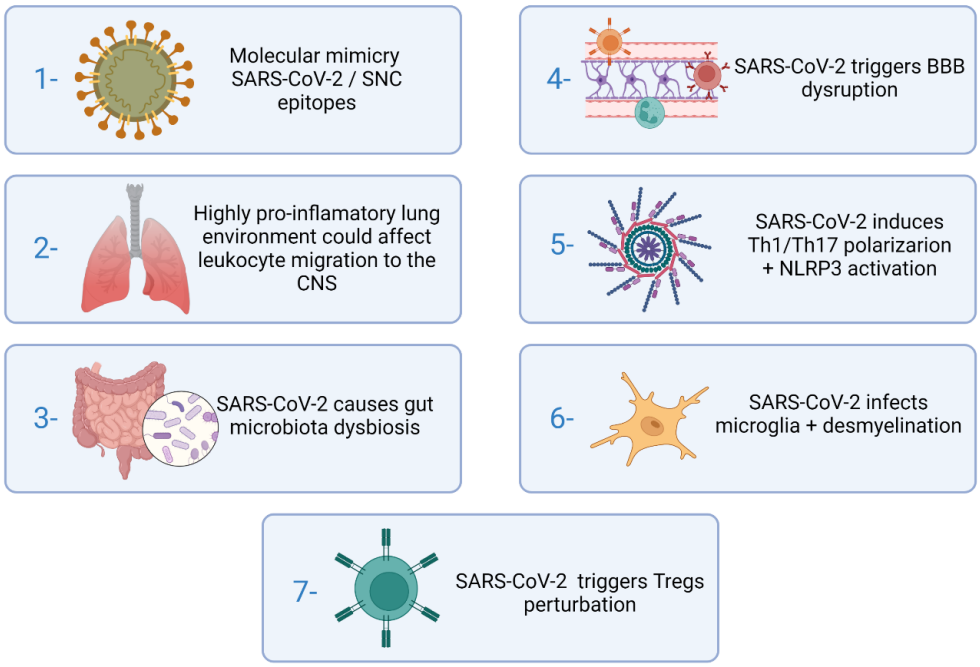
There is an overwhelming quantity of data concerning and proving the immune dysregulation during severe COVID-19. Because important dysregulations pathways coincide with immune alterations found in MS, it has been postulated that SARS-CoV-2 could be a risk factor for MS triggering or worsening in prone individuals. Through a system biology study, BELLUCCI et al., (2021), found expressive interaction of SARS-CoV-2 with genes associated to autoimmunity, especially MS. The three following crossroads: type-1 IFN response, Th17 axis, and inflammasome pathway, were considered more relevant and will be briefly commented on the basis of BELLUCCI et al., (2021), opinions and informations. A defective IFN-I

production has been described in both, COVID-19 and MS MEFFRE; IWASAKI, (2020), and the use of IFN-y, that is already a classical therapy for MS, decreases the risk of COVID-19 in people with MS (SORMANI et al., 2021). Contrastingly, people prone to develop MS are probably more susceptible to develop immunological alterations induced by SARS-CoV-2. This proposition is based on reports showing that severely ill COVID-19 patients would be infected with virus variants that impair IFN-I response ZHANG et al., (2020b) or present elevated levels of IFN-I neutralizing antibodies (BASTARD et al., 2020).

The Th1/Th17 axis, responsible for IFN-y and IL-17 production, respectively, is also shared by both pathologies. The contribution of these Th subsets is strongly supported by the literature (KASKOW; BAECHER-ALLAN, 2018). Interestingly, the Th17 subset develops in the intestine and its higher frequency correlates with microbiota alterations (COSORICH et al., 2017). Coincidentally, severe COVID-19 is associated with high levels of IFN-y, Th17 polarization BELLUCCI et al., (2021) and dysbiosis of the gut microbiota (YEOH et al., 2021). This scenario suggests that severe COVID-19 could affect MS development through the Th1/Th17 axis.

Another link existing at the crossroad between COVID-19 and MS is the inflammasome which is a complex molecular platform comprising a sensor protein, inflammatory caspases and, in some cases, an adapter protein that bridges the two other components. Its activation by danger- and pathogen-associated molecular patterns promotes IL-1□ and IL-18 production and pyroptosis (FRANCHI et al., 2009). Dysregulated inflammasome activation can be associated to infections and inflammatory pathologies. A strong contribution has been atributed to inflammasomes, specially the NLRP3, to the development of both, MS and EAE that is its corresponding animal model. According to BARCLAY & SHINOHARA (2017), NLRP3 activation is involved in various MS stages as initial inflammation, T cell polarization, CNS barriers breakdown and neurodegeneration. The ability of SARS-CoV-2 to also activate this platform

has been robustly demonstrated by many authors. RODRIGUES et al., (2021), for example, described that the NLRP3 inflammasome is activated during COVID-19, being higher activation levels associated with more aggressive disease. This association makes sense, considering that a significant cause of COVID-19 pathogenesis and subsequent severity is the cytokine storm associated with NLRP3 overactivation (DUTTA; LIU; XIONG, 2022). At the first year of the pandemics, DI STADIO; ROMANI; BERNITSAS, (2020), already speculated that MS could be worsened by NLRP3 inflammasome activation and the consequent cytokine storm triggered by SARS-CoV-2. The possible stages of MS immunopathogenesis that could most likely be deleteriously affected by SARS are suggested in **figure 2**.



Fonte: Created with Biorender.com

Figure 2. Connection between Multiple Sclerosis and SARS-CoV-2. Representative illustration of the possible stages of immunopathogenesis of multiple sclerosis, correlated with the possible deleterious effects caused by SARS-CoV-2 infection.

Despite this expectation of a deleterious effect of COVID-19 on MS

manifestations, a retrospective study conducted by ETEMADIFAR et al., (2021), led to the conclusion that, regardless of its severity, COVID-19 was not associated with an increased risk of relapse shortly after infection. The authors attributed these non-expected results to two possible causes: the post-COVID-19 lymphopenia and the use of immunomodulatory drugs to control MS. The authors also do not discard the possibility that COVID-19 delerious effects are observed later, that is, as sequelae associated to post-COVID conditions.

8.Connection between multiple sclerosis and vitamin D

Similarly to the approach used to analyze the conexion between COVID-19 and vitD, we also limited this complex subject to answer the three same questions: 1. Is there a vitD deficiency in MS patients and is this a risk factor to develop this disease and to present a more severe pathology? 2. Is vitD supplementation indicated for MS patients? 3. How is the vitD presumed to control MS pathogenesis?

Literature data concerning the relationship between vitD and MS supports the concepts that there is deficiency of this vitamin in MS patients, that this is a risk factor for disease development and that this deficit probably contributes to a more severe pathology. The hypothesis that adequate vitD levels were relevant to prevent MS development emerged from the realization that this disease was more prevalent in geographical regions with lower solar incidence where the production of vitD by the skin, stimulated by UV light, is low. In 1994, NIEVES et al., (1994), already described a high prevalence of vitD deficiency in MS patients. This finding was subsequently validated by many other authors as FAHMI; MOHAMED; ELSAID, (2014), that also showed a significant correlation among this deficiency, MRI load, and disease severity. Several studies have been conducted to establish if vitD supplementation could be considered an add-on therapy for MS. Some of the studies that showed beneficial effects in relapsing-remitting patients

are briefly referenced. SOILU-HÄNNINEN et al., (2012), studied efficacy of its supplementation as an add-on therapy in patients under IFN-□-1b. There was no difference in the annual relapse rate but vitD group presented a reduced disease activity indicated by MRI technique. LAURSEN et al., (2016), supplemented MS patients under natalizumab treatment. Supplementation corrected hypovitaminosis and decreased annualized relapse rate. As vitD efficacy could rely on high doses and considering that they could trigger serious effects as fatigue, muscle weakness, renal failure, and cardiac arrythmia, some clinical trials were designed to test these possible deleterious outcomes. KIMBALL et al., (2007), showed that even vitD supplements that elevated twice the top of physiological range did not elicit hypercalcemia, hypercalciuria or any other detrimental effect. The efficacy of high vitD dose was assayed by (DÖRR et al., 2020). The results were inconclusive, that is, neither support nor clearly discredit the potential benefit of high doses of this vitamin. According to BOLTJES et al., (2021), there is no substantial evidence up to know to approve adoption of vitD as an add-on therapy for MS. In spite of literature discrepancies, these authors recommend the implementation of standardized study desigs with very well-defined variables concerning the kind of vitD suplemment, its concentration, the composition of the coorts, the clinical and laboratory parameters to be evaluated, and the assembly of biological samples to build a biobank for further evaluation. Detailed reasoning and suggestions about their proposed study design are available in their publication. The immunomodulatory ability of vitD is clearly pleiotropic and reaches the majority of the immune cells in different phases of the immune response. This is explained by its interaction with the vitamin D receptor (VDR) that is expressed in immune cells including PMNs, macrophages, DCs, and B and T lymphocytes (WANG; ZHU; DELUCA, 2012). Even though vitD can increase some mechanisms associated with the innate arm of immunity, it is usually downmodulatory, that is, it works mainly as an anti-inflammatory modulator. The understanding of this considerable biological activity has sparked much

interest in two aspects: its level in patients with inflammatory diseases and the possibility of its supplementation as a therapeutic measure. The interaction of the active form of vitD with VDR and the main effects over the immune system are illustrated in figure 3. An overview of the main outcomes from vitD interaction with immune cells is presented in **table 1**. Its potential as an adjunct or alternative therapy in inflammatory diseases is exemplified in **table 2**.

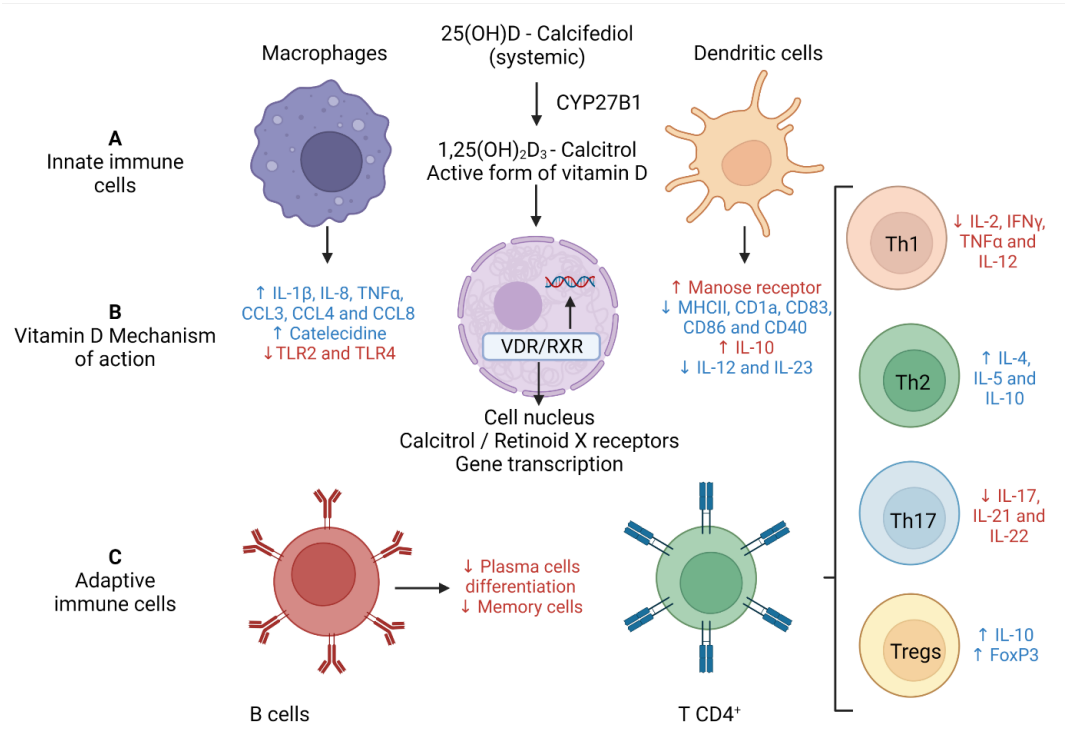


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Figure 3. Immunomodulatory effect of vitamin D. (A) Vitamin D in its form 25(OH)D3 - Calcifediol, to become active, goes through two hydroxylations, one of them is performed by 1α-hydroxylase (CY27B1), present in numerous cells of innate immunity exerting its immunomodulatory role on expression of receptors and synthesis of cytokines. (B) This immunomodulatory action is possible due to this hydroxylation sequence that results in vitamin D in its active form 1,25(OH)2D3 – Calcitrol, the hormone binds to the VDR receptor that conjugates with the Rethyroid Receptor X act on the inhibition or expression of genes linked to the responses of immunity against pathogens. (C) Adaptive immunity cells, in addition to being directed from vitamin D immunomodulation in innate immunity, also suffer the action of the hormone, as they also have VDR and CY27B1 receptor, and this induces

the adaptive response profile and its effector mechanism.

Table 1. Immunomodulatory effect of vitamin D on the cell. The table presents the immunomodulatory action of vitamin D on the different cell types, the experimental model used to observe this effect and how vitamin D was administered.

Immunomodulation by Vitamin D in the cell			
Cell Type / Source	**Experimental Model**	**Treatment**	**Main Outcome**
Dendritic cell PENNA & ADORINI 2000 BERER et al., 2000	Cell culture from peripheral blood monocytes	1,25 $(OH)_2D_3$ added to the culture medium	Inhibition of differentiation and maturation Apoptosis induction ↓ MHC II, CD40, CD80, CD86, IL-12 and IL-23 ↑ IL-10 expression
Macrophages VERWAY et al., 2013 LIU et al., 2006 GOMBARD; BORREGAARD; KOEFLER	Co-culture of macrophages and human lung epithelial Culture of bone marrow cells from humans and mice	1,25 $(OH)_2D_3$ added to the culture medium	↑ IL-β, IL-8, TNF-α, CCL3, CCL4 and CCL8 ↓ TLR2 and TLR4 Induces catelecidine Synthesis
Peripheral mononuclear cells KHOO et al. 2011	Human peripheral blood cell culture	1,25 $(OH)_2D_3$ added to the culture medium	↓ Dose-dependent IL-6, TNF-α and IFN-y ↑ catelecidine
Neutrophils YANG et al., 2015 CHEN; EAPEN; ZOSKI 2015 ARAZ-CIBRIAN; GIRALDO; URCUQUI-ICHIMA 2019	Human peripheral blood cell culture	1,25 $(OH)_2D_3$ added to the culture medium	↑ Apoptosis in chronic obstructive pulmonary disease ↑ IL-8 levels Enhancement of NETS
Eosinophils MATHEU et al., 2003	Knockout mice	Vitamin D subcutaneous injection	Eosinophilic narrowing of the Upper airways ↓ Synthesis of IL-5
Mast cells BIGGS et al., 2010	Knockout mice	Vitamin D subcutaneous injection	Eosinophilic narrowing of the Upper airways ↓ Synthesis of IL-5

Th1 cells inhibition SKROBOT; DEMKOW; WACHOWSKA 2018 RAUSCH-FAN et al. 2002	Human peripheral blood cell culture	1,25 $(OH)_2D_3$ added to the culture medium	Decreased synthesis of IL-2, IFN-y e TNF-α Inhibition of IL-12 synthesis
Induction of Th2 cell differentiation SKROBOT; DEMKOW; WACHOWSKA 2018 BOOSTRA et al., 2001	Knockout mice	?	↑ Synthesis of IL-4, IL-5 and IL-10 ↑ Transcriptional of GATA3
Reduced profile of Th17 cells IKEDA et al. 2010 JOSHI et al., 2011	Human peripheral blood cell culture Knockout mice (Tissue culture)	Intraperitoneal treatment with 1,25 $(OH)_2D_3$ 1,25 $(OH)_2D_3$ added to the culture medium	Inhibition of IL-17, IL-21 and IL-22 synthesis
Promotion of regulatory T cell differentiation URRY et al., 2012 KANG et al., 2012	Human peripheral blood cell culture Knockout mice (Tissue culture)	1,25 $(OH)_2D_3$ added to the culture medium	Increases the synthesis of IL-10 and the FoxP3 transcription factor
B cells CHEN et al., 2007	Human peripheral blood cell culture	1,25 $(OH)_2D_3$ added to the culture medium	Inhibits B cell maturation into plasma cells and memory ↓ Isotype change

Table 2. Immunomodulatory effect of vitamin D on autoimmune disease. The table presents the immunomodulatory action of vitamin D in different autoimmune diseases, the experimental model used to observe this effect and how vitamin D was administered.

Immunomodulation by Vitamin D in autoimmune disease

Disease / Source	Experimental Model	Treatment	Main Outcome
Multiple sclerosis SLOKA et al., 2011 CHAG et al., 2010 COSTA et al., 2016	Human peripheral blood cell culture Knockout mice (Tissue culture)	1,25 $(OH)_2D_3$ administered intraperitoneally and in mouse Water. 1,25 $(OH)_2D_3$ added to the culture medium	Th2 increase Decreased Th1, Th17, IFN-y and IL-17
Rheumatoid arthritis CANTORA & DeLUCA 1998 ZHOU et al., 2019	Knockout mice	Intraperitoneal treatment with 1,25 $(OH)_2D_3$ 1,25 $(OH)_2D_3$ introduced in the ration	Stopping disease progression in mice Decreased IL-17 and increased Tregs in mice
Systemic Lupus Erythematosus ABOU-RAYA[1]; ABOU-RAYA; HELMII 2013 PIANTONI et al., 2015	Measurement of serum calciferol levels in humans Human peripheral blood cell culture	Oral supplementation with cholecalciferol	↓ IL-8, IL-1, IL-6 and TNF-α ↑ Tregs
Inflammatory bowel disease DANIEL et al., 2008 BARTELS et al., 2007 CANTORNA et al., 2000	Human peripheral blood cell culture BALB/c mice	1,25 $(OH)_2D_3$ added to the culture medium Intraperitoneal treatment with 1,25 $(OH)_2D_3$	↑ IL-10, IL-4, TGF-β ↓ Th1, IFN-y and TNF-α
Airway Diseases PFEFFER & HAWRYLOWICZ 2018 BREHM et al., 2010 GUPTA et al., 2014 URRY et al., 2012 SUBRAMANINA; BERGMAN; NORMAK 2017	Serum dosage of $25(OH)D_3$ Asthmatic children Knockout mice	1,25 $(OH)_2D_3$ added to peripheral blood culture and to co-culture of neutrophils and pneumococcus	Low vitamin D levels are associated with severe asthma ↑ Tregs and IL-10 ↓ IgE

On the case of MS, its presumed protective effect will possibly happen in both, in the periphery and the CNS. As previously mentioned in this review, it has been suggested that the immune response against neuroantigens is initiated at the peripheral lymphoid organs. VitD could be already effective at this initial stage by modulating the differentiation and function of APCs. Is has been widely demonstrated that vitD affects

differentiation, maturation and function of DCs directing them to a tolerogenic profile (NAVARRO-BARRIUSO et al., 2019). These APCs will then modulate naïve TCD4+ lymphocytes towards a functional hyporresponsiveness. VitD-induced tolerogenic DCs are also capable to drive the differentiation of regulatory T cells. This findings were endorsed by FARIAS et al., (2013), demonstrating that the adoptive transference of vitD-induced IDO+ DCs, which are imature and tolerogenic, led to increased percentage of CD4+CD25+Foxp3+ regulatory T cells in the lymph nodes of rats with experimental autoimmune encephalomyelitis (EAE). T lymphocytes are another important target of vitD effects; it deceases both, their proliferation and differentiation. These effects are also been supported by using an animal model of MS. ZEITELHOFER et al., (2017), demonstrated that the protective effect detected in rats with EAE after vitD supplementation was associated with decreased proliferation of TCD4+ and lower frequency of Th17a cells. In a subsequent study, ZEITELHOFER et al., (2017) showed that these effects were associated with the downmodulation of various metabolic and signaling routes which are essential for Th1/Th17 polarization. This inhibition was concurrent with reduced DNA methylation and upregulation of many ncRNAs classes. The expected change from an inflammatory serum profile to an anti-inflammatory one upon vitD supplementation has not being entirely confirmed (MEHRABADI; ZAHEDI, 2021).

One of the critical stages in MS immunopathogenesis is the BBB breakdown which can be detected in patients by gadolinium enhanced MRI in the CNS (MARTÍN-AGUILAR et al., 2022). An effect at this level is expected considering that BBB endothelial cells express VDR. Our research team showed that calcitriol administration to a murine model of MS decreases neuroinflammation and reduces BBB dysruption (DE OLIVEIRA et al., 2020). Other experimental evidences have disclosed the molecular mechanisms underlying this protective effect. By using an in vitro system constituted by a human brain microvascular endothelial cell line (HBMEC)

stimulated with TNF-□ or sera from MS patients, TAKAHASHI et al., (2017), showed that vitD determined upregulation of tight junction proteins and downregulation of cell adhesion molecules. VitD can also downmodulates neuroinflammation by targeting additional CNS cells. It decreases, for example, the production of TNF-α, IL-1β and also the expression of IL-4 by astrocytes in vitro stimulated with LPS. This likely outcome was reproduced in neonatal rats injected with LPS (JIAO et al., 2017). Addition of vitD to stimulated microglial cells reduces the expression of Iba-1, MHC-II, CD86 and TLR-4 in vitro and in EAE (DE OLIVEIRA et al., 2020; GALOPPIN et al., 2022). GOMEZ-PINEDO et al., (2020), by using a model of demyelinaton in rats, described that VitD also operates at the level of remyelination. VitD promotes the proliferation and differentiation of neural stem cells and their migration to the lesion site where these cells subsequently differentiate into oligodendrocyte lineage cells and produce myelin basic protein. Lastly, the possibility that vitD is also acting through inflammasome inhibition must be considered. This system operates in the periphery during the initial induction/expansion of Th1/Th17 cells and also during the inflammatory process in the CNS. The demonstration that vitD negatively regulates the NLRP3 inflammasome via VDR signaling to effectively inhibit IL-1β secretion, by RAO et al., (2019), gives some support to this additional target of vitD protective in MS.

9.Experimental animal models to decipher the complex COVID-19 & MS interplay

Very relevant questions concerning the relationship between COVID-19 and MS had already been raised and partially answered by experts in the field. We believe, however, that experimental animal models could add more knowledge to the remaining gaps of this complex interplay between this new coronavirus and this autoimmune disease. Insightful and updated reviews

had been published regarding the most useful animal models to be applied to investigate these two pathologies (CONSTANTINESCU et al., 2011; FAN et al., 2022; MUÑOZ-FONTELA et al., 2020a; ROBINSON et al., 2014).

For the sake of objectivity, only the models that seem to be imediately available to investigate aspects related to these two pathologies will be briefly described. Syriam hamsters (Mesocricetus auratus) are widely used in the research of respiratory virus. In addition, their ACE2 receptor binds tighly to SARS-CoV-2 what makes then naturally susceptible to infection by this virus. The experimental transnasal inoculation of SARS-CoV-2 in 4–8-week-old hamsters triggers a very reproducible infection characterized by a short-term, self-limiting, epitheliotropic infection of the lungs and intestine with almost complete elimination of the virus before 14 days post infection. Details of these lesions that are very similar to the ones found in humans were described by (SHOU et al., 2021). This experimental disease can progress with different degrees of severity, depending upon the hamster strain (GRUBER et al., 2022). It was recently described by FRERE et al., (2022), that hamsters develop a condition that resembles very close the post-acute sequalae of COVID-19 also known as Long COVID. After virus clearance, these animals presented a clear inflammatory process in both, the olfactory bulb and the olfactory epithelium. This process included myeloid and T cells activation and production of pro-inflammatory cytokines, including IFN-y. Long COVID is a multi-organ disorder that can affect different organs and systems. Manifestations as fatigue, brain fog, headache, and cognitive disordes have being attributed to the involvement of the nervous system (STEFANOU et al., 2022). Even though not entirely understood, these symptoms are possibly linked to neuroinflammation and oxidative stress processes. A possible relation with Long COVID and neurological diseases, including MS, has been postulated. Data from a prospective and longitudinal observational study of the UK MS Register GARJANI et al., (2022), indicated a higher prevalence of long COVID in pwMS and also that those with higher levels of pre-COVID-19 neurological

impairment were at higher risk of long COVID. We believe that this is an interesting model to be explored in the context of these two diseases. The use of hamsters to model MS are scarce. However, older publications by MASSANARI (1981), showed that Syrian hamsters immunized with guinea pig spinal cord, in the presence of adjuvants, developed a chronic paralysis after 50-100 days, that was often relapsing. Seventy percent of these animals presented mononuclear cells infiltration and focal demyelination in the neuraxis. MASSANARI & WILSON (1983), also demonstrated that their susceptibility to develop EAE was highly dependent on the inbred strain, some developed acute paralysis around 10-21 days after immunization. Interesting, this model was already used to test the possible interference of a virus on EAE development. MASSANARI; PATERSON; LIPTON, (1979), showed that the persistence of a measles virus in the CNS exacerbated EAE manifestations.

Mice would be, for many reasons including the availability of reagents to perform immunological characterizations, the first choice for these investigations. However, wild type ACE2 does not bind adequately to the viral spike protein, rendering them resistant to the infection (WAN et al., 2020). Different strategies have been engendered to overcome this obstacle. We believe that transgenic mice expressing human ACE2 would be worthwhile to be tested, considering that COVID-19 severity could be controlled by the level of ACE2 expression MUÑOZ-FONTELA et al., (2020b) what could allow a more precise correlation with EAE aggravation. As will be further pointed to, these transgenic mice would need to have a C57BL/6 and SJL background to be also responsive to EAE induction. To the best of our knowledge, the suitability of ACE2 transgenic C57BL/6 and SJL mice to develop EAE was not tested yet. The most employed animal model for MS studies is experimental autoimmune encephalomyelitis (EAE). Murine EAE is usually induced by active immunization with myelin-derived peptides emulsified with complete Freund´s adjuvante in the presence of pertussis toxin. C57BL/6 and SJL/J mice strains immunized with specific

immunodominant peptides develop a chronic and a relapsing-remitting forms of the disease, respectively (PROCACCINI et al., 2015). EAE in mice is characterized by an ascending paralysis that starts by the tail, is followed by limb and forelimb paralysis and its clinical severity can be easily classified by a clinical score based in a 5-points scale RANGACHARI & KUCHROO, (2013), together with weight loss. The immunization of SJL/J mice with PLP139-151 can result in an initial paralytic attack, followed by multiple remissions and relapses whereas immunization of C57BL6/J mice with a high dose of MOG35-55 usually causes a chronic disease course in which an initial attack does not resolve.

Having in mind that most of COVID-19 pathogenesis is due to a hyperinflammatory reaction and that this process can affect MS, a few models of inflammation induction by virus antigens are also succinctly described. KHAN et al., (2021), observed that the spike protein is a strong inducer of inflammatory cytokines and chemokines, including IL-6, IL-1β, TNFα, CXCL1, CXCL2, and CCL2, but not IFNs, in human and mouse macrophages and lung epithelial cells. The potential of spike to induce inflammation was also showed in vivo by PUTHIA et al., (2022) by coadministration of aerosolized spike protein together with LPS to the lungs. This procedure triggered a severe pulmonary inflammation and a cytokine profile similar to that observed in COVID-19. The whole inactivated virus has also been employed to elicit inflammation. The intratracheal instillation of human ACE2-transgenic mice with formaldehyde-inactivated SARS-CoV-2 caused weight loss and pulmonary pathologic alterations as consolidation, hemorrhage, necrotic debris, and hyaline membrane formation. IL-1β, TNF-α, IL-6 and infiltration of of activated neutrophils, inflammatory monocyte-macrophages, and T cells were also detected in the the lungs (BI et al., 2021). We recently established a model of lung inflammation by intranasal instillation of inactivated SARS-CoV-2. This procedure triggered an exuberant inflammatory process composed of various cell types and mediators similar to lung inflammation associated with COVID-19. This

inflammatory process was significantly downmodulated by intranasal vitD administration, suggesting that this hormone has the potential to be an adjunct therapy for COVID-19 (Fernandes de Souza et al., 2022, submitted). In addition, considering our previous data that support a strong protective vitD effect on EAE development DE OLIVEIRA et al., (2020); GONÇALVES ZORZELLA-PEZAVENTO et al., (2022); MIMURA et al., (2021), we believe that IN vitD administration could downmodulate inflammatory reactions occurring simultaneously in the lungs and the CNS.

10.Conclusion

By focusing on aspects as inflammation, autoimmunity and CNS dysfunction, this review reinforces the existence of a strong relationship between COVID-19 and MS. It indicates that SARS-CoV-2 could, theoretically, affect MS development at different levels by employing several immunological mechanisms. In addition, this review highlights the fact that the emergence and severity of these two pathologies is highly associated with insufficient vitamin D levels and that its supplementation could be useful for their prevention and maybe therapy. Experimental disease models are also mentioned and discussed concerning their usefulness to complement the current and possible future uncertainties, bearing in mind that more severe neurological changes associated with long-term COVID are expected.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this review.

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MATERIAL COMPLEMENTAR

Efeito da imunização nas características clínicas da EAE

Como esperado, animais submetidos à indução de encefalomielite desenvolveram a paralisia típica da doença cujos escores clínicos atingiram o valor máximo em torno do vigésimo dia após a imunização com o autoantígeno. Também como previsto, o escore clínico diminuiu gradativamente à medida que a doença atingiu a fase crônica. A perda de peso que acompanha o desenvolvimento clínico da doença também foi evidente no grupo EAE. O grupo previamente imunizado com o vírus desenvolveu uma doença clínica muito similar à do grupo EAE mas estes animais não perderam tanto peso. Estes resultados são mostrados na figura 1.

Um experimento adicional confirmou a suspeita de que a imunização prévia com o vírus não só não agravava a EAE como parecia ter um efeito levemente protetor contra a doença. Este efeito protetor foi bastante discreto em relação ao score clínico mas mais acentuado quando se considerou a perda de peso corporal. Estes resultados são mostrados na figura 1.

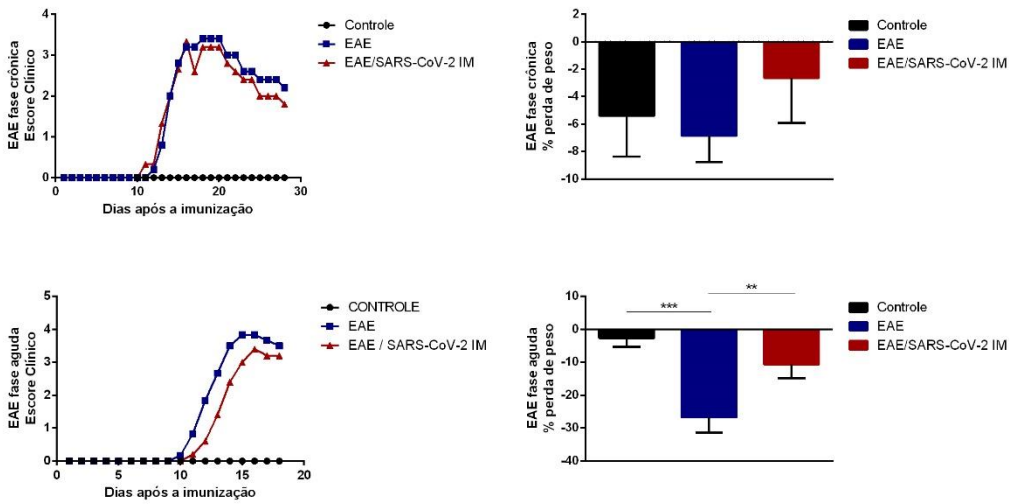
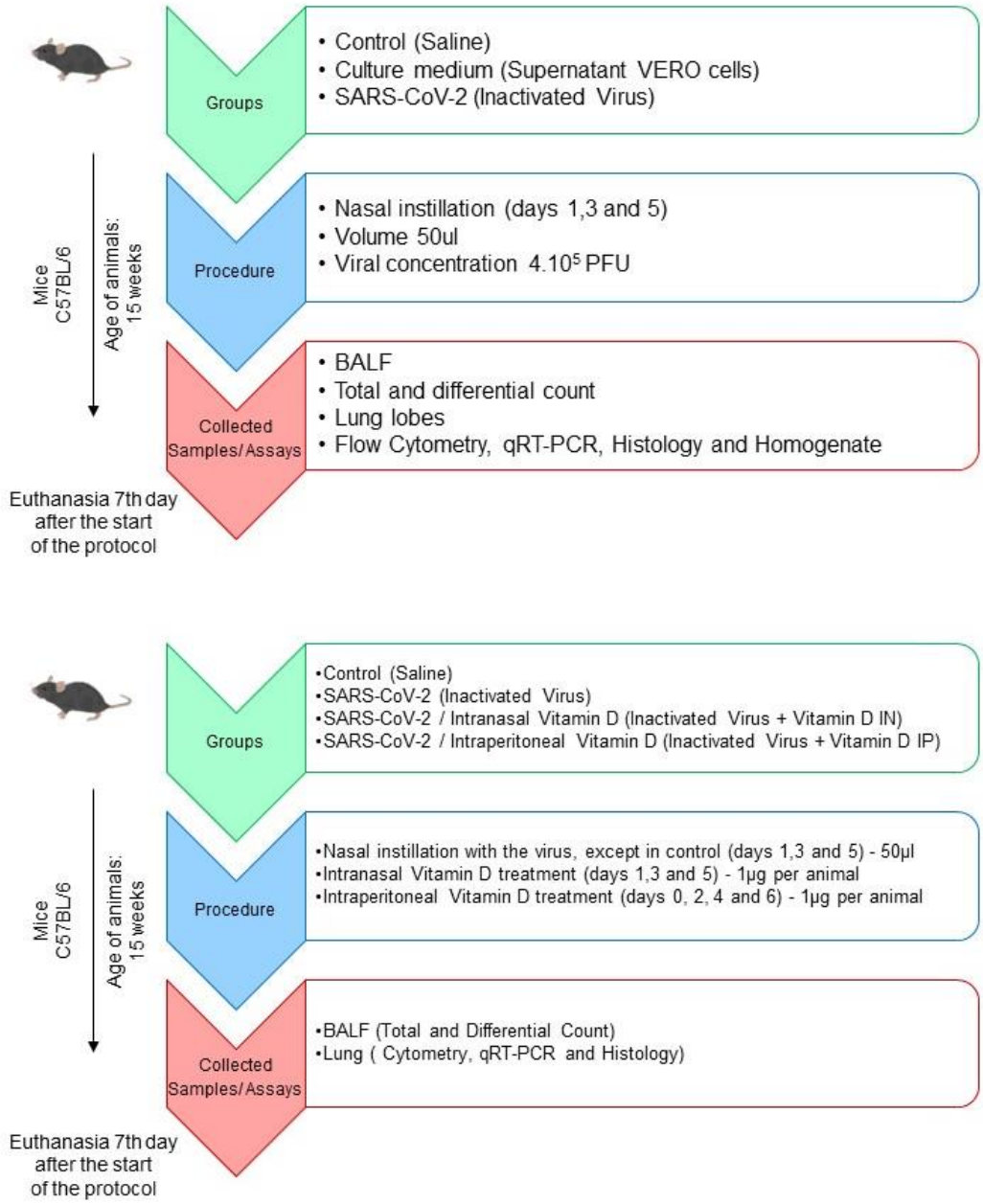
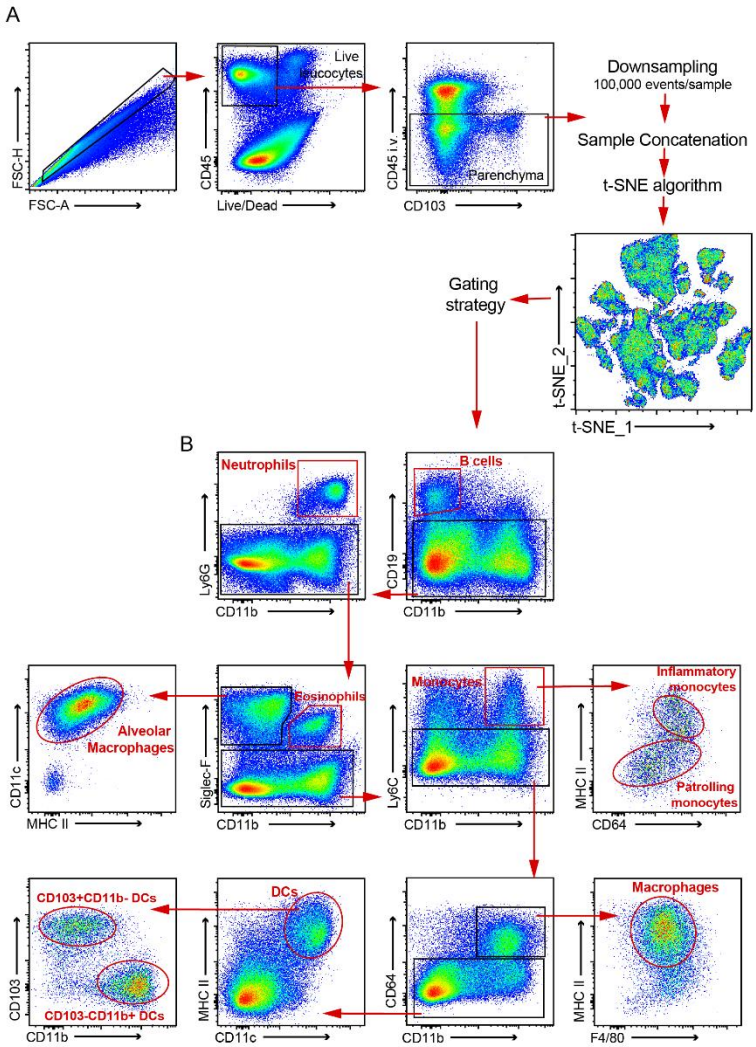


Figura 1: Efeito da imunização intramuscular com SARS-CoV2 nas características clínicas e na inflamação no SNC em camundongos com EAE. As figuras represantam a evolução clínica dos camundogos em relação ao peso (g) e escore clínico. A doença foi induzida por imunização subcutânea no dorso com 110 µg de MOG (Proteimax)

emulsificada com 50µL de Adjuvante Completo de Freund (Sigma) contendo *Mycobacterium tuberculosis* (2 mg/mL). No dia da imunização e 48 horas após, os animais foram inoculados com 180ng de toxina pertussis via intraperitoneal. A gravidade da doença foi definida segundo a seguinte escala: 0- normal; 1- cauda frouxa; 2- perda da tonicidade do movimento do quadril; 3- paralisia parcial das patas traseiras; 4- paralisia total das patas traseiras; 5- tetraplegia ou morte. Também foi avaliada a perda de peso corporal todos os dias durante o curso do experimento. Quanto ao virus os animais foram submetidos à imunização com 2 doses de SARS-CoV2 intraperitoneal associado ao alúmen. * P<0,05, ** P<0,01, *** P<0,001.

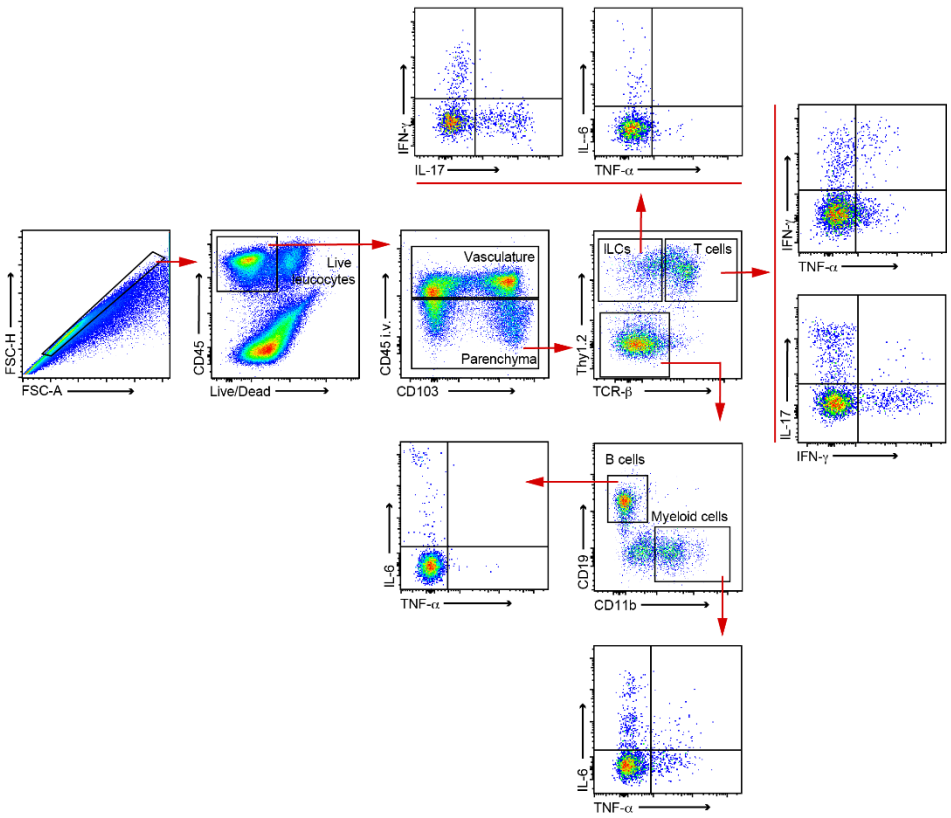


Supplementary Figure 1: General experimental design. Induction of lung inflammation by intranasal instillation of UV inactivated SARS-CoV-2 (A), evaluation of vitamin D therapeutic potential by its administration by intranasal and intraperitoneal delivery (B)

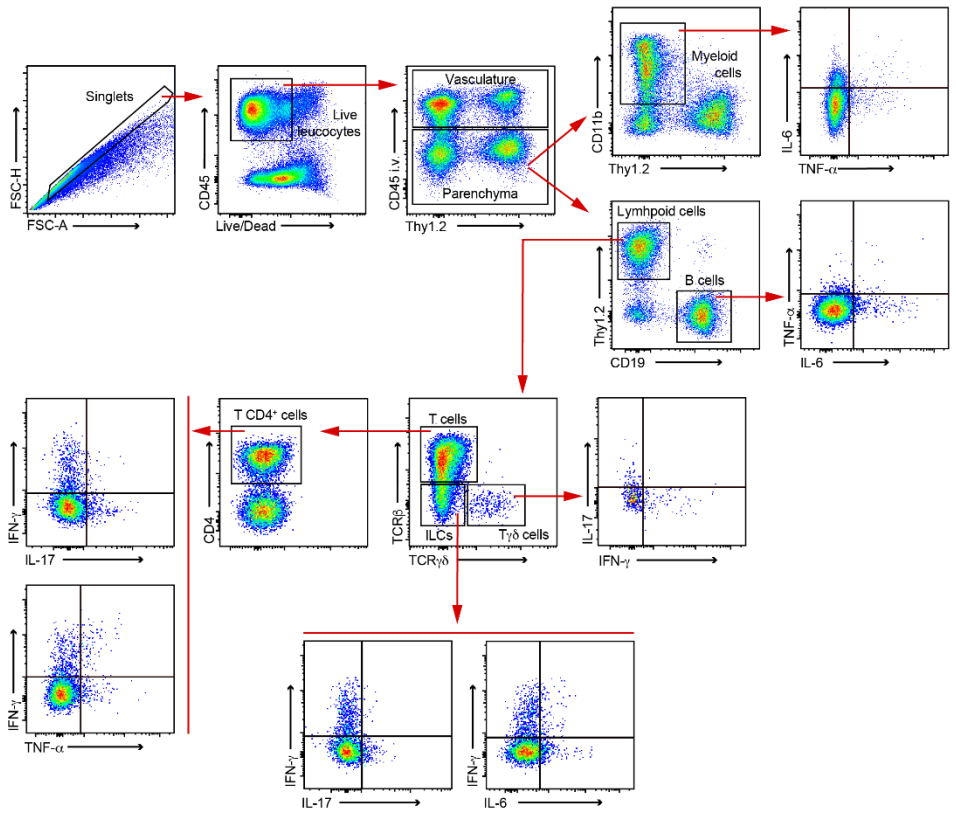


Supplementary Figure 2: Gate strategy and t-SNE analysis of cells isolated from the lung tissue of mice. Specific cell subsets infiltrating the lungs of mice were analyzed by flow cytometry. In order to differentiate the parenchyma-infiltrating leukocytes from the vasculature-associated fraction, mice were intravenously injected with FITC-labeled anti-CD45 antibody 3 min before euthanasia. (**A**) After doublets exclusion, total live (Live/Dead, LD negative) leukocytes were gated, and the vascular fraction of leukocytes was excluded from the analysis based on the anti-CD45 FITC intravenous (i.v.) staining. Next, 100,000 events from each sample were downsampled and concatenated in a file used for the t-SNE algorithm (2,000 interactions, 80 perplexity). (**B**) The cell subsets in each cluster were identified based on the following gate strategies: B cells ($CD45^+CD45i.v.^-CD19^+$), Neutrophils ($CD45^+CD45i.v.^-CD11b^+Ly6G^+$), Eosinophils ($CD45^+CD45i.v.^-CD11b^+Ly6G^-Siglec\text{-}F^+$), Alveolar Macrophages ($CD45^+CD45i.v.^-CD11b^-Ly6G^-Siglec\text{-}F^+CD11c^+MHCII^+$), Tissue-resident Macrophages ($CD45^+CD45i.v.^-CD11b^+Ly6G^-Siglec\text{-}F^-Ly6C^-$

$CD64^{+}F4/80^{+}MHCII^{+}$), Inflammatory Monocytes ($CD45^{+}CD45i.v.^{-}CD11b^{+}Ly6G^{-}Siglec\text{-}F^{-}Ly6C^{+}CD64^{+}MHCII^{+}$), Patrolling Monocytes ($CD45^{+}CD45i.v.^{-}CD11b^{+}Ly6G^{-}Siglec\text{-}F^{-}Ly6C^{+}MHCII^{-}$), Dendritic cells - DCs, ($CD45^{+}CD45i.v.^{-}CD11c^{+}MHCII^{+}CD19^{-}Ly6G^{-}Siglec\text{-}F^{-}Ly6C^{-}CD64^{-}F4/80^{-}CD103^{+/-}CD11b^{+/-}$) and respective DC subsets $CD11b^{+}$ and $CD103^{+}$.



Supplementary Figure 3: Gate strategy for cytokine detection by flow cytometry. Intracellular cytokine production was detected by flow cytometry in PMA/Ionomycin /brefeldin *in vitro* stimulated cells. After doublets exclusion, total live leukocytes were gated, and the vascular fraction of leukocytes was excluded from the analysis based on the anti-CD45 FITC staining (described in **Supplementary Figure 2**). The cell subsets in each cluster were identified based on the following gate strategies: Innate Lymphoid Cells (ILCs) ($CD45^+CD45i.v.^-Thy1.2^+TCRb^-CD11b^-$), T cells ($CD45^+CD45i.v.^-Thy1.2^+TCRb^+CD11b^-$), Myeloid cells ($CD45^+CD45i.v.^-Thy1.2^-TCRb^-CD11b^+$) and B cells ($CD45^+CD45i.v.^-CD19^+$).



Supplementary Figure 4: Gate strategy for cytokine detection by flow cytometry. Intracellular cytokine production was detected by flow cytometry in PMA/Ionomycin/brefeldin *in vitro* stimulated cells. After doublets exclusion, total live leukocytes were gated, and the vascular fraction of leukocytes was excluded from the analysis based on the anti-CD45 FITC staining (described in **Supplementary Figure 2**). The cell subsets in each cluster were identified based on the following gate strategies: Innate Lymphoid Cells (ILCs) ($CD45^+CD45i.v.^-Thy1.2^+TCRb^-TCRgd^-CD19^-$), $TCD4^+$ cells ($CD45^+CD45i.v.^-Thy1.2^+TCRb^+CD4^+$), Tgd cells ($CD45^+CD45i.v.^-Thy1.2^+TCRgd^+TCRb$) Myeloid cells ($CD45^+CD45i.v.^-Thy1.2^-$ $CD11b^+$) and B cells ($CD45^+CD45i.v.^-CD19^+Thy1.2$).