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**O Papel da ubiquitina ligase Marchf1
durante o desenvolvimento de
células dendríticas e na geração da
resposta imune antitumoral**

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LISTA DE ABREVIATURAS

APC – *Antigen presenting cell*; Célula apresentadora de antígeno

ATP – *Adenosine triphosphate*; Adenosina trifosfato

B16-OVA – *Murine metastatic melanoma cell line*; Linhagem de melanoma metastático murino que expressa ovalbumina

BM – *Bone marrow*; Medula óssea

CAR – *Chimeric antigen receptor*; Receptor quimérico de antígenos

CD – *Cluster of differentiation*; Grupo de diferenciação

cDC – *Conventional dendritic cell*; Célula dendrítica convencional

cDC1s – *Conventional dendritic cell type 1*; Célula dendrítica convencional do tipo 1

CDP – *Common DC progenitor*; Progenitor comum de células dendríticas

CMP – *Common myeloid progenitors*; Progenitor comum mielóide

CTLA-4 – *Cytotoxic T-lymphocyte associated protein 4*; Proteína citotóxica 4 associada a linfócitos T

DAMPs – *Damage-associated molecular pattern*; Moléculas associadas ao dano

DC – *Dendritic cell*; Células dendrítica

DC3 – *Dendritic cell type 3*; Células dendríticas não convencionais do tipo 3

dLN – *Draining lymph node*; Linfonodo drenante

ESCRT – *Endosomal sorting complexes required for transport*;

Flt3 – *fms-like tyrosine kinase 3*; Tirosina quinase do tipo fms 3

Flt3L – *fms-like tyrosine kinase 3 ligand*; Ligante tirosina quinase do tipo fms 3

GMP – *Granulocyte-macrophage progenitors*; Progenitor granulócitos-macrófagos

ICB – *Immune checkpoint blockade*; Inibidores de checkpoint imunológicos

IFN- γ – *Interferon gamma*; Interferon do tipo gama

mAbs – *Monoclonal antibodies*; Anticorpos monoclonais

Marchf – *E3-ligases associated ring-CH type finger*; E3-ligases associada a membrana

MC38 – *Murine colon adenocarcinoma cell line*; Linhagem celular de adenocarcinoma de cólon murino

MDP – *Macrophage/DC precursors*; Percursos de Macrófagos/Células Dendríticas

MHC – *Major histocompatibility complex*; Complexo principal de histocompatibilidade

moDC – *Dendritic cell derived monocyte*; Células dendríticas derivadas de monócitos

MST – *Median survival time*; Média de tempo de sobrevivência

PD-1 – *Programmed cell death protein 1*; Proteína de morte celular programada 1

pDC – *Plasmacytoid dendritic cells*; Células dendríticas plasmacitárias

PD-L1 - *Programmed death-ligand 1*; Ligante da proteína de morte celular programada 1

Poly-IC – *Polyinosinic:polycytidylic acid*; Ácido poliinosínico-policitidílico

PRR – *Pattern recognition receptors*; Receptores de reconhecido padrão

RING-E3 – *Really Interesting New Gene-E3*

TCR – *T-cell receptor*; Receptor de células T

Th2 – *T helper 2*; Resposta imune T do tipo 2

TIM-3 – *T-cell immunoglobulin and mucin domain 3*; Imunoglobulina com domínio mucina 3 de células T

TLR – *Toll-like receptors*; Receptores do tipo Toll

TME – *Tumor microenvironment*; Microambiente tumoral

TNF- α – *Tumor necrosis factor alpha*; Fator de necrose tumoral do tipo alfa

Treg – *Regulatory T cells*; Células T regulatórias

WT – *Wild-type*; Tipo Selvagem

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CONSIDERAÇÕES INICIAIS

Esta tese foi desenvolvida ao longo de 4 anos e 8 meses. O doutorado foi iniciado em janeiro de 2020, com o objetivo de investigar o envolvimento imunológico da proteína Marchf1 na progressão tumoral. No entanto, em março de 2020, a pandemia de COVID-19 foi declarada, e, por essa razão, o projeto teve que ser adiado. O laboratório da Prof.^a Cristina Bonorino, o LAIT, em colaboração com pediatras do Hospital Moinhos de Vento de Porto Alegre e financiamento do Ministério da Saúde, deu início a um novo estudo para compreender o perfil imunológico de crianças infectadas pelo SARS-CoV-2 em comparação com adultos, conhecido como projeto COVIDa. Esse projeto durou aproximadamente 2 anos e 6 meses, resultando em duas publicações científicas, nas quais dividi a primeira autoria (**Capítulo 3** e **Capítulo 4**).

O primeiro desses artigos, intitulado *"Pediatric COVID-19 patients in South Brazil show abundant viral mRNA and Strong specific anti-viral responses"*, foi publicado na revista *Nature Communications*. Nele, comparamos a resposta imunológica aguda de crianças infectadas com SARS-CoV-2 com a de adultos que apresentaram sintomas leves ou graves da doença (**Capítulo 3**). Observamos que, embora as crianças infectadas tivessem altos níveis de RNA viral e um perfil imune dominado por células *naïve* em comparação com os adultos, elas desenvolveram, como os adultos, uma forte resposta de anticorpos e células T específicas contra a proteína Spike (S) do vírus. Contudo, e principalmente, as crianças apresentaram uma resposta de células T CD8⁺TNF⁺ contra a proteína do nucleocapsídeo viral (N), que não era vista em adultos.

No segundo artigo, intitulado *"SARS-CoV-2 infected children form early immune memory responses dominated by nucleocapsid-specific CD8⁺ T cells and antibodies"*, publicado na *Frontiers in Immunology*, avaliamos a resposta imune de memória nas crianças, três e seis meses após a infecção inicial, com o objetivo de compreender a evolução dessa resposta (**Capítulo 4**). Observamos que já três meses após a infecção, as crianças desenvolveram memória imune para a proteína N, tanto de células T CD8⁺TNF⁺ quanto de anticorpos – diferentemente dos adultos, que focaram sua resposta de memória na proteína S. As crianças também desenvolveram resposta contra a proteína Spike. A resposta de anticorpos das crianças, entretanto, não foi capaz de neutralizar novas variantes do vírus circulantes na época, como Gama e Delta, sugerindo que a resposta

gerada pela infecção contra a variante original não protegeria completamente da possibilidade de reinfecção por essas cepas, evidenciando a necessidade de vacinação contra futuras infecções.

Após o término do projeto COVIDa, e da publicação destes dois artigos, decidimos retomar o projeto inicial sobre o papel da *Marchfl* na progressão tumoral. Com o apoio dos meus orientadores, Cristina Bonorino e Thiago Borges, e em colaboração com o Dr. Leonardo Riella, fui selecionada para realizar um estágio de 12 meses (setembro de 2022 a setembro de 2023) no *Center for Transplant Sciences*, no Massachusetts General Hospital (MGH)/Harvard Medical School, em Boston, EUA, com uma bolsa CAPES PDSE Doutorado Sanduíche. No MGH, utilizamos animais com deleção de *Marchfl* (*Marchfl^{-/-}*) e animais com deleção de *Marchfl* em células dendríticas (*CD11c^{Cre}Marchfl^{fl/fl}*). A maior parte dos resultados descritos nos **capítulos 1 e 2** desta tese foram obtidos durante esse período. O capítulo 1 compila o estudo que reúne o maior volume de dados, realizados com modelos tumorais; e o capítulo 2 foca em modelos de transplante.

Finalmente, além desses resultados, colaborei com o Dr. Borges em uma revisão publicada na *Transplantation Reviews* intitulada "*Inhibitory innate receptors and their potential role in transplantation*" (**Capítulo 5**). E contribuí em um segundo trabalho sobre transplantes intitulado "*Siglec-E is a Critical Inhibitory Receptor Controlling APC Activation and T Cell-Mediated Transplant Rejection*", atualmente sob revisão na *Science Translational Medicine* (**Capítulo 6**).

Nesta tese, optamos, portanto, por iniciar com os dados que ainda não foram publicados, relativos ao projeto de doutorado original; e organizamos os demais trabalhos já publicados em seguida.

RESUMO

Marchf1 ligases são enzimas da família das ubiquitina ligases E3, responsáveis por ubiquitinar proteínas e direcioná-las para a degradação pelo proteassomo. Marchf1 ubiquitina as moléculas MHC II e CD86, responsáveis pelos sinais 1 e 2 de ativação de células T *naive* pelas células apresentadoras de antígeno (APCs), como células dendríticas (DCs). Marchf1 tem um papel importante na regulação de respostas em modelos de asma e transplante, no entanto seu papel na regulação da resposta imune durante o crescimento tumoral ainda é desconhecido. Neste trabalho, investigamos o papel da Marchf1 nos modelos tumorais murinos de melanoma B16-OVA e adenocarcinoma de cólon MC38, usando animais geneticamente deficientes em Marchf1 (Marchf1^{-/-}). Observamos que a ausência de Marchf1 tanto no hospedeiro como especificamente em DCs (animais CD11c^{Cre}Marchf1^{fl/fl}) resultou em um maior crescimento tumoral nos dois modelos. Isso foi acompanhado por uma redução da resposta específica de células T CD8⁺ e de DCs convencionais do tipo 1 (cDC1s) no microambiente tumoral e em tecidos linfoides secundários. A resposta desses animais à imunoterapia com anticorpos anti-PD1 e anti-CTLA-4 também ficou diminuída. Uma investigação mais aprofundada revelou que a falta de Marchf1 nas cDC1s não altera sua capacidade de apresentar antígenos a células T específicas, nem resulta em diminuição da sua sobrevivência nos tecidos em comparação com os animais controle. Contudo, ao analisar o desenvolvimento das DCs em animais Marchf1^{-/-}, foi possível observar um menor número de precursores das cDCs (pré-cDCs) na medula óssea e nos tecidos linfoides secundários. O tratamento dos animais com a citocina Ftl3L resultou na restauração do controle do crescimento tumoral semelhante aos animais controles, bem como na recuperação da resposta T CD8⁺ efetora e de níveis normais de cDC1s. Nossos resultados indicam que o desenvolvimento normal de precursores de DCs na medula óssea, bem como a infiltração de cDC1s no microambiente tumoral é dependente de mecanismos que necessitam da expressão da ubiquitina ligase Marchf1 em DCs. E que ao menos alguns desses mecanismos podem ser restaurados com o tratamento com Ftl3L. Além do mais, observamos resultados similares em outro modelo dependente de células T CD8⁺, o modelo de transplante de coração. Portanto, podemos concluir que Marchf1 é necessária para a indução de uma resposta imune efetora funcional.

Palavras-chaves: Marchf1, células dendríticas, MHC II, cDC1s, tumor

ABSTRACT

Marchf ligases are enzymes belonging to the E3 ubiquitin ligase family, responsible for ubiquitinating proteins and directing them to proteasomal degradation. Marchf1 ubiquitinates MHC II molecules and CD86, which are crucial for the activation of naive T cells by antigen-presenting cells (APCs), such as dendritic cells (DCs). While Marchf1 plays an important role in regulating responses in asthma and transplant models, its role in immune regulation during tumor growth remains unknown. In this study, we investigated the role of Marchf1 in murine tumor models of B16-OVA melanoma and MC38 colon adenocarcinoma, using animals genetically deficient in Marchf1 (Marchf1^{-/-}). We observed that the absence of Marchf1, both in the host and specifically in DCs (CD11c^{Cre}Marchf1^{fl/fl} animals), resulted in increased tumor growth in both models. This was accompanied by a reduction in CD8⁺ T cell responses and conventional type 1 DCs (cDC1s) in the tumor microenvironment and secondary lymphoid tissues. The response of these animals to immunotherapy with anti-PD1 and anti-CTLA-4 antibodies was also diminished. Further investigation revealed that the lack of Marchf1 in cDC1s did not alter their capacity to present antigens to specific T cells, nor did it result in decreased survival in tissues compared to control animals. However, analysis of DC development in Marchf1^{-/-} animals showed a reduced number of cDC1 precursors (pre-cDCs) in the bone marrow and secondary lymphoid tissues. Treatment of these animals with the cytokine Flt3L restored tumor growth control to levels similar to control animals, as well as the recovery of CD8⁺ effector T cell responses and normal cDC1 levels. Our results indicate that the normal development of DC precursors in the bone marrow and the infiltration of cDC1s into the tumor microenvironment depend on mechanisms requiring Marchf1 ubiquitin ligase expression in DCs. Moreover, we observed similar results in another CD8⁺ T cell-dependent model, the heart transplant model. Therefore, we can conclude that Marchf1 is required for the induction of a functional effector immune response.

Keywords: Marchf1, dendritic cells, MHC II, cDC1s, tumor

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1. Introdução

1.1 Imunologia tumoral

A hipótese da vigilância imunológica, formulada por Burnet e Thomas, (1), propõe que o sistema imune patrulha o organismo à procura de células transformadas, sendo capaz de reconhecê-las e destruí-las. Dessa forma, o crescimento de um tumor seria um evento raro, proveniente de células que escaparam dessa vigilância, sugerindo assim a importância do sistema imune na biologia das neoplasias. No entanto, há situações em que o próprio sistema imune acaba favorecendo o crescimento tumoral, por ser fonte de fatores de crescimento, produtos oxidativos, e reguladores de angiogênese (Ribatti; Crivellato, 2009). Um fator importante para criar um microambiente favorável para a formação e crescimento tumoral é o rearranjo de componentes celulares através de uma interação dinâmica e mútua (Hanahan; Coussens, 2012a). Este microambiente tumoral é composto por células tumorais, células estromais do tumor que inclui fibroblastos, células endoteliais, células imunológicas e de componentes não celulares da matriz extracelular (Fridman *et al.*, 2012; Hanahan; Weinberg, 2011). Como principal elemento deste microambiente, as células tumorais controlam a função dos componentes celulares e não celulares através de redes de sinalização para estimular as células não-malignas a trabalharem em seu benefício. As consequências dessa interação são refletidas na formação e manutenção tumoral, assim como no auxílio da deficiência nas respostas a terapias ou resistência a multidroga. As células não-malignas no microambiente tumoral são conhecidas como favorecedoras da tumorigênese em todas as fases do desenvolvimento do câncer e na metástase (Hanahan; Coussens, 2012b). Uma forma de modular as células imunes de um perfil imunossupressor para um perfil de imuno ativação e efector contra o tumor é através do uso de terapias que restauram e aprimoram as atividades antitumorais dessas células.

Muitas terapias atualmente são focadas em restabelecer os mecanismos antitumorais operados por células T, fazendo com que elas reconheçam o tecido tumoral como diferente de células normais, e exerçam uma resposta citotóxica (Sun *et al.*, 2023). Um dos principais exemplos é a terapia com inibidores de *checkpoints* imunológicos (ICIs) (Figura 1). Os *checkpoints* imunes são essenciais na regulação da imunidade evitando a exacerbação de respostas imunes contra os próprios tecidos saudáveis, no entanto podem ser subvertidos em pacientes com câncer, dessa forma contribuindo para o crescimento tumoral. A remoção destes bloqueios no microambiente tumoral é uma

estratégia para intensificar a resposta imunológica, através da ativação de células T CD8⁺ (Reiss; Forde; Brahmer, 2014; Schwabe; Jobin, 2013). Hoje, há uma série de anticorpos monoclonais (mAbs) utilizados na modulação da ativação e tolerância imunes, que bloqueiam interações de moléculas co-inibitórias com seus ligantes, constituindo uma área extremamente eficaz de tratamento do câncer (Kothari *et al.*, 2024).

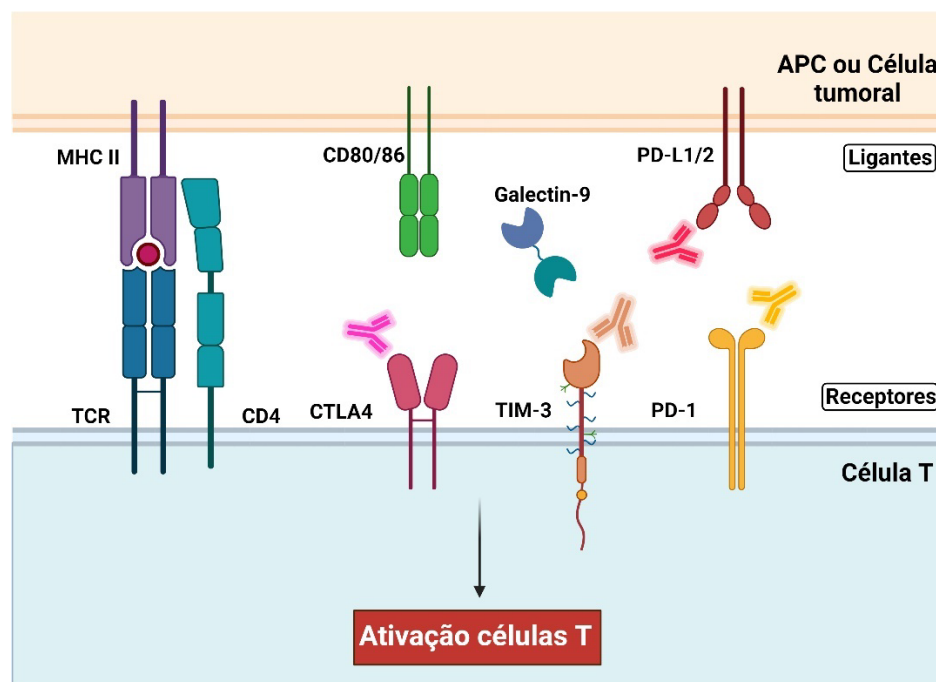


Figura 1. Bloqueio de *checkpoints* imunológicos inibitórios em células T utilizando anticorpos monoclonais específicos. Anticorpos específicos bloqueiam a ligação dos receptores *checkpoint* PD-1, CTLA4 e TIM-3 com os seus respectivos ligantes PD-L1/2, CD80/86 e Galectina-9. O bloqueio dessa interação favorece os sinais estimulatórios da sinapse imune e resulta na ativação das células T, restabelecendo atividades efetoras contra o tumor dessas células. *Criado no Biorender.com*

Outro exemplo de imunoterapia é a transferência adotiva de células T modificadas para expressão de receptores quiméricos que reconhecem antígenos tumorais, conhecida como células CAR (*chimeric antigen receptor*) T. De forma breve, as células T dos pacientes são isoladas e geneticamente modificadas para expressar os CARs, expandidas *in vitro* e transferidas de volta ao paciente (Garrido *et al.*, 2016). Essa terapia independe do reconhecimento de moléculas de histocompatibilidade (MHC) pelos linfócitos T nas

células tumorais, pois o receptor quimérico é em parte um anticorpo contra um marcador tumoral.

Além das terapias direcionadas às células T, a imunoterapia também explora outras populações imunológicas, como as células dendríticas (DCs). As DCs, reconhecidas por sua potente capacidade de apresentação de antígenos, são investigadas como vacinas que induzem respostas imunes robustas contra neoantígenos tumorais (Barbuto *et al.*, 2004; Constantino *et al.*, 2016). Essa estratégia envolve a geração *ex vivo* de DCs derivadas de monócitos do sangue periférico de pacientes, que são expostas a antígenos tumorais e ativadas com ligantes específicos e citocinas, para, posteriormente, serem reinfundidas nos pacientes, visando a indução de uma resposta imunológica antitumoral eficiente. (Constantino *et al.*, 2016). Outra estratégia terapêutica visa estimular o aumento do número de DCs em pacientes com câncer por meio do uso do ligante da tirosina quinase *fms* (Flt3), o Flt3L. O Flt3 é uma proteína de superfície expressa em altos níveis nas células-tronco hematopoéticas, e a ligação de Flt3L a Flt3 promove a expansão dessas células, resultando em um aumento no número de DC (Cueto; Sancho, 2021). Essa terapia tem sido explorada em combinação com radioterapia e inibidores de *checkpoints* imunológicos (ICIs) (Brody, 2024), buscando potencializar a resposta imunológica antitumoral e melhorar os resultados clínicos dos pacientes. Essas terapias imunológicas, ao modular diferentes componentes do sistema imunológico, apresentam-se hoje entre as principais abordagens na oncologia, destacando a relevância do microambiente tumoral para a eficácia terapêutica e abrindo constantemente novas perspectivas para o tratamento de neoplasias.

1.2 Células dendríticas e geração de respostas T

Originalmente descritas por Ralph Steinman e Zanvil Cohn em 1973, as células dendríticas (DCs) desempenham um papel crítico na mediação das respostas imunes inatas e na indução de respostas imunes adaptativas. Desde essa descoberta, as DCs têm sido referenciadas como "adjuvantes naturais", reconhecidas como as células apresentadoras de antígenos (APCs) mais potentes, capazes de ativar tanto respostas imunes *naïve* quanto de memória. As DCs constituem uma população heterogênea de leucócitos derivados da medula óssea, distribuídos por diversos tecidos. Essas células são especializadas em adquirir, transportar, processar e apresentar antígenos às células T, bem

como fornecer a elas os sinais de ativação e diferenciação. Desempenham, assim, um papel central na coordenação da resposta imunológica adaptativa (Shortman; Liu, 2002).

Para induzir uma resposta imune adaptativa eficaz, envolvendo células T CD4⁺ e CD8⁺ contra tumores (**Figura 2**), as DCs capturam e processam material tumoral presente no microambiente tumoral (*tumor microenvironment* - TME). Posteriormente, essas células apresentam antígenos tumorais em moléculas do complexo maior de histocompatibilidade (MHC) de classe I ou II em sua superfície, formando um complexo peptídeo, conhecido como sinal 1 da ativação das células T. As DCs, por meio de receptores especializados, como os receptores de reconhecimento padrão (PRRs), são capazes de detectar sinais de dano no TME. A ativação dos PRRs desencadeia a ativação das DCs e induz a expressão de moléculas coestimuladoras como CD80 e CD86, caracterizando o sinal 2, ou inibitórias, como o PD-L1 (Chen; Flies, 2013).

Durante o processo de maturação, as DCs migram do TME para os linfonodos drenantes, guiadas por quimiocinas e citocinas. Nos linfonodos, ocorre a sensibilização (o *priming*) inicial das células T *naïve*, em que as DCs precisam fornecer a combinação do sinal 1 e sinal 2, juntamente com a secreção de citocinas, que constituem o sinal 3. Esse sinal 3 orienta a polarização das células T em diferentes subtipos. Caso algum desses sinais seja interrompido, as células T podem não ser ativadas de forma completa, resultando em um estado de anergia ou até em apoptose (Chen; Flies, 2013). As células tumorais presentes no TME podem inibir as funções das DCs, modulando sua ativação para favorecer a imunossupressão e a tolerância. Nessa condição, as DCs podem impedir ativamente a imunidade anticâncer e induzir uma resposta das células T que favoreça o crescimento tumoral (Bandola-Simon; Roche, 2019; Wculek *et al.*, 2020; Xiao *et al.*, 2023).

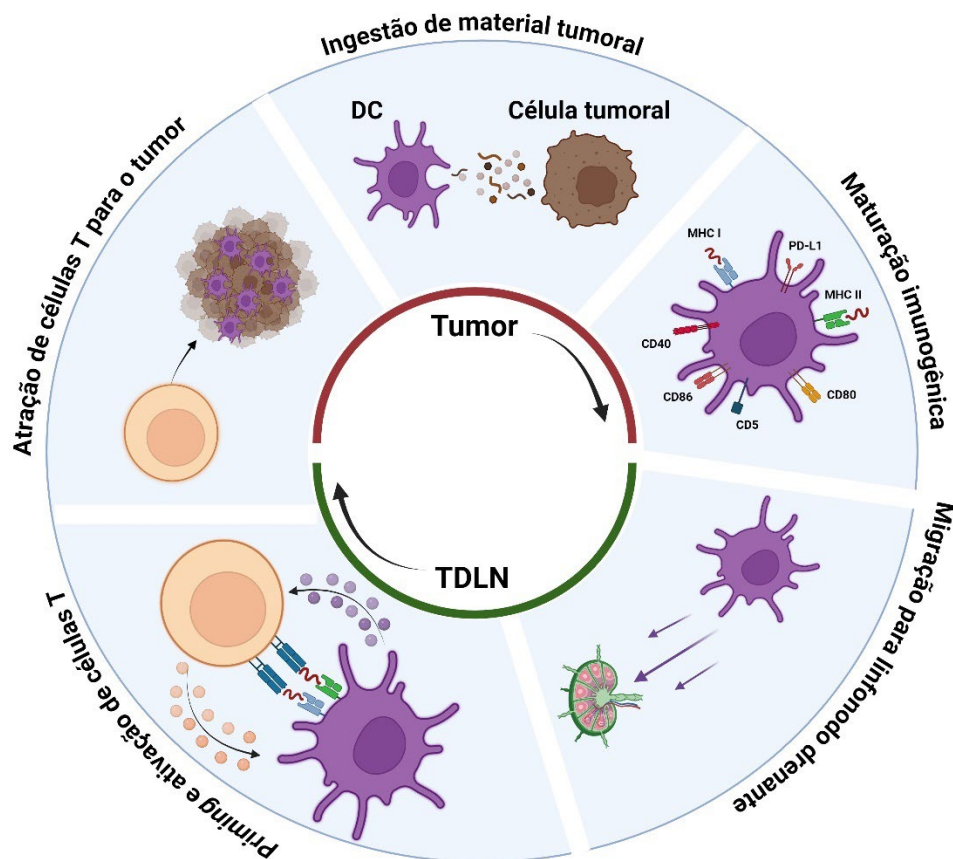


Figura 2. Processo de maturação das DCs e ativação da resposta de células T contra o tumor.

As células dendríticas (DCs) no microambiente tumoral (TME) desempenham um papel crucial na imunidade antitumoral. Elas capturam antígenos tumorais, detectam sinais de perigo, processam os antígenos para apresentação e sofrem ativação imunogênica antes de migrar para os linfonodos drenantes do tumor (TDLN). No TDLN, as DCs apresentam os antígenos às células T *naïve*, juntamente com sinais coestimulatórios e citocinas solúveis. Após a ativação das células T, as DCs podem secretar fatores que atraem essas células T de volta ao tumor, promovendo uma resposta imune direcionada. Adaptado de Heras-Murillo et al e criado no Biorender.com

A complexidade nos subtipos de DCs é grande, sendo esses subtipos diferenciados com base na expressão diferencial de marcadores de superfície, na sua localização anatômica e nas funções que desempenham (Heath; Carbone, 2009) (**Figura 3**). As DCs convencionais (*conventional DCs*, cDCs) se desenvolvem de progenitores comuns de DCs (CDPs) na medula óssea e são subdivididas em duas grandes categorias, cDC1s e cDC2s (Cabeza-Cabrero *et al.*, 2021; Nakamizo *et al.*, 2021; Villani *et al.*, 2017). As cDC1s são funcionalmente homogêneas, caracterizadas pela expressão de XCR1 e CLEC9A em humanos e murinos, e induzem resposta T CD8⁺ a

antígenos extracelulares através da apresentação cruzada de pelo MHC I (Böttcher; Reis e Sousa, 2018). Já a população de cDC2s é heterogênea, com presença de diversos subgrupos identificados com base na expressão de fatores de transcrição específicos. De acordo com essa classificação, as cDC2s podem ser subdivididas com base na expressão de Notch (Villani *et al.*, 2017), KLF4 (Fernandes Rodrigues *et al.*, 2023), ou na presença do T-bet, resultando nas subpopulações T-bet⁺ cDC2sAs e T-bet⁻ cDC2sBs (Brown *et al.*, 2019). As cDC2s são consideradas boas indutoras de respostas T CD4⁺ apresentando antígenos extracelulares pela via clássica no MHC II (Kvedaraite; Ginhoux, 2022).

Além dessas subpopulações convencionais, também é possível identificar as DCs não convencionais do tipo 3 (DC3), que se distinguem das cDC1s e cDC2s tanto a nível fenotípico como ontogenético. Ao contrário das cDCs, que se desenvolvem a partir das progenitoras de DCs (CDP), as DC3s originam-se de progenitores de DCs e monócitos (MDP) expressando o marcador Ly6C⁺ (Liu *et al.*, 2023; Nakamizo *et al.*, 2021). Funcionalmente, as DC3s são capazes de sensibilizar células T *naïve* e polarizar células T CD8⁺ em células T CD8⁺ residentes de memória (Bourdely *et al.*, 2020). Outra subpopulação importante inclui as DCs plasmocitóides (pDCs), que possuem uma origem dual, tanto mieloide (MDP) quanto linfóide (CLP) (Sathe *et al.*, 2013). Embora as pDCs tenham capacidade limitada de apresentar antígenos, sua principal característica é a produção rápida e abundante de interferon tipo I (IFN α/β), especialmente em resposta a infecções virais (Krug *et al.*, 2001; Swiecki *et al.*, 2010). As DCs derivadas de monócitos (moDCs), apesar de se originarem de forma distinta das cDCs e pDCs, compartilham características fenotípicas e funcionais com essas células, sendo capazes de apresentar antígenos tanto para células T CD8⁺ quanto para células T CD4⁺, e de induzir respostas do tipo Th2 e Th17 (Schlitzer; McGovern; Ginhoux, 2015). Além disso, as células de Langerhans representam uma população fenotipicamente distinta de DCs, originando-se de progenitores embrionários e residindo nas camadas basais de tecidos epiteliais, como a epiderme, onde desempenham um papel crucial na vigilância imunológica (Merad; Ginhoux; Collin, 2008). Recentemente, foi descrita uma nova subpopulação denominada DC transicional (tDC), que apresenta um fenótipo intermediário entre pDCs e cDC2s, funcionalmente caracterizada tanto em camundongos quanto em humanos (Sulczewski *et al.*, 2023; Villani *et al.*, 2017).

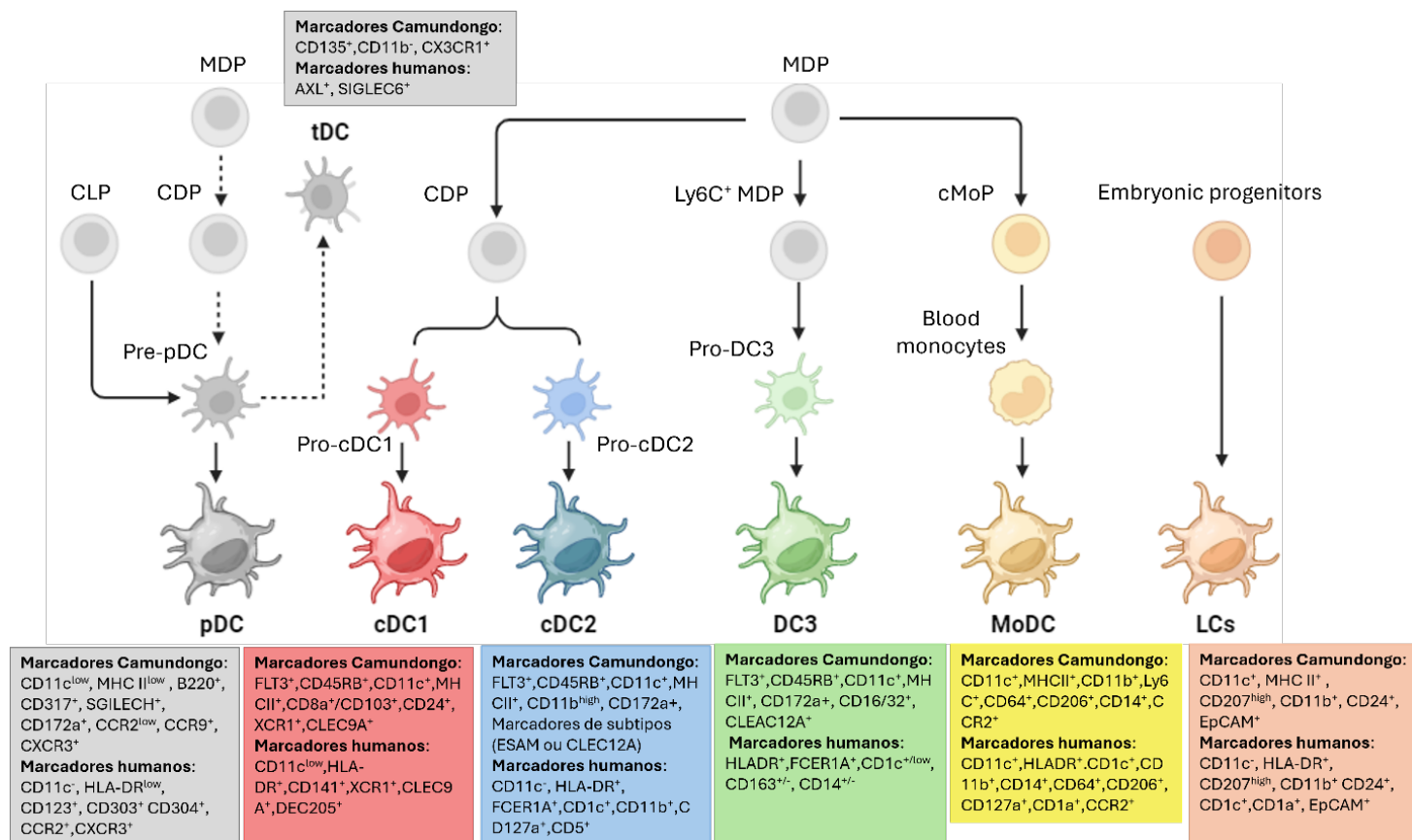


Figura 3. Desenvolvimento e heterogeneidade das DCs. Representação dos tipos de células dendríticas (DCs), suas origens celulares e os marcadores específicos de superfície utilizados para sua identificação em camundongos e humanos. As DCs plasmocitóides (pDCs) derivam de progenitores comuns linfoides (CLPs) ou, alternativamente, se diferenciam a partir de progenitores comuns de DCs (CDPs) — uma origem compartilhada com as DCs convencionais tipo 1 (cDC1s) e tipo 2 (cDC2s). As DCs transicionais (tDCs) compartilham a mesma origem com as pDCs e apresentam um fenótipo que abrange características tanto de pDCs quanto de cDC2s. As cDC1s e cDC2s desenvolvem-se a partir de pré-cDC1s e pré-cDC2s, respectivamente, que se diferenciam dos CDPs antes de migrarem da medula óssea. As DCs tipo 3 (DC3s) diferenciam-se a partir de pro-DC3s, que, assim como os CDPs, derivam de progenitores de DCs de monócitos (MDPs). As DCs derivadas de monócitos sanguíneos clássicos (MoDCs) também se originam de MDPs, mas através de progenitores de monócitos comuns (cMoPs). As células de Langerhans (LCs), apesar de residirem na epiderme como macrófagos autossustentáveis, são tradicionalmente incluídas na família das DCs devido à sua funcionalidade similar. Adaptado de Heras-Murillo et al

Em humanos, a relevância das DCs no combate a tumores é evidenciada pela correlação entre sua abundância no microambiente tumoral e um prognóstico favorável,

além de uma melhor resposta aos ICIs (Marciscano; Anandasabapathy, 2021; Wculek *et al.*, 2020). Avanços tecnológicos têm proporcionado conhecimentos significativos sobre o estado funcional das DCs em diferentes tipos de câncer, expandindo a compreensão sobre a diversidade dos papéis desempenhados pelos subtipos de DCs na doença. Esse acúmulo de conhecimento destaca o potencial das DCs como alvos terapêuticos ou como agentes preditores de resposta à imunoterapia. Entre os subtipos de DCs, as cDC1s são reconhecidas como potentes indutoras da imunidade antitumoral, sendo mais eficazes no transporte de antígenos tumorais para os linfonodos drenantes do que outros subtipos de DCs (Barry *et al.*, 2018; Böttcher *et al.*, 2018; Broz *et al.*, 2014; Iwanowycz *et al.*, 2021; Roberts *et al.*, 2016). A depleção de cDC1s em camundongos deficientes em Batf3 comprometeu a capacidade dos animais de rejeitar tumores imunogênicos transplantáveis e resultou em uma redução das respostas mediadas por células T à imunoterapia tumoral, incluindo tratamentos com ICIs (Liu *et al.*, 2019). Além disso, foi observada uma correlação positiva entre a densidade de cDC1s no tecido e a resposta a terapias, bem como a sobrevivência de pacientes em diferentes tipos de tumores (Böttcher; Reis e Sousa, 2018). No entanto, cDC2s também desempenham um papel importante na geração de respostas imunes antitumorais, embora tais mecanismos específicos de ação ainda sejam menos compreendidos em comparação com as cDC1s, provavelmente devido à heterogeneidade dessa subpopulação bem como à dificuldade de realizar uma identificação definitiva com base nos marcadores definidos atualmente (Murphy; Murphy, 2022; Wculek *et al.*, 2020).

Um aspecto fundamental no controle das respostas imunológicas envolve modificações pós-traducionais em moléculas chave que regulam as funções das DCs. A ubiquitinação, por exemplo, é um mecanismo regulador que pode modular a funcionalidade dessas células, afetando a expressão das moléculas de MHC II e CD86, correspondentes aos sinais 1 e 2 na ativação das células T.

1.3 Ubiquitinação

A ubiquitinação é uma modificação pós-traducional que exerce um papel crucial na regulação da estabilidade, localização celular e atividade biológica de proteínas transmembranas, impactando diretamente o controle das respostas imunológicas (Ishido *et al.*, 2009). Esse processo envolve a adição de uma ou mais moléculas de ubiquitina, uma pequena proteína composta por 76 resíduos de aminoácidos, que pode levar a várias

consequências biológicas, como a degradação proteica pelo proteassoma, progressão do ciclo celular, regulação da transcrição, reparo de DNA e transdução de sinais.

O processo de ubiquitinação ocorre de maneira sequencial e é mediado por três tipos de enzimas: a enzima ubiquitina-ativadora E1, a enzima ubiquitina-conjugadora E2 e a ubiquitina-ligase E3 (**Figura 4**) (Komander, 2009). Inicialmente, a enzima E1 ativa a molécula de ubiquitina em uma reação dependente de ATP. Em seguida, a ubiquitina ativada é transferida da E1 para a enzima E2. Na etapa final, a ubiquitina é conjugada à proteína substrato por meio da enzima E3, que atua como uma plataforma de ligação, facilitando a interação entre a E2 conjugada à ubiquitina e o substrato alvo (Myung; Kim; Crews, 2001).

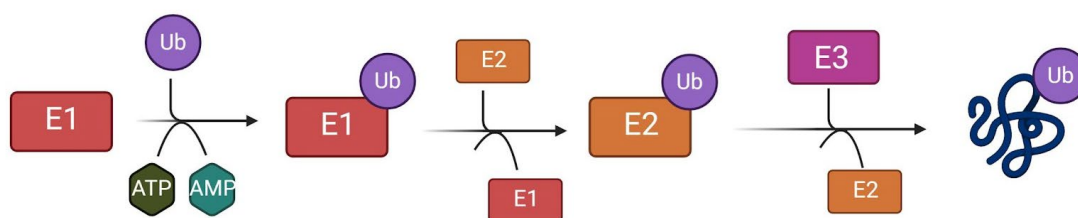


Figura 4. Via da ubiquitinação-proteassomo. O processo de ubiquitinação envolve a transferência sequencial da molécula de ubiquitina, começando pela enzima E1-ubiquitina-ativadora, seguida pela E2-ubiquitina-conjugadora, e, finalmente, pela E3-ubiquitina-ligase, que reconhece e conjuga a ubiquitina à proteína substrato alvo. Criado com BioRender.com

As enzimas E3 ligases representam o componente final na cascata de ubiquitinação E1-E2-E3, desempenhando o papel crítico de transferir a ubiquitina do complexo E2-ubiquitina para a proteína substrato específica. Essas enzimas podem ser agrupadas em três grandes famílias: *E6-AP carboxyl terminus* (HECT-E3), *RING-between-RING* (RBR-E3) e *Really Interesting New Gene-E3* (RING-E3) ligases (Morokuma *et al.*, 2007). A família RING-E3 é a maior entre as E3 ligases, com mais de 600 enzimas caracterizadas em humanos, e é capaz de mediar a transferência da ubiquitina diretamente da enzima E2 para a proteína substrato (Nathan *et al.*, 2008). Essa família é caracterizada pela presença de um domínio RING, que contém oito resíduos de cisteínas e histidinas coordenadas com dois átomos de zinco no interior da proteína (Deshaies; Joazeiro, 2009). As principais funções das RING-E3 incluem fornecer uma estrutura que permite a ligação simultânea da E2 e da proteína substrato e induzir uma conformação fechada da E2-ubiquitina, que é preparada para o ataque nucleofílico do

substrato (Flach *et al.*, 2014). Dessa forma, as E3 ligases conferem especificidade à via de ubiquitinação, sendo potenciais alvos primários para o desenvolvimento de fármacos (Jabbour *et al.*, 2009). Nesta tese, será explorada uma subfamília específica de E3 ligases, as ligases associadas à membrana com domínio RING-CH, conhecidas como Marchf ligases.

1.4 Marchf ligases

As ligases E3 associadas à membrana, conhecidas como ligases Marchf, foram inicialmente identificadas em vírus como imunoevasinas capazes de reduzir a expressão das moléculas do Complexo Principal de Histocompatibilidade de Classe I (MHC I) (Bartee *et al.*, 2004; Herr *et al.*, 2012; Stevenson *et al.*, 2000). Através de análises estruturais, foram identificados 11 homólogos contendo um domínio RING-CH, denominados Marchf1 a Marchf11 (Bartee *et al.*, 2004; Jahnke; Trowsdale; Kelly, 2012). Todas as Marchfs, exceto Marchf7 e Marchf10, possuem domínios transmembrana, sendo organizadas estruturalmente com um domínio RING-CH N-terminal seguido por dois ou mais domínios transmembrana (**Figura 5**). Esses domínios desempenham um papel crucial no reconhecimento de substratos, interagindo com as regiões transmembrana das proteínas-alvo (Bartee *et al.*, 2004; Jahnke; Trowsdale; Kelly, 2012). Análises filogenéticas permitiram dividir as Marchfs em cinco grupos: Marchf1 junto com Marchf8, Marchf2 com Marchf3, Marchf5 com Marchf6, Marchf7 com Marchf10, e Marchf4 junto com Marchf9 e Marchf11 (Bauer *et al.*, 2017).

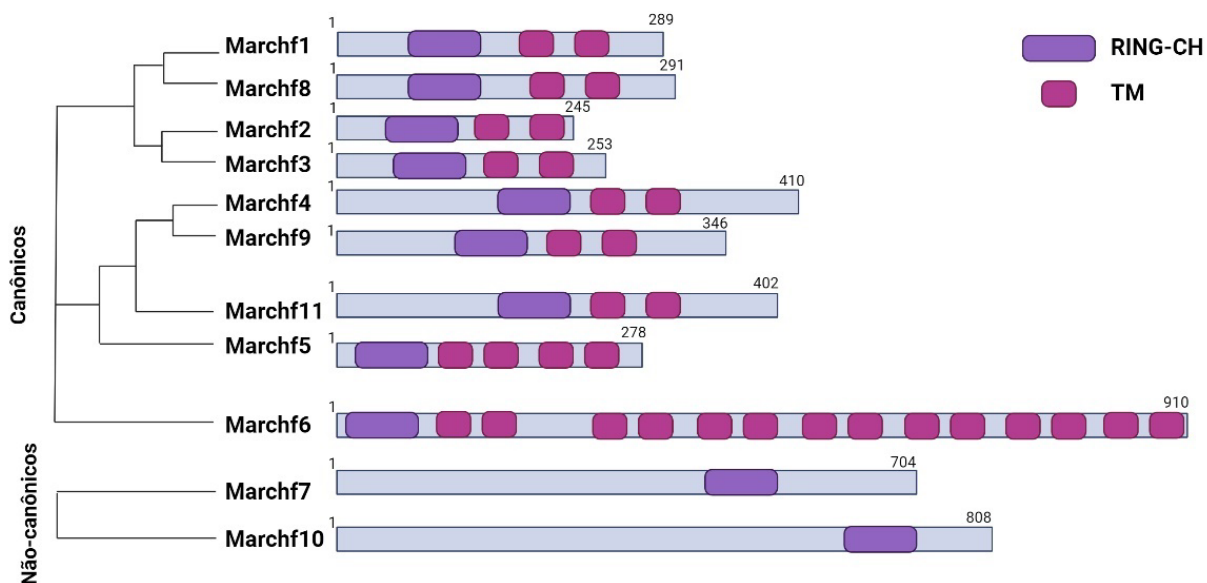


Figura 5. Estrutura das Marchf E3 ligases. Distribuição filogenética das ligases Marchf1, baseada em sua estrutura. RING-CH: domínio RING-CH; TM: domínio transmembrana. Criado no BioRender.com adaptado de Lin et al (Lin; Li; Shu, 2019).

Um dos principais papéis das proteínas Marchf é a regulação das respostas imunológicas, catalisando a poliubiquitinação de receptores imunes (Shortman; Liu, 2002). As Marchfs envolvidas na imunidade incluem Marchf1, Marchf2, Marchf3, Marchf4, Marchf8 e Marchf9 (Samji; Hong; Means, 2014), que possuem alta expressão em diversas populações imunes como células T, células B, monócitos, macrófagos e DCs (Chattopadhyay; Shevach, 2013; De Gassart *et al.*, 2008; Galbas *et al.*, 2012; Goto *et al.*, 2003; Nathan *et al.*, 2008; Tada *et al.*, 2015; Thibodeau *et al.*, 2008; Yonashiro *et al.*, 2006). Dada a sua expressão elevada em células imunológicas e seu papel na regulação imune, este estudo se concentrará na Marchf1.

1.5 Marchf1

A ubiquitina E3-ligase associada à membrana 1 (Marchf1) é expressa em tecidos linfoides secundários (Deshaies; Joazeiro, 2009; Oh *et al.*, 2013a; Samji; Hong; Means, 2014), principalmente em células imunes como células B, monócitos e DCs (Matsuki *et al.*, 2007; Thibodeau *et al.*, 2008; Young *et al.*, 2008). Em monócitos e DCs, a transcrição gênica de Marchf1 pode ser induzida pela interleucina 10 (IL-10) (Thibodeau *et al.*, 2008). Durante a maturação das DCs a expressão de Marchf1 é reduzida, o tratamento com ligantes de receptores do tipo Toll (TLR) em DCs humanas e murinas resulta na diminuição da expressão de Marchf1 e, conseqüentemente, na reversão da poliubiquitinação do MHC II e do CD86 (Baravalle *et al.*, 2011; Carreno; Collins, 2002; Galbas; Thibodeau, 2012; Tada *et al.*, 2015; Young *et al.*, 2008). Esses achados sugerem que Marchf1 desempenha um papel crucial na regulação da apresentação de antígenos e na maturação das DCs.

Um papel fundamental da Marchf1 é a ubiquitinação da molécula de MHC de Classe II (MHC II), reduzindo sua expressão na superfície celular. Enquanto o MHC I é essencial para a ativação de células T CD8 pelas DCs, as moléculas de MHC II são cruciais para o desenvolvimento e ativação de células T CD4 (Lehner; Cresswell, 2004). A poliubiquitinação do MHC II pela Marchf1 ocorre em um resíduo de lisina conservado na cauda citoplasmática da cadeia β do MHC II em células B (Matsuki *et al.*, 2007), DCs

pDCs, cDCs (Young *et al.*, 2008) e monócitos (Thibodeau *et al.*, 2008) (**Figura 6**). O MHC II poliubiquitinado é reconhecido pelos complexos de classificação endossomal necessários para transporte e direcionado a endossomos tardios e lisossomos para destruição (ten Broeke; Wubbolts; Stoorvogel, 2013). A inibição de Marchf1 resulta em um aumento de 5 a 10 vezes na expressão de MHC II na superfície celular (Thibodeau *et al.*, 2008; Young *et al.*, 2008). Quando ativadas por ligantes de TLR, como CpG, as células B, pDCs, cDCs ou monócitos apresentam diminuição ou cessação da ubiquitinação do MHC II devido à redução parcial ou total da transcrição de Marchf1 (Galbas; Thibodeau, 2012).

Outra molécula que é ubiquitinada por Marchf1 é o CD86 (**Figura 6**), um regulador imunológico essencial expresso em APCs, que fornece um dos principais sinais co-estimulatórios para ativação e sobrevivência de células T (Carreno; Collins, 2002). A poliubiquitinação de CD86 ocorre no resíduo de lisina na posição 267 (K267), induzindo sua degradação (Baravalle *et al.*, 2011). Consequentemente, CD86 é expresso de forma estável na superfície de DCs ausentes da expressão de Marchf1 (Baravalle *et al.*, 2011), indicando que a poliubiquitinação de CD86 mediada por Marchf1 é crucial na regulação da apresentação de antígenos pelas DCs.

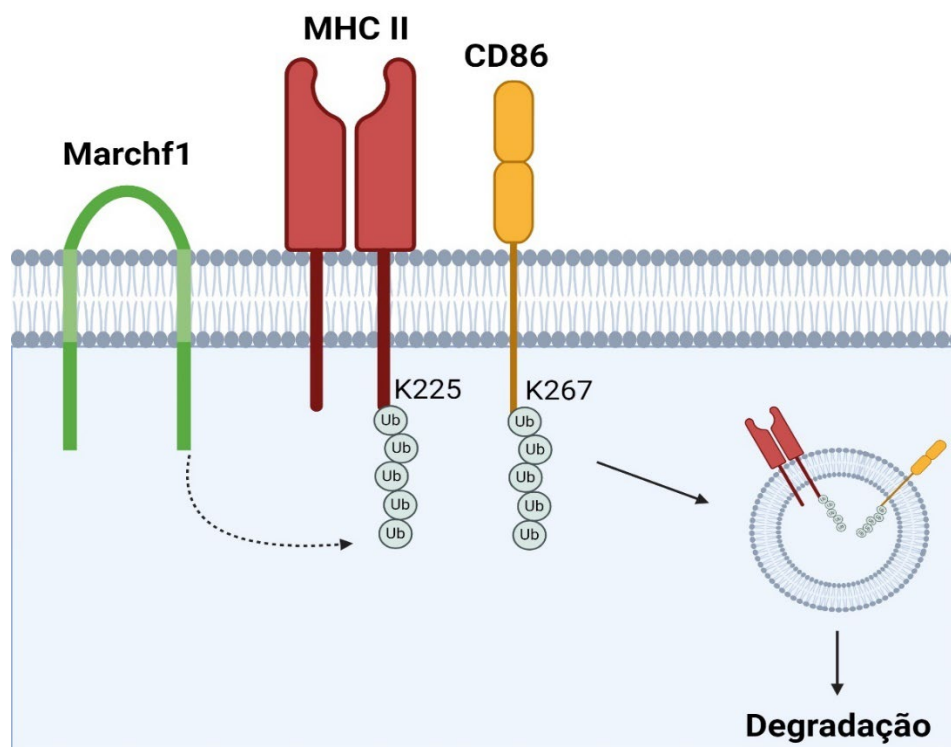


Figura 6. Regulação da expressão de MHC II e CD86 por *Marchf1*. Representação esquemática da poliubiquitinação nos resíduos do aminoácido lisina (K) dos imunoreceptores MHC II e CD86. O domínio RING-CH da *Marchf1* interage com lisinas, mediando a ubiquitinação e subsequente endocitose, levando a degradação. Criado no BioRender.com

A ativação das DCs resulta na diminuição da expressão de *Marchf1*, um mecanismo regulado por CD83, estabilizando os níveis de MHC II e CD86 na superfície celular (Tze *et al.*, 2011). No entanto, as moléculas MHC I e CD80, que atuam como coestimuladoras junto com CD86 e MHC II, não são ubiquitinadas pela *Marchf1*. (Motta *et al.*, 2007).

Nosso grupo de pesquisa estudou a redução na expressão de MHC II e CD86 em DCs causada pelo tratamento com a proteína DnaK de *Mycobacterium tuberculosis* (Borges *et al.*, 2010, 2012, 2016; Lopes *et al.*, 2014, 2016; Motta *et al.*, 2007). No estudo de Borges e colaboradores (Borges *et al.*, 2018), descrevemos o mecanismo pelo qual essa diminuição ocorre em DCs e outras células do sistema imunológico inato. Demonstramos que a DnaK se liga a um receptor de superfície que sinaliza via TLR2, induzindo IL-10 que ativa *Marchf1*, responsável por ubiquitinar MHC II e CD86, levando à inibição da ativação de linfócitos T e da rejeição do transplante de pele em camundongos (Borges *et al.*, 2018).

1.6 *Marchf1* e modulação imunológica

O papel imunológico da *Marchf1* ainda é pouco explorado. Sabe-se que na sua ausência há um aumento na expressão de MHC II e CD86 na membrana celular das APCs (Baravalle *et al.*, 2011). Contudo, camundongos geneticamente deficientes em *Marchf1* (*Marchf1*^{-/-}) possuem números reduzidos dos subtipos de DCs, cDC1s e cDC2s, no baço (Schriek *et al.*, 2022). O mecanismo pelo qual esses animais possuem essa diminuição não está totalmente esclarecido.

Além dos números reduzidos de cDC1s e cDC2s, uma outra população imune que é afetada pela expressão de *Marchf1* é a das células T regulatórias (Treg). Animais *Marchf1*^{-/-} possuem números reduzidos de células Treg tímicas (Oh *et al.*, 2013b), e DCs deficientes em *Marchf1* (CD11c^{Cre}*Marchf1*^{fl/fl}) ou sem a capacidade de ubiquitinar MHC II devido a uma mutação pontual (K225R – MHC II^{K>R}) falham em gerar Tregs antígeno específicas, tanto *in vitro* quanto *in vivo* (Oh *et al.*, 2018), sem impactar o desenvolvimento de células T CD4⁺ e T CD8⁺ (Liu *et al.*, 2020). Os autores propuseram que essa redução de Tregs seria causada por uma acumulação excessiva de MHC II na

membrana plasmática, afetando as balsas lipídicas e rede de tetraspaninas, comprometendo a capacidade das DCs de interagir adequadamente com timócitos, prejudicando assim o processo de diferenciação de Tregs (Oh et al., 2018).

Marchfl é essencial para o desenvolvimento de uma resposta imune do tipo Th2, contribuindo para patologias associadas à asma alérgica, infecção fúngica e parasitária. A ausência de Marchfl em DCs (CD11c^{Cre}Marchfl^{fl/fl}) correlacionou com uma diminuição do número de eosinófilos, concentrações séricas de IgE e da produção de IL-4 e IL-13 pelas células T CD4 (Castellanos *et al.*, 2022). Essas alterações foram acompanhadas por um aumento da expressão de marcadores de exaustão nas células T CD4⁺ efectoras, como PD-1, CTLA-4, TIGIT e TIM-3 (Castellanos *et al.*, 2022). Mais recentemente, foi relatado que a proteína *ubiquitin-like protein 3* (UBL3) é necessária para os mecanismos de imunidade adaptativa regulados pela Marchfl (Liu *et al.*, 2022).

Surpreendentemente, um estudo (Wilson *et al.*, 2018a) relatou que o aumento de MHC II em animais deficientes de Marchfl era acompanhado por uma redução dos níveis de MHC I em DCs e células B, mas não caracterizou os mecanismos subjacentes a essa observação. Nesse estudo, os autores verificaram que DCs provenientes de animais com mutação MHC II^{K>R}, assim impedindo que o MHC II seja ubiquitinado pela Marchfl, impactava a apresentação cruzada de peptídeos para células T CD8⁺ e consequentemente sua ativação e proliferação *in vitro* (Wilson *et al.*, 2018a)

Conjuntamente, esses resultados indicam que a expressão de Marchfl e a ubiquitinação das moléculas de MHC II e CD86 afetam a regulação das respostas imunes efectoras, tanto CD4⁺ como CD8⁺. Contudo, os mecanismos pelos quais a expressão de Marchfl nas DCs afeta o desenvolvimento da resposta imunológica ao crescimento tumoral ainda não foram explorados. Este trabalho visa contribuir para a compreensão do papel da expressão de Marchfl em DCs na geração de respostas imunes antitumorais.

2. Justificativa

As imunoterapias oncológicas de inibição de *checkpoint* visam modular sinais de ativação e inibição de células T. No entanto, outras estratégias terapêuticas focam na população celular que é a contraparte dos linfócitos T na sinapse imune: as células dendríticas (DCs). As DCs desempenham um papel crucial na apresentação de peptídeos tumorais através do primeiro sinal (MHC) e na estimulação da ativação de células T. Este estudo tem como objetivo investigar o envolvimento da proteína Marchf1 em DCs durante a geração de respostas imunes a tumores, utilizando modelos tumorais murinos. Marchf1 é responsável por poliubiquitinar e direcionar para degradação os receptores imunológicos MHC II e CD86, que correspondem ao sinal 1 e 2 fornecidos por DCs para a ativação de células T. Nossa hipótese é de que, na ausência de Marchf1, ocorrerá um aumento na expressão de MHC II e CD86 nas DCs, e uma diminuição de células Treg, resultando em uma maior ativação das respostas imunes mediadas por células T, gerando uma resposta antitumoral mais eficaz. Este trabalho visa contribuir para um melhor entendimento mecanístico do desenvolvimento da resposta antitumoral, investigando a Marchf1 como um potencial alvo para terapias e diagnósticos no tratamento de câncer.

3. Objetivos

3.1 Objetivo Geral

Investigar o papel da expressão ubiquitina ligase Marchf1 em células dendríticas (DCs) no controle do crescimento tumoral.

3.2 Objetivos Específicos

3.2.1. Analisar o efeito da deleção de Marchf1 em DCs durante o crescimento tumoral na geração de respostas imunes a tumores.

3.2.2. Investigar o impacto da inibição da expressão de Marchf1 em DCs na resposta ao tratamento com inibidores de checkpoint imunológicos.

3.2.3. Avaliar a capacidade funcional das cDC1s sem Marchf1 em induzir a ativação de células T específicas.

3.2.4. Examinar o desenvolvimento de progenitores de DCs na medula óssea de animais geneticamente deficientes em Marchf1.

4. Capítulo 1

Manuscrito de Artigo Científico

The E3 ubiquitin ligase Marchf1 controls DC development and anti-tumor effector T cell responses

Autores: Karina Lima, Rodrigo B. Gassen, Kaifeng Liu, Guilherme T. Ribas, Hugo L. Corrêa, James J. Moon, Leonardo Riella, Cristina Bonorino, Thiago J Borges

Situação: Em preparação

Motivação: Essa é a primeira versão desse manuscrito, em que investigamos a importância da Marchf1 nas células dendríticas (DCs) para o controle do crescimento tumoral. Considerando o papel crucial da Marchf1 na modulação das células imunológicas por meio da poliubiquitinação de MHC II e CD86, afetando assim a ativação de células T, nossa hipótese inicial era de que a ausência de dessa proteína poderia resultar em uma maior ativação de células T contra o tumor, levando consequentemente à sua redução. Para testar essa hipótese, utilizamos diferentes modelos animais, incluindo camundongos geneticamente deficientes em Marchf1 (Marchf1^{-/-}) ou sem Marchf1 especificamente em DCs (CD11c^{Cre}Marchf1^{fl/fl}), em dois modelos tumorais diferentes, como B16-OVA e MC38.

The E3 ubiquitin ligase *Marchf1* controls DC development and anti-tumor effector T cell responses

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Summary

High MHC II and CD86 levels on dendritic cells (DCs) are critical for the induction of antitumor responses, ensuring efficient antigen presentation, and shaping T-cell immunity. The membrane-associated RING-CH-1 (*Marchf1*) ubiquitin ligase is responsible for the polyubiquitination and consequent degradation of these two receptors, modulating adaptive immune responses. Little is known, however, about the role of *Marchf1* in tumor progression. Here, we show that conditional depletion of *Marchf1* in dendritic cells (CD11c⁺) unexpectedly results in enhanced tumor growth. Mice lacking *Marchf1* in DCs show a remarkable reduction of cDC1s cell infiltration, a consequent defective CD8⁺ T cell response in the tumor microenvironment, and an inefficient response to immune checkpoint blockade (ICB) therapy. While antigen presentation is not affected in *Marchf1*^{-/-} DCs, *Marchf1*-deficient mice have reduced cDC1s precursors in the bone marrow. This is accompanied by an impaired distribution of pre-cDCs in the lymphoid tissues. CD8⁺ T cell responses can be restored in these mice by Flt3L injection, increasing the numbers of cDC1s and resulting in tumor rejection. Collectively, our results indicate that *Marchf1* expression in DCs is required for normal DC development and further DC-precursor distribution in both lymphoid tissues and the tumor microenvironment, revealing *Marchf1* as an important new player in the generation of anti-tumor immunity.

Introduction

Dendritic cells (DCs) are crucial for the induction of tumor-specific T-cell immunity¹. Upon encountering and capturing tumor antigens in the tissues, they are activated, migrating to the draining lymph nodes². DCs are a heterogeneous population comprising different subsets distinguished by their development, phenotypic differences, localization, and functional specialization^{3–5}. Migratory DCs present antigenic peptides to naïve T cells through MHC:peptide complexes, priming T cells and inducing their differentiation and activation to an effector state⁶. Conventional DCs (cDCs) are migratory DCs that play an essential role in inducing antitumoral immunity. They can be divided into two distinct subsets, type 1 cDC (cDC1s) and type 2 cDC (cDC2s)⁷ based on their ability to present tumor antigens and prime different subsets of T cells. While cDC2s conventionally activate CD4⁺ T cells through MHC II, the cDC1s subset can prime CD8⁺ T cells to exogenous antigens presenting them via MHC I^{8–13}.

One of the main mechanisms governing DC function is the regulation of surface immunoreceptors through ubiquitination¹⁴. This process controls the function of a target protein through a series of reactions involving E1-activating, E2-conjugation, and E3-ligating enzymes, resulting in endosomal degradation, therefore reducing target protein cell surface abundance¹⁵. The membrane-associated RING-CH-1 E3 ligase (Marchf1) ubiquitinates MHC II^{16,17} and CD86^{16,18} by transferring ubiquitin moieties to a cytoplasmatic lysine amino acid¹⁹ and regulates adaptive immunity response. Genetic ablation of Marchf1 leads to high surface levels of MHC II and CD86 expression in DCs and other conventional and non-conventional APCs^{20,21}, which could lead to an indirect downregulation of MHC I²². These alterations in DCs interfere with adaptive responses by the inefficient presentation of peptide and protein antigens via MHC I to CD8 T cells, affecting activation and proliferation *in vitro*²². Marchf1 is also required for Th2 responses to allergenic inflammation²³, controlling an exhaustion program of effector CD4 T cells²⁴. Moreover, the absence of Marchf1 in thymic cDCs can disrupt regulatory T (Treg) cell selection and decrease its numbers^{25,26}. However, the role of Marchf1 in the modulation of anti-tumor immune responses has not been explored.

Here, we investigated the role of Marchf1 expressed by DCs in the induction of anti-tumor immune responses using two mouse tumor models. Our findings demonstrate that genetic ablation of Marchf1 in mouse DCs leads to loss of tumor growth control. A defective tumor-specific CD8⁺ T cell response was observed that failed to be activated

due to a lower number of cDC1s in the tumor microenvironment and lymphoid tissues. This correlated with reduced numbers of DC precursors in the bone marrow, and pre-cDC distribution to lymphoid tissues. Additionally, *Marchfl* expression by DCs was required to induce an optimal immune checkpoint blockade response, underlying the importance of engaging cDC1s in tumor control. This impairment was reversed by injecting *Ftl3L* *in vivo*, increasing cDC1s numbers, and restoring anti-tumor CD8⁺ T cell responses and control of tumor growth. This study identifies *Marchfl* in dendritic cells as a prominent target for inducing effective CD8⁺ T-cell immunity against tumors by modulating key aspects of DC development and function.

Methods

Mice

C57BL/6J (B6, stock no: 000664), CD11c^{Cre} (stock no: 008068), and OT-I (C57BL/6-Tg (Tcr α Tcr β)1100Mjb/J, stock no: 003831) described in ²⁷ transgenic mice were purchased from The Jackson Laboratory. *Marchfl*-deficient mice (*Marchfl*^{-/-}) and *Marchfl*^{fl/fl} were a gift from Dr. Jeoung-Sook Shin (University of California, San Francisco, USA) and described in ^{23,28}. *Marchfl*^{-/-} were generated by the addition of loxP sequences flanking the RING domain on the exon 6 and crossed with CAG-cre mice to delete exon 6. CD11c^{Cre}*Marchfl*^{fl/fl} mice were generated by breeding CD11c^{Cre} mice with *Marchfl*^{fl/fl} for at least three generations before use. *Marchfl*^{fl/fl} and CD11c^{Cre}*Marchfl*^{fl/fl} were maintained as breeding colonies in the Massachusetts General Hospital (MGH) animal facility with water and food *ad libitum*. All mice used in the experiments were equal proportion of females and males, between 7 to 12 weeks old. Following the Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH) Animal Care guidelines, mice were bred and housed in individual and standard mini-isolators under specific pathogen-free conditions. The MGHIACUC approved all experiments under protocol numbers 2020N000085 and 2020N000125.

Tumor Models

The C57BL/6-derived melanoma cell line B16-OVA (Sigma-Aldrich – SCC420) and colon adenocarcinoma MC38 (Sigma-Aldrich – SCC172) were maintained at 37°C with 5% CO₂ in DMEM medium (Gibco) supplemented with 10% heat-inactivated FBS

(Gibco), penicillin, and streptomycin (Gibco). 1×10^6 tumor cells were injected subcutaneously in 100 μ l PBS on the dorsal portion of the animals. Tumor size was determined by the formula $L \times W^2 \times 0.5$, where L = length and W = width²⁹.

Isolation of tumor-infiltrating immune cells

Single-cell suspension was obtained after tumor digestion with type D collagenase (Roche) at 37°C for 30 min. Infiltrating immune cells were enriched by density gradient centrifugation with Lymphocyte Cell Separation Media Mouse (Cedarlane) for 20 min at 400g.

Flow cytometry and sorting

Cells were first incubated with TruStain FcX (anti-mouse CD16/32 - Biolegend) for 20 min in phosphate-buffered saline (PBS) containing 2% FBS and stained with antibodies on ice for 30 min. For intracellular cytokine detection, cells were stimulated for 4h with Cell Activation Cocktail with Brefeldin A (Biolegend) or OVA₂₅₇₋₂₆₄ and OVA₃₂₃₋₃₃₉ peptides before staining with eBioscience FOXP3/Transcription Factor Staining Buffer Set (Invitrogen) according to the manufacturer's instructions. Flow cytometry data were acquired on an LSR Fortessa X-20 (BD Biosciences) using BD FACSDiva software and FlowJo software analyses (Tree Star, v10.8.1). LIVE/DEAD™ Fixable Cell Stain (ThermoFisher) was used for dead-cell exclusion. The following fluorescent conjugate-labelled antibodies were used: CD4 BUV395 (SK3 – BD Biosciences), CD8 α BUV737 (SK1 – BD Biosciences), Ki-67 AF700 (18A8 - Biolegend), IFN- γ BV650 (XMG1.2 - Biolegend), TNF- α PE-Cy7 (MP6-XT22 – Biolegend), XCR1 PE (ZET - Biolegend), CD11b BV711 (M1/70 – Biolegend), Granzyme B APC (QA16A02 - Biolegend). CD45 APC-H7 (30-F11 – Biolegend), CD3 APC (145-2C11 – Biolegend), CD11c FITC (N418 – Biolegend), MHCII AF700 (M5/114.15.2–Biolegend), B220 BV510 (RA3-6B2 – Biolegend), CD19 BV711 (6D5 – Biolegend) , F4/80 Pacific Blue (BM8 – Biolegend) , CD11b APC (M170 – Biolegend), Ly-6C BV510 (HK1.4 – Biolegend), OVA tet PE, CD44 PE (IM7 – Biolegend), CD62L APC (MEL-14, Biolegend), Sca1 APC-H7 (D7 – Biolegend), Linage FITC (Biolegend), c-kit APC (2B8- Biolegend), CXCR1 Pacific blue (SA011F11 – Biolegend), Csf-1R BV605 (AFS98 – Biolegend), Flt3 (A2F10 –

Biolegend), MHCII BV785 (M5/114.15.2 – Biolegend) and Sirp α Percp-Cy5.5 (P84 – Biolegend). The PE ovalbumin tetramers were provided by the NIH Tetramer Core Facility. Splenic cDC1s (CD11c⁺MHC II⁺CD8 α ⁺CD11b⁻) were sorted with a FACS Symphony S6 (BD Biosciences) from WT and Marchfl^{-/-} mice for cDC1s cross-presentation assays.

Immunohistochemistry and microscopy

Tumor tissue sections (5 μ m) were stained using Opal 3-Plex Anti-Rb Detection Kit (Akoya Biosciences). The following fluorescent conjugate-labeled antibodies were used: CD3 ϵ (D4V8L – Cell Signaling), CD11c (D1V9Y – Cell Signaling), CD103 (EPR22590-27 – Abcam). Stained sections were photographed using an Akoya Phenolmager HT multi-spectral slide and processed with QuPath software (version 5.1).

OT-I proliferation assay

OT-I naïve CD8 were magnetically purified (MojoSort Mouse CD8 Naïve T cell – Biolegend) and labeled with surface antibodies to confirm the purification of CD44^{low}CD62L^{high} naïve cells by flow cytometry. Flow-sorted cDC1s were pulsed with 200 μ g/ml OVA protein (InvivoGen) for 2h, then washed twice and cultured with Cell trace violet (CTV, Thermo Fisher) stained for three days. The proliferation of OT-I cells was examined using CTV dilution with flow cytometry.

For induced CD103⁺ cDC1s-mediated cross-presentation, CD103⁺ cDC1s were generated as described³⁰. Briefly, bone marrow DCs (BMDCs) were cultured for 16 days in RPMI1640 media (Gibco) with 10% heat-inactivated FBS (Gibco), penicillin/streptomycin (Gibco), 50 μ M β -mercaptoethanol supplemented with Ftl3L (Biolegend) combined with granulocyte-macrophage colony-stimulation factor (GM-CSF- Biolegend). Afterward, cells were harvested and co-incubated in different concentrations with OT-I CD8 purified (MojoSort Mouse CD8 T cell – Biolegend) for three days. The proliferation of OT-I cells was examined using CTV dilution with flow cytometry.

In vivo treatments

For the Ftl3L experiment, recombinant Ftl3L-Ig (15 ug in 100 μ l PBS, Bio X Cell) or PBS was i.p. injected (nine daily injections) starting on day 3 post-tumor injection or transplant, as described in³¹. High molecular weight Polyinosinic-polycytidylic acid (Poly-IC) (50 ug in 50 μ l, InvivoGen) was injected intratumorally on days 5 and 11.

For immune checkpoint blockade experiments, 200 μ g of anti-PD-1 (clone RMP1-14, Bio X cell, RRID: AB_10949053) in combination with 200 μ g anti-CTLA4 (9H10, Bio X cell, RRID: AB_10950184), or with Rat IgG2a control (Bio X cell, RRID: AB_1107769) were intraperitoneally administered on the indicated days.

Transcriptional analyses of human samples

Survival information of 464 skin cancer melanoma patients was obtained from the Cancer Genome Atlas (TCGA) Database (<https://www.tcgasurvival.com/>) based on the expression of *MARCHF1* in the primary or metastatic melanoma, by RNA sequencing, where high expression was defined as gene value higher than 5.0, determined by transcript models corresponding to the TCGA GAF2.1 and normalized within-sample to a fixed upper quartile³². For *MARCHF1* expression analysis, all samples were downloaded from the public GEO NCBI database. For the melanoma samples biopsy analysis, bulk RNAseq (GSE78220 – ³³) we downloaded the expression matrix, and we used the Brunner-Munzel test from Scipy v1.13.0 to calculate the statistical difference between conditions for the *MARCHF1* gene.

Statistic and Survival Curve

Kaplan-Meier survival curves were constructed, and a log-rank test was used to compare the patient survival between groups. For independent 2-group comparisons, we used the unpaired 2-tailed Student's *t*-test for analysis. Two-tailed *t*-tests were corrected for multiple comparisons using the Holm-Sidak method. The 1-way ANOVA or 2-way ANOVA test was used for multiple group comparisons to determine differences

depending on the number of comparison groups. Multiple comparisons between levels were checked with the Tukey post hoc test for a 1-way ANOVA and the Sidak test for a 2-way ANOVA. Differences were considered significant at $P \leq 0.05$. Prism software was used for data analysis and graph drawing (GraphPad Software, Inc.).

Results

*Ablation of *Marchfl* in DCs favors tumor growth in vivo*

The absence of *Marchfl* on DCs leads to higher MHC II and CD86 surface expression. High MHC II and CD86 levels in the tumor microenvironment (TME) are associated with enhanced T cell activation³⁴ and improved patient outcomes³⁵. We thus hypothesized that knocking out *Marchfl* in DCs would result in a more robust immune response against the tumor by increasing MHC II and CD86 levels. To investigate the role of *Marchfl* in tumor development, we first analyzed tumor progression in mice that lack expression of *Marchfl* (*Marchfl*^{-/-}). As described, these mice have a higher surface expression of MHC II and CD86 in all APCs (**Suppl Fig 1A**). Nevertheless, when we injected the murine melanoma tumor cells B16-OVA, we observed larger and heavier tumors in the *Marchfl*^{-/-} mice compared to WT controls (**Fig 1A and 1B**). To investigate whether this effect was tumor-specific, we injected the murine colon carcinoma model MC38 and observed larger tumors in *Marchfl*^{-/-} mice compared to the control group (**Fig 1C and 1D**). These results indicated that the absence of *Marchfl* favors tumor growth, even with an increased expression of MHC II and co-stimulatory molecules in APCs.

Marchfl acts by polyubiquitinating MHC II and CD86, leading to the degradation of these immune receptors, thus affecting T cell activation¹⁶. Because DCs have an essential role in presenting tumor antigens to naïve T cells in draining lymph nodes², we investigated the effect of tumor growth in a conditional *knockout* of *Marchfl* in DCs (CD11c⁺) using the CD11c^{Cre}*Marchfl*^{fl/fl} mice. *Marchfl*^{fl/f} mice were used as controls. In the conditional knockout mice, the sole population with increased MHC II and CD86 was the DCs (**Suppl Fig 1B**). Following the inoculation of B16-OVA tumor cells, larger tumors were observed in the CD11c^{Cre}*Marchfl*^{fl/fl} mice, recapitulating the results in the *Marchfl*^{-/-} animals (**Fig 1E and 1F**). This effect was not restricted to the control of B16OVA tumors. MC38 tumors were also larger in CD11c^{Cre}*Marchfl*^{fl/fl} hosts compared to controls (**Fig 1G and 1H**). These results underscore the critical role of *Marchfl* expression in DCs in immune

regulation of tumor growth *in vivo*. To explore the potential clinical relevance of our findings, we assessed *MARCHF1* gene expression in human tumor samples through RNA sequencing data from The Cancer Genome Atlas (TCGA). Consistent with our mouse data, we observed a significant overall survival advantage for patients with higher *MARCHF1* expression (**Fig 1I**) in skin cancer melanoma patients. Although the TCGA data is not restricted to tumor-infiltrating DCs, these findings support a role for *MARCHF1* expression in the control of tumor progression.

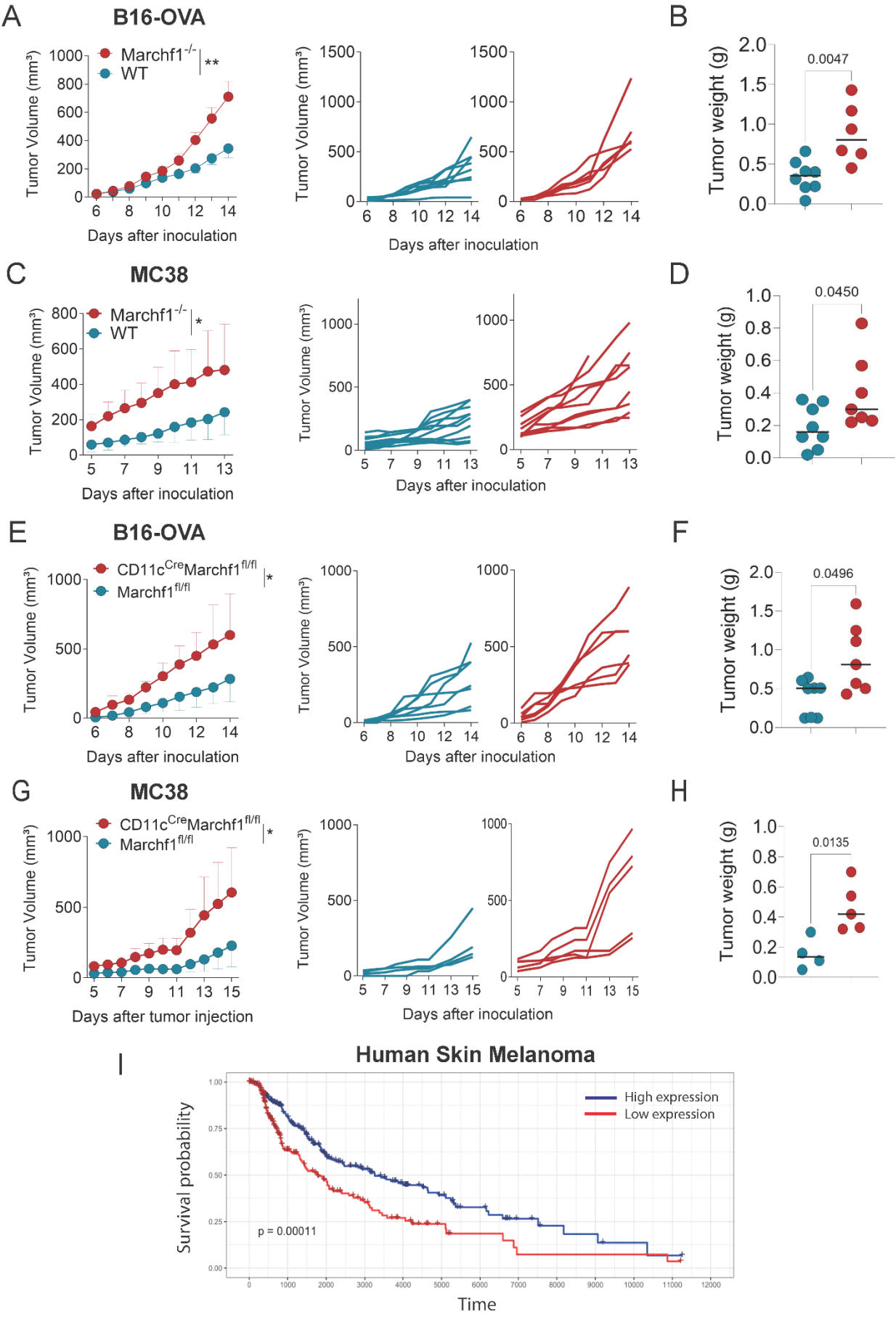


Figure 1. *Marchfl* deficiency in dendritic cells correlates with increased tumor growth.

(A) Tumor growth and (B) tumor weight in B16-OVA tumor-bearing WT and *Marchfl*^{-/-}, followed for 14 days. Data are shown as mean $\pm$ SEM, ***p* < 0.001. Tumor weight shows individual values and data are shown as mean $\pm$ SD, pooled from two independent experiments (*n* = 6-8/per group). (C) Tumor growth and (D) tumor weight in MC38 tumor-bearing WT and *Marchfl*^{-/-}, followed for 13 days. Shown in the mean $\pm$ SEM of two independent experiments (*n* = 7-8 /per group), **p* < 0.05. Tumor weights are shown as individual data points. (E-F) Tumor growth and weight in B16-OVA tumor-bearing *Marchfl*^{fl/fl} and CD11c^{Cre}*Marchfl*^{fl/fl} mice. Tumor growth was followed for 14 days. Data are presented as mean $\pm$ SEM of two independent experiments (*n* = 7 /per group). **p* < 0.05. The tumor weight graph shows individual values. (G-H) Tumor growth and weight in MC38 tumor-bearing *Marchfl*^{fl/fl} and CD11c^{Cre}*Marchfl*^{fl/fl} mice. Tumor growth was followed for 15 days. Shown in the mean $\pm$ SEM of one experiment (*n* = 4-5 /per group), values are mean $\pm$ SD **p* < 0.05. The tumor weight graph shows individual values. (I) Kaplan-Meier analysis of overall survival in patients with skin cancer melanoma, stratified by *MARCHF1* expression (cut-off value = 5).

Marchfl deletion in DCs affects antitumoral CD8⁺ T cell responses

Because DCs are essential for T cell activation, we next asked if T cell responses in the tumor microenvironment (TME) could be altered, contributing to the larger tumors observed in the CD11c^{Cre}*Marchfl*^{fl/fl} mice. Flow cytometry analysis of the B16-OVA infiltrating cells showed that, while the number of tumor-infiltrating CD4⁺ T cells remained unchanged in CD11c^{Cre}*Marchfl*^{fl/fl} mice relative to controls (**Fig. 2A**), there was a reduction in the total number of infiltrating CD8⁺ T cells (**Fig. 2B**). Similar results were observed when OVA-specific responses in the TME were analyzed, with no difference in infiltrating CD4⁺ T cell levels (**Fig 2C**) but reduced numbers of antigen-specific CD8⁺ T cells (**Fig 2D**) in the CD11c^{Cre}*Marchfl*^{fl/fl} animals. In addition, *ex-vivo* challenge of CD8⁺ T cells from the TME with OVA peptides resulted in a significantly impaired cytotoxic response in these mice, revealing a decrease in proliferating capacity of OVA-specific CD8⁺ T cells (**Fig 2E**) as well as a reduced percentage of IFN- γ ⁺TNF- α ⁺ CD8⁺ T cells within the TME compared to controls (**Fig 2F**). This indicated that the priming of these antigen-specific CD8⁺ T cells had been suboptimal *in vivo*.

Given the differences between tumor-specific CD4⁺ and CD8⁺ T cell responses in CD11c^{Cre}*Marchfl*^{fl/fl} tumor-bearing mice, we wondered if *Marchfl* ablation could differentially affect cDC2s and cDC1s populations, which have distinct priming abilities when presenting tumor antigens in the draining lymph nodes to naïve CD4⁺ and CD8⁺ T cells⁷. We observed that while the cDC2s cell numbers in the TME did not differ between the groups (**Fig 2G**), the cDC1s were significantly reduced in the TME of the

CD11c^{Cre}Marchfl^{fl/fl} animals (**Fig 2H and 2I**). Further analysis revealed that the cDC1s population was also reduced within the secondary lymphoid organs of tumor-bearing CD11c^{Cre}Marchfl^{fl/fl} mice (**Suppl Fig 2A and 2B**). These results suggest that the absence of Marchfl expression in DCs decreases functional antigen-specific CD8⁺ T but not CD4⁺ T cell responses, and this could be related to the lower numbers of cDC1s cells in the TME and secondary lymphoid tissues.

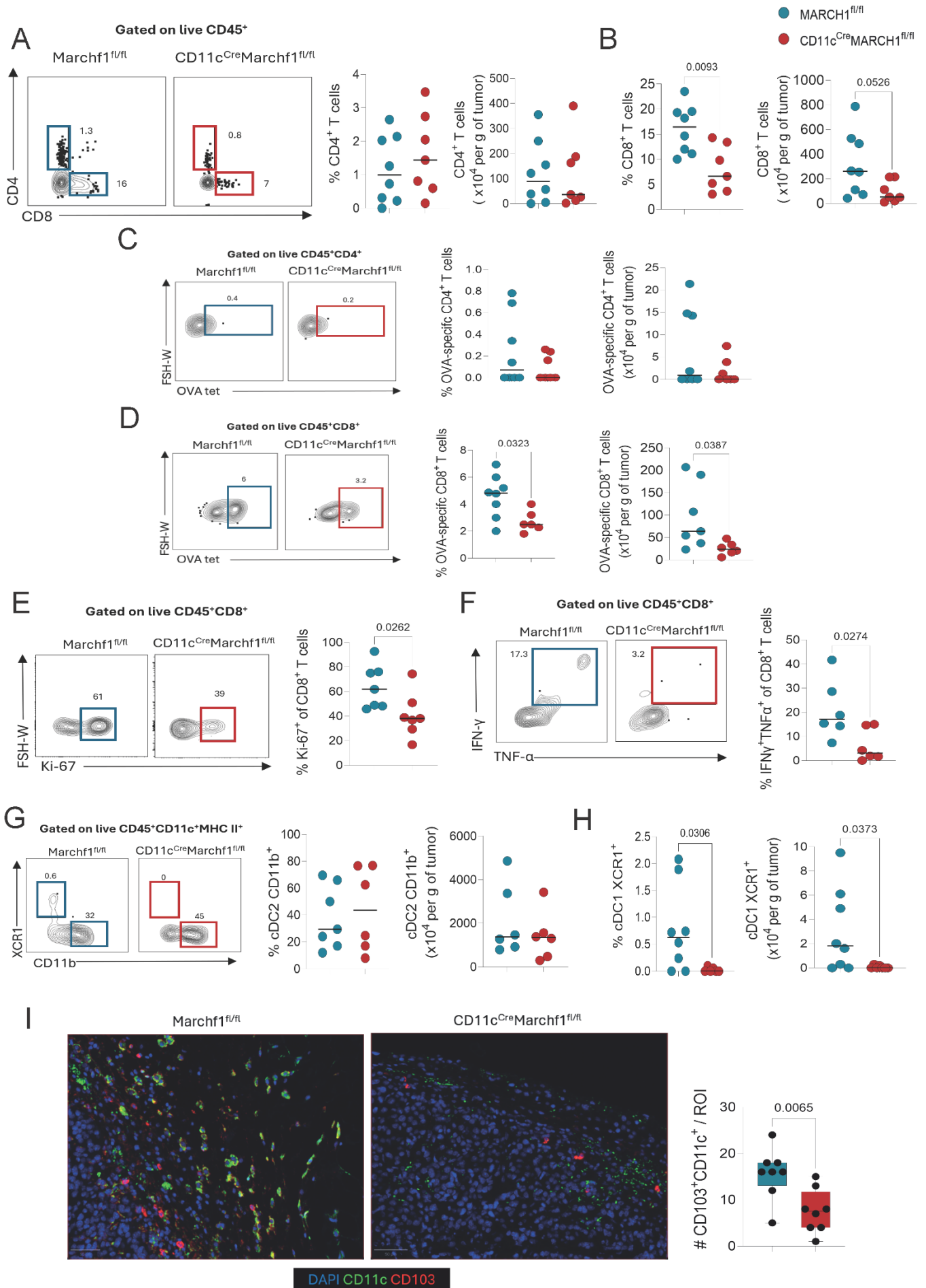


Figure 2. The absence of *Marchfl* in DCs results in reduction of cDC1s and CD8 T cells within the B16-OVA tumor microenvironment

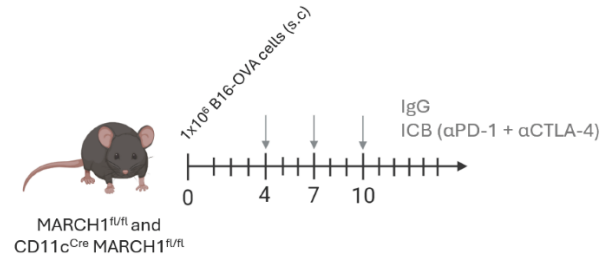
(A-B) Representative dot plots (left) and quantification (right) of frequency and absolute numbers of live CD4 and CD8 T cells infiltrating B16-OVA tumors 14 days post-injection in CD11c^{Cre}Marchfl^{fl/fl}. (C-D) Representative dot plots (left) and quantification (right) of frequency and absolute numbers of live OVA-specific CD4 (C) and CD8 (D) T cells infiltrating tumor. (E) Representative dot plots (left) and frequency (right) of proliferating Ki-67⁺ CD8 T cells after 4-h OVA peptide stimulation *ex vivo*. (F) Representative dot plots (left) and frequency (right) of IFN- γ and TNF- α production in CD8 T cells after 4-h OVA peptide stimulation *ex vivo*. (G-H) Representative dot plots (left) and quantification (right) of frequency and absolute numbers of tumor-infiltrating cDC1s XCR1⁺ (G) and cDC2s CD11b⁺ cells (H). (I) Representative 20x immunofluorescence images of cDC1s cells (CD11c+CD103+) with Phenochart software (right) and quantification of cDC1s cells in four tiled regions of interest (ROI). Data was collected 14 days post-tumor injection. Graphs show individual values of two independent experiments (n = 7-8).

Marchfl expression in DCs is required for effective immune checkpoint blockade response.

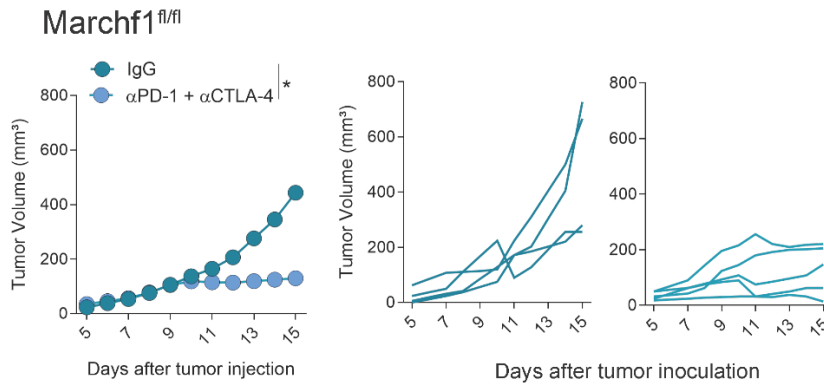
A deficient CD8⁺ T cell response in tumor-bearing individuals can be rescued upon immune checkpoint blockade (ICB) treatment with anti-PD-1 and anti-CTLA-4 blocking antibodies³⁶. Different studies have shown that DCs are implicated in the success of ICB therapy^{10,36}. We thus asked if anti-PD-1 and anti-CTLA-4 antibodies could recover cytotoxic T cell responses in CD11c^{Cre}Marchfl^{fl/fl} mice bearing B16-OVA tumors. (**Fig 3A**). While ICB treatment reduced the tumor growth (**Fig 3B**) and tumor weight (**Fig 3C**) in Marchfl^{fl/fl} control mice, it was ineffective in CD11c^{Cre}Marchfl^{fl/fl} mice (**Fig 3D and 3E**), suggesting that Marchfl in DCs has an important role in the effective response of ICB treatment in the melanoma model.

To extend these findings to humans, we analyzed *MARCHF1* expression in a public bulk RNA-seq dataset from tumor tissues isolated from melanoma patients characterized as responders or non-responders to ICB treatment (**Fig 3F**, ³³). Patients who exhibited a complete response to anti-PD-1 therapy had significantly higher *MARCHF1* expression compared to those with partial responses (**Fig 3G**). These observations support the notion that *MARCHF1* expression in the TME is associated with favorable outcomes in ICB-treated patients, underscoring the possible importance of Marchfl for effective antitumor responses during ICB therapy.

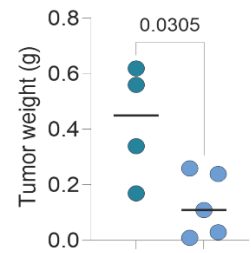
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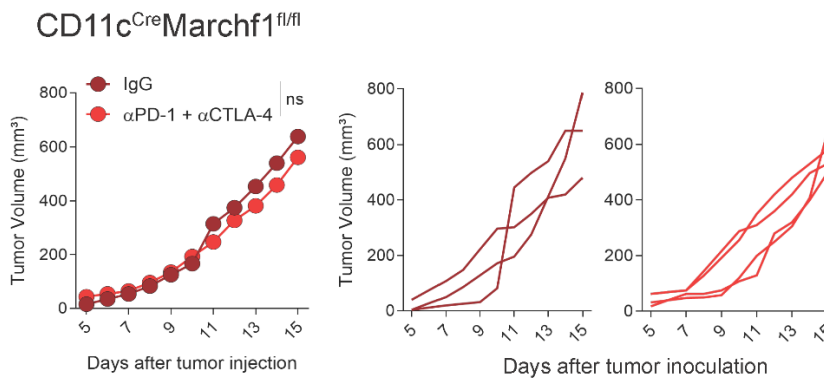
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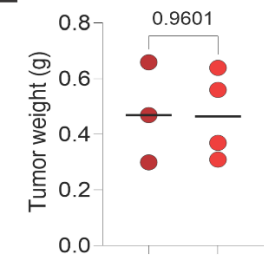
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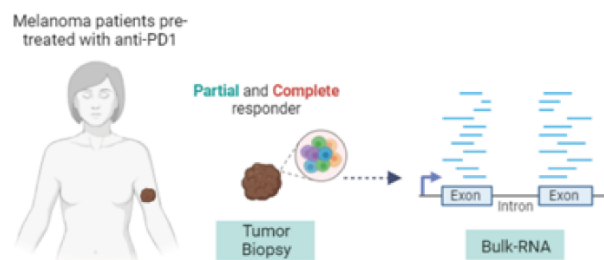
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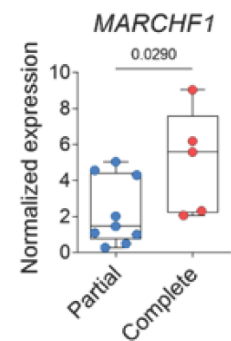


Figure 3. Marchf1 deficiency in DCs impairs responsiveness to immune checkpoint blockade (ICB) treatment.

(A) Experimental design showing treatment with a combination of ICBs (150μg of αPD-1 and αCTLA-4) or control IgG in B16-OVA tumor-bearing Marchf1^{fl/fl} and CD11c^{Cre}Marchf1^{fl/fl} mice. Cartoon created with Biorender.com. (B) Tumor growth in Marchf1^{fl/fl} mice treated with ICBs or control IgG over 15 days, shown as mean ± SEM (left) and individual tumor growth by groups

(right). Data represent one experiment (n = 4-5). (C) Tumor weight graph showing individual values. (D) Tumor growth of CD11c^{Cre}Marchf1^{fl/fl} treated with ICBs or control IgG over 15 days. The mean ± SEM (left) and individual tumor growth by groups (right) are shown. Data from one experiment (n = 7). (E) Tumor weight in CD11c^{Cre}Marchf1^{fl/fl} mice, graph shows individual values. (F-G) Experimental design (F) and MARCHF1 expression (G) of bulk-RNA analysis of tumor biopsies from melanoma patients treated with anti-PD1 separated the patients based on the complete or partial response to treatment. Data was obtained from one human public database (d GSE78220)

Marchf1 deficiency does not intrinsically affect cross-presentation by cDC1s

Tissue cDC1s cells encounter, capture, and process tumor cells while migrating to draining lymph nodes (dLN). This is followed by the presentation of tumor antigens and the priming of naïve CD8⁺ T cells⁸. Our *in vivo* and *ex vivo* results indicated that the priming of tumor-specific naïve CD8⁺ T cells was defective in CD11c^{Cre}Marchf1^{fl/fl} mice. We reasoned that Marchf1 deficiency could lead to an intrinsic impaired ability of these DCs to induce T cells. Alternatively, the reduced numbers of cDC1s *in vivo* could affect the ability of Marchf1-deficient mice to mount efficient CD8⁺ T cell responses.

To investigate whether the defective CD8⁺ T response observed in the tumor microenvironment of CD11c^{Cre}Marchf1^{fl/fl} mice was due to an intrinsic functional defect in the cDC1s population, we performed cross-presentation assays by analyzing OVA-specific T-cell proliferation *in vitro*. We differentiated cDC1s from WT or Marchf1^{-/-} bone marrow cells with FMS-like tyrosine kinase 3 ligand (Flt3L) and GM-CSF for 16 days³⁰. After differentiation, CD103⁺ cDC1s were pulsed with OVA protein and co-cultured with naïve OT-I cells (**Fig 4A**). We observed no significant differences in total OT-I proliferation (**Fig 4B**), and both groups presented a similar proliferation curve (**Fig 4C**). To confirm these findings, we flow-sorted splenic cDC1s cells (CD11c⁺MHC II⁺CD8α⁺) from naïve Marchf1^{-/-} or WT mice, pulsed these cells with OVA protein, and co-cultured them with naïve OT-I cells (**Fig 4D**). Similarly to the bone-marrow cDC1s, we found no differences in total OT-I proliferation (**Fig 4E**) and effector memory CD44⁺ cells (**Fig 4F**), demonstrating that Marchf1^{-/-} cDC1s are functionally able to perform cross-presentation *in vitro*.

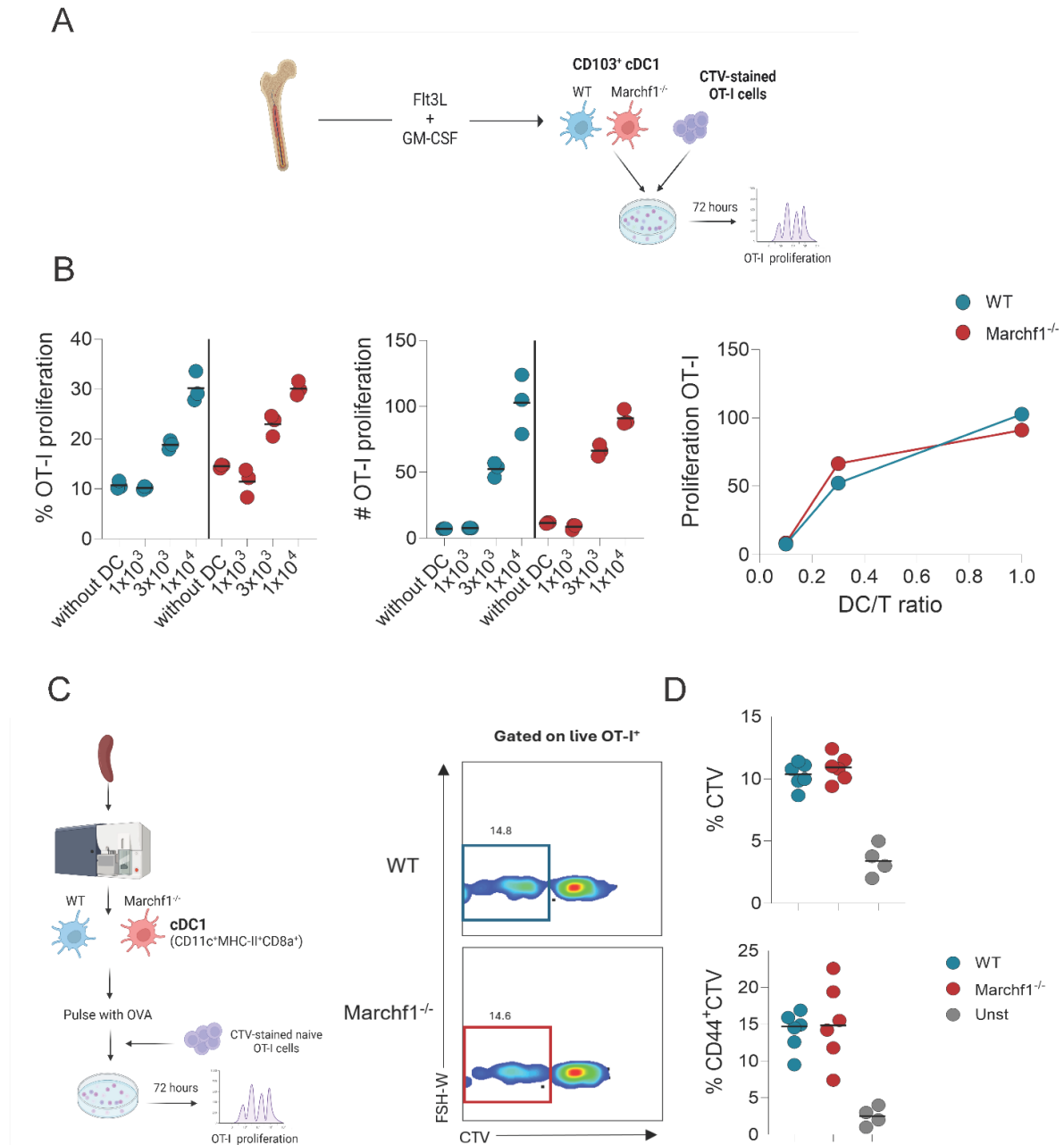


Figure 4. *Marchf1* deficiency does not impair cross-presentation by cDC1s cells. (A) Experimental design of OT-I proliferation assay, briefly bone marrow dendritic cells (BMDCs) were extracted from the bone marrow and stimulated during 16 days with Flt3L and GM-CSF to become cDC1s $CD103^+$. Afterward, cDC1s $CD103^+$ was pulsed with OVA protein and co-incubated with Cell trace violet-stained (CTV) OT-I cells. (B) Quantification of CTV-stained OT-I cell proliferation, shown as frequency (left), absolute numbers (middle), and correlation of OT-I proliferation with the DC cell ratio (right). Graphs show individual values of one experiment in triplicate. (C) Experimental design of OT-I proliferation assay (left), briefly splenic $CD11c^+MHC-II^+CD8\alpha^+$ cDC1s population from *Marchf1*^{-/-} and WT were FACS sorted, pulse with

OVA protein and co-incubated with Cell trace violet-stained (CTV) naïve OT-I cells. Plots show CTV-stained OT-I dilution via flow cytometry (right). **(D-E)** Frequency of CTV-stained OT-I cells and CD44⁺OT-I cells. Data represent individual values from two independent experiments in triplicate. All cartoons were created with Biorender.com

*Genetic ablation of *Marchf1* impairs pre-cDC development*

We next explored the underlying mechanisms leading to the observed reduction in cDC1s numbers in *Marchf1*-deficient mice. After ruling out the possibility that DCs from mice lacking *Marchf1* were more prone to cell death (**Suppl Fig 3**), we asked whether the deletion of *Marchf1* would result in defective DC development. Analysis of bone marrow (BM) progenitors in WT or *Marchf1*^{-/-} B16-OVA tumor-bearing mice revealed alterations in the early stages of DC development (**Fig 5A**). We observed that tumor-bearing *Marchf1*^{-/-} mice have an increase in the frequency of the granulocyte-macrophage progenitors (GMP) population (**Fig 5B**) but a reduction in the frequency and numbers of the macrophage-DC (MDP) (**Fig 5C**) and common DC (CDP) precursors (**Fig 5D**). These alterations are already present in *Marchf1*^{-/-} mice without tumors (**Suppl Fig 4**), indicating that this is not an intrinsic effect of the tumor model in these mice. Furthermore, when we analyzed the pre-cDC population, which differentiates into cDC1s or cDC2s^{37,38}, no differences were observed in the BM, but a significant reduction in pre-cDCs within the spleen and dLNs of *Marchf1*^{-/-} tumor-bearing mice (**Fig 5E**). These findings indicate that *Marchf1* deficiency leads to impaired DC development in the BM and disrupted pre-cDC distribution to peripheral tissues.

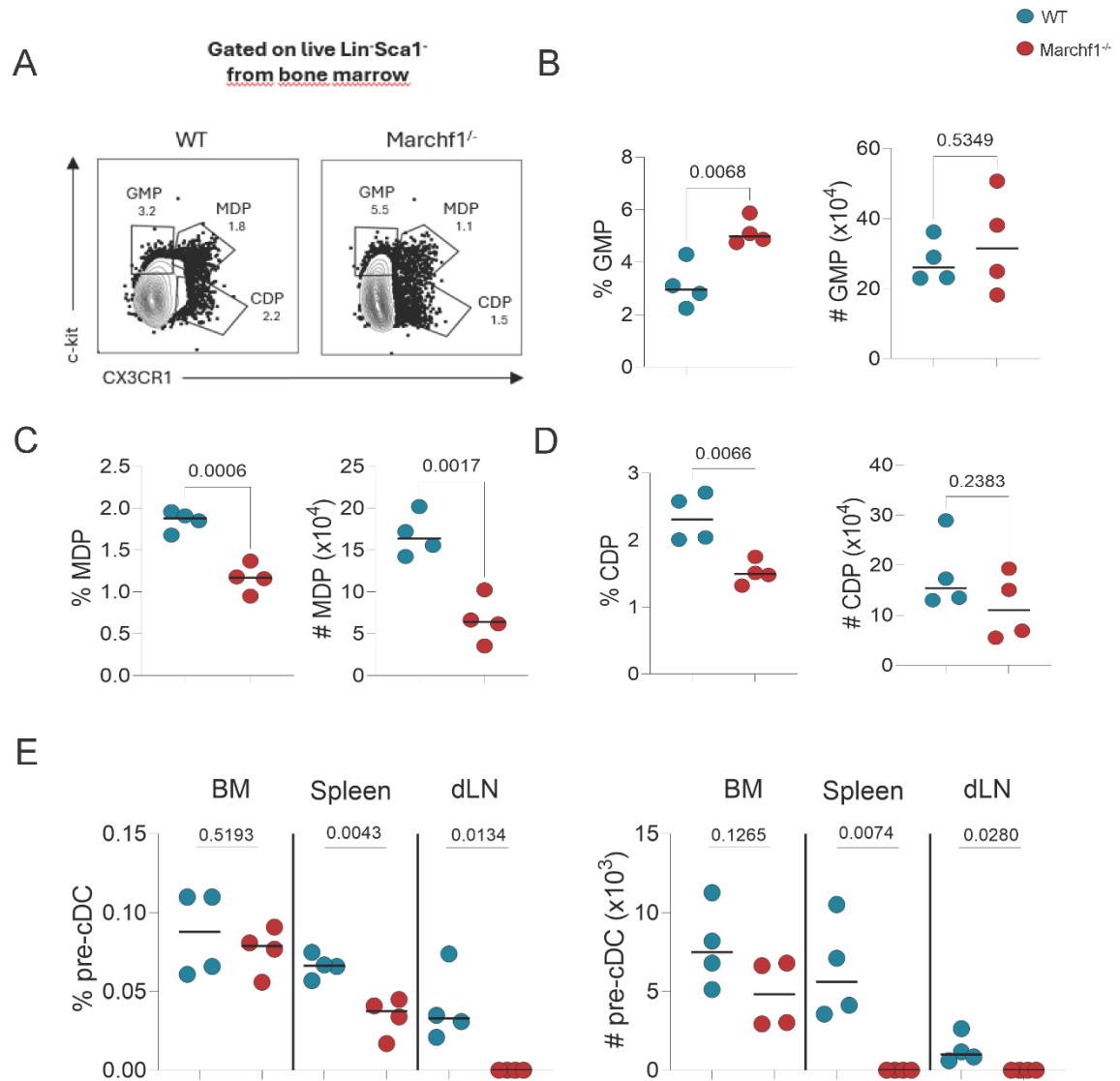


Figure 5. Marchf1 deficiency impairs DC development in tumor-bearing mice.

(A) Representative contour plots of DC progenitors from the bone marrow B16-OVA tumor-bearing WT or Marchf1^{-/-} mice on day 14 post-tumor inoculation. (B) Frequency (left) and absolute numbers (right) of GMP in the bone marrow of WT and Marchf1^{-/-} tumor-bearing mice, individual values are shown (n=4/group). (C) Frequency (left) and absolute numbers (right) of MDP in the bone marrow of tumor-bearing WT and Marchf1^{-/-} mice, individual values are shown (n=4/group). (D) Frequency (left) and absolute numbers (right) of CDP in the bone marrow of tumor-bearing WT and Marchf1^{-/-} mice, individual values are shown (n=4/group). (E) Frequency (left) and absolute numbers (right) of the pre-cDC population from tumor-bearing WT and Marchf1^{-/-} mice in the BM, spleen, and dLN. Individual values are shown (n=4/group). GMP – granulocyte-monocyte-DC progenitor; MDP – monocyte-DC progenitor; CDP – common DC progenitor populations; BM – bone marrow; dLN – draining lymph node.

Restoration of cDC1s numbers restores tumor growth control in CD11c^{Cre}Marchfl^{fl/fl} mice

Given the impairment in the development of DC progenitors in the bone marrow of Marchfl deficient mice and in the distribution of pre-cDCs to secondary lymph organs, we next asked whether inducing the development of cDC1s in the CD11c^{Cre}Marchfl^{fl/fl} mice could prevent the pronounced tumor growth previously observed. For this, we treated CD11c^{Cre}Marchfl^{fl/fl} tumor-bearing mice with Flt3L. Flt3L is a cytokine that promotes hematopoietic progenitor commitment to the DC lineage, expanding DCs and helping their survival and proliferation in the peripheral tissues³⁷. Treatment with nine injections of Flt3L combined with intratumoral injections of Poly-IC was previously demonstrated to preferentially expand cDC1s progenitors in the tumor, enhancing responses to immunotherapies³¹. Consistently with those findings, we observed a selective increase in the cDC1s population in the spleen of both Flt3L-treated CD11c^{Cre}Marchfl^{fl/fl} and Marchfl^{fl/f} mice (**Fig 6A-B**). Flt3L treatment abrogated the differences in melanoma tumor growth (**Fig 6C**) and tumor weight (**Fig 6D**) between the two groups of mice. The cDC1s population in the tumor microenvironment of Flt3L-treated CD11c^{Cre}Marchfl^{fl/fl} animals was also increased (**Fig 6E**), suggesting that the expanding cDC1s alone can reverse the loss of tumor growth control in CD11c^{Cre}Marchfl^{fl/fl} mice, leading to an increase in tumor-infiltrating CD8⁺ T cells, both total (**Fig 6F**) and antigen-specific (**Fig 6G**). CD8⁺ T cells were not only increased in the TME but were also more functional, characterized by greater production of granzyme B (**Fig 6H**) and IFN- γ and TNF- α (**Fig 6I**) when stimulated with OVA peptides. Thus, the absence of Marchfl in DCs leads to lower numbers of cDC1s in the tumor microenvironment, resulting in poor CD8⁺ T cells mediated anti-tumor immune responses. These responses can be restored by stimulating the development and increasing the number of cDC1s with Flt3L treatment.

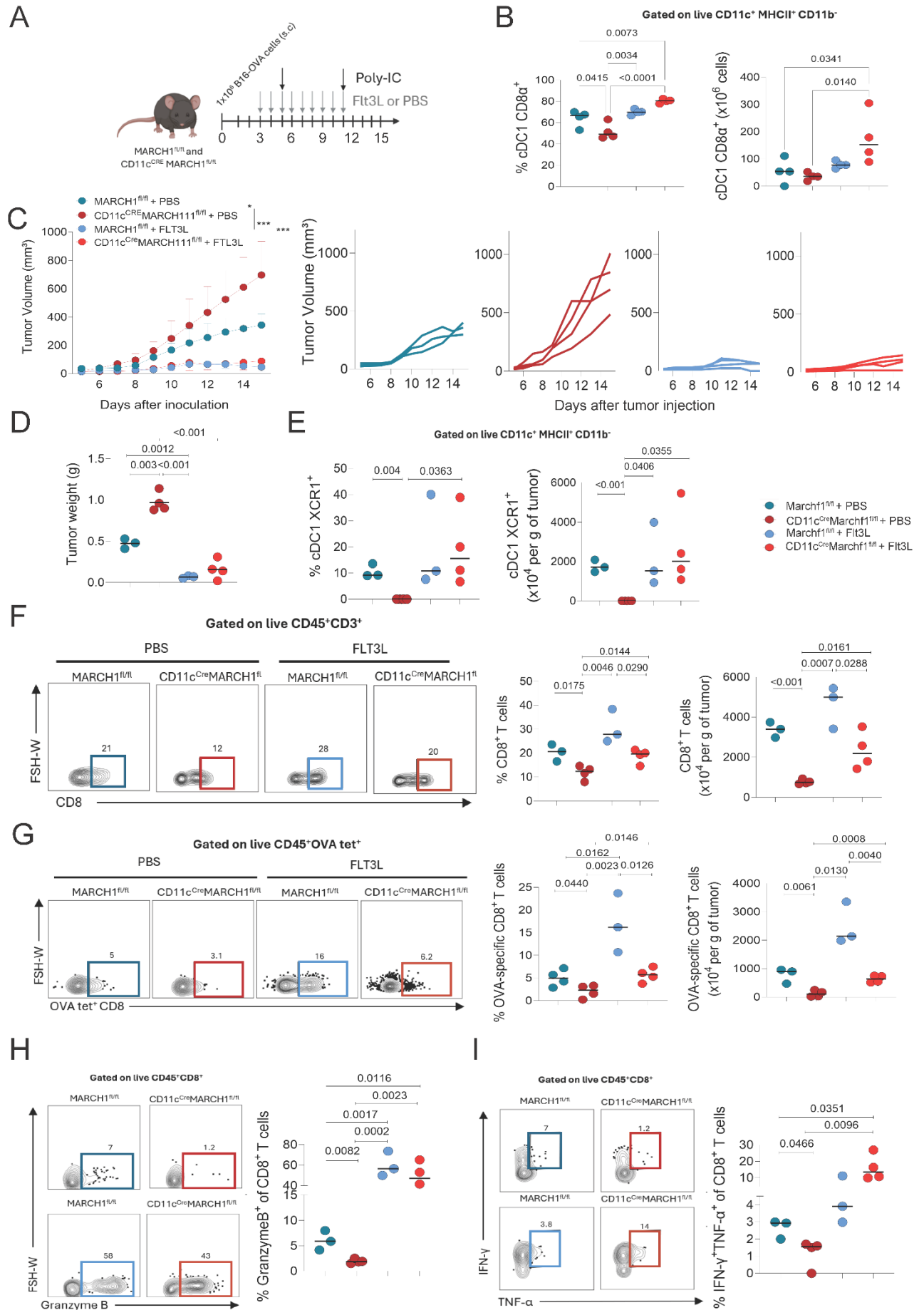


Figure 6. Treatment with Flt3L rescues deficient CD8⁺ T cell responses in *Marchf1*-deficient mice.

(A) Experimental design of B16-OVA tumor-bearing *Marchf1*^{fl/fl} and *CD11c*^{Cre}*Marchf1*^{fl/fl} mice treated for 15 days with nine i.p doses of FLT3L (15ug/dose) and two i.t doses of Poly-IC (50/ug). Cartoon created with Biorender.com. (B) Quantification of live cDC1s CD8 α ⁺ in the spleen. Frequency (left) and absolute numbers (right). (C) Tumor growth was followed for 15 days. The mean $\pm$ SEM (left) and individual tumor growth by groups (right) are shown. *** $p < 0.0001$; ** $p < 0.001$; * $p < 0.05$.; Data from one experiment ($n = 3-4$ /per group) (D) Tumor weight, shown as individual values by group. (E) Quantification of live tumor-infiltrating cDC1s XCR1⁺. Frequency (left) and absolute numbers (right). (F) Representative dot plots (left) and frequency and absolute numbers of live tumor-infiltrating CD4 and CD8 T cells (right). (G) Representative dot plots (left) and quantification of frequency and absolute numbers (right) of CD8 cells OVA tetramer⁺. (H) Quantification of Granzyme B production in CD8 T cells after 4-h OVA peptide stimulation ex vivo. Representative dot plots (left) and frequency (right). (I) Analysis of IFN- γ and TNF- α production in CD8⁺ T cells post-OVA peptide stimulation ex vivo, shown as representative dot plots (left) and frequency (right).

Discussion

Marchf1 has been identified as a critical factor in the development of Type 2 immunity associated with allergic asthma; however, its deletion did not influence the differentiation of Type 1 T helper cells²³. Moreover, the same study reported no significant role for *Marchf1* in cytotoxic CD8⁺ T cell responses during viral infection²³. Our initial hypothesis was that high MHC II and CD86 levels in APCs, due to the absence of *Marchf1*, would induce more T cell activation and, consequently, an enhanced antitumoral response. Surprisingly, we observed the opposite. *Marchf1* deficiency in the DC compartment led to a reduction in the cDC1s population and a consequent ineffective CD8⁺ T cell response that was unrecoverable with ICB therapy. The reduced cDC1s numbers were explained by an impairment in DC development, evidenced by reduced bone marrow-derived monocyte-dendritic progenitors (MDPs) and common dendritic progenitors (CDPs). This affected the pre-cDCs distribution both in the secondary lymphoid organs and TME. When the numbers of cDC1s were restored by the injection with Flt3L, the antitumor cytotoxic responses were restored, and tumor growth was reverted.

This study revealed that the expression of *Marchf1* is necessary for cDC1s development. Hematopoietic stem cells (HSCs) in the bone marrow differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), with CMPs giving rise to DC-fate restricted progenitors (CPDs), which in turn generate pre-cDCs and pre-pDCs³⁷. The differentiation process is driven by Flt3L binding to its receptor Flt3³⁸. Pre-cDCs

are released into the blood to reside in the peripheral tissues and lymphoid organs, where they differentiate into cDC1s and cDC2s³⁸. In *Marchfl*^{-/-} tumor-bearing mice, we observed a reduction in MDP, CDP, and pre-cDC precursors, leading to impaired pre-cDCs distribution in secondary lymphoid tissues. Additionally, we observed lower cDC1s numbers in the conditional knockout *CD11c*^{Cre}*Marchfl*^{fl/fl}. Since *CD11c* expression occurs during the final step of the bone marrow in the pre-cDC population³, the ability to stimulate cDC1s proliferation in response to Flt3L suggests that *Marchfl*-deficient *CD11c*⁺ cells retain the molecular machinery required for cDC1s development. Our data reveals the importance of *Marchfl* as a quality control checkpoint during DC development. Further studies are necessary to clarify the molecular mechanisms driving the defective DC development in the absence of *Marchfl*.

Our data also indicate that *Marchfl* is essential for an efficient antitumoral CD8 response within the TME. In addition to the lower numbers of cDC1s, other factors could contribute to this result. One potential explanation is that MHC II ubiquitination by *Marchfl* indirectly impacts MHC I. It has been reported that *Marchfl* ablation decreases MHC I surface in DCs, which leads to an inefficient presentation of peptides via MHC I to CD8⁺ T cells *in vitro*²². However, the mechanisms underlying this effect remain unclear. In our study, however, we observed no differences in MHC I expression by DCs *in vivo* (data not shown). Furthermore, *in vitro* cross-presentation assays demonstrated that WT or *Marchfl*^{-/-} cDC1s were equally efficient in inducing the proliferation of OT-I cells, suggesting that the *Marchfl* deficient cell population is not functionally impaired.

It is possible that the recovery effect seen with Flt3L treatment is not solely due to the restoration of cDC1s numbers. Flt3L is also necessary for the development of other DC subpopulations, such as pDCs³⁹. A subset of pDCs expressing OX40L^{hi} has been implicated in anti-tumor immunity and the generation of CD8⁺ T cells⁴⁰. Also, Flt3L could be not only restoring the numbers of cDC1s cells, but also activating them⁴¹. Flt3-mediated PI3K activation has been demonstrated in mature DCs⁴². It has been recently shown that Flt3L can enhance fully differentiated DCs when constitutively expressed⁴³. However, a different study found that Flt3L did not affect the activation state of tumor-infiltrating cDC1s because it did not affect the CD40, CD86, or MHCII levels in this cell population³¹. A more precise way to analyze the exclusive effect of *Marchfl* in cDC1s is to use *Xcr1*^{Cre} animals. Further investigation on the extent to which there is a reduction of cDC1s in mice lacking *Marchfl* is required, and a more comprehensive analysis of

DCs development in the bone marrow of these mice must be performed. Finally, it is possible that other populations of immune cells are affected by the lack of *Marchf1* in DCs - and that they are also rescued by Flt3L treatment. Flt3L^{-/-} mice lack not only DCs but also activated NK cells, leading to susceptibility to *Toxoplasma gondii* infection⁴⁴. Overall, our data reveals *Marchf1* as a major target for the efficient generation of tumor specific CD8⁺ T cell responses, as well as for the optimization of ICB treatment.

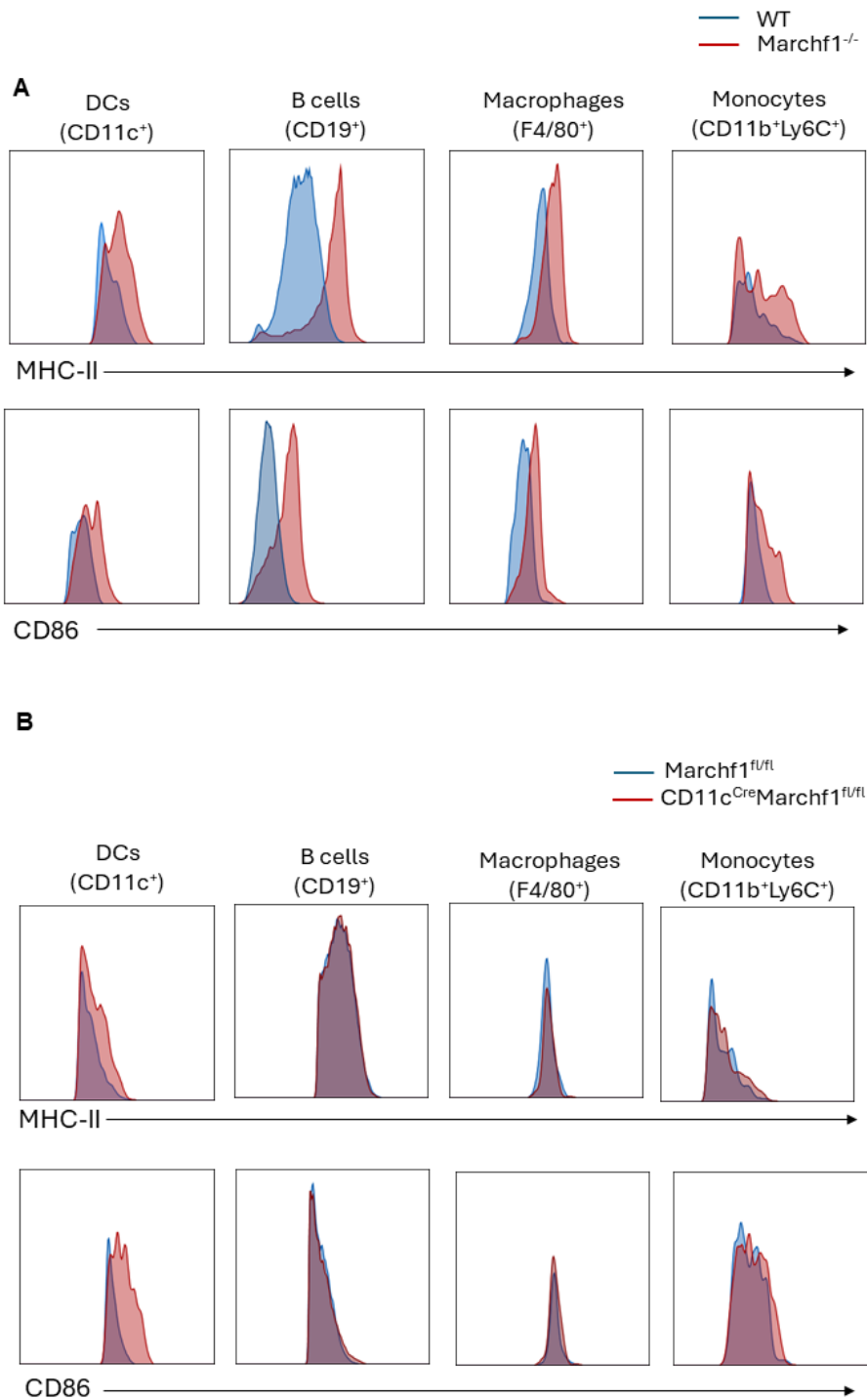
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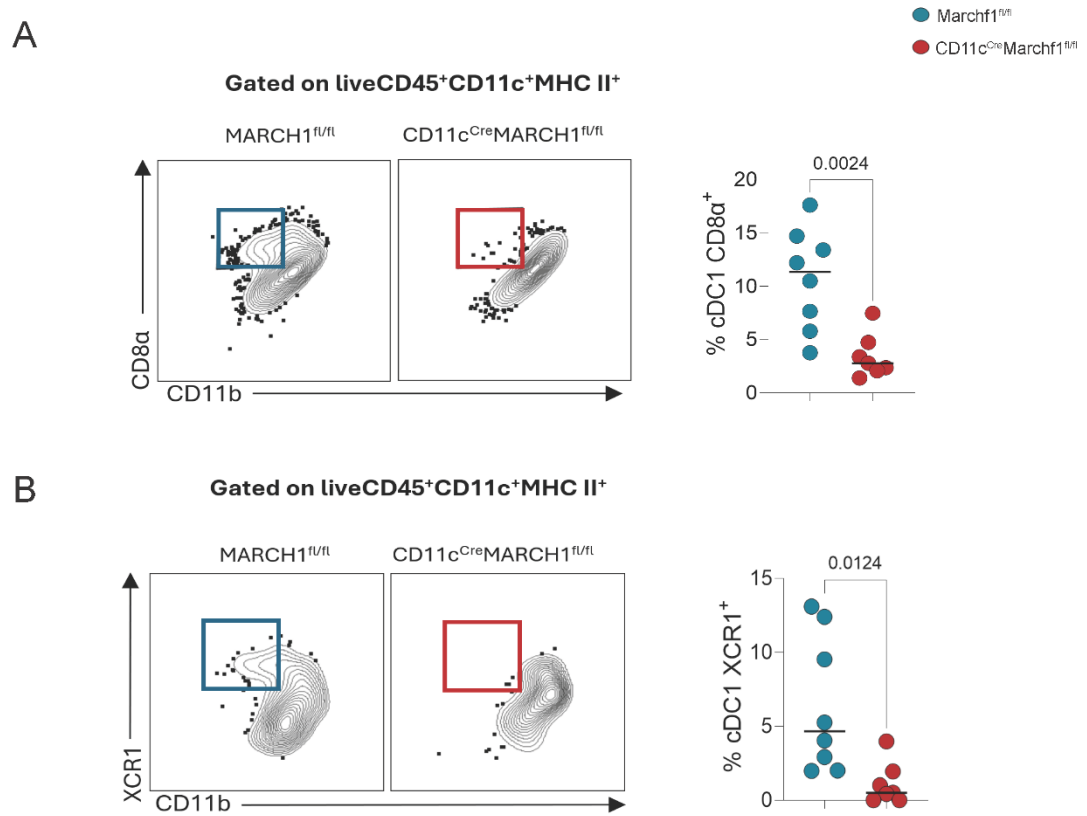
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Supplementary Figures



Supplementary Figure 1. Elevated MHC II and CD86 expression in splenic APC on Marchf1^{-/-} and CD11c^{Cre}Marchf1^{fl/fl} mice.

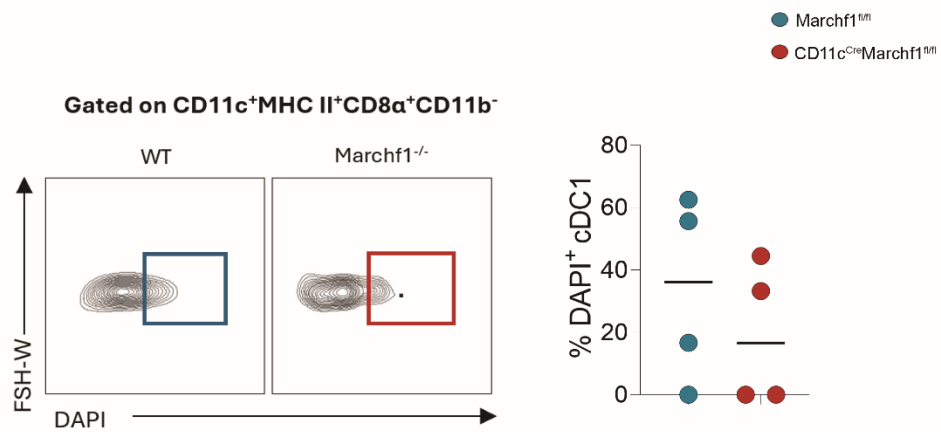
(A) Representative histograms displaying MHC II and CD86 expression levels in splenic APCs from Marchf1^{-/-} and WT mice. (B) Representative histograms displaying MHC II and CD86 expression levels in splenic APCs from CD11c^{Cre}Marchf1^{fl/fl} and Marchf1^{fl/fl} mice



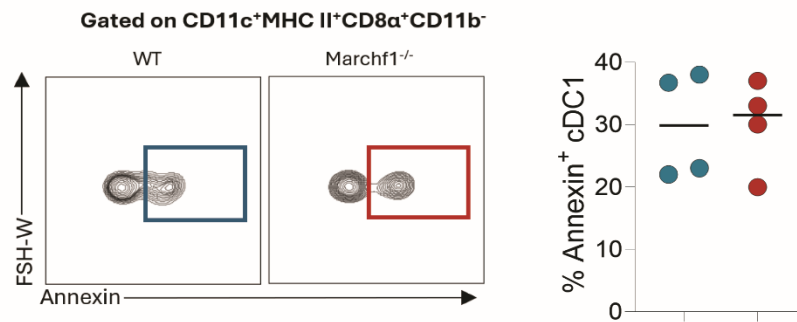
Supplementary Figure 2. cDC1s cells are reduced in secondary lymphoid tissues of mice lacking *Marchf1* in dendritic cells.

(A) Quantification of live splenic cDC1s cells expressing CD8α⁺ in *Marchf1*^{fl/fl} and *CD11c*^{Cre}*Marchf1*^{fl/fl} mice. Representative dot plots (left) and quantification of frequency (right) are shown. (B) Quantification of live cDC1s cells expressing XCR1⁺ in the draining lymph node of *Marchf1*^{fl/fl} and *CD11c*^{Cre}*Marchf1*^{fl/fl} mice. Representative dot plots (left) and quantification of frequency (right) are shown.

A

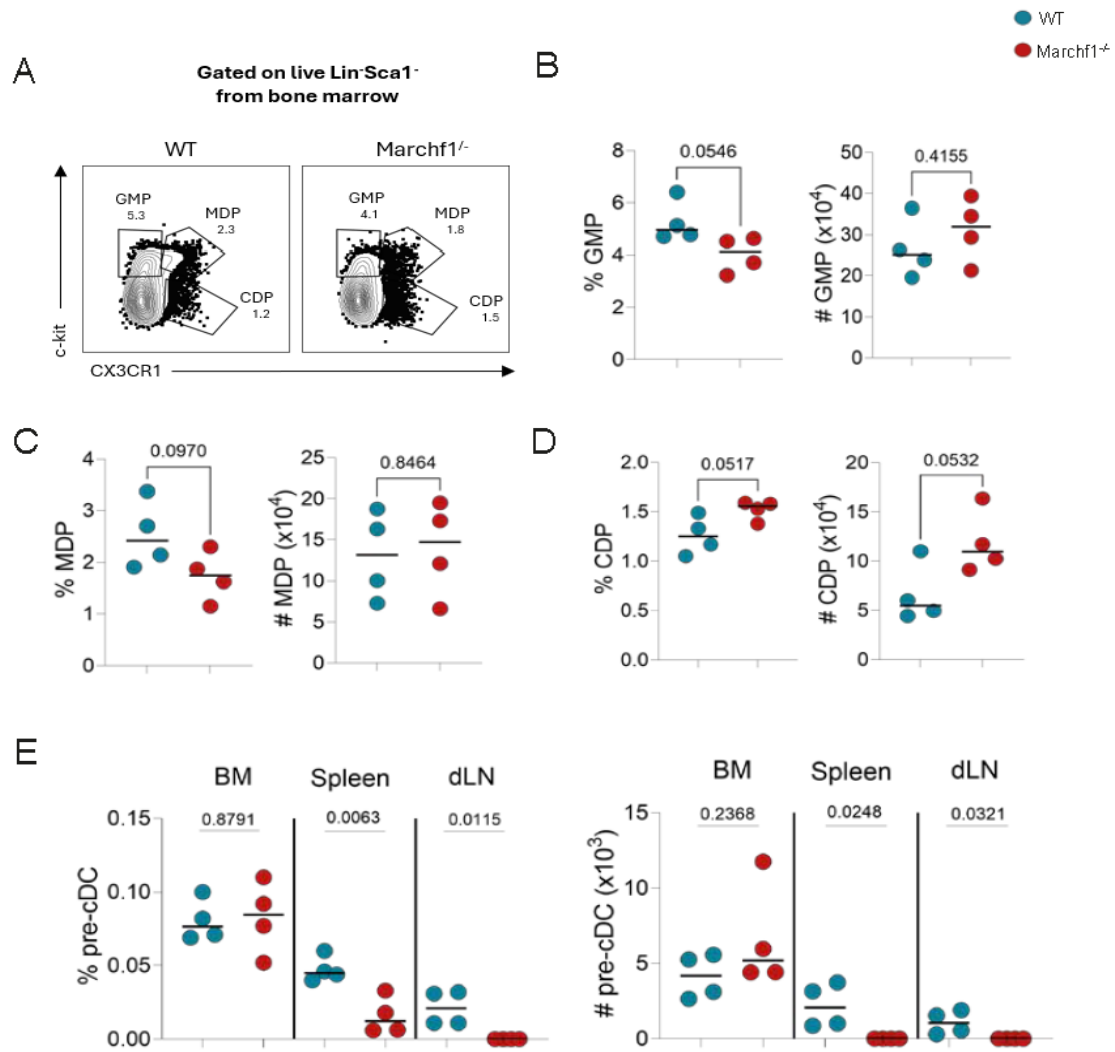


B



Supplementary Figure 3. cDC1s cell death levels remain unchanged in *Marchf-1* deficient mice versus WT mice.

(A) Quantification of DAPI⁺ cells in splenic cDC1s from $Marchf1^{-/-}$ and WT. Representative dot plots (left) and quantification of frequency (right) are shown. (B) Quantification of AnnexinV⁺ cells in splenic cDC1s expressing in $Marchf1^{-/-}$ and WT. Representative dot plots (left) and quantification of frequency (right) are shown.



Supplementary Figure 4: DC development is also impaired in the naïve mice with the absence of *Marchf1* expression.

(A) Representative contour plots of DC progenitors from the bone marrow of naïve WT or *Marchf1*^{-/-} mice. (B) Frequency (left) and absolute numbers (right) of GMP in the bone marrow of naïve WT and *Marchf1*^{-/-}, individual values are shown (n=4/group). (C) Frequency (left) and absolute numbers (right) of MDP in the bone marrow of naïve WT and *Marchf1*^{-/-} mice, individual values are shown (n=4/group). (D) Frequency (left) and absolute numbers (right) of MDP in the bone marrow of naïve WT and *Marchf1*^{-/-} mice, individual values are shown (n=4/group). (E) Frequency (left) and absolute numbers (right) of the pre-cDC population from naïve WT and *Marchf1*^{-/-} mice in the BM, spleen, and dLN. Individual values are shown (n=4/group). GMP – granulocyte-monocyte-DC progenitor; MDP – monocyte-DC progenitor; CDP – common DC progenitor populations; BM – bone marrow; dLN – draining lymph node.

5. Capítulo 2

Papel da *Marchf1* em modelos de transplante cardíaco

Situação: Dados preliminares

Motivação: Nossas observações anteriores no modelo tumoral indicaram que a ausência de *Marchf1* compromete a resposta efetiva das células T CD8⁺ devido à redução das células dendríticas convencionais de tipo 1 (cDC1s). Isso nos levou a questionar se a ausência de *Marchf1* em DCs teria o mesmo efeito em outros modelos de respostas imunes dependentes de células T CD8⁺. Decidimos, portanto, explorar o papel da *Marchf1* em modelos de transplante, com foco específico no modelo de transplante cardíaco, modelo estudado no laboratório do Massachusetts General Hospital (MGH – Boston, EUA) durante o doutorado sanduíche.

Introdução

O transplante cardíaco é atualmente a intervenção terapêutica mais eficaz para pacientes com insuficiência cardíaca em estágio terminal. No entanto, durante o procedimento, o enxerto é submetido a um período de hipóxia e isquemia durante sua preservação, resultando em disfunção metabólica e resposta ao estresse celular (Todd; Palmer, 2017). Esse processo desencadeia a produção de mediadores inflamatórios que exacerbam o dano tecidual e promovem a morte celular (Braza *et al.*, 2016). As moléculas associadas ao dano (DAMPs), liberadas devido ao estresse ou à morte celular, são expostas na superfície das células ou no ambiente extracelular, e, ao serem reconhecidas pelos receptores de reconhecimento padrão (PRRs) das células imunológicas do hospedeiro, desencadeiam uma resposta inflamatória (Li; Lan, 2023). Essa interação entre DAMPs e PRRs ativa uma cascata de sinalização intracelular, promovendo a liberação de citocinas e quimiocinas pró-inflamatórias, intensificando o dano ao enxerto. Além de sua participação na resposta imune inata, os DAMPs gerados durante a isquemia e reperfusão também podem iniciar a resposta imune adaptativa por meio da ativação das DCs (Celli; Albert; Bousso, 2011) (**Figura 1**).

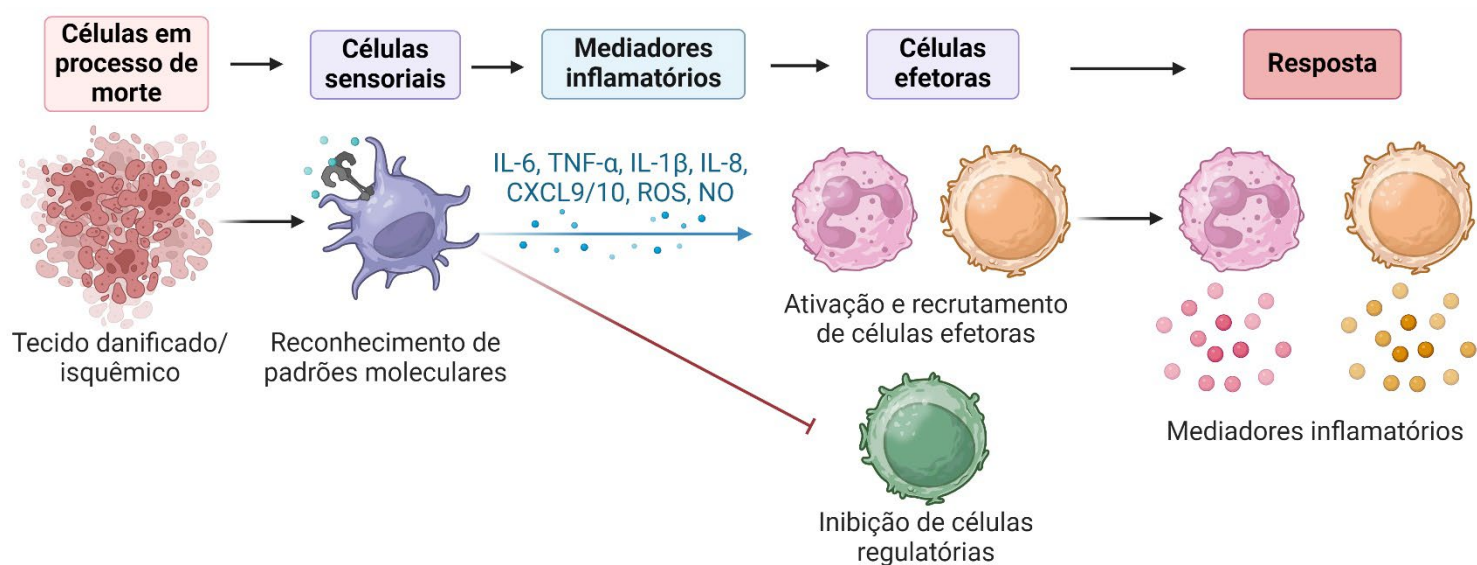


Figura 1. Desenvolvimento da resposta inflamatória após transplante de órgão sólido. O desenvolvimento da resposta começa com a isquemia no órgão transplantado, resultando na liberação de DAMPs. Esses DAMPs interagem com receptores do tipo Toll (TLRs), nas DCs, ativando-as e desencadeando uma resposta inflamatória caracterizada pela secreção de

citocinas e quimiocinas pró-inflamatórias. Esse ambiente inflamatório favorece o *priming* e a ativação de linfócitos T alorreativos. Além disso, a ativação das vias de sinalização dos TLRs nas células dendríticas prejudica o desenvolvimento e a função das células T reguladoras (Tregs), que normalmente suprimem as respostas imunológicas. A ausência de uma regulação eficaz mediada por Tregs amplifica ainda mais a resposta aloimune, contribuindo para a rejeição do órgão transplantado.

As DCs, ao apresentar peptídeos antigênicos associados às moléculas do complexo principal de histocompatibilidade (MHC) para os receptores de células T (TCR), fornecem os sinais coestimuladores necessários para a proliferação e diferenciação dos linfócitos T (Liu; Nussenzweig, 2010). No contexto do transplante, as DCs do doador migram para os tecidos linfoides secundários do receptor, onde iniciam a resposta aloimune, interagindo com linfócitos T alorreativos do hospedeiro ou transferindo aloantígenos para as DCs do receptor (Zhuang; Lakkis, 2015). Os aloantígenos podem ser transferidos intactos (apresentação semidireta) ou processados e apresentados como peptídeos não próprios ligados a moléculas MHC próprias (apresentação indireta) (Herrera *et al.*, 2004; Sivaganesh *et al.*, 2013). Embora os órgãos transplantados eventualmente percam as DCs do doador, pois a meia-vida dessas células é de dias, essas células são rapidamente reconstituídas por DCs do receptor (Grau Birgit Herbst *et al.*, 1998; Penfield *et al.*, 1999). O papel dessas DCs do receptor ainda não é completamente compreendido, mas estudos sugerem que elas podem intensificar a aloimunidade ao capturar antígenos do enxerto e ativar linfócitos T adicionais nos tecidos linfoides secundários ou exercer sua função localmente, ativando linfócitos T efetores no próprio enxerto (Celli; Albert; Bousso, 2011). Apesar do uso de imunossupressores aumentar significativamente a sobrevida dos órgãos transplantados, essas terapias, de forma similar às usadas em tratamentos oncológicos, focam predominantemente na inibição das células T, como o uso do CTLA-4-Ig (Iglesias *et al.*, 2022), negligenciando o papel crucial da imunidade inata. Assim, a modulação da resposta inflamatória inicial, mediada pelo sistema imune inato, emerge como uma estratégia promissora para reduzir as taxas de rejeição e melhorar os desfechos em transplantes cardíacos.

Resultados

Inicialmente, examinamos a expressão de *Marchf1* em nível transcriptômico utilizando dados públicos de dois estudos distintos (GSE9377 -ref e GSE203548- ref), que incluíam biópsias de corações transplantados de pacientes categorizados em biópsias estáveis e biópsias com rejeição (**Figura 2A**). Os resultados mostraram que a expressão de *MARCHF1* estava significativamente aumentada nas amostras de pacientes que sofreram rejeição (**Figura 2B**), sugerindo uma possível influência de *Marchf1* na rejeição de aloenxertos.

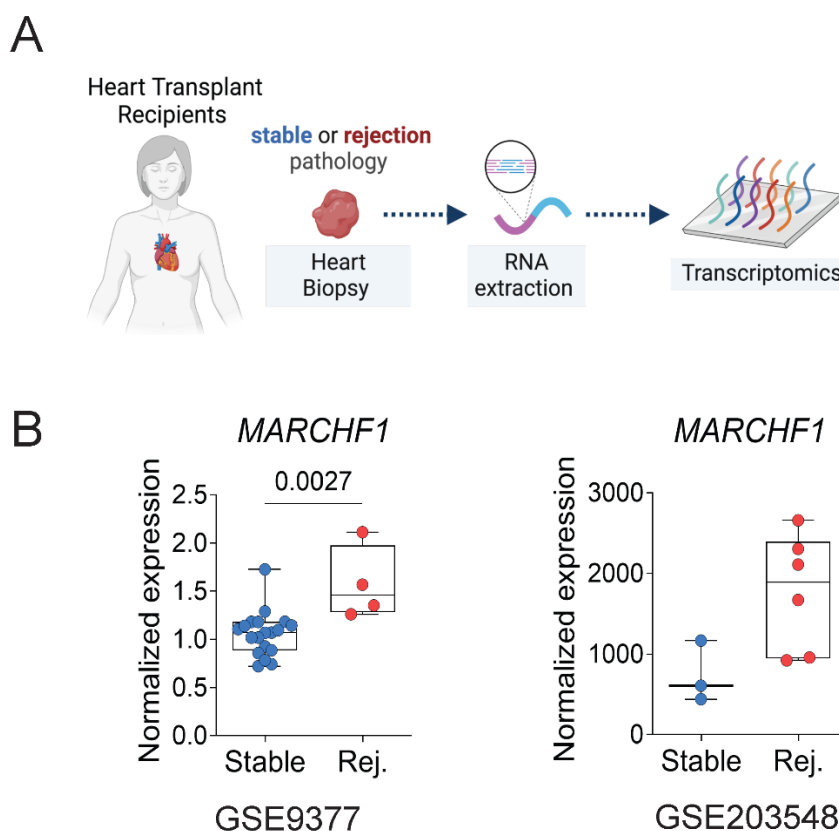


Figura 2. Expressão de *MARCHF1* em pacientes transplantados. (A-B) Análise da expressão gênica de *MARCHF1* em biópsias de pacientes submetidos a transplante de coração obtido de dados públicos de dois diferentes estudos – GSE9377 e GSE203548. As amostras foram separadas em amostras com aloenxerto estável e amostras com rejeição.

Para validar o papel da *Marchf1* em rejeição de aloenxerto, realizamos experimentos utilizando modelos murinos de transplante cardíaco. Corações de camundongos BALB/c (H-2^d) foram transplantados na cavidade abdominal de

camundongos C57BL/6 selvagens (WT) e C57BL/6 *Marchf1*^{-/-} (H-2^b). Dois dias após o transplante, os animais receberam uma dose única de bloqueio de coestimulação (CTLA-4-Ig) para induzir rejeição subaguda (**Figura 3A**). Observamos um aumento significativo no tempo médio de sobrevivência do enxerto (MST) nos receptores *Marchf1*^{-/-} (MST >150 dias) em comparação com os WT (MST 28 dias) (**Figura 3B**), sugerindo uma deficiência na resposta imunológica efetora nos animais deficientes em *Marchf1*.

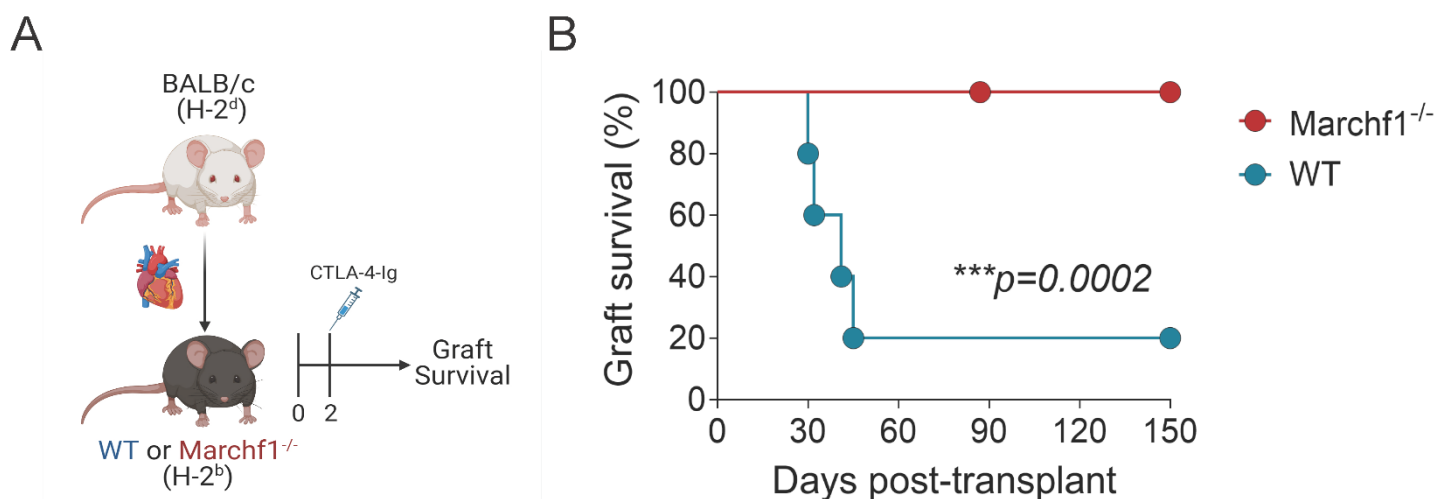


Figura 3. Ausência de *Marchf1* induz em um aumento da sobrevivência do enxerto em modelo completo de incompatibilidade. (A) Desenho experimental transplante de coração na região abdominal em animais *Marchf1*^{-/-} e WT. Animais BALB/c foram usados como doador. Dois dias após o transplante, os animais transplantados foram tratados com uma dose única de 250 µg CTLA-4-Ig pela via intraperitoneal. O enxerto foi monitorado baseado na palpitação. (B) Curva de Kaplan-Meier de sobrevivência do enxerto. N = 12/grupo.

Para avaliar se a sobrevivência prolongada do enxerto cardíaco nos camundongos *Marchf1*^{-/-} era específica ao antígeno, desafiamos esses animais, que tinham o primeiro transplante ativo no dia 150, com um segundo transplante na região cervical de um coração doador BALB/c, ou singênico C57BL/6 ou de camundongos C3H (H-2^k) (**Figura 4A**). O transplante com um segundo coração de BALB/c resultou em aceitação do enxerto a longo prazo sem a necessidade de imunossuppressores, enquanto o transplante de corações de C3H resultou em rejeição acelerada (MST de 10 dias) (**Figura 4B**). Esses dados indicam que a ausência de *Marchf1* nos animais receptores promove uma resposta imunológica de memória específica ao enxerto.

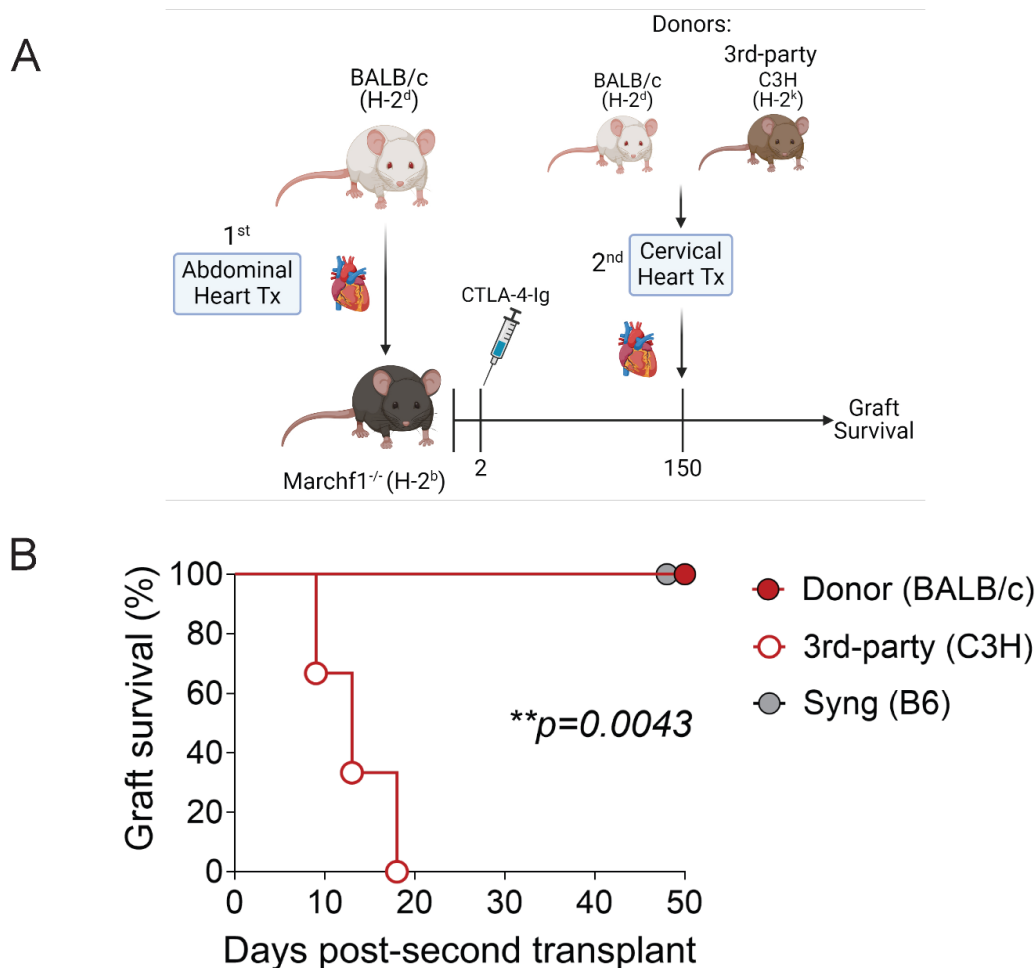


Figura 4. Especificidade Antigênica da Tolerância em *Marchf1*^{-/-}. (A) Desenho experimental do cotransplante na região abdominal e cervical em animais *Marchf1*^{-/-}. Animais *Marchf1*^{-/-} (H-2^b) foram transplantados na região abdominal com coração de animais BALB/c e tratados com 250 ug of CTLA-4-Ig no dia 2 após o transplante. Cento de cinquenta dias após o transplante, os mesmos animais receberam um segundo transplante na região cervical com coração de animais BALB/c (H-2^d), ou C57BL/6 (H-2^b) ou C3H (H-2^k). O enxerto foi monitorado baseado na palpitação por 50 dias. (B) Curva de Kaplan-Meier de sobrevivência do enxerto. N = 3-5/grupo.

Para descartar potenciais efeitos secundários dos agentes imunossupressores, realizamos transplantes de coração utilizando um modelo de incompatibilidade parcial, em que os mecanismos de rejeição são proporcionalmente menos intensos em comparação com incompatibilidade total, permitindo monitoramento do enxerto sem o uso de drogas. Neste modelo, camundongos híbridos F1, resultantes do cruzamento entre fêmeas BALB/c e machos C57BL/6, foram utilizados como doadores. Corações de

animais B6 x BALB/c F1 (H-2^{d/b}) foram transplantados em camundongos WT ou *Marchf1*^{-/-} (**Figura 5A**). Semelhante aos resultados observados com corações de doadores BALB/c, os camundongos *Marchf1*^{-/-} apresentaram uma maior sobrevivência do aloenxerto (MST >100 dias) comparado aos WT (MST 20 dias), sem a necessidade de imunossupressão (**Figura 5B**), sugerindo que a ausência de *Marchf1* induz tolerância e aceitação do aloenxerto cardíaco.

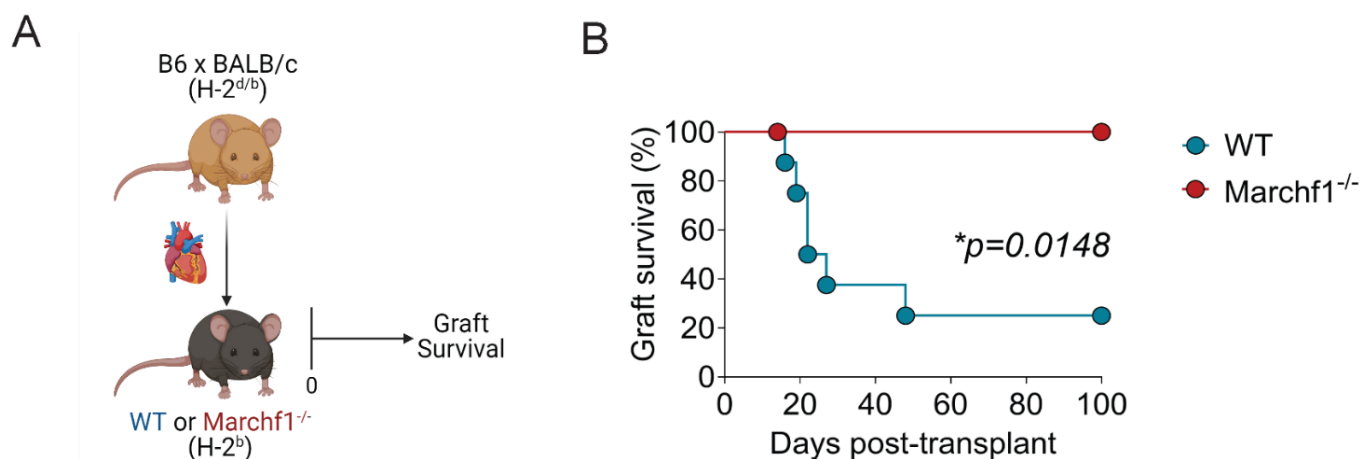


Figura 5. Ausência de *Marchf1* em DCs induz em um aumento da sobrevivência do enxerto em modelo de incompatibilidade parcial (A) Desenho experimental transplante de coração na região abdominal em animais *Marchf1*^{-/-} e WT. Animais B6 x BALB/c F1 foram usados como doador. O enxerto foi monitorado baseado na palpitação. (B) Curva de Kaplan-Meier de sobrevivência do enxerto. N = 5/grupo.

Para investigar o papel específico da *Marchf1* nas DCs, utilizamos camundongos *CD11c*^{Cre}*Marchf1*^{fl/fl} como receptores do aloenxerto cardíaco. O transplante de coração de animais BALB/c foi realizado em camundongos controle *Marchf1*^{fl/fl} e *CD11c*^{Cre}*Marchf1*^{fl/fl}, com tratamento de uma dose única de CTLA-4-Ig no segundo dia pós-transplante (**Figura 6A**). Observamos uma sobrevivência prolongada do enxerto nos camundongos *CD11c*^{Cre}*Marchf1*^{fl/fl} (MST 80 dias) em comparação com os controles (MST 50 dias) (**Figura 6B**). Resultados similares foram observados quando utilizamos os animais BALB/c x C57BL/6 F1 como doadores (**Figura 6C**), um prolongamento do

enxerto nos animais com depleção de *Marchf1* nas DCs (MST >50 dias) comparado com os animais controles (MST 30 dias) (**Figura 6D**).

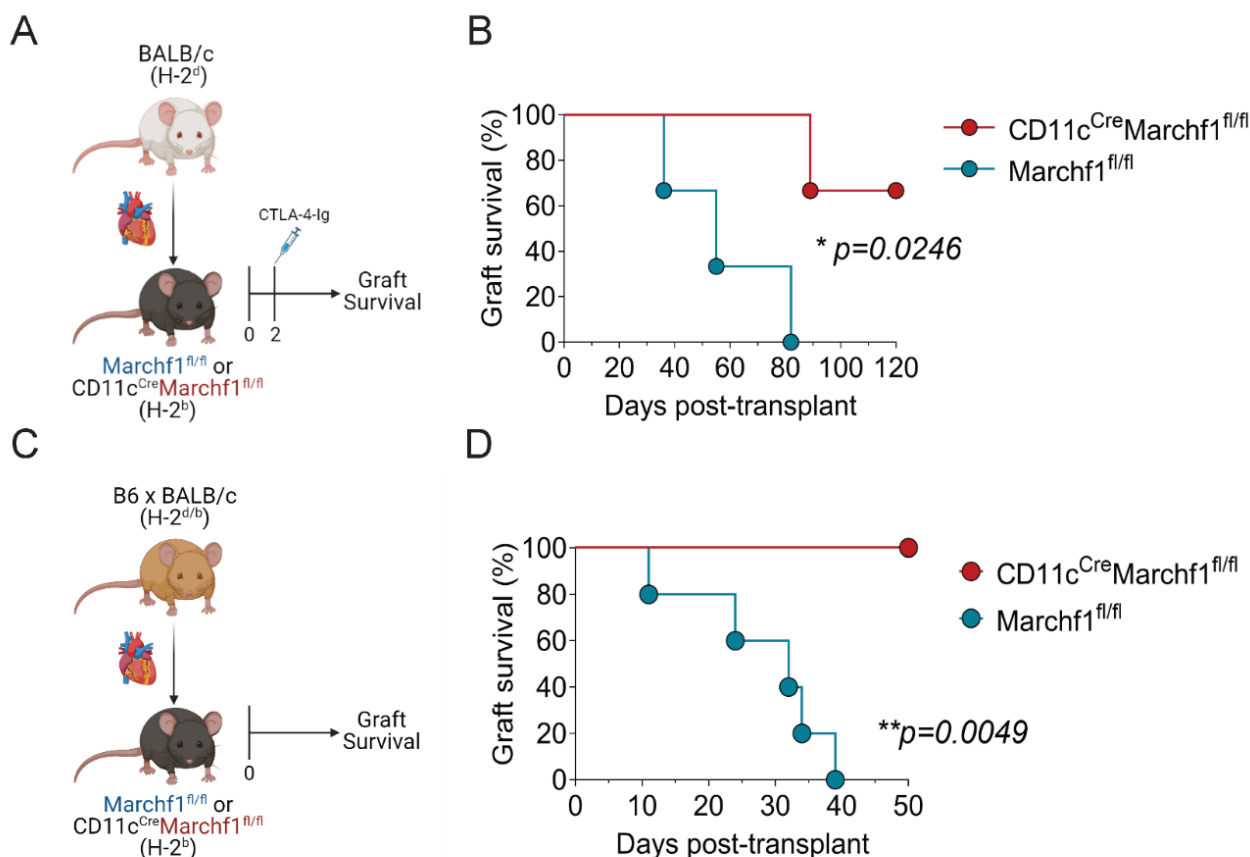


Figura 6. Ausência de *Marchf1* em DCs aumenta a sobrevida do enxerto. (A) Desenho experimental transplante de coração na região abdominal em animais *CD11c*^{Cre}*Marchf1*^{fl/fl} e *Marchf1*^{fl/fl}. Animais BALB/c foram usados como doador. Dois dias após o transplante, os animais transplantados foram tratados com uma dose única de 250 µg CTLA-4-Ig pela via intraperitoneal. O enxerto foi monitorado baseado na palpitação. (B) Curva de Kaplan-Meier de sobrevivência do enxerto. N = 5/grupo. (C) Desenho experimental transplante de coração na região abdominal em animais *CD11c*^{Cre}*Marchf1*^{fl/fl} e *Marchf1*^{fl/fl}. Animais BALB/c x C57BL/c foram usados como doador. O enxerto foi monitorado baseado na palpitação. (D) Curva de Kaplan-Meier de sobrevivência do enxerto. N = 5/grupo.

Uma vez que os estudos em modelos tumorais haviam evidenciado que a ausência de *Marchf1* em camundongos *CD11c*^{Cre}*Marchf1*^{fl/fl} resulta em uma redução das cDC1s e consequente prejuízo na resposta efetora de células T CD8⁺, testamos se o aumento dos números de cDC1s, utilizando Flt3L, poderia acelerar a rejeição do aloenxerto.

Transplantamos corações de B6 x BALB/c F1 em camundongos *Marchf1*^{-/-} e, após o transplante, os animais foram tratados com Flt3L combinado com Poly-IC para aumentar o número de cDC1s (**Figura 7A**). Todos os receptores tratados com Flt3L rejeitaram os enxertos por volta do dia 20 pós-transplante, enquanto os *Marchf1*^{-/-} não tratados mantiveram o enxerto sem rejeição (**Figura 7B**).

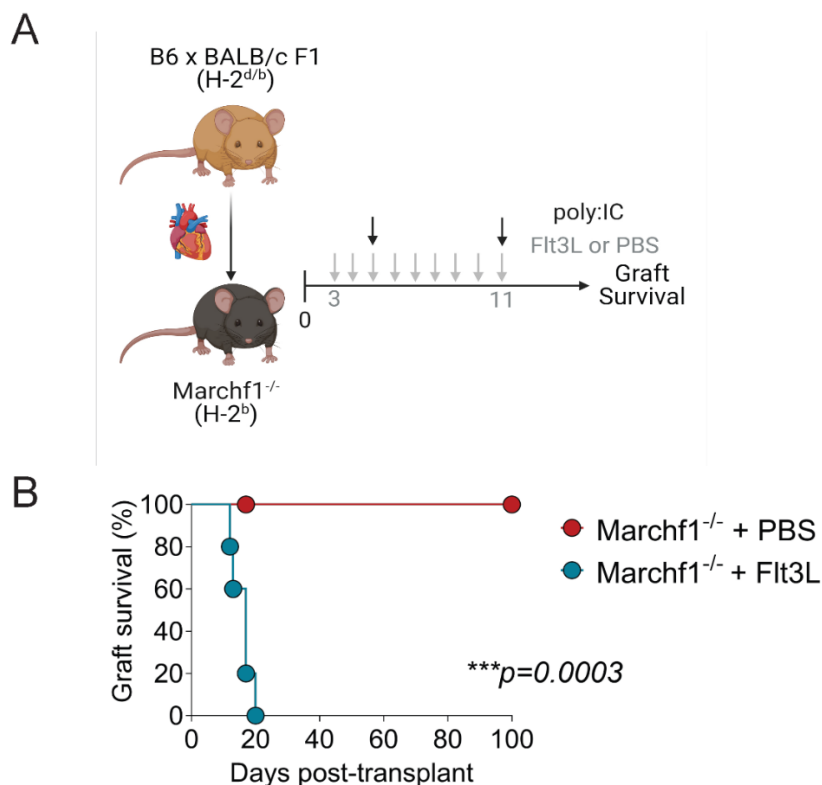


Figura 7. Aumento de cDC1s em animais ausentes de *Marchf1* reverte a sobrevida do enxerto. (A) Desenho experimental do tratamento com Flt3L em camundongos ausentes de *Marchf1*. Animais BALB/c x C57BL/6 F1 foram usados como doador. Os animais que receberam o transplante foram tratados diariamente por 8 dias, começando no dia 3 após o transplante, com 15 µg of Flt3L ou PBS pela via intraperitoneal. Nos dias 5 e 11 após o transplante todos os animais receberam injeção pela via intravenosa de 50 µg of Poly:IC. N = 5/grupo.

Esses resultados sugerem que a ausência de *Marchf1*, especialmente nas DCs, compromete a resposta de células T também em um modelo de transplante, particularmente no compartimento de células T CD8⁺, sendo que o tratamento com Flt3L que leva a um aumento de cDC1s restaura a resposta imunológica de forma semelhante em modelos de tumor e de transplante cardíaco.

6. Capítulo 3

Artigo Científico

Pediatric COVID-19 patients in South Brazil show abundant viral mRNA and strong specific anti-viral responses

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Motivação: Estudar a resposta imunológica aguda de crianças infectadas com o vírus SARS- CoV-2 comparando com adultos com doença leve e severa.

ARTICLE


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OPEN

Pediatric COVID-19 patients in South Brazil show abundant viral mRNA and strong specific anti-viral responses

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COVID-19 manifests as a milder disease in children than adults, but the underlying mechanisms are not fully characterized. Here we assess the difference in cellular or humoral immune responses of pediatric and adult COVID-19 patients to see if these factors contribute to the severity dichotomy. Children's non-specific immune profile is dominated by naïve lymphocytes and HLA-DR^{high}CX3CR1^{low} dendritic cells; meanwhile, children show strong specific antibody and T cell responses for viral structural proteins, with their T cell responses differing from adults by having weaker CD8⁺TNF⁺ T cells responses to S peptide pool but stronger responses to N and M peptide pools. Finally, viral mRNA is more abundant in pediatric patients. Our data thus support a scenario in which SARS-CoV-2 infected children contribute to transmission yet are less susceptible to COVID-19 symptoms due to strong and differential responses to the virus.

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Coronavirus disease 2019 (COVID-19) is a complex disease with multisystemic involvement, and an array of clinical manifestations that can vary from asymptomatic to severe outcomes leading to death¹ constituting an ongoing worldwide emergency². Epidemiological evidence of less severe forms of the disease and reduced mortality in children upon infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is consistent^{3,4}, except for a multisystem inflammatory syndrome (MISC) associated with co-morbidities in a relatively low percentage of children⁵. The pediatric population (0–19 years old) represents more than 25% of the Brazilian population, however, it is observed that this group corresponds to only 1.9% (19,589/989,170) of all cases of COVID-19 reported in the past 12 months. Mortality (case fatality rate, or CFR—the proportion of deaths in identified confirmed cases), among children, represented 0.5% (1564/321,659) of all deaths due to the disease reported in the same period. The lethality in children and adolescents hospitalized due to SARS by COVID-19 was 8.0% (1574/19,589), while the overall lethality in all age groups was 32.5% (321,659/989,170), in the observed period (data from SIVEP-Gripe/Influenza Epidemiological Surveillance Information System, Brazilian Ministry of Health). Thus, a significantly lower number of children and adolescents have severe clinical presentations with the need for hospitalization, or that will lead to death when compared to other age groups.

Different hypotheses are used to explain this phenomenon^{6,7}. Milder disease in children can result from a reduced expression of the viral receptor Angiotensin-converting enzyme 2 (ACE2), leading to lower levels of viral replication⁸. Alternatively, a differential immune response in children leads to a distinct infection course from adults⁹, or yet the pre-existence of neutralizing antibodies to seasonal coronaviruses could confer some cross-protection against SARS-CoV-2 induced disease. Children are considered one of the main reservoirs for these viruses¹⁰, even though some studies show large circulation also among college students¹¹. At present, the scarcity of data prevents a clear understanding of the striking differences between the pediatric and adult outcomes after infection by SARS-CoV-2.

Comprehensive studies have characterized immune responses in adults with mild or severe forms of COVID-19^{12–15}. However, considerably fewer studies have focused on pediatric patients. This is a subject of paramount importance, not only because it is central to the design of public policies regulating school opening (and all the activities associated with it) during the pandemic, but also because understanding the milder disease presentation in children may provide important clues for the design of prevention strategies as well as novel therapeutic pathways for the management of COVID-19.

Here, we present a detailed characterization of plasma and peripheral blood mononuclear cells (PBMCs) from adult and pediatric COVID-19 patients by multi-parameter flow cytometry, defining 78 immune cell subsets. Using a systems approach, we analyze 38,670 data points, including anti-SARS-CoV-2 IgA and IgG antibodies, and frequencies of specific effector T cells. Taken together, our findings suggest that children produce a strong, yet differential immune response when compared to adults, which associates with the mild manifestation in pediatric COVID-19.

Results

Unsupervised analysis of non-specific immune responses in pediatric patients and adults with mild or severe disease. The study design is summarized in Fig. 1A. We have recruited a total of 92 patients (25 children; 34 adults with mild disease—AMD; and 33 adults with severe disease—ASD). All subjects had COVID-19 confirmed by PCR detecting SARS-CoV-2 infection.

All children had mild disease and were treated as outpatients. Their characteristics are described in Table 1. The youngest individual enrolled was 7 months old—which does not appear in the table because only the interquartile interval (IQR) is shown. Most individuals were Caucasian. As expected, comorbidities were concentrated in the group with severe disease, which was also the group with a higher mean age. Some symptoms are probably not accurately assessed in some children, such as anosmia or dysgeusia, due to the age of some individuals in this group. Dyspnea was significantly less frequent in children. Median cycle threshold (Ct) levels for all three probes used in PCR were higher in AMD, and not different between ASD and children.

Comprehensive immune profiling of PBMCs from pediatric and adult patients was performed using flow cytometry, generating 77 variables (frequencies of cell subpopulations and geometric Mean Fluorescent Intensity/gMFI of activation markers). Gating strategies are detailed in Supplementary Fig. 1A–H). To reduce the dimensionality of the numerous variables, a PCA analysis was carried out. A biplot graphic representation of principal component scores PC1 and PC2 indicated that some of the pediatric patients were separated from the mild adult patients (AMD), and the severe adult patients (ASD) (Fig. 1B). Although some overlapping between groups was seen for a few individuals from each group, PC1 separated most of the ASD patients from the pediatric patients, while PC2 separated AMD patients from severe ones and pediatric patients (Fig. 1B). The absence of a perfect separation among groups of patients was not unexpected since some of the individuals from different groups may have similar cell frequency profiles. The PC scores from the three groups were plotted and the significance of the differences was evaluated by a KW test (Fig. 1C). Children had the highest mean score value for PC1 (2.765), followed by AMD (0.142) and ASD (−2.351), and these differences were highly significant (Fig. 1C). For PC2, AMD had a mean score of 2.079, higher ($p < 0.0001$) than children and ASD (mean scores of −1.585 and −1.180, respectively, $p > 0.999$). To further assess the immune responses of each group, we performed a hierarchical clustering analysis based on the original data. The results (Fig. 1E) showed three main clusters—highlighted in different shades of gray. The first cluster is mainly composed of adults with mild disease; the second cluster, mainly of children; and the third, mainly of adults with severe disease. In spite of the resulting clusters not being composed uniquely by individuals of the same group of patients, mild cases represent 72% of the cases in the first cluster whereas children slightly predominate in the second one, and ASD in the third one. Thus, the hierarchical clustering analysis supported the findings from PCA, showing that the immune responses of most of the children differ from adult individuals with severe COVID-19. We also examined principal components PC3, PC4 and PC5 scores for additional information about the differences in the profiles of child and adult patients. PC3 separated children from ASD, as already seen using PC1, whereas PC4 and PC5 did not separate the groups at all (Supplementary Fig. 2A). Principal components are calculated based on correlations among variables, and to interpret the meaning of each PC we analyzed the positive or negative contributions (loadings) of each variable in each PC. The variables with the main positive and negative contributions (loadings) are identified in Fig. 1D, for PC1 and PC2. Respective loadings values are listed in Supplementary Table 1. PC1 had positive inputs mainly by IgM⁺ memory B cells, naïve B cells, and cDC1 DR expression; and main negative contributions by proliferating B cells; plasmablasts; and CX3CR1⁺ expression in dendritic cells (DC). That indicated that the group with the highest mean scores for PC1 (children) would be characterized by a profile of predominantly naïve or low-affinity memory B cells,

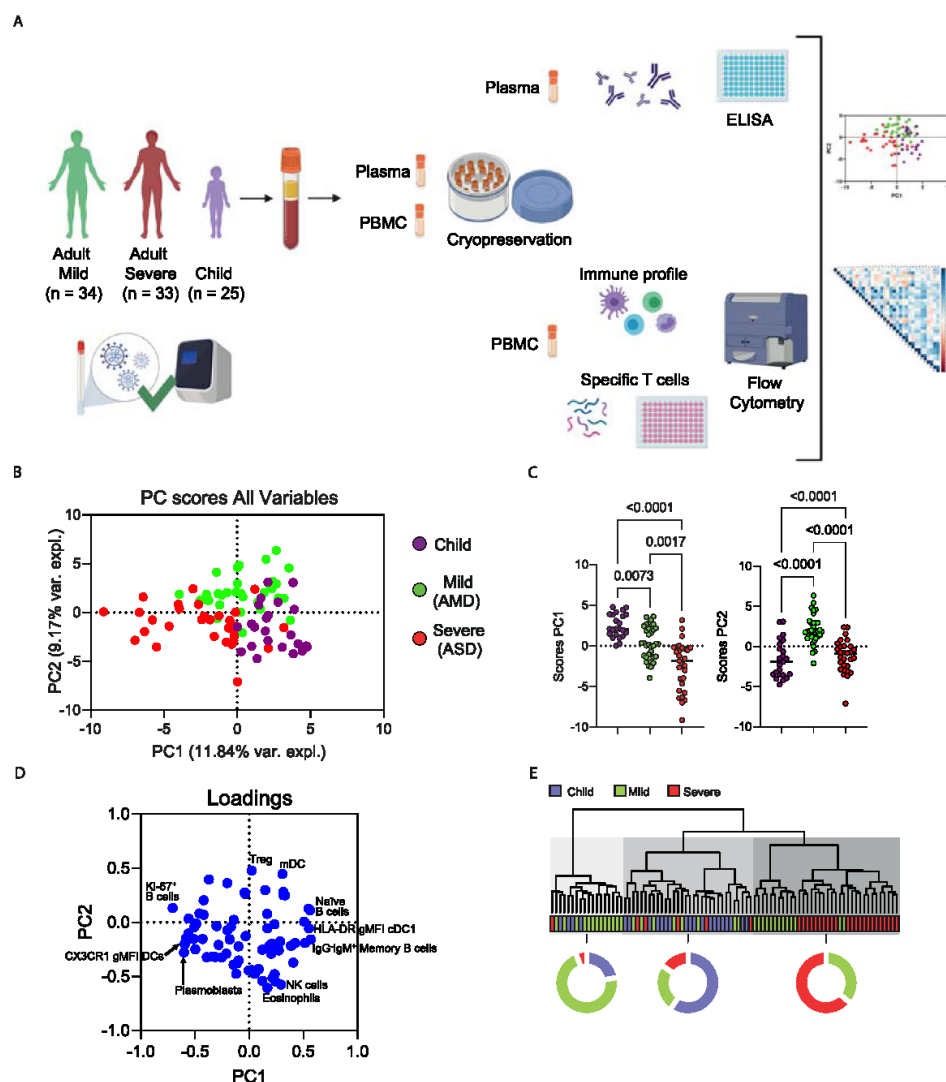


Fig. 1 Experimental approach and differential immune profile of children, mild and adult patients by principal component analysis. **A** Graphical representation of the study design; **B–E** Principal component analysis of the clusters of pediatric (purple) and adult patients with mild (green) and severe (red) disease; each dot represents a patient, color coded. **B** Distribution of clusters by PC1 and PC2; **C** comparison of scores for each PC by analysis of variance (Kruskal–Wallis) with *P* values indicated over brackets, the horizontal bar representing the median. **D** Contribution of variables (loadings) to PC1xPC2. Each blue dot is a variable. **E** Hierarchical clustering analysis of immune variables. Each shade of gray is a cluster with a representative distribution between groups. Graphical study design was created with biorender.com (accessed on 20 October 2021). Source data are provided as a Source Data file.

not activated/differentiated; and their dendritic cells would be high in DR, but low in CX3CR1. The opposite would be true for individuals with the lowest mean scores (ASD), while AMD would be characterized by an intermediate profile for these variables. The main positive influences for PC2 were T regs, myeloid dendritic cells (mDCs), and TEMRA cells, indicating mild adult patients present significantly higher frequencies of these cell subpopulations. The main negative influences for PC2 were eosinophils, natural killer (NK) cells, and granulocytes, and these should be the lowest in AMD. To verify these PCA interpretations, we compared them to the analysis of variance

(KW) results performed among the three groups of patients regarding the three most relevant, positive and negative (Fig. 2) influencers, variables for PC1 and PC2. Comparisons of the three groups for their variances regarding each variable agreed with the differences among them detected by PCA. What emerged from these two combined analyses was that AMD, ASD, and children differed based mostly on the state of activation of B and T lymphocytes, and in targeting innate inflammatory responses to inflamed tissues.

The percent of the total variability explained by these first principal components was low; the first two PCs together

Table 1 Clinical characteristics of all patients in this study.

Characteristics	Mild (n = 34)	Severe (n = 33)	Children (n = 24)	P-value
Age (y), median (IQR)	37.8 (27.0–44.6)	60.8 (38.8–75.9)	7.4 (2.5–13.8)	2.20e-14^a
Female sex, n (%)	22 (64.7)	17 (51.5)	10 (41.7)	0.21 ^b
Active or passive smoking, n (%)	2 (5.9)	6 (18.2)	3 (12.5)	0.08 ^b
Racial or ethnic group				
Caucasian, n (%)	28 (82.4)	20 (60.6)	17 (70.8)	0.22 ^b
Non-caucasian, n (%)	4 (11.8)	1 (3.0)	5 (20.8)	
Days from symptom onset to sample collection				
Days, median (IQR)	18.0 (16.0–20.5)	10.0 (7.5–14.0)	15.0 (7.8–17.0)	1.00e-04^a
Symptoms				
Headache, n (%)	32 (94.1)	23 (69.7)	13 (54.2)	0.02^b
Myalgia, n (%)	30 (88.2)	20 (60.6)	8 (33.3)	1.83e-03^b
Malaise, n (%)	28 (82.4)	31 (93.9)	14 (58.3)	1.26e-03^b
Coryza, n (%)	26 (76.5)	18 (54.5)	15 (62.5)	0.21 ^b
Cough, n (%)	25 (73.5)	30 (90.9)	16 (66.7)	0.07 ^b
Fever, n (%)	23 (67.6)	26 (78.8)	19 (79.2)	0.51 ^b
Chills, n (%)	21 (61.8)	20 (60.6)	9 (37.5)	0.12 ^b
Dyspnea, n (%)	20 (58.8)	22 (66.7)	4 (16.7)	4.57e-04^b
Dysgeusia, n (%)	20 (58.8)	12 (36.4)	6 (25.0)	0.19 ^b
Sore throat, n (%)	19 (55.9)	12 (36.4)	10 (41.7)	0.32 ^b
Appetite loss, n (%)	19 (55.9)	21 (63.6)	12 (50.0)	0.56 ^b
Anosmia, n (%)	19 (55.9)	11 (33.3)	6 (25.0)	0.21 ^b
Stuffy nose, n (%)	17 (50.0)	11 (33.3)	13 (54.2)	0.33 ^b
Conjunctivitis, n (%)	16 (47.1)	10 (30.3)	7 (29.2)	0.28 ^b
Nausea, n (%)	14 (41.2)	12 (36.4)	6 (25.0)	0.64 ^b
Sputum production, n (%)	12 (35.3)	10 (30.3)	6 (25.0)	0.70 ^b
Diarrhea, n (%)	12 (35.3)	16 (48.5)	7 (29.2)	0.25 ^b
Vomiting, n (%)	2 (5.9)	4 (12.1)	4 (16.7)	0.42 ^b
Skin rash, n (%)	1 (2.9)	1 (3.0)	1 (4.2)	0.97 ^b
Underlying medical conditions				
Obesity, n (%)	10 (29.4)	13 (39.4)	0 (0.0)	0.01^b
Hypertension, n (%)	6 (17.6)	15 (45.5)	0 (0.0)	1.00 ^b
Asthma, n (%)	1 (2.9)	2 (6.1)	5 (20.8)	3.77e-05^b
Diabetes mellitus, type 1 and 2, n (%)	1 (2.9)	11 (33.3)	0 (0.0)	0.04^b
Cancer, n (%)	1 (2.9)	2 (6.1)	0 (0.0)	0.73 ^b
Tuberculosis, n (%)	0 (0.0)	1 (3.0)	0 (0.0)	0.73 ^b
Stroke/CVA, n (%)	0 (0.0)	4 (12.1)	0 (0.0)	0.26 ^b
COPD, n (%)	0 (0.0)	4 (12.1)	1 (4.2)	0.43 ^b
Heart failure, n (%)	0 (0.0)	2 (6.1)	0 (0.0)	0.53 ^b
Congenital heart disease, n (%)	0 (0.0)	1 (3.0)	0 (0.0)	0.73 ^b
Ct value (median, IQR)				
ORF1ab	19.3 (16.7–22.6)	24.3 (19.9–29.7)	19.0 (15.4–27.5)	0.05 ^a
S	19.8 (16.7–23.0)	25.3 (21.9–27.7)	19.0 (13.2–28.3)	0.02^a
N	18.7 (16.1–23.5)	24.0 (20.6–28.6)	19.0 (14.1–29.2)	0.02^a
Oxygen use				
Oxygen use during hospitalization, n (%)	0 (0.0)	22 (66.7)	0 (0.0)	8.44e-12^b

IQR = interquartile range.

^aKruskal–Wallis test.^bPearson's Chi-squared test; Significance P-value are bold.

explained only 21% of the variance (Supplementary Table 1). This indicated that these 77 variables were not highly correlated. A correlation analysis using Spearman's coefficient confirmed this observation (Supplementary Fig. 2), revealing a general pattern of moderate to weak correlations, but also identifying clusters of variables that were more correlated than others. These clusters represented six types of immune “signatures”: proliferating/activated T cells; DCs; granulocytes + monocytes; NK cells; B cells; and memory T cells. Follicular helper T cells (T_{fh}) related variables were weakly correlated and were not considered as a cluster. To further identify differences and/or similarities of the general immune responses among the three groups of COVID-19 patients we performed six separated PCAs for the identified clusters of variables. In this analysis, the first two PCs for each cluster were now explained a larger portion of the total variance (44.19, 63.17, 50.37, 67.35, 49.56, and 42.49%, respectively -

Supplementary Table 1) among the three types of patients, and their distributions in children, AMD and ASD were analyzed. The results are shown in Fig. 3A–D and Supplementary Fig. 3A–C, with the respective graphic representations for scores and loadings. We started by analyzing PCs formed by the innate cells' signatures. Principal components for the clusters of Granulocytes + Monocytes (Fig. 3A) and NK cells (Fig. 3B), although derived from expressive correlations among their respective variables, did not separate the three groups of patients, indicating that the individuals were not significantly different for the variables that composed these PCs, even though they were highly correlated (Supplementary Fig. 2B, C). That was intriguing because those variables had, as stated above, important contributions for the PC2 of all variables (Fig. 1), but it also indicated that this contribution helped separate the groups mostly based on their correlations with other variables in the group of all variables,

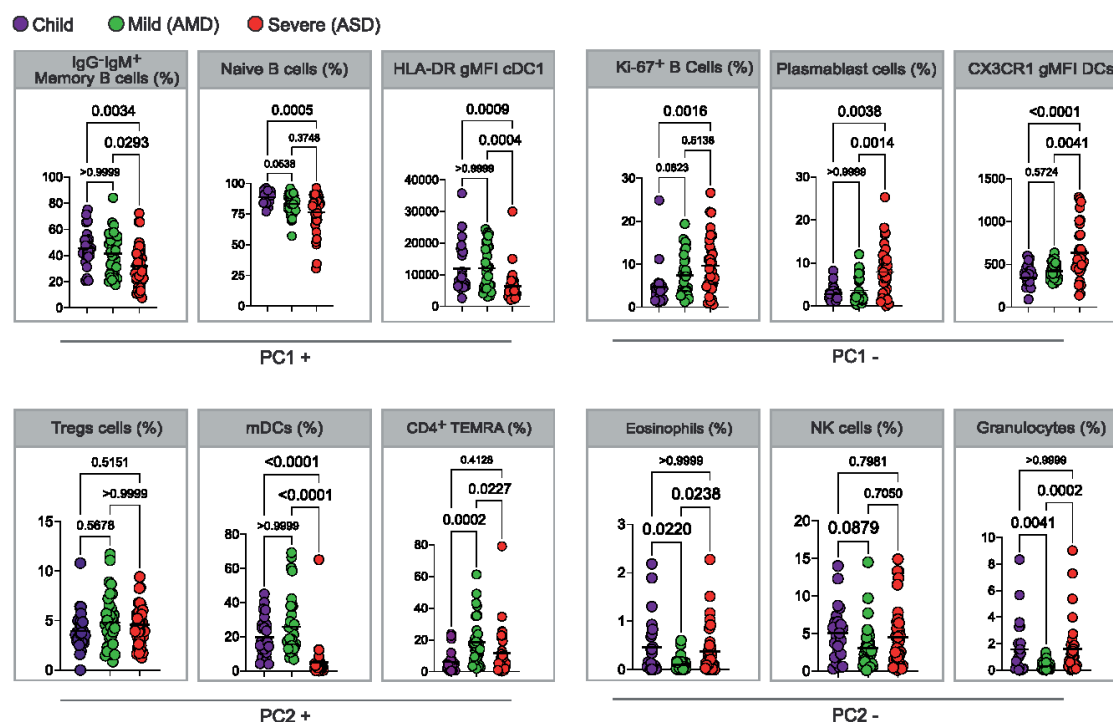


Fig. 2 Analysis of variance of the main variables contributing to principal components. A, B Kruskal–Wallis tests comparing values of each of the three immune variables that presented the highest influences—either positive (**A**) or negative (**B**) for PC1, PC2 and PC3. Each dot represents a patient, color coded: children—purple, adult with mild disease—green, and adult with severe disease—red. *P* values are indicated over brackets, the horizontal bar representing the mean. Source data are provided as a Source Data file.

and not on the differences among groups for those variables alone.

Findings in PCA for the DCs signature (Fig. 3C) indicated that children were significantly separated from ASD, but not from AMD, with lower scores for PC1 (mostly CX3CR1 expression in DCs) and higher scores for frequencies of DCs (Fig. 3C). HLA-DR expression in DCs subpopulations constituted negative contributions for PC1. This indicated that children, as well as AMD, would have more DCs than ASD, high in HLA-DR and low in CX3CR1. That was confirmed by the KW analysis of the three groups (Fig. 3C). Spearman correlation analysis (Fig. 3D) evidenced that CX3CR1 expression was negatively correlated with HLA-DR expression. These results suggested that high DCs frequencies in blood, with low CX3CR1, but high HLA-DR expression could be involved, or at least serve as markers, for mild disease. Conversely, low DCs frequencies, with high CX3CR1 expression, could be associated with more severe disease.

PCA for the clusters involving adaptive cells variables was performed next and are shown in Supplementary Fig. 3. B cells (Supplementary Fig. 3A, Supplementary Table 1) and T cell activation/proliferation (Supplementary Fig. 3B, Supplementary Table 1) PCA corroborated a general pattern of response in children, either separating from the other groups (only sometimes grouping with AMD, apart from ASD). KW analysis of mean scores for each PC showed that in some cases children presented some significant differences from AMD. For example, PC1 of B cells recapitulated findings from the first analysis, mainly positively influenced for IgM⁺ B cells and naive cells, and showed children with significantly higher scores, compared to mild and ASD ($p < 0.01$). Children were grouped with AMD ($p = 0.1599$), and apart from ASD ($p < 0.0001$), regarding PC1 of

T cell activation/proliferation (Supplementary Fig. 3B). The ASD had the highest scores for this PC, highly influenced by activated and proliferating CD4⁺ and CD8⁺ T cells, suggesting that adults with severe disease were characterized by higher frequencies of activated, Ki67⁺ T cells, and the opposite would be observed for children and AMD—though this was not always corroborated by the KW analysis. Finally, PCA for T cell memory clustered variables showed a trend to separate the three groups (Supplementary Fig. 3C). PC1 scores, strongly positively influenced by naive T cells, but negatively influenced by effector memory T cells (TEM), significantly separated children from AMD ($p = 0.0005$), and these somewhat separated from ASD ($p = 0.0410$), indicating children and AMD would have lower frequencies of TEM and higher frequencies of naive T cells compared to ASD. Terminally differentiated memory CD4⁺ and CD8⁺ T cells (TEMRA) were strong positive influences for PC2, while expression of CD69 and CD137 in TEM cells negatively influenced PC2. Children and AMD, with high scores for PC2, did not differ from each other, suggesting they would both be characterized by higher frequencies of TEMRA (especially CD4⁺ TEMRA) cells than ASD, which in turn would have higher frequencies of activated, CD69⁺, CD137⁺, TEM cells. Confirmations of the interpretations of these PCs were again sought in the KW analysis for individual variables next to each PCA result (Supplementary Fig. 3C), and in Supplementary Fig. 4—which compiles all the remaining variables KW analyses results. This led us to note that for TEMRA, children differed from AMD and not from ASD. CD45RA, a marker upregulated both in naive and TEMRA cells, has been reported to show varied expression during the generation of memory pools of chronic infections¹⁹ as well as in response to vaccination²⁰. CD4⁺ TEMRA cells associated with

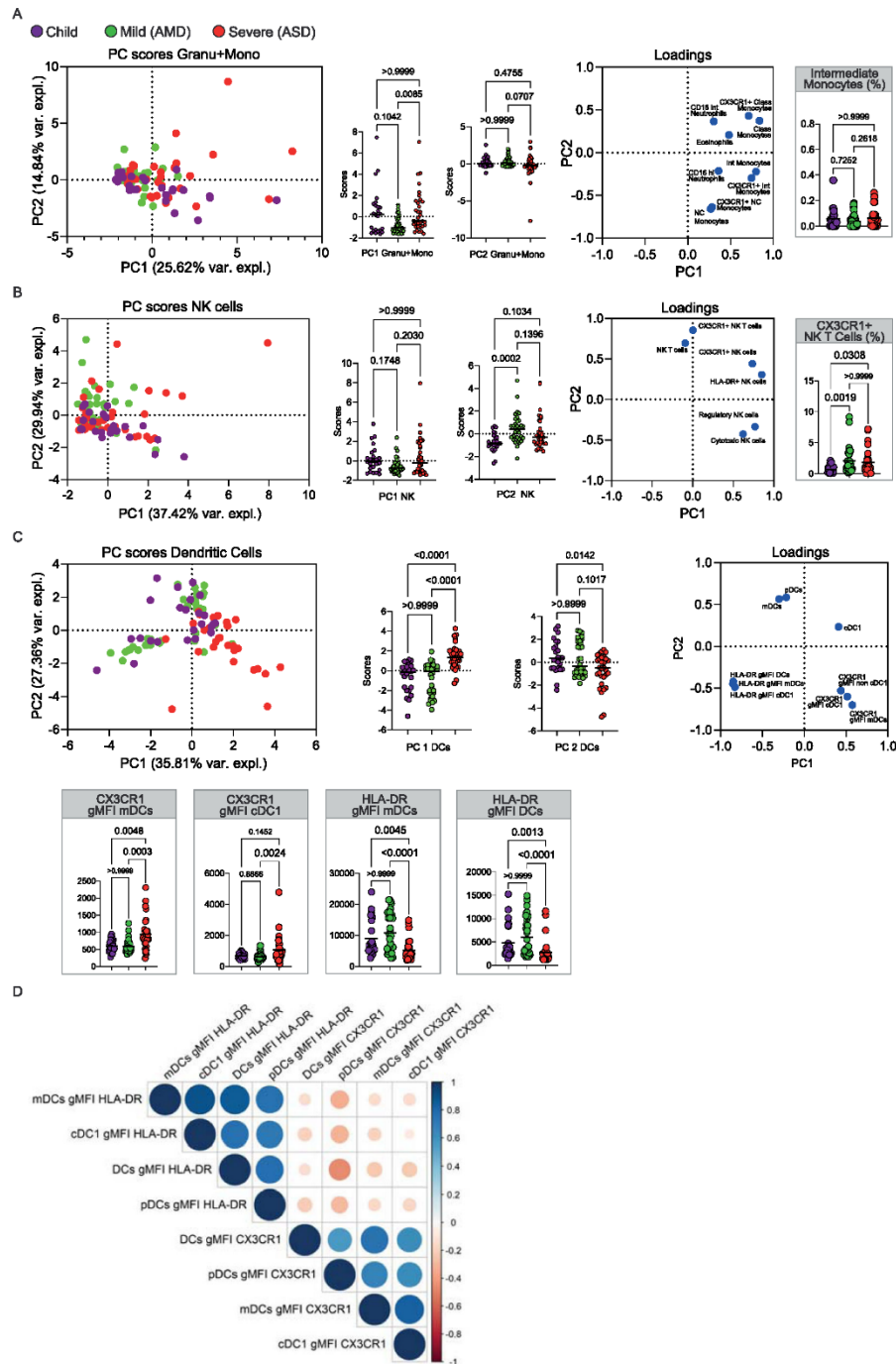


Fig. 3 Principal Component Analysis of innate cells immune signatures. A–G Principal component analysis of the clusters of patients (each dot representing a patient color coded), according to the immune signatures (**A** Granu+Mono, Granulocytes and Monocytes; **B** NK cells; **C** Dendritic Cells; **D** Spearman correlation analysis of HLA-DR and CX3CR1 expression in DCS. For each signature, are displayed the PCA plot of PC1xPC2, the differences in scores of individuals for each PC; the loadings of the main variables contributing to each PC and Kruskal–Wallis tests comparisons of the scores of the major contributing variables values for each group of patients. *P* values are indicated over brackets, the horizontal bar representing the median for scores; and the mean for percentages and gMFI. Source data are provided as a Source Data file.

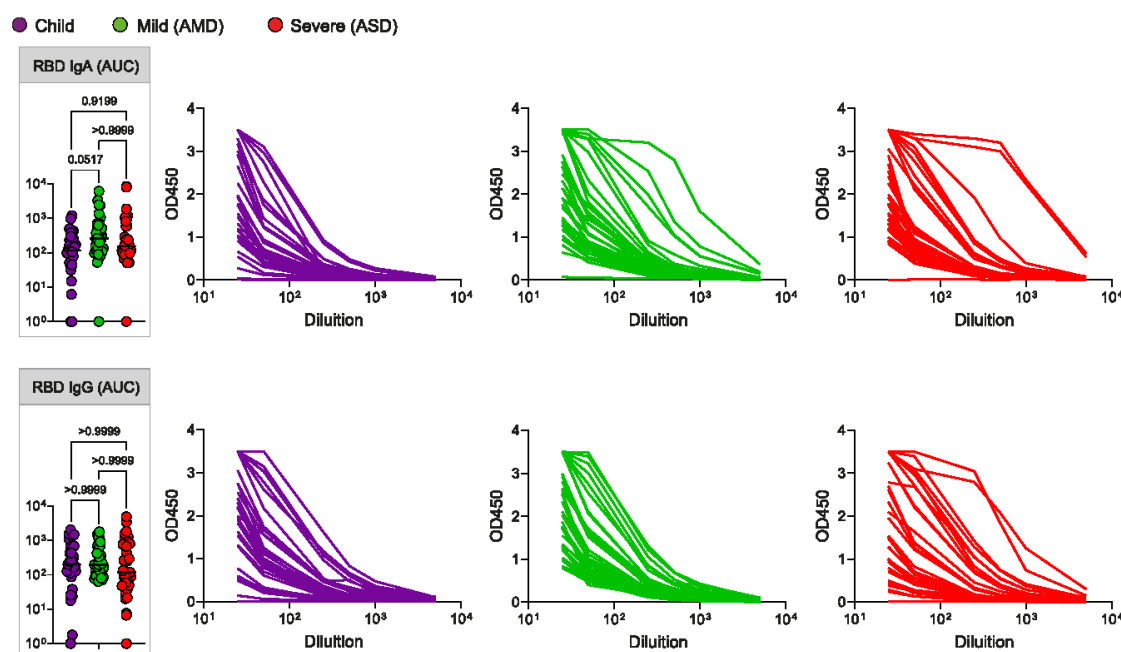


Fig. 4 Antibody responses. SARS-CoV-2 spike RBD IgA and IgG antibody titers determined by ELISA using serial dilutions of plasma. Individual titration curves for individuals (represented by a line, color coded) and analysis of variance (Kruskal-Wallis) of the values calculated as the area under the curve (AUC) for IgA (**A**) and IgG (**B**) are displayed. P values are displayed over brackets, the horizontal bar representing the median. Source data are provided as a Source Data file.

protection in dengue²¹. Thus, the PCA could indicate possible differences for children and AMD, compared to ASD, in pathways for the generation of memory. In the KW comparisons, CD4⁺ TEMRA cells were lower in children than in AMD, and only AMD differed from ASD significantly. For central memory T cells (TCM), there were no differences among groups (Supplementary Fig. 4A). Altogether, the patterns revealed by PCA indicated that children have higher frequencies of non-specific antigen inexperienced B and T cells and DCs, with high HLA-DR and low CX3CR1 expression. In some cases, children and AMD shared not only a mild presentation of the disease but also a similar immune profile. The immune profile of ASD was characterized by higher frequencies and markers of T and B cell activation and proliferation, TEM cells, and lower DCs with high expression of CX3CR1. Finally, we investigated the production of inflammatory plasma cytokines (Supplementary Fig. 4B). Mostly, only subjects with severe disease showed, at least on average, significant higher levels of detectable cytokines in blood, except for IL-4, which was increased in children compared to AMD.

SARS-CoV-2 specific T cells and antibodies responses in children are comparable to the ones of adult patients. The characteristics of the non-specific immune profile of children led us to ask if they had effectively formed SARS-CoV-2 specific responses upon infection. Seroconversion after infection with SARS-CoV-2 patients with all forms of the disease has been described by several studies—reviewed in²². Antibodies to the S protein, and more specifically to the RBD of this protein, are clinically considered a hallmark of infection, and frequently proposed as a correlate of protection. We thus compared children, AMD, and ASD for their RBD specific—IgA and IgG titers. On average, children presented levels of both IgG and IgA comparable to the adult patients

(Fig. 4). In our cohort, although some individuals from the ASD group presented higher levels of antibodies, differences among the groups were non-significant. Our results revealed that, even though children presented a generally naïve, non-activated, immune profile, they had efficiently generated SARS-CoV-2 specific antibody responses, in levels that did not differ from the ones in AMD ($p < 0.103$ for IgA; $p > 0.999$ for IgG) or ASD ($p < 0.916$ for IgA; $p > 0.999$ for IgG) COVID-19 patients.

We next asked if children had generated specific effector T cell responses to SARS-CoV-2. There are four structural proteins in SARS-CoV-2: the spike glycoprotein (S), the envelope (E) protein, the membrane (M) protein, and the nucleocapsid (N) protein. Specific effector T cell responses have been described in adult COVID-19 patients, both with mild and severe disease^{23,24}, however fewer studies have focused on specific immune responses in pediatric patients infected with SARS-CoV-2. We measured the frequencies of CD4⁺ T and CD8⁺ T cells expressing tumor necrosis factor (TNF), gamma-interferon (IFN γ), or interleukin-17 (IL-17) in response to stimulation by peptide pools of the S, N, and M proteins of the virus (Fig. 5). Figure 5A shows representative flow cytometry plots of cytokine-producing CD4⁺ or CD8⁺ T cells upon stimulation with SARS-CoV-2 peptide pools. Negative (DMSO) and positive (PMA + ionomycin) control representative plots can be seen in Supplementary Fig. 5. Children presented detectable CD4⁺ and CD8⁺ T cell responses upon stimulation with all three peptide pools (Fig. 5B). When we compared types of responses in each group for the different peptide pools, children showed a significantly higher TNF+CD8⁺ T response for the M ($p < 0.005$) and for the N ($p < 0.0409$) peptide pools than for the S pool (5B, upper right panel). This was not seen in adults, and although there was a trend for lower CD4⁺TNF⁺ responses in children, it was not significant. Supplementary Fig. 6 shows the responses compared

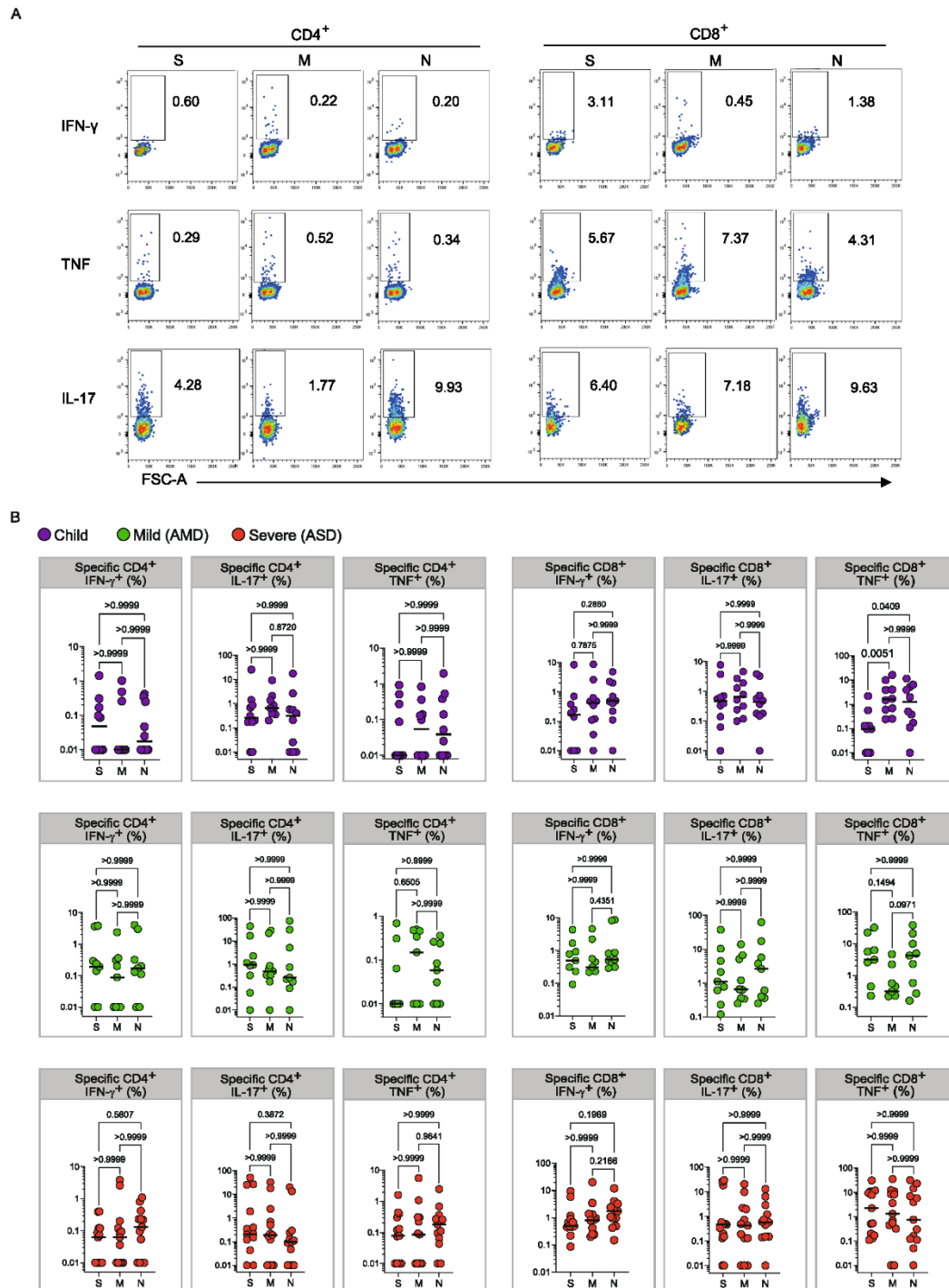


Fig. 5 Specific T cell responses. **A** Gating strategies and typical plots of CD4⁺ and CD8⁺ T cells stimulated with peptide pools from structural proteins spike (S), membrane (M) and nucleocapsid (N), and analyzed by flow cytometry for cytokine production. **B** Comparisons of effector T cells in each group—percentages of CD4⁺ or CD8⁺ cells, producing IFN γ , TNF or IL-17 in response to stimulation by each peptide pool. Each dot represents a patient, color coded: purple for children; green for adults with mild disease and red for adults with severe disease. All analyses are Kruskal-Wallis tests, and the *p* values are indicated over brackets (*n* children = 10, *n* AMD = 9, *n* ASD = 13). Significant differences are indicated by *p* values in a higher font, the horizontal bar representing the median. Source data are provided as a Source Data file.

among the groups. About 30% of individuals—of all groups—did not show responding CD4⁺ T cells to the peptide pools; a higher frequency of individuals did not respond to the S pool compared to the M and N pools. In the ones that responded, CD4⁺ IL-17⁺ T cell responses were higher for all three peptide pools (about 1 log higher than IFN γ and TNF CD4⁺ T cell responses). CD8⁺ T cell responses were, in general, more robust, although for S and M peptide pools there were still some individuals, though fewer, that did not respond to stimulation. The absence of response, in our sample, did not correlate with the early time of collection, as reported by¹⁹. TNF⁺CD8⁺ T cell responses were about 1 log higher than what was detected for CD4⁺ T cells, for all peptide pools (Supplementary Fig. 6). The IL-17⁺CD4⁺ T cell responses to stimulation by all three pools were higher than the TNF⁺CD4⁺ and IFN γ ⁺CD4⁺ for all three groups. The differential TNF⁺ cytotoxic response to M and N peptides seen in children led us to investigate levels of anti-N antibodies. Children made strong anti-N IgG levels, not different from AMD and ASD (Fig. 6A). Anti-RBD IgA, but not IgG levels, correlated positively with CD4⁺ IFN γ ⁺ responses (Fig. 6B). Interestingly, the TNF⁺ cytotoxic responses to M and N peptide pools were inversely correlated with levels of anti-RBD and anti-N antibodies (Fig. 6B). Anti-N antibody levels correlated positively with anti-N CD4⁺ IFN γ ⁺ responses (Fig. 6B). Taken together, these results indicate that children do generate specific humoral and effector cell responses upon infection with SARS-CoV-2, with a differential, higher cytotoxic response against proteins M and N, not associated with antibody responses to the spike protein.

Discussion

It is clear from our study as well as from others²⁵ that children do get infected by SARS-CoV-2, and thus possibly contribute to the community-based spread of the virus, contrary to what is suggested by studies on the low nasal ACE2 expression in children²⁶. The lower rates of infection in children can be biased by lower testing, as pointed out by²⁷, and should be more carefully studied, given its importance for planning school openings. Our findings on the more naïve, non-specific lymphocyte profile presented by in children, if taken isolated from the other results in this study, may indicate that a naïve immune system does better than an old one, as has been suggested⁹ and that children would be better equipped to mount fast and efficient immune responses to rapidly clear the virus. However, a prediction from this hypothesis is that children would be more likely to present mild forms of all viral diseases, and this is not the case. While milder manifestations are observed in Middle East respiratory syndrome (MERS), SARS, and varicella, the opposite is observed for infection with polio-virus, and respiratory viruses, especially influenza and respiratory syncytial virus (RSV) - reviewed in⁶.

Our data indicate that not only the adaptive but also their innate immune system has characteristics that enable children to mount an immune response that controls the infection. The differences observed for dendritic cells might offer an important clue. DCs play crucial roles in initiating and shaping the adaptive response, and subpopulations of DCs, especially pDC, are determinant for the generation of efficient antiviral responses, being one of the main sources of type I interferon²⁸. A previous study in COVID-19 adult patients indicated decreased activation and numbers of DCs²⁹. In our study, children consistently show higher frequencies of DCs, including pDC, compared to adults. High HLA-DR expression is characteristic of mature DCs; as they become activated by engagement of pattern recognizing receptors, HLA-DR first increases, and then decreases as DCs migrate to draining lymph nodes^{28,30}. Low HLA-DR expression in children is associated with immune suppression⁴ and acute inflammatory conditions³¹.

The inverse correlation of HLA-DR with CX3CR1 in DCs is intriguing. CX3CR1, also known as the fractalkine receptor, is considered a homing marker for inflamed tissue and plays a role in pathology in Japanese virus-induced encephalitis³² and peritoneal vasculitis in a sepsis model³³. The high levels of HLA-DR in children's DCs indicate that their cells are not poorly activated, but mature and able to generate efficient immune responses. Heinonen et al. integrated blood transcriptomics and cell profiling with clinical data in a cohort of 190 children with RSV and found low HLA-DR in monocytes to be associated with severe disease³⁴. The low expression of CX3CR1 in our samples suggests that children's DCs are not targeted to inflamed sites, as they seem to be in ASD. Our results indicate that expression of CX3CR1 in circulating DCs could associate, or serve as a marker for, pathological mechanisms in severe COVID-19, suggesting that inflammation to specific sites such as the lung may be affected by age, and that low CX3CR1 expression in DCs might favor the generation of antiviral responses. Loske et al.³⁵, in a single-cell transcriptomics profile of nasal samples, suggested a pre-activated state of the antiviral innate immune cells in children - DCs expressing higher levels of MDA5 and RIG I, two pattern recognition receptors relevant for antiviral responses. Also, children showed a KLRG1-expressing population of memory CD8⁺ T cells. Both data concur with our findings, which support a role for potentially responsive DC and effector cytotoxic T cell populations in the differential, possibly protective, immune response in children that is associated with milder disease.

Pediatric patients in our sample presented SARS-CoV-2 specific antibodies and T cell responses in levels comparable to adult patients. The higher TNF⁺CD8⁺ responses for the M and N proteins could be associated with a protective response in children. The M protein is the most abundant structural protein on the surface of the virus³⁶, potentially constituting an important target of immune responses. A study by Thieme et al. found anti-M CD4⁺ T cells as the highest T cell response in critical COVID-19 patients³⁷. In that study, CD4⁺ T, rather than CD8⁺ T cell immunity to SARS-CoV-2 proteins dominated the response in severe and critical patients, indicating that a robust CD4⁺ T cell response to these antigens did not correlate with protection. The nucleocapsid (N) protein is structural, abundantly produced upon infection, and highly conserved among beta coronaviruses³⁸. It was a major target for early B cell responses in the SARS epidemic of 2003³⁹. Recent studies^{18,40} found strong anti-N T cell responses in SARS-CoV-2 patients, and in individuals who recovered from SARS, supporting a relevant role for structural N protein as an immune target in SARS-CoV-2 infection. We found robust antibody responses in children both against the S RBD and N protein, while Weisberg et al.⁹ found antibodies to the S, but not the N protein; and Cohen et al.⁴¹ found lower responses in children in general. These differences, as well as a lower response to the S protein in general in our sample, may reflect differences in HLA between American and Brazilian populations, or even a difference in immunization history, given a tradition in vaccination programs for children in Brazil. The correlations of antibody responses with CD4⁺ T cells are somewhat expected, given the help needed for antibody production, and indicate that these responses are somewhat coordinated, but also that not all antibodies produced are linked to TNF or IFN γ help. The inverse correlation between specific CD8⁺ T cell responses and antibodies may indicate a relevant role for cytotoxic immunity against SARS-CoV-2, beyond antibody production.

A hypothesis frequently raised to explain milder disease in children with COVID-19 is that the presence of neutralizing antibodies to such viruses could cross-protect them upon infection with SARS-CoV-2. However, a recent study in adults found no evidence of cross-protection associated with levels of these antibodies⁴². Alternatively, protection could be conferred

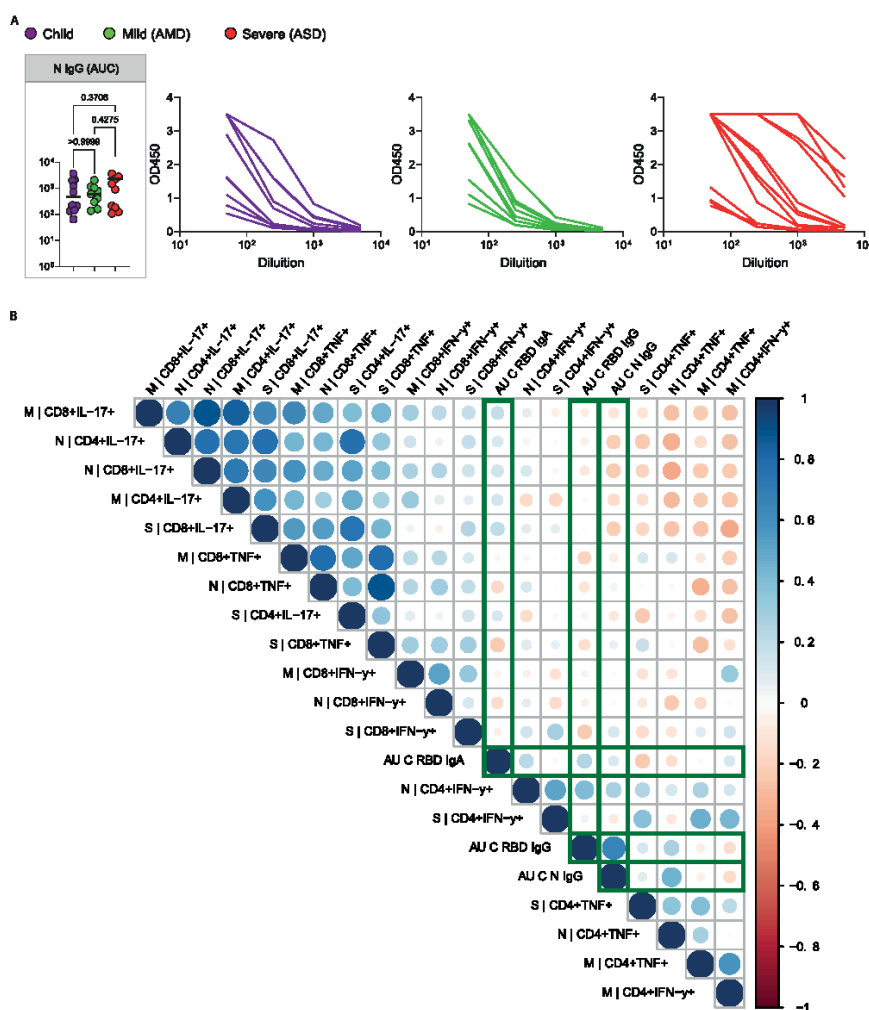


Fig. 6 Anti-N IgG response and correlation of specific responses. **A** SARS-CoV-2 anti-N IgG antibody titers determined by ELISA using serial dilutions of plasma. Individual titration curves for individuals (represented by a line, color coded) and analysis of variance (Kruskal-Wallis) of the values calculated as the area under the curve (AUC) for IgG (**A**) are displayed. *P* values are displayed over brackets, the horizontal bar representing the median. **B** Matrix representing a Spearman correlation analysis of specific effector T cells responses (in percentages of CD4⁺ and CD8⁺ cells expressing cytokines in response to peptide pools) and the antibody response to the RBD of the spike protein and to the N protein (represented as values for the AUC). Correlations between the specific effector CD8⁺ T and CD4⁺ T cells frequencies, and the antibody (AUC—area under the curve) values for the RBD and N protein are highlighted. Source data are provided as a Source Data file. *n* children = 10, *n* AMD = 9, *n* ASD = 13.

not by cross-reactive antibodies, but rather by pre-existing N-protein-specific T cells. Most studies—and most vaccines—have so far focused on protection against SARS-CoV-2 infection by antibodies to the spike protein. Both screen studies by Ferretti et al.⁴⁰ and Ng et al.¹⁰ indicated that T cell immunity to SARS-CoV-2 infected individuals includes many targets outside the spike protein and that they are not conserved among coronaviruses that cause the common cold. These findings agree with the ones of the Le Bert study²⁴. Our results support that the role of T cell responses to the N protein must be further investigated, with a more detailed T cell epitope mapping. Such work might reveal additional correlates of protection, and/or epitopes to add in the next generation of COVID-19 vaccines. A recent report⁴³ indicated that T cell immunity was not markedly affected, so far, by the emergence of new variants, supporting the identification of T cell epitopes to be added to the next vaccines.

The main limitation of this study is that it is mostly an exploratory, descriptive one, and compares individuals in different age groups. A second important limitation is the small sample size. We believe this was a valid approach given the magnitude of what is still unknown regarding pediatric immunity in COVID-19. Biomarkers in peripheral blood are only useful when highly correlated with outcomes. Yet, most studies that seek to understand how immune responses can correlate with protection compare adults with mild and severe disease, and it is known that these are in different age groups. At present, the best age-matched controls for SARS-CoV-2 infected patients are still unknown. Certainly, the absence of a pre-pandemic healthy children control group is a limitation of this study and all the other ones that focused on the general, non-specific immune profile of children with COVID-19. There is still much to be understood about immunological differences not only between pediatric and adult COVID-19 patients but also in other diseases. At the time the

project started, COVID-19 numbers in Brazil were still not high and the frequency of MISC patients or children with severe manifestations of the disease was still too low to include. The study on this cohort is still ongoing, with two more points of sample collection. We expect that further analysis of our data, as well as other studies, on immune profile data and specific responses, will bring relevant information on the generation of immune memory in pediatric COVID-19.

Methods

Ethics statement. This study was approved by the Institutional Review Board (IRB 30749720.4.1001.5330) at Hospital Moinhos de Vento and Ethics Committee from Fundação Faculdade Federal de Ciências Médicas de Porto Alegre (CEP-UPCSPA) (CAAE 30749720.4.3001.5345). Informed consent was obtained from all participants or their legal guardians. The study was conducted according to good laboratory practices and following the Declaration of Helsinki.

Patients. A prospective cohort study was carried out at Hospital Moinhos de Vento and at Hospital Restinga e Extremo Sul, both in Porto Alegre, southern Brazil. A convenience sample of adults and children older than 2 months were enrolled from June to December 2020 at either the outpatient clinics (OPC), emergency rooms (ER), or hospitalized. Subjects were screened if presenting cough and/or axillary temperature $\geq 37.8^\circ\text{C}$ and/or sore throat. Both blood samples and respiratory samples collected through nasopharyngeal swabs were obtained at enrollment. Only patients with the clinical diagnosis of COVID-19 and SARS-CoV-2 infection confirmed by RT-PCR were included in the study. Clinical and demographic data were collected at inclusion, following a standardized protocol. Disease severity was classified according to the World Health Organization classification following a standardized protocol. Disease severity was classified accordingly after completing the follow-up questionnaire¹⁶.

SARS-CoV-2 RT-qPCR. A qualitative RT-PCR assay to SARS-CoV-2 was performed for all participants. Bilateral nasopharyngeal and oropharyngeal swabs were collected and placed in the same transport medium with saline solution and RNAlater[®], RNA Stabilization Solution (Catalog number AM7021, Invitrogen[™]). MagMax[™] Viral/Pathogenic Nucleic Acid Isolation Kit (Applied Biosystems) was used to extract viral RNA in the KingFisher Duo Prime System (ThermoFisher, USA) automated platform. The RT-PCR assay was performed in 10 μL total reaction, using Path[™] 1-Step RT-qPCR Master Mix, CG (catalog number A15299, Applied Biosystems) and TaqMan[™] 2019-nCoV Assay Kit v1 (catalog number A47532, Applied Biosystems) which comprises the SARS-CoV-2-specific targets (gene ORF1ab, gene S and gene N). As reaction control, we used 5 μL (200 copies/ μL) of the TaqMan[™] 2019-nCoV Control Kit v1 (catalog number A47533, Applied Biosystems). QuantStudio 5 (ThermoFisher Scientific, USA) was used to perform the PCR.

PBMC isolation and cryopreservation. Blood was collected in EDTA tubes (Firstlab, PR, Brazil) and stored at room temperature before processing for PBMC isolation and plasma collection. Plasma was separated by centrifugation and cryopreserved. PBMCs were next isolated by density-gradient centrifugation using Ficoll-Paque[™] PLUS (GE Healthcare[®]), and either studied directly or resuspended in fetal bovine serum (FBS) 5% DMSO and stored in liquid nitrogen until use.

Flow cytometry. Cells were thawed by diluting them in 5 mL pre-warmed complete RPMI1640 medium (Sigma-Aldrich - R8758) containing 5% FBS and spun at 1500 rpm for 5 min. Supernatants were carefully removed, and cells were resuspended in PBS. After, were stained with the BD Horizon[™] Fixable Viability Stain 510 together with antibodies for surface markers, as follows: anti-CD3-APC-H7 (clone SK7), anti-CD24-APC-H7 (clone ML5), anti-HLA-DR-APC-H7 (clone G46-6), anti-CD4-PerCP-Cy5.5 (clone RPA-T4), anti-CD27-PerCP-Cy5.5 (clone M-271), anti-CD11c-PerCP-Cy5.5 (clone B-ly6), anti-CD14-PerCP-Cy5.5 (clone M5E2), anti-CD8-FITC (clone HIT8a), anti-IgG-FITC (clone G18-145), Lineage 2-FITC (cat. 643397), anti-CD16-FITC (clone 3G8), anti-CXCR5 (CD185)-BB515 (clone RF8B2), anti-CD19-APC (clone H1B19), anti-CD127-Alexa 647 (clone HIL-7R-M21), anti-CX3CR1-Alexa647 (clone 2A9-1), anti-CD69-APC (clone FN50), anti-CD38-PE (clone HIT2), anti-ICOS (CD278)-PE (clone DX29), anti-CD141-PE (clone 1A4), anti-CD66b-PE (clone G10F5), anti-CD137 (4-1BB)-PE (clone 4B4-1), anti-HLA-DR-PE-Cy7 (clone G46-6), anti-CD19-PE-Cy7 (clone SJ25C1), anti-CD25-PE-Cy7 (clone 2A3), anti-CD45RA-PE-Cy7 (clone L48), anti-IgM-BV421 (clone G20-127), anti-PD-1 (CD279)-BV421 (clone MIH4), anti-CD303-BV421 (clone V24-785), anti-CD56-BV421 (clone NCAM 16), anti-CCR7-BV421 (clone 2-L1-A) antibodies. For intracellular staining, cells were first stained for surface markers and subsequently fixed and permeabilized using the Transcription Factor Buffer Set (BD Biosciences-Pharmingen, USA), then stained with anti-Ki-67-BV421 (clone B56), anti-Perforin-Alexa 647 (clone δG9), and anti-Granzyme B-BV421 (clone GB11) antibodies. Following in vitro stimulation assays with specific peptides, cells were stained with the BD Horizon[™] Fixable Viability Stain 510 and

anti-CD3-PE-Cy7 (clone SK7), anti-CD4-PerCP-Cy5.5 (clone RPA-T4), anti-CD8-APC-H7 (clone SK1), anti-CCR7-BV421 (clone 2-L1-A), and subsequently fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences-Pharmingen, USA), then stained with anti-IFN γ -FITC (clone 4 S.B3), anti-TNF (clone MAb11) and anti-IL-17-PE (clone SCPL1362) antibodies. Antibody dilutions are available in supplementary Table 2. All samples were analyzed using BD Biosciences - FACSCanto II and FlowJo 10.7.1 software.

Th1/Th2/Th17 cytokine measurements. The concentration of seven cytokines, IFN- γ , TNF, IL-2, IL-4, IL-6, IL-10, and IL-17A in serum were measured using a commercial BD CBA Human Th1/Th2/Th17 cytokines kit (cat 560484, BD Inc., USA). Briefly, a mixture of seven capture beads (with distinct fluorescent intensities) coated with capture antibodies specific for each cytokine and a phycoerythrin (PE) detection reagent were used as the manufacturer's instruction. Then, samples (plasma were heat-inactivated at 56°C for 60 min) were acquired and measured on the BD FACS Canto II flow cytometer and analyzed by PCAP Array software 3.0. Individual cytokine concentrations were indicated by their fluorescent intensities. The value of each cytokine was normalized by the limit of detection.

In vitro T cells stimulation assays. PBMC were thawed, assayed for viability, counted, and plated in 96-well plates at 3×10^6 PBMCs/mL, 100 μL /well in RPMI1640 medium (Sigma-Aldrich - R8758) supplemented with 10% FBS (100 IU of penicillin/mL, 100 μg of streptomycin/mL (Lonza, Belgium) and 2 mM L-glutamine (Lonza, Belgium) (R10H medium), and subsequently stimulated with peptide PepTivator SARS-CoV-2 Prot S (130-126-700 - Miltenyi Biotec, Germany), PepTivator SARS-CoV-2 Prot N (130-126-698 - Miltenyi Biotec, Germany) and PepTivator SARS-CoV-2 Prot M (130-126-702 - Miltenyi Biotec, Germany) at 1 μg /mL. Phorbol 12-myristate 13-acetate (PMA/50 ng/mL, Sigma, USA) plus ionomycin (1 μg /mL, Cayman chemical company, USA) and DMSO were used as positive and negative controls for stimulation, respectively. Stimulation with a cytomegalovirus (CMV) peptide pool at 2 μg /mL (Mabtech, Sweden) was also performed, as a positive control for the assay. All treatments were submitted for 18 h at 37°C and 5% CO_2 , 3 h before harvesting. Golgi Plug (BD Biosciences, USA) 1 μg /mL was added to each well. Cells were stained and analyzed for phenotype as described above.

Enzyme-linked immunosorbent assay (ELISA). Plasma was tested for IgG and IgA antibodies to S-RBD protein (#RP-87678 - Invitrogen, USA) and N protein (kindly provided by Dr. Ricardo Gazinelli - Fiocruz Belo Horizonte, Brazil) using a protocol described in¹⁷. Briefly, ELISA plates (Kasvi, Brazil) were coated overnight with 1 μg /mL of SARS-CoV-2 Spike Protein (S-RBD; Catalog nr. RP-87678 - Invitrogen). On the following day, plates were blocked for 1 h at room temperature with blocking buffer (3% skim milk powder in phosphate-buffered saline (PBS) containing 0.05% Tween-20). Plasma samples were heat-inactivated at 56°C for 60 min and then serially diluted in 1% milk in 0.05% PBS-Tween 20 starting at a 1:25. Plasma was incubated for 2 h at 37°C . Secondary antibodies were diluted in 0.05% PBS-Tween and incubated for 1 h at room temperature. For both IgG, anti-human peroxidase produced in rabbit (#IC-1H01 - Rhea Biotec, Brazil), and IgA, anti-human peroxidase produced in goat (#A18781 - Invitrogen, USA), was used at a 1:10,000 dilution. The assay was developed with TMB Elisa Substrate - High Sensitivity (Abcam, United Kingdom) for 30 min, and the reaction stopped with 1 M chloric acid. Readings were performed in an ELISA reader (Biochrom EZ 400), and O.D. at 450 nm was used to calculate the area under the curve (AUC), using a baseline of 0.07 for peak calculation¹⁸.

Statistics and systems analysis. Percentages were used to describe categorical variables. Pearson's Chi-square test was used to evaluate proportions among children, AMD and ASD. Data normality assumptions were verified for continuous variables and summarized in terms of the median and interquartile range (IQR). A two-tailed Kruskal-Wallis (KW) test followed by Benjamini-Hochberg correction for multiple comparisons was used to compare p-values among the groups. Principal Component Analysis (PCA) was employed to reduce the dimensions of 78 immunological variables generated by flow cytometry analysis, to explain the total variability with a smaller, new set of variables. Spearman correlations were performed between all variables (every set of two variables) and within sets of variables to identify clusters of correlated variables. In PCA of the variables grouped in clusters, variables containing redundant information were excluded. Comparison among groups regarding single variables or PC values was performed by non-parametric Kruskal-Wallis test, followed by 2 by 2 multiple comparisons with p-values adjusted accordingly. Analyses were performed either in GraphPad Prism v.9 or R and sometimes confirmed in Python. Hierarchical clustering analysis was performed using R and a dendrogram was created for the COVID-19 patients using Canberra as the distance measure and Ward's (ward.D) as agglomeration method. The heights used to merge clusters were manually defined.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding authors on reasonable request. Source data are provided with this paper.

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Author contributions

T.F., K.L., P.O.S. and J.C.F. all designed and performed experiments, analyzed data, wrote and edited the manuscript. G.H., R.Z., V.N.P., J.C.F.N., A.F.H., I.G., A.C.O. and R.S.M. assisted in experiments. M.B.B.G. performed computational analysis. R.B.G. assisted in flow cytometry analysis and interpret the results. L.D.C., A.B. and R.E.M. helped with data collection. S.M.C.J. coordinated all statistical analysis; I.T.S.S. helped in statistical analysis. L.R.J. helped design the study and edited the manuscript. G.O.Z., I.R.F., F.H.V. and M.P.B. helped collect patients' samples and performed RT-PCR analysis. C.S.G. helped collect patients' samples. H.I.N. performed clustering analysis. T.J.B. helped design the study, analyzed data and edited the manuscript. M.S.C.C., J.A.S. assisted with epidemiological information. M.C.S. and R.T.S. coordinated the cohort, conceived and designed the study, wrote and edited the manuscript. C.B., conceived and designed the study, performed data analysis, performed principal components analysis, wrote and edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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7. Capítulo 4

Artigo Científico

SARS-CoV-2 infected children form early immune memory responses dominated by nucleocapsid-specific CD8⁺ T cells and antibodies

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SARS-CoV-2 infected children form early immune memory responses dominated by nucleocapsid-specific CD8+ T cells and antibodies

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This is the third year of the SARS-CoV-2 pandemic, and yet most children remain unvaccinated. COVID-19 in children manifests as mostly mild or asymptomatic, however high viral titers and strong cellular and humoral responses are observed upon acute infection. It is still unclear how long these responses persist, and if they can protect from re-infection and/or disease severity. Here, we analyzed immune memory responses in a cohort of children and adults with COVID-19.

Important differences between children and adults are evident in kinetics and profile of memory responses. Children develop early N-specific cytotoxic T cell responses, that rapidly expand and dominate their immune memory to the virus. Children's anti-N, but not anti-S, antibody titers increase over time. Neutralization titers correlate with N-specific antibodies and CD8⁺T cells. However, antibodies generated by infection do not efficiently cross-neutralize variants Gamma or Delta. Our results indicate that mechanisms that protect from disease severity are possibly different from those that protect from reinfection, bringing novel insights for pediatric vaccine design. They also underline the importance of vaccination in children, who remain at risk for COVID-19 despite having been previously infected.

KEYWORDS

Memory T cell, antibodies, neutralizing antibodies, variants of concern, N protein, COVID-19

Introduction

After almost three years of the SARS-CoV-2 pandemic, most children remain yet to be vaccinated against COVID-19. Children are often asymptomatic or develop mild disease upon infection. Some have questioned the need or urgency to vaccinate those under 18 years old, arguing that immunity to infection is sufficient for protection against the virus. Nevertheless, a recent study from the Center for Disease Control (CDC) (1) reports that the greatest increase in seroprevalence in the United States during 2022 was in children: 44.2 to 75.2% (0-11 years old) and 45.6 to 74.2% (12-18 years old). A recent US study reported an incidence of 316 cases of Multisystem inflammatory syndrome (MIS-C) per million of infected children (2). In Brazil, in 2022, until epidemiological week 24, 11,453 children (0-19 years old) were hospitalized for covid-19 and of these, 538 died. These epidemiological data are relevant and show the impact of SARS-CoV-2 infection for children not only for risk of COVID-19 disease, but also for possible and still unknown effects of SARS-CoV-2 infection (3). Some studies provide evidence that children may develop long COVID-19 (4, 5), reinforcing the need for a more thorough analysis of children's immunity to infection by SARS-CoV-2.

Most of the information available on immune memory to the virus, whether generated by infection or vaccination, comes from studies on adults. Also, most of these studies focus on the responses against the Spike protein (S) of the virus, especially against its receptor binding domain (RBD). These currently constitute the source of data used to estimate the protective potential of immune responses. While different studies highlight

the important role of innate immunity, especially of type I interferon (6), mechanisms and relative contributions of specific adaptive responses are less clear. Some propose neutralizing antibodies (NAbs) to the RBD as the best candidate correlate of protection (7) and others have shown that even when neutralization capacity wanes, T cells can still cross-recognize variants and protect against different SARS-CoV-2 variants (8), mostly because of T cell epitope conservation (9), with many of these epitopes come from sequences outside the S-protein coding region (10).

Fewer studies have focused on immune responses in COVID-19-affected children. We have previously compared immune responses in a sample of children and adults infected by SARS-CoV-2 (11), which revealed differences that might be related to protection from disease. The main difference observed was a predominant CD8⁺TNF- α ⁺ T cell response against the nucleocapsid (N) protein in children. Here, we performed a longitudinal follow-up analysis of immune memory responses 3 and 6 months after infection. Children developed an early and sustainable CD8⁺TNF- α ⁺ memory response to the N protein that is preferentially differentiated into terminal memory cells. This response is positively correlated with anti N, but not anti-S, antibody titers. Children produce durable antibody responses, however the neutralizing antibodies generated by infection do not efficiently cross neutralize other SARS-CoV-2 Variants of Concern (VOCs), such as Gamma and Delta lineages. Our results underline the need for a functional characterization of which responses generated by infection can prevent disease. They also reinforce the need for vaccination against COVID-19 in children, who even after infection remain at risk against new emerging SARS-CoV-2 variants.

Material and methods

Ethics statement

This study was approved by the Institutional Review Board (IRB 30749720.4.1001.5330) at Hospital Moinhos de Vento and Research Ethics Committee from Fundação Faculdade Federal de Ciências Médicas de Porto Alegre (CEP-UFCSPA) (CAAE 30749720.4.3001.5345). Legal consent was obtained from all participants or their legal guardians. The study was conducted according to good laboratory practices and following the Declaration of Helsinki.

Patients

A prospective cohort study was carried out at Hospital Moinhos de Vento and at Hospital Restinga e Extremo Sul, both in Porto Alegre, southern Brazil, and their immune status at primary infection was assessed as described (11). Study participants, adults and children older than 7 months, were asked to return for a 3- and 6-month follow-up visit to the respective hospital between October 2020 and April 2021 for blood sample collection.

PBMC isolation and *in vitro* T cells stimulation assays

Blood was collected in EDTA tubes (Firstlab, PR, Brazil) and stored at room temperature before processing for PBMC isolation and plasma collection. Plasma was separated by centrifugation and cryopreserved. PBMCs were next isolated by density-gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare®), and either studied directly or resuspended in fetal bovine serum (FBS) 5% DMSO and stored in liquid nitrogen until use. For *in vitro* T cell stimulation, cells were thawed, assayed for viability, counted, and plated in 96-well plates at 3×10^5 PBMCs/mL, at 100 μ L/well in RPMI1640 medium (Sigma-Aldrich - R8758) supplemented with 10% FBS and 100 IU of penicillin/mL, 100 μ g of streptomycin/mL (Lonza, Belgium) and 2 mM L-glutamine (Lonza, Belgium) (R10H medium). Subsequently, cells were stimulated with peptide PepTivator SARS-CoV-2 Prot S (130-126-700 - Miltenyi Biotec, Germany), PepTivator SARS-CoV-2 Prot N (130-126-698 - Miltenyi Biotec, Germany) and PepTivator SARS-CoV-2 Prot M (130-126-702 - Miltenyi Biotec, Germany) at 1 μ g/mL. Anti-CD3/anti-CD28 (4 μ g/mL/2 μ g/mL, BD Biosciences) were used as a positive control for stimulation and used to normalize values obtained by peptide stimulation. All treatments were

incubated for 18h at 37°C and 5% CO₂. Three hours before harvesting, Golgi Plug (BD Biosciences, USA) 1 μ g/mL was added to each well. Cells were stained and phenotypes were analyzed by flow cytometry.

Flow cytometry analysis

After *in vitro* stimulation, cells were stained with BD Horizon™ Fixable Viability Stain 510 together with antibodies for surface markers, as follows: anti-CD4-PerCP-Cy5.5 (clone RPA-T4), anti-CD8-APC-H7 (clone SK1), anti-CCR7-BV421 (clone 2-L1-A), CD45RA-PE-Cy7 (clone L48). They were subsequently fixed and permeabilized using Cytofix/Cytoperm kit (BD Biosciences-Pharmingen, USA), then stained with anti-IFN γ -FITC (clone 4S.B3), anti-TNF (clone MAb11) and anti-IL-17-PE (clone SCPL1362) antibodies. Antibody dilutions are available upon request. All samples were analyzed using BD Biosciences - FACSCanto II and FlowJo 10.7.1 software.

Enzyme-linked immunosorbent assay

Plasma was tested for IgG and IgA antibodies to S-RBD protein (#RP-87678 - Invitrogen, USA), trimeric SARS-CoV-2 spike (S), S protein from variants Delta and Gamma (kindly provided by Dr. Leda Castilho - Federal University of Rio de Janeiro - UFRJ, Brazil) and N protein (kindly provided by Dr. Ricardo Gazinelli - Fiocruz Belo Horizonte, Brazil) using a protocol described in 17. Briefly, ELISA plates (Kasvi, Brazil) were coated overnight with 1 μ g/mL of SARS-CoV-2 proteins. On the following day, plates were blocked for 1 h at room temperature with a blocking buffer (3% skim milk powder in phosphate-buffered saline (PBS) containing 0.05% Tween-20). Plasma samples were heat-inactivated at 56°C for 60 minutes and then serially diluted in 1% milk in 0.05% PBS-Tween 20 starting at 1:25. Plasma was incubated for 2 h at 37°C. Secondary antibodies were diluted in 0.05% PBS-Tween and incubated for 1 h at room temperature. For both IgG, anti-human peroxidase produced in rabbit (#IC-1H01 - Rhea Biotec, Brazil), and IgA, anti-human peroxidase produced in goat (#A18781 - Invitrogen, USA), was used at a 1:10,000 dilution. TMB Elisa Substrate - High Sensitivity (Abcam, United Kingdom) was added for 30 minutes, and the reaction stopped with 1M chloric acid. Results were documented in an ELISA reader (Biochrom EZ 400), and O.D. at 450 nm was used to calculate the area under the curve (AUC), using a baseline of 0.07 for peak calculation 18. For average AUC calculations, adult individuals who were vaccinated between the 3 and 6-months timepoint were excluded.

Viruses and cells

The present study was conducted using three different SARS-CoV-2 lineages: The B original lineage (Genbank HIAE-02 SARS-CoV-2/SP02/human/2020/BRA) isolated from the second case of COVID-19 in Brazil and kindly provided by Prof. Edison Durigon; Gamma (P.1) (GISAID: EPI_ISL_1708318) isolated from a patient seen in Campinas, Brazil; and Delta (B.1.617.2-like) (GISAID: EPI_ISL_3461104) isolated from a patient seen in Porto Alegre, Brazil. SARS-CoV-2 lineages were isolated and propagated in Vero cells (CCL-81; ATCC, Manassas, VA, USA) as described elsewhere (Clarke et al., 2022). Viral titers of the viral stocks used in the study were determined by plaque-forming unit (PFU) assay as previously described (12). All experiments with viable SARS-CoV-2 were performed under biosafety level 3 (BSL-3) in the Laboratory of Emerging Viruses (LEVE) of the University of Campinas, Brazil.

Plaque reduction neutralization test

Patient's serum neutralizing activity against B, Gamma and Delta isolates of SARS-CoV-2 were evaluated using an in-house Plaque Reduction Neutralization Test (PRNT) as previously described (13). Briefly, plasma samples from infected patients were serially diluted in a 1:2 dilution factor (1/10, 1/20, 1/40, 1/80, 1/160 and 1/320) in DMEM and incubated with a solution containing 2×10^3 PFU/mL of SARS-CoV-2 isolates for 1h at 37°C with 5% CO₂. Virus-serum mixtures were added to Vero cells monolayers and incubated for an additional hour for viral adsorption. Supernatants were then discarded, and Vero cells were covered with carboxymethylcellulose (CMC) 1% in DMEM containing 5% FBS. Cells were incubated for 72h to 96h at 37°C with 5% CO₂. CMC/DMEM medium was removed, cells were fixed with 8% paraformaldehyde and stained with 1% methylene blue (Sigma-Aldrich). After staining, the number of lysis plaques in all wells were manually counted and the plaque reduction was calculated for each patient sample after normalization with the plaque numbers observed in SARS-CoV-2 positive controls, without serum. PRNT₅₀ for each patient was calculated as the dilution that showed approximately a 50% reduction in the number of plaques when compared with the SARS-CoV-2 infected positive controls.

Statistics

Patients' demographic or clinical characteristics measured as categorical variables were described using percentages and compared among groups using Pearson chi-square test. Continuous clinical characteristics were summarized in terms of median and interquartile range (IQR) and compared among

groups using Kruskal-Wallis test. Differences among groups of patients for time variation in levels (area under the curve, AUC) of IgG and IgA antibodies were analyzed using Generalized Estimating Equations (GEE). The models considered gamma distribution for the data, log link and unstructured working correlation matrix. Sequential Sidák's adjustment was used to adjust p-values for pairwise multiple comparisons. These calculations were done using SPSS v.18. For memory T cell data, the results observed in the three time points were compared using nonparametric Friedman test in each patient's group. If the result was statistically significant, Wilcoxon test for paired samples was then employed to compare 3- and 6-month. Memory T cell difference among groups in selected time points of collection were analyzed using Kruskal-Wallis nonparametric analysis of variance, followed by Dunn's test for pairwise comparisons between groups. Spearman's rank correlation matrices were obtained using R package *corrplot* (14).

High-dimensional data and heatmap analysis of flow cytometry data

t-SNE and FlowSOM analyzes were performed on Flowjo v.10.8.1. Cells populations were downsampled from live cells, using the Downsample v.3.0.018 plugin for Flowjo to 2000 cells and subsequently concatenated. FlowSOM clustering with the FlowSOM v3.0.18 plugin was performed on the concatenated file with n= 6 clusters for the memory T cells populations. The FlowSOM output was surface markers expression and the percentage of cells in each cluster between groups. The t-SNE map was built with a vantage point tree algorithm with 550 iterations. Cluster identification in the map was performed with the help of the Cluster Explorer v1.6.3 plugin through the heatmap expression of surface markers CD4, CD8, CD45RA, and CCR7. Heatmap analyses were performed using the ggplot2 package v3.3.6 (15), where data was normalized through z-score, considering CD8, CD4, CD45RA, CCR7, TNF- α , IFN- γ and IL-17 markers.

Results

The study design is summarized in Figure 1A. The cohort included children, adults with mild and severe disease, with blood collected between July 2020 and April 2021. All children only had mild symptoms, as previously described in (11). Patients, or their legal guardians, consented to donate blood samples approximately 3 and 6 months after the first sample was collected at diagnosis (11). Samples were cryopreserved and later analyzed for memory markers including specific antibodies and T cell populations. Demographic and clinical data of patients that remained in the study for six months are presented in

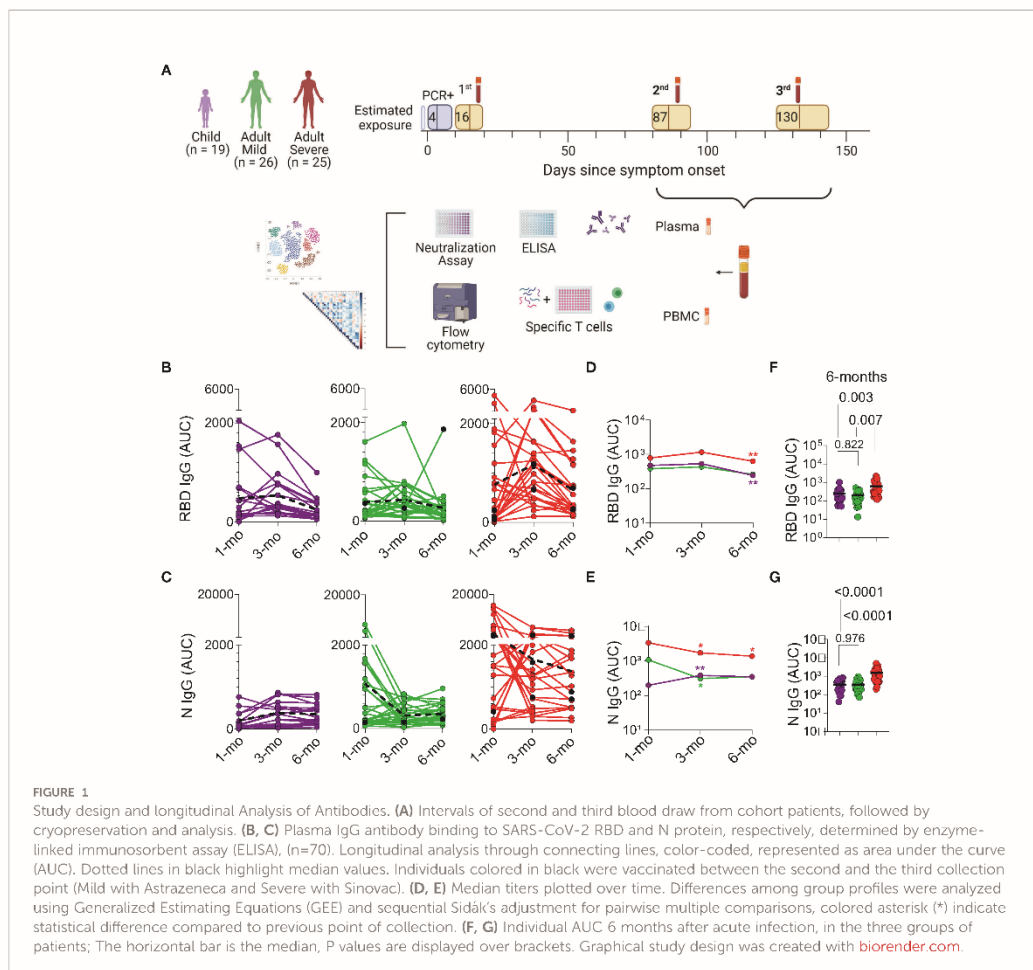


Table 1. The percentage of absence between the sample studied for acute infection (11) versus memory response study, was 21% in children, 8% in adults with mild diseases and 24% in adults with severe disease.

IgG titers to the N, but not the S protein, increase over time in children

Antibodies from blood samples are currently the main surrogate markers of memory of infection and/or vaccination. Plasma titers of IgG antibody binding to SARS-CoV-2 RBD (Figures 1B, D, F) and N (Figures 1C, E, G) proteins, as well as of

IgA (Figure S1) were determined by ELISA immediately after acute infection and at the 3 and 6 months after infection. These two later time points values were compared to the those detected at diagnosis. Considerable individual variation in responses to both antigens, a hallmark of infection, can be observed in each group (Figures 1B, C). Remarkably, children's anti-N IgG titers show a significant increase ($p=0.001$) at the 3 months timepoint, and which is maintained until 6 months (Figure 1E), while their anti-S-IgG titers decrease significantly ($p<0.001$) (Figure 1D). Although adults with severe disease show significantly higher titers of both anti-N and anti-S antibodies throughout time, a significant decrease in both antibodies is observed at the 6 months post-acute infection (anti-S IgG $p<0.001$; anti-N IgG $p=0.026$) (Figures 1D–G).

TABLE 1 Clinical characteristics of patients.

Characteristics	Child(N = 19)	Mild(N = 26)	Severe(N = 25)	P-value
Age (y), median (IQR)	12.0 (3.7–15.2)	42.5 (29.7–47.5)	51.0 (38.0–70.5)	<0.001
Female sex, n (%)	10 (52.6)	16 (61.5)	12 (48.0)	0.616
Days from symptom onset to sample collection				
1 st point, days, median (IQR)	16 (12.0–42.0)	18 (16.0–22.5)	11 (9.5–15.5)	0.063
2 nd point, days, median (IQR)	87 (83.0–106.0)	92 (84.0–100.0)	82 (74.5–88.0)	0.019
3 rd point, days, median (IQR)	131 (119.0–155.0)	133 (127.0–143.5)	125 (121.5–130.5)	0.080
Days from symptoms to SARS-CoV-2 positive PCR test, median (IQR)	3 (2–8)	3 (2–5)	7 (3.5–10.0)	0.012
Number of symptoms, median (IQR)	7 (4–11)	14 (10–19)	12 (8–17)	0.003
Number of underlying medical conditions, median (IQR)	0 (0–1)	0 (0–1)	2 (1–6)	0.005

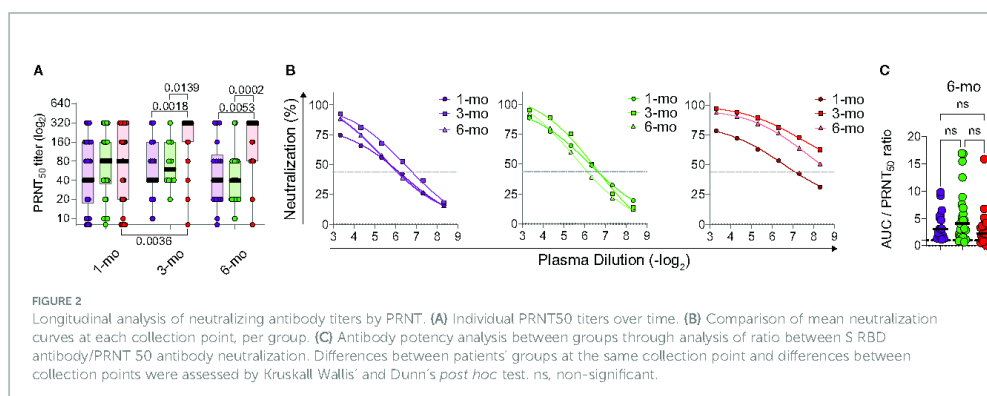
Quantitative variables were compared using Kruskal-Wallis test and for categorical variables Pearson's Chi-squared test was used; IQR, interquartile range. Bold values indicate P-value < 0.05

IgA titers to the RBD dropped significantly in all groups at this time point, while anti-N IgA antibodies increase slightly in children, and significantly in adults which had mild disease ($p < 0.001$) (Figure S1C and S1D). These results indicate that a test based solely on anti-IgG RBD might not constitute the best assay to detect antibody memory in most of the population, especially in children.

Vaccinated individuals are depicted in black (Figures 1B, C; and Figures S1A, S1B). Their titers are shown for comparison but were excluded from the average AUC calculations. One adult with mild disease was vaccinated and received the Astra Zeneca vaccine; the adults with severe disease that were vaccinated received the Sinovac vaccine. The latter showed no obvious increases in antibodies. Among individual children, which were all unvaccinated at that time, no obvious increases in antibody titers were detected at the analyzed timepoints. This is consistent with the time in which the study was carried out, that is, on lockdown and could indicate that these children were not reinfected during that six-month period.

Memory neutralizing antibodies in children

More than total specific antibody titers, the detection of neutralizing antibodies (NABs) is frequently proposed as the gold standard for protective immunity to different viruses, including SARS-CoV-2. We thus performed a longitudinal analysis of NAB production in the cohort members (Figure 2). Once again, considerable individual variation can be observed. All three groups show higher averages in PRNT50 titers at the 3 months timepoint (Figures 2A, B) in comparison with the diagnosis timepoint, although this increase is significantly higher in adults who had severe disease ($p < 0.01$). At the 6 months timepoint, NABs titers are maintained, and still higher in the adults who experienced severe disease (Figures 2A, B). Finally, the ratio between total and neutralizing antibodies, at the 6 months timepoint (Figure 2C) was not different among the groups. Altogether, these results suggest that SARS-CoV-2 infected children make and sustain neutralizing antibodies at levels that are comparable those observed in adults.



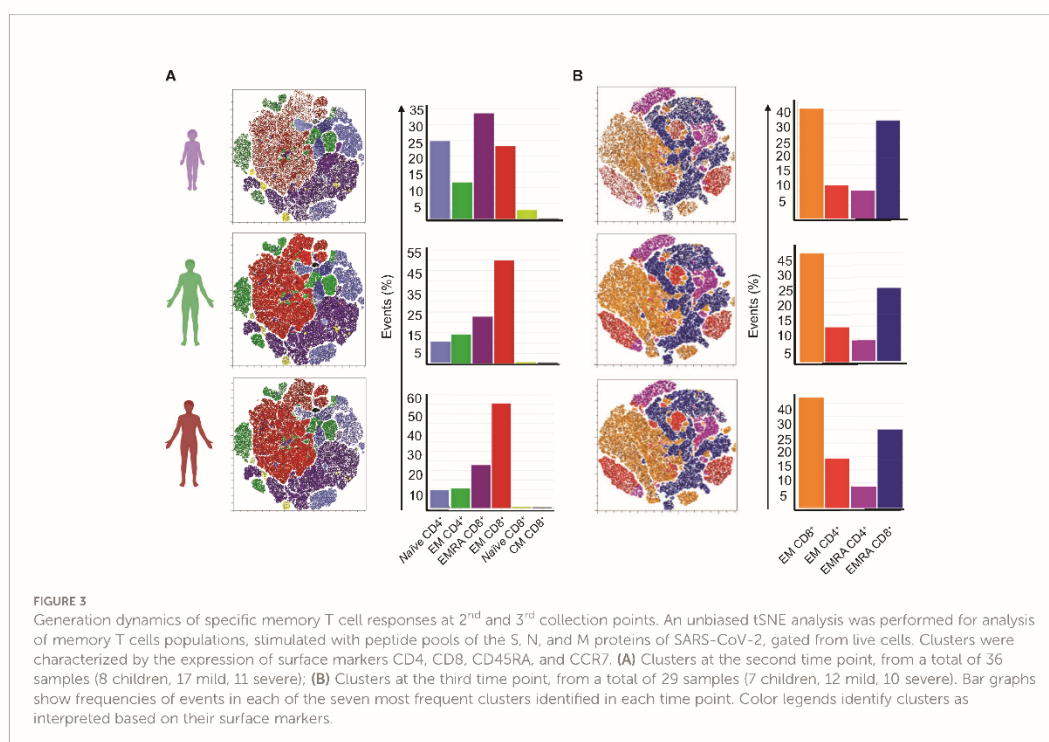
Dynamics and quality of specific T cell memory generation to SARS-CoV-2 are different in children compared to adults

The low antibody titers shown by children over time indicate that the mild form of disease they experience might be explained by other mechanisms, particularly those mediated by T cells. To investigate the generation of memory T cell responses in the cohorts, we stimulated PBMCs from 3 and 6 months after the acute infection with peptide pools of SARS-CoV-2 proteins S, M or N. SARS-CoV-2 specific T cells were then analyzed by flow cytometry for T cell memory (using CD45RA and CCR7 expression) and functional markers through the production of cytokines (IFN- γ , IL-17 and TNF- α).

An unbiased tSNE analysis of SARS-CoV-2 specific stimulated T cells (CD4 and CD8) was performed. Seven main clusters in the 3- and 6-months points were identified. At 3 months (Figure 3A), children show generally lower frequencies of specific T cells than adults. Both adult groups show a higher frequency of a cluster of CD45RA⁺CCR7⁻ CD8⁺ T cells – which we named effector CD8⁺ T cells (EM CD8⁺). Children presented fewer events for this cluster and more events for the clusters of CD45RA⁺CCR7⁺ CD4⁺ as well as CD45RA⁺CCR7⁺ CD8⁺, named by us *naïve* CD4⁺ and CD8⁺ T cells, respectively.

Notably, one cluster of CD45RA⁺CCR7⁻ CD8⁺ cells, identified by us as terminally differentiated memory (EMRA) T cells that emerge at this point is most frequent in children compared to either of the adult groups. At 6 months, clusters were qualitatively different (Figure 3B). Frequencies of events in each of the identified clusters were more similar between children and adults. Children no longer present prominent clusters of *naïve* cells. Instead, CD8⁺ EMRA cells were dominant in children, while the cluster characterized by effector cells is more evident in adults. In sum, these results indicated that memory CD8⁺ T cell responses in children were faster, and more intense, than in adults.

We next proceeded to a more quantitative and functional analysis of these memory T cell responses. We had previously shown that anti-N CD8⁺TNF- α ⁺ T cells were generated upon acute infection in children, while adults focused that response on S peptides (11). To investigate if this was the main response that was differentiated into memory in children, we performed a longitudinal analysis of the percentages of specific cells producing TNF- α , IL-17 or IFN- γ in response to the different peptide pools (Figure S2). The results are shown in Figure 4 and Supplementary Figures 3 and 4. Figure 4A shows that after 3 months most children show central memory CD8⁺TNF- α ⁺ T cells responding to N peptides. Some children already show



effector memory and even EMRA cells with these characteristics. At the 6-month time point, most children had developed CD8⁺TNF- α ⁺ effector memory and EMRA cells specific for N peptides, but not for the S and M peptide pools. This does not happen for IFN- γ and IL-17 CD8 memory responses (Figures S3A, S3B). While most children and adults generate memory cells producing these two cytokines in response to all three proteins at the 3 months post-acute infection, most of these responses are lost at 6 months. Supplementary Figure 4 shows quantification of functional specific CD4⁺ T cell responses at the two timepoints. It is remarkable that most children lose at 6 months all the detectable circulating responses seen at 3 months post-acute infection. These results suggest that children, but not adults, develop early and long-lasting T cell memory responses, and that CD8⁺TNF- α ⁺ cells specific for the N protein constitute a major, if not the main, component of those responses.

To further investigate which children are forming memory responses to the viral peptides, we compared their individual responses through heatmaps (Figure 4B). Most children focused their memory responses on CD8⁺TNF- α ⁺, especially to the N protein, with fewer individuals targeting the M and S peptide pools, at 6 months after acute infection.

A Spearman correlation analysis (Figure 5) evidenced that in children, but not adults, anti-N IgG and IgA titers correlated positively with N-specific CD8⁺TNF- α ⁺ T cells – especially EMRA cells. PRNT50 titers in children, but not in adults, also correlated positively with CD8⁺TNF- α ⁺ EMRA cells. This positive correlation was not observed for protein S (Figure S5). Altogether, these results indicate that children generate distinctive memory T and B cell responses to SARS-CoV-2 infection, in kinetics, target and function.

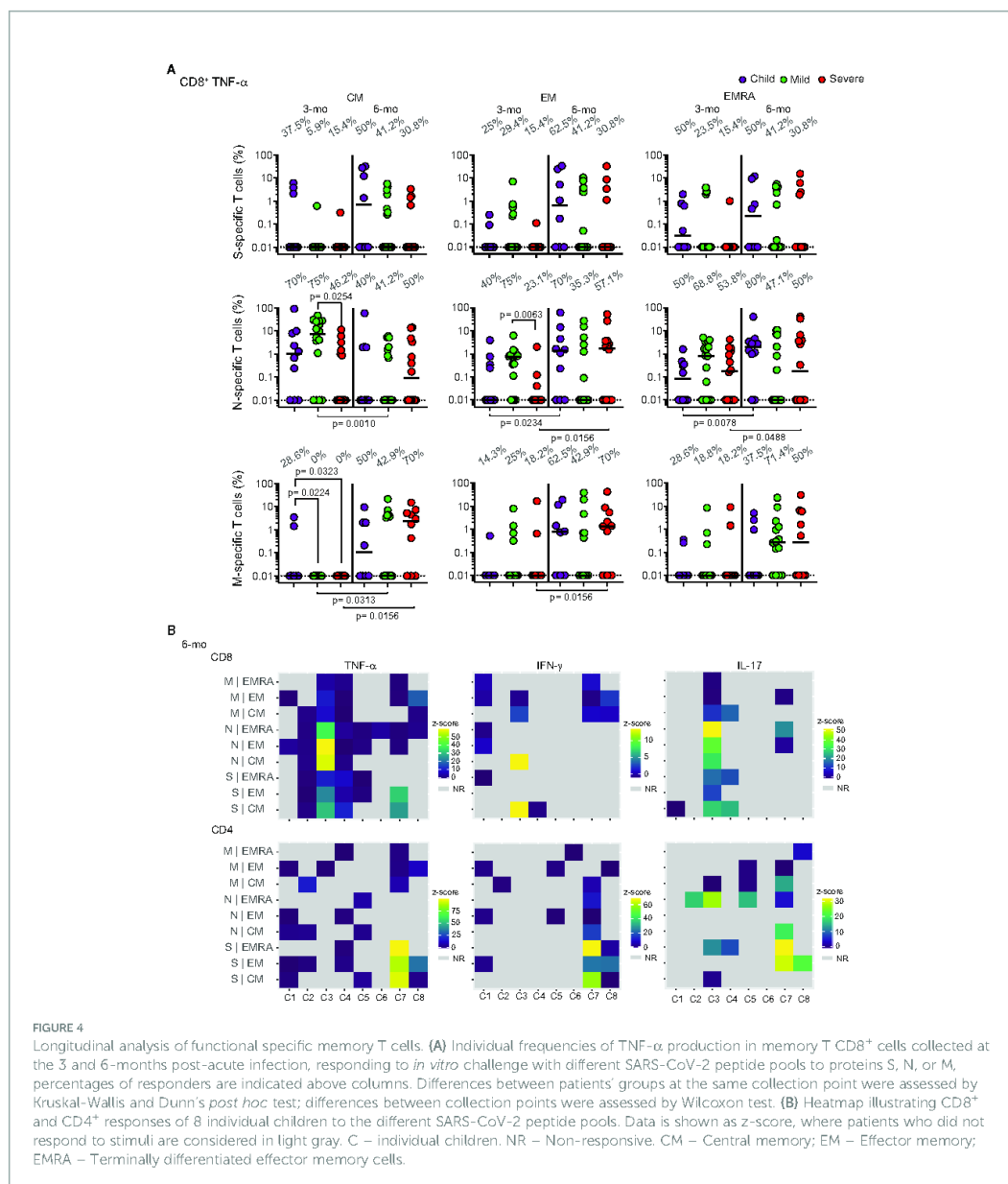
Memory NABs in children do not efficiently cross-neutralize infection by emerging SARS-CoV-2 variants

At the time we ended sample collections for this study, the original variant, B, was being substituted for more prevalent variants of concern (VOCs), namely Gamma (16), and later, Delta (17). The neutralizing antibodies generated by vaccination encoding the RBD of the S protein were shown to retain protection potential against VOCs (16). Most of the children, however, have not been vaccinated. We asked if the children's memory antibody responses to SARS-CoV-2 infection would be protective against infection by the emerging variants. To investigate that, we analyzed the recognition and neutralizing potential of the memory antibodies present at 6 months post-diagnosis. We assayed these antibodies against the trimeric Spike of the variants by ELISA and performed PRNT tests using the Gamma and Delta lineages (Figure 6). Responses to the trimeric S of the original variant (B) were always higher in titers than responses to the RBD (Figure 6A). For almost every individual,

total IgG antibodies to the B trimeric S are still able to recognize the trimeric S of variants Gamma and Delta. However, this does not hold for neutralizing antibodies (Figures 6B–D). Only adults with severe disease maintained significant higher potential for neutralization of the Gamma VOC compared to children and adults with mild disease. For Delta, a later-emerging VOC, marked reductions in neutralization are observed for all groups. These results indicate that neutralizing antibodies generated by infection with the original B variant in children have low or no protective potential upon encounter with emerging VOCs.

Discussion

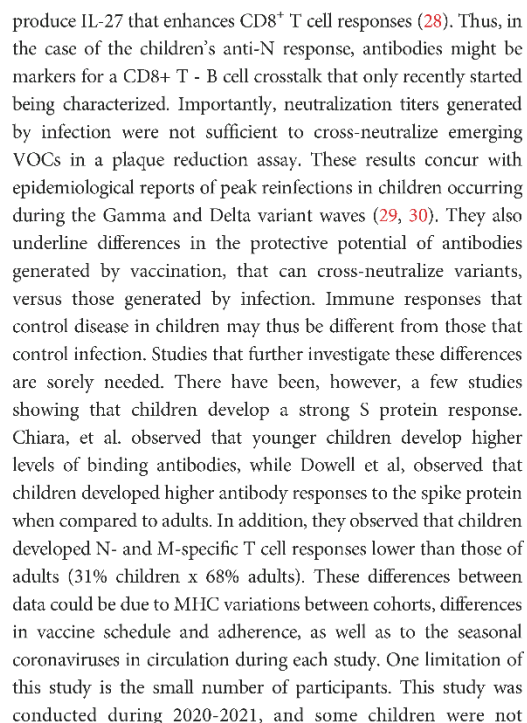
Throughout the SARS-CoV-2 pandemic, children have been regarded as having a minor risk of disease, mostly because of the mild presentation of COVID-19 observed in so many of them. They have thus become the least studied age group, and the last to be vaccinated. However, the immune responses they develop to infection might hold important information on what is necessary to generate fast and efficient protection against disease. In this study, we identified key differences in the memory immune response generated by infected children when compared to adults. The increase of anti-N antibodies three months after diagnosis and the early appearance of memory N-specific CD8⁺TNF- α ⁺ cells could reflect a higher precursor frequency of N-peptide-recognizing cells in children. This could be due to pre-existing cross-reactive T cells and might explain the mild disease generally presented by SARS-CoV-2 infected children. The N gene has a higher degree of conservation among different lineages of SARS-CoV-2 than the S gene, accumulating fewer mutations (18). It is highly expressed during infection (19) and is associated with the protection of the viral RNA genome, resulting in specific T cells that are long-lasting (20). In adults, cross-reactive memory T cells were shown to be associated with protection from COVID-19 (21). In that study, the peptides recognized by cells in protected individuals were mainly from the nucleocapsid (N) protein. Most, but not all, individuals who recover from COVID-19 generate T cell responses to multiple viral epitopes (22) and a strong activation of these cross-reactive clones has been linked to protection (23). It has been suggested that, in children, a high frequency of antibodies and T cells recognizing antigens from seasonal coronaviruses could be due to repeated reinfections (24), though the protective immunity generated by infection with these endemic coronaviruses is not usually long-lasting (25). Studies on precursor frequencies of T cells recognizing coronaviruses epitopes, however, are lacking. This makes it difficult to support such a hypothesis with evidence. Interestingly, the efficacy of RBD-encoding mRNA vaccines drops from over 90% in adults to 51% (Moderna) and 75% (Pfizer) in young children (26). It is possible that a better



tailoring of vaccines for children, including N peptides, could stimulate this N-specific response, thus increasing efficacy in this age bracket.

The fact that the N-specific antibodies in children, but not adults, correlated with anti-N CD8⁺TNF- α ⁺ memory T cells may evidence functional mechanisms of protection that have not been

characterized for COVID-19. While a correlation between antibodies and CD4⁺ T cells, mainly follicular T cells (Tfh), would be expected, a recent study by Nelson et al. (27) analyzed memory T cell responses using peptide:HLA tetramers and did not find high associations between peak antibodies to SARS-CoV-2 and Tfh cells. Klarquist et al. found that B cells are necessary to



brought back for all blood draws at the follow-up time points. Nevertheless, the consistency of responses found in all our analyses indicates important aspects of pediatric COVID-19 immunity, that may inform vaccination strategies for this age group (31, 32).

Data availability statement

The data presented in the study are deposited in the Mendeley repository, found here: <https://data.mendeley.com/datasets/pfkhyr6wt/1>. Further inquiries can be directed to the corresponding author/s.

Ethics statement

This study was reviewed and approved by Institutional Review Board (IRB 30749720.4.1001.5330) at Hospital Moinhos de Vento and Research Ethics Committee from Fundação Faculdade Federal de Ciências Médicas de Porto Alegre (CEP-UFCSPA) (CAAE 30749720.4.3001.5345). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

KL, JF, PS, TF, all designed and performed experiments, analyzed data, wrote, and edited the manuscript. KL, performed and analyzed t-distributed stochastic neighbor embedding (tSNE) analysis. JF, performed and analyzed the correlation and heatmap matrices. GH, RZ, assisted in experiments. LR, helped design the study. LC, AB, KB-d-S, FG, RM, performed Plaque Reduction Assay (PRNT). GZ, IF, FV, MP-B helped collect patients. MT, GC, WA, assisted with epidemiological information. TB helped design the study and edited the manuscript. HN helped with tSNE and heatmap analysis and edited the manuscript. JLP, coordinated and performed PRNT analysis and edited the manuscript. SC-J coordinated all statistical analysis and edited the manuscript. MS, RS coordinated the cohort, conceived, and designed the study and edited the manuscript. CB, conceived and designed the study, performed data analysis, wrote, and edited the manuscript. All

authors contributed to the article and approved the submitted version.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.1033364/full#supplementary-material>

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8. Capítulo 5

Artigo de Revisão

Inhibitory innate receptors and their potential role in transplantation

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Motivação: Revisão escrita durante período de doutorado sanduíche no Massachusetts General Hospital (MGH)/ Harvard Medical School – EUA. Descrição de receptores inibitórios presentes em células inatas e análise funcional em transplante de órgãos sólidos.



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Review article

Inhibitory innate receptors and their potential role in transplantation

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ABSTRACT

The regulatory arm of the immune system plays a crucial role in maintaining immune tolerance and preventing excessive immune responses. Immune regulation comprises various regulatory cells and molecules that work together to suppress or regulate immune responses. The programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) are examples of inhibitory receptors that counteract activating signals and fine-tune immune responses. While most of the discoveries of immune regulation have been related to T cells and the adaptive immune system, the innate arm of the immune system also has a range of inhibitory receptors that can counteract activating signals and suppress the effector immune responses. Targeting these innate inhibitory receptors may provide a complementary therapeutic approach in several immune-related conditions, including transplantation. In this review, we will explore the potential role of innate inhibitory receptors in controlling alloimmunity during solid organ transplantation.

1. Introduction

The regulation of immune responses is essential to maintain tolerance and prevent uncontrolled harmful responses [1]. Ideally, an effector immune response must be able to resolve any tissue trauma or infection with no deleterious impact on the target tissue. Importantly, the interplay of innate activating and inhibitory signals will go on to shape the adaptive immune responses that ensue [2]. A network of regulatory cells and molecules closely cooperate to control and fine-tune immune responses. Inhibitory receptors like the programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) play a crucial role in this regulation by counteracting activating signals in effector immune responses. These inhibitory receptors, expressed mainly by T cells, can suppress effector T cell activation and proliferation. The inhibition of these pathways using monoclonal antibodies (immune checkpoint blockade) remarkably changed therapeutic approaches in oncology and has been largely employed in the treatment

of various metastatic cancers.

Similarly to the adaptive arm of the immune system, the innate immune system also has a range of inhibitory receptors that are essential in keeping immune homeostasis. Innate inhibitory receptors are potential targets in transplantation rejection, representing a new avenue for therapeutic intervention that can be used in combination with existing therapies to achieve drug minimization and improve graft survival. In this review, we will explore the potential role of innate inhibitory receptors in controlling alloimmunity during solid organ transplantation. The main inhibitory receptors covered in this review, by which cells they are expressed, and their potential function and/or disease relevance are summarized in Fig. 1 and Table 1.

2. Innate immune cells initiate alloimmunity

The innate immune system is the first level of defense of the immune system and can quickly detect and fix disturbances in tissue homeostasis.

Abbreviations: APCs, antigen-presenting cells; CEACAM, carcinoembryonic antigen-related cell-adhesion molecule; CTLA-4, cytotoxic T lymphocyte-associated protein 4; DAMPs, damage-associated molecular patterns; DCs, dendritic cells; HLA, human leukocyte antigen; Hsps, heat shock proteins; ITIM, tyrosine-based inhibitory motifs; IRI, ischemia-reperfusion injury; LAIR, leukocyte-associated immunoglobulin-like receptor; LILR, Leukocyte Ig-like receptor; MDSCs, myeloid-derived suppressor cells; MoDCs, monocyte-derived DCs; NK cells, natural killer cells; OLT, orthotopic liver transplantation; PAMPs, pathogen-associated molecular patterns; PD-1, programmed cell death protein 1; PRRs, pattern-recognition receptors; SHP, Src-homology 2 domain-containing phosphatase; Siglec, sialic-acid-binding immunoglobulin-like lectin; SLOs, secondary lymphoid organs; Tregs, regulatory T cells-human leukocyte antigen.

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The innate immune cells, including antigen-presenting cells (APCs) and natural killer (NK) cells, express pattern-recognition receptors (PRRs) that act as sensors of tissue injury or defects. These receptors recognize conserved ligands that vary from pathogen-associated molecular patterns (PAMPs) to damage-associated molecular patterns (DAMPs) derived from microorganisms or injured tissues, respectively. Upon engagement, PRRs trigger downstream inflammatory signaling pathways, including the transcription factor NF- κ B and the inflammasome complex that will lead to the expression of costimulatory molecules (e.g. CD80, CD86, CD40) and the production of inflammatory cytokines [3]. Once fully activated, tissue APCs will initiate and instruct adaptive immune responses by migrating to secondary lymphoid organs (SLOs) and eliciting antigen-specific T and B cell responses.

The initial immunological insult in solid organ transplantation involves the tissue ischemic injury, which activates resident APCs through the release of DAMPs and the engagement of PRRs with consequent inflammation [4,5]. The release of DAMPs and chemokines by the ischemic injury also recruits and activates neutrophils, monocytes and macrophages that amplify the activation of the APCs [6,7]. After their activation, APCs migrate from the tissue graft to the recipient's SLOs and deliver the required signals to induce the activation of alloreactive T cells [8,9]. Innate immune activation will initiate and potentiate the adaptive response against the allograft in part by enhancing organ immunogenicity through upregulation of (i) donor alloantigen

presentation, (ii) costimulatory molecules, and (iii) cytokine secretion. In the setting of alloimmune-mediated transplant injury late after transplant, innate immune activation may enhance and perpetuate the alloreactivity [10–12], ultimately contributing to graft fibrosis and failure. Furthermore, recent studies have indicated that innate immune activation may prevent and break tolerance in preclinical models [13–17].

Suppression of the immune activation against the transplanted organ is crucial to prevent rejection. Immunosuppressive drugs used in the clinic primarily target T cells and are associated with many systemic side effects. Therefore, an unmet need exists to develop effective therapies to modulate innate immunity in transplantation. Ultimately, the engagement of innate inhibitory receptors and/or their ligands is a potential complementary strategy for reducing rejection rates and improving long-term graft survival.

2.1. Inhibitory innate receptors and their ligands

Inhibitory immune receptors are expressed by all innate cells and are thought to counteract and/or fine-tune PRR-induced inflammatory signals (Fig. 1). They can control the activation levels of innate cells, mitigating cellular responses and keeping the balance of immune responses. Genetic ablation of these receptors increases the susceptibility of animals to autoimmunity and exacerbates immune responses during

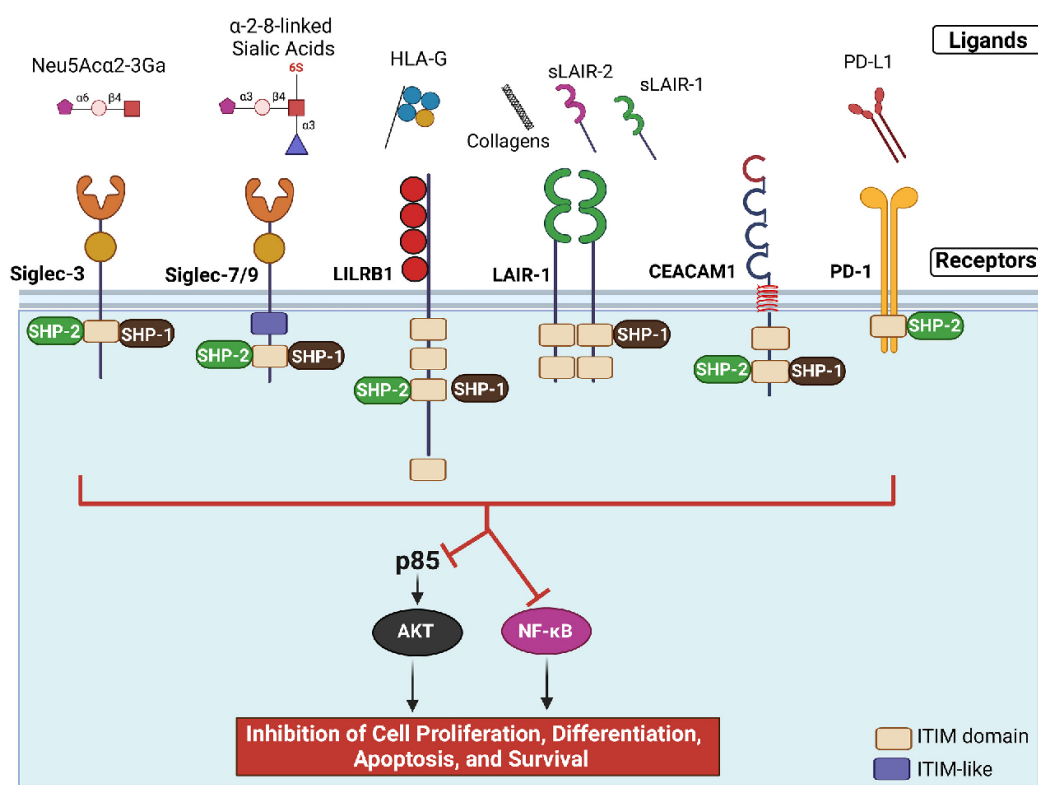


Fig. 1. Overview of inhibitory innate receptors and their intracellular downstream signaling pathway. Schematic illustration of inhibitory receptors and their ligands regulating innate immunity. After the engagement of the receptors by their ligands, the intracellular ITIM or ITIM-like motifs recruit SHP1/SHP2 inhibiting downstream signaling of inflammatory factors like NF- κ B and AKT. This inhibition of inflammatory cascades affects cell proliferation, differentiation, and survival. ITIM: immunoreceptor tyrosine-based inhibitory motif; Siglec: sialic-acid-binding immunoglobulin-like lectins; LILRB1: leukocyte Ig-like receptor; HLA-G: human leukocyte antigen G; LAIR-1: leukocyte-associated immunoglobulin-like receptor-1; sLAIR-1: soluble LAIR-1; sLAIR-2: soluble LAIR-2; CEACAM1: carcinoembryonic antigen-related cell-adhesion molecule 1; PD-1: programmed cell death protein 1; PD-L1: programmed cell death protein ligand-1; SHP-1: Src-homology 2 domain-containing phosphatase 1; SHP-2: Src-homology 2 domain-containing phosphatase 2; AKT: protein kinase B; NF- κ B: Nuclear factor kappa B. Cartoon created with BioRender.com.

Table 1
Inhibitory innate receptors expression by different immune cells and their related clinical disorders.

Receptor	Ligands	Expression	Pathology
Siglec-2	NeuGC $\alpha 6$ -GalNAc- $\beta 4$ -GlcNAc	B cells cDC Mast cells	B-cell lymphoma [99,100] Rheumatoid arthritis [101,102] Type 1 diabetes [103]
Siglec-3	NeuAc- $\alpha 6$ -GalNAc- $\beta 4$ -GlcNAc	Macrophages Myeloid progenitors Monocytes Microglia	Acute and chronic myeloid leukemia [104–106] Alzheimer's disease [107]
Siglec-5	NeuAc- $\alpha 6$ -GalNAc	Neutrophils Monocytes	Neutrophil disorders [108]
Siglec-6	NeuAc $\alpha 8$ -NeuAc- $\alpha 3$ -Gal NeuAc- $\alpha 6$ -GalNAc	B cells4 Mast cells B cells Mast cells Trophoblasts	Preeclampsia [109,110]
Siglec-7	NeuAc $\alpha 8$ -NeuAc- $\alpha 3$ -Gal	Natural Killer cells Monocytes T cells Mast cells	Mast cell leukemia [111] Cancers [112,113]
Siglec-8	NeuAc $\alpha 3$ -Gal6S- $\beta 4$ -GlcNAc	Eosinophils Basophils Mast cells	Asthma [114,115] Eosinophilic gastritis [116]
Siglec-9	NeuAc $\alpha 3$ -Gal- $\beta 4$ -GlcNAc6S-Fuc	Monocytes Neutrophils Conventional Dendritic cells Natural Killer cells T cells	Cancer [117,118] Chronic obstructive pulmonary disease [119] Rheumatoid arthritis [120] Cancer [121,122]
Siglec-10	NeuAc- $\alpha 6$ -GalNAc- $\beta 4$ -GlcNAc	B cells Monocytes Eosinophils	
Siglec-11	NeuAc $\alpha 8$ -NeuAc- $\alpha 3$ -Gal	Macrophages Microglia	Neurological disorders [123–125] Cancer [126–128] Type 1 diabetes [129]
CEACAM1	CEACAM1 Microbial ligands PD-1	Endothelial Epithelial T cells B cells Natural Killer cells Dendritic Cells	
LAIR-1	Collagens sLAIR-1 sLAIR-2 Surfactant protein D C1q	T cells B cells Natural Killer cells Monocytes Monocyte-derived Dendritic cells (MoDCs) Eosinophils Basophils Mast cells	Autoimmune diseases [130] Leukemia [131] Viral infection [62]
LAIR-2	Collagens C1q	T cells B cells NK cells Monocytes Neutrophil Basophil	Rheumatoid Arthritis [65,132] Autoimmune Thyroid Disease [133] Cancer [134] Cancer [135]
LILRB1	Classical HLA class I (HLA-A, HLA-B, HLA-C) Non-classical HLA class I (HLA-E, HLA-F, HLA-G and HLA-H) Non-HLA angiopoietin-like (ANGPTL) S100A8/S100A9	Natural Killer cells T cells B cells Monocytes Macrophages Dendritic cells Granulocytes	Cancer [136] Viral Infection [136]
PD-1	PD-L1	T cells B cells Natural Killer cells	Cancer [89] Sepsis [137] Bacterial Infection

Table 1 (continued)

Receptor	Ligands	Expression	Pathology
		T regulatory T cells T follicular helper (Tfh) Dendritic cells Macrophages Neutrophils	[138] Viral infection [139]

infections [1,18]. In humans, the side effects of the immune checkpoint blockade in cancer patients highlights the crucial role of these receptors in maintaining immune homeostasis and tolerance. One key feature of these inhibitory receptors is the presence of tyrosine-based inhibitory motifs (ITIM) motifs in their intracellular portion. Once the inhibitory receptors are engaged by their ligands, the ITIMs recruit tyrosine phosphatases (e.g. Src-homology 2 domain-containing phosphatase (SHP) 1 or 2), dephosphorylating essential inflammatory signaling molecules, like the NF- κ B or the cytoplasmic tail of inflammatory PRRs.

The function of different inhibitory receptors can vary according to their expression pattern, the ligands availability and other co-signaling pathways that are being triggered at the same time. Their functional categories were recently described by Rumpert *et al* [18]. The understanding of how the activation of different co-receptors and signaling pathways orchestrate/regulate inflammatory disorders, including transplant rejection, is crucial to develop innate-focused therapeutic targets. Another important aspect of this immune regulation is the tissue expression pattern of different ligands to those inhibitory innate receptors under pathological conditions. Although several ligands have been recently identified, their functional role in a pathological context, such as transplant rejection, is not well-defined. Therefore, we propose that there are three arms of regulation that could impact the rejection responses and be therapeutically targeted: 1) the expression and activation status of the innate inhibitory receptors, 2) their interaction with co-receptors, and 3) the tissue expression of its ligands on the graft tissue. The upregulation of innate inhibitory receptors and/or their ligands in the tissue graft could comprise a complementary novel strategy to prevent rejection. Here, we describe the potential implications of different innate inhibitory receptors in the context of solid organ transplantation.

3. Families

3.1. Siglec family

Immune cells can express several types of sugar-binding proteins, called lectins, on their cell surface [19]. The engagement of some of these lectins can induce potent inhibitory signal transduction cascades that reduce the ability of the immune cells to recognize and eliminate target cells. Sialic-acid-binding immunoglobulin-like lectins (Siglecs) are a class of immune checkpoints that can initiate signal transduction via protein-glycan interactions [20]. They can be evolutionarily divided based on their conservation or their function – inhibitory, activating, and non-signaling. Most Siglecs bind to conjugated sugar motifs (sialic acids), resulting in robust anti-inflammatory effects [21] (Fig. 1, Table 1). Recently, Siglecs were described to have non-sialic ligands, such as Heat shock proteins (Hsps) [22,23]. Based on evolution, Siglecs are divided into isoforms that are highly conserved with minimal changes between species, like the CD22, MAG and Siglec-5, or the CD33-related Siglecs that suffered rapid evolution in mammals. Due to this divergence, rodent Siglecs are assigned with letters and human Siglecs with numbers. Inhibitory Siglecs contain an intracellular ITIM or an ITIM-like motif that is phosphorylated after receptor engagement and leads to the recruitment of either SHP-1 or SHP-2, which dephosphorylate downstream components of stimulatory pathways, thus inhibiting cell activation [24] (Table 1). Below, we will cover some examples of

Siglecs that might be relevant for the alloimmune response.

One of the most studied Siglecs is the murine Siglec-E (the human Siglec-7 and Siglec-9 counterpart). DCs lacking Siglec-E produce more IL-6 and TNF- α when treated with TLR ligands than wild-type (WT) controls, demonstrating that Siglec-E is a negative modulator of the TLR signaling [25]. Mechanistically, Siglec-E recruits SHP-1/2 which will dephosphorylate NF- κ B, inhibiting its activation triggered by activating PPRs, including TLRs [26]. Interestingly, mice lacking Siglec-E have an exacerbated recruitment of neutrophils to the lung in a pulmonary inflammation model [27], and female mice develop a lupus-like phenotype when older than 6 months of age [28]. Siglec-E was also described to regulate the activation of the innate receptor CD36 and control inflammation and foam cell formation in a mouse model of atherosclerosis [29]. Targeting Siglec-E has immunosuppressive effects in murine models of sepsis [30] and asthma [31] through the modulation of macrophages and DCs. Although the precise role of Siglec-E or its human counterparts Siglec-7 and -9 in transplantation still needs to be elucidated, these data indicate that Siglec-E/7/9 might be potential candidates to target during transplantation.

Other Siglecs might also have immune-modulating roles in transplantation. In human kidney transplant recipients, circulating Siglec-3⁺ myeloid-derived suppressor cells (MDSCs) significantly increased in frequency and absolute numbers at 3, 6, and 12 months post-transplantation compared to baseline (time of transplantation) [32]. Those CD11b⁺Siglec-3⁺HLA-DR⁻ cells isolated from patients were capable of inhibiting CD4⁺ T cell proliferation *in vitro*, suggesting that these cells are functionally suppressive. Moreover, Siglec-3⁺ MDSCs had a significant positive association with Foxp3⁺ regulatory T cells (Tregs) 12 months post-transplantation. When patients-derived MDSCs were co-cultured with CD4⁺ naive T cells from the same patient, it was observed an increase in the cell number of CD4⁺ Foxp3⁺ T cells (Tregs) [32]. Similarly to Siglec-E, engaging or delivering antigens via Siglec-3 on MDSCs is a promising approach to decrease effector T cells and increase Treg responses. Thus, targeting Siglec receptors comprise a promising approach to induce immune regulation during the transplantation of solid organs.

3.2. CEACAM family

The carcinoembryonic antigen-related cell-adhesion molecule 1 (CEACAM1, also known as CD66a) is a cell-surface anchored glycoprotein expressed by endothelial, epithelial and immune cells like T cells, B cells, NK cells, and DCs [33,34]. As a result of differential splicing, CEACAM1 can be expressed as different isoforms that differ in their structure and function. Interestingly, the length of their cytoplasmic tails can also determine differences in their responses. Isoforms with a long cytoplasmic tail (CEACAM1-L), which contains two ITIM domains, generally transmit inhibitory signals. By contrast, CEACAM1 isoforms with a short (CEACAM1-S) cytoplasmic tail lack ITIM sequences. CEACAM1s have well-described non-immune roles in vascular neogenesis [35], insulin metabolism [36], tumor development [37] and apoptosis [38].

Regarding immune responses, ligated CEACAM1-L recruits SHP-1 and SHP-2 to its cytoplasmic tail in an ITIM-dependent manner [39,40] (Fig. 1, Table 1). Its anti-inflammatory roles *in vivo* are evidenced by studies employing animals lacking *Ceacam* gene expression (*Ceacam*^{-/-} mice). These animals develop a systemic pro-inflammatory state, marked by elevated tissue-associated macrophages, TNF- α and IFN- γ levels in the liver and adipose tissue [41]. Also, CEACAM1 is critical for B cell survival and is essential for antibody production [42]. In tumor models, the silencing of CEACAM1 in mouse and human cancer cells upregulates NK cell activating ligands on the cell surface [43] and diminished the recruitment of MDSCs [44].

In a mouse model of liver transplantation, CEACAM1 affected the susceptibility of liver grafts to peritransplant stress, influencing clinical outcomes, showing the functional role of CEACAM1 signaling to

alleviate graft damage during cold preservation, mitigate ischemia-reperfusion injury (IRI), and improve early clinical outcomes [45]. The ASK1-p38 signaling pathway is a key molecular mechanism in CEACAM1-mediated liver graft protection. Cold-stored human donor livers of 60 liver transplant recipients showed decreased CEACAM1 protein levels and increased ASK1 signaling with inferior post-orthotopic liver transplantation (OLT) function [45]. Reduced hepatic CEACAM1 expression was identified as one of the independent predictors of early allograft dysfunction in humans, demonstrating that CEACAM1 may serve not only as a target for therapeutic OLT modulation but also as a biomarker of donor liver tissue quality. In the same cohort of liver recipients, pre-transplant CEACAM1 expression correlated negatively with soluble aspartate aminotransferase (sAST) and soluble alanine aminotransferase (sALT) levels at postoperative day (POD) 1 [45]. In addition, low CEACAM1 cases exhibited significantly higher sAST at POD1–5 and sALT at POD1–7. Pretransplant low CEACAM1 livers exhibited increased mRNA levels of immune activation markers, associating with enhanced post-reperfusion innate/adaptive immune response and increased hepatocellular damage in the early post-OLT period [45].

Kidney transplant recipients with stable grafts have low expression of CEACAM1 protein, suggesting a constitutive expression in renal transplantation [46]. However, in patients with acute rejection, CEACAM1 was markedly upregulated. The acute vascular rejection revealed the highest tubular CEACAM1 levels, whereas interstitial rejection showed the highest glomerular expression [46]. These results indicate that the up-regulation of CEACAM1 is an indicator of acute inflammatory processes in biopsy specimens from patients with acute renal allograft rejections and, therefore, might be used as a clinical marker. Biologically, CEACAM1 upregulation during rejection could be a mechanism to counteract inflammatory responses in the kidney.

3.3. LAIR family

The leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1, also known as CD305) is a type I transmembrane glycoprotein containing a single extracellular C2-type Ig-like domain and two immunoreceptor tyrosine-base inhibitory motifs in its cytoplasmic tail [47]. *LAIR1* gene consists of 10 exons and shows considerable homology to LAIR-2. The *LAIR2* gene encodes a protein that is ~84% homologous to human LAIR-1 but lacks transmembrane and intracellular domain [47], and it has been found only in humans [48]. LAIR-1 is expressed on almost all cells of the immune system, including NK cells, T cells, B cells, monocytes [47], monocyte-derived DCs (MoDCs) [49], eosinophils [50], basophils and mast cells [51] (Table 1). Upon its engagement, LAIR-1 delivers a potent inhibitory signal capable of inhibiting cell lysis, cytotoxic activity, antigen stimulation, and cytokine-mediated signals [49,52–58] (Fig. 1, Table 1). Differently from other inhibitory receptors that the ligands are cell-bound, implying a role in cell-cell interactions, LAIR-1 ligands are collagens, showing a role for extracellular matrix proteins in immune regulation. The regulation of LAIR-1-mediated inhibition might be dependent on different factors such as the strength of the activation signals, and the levels of expression of the receptor, but also on soluble LAIR-1 and LAIR-2 molecules [59]. In addition, LAIR-1 also recognizes proteins that have collagen domains, such as surfactant protein D [60], and C1q, a component of the classical complement pathway [61].

Animals lacking the *Lair1* gene (*Lair1*^{-/-} mice) showed enhanced airway inflammation accompanied by increased neutrophil and lymphocyte recruitment to the airways [62], highlighting its immune downregulatory role *in vivo*. LAIR-1 also demonstrated some involvement in ischemia-reperfusion injury. In the subacute phase of ischemia-reperfusion injury, neutrophils and monocytes play an important role in the increase in infarct size. LAIR-1 expression was increased in those cells 3 days post-heart failure after myocardial infarction [63]. Moreover, aberrant LAIR-1 expression has been associated with autoimmune

diseases [64,65], leukemia [66], and viral infection [67].

It has been well known that many trans-membrane receptors can be shed from the cell surface and be released into the circulation in a soluble form when lymphocytes, endothelial, and other immune cells are activated. These soluble receptors can be used as markers of lymphocyte activation in transplant patients. For example, soluble LAIR-1 (sLAIR-1) levels were higher in the patients' sera on days 3–7 post-kidney transplantation compared with healthy individuals [68]. Also, patients with biopsy-proven chronic rejection showed higher serum levels of soluble LAIR-1 than those without rejection. Mechanistically, high levels of serum sLAIR-1 may block the interaction of membrane LAIR-1 with its ligand, which may lead to persistent activation of effector T cells [68]. These results suggest the role of LAIR-1 in inhibiting graft rejection and that soluble LAIR-1 may be used as a predictor for monitoring immune responses to organ transplants.

The inhibitory characteristic of the LAIR family has also been explored therapeutically. The use of LAIR-2 fused with the N-terminus portion of the human IgG1 Fc domain (LAIR-2-Fc) can inhibit the complement system activation [69]. The complement system is involved in the antibody-mediated rejection (AMR) of transplant organs by anti-HLA or anti-ABO antibodies [70]. C1q is a ligand for LAIR-1 on DCs, by which the binding to collagen-like regions of C1q leads to the activation of a negative intracellular signaling pathway mediated by the recruitment of SHP-1 [59]. When LAIR-2-Fc binds to C1q, it interferes with the activation of the classical pathways of the complement system. LAIR-2-Fc inhibited complement-mediated lysis of cells sensitized with anti-HLA antibodies in an *ex vivo* model for antibody-mediated rejection [69]. Therefore, LAIR-2-Fc is suggested to be an effective novel complement inhibitor for the treatment and prevention of antibody-mediated allograft rejection and antibody-mediated clinical conditions.

3.4. LILRs family

Leukocyte Ig-like receptors (LILRs, also called LIRs, ILTs, and CD85) are a family of 11 immunoregulatory receptors that comprise two functional classes: activating LILRs (LILRA1–6) and inhibitory LILRs (LILRB1–5) [71,72]. LILRs share homology to the human killer cell inhibitory receptor (KIR) family, with similar compositions of both their cytoplasmic domains and Ig-like structure [71]. The main difference is that KIRs are almost exclusively expressed in natural killer (NK) cells [73], while LILRs are found on most leukocyte subsets, including NK cells, T lymphocytes, B lymphocytes, and cells of the myeloid lineage – monocytes, macrophages, DCs, and granulocytes [73].

Regarding its function, the activation of LILRBs promotes tolerance and immunosuppression, whereas their antagonism induced an immunostimulatory milieu capable of generating potent effector immune responses [74]. LILRs predominantly modulate the function and fate of professional APCs such as macrophages, DCs and B cells [74–76]. LILRBs elicit inhibitory signaling via their long cytoplasmic tails that contain up to four ITIMs [71], which use the enzymatic phosphatase action of SHP-1 and SHP-2 proteins to suppress downstream signaling pathways, such as AKT and ERK, that normally promote effector functions [74,76] (Fig. 1, Table 1). Studies using genetically modified mice highlight the inhibitory role of this family of receptors *in vivo*. MDSCs from mice lacking PIR-B (mouse ortholog of LILRB2) underwent a specific transition to inflammatory M1-like cells when entering the periphery from bone marrow, resulting in decreased suppressive function and Treg activation activity [77]. PIR-B deficiency affected the maturation of DCs and the defective regulation of receptor-mediated activation in APCs [78]. This led to exacerbated Th2 immune responses and B cell hypersensitivity [78], suggesting the LILRB2 has a role in immunologic homeostasis and maintenance of self-tolerance.

The LILRB1 receptor (also known as LIR1, ILT2, CD85j) and its close relation to HLA is of interest in transplantation. LILRB1 engagement renders DCs tolerogenic, hindering the onset of adaptive immunity and promoting immune evasion [79–82]. LILRB1 was originally shown to

broadly bind to human leukocyte antigen (HLA) class I molecules (HLA-A, HLA-B, HLA-C) and non-classical HLA class I (HLA-E, HLA-F, HLA-G, and HLA-H) [75,83]. But it can also bind to other ligands including the non-HLA angiopoietin-like protein family [84] and S100A8/S100A9 [85]. Among the non-classical HLA class I molecules, HLA-G expression during allogeneic recognition is associated with a better graft acceptance [86]. The serum levels of soluble HLA-G dimers were demonstrated to be higher in stable kidney transplant recipients compared with those that had a rejection. Interestingly, these soluble HLA-G dimers were responsible for reducing granzyme B production by CD8⁺ T cells in a mechanism dependent on the LILRB1 receptor [86]. In addition, *in vivo* overexpression of LILRB1 leads to an expansion of an MDSC population with suppressive activity upon engagement by its HLA-G ligand [87]. When these MDSCs were adoptively transferred, they generated a LILRB1-dependent prolongation of skin allograft survival [87], suggesting that induction of MDSCs using LILRB1 inhibitory receptor with HLA-G ligand may be an attractive strategy for preventing rejection of highly immunogenic organs and tissues.

3.5. PD-1 expression by myeloid cells

PD-1 is a major target of immune checkpoint therapy because it is expressed by activated T cells, inhibiting their effector functions [88]. However, PD-1 can also be expressed by myeloid cells, which have limited knowledge of its role in several conditions (Fig. 1, Table 1). In a cancer context, all myeloid cell subsets upregulated PD-1 expression in two mice tumor models, B16-F10 (melanoma) and MC38 (colon carcinoma). PD-1 signaling can regulate myeloid responses and has implications for antitumoral immunity [89]. Also, mice lacking PD-1 expression in the myeloid compartment showed a shift in the myeloid cell fate from MDSCs towards differentiated monocytes, macrophages, and DCs. Interestingly, this led to more effective inhibition of tumor growth than T cell-specific PD-1 ablation [89]. The inhibitory receptor PD-1 blocks T-cell activation through the recruitment of the phosphatase SHP-2 to its cytoplasmic tail. The ablation of either SHP-2 or PD-1 in myeloid cells resulted in enhanced differentiation, activation, phagocytosis, and inflammatory responses in myeloid cells of tumor-bearing mice, leading to diminished tumor growth [90]. Thus, PD1:SHP-2 signaling restrains myelocyte differentiation resulting in a myeloid landscape that suppresses antitumor immunity [91]. These works in tumors show that PD-1 expression in myeloid cells can be effective in the development of antitumor immunity. The investigation of the function of PD-1 in myeloid cells after transplantation could be harnessed for the development of novel targets to tame rejection.

4. Inhibitory receptors and blood transcriptomics in transplant recipients

To illustrate the involvement of inhibitory innate receptors, we analyzed the gene expression of 14 human inhibitory innate receptors (*SIGLEC2*, *SIGLEC3*, *SIGLEC5*, *SIGLEC6*, *SIGLEC7*, *SIGLEC8*, *SIGLEC9*, *SIGLEC10*, *SIGLEC11*, *SIGLEC12*, *LILRB1*, *LAIR1*, *LAIR2*, and *CEACAM1*) using microarray analysis (GSE15296 [92]) based on the peripheral blood. We compared their expression during rejection and non-rejection episodes in kidney transplant recipients (Fig. 2). We found that all the genes were either down- or up-regulated during rejection events (Fig. 2), suggesting their putative involvement in alloimmunity. Although these data have the limitation that they may not reflect the precise alterations in the expression pattern of these receptors in the graft or lymphoid tissues, it strongly suggests the involvement of these receptors in transplantation.

5. Current immunosuppressive drugs and inhibitory receptors

Commonly used immunosuppressive drugs for transplant rejection primarily target adaptive immunity, with minor effects on innate

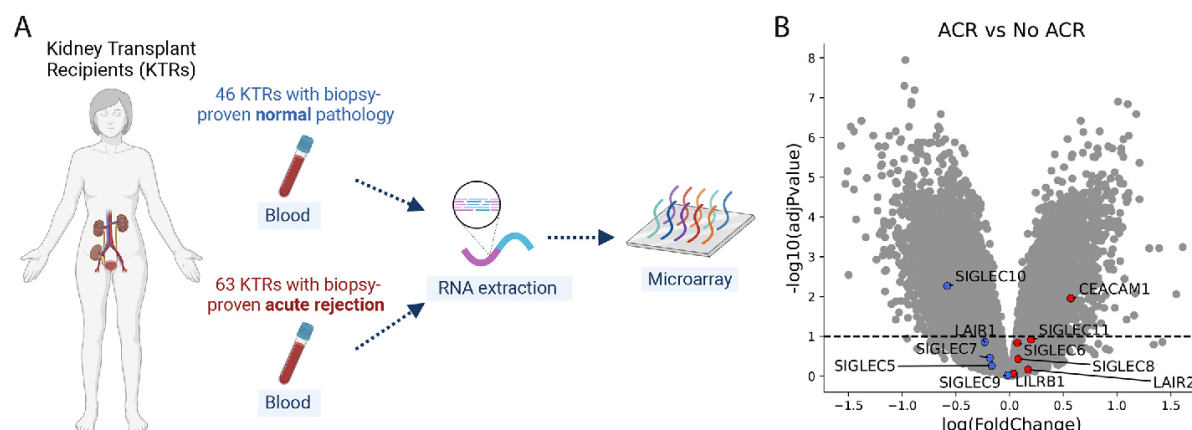


Fig. 2. (A) Schematic cartoon and (B) volcano plot showing the gene expression of different inhibitory innate receptors in acute cellular rejection (ACR) and non-rejection (No ACR) episodes from a microarray study based on peripheral blood of kidney transplant recipients (GSE15296 [92]). Cartoon created with BioRender.com.

immunity (reviewed by Zaza et al. [93]). Immunosuppression after transplantation includes induction agents (rabbit anti-thymocyte globulin and basiliximab) and maintenance drugs, such as calcineurin inhibitors (cyclosporine A and tacrolimus), inhibitors of the mTOR pathway (rapamycin and everolimus), antiproliferative agents (mycophenolate mofetil and azathioprine), costimulation blockade (Belatacept), and corticosteroids. While there is evidence that many of the immunosuppressive drugs are deleterious to regulatory T cells [94], recent reports suggest that some of these therapies can impact the expression of innate inhibitory receptors. In one study, the expression of Siglec receptors by primary human NK cells was increased upon *in vitro* incubation with cyclosporine A, tacrolimus, mycophenolate or corticosteroids. The combination of tacrolimus, mycophenolate and corticosteroids enhanced even more the expression of Siglecs by human NK cells [95]. Moreover, immunosuppressive drugs can influence the expression of the HLA-G (LILRB1 ligand). mTOR inhibitors [96,97] and Belatacept [98] treatments were associated with enhanced release of soluble HLA-G molecules. Despite those limited reports, the precise role of the immunosuppressants on the expression of innate inhibitory receptors, its downstream signaling pathways, and interaction with co-receptors and ligands still need to be further elucidated. This will be crucial to translate novel therapeutic approaches in combination with current agents aiming for drug minimization and improvement of graft survival.

6. Concluding remarks

The control of effector immune responses against the allograft is essential to prevent rejection. This can be achieved with immunosuppressive drugs that target mainly T cells and are linked with systemic side effects. The innate system has several inhibitory receptors that can counteract and/or fine-tune activating signals and downmodulate the effector adaptive responses. Targeting inhibitory receptors from the innate arm of the immune system is a potentially promising complementary strategy to overcome this problem.

Finally, despite the progress in identifying these innate inhibitory receptors, the biological context and their functional role in which they modulate effector immune cell functions in a pathological context, such as transplant rejection, is still poorly understood. Particularly, the better understanding of their expression kinetics and pattern in different cell subsets pattern, interaction with co-receptors, the detailed signaling pathways they interact with, location, and ligand availability in different tissues are still not well defined for these inhibitory receptors.

This knowledge and future studies are of paramount importance to developing novel innate-focused therapeutic targets in transplantation.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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9. Capítulo 6

Artigo Científico

Siglec-E is a Critical Inhibitory Receptor Controlling APC Activation and T Cell-Mediated Transplant Rejection

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Motivação: Desenvolvido durante o período de doutorado sanduíche no Massachusetts General Hospital (MGH)/Harvard Medical School – EUA. Estudar o envolvimento do receptor inibitório Siglec-E em rejeição de transplante de órgão sólido.

Summary

Early after transplantation, inflammation and tissue injury release danger signals that activate myeloid innate immune cells like DCs, driving adaptive immune responses and acute rejection. Current immunosuppressive drugs primarily target T cells, leaving innate immunity inadequately controlled. Siglec-E (SigE), an innate inhibitory receptor, binds sialic acid-carrying ligands to suppress inflammatory responses. In mouse heart transplants, SigE is upregulated in graft-infiltrating myeloid cells, including DCs. Human homologs Sig-7 and Sig-9 are also upregulated in rejecting heart and kidney transplant biopsies. Genetic ablation of SigE in recipients accelerates acute rejection of heart, kidney, and skin allografts. SigE-deficient DCs are more susceptible to activation by danger signals and show enhanced NF- κ B activation and TNF- α production, increasing alloreactive T-cell responses. Overexpressing SigE on DCs reduces their activation by DAMPs and their T-cell allostimulatory capacity. Thus, SigE is a crucial inhibitory receptor controlling APC activation and T cell-mediated transplant rejection.

Introduction

The initial immunological challenge in solid organ transplantation entails activating the innate immune cells through danger signals or damage-associated molecular patterns (DAMPs) released by the tissue ischemic injury. This innate activation leads to an early inflammatory cascade culminating in the differentiation and stimulation of antigen-presenting cells (APCs) ^{1,2}. Following their activation, APCs deliver the required signals to induce alloreactive T cells in secondary lymphoid organs ^{3,4}. Exacerbated innate immune responses may hinder and break tolerance in preclinical models ⁵⁻⁷, enhance the adaptive immune response, and contribute to late events like graft fibrosis and failure ⁸⁻¹⁰. Hence, regulating innate immune activation is a crucial step in the prevention of transplant rejection and improvement in transplant outcomes. However, current immunosuppressive regimens primarily target T cells, while innate immunity is not efficiently controlled in transplantation. Therefore, an unmet need exists to define underlying suppressive mechanisms and develop effective strategies to modulate innate immunity.

Immune regulation of innate immunity is vital for maintaining a balanced immune response, preventing excessive inflammation, and avoiding tissue damage. Specifically, innate inhibitory receptors can counteract and fine-tune inflammatory signals triggered by DAMPs through pattern recognition receptors ¹¹. Understanding how different inhibitory innate receptors regulate inflammation in transplant rejection is crucial to developing innate-focused therapies. Indeed, myeloid cells with suppressive functions can inhibit effector T cells and induce Tregs, enabling immune regulation in experimental models^{12,13}. Nevertheless, the mechanisms governing myeloid suppressive functions in transplantation still need to be fully understood.

Siglec (sialic acid-binding immunoglobulin-like lectin)-E, or SigE, is an innate receptor that down-modulates inflammation. SigE is expressed by myeloid cells¹⁴, binds mainly to ligands carrying sialic acids, and inhibits TLRs-triggered inflammatory responses^{15,16}.

Here, we identified a crucial inhibitory mechanism in transplantation mediated by SigE. SigE deficiency accelerates acute cellular rejection of heart, kidney, and skin allografts. Mechanistically, SigE-deficient DCs are more susceptible to activation by a danger signal and can induce exacerbated alloreactive T-cell responses, precipitating rejection. In human transplant recipients, Sig7 and 9 – the human counterparts of SigE – were upregulated in heart and kidney biopsies during rejection. Moreover, Sig9-silenced human Mo-DCs recapitulated our findings using murine APCs, leading to higher allo-T cell activation. These data undercover SigE/7/9 as an attractive potential target for reducing rejection rates and improving long-term graft survival.

Results

Siglec-E is differently expressed on dendritic cells and Ly6C^{hi} monocytes upon heart transplantation

To characterize Siglec-E expression by myeloid cells accumulating in allografts during rejection, we transplanted BALB/cJ hearts (H-2^d) into fully mismatched C57BL6/J (H-2^b) recipients. We harvested cardiac allografts on days 3, 5 and 7 post-transplantation and analyzed SigE expression by graft-infiltrating leukocytes by flow cytometry (**Figure 1A, Suppl Figure 1A**). When we gated on live CD45⁺ heart allograft cells, we identified four major populations based on differential expression patterns of CD11c, CD11b, Ly6C and Ly6G (**Figure 1B**), as previously reported in Conde *et al.*¹². SigE was significantly upregulated in total CD45⁺ leukocytes following transplantation (**Figure 1C**). From the infiltrating cells, quantitative analysis revealed a higher SigE upregulation in DCs (**Figure 1D**) and Ly6C^{hi} monocytes (**Figure 1E**) on day 7 after transplantation when allografts start to reject. SigE expression remained stable in Ly6C^{int/low} monocytes (**Figure 1F**) and neutrophils (**Figure 1G**) over time. Immunofluorescence analysis confirmed SigE upregulation in rejecting heart allografts (**Figure 1H**). We next sought to assess the potential clinical relevance of these results. We investigated the expression of *SIGLEC7* and *SIGLEC9* (SigE homologs in humans) transcripts in heart biopsies from transplanted recipients during stable or rejecting time points in datasets previously described^{17,18}. In humans, both *SIGLEC7* and *SIGLEC9* genes were upregulated during rejection episodes (**Figure 1I**). These findings are consistent with the negative feedback mechanism, in which SigE is upregulated to control inflammatory responses.

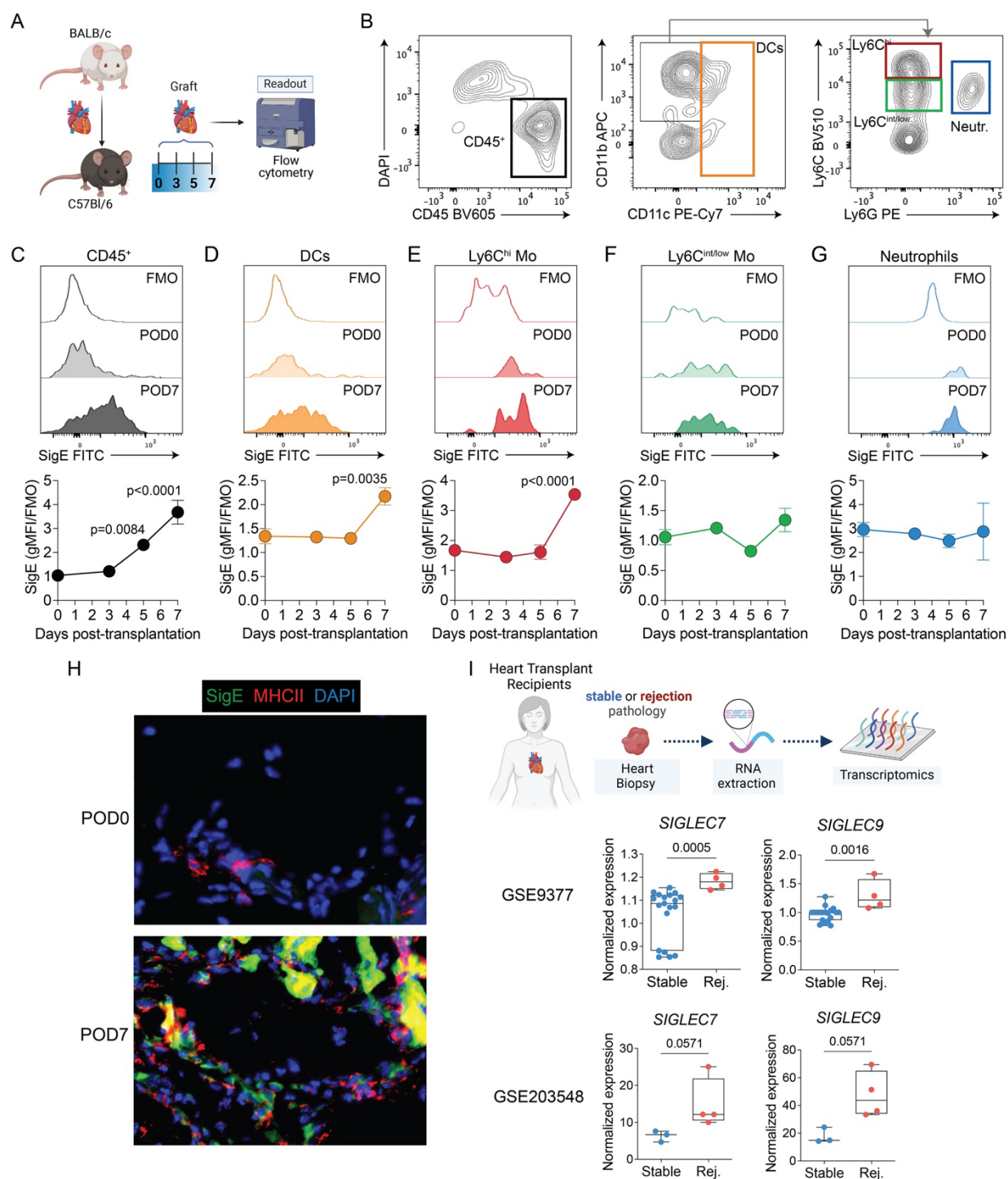


Figure 1. Siglec-E is upregulated in graft-infiltrating myeloid cells during heart transplant rejection. (A) Schematic of experimental design for (B - H). BALB/c hearts were transplanted into C57BL/6 recipients. Cardiac allografts and spleens were harvested for flow cytometry analyses on days 3, 5 and 7 post-transplantation. (B) Gating strategy for graft-infiltrating innate myeloid cell populations. Representative histograms and normalized SigE expression (gMFI: geometric mean fluorescence intensity/FMO: fluorescence minus one) over time on (C) total CD45⁺, (D) DCs, (E) Ly6C^{hi}, (F) Ly6C^{int/low} and (G) neutrophils from heart allografts at different time points. Statistics were performed by one-way ANOVA with Tukey's multiple comparisons test (n = 4 - 6 mice/time point). (H) SigE and MHC II immunofluorescence analyses in naïve

hearts and rejecting cardiac allografts on day 7 post-transplantation (POD: postoperative day). (A-H) Representative of two independent experiments. (I) *SIGLEC7* and *SIGLEC9* gene expression in heart allograft biopsies during stable or rejection episodes from two public studies in heart transplant recipients (GSE9377 and GSE203548). Statistical analyses using the Mann-Whitney test.

The inhibitory Siglec-E receptor controls allograft rejection

To evaluate the suppressive function of SigE *in vivo*, we transplanted BALB/c hearts (H-2^d) into WT or SigE^{KO} (both H-2^b) fully allogeneic recipients. We treated them with a single dose of costimulation blockade (**Figure 2A**). Treating WT recipients with a single dose of CTLA-4-Ig on day 2 resulted in a subacute rejection with a median survival time (MST) of 58 days. Interestingly, SigE^{KO} recipients have accelerated rejection with an MST of 19 days ($p < 0.0001$, **Figure 2B**). Histopathology analyses of the allografts from SigE^{KO} recipients revealed more inflammation, endarteritis (**Figure 2C**) than WT controls. We next investigate whether SigE^{KO} transplanted mice have increased alloreactive T cell responses on day 21 after transplant by ELISPOT (**Figure 2D**). SigE^{KO} recipients showed more IFN- γ -producing alloreactive T cells than controls (**Figure 2E**). Moreover, grafts isolated from SigE^{KO} mice weighted significantly more than WT controls (**Figure 2F**). Similarly, the numbers of graft-infiltrating CD45⁺ lymphocytes were significantly higher in SigE^{KO} recipients (**Figure 2G**, **Suppl Figure 1B**). Cardiac grafts from SigE^{KO} mice had a significantly higher frequency and number of IFN- γ +TNF- α + CD4⁺ (**Figure 2H**) and CD8⁺ T cells (**Figure 2I**), confirming our ELISPOT data. This was accompanied by increased graft-infiltrating Ki67⁺GranzymeB⁺ cytotoxic CD8⁺ T cells (**Figure 2J**).

We employed a semi-allogeneic model in which BALB/c $\times$ B6 F1 (CB6F1/J, H-2^{d/b}) donor hearts were transplanted into WT or SigE^{KO} hosts (**Suppl Figure 2A**) to exclude the costimulation blockade as a co-founder. In this model, the genetic ablation of SigE also leads to decreased CB6F1/J cardiac allograft survival (MST: 24 vs. 11 days, $p = 0.0008$, **Suppl Figure 2B**). Next, we investigated whether SigE inhibitory effects are universal or restricted to heart allografts. We analyzed the expression of *SIGLEC7* and *SIGLEC9* transcripts in biopsies of human kidney transplanted recipients and found upregulation of both genes during rejection (**Suppl Figure 3A**). We then tested the role of SigE in a mouse model of kidney transplantation (**Suppl Figure 3B**), previously reported by our group¹⁹. Like the heart transplant models, SigE^{KO} kidney recipients had severe acute cellular rejection characterized by tubulointerstitial inflammation and tubulitis (**Suppl Figure 3C**), and higher acute cellular rejection scores (**Suppl Figure 3D**). Spleens of SigE^{KO} transplanted mice were enlarged (**Suppl Figure 3E**), and grafts had significantly higher numbers of CD45⁺ cells (**Suppl Figure 3F**) along with cytotoxic CD8⁺

T cells (**Suppl Figure 3G**). Additionally, SigE^{KO} recipients rapidly rejected skin allografts from Act-mOVA donors in a minor mismatch skin mouse transplantation model (MST: 21.5 vs. 12.5 days, $p = 0.0021$, **Suppl Figure 4**). Thus, the absence of SigE exacerbates acute T cell-mediated rejection, leading to shortened allograft survival. Our data demonstrate that SigE has inhibitory functions in controlling rejection in different mouse transplantation models, including heart, kidney and skin.

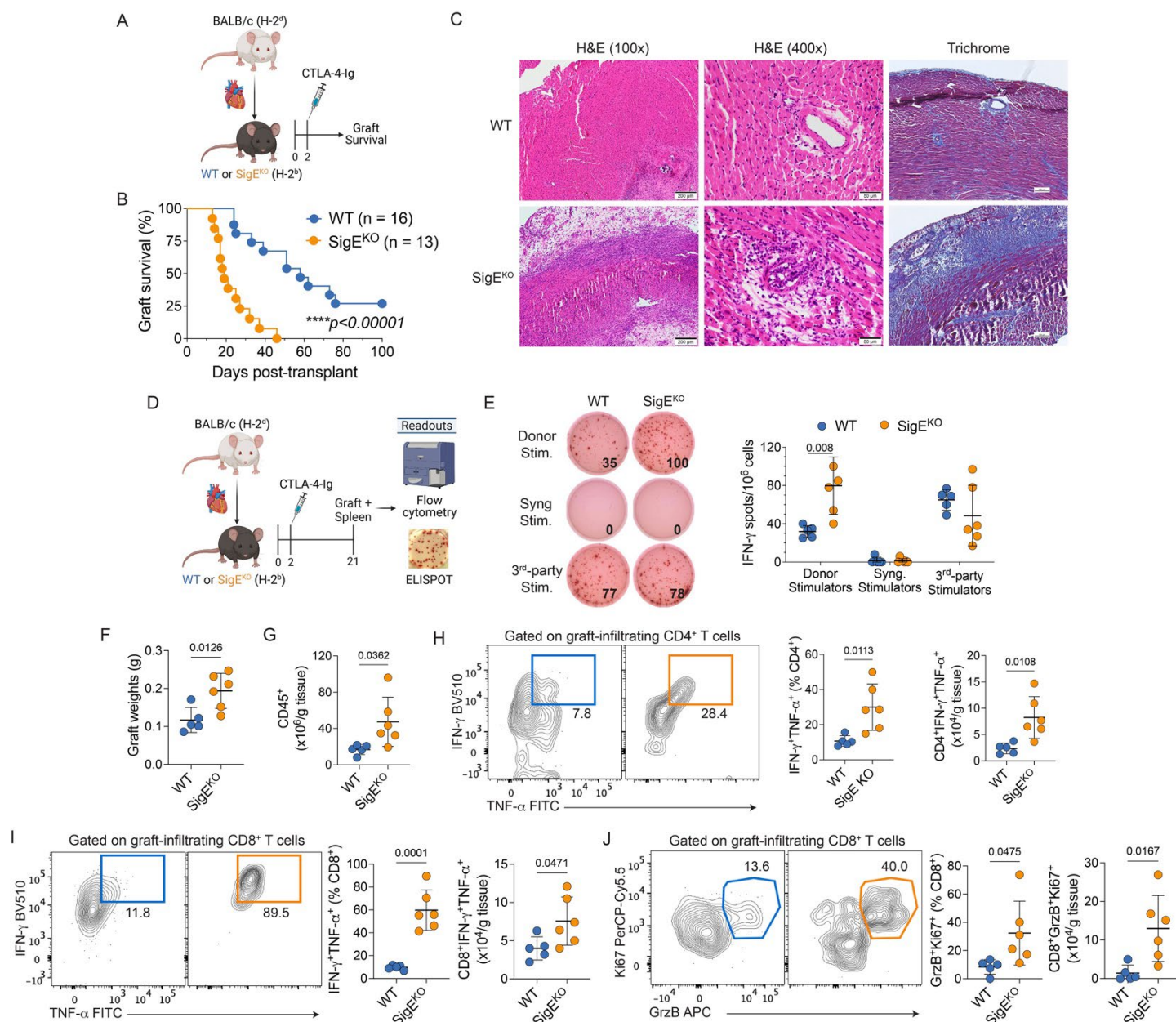


Figure 2. Siglec-E inhibits T cell-mediated rejection. (A) Schematic of experimental design in which BALB/c hearts were transplanted into WT or SigE^{KO} C57BL/6 recipients treated with a single dose of CTLA-4-Ig (250 μ g on day 2 after transplant). (B) Kaplan-Meier curves of cardiac

allograft survival. The p-value was determined by a log-rank test ($n = 13 - 16$ mice/group). (C) Representative images of H&E- or trichrome-stained sections of heart allografts harvested from each group on day 21 after transplant. $\times 100$ or 400 magnifications. (D) Twenty-one days after heart transplants, splenocytes and heart allografts populations from WT or SigE^{KO} mice were analyzed by flow cytometry and ELISPOT. (E) Splenic T cells were magnetically isolated and cultured with allogeneic (donor; BALB/c), syngeneic (syng; B6), or 3rd-party (C3H) irradiated splenocytes for 24 h in anti-mouse IFN- γ -coated plates. IFN- γ production by T cells was measured by ELISPOT (number of spots per 10^6 T cells $\pm$ SD in quintuplicates). (F) Harvested graft weights and (G) numbers of heart allografts total CD45⁺ cells in each group. Representative contour plots, percentages and absolute numbers of graft-infiltrating (H) IFN- γ ⁺TNF- α ⁺CD4⁺ T cells, (I) IFN- γ ⁺TNF- α ⁺CD8⁺ T cells, and (J) Ki67⁺GranzymeB⁺CD8⁺ T cells. (F – J) Statistical analyses were done by unpaired t-test ($n = 5 - 6$ mice/group).

The absence of the inhibitory Siglec-E receptor in the innate compartment accelerates cardiac graft rejection

Given that SigE is mainly upregulated by DCs and allografts are rapidly rejected by SigE-deficient mice, we sought to investigate whether DCs from transplanted mice are more activated in the absence of this inhibitory receptor. Indeed, graft and splenic DCs from SigE^{KO} heart allograft recipients express higher levels of MHCII and CD86 on day 10 post-transplantation (**Figure 3A-B**). Cardiac grafts and spleens from SigE^{KO} mice had a significantly higher frequency and number of MHC^{hi}CD86^{hi} activated DCs (**Figure 3C-D**) that were producing TNF- α (**Figure 3E-F**), confirming our ELISPOT data. Similar findings were observed in the kidney transplant model (**Suppl Figure 3H**). We next asked whether antigen-presentation in the absence of SigE leads to enhanced activation of antigen-specific T cells. To answer this question, we crossed Rag1^{KO} mice to SigE^{KO} to generate a Rag1^{KO}SigE^{KO} strain. Flow cytometry experiments confirmed that Rag1^{KO}SigE^{KO} mice have SigE expression ablated on four major myeloid populations (**Suppl Figure 5A-B**), including DCs (**Suppl Figure 5C**), Ly6C^{hi} monocytes (**Suppl Figure 5E**), Ly6C^{int/low} monocytes (**Suppl Figure 5G**), and neutrophils (**Suppl Figure 5I**). Interestingly, while these animals have similar levels of DCs (**Suppl Figure 5D**), they presented increased levels of Ly6C^{hi} monocytes (**Suppl Figure 5F**), Ly6C^{int/low} monocytes (**Suppl Figure 5H**), and neutrophils (**Suppl Figure 5J**) when compared to Rag1^{KO} controls, underlying SigE inhibitory effects. To investigate SigE role in T cell-mediated rejection, we transplanted Act-mOVA heart grafts (H-2^b) into Rag1^{KO} or Rag1^{KO}SigE^{KO} (H-2^b) recipients and adoptively co-transferred OVA-specific OT-I and OT-II at a 1:1 ratio 5 days after transplantation (**Figure 3G**). As previously described, most Act-mOVA grafts were not rejected (**Figure 3H**) and had a normal pathology (**Figure 3I**) because they failed to mount an allogeneic non-self innate immune response²⁰. Surprisingly, Act-mOVA cardiac grafts were rapidly rejected by Rag1^{KO}SigE^{KO} mice

(MST: >50 vs. 12 days, $p < 0.0001$, **Figure 3H**). Grafts harvested from $\text{Rag1}^{\text{KO}}\text{SigE}^{\text{KO}}$ recipients showed extensive necrosis and a higher acute cellular rejection score (**Figure 3I**). The genetic ablation of SigE led to higher numbers of intra-graft $\text{MHC}^{\text{hi}}\text{CD86}^{\text{hi}}$ activated DCs producing more TNF- α (**Figure 3J**) on day 7 post-transplant. Graft-infiltrating OT-I and OT-II cells were analyzed based on their expression of Va2 and Vb5 TCR chains, (**Suppl Figure 1C**). We found greater number of graft-infiltrating total and IFN- γ ⁺TNF- α ⁺ OT-I and OT-II cells in $\text{Rag1}^{\text{KO}}\text{SigE}^{\text{KO}}$ mice (**Figure 3K**), consistent with a more potent acute T cell-mediated cellular rejection. These findings imply that DAMPs released during tissue ischemia can activate innate immune cells in SigE-deficient mice, triggering functional antigen-specific adaptive immune responses that cause graft rejection. Our data suggest that lacking an important inhibitory receptor such as SigE can overcome the allogeneic non-self component required to initiate an innate immune response in transplantation²⁰.

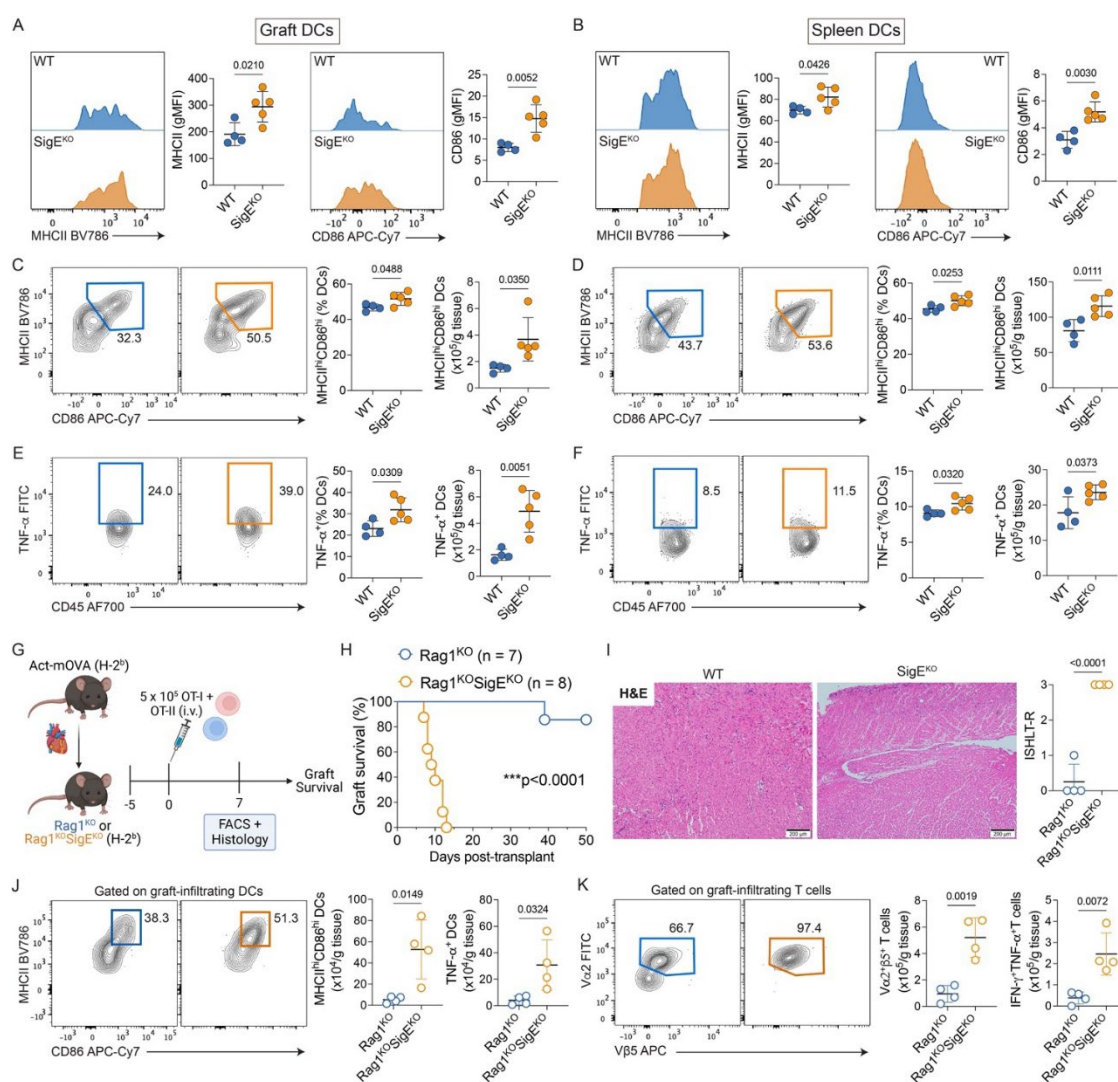


Figure 3. The absence of Siglec-E enhances innate activation and precipitates graft rejection. BALB/c hearts were transplanted into WT or SigE^{KO} C57BL/6 recipients treated with

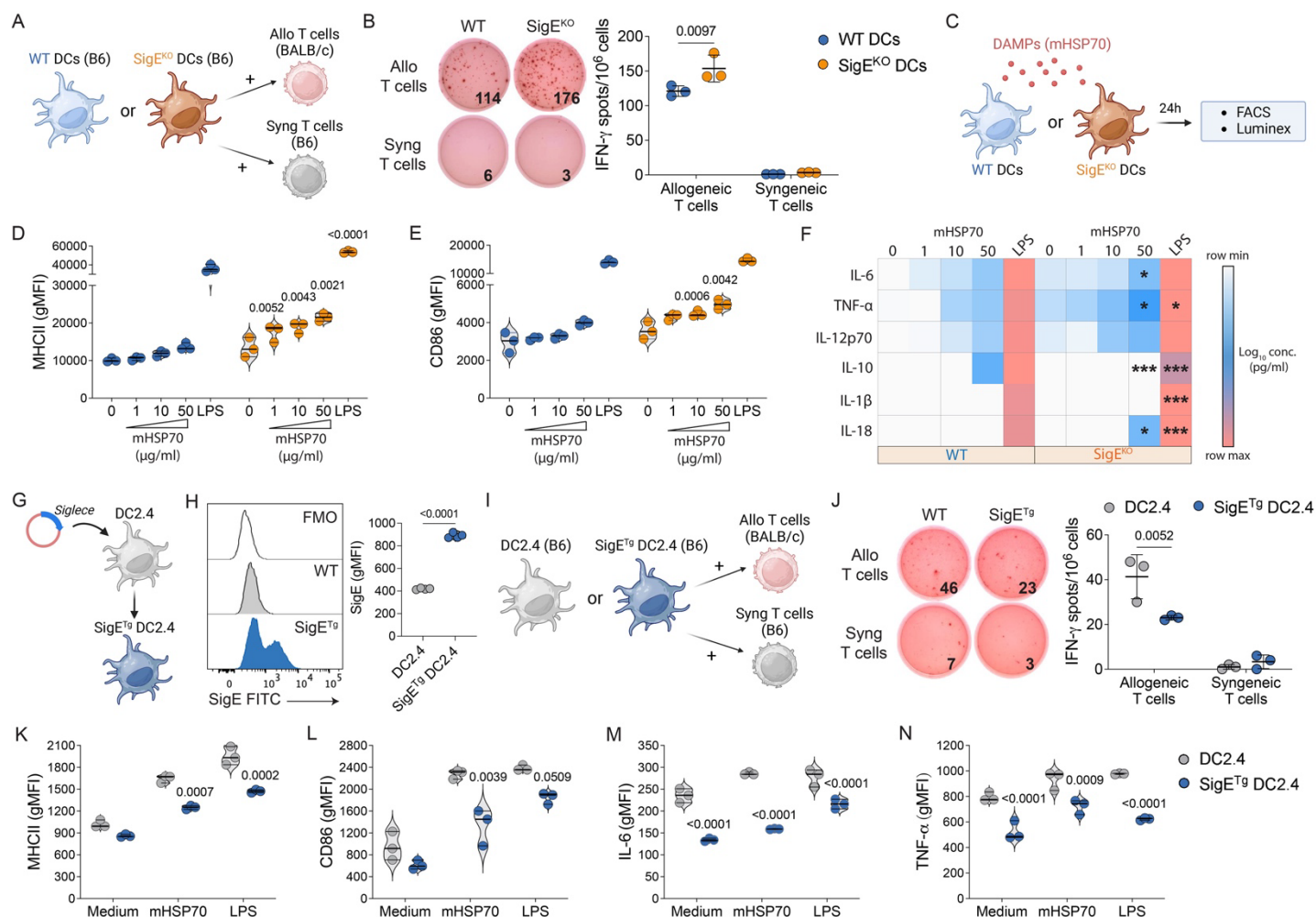
a single dose of CTLA-4-Ig (250µg on day 2 after transplant). Splenocytes and heart allografts populations were analyzed by flow cytometry on day 10 post-transplantation. (A) MHCII and CD86 geometric mean fluorescence intensity (gMFI) on (A) graft or (B) splenic DCs. Representative contour plots, percentages and absolute numbers of MHCII^{hi}CD86^{hi} (C) graft or (D) splenic DCs. Representative contour plots, percentages and absolute numbers of TNF-α⁺ (E) graft or (F) splenic DCs. (G) Act-mOVA heart grafts were transplanted into B6 Rag1^{KO} or Rag1^{KO}SigE^{KO} recipients, and mice received 5x10⁵ OT-I and 5x10⁵ OT-II cells 5 days after transplantation. (H) Graft survival as in (G). The p-value was determined by a log-rank test (n = 7 - 8 mice/group). (I) Representative images of H&E-stained sections and rejection scores of heart allografts harvested from each group on day 7 after adoptive transfer. ×100 magnification. (J) Representative contour plots and absolute numbers of MHCII^{hi}CD86^{hi} graft-infiltrating DCs, along with absolute numbers of TNF-α⁺ DCs. (K) Adoptively transferred OT-I and OT-II cells were analyzed based on their expression of Va2 and Vb5 TCR chains. Representative contour plots and absolute numbers of total Va2⁺Vb5⁺ graft-infiltrating T cells, along with absolute numbers of Va2⁺Vb5⁺IFN-γ⁺TNF-α⁺ T cells. (A - K) Statistical analyses were done by unpaired t-test (n = 4 - 5 mice/group).

Siglec-E regulates DC activation induced by DAMPS and controls the induction of alloreactive T cell responses

We reasoned that SigE controls DC activation induced by danger signals, modulating the activation of alloreactive T cells. To investigate that, we differentiated bone marrow-derived DCs (BMDCs) from WT or SigE^{KO} mice and co-culture them with allogeneic (BALB/c) or syngeneic (B6) T cells in an IFN-γ-coated ELISPOT plate for 3 days (**Figure 4A**). SigE^{KO} DCs induced a higher production of IFN-γ spots by allo-T cells compared to WT DCs (**Figure 4B**), suggesting an increase in the priming of alloreactive cells. To study whether SigE^{KO} DCs are more susceptible to activation by DAMPs, we treated WT or SigE^{KO} BMDCs with increasing doses of mHSP70 – a danger signal well-characterized by our group^{21,22} (**Figure 4C**). LPS was used as a positive control. mHSP70 increased the expression of DC activation markers MHC II (**Figure 4D**) and CD86 (**Figure 4E**) in a dose-dependent manner. This elevation was notably more significant in the absence of Siglec-E (**Figure 4D-E**). Upon DAMP stimulation, SigE^{KO} BMDCs produced more IL-6, TNF-α and IL-18 and less IL-10 than WT cells (**Figure 4F**). Therefore, the absence of the inhibitory SigE receptor potentiates the expression of maturation markers and inflammatory cytokines production by BMDCs stimulated with a danger signal.

To confirm that SigE was negatively regulating the activation of DCs and their ability to stimulate alloreactive T cells, we generated murine DCs that overexpressed SigE using the DC cell line DC2.4 (SigE^{Tg}, **Figure 4G-H**). We then co-cultured irradiated SigE^{Tg} or control DC2.4 DCs with allogeneic (BALB/c) or syngeneic (B6) T cells in an IFN-γ-coated ELISPOT plate (**Figure 4I**). SigE^{Tg} DCs stimulated significantly less IFN-γ-producing allo-T cells (**Figure 4J**), demonstrating SigE's role in controlling DC allo-stimulatory ability. Next, we investigated whether the overexpression of SigE on DCs would inhibit DAMP-triggered DC activation. SigE^{Tg}

or control DC2.4 cells were treated with mHSP70 or LPS. DC activation was determined by evaluating the surface levels of MHC II and CD86, and the cytokine profile by flow cytometry. We observed that control DC2.4 cells had a more pronounced increase in the expression of MHC II (**Figure 4K**) and CD86 (**Figure 4L**) when compared to SigE^{Tg} DC2.4 cells. Also, the increase in the production of IL-6 (**Figure 4M**) and TNF- α (**Figure 4N**) was lower in SigE^{Tg} DCs. Our findings demonstrate that SigE regulates and fine-tunes inflammatory effects induced by danger signals, impacting the subsequent adaptive immune response.



represent two repetitions performed in triplicates of a pool of 2 mice per genotype. **(G)** DC2.4 cells were lentivirally transduced with a *Siglece* gene encoding plasmid. **(H)** SigE expression in control and SigE-overexpressing (SigE^{Tg}) DC2.4 cells. gMFI: geometric mean fluorescence intensity. **(I)** Control or SigE^{Tg} DC2.4 cells were irradiated and co-cultured with allogeneic (allo, BALB/c) or syngeneic (syng, B6) T cells for 5 days in anti-mouse IFN- γ -coated plates. **(J)** IFN- γ production by T cells was measured by ELISPOT. Numbers of spots per 10⁶ T cells $\pm$ SD in triplicates. Control or SigE^{Tg} DC2.4 cells were treated with 50 μ g/ml of mHSP70 or 250 ng/ml of LPS for 24h, and **(K)** MHC II, **(L)** CD86, **(M)** IL-6, and **(N)** levels were evaluated by flow cytometry. **(J - N)** Experiments were performed in triplicates, and statistical analyses were done by unpaired t-tests.

Transcriptional Profiling Reveals Upregulation of Pro-Inflammatory Immune Pathways in SigE-Deficient Dendritic Cells

A prediction from our findings is that SigE deficiency triggers key pro-inflammatory signaling pathways on DCs. We performed bulk RNA-seq analyses to assess the transcriptional identity of DCs from WT or SigE^{KO} heart recipient mice in more detail. Splenic DCs (CD11c⁺MHCII⁺CD3⁻CD19⁻) were flow-sorted on day 10 post-transplant (**Figure 5A**). When analyzing differentially expressed genes (DEGs), we observed 428 were upregulated and 134 downregulated genes in SigE^{KO} DCs compared to WT (**Figure 5B**). Next, we performed gene set enrichment analysis (GSEA) to better understand the transcriptional changes in SigE^{KO} vs WT DCs. The DEGs in SigE^{KO} DCs corresponded to innate immune-related pathways like TNF- α signaling via NF- κ B (**Figure 5, C and D**), natural killer cell-mediated cytotoxicity (**Figure 5, C and E**), allograft rejection (**Figure 5, C and F**) and inflammatory response (**Figure 5, C and G**), among other categories related to immune responses. Together, these data suggest that SigE plays a role in inhibiting innate pro-inflammatory genes in DCs upon heart transplantation.

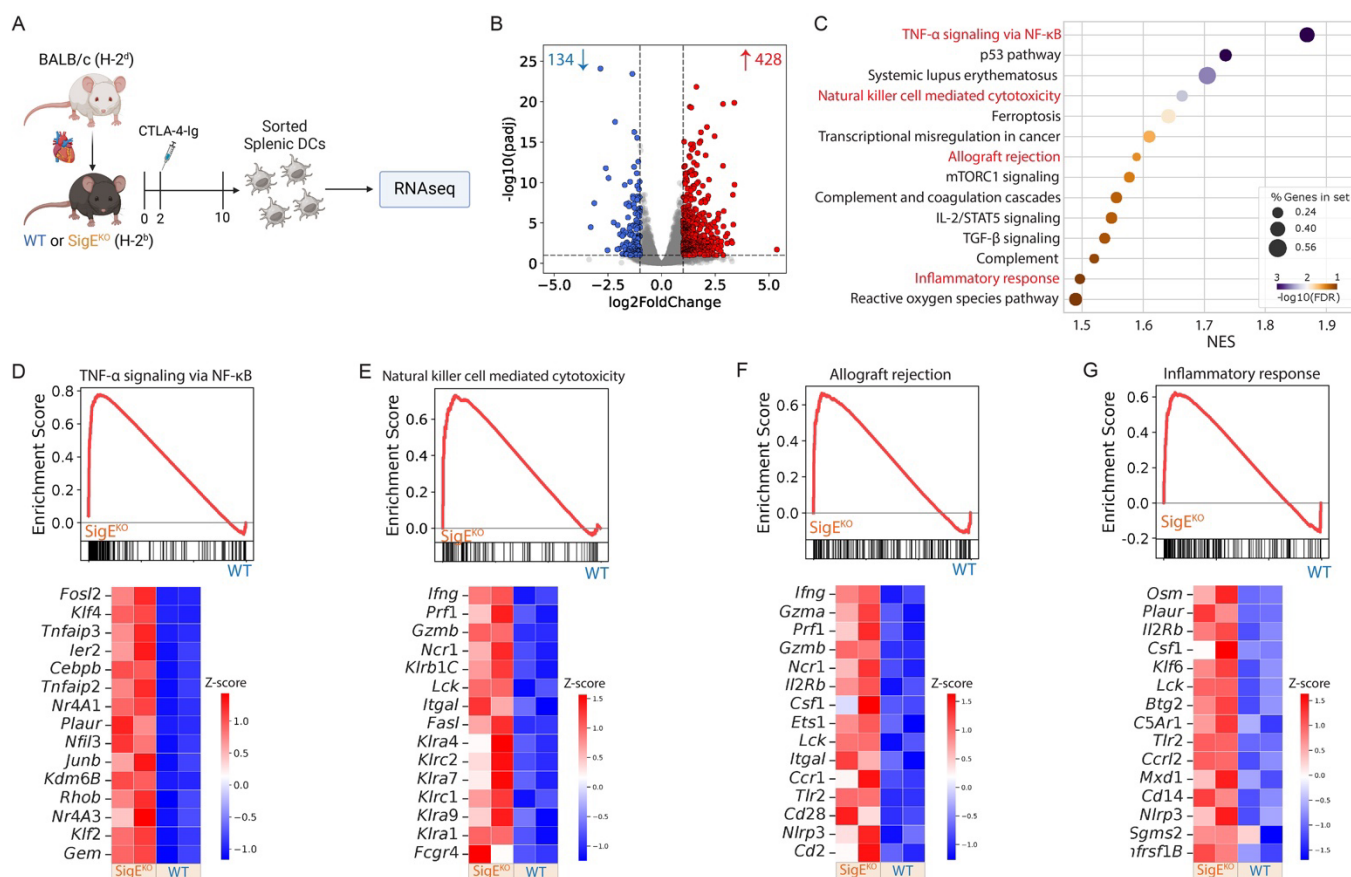


Figure 5. Siglec-E ablation leads to the upregulation of a pro-inflammatory transcriptional profile in DCs. (A) BALB/c hearts were transplanted into WT or SigE^{KO} C57BL/6 recipients treated with a single dose of CTLA-4-Ig (250μg on day 2 after transplant). Splenic DCs were flow-sorted on day 10 post-transplantation for RNA-seq analysis. (B) Volcano plot showing significantly upregulated (red, 428) and downregulated (blue, 134) genes were identified by comparing SigE^{KO} to WT DCs. (C) Top 14 gene ontology terms for DEGs in SigE^{KO} group compared to WT. Heatmap and gene set enrichment analysis plot of genes involved in (D) TNF-α signaling via NF-κB, (E) natural killer cell-mediated cytotoxicity, (F) allograft rejection, and (G) inflammatory response pathways. The experiments represent two duplicates of a pool of 5 mice per genotype.

Blocking the NF-κB:TNF-α axis abrogates the pro-inflammatory effects triggered by Siglec-E deficiency

Previous studies suggested that SigE may inhibit DAMP-triggered inflammatory signaling by recruiting SH2-domain-containing protein tyrosine phosphatases SHP-1 and SHP-2 through its tyrosine-based inhibitory motifs (ITIM)²³, thus dephosphorylating NF-κB and dampening TNF-α production^{15,16} (**Figure 6A**). Given that NF-κB signaling on DCs is essential to induce alloreactive T cell responses²⁴, we evaluated its levels in WT or SigE^{KO} BMDCs by flow cytometry. In fact, SigE^{KO} BMDCs had higher levels of phosphorylated p65 (p-p65, activated NF-κB) compared to control DCs (**Figure 6B**). TNF-α production is an important cytokine associated with early

inflammatory events following transplantation²⁵. Because SigE^{KO} DCs produced more TNF- α and the NF- κ B:TNF- α signaling axis was transcriptionally upregulated, we next investigated whether NF- κ B or TNF- α were required for the enhanced allo-stimulatory ability found in SigE^{KO} DCs. To investigate that, we initially performed an ELISPOT assay in which we treated SigE^{KO} BMDCs with the NF- κ B inhibitor PDTC²⁴ (**Figure 6C**) or an anti-mouse TNF- α blocking antibody (**Figure 6E**), and co-cultured them with allogeneic (BALB/c) or syngeneic (B6) T cells. SigE^{KO} BMDCs were not able to induce a higher production of IFN- γ spots by allogeneic T cells when NF- κ B was inhibited (**Figure 6D**), or upon TNF- α blockade (**Figure 6F**), suggesting a negative regulation of this pro-inflammatory pathway by SigE. Our data suggest that enhanced NF- κ B activation and TNF- α production could contribute to the accelerated graft rejection observed in SigE^{KO} recipient mice. We attempted to circumvent these effects by *in vivo* blocking NF- κ B with PDTC inhibitor or anti-mouse TNF- α antibody XT3.11 in the first-week post-transplant (**Figure 6G**). This strategy was chosen to target initial innate immunity activation early after the heart transplantation. Administration of either NF- κ B inhibitor PDTC (MST of 96.5 days, $p = 0.0007$) or anti-TNF- α mAb (MST of 75 days, $p = 0.0011$) significantly prolonged graft acceptance in SigE^{KO} heart recipients (Controls, MST of 32 days, **Figure 6H**).

Next, we used our SigE^{Tg} DCs to study whether SHP-2-mediated SigE inhibitory functions suppress alloreactive T cell generation. As expected, SigE^{Tg} DCs have higher baseline levels of SHP-2 (**Figure 6I**). To inhibit SHP-2, we treated SigE^{Tg} DCs with the SHP099 inhibitor for 24h before co-culturing them with allogeneic (BALB/c) or syngeneic (B6) T cells in an IFN- γ -coated ELISPOT plate (**Figure 6J**). The decreased ability of SigE^{Tg} DCs in inducing the production of IFN- γ spots by allogeneic T cells was abolished when SHP-2 was inhibited (**Figure 6K**), consistent with its inhibitory functions. Thus, SigE controls NF- κ B activation and the subsequent TNF- α production by the recruitment of SHP-2, fine-tuning the activation of allogeneic adaptative immune responses.

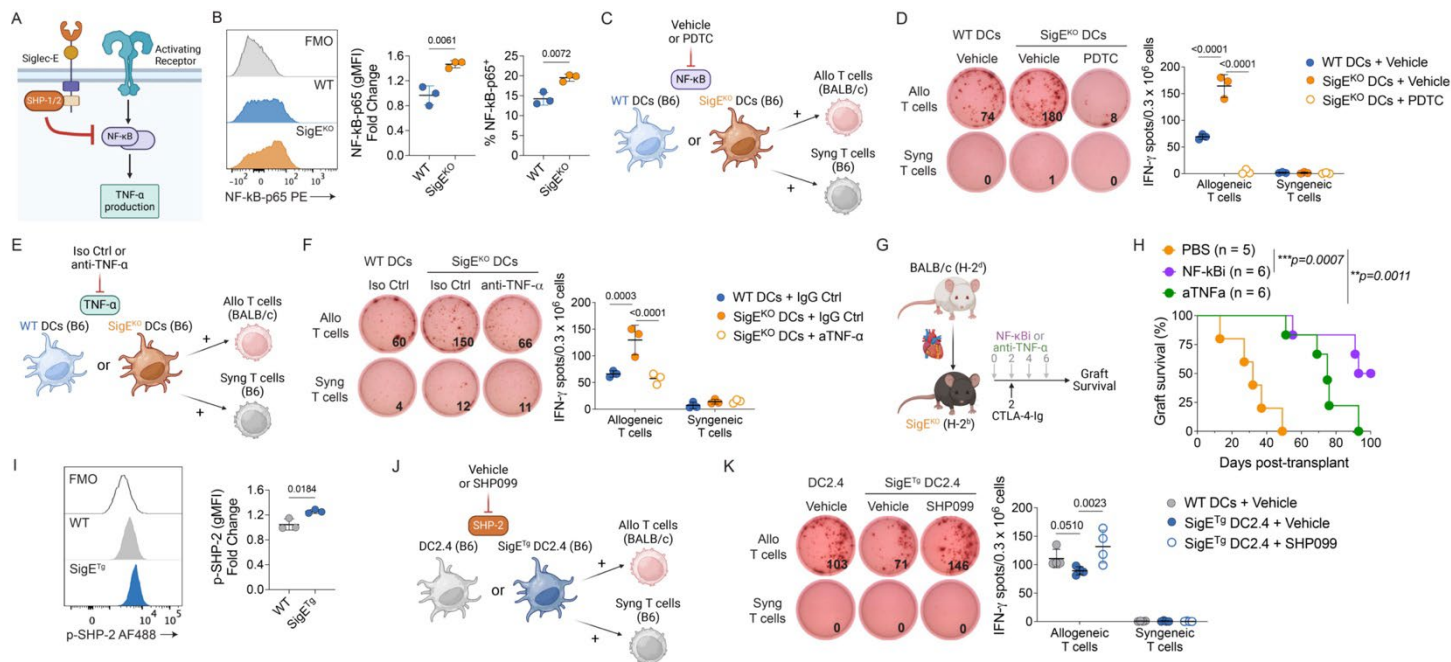


Figure 6. NF-κB and TNF-α blockade attenuate inflammatory effects in SigE-deficient DCs.

(A) SigE recruits SHP-1/2, inhibiting NF-κB activation and the following TNF-α production. (B) WT or SigE^{KO} BMDCs were treated for 30 min with PMA+ ionomycin and levels of phosphorylated p65 (p-p65, activated NF-κB) were analyzed by flow cytometry. Representative histograms, gMFI levels and percentages of p-p65⁺ cells. Experiments were performed in triplicates, and statistical analyses were done by unpaired t-tests. (C) WT or SigE^{KO} BMDCs pre-treated with vehicle or PDTC (NF-κB inhibitor) were irradiated and co-cultured with allogeneic (allo, BALB/c) or syngeneic (syng, B6) T cells for 3 days in anti-mouse IFN-γ-coated plates. (D) IFN-γ production by T cells from (C) was measured by ELISPOT. Numbers of spots per 0.3 x 10⁶ T cells ± SD in triplicates. (E) WT or SigE^{KO} BMDCs pre-treated with isotype control (iso ctrl) or anti-mouse TNF-α were irradiated and co-cultured with allogeneic (allo, BALB/c) or syngeneic (syng, B6) T cells for three days in anti-mouse IFN-γ-coated plates. (F) IFN-γ production by T cells from (E) was measured by ELISPOT. Numbers of spots per 0.3 x 10⁶ T cells ± SD in triplicates. (D and F) Experiments were performed in triplicates, and statistical analyses were performed using by one-way ANOVA with Tukey post-test. (G-H) Graft survival of WT or SigE^{KO} C57BL/6 recipients transplanted with BALB/c hearts and treated with a single dose of CTLA-4-Ig in combination with PDTC (NF-κB inhibitor) or anti-mouse TNF-α treatments on days 0, 2, 4 and 6 after transplant. The p-value was determined by a log-rank test (n = 5 - 6 mice/group). (I) Baseline levels of phosphorylated SHP-2 (p-SHP-2) in control or SigE^{Tg} DC2.4 cells. Experiments performed in triplicates and statistical analyses were done by unpaired t-tests. (J) Control or SigE^{Tg} DC2.4 cells pre-treated with vehicle or SHP099 (SHP-2 inhibitor) were irradiated and co-cultured with allogeneic (allo, BALB/c) or syngeneic (syng, B6) T cells for 5 days in anti-mouse IFN-γ-coated plates. (K) IFN-γ production by T cells from (J) was measured by ELISPOT. Numbers of spots per 0.3 x 10⁶ T cells ± SD in triplicates. Experiments were performed in triplicates, and statistical analyses using by one-way ANOVA with Tukey post-test. gMFI: geometric mean fluorescence intensity; FMO: fluorescence minus one.

Genetic knockdown of SIGLEC9 leads to enhanced activation and allostimulatory capacity of human Mo-DCs

Siglec-9 (Sig9) is the human counterpart of SigE^{26,27}. To study the relevance of Sig9 on human DCs, we differentiated monocyte-derived DCs from healthy volunteers and knocked down the *SIGLEC9* gene using siRNAs (**Figure 7A**). A ~54% reduction in surface Sig9 protein levels was observed in primary human Mo-DCs incubated with *SIGLEC9* siRNAs (Sig9^{KD}) compared to control siRNAs (Ctrl, **Figure 7B**). To investigate the impact of Sig9 on the activation of human DCs, we treated Mo-DCs with LPS (**Figure 7C**). Upon LPS stimulation, activation markers HLA-DR (MHC II, **Figure 7D**), HLA-A/B/C (MHC I, **Figure 7E**) and CD86 (**Figure 7F**) were more upregulated in Sig9^{KD} DCs compared to controls, while CD40 remained at similar levels (**Figure 7G**). Moreover, Sig9-silenced human Mo-DCs produced remarkably more IL-6, IL1 β , IL-8 and IFN- γ than control cells (**Figure 7H**). Furthermore, to functionally assess Sig9's ability to inhibit the allo-stimulatory capacity of human DCs, control or Sig9^{KD} Mo-DCs were co-cultured with third-party human T cells and IFN- γ production was measured by ELISPOT (**Figure 7I**). Sig9^{KD} human Mo-DCs induced significantly more alloreactive T cell IFN- γ production than those cultured with control Mo-DCs (**Figure 7J**). Overall, these data further support the conclusion that Sig9 negatively regulates DC activation, inhibiting their ability to promote human alloimmune responses.

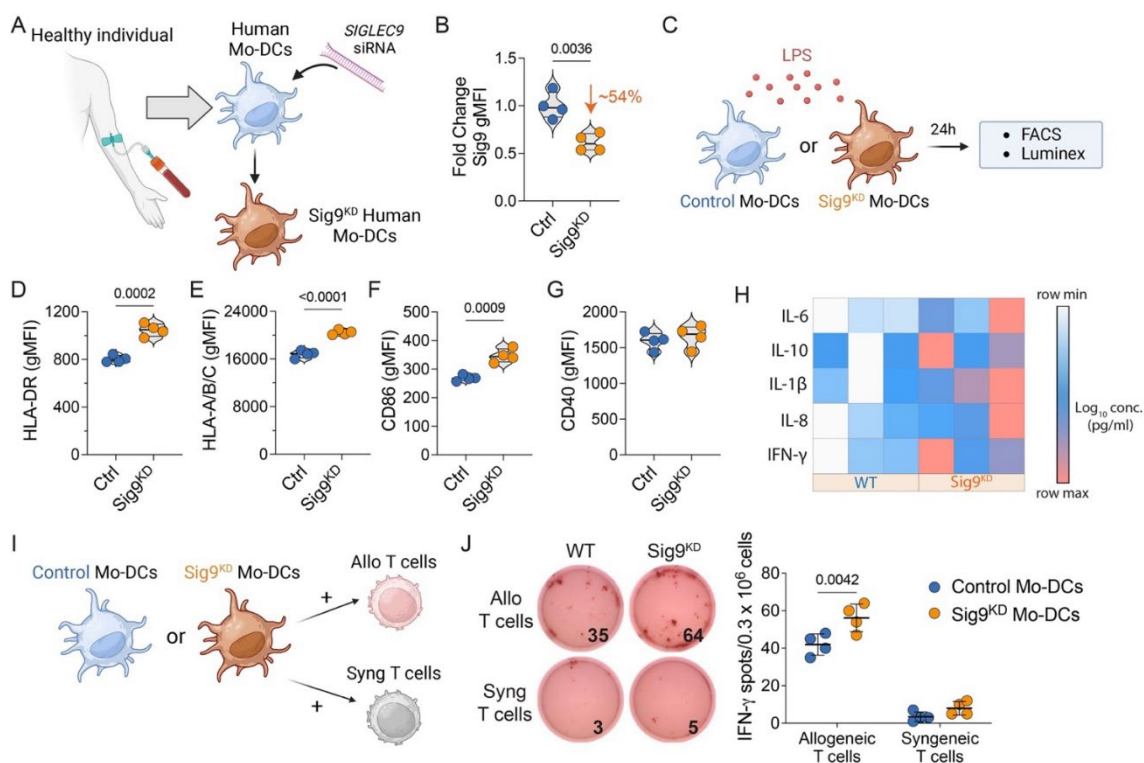


Figure 7. Siglec-9 controls human DC activation and the generation of alloreactive T cells. (A) Human DCs were differentiated from monocytes (Mo-DCs) of healthy individuals and Sig9 was knocked down using siRNAs. (B) Fold change Sig9 expression in control (ctrl) and Sig9-

silenced (Sig9^{KD}) Mo-DCs. gMFI: geometric mean fluorescence intensity. (C) Control or Sig9^{KD} Mo-DCs were treated with 250 ng/ml of LPS for 48h. (D) HLA-DR, (E) HLA-A/B/C levels, (F) CD86, and (G) CD40 were evaluated by flow cytometry in both groups. (F) Cytokine profile from culture supernatants analyzed by LUMINEX. (I) Control or Sig9^{KD} Mo-DCs were irradiated and co-cultured with allogeneic (third-party donor) or syngeneic (autologous) T cells in anti-humane IFN- γ -coated plates for five days. (J) IFN- γ production by T cells was measured by ELISPOT. Numbers of spots per 0.3×10^6 T cells $\pm$ SD in quadruplicates. (B - J) Experiments were performed in triplicates and statistical analyses using the Mann-Whitney test.

Discussion

Here, we identified SigE as an innate immune inhibitory receptor in transplantation with a crucial role in controlling T cell-mediated rejection. We demonstrate that the deletion of SigE in transplant recipients leads to accelerated acute cellular rejection. As the main mechanism, we show that murine SigE deletion or human Sig9 knockdown leads to increased DC activation by danger signals and an enhanced stimulation of allogeneic T cells. This effect was mediated by an elevated expression of NF- κ B and TNF- α production.

Innate immunity is crucial to initiate effector responses to the transplanted organ^{20,28}. Early exacerbated innate immune responses may enhance the adaptive immune response, hindering tolerance development and suppressing immune regulation in preclinical models⁵⁻⁷. The late activation of innate immune cells can also contribute to chronic rejection²⁹⁻³¹, graft fibrosis and failure⁸⁻¹⁰. Among immune cells, activated DCs are the most efficient APCs in mice and humans. Following transplantation, DCs are activated by inflammatory tissue cues, triggering their migration to host secondary lymphoid tissues and the graft itself, where they will prime T cells³²⁻³⁴. Corroborating these preclinical findings, a high density of graft-infiltrating DCs correlated with increased T-cell infiltration, poor allograft function and survival in kidney transplant recipients³⁵.

Understanding the required signals that control DC activation *in vivo* may enable effective immune modulation with lower doses of immunosuppressants, thereby minimizing side effects and toxicity. The strength of an immune response to damage is determined by the balance between the engagement of activating and inhibitory innate receptors and the crosstalk between their downstream signals. This is evidenced by our findings showing that SigE^{KO} DCs are more activated following transplantation, and when stimulated by a classical DAMP – mHSP70 *in vitro*. The simplest explanation for these results is that the absence of SigE leads to ineffective control of DC activation, unbalancing the response to a more pro-inflammatory profile. Corroborating this idea, although recipients' DCs are described to recognize nonself-allogeneic components and become activated independently of danger signals²⁰, we found that mice lacking SigE can trigger T cell-mediated rejection even in the absence of an allo-MHC component (**Figure 3G-K**).

Therefore, we propose that SigE can control the threshold for innate activation by DAMPs, which contributes to distinguishing allogeneic signals from noise triggered by self-molecules ¹¹. In a transplantation context, the magnitude of the inflammatory signaling pathways triggered by allo-stimulation might exceed this activation threshold. However, this threshold is decreased when SigE is absent, and innate cells become more susceptible to the activation by DAMPs and allo-MHC.

Despite the clear role of innate immunity in promoting transplant rejection, immunosuppressive drugs used in clinical practice primarily target T cells and are associated with several systemic side effects. Hence, there is a pressing need to develop effective therapies that modulate innate immunity in transplantation. Besides controlling DC activation by danger signals, we demonstrated that overexpression of SigE can also be targeted to downregulate MHC II and CD86 by DCs (**Figure 4K-L**), reducing their ability to stimulate alloreactive T cells (**Figure 4J**). Reduced MHC II expression decreases signal 1 to T cells and is involved in tolerogenic responses ³⁶. Regulatory DCs (DCregs) exhibit low levels of surface MHC molecules and limited expression of costimulatory molecules, making them less responsive to danger signals and more likely to induce apoptosis of effector T cells and generate Tregs ³⁷. In a clinical setting, strategies have been explored to utilize the therapeutic potential of these cells, such as administering donor DCregs ³⁸ or recipient DCregs loaded with donor antigens before transplantation ³⁹. Nevertheless, there are still obstacles to overcome in cellular therapy, including cost, determining the best cell source and dosage, a potential sensitization to donor antigens, and finding ways to enhance the short-term survival of cells post-adoptive transfer ^{40,41}. Targeting innate inhibitory receptors, like SigE, offers an attractive alternative method to mitigate these risks by inhibiting the activation of APCs crucial for T cell activation and ultimately contributing to reducing rejection rates and promoting long-term graft survival. By engaging SigE during transplantation, we aim to overcome the strong initial innate activation and decrease the production of allo-specific T cells. Further investigation is needed to determine if this approach could induce tolerance to donor antigens when combined with other immunosuppressive medications.

SigE immune regulatory effects have been demonstrated in other preclinical models, such as infection and cancer. To evade effector immune responses, cancer cells often overexpress sialic acids on glycans in a process called hypersialylation ^{42,43}. By doing that, they engage SigE on myeloid cells to downmodulate the innate and adaptive immunity to tumors ^{44,45}. Thus, SigE is an inhibitory immune checkpoint targeted to enhance therapeutic antitumor immune effector responses *in vivo* ^{26,50}. In an inflammatory context, targeting SigE with sialic acid-coated nanoparticles demonstrated immunosuppressive effects in murine models of sepsis by modulating macrophages ⁴⁶. Mice deficient in SigE exhibit increased neutrophil recruitment to the lungs in a

pulmonary inflammation model⁴⁷. Female SigE^{KO} mice over six months old also develop a lupus nephritis phenotype⁴⁸. SigE is also implicated in regulating inflammation and foam cell formation in a mouse model of atherosclerosis⁴⁹. We extend these findings here by demonstrating a critical role for SigE in controlling acute cellular rejection in transplantation.

Inhibitory Siglecs contain an intracellular ITIM or an ITIM-like motif that is phosphorylated after receptor engagement and leads to either SHP-1 or SHP-2 recruitment⁴⁷. DCs lacking SigE produce more IL-6 and TNF- α when treated with TLR ligands than wild-type (WT) controls, demonstrating that SigE is a negative modulator of the TLR signaling¹⁶. Mechanistically, SigE recruits SHP-1/2, which will dephosphorylate NF- κ B, inhibiting innate immune cell activation triggered by activating TLRs¹⁵. A similar mechanism was observed in a preclinical model of glioblastoma, in which SigE deletion led to elevated expression of inflammatory genes regulated by NF- κ B in myeloid-derived cells and increased effector responses in the tumor microenvironment⁵⁰. Besides TLRs, we recently demonstrated that SigE can regulate the downstream responses of LOX-1 when it is engaged by the mHSP70 or oxLDL ligands⁵¹. SigE can also counteract the activation of the innate receptor CD36 by recruiting SHP1⁴⁹. The precise identity of activating receptors and their ligands targeted by SigE during transplantation still needs to be determined. However, our data suggest a conserved SigE downstream signaling pathway since the ability of murine SigE to control innate immunity activation following transplantation is mainly a negative regulator of important inflammatory mediators like NF- κ B and the subsequent production of TNF- α . Other innate receptors were implicated in controlling effector alloimmune responses. APCs expressing the C-type lectin DC-SIGN have an inferior ability to induce effector alloimmune responses *in vitro*^{52,53}. Moreover, murine DC-SIGN⁺ macrophages can promote IL-10-mediated transplantation tolerance in an experimental heart transplantation model¹². Donor livers lacking the expression of the inhibitory receptor CEACAM1 have a decreased survival due to enhanced sterile inflammation triggered by cold stress⁵⁴. Whether the inhibitory innate receptors required to induce immune regulation in transplantation are tissue-specific and whether these receptors interact with SigE still needs to be determined. Overall, our study highlights the role of the innate receptor Siglec-E/9 as a critical regulatory pathway in controlling APC activation and the subsequent effector alloreactive T cell-mediated immunity. These results build on a growing interest in designing combination immunosuppressive regimens targeting both innate and adaptive arms of the immune system to generate a more tolerogenic environment, reduce the intensity of the early immune response, and allow minimization of systemic immunosuppression.

Limitations of the study

Our study did not address functional differences of SigE in different DC subsets, including cDC1, cDC2 and pDCs. Considering the phenotypical and functional differences, it is possible that SigE is differently required by DC subsets. Moreover, although progress has been made in identifying innate inhibitory receptors, their biological context and functional role in modulating effector immune cell functions during transplant rejection remains poorly understood. Specifically, the expression kinetics of these receptors in different immune cell subsets, their interactions with co-receptors, the signaling pathways they are involved in, their locations, and the availability of ligands in different tissues are still not well-defined.

Methods

Mice

C57BL/6J (B6, H-2^b, stock no: 000664), BALB/cJ (H-2^d, stock no: 000651), CB6F1/J (H-2^{b/d}, stock no: 100007), and C3H/HeJ (H-2^k, stock no: 000659) were purchased from The Jackson Laboratory. Act-mOVA (C57BL/6-Tg(CAG-OVAL)916Jen/J, stock no: 005145) described in Ehst *et al.* ⁵⁵, OT-I (C57BL/6-Tg(Tcr α Tcr β)1100Mjb/J, stock no: 003831) described in Hogquist *et al.* ⁵⁶, and OT-II (B6.Cg-Tg(Tcr α Tcr β)425Cbn/J, stock no: 004194) transgenic mice, described in Barnden *et al.* ⁵⁷, were also purchased from The Jackson Laboratory. Siglec-E-deficient mice (SigE^{KO}, Siglece^{tm1.2Avrk}) were a generous gift from Dr. Ajit Varki (UCSD) and described in Uchiyama *et al.* ⁵⁸. and Rag1^{KO} (B6.129S7-Rag1^{tm1Mom}/J, stock no: 002216) were obtained from The Jackson Laboratory and bred to SigE^{KO} strain (Rag1^{KO}SigE^{KO}). All mice used in the experiments were 7 to 12 weeks old, from both sexes, and we tried to keep a consistent ratio of ~50%:50%. Similar findings are reported for both sexes. Mice were bred and housed in individual and standard mini-isolators under specific pathogen-free conditions in the Massachusetts General Hospital (MGH) animal facility with water and food *ad libitum*, following the Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH) Animal Care guidelines. The MGH Institutional Animal Care and Use Committee (IACUC) approved all experiments under protocol numbers 2020N000085 and 2020N000125.

Cell lines

WT DC2.4 cell line (H-2^b) was grown in RPMI medium (Gibco) supplemented with 10% FBS (Gibco), 100 U/mL of Penicillin and 100 mg/mL of Streptomycin (both from Gibco) at 37°C, 5% CO₂. DC2.4 Siglec-E⁺ (SigE^{Tg}) cell line was produced through lentiviral transduction of Siglec-E construct with CLIP tag at N-terminus. Siglec-E expression was driven by the SFFV promoter.

Murine heterotopic heart transplantation

Heterotopic vascularized cardiac transplantation was performed as previously described ⁵⁹. Cardiac allografts were isolated from the donor mice after adequate cannulation and preserved with heparinized saline on ice until the recipients were ready for transplantation. Subsequently, the recipient mice were anesthetized, and the heart graft was transplanted into the recipient using microsurgical techniques. In abdominal transplantation, the allograft's ascending aorta and pulmonary artery were end-to-side anastomosed to the recipient's abdominal aorta and inferior vena cava, respectively. In cervical transplantation, the graft's ascending aorta and pulmonary artery were end-to-end anastomosed to the recipient's right external carotid artery and vein, respectively. The time of cold ischemia of the graft was 25 min, and the warm ischemia was 30 to 35 min. Functionality of the cardiac grafts was monitored daily by palpation. Rejection was determined by complete cessation of palpable heartbeat and was confirmed by direct visualization after laparotomy. Graft survival is shown as the MST in days. Loss of graft function within 48 h after transplantation was classified as technical failure and animals in which this occurred were omitted from the analysis.

Murine kidney transplantation

Kidney transplantation was performed as previously described ⁶⁰. Briefly, the donor's left kidney, including the left renal artery, abdominal aorta, and left renal vein were isolated from the donor mice. Under isoflurane anesthesia, the kidney allograft was transplanted into the recipients' abdomen by anastomosing graft abdominal aorta and left renal vein to the recipient's abdominal aorta and inferior vena cava, respectively. The recipient's right native kidney was removed during the surgery to make space for graft implantation, and the left kidney remained untouched. The ureter was directly anastomosed into the bladder by using a pull-through technique. The time of cold ischemia of the graft was 35 min, and the warm ischemia was 30 to 35 min.

Skin transplantation

We performed a fully MHC-mismatched murine skin allograft model ⁶¹. Briefly, 1 cm² sections of tail skin were removed. Recipients were anesthetized, and fur was shaved off the dorsal trunk. At the shaved area, 1 cm² of skin was removed in each recipient mouse, and the donor tail skin fragment was sutured to the exposed tissue of each recipient. The state of graft acceptance was

recorded daily. The observation of cyanosis, erythema, erosion, and skin graft loss confirmed graft rejection.

In vivo treatments

On day 2 after heart transplantation, 250 µg of CTLA4-Ig (Abatacept, Bristol-Myers Squibb) was given by i.p. injection. For skin transplantation, recipients received 250 µg of CTLA4-Ig (Abatacept, Bristol-Myers Squibb) by i.p. injection on days 0, 2, 4 and 6 post-transplant. For the *in vivo* inhibition of NF-κB, 2.5 mg of Pyrrolidinedithiocarbamate (PDTC, MedChemExpress) was given by i.p. injection on days 0, 2, 4 and 6 after heart transplantation. TNF-α was blocked by i.p. administration of 250 µg of anti-mouse TNF-α (clone XT3.11, BioXCell) on days 0, 2, 4 and 6 post-transplant.

Adoptive cell transfers

To evaluate the effects of antigen presentation in the absence of SigE, we used a previously described system in which OVA-specific transgenic T cells in naive animals are adoptively transferred to recipients transplanted with an Act-mOVA-expressing heart ²⁰. For the adoptive transfer, WT OT-I and OT-II transgenic cells were harvested from spleens of naïve mice. CD8⁺ T cells were magnetically isolated from OT-I spleens by negative selection using the MojoSort Mouse CD8 T Cell Isolation Kit (Biologend). CD4⁺ T cells were magnetically isolated from OT-II spleens by negative selection using the MojoSort Mouse CD4 T Cell Isolation Kit (Biologend). The purified cells were resuspended in PBS 1x and adoptively transferred into Act-mOVA heart recipients 5 days after the transplantation. The purity (≥95%) of isolated OT-I and OT-II cells was controlled by flow cytometry.

Isolation of organ-infiltrating lymphocytes

To isolate cells from spleens and cardiac allografts, organs were excised, minced, and digested with 500 U/mL collagenase D (Roche) for 30 minutes at 37°C, followed by incubation with 0.1 M EDTA in PBS, pH 7.2, buffer for 5 minutes before a final suspension in 5 mM EDTA (Gibco), 1% FBS (Gibco) in PBS 1x, pH 7.2 (Boston BioProducts). Isolated cells were then mechanically dissociated through a 70 µm cell strainer (Corning) in a 50 ml tube, and RBCs were lysed using hypotonic ACK buffer (Gibco).

For kidney allografts, organs were excised, minced, and digested with a dissociation solution with 1 mg/mL of collagenase type I (Gibco) and 0.1 mg/mL of DNAase I (Stem Cell)

for 30 minutes at 37°C. Isolated cells were then mechanically dissociated through a 70 µm cell strainer (Corning) in a 50 ml tube, washed and resuspended in 1x PBS. Leukocytes were enriched using Mouse Cell Separation media (Cedarlane) in 15 ml tubes, following the manufacturer's instructions.

Murine dendritic cell cultures

DCs were grown from WT or SigE^{KO} bone marrows (BMDCs) in the presence of 40 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (both from Peprotech or Biolegend). Cells were cultured in 24-well plates in DMEM (Gibco), 10% FBS (Gibco) and pen/strep (Gibco) at 37°C, 7% CO₂. After six days in culture, non-adherent cells (DCs) were separated from adherent cells and were plated with 10 ng/ml of GM-CSF and IL-4. On the next day, BMDCs were incubated in medium (control) or different concentrations of low endotoxin recombinant murine HSP70 (mHSP70) produced in eukaryotic HEK293 cells (Sino Biological) for 24h at 37°C, 7% CO₂. Cells were analyzed by flow cytometry. Supernatants were collected, and cytokines were analyzed by Luminex.

Flow cytometry

We stained the isolated kidney cells and splenocytes for flow cytometry. Isolated cells were Fc-blocked (TruStain FcX, anti-mouse CD16/32, clone 93, Biolegend) for 20 min before staining for surface markers for 30 minutes in FACS buffer (2% FBS in PBS 1x) on ice. We used the following anti-mouse antibodies: CD45-BV605 (1:50, clone 30-F11, Biolegend), CD11b-APC (1:66, clone M1/70, Biolegend), CD11c-PE-Cy7 (1:66, clone N418, Biolegend), LyG-PE (1:100, clone 1A8, Biolegend), Ly6C-BV510 (1:25, clone HK1.4, Biolegend), SigE-FITC (1:200, clone M1304A01, Biolegend), F4/80-PacBlue (1:100, clone BM8, Biolegend), CD45-AF700 (1:100, clone 30-F1, Biolegend), CD3-BV605 (1:100, clone 145-2C11, Biolegend), CD8-AF594 (1:100, clone 53-6.7, Biolegend), CD4-APC-Cy7 (1:100, clone RM4-5, Biolegend), Foxp3-PE (1:20, clone FJK-16s, Thermo Fisher), IFN-γ-BV510 (1:25, clone XMG1.2, Biolegend), TNF-α-FITC (1:100, clone QA17A29, Biolegend), Ki67-PerCP-Cy5.5 (1:100, clone 11F6, Biolegend), GrzB-APC (1:20, clone QA16A02, Biolegend), CD3-PacBlue (1:200, clone 17A2, Biolegend), CD19-PacBlue (1:200, clone 6D5, Biolegend), CD11c-PacBlue (1:200, clone N418, Biolegend), CD11c-Spark UV387 (1:500, clone N418, Biolegend), F4/80-PerCP-Cy5.5 (1:100, clone BM8, Biolegend), MHCII(I-A/I-E)-BV786 (1:83, clone M5/114.15.2, Biolegend), CD86-APC-Cy7 (1:100, clone GL-1, Biolegend), NK1.1-BV786 (1:200, clone PK136, BD Biosciences), TCRVa2-FITC (1:100, clone B20.1, BD Biosciences), TCRVb5-APC (1:100, clone 22F6, Biolegend). For intracellular

cytokine staining, cell suspensions were preincubated for 4 h with 50 ng/mL PMA, 500 ng/mL ionomycin, and Brefeldin A (all from Biolegend) at 37°C, 5% CO₂ in 10% FBS RPMI before Fc blocking, followed by surface staining, permeabilization, and intracellular staining. Intracellular staining was performed after treating cells with the eBioscience Foxp3 Fixation/Permeabilization solution (Thermo Fisher). Cells were incubated overnight at 4°C with intracellular markers.

For NF- κ B signaling experiments, WT or SigE^{KO} BMDCs were stimulated with 50 ng/mL of PMA plus 500 ng/mL of ionomycin for 30 min at 37°C, 5% CO₂. For SHP-2 detection, WT or SigE^{Tg} DC2.4 cells were stained directly from the cell cultures. Cells were then fixed with 4% PFA for 10 min at room temperature and permeabilized with True-Phos Perm Buffer (Biolegend) for 20 min on ice. Then, cells were Fc-blocked followed by staining for phospho-NF- κ B-p65-PE (1:100, clone D14E12, Cell Signaling), phospho-SHP-2 (Tyr542)-FITC (1:20, clone E8D6V, Cell Signaling) or rabbit (DA1E) IgG Isotype Control (Cell Signaling) for 30 min at room temperature. Stained cells were analyzed on an LSR Fortessa X-20 flow cytometer (BD Biosciences) with FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar). Viable cells were selected based on the staining with LIVE/DEAD Fixable Blue Dead Cell Stain Kit (1:1000, Thermo Fisher) or Fixable Viability Dye eFluor 780 (eBioscience) before the Fc-blocking.

To isolate splenic DCs, spleens from heart-transplanted mice were magnetically depleted for CD3 and CD19 using positive selection kits from Biolegend (MojoSort mouse CD3 Selection Kit, and MojoSort mouse CD19 nanobeads). Enriched cells were stained and sorted using a FACSARIA cell sorter (70 μ M nozzle) using optimal purity settings. DCs were gated as CD3⁻CD19⁻MHCII⁺CD11c⁺ cells.

Histopathology and immunofluorescence analyses

For histopathology, cardiac and kidney graft samples from transplanted mice were harvested from both WT and SigE^{KO} groups at 14 or 21 days after transplantation. In Rag1^{KO} and Rag1^{KO}SigE^{KO} groups, cardiac grafts were harvested on day 7. Grafts were then fixed in 10% formalin, embedded in paraffin, transversely sectioned, and stained with hematoxylin and eosin (H&E), and Masson Trichrome for heart samples. Kidney grafts were stained with H&E or Periodic acid-Schiff (PAS) stain. A transplant pathologist blinded to the experimental groups graded the degree of the rejections. Heart rejection grades (from 0 to 3) were performed using the revised ISHLT classification⁶². Photomicrographs were taken using an Olympus BX53 microscope with a DP27 camera and cellSens Imaging Software (Olympus Life Science Solutions).

For the Siglec-E colocalization with the MHC II (I-A^b), frozen sections (8 μ m) from cardiac allograft and spleen tissues were fixed with acetone and washed three times with PBS.

The sections were blocked with PBS containing 1% bovine serum albumin (BSA) at room temperature for 1 hour. The sections were stained with the primary antibody diluted with PBS containing 1% BSA, followed by the specific secondary antibody for 1 hour at room temperature. Stained sections were photographed using an Axio Imager M2 microscope (Zeiss) and processed with ImageJ and Photoshop software. Primary antibodies used were: rat anti-mouse MHC II (I-A/I-E, clone M5/114.15.2, Cell Signaling) as 1:100, and goat anti-mouse Siglec-E (polyclonal IgG, R&D Systems) as 1:150. All secondary antibodies were purchased from Jackson ImmunoResearch and used as 1:100.

Bulk RNA sequencing

Total RNA was isolated from flow-sorted splenic DCs using RNAeasy Plus (Qiagen). Dual-indexed libraries were generated using Takara/Clontech Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian reagents (Takara). RNA sequencing (RNA-seq) reactions were sequenced on a NovaSeq sequencer (Illumina) according to the manufacturer's instructions in pair-ended 2 x 150 bp reads. All expected barcodes were detected and mean depth is ≥ 40 M reads. We used Fatsp 0.22.0⁶³ to detect and trim the adaptor sequences and to keep only reads with a minimum of 60% of bases with a phred quality equals or greater than 15. After quality control, we estimated the transcription abundance with Salmon 1.9.0⁶⁴ using the reference mouse transcriptome and annotation GRCm39 from release M34-Ensembl 111 from Gencode⁶⁵ portal (<https://www.gencodegenes.org/mouse/>). We used the R 4.2.1 package tximport 1.26.1⁶⁶ to summarize the transcript-level abundances to use in DESeq2 1.38.3⁶⁷ to perform the differential expression analysis. We used a Python 3.12.1 package of GSEA⁶⁸ to perform the enrichment analysis. The graphs were made with matplotlib 3.8.0⁶⁹ and seaborn 0.11.2⁷⁰.

Transcriptional analyses of human tissue biopsies

We took advantage of publicly available datasets. All samples were downloaded from the public GEO NCBI database. The microarray RNAseq (GSE9377)¹⁸ expression matrix was downloaded via GEOquery R package v 2.66.0⁷¹ and the quantile normalization was performed with in-home script in R 4.2.1. For bulk RNAseq (GSE131179)⁷² we downloaded the expression matrix and, for both studies, we used the Brunner-Munzel test from Scipy v 1.13.0⁷³ to calculate the statistical difference between conditions for *SIGLEC7* and *SIGLEC9* genes. For the single-nuclear RNA-seq study (GSE203548)¹⁷ we downloaded the available matrix and analyzed it with Scanpy v 1.10.1⁷⁴, we calculated a pseudo bulk analysis with DESeq2 v 1.38.3⁶⁷ to perform a differential expression analysis.

PBMCs isolation from healthy volunteers' blood

Peripheral blood samples were obtained from healthy subjects and peripheral blood mononuclear cells (PBMCs) were then isolated by density gradient centrifugation using Ficoll-Paque solution (GE Healthcare). Cells were washed in PBS and plated for dendritic cell differentiation. Human total T cells were magnetically isolated by negative selection using a MojoSort Human CD3 T Cell Isolation Kit from Biolegend. All enrolled subjects provided written informed consent to participate in the study, as approved by the MGH human research committee (Institutional Review Board No: 2017P000298).

Generation of human monocyte-derived dendritic cells

Monocytes were isolated from PBMCs by adherence to plastic at 37 °C, 5% CO₂ for 4 h. Adherent cells were cultured in 24-well plates in DMEM medium (Gibco) supplemented with 10% FBS (Gibco), 10% FBS (Gibco), pen/strep (Gibco), and the addition of human GM-CSF and IL-4 (both at 50 ng/ml from Biolegend) at 37°C, 5% CO₂. After 5 days, human TNF- α (500 UI/ml, from Biolegend) was added for Mo-DCs maturation. Siglec-9 knockdown was performed using siRNAs with Mo-DCs from day 7 of culture. Briefly, 1x10⁶ cells were plated in 6-well plates in complete RPMI medium and incubate with combination of siRNAs against *Homo sapiens SIGLEC9* gene (at 10 mM, ID s25960 and s25961, Thermo Fisher) using the Lipofectamine/RNAiMAX Transfection Reagent (Thermo Fisher) with Opti-MEM medium (Thermo Fisher) for 4h at 37°C, 5% CO₂. A combination of Silence Select negative control no. 1 and 2 siRNAs (Thermo Fisher) were used as controls. After incubation, we added complete DMEM supplemented with GM-CSF and IL-4 (both from Biolegend, at 10 and 20 ng/ml, respectively). The reduction of Siglec-9 expression was analyzed using flow cytometry 48 h later. For activation experiments, Mo-DCs were stimulated with 300 ng/ml of LPS (InvivoGen) for 24h at 37°C, 7% CO₂. After that, cells were analyzed by flow cytometry. Supernatants were collected, and cytokines were analyzed by Luminex.

Mouse and human IFN- γ ELISPOT assays

Mouse IFN- γ ELISPOT assays were performed using kits from BD Biosciences according to the manufacturer's protocol. Briefly, 0.45 μ m hydrophobic, high-protein-binding Immobilon-P membrane plates (Millipore) were coated with IFN- γ capture antibody at 4°C overnight, followed by blocking with 10% FBS-supplemented RPMI1640 (Gibco) for 1 hour at room temperature. In

a set of experiments, lymphocytes were magnetically isolated from transplanted mouse spleens on day 21 by negative selection using the MojoSort Mouse CD3 T Cell Isolation Kit (Biolegend). We incubated 3×10^5 T cells with 5×10^5 irradiated donor (BALB/c), syngeneic (B6), or third-party (C3H) splenocytes at 37°C and 5% CO₂ for 24 h. Spots were detected and counted using an ImmunoSpot analyzer (Cellular Technology).

In another set of experiments, 3×10^5 BALB/c (allogeneic) or B6 (syngeneic) T cells were incubated with 3×10^4 irradiated (30 Gy) WT or SigE^{KO} BMDCs at 37°C 5% CO₂ for 3 days in RPMI medium (Gibco) supplemented with 10% FBS (Gibco), pen/strep (Gibco). Alternatively, 3×10^4 irradiated WT or SigE^{Tg} DC2.4 cells were co-cultured with 3×10^5 BALB/c (allogeneic) or B6 (syngeneic) T cells were incubated at 37°C 5% CO₂ for 5 days in the same medium than above. For NF-κB inhibition, BMDCs were incubated with 10 mM of PDTC (MedChemExpress) for 4h as described in ²⁴, and washed twice before irradiation. For TNF-α inhibition, BMDCs were treated with 25 mg/ml of anti-mouse TNF-α (clone XT3.11, BioXCell) for 24h as described in ⁷⁵, and washed twice before irradiation. For SHP-2 inhibition, were incubated with 20 mM of SHP099 (MedChemExpress) for 24h as described in ⁷⁶, and washed twice before irradiation.

Human IFN-γ ELISPOT assays were similarly performed as above, using a human IFN-γ ELISPOT kit (BD Biosciences). 3×10^4 human purified T cells were cultured together with 3×10^5 irradiated human Mo-DCs prepared as above at 37 °C 5% CO₂ for 3 days. Spots were detected and counted using an ImmunoSpot analyzer (Cellular Technology).

Cytokines measurements

Mouse cytokine profiles were determined from culture supernatants using a ProcartaPlex 11-Plex Kit from Thermo Fisher, containing the following cytokines: IL-6, TNF-α, IL-12p70, IL-10, IL-1β, IL-18, GM-CSF, IL-4, IL-5, IL-13, and IFN-γ. Human cytokine profiles were determined from culture supernatants using a Cytokine Human Magnetic 10-Plex Panel Kit from Thermo Fisher, containing the following cytokines: IL-6, IL-10, IL-1β, IL-8, IFN-γ, GM-CSF, IL-5, TNF-α, IL-2, and IL-4. Data are expressed as Log₁₀ concentration (pg/ml) in a heatmap created with the Morpheus matrix visualization and analysis tool from the Broad Institute (<https://software.broadinstitute.org/morpheus>).

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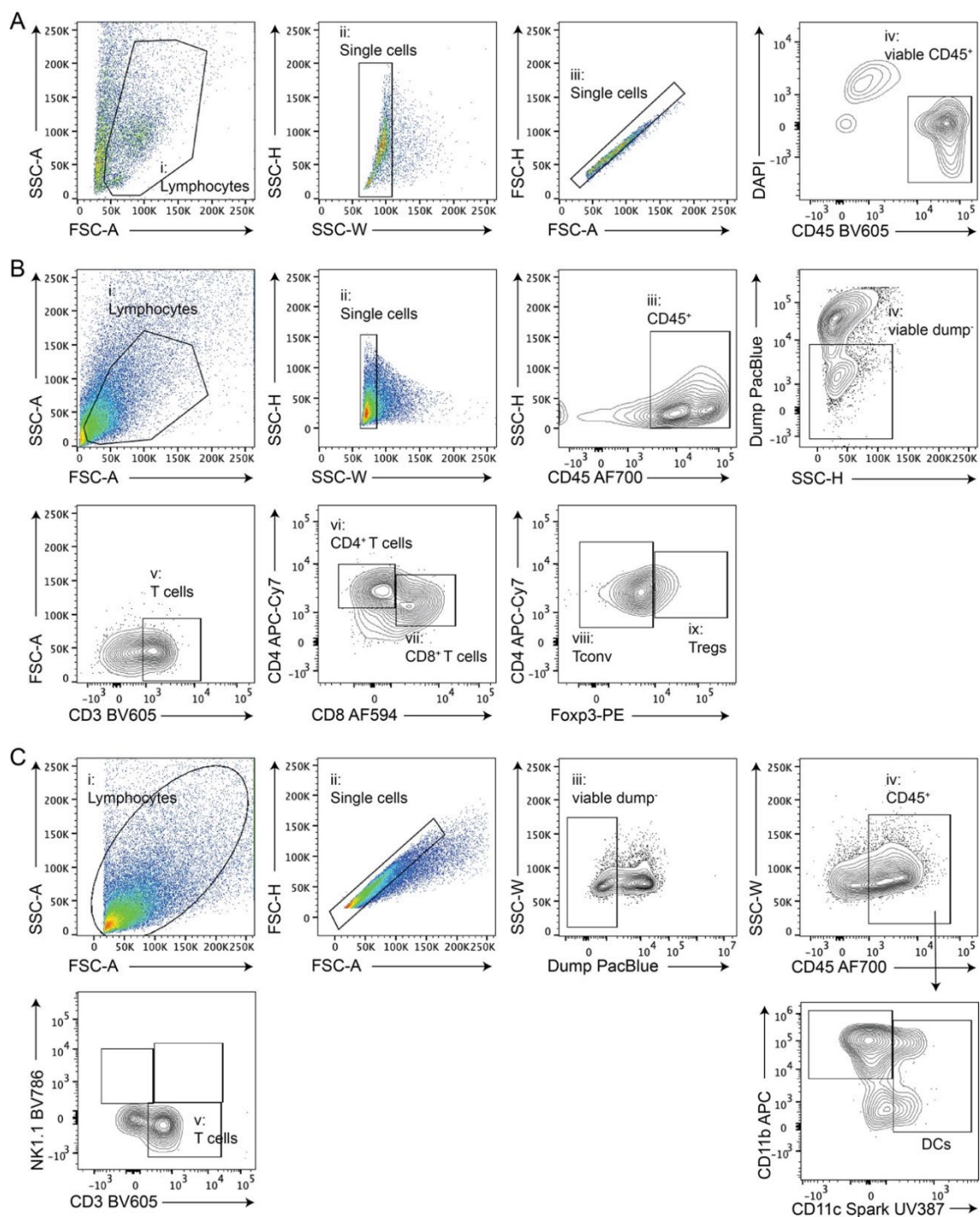
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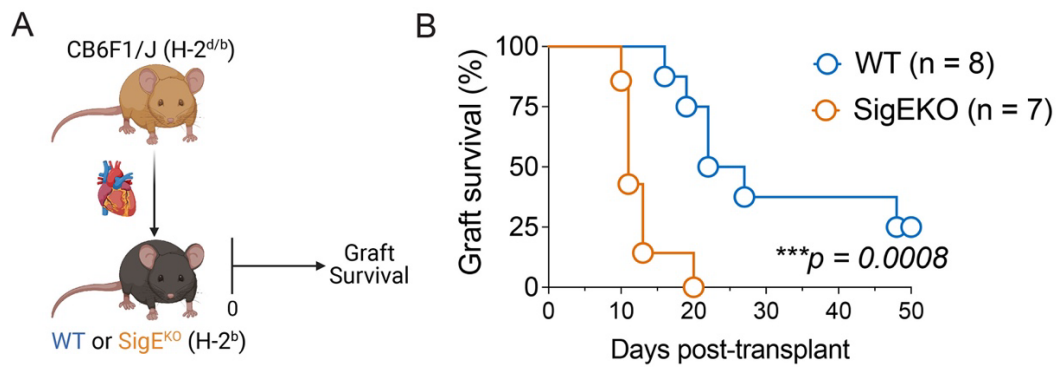
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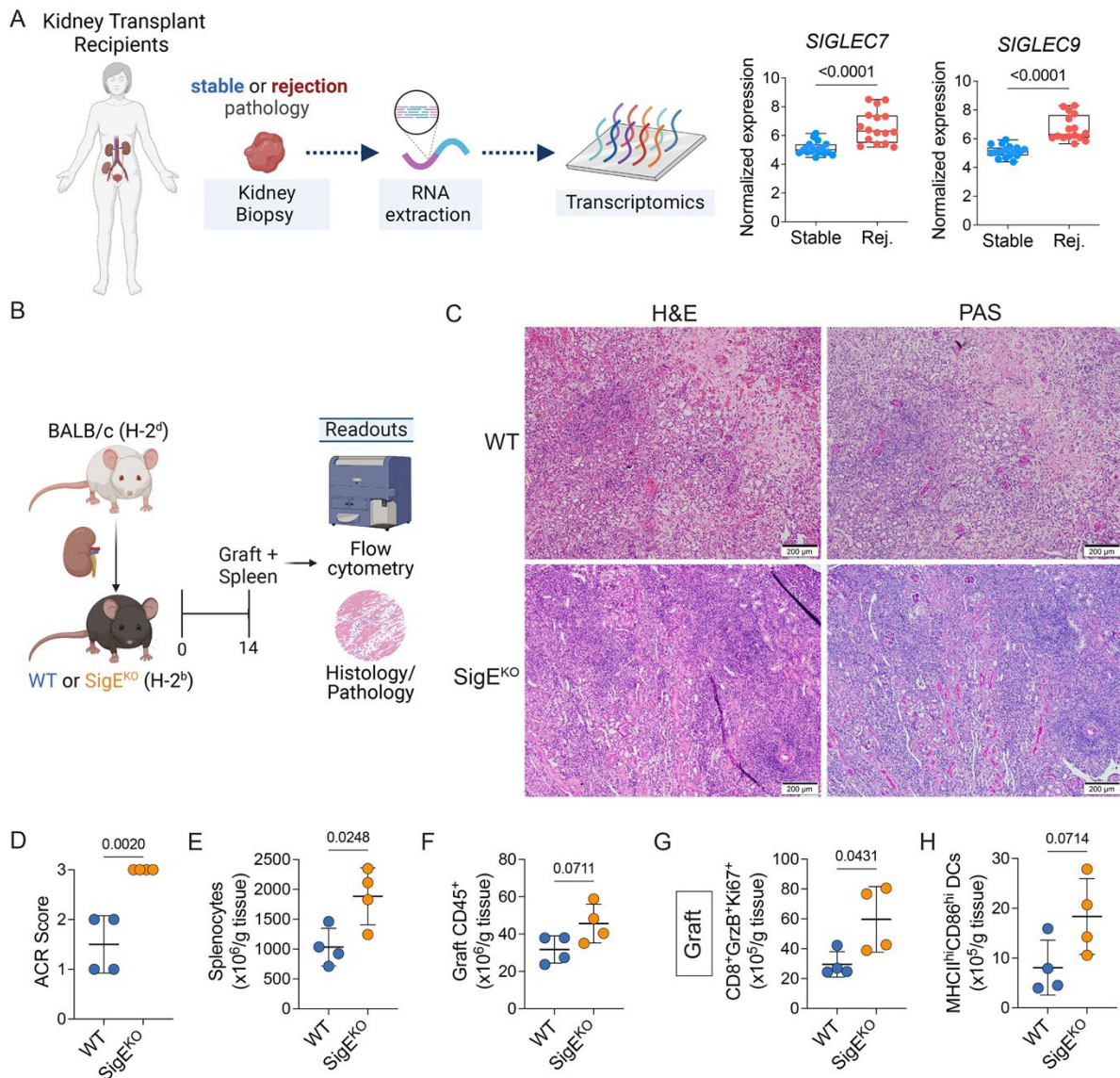
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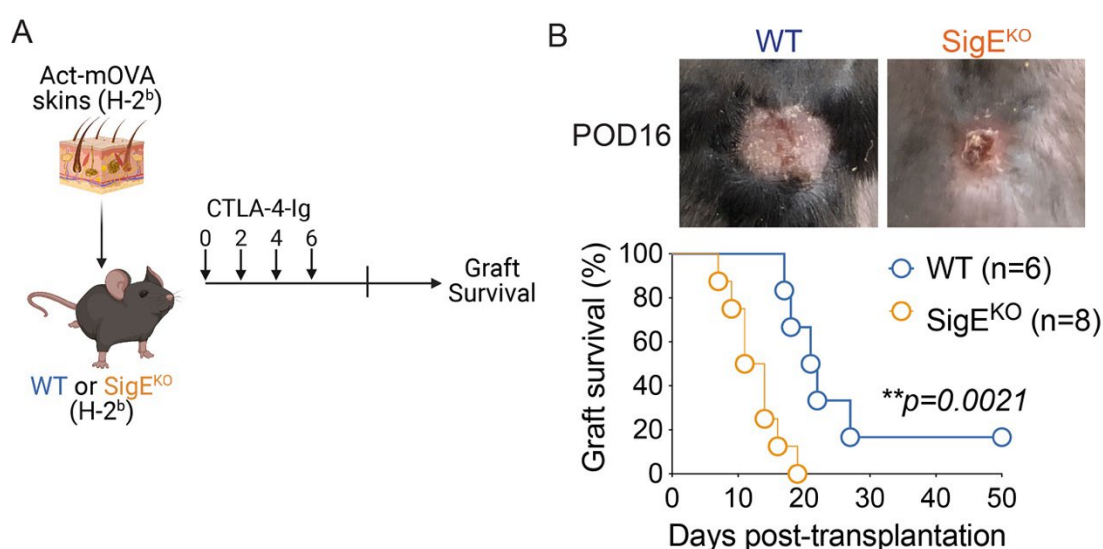
Suppl Figure 1. (A) Gating strategy for the detection of cardiac allograft-infiltrating lymphocytes over time (related to **Fig. 1A-G**). (B) Gating strategy for the detection of cardiac allograft-infiltrating T cell subsets on day 21 post-transplant (related to **Fig. 2H-J**). (C) Gating strategy for the detection of adoptively transferred OT-I and OT-II cells as well as DCs infiltrating heart grafts (related to **Fig. 3J-K**).



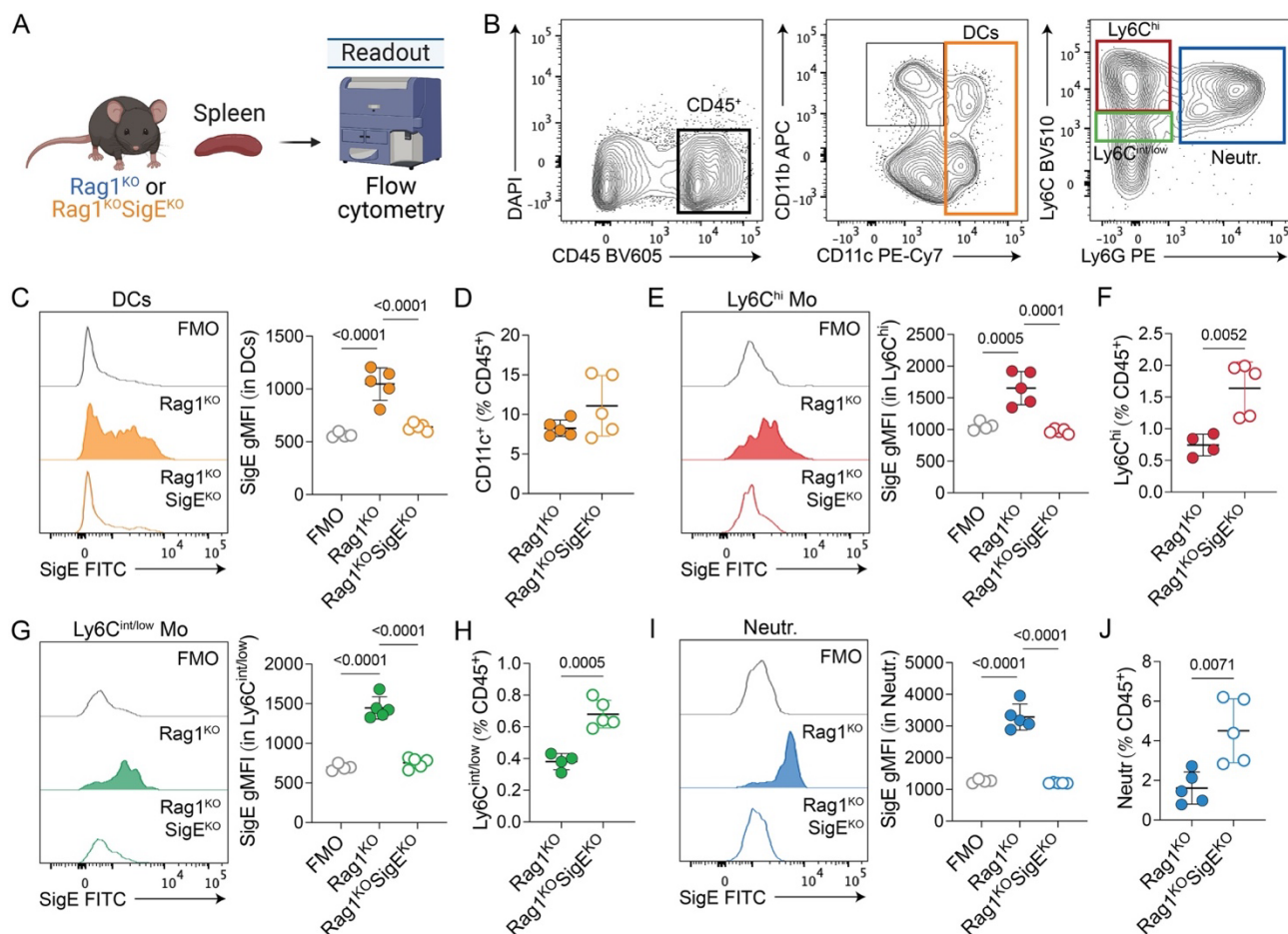
Suppl Figure 2. Siglec-E ablation precipitates cardiac graft rejection. (A) Schematic of experimental design in which BALB/c x B6 F1 (CB6F1/J) hearts were transplanted into WT or SigE^{KO} C57BL/6 recipients. (B) Kaplan-Meier curves of cardiac allograft survival. The p-value was determined by a log-rank test (n = 7 - 8 mice/group).



Suppl Figure 3. Siglec-E^{KO} kidney recipients have pronounced acute cellular rejection. (A) *SIGLEC7* and *SIGLEC9* gene expression in kidney allograft biopsies during stable or rejection episodes from a public dataset of kidney transplant recipients (GSE131179). (B) Schematic of experimental design in which BALB/c kidneys were transplanted into WT or SigE^{KO} C57BL/6 recipients. Splenocytes and kidney allografts =from WT or SigE^{KO} mice were analyzed by flow cytometry and histology. (C) Representative images of H&E- or PAS-stained sections of kidney allografts harvested from each group on day 14 after transplant. ×100 magnification. (D) Acute cellular rejection score for each group. (E) Spleen cellularity, (F) numbers of kidney allografts total CD45⁺ cells, (G) intragraft Ki67⁺GranzymeB⁺CD8⁺ T cells and (H) intragraft MHCII^{hi}CD86^{hi} DCs in each group. (D – H) Statistical analyses were done by unpaired t-test (n = 5 - 6 mice/group).



Suppl Figure 4. Accelerated skin graft rejection in Siglec-E^{KO} recipients. (A) Schematic of experimental design: Act-mOVA skins were transplanted onto WT or SigE^{KO} C57BL/6 recipients treated with CTLA-4-Ig (250µg on days 0, 2, 4, and 6 after transplant). (B) Visual aspects on day 16 and Kaplan-Meier curves of skin graft survival. The p-value was determined by a log-rank test (n = 6 - 8 mice/group).



Suppl Figure 5. Characterization of $Rag1^{KO}SigE^{KO}$ mice. (A) Schematic of experimental design for (B - J). Spleens from naïve $Rag1^{KO}$ or $Rag1^{KO}SigE^{KO}$ were harvested and analyzed by flow cytometry. (B) Gating strategy for splenic myeloid cell populations. Representative histograms and SigE expression (gMFI: geometric mean fluorescence intensity; FMO: fluorescence minus one) on (C) DCs, (E) $Ly6C^{hi}$, (G) $Ly6C^{int/low}$ and (I) neutrophils. Statistics were performed by one-way ANOVA with Tukey's multiple comparisons test ($n = 4 - 5$ mice/group). Percentages of splenic (D) DCs, (F) $Ly6C^{hi}$, (H) $Ly6C^{int/low}$ and (J) neutrophils in $Rag1^{KO}$ or $Rag1^{KO}SigE^{KO}$ naïve mice. Statistics were performed by unpaired t-test ($n = 5$ mice/group).

10. Discussão geral e considerações finais

O foco desta tese foi explorar, pela primeira vez, o papel da *Marchf1* em células dendríticas (DCs) na geração de respostas imunes durante o crescimento tumoral. Esses são aspectos que não foram abordados por estudos anteriores, concentrados principalmente no papel dessa proteína em células tumorais.

Estudos prévios observaram um aumento de *Marchf1* em amostras tumorais de pacientes com câncer de ovário e hepatocarcinoma, em comparação com tecidos normais, em análises histológicas (Meng *et al.*, 2016; Xie *et al.*, 2019). O silenciamento de *Marchf1* em linhagens celulares destes tipos de tumores resultou na supressão da proliferação, migração e invasão das células tumorais, mediada pela regulação negativa das vias NF- κ B e Wnt/ β -catenina (Xie *et al.*, 2019). Em contrapartida, a superexpressão de *Marchf1*, por meio de transfecção gênica, promoveu o aumento da proliferação das células tumorais (Meng *et al.*, 2016), sugerindo um papel direto de *Marchf1* na progressão tumoral. Esses dados reforçam a hipótese de que *Marchf1* exerce funções importantes na biologia das células tumorais.

Neste trabalho, focamos na importância da *Marchf1* no sistema imunológico, particularmente no desenvolvimento e função das DCs em gerar uma resposta T CD8⁺ contra o tumor. Estudos prévios demonstraram que a *Marchf1* é crucial para a resposta imune do tipo 2 (T_H2) (Castellanos *et al.*, 2021), evidenciando que camundongos ausentes da expressão de *Marchf1* (*Marchf1*^{-/-}) falham em desenvolver esse tipo de resposta. No entanto, o mesmo estudo sugeriu que a *Marchf1* não era necessária para a geração de respostas imunes do tipo 1 (T_H1), T CD8⁺ citotóxicas e do tipo 17 (T_H17) em modelos virais e de autoimunidade. Esses resultados contrastam com os resultados obtidos nesta tese, onde a expressão de *Marchf1* mostrou-se essencial para a resposta das células T CD8⁺ citotóxicas antitumorais, mediada pela ativação das células cDC1s.

Nossa hipótese inicial era de que a ausência de *Marchf1* poderia levar a uma ativação imune aumentada e, conseqüentemente, a uma melhor resposta antitumoral. Contudo, nossos resultados indicaram o oposto. Observamos que a ausência dessa proteína em DCs resulta em um aumento significativo do crescimento tumoral em dois modelos: melanoma metastático murino (B16-OVA) e adenocarcinoma de cólon (MC38). Embora a expressão de MHC II e CD86 estivesse aumentada nos animais deficientes em *Marchf1*, isso não se traduziu na esperada ativação de células T, contrariando nossa hipótese inicial. Esses

resultados observados em modelos murinos foram em concordância com os resultados de sobrevivência de pacientes com melanoma, em que pacientes com uma expressão alta de *Marchfl* apresentaram uma melhor sobrevida comparado com os pacientes com expressão reduzida. No entanto, com esses dados de sobrevivência do TCGA é difícil de concluir quanto à identificação do tipo de célula em que a expressão de *Marchfl* está sendo analisada, por ser uma análise geral do microambiente tumoral. No entanto, de maneira geral, é possível observar uma tendência da expressão de *Marchfl* influenciado a sobrevida dos pacientes.

Sabe-se que as interações entre os componentes do microambiente tumoral são cruciais para o desenvolvimento de uma resposta eficiente contra o tumor, mesmo que as células tumorais exerçam inibição das funções de algumas células antitumorais no microambiente, para facilitar seu crescimento (ref). Observamos que nos animais com depleção de *Marchfl* em DCs (*CD11c^{Cre}Marchfl^{fl/fl}*), no modelo de B16-OVA, o aumento do tumor estava acompanhado por uma deficiência numérica e funcional das células T *CD8⁺* presentes no microambiente tumoral. Essas células conhecidas como células T citotóxicas, desempenham o principal papel efetor no controle e na eliminação do tumor (Raskov *et al.*, 2021). Resultados similares são documentados em estudos com humanos. Pacientes com câncer que apresentam uma maior proporção de células T *CD8⁺* tendem a ter uma sobrevida prolongada e a responder melhor às imunoterapias (Jin *et al.*, 2021). Durante sua ativação, as células T *CD8⁺* recebem apresentação de antígenos tumorais via TCR, através da interação com o MHC I das DCs (Rossjohn *et al.*, 2015). A subpopulação de DCs responsável por apresentar antígenos tumorais via MHC I para as células T *CD8⁺*, dessa forma ativando-as, são as cDC1s (Den Haan; Lehar; Bevan, 2000).

Pacientes com câncer que possuem grandes quantidades de cDC1s no microambiente tumoral apresentam maior sobrevida (Böttcher *et al.*, 2018; Broz *et al.*, 2014; Michea *et al.*, 2018). A relevância dessa subpopulação para o controle do crescimento tumoral é corroborada por diversos estudos com camundongos deficientes em cDC1s, como no modelo *Batf3^{-/-}*, além de outros modelos *in vivo* de depleção de cDC1s (Hildner *et al.*, [s. d.]; Liu *et al.*, 2019). Tais estudos demonstraram, de forma consistente, a incapacidade de animais *Batf3^{-/-}*, de rejeitar tumores imunogênicos e em responder a terapias imunes baseadas em células T, como a terapia adotiva (Liu *et al.*, 2019). Além do mais, já se foi observado que ao reprogramar geneticamente células tumorais para expressarem fatores de transcrição clássicos de cDC1s (PU.1, IRF8 e BATF3), as células tumorais começam

apresentar antígenos dessa forma remodelando o microambiente tumoral e recrutando células T citotóxicas (Ascic *et al.*, 2024).

No microambiente tumoral dos camundongos CD11c^{Cre}Marchfl^{fl/fl} observamos uma ausência dessa subpopulação de DCs, o que seria um fator chave na diminuição da ativação das células T CD8⁺ e, conseqüentemente, na piora da resposta imune antitumoral nesses animais. As cDC1s são essenciais para uma resposta eficiente ao tratamento com inibidores de checkpoints imunológicos, como o anti-PD1 e o anti-CTLA-4 (81,82), que se mostraram ineficazes em animais com depleção de Marchfl em DCs. Isso sugeriu que Marchfl tem um papel crucial também na resposta a esses tratamentos, provavelmente atuando na biologia das cDC1s. Esses resultados em modelo murino coincidiram com dados observados em humanos, em que ao analisar dados públicos de um estudo de pacientes com melanoma tratados com anti-PD-1 observamos que aqueles que apresentaram resposta completa ao tratamento exibiram níveis aumentados de Marchfl em comparação com os pacientes que responderam parcialmente.

Investigamos ainda se a resposta T CD8⁺ estava diminuída não apenas devido ao menor número de DCs nos animais ausentes de Marchfl, mas de que essa deleção genética resultaria também em defeitos funcionais nas DCs. Isso seria inclusive esperado, dados o estudo anterior que havia observado uma diminuição da expressão de MHC I nas DCs e em células B de animais deficientes de Marchfl (Wilson *et al.*, 2018b). Em nossas mãos, os níveis de MHC I não mostraram alteração nas DCs dos animais sem Marchfl, e essas DCs mantiveram uma capacidade de apresentar peptídeos tumorais semelhante à observada em camundongos selvagens. Além disso, não detectamos nenhum outro defeito funcional intrínseco quanto à apresentação de antígenos nos animais deficientes de Marchfl. Utilizando a proteína OVA e células T específicas (OT-I), observamos que as cDC1s dos camundongos sem Marchfl mantiveram sua funcionalidade. Elas foram capazes de estimular as células T CD8⁺ específicas *in vitro*, conforme evidenciado pela análise de proliferação utilizando o corante *Cell Trace Violet* (CTV), após o estímulo com o antígeno, de maneira comparável aos animais controle.

Uma vez excluído esse defeito funcional das DCs como causa da resposta T CD8⁺ diminuída, hipotetizamos que as DCs poderiam estar morrendo mais *in vivo*. Um estudo (Schriek *et al.*, 2022) reportou que animais deficientes em Marchfl possuem um menor número de cDC1s e cDC2s no baço, sem alteração em outras populações como pDC, células B e T CD4⁺/CD8⁺. Os autores sugeriram que o aumento de MHC II na superfície

de células cDCs devido à ausência de *Marchfl* induziria a ligação do componente 3 do sistema complemento (C3). C3 é reconhecido pelo seu receptor CR2 presentes em células B, e através do processo de trogocitose essas células B sequestram o MHC II da membrana celular das cDCs. Por ter um excesso de MHC II presente na membrana, e consequentemente muito C3 conjugado a essa molécula, as células B realizariam o processo de “*trogoptose*”, combinando trogocitose e apoptose, resultando na morte das cDCs. Contudo, em nossos experimentos, detectamos que cDC1s no baço dos animais ausentes de *Marchfl* não parecem estar morrendo em níveis aumentados em comparação com animais controles.

Um dos principais achados do nosso estudo foi verificar que a ausência de *Marchfl* resulta em alterações no desenvolvimento de precursores de DCs na medula óssea. Especificamente, verificamos uma diminuição significativa nos MDPs e CDPs, com uma tendência de redução na população de pré-cDCs. Além disso, ao examinar essas células em tecidos linfoides secundários, como o baço e os linfonodos de drenagem do tumor, também detectamos uma redução significativa nas pré-cDCs. Estudos mais aprofundados irão revelar que outras alterações ocorrem nos precursores de DCs na medula óssea desses animais que possam influenciar inclusive o transporte das pré-cDCs da medula óssea para os tecidos linfoides.

No entanto, há necessidade de aprofundar o entendimento de como está o desenvolvimento das DCs em animais $CD11c^{Cre}Marchfl^{fl/fl}$. Em nosso estudo, vimos que o crescimento tumoral tanto em animais *Marchfl*^{-/-} como $CD11c^{Cre}Marchfl^{fl/fl}$, estava aumentado. Embora uma explicação para isso nos animais *Marchfl*^{-/-} seja o defeito no desenvolvimento, isso não explicaria completamente o fenótipo em animais $CD11c^{Cre}Marchfl^{fl/fl}$. Principalmente porque o início da expressão de CD11c ocorre somente na etapa final da medula óssea na população pré-cDC (Dhodapkar et al., 1999). Dessa forma, é possível que mais de um mecanismo, ou outros mecanismos, como a diminuição na distribuição das pré-cDCs para os tecidos linfóides, explique esse crescimento tumoral aumentado.

O desenvolvimento e a manutenção das DCs, na medula óssea, são dependentes da via de sinalização mediada pelo receptor tirosina quinase Flt3 e seu ligante, o fator ligante de Flt3 (Flt3L) (Cueto; Sancho, 2021). Observamos em nossos resultados que sob estimulação com Flt3L, os animais com depleção de *Marchfl* em DCs ainda possuem a capacidade de ativar as vias necessárias para o desenvolvimento adequado dessas células.

Diversos ensaios clínicos em pacientes com câncer têm investigado o uso de Flt3L em combinação com radioterapia e inibidores de checkpoints imunológicos (Brody, 2024), com o objetivo de aumentar o número de DCs e promover sua ativação, favorecendo uma resposta imune antitumoral mais eficaz. Nos animais $CD11c^{Cre}Marchfl^{fl/fl}$, o tratamento com Flt3L durante nove dias resultou em um aumento significativo de cDC1s no baço e no tumor, revertendo a resposta reduzida de células T $CD8^+$ e controlando o crescimento tumoral. Estudos mais detalhados precisam ser realizados para determinar se o tratamento com Flt3L funciona apenas por aumentar o número de DCs, ou se aciona adicionalmente alguma via de sinalização que resulta na ativação de DCs. No entanto, estudos anteriores (Salmon *et al.*, 2016) não observaram um aumento de MHC classe II ou CD86 induzido por Flt3L, sugerindo que, na ausência de Marchfl, apenas o aumento numérico de DCs é suficiente para a recuperação da resposta citotóxica anti-tumoral.

Diversos fatores presentes no microambiente tumoral, como citocinas, quimiocinas e produtos metabólicos derivados de células tumorais conseguem induzir disfunções nas DCs, afetando processos cruciais como diferenciação, maturação e apresentação de antígenos (Jhunjhunwala; Hammer; Delamarre, 2021). No entanto, no contexto da proteína Marchfl, é bem estabelecido que, *in vivo*, Marchfl somente poliubiquitina MHC II e CD86, regulando assim a expressão dessas moléculas na superfície das DCs (Liu *et al.*, 2022). A ausência de Marchfl pode resultar em um aumento de MHC II em células precursoras durante o desenvolvimento das DCs na medula óssea, o que pode ativar um sistema de controle de qualidade celular, afetando o desenvolvimento adequado dessas células. Contudo, essa hipótese ainda requer uma investigação mais aprofundada para ser confirmada.

Um melhor entendimento da composição do microambiente tumoral dos animais com depleção de Marchfl nas DCs é necessária para compreender os mecanismos pelos quais a deleção dessa proteína resulta em uma resposta citotóxica antitumoral ineficaz. Uma abordagem é a utilização da tecnologia de sequenciamento de RNA de células individuais (*single-cell RNA sequencing*) de células hematopoiéticas $CD45^+$. Atualmente, estamos realizando essa análise para melhor compreender que fatores e tipos de células estão alterados entre os animais do grupo experimental e os controles. Além disso, planejamos investigar de uma forma adicional se os efeitos observados se dão através da diminuição especificamente do número das cDC1s. Para isso, iremos diferenciar células hematopoiéticas da medula óssea de camundongos $CD11c^{Cre}Marchfl^{fl/fl}$ ou controles

Marchfl^{fl/fl} em cDC1s, ativando-as *ex vivo* com a proteína OVA e Poly-IC, posteriormente realizando a transferência adotiva das cDC1s para os animais CD11c^{Cre}Marchfl^{fl/fl} e Marchfl^{fl/fl} com tumor B16-OVA. Dessa forma, iremos responder duas perguntas: 1) se aumento do número de apenas as cDC1s no microambiente dos animais CD11c^{Cre}Marchfl^{fl/fl} irá induzir uma resposta imune antitumoral e dessa forma inibir o crescimento do tumor; e (2) se as cDC1s dos animais CD11c^{Cre}Marchfl^{fl/fl} são capazes de induzir respostas antitumorais da mesma forma que os animais controles Marchfl^{fl/fl}. Esse tratamento irá excluir possíveis efeitos adicionais do Flt3L, que também é necessário para o desenvolvimento de pDCs. Para estudar um efeito mais específico de Marchfl nas cDC1s, também planejamos utilizar animais com depleção nas células XCR1⁺. Baseado nessas análises, será possível mostrar de uma forma mais específica e direta a relação de Marchfl e ubiquitinação de MHC II e CD86 na população de cDC1s com a indução de respostas de células T CD8 efetivas.

Além dos resultados obtidos no modelo tumoral, decidimos explorar se o efeito da deleção de Marchfl em DCs também afetaria a resposta de células T CD8⁺, decorrente da redução no número de cDC1s, em modelo de transplante. Inicialmente, observamos que pacientes submetidos a transplante cardíaco que desenvolveram episódios de rejeição apresentaram um aumento nos níveis de Marchfl em análises transcriptômicas, sugerindo um papel da proteína no processo de rejeição. Nos modelos murinos, constatamos que tanto os animais deficientes em Marchfl (Marchfl^{-/-}) quanto os animais com depleção de Marchfl nas DCs (CD11c^{Cre}Marchfl^{fl/fl}) que receberam transplantes cardíacos de doadores BALB/c com incompatibilidade total ou BALB/c x C57BL/6 F1 com incompatibilidade parcial, demonstraram uma maior aceitação do aloenxerto em comparação com os animais controle. Além disso, os animais Marchfl^{-/-} apresentaram uma resposta antígeno-específica de aceitação quando submetidos a um segundo transplante com coração de doador idêntico ao do primeiro transplante.

Estudos prévios já haviam demonstrado uma correlação entre o número de cDC1s e a gravidade de doenças renais, mostrando que amostras saudáveis de rim continham números reduzidos de cDC1s, enquanto pacientes com subtipos variados de doenças renais crônicas, que eventualmente necessitam de transplante, apresentavam números aumentados de cDC1s, correlacionando-se com a gravidade da doença e com células T CD8⁺ (Zhuang; Lakkis, 2015). Para avaliar o efeito do aumento de cDC1s na rejeição do aloenxerto, tratamos os animais Marchfl^{-/-} com uma combinação de Flt3L e Poly:IC após

o transplante, similar ao protocolo utilizado no modelo tumoral. Observamos que o aumento de cDC1s induziu uma resposta rápida contra o aloenxerto, resultando na rejeição do órgão em todos os animais tratados poucos dias após o término do tratamento.

Esses resultados reforçam o papel crucial da *Marchf1* na modulação da resposta imune e sugerem que o controle do número de cDC1s pode influenciar diretamente o sucesso ou falha de transplantes.

Nesta tese, demonstramos a relação entre *Marchf1* em DCs como um fator para induzir resposta imunes de células T $CD8^+$ contra antígenos tumorais e de aloenxertos por meio do desenvolvimento de cDC1s. Em adição a esse fator biológico no desenvolvimento de cDC1s, é possível também sugerir *Marchf1* como um marcador para prever sobrevivência e uma resposta completa à imunoterápicos, e provavelmente um alvo importante para imunoterapia oncológica.

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12. Currículo Lattes



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Última atualização do currículo em 01/06/2024

Karina Lima é bacharel (2017) em Biotecnologia Molecular pela Universidade Federal do Rio Grande do Sul (UFRGS) e mestre em Biologia Celular e Molecular do Programa de Pós-Graduação em Biologia Celular e Molecular na Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), onde trabalhou com co-chaperona da Hsp70 a HspBP1, com o intuito de identificar mecanismos pela qual essa proteína possui um efeito antitumoral. A sua pesquisa foi focada em produzir a HspBP1 de forma recombinante e verificar a função in vivo. Teve um breve envolvimento com a pesquisa clínica no âmbito do estudo dos efeitos da vacina da dengue, coordenado pelo Instituto Butantan e desenvolvido no Centro de Pesquisa Clínica do Hospital São Lucas da PUCRS. Atuou como pesquisadora assistente na PUCRS trabalhando no projeto "Cavalos de Tróia": Desenvolvimento de Novos Anticorpos Conjugados a Drogas (ADCs) para Tratamento de Tumores Primários e Micrometástases, sendo responsável pela produção e caracterização de anticorpos monoclonais. Aluna de doutorado na Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), investigando o papel das enzimas da família MARCH ligases na progressão tumoral. Realizou seu doutorado sanduíche no Massachusetts General Hospital - Harvard Medical School (EUA) por um período de 12 meses. (Texto informado pelo autor)

Identificação

Nome

Karina Rodrigues Lima

Nome em citações bibliográficas

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RODRIGUES; LIMA, KARINA

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Formação acadêmica/titulação

2020

Doutorado em andamento em BIOCÊNCIAS.
 Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.
 com **período sanduíche** em Massachusetts General Hospital (Orientador: Thiago de Jesus Borges).
 Orientador: Cristina Beatriz Cazabuena Bonorino.
 Coorientador: Thiago de Jesus Borges.
 Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasil.

2017 - 2018

Mestrado em Biologia Celular e Molecular.
 Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Brasil.
 Título: Avaliação in vivo do potencial antitumoral da co-chaperona HspBP1☹️, Ano de Obtenção: 2019.
 Orientador: 😊 Moises Evandro Bauer.
 Coorientador: Cristina Beatriz Cazabuena Bonorino.
 Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, Brasil.
 Palavras-chave: HspBP1; Hsp70.
 Grande área: Ciências Biológicas
 Grande Área: Ciências Biológicas / Área: Bioquímica / Subárea: Biologia Molecular.
 Grande Área: Ciências Biológicas / Área: Biologia Geral / Subárea: Estresse celular.
 Setores de atividade: Pesquisa e desenvolvimento científico.

2013 - 2016

Graduação em Biotecnologia Molecular.
 Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.
 Título: Avaliação de parâmetros de estresse oxidativo em animais tratados com dexametasona e submetidos ao kindling pelo pentilenotetrazol.
 Orientador: Adriana Simon Coitinho.
 Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, Brasil.

Formação Complementar

2021 - 2021

Curso Teórico-Prático de FlowJo - Módulo Básico. (Carga horária: 8h).
BD Bioscience, BD, Brasil.

2021 - 2021

Curso Teórico-Prático de FlowJo - Módulo Avançado. (Carga horária: 8h).
BD Bioscience, BD, Brasil.

2020 - 2020

Treinamento operacional - BD FACSCanto II. (Carga horária: 8h).
BD Bioscience, BD, Brasil.

2016 - 2016

Revendo Fisiologia. (Carga horária: 50h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2014 - 2014

Sequenciamento de DNA: Perspectivas na Biotecnologia. (Carga horária: 20h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

Atuação Profissional

Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.

Vínculo institucional

2019 - Atual

Vínculo: , Enquadramento Funcional:

Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

Vínculo institucional

2013 - 2016

Vínculo: Bolsista, Enquadramento Funcional: Iniciação Científica, Carga horária: 20, Regime: Dedicação exclusiva.

Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Brasil.

Vínculo institucional

2019 - 2020

Vínculo: Celetista, Enquadramento
Funcional: Pesquisador Assistente, Carga
horária: 40

**Outras
informações**

Pesquisadora assistente do projeto ?
Cavalos de Tróia?: Desenvolvimento de
Novos Anticorpos Conjugados a Drogas
(ADCs) para Tratamento de Tumores
Primários e Micrometástases. Trabalhando
com produção e caracterização de
anticorpos monoclonais.

Projetos de pesquisa

2019 - Atual

MARCH1 como alvo em imunoterapia para
cancer;

Descrição: Identificar mecanismos
imunossupressores da molécula MARCH1
que regula a expressão de MHC II e CD86
em células dendríticas de indivíduos com
tumores.

Situação: Em andamento; Natureza:
Pesquisa.

Alunos envolvidos: Doutorado: (2) .

Integrantes: Karina Rodrigues Lima -
Integrante / Cristina Bonorino -
Coordenador / Julia Crispim da Fontoura -
Integrante / Thiago de Jesus Borges -
Integrante.

Áreas de atuação

1.

Grande área: Ciências Biológicas / Área:
Biotecnologia.

2.

Grande área: Ciências Biológicas / Área:
Biotecnologia / Subárea: Biologia
Molecular.

3.

Grande área: Ciências Biológicas / Área:
Biotecnologia / Subárea: Imunologia.

4.

Grande área: Ciências Biológicas / Área:
Biologia Geral / Subárea: Biologia Celular.

5.

Grande área: Ciências Biológicas / Área:
Biologia Geral / Subárea: Genética.

Idiomas

Inglês

Compreende Bem, Fala Bem, Lê Bem,
Escreve Razoavelmente.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica



1.

★ **LIMA, KARINA**; RIBAS, GUILHERME T. ; RIELLA, LEONARDO V. ; BORGES, THIAGO J. . Inhibitory innate receptors and their potential role in transplantation. Transplantation Reviews **JCR**, v. 37, p. 100776, 2023. **Citações:** **WEB OF SCIENCE** = 1

2.

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Bancas

Participação em bancas de trabalhos de conclusão

Trabalhos de conclusão de curso de graduação

1.

LIMA, K. R.; PERES, A.. Participação em banca de Verônica Toniazzi Brunetto. Investigação da plasticidade e da participação de neutrófilos associados ao câncer colorretal. 2022. Trabalho de Conclusão de Curso (Graduação em Biomedicina) - Fundação Universidade Federal de Ciências da Saúde de Porto Alegre.

Eventos

Participação em eventos, congressos, exposições e feiras

1.

XII Simpósio Sul de Imunologia. Avaliação in vivo do potencial antitumoral da co-chaperona HspBP1 produzida de forma recombinante. 2019. (Simpósio).

2.

Second AACR International Conference on Translational Cancer mEDICINE. Antitumoral effect of the co-chaperone HspBP1. 2018. (Congresso).

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XI Simpósio Sul de Imunologia (ImunoSul).Avaliação in vivo do potencial antitumoral da co-chaperona HspBP1 produzida de forma recombinante. 2018. (Simpósio).

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X Simpósio Sul de Imunologia (ImunoSul).Expressão de TDO e IDO in vitro e in vivo em linhagens tumorais. 2017. (Simpósio).

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