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RESPUESTA INMUNITARIA A LA INFECCIÓN POR SARS-CoV-2 Y A LA VACUNA CONTRA LA COVID-19

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

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ABREVIATURAS

ARN: Ácido ribonucleico

ARNm: Ácido ribonucleico mensajero

ART: Tratamiento antirretroviral

CD: células dendríticas.

CDm: células dendríticas mieloides

CDp: células dendríticas plasmacitoides

CMV: citomegalovirus

COVID-19: Enfermedad por Coronavirus 2019 (del inglés *Coronavirus disease 2019*)

EBV: Epstein-barr virus

ECA2: Enzima convertidora de angiotensina 2

GM-CSF: Factor estimulante de colonias de granulocitos y macrófagos (del inglés *Granulocyte Macrophage Colony-Stimulating Factor*)

HCoV: Coronavirus humano (del inglés *human coronavirus*)

IDO: Indoleamine 2,3 dioxygenase

IFN: Interferón

IL: interleucina

MERS-CoV: Coronavirus del síndrome respiratorio de Oriente Medio (del inglés *Middle East Respiratory Syndrome Coronavirus*)

NK: célula asesina natural (del inglés, *natural killer cells*)

OMEC: Oxigenación por membrana extracorpórea

OMS: Organización Mundial de la Salud

SARS: Síndrome respiratorio agudo grave (del inglés *severe acute respiratory syndrome*)

SARS-CoV-2: Coronavirus de tipo 2 causante del síndrome respiratorio agudo severo (del inglés *severe acute respiratory syndrome coronavirus 2*)

SDRA: distrés respiratorio agudo

TIGIT: inmunoglobulina de células T y dominio inhibidor basado en tirosina inmunorreceptora (*T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain*)

TLR: Receptores tipo Toll (del inglés *Toll-like receptor*)

TMPRSS2: Proteasa transmembrana de serina 2 (del inglés *Transmembrane serine protease 2*)

TNF: Factor de necrosis tumoral (del inglés *tumor necrosis factor*)

VIH: Virus de la inmunodeficiencia humana

VOC: variante preocupante (del inglés *variant of concern*).

RESUMEN

El SARS-CoV-2 es un β-coronavirus que causa la enfermedad COVID-19, provocando en los casos más extremos un síndrome respiratorio agudo severo o incluso la muerte. Conocer y entender la respuesta inmunitaria innata y adaptativa al virus se convirtió en uno de los principales retos al inicio de esta pandemia y fueron numerosos los estudios que la empezaron a describir. En los trabajos publicados hasta el momento en el que se inició esta tesis doctoral, se detectaron alteraciones en el sistema inmunitario innato como un déficit en el número y función de células dendríticas (CD), además de algunas características de la respuesta específica humoral y la mediada por linfocitos T. Estas y otras alteraciones inmunitarias desencadenaban una infamación exacerbada, que en los individuos más graves y debido a la falta de tratamientos eficaces, desembocaba en patologías severas. Para completar la información que hasta ese momento se conocía y caracterizar mejor la respuesta al SARS-CoV-2, consideramos necesario analizar pormenorizadamente las alteraciones inmunitarias en células innatas y la calidad de la respuesta específica de las células T, tanto en el momento de la infección aguda como siete meses después de la infección. Otro de los factores clave para comprender mejor la respuesta inmunitaria al SARS-CoV-2 es la respuesta heteróloga o cruzada con coronavirus endémicos, por lo que también estudiamos este factor en una cohorte de individuos sanos prepandemia.

Una de las principales poblaciones afectadas por la progresión más grave de la COVID-19 fueron individuos de edad avanzada. Esto hizo que se convirtieran en uno de los primeros focos de atención a la hora de suministrar vacunas que les protegieran de esta enfermedad. Sin embargo, estudios en vacunas frente a otros virus han demostrado que las personas de mayor edad muestran una menor respuesta vacunal. Esto se debe en gran parte al agotamiento que sufre el sistema inmunitario con la edad, denominado: inmunosenescencia. Por lo tanto, vimos necesario describir cuáles eran los defectos inmunitarios asociados a la edad que afectaban a que hubiera una menor respuesta a la vacuna SARS-CoV-2 más utilizada hasta el momento en nuestro país, la vacuna de Pfizer-BioNTech (BNT162b2), comparando una cohorte de individuos jóvenes y otra de personas de mayor edad.

Los objetivos de la presente tesis doctoral fueron los siguientes: i) describir los defectos en células dendríticas (CD) asociados a la infección por SARS-CoV-2 y a la gravedad de la COVID-19 y conocer si estos defectos en las CD perduraban siete meses tras la infección, ii) estudiar la calidad de la respuesta SARS-CoV-2 específica mediada por linfocitos T, si se asocia a la gravedad de la COVID-19 en infección aguda, si ésta perduraba siete meses tras la infección y si se relacionaba con la respuesta a coronavirus endémicos (HCoV), y iii) Analizar factores de la inmunidad innata y

adaptativa asociados a menor respuesta a la vacuna BNT162b2 contra SARS-CoV-2 en personas mayores de 60 años.

En la infección aguda observamos que los pacientes infectados por SARS-CoV-2 hospitalizados mostraron bajos niveles de células dendríticas mieloides (CDm) CD1c+ y células dendríticas plasmacitoides (CDp), asociados a una menor producción de interferón (IFN)-α y un patrón alterado de marcadores inflamatorios. Además, el déficit en la producción de IFN-α se asoció a la gravedad de la infección por SARS-CoV-2 en pacientes hospitalizados. Siete meses tras la infección describimos que algunos de estos defectos observados en CD, sobre todo aquellos asociados a marcadores de activación (CD86+ y CD4+), únicamente se recuperan en pacientes previamente no hospitalizados tras siete meses de la infección. Sin embargo, los bajos niveles de CD (CD1c+ CDm y CDp) y defectos asociados a marcadores de migración a mucosa intestinal (integrina α4β7) y tolerogénesis (*Indoleamine 2,3 dioxygenase* o IDO) no se recuperaron siete meses tras la infección tanto en pacientes previamente hospitalizados como no hospitalizados.

Por otro lado, en cuando a la respuesta T específica de SARS-CoV-2 se refiere, observamos que las combinaciones de citoquinas con únicamente IFN-γ se asociaban a mayor gravedad de la COVID-19, mientras que aquellas que presentaron interleucina (IL)-2 se asociaron con un mejor curso de la infección aguda. Además, los pacientes leves presentaban una respuesta T específica de SARS-CoV-2 más polifuncional. Por último, encontramos en pacientes graves una mayor producción de anticuerpos asociada de forma inversa a la expresión de combinaciones que incluían IL-2. Nos gustaría destacar que siete meses tras la infección, aun se detectó respuesta celular y humoral, observándose una calidad de la respuesta T similares a los de infección aguda. No obstante, los sujetos previamente hospitalizados presentaban un mayor agotamiento (*T cell immunoglobulin and ITIM domain* o TIGIT) en células T. Por último, se encontró una asociación entre la respuesta a coronavirus endémicos y SARS-CoV-2 principalmente mediada por la expresión IL-2.

Adicionalmente, cuando estudiamos la respuesta a la vacuna de BNT162b2 (Pfizer-BioNTech) en sujetos de mayor edad, encontramos que la disfunción tímica y la consecuente alteración de la homeostasis de las células T (menos linfocitos T naïve y más memoria) se relacionaba con una menor respuesta T específica del SARS-CoV-2 tras la vacunación. Además, observamos que una menor activación, migración y función de las CD (por ejemplo, menos producción de IFN-α y menor expresión de moléculas co-estimuladoras como CD86) y un perfil proinflamatorio mediado por monocitos (por

ejemplo, mayor producción de IL-6 y TNFα) en individuos de mayor edad, se asociaba con una menor respuesta a la vacuna SARS-CoV-2.

En conclusión, estos hallazgos contribuyen a un mejor entendimiento de las alteraciones del sistema inmunitario que inducen una sintomatología grave de la COVID-19, las secuelas inmunológicas inducidas por esta enfermedad, además de los defectos innatos y adaptativos asociados a la edad que dificultan una respuesta vacunal efectiva frente al SARS-CoV-2. Estos descubrimientos pueden ayudar a identificar biomarcadores de progresión de la enfermedad, contribuir al diseño de nuevas dianas terapéuticas, así como para mejorar el diseño de nuevos prototipos de vacunas o nuevas estrategias de vacunación con el fin de lograr una protección más amplia y duradera contra el COVID-19.

INTRODUCCIÓN

1. Aspecto virológicos, epidemiológicos y clínicos de la COVID-19

Los primeros casos de infección por el SARS-CoV-2 (en inglés, ***S**evere **A**cute **R**espiratory **S**yndrome **Co**rona**v**irus **2***) se dieron en el mes de diciembre de 2019 en Wuhan (China). Este virus ha sido el causante de una de las pandemias más importantes registradas a nivel global. En febrero de 2020, fue la Organización Mundial de la Salud (OMS) quien la denominó COVID-19 (en inglés, ***Co**rona**vi**rus **d**isease – 19*) (1). Esta enfermedad es la responsable de 764.474.387 infectados y 6.915.286 muertes a nivel mundial (2), 13.798.747 infectados y 120.426 fallecidos en España (3).

El SARS-CoV-2 pertenece a la familia *Coronaviridae*. Los coronavirus conforman una gran familia de virus que causan enfermedades en animales y humanos. Existen cuatro coronavirus que son endémicos en humanos (coronavirus humano (HCoV)-NL63, HCoV-229E, HCoV-OC43 y HCoV-HKU1) y preferentemente infectan el tracto respiratorio superior y causan síntomas de resfriado común, mientras que hay otros que causan enfermedades más graves, como son los coronavirus que causan el síndrome respiratorio de Oriente Medio (MERS-CoV) y el síndrome respiratorio agudo severo (SARS-CoV-1). Los coronavirus endémicos son estacionales y comunes a nivel mundial y fueron responsables de un 10-20% de las infecciones virales respiratorias en 2019, causando predominantemente enfermedad leve (4). Por el contrario, el MERS-CoV, el SARS-CoV-1 y el SARS-CoV-2 han surgido en los últimos 20 años y pueden causar una enfermedad grave y potencialmente mortal (5). Todos los coronavirus humanos expresan las proteínas estructurales, proteínas accesorias (5–10) y proteínas no estructurales (NSPs) (11). Estos coronavirus endémicos comparten una homología de secuencia genética del 69 % (OC43), 68 % (HKU1) y 65 % (NL63 y 229E) (6) con el SARS-CoV-2 (mientras que el virus SARS-CoV-1 comparte un 79% y el virus MERS un 50%) (12), la homología entre sus proteínas estructurales y NSP (4,6) y epítopos potenciales entre estos coronavirus pueden provocar respuestas inmunitarias adaptativas tras una infección por SARS-CoV-2.

El SARS-CoV-2 es un β-coronavirus con una cadena sencilla de ARN y una estructura genómica de alrededor de 30.000 bases. Codifica para unos 9.860 aminoácidos y presenta un virión espiculado de 60-140 nm. En comparación con otros β-coronavirus, el SARS-CoV-2 presenta el primer marco de lectura que ocupa dos tercios de la secuencia del virus, y traduce las proteínas pp1a, pp1b que, a su vez, se escindirán en 16 NSP. Además, el virus expresa cuatro proteínas estructurales esenciales, la glicoproteína de superficie Spike (S), la proteína pequeña de la envuelta (E), la proteína matriz de la membrana (M) y la proteína de la nucleocápside (N), además

de una serie de proteínas capaces de interferir en el funcionamiento del sistema inmunitario del huésped (13–15).

El SARS-CoV-2 se transmitió en primer lugar de animales a humanos (16), pero enseguida se comprobó que existía la transmisión entre personas (17). La vía de contagio entre humanos se produce a través del contacto directo con las secreciones respiratorias que se producen sobre todo al respirar, toser o estornudar; también puede darse mediante contacto con las manos y otros objetos contaminados por estas secreciones, y tras tocarse la boca, la nariz o los ojos. La transmisión de persona a persona tras el contacto con una persona infectada por SARS-CoV-2 aumenta con la cercanía y la duración del contacto, acentuándose en entornos cerrados, así como con una mayor cantidad de virus en las secreciones respiratorias que actúan como vector (18).

La cepa emergente de SARS-CoV-2 de 2019, normalmente denominada cepa original o "de tipo salvaje", ha mutado con el tiempo. Estas mutaciones han dado lugar a nuevas variantes. Cuando estas mutaciones tienen el potencial de aumentar la facilidad con la que el virus se propaga e inducen mecanismos de escape del sistema inmunitario causando una enfermedad más grave, la variante se designa como variante preocupante (en inglés, **V**ariant **o**f **C**oncern (VOC)). El 31 de mayo de 2021, la OMS recomendó un sistema de denominación para las variantes del SARS-CoV-2 que utiliza el alfabeto griego.

La mayoría de las presentaciones clínicas son leves cuya sintomatología es similar a la causada por el virus de la gripe, que incluye fiebre, tos, malestar, mialgia, cefalea y alteraciones o pérdida del gusto (ageusia) y el olfato (anosmia). Sin embargo, la enfermedad grave suele comenzar aproximadamente una semana después de la aparición de los síntomas. Esta es desencadenada por una inflamación exacerbada que desemboca en la disnea, que es consecuencia de la hipoxemia (19,20). Es causada principalmente por un daño alveolar difuso bilateral, formación de membranas hialinas, infiltrados inflamatorios mononucleares intersticiales y descamación, congruentes con el síndrome de distrés respiratorio agudo (SDRA), y es similar a la patología pulmonar observada en el MERS y el SARS (21–24). Estos individuos requieren asistencia respiratoria u oxigenoterapia, pudiendo llegar a la muerte del individuo infectado.

En función de la gravedad en la enfermedad y el tiempo de recuperación desde el primer día hasta los 28 siguientes al diagnóstico de la infección, se estableció una escala ordinal de ocho categorías: **1**, no hospitalizado y sin limitación de actividades; **2**, no hospitalizado, con limitación de actividades, necesidad de oxígeno a domicilio, o

ambas; **3**, hospitalizado, sin necesidad de oxígeno suplementario y que ya no requiere atención médica continua; **4**, hospitalizado, sin necesidad de oxígeno suplementario pero con necesidad de atención médica continuada; **5**, hospitalizado, con necesidad de oxígeno suplementario; **6**, hospitalizado, con necesidad de ventilación no invasiva o uso de dispositivos de oxígeno de alto flujo; **7**, hospitalizado, que recibe ventilación mecánica invasiva u oxigenación por membrana extracorpórea (OMEC); y **8**, fallecimiento (25).

La evolución clínica de la infección por SARS-CoV-2 se puede dividir en las siguientes etapas (26):

Fase 1. Infección temprana. La fase inicial se produce en el momento de la inoculación y el establecimiento temprano de la enfermedad. Para la mayoría de las personas, se trata de un periodo de incubación asociado a síntomas leves y a menudo inespecíficos, como malestar general, fiebre y tos seca (27). Durante este periodo, el SARS-CoV-2 se replica y se instala en el huésped, principalmente en el sistema respiratorio. Es en este periodo donde se da el mayor potencial infeccioso en los primeros cinco días del inicio de los síntomas (28) (Figura 1).

Para llevar a cabo el proceso de infección, el SARS-CoV-2 se une al receptor de la enzima convertidora de angiotensina 2 (ECA2) en células humanas, abundantes en el epitelio de pulmón e intestino delgado y endotelio vascular, mediante la unión de la glicoproteína S del SARS-CoV-2. Esta está formada por tres unidades idénticas organizadas en forma de círculo, afín al receptor ECA2, mediando la fusión de la cubierta membranosa del virus con la membrana de la célula que está siendo infectada. La unión entre el receptor ECA2 y la glicoproteína S se produce gracias a la activación de esta última. La activación de la proteína S está mediada por la enzima celular proteasa transmembrana de serina 2 (del inglés *transmembrane serine protease,* TMPRSS2), que suele localizarse cerca de ECA-2. TMPRSS2 corta la proteína S, lo que activa proteínas de la envoltura viral que favorecen la fusión con la membrana celular. De este modo, los virus entran en la célula rodeados de membrana celular, formando endosomas. En estos pequeños compartimentos, se liberan catepsinas, otras proteínas que modifican de nuevo la proteína S, y proteasas que favorecen la liberación del ARN viral al citoplasma. La replicación del ARN viral y la síntesis de proteínas virales ocurren en el retículo endoplasmático y aparato de Golgi, dando lugar al virión maduro que se libera al espacio intersticial (29).

En un proceso fisiológico de un individuo sano, ECA2 se encarga de hidrolizar diferentes angiotensinas (30,31) en diferentes productos que favorecen la vasodilatación y estados antitrombóticos, antiinflamatorios, antifibróticos y antiproliferativos (32). Por este motivo,

que el receptor ECA2 se encuentre bloqueado por el SARS-CoV-2 disminuiría su propia actividad, promoviendo un estado proinflamatorio, protrombótico y profibrótico en el individuo (33,34).

Fase 2. Pulmonar o aguda. En esta fase continúa la replicación viral y se da una inflamación principalmente localizada en el pulmón. En la mayoría de los casos los pacientes desarrollan una neumonía vírica con tos, fiebre y finalmente hipoxia (Figura 1).

Fase 3. Inflamación y coagulopatía. Una minoría de pacientes con COVID-19 pasa a sufrir esta fase, que se manifiesta como un síndrome de hiperinflamación sistémica extrapulmonar. Se caracteriza por una disminución de células T y niveles exacerbados de citoquinas proinflamatorias, entre otras alteraciones inmunitarias. En esta fase, se aprecia vasoplejía, insuficiencia respiratoria e incluso colapso cardiopulmonar o la afectación orgánica sistémica, como miocarditis (Figura 1).

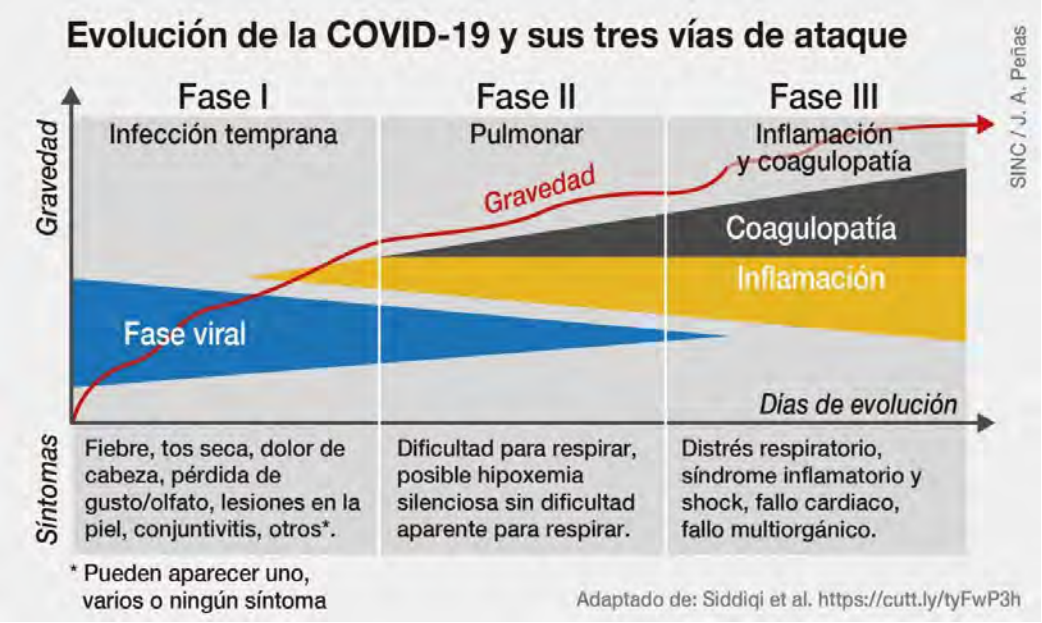

Figura 1. *Fases de la COVID-19 y evolución de la infección en función de la gravedad.*

2. Respuesta inmunitaria asociada a la infección por SARS-CoV-2

Cuando se comenzó la presente tesis doctoral, únicamente se conocían aspectos muy generales de la respuesta humoral y celular específica frente al SARS-CoV-2. La respuesta inmunitaria tanto innata como adaptativa tienen un papel crucial en el control de las infecciones virales incluyendo el SARS-CoV-2. Sin embargo, las respuestas inmunitarias descontroladas pueden provocar una inmunopatología significativa.

Como en cualquier otra infección viral, cuando el SARS-CoV-2 infecta una célula mediante el receptor ECA2, en primer lugar, las células dendríticas (CD) lo detectan y lo presentan a los linfocitos T CD4+ y CD8+. Estos linfocitos específicos del virus se activan y se multiplican, los CD4+ producen citoquinas que atraen a las células del sistema inmunitario innato, como los neutrófilos y los monocitos, que producen una respuesta inflamatoria. Los linfocitos T CD8+, también conocidos como células citotóxicas, eliminan las células infectadas junto con las células asesinas naturales (NK). Además, se desarrolla una respuesta humoral mediada por las células B, a su vez favorecida por los linfocitos T CD4+, que producen anticuerpos que pueden neutralizar el virus.

La gravedad de la enfermedad depende del equilibrio entre la respuesta inmunitaria, la replicación viral y el daño de tejidos y órganos. En la COVID-19 grave se produce un desequilibrio de esta respuesta, caracterizada por una hiperinflamación impulsada por la inmunidad innata, y por niveles muy elevados de citoquinas y otras moléculas proinflamatorias (35,36).

2.1. Respuesta inmunitaria innata: Tras la entrada del virus, la respuesta inflamatoria inicial atrae células T específicas del virus al lugar de la infección, donde las células infectadas son eliminadas antes de que el virus se extienda, lo que conduce a la recuperación en la mayoría de las personas. En los pacientes que desarrollan una enfermedad grave, el SARS-CoV-2 provoca una respuesta inmunitaria exacerbada, que implica la sobreproducción de citoquinas proinflamatorias como interleucina (IL)-1, IL-6 y factor de necrosis tumoral (en inglés, tumor necrosis factor, TNF) (21,37,38) que se acumulan en los pulmones y acaban dañando el parénquima pulmonar (21–24). Éstas inducen la producción de otras citoquinas por parte de las células T y NK, como IL-2, factor estimulante de colonias de granulocitos y macrófagos (en inglés, Granulocyte Macrophage Colony-Stimulating Factor, GM-CSF) e interferón (IFN)-γ. Estos altos niveles de citoquinas provocan la movilización de diversas células inmunitarias, como neutrófilos, macrófagos, monocitos, CD y linfocitos T, desde la circulación sanguínea hacia el tejido infectado, lo que provoca daño alveolar difuso, daño de la barrera vascular, daño multiorgánico y, en última instancia, la muerte (39,40).

Las primeras células que rápidamente se extravasan son los neutrófilos y los monocitos. Los monocitos son células inmunitarias innatas fagocíticas que circulan por la sangre y se dividen clásicamente en tres subconjuntos en función de su expresión respectiva de CD14 y CD16, dividiéndose en monocitos clásicos (CD14++CD16-), intermedios (CD14++CD16+) y no clásicos (CD14+CD16++) (41,42). En condiciones patológicas, incluida la infección por SARS-CoV-2, los monocitos activados y reclutados por

mediadores inflamatorios, se infiltran en los tejidos afectados y adquieren fenotipos inflamatorios de tipo macrófago y CD para desempeñar sus funciones efectoras de actividades pro- y antiinflamatorias, presentación de antígenos y remodelación tisular (39).

Otro de los tipos de células inmunitarias innatas que puede desempeñar un papel fundamental en la respuesta contra el SARS-CoV-2 son las CD (43). A pesar de que las CD son muy heterogéneas, se han descrito las siguientes subpoblaciones: CD convencionales o mieloides (CDm) y las células dendríticas plasmocitoides (CDp). Las CDm a su vez incluyen las subpoblaciones CD1c+ (principalmente median la respuesta de linfocitos T CD4+), CD141+ (principalmente median la respuesta de linfocitos T CD8+ y la presentación cruzada) y CD16+ CDm (modulan la respuesta inflamatoria). Las CDp son las principales productoras de IFN-α (44). En general, las CD participan en la presentación antigénica, la producción de citoquinas, el control de las respuestas inflamatorias, generación de tolerancia inmunológica, el reclutamiento de otras células inmunitarias y la diseminación vírica (44).

Sin embargo, el papel de estas células en la respuesta a la infección aguda por SARS-CoV-2 no estaba bien caracterizado. Durante el desarrollo de los estudios que componen esta tesis doctoral, se conocía que había alteraciones en las CD consistentes en una disminución del número de CD en respuesta a la infección en sangre periférica (45) y su asociación con la gravedad de la COVID-19 (43), el agotamiento de CDp asociado a un mal pronóstico de la enfermedad (46) y el deterioro de la respuesta provocada por falta de producción de IFN-I (47).

De este modo, se planteó el **Objetivo 1a** del Artículo 1 de la presente tesis doctoral, que fue analizar el papel de las CD en el pronóstico de la COVID-19 y los potenciales defectos de la CD asociados a la infección por SARS-CoV-2.

2.2. Respuesta adaptativa: Las células T CD4+ y CD8+ desempeñan un papel antiviral crítico a través de la inducción de células B y consecuente secreción de anticuerpos específicos contra el patógeno y la eliminación de las células infectadas por el SARS-CoV-2 (48,49).

Estudios en modelos de infección por SARS-CoV-1 demostraron que tanto la respuesta de células T CD4+ como CD8+ estaban implicadas en la protección y la eliminación del virus en la infección aguda (50,51). Durante el desarrollo de esta tesis, se observó que en la infección por SARS-CoV-2, los niveles elevados de respuesta de células T CD4+ se habían asociado a una enfermedad leve y a una eliminación precoz del virus en la infección aguda, mientras que la ausencia de esta respuesta se asoció a un desenlace

mortal (52,53). Aunque a un nivel de magnitud inferior, la respuesta específica de células T CD8+ frente al SARS-CoV-2 en coordinación con la respuesta de células T CD4+ en la infección aguda parecía ser esencial para un buen pronóstico (52). Sin embargo, también se había descrito que una respuesta T de mayor magnitud y unos niveles más elevados de anticuerpos frente al SARS-CoV-2, se asociaban a un mal pronóstico de la enfermedad (54,55). A pesar de todos estos hallazgos, la información sobre la calidad de la respuesta de las células T en la infección aguda era escasa y contradictoria. Por ese motivo se hizo necesario un análisis detallado, de no solo la magnitud, sino también de la calidad de la respuesta T frente al virus del SARS-CoV-2 en relación con la gravedad de la enfermedad, **Objetivo 2a** abordado en el Artículo 2 de la presente tesis doctoral.

Otro factor importante para comprender mejor la respuesta inmune adaptativa frente al SARS-CoV-2 es la posible reactividad cruzada con coronavirus endémicos. A este fenómeno de reactividad cruzada entre coronavirus endémicos y SARS-CoV-2, le atribuimos el término de respuesta heteróloga, que se da cuando la respuesta específica mediada por los linfocitos T o B frente a SARS-CoV-2 en un individuo es afectada por la exposición previa a coronavirus endémicos. Tay et al. (2020) observaron un alto nivel de reactividad cruzada entre los epítopos de células B y T y los anticuerpos contra proteínas estructurales de virus similares al SARS-CoV-1 y los cuatro coronavirus endémicos (13). Durante el desarrollo de esta tesis, en cohortes de distintos puntos geográficos, se ha detectado hasta un 50 % de individuos que tienen este tipo de respuesta heteróloga (4,56,57), lo que lleva a pensar que las respuestas inmunitarias reactivas cruzadas que existen en los individuos antes de la exposición al SARS-CoV-2 también pueden afectar a la susceptibilidad a la infección y a la gravedad de la enfermedad (4,58–64). Sin embargo, las consecuencias de la reactividad cruzada preexistente entre coronavirus endémicos y SARS-CoV-2 se desconocían y eran de considerable interés. De este modo, hay indicios claros de que el contacto previo con coronavirus endémicos puede ser favorable para afrontar la infección por SARS-CoV-2 con un mejor pronóstico. Caracterizar la respuesta frente a HCoV y SARS-CoV-2 fue el **Objetivo 2c** del Artículo 2 de la presente tesis doctoral.

3. Respuesta inmunitaria frente al SARS-CoV-2 tras la infección aguda

Conocer adecuadamente la respuesta inmunitaria frente al virus a medio largo plazo era crucial para comprender si alteraciones inmunológicas que se habían producido durante la infección aguda llegaban a normalizarse. Aunque inicialmente estos aspectos eran desconocidos, durante el desarrollo de los estudios de esta tesis

doctoral se fue conociendo que la duración de la respuesta específica de las células T CD4+ y de las células B de memoria contra la proteína S del SARS-CoV-2 parecía ser estable, mientras que la respuesta de las células T CD8+ disminuía a la mitad a los 6-8 meses después de la infección (65). Además, los pacientes con síntomas tras la infección aguda mostraron una disminución de la producción de IFN-γ en las células T CD8+ N-específicas 4 meses después de la infección (55).

Respecto a la inmunidad humoral, una vez que los individuos superaban la infección, la mayoría de ellos desarrollaron anticuerpos séricos detectables contra el dominio de unión al receptor de la proteína S y con actividad neutralizante asociada durante al menos 21 días tras el inicio de síntomas (66,67). Otros estudios también identificaron células B específicas de SARS-CoV-2, que aumentaron durante los meses posteriores a la infección, así como células plasmáticas virus-específicas, lo que sugería una respuesta humoral de largo plazo (65,68–70).

Por otro lado, la recuperación de los defectos de las CD tras la COVID-19 podría ser muy relevante, ya que la normalización del sistema inmunitario innato tras la infección aguda afectaría a su capacidad para responder a nuevos desafíos víricos y bacterianos. Sin embargo, se desconocía la recuperación del número y la función de las CD tras la COVID-19.

Todos estos resultados de los diferentes brazos de la inmunidad pasada la infección, podrían ser muy relevantes ya que, aunque la mayoría de las personas se recuperaban completamente de la COVID-19 tras la infección aguda y resolvían los síntomas, con el paso del tiempo se han observado muchas secuelas clínicas a largo plazo. A estas secuelas se les ha denominado "COVID-19 de larga duración" o "síndrome post-COVID", que incluye: la fatiga crónica, la dificultad para respirar, dolor en el pecho y cabeza, dificultades cognitivas, pérdida de olfato y/o gusto, problemas cardíacos, pulmonares o neurológicos, entre otras (71–75). Estas secuelas clínicas podrían estar asociadas a estas secuelas inmunológicas.

Por lo tanto, además de estudiar cómo se comportaba el sistema inmunitario durante la infección aguda, decidimos estudiar el papel de la respuesta inmunitaria innata y adaptativa a largo plazo después de la infección y analizar si los defectos presentes durante la infección aguda se normalizaban, analizando de manera detallada distintas características de las CD y la calidad de la respuesta SARS-CoV-2-específica por parte de las células T. Estas cuestiones se abordaron en **Objetivo 1b** del Artículo 1 y al **Objetivo 2b** del Artículo 2 de la presente tesis doctoral, respectivamente.

4. Respuesta vacunal e inmunosenescencia en infección por SARS-CoV-2

Las vacunas son consideradas las herramientas más eficientes para prevenir la progresión de la COVID-19 y frenar la pandemia. Varias vacunas están disponibles a nivel mundial. Las vacunas COVID-19 se han desarrollado y se están desarrollando utilizando varias plataformas diferentes con distintos grados de eficacia e inmunogenicidad (76). Algunas de estas estrategias consisten en la utilización de virus inactivados o virus vivos atenuados, subunidades de proteínas, o innovadoras técnicas como el uso de ADN o ARN mensajero (ARNm).

La vacuna de ARNm BNT162b2, comúnmente conocida como vacuna de Biontech/Pfizer, demostró una gran seguridad y eficacia para prevenir la evolución grave de la COVID-19 (77). Se administra por vía intramuscular (o intradérmica) con dos dosis de 30 µg administradas con tres a ocho semanas de diferencia. Actúa estimulando una respuesta inmunológica contra la proteína S del virus, y se ha demostrado que previene las hospitalizaciones y el curso grave de la enfermedad. La vacunación con dos dosis contra el SARS-CoV-2 induce una fuerte respuesta humoral medida por anticuerpos frente a la proteína S del coronavirus con capacidad neutralizante (78,79). Además, se observaron notables respuestas de células T CD4+ y CD8+ específicas de SARS-CoV-2 tras la vacunación con BNT162b2 (80–82).

A menudo, se observa que la respuesta a las vacunas es menor en los adultos de mayor edad o ancianos (83–85). Nosotros especulamos que con la vacuna frente a la COVID-19 debía ocurrir lo mismo. Se han identificado varios factores como predictores de la gravedad o progresión de las infecciones en general, como la edad y la presencia de distintas comorbilidades (86–90). Esta disfunción inmunitaria relacionada con la edad se define en general como inmunosenescencia, que depende de múltiples factores y varía mucho entre individuos, e incluye (i) la disminución de la capacidad de respuesta a nuevos antígenos, (ii) la disminución de la capacidad de respuesta de las células T de memoria y (iii) una inflamación crónica de bajo grado persistente. Otro desencadenante de la inmunosenescencia es la involución del timo, que provoca la disminución gradual del aporte de nuevos linfocitos T a la sangre periférica por parte de este órgano (91–96). Este envejecimiento inmunitario o inmunosenescencia se sustenta en una remodelación multifacética de la inmunidad innata y adaptativa que se ha asociado a baja respuesta vacunal (97).

La reducción del éxito vacunal en los adultos de mayor edad es especialmente conocida en el caso de las vacunas contra la hepatitis B, el neumococo y la gripe (86,98). En el caso de las vacunas BNT162b2, se notificó, durante el desarrollo de esta tesis,

una menor eficacia en términos de síntomas de COVID-19, ingresos hospitalarios y fallecimientos tras la vacunación en personas de edad avanzada (99–101). Además, otros estudios han descrito niveles más bajos de anticuerpos neutralizantes en ancianos vacunados en comparación con participantes más jóvenes, especialmente seis meses después de la segunda dosis (83,102–105). De igual modo, se observaron niveles bajos de respuesta de células T SARS-CoV-2-específicas tras la vacunación en ancianos (83). Sin embargo, no se habían caracterizado los factores inmunitarios adaptativos e innatos asociados a la menor respuesta vacunal en ancianos.

De este modo, se planteó el **Objetivo 3** del Artículo 3 de la presente tesis doctoral, donde se quiso estudiar las principales alteraciones inmunitarias, tanto en términos de inmunidad innata como adaptativa específica del SARS-CoV-2, asociadas a una menor respuesta a la vacuna BNT162b2 de ARNm en personas de edad avanzada.

HIPÓTESIS Y OBJETIVOS

Hipótesis 1. Al no existir tratamientos antivirales efectivos y haber persistencia viral se producen déficits en el número y función de células dendríticas (CD) por migración continua a foco inflamatorio. Estos defectos serán mayores a mayor gravedad de la COVID-19. Una vez resuelta la infección estos déficits inmunológicos se recuperarán, al menos en sujetos que cursaron la COVID-19 de manera leve.

Objetivo 1a. Investigar los defectos en CD asociados a la infección por SARS-CoV-2 y a la gravedad de la COVID-19.

Objetivo 1b. Estudiar si estos defectos en las CD perduran siete meses tras la infección.

Estos objetivos se abordaron en el trabajo: **Pérez-Gómez A et al. Dendritic cell deficiencies persist seven months after SARS-CoV-2 infection. *Cell Mol Immunol.* 2021 Sep;18(9):2128-2139. doi: 10.1038/s41423-021-00728-2. Epub 2021 Jul 21. PMID: 34290398; PMCID: PMC8294321.**

Hipótesis 2. La calidad de la respuesta SARS-CoV-2 específica mediada por linfocitos T está asociada a la gravedad de la COVID-19 en infección aguda y perdura siete meses tras la infección y se relaciona con la respuesta a coronavirus endémicos (HCoV).

Objetivo 2a. Evaluar la calidad de la respuesta T SARS-CoV-2 específica asociada a la progresión de la enfermedad en la infección aguda.

Objetivo 2b. Estudiar si esta respuesta T perdura en el tiempo y se asocia al curso de la infección durante la etapa aguda.

Objetivo 2c. Medir la asociación entre la respuesta específica frente a SARS-CoV-2 y HCoV.

Estos objetivos se abordaron en la publicación: **Pérez-Gómez A et al. Deciphering the quality of SARS-CoV-2 specific T-cell response associated with disease severity, immune memory and heterologous response. *Clin Transl Med.* 2022 Apr;12(4):e802. doi: 10.1002/ctm2.802. PMID: 35415890; PMCID: PMC9005926.**

Hipótesis 3. La respuesta frente a la vacuna BNT162b2 (Pfizer) frente a la COVID-19 es inferior en los ancianos debido a alteraciones inmunitarias asociadas a la edad, tanto en lo que respecta a la inmunidad innata como a la adaptativa específica del SARS-CoV-2.

Objetivo 3a. Estudiar la magnitud y calidad de la respuesta específica de SARS-CoV-2 en sujetos mayores de 60 años durante y dos meses después de la vacunación con BNT162b2.

Objetivo 3b. Determinar si alteraciones centrales (función tímica) y periféricos en las subpoblaciones de células T en personas mayores de 60 años se asociaban a la respuesta vacunal frente al SARS-CoV-2.

Objetivo 3c. Analizar si modificaciones a nivel fenotípico y funcional de células innatas, incluyendo células dendríticas y monocitos, en personas mayores de 60 años se asociaban a la respuesta vacunal frente al SARS-CoV-2.

Este objetivo se abordó en el trabajo: **Vitallé J, Pérez-Gómez A et al. Immune defects associated with lower SARS-CoV-2 BNT162b2 mRNA vaccine response in aged people. *JCI Insight.* 2022 Sep 8;7(17):e161045. doi: 10.1172/jci.insight.161045. PMID: 35943812; PMCID: PMC9536264.**

MATERIAL, MÉTODOS Y RESULTADOS

Dendritic cell deficiencies persist seven months after SARS-CoV-2 infection

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ARTICLE

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Dendritic cell deficiencies persist seven months after SARS-CoV-2 infection

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Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)-2 infection induces an exacerbated inflammation driven by innate immunity components. Dendritic cells (DCs) play a key role in the defense against viral infections, for instance plasmacytoid DCs (pDCs), have the capacity to produce vast amounts of interferon-alpha (IFN-α). In COVID-19 there is a deficit in DC numbers and IFN-α production, which has been associated with disease severity. In this work, we described that in addition to the DC deficiency, several DC activation and homing markers were altered in acute COVID-19 patients, which were associated with multiple inflammatory markers. Remarkably, previously hospitalized and nonhospitalized patients remained with decreased numbers of CD1c+ myeloid DCs and pDCs seven months after SARS-CoV-2 infection. Moreover, the expression of DC markers such as CD86 and CD4 were only restored in previously nonhospitalized patients, while no restoration of integrin β7 and indoleamine 2,3-dyoxigenase (IDO) levels were observed. These findings contribute to a better understanding of the immunological sequelae of COVID-19.

Keywords: SARS-CoV-2; COVID-19; Dendritic cell; Long-COVID

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INTRODUCTION

Coronavirus disease 19 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and may progress with mild symptoms or asymptomatically in most of the individuals, while others experience an acute respiratory distress syndrome (ARDS) and poorer prognosis, including death [1]. Disease severity depends on the balance between host immune response, viral replication and tissue and organ damage. In severe COVID-19 there is a deregulation of this response, characterized by an hyperinflammation driven by innate immunity, characterized by very high levels of cytokines and pro-inflammatory biomarkers, also known as cytokine storm [2, 3].

One of the innate immune cell types that may play a pivotal role in the response against SARS-CoV-2 are the dendritic cells (DCs). There are two main DC types, conventional or myeloid DCs (mDCs) which include CD1c+, CD16+, and CD141+ mDC subsets, and plasmacytoid dendritic cells (pDCs). In general, DCs participate in antigen presentation, cytokine production, control of inflammatory responses, tolerance induction, immune cell recruitment, and viral dissemination. However, the role of these cells in response to acute SARS-CoV-2 infection and the recovery in convalescent subjects is not fully characterized. Some studies have shown a decrease of DC numbers in response to infection in peripheral blood [4] and also an association with disease severity [5]. This deficiency seems to be due to the migration of some DC subsets, such as CD1c, to the lung [6], and probably to other inflammatory foci. pDCs also seems to play a key role in COVID-19 [7]. pDCs are the main type I interferon (IFN-I) producers, with 1000-fold production compared to other immune cell types [8]. IFN-I is known to have an essential role in viral infections [9]. Significantly, pDCs depletion has been associated with poor COVID-19 prognosis [10]. Moreover, critical patients showed a highly impaired IFN-I response [7] associated with high viral load and aggravated inflammatory response [11].

The recovery of DC defects after COVID-19 could be crucial, since the normalization of the innate immune system after the acute insult would mean the system's readiness to respond to new viral and bacterial challenges. However, the recovery of DC cell numbers and function after COVID-19 is unknown. This recovery is

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also important in the sense that a variable proportion of people who have overcome COVID-19 show clinical sequelae [12] which relation with innate immune defects needs to be clarified. Thus, the aim of the study was to analyze DC defects associated with SARS-CoV-2 infection, COVID-19 severity and whether these defects were restored after a median of 7 months after the resolution of the infection.

RESULTS

Patients with acute SARS-CoV-2 infection show a considerable decrease in DC percentages and TLR9-dependent IFN-α production

In order to investigate the effect of SARS-CoV-2 infection on the innate immune system, we first analyzed the percentages of total DCs and the different subsets in acute SARS-CoV-2 infected patients (COVID-19 patients) compared with age and sex matched healthy donors (HD). Specifically, we measured mDCs (CD123− CD11c+), including CD1c+, CD16+, and CD141+ mDC subsets, and pDCs (CD123+ CD11c−) (Supplementary Fig. 1a). Our results showed that acute COVID-19 patients exhibited a significant decrease in the percentages of total mDCs mainly due to CD1c+ mDCs decreased in comparison with HD. Meanwhile CD16+ and CD141+ mDCs remained at similar levels of HD (Fig. 1A). Remarkably, the percentage of pDCs in acute COVID-19 patients was considerably diminished with respect to HD (Fig. 1B left). Then, we calculated the ratio mDC/pDC in the different subjects, which was much lower in HD that in COVID-19 patients (Supplementary Fig. 2a). Additionally, based on previously published results [13], the following pDC subsets were analyzed: P1-pDC (CD86−PD-L1+), P2-pDC (CD86+PD-L1+) and P3-pDC (CD86+PD-L1−). Here, a lower percentage of P2- and P3-pDCs was observed in acute COVID-19 patients than in HD (Supplementary Fig. 2b). pDCs are known to be the main producers of IFN-α [8]. Therefore, to study their function in SARS-CoV-2 infection, we stimulated peripheral blood mononuclear cells (PBMCs) with CpG oligodeoxynucleotides class A (CpG)-A, a Toll-like receptor (TLR)-9 dependent stimulation, and we analyzed IFN-α production. We found that IFN-α production in acute COVID-19 was much lower than in HD (Fig. 1B right). To clarify if the decreased IFN-α production was due to a diminished percentage of pDCs, we performed a correlation analysis and we found that the IFN-α production was positively associated with the percentage of pDCs in both acute COVID-19 patients and HD (Fig. 1C). In conclusion, patients with acute SARS-CoV-2 infection exhibit a deficit in DC numbers and also decreased TLR9-dependent IFN-α production.

Acute SARS-CoV-2 infected patients show an altered pattern of DC activation markers

Afterwards, we analyzed the expression of DC activation markers in acute COVID-19 patients and HD. We measured the expression of homing receptors ((integrin-β7 (β7) and C-C chemokine receptor type 7 (CCR7)), co-stimulatory molecules (CD86 and CD4), and markers of immune tolerance and suppression ((Indoleamine 2,3-dioxygenase (IDO) and Programmed Death-ligand 1 (PD-L1), respectively) (Supplementary Fig. 1b). Most of the DC subpopulations, presented lower percentage of β7, specially total mDCs, CD1c+ mDCs, and pDCs and a higher percentage of CCR7+ DCs in acute COVID-19 patients compared with HD (Table 1). We also found lower percentage of CD86+ cells in acute patients in CD1c+ and CD16+ mDCs and pDCs. No differences were found in CD4+ DC levels (Table 1). Lastly, acute COVID-19 patients showed higher percentage of IDO+ cells within CD1c+ and CD16+ mDCs compared with HD, while a lower percentage PD-L1+ was seen within pDCs (Table 1). These results are indicative of alterations in different homing and activation patterns of DCs in response to SARS-CoV-2 infection.

IFN-α production is associated with COVID-19 severity

The next step of this study was to investigate whether DC numbers and their function might be different in acute COVID-19 depending on disease severity. Therefore, we classified acute COVID-19 patients in two groups: severe ((high oxygen support requirement and Intensive Care Unit (ICU) admission or death)) and mild (low oxygen requirement and no ICU admission) (Supplementary Table 1). Our results did not show any significant difference in the percentage of mDCs and subpopulations and pDCs between severe and mild COVID-19 patients (Fig. 2A). However, we found increased levels of total CCR7+ mDCs and PD-L1+ CD141+ mDCs in severe patients (Supplementary Fig. 3). Importantly, we did find a considerable decrease in TLR9-dependent IFN-α production in severe subjects compared to mild patients (Fig. 2B). In summary, acute SARS-CoV-2 infected patients with severe symptoms exhibit a lower capacity to produce IFN-α than patients with mild symptoms.

DC parameters are differentially associated to inflammation markers in mild and severe acute SARS-CoV-2 infected patients

DC numbers and activation markers were correlated to multiple inflammatory marker levels, including clinical biomarkers ((C-reactive protein (CRP), D-dimers and lactate dehydrogenase (LDH)), pro-inflammatory cytokines ((tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL1-β, macrophage inflammatory protein (MIP1)-α, MIP1-β, interferon inducible protein (IP)-10 and interferon (IFN)-γ) and soluble (sCD25)), and neutrophil numbers. These correlations were done in the overall group of patients during acute infection and also dividing in both severe and mild COVID-19. In the overall population, we observed correlations of dendritic cell subset levels with different pro-inflammatory cytokines and clinical biomarker levels (Supplementary Fig. 4). Interestingly, we observed a different correlation pattern in severe and mild patients and of note, more associations were found in mild patients (Fig. 3). On one hand, regarding COVID-19 patients with mild symptoms, the percentages of DC subpopulations were inversely correlated with D-dimers, IL-6, IL-8, sCD25 levels and neutrophil numbers, while they were positively correlated with TNF-α, IL-1β, MIP-1α, MIP1-β, and IFN-γ levels, with the exception of CD16+ mDCs that were negatively correlated with most of the inflammatory parameters. It is remarkable, that the percentage of pDCs showed a strong inverse correlation with D-dimer levels and neutrophil numbers. Focusing on DC homing and activation markers, regarding the expression of β7 in DCs, inverse associations prevailed, highlighting the strong correlations found in CD16 + β7+ mDCs with D-dimers and in β7+ pDCs with IL1-β. In contrast, the expression of CD86 and IDO in DCs was predominantly positively associated to several inflammatory markers, mainly in the case of CD141+ mDCs and pDCs (Fig. 3A). On the other hand, in severe COVID-19, many associations were lost (e.g., IDO expression) and others were opposite (e.g., CD86), comparing with mild patients. For instance, remarkably, the DC percentages and the expression of β7 and CD86, the associations found with inflammatory marker levels showed an opposite trend (Fig. 3B). Therefore, we conclude that DC levels and activation markers are associated to the inflammatory status of acute SARS-CoV-2 infected subjects, with a differential profile between patients with severe symptoms compared to those with mild symptoms.

CD1c+ mDC and pDC levels and IFNα production are not normalized 7 months after SARS-CoV-2 infection

Apart from COVID-19 patients in acute phase, we also studied patients after 7 months of SARS-CoV-2 infection median 208 (interquartile range [IQR] [189–230]) days after symptoms' onset, (Supplementary Table 1). Some of these patients were hospitalized during acute infection (Hosp 7M), while others were not (No Hosp 7M). We analyzed the percentages of DC subpopulations in these

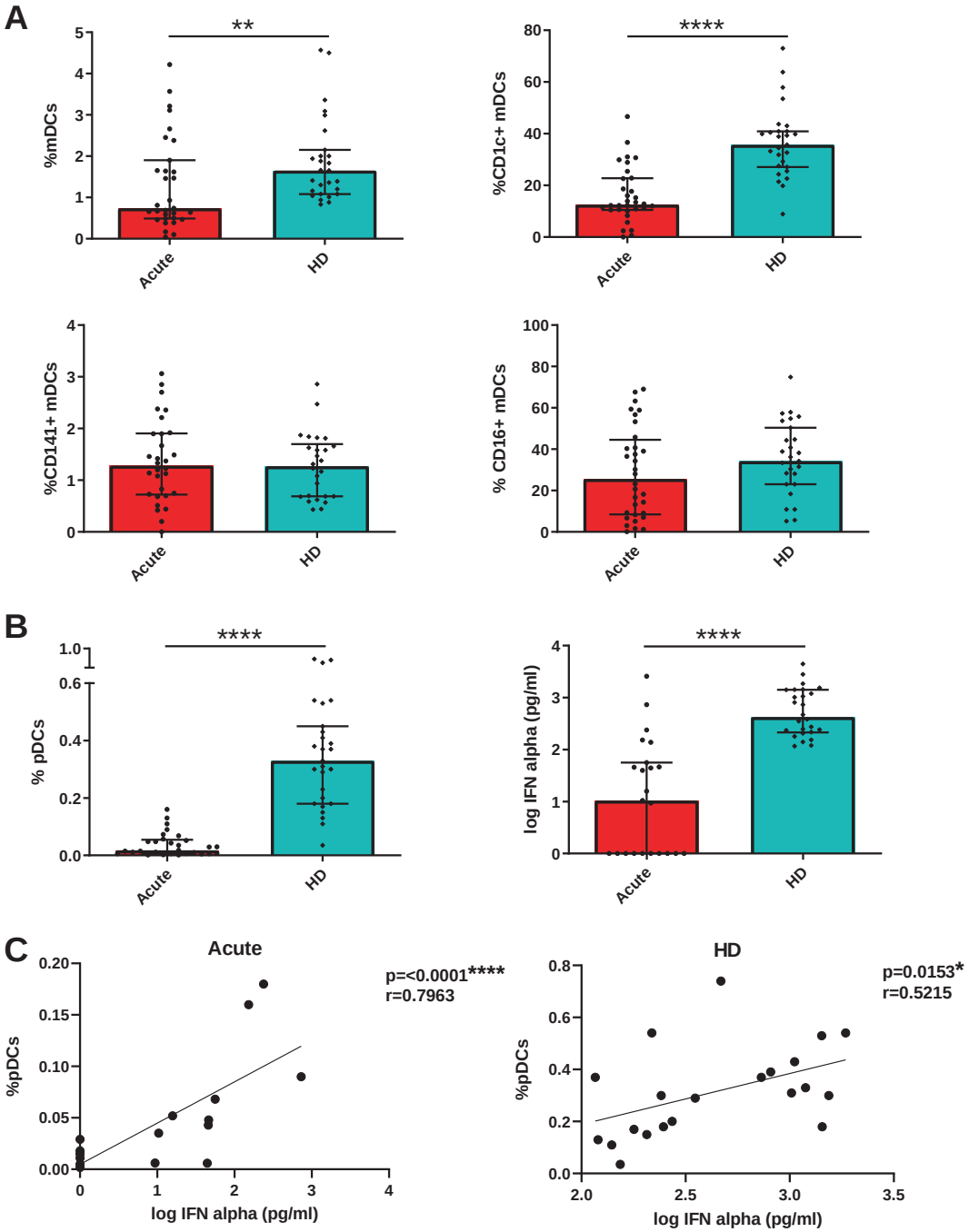

Fig. 1 Patients with acute SARS-CoV-2 infection show a considerable decrease in DC percentages and TLR9-dependent IFN-α production. Bar graphs representing the percentage of total mDCs, CD1c+, CD141+, and CD16+ mDCs (**A**) and the percentage of pDCs and IFN-α production in response to CpG-A in acute SARS-CoV-2 infected patients (acute) and healthy donors (HD) (**B**). The median with the interquartile range is shown. Correlation between the percentage of pDCs and IFN-α production in acute patients and HD. Each dot represents an individual (**C**). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Mann–Whitney *U* test was used for groups' comparisons and Spearman test for nonparametric correlations

two groups and compared with HD's levels. First, we observed a higher percentage of total mDCs on previously hospitalized patients compared with HD (Fig. 4A). Regarding mDC subpopulations, while the percentages of CD141+ and CD16+ were not altered, the percentage of CD1c+ mDCs remained lower in patients after 7 months compared with HD (Fig. 4B–D). Remarkably, the percentage of pDCs also persisted very low and was not restored 7 months after the infection in these both groups (Fig. 4E), confirmed by the mDC/pDC ratio (Supplementary Fig. 5a). Moreover, the percentage of P1-pDCs (CD86− PD-L1+) was only reduced in previously hospitalized patients comparing with HD, unlike P2− (CD86+PD-L1+) and P3-pDCs (CD86+PD-L1−), that were decreased in both hospitalized and nonhospitalized ones (Supplementary Fig. 5b). Next, to corroborate that our results were reproducible

Table 1. Acute SARS-CoV-2 infected patients show an altered pattern of DC homing and activation markers

Activation markers	Dendritic cells	Acute	HD	*p*
Beta7	**mDCs**	**5.7 [3.2–11.1]**	**22.1 [15.7–33.8]**	**<0.0001**
	CD1c+ mDCs	**43.8 [26.2–62.2]**	**62.0 [38.8–69.7]**	**0.0340**
	CD16+ mDCs	0.0 [0.0–0.0]	0.03 [0.00–0.0]	0.0851
	CD141+ mDCs	15.6 [9.9–28.2]	25.9 [18.8–33.3]	0.0547
	pDCs	**2.2 [0.5–3.3]**	**6.1 [3.2–10.4]**	**0.0004**
CCR7	**mDCs**	**3.1 [1.4–21.7]**	**0.9 [0.3–1.5]**	**<0.0001**
	CD1c+ mDCs	**18.2 [6.4–94.3]**	**4.5 [2.4–8.1]**	**<0.0001**
	CD16+ mDCs	**3.1 [0.3–14.4]**	**0.3 [0.1–0.5]**	**0.0015**
	CD141+ mDCs	**11.0 [3.1–18.5]**	**1.9 [0.6–4.6]**	**0.0005**
	pDCs	**0.9 [0.2–15.6]**	**0.0 [0.0–0.0]**	**<0.0001**
CD86	mDCs	64.9 [37.9–77.4]	57.7 [50.4–65.2]	0.4447
	CD1c+ mDCs	**5.5 [2.8–11.2]**	**12.8 [6.7–17.4]**	**0.0053**
	CD16+ mDCs	**95.8 [88.5–97.5]**	**98.1 [96.7–98.7]**	**0.0034**
	CD141+ mDCs	7.6 [0.0–22.3]	5.9 [2.6–13.6]	0.9075
	pDCs	**0.3 [0.0–0.9]**	**1.4 [0.4–2.2]**	**0.0024**
CD4	mDCs	8.8 [2.5–24.1]	5.6 [4.0–10.6]	0.3229
	CD1c+ mDCs	27.0 [7.7–61.5]	23.0 [15.7–51.1]	0.6457
	CD16+ mDCs	8.5 [2.2–16.5]	10.7 [6.6–14.6]	0.3194
	CD141+ mDCs	21.3 [10.6–51.1]	40.0 [21.1–47.7]	0.1109
	pDCs	57.3 [46.6–77.9]	70.5 [59.0–87.9]	0.0750
IDO	mDCs	1.3 [0.8–3.1]	2.0 [1.5–2.8]	0.1469
	CD1c+ mDCs	**9.1 [2.4–23.2]**	**2.2 [1.6–4.5]**	**0.0039**
	CD16+ mDCs	**6.8 [0.1–27.2]**	**0.4 [0.0–0.7]**	**0.0004**
	CD141+ mDCs	72.1 [56.9–85.7]	69.2 [59.4–79.5]	0.6460
	pDCs	0.0 [0.0–0.1]	0.1 [0.0–0.3]	0.2071
PD-L1	mDCs	17.4 [5.1–34.4]	21.8 [10.1–41.3]	0.2531
	CD1c+ mDCs	0.9 [0.4–5.2]	0.6 [0.2–1.7]	0.1810
	CD16+ mDCs	28.6 [13.8–47.9]	21.0 [6.1–41.0]	0.2107
	CD141+ mDCs	0.0 [0.0–2.0]	0.9 [0.0–2.7]	0.2166
	pDCs	**0.8 [0.2–2.1]**	**5.6 [3.1–8.7]**	**<0.0001**

Percentages of dendritic cells positive for activation markers in acute SARS-CoV-2 infected patients (Acute) and healthy donors (HD) are presented. The median with interquartile ranges [IQR] is shown. Significant differences are indicated in bold

applying a paired analysis, we studied DC kinetic in a subgroup of subjects with available paired samples, analyzing the percentages of DC subpopulations in the acute phase, 6–8 months later and comparing them with HD. Even though the sample size was lower because of the sample availability, these results reproduced the analysis with unpaired samples (Supplementary Fig. 6).

When we measured the TLR9-dependent IFN-α production, we found that hospitalized patients 7 months after the infection showed a lower IFNα production than HD, unlike nonhospitalized patients, which display a similar production comparing with HD (Fig. 4F). Here, we conclude that the deficit of CD1c+ mDCs and pDCs is maintained 7 months after SARS-CoV-2 infection independently of whether the patients were or not previously hospitalized, and that IFNα production is not restored in previously hospitalized patients 7 months after infection.

Some DC activation markers are not normalized in previously hospitalized patients 7 months after SARS-CoV-2 infection

Afterwards, we measured the DC activation and homing markers in previously hospitalized and nonhospitalized patients 7 months after infection, and we compared them with the ones from HD. We observed that the expression of CD86 was lower in CD16+ and CD1c+ mDC subsets from hospitalized patients than in non-hospitalized ones and HD (Fig. 5A, B). Similar results were found in the expression of PD-L1 in total mDCs (Fig. 5C). Furthermore, hospitalized patients also showed lower levels of CD4 in total mDCs, CD1c+, and CD141+ mDCs and pDCs (Fig. 5D–G). In contrast, pDCs from hospitalized patients exhibited higher percentage of CCR7+ cells within pDCs compared with non-hospitalized ones and HD (Fig. 5H). In summary, these results show a recovery of some DC activation markers, mainly CD86 and CD4, only in previously nonhospitalized patients, while in more severe patients who required hospitalization, the defects in these markers persisted 7 months after infection.

Some DC activation markers are not normalized neither in previously hospitalized nor in nonhospitalized patients 7 months after SARS-CoV-2 infection

Importantly, when we focused on the expression of other DC activation markers, we observed a lower percentage of β7+ cells in all mDCs and pDCs from both hospitalized and nonhospitalized patients after 7 months of infection compared to HD (Fig. 6A–E). The levels were also lower for IDO+ in total mDCs, CD1c+, and CD141+ mDCs and pDCs (Fig. 6F–I). Lastly, we also found that

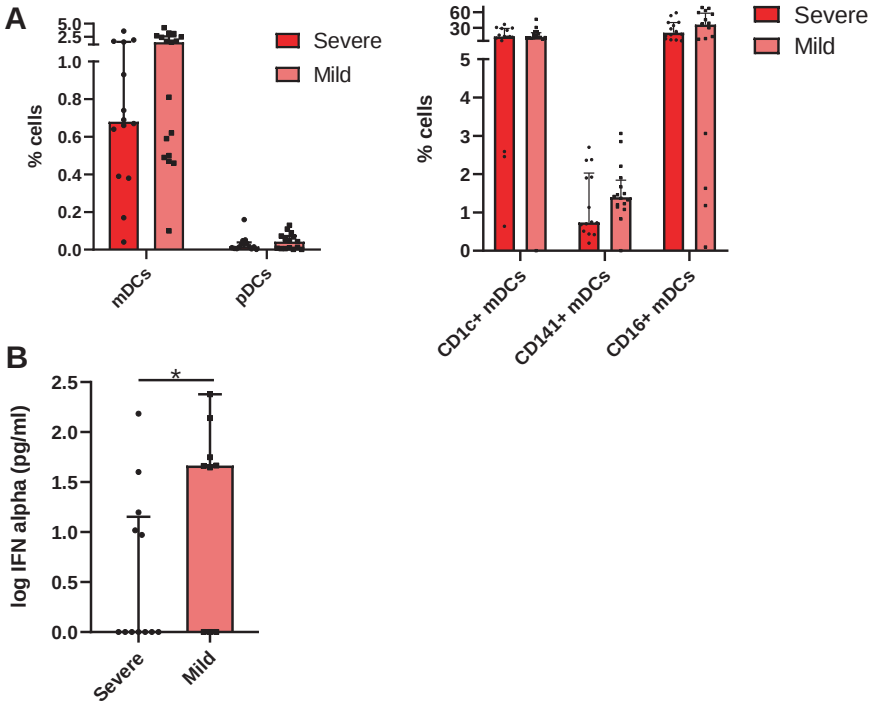

Fig. 2 IFN-α production is associated with COVID-19 severity. Bar graphs representing the percentage of pDCs and total mDCs, CD1c+, CD141+ and CD16+ mDCs subsets (**A**) and the percentage of IFN-α production in response to CpG-A (**B**) in acute severe and mild SARS-CoV-2 infected patients. The median with the interquartile range is shown and each dot represents an individual. *$p < 0.05$. Mann–Whitney *U* test was used for groups' comparisons

both hospitalized and nonhospitalized patients 7 months after infection showed lower percentages of CCR7+ and CD4+ cells within CD16+ mDCs and PD-L1+ cells within pDCs compared to HD (Fig. 6J–L). In conclusion, we demonstrated that the alterations in integrin β7 and IDO, associated with migration and tolerance, are not restored to normal levels neither in previously hospitalized nor in nonhospitalized patients 7 months after SARS-CoV-2 infection.

DISCUSION

The present study revealed that the deficits observed in CD1c+ mDCs and pDCs levels associated with altered homing and activation patterns in SARS-CoV-2 infected subjects in acute phase, were not restored beyond 7 months after infection. Importantly, this long-term defects related to DC migration and tolerogenesis (integrin β7 and IDO expression) were present independently of whether or not the patients were previously hospitalized. In addition, hospitalized patients showed additional deficiencies related with DC activation.

pDCs are known to have an important role in the first line of defense against viral replication, which mainly resides in their capacity to produce IFN-I via TLR-7/8 stimulation [14]. In this study, we first observed that acute SARS-CoV-2 infected patients displayed a dramatic decrease in pDC levels and a considerable reduction of IFN-α production. The strong direct correlation between pDC levels and IFN-α production suggested that this cell type was the main producer of this cytokine as it happens in other viral infections [15]. In fact, SARS-CoV-2 is known to induce pDC activation, accompanied by a high production of IFN-I and other cytokines, which is critically depended on IRAK4 and UNC93B [16]. The observed reduction of IFN-α is in accordance with previous studies in animal models of SARS-CoV-1 infection, which associated this deficit with lethal pneumonia [17] and is also consistent with recently published data following transcriptomic approaches [11] and intracellular cytokine staining after TLR stimulation in SARS-CoV-2 infection [7]. Importantly, the low IFN-α production was the main parameter associated with disease severity, in agreement with previous studies [7, 11], highlighting the potential use of this measurement as an early biomarker of disease progression. The mechanisms behind the attenuated IFN response have been related with viral antagonism of STAT1 (Signal transducer and activator of transcription 1) phosphorylation [18] and significantly, life-threatening ARDS in COVID-19 patients have been associated with neutralizing auto-antibodies against IFN-I [19, 20] and other inborn errors of IFN-I immunity [21]. Furthermore, single cell RNA sequencing of antigen-presenting cells revealed a lower expression of IFNAR1 and 2 in severe COVID-19 patients, suggesting a defect in IFN-α signaling, and also a downregulation of IFN-stimulated genes in both moderate and severe patients [22]. All these results support the essential role of IFN-I production in the first line of defense in COVID-19 for avoiding disease progression and point out to early immunotherapeutic strategies targeting this pathway. Remarkably, our results showed that, 7 months after SARS-CoV-2 infection, the IFNα production is not completely restored to normal levels, but only in previously hospitalized patients. This might be associated to the deficit in P1-pDCs found in hospitalized patients but not in nonhospitalized ones, being this pDC subset the main source of IFN-I [13]. Thus, our findings are indicative of a deficiency not only in pDC numbers but also in their function 7 months after SARS-CoV-2 infection in patients that were previously hospitalized.

Apart for IFN-I deficiency, one of the hallmarks of acute COVID-19 is the detection in plasma of heightened levels of soluble pro-inflammatory cytokines inducing a cytokine storm [23]. Here, we found multiple correlations between DC numbers and DC activation markers with inflammatory marker and cytokine levels in acute SARS-CoV-2 infected patients. It was remarkable that the lower percentage of DCs was associated to higher levels of IL-6 and higher neutrophil numbers. High levels of IL-6 in COVID-19 patients have been widely related to a poorer disease progression [10]. Moreover, neutrophils have been described as crucial drivers

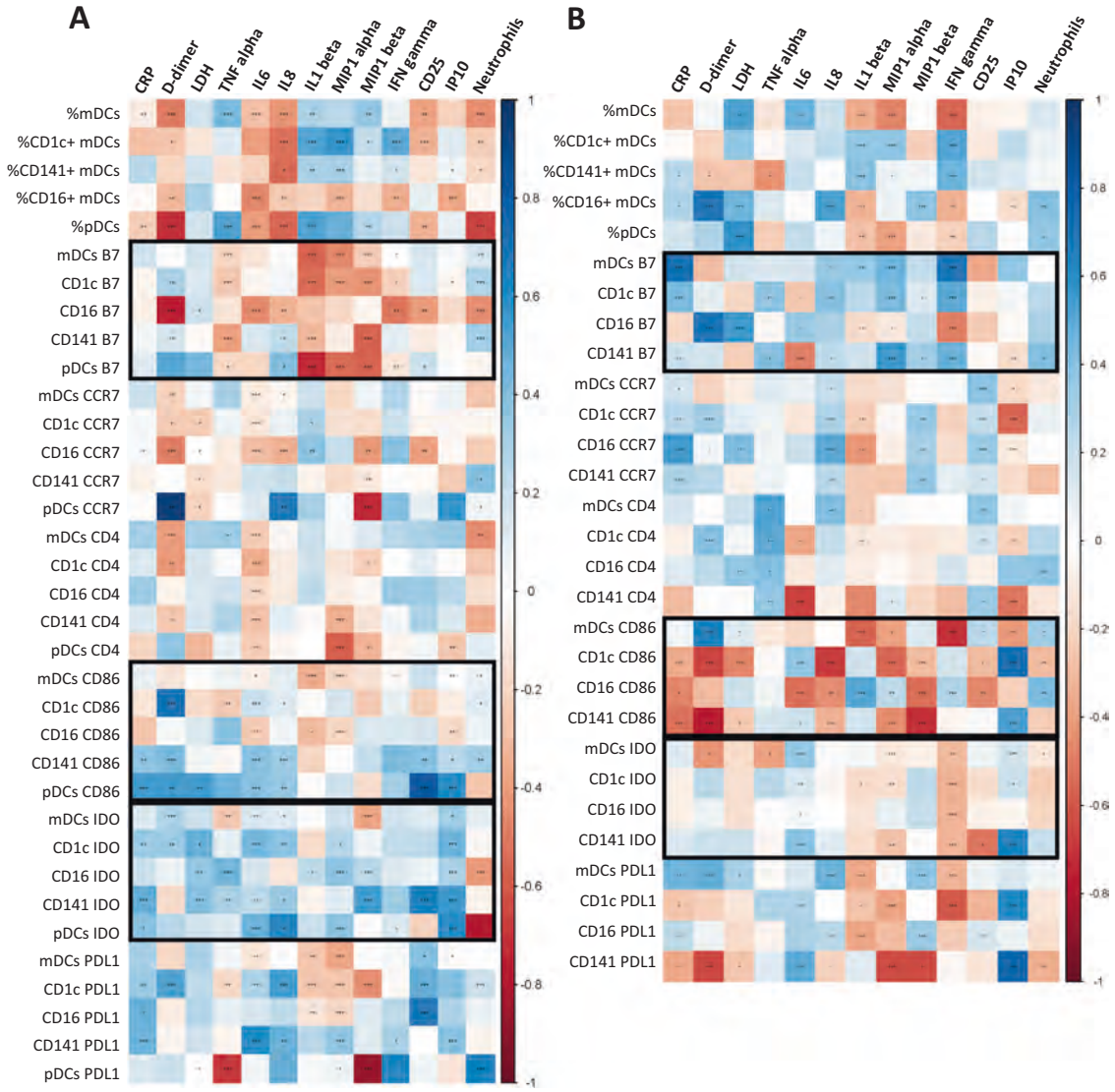

Fig. 3 DC parameters are differentially associated to inflammatory markers in mild and severe acute SARS-CoV-2 infected patients. Heatmap graphs representing correlations between the percentages of DC subpopulations and the percentages of DCs expressing activation and homing markers with inflammatory marker levels including CRP, D-dimer, LDH, TNF-α, IL-6, IL-8, IL1-β, MIP1-α, MIP1-β, IFN-γ, CD25, IP-10, and neutrophil numbers, in mild (**A**) and severe (**B**) SARS-CoV-2 infected patients. Blue color represents positive correlations and red color shows negative correlations. The intensity of the color indicates the R coefficient. The most relevant data are highlighted with black squares. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Spearman test was used for nonparametric correlations

of hyperinflammation in COVID-19 [24]. It has to be also underlined, that the percentage of DCs expressing integrin β7 was inversely correlated to numerous inflammatory marker levels. These results suggest the hypothesis that not DC per se but DC migration to inflammatory sites may importantly contribute to the cytokine storm observed in SARS-CoV-2 infected patients. Our results also showed that patients with distinct level of disease severity displayed different associations of DC numbers and DC activation markers with inflammation. Therefore, DCs might be important contributors to the high inflammatory status characteristic of COVID-19 patients and this may dictate subsequent clinical progression.

The decreased numbers of total mDCs, CD1c+ mDCs and pDCs found in acute SARS-CoV-2 infected patients were in accordance with previous publications [5, 6]. This fact might be explained by different mechanisms, including apoptosis due to increased inflammatory mediators produced by abortive SARS-CoV-2 infection of myeloid cells [25]. Another nonexclusive explanation could be that DCs migrate from peripheral blood to tissues or inflammatory sites, such as CD1c+ mDCs preferential migration to the lungs in patients with severe COVID-19 [6]. These defects were accompanied by alterations mainly found in activation, migration and tolerogenic markers that importantly persisted 7 months after infection in previously hospitalized and also in nonhospitalized patients. Especially persistent in the total and DC subsets was the decreased expression of integrin β7. The expression of αEβ7 defines migration to antigen presentation sites within lymph nodes [26] and α4β7 on mDCs and pDCs is indicative of migration of these cells to gut [27]. Remarkably, SARS-CoV-2 has been shown to infect and productively replicate in human small intestinal organoids, increasing cytokine production and human angiotensin-converting enzyme 2 expression [28]. It has been also reported, that the disruption in gut barrier integrity contributes to COVID-19 severity [29]. Thus, the lower percentage of DCs expressing integrin β7 in peripheral blood might be a consequence of ongoing DC migration to the gut or other tissues or inflammatory sites up to 7 months after infection. In fact, necropsy studies in SARS-CoV-2 infected patients have shown mononuclear inflammatory infiltrates in different organs [30]. Also

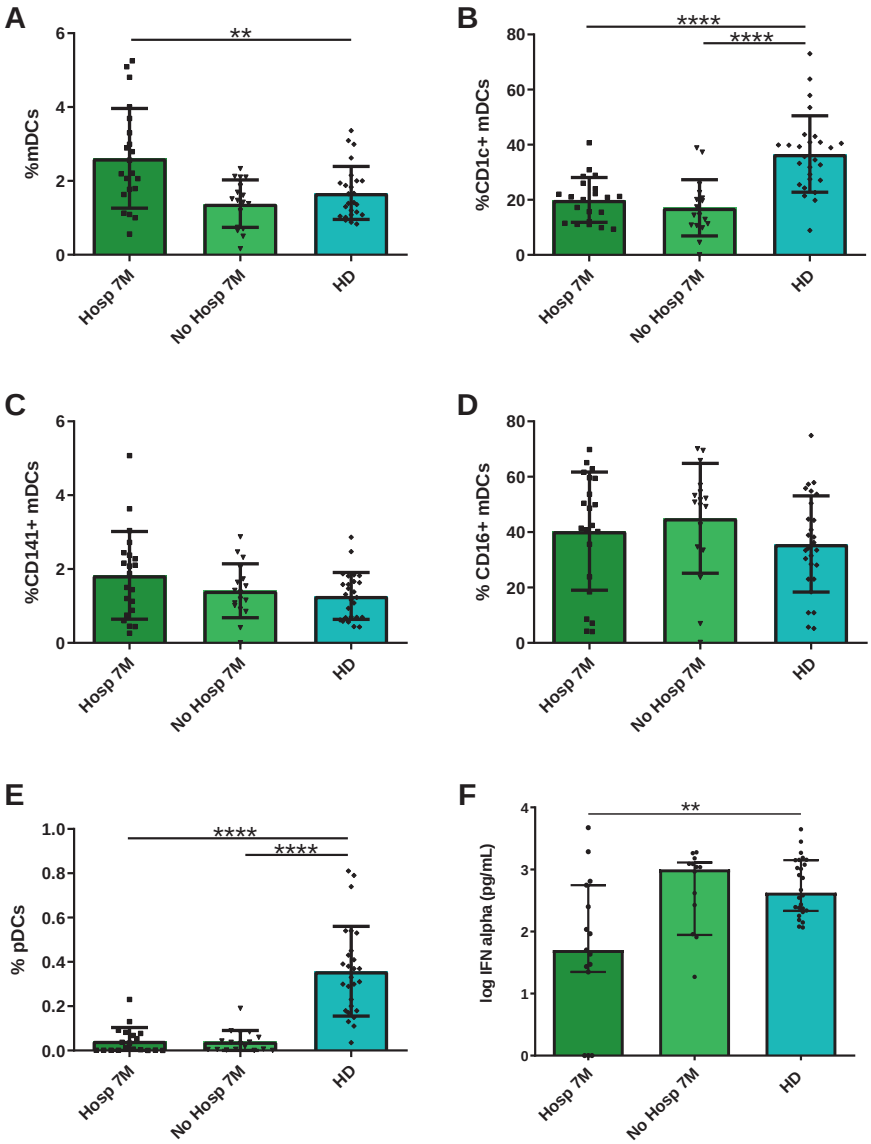

Fig. 4 CD1c + mDC and pDC levels and IFNα production are not normalized 7 months after SARS-CoV-2 infection. Bar graphs representing the percentage of total mDCs, CD1c+, CD141+, and CD16+ mDCs, pDCs (**A–E**) and the IFNα production (**F**) in previously hospitalized (Hosp 7M) or previously nonhospitalized (No Hosp 7M) patients 7 months after SARS-CoV-2 infection and in healthy donors (HD). The median with the interquartile range is shown and each dot represents an individual. $^{**}p < 0.01$; $^{****}p < 0.0001$. Mann–Whitney *U* test was used for groups' comparisons

prominent was the deficit in IDO expressing DCs 7 months after infection. In contrast, IDO+ CD1c+ and CD16+ mDC levels in acute infection were dramatically increased compared to HD. This is in agreement with other acute respiratory infections such influenza [31] and respiratory syncytial virus [32] in which IDO expression is increased in order to counteract excessive inflammation as happen after acute SARS-CoV-2 infection. However, in this infection, the tissue damage in low respiratory tract is prominent [33] and may persist at the long-term what may cause the exhaustion of IDO producing DCs and/or migration of these cells to inflammatory focus even after 7 months after infection. Although these defects were present independently of whether or not the participants were previously at the hospital, hospitalized patients showed additional defects. These were, lower expression of the co-stimulatory molecule CD86, found in acute infection also by other authors [5, 7, 24] that persisted 7 months after infection together with lower levels of CD4 + DCs. Low levels of activation molecules, such as CD86 have been related with a possible impairment in T cell and DCs response to the virus. Specifically, we and others have found pDC hyporesponsiveness to HIV after CD4 downregulation in this cell type [15, 34]. On the contrary, CCR7+ pDCs remained at high levels even after 7 months after infection indicating again ongoing migration to lymph node or other inflammatory foci. In this line, the higher expression of other chemokine receptors such as CCR1, CCR3, and CCR5 has previously described in SARS-CoV-1 infected monocyte derived DCs [35].

It is unknown whether these defects in the DCs compartment will be reversible after longer follow up or specific therapies may be needed for the normalization of these defects. What is clear is that persisting symptoms and unexpected substantial organ dysfunction are observed in an increasing number of patients who have recovered from COVID-19 [12]. Actually, Huang et al. recently described that 7 months after illness onset, 76% of the SARS-CoV-2 infected patients reported at least one symptom that persisted, being fatigue or muscle weakness the most frequently reported symptoms [36]. In addition, many of those previously hospitalized patients

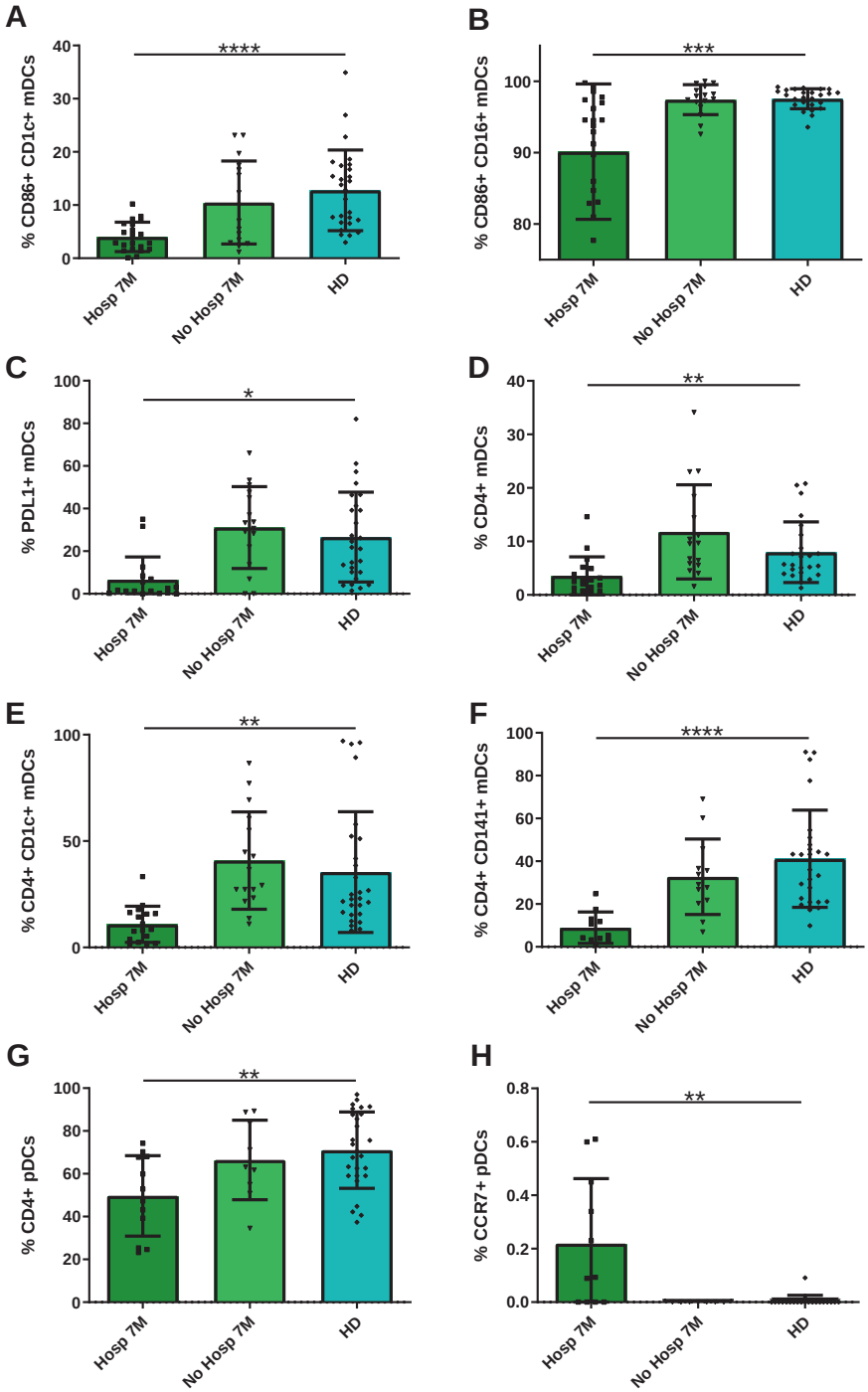

Fig. 5 Some DC activation markers are not normalized in previously hospitalized patients 7 months after SARS-CoV-2 infection. Bar graphs representing the percentage of DC subpopulations expressing CD86 (**A**, **B**), PD-L1 (**C**), CD4 (**D–G**), and CCR7 (**H**) in previously hospitalized (Hosp 7M) or previously nonhospitalized (No Hosp 7M) patients 7 months after SARS-CoV-2 infection and in healthy donors (HD). The median with the interquartile range is shown and each dot represents an individual. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Mann–Whitney *U* test was used for groups' comparisons

presented residual chest imaging abnormalities, impaired pulmonary diffusion capacity and other extrapulmonary manifestations as a low estimated glomerular filtration rate [36]. The immune mechanisms that might be involved in the development of these persisting symptoms are still unknown. However, it would be expected that 7 months after SARS-CoV-2 infection there is still an inflammatory response due to persistent tissue damage or persistence presence of viral antigens in the absence of viral replication which may cause these deficits in DC. In fact, it has been reported that SARS-CoV-2 can persist in the intestines up to 7 months following symptoms resolution [37]. Thus, we postulate that the decrease in peripheral DCs numbers, along with the alterations in DC homing and activation markers 7 months after the infection might be indicative of DC migration to inflammatory sites which may be contributing to long-term symptoms, a phenomenon also known as long COVID.

One of the limitations of this study might be that, for a more precise identification of pDCs, CD2+, CD5+, and AXL+ cells should have been excluded [38]. Nevertheless, since these cell populations

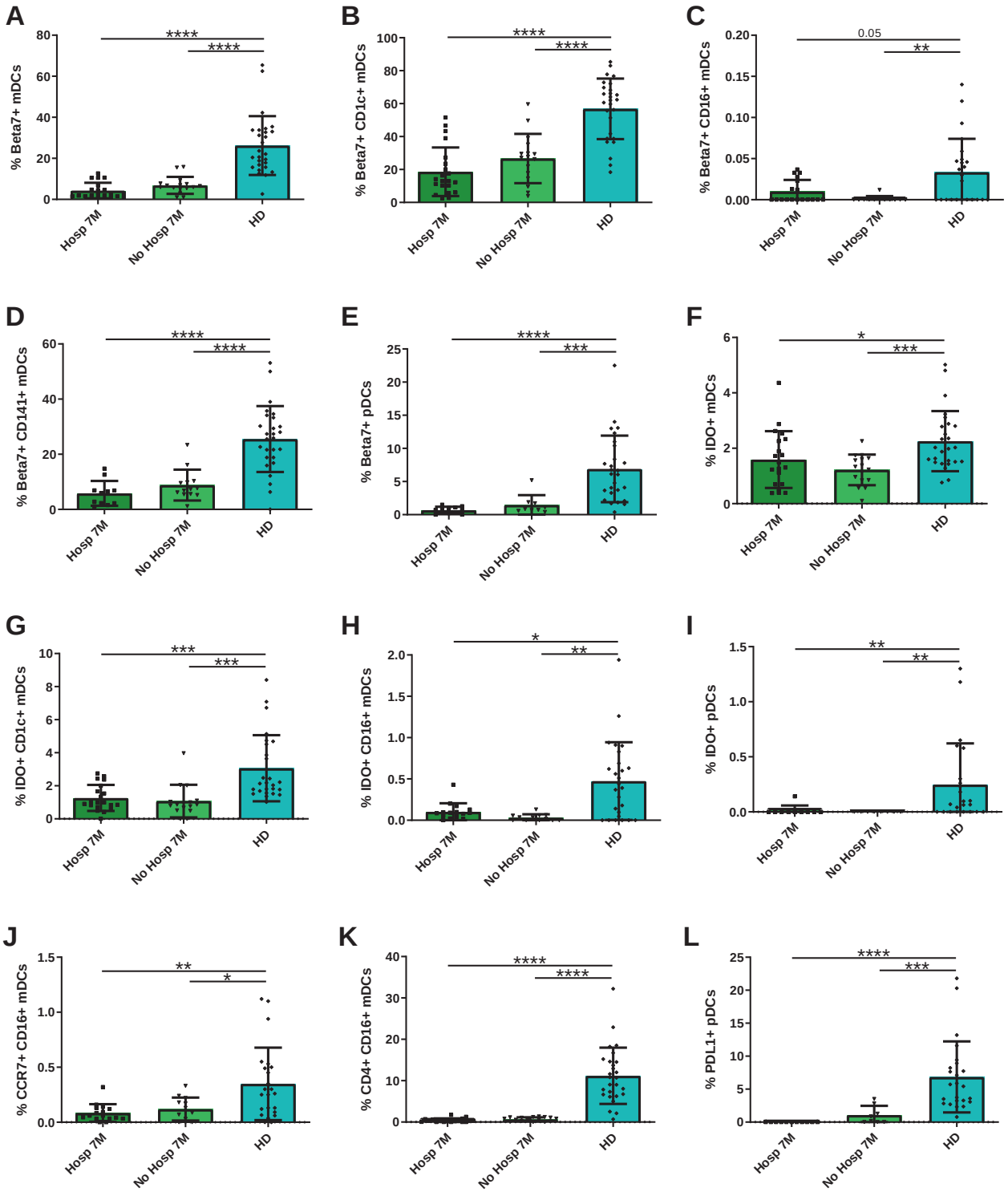

Fig. 6 Some DC activation markers are not normalized neither in previously hospitalized nor in nonhospitalized patients 7 months after SARS-CoV-2 infection. Bar graphs representing the percentage of DC subpopulations expressing β7 (**A–E**), IDO (**F–I**), CCR7 (**J**), CD4 (**K**), and PD-L1 (**L**) in previously hospitalized (Hosp 7M) or previously nonhospitalized (No Hosp 7M) patients 7 months after SARS-CoV-2 infection and in healthy donors (HD). The median with the interquartile range is shown and each dot represents an individual. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Mann–Whitney *U* test was used for groups' comparisons

are barely represented within PBMCs, the showed results correspond mainly to pDCs, although some contamination with AS-DC cannot be excluded. The same happened with CD123+ mDCs, which were not included in our gating strategy, however, the levels of this subset was so low that did not change total mDC levels (data not shown). Moreover, a limitation of this work might be that all patients included in this study belong to the first wave of COVID-19 in Spain. It would have been interesting to have access to tissue samples, however due to safety issues at that moment of the pandemic it was not possible. At that time, different experimental

treatments with very limited but transitory immunosuppressive effects were administered what may have affected the levels of immune parameters. However, the agreement of our observations with other data in the literature during acute infection and the persistence of these defects 7 months after infection minimized the potential bias of these treatments in our results.

In summary, we have demonstrated that SARS-CoV-2 infected patients showed a deficit in some DC subsets and alterations in DC homing and activation markers, which are not restored more than 7 months after the infection independently of previous hospitalization. Our results suggest that there is an ongoing inflammation which could be partially induced by DCs, these findings might contribute to a better understanding of the immunological sequelae of COVID-19.

MATERIALS AND METHODS

Study participants

Seventy one participants with confirmed detection of SARS-CoV-2 by reverse-transcription polymerase chain reaction (RT-PCR) were included. Out of these 71, 33 were hospitalized in acute phase of COVID-19 from March 25th to May 8th 2020, while 38 participants were recruited 7 months after being diagnosed with COVID-19, from September 9th to November 26th 2020. These participants came from the COVID-19 patients' Cohort Virgen del Rocio University Hospital, Seville (Spain) and the COVID-19 Cohort IIS Galicia Sur (CohVID GS), Vigo (Spain). Twenty-seven healthy donors (HD), with cryopreserved pre-COVID-19 samples (May 12th to July 18th 2014) were included from the HD cohort, collection of samples of the Laboratory of HIV infection, Andalusian Health Public System Biobank, Seville (Spain) (C330024). Written or oral informed consent was obtained from all participants. The study was approved by the Ethics Committee of the Virgen del Rocio University Hospital (protocol code "pDCOVID"; internal code 0896-N-20). Hospitalized participants during the acute phase of infection were divided in Mild ($n = 17$) or Severe ($n = 16$), based on the highest grade of disease severity during course of hospitalization. Severe participants were those who required Intensive Care Unit admission, or having ≥6 points in the score on ordinal scale based on Beigel et al. [39] or death. Blood samples were collected at a median of 3 [interquartile range (IQR) 2–23] days after hospitalization and 14 [9–31] days after symptoms onset (Supplementary Table 1). The group of participants discharged after infection, included previously hospitalized ($n = 21$) and previously nonhospitalized subjects ($n = 17$). The samples from these participants were collected after a median of 201 [181–221] days after hospitalization and 208 [189–230] days after symptoms onset (Supplementary Table 1). COVID-19 participants in the different groups were age and sex matched with HDs' group (Supplementary Table 1).

Cell and plasma isolation

PBMCs from healthy donors and participants were isolated from fresh blood samples using BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes (with Sodium Heparin, BD Cat# 362780) in a density gradient centrifugation at the same day of blood collection. Afterwards, PBMCs were cryopreserved in freezing medium (90% of fetal bovine serum (FBS) + 10% dimethyl sulfoxide) in liquid nitrogen until further use. Plasma samples were obtained using BD Vacutainer™ PET EDTA Tubes centrifugation, aliquoted and cryopreserved at −80 °C until further use.

Dendritic cell immunophenotyping

For DCs flow cytometry, PBMCs were centrifuged, pelleted and washed with Phosphate-buffered saline (PBS) and stained for 35 min at room temperature with LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies), BV421 CD86, BV650 CD11c, BV711 HLA-DR, BV786 CCR7 (CD197), FITC Lin-2 (CD3, CD14, CD19, CD20, CD56), BV605 CD16, PeCF594 PD-L1 (CD274), APC Integrin-β7 (BD Biosciences), PerCPCy5,5 CD4, APCCy7 CD1c, PeCy7 CD141 (BioLegend) and AF700 CD123 (R&D, San Diego, CA) antibodies. Then PBMCs were washed with Permeabilization Buffer 10X diluted 1:10 (eBioscience™), permeabilized by Fixation/Perm buffer (eBioscience™), and intracellularly stained with PE IDO (eBioscience, San Diego, CA, USA) antibody. DCs were gated based on Lin-2 HLA-DR expression. Each subset (mDCs and pDCS) was gated based on CD123 and CD11c expression. mDCs subsets were gated by using CD16, CD1c and CD141 staining, for gating strategy see Supplementary Fig. 1. Flow cytometry analyses were performed on an LRS Fortessa flow cytometer using FACS Diva software (BD Biosciences). Data were analyzed using the FlowJo software (Treestar, Ashland, OR). At least 1×10^6 events were acquired per sample.

Cell culture and IFN-α quantification

1×10^6 thawed PBMCs were incubated at 37 °C and 5% CO_2 during 18 h in RPMI with 10% FBS without any stimuli or with 1 μM CpG-A (ODN 2216; InvivoGen). After incubation, cells were pelleted and the supernatants conserved for the subsequent quantification of IFN-α production at −80 °C. The amount of IFN-α in cell culture supernatants was assessed by an IFN-α multisubtype enzyme-linked immunosorbent assay kit (PBL Interferon Source Cat# 41105) according to the manufacturer's instructions.

Cytokine quantification in plasma

Plasmas previously collected were used for the quantitative determination of cytokines. We used 3 different kits to quantify sCD25 by Human CD25/IL-2R alpha Quantikine ELISA Kit (R&D System, Cat# DR2A00), IP-10 by Human IP-10 ELISA Kit (CXCL10) (Abcam, Cat# ab173194) and IL-6, IL-8, IL-1β, TNF-α, IFN-γ, MIP-1α, and MIP-1β by MILLIPLEX MAP Human High Sensitivity T Cell Panel (Merck Cat# HSTCMAG-28SK) according to the manufacturer's instructions.

Statistics

Statistical analyses were performed by using Statistical Package for the Social Sciences software (SPSS 25.0; SPSS, Inc., Chicago, IL) and R environment 4.0.3 (2020-10-10), using RStudio Version 1.3.959 as the work interface and GraphPad Prism, version 8.0 (GraphPad Software, Inc.). ROUT method was utilized to identify and discard outliers. Differences between conditions among different groups were analyzed by two-tailed Mann–Whitney *U* test. The Wilcoxon test was used to analyze paired samples. The Spearman test was used to analyze correlations between variables. All differences with a *P* value of <0.05 were considered statistically significant.

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AUTHOR CONTRIBUTIONS

APG, JV, and MGC performed the experiments, analyzed and interpreted the data and participated in writing of the paper. AGV, MTR, ASG, and EMM participated in data collection, data analysis and interpretation and performed experiments, MRJL, MRIB participated in paper data interpretation, IRJ, CI, and JCC participated in data collection, CS, CRO, NE, AFV, and MC participated in data collection and paper interpretation. LFLC and EP participated in paper data analysis, patient and data collection, interpretation/ discussion of the results and coordination. ERM, participated in data analysis and interpretation, writing, conceived the idea and coordinate the project. APG, JV, and MCGC contributed equally to this work.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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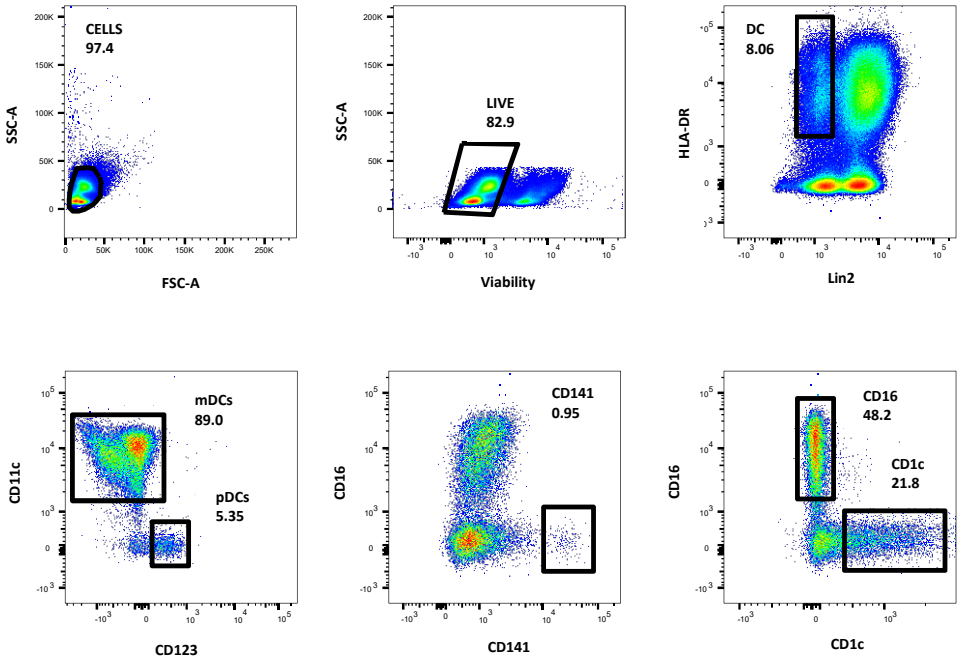
Figure S1

A


CELLS
97.4
SSC-A
FSC-A
LIVE
82.9
SSC-A
Viability
DC
8.06
HLA-DR
Lin2
mDCs
89.0
pDCs
5.35
CD11c
CD123
CD141
0.95
CD16
CD141
CD16
48.2
CD1c
21.8
CD16
CD1c

B

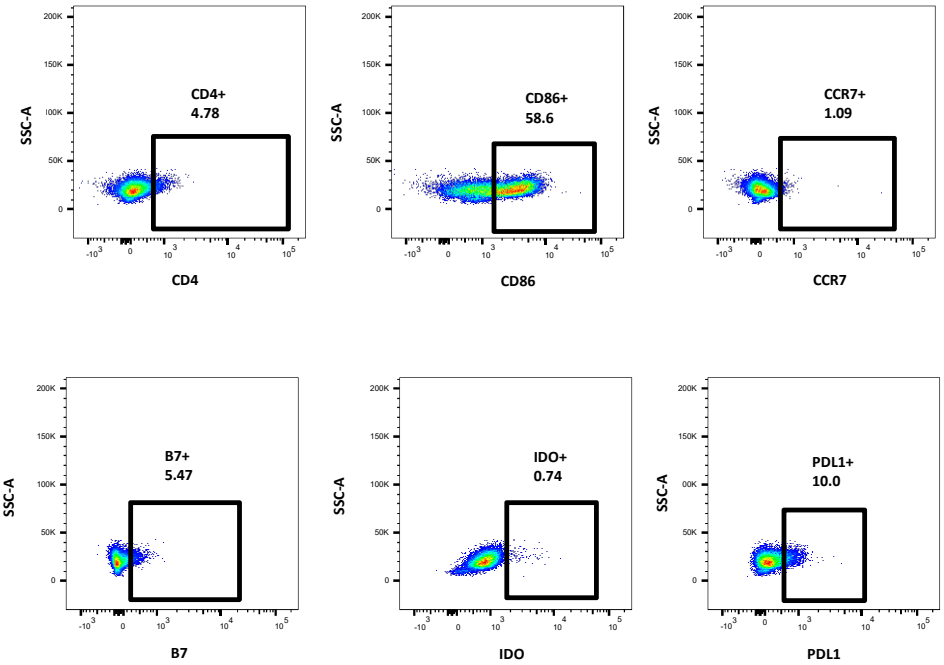

CD4+
4.78
SSC-A
CD4
CD86+
58.6
SSC-A
CD86
CCR7+
1.09
SSC-A
CCR7
B7+
5.47
SSC-A
B7
IDO+
0.74
SSC-A
IDO
PDL1+
10.0
SSC-A
PDL1

Figure S2

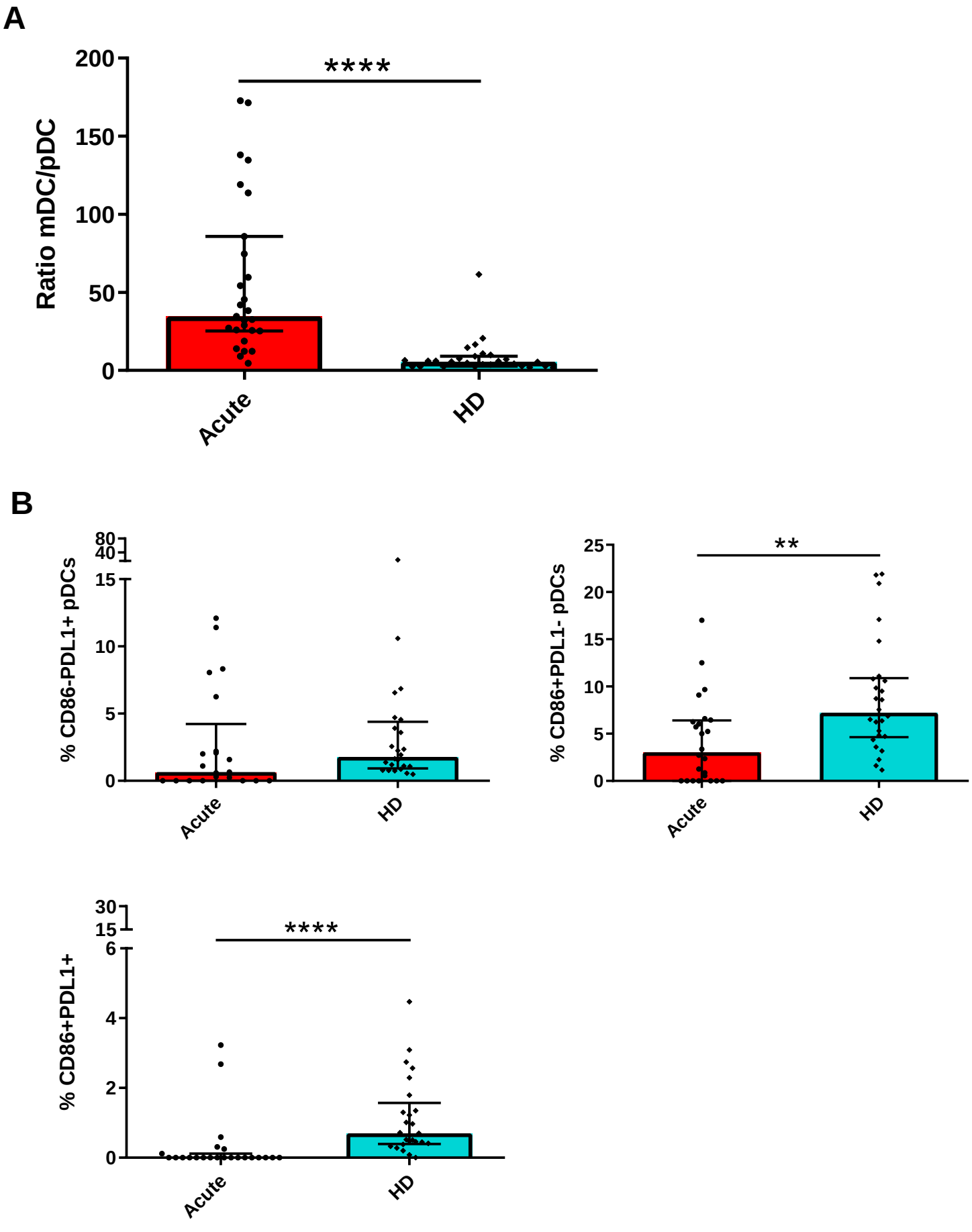

A
Ratio mDC/pDC
200
150
100
50
0
****
Acute
HD
B
% CD86-PDL1+ pDCs
80
40
15
10
5
0
Acute
HD
% CD86+PDL1- pDCs
25
20
15
10
5
0
**
Acute
HD
% CD86+PDL1+
30
15
6
4
2
0
****
Acute
HD

Figure S3

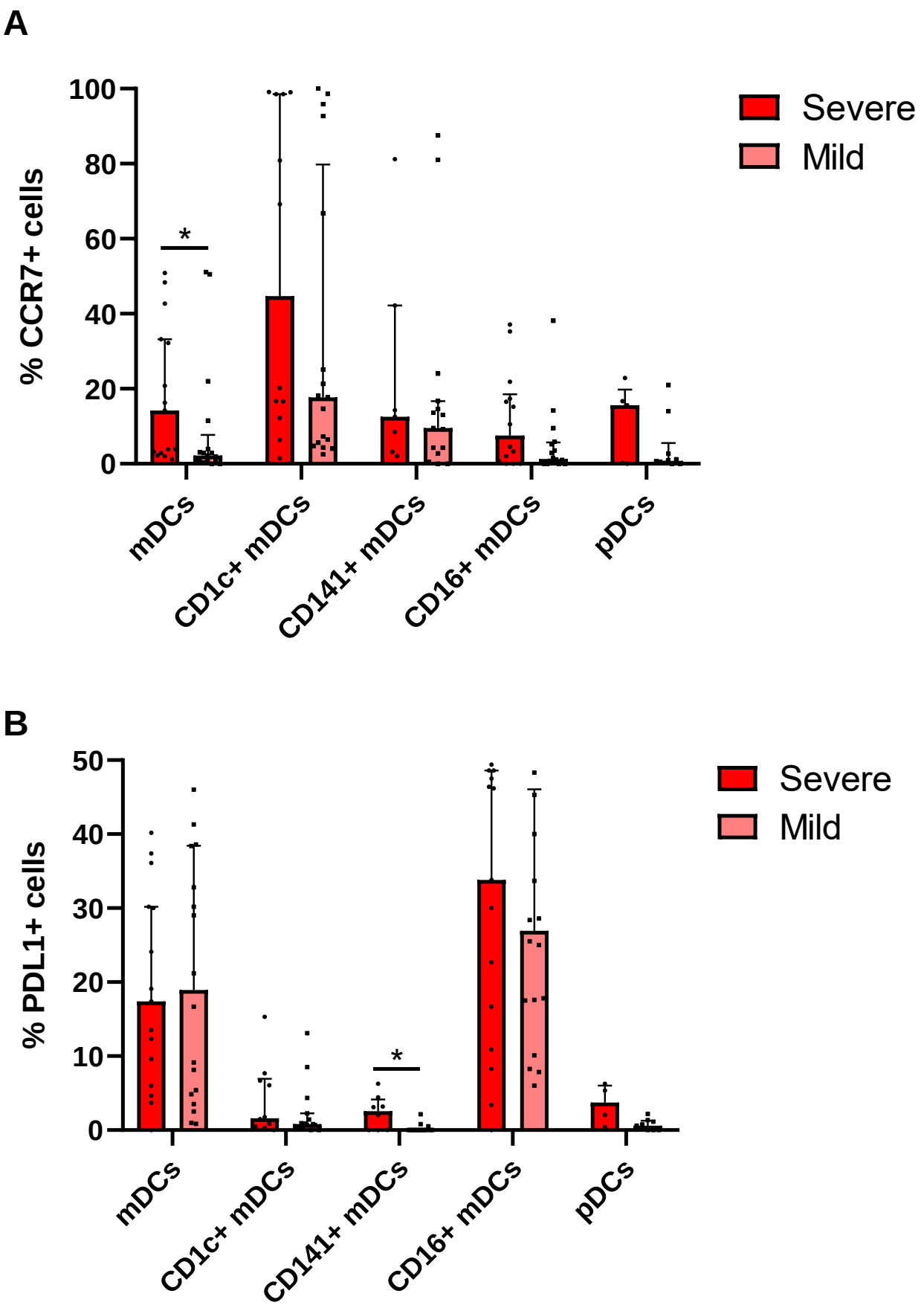
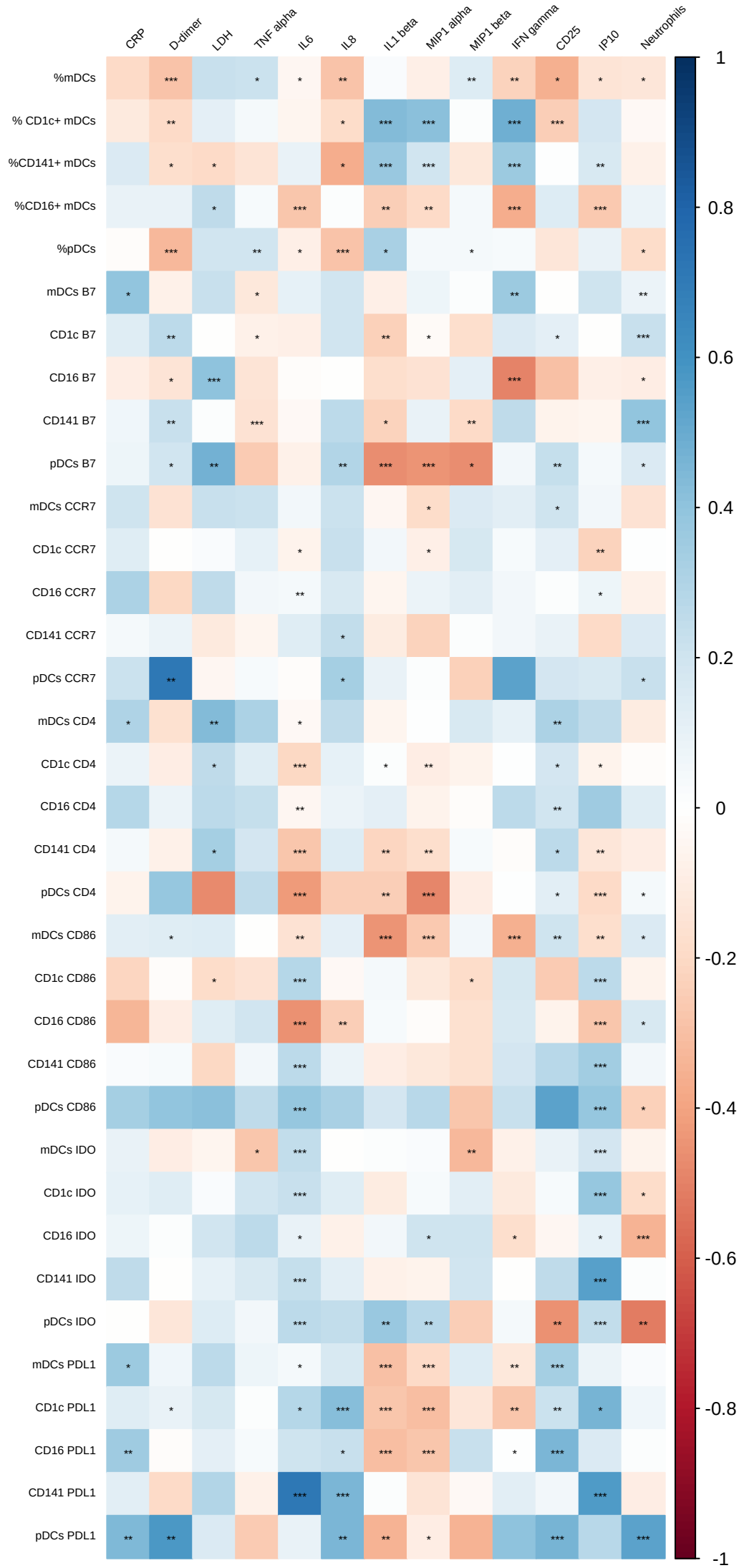

A
% CCR7+ cells
100
80
60
40
20
0
*
Severe
Mild
mDCs
CD1c+ mDCs
CD141+ mDCs
CD16+ mDCs
pDCs
B
% PDL1+ cells
50
40
30
20
10
0
*
Severe
Mild
mDCs
CD1c+ mDCs
CD141+ mDCs
CD16+ mDCs
pDCs

Figure S4


CRP
D-dimer
LDH
TNF alpha
IL6
IL8
IL1 beta
MIP1 alpha
MIP1 beta
IFN gamma
CD25
IP10
Neutrophils
%mDCs
% CD1c+ mDCs
%CD141+ mDCs
%CD16+ mDCs
%pDCs
mDCs B7
CD1c B7
CD16 B7
CD141 B7
pDCs B7
mDCs CCR7
CD1c CCR7
CD16 CCR7
CD141 CCR7
pDCs CCR7
mDCs CD4
CD1c CD4
CD16 CD4
CD141 CD4
pDCs CD4
mDCs CD86
CD1c CD86
CD16 CD86
CD141 CD86
pDCs CD86
mDCs IDO
CD1c IDO
CD16 IDO
CD141 IDO
pDCs IDO
mDCs PDL1
CD1c PDL1
CD16 PDL1
CD141 PDL1
pDCs PDL1
1
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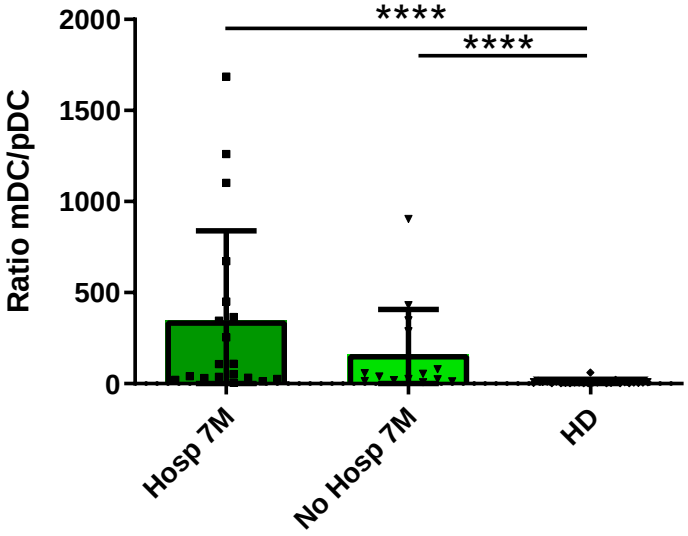
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Figure S5

A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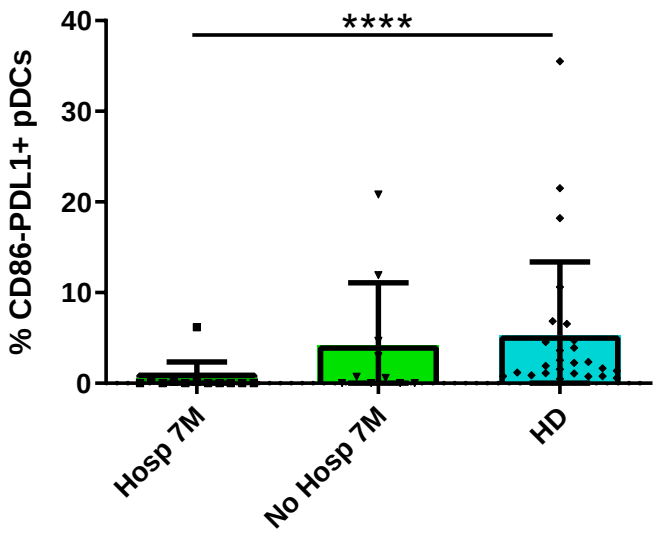
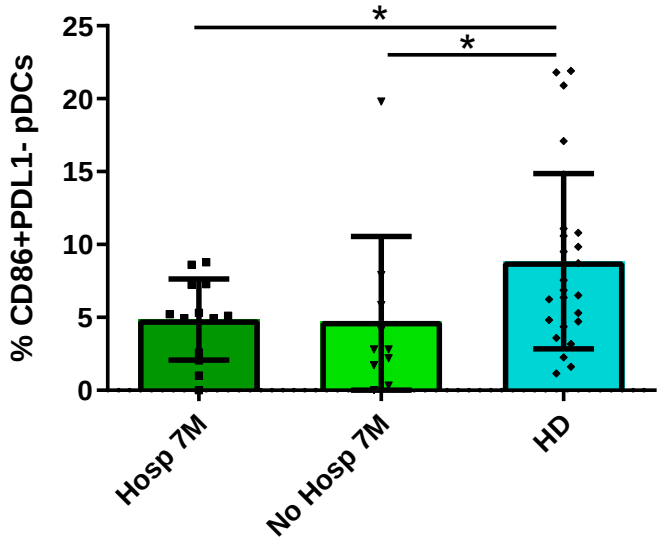
2000
1500
1000
500
0
****
****
Hosp 7M
No Hosp 7M
HD

B


% CD86-PDL1+ pDCs
40
30
20
10
0
****
Hosp 7M
No Hosp 7M
HD


% CD86+PDL1- pDCs
25
20
15
10
5
0
*
*
Hosp 7M
No Hosp 7M
HD

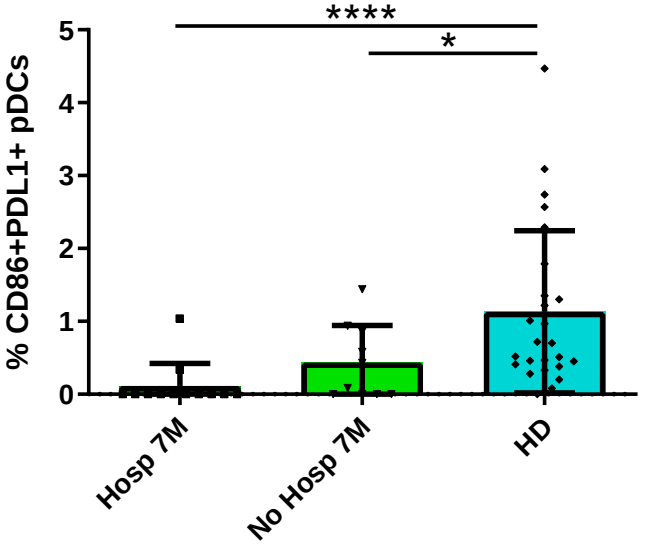
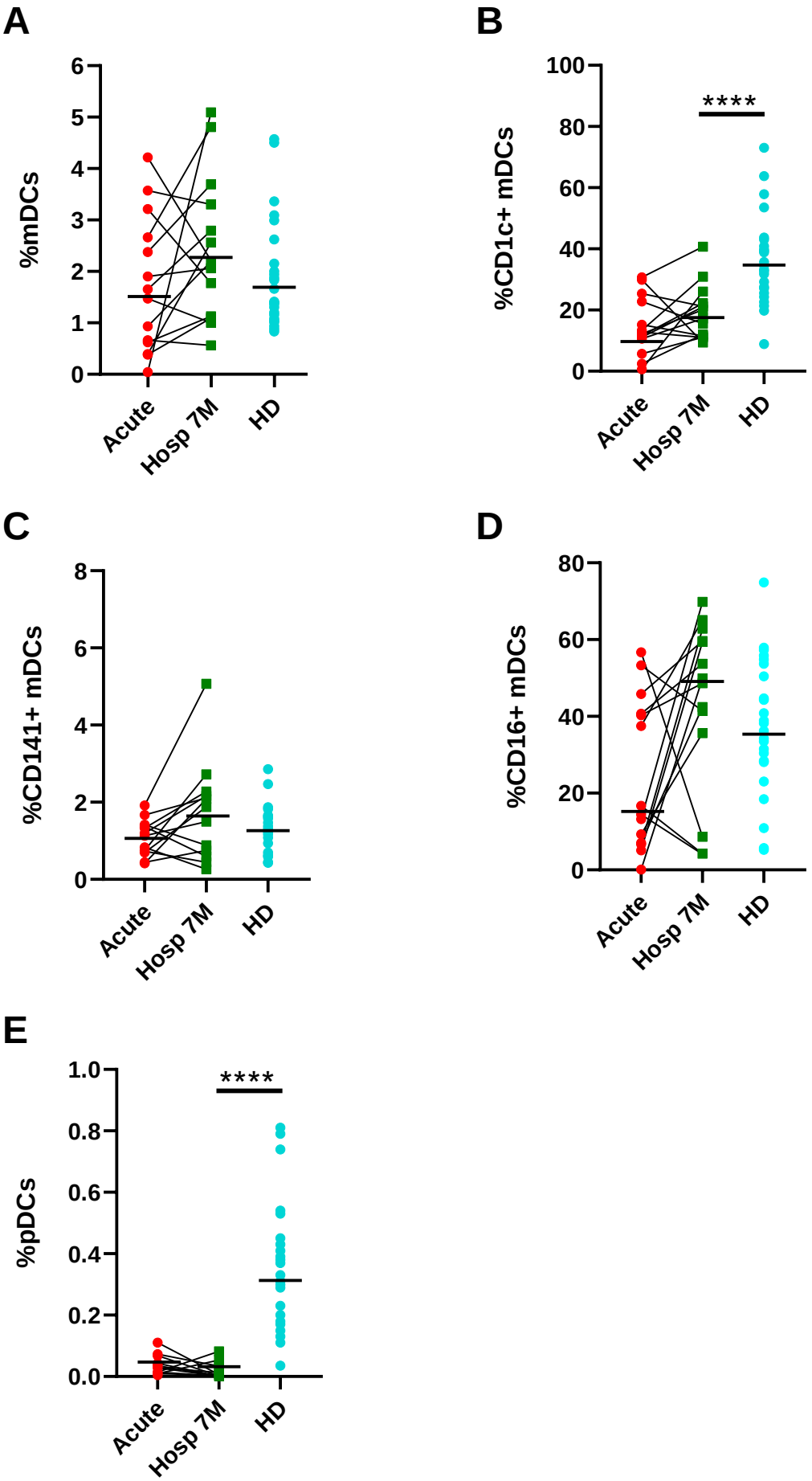

% CD86+PDL1+ pDCs
5
4
3
2
1
0
****
*
Hosp 7M
No Hosp 7M
HD

Figure S6


A
%mDCs
6
5
4
3
2
1
0
Acute
Hosp 7M
HD
B
%CD1c+ mDCs
100
80
60
40
20
0
****
Acute
Hosp 7M
HD
C
%CD141+ mDCs
8
6
4
2
0
Acute
Hosp 7M
HD
D
%CD16+ mDCs
80
60
40
20
0
Acute
Hosp 7M
HD
E
%pDCs
1.0
0.8
0.6
0.4
0.2
0.0
****
Acute
Hosp 7M
HD

SUPPLEMENTAL INFORMATION

Figure S1. Gating strategy for the identification of DC subpopulations and activation markers

Pseudocolor dot plots showing the gating strategy used for the identification of studied populations in a representative SARS-CoV-2 infected patient. (A) Mononuclear cells were selected according to their size (FSC-A) and complexity (SSC-A) and dead cells were discarded using a viability marker. DC and subpopulations were identified as follows: total DCs (Lin2- HLA-DR+), total mDCs (CD11c+ CD123-), pDCs (CD11c- CD123+), and within mDCs: CD1c+, CD141+ and CD16+. (B) Representative data of total mDCs showing selected gates to analyze the percentages of CD4+, CD86+, CCR7+, β7+, IDO+ and PD-L1+ cells.

Figure S2. mDC/pDC ratio and the percentages of pDC subsets in acute SARS-CoV-2 infected patients and healthy donors

Bar graphs representing (A) the ratio mDC/pDC and (B) the percentages of P1-pDC (CD86-PD-L1+), P2-pDC (CD86+PD-L1+) and P3-pDC (CD86+PD-L1-) subsets in acute SARS-CoV-2 infected patients (acute) and healthy donors (HD). The median with the interquartile range is shown and each dot represents one individual. ****$p < 0.0001$, **$p < 0.01$. Mann-Whitney U test was used for groups' comparisons.

Figure S3. DC markers expression in SARS-CoV-2 infected patients with severe and mild symptoms.

Bar graphs representing the percentage of DCs expressing CCR7 (A) and PD-L1 (B) in acute severe and mild SARS-CoV-2 infected patients. The median with the interquartile range is shown and each dot represents an individual. *p < 0.05. Mann-Whitney U test was used for groups' comparisons.

Figure S4. Associations of DC numbers and activation markers with inflammatory markers in acute SARS-CoV-2 infected patients

Heatmap graphs representing correlations between the percentages of DC subpopulations and the percentages of DCs expressing activation and homing markers with inflammatory markers including CRP, D-dimer, LDH, TNF-α, IL-6, IL-8, IL1-β, MIP1-α, MIP1-β, IFN-γ, sCD25, IP-10 and neutrophil numbers, in acute SARS-CoV-2 infected patients. Blue color represents positive correlations and red color shows negative correlations. The intensity of the color indicates the R coefficient. *p < 0.05, **p < 0.01, ***p < 0.001. Spearman test was used for non-parametric correlations.

Figure S5. mDC/pDC ratio and the percentages of pDC subsets seven months after SARS-CoV-2 infection

Bar graphs representing (A) the ratio mDC/pDC and (B) the percentages of P1-pDC (CD86-PD-L1+), P2-pDC (CD86+PD-L1+) and P3-pDC (CD86+PD-L1-) subsets in previously hospitalized (Hosp 7M) or previously non-hospitalized (No

Hosp 7M) patients seven months after SARS-CoV-2 infection and in healthy donors (HD). The median with the interquartile range is shown and each dot represents an individual. ****p < 0.0001, *p < 0.05. Mann-Whitney U test was used for groups' comparisons.

Figure S6. Paired analysis of DC subsets of SARS-CoV-2 infected patients in acute phase and seven months after the infection

Before and after graphs representing the paired analysis of the percentage of total mDCs, CD1c+, CD141+ and CD16 mDCs and pDCs (A - E) in patients in acute phase (Acute) and seven months after SARS-CoV-2 infection (Hosp 7M) and in healthy donors (HD). The median is shown and each dot represents an individual. ****p < 0.0001. Wilcoxon test was used for paired samples and Mann-Whitney U test was used for groups' comparisons.

Supplementary table 1. Characteristics of the study patients.

	Acute Infection			**Discharged (6-8 months after diagnosis)**			**Healthy Donors**
	All (n=33)	**Mild (n=17)**	**Severe* (n=16)**	**All (38)**	**Previously Hospitalized (n=21)**	**Previously Non Hospitalized (n=17)**	**(n=27)**
Age (years)	66 [59-77]	62 [57-78]	69 [63 – 73]	67 [60 – 72]	68 [63 – 73]	65 [58 – 71]	62 [39 – 84]
Sex (Female sex), n (%)	12 (36)	7 (41)	5 (31)	17 (48)	7 (33)	10 (59)	11 (41)
Oxygen Saturation ($SatO_2$), (%)	95 [91 – 98]	96 [95 – 99]	92 [90 – 95]	N/A	N/A	N/A	N/A
Time since hospitalization, (days)	3 [2 – 23]	2 [1 – 3]	20 [3 – 31]	201 [181 – 221]	183 [168 – 197]	221 [219 – 228]	N/A
Time since symptoms onset, (days)	14 [9 – 31]	11 [5 – 14]	31 [19 – 38]	208 [189 – 230]	192 [179 – 203]	230 [224 – 235]	N/A
Time hospitalized, (days)	16 [7 – 34]	7 [5 – 10]	28 [20 – 43]	N/A	16 [8 – 40]	0	N/A
Comorbidities, n (%)	26 (79)	13 (77)	13 (81)	19 (50)	16 (76)	3 (18)	N/A
Diabetes mellitus	8 (24)	4 (24)	4 (25)	6 (16)	5 (24)	1 (6)	N/A
Hypertension	19 (57)	8 (47)	11 (70)	15 (40)	13 (62)	2 (12)	N/A
Cardiovascular disease	7 (21)	4 (24)	3 (19)	8 (21)	6 (29)	2 (12)	N/A
Obstructive pulmonary disease	5 (15)	3 (18)	2 (13)	4 (11)	4 (19)	0	N/A
Malignancy	2 (6)	1 (6)	1 (6)	2 (5)	2 (10)	0	N/A
Symptoms at admission (%)							
Cough	20 (61)	10 (59)	10 (63)	29 (76)	19 (91)	10 (59)	N/A
Fever	22 (67)	10 (59)	12 (75)	28 (74)	16 (76)	12 (71)	N/A
Dyspnea	14 (43)	7 (41)	7 (44)	21 (55)	15 (71)	6 (35)	N/A
Anosmia	6 (18)	3 (18)	3 (19)	4 (11)	4 (19)	0	N/A
Diarrhoea	7 (21)	4 (24)	3 (19)	12 (32)	9 (43)	3 (18)	N/A
Muscle pain	6 (18)	2 (12)	4 (25)	4 (11)	3 (14)	1 (6)	N/A
Treatment during hospitalization; n (%)							
Hydroxychloroquine	28 (85)	13 (77)	15 (94)	N/A	20 (95)	N/A	N/A
Lopinavir/Ritonavir	20 (61)	7 (41)	13 (81)	N/A	15 (71)	N/A	N/A
Beta Interferon	10 (30)	1 (6)	9 (56)	N/A	9 (43)	N/A	N/A
Corticoids	16 (49)	4 (24)	12 (75)	N/A	8 (38)	N/A	N/A
Remdesivir	3 (9)	3 (18)	0	N/A	0	N/A	N/A

Tocilizumab	9 (27)	0	9 (56)	N/A	7 (33)	N/A	N/A

[a]Categorical variables are expressed as number and percentages (%), and continuous variables are expressed as median (interquartile ranges [IQR]). N/A, not applicable. The different groups (acute infection, discharged patients and healthy donors) were age and sex matched. Chi-square test and a Mann-Whitney U test were used to compare categorical and continuous variables, respectively. Analysis by age; acute infection vs HD (p=0.259); discharged patients vs HD (p=0.440); Previously Hospitalized patients vs HD (p=0.488); Previously Non Hospitalized patients vs HD (p=0.604). Analysis by sex; acute infection vs HD (p=0.793); discharged patients vs HD (p=0.803); Previously Hospitalized patients vs HD (p=0.765); Previously Non Hospitalized patients vs HD (p=0.354). *Participants were divided in Mild or Severe, based on the highest grade of disease severity during course of hospitalization. Severe participants were those who required Intensive Care Unit admission, or having ≥6 points in the ordinal scale score based on Beigel et al. (Beigel New Engl J Med 2020) or death.

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RESEARCH ARTICLE

Deciphering the quality of SARS-CoV-2 specific T-cell response associated with disease severity, immune memory and heterologous response

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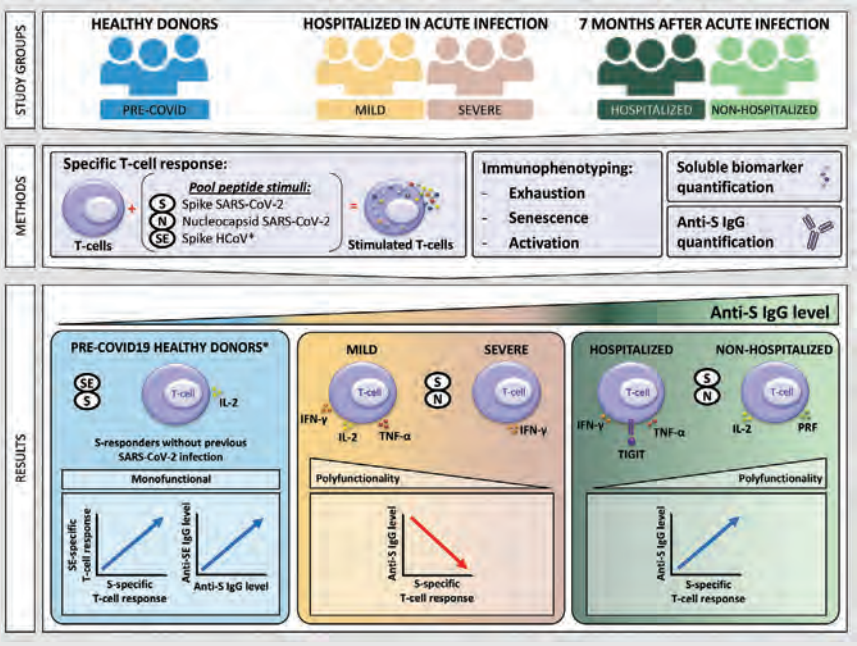
Graphical Abstract



"In acute infection, combinations with only IFN-γ were deleterious and those with IL-2 were associated with a better course of acute infection. Additionally, mild patients had a more polyfunctional T response. In severe

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patients, there was increased antibody production inversely associated with the expression of combinations including IL-2. Seven months after infection, cellular and humoral responses were present, with T-cell response quality similar to acute infection. However, previously hospitalised subjects had higher T-cell exhaustion. Finally, an association was found between the response to HCoV and SARS-CoV-2 mainly mediated by IL-2 expression in pre-COVID-19 participants."

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RESEARCH ARTICLE

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Abstract

SARS-CoV-2 specific T-cell response has been associated with disease severity, immune memory and heterologous response to endemic coronaviruses. However, an integrative approach combining a comprehensive analysis of the quality of SARS-CoV-2 specific T-cell response with antibody levels in these three scenarios is needed. In the present study, we found that, in acute infection, while mild disease was associated with high T-cell polyfunctionality biased to IL-2 production and inversely correlated with anti-S IgG levels, combinations only

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including IFN-γ with the absence of perforin production predominated in severe disease. Seven months after infection, both non-hospitalised and previously hospitalised patients presented robust anti-S IgG levels and SARS-CoV-2 specific T-cell response. In addition, only previously hospitalised patients showed a T-cell exhaustion profile. Finally, combinations including IL-2 in response to S protein of endemic coronaviruses were the ones associated with SARS-CoV-2 S-specific T-cell response in pre-COVID-19 healthy donors' samples. These results could have implications for protective immunity against SARS-CoV-2 and recurrent COVID-19 and may help for the design of new prototypes and boosting vaccine strategies.

KEYWORDS
COVID-19, endemic coronaviruses, IL-2, nucleocapsid, polyfunctionality, SARS-CoV-2, Spike, T-cell response

1 | INTRODUCTION

Host immune response against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection is a key factor in the progression of Coronavirus Disease 2019 (COVID-19)[1] and its deregulation results in fatal disease in hospitalised COVID-19 patients.[2,3] The coordination of different branches of adaptive immunity, such as CD4+, CD8+ T-cell and antibody responses, is essential for the resolution of COVID-19.[4] Despite the already known role of T-cell response against SARS-CoV-2 infection, there are still gaps that need to be clarified in relation to the quality of this response and its association with: (i) disease severity in acute infection, (ii) long-lasting immune memory and (iii) the heterologous response found in healthy donors (HDs).[5]

Seminal studies in SARS-CoV-1 infection models showed that both CD4+[6] and CD8+[7] T-cell response were involved in protection and virus clearance in acute infection. In SARS-CoV-2 infection, high CD4+ T-cell response levels have been associated with mild disease and enhanced early virus clearance in acute infection, while the absence of this response was associated with fatal COVID-19 outcome.[4,8–10] Although at a lower level of magnitude, SARS-CoV-2 specific CD8+ in coordination with CD4+ T-cell response in acute infection seems to be essential for a good prognosis.[4] Opposite to these findings, a higher magnitude and broader overall T-cell response[11,12] and higher antibody levels against SARS-CoV-2[13] have been associated with poor disease outcome. Despite all these findings, the information about the quality and polyfunctionality of T-cell response in acute infection is scarce. The detailed and comprehensive analysis by intracellular staining (ICS) may clarify existing paradoxes about the role of T-cell response in acute infection and may provide additional immune correlates of protection.

Equally important for immune protection and recurrent COVID-19 is to analyse the immune memory after SARS-CoV-2 infection. The longevity of CD4+ T-cell and memory B cell response against the spike protein (S) seems to be stable, while CD8+ T-cell response lowered by half at 6–8 months after infection[14]. Moreover, it is very important to know whether the disease severity during acute infection may dictate the quality and the magnitude of long-term immune memory. Patients with post-acute symptoms showed a trend to decline IFN-γ production in *N*-specific CD8+ T-cells 4 months after infection.[12] However, a detailed analysis of the quality of T-cell response at the longer term after infection is lacking. These analyses may have important implications on current vaccination strategies.

The immune memory response is not always triggered by a previous contact with SARS-CoV-2. Heterologous response in unexposed HDs has been found due to the sequence homology between common cold coronaviruses (HCoV) and SARS-CoV-2.[8,15–17] A detailed analysis of the

correlation and the qualities of these responses is needed in order to know potential correlates of protection and vaccine responses.

In the present study, using an integrative approach combining antibody levels, SARS-CoV-2 specific CD4+ and CD8+ T-cell response, we found specific magnitude and polyfunctionality features of this response associated with disease severity in acute infection, with long-term immune memory in previously hospitalised and non-hospitalised patients and also associated with heterologous response to endemic coronaviruses.

2 | MATERIAL AND METHODS

2.1 | Study participants

Seventy participants with confirmed detection of SARS-CoV-2 by reverse-transcription polymerase chain reaction (RT-PCR) as previously described[18] were included. Out of these 70, 37 were hospitalised in acute phase of COVID-19 from March 25 to May 8, 2020, while 33 participants were recruited 7 months after being diagnosed with COVID-19, from September 9 to November 26, 2020. These participants came from the COVID-19 patients' Cohort Virgen del Rocio University Hospital, Seville (Spain) and the COVID-19 Cohort IIS Galicia Sur (CohVID GS), Vigo (Spain).[19] Thirty-three HDs, pre-COVID-19 cryopreserved samples (May 12 to July 18, 2014) were included in the HD cohort, collected at Laboratory of HIV infection, Andalusian Health Public System Biobank, Seville (Spain) (C330024).[19] Written or oral informed consent was obtained from all participants. The study was approved by the Ethics Committee of the Virgen Macarena and Virgen del Rocio University Hospital (protocol code "pDCOVID"; internal code 0896-N-20). Hospitalised participants during the acute phase of infection were divided in mild ($n = 18$) or severe ($n = 19$), based on the highest level of disease severity during course of hospitalization. Severe participants were those who required Intensive Care Unit admission, or having ≥6 points in the score on ordinal scale[20] or death. The remaining acutely infected individuals by SARS-CoV-2 were considered mild. Blood samples were collected at a median of 3 days [interquartile range (IQR) 2.0 – 21.5] after hospitalisation and 17 days [7.0–31.5] after symptoms onset (Table S1). The group of participants discharged after infection, included previously hospitalised ($n = 19$) and previously non-hospitalised subjects ($n = 14$). The samples from these participants were collected after a median of 201 days [180.5–221] after hospitalisation and 208 days [190–232] after symptoms onset (Table S1). Clinical and demographic data from both HD and infected subjects are described in Table S1. Acutely SARS-CoV-2 infected patients and COVID-19 convalescent (previously hospitalised and not) participants were age and sex matched with HDs' group (Table S1).

2.2 | Cell and plasma isolation

Peripheral blood mononuclear cells (PBMCs) from HDs and participants were isolated from peripheral blood samples using BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes (with Sodium Heparin) by density gradient centrifugation at the same day of blood collection. Afterwards, PBMCs were cryopreserved in freezing medium (90% of fetal bovine serum (FBS) + 10% dimethyl sulphoxide (DMSO)) in liquid nitrogen until further use. Plasma samples were obtained using BD Vacutainer™ PET ethylenediamine tetraacetic acid (EDTA) centrifugation tubes and were cryopreserved at −80°C until further use.

2.3 | Cell stimulation

PBMCs were thawed, washed and rested for 1 h in 0.25 μl/ml DNase I (Roche Diagnostics, Indianapolis, IN)-containing R-10 complete medium (RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin G, 100 l/ml streptomycin sulphate, and 1.7 mM sodium L-glutamine). 1.5×10^6 PBMCs were stimulated in vitro for 6 h with overlapping peptides of protein S (PepMix™ SARS-CoV-2; Spike Glycoprotein, from JPT, Berlin, Germany), 1.5×10^6 with N (PepMix™ SARS-CoV-2; Nucleocapsid Protein, from JPT, Berlin, Germany) and 1.5×10^6 with protein S of an optimised peptide pool of endemic coronavirus (SE).[21] 1.5×10^6 PBMCs incubated with the proportional amount of DMSO were included as negative control for all the samples and 1.5×10^6 PBMCs stimulated with staphylococcal enterotoxin B (SEB) for each batch of experiments as a positive control. The stimulation was performed in the presence of 10 μg/ml of brefeldin A (Sigma Chemical Co, St. Louis, MO) and 0.7 μg/ml of monensin (BD Biosciences) protein transport inhibitors, anti-CD107a-BV650 (clone H4A3; BD Biosciences, USA) monoclonal antibody and purified CD28 and CD49d as previously described.[22] T-cell specific response was defined as the frequency of cells expressing intracellular cytokines and/or degranulation markers after stimulation with S, N and SE peptides, normalised with the unstimulated condition (background subtraction). The study of the specific T-cell response to the Spike peptide pool was prioritised over that of the nucleocapsid peptide pool according to the available number of cells.

2.4 | Immunophenotyping and intracellular cytokine staining

Both cultured PBMCs and cells for phenotypical analysis were washed (1800 rpm, 5 min, room temperature) with phosphate-buffered saline (PBS) and incubated 35 min at room temperature (RT) with LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies), anti-CD14-BV510 (clone MφP9), anti-CD19-BV510 (clone SJ25C1), anti-CD56-BV510 (clone NMCAM16.2), anti-CD3-BV711 (clone SP34-2), anti-CD45RA-FITC (clone L48), anti-CD8-APC (clone SK-1), anti-CD27-APCH7 (clone M-T271), anti-PD-1-BV786 (CD279, clone EH12-1), anti-CD38 (clone HIT2), anti-CD28 (clone CD28.2) (all of them from BD Bioscience); anti-(T cell immunoreceptor with Ig and ITIM domains, TIGIT)-PerCPCy5.5 (clone A15153G) and anti-HLA-DR (clone L243) (from BioLegend). PBMCs were washed with PBS and fixed and permeabilised with BD Cytofix/CytoPerm following manufacturer's protocol (Cat. No. 554714, BD Bioscience), and intracellularly stained at 4°C for 30 min with anti-(interleukin, IL)-2-BV421 (clone MQ1-17H12), anti-(interferon, IFN)-γ-PE-Cy7 (clone B27) (BD Bioscience), anti-(tumor necrosis factor, TNF)-α-AF700 (clone Mab11) (BD Pharmingen), anti-Perforin-PE (clone B-D48) (BioLegend). T cells were gated based on the CD3 and CD8 expression. Each subset (total memory, MEM; central memory, CM; effector memory, EM; and terminally differentiated effector memory, TEMRA) was gated based on CD45RA and CD27 expression (for gating strategy, see Figure S12). The specific T-cell response to each stimuli was determined by the sum of the expression of each cytokine (IFN-γ, IL-2 and TNF-α) in the different T-cell subsets. To classify an individual as a responder, this value must be higher than 0.05.[22,23] Flow cytometry analyses were performed on an BD LSR Fortessa™ Cell Analyzer flow cytometer using FACS Diva software (BD Biosciences). For this analysis, at least 1×10^6 events were acquired per sample and a median of 4.72×10^5 live T-cells were gated. Data were analysed using the FlowJo 10.7.1 software (Treestar, Ashland, OR).

2.5 | Cytokine quantification

Cytokine levels were assayed in plasma samples using three different kits. sCD25 were measured by Human CD25/IL-2R alpha Quantikine ELISA Kit (R&D System, Cat# DR2A00), using 1:2 plasma dilution; and IP-10 by Human IP-10 ELISA Kit (CXCL10) (Abcam, Cat# ab173194), where plasma was diluted from 1:2 to 1:4. In order to quantify IL-6, IL-8, IL-1β, TNF-α, IFN-γ, MIP-1α, MIP-1β, MILLIPLEX MAP Human High Sensitivity T Cell Panel (Merck Cat# HSTCMAG-28SK) were used, where plasma was diluted 1:2. Samples were assayed in duplicate. All of these kits were utilised according to the manufacturer's instructions.

2.6 | Quantification of anti-S SARS-CoV-2 and endemic coronaviruses IgG antibodies

Anti-S IgG SARS-CoV-2 and endemic coronaviruses (NL63, OC43, 229E and HKU1) levels were measured by ELISA as previously described.[4,16,24,25] Briefly, Nunc Maxisorp flat-bottomed 96-well plates (ThermoFisher Scientific #3690) were coated with 1μg/ml of recombinant SARS-CoV-2 (Sino Biological, #40589-V08B1), NL63 (Sino Biological, #40604-V08B), OC43 (Sino Biological, #40607-V08B), 229E (Sino Biological, #40605-V08B) and HKU1 (Sino Biological, #40606-V08B) Spike protein, overnight at 4°C. The following day, plates were blocked with 3% milk in PBS containing 0.05% Tween-20 for 120 min at RT. Plasma samples were heat inactivated at 56°C for 45 min. Plasma was diluted 1:50 for endemic coronaviruses and 1:50 or 1:100 for SARS-CoV-2 in 1% milk containing 0.05% Tween-20 in PBS and incubated for 90 min at RT. Plates were washed four times with 0.05% PBS-Tween-20. Secondary antibodies, streptavidin-horseradish peroxidase-conjugated mouse anti-human IgG (Hybridoma Reagent Laboratory, Baltimore, MD, #HP6043-HRP) was used at 1: 2,000 dilutions in 1% milk containing 0.05% Tween-20 in PBS. Plates were washed four times with 0.05% PBS-Tween-20. The plates were developed using fast o-phenylenediamine Peroxidase Substrate (Merck, #P9187), the reaction was stopped using 1M HCl, and the optical density (OD) at 490 nm (OD490) was read on a Multiskan GO Microplate Spectrophotometer (ThermoFisher Scientific) within 2 h. Two technical replicates were performed per sample. In order to validate the assays, stringent cutoff value of the SARS CoV-2 S specific IgG signal was determined as the average of the OD of plasma samples collected from pre-COVID HDs plus the SD multiplied by the factor 3, based on readings obtained from 21 serum samples of HDs (negative controls).[26] The cutoff value was found to be 0.23 (mean = 0.066, SD = 0.056). Due to detectable S-IgG levels of plasma samples from pre-COVID HDs using the recombinant S protein from the human endemic coronaviruses, the cut-off values for HCoV-NL63, -OC43, -229E and -HKU1 ELISAs were set at arbitrary value = blank mean + 3SD). The cutoff value was found to be 0.06 (mean = 0.06, SD = 0.001) for HCoV-NL63; 0.07 (mean = 0.06, SD = 0.003) for HCoV-OC43; 0.09 (mean = 0.08, SD = 0.004) for HCoV-229E and 0.07 (mean = 0.07, SD = 0.001) for HCoV-HKU.

2.7 | Statistical analysis

Non-parametric statistical analyses were performed using Statistical Package for the Social Sciences software (SPSS 25.0; SPSS, Inc., Chicago, IL), RStudio Version 1.3.959 and GraphPad Prism version 8.0 (GraphPad Software, Inc.). Polyfunctionality was defined as the percentage of lymphocytes producing combinations of cytokines (IL-2, TNF-α and IFN-γ), the degranulation marker CD107a and perforin (PRF). The simultaneous expression of the three cytokines, were also named as three functions, plus CD107a and/or PRF, as four and five functions, respectively. Polyfunctionality pie charts were constructed using Pestle version 1.6.2 and Spice version 6.0 (provided by M. Roederer, NIH, Bethesda, MD) and was quantified with the polyfunctionality index algorithm[27] employing the 0.1.2 beta version of the FunkyCells Boolean Dataminer software provided by Martin Larson (INSERM U1135, Paris, France). Median and interquartile ranges were used to describe continuous variables and percentages to describe categorical variables. The ROUT method was utilised to identify and discard outliers. Differences between different groups were analysed by two-tailed Mann–Whitney *U*-test. The Wilcoxon signed-rank test was used to analyse paired samples. Categorical variables were compared using the χ^2 test or the Fisher's exact test. The Spearman test was used to analyse correlations between variables. All differences with a *P*-value of < 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Hospitalised patients with acute SARS-CoV-2 infection showed an altered T-cell phenotypic profile

Patients hospitalised with acute SARS-CoV-2 infection showed higher CD4+ and lower CD8+ T-cells levels compared with sex- and age-matched pre-COVID-19 HDs, which resulted in higher CD4:CD8 T-cell ratio (Figure 1A, left panel). SARS-CoV-2 infection was also associated with lower EM and TEMRA CD4+ T-cell levels (Figure 1A, middle panel), while no differences were observed in CD8+ T-cell subset levels (Figure S1A). CD4:CD8 TEMRA ratio was lower in acute COVID-19 patients compared with HD (Figure 1A, right panel). Analyses of T-cell activation by HLA-DR and CD38 co-expression revealed higher levels in all of CD8+ T-cell subsets and TEMRA CD4+ T-cells in SARS-CoV-2 infected patients (Figure 1B). This was also observed for CD38 single expression in all CD8+ T-cell subsets (Figure S1B) but not for HLA-DR single expression (Figure S1C,D). The levels of senescent CD4+ (CD57+CD28−), but not CD8+ T-cell subsets, were lower in acute infection (Figure 1C). However, T-cell exhaustion, assayed by PD-1 and TIGIT expression and co-expression of both markers, was higher in acute SARS-CoV-2 infection in most of the T-cell subsets (Figure 1D–F).

3.2 | Characteristics of SARS-CoV-2 specific T-cell response in acute hospitalised patients and healthy donors

We assayed SARS-CoV-2 specific T-cell response by intracellular cytokine staining (ICS), this technique is a well-established method for evaluating virus-specific T-cell response.[22,28] ICS, despite of using a high number of cells, allowed us to get more information about several cytokines to assay the magnitude and quality of the T-cell response. We assessed CD4+ (CD3+CD8−) and CD8+ T-cell response specific to spike (S) and nucleocapsid (N) peptide pools. The specific T-cell response to each stimuli was determined by the sum of the expression of each assayed cytokine (IFN-γ, IL-2 and TNF-α). To classify an individual as a responder, we consider a threshold higher than 0.05%, as previously published.[22] First, as expected, we observed a higher magnitude of the response in most of T-cell subsets for both peptide pools (N and S) in hospitalised acute SARS-CoV-2 infected patients (acute) compared to HD samples (Figure 2A,B, top panels). However, there were differences neither in the magnitude of the response nor in the proportion of responders in the TEMRA subset for both CD4+ and CD8+ T-cells and for S and N stimuli (Figure 2A,B). In fact, there were no differences in the levels of responders for all CD8+ T-cell subsets for N peptides (Figure 2B, bottom panels). Overall, 75% and 82% of HD had SARS-CoV-2 specific CD4+ and CD8+ T-cell response, respectively, considering S+N peptides and all T-cell subsets (Figure 2C,D).

Second, comparing the response to S and N peptide pools, there was a higher magnitude of response to S compared to N stimulus in CM CD4+ T-cell ($p = 0.042$) and a trend in MEM CD4+ T-cells ($p = 0.126$) (Figure 2A, top panels), however there were no differences for CD8+ T-cell subsets (Figure 2B, top panels). Additionally, a cumulative SARS-CoV-2-specific T-cell measurement was calculated as the sum of the S and N responses (Figure S2). Our data show that all the patients had detectable SARS-CoV-2 specific T-cell response considering together the response against S and N peptide pools and to all the CD4+ and CD8+ T-cell subsets (Figures 2C,D, Figure S2).

Finally, when comparing CD4+ and CD8+ T-cell response in hospitalised patients (acute), the magnitude of SARS-CoV-2 specific MEM CD4+ T-cell response (Figure 2A, top panel) was higher compared MEM CD8+

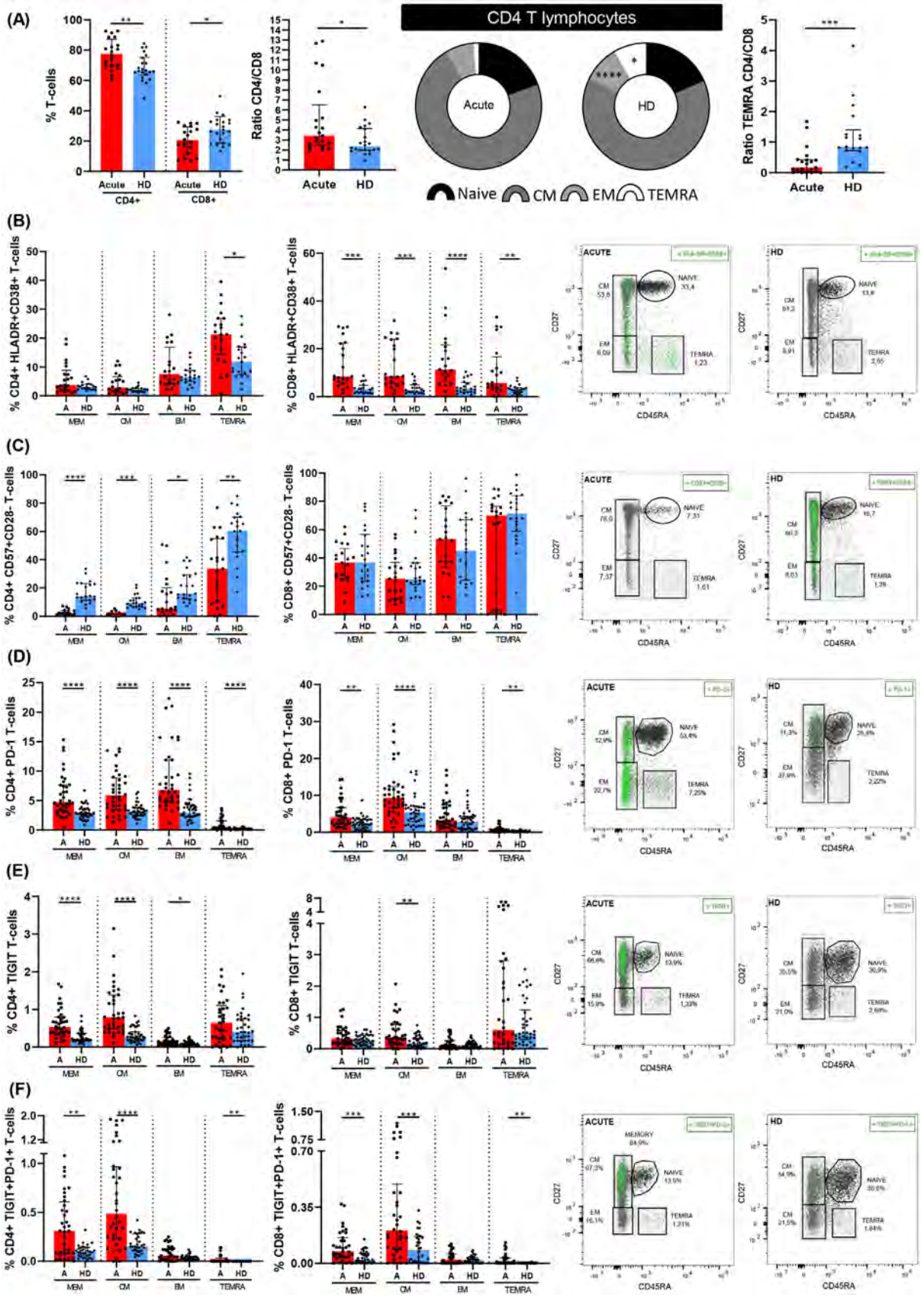


FIGURE 1 Altered CD4+ T-lymphocyte maturation phenotype and markers of T-cell activation, senescence and exhaustion in patients with acute SARS-CoV-2 infection. (A) Bar graphs representing the percentage of total CD4+ and CD8+ T cells (left panel); ratio between CD4+ and CD8+ (middle panel); and, CD4:CD8 ratio in TEMRA T-cell subset (right panel). Pie graphs show medians of each CD4+ T-cell

T-cell response (Figure 2B, top panel) for protein S ($p = 0.048$), but not different for the rest of subsets and for protein N (Figure 2A,B, top panels). In the same way, there was a higher percentage of responders for MEM CD4+ T-cells compared to MEM CD8+ T-cells in S protein ($p = 0.025$), there were no differences in the proportion of responders for the rest of subsets for S and N proteins (Figure 2A,B, bottom panels).

3.3 | IFN-γ and IL-2 polyfunctional response in S-specific CD4+ T-cells was differentially associated with disease severity while IL-2 production in S-specific CD8+ T-cells was associated with mild disease

We next analysed in acute infection the association of S-specific CD4+ T-cell response with disease severity in hospitalised patients segregated as mild and severe patients. The S-specific CD4+ T cell response was significantly higher in the TEMRA CD4+ T-cell subset in severe compared to mild patients (Figure 3A). When individual cytokine production was analysed, these higher levels were attributed to IFN-γ production in S-specific TEMRA CD4+ subset (Figure 3B; Figure S3A), but not for IL-2 or TNF-α (Figure S3B). Multiple combination of cytokines, together with CD107a and perforin expression revealed that combinations only including IFN-γ+ CM (Figure 3C, Figure S3C) and TEMRA cells (Figure S3D) were increased in severe compared with mild patients. The same occurred for combinations including IFN-γ+ and TNF-α+ CM cells (Figure S3E). However, combinations including IL-2, such as IL-2+TNF-α+ MEM cells were increased in mild compared to severe patients (Figure 3D). In fact, S-specific MEM CD4+ T-cell polyfunctionality was higher in mild patients, mainly because of increased bifunctional combinations including IL-2 (IL-2+TNF-α+, IL-2+IFN-γ+ and IL-2+perforin+) that were not present in severe patients, where IFN-γ+TNF-α+ combination was predominant (Figure 3E). It is also important to highlight that perforin expression was higher in MEM and EM subsets of mild patients in comparison with severe patients (Figure S3F). This was reflected in a higher S-specific MEM polyfunctional index in mild compared to severe patients (Figure 3F). Additionally, we observed that the bulk of S-specific CD4+ T-cell response in the different subsets was inversely associated with different inflammatory markers (Figure S4A,B, Table S2), while specific combinations including IFN-γ were directly associated with plasmatic IP-10 levels (Figure S4A–C, Table S2). Overall, a high polyfunctional S-specific CD4+ T-cell response biased to IL-2 production was associated with mild disease, while combinations only including IFN-γ were associated with severe disease outcomes.

In relation to S-specific CD8+ T-cells, the bulk of CM CD8+ T-cell response was higher in mild compared to severe patients (Figure 3G). We observed that the cytokine responsible of these differences was IL-2, which presented higher levels in MEM, CM and EM S-specific CD8+ T-cells in mild subjects (Figure 3H; Figure S5A), while very low levels and no differences were observed in IFN-γ+ and TNF-α+ production (Figure S5B). These results were confirmed by combinations only including IL-2 and with no expression of the rest of the cytokines, CD107a and perforin, in the same subsets: MEM, CM and EM (Figure 3I). Similar results were observed for three and four functions (Figure S5C). In summary, IL-2 production in not terminally differentiated S-specific CD8+ T-cells was associated with mild disease progression in hospitalised acute SARS-CoV-2 infected patients.

3.4 | Polyfunctional N-specific CD4+ T-cell response was associated with mild disease in acute SARS-CoV-2 hospitalised patients

We also analysed in detail the quality of N-specific T-cell response. MEM and CM IL-2+ and EM TNF-α+ N-specific CD4+ T cell levels were higher in mild compared to severe patients (Figure 4A,B). We did not observe differences for the bulk of IFN-γ+ N-specific CD4+ T-cell response (Figure S6A,B). Following the same profile of S-specific CD4+ T-cell response, combinations including only IL-2+ and TNF-α+ in MEM, CM and EM N-specific

subset in acute SARS-CoV-2 infected individual (acute) and healthy donor (HD) groups. Each subset from both groups were compared. Next bar graphs together with the representative dot-plots of each group mentioned above show the expression of each biomarker: (B) HLA-DR+CD38+ (activation marker); (C) CD57+CD28- (immune senescence marker); (D) PD-1+; (E) TIGIT+ and (F) PD-1+TIGIT+ T-cells (exhaustion markers). Total memory T-cell subset includes central memory T-cells (CM), effector memory T-cells (EM) and terminally differentiated effector memory T cells (TEMRA) subsets. The medians with the interquartile ranges are shown. For dot-plots, green points are positive events of each biomarker. ROUT method was utilised to identify and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Mann–Whitney U-test was used for groups' comparisons and Spearman test for non-parametric correlations. Categorical variables were compared using the $\chi 2$ test or the Fisher's exact test. (Acute, $n = 37$; HD, $n = 33$)

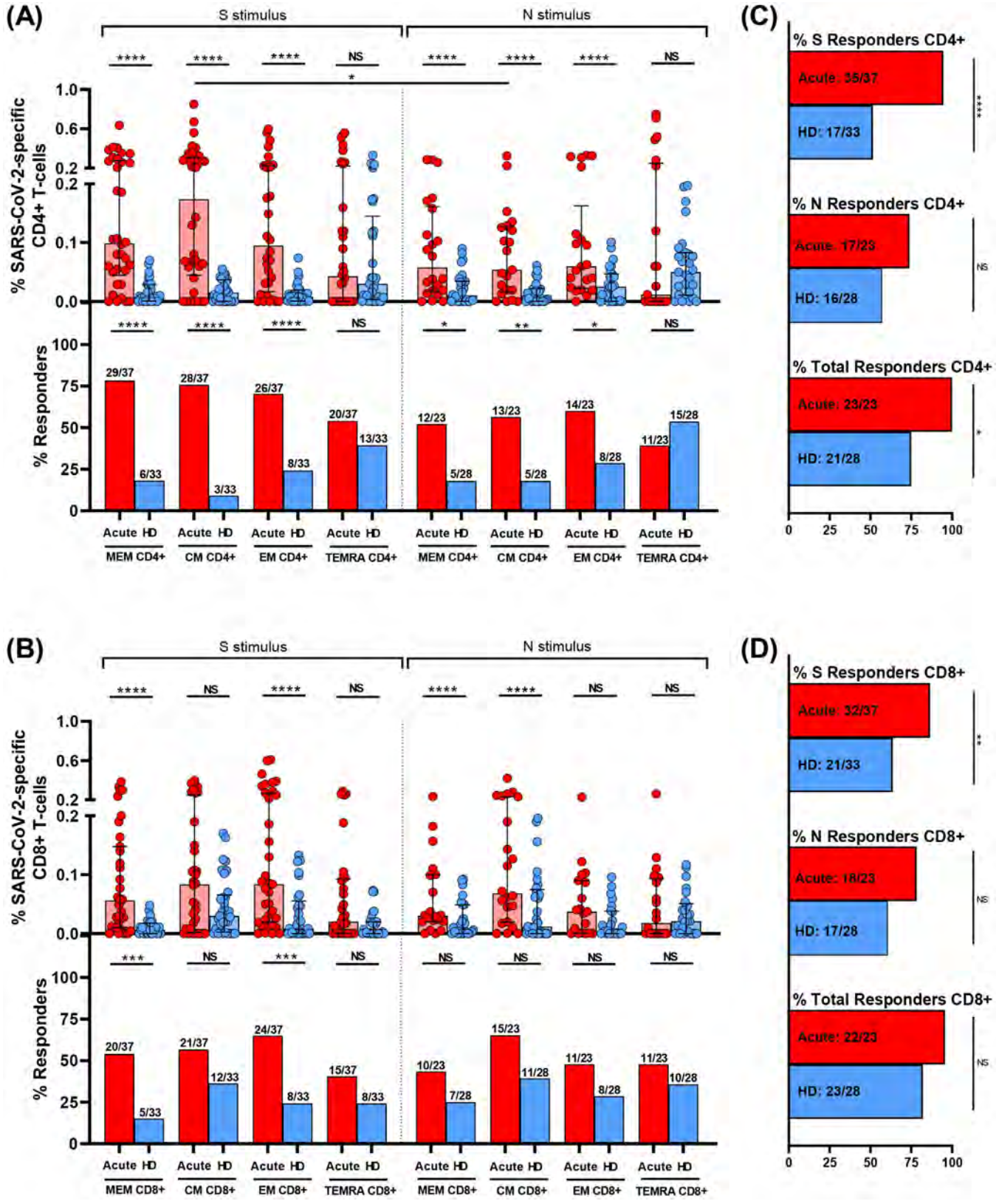


FIGURE 2 S and N specific CD4+ and CD8+ T-cell response in acute SARS-CoV-2 infection individuals and healthy donors. (A,B) Bar graphs in top panels represent percentage of S and N specific CD4+ and CD8+ T-cell response in SARS-CoV-2 infected patients (red) and healthy donors (blue) (upper panels). Bar graphs in low panels also show the number and percentage of responders, considering a responder subjects as those with the percentage of SARS-CoV-2-specific T-cells higher than 0.05% considering the sum of IFN-γ, TNF-α and IL-2 production. (C,D) Bar graphs describe the number and percentage of responders for S peptide pool, as the sum of any CD3+CD4+ or

CD4+ T-cells were associated with mild disease progression (Figure 4C). Besides, we observed higher levels of combinations with triple cytokine positive MEM, CM and EM CD4+ T-cells in mild compared to severe patients (Figure 4D). In fact, MEM N-specific response showed a higher proportion of triple and a variety of double combinations (Figure 4E), likewise a higher MEM polyfunctional index in mild compared to severe patients (Figure 4F). These results were reproduced in polyfunctionality of CM and EM subsets with three and four functions that were also associated with mild disease progression (Figure S6C). We did not observe great differences in N-specific CD8+ T-cell response according with disease severity, only higher levels of combinations with only IFN-γ+ T-cells in severe compared to mild patients (Figure S6D).

3.5 | Similar magnitude of SARS-CoV-2 specific T-cell response in previously hospitalised and non-hospitalised patients seven months after infection

In addition to the analyses in the acute phase, we analysed the magnitude of SARS-CoV-2 specific T-cell response seven months after SARS-CoV-2 infection in two group of individuals: (i) those previously hospitalised during acute infection and (ii) without previous hospitalisation. First, we analysed CD4+ T-cell response. No differences were observed in the magnitude of N- and S-specific T-cell response, except for higher S-specific TEMRA CD4+ T-cell levels in non-hospitalised patients compared to previously hospitalised patients (Figure 5A, top panel). Despite the 18% lower frequency of TEMRA CD4+ T-cells in previously hospitalised responders, this difference was not statistically significant (Figure 5A, bottom panel). No differences in the proportion of responders were observed in the rest of subsets and neither for S- nor N-protein (Figure 5A, bottom panel). Considering the cumulative SARS-CoV-2-specific CD4+ T-cell response (sum of the S and N response, Figure S7A) in all the T-cell subsets, all patients, with the exception of one in the group of previously hospitalised patients, had detectable CD4+ T-cell response (Figure 5B). We also compared the magnitude of S- versus N-specific CD4+ T-cell response in both groups. We found that the magnitude of MEM and CM response was higher in S compared to N in both, previously hospitalised ($p = 0.008$; $p = 0.008$, respectively) and non-hospitalised patients ($p = 0.014$; $p = 0.009$, respectively) 7 months after SARS-CoV-2 infection (Figure 5A, top panel). Second, we analysed the magnitude of SARS-CoV-2 specific CD8+ T-cell response and we found that previously hospitalised patients presented higher S-specific EM T-cells compared to non-hospitalised patients (Figure 5C, top panel). There were no differences for the rest of subsets or stimuli between both groups (Figure 5C, top panel). We also did not find differences in the percentage of responders (Figure 5C, bottom panel). The analysis of the cumulative SARS-CoV-2-specific CD8+ T-cell response (Figure S7B) of previously hospitalised and non-hospitalised, showed that 88.9% and 92.8%, respectively, had detectable SARS-CoV-2-specific CD8+ T-cell response considering all T-cell subsets and S or N stimuli (Figure 5D). Furthermore, we found that the magnitude of MEM and EM response was higher in N compared to S peptides in non-hospitalised patients ($p = 0.026$; $p = 0.024$, respectively), while no differences were found in previously hospitalised patients 7 months after SARS-CoV-2 infection (Figure 5C). Finally, we compared the magnitude of response between CD4+ and CD8+ T-cells. We found that in both groups, MEM and CM S-specific CD4+ T-cell response was higher than in CD8+ T-cells ($p = 0.005$ and $p = 0.036$ for previously hospitalised; $p = 0.004$ and $p = 0.013$ for non-hospitalised, respectively), while no differences were found for N stimulus (Figure 5A–C).

3.6 | Higher CD4+TIGIT+ T-cell and differential quality of S-specific CD4+ CM T-cell levels in previously hospitalised compared to non-hospitalised patients 7 months after infection

After finding a similar magnitude of SARS-CoV-2 specific T-cell response in both groups of individuals 7 months after infection, we assayed the quality of T-cell response and exhaustion markers in previously hospitalised and non-hospitalised patients. The TIGIT expression in all the CD4+ T-cell subsets were higher in previously hospitalised than in non-hospitalised patients (Figure 6A). We did not find differences between groups in TIGIT+ CD8+ T-cells (Figure S8A). Likewise, PD-1 expression was similar in all T-cell subsets in both groups (Figure S8B,C). When

CD3+CD8+ T-cell subset (%S Responders); for N peptide pool, as the sum of any CD3+CD4+ or CD3+CD8+ T-cell subset (%N responders) and the total responders as the sum of CD3+CD4+ or CD3+CD8+ S and N responses (% of total responders). The medians with the interquartile ranges are shown. Each dot represents an individual. ROUT method was utilised to identify and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Mann–Whitney U-test was used for groups' comparisons. Categorical variables were compared using the $\chi 2$ test or the Fisher's exact test. (Acute, $n = 37$; HD, $n = 33$)

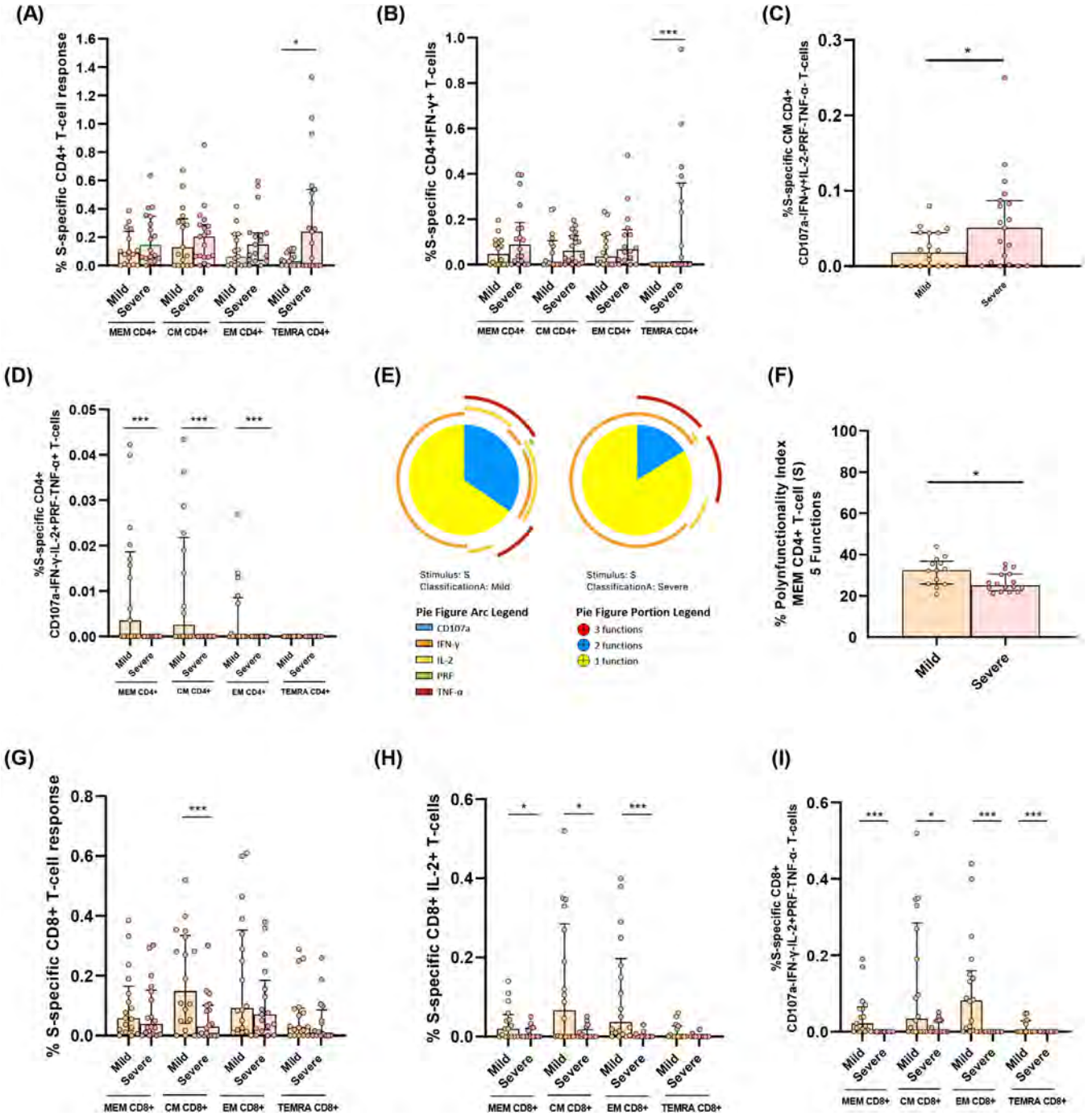


FIGURE 3 S-specific CD4+ and CD8+ T-cell response is associated with disease severity in acute SARS-CoV-2 infection. (A) Bar graphs show S-specific CD4+ T-cell response, considering the sum of IFN-γ, TNF-α and IL-2 production, in the different CD4+ T-cell subsets, in mild and severe acute patients' groups. (B) S-specific CD4+ T-cell response considering the levels of cells producing IFN-γ. (C) S-specific CM CD4+ T-cell levels of combinations only including IFN-γ+ cells for five (IFN-γ, TNF-α, IL-2, CD107a and PRF) functions. (D) S-specific CD4+ T-cell levels in the different T-cell subsets of combinations including IL-2+ and TNF-α+ cells for five (IFN-γ, TNF-α, IL-2, CD107a and PRF) functions. (E) S-specific MEM CD4 T-cell polyfunctionality pie charts. Each sector represents the proportion of S-specific CD4 T-cells producing two (blue) and one (yellow) function. Arc represents the type of function (IFN-γ, TNF-α, IL-2, CD107a and PRF) expressed in each sector. Permutation test, following the Spice version 6.0 software was used to assess differences between pie charts. (F) Polyfunctional index bar graph of S-specific MEM CD4+ polyfunctionality, for five functions. (G) Bar graphs show S-specific CD8+ T-cell response, considering the sum of IFN-γ, TNF-α and IL-2 production, in the different CD8+ T-cell subsets, in mild and severe acute patients' groups, (H) S-specific CD8+ T-cell response considering the levels of cells producing IL-2 and (I) S-specific CD8+ T-cell levels of combinations only including IL-2+ cells for five (IFN-γ, TNF-α, IL-2, CD107a and PRF) functions. The medians with the interquartile ranges are shown. Each dot represents a patient. ROUT method was utilised to identify and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Mann–Whitney U test was used for groups' comparisons. (Mild, $n = 18$; severe, $n = 19$)

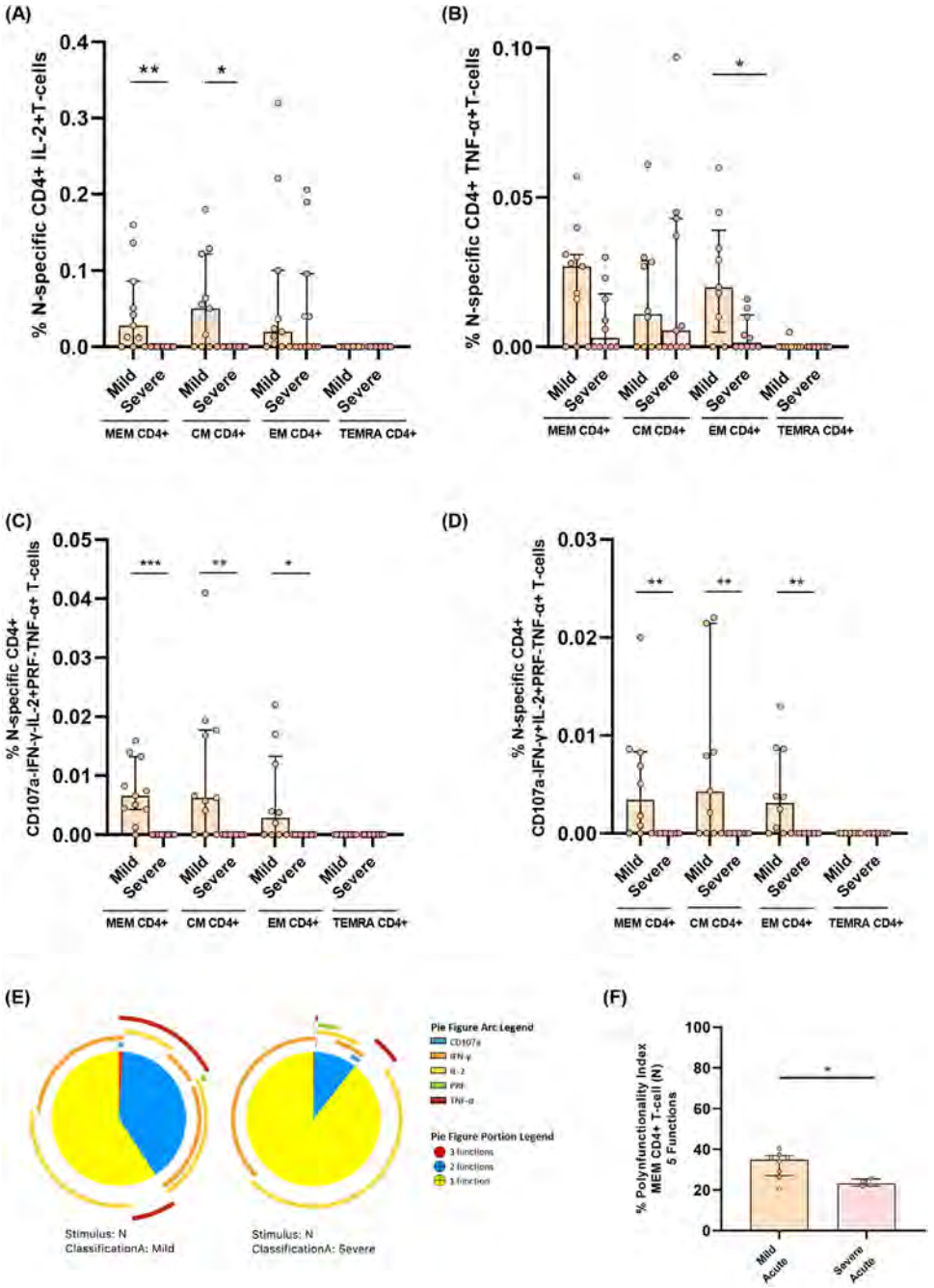


FIGURE 4 Cytokine combinations and polyfunctional N-specific CD4+ T-cell response are associated with COVID-19 progression. (A) N-specific CD4+ T-cell response considering the levels of cells producing IL-2 in the different CD4+ T-cell subsets, in mild and severe acute patients' groups. (B) N-specific CD4+ T-cell response considering the levels of cells producing TNF-α in the different CD4+ T-cell subsets, in mild and severe acute patients' groups. (C) N-specific CD4+ T-cell levels in the different T-cell subsets of combinations including IL-2+ and TNF-α+ cells for five (IFN-γ, TNF-α, IL-2, CD107a and PRF) functions. (D) N-specific CD4+ T-cell levels in the different T-cell subsets of combinations including IL-2+, TNF-α+ and IFN-γ+ T-cells for five (IFN-γ, TNF-α, IL-2, CD107a and PRF) functions. (E) N-specific MEM CD4 T-cell polyfunctionality pie charts. Each sector represents the proportion of N-specific CD4+ T-cells producing three (red), two (blue) and one (yellow) function. Arc represents the type of function (IFN-γ, TNF-α, IL-2, CD107a and PRF) expressed in each sector. Permutation test, following the Spice version 6.0 software was used to assess differences between pie charts. (F) Polyfunctional index bar graph of N-specific MEM CD4+ polyfunctionality, for five functions. The medians with the interquartile ranges are shown. Each dot represents a patient. ROUT method was utilised to identify and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Mann–Whitney U-test was used for groups' comparisons. (Mild, $n = 11$; severe, $n = 11$)

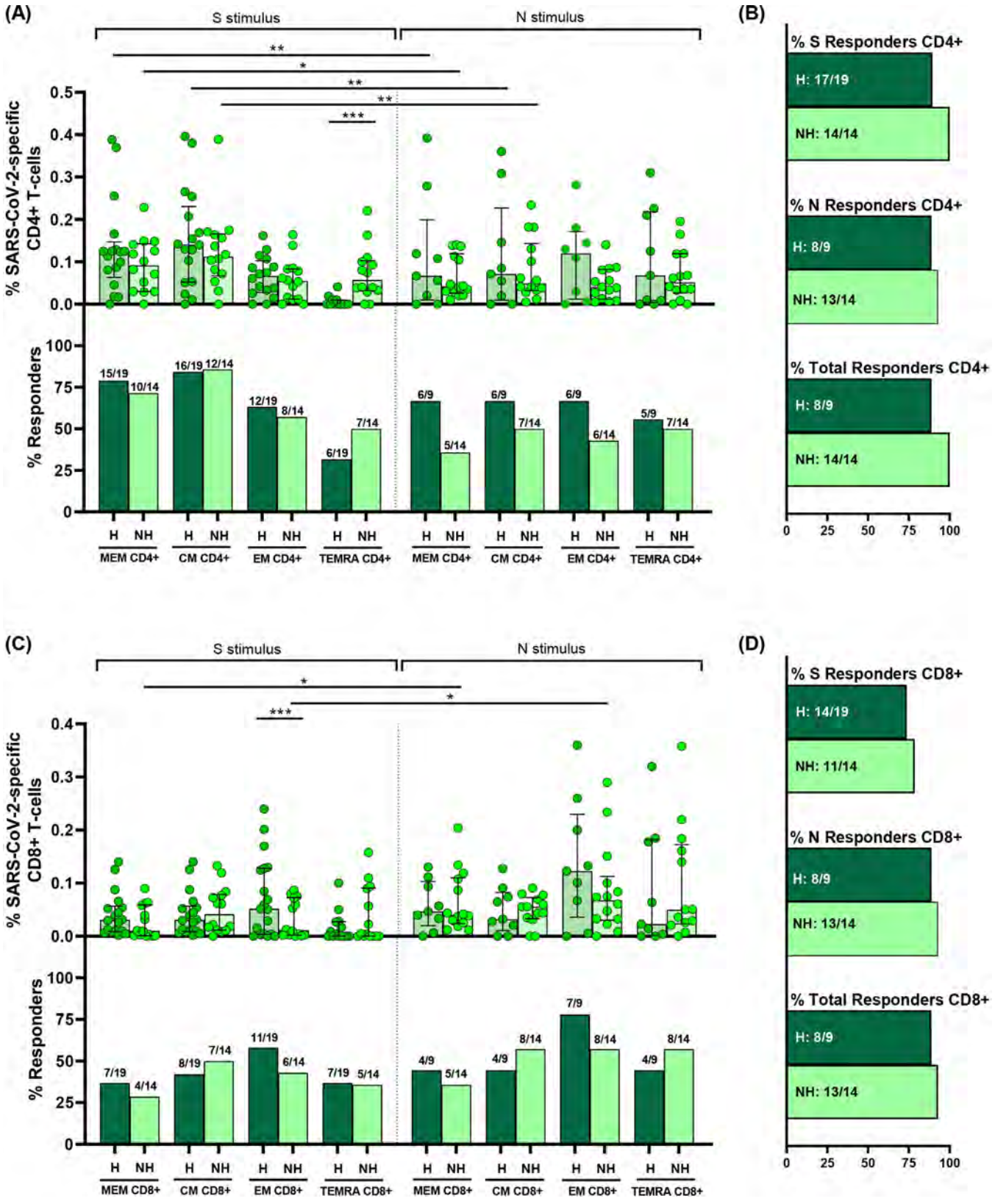


FIGURE 5 S and N specific CD4+ and CD8+ T-cell response are present in previously hospitalised (H) and non-hospitalised (NH) patients 7 months after SARS-CoV-2 infection. (A,C) Bar graphs represent percentage of S and N specific CD4+ and CD8+ T-cell response in previously hospitalised (H) (dark green) and non-hospitalised (NH) (light green) subjects (top panels). Bar graphs also show the number and percentage of responders, considering a responder subjects as those with the percentage of SARS-CoV-2-specific T-cells higher than 0.05% considering the sum of IFN-γ, TNF-α and IL-2 production (bottom panels). (B,D) Bar graphs describe the number and percentage of

we analyse multiple combination of cytokines, previously hospitalised patients showed higher levels of S-specific CM CD4+ T-cell with combinations only including TNF-α (Figure 6B, left panel; Figure S8D). Furthermore, S-specific CM CD4+ T-cell response was more polyfunctional in non-hospitalised patients compared with those previously hospitalised (Figure 6B, right panel). In the same line, N-specific T-cell response also contained higher levels of combinations only including IFN-γ for EM CD4+ T-cells and only including TNF-α for CM CD8+ T-cells in previously hospitalised than in non-hospitalised patients (Figure 6C,D; Figure S8E,F). However, non-previously hospitalised convalescent patients showed N-specific CM and EM CD8+ T-cells with higher production of IL-2 (Figure 6D,E, right panel) and perforin (Figure 6F), respectively.

3.7 | Anti-S IgG levels were associated with disease severity and differentially with SARS-CoV-2-specific T-cell response in acute and convalescent subjects seven months after infection

Next, we analysed antibody levels against S protein and the association of this humoral response with disease severity and T-cell immunity. In acute infection, we observed a trend to increased antibody levels in severe compared to mild patients (Figure 7A). Seven months after SARS-CoV-2 infection anti-S IgG levels remained high, similar to severe patients in acute infection and at higher levels compared to mild patients in both previously hospitalised and in non-hospitalised patients (Figure 7A). As expected, all the groups had higher antibody levels compared to HD (Figure 7A). In relation to T-cell response, in general, SARS-CoV-2 specific T-cell response was inversely associated with anti-S IgG levels in acute infection (Figure S9A), while a direct correlation was observed 7 months after infection (Figure S9B). A representative example was the moderated inverse correlation of S-specific EM CD4+ T-cell producing IL-2 in acute infection (Figure 7B) compared to the direct correlation found 7 months after infection (Figure 7C).

3.8 | Heterologous SARS-CoV-2 response was associated with the magnitude and the quality of endemic coronavirus response

Similar to previously reported,[21] we found that a high percentage of HD (pre-COVID-19 samples) presented detectable CD4+ and CD8+ SARS-CoV-2 specific T-cell response (75% and 82%, respectively, Figure 2). In order to characterise this immune response, we performed anti-S IgG levels and specific T-cell response by ICS using an optimised peptide pool for the four human endemic coronaviruses[21]. In acute SARS-CoV-2 infected participants we observed a direct correlation of anti-S SARS-CoV-2 IgG levels with those of three out of the four endemic coronaviruses (HCoV-NL63, -OC43 and -HKU1) (Figure S10, Table S3). When we split this group, we only found a positive correlation of anti-S SARS-CoV-2 IgG and anti-S HCoV-NL63 and -OC43 levels in severe (Figure S10, Table S3) but no correlation was found in mild patients (Figure S10, Table S3). In HD, we also found a positive correlation of anti-S SARS-CoV-2 IgG and anti-S HCoV-OC43, -229E and HKU-1 levels (Figure S10, Table S3). Finally, in all the groups together, anti-S SARS-CoV-2 IgG levels were directly associated with anti-S IgG levels of the beta-coronaviruses HCoV-OC43 and -HKU1 (Figure S10, Table S3). After that, we performed S-specific T-cell response to the optimised peptide pool of endemic coronaviruses (SE) in HD. We found detectable SE T-cell response in all the CD4+ and CD8+ T-cell subsets (Figure 8A). Analysing the bulk of SE T-cells reported higher levels of response in the TEMRA and CM subsets, in CD4+ and CD8+ T-cells, respectively. Overall, the proportion of responders was 55.6% for CD4+ and 72% for CD8+ T-cells and 80% the global response (Figure 8A). The response to human endemic coronaviruses correlated to S-specific for SARS-CoV-2 in CM and EM CD4+ subsets (Figure 8B,C) and in CM CD8+ subset (Figure 8D). Attending to the quality of this response, it was mainly monofunctional and IL-2 production prevailed in CD4+ and CD8+ T-cells respect to other cytokines (Figure 8E; Figure S11A). Interestingly, combinations including IL-2, but not IFN-γ, in response to human endemic coronaviruses correlated with S-specific SARS-CoV-2 response, for CD4+ MEM (Figure 8F), CM

responders for S peptide pool, as the sum of any CD3+CD4+ or CD3+CD8+ T-cell subset (% S responders); for N peptide pool, as the sum of any CD3+CD4+ or CD3+CD8+ T-cell subset (% N responders) and the total responders as the sum of CD3+CD4+ or CD3+CD8+ S and N responses (% of total responders). The medians with the interquartile ranges are shown. Each dot represents an individual. ROUT method was utilised to identify and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Mann–Whitney U-test was used for groups' comparisons. Categorical variables were compared using the $\chi 2$ test or the Fisher's exact test. (H, $n = 19$; NH, $n = 14$)

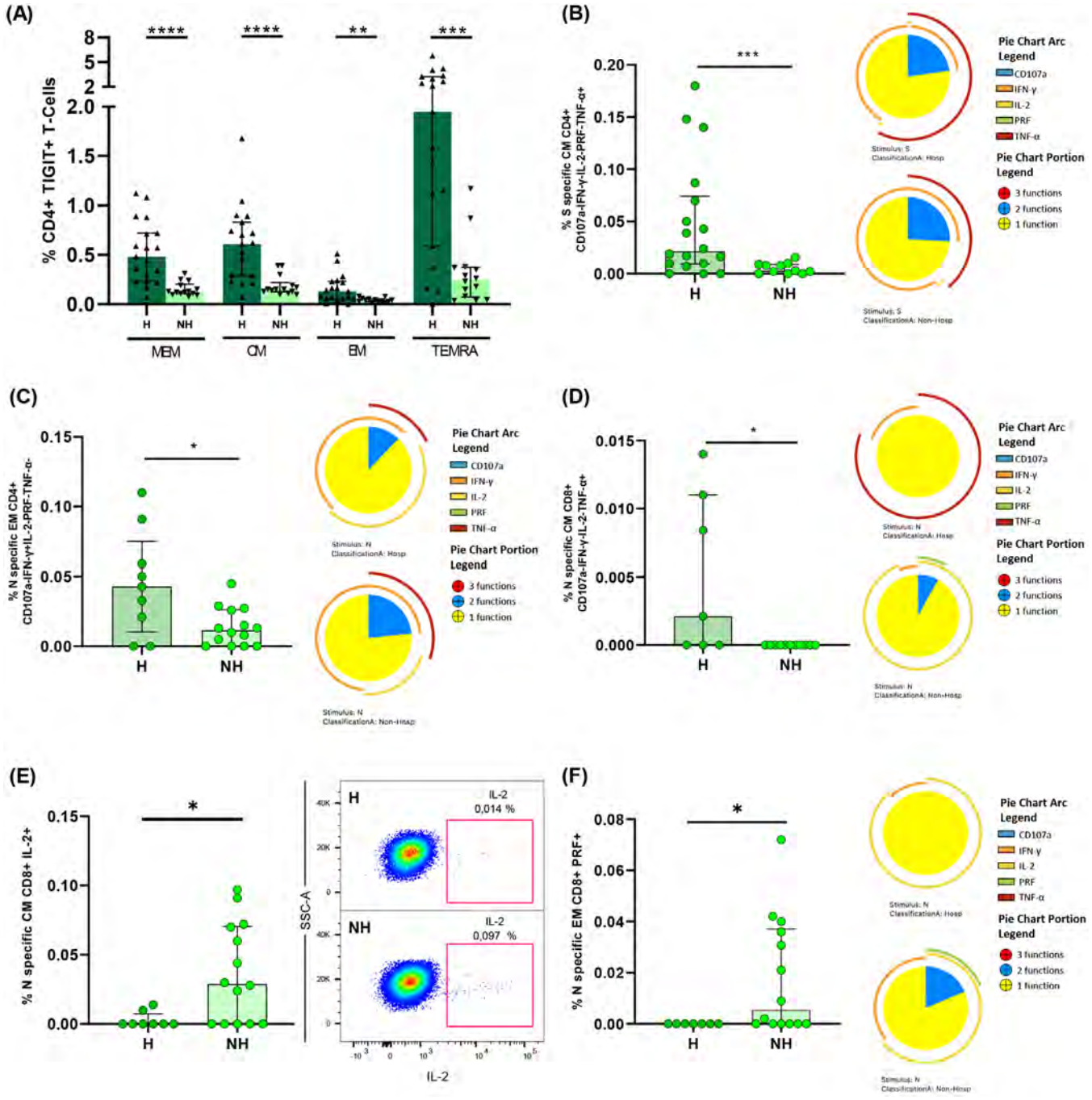


FIGURE 6 Previously hospitalised subjects during acute infection showed higher TIGIT+ CD4+ T-cell levels and lower polyfunctional S- and N-specific T-cell response than non-hospitalised subjects 7 months after SARS-CoV-2 infection. (A) TIGIT expression in each CD4+ T-cell subset in previously hospitalised and non-hospitalised subjects 7 months after SARS-CoV-2 infection. (B) S-specific CM CD4+ T-cell levels of combinations only including TNF-α+ cells for five (IFN-γ, TNF-α, IL-2, CD107a and PRF) functions (left panel). S-specific CM CD4 T-cell polyfunctionality pie charts (right panel). (C) N-specific EM CD4+ T-cell levels of combinations only including IFN-γ+ cells for five (IFN-γ, TNF-α, IL-2, CD107a and PRF) functions (left panel). N-specific EM CD4 T-cell polyfunctionality pie charts (right panel). (D) N-specific CM CD8+ T-cell levels of combinations only including TNF-α+ cells for five (IFN-γ, TNF-α, IL-2, CD107a and PRF) functions (left panel). N-specific CM CD8 T-cell polyfunctionality pie charts (right panel). (E) N-specific CM CD8+ T-cell levels of cells producing IL-2 (left panel). Representative dot plot showing IL-2 production in N-specific CM CD8+ T-cells (right panel). (F) N-specific EM CD8+ T-cell levels of cells producing PRF (left panel). N-specific EM CD8 T-cell polyfunctionality pie charts (right panel). For all the pie charts, each sector represents the proportion of SARS-CoV-2-specific T-cells producing two (blue) and one (yellow) function. Arc represents the type of function (IFN-γ, TNF-α, IL-2, CD107a and PRF) expressed in each sector. Permutation test, following the Spice version 6.0 software was used to assess differences between pie charts. Each dot represents an individual. ROUT method was utilised to identify and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Mann–Whitney U-test was used for groups' comparisons. (H, $n = 19$; NH, $n = 14$)

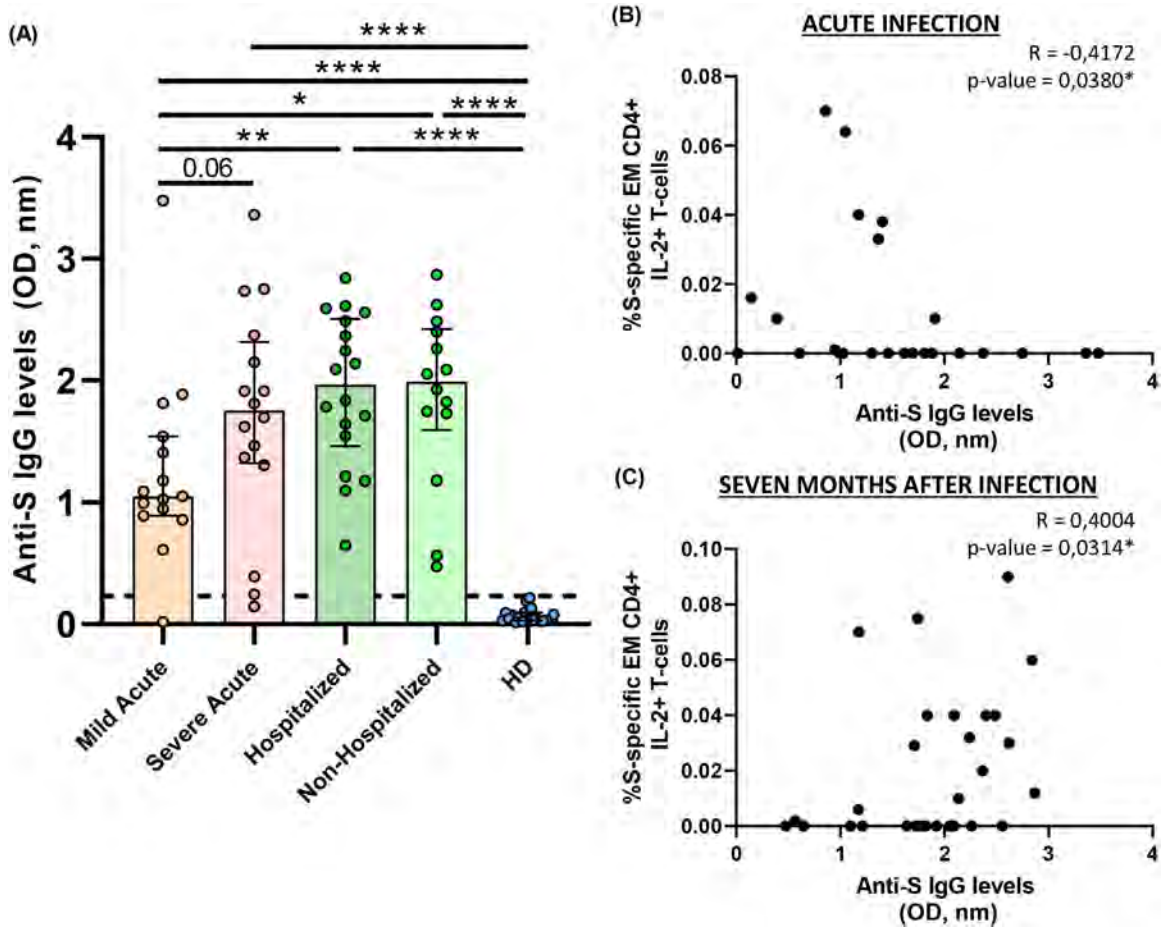


FIGURE 7 Anti-S IgG levels are associated with COVID-19 severity and correlate with S-specific T-cell response. (A) Bar graphs represent anti-S IgG levels in each study group. The dotted line indicates the cut-off value (0.23 OD, nm). (B) Correlation graphs between anti-S IgG levels and the percentage of S-specific EM CD4+ IL-2+ T-cell response in acute infection. (C) Correlation graphs between anti-S IgG levels and the percentage of S-specific EM CD4+ IL-2+ T-cell response previously hospitalised patients 7 months after SARS-CoV-2 infection. Plasma sample was used at 1:100 dilution. Each dot represents an individual. ROUT method was utilised to identify and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Mann–Whitney *U*-test was used for groups' comparisons and Spearman test for non-parametric correlations. (Mild acute, $n = 15$; severe acute, $n = 16$; hospitalised, $n = 18$; non-hospitalised, $n = 14$, HD, $n = 21$)

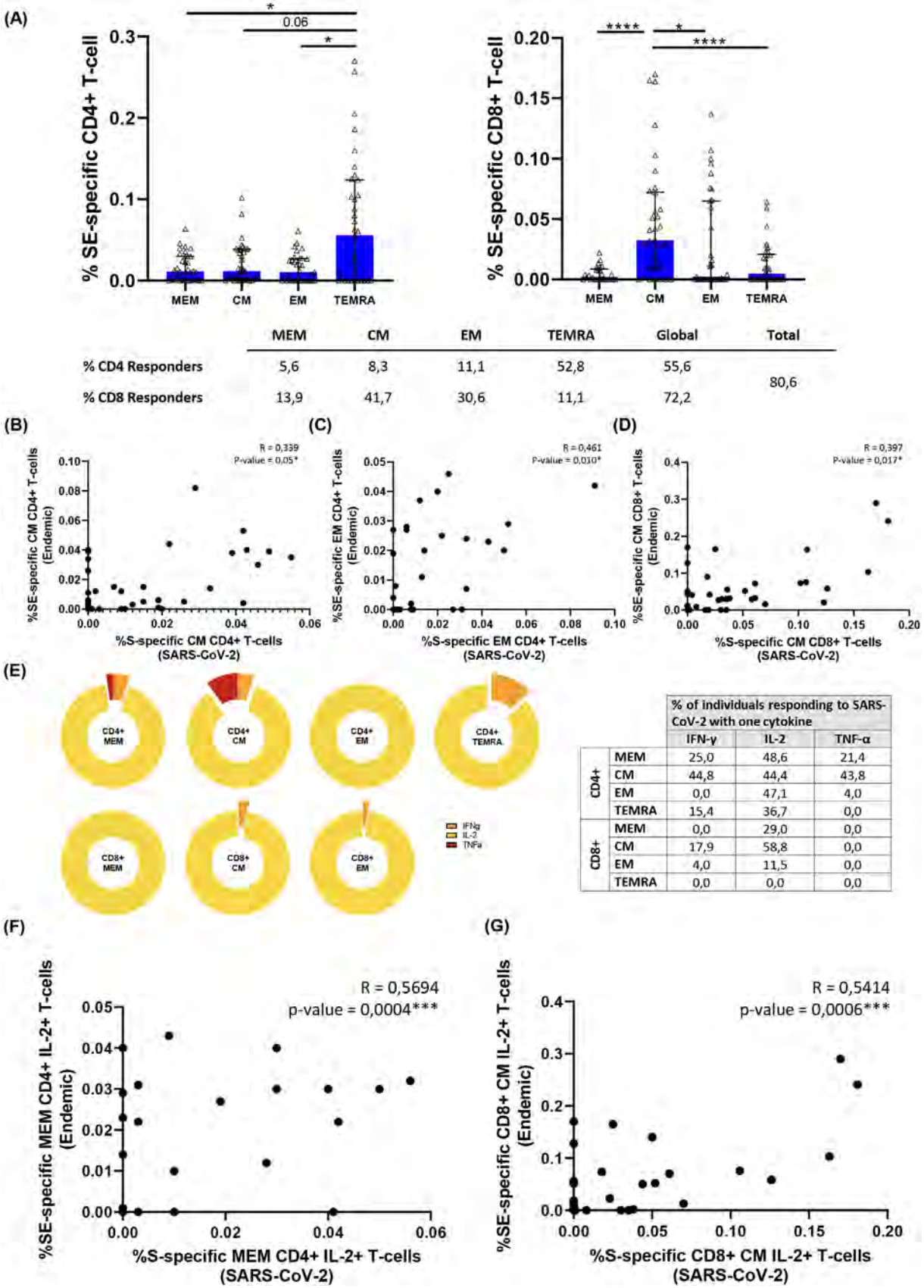
and EM subsets (Figure S11B,C) and CD8+ CM T-cells (Figure 8G).

4 | DISCUSSION

In the present study, analysing 103 subjects, we describe features of SARS-CoV-2 specific humoral and T-cell response differentially associated with disease severity in hospitalised patients during acute infection. This response is long-lasting seven months after infection independently whether patients were previously hospitalised or not, although previous hospitalisation was associated with exhausting T-cell features present in acute infection. Finally, we comprehensively analysed the features of the high levels of cross-reactive response between SARS-CoV-2 and human endemic coronaviruses in HDs.

We used ICS for the systematic analysis of SARS-CoV-2 specific T-cell response. ICS is a technique commonly used for analysing T-cell response against viral infections[22,28] and can be complementary to other strategies as T-cell receptor dependent activation induced marker (AIM).[16,29,30] Although a high amount of cells is needed, a comprehensive cytokine-dependent functional characterisation of virus specific T-cell response can be achieved.[5] We analysed the response against protein S and N, because these are the main targets, in terms of magnitude, of SARS-CoV-2 specific T-cell response.[16]

First, we found that all patients in acute infection, independently of disease severity, had detectable SARS-CoV-2 specific T-cell response, as a summation of S+N response and considering all CD4+ and CD8+ T-cell subsets. These data were remarkable based on the high activation and inhibitory receptor T-cell levels found in the present study



	MEM	CM	EM	TEMRA	Global	Total
% CD4 Responders	5,6	8,3	11,1	52,8	55,6	80,6
% CD8 Responders	13,9	41,7	30,6	11,1	72,2	

		% of individuals responding to SARS-CoV-2 with one cytokine		
		IFN-γ	IL-2	TNF-α
CD4+	MEM	25,0	48,6	21,4
	CM	44,8	44,4	43,8
	EM	0,0	47,1	4,0
	TEMRA	15,4	36,7	0,0
CD8+	MEM	0,0	29,0	0,0
	CM	17,9	58,8	0,0
	EM	4,0	11,5	0,0
	TEMRA	0,0	0,0	0,0

FIGURE 8 Characteristics of the cross-reactive T-cell response quality between SARS-CoV-2 and endemic coronaviruses. (A) Bar graph represents the SE-specific T-cell response in each CD4+ (left panel) and CD8+ (right panel) T-cell subsets. The table shows the percentage of responders considering a responder subjects as those with the percentage of SE-specific T-cells higher than 0.05% considering the sum of

in response to SARS-CoV-2 infection. Patients in acute infection showed low CD8+ T-cell levels and consequently high CD4+:CD8+ T-cell ratio compared to HD together with high T-cell inhibitory receptor levels, such as high levels of PD-1, as previously reported,[9,31] and TIGIT, likewise high levels of activation in all the CD8+ T-cell subsets but only for terminally differentiated CD4+ T-cells. This scenario was compatible with a prominent T-cell migration to damaged tissue[32,33] which was associated with lower TEMRA CD4+:CD8+ T-cell ratio, because of high levels of TEMRA CD4+ HLA-DR+CD38+ T-cells accompanied by low peripheral CD4+ T-cell senescent levels, pointing out to preferential tissue recruitment of these cells.

According with previous studies, we found higher levels of response in CD4+ compared to CD8+ T-cells and against S than N protein in CD4+ T-cells.[31] This was confirmed in the MEM subset. The higher CD4+ compared to CD8+ T-cell response levels may be due to the use of optimised peptide pools for MHC-II. However, higher CD4+ compared to CD8+ T cell response levels have been traditionally associated with control of SARS-CoV-1 infection.[6,34] In our cohort, the high number of responders could be mainly due to CD8+ T-cells (96%), while other cohorts only found 53% of responders in this subset.[4] In this sense, the use of ICS for assaying T-cell response provides more information about the expression of other cytokines than IFN-γ in comparison to other methods.

In acute infection, some studies have associated a deleterious effect of SARS-CoV-2 specific T-cell response with disease progression,[11,12] while others have shown a beneficial role associated with mild disease in acute hospitalised patients.[9] Results presented herein may clarify this paradox. Severe compared to mild patients showed higher IFN-γ but lower IL-2+TNF-α+ S- and N-specific CD4+ T-cell levels. These results point out that studies using only IFN-γ ELISPOT technology[11,35] would show that SARS-CoV-2-specific T-cell response in acute infection is deleterious. However, we found that combinations including IL-2 were polyfunctional, including the cytotoxic marker perforin, pointing out to a higher antiviral activity with a classical signature of canonical Th1 cells[4,5,8,11] associated with mild disease in hospitalised patients. This fact was supported by S-specific CD8+ T-cell response, which mainly consisted in IL-2 production, in this case in monofunction, at higher levels in mild patients. Besides, it is important to highlight the higher polyfunctionality found in N compared to S protein. Polyfunctional combinations of three functions (IFN-γ+IL-2+TNF-α+) were at higher levels in MEM, CM and EM CD4+ T-cells in mild compared to severe patients in response to N protein. A successful outcome of acute disease may come for the combination and coordination of CD4+, CD8+ T-cell response and antibody production against SARS-CoV-2[4]. For having an integrated picture of acute anti-SARS-CoV-2 response, we also assayed anti-S IgG levels that in accordance with previous studies,[9,13] were at higher levels in severe patients. Although overall, in acute infection, as previously reported,[9,11] S-specific T-cell response was directly associated with anti-S IgG levels, we found that the production of IL-2 by EM S-specific T-cells was inversely associated with antibody production. In the same line, overall S-specific CD4+ T-cell response was inversely associated with inflammatory markers; however, combinations including IFN-γ were directly associated with IP-10 plasmatic levels which has previously associated with disease progression.[36,37] Altogether, these results define two different quality profiles of humoral and S/N-specific T-cell response associated with diseases progression in hospitalised patients: (i) a mild disease progression profile associated with IL-2 production, inversely correlated with anti-S IgG levels and associated with a higher T-cell polyfunctionality, which should promote CD4+ T-cell proliferation and CD4+ T-cell help to CD8+ T-cells together with antiviral potential enabling rapid virus clearance; and (ii) a severe disease progression profile consisting in high anti-S IgG levels and combinations only including IFN-γ, mainly in terminally differentiated T-cells with absence of perforin production, no CD8+ T-cell help and limited antiviral potential what may favour the failure to early control of the virus and poor disease outcome.

Next, we sought to analyse the immune memory to SARS-CoV-2 7 months after infection in two groups of subjects with different course of the disease: patients that overcame the disease without the need of hospitalisation and previously hospitalised patients. We observed that in both groups, all subjects displayed detectable T-cell response,

IFN-γ, TNF-α and IL-2 production. (B) Correlation between S-Specific and SE-specific CM CD4+ T-cell levels. (C) Correlation between S-Specific and SE-specific EM CD4+ T-cell levels and (D) correlation between S-Specific and SE-specific CM CD8+ T-cell levels in healthy donors. (E) Pie graphs represent IFN-γ, IL-2 and TNF-α expression in each T-cell subset, where median percentages of this expression are shown in right table. (F) Correlation between S-Specific and SE-specific MEM CD4+ IL-2+ T-cells and (G) S-specific and SE-specific CM CD8+ IL-2+ T-cell levels. Each dot represents an individual. ROUT method was utilised to identify and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Mann–Whitney U-test was used for groups' comparisons and Spearman test for non-parametric correlations. (HD, $n = 33$)

considering S+N response and all CD4+ and CD8+ T-cell subsets. This is in accordance with immune memory found to SARS-CoV-1 infection, which have been shown to last for years[38] and agreed with the magnitude of T-cell response found 8 months after SARS-CoV-2 infection in previously non-hospitalised subjects.[14] Although in that study using AIM they found only SARS-CoV-2 specific CD8+ T-cell response in 50%, while we observed 91% of responders but 75% in previously hospitalised patients.[14] Despite the general absence of difference in the magnitude of T-cell response between both groups, in terms of quality of this response, previously hospitalised patients showed higher T-cell exhaustion levels (TIGIT and PD-1 expression) and higher S and N-specific T-cell levels of combinations of only including IFN-γ and TNF-α production compared to non-hospitalised patients. Additionally, non-hospitalised patients presented higher IL-2 and perforin production in N-specific CD8+ T-cells compatible with a preserved antiviral activity. This profile is reminiscent of the one found in severe compared to mild patients in acute infection. However, on the contrary to what happened in acute disease, 7 months after infection in previously hospitalised subjects, anti-S IgG levels were directly, not inversely, associated with SARS-CoV-2 specific T-cell response, especially that enriched in IL-2 production which was associated with a good prognosis in acute infection. These results demonstrate that anti-SARS-CoV-2 humoral and cellular response are long-lasting and robust at least 7 months after infection in both non-hospitalised and previously hospitalised patients. However, previously hospitalised patients showed T-cell exhaustion and some signs of SARS-CoV-2 specific T-cell response associated with disease progression in acute infection, although this response was more IL-2 biased, which was associated with good prognosis. This defects found in previously hospitalised patients 7 months after infection may be selectively associated with long-COVID symptoms, as has been recently reported 4 months after infection;[12] however, we cannot confirm it because information about long-lasting symptoms were not recorded in this cohort as this was not the aim of the present study.

Finally, we found high levels of cross-reactive CD4+ and CD8+ T-cell response to SARS-CoV-2 (75% and 82%, respectively) in pre-COVID-19 HD samples. These levels were even higher than those found in previous cohorts showing 20%–50% of cross-reactivity.[8,16,38,39] Using an optimise peptide pool[21] for the four endemic coronaviruses: NL63, OC43, 229E and HKU1, we found that endemic S-specific CD4+ and CD8+ T-cell response was directly correlated with SARS-CoV-2 S-specific CD4+ and CD8+ T-cell response in EM and CM subsets. These results confirm cross-reactive SARS-CoV-2 specific T-cell response with endemic coronavirus. Comprehensively analyses of endemic S-specific T-cell response was mainly induced by TEMRA CD4+ T-cells and CM CD8+ T-cells and we found that the response was totally biased to IL-2 production, what may explain some previously published results using IFN-γ ELISPOT that did not find cross-reactive response.[11] In fact, combinations including IL-2, but not IFN-γ, were the ones associated with SARS-CoV-2 S-specific CD4+ and CD8+ T-cell response. These results suggest that this pre-existing T-cell memory could reduce the likelihood of suffering from COVID-19, although this cannot be confirmed based on the present results. However, whether a different quality of endemic T-cell specific response, based mainly in IL-2 or IFN-γ production, may contribute to variations in COVID-19 progression is currently unknown.

One limitation of this study is that patients included were mostly elderly subjects (71 [62–90] years old) all recruited in the first wave of COVID-19 in Spain, in those dates, samples were difficult to obtain and experimental therapies with very limited but transitory immunosuppressive effects were administered what may have affected the levels of immune parameters in acute infection. However, in those patients who were treated with IFN-β and corticosteroids, samples were collected time enough after these therapies to reverse the potential effects (24 days [7–28] and 9 days [1–21], respectively) what may have not affected the results presented herein. It is important to note that despite of the different time of recruitment since hospitalisation between severe and mild patients in acute infection, the same differences in SARS-CoV-2 specific T-cell parameters were found between mild patients (3 [2–3] days) and a subgroup of severe patients (2.5 [1.0–3.3] days) with the same time since hospitalisation (data not shown). According to these results, the time of sample collection is independent of the clinical phenotype (mild vs. severe). Seven-months post-infection, one potential bias may come from the group of hospitalised subjects that were composed by patients with previous mild (42%) or severe (58%) disease, however, as no differences were found in any parameter associated to T-cell response (data not shown) between these two subgroups, they formed part of the same group of previously hospitalised and were compared with non-hospitalised patients. ICS needs a notable amount of cells to be assayed, this avoids us to perform endemic virus-specific T-cell response in COVID-19 samples; however, it has allowed us to obtain comprehensive data about the quality of SARS-CoV-2 specific T-cell response. Finally, anti-S IgG levels were assayed against the whole S protein and not for the Receptor Binding Protein (RBD), cross-reactive reaction cannot be excluded and results have to be

interpreted taking this into account. In the same way, further research is needed to confirm and correlate our T-cell response profile with neutralising antibody levels and B-cell polyfunctionality.

In summary, our results gain insights in the characteristics of T-cell response associated with disease severity in acute infection, supporting important information about correlates of immune protection, such as a broader polyfunctional CD4+ T-cell response with predominance of IL-2 production also present in SARS-CoV-2 specific CD8+ T-cell response, distinguished mild disease progression from severe COVID-19 characterised by an inefficient monofunctional IFN-γ+ CD4+ T-cell response in acute hospitalised patients. However, independently of previous hospitalization, SARS-CoV-2 specific T-cell response was robust 7 months after infection, although some defects associated with T-cell exhaustion were observed in previously hospitalised patients.

These results could have implications for protective immunity against SARS-CoV-2 and recurrent COVID-19 and may help to identify populations, apart from the classical risk ones, that are in the need of new boosting of existing vaccines, by thoroughly assessing the magnitude and quality of their specific T-cell and humoral response to SARS-CoV-2, as well as for improving the design of new prototypes looking for the quality profile of the specific T-cell response to SARS-CoV-2 presented herein in ongoing vaccine clinical trials in order to achieve of broader long-lasting protection against COVID-19.

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CONFLICT OF INTERESTS

Alessandro Sette is a consultant for Gritstone, Flow Pharma, Arcturus, Immunoscape, CellCarta, OxfordImmunotech and Avalia. LJI has filed for patent protection for various aspects of T cell epitope and vaccine design work. All other authors declare that they have no competing financial interests.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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SUPPLEMENTARY MATERIALS

Deciphering the quality of SARS-CoV-2 specific T-cell response associated with disease severity, immune memory and heterologous response

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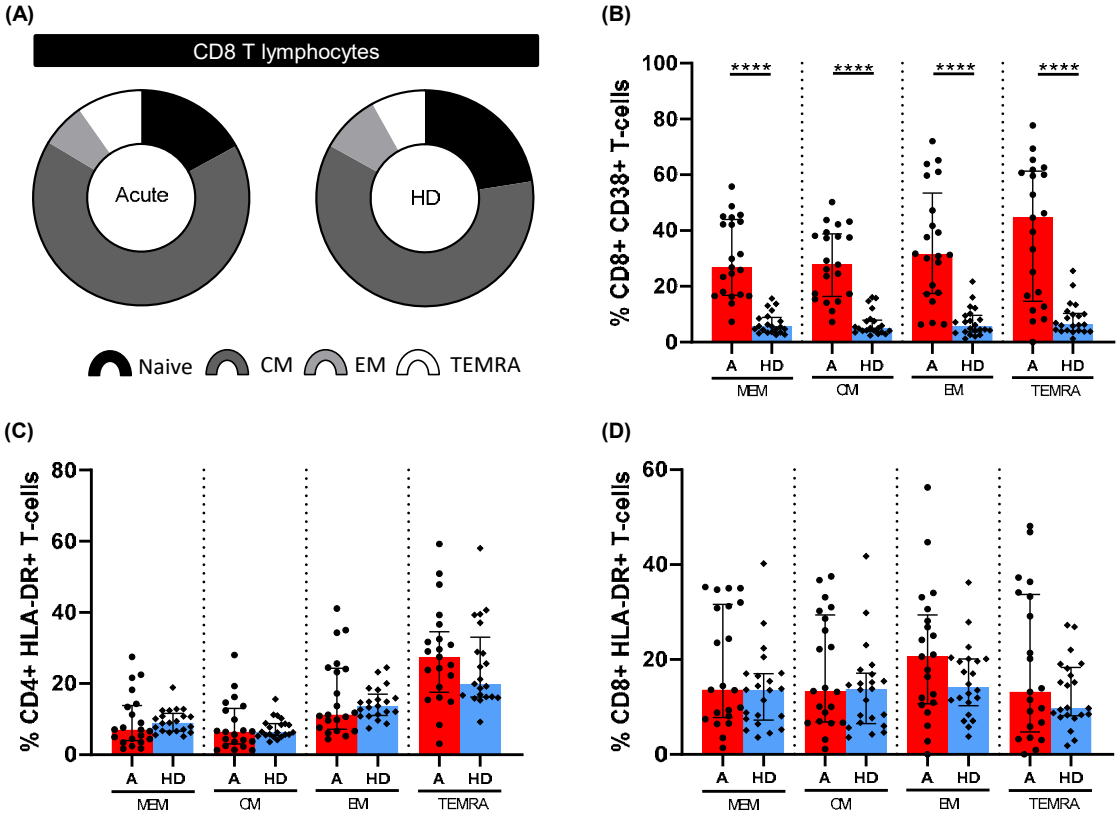
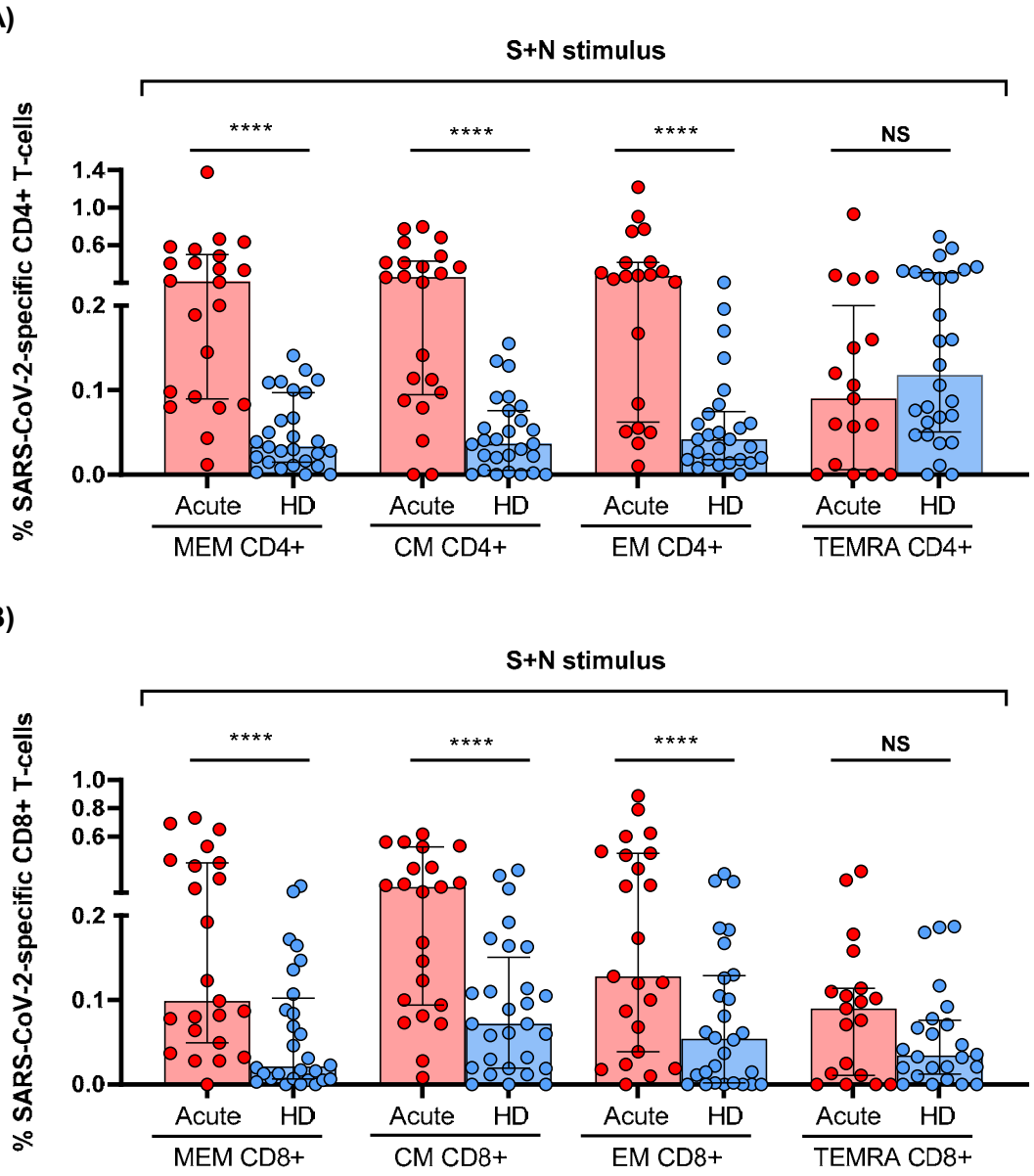
SUPPLEMENTARY MATERIALS

Supplementary Figure 1



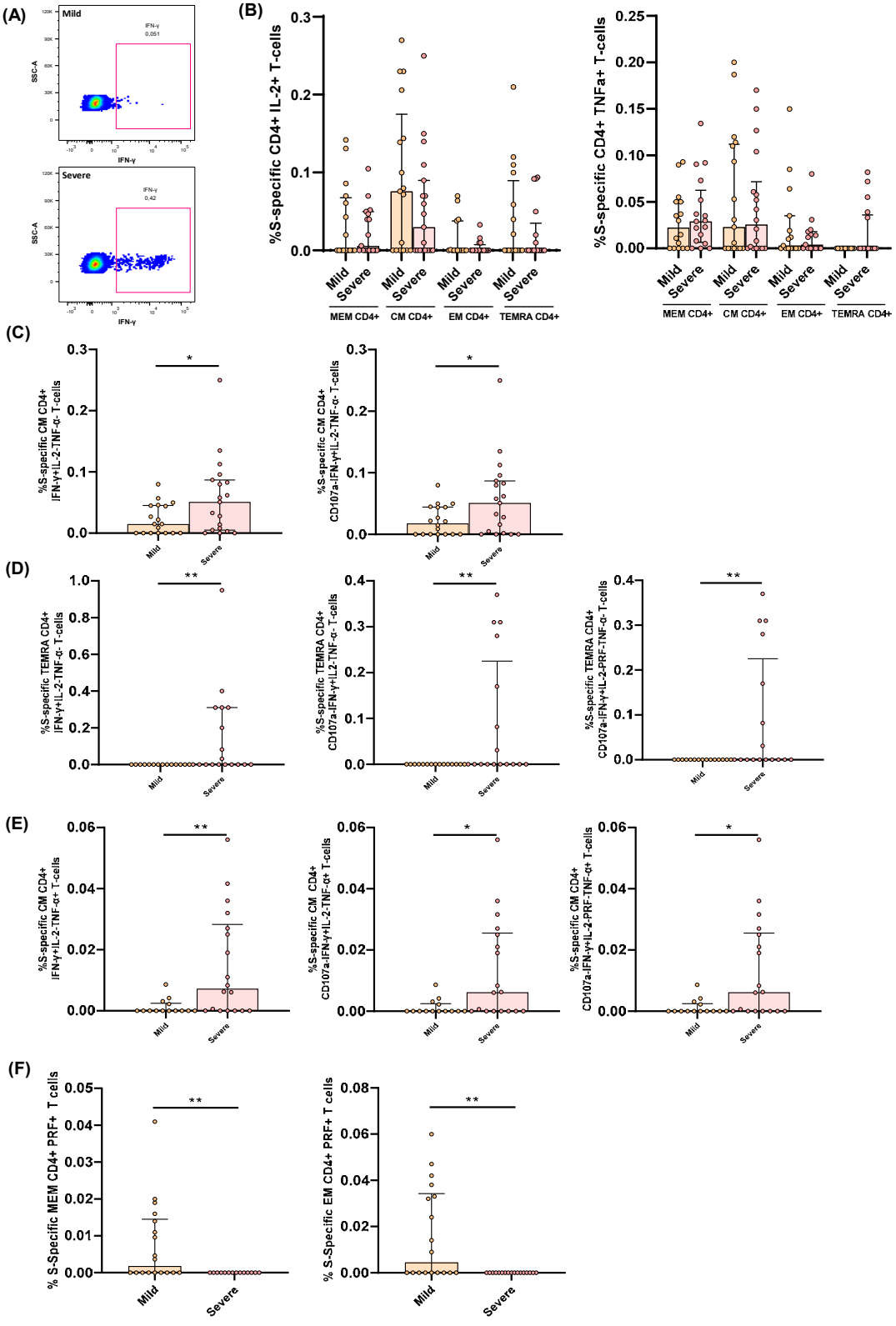
Supplementary Figure 1. CD8+ T-cells maturation phenotype and activation markers. (A) Pie graphs show medians of each CD8+ T-cell subset in acute SARS-CoV-2 infected individuals and healthy donor groups. Bar graphs represent the percentage of **(B)** CD8+CD38+, **(C)** CD4+HLA-DR+ and **(D)** CD8+HLA-DR+ T-cells. The medians with the interquartile ranges are shown. ROUT method was utilized to identify and discard outliers. *p < 0.05, **p <0.01, ***p < 0.001, ****p < 0.0001. Mann-Whitney U test was used for groups' comparisons and Spearman test for non-parametric correlations. (Acute, n= 37; HD, n= 33)

Supplementary Figure 2



Supplementary Figure 2. Combined SARS-CoV-2 specific T-cell response to N and S proteins in acute SARS-CoV-2 infected individuals and healthy donors. (A) Bar graphs represent the percentage of S plus N-specific CD4+ T-cell response. **(B)** Bar graphs represent the percentage of S plus N-specific CD8+ T-cell response. The medians with the interquartile ranges are shown. Each dot represents an individual. ROUT method was utilized to identify and discard outliers. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Mann-Whitney U test was used for groups' comparisons. (Acute, n= 37; HD, n= 33)

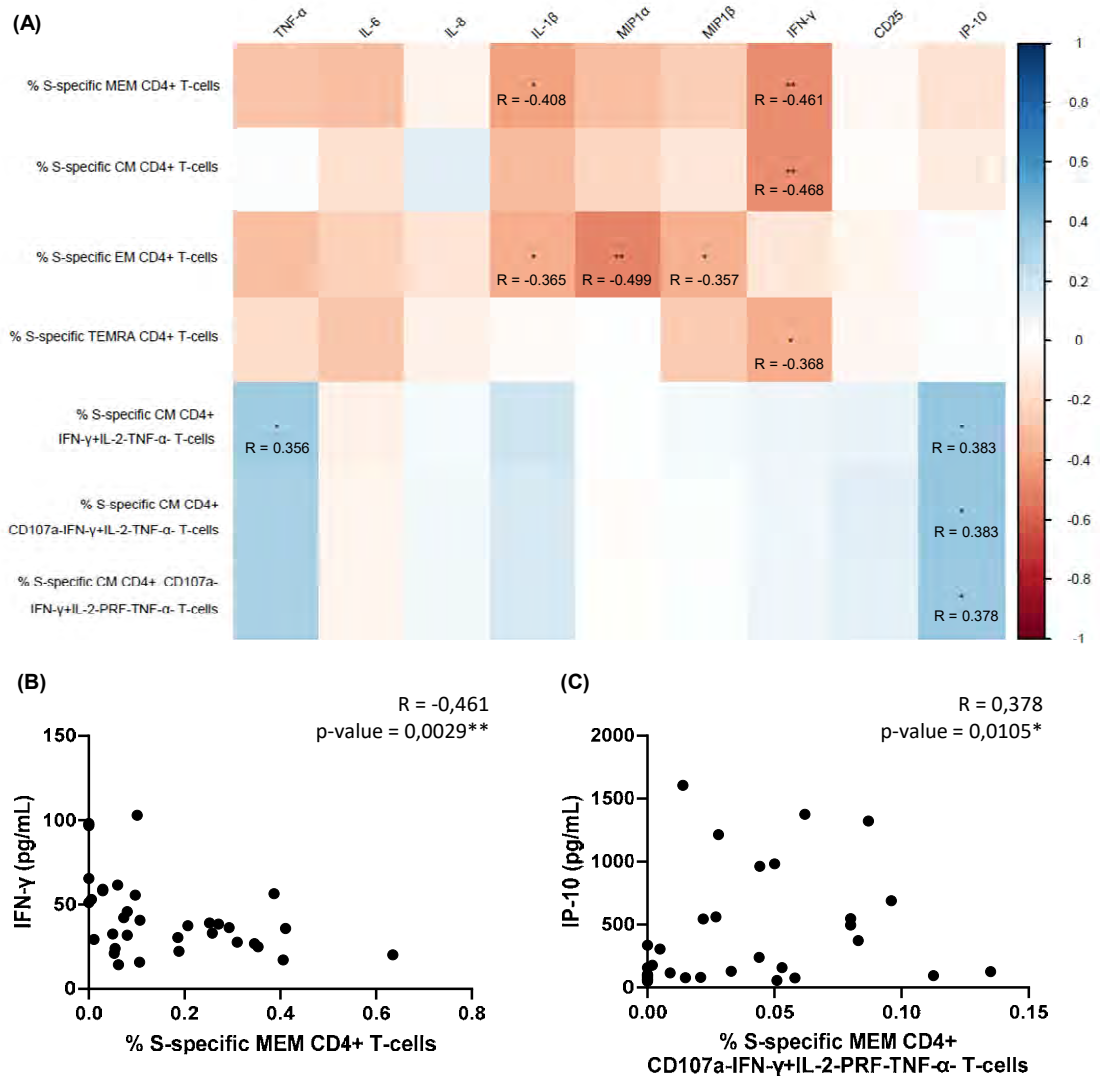
Supplementary Figure 3



Supplementary Figure 3. Additional S-specific CD4+ T-cell response features associated with disease severity in acute SARS-CoV-2 infection. (A) Representative dot-plot of expression of IFN-γ+ in CD4+ TEMRA cells. **(B)** Bar graphs represent S-

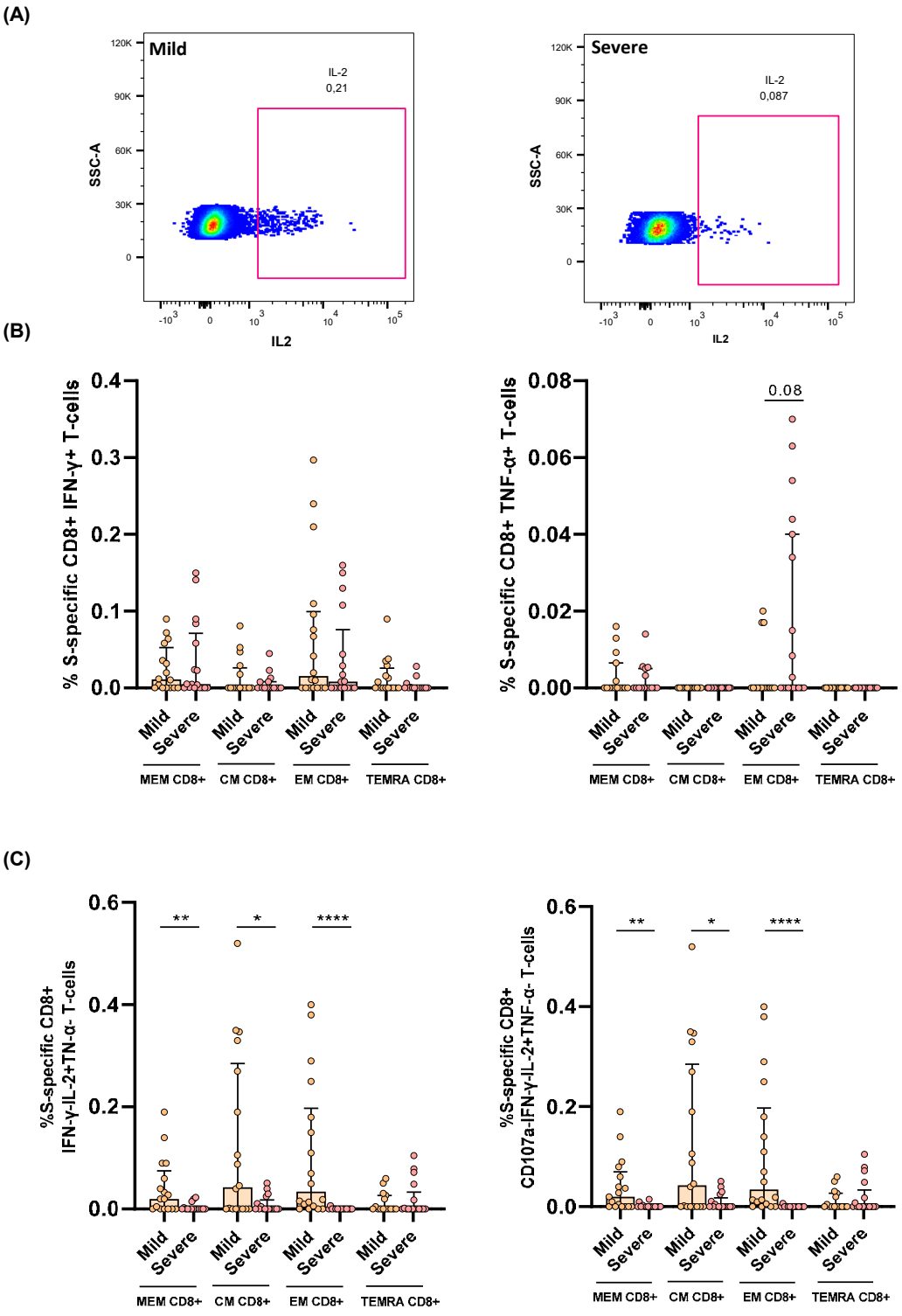
specific CD4+ T-cell response considering the levels of cells producing IL-2 (left panel) and TNF-α (right panel). **(C)** S-specific CM CD4+ T-cell levels of combinations only including IFN-γ+ cells for three (IFN-γ, TNF-α and IL-2) and four (IFN-γ, TNF-α, IL-2 and CD107a) functions. **(D)** S-specific TEMRA CD4+ T-cell levels of combinations only including IFN-γ+ cells for three (IFN-γ, TNF-α and IL-2), four (IFN-γ, TNF-α, IL-2 and CD107a) and five (IFN-γ, TNF-α, IL-2, CD107a and PRF) functions. **(E)** S-specific CM CD4+ T-cell levels of combinations including IFN-γ+ and TNF-α+ cells for three (IFN-γ, TNF-α and IL-2), four (IFN-γ, TNF-α, IL-2 and CD107a) and five (IFN-γ, TNF-α, IL-2, CD107a and PRF) functions. **(F)** Percentage of S-specific production of: PRF+ in CD4+ total memory (MEM) and central memory (CM) T-cell subsets. The medians with the interquartile ranges are shown. Each dot represents a patient. ROUT method was utilized to identify and discard outliers.*$p < 0.05$, **$p < 0.01$. Mann-Whitney U test was used for groups' comparisons. (Mild, n= 18; Severe, n= 19)

Supplementary Figure 4



Supplementary Figure 4. S-specific T-cell response is differentially associated to inflammatory markers in acute SARS-CoV-2 infected patients. Correlation matrix representing associations between representative parameters of S-specific CD4+ T-cell response with inflammatory markers including TNF-α, IL-6, IL-8, IL1-β, MIP1-α, MIP1-β, IFN-γ, CD25 and IP-10, in acute SARS-CoV-2 infected patients. Blue color represents positive correlations and red color shows negative correlations. The intensity of the color indicates the R coefficient. ROUT method was utilized to identify and discard outliers. *p < 0.05, **p < 0.01, ***p < 0.001. Spearman test was used for non-parametric correlations. (Acute, n=34)

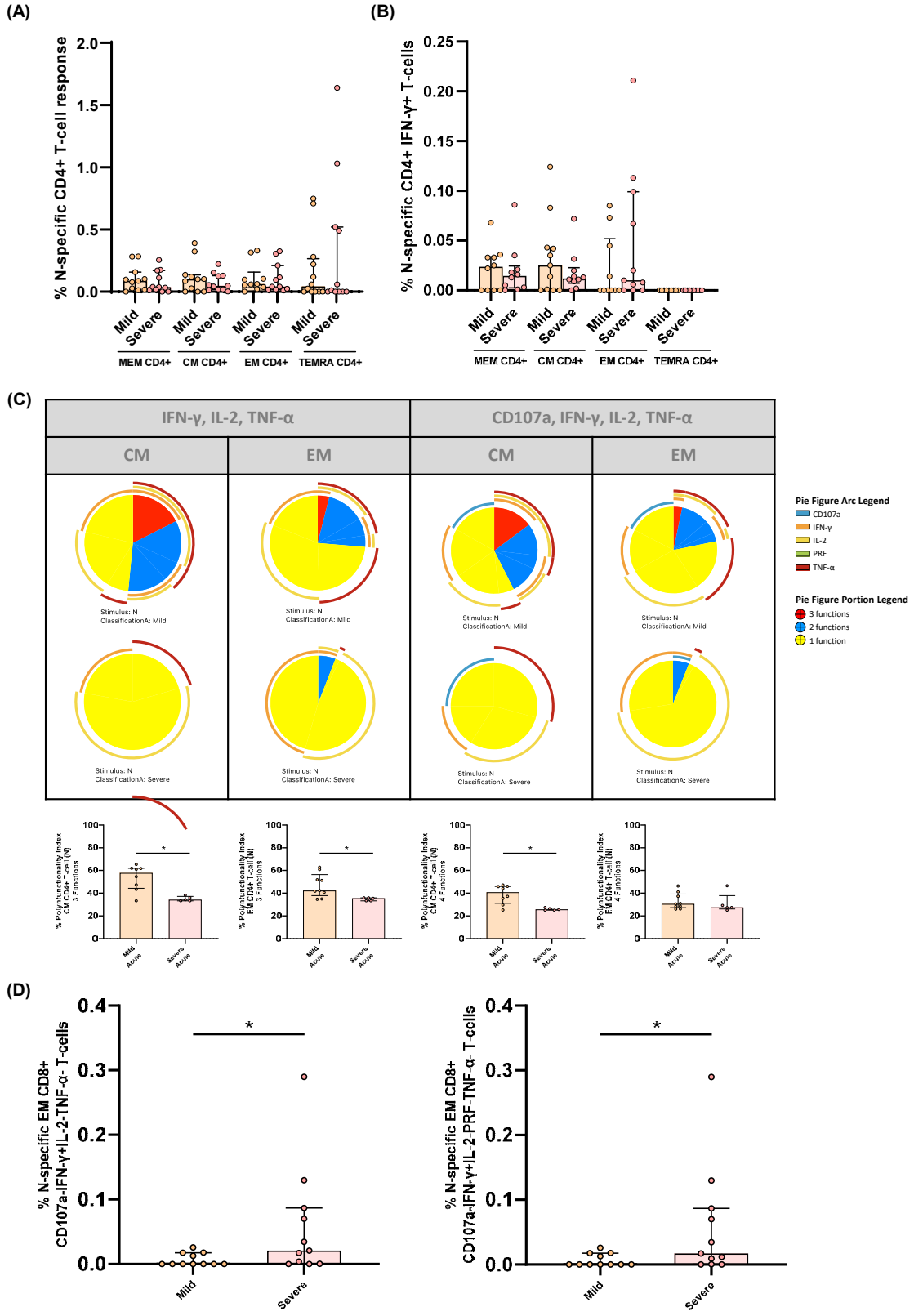
Supplementary Figure 5



Supplementary Figure 5. Additional features of S-specific CD8+ T-cell response associated with disease severity. (A) Representative dot-plot of IL-2 expression in S-specific CM CD8+ T-cells. **(B)** Bar graphs represent S-specific CD8+ T-cell response

considering the levels of cells producing IFN-γ (left panel) and TNF-α (right panel). **(C)** S-specific CD8+ T-cell levels of combinations only including IL-2+ cells for three (IFN-γ, TNF-α and IL-2 and CD107a) and four (IFN-γ, TNF-α, IL-2 and CD107a) functions. The medians with the interquartile ranges are shown. Each dot represents a patient. ROUT method was utilized to identify and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Mann-Whitney U test was used for groups' comparisons. (Mild, n= 18; Severe, n= 19)

Supplementary Figure 6

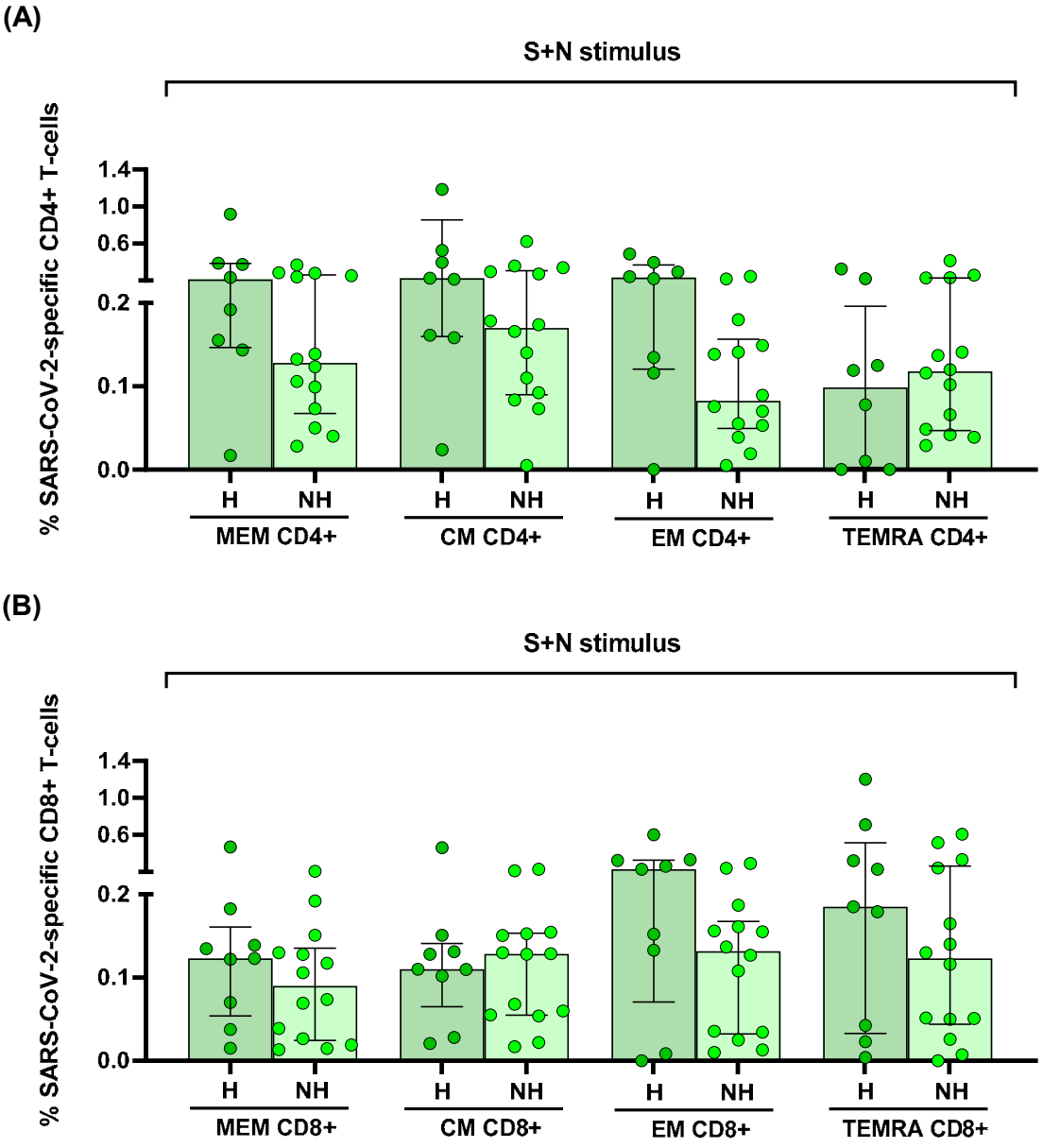


Supplementary Figure 6. Additional features of N-specific T-cell response associated with disease progression. (A) Bar graphs show N-specific CD4+ T-cell response,

74 considering the sum of IFN-γ, TNF-α and IL-2 production, in the different CD4+ T-cell
75 subsets, in mild and severe acute patients' groups, **(B)** N-specific CD4+ T-cell response
76 considering the levels of cells producing IFN-γ. **(C)** N-specific CM and EM CD4 T-cell
77 polyfunctionality pie charts for three and four functions. Each sector represents the
78 proportion of N-specific CD4 T-cells producing three (red), two (blue) and one (yellow)
79 function. Arcs represents the type of function (IFN-γ, TNF-α, IL-2, CD107a and PRF)
80 expressed in each sector. Permutation test, following the Spice version 6.0 software was
81 used to assess differences between pie charts. **(D)** N-specific EM CD8+ T-cell levels of
82 combinations only including IFN-γ+ cells for four (IFN-γ, TNF-α, IL-2 and CD107a) and
83 five (IFN-γ, TNF-α, IL-2, CD107a and PRF) functions. The medians with the interquartile
84 ranges are shown. Each dot represents a patient. ROUT method was utilized to identify
85 and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Mann-Whitney U test was used
86 for groups' comparisons. (Mild, n= 11; Severe, n= 11)

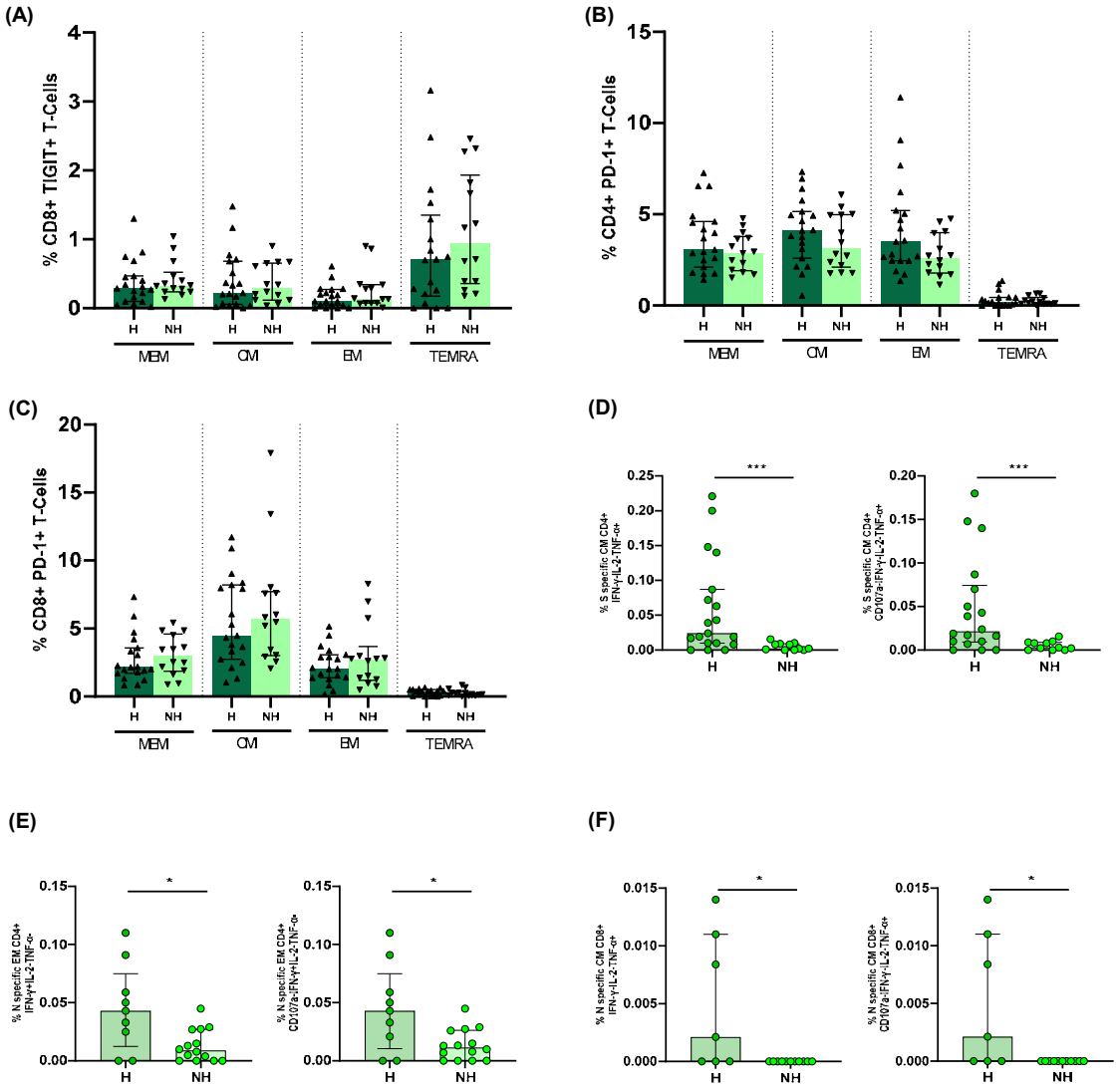
87

Supplementary Figure 7



Supplementary Figure 7. Combined SARS-CoV-2 specific T-cell response to N and S proteins in previously hospitalized and non-hospitalized subjects seven months after SARS-CoV-2 infection. (A) Bar graphs represent the percentage of S plus N-specific CD4+ T-cell response. **(B)** Bar graphs represent the percentage of S plus N-specific CD8+ T-cell response. Each dot represents an individual. ROUT method was utilized to identify and discard outliers. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Mann-Whitney U test was used for groups' comparisons. (H, n= 19; NH, n= 14)

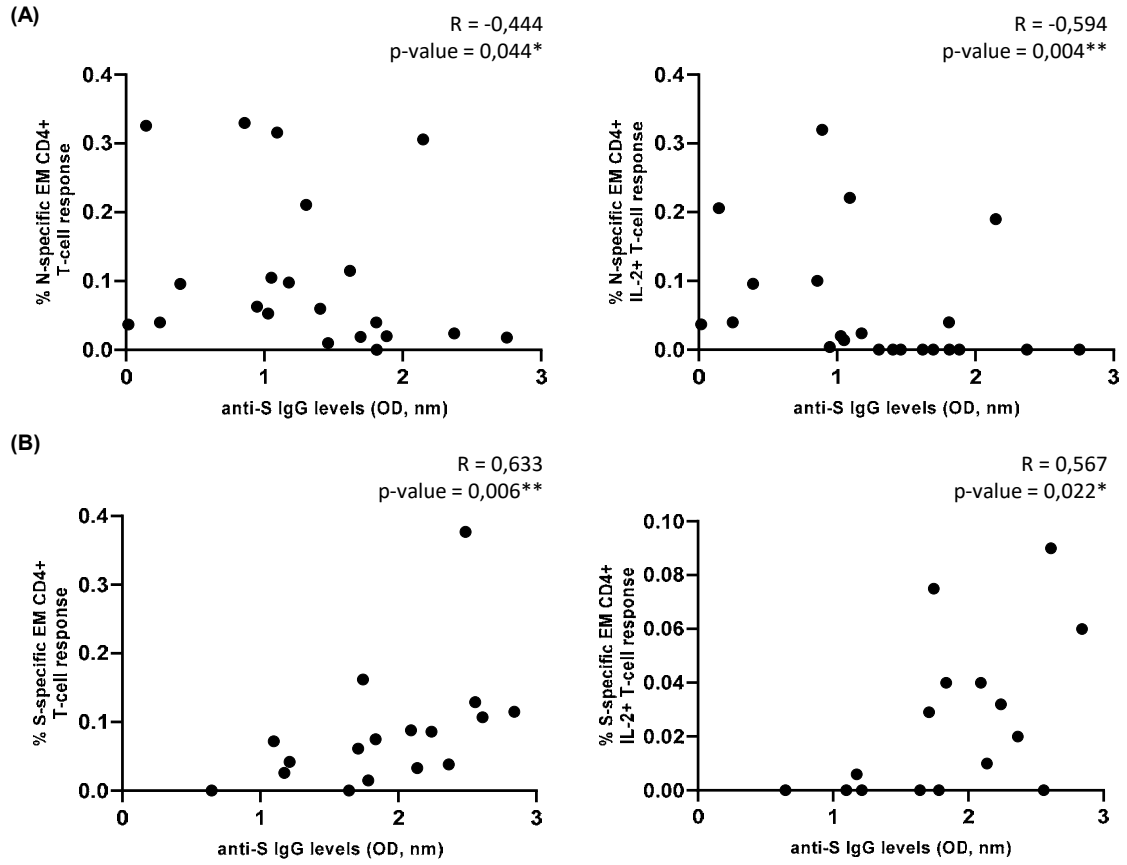
Supplementary Figure 8



Supplementary Figure 8. Additional features of T-cell exhaustion and SARS-CoV-2-specific T-cell response in previously hospitalized and non-hospitalized subjects seven months after SARS-CoV-2 infection. (A) TIGIT expression in each CD8+ T-cell subset, **(B)** PD-1 expression in each CD4+ T-cell subset, **(C)** PD-1 expression in each CD8+ T-cell subset, in previously hospitalized and non-hospitalized subjects seven months after SARS-CoV-2 infection. **(D)** S-specific CM CD4+ T-cell levels of combinations only including TNF-α+ cells for three (IFN-γ, TNF-α, and IL-2) (left panel) and four (IFN-γ, TNF-α, IL-2 and CD107a) functions (right panel). **(E)** N-specific EM CD4+ T-cell levels of combinations only including INF-γ+

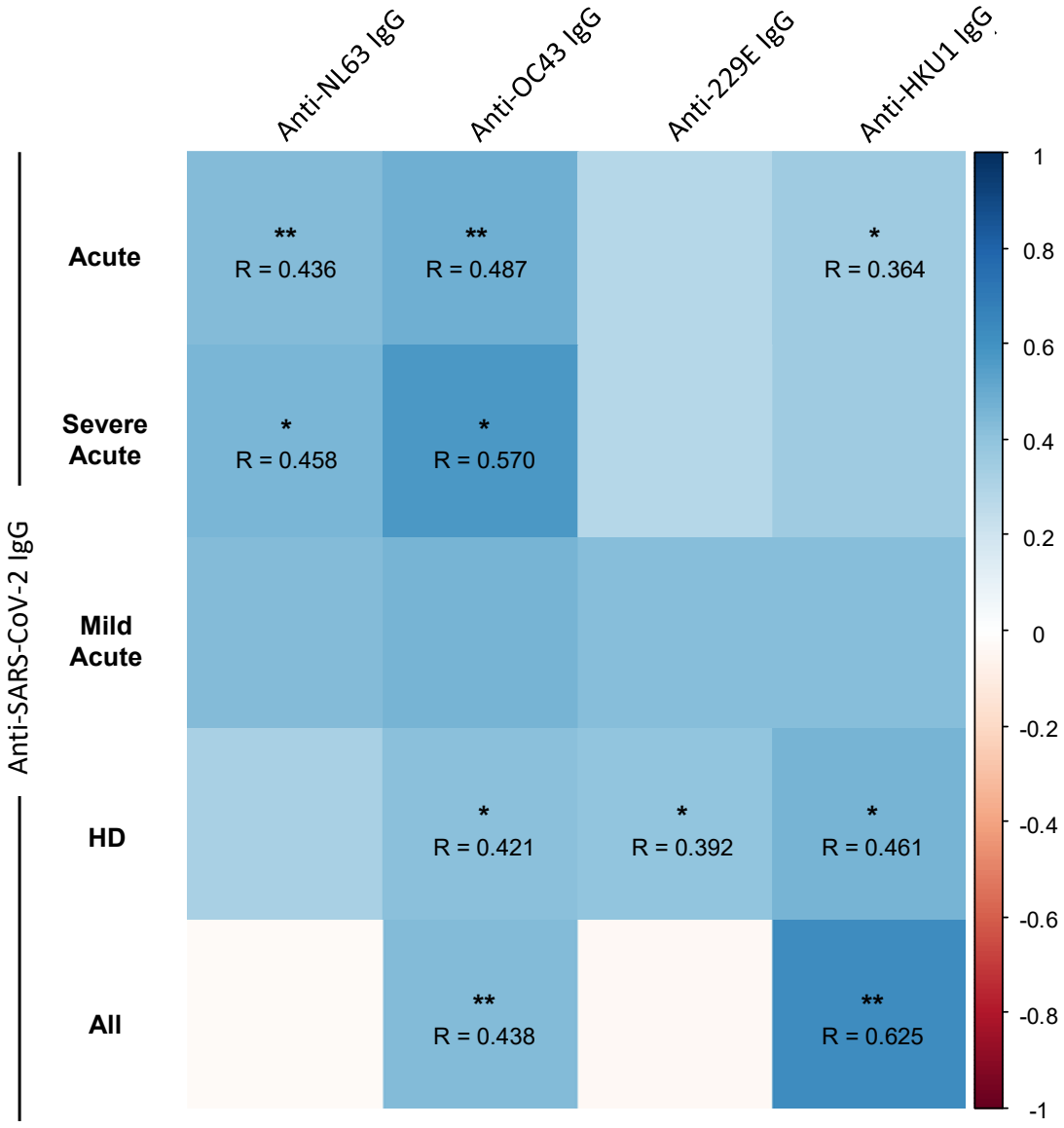
cells for three (IFN-γ, TNF-α, and IL-2) (left panel) and four (IFN-γ, TNF-α, IL-2 and CD107a) functions (right panel). **(F)** N-specific CM CD8+ T-cell levels of combinations only including TNF-α+ cells for three (IFN-γ, TNF-α, and IL-2) (left panel) and four (IFN-γ, TNF-α, IL-2 and CD107a) functions (right panel). The medians with the interquartile ranges are shown. Each dot represents an individual. ROUT method was utilized to identify and discard outliers. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Mann-Whitney U test was used for groups' comparisons. (H, n= 19; NH, n= 14)

Supplementary Figure 9



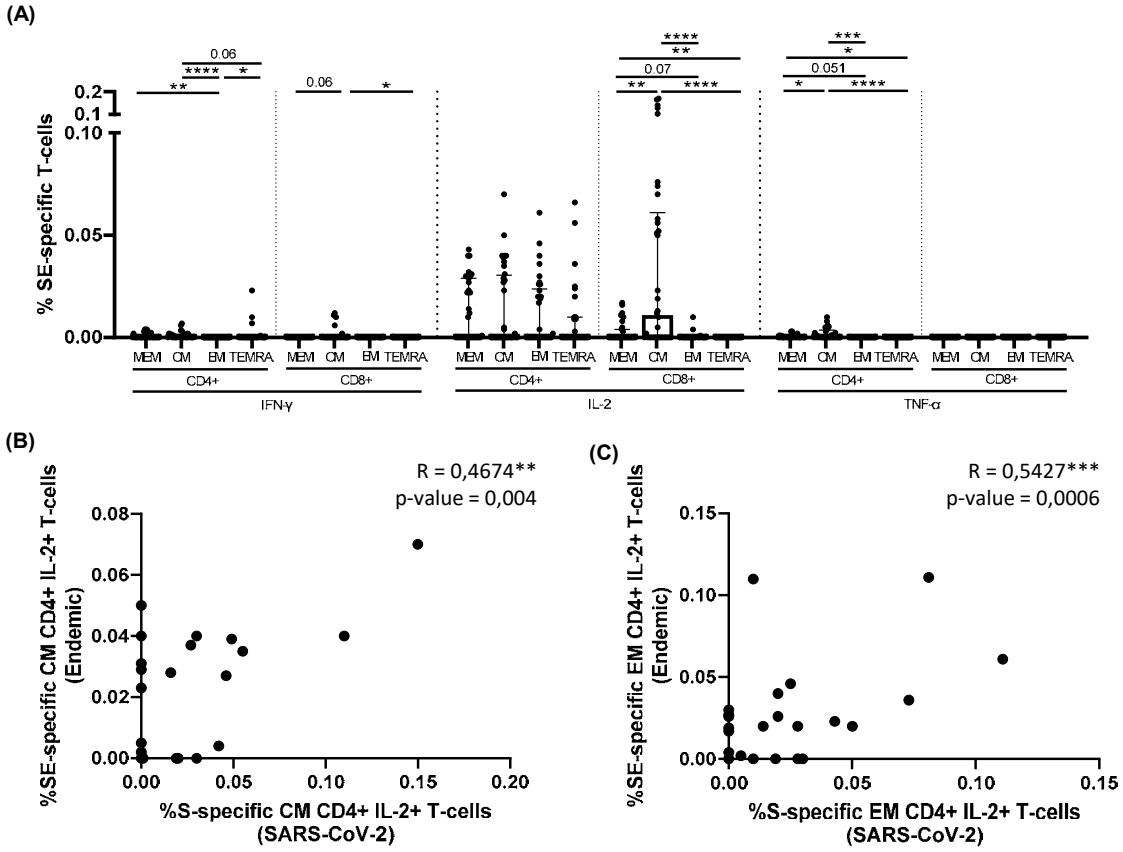
Supplementary Figure 9. Anti-S IgG levels are directly correlated with S-specific T-cell response in acute infection but this correlation is inverse seven months after SARS-CoV-2 infection in previously hospitalized patients. (A) Correlation graphs between anti-S IgG levels and the percentage of N-specific EM CD4+ T-cells (left panel) and N-specific CD4+ EM IL-2+ T-cells in acute SARS-CoV-2 infection. **(B)** Direct correlation between anti-S IgG levels and the percentage of S-specific EM CD4+ T-cells (left panel) and S-specific CD4+ EM IL-2+ T-cells in previously hospitalized patients seven months after SARS-CoV-2 infection (right panel). Each dot represents an individual. ROUT method was utilized to identify and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Spearman test was used for non-parametric correlations. (Acute, n= 31; Hospitalized, n= 17)

Supplementary Figure 10



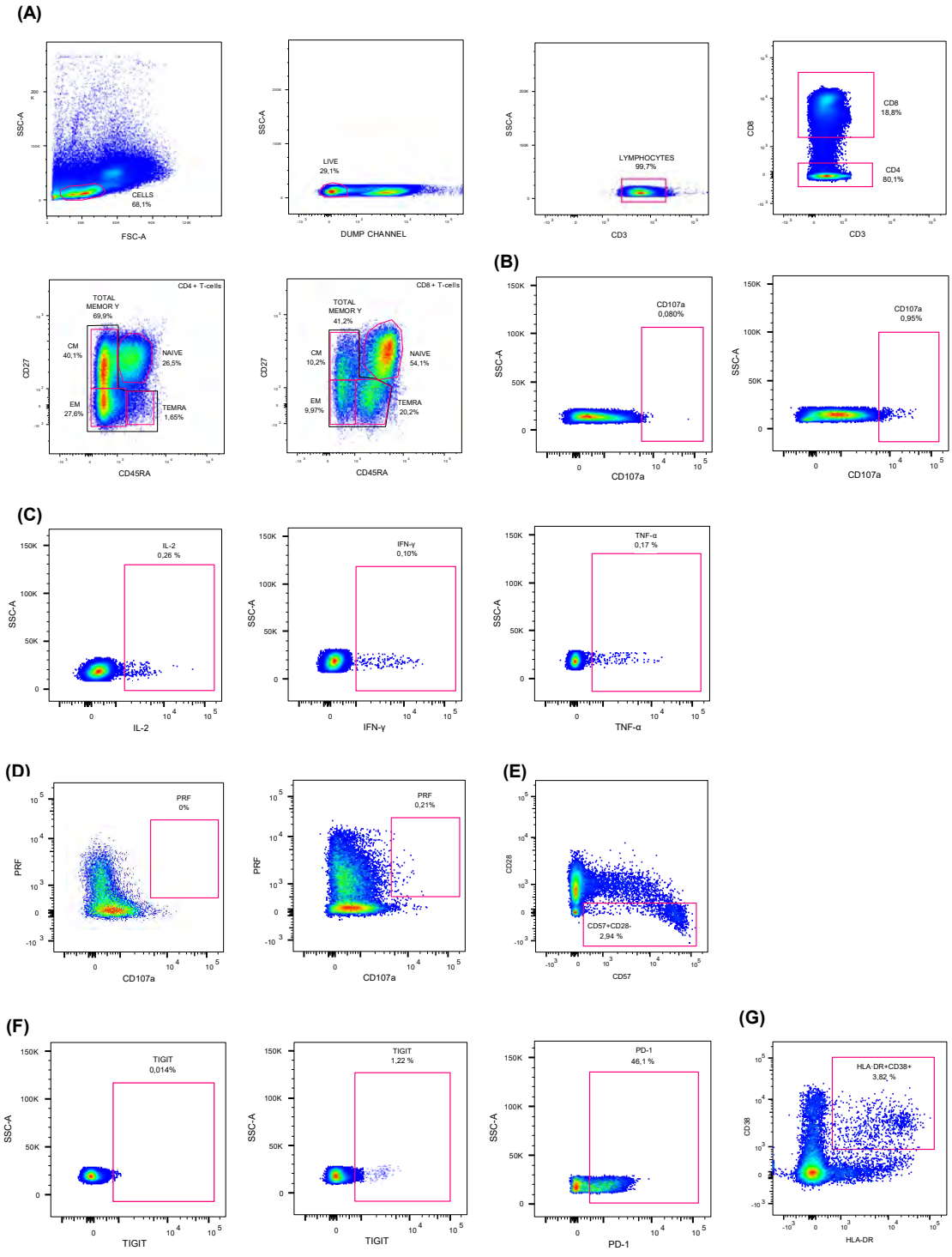
Supplementary Figure 10. Anti-S IgG levels against endemic coronaviruses are associated with anti-S IgG levels against SARS-CoV-2. Correlation matrix between anti-S IgG levels against SARS-CoV-2 and endemic coronaviruses (NL63, OC43, 229E and HKU1). Plasma sample was used at 1: 50 dilution. Blue color represents positive correlations and red color shows negative correlations. The intensity of the color indicates the R coefficient. ROUT method was utilized to identify and discard outliers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Spearman test was used for non-parametric correlations. (Mild, n=18; Severe, n=19; HD, n=28)

Supplementary Figure 11



Supplementary Figure 11. S-specific T-cell response in HD to endemic coronaviruses is mainly mediated by IL-2 production. (A) Bar graph represent the SE-specific T-cell response in each T-cell subset for each cytokine. **(B)** Correlation between S-Specific and SE-specific CM CD4+ IL-2+ T cell levels and **(C)** Correlation between S-Specific and SE-specific EM CD4+ IL-2+ T cell levels. Each dot represents an individual. ROUT method was utilized to identify and discard outliers. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Mann-Whitney U test was used for groups' comparisons and Spearman test for non-parametric correlations. (HD, n = 33)

Supplementary Figure 12



Supplementary Figure 12. Schematic diagram of the gating strategy. (A) Phenotyping of CD4+ and CD8+ T-lymphocyte subsets, including naïve (NAIVE); Total Memory (MEM), Central Memory (CM), Effector Memory (EM) and terminally differentiated memory (TEMRA) T cells. **(B)** Degranulation factor, CD107a. where

153 negative control staining is on the left and positive sample on the right. **(C)** Gating of
154 intracellular cytokine production, including interferon gamma (IFN-γ), interleukin-2
155 (IL-2) and tumor necrosis factor alpha (TNF-α). **(D)** Cytolytic enzime, mature perforin
156 (PRF), where negative control staining is on the left and positive sample on the right. **(E)**
157 Senescence, CD57+CD28-. **(F)** Exhaustion markers, T cell immunoreceptor with Ig and
158 ITIM domains (TIGIT), where negative control staining is on the left and positive sample
159 on the right; and programmed death 1 molecules (PD-1) and **(G)** Activation,
160 HLA-DR+CD38+.

161

Supplementary Table 1. Characteristics of the study subjects.

	Acute Infection			Discharged (6-8 months after diagnosis)			Healthy Donors		
	All (n=37)	**Mild (n=18)**	**Severe (n=19)**	**All (n=33)**	**Previously Hospitalized (n=19)**	**Previously Non Hospitalized (n=14)**	**All (n=33)**	**Old HD (n=19)**	**Young HD (n=14)**
Age (years)	71 [61.5 – 90]	70 [57.75 – 76.5]	72 [63 – 77]	66 [58 – 74]	71 [59 – 77]	65.5 [56.75 – 71.5]	62 [38.5 – 87]	84 [71 – 90]	38 [33.75 – 40.75]
Sex (Female sex), n (%)	13 (35.1)	6 (33.3)	7 (36.8)	12 (36.4)	5 (26.3)	7 (50)	14 (42.4)	10 (52.6)	4 (28.6)
Oxygen Saturation ($SatO_2$), (%)	93 [91 – 97]	96 [93 – 98.25]	91 [87 – 95]	N/A	N/A	N/A	N/A	N/A	N/A
Time since hospitalization, (days)	3 [2 – 21.5]	2.5 [1 – 3.25]	18 [3 – 28]	201 [180.5 – 221]	187 [173 – 193]	221 [218 – 230.75]	N/A	N/A	N/A
Time since symptoms onset, (days)	17 [7 – 31.5]	7.5 [4 – 19.5]	31 [17 – 37]	208 [190 – 232]	186 [195 – 202]	232 [224.5 – 239.25]	N/A	N/A	N/A
Time hospitalized, (days)	16 [7.5 – 29]	8 [6.5 – 12.25]	26 [17 – 36]	N/A	19 [9 – 37]	0	N/A	N/A	N/A
Comorbidities, n (%)	29 (78.4)	15 (83.3)	14 (73.7)	19 (57.6)	15 (78.9)	3 (21.4)	N/A	N/A	N/A
Diabetes mellitus	12 (32.4)	7 (38.9)	5 (26.3)	4 (12.1)	3 (15.8)	1 (7.1)	N/A	N/A	N/A
Hypertension	25 (67.6)	12 (66.7)	13 (68.4)	12 (36.4)	10 (52.6)	2 (14.3)	N/A	N/A	N/A
Cardiovascular disease	11 (29.7)	7 (38.9)	4 (21.1)	6 (18.2)	4 (21.1)	2 (14.3)	N/A	N/A	N/A
Obstructive pulmonary disease	4 (10.8)	2 (11.1)	2 (10.5)	0	0	0	N/A	N/A	N/A
Malignancy	4 (10.8)	2 (11.1)	2 (10.5)	0	0	0	N/A	N/A	N/A
Symptoms at admission (%)									
Cough	24 (64.9)	11 (61.1)	13 (68.4)	22 (66.7)	12 (63.2)	10 (71.4)	N/A	N/A	N/A
Fever	27 (73)	11 (61.1)	16 (84.2)	25 (75.8)	14 (73.7)	11 (78.6)	N/A	N/A	N/A
Dyspnea	19 (51.4)	7 (38.9)	12 (63.2)	16 (48.5)	10 (52.6)	6 (42.9)	N/A	N/A	N/A
Anosmia	5 (13.5)	0	5 (26.3)	6 (18.2)	6 (31.6)	0	N/A	N/A	N/A
Diarrhoea	6 (16.2)	3 (16.7)	3 (15.8)	9 (27.3)	7 (36.8)	2 (14.3)	N/A	N/A	N/A
Muscle pain	3 (8.1)	1 (5.6)	2 (10.5)	6 (18.2)	5 (26.3)	1 (7.1)	N/A	N/A	N/A
Treatment during hospitalization; n (%)									
Hydroxychloroquine	33 (89.2)	15 (83.3)	18 (94.7)	N/A	19 (100)	N/A	N/A	N/A	N/A
Lopinavir/Ritonavir	24 (64.9)	8 (44.4)	16 (84.2)	N/A	16 (84.2)	N/A	N/A	N/A	N/A
Beta Interferon	12 (20)	2 (11.1)	10 (52.6)	N/A	7 (36.8)	N/A	N/A	N/A	N/A
Corticoids	18 (48.6)	4 (22.2)	14 (73.7)	N/A	12 (63.2)	N/A	N/A	N/A	N/A
Remdesivir	3 (8.1)	3 (16.7)	0	N/A	0	N/A	N/A	N/A	N/A
Tocilizumab	10 (27)	1 (5.6Y)	9 (47.4)	N/A	10 (52.6)	N/A	N/A	N/A	N/A

Categorical variables are expressed as number and percentages (%), and continuous variables are expressed as median (interquartile ranges [IQR]). N/A, not applicable. The different groups (acute infection, discharged patients and healthy donors) were age and sex matched. Chi-square test and a Mann-Whitney U test were used to compare categorical and continuous variables, respectively. Analysis by age; acute infection vs HD (p=0.423); discharged patients vs HD (p=0.700); Previously Hospitalized patients vs HD (p=0.635); Previously Non Hospitalized patients vs HD (p=0.907). Analysis by sex; acute infection vs HD (p=0.532); discharged patients vs HD (p=0.614); Previously Hospitalized patients vs HD (p=0.245); Previously Non Hospitalized patients vs HD (p=0.633). Severe participants were those who required Intensive Care Unit admission, or having ≥6 points in the ordinal scale score based on Beigel et al. (1) or death.

Supplementary Table 2. Statistical description of variables from matrix correlation of figure S4.

	Acute			Mild Acute			Severe Acute		
	n	Median	IQR	n	Median	IQR	n	Median	IQR
TNF-α	36	10.51	5.920-12.790	17	7.9	5.095-11.950	19	10.97	6.250-13.110
IL-6	36	5.935	2.908-12.950	17	6.38	3.365-12.680	19	5.31	2.490-26.930
IL-8	36	10.4	5.683-20.030	17	7.69	4.770-19.490	19	10.94	8.340-21.650
IL-1β	36	1.15	0.545-1.748	17	1.25	0.355-1.935	19	1.06	0.6-1.600
MIP-1α	36	8.535	0.0375-16.630	17	10.81	0.150-21.930	19	6.88	0-15.110
MIP-1β	36	14.8	8.293-20.090	17	9.47	6.825-17.640	19	16.67	11.140-30.820
IFN-γ	36	36.03	25.380-54.960	17	38.47	28.030-60.970	19	35.8	21.090-42.130
CD25	37	2140	1252-3628	18	1637	842.400-3156	19	2276	1649-3883
IP-10	36	157.7	90.230-557.900	17	158.2	78.060-553.300	19	157.2	101.700-688.900

Continuous variables are expressed as median (interquartile ranges [IQR]).

Supplementary Table 3. Anti-hCoV IgG antibodies OD (nm) raw data.

			hCoV IgG levels			
Groups		Donor	anti-NL63	anti-OC43	anti-229E	anti-HKU1
ACUTE INFECTION	MILD	M01	2.06050	2.96355	1.66870	1.67365
		M02	3.11130	3.31210	2.77500	3.10020
		M03	2.55805	3.44265	2.00490	3.00435
		M04	2.07290	3.44095	2.06930	3.13145
		M05	0.00000	0.00000	2.15405	3.23870
		M06	3.10805	3.68865	3.06160	3.42290
		M07	2.83610	2.58745	2.12805	1.53900
		M08	1.91075	3.32160	2.14930	2.88035
		M09	2.94100	2.24360	1.80360	1.54500
		M10	1.44245	2.07775	2.41785	0.62415
		M11	1.39020	3.23805	1.15420	1.91420
		M12	2.51375	1.51760	2.36260	1.48930
		M13	2.94055	3.35270	2.19175	2.77405
		M14	1.24945	3.08910	1.94425	2.16265
		M15	3.11155	3.24540	2.44125	2.61405
		M16	2.63170	3.48915	2.76435	3.21230
		M17	1.13435	3.23520	1.92100	2.87755
		M18	2.57915	3.24715	2.42505	2.73475
	SEVERE	S01	2.00120	2.89995	1.50810	1.75290
		S02	0.91690	2.94775	1.38830	1.80175
		S03	2.81545	3.50290	1.92365	2.99790
		S04	1.90535	3.17535	1.43945	1.92655
		S05	2.80295	3.12565	2.83170	2.70280
		S06	2.09870	3.26910	2.88970	2.55740
		S07	2.54025	3.27885	2.37210	2.69690
		S08	1.15630	3.20725	1.68495	2.21965
		S09	3.21110	3.47330	2.58010	3.14900
		S10	2.53980	2.71260	2.93990	2.35875
		S11	3.01670	3.40830	1.48055	2.84185
		S12	3.01035	3.07690	2.59025	2.72425
		S13	3.19805	3.40095	2.31100	2.97760
		S14	2.30980	3.01010	0.61455	2.12250
		S15	0.82135	3.44165	0.12615	3.15965
		S16	1.65550	2.96715	0.80200	3.01000
		S17	0.54665	2.92455	1.37010	2.45750
		S18	2.52410	2.96640	2.15910	2.80970
		S19	2.27360	2.84940	1.86385	2.31930

174

Continuation of Supp Table 3

		hCoV IgG levels			
Groups	**Donor**	**anti-NL63**	**anti-OC43**	**anti-229E**	**anti-HKU1**
HEALTHY DONORS	**HD01**	2.78740	2.76360	2.16610	2.18175
	HD02	1.56065	1.08055	1.85665	1.10430
	HD03	3.46150	3.34870	2.18810	2.31240
	HD04	3.32320	3.34310	2.34810	2.24415
	HD05	2.84550	3.12610	2.78980	1.08700
	HD06	2.48170	1.89540	2.35595	0.77970
	HD07	3.14250	3.10555	3.07435	1.58460
	HD08	2.41505	1.79415	2.76840	1.10670
	HD09	1.41850	1.61420	1.38180	1.94045
	HD10	1.76910	0.92290	1.50620	0.28585
	HD11	2.18840	1.68420	2.74380	1.10005
	HD12	0.43990	0.97100	0.64410	0.34825
	HD13	3.23670	2.91245	2.68235	2.20570
	HD14	2.71950	3.06770	2.50610	2.08350
	HD15	3.21100	2.91520	2.00115	1.84450
	HD16	3.00565	3.44410	2.60100	2.94880
	HD17	3.23340	3.46235	2.74580	3.01430
	HD18	3.07415	2.46865	2.18675	2.59050
	HD19	2.17750	2.12810	2.48465	2.04760
	HD20	2.74580	3.44920	2.43030	1.96845
	HD21	3.45685	3.40485	1.90945	2.42975
	HD22	2.90100	3.04070	3.00650	2.35845
	HD23	2.71295	2.90215	3.09800	1.81250
	HD24	3.29385	3.35510	3.07445	2.28220
	HD25	2.32495	1.62785	1.64240	0.97640
	HD26	3.10550	3.10445	1.88390	1.73530
	HD27	3.30835	2.62560	2.83390	1.35510
	HD28	1.92085	3.46900	2.09340	1.36995

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177 **REFERENCES**

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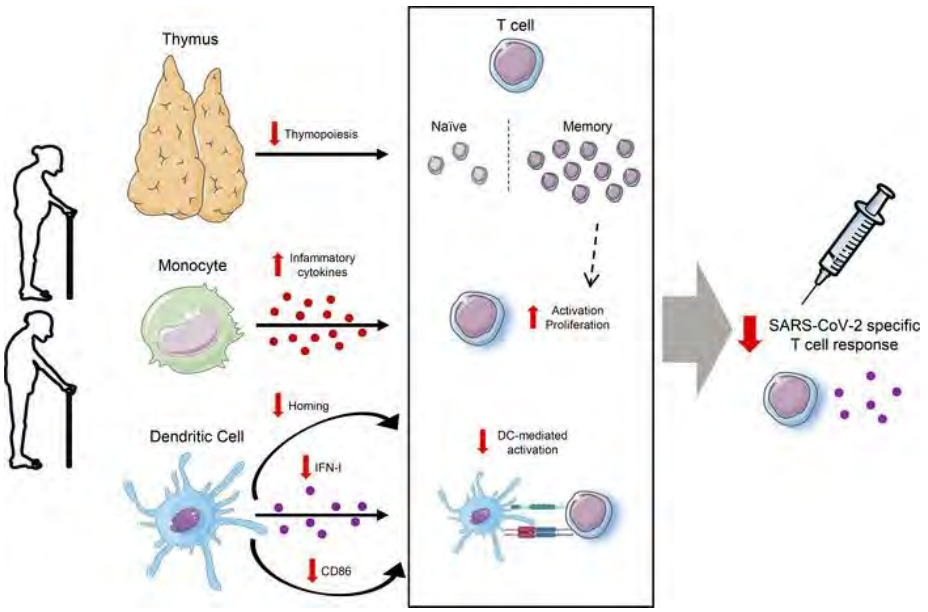
Research Article | Immunology | Vaccines

Graphical abstract



JCI insight

RESEARCH ARTICLE

Immune defects associated with lower SARS-CoV-2 BNT162b2 mRNA vaccine response in aged people

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The immune factors associated with impaired SARS-CoV-2 vaccine response in elderly people are mostly unknown. We studied individuals older than 60 and younger than 60 years, who had been vaccinated with SARS-CoV-2 BNT162b2 mRNA, before and after the first and second dose. Aging was associated with a lower anti–RBD IgG levels and a decreased magnitude and polyfunctionality of SARS-CoV-2–specific T cell response. The dramatic decrease in thymic function in people > 60 years, which fueled alteration in T cell homeostasis, and their lower CD161$^+$ T cell levels were associated with decreased T cell response 2 months after vaccination. Additionally, deficient DC homing, activation, and TLR-mediated function, along with a proinflammatory functional profile in monocytes, were observed in the > 60-year-old group, which was also related to lower specific T cell response after vaccination. These findings might be relevant for the improvement of the current vaccination strategies and for the development of new vaccine prototypes.

Introduction

Immune aging is sustained by multifaceted remodeling of innate and adaptive immunity, and it includes a diminished response to new antigens, a decreased memory T cell response, and a persistent chronic inflammation (1–4). Immune aging leads to more severe consequences of viral infections, as well as lower protection following vaccination (5). SARS-CoV-2 infection and its associated disease, COVID-19, are known to have a higher impact in aged people. In fact, delayed viral clearance, prolonged disease, and higher COVID-19 fatality rate have been related to age (6), and approximately 80% of hospitalizations involved people older than 65 years (7, 8).

Vaccination is the most effective tool for the prevention of the serious symptomatology caused by SARS-CoV-2 and other viral infections, especially for vulnerable populations such as elderly people (9, 10). The BNT162b2 mRNA vaccine, commonly known as the Biontech/Pfizer vaccine, has shown high safety and efficacy against severe outcomes of COVID-19 (11). The 2-dose vaccination of this SARS-CoV-2 vaccine induces a strong humoral response measured by the magnitude of binding antibodies to coronavirus Spike (S) protein and the neutralization capacity of the antibodies (11, 12). In addition, notable SARS-CoV-2 S–specific CD4$^+$ and CD8$^+$ T cell responses have been observed after BNT162b2 vaccination (13–15).

In spite of the promising results of the BNT162b2 vaccination, a lower effectiveness — in terms of COVID-19 symptoms, admissions to hospital, and deaths after the vaccination — has been reported in elderly people (16–18). Moreover, other studies have described lower levels of neutralizing antibodies in vaccinated elderly people compared with younger participants (19–21), especially 6 months after the second dose (22, 23). In addition, low levels of S-specific T cell response after vaccination have been shown in the elderly (20). However, the adaptive and innate immune factors associated with the lower vaccine response in elderly people are not yet characterized.

Conflict of interest: The authors have declared that no conflict of interest exists.

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A better understanding of the age-related immune dysfunction to the SARS-CoV-2 vaccine is crucial for future vaccination strategies to improve older adults' protection against this virus. Thus, the aim of this study was to investigate the major immune alterations in aged people, in terms of both SARS-CoV-2–specific adaptive and innate immunity, associated with a lower response to the SARS-CoV-2 BNT162b2 mRNA vaccine.

Results

Association of SARS-CoV-2–specific IgG levels with age. In this study, we included 54 healthy adults vaccinated with the BNT162b2 mRNA vaccine against SARS-CoV-2, classified according to their age: 33 young people < 60 years of age (median, 29 years [interquartile range (IQR) 26–49 years]) and 21 aged people > 60 years of age (median 73 years [IQR 72–74 years]). Comorbidities of all donors are presented in Supplemental Figure 1 (supplemental material available online with this article; https://doi.org/10.1172/jci.insight.161045DS1). As it was expected, the aged group showed a higher percentage of donors with comorbidities, with cardiovascular diseases and arthrosis appearing most prominently (Supplemental Figure 1). Three participants were excluded from the study due to a positive result for SARS-CoV-2 RNA PCR or antibodies against receptor-binding domain (RBD) of the S protein of SARS-CoV-2 prior to vaccination. The innate and adaptive immunity parameters were analyzed before vaccination (PRE); 3 weeks after the first dose, just before the administration of the second dose (1D); and 2 months after the second dose (2D) (Supplemental Figure 1).

Firstly, anti–RBD SARS-CoV-2 IgG levels were quantified by RBD-specific ELISA in > 60-year-old and < 60-year-old people at the 3 time points described above. In accordance to previous studies (11, 20), the BNT162b2 mRNA vaccine induced the production of SARS-CoV-2–specific IgG levels, and these levels were much higher after the second dose compared with the first dose (Figure 1A). Although no significant differences were observed between aged and young participants, young people tended to produce higher levels of specific antibodies after the first dose (Figure 1A). This tendency was also observed when we correlated anti–RBD IgG levels and age after the administration of the first dose (Figure 1B, left) and after the second dose (Figure 1B, right). In addition to SARS-CoV-2–specific antibodies, we also determined IgG autoantibodies against IFN-α in plasma of the studied donors. In fact, anti–IFN-Is have been previously observed in severe COVID-19 patients (24), but the role of these antibodies in the context of SARS-CoV-2 vaccination remains unknown. anti–IFN-α IgGs were observed only in 2 donors; these autoantibodies were found before and after SARS-CoV-2 vaccination (Supplemental Figure 2). Therefore, only 2 donors were positive for anti–IFN-α autoantibodies, and this was not related to the response to SARS-CoV-2 vaccination.

Aged people show a lower and less polyfunctional SARS-CoV-2 S–specific CD4$^+$ and CD8$^+$ T cell response after vaccination. We investigated the magnitude and polyfunctionality of SARS-CoV-2 S–specific CD4$^+$ and CD8$^+$ T cell response through intracellular cytokine staining. We analyzed these parameters in total memory (Memory), central memory (CM), effector memory (EM), and terminal differentiated effector memory (TEMRA) CD4$^+$ and CD8$^+$ T cells (Supplemental Figure 2A). Three weeks after the administration of the first dose, CD4$^+$ T cells principally produced IFN-γ and TNF-α as acute responses to SARS-CoV-2 S protein, but they also expressed low levels of the degranulation marker CD107a, perforin (PRF), and IL-2 (Supplemental Figure 2B). Importantly, CD4$^+$ T cells from people > 60 years old showed a lower SARS-CoV-2 S–specific IFN-γ production and cytotoxic response, reflected in the percentage of CD107a$^+$ and PRF$^+$ cells, after the first dose of vaccination — but mainly after the second dose (Figure 2A). In fact, the second dose of vaccination induced an increase in the cytotoxic function by CD4$^+$ T cells in people < 60 years old but not in people > 60 years old (Figure 2A and Supplemental Figure 2C). Regarding CD8$^+$, as it was observed in CD4$^+$ T cells, aged people showed a lower SARS-CoV-2 S–specific CD8$^+$ T cell response, based mainly on the production of IFN-γ and the cytotoxic capacity (CD107a$^+$ and PRF$^+$), 2 months after the second dose (Figure 2B).

To determine the quality of the specific T cell response to the SARS-CoV-2 vaccine, we analyzed the polyfunctionality of CD4$^+$ and CD8$^+$ T cells, which is defined by those cells that simultaneously produce multiple cytokines and degranulate (functions). In general, a low polyfunctional T cell response was observed after the vaccination with BNT162b2 mRNA vaccine in both aged and young participants (Figure 2C). However, a more polyfunctional memory CD4$^+$ T cell response to SARS-CoV-2 was observed in people < 60 years old compared with people > 60 years old after the first dose, and a similar trend was observed after the second dose (Figure 2C). The polyfunctional profile of the rest of CD4$^+$ T cell subsets showed a similar pattern, with the exception of TEMRA CD4$^+$ T cells that presented higher polyfunctionality after the second dose in

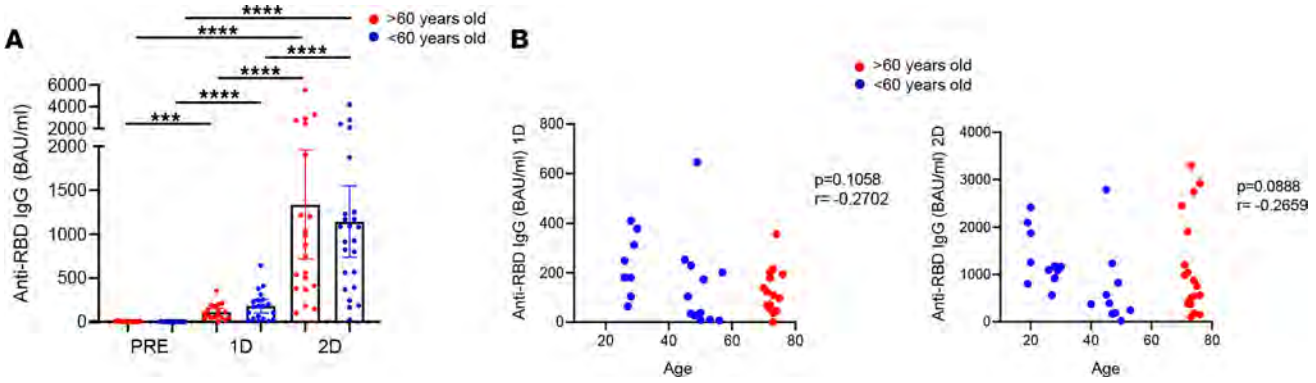


Figure 1. **Association of SARS-CoV-2–specific IgG levels with age**. (**A**) Anti–RBD IgG levels (binding antibody units [BAU]/mL) in participants > 60 years old (red) and those < 60 years old (blue) before SARS-CoV-2 vaccination (PRE), 3 weeks after the first dose (1D), and 2 months after the second dose (2D). (**B**) Correlation of anti–RBD IgG levels with age in all the study participants after the first dose (left) and after the second dose (right). Mann-Whitney *U*, Wilcoxon, and Spearman tests were used ($n = 54$). Friedman test was applied in **A** (>60-year-old, ****$P \leq 0.0001$; <60-year-old, ****$P \leq 0.0001$).

young people (Supplemental Figure 2D). To further characterize this SARS-CoV-2–specific T cell response to vaccination, we analyzed different combinations of the studied functions. $CD4^+$ T cells expressing CD107a and PRF simultaneously were enriched in young people < 60 years old 2 months after the second dose of vaccination, confirming the higher T cell cytotoxic capacity of young people (Figure 2, C and D). Combinations including both IFN-γ and TNF-α and the ones including only IFN-γ were mainly observed in young people after the first and second dose of vaccination (Figure 2D). Furthermore, the percentages of T cells expressing 3 functions at the same time (e.g., IFN-γ^+CD107a$^+$PRF$^+$ or IFN-γ^+IL-2$^+$TNF-α^+) and other 2-function combinations (e.g., IFN-γ^+IL-2$^+$) were also higher in the < 60-year-old group than in > 60-year-old group (Supplemental Figure 2E). Therefore, the SARS-CoV-2 S–specific T cell response is lower and less polyfunctional in aged people after vaccination with the BNT162b2 mRNA vaccine.

Lower thymic function and altered T cell homeostasis found in aged people are associated with a lower T cell response to the SARS-CoV-2 vaccine. Once we demonstrated that aged people displayed a lower SARS-CoV-2–specific T cell response after vaccination, we investigated the immune defects that might be involved in the diminished response of this vulnerable population. In our group, we previously reported that thymic function failure and inflammation levels independently predict all-cause mortality in healthy elderly people (25). Thus, we studied if these factors could be associated with a lower SARS-CoV-2 vaccine response in aged participants. Thymic output can be measured through the presence of T cell receptor rearrangement excision circles (TREC) in naive T cells, which are indicators of recent thymic emigrants in humans (26). Thus, to determine the thymic function, we have adapted signal joint TREC (sjTREC) measurement with droplet digital PCR (ddPCR). Our results showed a considerably lower level of thymic function in people > 60 years old in comparison with participants < 60 years old; accordingly, there was a strong association between thymic function and age (Figure 3A). We observed a correlation between thymic function with naive $CD4^+$ and $CD8^+$ T cells and with the naive $CD4^+/CD8^+$ ratio (Figure 3B). In addition, decreased thymic function has been related to the phenomenon called memory inflation, which includes the alteration of the naive and memory T cell proportions in the periphery skewing toward memory T cells (27). This phenomenon was reflected in $CD4^+$ and $CD8^+$ T cell subset distribution in aged people > 60 years old, which displayed lower percentages of naive T cells and higher percentages of memory T cells than young participants (Figure 3C). Three weeks after the vaccination with the first dose, young participants < 60 years old showed a trend to decrease the percentages of naive T cells and to increase memory T cells and showed the restoration of T cell subset distribution that occurred 2 months after the second dose (Figure 3C). Thus, the differences in naive and memory T cells between aged and young people observed prior to vaccination were lost after the first dose but were restored 2 months after the second dose (Figure 3C).

According to lower thymic function and high memory inflation, elevated levels of T cell homeostatic proliferation and activation were found in the elderly people. Specifically, we observed that, prior to vaccination, people > 60 years old displayed a higher percentage of activated (HLA-DR$^+$) and proliferating (Ki67$^+$) memory and CM $CD4^+$ T cells compared with people < 60 years old (Figure 3, D and E). After vaccination with 2 doses, an increase of HLA-DR$^+$ and Ki67$^+$ $CD4^+$ T cells was observed in young participants, while

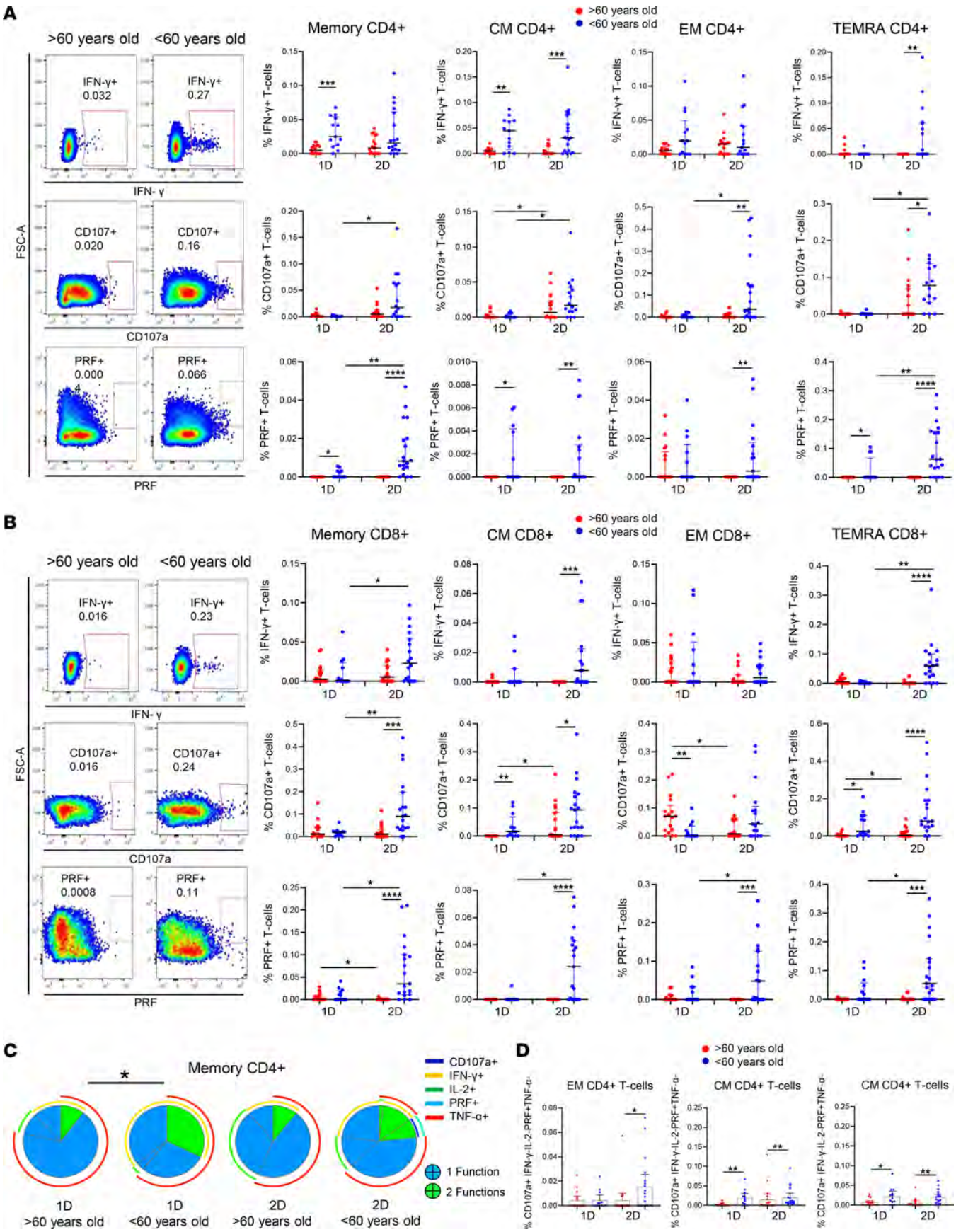


Figure 2. Aged people show a lower and less polyfunctional SARS-CoV-2 S–specific CD4⁺ and CD8⁺ T cell response after the vaccination. (**A** and **B**) Dot plots presenting the percentage of IFN-γ⁺, CD107a⁺, and PRF⁺ cells within memory, CM, EM, and TEMRA CD4⁺ (**A**) and CD8⁺ (**B**) T cells after S-specific SARS-

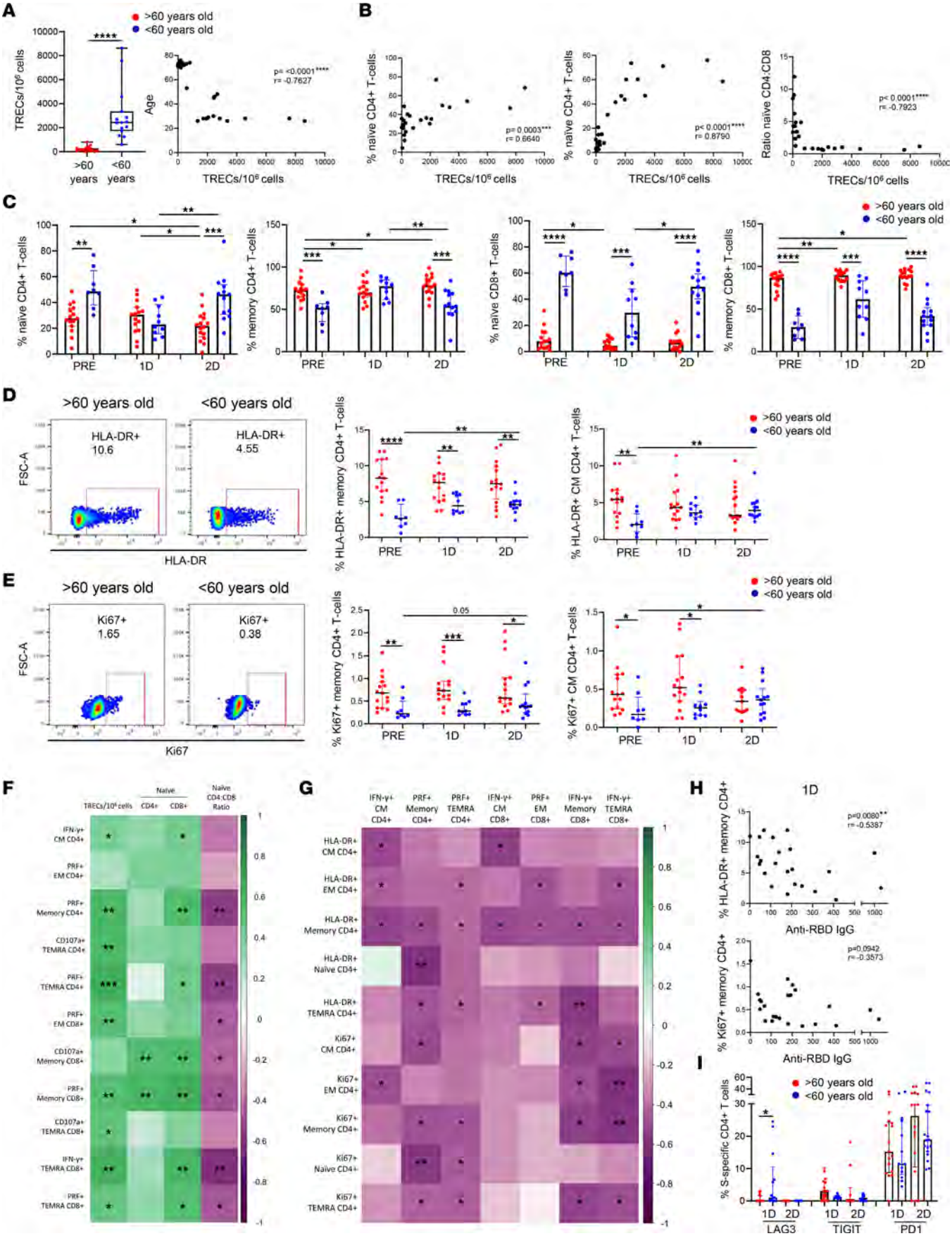
CoV-2 stimulation, comparing participants > 60 years old (red) and < 60 years old (blue) 3 weeks after the first dose (1D) and 2 months after the second dose (2D) of SARS-CoV-2 vaccine (right). Pseudocolor dot plot graphs show a representative data of memory $CD4^+$ T cells from a > 60-year-old and a < 60-year-old donor 2 months after vaccination (left). (**C**) Pie charts representing SARS-CoV-2 S–specific memory $CD4^+$ T cell polyfunctionality. Each sector represents the proportion of S-specific $CD4^+$ T cells producing 2 (green) or 1 (blue) functions. Arcs represent the type of function (CD107a, IFN-γ, IL-2, PRF, and TNF-α) expressed in each sector. (**D**) Bar graphs showing the percentage of EM and CM $CD4^+$ T cells expressing different combinations of studied functions (CD107a, IFN-γ, IL-2, PRF, and TNF-α) comparing > 60-year-old (red) and < 60-year-old (blue) participants after the first (1D) and the second (2D) dose. Mann-Whitney *U* (**A**, **B**, and **D**), Wilcoxon (**A**, **B**, and **D**), and Permutation (**C**) tests were used ($n = 41$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

older people showed a lack of further activation and proliferation through vaccination (Figure 3, D and E). Similar results were found in the rest of $CD4^+$ T cell subsets (Supplemental Figure 3, A and B) and in some of the $CD8^+$ T cell subsets (data not shown). Thymic function was negatively correlated with T cell activation in most of the subsets and was negatively correlated with proliferation in naive $CD8^+$ T cells (Supplemental Figure 3C). Importantly, thymic dysfunction and the related defects in the homeostasis of a T cell compartment found in aged people prior to vaccination — expressed as higher T cell activation and proliferation and memory inflation — and memory inflation were correlated with a lower SARS-CoV-2–specific $CD4^+$ and $CD8^+$ T cell response after the 2-dose vaccination (Figure 3, F and G). Furthermore, the percentage of activated ($HLA\text{-}DR^+$) memory $CD4^+$ T cells was also inversely associated with anti–RBD IgG levels after the first dose, and the same trend was observed regarding proliferating ($Ki67^+$) T cells (Figure 3H). Moreover, the vaccination altered the expression of immune checkpoints as LAG-3, PD-1, and TIGIT on $CD4^+$ and $CD8^+$ T cells being higher in the < 60-year-old group compared with the > 60-year-old group after the first or second dose in most of the T cell subsets (Supplemental Figure 3, D–F). The same result was observed regarding LAG-3 expression within SARS-CoV-2–specific $CD4^+$ T cells, where this expression was higher in young participants after the first dose (Figure 3I). In contrast, PD-1 and TIGIT tended to be lower in the SARS-CoV-2–specific $CD4^+$ T cells from < 60-year-old group compared with the > 60-year-old group (Figure 3I). In addition, virus-specific T cell response after vaccination was also inversely correlated with the expression of PD-1 in bulk $CD4^+$ T cells before vaccination (Supplemental Figure 3G).

Other T cells that notably differed between aged and young participants were $CD161^+$ T cells. These cells mainly produce IL-17 and are known to have an important contribution in pathogen clearance (28). $CD161^+$ T cells presented higher levels in people < 60 years old compared with people > 60 years old, independently on the vaccination (Figure 4A). The percentage of CD161-expressing $CD4^+$ and $CD8^+$ T cells was positively associated with SARS-CoV-2 S–specific T cell response 2 months after the vaccination (Figure 4B).

An impaired DC homing and functional capacity are associated with a lower T cell response to SARS-CoV-2 vaccine in aged people. In addition to the alteration of the adaptive immunity associated with lower response to the vaccine in the elderly, there is a remodeling of the innate immune system with aging (29). Thus, we next studied plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) (Supplemental Figure 4A), innate immune cells with a key role in the modulation of T cell response (30). We first observed a decrease in pDC percentages 2 months after the second dose (Supplemental Figure 4B). Although we did not find differences between people > 60 and < 60 years old in pDC levels (Supplemental Figure 4B), we observed a considerable difference in pDC functional capacity (Figure 5A). When cells were stimulated in a TLR-9–dependent manner by CpG-A, a lower IFN-α production was observed in aged people compared with young participants, both after the first and second dose of the SARS-CoV-2 vaccine (Figure 5A, left panel). Interestingly, we also observed how this functional capacity of the pDCs was associated with anti–RBD IgG levels after the first dose (Figure 5A, right panel).

Next, we focused on mDC subsets, including $CD1c^+$, $CD16^+$, and $CD141^+$ mDCs (Supplemental Figure 4A). Our results showed a higher percentage of $CD1c^+$ mDCs in < 60-year-old than in > 60-year-old people prior to vaccination and after the first dose (Figure 5B), a mDC subpopulation that modulates $CD4^+$ T cell response (30). In contrast, a notable decrease in $CD1c^+$ mDC percentage was observed only in young people 2 months after the second dose of the SARS-CoV-2 vaccine (Figure 5B). The same result was found regarding the percentage of integrin-β7–expressing $CD1c^+$ mDCs (Supplemental Figure 4C, left panel), with integrin-β7 as a maker of cell homing to gut. It is remarkable, that the decrease in integrin-$\beta7^+CD1c^+$ mDCs was correlated with a higher IFN-γ production by TEMRA $CD4^+$ and $CD8^+$ T cells in response to SARS-CoV-2 two months after vaccination (Supplemental Figure 4C, right panel). Furthermore, higher expression of indoleamine 2,3-dioxygenase (IDO) was found on $CD1c^+$ mDCs from young people < 60 years old in most of the studied time points (Figure 5C), and the percentage of $CD1c^+$ and IDO^+CD1c^+ mDCs prior to vaccination were correlated with SARS-CoV-2–specific T cell response 2 months after the


A
>60 years old
<60 years old
TRECs/10⁶ cells
Age
TRECs/10⁶ cells
p= <0.0001****
r= -0.7627
B
% naive CD4+ T-cells
TRECs/10⁶ cells
p= 0.0003***
r= 0.6640
p< 0.0001****
r= 0.8790
Ratio naive CD4:CD8
p< 0.0001****
r= -0.7923
C
% naive CD4+ T-cells
% memory CD4+ T-cells
% naive CD8+ T-cells
% memory CD8+ T-cells
PRE
1D
2D
D
>60 years old
<60 years old
HLA-DR+ 10.6
HLA-DR+ 4.55
FSC-A
HLA-DR
% HLA-DR+ memory CD4+ T-cells
% HLA-DR+ CM CD4+ T-cells
E
Ki67+ 1.65
Ki67+ 0.38
Ki67
% Ki67+ memory CD4+ T-cells
% Ki67+ CM CD4+ T-cells
F
TRECs/10⁶ cells
Naive CD4+
Naive CD8+
Naive CD4:CD8 Ratio
IFN-γ+ CM CD4+
PRF+ EM CD4+
PRF+ Memory CD4+
CD107a+ TEMRA CD4+
PRF+ TEMRA CD4+
PRF+ EM CD8+
CD107a+ Memory CD8+
PRF+ Memory CD8+
CD107a+ TEMRA CD8+
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HLA-DR+ Memory CD4+
HLA-DR+ Naive CD4+
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H
1D
% HLA-DR+ memory CD4+
p=0.0080**
r= -0.5387
Anti-RBD IgG
% Ki67+ memory CD4+
p=0.0942
r= -0.3573
Anti-RBD IgG
I
% S-specific CD4+ T cells
LAG3
TIGIT
PD1

Figure 3. Lower thymic function and altered T cell homeostasis found in aged people are associated with a lower T cell response to the SARS-CoV-2 vaccine. (**A** and **B**) Bar graphs showing TREC/1 × 10^6 cells as a measure of thymic function in > 60-year-old and < 60-year-old participants prior to vaccination (**A**, left) and the correlation of the TREC/1 × 10^6 cells with age (**A**, right), naive CD4$^+$ T cells (**B**, left), naive CD8$^+$ T cells (**B**, middle), and naive CD4$^+$/CD8$^+$ T cell ratio (**B**, right). (**C**) Bar graphs representing the percentage of naive and memory CD4$^+$ and CD8$^+$ T cells in > 60-year-old (red) and < 60-year-old (blue) participants before SARS-CoV-2 vaccination (PRE), 3 weeks after the first dose (1D), and 2 months after the second dose (2D). (**D** and **E**) Dot plots representing the percentage of memory and CM CD4$^+$ T cells expressing HLA-DR (**D**) and Ki67 (**E**) > 60-year-old (red) and < 60-year-old (blue) participants at the 3 time points (right). Pseudocolor dot plot graphs show representative data of memory CD4$^+$ T cells from a > 60-year-old (red) and < 60-year-old (blue) donor expressing HLA-DR (**D**) and Ki67 (**E**) before vaccination (left). (**F** and **G**) Correlation matrixes representing associations of SARS-CoV-2 S–specific CD4$^+$ and CD8$^+$ T cells expressing IFN-γ or cytotoxicity markers 2 months after the second dose of vaccination with TREC/1 × 10^6 cells and naive T cells (**F**) and with the percentage of HLA-DR$^+$ and Ki67$^+$ CD4$^+$ T cells (**G**) before vaccination in all participants. (**H**) Correlation plots of anti–RBD IgG levels after the first dose of vaccination with the percentage of HLA-DR$^+$ and Ki67$^+$ CD4$^+$ T cells before vaccination. (**I**) Bar graphs representing the percentage of SARS-CoV-2–specific CD4$^+$ T cells expressing LAG-3 in > 60-year-old (red) and < 60-year-old (blue) participants at the 3 follow-up time points. Mann-Whitney *U* (**A**, **C**, **D**, **E**, and **I**), Wilcoxon (**A**, **C**, **D**, **E**, and **I**), and Spearman (**A**, **B**, **F**, **G**, and **H**) tests were used (n = 32). Friedman test was applied in **C** (Naive CD4$^+$ T cells: > 60-year-old, P = 0.06, and < 60-year-old, P = 0.074; Memory CD4$^+$ T cells: > 60-year-old, P = 0.071, and < 60-year-old, P = 0.074; Naive CD8$^+$ T cells: > 60-year-old, P = 0.307, and < 60-year-old, P = 0.015; Memory CD8$^+$ T cells: > 60-year-old, P = 0.035, and < 60-year-old, P = 0.091), **D** (Memory CD4$^+$ T cells: > 60-year-old, P = 0.441, and < 60-year-old, P = 0.022; CM CD4$^+$ T cells: > 60-year-old, P = 0.529, and < 60-year-old, P = 0.022), and **E** (Memory CD4$^+$ T cells: > 60-year-old, P = 0.273, and < 60-year-old, P = 0.074; CM CD4$^+$ T cells: > 60-year-old, P = 0.657, and < 60-year-old, P = 0.091). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

second dose (Figure 5D). Interestingly, although no significant correlation was found, a trend was observed between IDO expression on CD1c$^+$ and CD16$^+$ mDCs and proliferating (Ki67$^+$) memory CD4$^+$ T cells (Supplemental Figure 4D). The expression of CD86, PD-L1, and CD4 on CD1c$^+$ mDCs, markers related to the modulation of T cell response, was also higher in young people (Figure 5, E–G).

It is also known that CD141$^+$ mDCs are involved in the regulation of CD8$^+$ T cell response (30). In this study, higher CD141$^+$ mDC levels were found in the < 60-year-old group after the first dose, compared with > 60-year-old group (Supplemental Figure 4E). As occurred with CD1c$^+$ mDCs, young people also displayed a higher expression of the costimulatory molecule CD86 (Figure 5H, left panel) after vaccination in this subset, and this was correlated with SARS-CoV-2–specific cytotoxic response (PRF$^+$) by TEMRA T cells (Figure 5H, right panel). In order to study the functional capacity of mDCs, cells were stimulated with Poly(I:C), an agonist of TLR-3. In all participants, the percentages of activated CD141$^+$ mDCs (CD86$^+$CD40$^+$ and CD83$^+$) were increased following TLR-3 stimulation, before vaccination and after the first dose (Supplemental Figure 4F). Nevertheless, 2 months after the second dose, CD141$^+$ mDCs were not successfully stimulated via TLR-3, since they were already activated by the vaccination (Supplemental Figure 4E). This effect was also observed in the rest of mDC subsets (Supplemental Figure 4, G and H). Even though no differences were found between aged and young people in CD141$^+$ mDC response, a higher functional capacity of CD141$^+$ mDCs prior to vaccination was positively associated with SARS-CoV-2–specific T cell response after vaccination (Figure 5I). Additionally, other DC markers were altered after the vaccination on different subsets. CCR7, a chemokine receptor that orchestrates DC migration to draining lymph nodes (31), was highly expressed in aged people > 60 years old before vaccination, and its expression was increased after the first and second dose mostly in young people, with no response in elderly people (Figure 5J, left and middle panels). A higher CCR7 expression on DCs prior to vaccination was associated with a lower SARS-CoV-2–specific T cell response (Figure 5J, right panel).

Due to their role in the modulation of inflammatory responses, CD16$^+$ mDCs were also studied (32). In general, people > 60 years old displayed higher percentages of CD16$^+$ mDCs than participants < 60 years old (Figure 6A). Focusing on the effect of SARS-CoV-2 vaccination, an increase in CD16$^+$ mDC percentages was observed mainly after the first dose but also after the second dose only in aged participants (Figure 6A). The percentage of CD16$^+$ mDCs and the percentages of CD16$^+$ mDCs expressing integrin-β7, IDO, and CCR7 were inversely associated with S-specific T cell response (Figure 6B). Hence, our results indicate that the higher levels of CD16$^+$ mDCs, which have a proinflammatory function, along with the impaired DC homing and functional capacity found in aged people were associated with a lower T cell response to SARS-CoV-2 vaccine.

Higher monocyte-mediated proinflammatory profile found in aged people is associated with a lower T cell response to SARS-CoV-2 vaccine. As one of the main players in inflammatory responses and the inflammaging phenomenon (29, 33), monocytes were analyzed in this study. Specifically, previously studied activation and homing markers (34, 35) were analyzed in classical (CD14^{++}CD16$^-$), intermediate (CD14^{++}CD16$^+$) and nonclassical monocytes (CD14$^+$CD16^{++}) (Supplemental Figure 5A). Our results show that, in all participants, SARS-CoV-2 vaccination induced an increase in the expression of activation markers and TLR in classical and intermediate monocytes, including CD40, TLR-4 (Figure 7A, left panel, and Supplemental Figure 5B), TLR-2, and

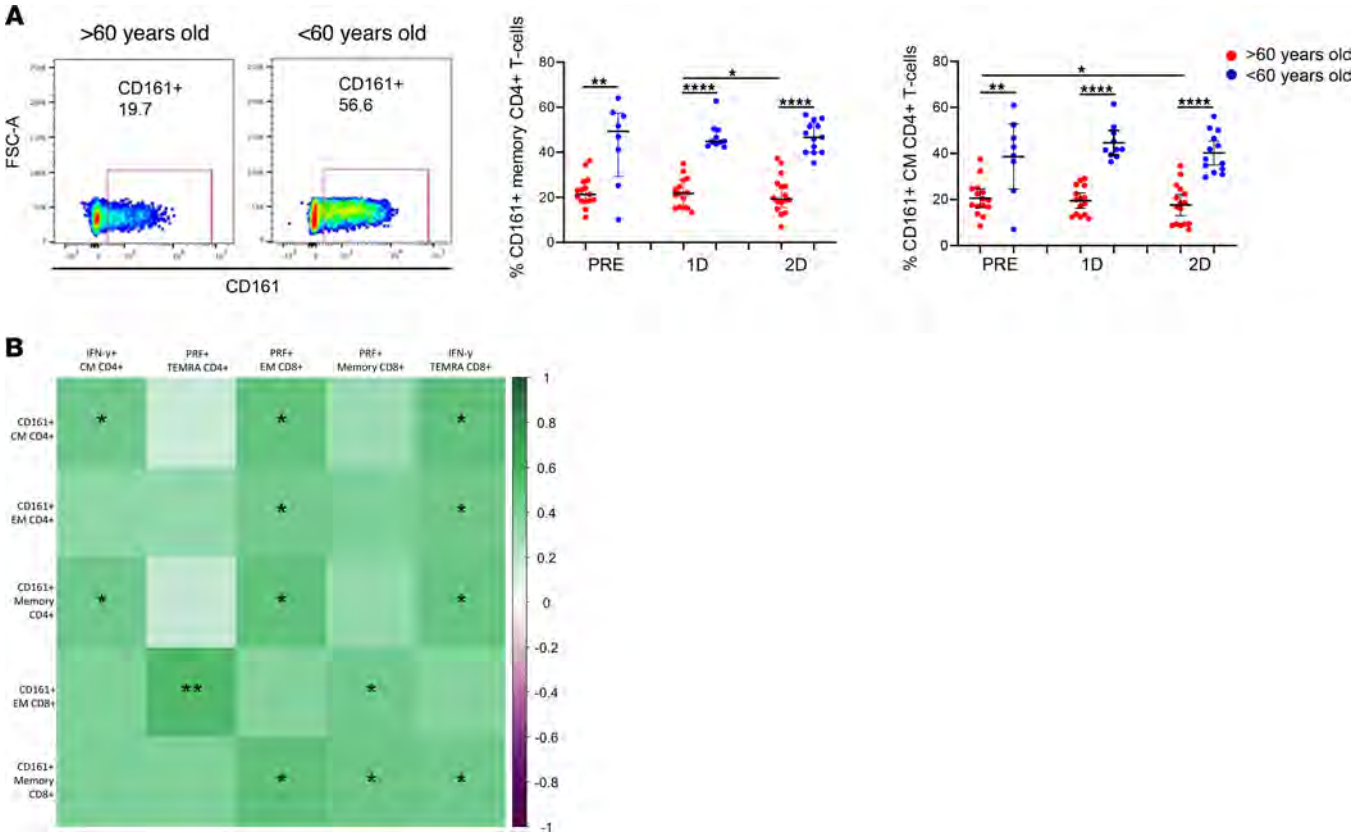

Figure 4. CD161-expressing T cell levels are associated with a higher SARS-CoV-2 vaccine T cell response. (**A**) Dot plots representing the percentage of memory and CM CD4$^+$ T cells expressing CD161 in > 60-year-old (red) and < 60-year-old (blue) participants before SARS-CoV-2 vaccination (PRE), 3 weeks after the first dose (1D), and 2 months after the second dose (2D) (right). Pseudocolor dot plot graphs show representative data of memory CD4$^+$ T cells from a > 60-year-old and < 60-year-old donor expressing CD161 before vaccination (left). (**B**) Correlation matrix representing associations of the percentage of CD161$^+$ T cells before vaccination with SARS-CoV-2 S–specific CD4$^+$ and CD8$^+$ T cells expressing IFN-γ or cytotoxicity markers 2 months after the second dose of vaccination in all participants. Mann-Whitney *U* (**A**), Wilcoxon (**A**), and Spearman (**B**) tests were used (n = 32). Friedman test was applied in **A** (Memory CD4$^+$ T cells: > 60-year-old, P = 0.091, and < 60-year-old, P = 0.368; CM CD4$^+$ T cells: > 60-year-old, P = 0.159, and < 60-year-old, P = 0.549).

CD49d (Supplemental Figure 5, C and D). Nevertheless, the monocyte activation levels after the first dose were higher in people < 60 years old than people > 60 years old (Figure 7A and Supplemental Figure 5B). The expression levels of CD40 and TLR-4 before vaccination were positively correlated with SARS-CoV-2–specific T cell response 2 months after the second dose (Figure 7A, right panel). The expression of CCR5, a monocyte chemokine receptor, is modulated after activation (36). Our results show that CCR5 expression before vaccination was higher in monocytes from young people compared with those from aged people (Figure 7B, left panel). However, CCR5 expression was downregulated after the first dose of SARS-CoV-2 vaccination, with this decrease being less pronounced in aged people (Figure 7B, right panel). Importantly, basal CCR5 expression was also directly correlated to IFN-γ production by CM CD4$^+$ and TEMRA CD8$^+$ T cells after SARS-CoV-2 vaccination (Figure 7C). Regarding other monocyte chemokine receptors, 2-dose vaccination induced a decrease in the percentage of intermediate monocytes expressing CCR2 and CD11b and nonclassical monocytes expressing CX3CR1; these percentages were lower in people < 60 years old than in people > 60 years old (Figure 7, C and D, and Supplemental Figure 5E). The lower percentages of monocytes expressing these chemokine receptors 2 months after the second dose of vaccination were inversely correlated with SARS-CoV-2 T cell response, and CD11b was also negatively correlated with anti–RBD IgG levels (Figure 7, E–G). Furthermore, the expression of CX3CR1 in nonclassical monocytes prior to vaccination was also inversely associated with the SARS-CoV-2–specific T cell response (Figure 7F). Moreover, monocyte tissue factor (CD142) expression is known to be induced by several inflammatory stimuli (37, 38). Here, we found an increase in the expression of tissue factor on classical monocytes after SARS-CoV-2 vaccination in both groups (Supplemental Figure 5F, left panel). In addition, tissue factor expression levels were higher on intermediate monocytes from young participants after the first dose (Supplemental Figure 5F, right panel).

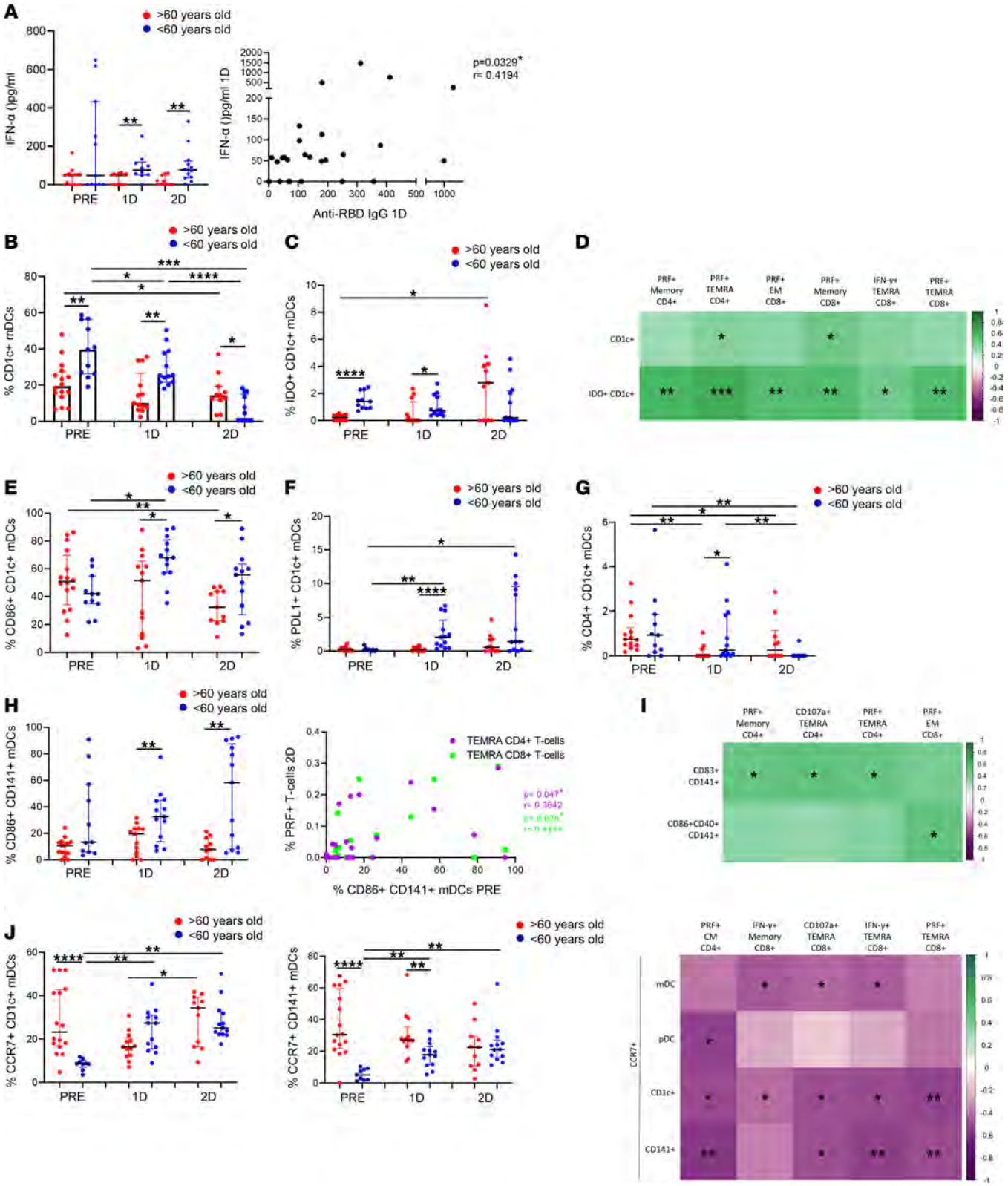

Figure 5. An impaired DC homing and functional capacity are associated with a lower T cell response to the SARS-CoV-2 vaccine in aged people. (**A**) Dot plots showing IFN-α production through CpG-A stimulation for 18 hours in > 60-year-old (red) and < 60-year-old (blue) participants before SARS-CoV-2 vaccination (PRE), 3 weeks after the first dose (1D), and 2 months after the second dose (2D) (left). Correlation analysis of IFN-α production with anti–RBD IgG levels 3 weeks after the first dose of vaccination (right). (**B** and **C**) Bar graphs representing the percentage of CD1c$^+$ and IDO$^+$CD1c$^+$ mDCs in > 60-year-old (red) and < 60-year-old (blue) participants at the 3 follow-up time points. (**D**) Correlation matrix showing associations between the percentages of CD1c$^+$ mDCs and IDO$^+$CD1c$^+$ mDCs before vaccination with SARS-CoV-2 S–specific T cells expressing cytokines or cytotoxicity markers 2 months after the second dose. (**E**–**G**) Dot plots showing the percentage of CD1c$^+$ mDCs expressing CD86 (**E**), PD-L1 (**F**), and CD4 (**G**) in > 60-year-old (red) and < 60-year-old (blue)

participants at the 3 time points. (**H**) Dot plots showing the percentage of CD141$^+$ mDCs expressing CD86 (left) in > 60-year-old (red) and < 60-year-old (blue) participants at the 3 time points. Correlation plot between the percentage of CD86$^+$CD141$^+$ mDCs before vaccination and the percentage of S-specific PRF$^+$ TEMRA CD4$^+$ and CD8$^+$ T cells 2 months after the second dose (right). (**I**) Correlation matrix showing associations between the percentage of CD141$^+$ mDCs expressing activation markers after TLR-3 stimulation for 24 hours with SARS-CoV-2 S–specific CD4$^+$ and CD8$^+$ T cells expressing cytotoxicity markers. (**J**) Dot plots showing the percentage of CCR7$^+$ mDCs in > 60-year-old (red) and < 60-year-old (blue) participants in the 3 follow-up time points (left and middle panels), and a correlation matrix representing associations of the percentage of mDCs expressing CCR7 with SARS-CoV-2 S–specific CD4$^+$ and CD8$^+$ T cells expressing cytokines or cytotoxicity markers 2 months after the second dose (right panel). Mann-Whitney *U* (**A**, **B**, **C**, **E**, **F**, **G**, **H**, and **J**), Wilcoxon (**A**, **B**, **C**, **E**, **F**, **G**, **H**, and **J**), and Spearman (**A**, **D**, **H**, **I**, and **J**) tests were used (n = 32). Friedman test was applied in **A** (> 60-year-old, P = 0.801; < 60-year-old, P = 0.717), **B** (> 60-year-old, P = 0.169; < 60-year-old, P = <0.0001), **C** (> 60-year-old, P = 0.147; < 60-year-old, P = 0.027), **E** (> 60-year-old, P = 0.018; < 60-year-old, P = 0.086), **F** (> 60-year-old, P = 0.381; < 60-year-old, P = 0.013), **G** (> 60-year-old, P = 0.042; < 60-year-old, P = 0.034), **H** (> 60-year-old, P = 0.223; < 60-year-old, P = 0.234), and **J** (CD1c mDCs: > 60-year-old, P = 0.121; < 60-year-old, P = 0.001 and CD141 mDCs: > 60-year-old, P = 0.459; < 60-year-old, P = 0.001).

It has been previously reported that intermediate monocytes are expanded in the blood of patients with systemic infections (39). Here, we found a considerable increase in the percentage of intermediate monocytes, along with a decrease in classical monocytes, after the 2-dose SARS-CoV-2 vaccination only in people > 60 years old (Figure 8A). No differences were found prior to vaccination or after the first dose (Supplemental Figure 5G). Intermediate monocytes are known to secrete TNF-α, IL-1β, IL-6, and CCL3 upon TLR stimulation (39). In this study, we stimulated cells in a TLR-4–dependent manner by adding LPS. Importantly, vaccinated aged people showed a higher production of IL-6, IL-1α, and TNF-α by monocytes upon LPS stimulation compared with young participants, mainly after the second dose (Figure 8B). Lastly, we discovered that participants who did not show a cytotoxic SARS-CoV-2–specific T cell response (PRF$^-$) produced higher levels of inflammatory cytokines by monocytes after vaccination compared with the ones presenting cytotoxic T cells (PRF$^+$) after SARS-CoV-2 vaccination (Figure 8C). In summary, although monocytes from aged people showed lower levels of activation and homing after vaccination, they produced higher levels of proinflammatory cytokines upon LPS stimulation, and this was inversely associated with SARS-CoV-2–specific T cell response.

Discussion

Aging is associated with impaired COVID-19 vaccine response (20). We confirmed and extended that the age-related immunological defects were characterized mostly by a lower magnitude and polyfunctionality of SARS-CoV-2–specific T cell response. The adaptive and innate immune factors behind these defects in aged people included an alteration in T cell homeostasis parameters fueled by lower thymic function and higher T cell activation and proliferation, DC dysfunction, and a higher proinflammatory profile in circulating monocytes.

In spite of the efficacy of BNT162b2 mRNA vaccine to prevent severe COVID-19 outcomes, vulnerable populations such as elderly people remain at risk. Several studies have reported lower humoral response in aged participants following vaccination (19–23). In accordance to these findings, we observed an inverse association between anti–RBD IgG levels with age at 2 months after vaccination. Moreover, we also found a diminished specific T cell response to SARS-CoV-2 vaccine in aged people, confirming previous results (20, 21). In addition to the magnitude, high quality of SARS-CoV-2–specific T cell function is required to achieve an effective vaccine response, which has not been completely determined yet. We found that specific T cells, in addition to producing different cytokines, exhibited a cytotoxic response to the vaccination, which is diminished in the elderly. Although young people showed a higher polyfunctional response than aged people, the BNT162b2 mRNA vaccine did not induce a high polyfunctional T cell immunity in general. This could be one of the key factors that might explain the absence of vaccine efficacy to avoid new infections over time, independent of the virus variant of concern (VOC).

Age-related changes in the immune system, known as immunosenescence, cause a subclinical immune deficiency that involves a reduced antiviral function and vaccine response (5). One of the most known changes of the immune aging is the involution of the thymus (40, 27). In our study, aged people exhibited thymic dysfunction and the subsequent memory inflation, reproducing previous findings (41). Remarkably, it has been described that thymic function failure predicts all-cause mortality in healthy aged people (25) and has a relevant role in viral infections such as HIV-1 infection (26, 42, 43). Moreover, it has been suggested that thymic aging might have an important implication in COVID-19 disease severity (44, 45). Importantly, we discovered that the thymic dysfunction, along with the memory inflation and a higher homeostatic T cell proliferation and activation found in aged people, correlated with a lower response to SARS-CoV-2 vaccination. Accordingly, a lower activity of the thymus has been previously associated with diminished responsiveness to

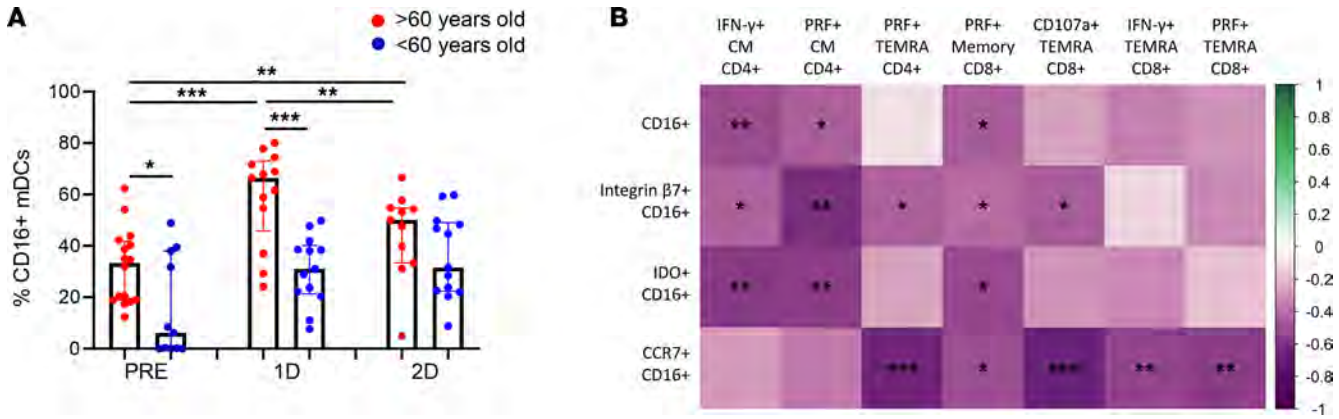

Figure 6. Higher CD16⁺ myeloid DC percentage from aged people is associated with a lower T cell response to the SARS-CoV-2 vaccine. (**A**) Bar graphs representing the percentage of CD16⁺ mDCs in > 60-year-old (red) and < 60-year-old (blue) participants before SARS-CoV-2 vaccination (PRE), 3 weeks after the first dose (1D), and 2 months after the second dose (2D) of vaccination. (**B**) Correlation matrix showing associations between the percentage of CD16⁺ mDCs and CD16⁺ mDCs expressing integrin-β7 and IDO with SARS-CoV-2 S–specific CD4⁺ and CD8⁺ T cells expressing cytotoxicity markers. Mann-Whitney *U* (**A**), Wilcoxon (**A**), and Spearman (**B**) tests were used (n = 32). Friedman test was applied in **A**, left (> 60-year-old, P = 0.01; < 60-year-old, P = 0.148).

vaccination against other viruses, such as yellow fever virus (46). In addition, thymic dysfunction was associated with a higher homeostatic T cell proliferation and activation. In fact, the age-dependent shift in the T cell population from naive to memory phenotype induces homeostatic T cell proliferation to compensate for the diminished T cell thymic output (47, 48). The higher T cell activation status found in aged people prior vaccination might be the reason why there is a lack of further activation of T cells after SARS-CoV-2 vaccination in this population. This was reflected in a lower increase in the expression of T cell activation markers, such as HLA-DR, in aged people after vaccination, as well as lower expression of checkpoint receptors as LAG-3 in both bulk and SARS-CoV-2–specific CD4⁺ T cells, a marker that is overexpressed after cellular activation (49).

One of the most remarkable findings of our study is the tight association of CD161 expression levels with age. CD161 is a C-type lectin receptor that is expressed in both T and NK cells (50). CD161⁺ T cells has been associated with IL-17 production and, hence, with pathogen clearance (51). In fact, we have previously observed that CD161⁺CD8⁺ T cells were related with HIV and hepatitis C virus–specific (HCV-specific) T cell polyfunctionality, which is essential for HIV spontaneous control and HCV spontaneous clearance (28). Results presented herein suggest the absence of a CD161 marker as a hallmark of T cell immunosenescence in accordance with recent findings, which show an inverse correlation of CD161 expression on CD8⁺ T cells with age, independent of CMV infection (52). Importantly, a reduction in the frequency of CD161⁺CD8⁺ T cells was found in peripheral blood of severe COVID-19 patients (53). In this study, we found that CD161 expression on T cells was tightly associated with SARS-CoV-2 vaccine response, highlighting this molecule as a potential target for immunotherapeutic strategies for age-related disease therapies and vaccine response in the elderly.

The regulation of the immune response highly depends on the function of DCs (30); however, their role in immune aging needs to be better understood. In this work, we found an altered DC subset distribution and an impaired DC homing and function with aging. The levels of CD1c⁺ mDCs, cells responsible for the modulation of CD4⁺ T cell response, were lower in aged people prior to vaccination, and this was related to a lower SARS-CoV-2 T cell response after vaccination. Accordingly, Schulz et al. previously observed an association between DC numbers and the response to yellow fever vaccine (46). Furthermore, the lower numbers of CD1c⁺ mDCs and integrin-β7–expressing CD1c⁺ mDCs observed after the second dose indicates that vaccination may induce a CD1c⁺ mDC migration to gut and probably to other tissues. A decrease in peripheral blood CD1c⁺ mDC numbers has been found in patients with severe COVID-19, due to a migration of these cells from blood to the lungs (54, 55). Interestingly, the vaccine-induced CD1c⁺ homing was barely observed in aged people, and this was also associated with a decreased SARS-CoV-2–specific T cell response, suggesting that aging might affect the capacity of CD1c⁺ mDCs to migrate and might consequently affect their ability to modulate T cell function. A lower expression of receptors such as CD86 in aged people could be indicative of a less efficient CD1c⁺ mDC activation and T cell costimulation after vaccination.

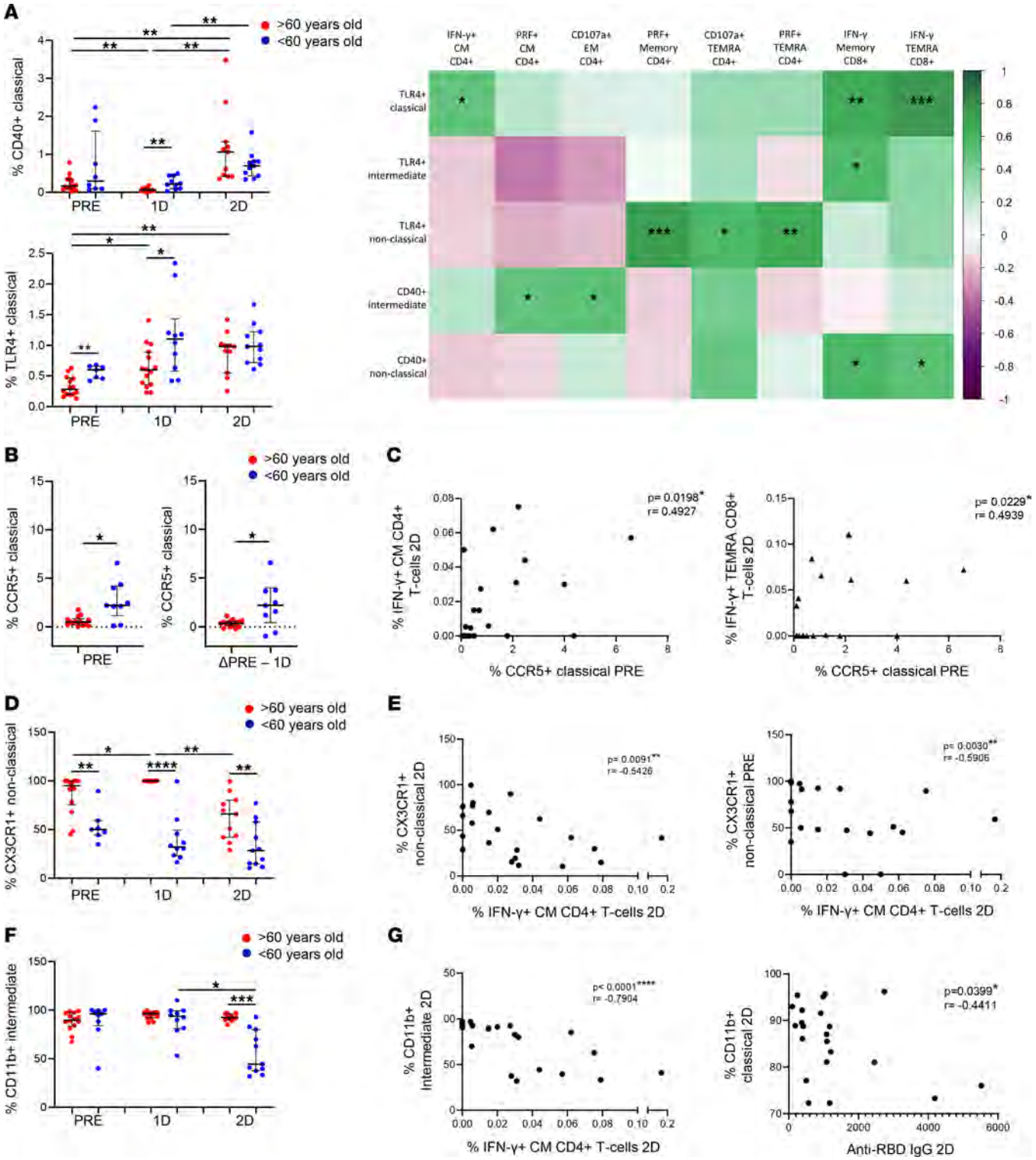

Figure 7. Diminished monocyte activation and homing found in aged people are related to a lower SARS-CoV-2 vaccine response. (**A**) Dot plots representing the percentage of classical monocytes expressing CD40 (top) and TLR-4 (bottom) in > 60-year-old (red) and < 60-year-old (blue) participants before SARS-CoV-2 vaccination (PRE), 3 weeks after the first dose (1D), and 2 months after the second dose (2D) of vaccination (left). A correlation matrix representing the percentage of monocytes expressing CD40 and TLR-4 prior to vaccination with SARS-CoV-2 S–specific $CD4^+$ and $CD8^+$ T cells expressing cytokines or cytotoxicity markers 2 months after the second dose (right). (**B** and **C**) Dot plots representing the percentage of classical monocytes expressing CCR5 before vaccination (**B**, left), the fold of decrease in the percentage of $CCR5^+$ cells after the first dose (**B**, right), and correlation analysis of the percentage of $CCR5^+$ classical monocytes prior to vaccination with SARS-CoV-2 S–specific $CD4^+$ and $CD8^+$ T cells expressing IFN-γ 2 months after the second dose (**C**). (**D** and **E**) Dot plots showing the percentages of nonclassical monocytes expressing CX3CR1 in > 60-year-old (red) and < 60-year-old (blue) participants at the 3 follow-up time points (**D**). Correlations of the percentage of nonclassical monocytes expressing CX3CR1 2 months after the second dose (**E**, left) and prior vaccination (**E**, right) with SARS-CoV-2 S–specific IFN-γ^+ $CD4^+$ T cells after the second dose. (**F** and **G**) Dot plots showing the percentages of

monocytes expressing CD11b in > 60-year-old (red) and < 60-year-old (blue) participants at the 3 follow-up time points (F). Correlation plots between the percentage of CD11b$^+$ classical monocytes with SARS-CoV-2 S–specific IFN-γ$^+$ CD4$^+$ T cells (**G**, left) and anti–RBD IgG levels (**G**, right) 2 months after the second dose. Mann-Whitney *U* (**A**, **B**, **D**, and **F**), Wilcoxon (**A**, **B**, **D**, and **F**), and Spearman (**A**, **C**, **E**, and **G**) tests were used (n = 28). Friedman test was applied in **A** (CD40: > 60-year-old, P = 0.006, and < 60-year-old, P = 0.050; TLR4: > 60-year-old, P = 0.042, and < 60-year-old, P = 0.779), **D** (> 60-year-old, P = 0.002; < 60-year-old, P = 0.717) and **F** (> 60-year-old, P = 0.607; < 60-year-old, P = 0.368).

In addition to homing, DC function is also altered with aging. In response to TLR-3, TLR-7/8, and TLR-9 ligand stimulation, CD141$^+$ mDCs and pDCs from aged participants secreted lower levels of IL-6, IL-12, and TNF-α (56). Other studies have reported that, in response to influenza A virus infection and West Nile infection, pDCs from older adults produced less type I IFN (57). Accordingly, our results show lower TLR-9–dependent IFN-α production by pDCs in aged people, both before and after the vaccination, against SARS-CoV-2. According to this, patients with COVID-19 presented lower TLR-9–mediated IFN-α production than healthy donors (54). This might be of great importance, since type I IFNs control the innate and adaptive immune system and induce cells' antiviral state via the upregulation of IFN-stimulated genes that inhibit the replication and spread of viruses (58). Remarkably, we found that CD141$^+$ mDCs may also have an important implication in SARS-CoV-2 vaccination immunity, which is one of the cell types that is known to be depleted in COVID-19 patients and which is important for disease progression (55, 59). Here, we discovered that TLR-3–mediated CD141$^+$ mDC activation capacity was directly associated with SARS-CoV-2 T cell response following vaccination. This is in accordance with the important function of CD141$^+$ mDCs in antigen presentation to T cells (60, 61).

Other important innate immune cells that might have relevant roles in vaccine response are monocytes. In this study, we reported that SARS-CoV-2 vaccination caused monocyte activation and homing, reflected by higher expression of activation markers (CD40, TLR-4, TLR-2, and CD49d) and lower percentages of CCR2-, CD11b-, and CX3CR1-expressing intermediate and nonclassical monocytes after vaccination. It has been described that, in addition to DCs, these monocyte subsets also migrate from bloodstream to lungs in patients with COVID-19 (55). However, vaccine-induced monocyte homing was found mainly in young people. As we observed in CD1c$^+$ mDCs, less monocyte homing was associated with a lower specific T cell response to vaccination. Although monocytes do not have a main role in the modulation of T cell response, monocytes are known to have the ability to prime tissue-resident T cells via cytokine production (62). Thus, a deficit in monocyte migration to inflammatory sites might also negatively affect T cell response.

One of the key age-related immune defects is the phenomenon called inflammaging, which is a persistent increase in basal proinflammatory phenotype found in the elderly (4). Monocytes are one of the principal players of inflammaging (29, 33). Inflammation is a critical factor in COVID-19 progression, where a monocyte-driven cytokine storm induces a hyperinflammatory phenotype, leading to a more severe symptomatology in COVID-19 patients (63). Here, we discovered higher TLR-4–mediated proinflammatory cytokine production by monocytes from aged people after SARS-CoV-2 vaccination; importantly, this cytokine production was inversely correlated with specific T cell response. This is in accordance with previously published studies, describing the role of inflammatory monocytes in the suppression of vaccine responses (64, 65). In fact, increased gene expression of inflammatory responses and TNF-α signaling via NF-κB were reported after COVID-19 vaccination (66). Importantly, plasma TNF-α levels in aged people were associated with a poorer antibody response following SARS-CoV-2 vaccination (21). According to our results from monocytes, aged people showed increased numbers of CD16$^+$ mDCs, a DC subset that also participates in inflammatory responses (32). These higher CD16$^+$ mDC levels were inversely associated with SARS-CoV-2–specific T cell response after vaccination. Altogether, a higher capacity of monocytes to produce proinflammatory cytokines, increased CD16$^+$ mDC numbers, and even the higher T cell activation status found in aged people are probably associated with the inflammaging phenomenon, which — at the same time — is related to lower vaccine immunity.

In the present study, numerous age-related immune deficits associated with a lower SARS-CoV-2 vaccination response were described. It may be interesting to carry out the same determinations long-term after SARS-CoV-2 vaccination, to study how these immune alterations might be contributing to a less-durable protection after COVID-19 vaccination found in aged people. Nevertheless, the described age-associated immune defects are already observed 2 months after the second dose; therefore, these defects are expected to be maintained or even increase with a longer follow-up and might explain why waning in mRNA

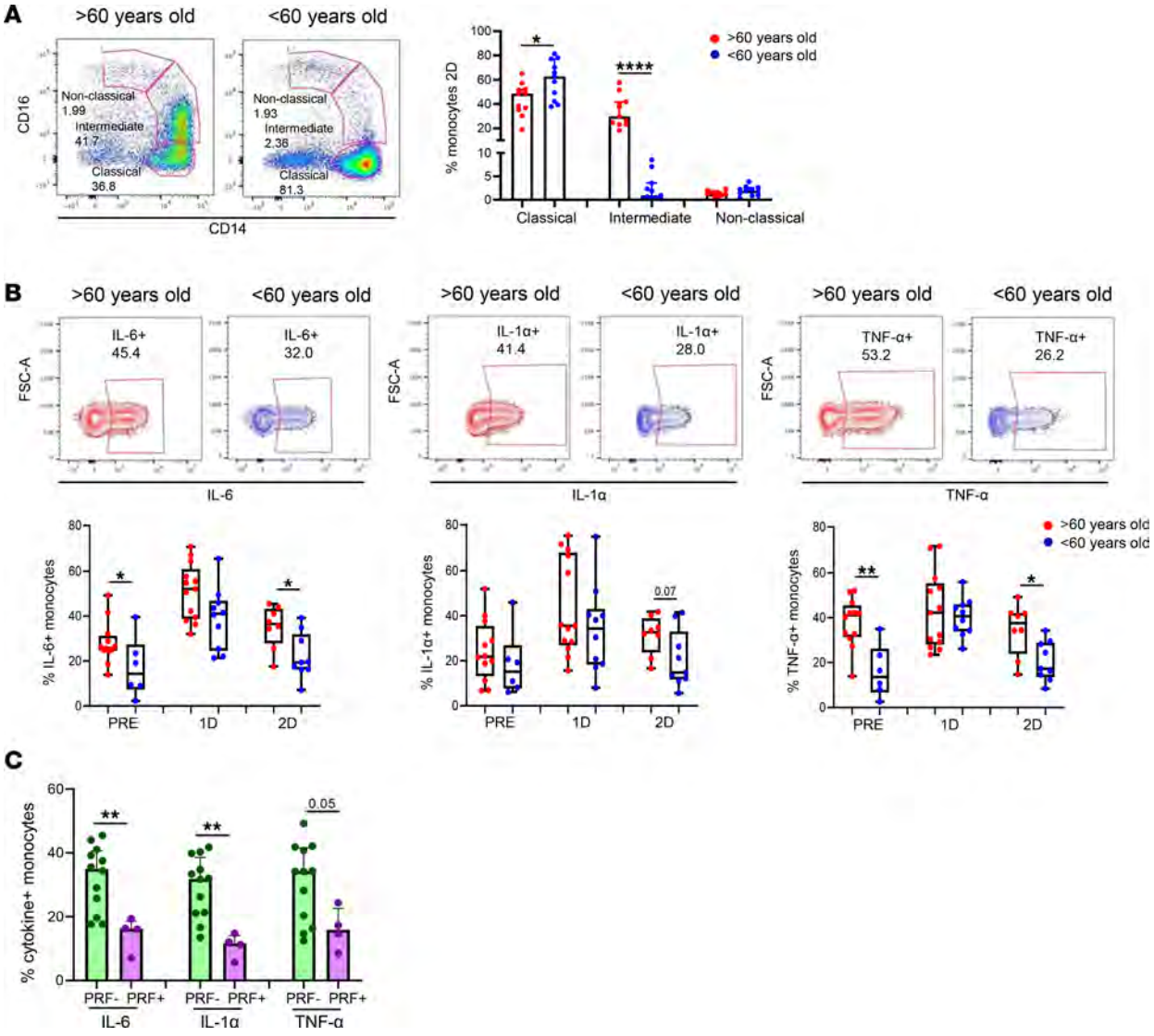

Figure 8. Higher monocyte-mediated proinflammatory profile found in aged people is associated with a lower T cell response to SARS-CoV-2 vaccine. (**A**) Bar graphs representing the percentage of classical, intermediate, and nonclassical monocytes in > 60-year-old (red) and < 60-year-old (blue) participants before SARS-CoV-2 vaccination (PRE), 3 weeks after the first dose (1D), and 2 months after the second dose (2D) of vaccination (right). Pseudocolor plots showing representative data of a > 60-year-old and < 60-year-old participant 2 months after the second dose (left). (**B**) Box-and-whisker plots (min to max) representing the percentage of IL-6$^+$, IL-1α$^+$, and TNF-α$^+$ monocytes upon TLR-4 stimulation in > 60-year-old (red) and < 60-year-old (blue) participants at the 3 time points (bottom). Contour plots showing representative data of the percentage of cytokine$^+$ monocytes from a > 60-year-old and < 60-year-old subject 2 months after the second dose (top). (**C**) Bar graphs showing the percentage of cytokine (IL-6, IL1-α, and TNF-α) producing monocytes on individuals with a cytotoxic SARS-CoV-2 S–specific T cell response and the ones with a negative response. The percentage of specific PRF$^+$ T cells higher than 0.01 was considered as a positive cytotoxic T cell response. Mann-Whitney *U* and Wilcoxon tests were used (n = 26). Friedman test was applied in **B**, (IL-6: > 60-year-old, P = 0.223, and < 60-year-old, P = 0.368; IL-1α: > 60-year-old, P = 1.00, and < 60-year-old, P = 0.368; TNF-α: > 60-year-old, P = 0.223, and < 60-year-old, P = 0.368).

vaccine effectiveness occurs at a greater rate among older people. Another limitation of the study might be the sample size. However, we performed an exhaustive and comprehensive analysis of age-associated immune deficits, and the differences and associations among the contrasts were clear. Moreover, functional assays were performed with peripheral blood mononuclear cells (PBMCs), instead of isolated cells; therefore, some of the alterations found in the studied cells' responses might be influenced by bystander cells. However, the analysis of functional capacity using PBMCs reflects better what is occurring in physiological conditions. Lastly, another limitation of this work could be that we only studied the response to BNT162b2 mRNA vaccine. Nevertheless, it is one of the most administered COVID-19 vaccines, and it would not be surprising that other vaccines present similar results, especially other mRNA vaccines.

In conclusion, we describe age-related innate and adaptive immune deficits associated with a lower SARS-CoV-2 vaccination response. Based on the results from this study, we suggest that the putative causal drivers of the lower vaccine response in aged people include: (a) thymic dysfunction, which induces a memory inflation (less naive T cells and higher memory T cells) and directly influences T cell response to the vaccine in aged people; (b) defective DC migration and activation, which cause a lower DC-mediated T cell costimulation and, therefore, a lower T cell response to the vaccine; and (c) the inflammaging, induced by a higher production of proinflammatory cytokines by monocytes and accompanied by a higher activation of the immune system, which causes an inefficient further activation of the immune cells in response to new antigens or, in this case, to the vaccine. These findings contribute to a better understanding of why aged people are less capable of responding to SARS-CoV-2 vaccination and might be relevant for the improvement of the current vaccination strategies — especially in this vulnerable population — and for the development of more efficient prototypes for the general population.

Methods

Study participants. Fifty-four participants vaccinated with the BNT162b2 mRNA vaccine against SARS-CoV-2 were included in this study. Participants were stratified by age: participants < 60 years old were categorized as young (n = 33) and those > 60 years old categorized as aged (n = 21); the median ages of young and aged participants were 29 (IQR 26–49) and 73 (IQR 72–74), respectively. Young participants were workers from the IBiS, and aged participants were community volunteers from Seville, Dos Hermanas (both in Seville, Spain), and Rota (Cadiz, Spain). Inclusion criteria included participants with self-sufficient health status, and participants were excluded if they had a diagnosis of dementia, had active infections, or were admitted to a hospital during the last 6 months. Three young participants were excluded from the study due to a positive result for SARS-CoV-2 PCR or SARS-CoV-2 RBD–specific antibodies prior to vaccinaton. Peripheral blood samples were extracted from February to November 2021.

Cell and plasma isolation. PBMCs and plasma were isolated from study participants' blood. PBMCs were isolated using BD Vacutainer CPT Mononuclear Cell Preparation Tubes (with sodium heparin, BD Biosciences) in a density gradient centrifugation at the same day of blood collection. CPTs were centrifuged at 1,811*g* for 20 minutes at room temperature (RT). Afterward, PBMCs were cryopreserved in freezing medium (90% of FBS [Thermo Fisher Scientific] + 10% dimethyl sulfoxide [DMSO; PanReac AppliChem]) in liquid nitrogen until further use. Plasma samples were obtained using BD Vacutainer PET EDTA Tube centrifugation at 1,811*g* for 5 minutes at room temperature, were aliquoted, and were cryopreserved at –80°C until further use.

Cell stimulation. PBMCs were thawed, washed using RPMI 1640 (Thermo Fisher Scientific), and rested for 1 hour in 0.25 μL/mL DNase I (Roche Diagnostics) containing R-10 complete medium (RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin G, 100 L/mL streptomycin sulfate [Thermo Fisher Scientific], and 1.7 mM sodium L-glutamine [Lonza]).

SARS-CoV-2–specific T cell response. To analyze the specific T cell response to SARS-CoV-2, 1.5×10^6 PBMCs were in vitro stimulated for 6 hours at 37°C in R-10 medium with overlapping peptides of protein S (PepMix SARS-CoV-2; Spike Glycoprotein, from JPT Peptide Technologies). In total, 1.5×10^6 PBMCs incubated with the proportional amount of DMSO were included in each experiment as a negative control. The stimulation was performed in the presence of 10 μg/mL of brefeldin A (Sigma-Aldrich) and 0.7 μg/mL of monensin (Golgi Stop, BD Biosciences) protein transport inhibitors, anti–CD107a-BV650 (clone H4A3; BD Biosciences) monoclonal antibody, and purified CD28 (clone CD28.2) and CD49d (clone 9F10) (both from BD Biosciences), as previously described (67, 68). Intracellular cytokines and cytotoxicity markers were analyzed by multiparametric flow cytometry. Specific T cell response was defined as the frequency of cells expressing intracellular cytokines and/or cytotoxicity markers after the stimulation with S peptides minus the levels of this response in the unstimulated condition (background subtraction).

Monocyte stimulation. In total, 1×10^6 PBMCs were in vitro stimulated in a 48-well plate for 5 hours at 37°C with 0.5 μL/mL of LPS (Invivogen) in R-10 medium, including 1×10^6 PBMCs without any stimulation as a negative control. A total of 0.7 μg/mL of monensin (Golgi Stop, BD Biosciences) was added to all experimental conditions. Intracellular cytokines were analyzed by flow cytometry.

mDC stimulation. PBMCs (0.5×10^6) were in vitro stimulated in a 24-well plate for 24 hours at 37°C with 2 μL/mL of Poly(I:C) (InvivoGen) in R-10 medium. PBMCs (0.5×10^6)incubated without stimulus were included as a negative control. Surface expression of activation markers were analyzed by flow cytometry.

pDC stimulation culture and quantification of IFN-α production. Thawed PBMCs (0.5×10^6)were incubated at 37°C and 5% CO_2 during 18 hours in R-10 medium with or without 1 μM of the TLR9 agonist CpG-A (InvivoGen). After incubation, cells were pelleted, and the supernatants were conserved at –80°C for the subsequent quantification of IFN-α production by ELISA according manufacturer's instructions (PBL Interferon Source).

Multiparametric flow cytometry. In general, for ex vivo phenotyping and functional assays, PBMCs were washed (652*g* for 5 minutes at RT) with phosphate-buffered saline (PBS; Thermo Fisher Scientific). PBMCs were then incubated 35 minutes at RT with a viability marker (LIVE/DEAD Fixable Aqua or Violet Dead Cell Stain; Invitrogen) and all the extracellular antibodies, as mentioned below. PBMCs were washed and fixed and permeabilized with BD Cytofix/CytoPerm (BD Biosciences) at 4°C for 20 minutes or Fixation/Permeabilization Buffer Set (eBioscience) at 4°C for 45 minutes, following the manufacturer's protocol. Then, cells were stained at 4°C for 30 minutes with intracellular antibodies (as shown below) and washed. Finally, cells were fixed for 20 minutes at 4°C with 4 % paraformaldehyde solution (PFA; Sigma-Adrich).

To assay T cell–specific response, PBMCs were extracellularly stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen), anti–DUMP-channel-BV510 (CD14 clone MφP9, CD19 clone SJ25C1, CD56 clone NMCAM16.2), anti–CD8-APC (clone SK-1), anti–CD3-BV711 (clone SP34-2), anti–CD45RA-FITC (clone L48), anti–CD27-APCH7 (clone M-T271) anti–PD-1–BV786 (CD279, clone EH12-1) (all from BD Bioscience), as well as anti–TIGIT-PerCPCy5.5 (clone A15153G) and anti–LAG-3–BV605 (clone 11C3C65) (both from BioLegend). They were permeabilized and fixed with Cytofix/CytoPerm buffer (BD Bioscience). Cells were intracellularly stained with: anti–IL-2–BV421 (clone MQ1-17H12), anti–IFN-γ–PE-Cy7 (clone B27) (BD Bioscience), anti–TNF-α–AF700 (clone Mab11) (all from BD Pharmingen), and anti–PRF-PE (clone B-D48) (BioLegend). For T cell phenotyping, cells were extracellularly stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen), anti–CD8-PerCP-Cy5.5 (clone SK1), anti–CD45RA-PeCy7 (clone L48), anti–CD3-BV711 (clone SP34-2) (BD Bioscience), anti–HLA-DR–BV570 (clone L243), and anti–CD161-BV421 (clone HP-3G10) (all from BioLegend); they were permeabilized and fixed with fixation/permeabilization buffer (eBioscience) and intracellularly stained with anti-Ki67 FITC (clone 11F6) (BioLegend). T cells were gated based on the CD3 and CD8 expression. Each subset (Memory, CM, EM, and TEMRA) was gated based on CD45RA and CD27 expression (Supplemental Figure 2A).

To assay monocytes functionality, PBMCs were extracellularly stained with LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen), anti–DUMP-channel-V450 (CD3 clone SP34-2, CD19 clone HIB19, CD20 clone L27, CD56 clone B159), anti–CD14-BV650 (clone M5E2), anti–CD16-PeCF594 (clone 3G8), and anti–HLA-DR–BV570 (clone L243) (all from BioLegend); they were permeabilized and fixed with BD Cytofix/CytoPerm (BD Biosciences) and intracellularly stained with anti–IL-6–Pe (clone MQ2-6A3), anti–IL-1α–FITC (clone AS5), and anti–TNF-α–AF700 (clone MAb11) (all from BD Biosciences). To assay monocytes phenotyping ex vivo, PBMCs were extracellularly stained with LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen), anti–DUMP-channel-V450 (CD3 clone SP34-2, CD19 clone HIB19, CD20 clone L27, CD56 clone B159), anti–CD14-BV650 (clone M5E2), anti–CD16-PeCF594 (clone 3G8), anti–TLR4-BV786 (clone TF901), anti–CD142-Pe (clone HTF-1), and anti–CCR5-APC-Cy7 (clone 2D7/CCR5) (all from BD biosciences), as well as anti–HLA-DR-BV570 (clone L243), anti–TLR2-FITC (clone TL2.1), anti–CD40-APC (clone HB14), anti–CX3CR1-PerCPCy5,5 (clone 2A9-1), anti–CCR2-BV605 (clone K036C2), and anti–CD49d-BV711 (clone 9F10) (all from BioLegend) and anti–CD11b-AF700 (clone VIM12) (Thermo Fisher Scientific). Monocytes were gated based on the CD14 and HLA-DR markers, and nonclassical, intermediate, and classical subsets were gated based on CD14 and CD16 expression (Supplemental Figure 5A).

To assay mDC functionality, PBMCs were extracellularly stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen), anti–CD11c-BV650 (clone B-ly6), anti–HLA-DR-BV570 (clone L243), anti–Lin-2-FITC (CD3 clone SK7, CD19 clone SJ25C1, CD20 clone L27, CD14 clone MφP9, and CD56 clone NCAM16.2), and anti–CD16-BV605 (clone 3G8) (all from BD Biosciences), as well as anti–CD1c-APCCy7 (clone L161), anti–CD141-PeCy7 (clone M80), anti–CD86-BV421 (clone 2331 -FUN-1]), and anti–CD40-APC (clone HB14) (all from BioLegend) and anti–CD83-AF700 (clone HB15) (Invitrogen). They were permeabilized and fixed with BD Cytofix/CytoPerm (BD Biosciences) and intracellularly stained with anti–IDO-Pe (clone eyedio) (eBioscience). For ex vivo DC phenotyping, PBMCs were extracellularly stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen), anti–CD11c-BV650 (clone B-ly6), anti–HLA-DR–BV711 (clone G46-6), anti–Lin-2–FITC, anti–CD16-BV605 (clone 3G8), anti–CCR7-BV786 (CD197) (clone 3D12), anti–CD86-BV421 (clone 2331 [FUN-1]), anti–PD-L1–PeCF594 (CD274) (clone MIH1), and anti–integrin-β7–APC (clone FIB504) (all from BD Biosciences), as well as anti–CD4-PerCPCy5,5 (clone OKT4), anti–CD1c-APC-

Cy7 (clone L161), and anti–CD141-PeCy7 (clone M80) (all from BioLegend) and anti–CD123-AF700 (clone 32703) (R&D Systems) antibodies. Cells were permeabilized and fixed with Fixation/Permeabilization buffer (eBioscience) and intracellularly stained with anti–IDO-Pe (clone eyeido) (eBioscience). DCs were identified by the expression of HLA-DR and the lack of expression of Lin-2. pDCs and mDCS were gated based on the CD123 and CD11c expression, respectively. mDCs subsets were gated using according to CD16, CD1c, and CD141 expression (Supplemental Figure 4A).

Multiparametric flow cytometry were performed on an LRS Fortessa flow cytometer using FACS Diva software (BD Biosciences), acquiring 0.5 to 1 × 10^6 events. Data were analyzed using the FlowJo 10.7.1 software (TreeStar).

Quantification of anti–RBD IgG and anti–IFN-α IgG levels. Anti–RBD IgG SARS-CoV-2 levels were measured by recombinant RBD–specific (rRBD-specific) ELISA as previously described (69, 70, 71, 72). Briefly, Nunc Maxisorp flat-bottomed 96-well plates (Thermo Fisher Scientific) were coated with 1 μg/mL of rRBD protein of the S antigen of SARS-CoV-2 (Sino Biological, 40592-V08H) overnight at 4°C. The following day, plates were blocked with 3% milk in PBS containing 0.05% Tween-20 for 120 minutes at RT. Plasma samples were heat inactivated at 56°C for 20 minutes of complement activity. Human plasma samples were diluted at 1:50, 1:100, 1:200, 1:400, or 1:800 in 1% milk containing 0.05% Tween-20 in PBS and incubated for 90 minutes at RT. Plates were washed 4 times with 0.05% PBS–Tween-20. Human serum standard reference material of anti–SARS-CoV-2 immunoglobulin (first WHO International Standard and International Reference Panel for anti–SARS-CoV-2 immunoglobulin from NIBSC, United Kingdom, NIBSC sample: 20/150) was used as standard curve to titer anti–SARS-CoV-2 IgG antibody in plasma samples. Human serum standard was added to the plates and serially diluted (2-fold dilutions) in 1% milk containing 0.05% Tween-20 in PBS. Pooled plasma samples (NIBSC, United Kingdom, NIBSC sample: 20/142) obtained from healthy blood donors before 2019 was used as negative control plasma. Secondary antibodies, streptavidin-horseradish peroxidase–conjugated mouse anti–human IgG (Hybridoma Reagent Laboratory) was used at a 1:5,000 dilution in 1% milk containing 0.05% Tween-20 in PBS. Plates were washed 4 times with 0.05% PBS–Tween-20. The plates were developed using fast o-Phenylenediamine dihydrochloride Peroxidase Substrate (Sigma-Aldrich); the reaction was stopped using 3 M HCl, and the optical density at 490 nm (OD490) was read on a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific) within 2 hours. Anti–SARS-CoV-2 IgG antibody titers for each donor were calculated as binding antibody units (BAU)/mL according to the manufacturers' information regarding the WHO Standard and was determined based on sigmoidal dose-response nonlinear regression, 4 parametric logistic (4PL), using GraphPad Prism, version 8.0 (GraphPad Software). For the measurement of anti–IFN-α IgGs, another ELISA assay was performed as previously described (73). In this case, plates were coated with 2 μg/mL of IFN-α2a (Miltenyi Biotec), and plasma samples were diluted 1:10. Eight nonvaccinated healthy donors and 3 patients with severe COVID-19 from a previous study of the group (68) were used as negative and positive controls, respectively.

DNA extraction and TREC measurement by ddPCR. The extraction of the genomic DNA from frozen PBMCs was performed using a blood DNA minikit (Omega; Bio-Tek). The DNA concentration was determined by Qubit assay according to the manufacturer's protocol (Thermo Fisher Scientific).

TRECs were quantified from extracted DNA by ddPCR (Bio-Rad) based on a previously modified method (74). In total, 20 μM of primer for sjTREC (DTF7, 5′→3′: AGGCTCTGTCTAGTGTGATAAC; DTR66, 5′→3′: TGACATGGA GGGCTGAAC), 10 μM of probe (PB1, 5′→3′: 6FAM-TGGGAGTTG-GGACCGCCAGAGAGG-BHQ1; SD1, 5′→3′: HEX-CACCCCTCTGTTCCCCACA- BHQ1), and ddPCR Supermix for probes no dUTP (Bio-Rad) were used. The reference gene used was RPP30 (2 copies per cell) (forward, 5′→3′: GATTTGGACCTGCGAGCG; reverse, 5′→3′: GCGGCTGTCTCC ACAAGT; Probe, 5′→3′: VIC-CTGACCTGAAGGCTCT-BHQ1). The ddPCR conditions were: 10 minutes at 95°C, 40 cycles of 30 seconds at 94 °C, 1 minute at 59°C, and 10 minutes at 98°C. Bio-Rad QuantaSoft software v.1.7.4 was used for determining the TRECs/1 × 10^6 cells.

Statistics. Nonparametric statistical analyses were performed using Statistical Package for the Social Sciences software (SPSS 25.0), RStudio Version 1.3.959, and GraphPad Prism version 8.0 (GraphPad Software). Polyfunctionality pie charts were constructed using Pestle version 1.6.2 and Spice version 6.0 (75). Median and IQRs were used to describe continuous variables, and percentages were used to describe categorical variables. The ROUT method was utilized to identify and discard outliers. Differences between aged and young groups were analyzed by 2-tailed nonparametric Mann-Whitney *U* test.

The nonparametric Wilcoxon test was used to analyze differences between time points. The Spearman test was used to analyze correlations between variables. For multiple comparisons, Friedman test was applied, including Bonferroni correction. Permutation test was used to assess differences between pie charts using Spice software. Hmisc and corrplot packages were used in R by the Spearman method to calculate correlations between pairs of variables and to plot the correlation matrix figures. Lateral intensity bar from red to blue, next to correlation matrixes, represents the ρ coefficient value of the Spearman test. All differences with $P < 0.05$ were considered statistically significant.

Study approval. The study was approved by the Ethics Committee of the Virgen del Rocio University Hospital (COVIMARATON; 0896-N-20). Written informed consent was received from all the participants of the study.

Author contributions

ERM conceived and designed the research. APG, CGC, IRJ, MDMSS, AMRM, MAMS, MREIB, and ERM participated in sample collection and processing. JV, APG, FJO, CGC, and MRJL performed the experiments. JV carried out the data analysis. JV, SB, LFLC, MREIB, and ERM participated in the interpretation/discussion of the results. JV, APG, and ERM wrote the manuscript. ERM and JV coordinated the project. All authors reviewed and discussed the manuscript.

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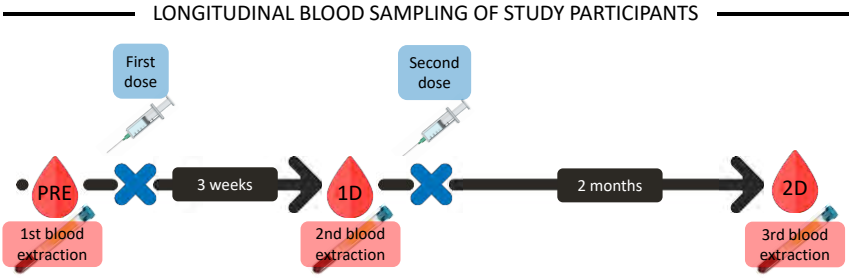
Figure S1

A



	All participants (n=54)	Elderly participants (n=21)	Young participants (n=33)	p-value
Age (years)	49.5 [28 – 73]	73 [72 – 74]	29 [26 –48.5]	<0.001
Sex (Female sex), n (%)	35 (64.8)	13 (61.9)	22 (66.7)	0.721
Comorbidities, n (%)	26 (48.10)	16 (76.2)	10 (30.30)	0.007
Cardiovascular disease	*10 (18.50)*	*6 (28.6)*	*4 (12.12)*	*0.129*
Cancer	*1 (1.85)*	*1 (4.76)*	*0 (0)*	*0.206*
Thyroid disease	*2 (3.7)*	*1 (4.76)*	*1 (3.03)*	*0.743*
Allergies	*5 (9.25)*	*1 (4.76)*	*4 (12.12)*	*0.363*
Arthritis/Arthrosis	*6 (10)*	*6 (28.6)*	*0 (0)*	*0.001*
Other	*2 (3.7)*	*1 (4.76)*	*1 (3.03)*	*0.743*
None	*26 (48.10)*	*5 (23.8)*	*21 (63.64)*	*0.004*
Unknown	*2 (3.7)*	*0 (0)*	*2 (6.06)*	*0.250*

B

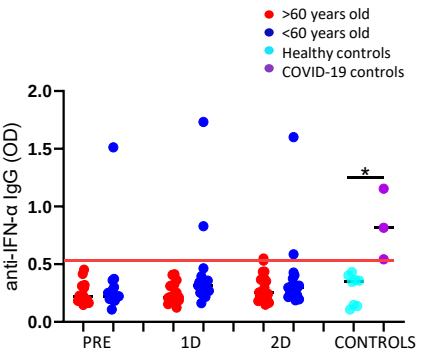


Figure S2

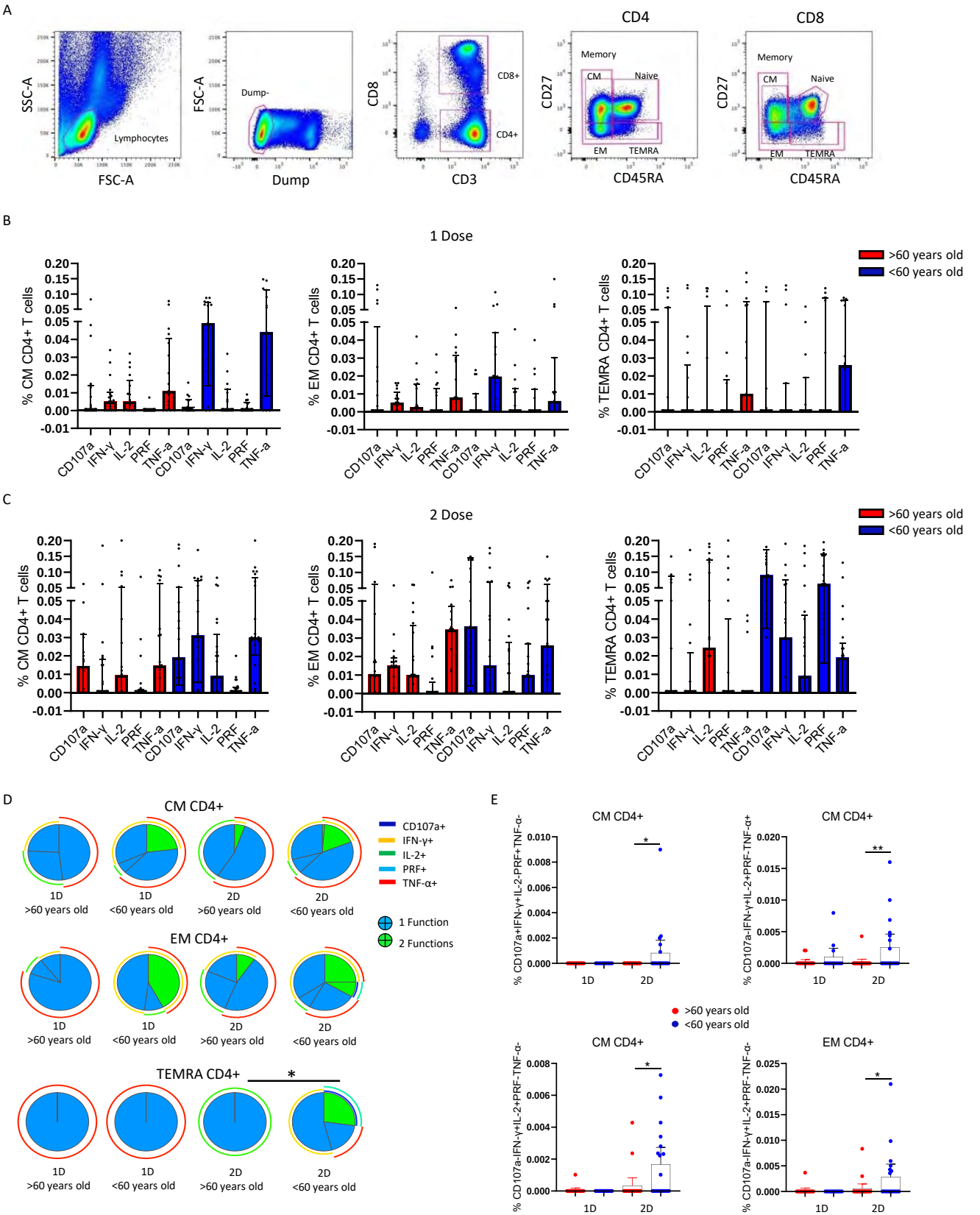

A
SSC-A
FSC-A
Lymphocytes
FSC-A
Dump
Dump-
CD8
CD3
CD8+
CD4+
CD4
CD27
CD45RA
Memory
CM
Naive
EM
TEMRA
CD8
B
1 Dose
% CM CD4+ T cells
% EM CD4+ T cells
% TEMRA CD4+ T cells
>60 years old
<60 years old
CD107a
IFN-γ
IL-2
PRF
TNF-a
C
2 Dose
D
CM CD4+
EM CD4+
TEMRA CD4+
1D
2D
>60 years old
<60 years old
CD107a+
IFN-γ+
IL-2+
PRF+
TNF-α+
1 Function
2 Functions
E
CM CD4+
EM CD4+
% CD107a+IFN-γ+IL-2-PRF+TNF-α-
% CD107a+IFN-γ+IL-2+PRF-TNF-α+
% CD107a-IFN-γ+IL-2+PRF-TNF-α-
% CD107a-IFN-γ+IL-2+PRF-TNF-α-
1D
2D
>60 years old
<60 years old

Figure S3

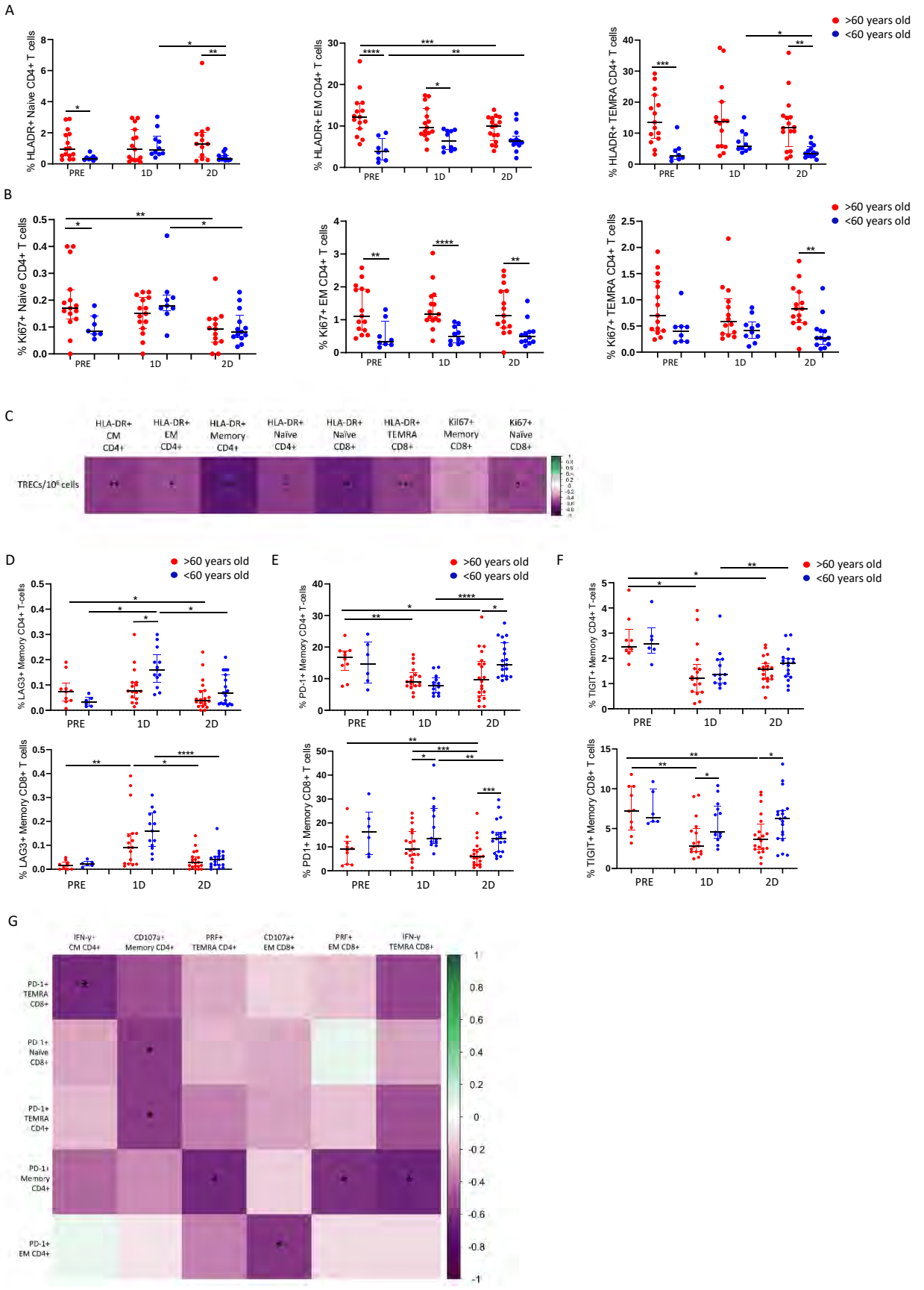

A
% HLADR+ Naive CD4+ T cells
% HLADR+ EM CD4+ T cells
% HLADR+ TEMRA CD4+ T cells
>60 years old
<60 years old
PRE
1D
2D
B
% Ki67+ Naive CD4+ T cells
% Ki67+ EM CD4+ T cells
% Ki67+ TEMRA CD4+ T cells
C
HLA-DR+ CM CD4+
HLA-DR+ EM CD4+
HLA-DR+ Memory CD4+
HLA-DR+ Naive CD4+
HLA-DR+ Naive CD8+
HLA-DR+ TEMRA CD8+
Kil67+ Memory CD8+
Ki67+ Naive CD8+
TRECs/10⁶ cells
D
% LAG3+ Memory CD4+ T-cells
% LAG3+ Memory CD8+ T cells
E
% PD-1+ Memory CD4+ T-cells
% PD1+ Memory CD8+ T cells
F
% TIGIT+ Memory CD4+ T-cells
% TIGIT+ Memory CD8+ T cells
G
IFN-γ+ CM CD4+
CD107a+ Memory CD4+
PRF+ TEMRA CD4+
CD107a+ EM CD8+
PRF+ EM CD8+
IFN-γ TEMRA CD8+
PD-1+ TEMRA CD8+
PD-1+ Naive CD8+
PD-1+ TEMRA CD4+
PD-1+ Memory CD4+
PD-1+ EM CD4+

Figure S4

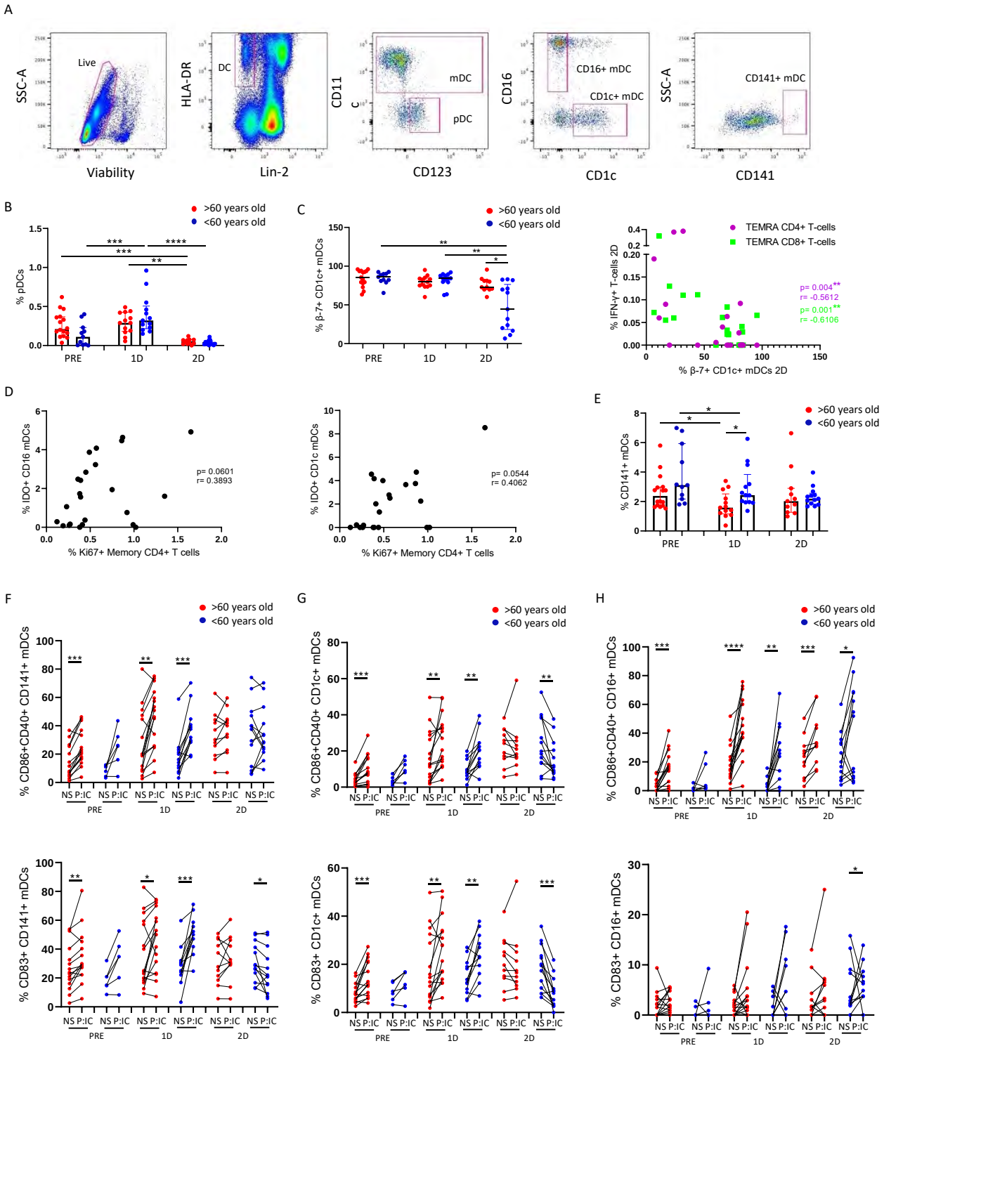

A
SSC-A
Live
Viability
HLA-DR
DC
Lin-2
CD11c
mDC
pDC
CD123
CD16
CD16+ mDC
CD1c+ mDC
CD1c
SSC-A
CD141+ mDC
CD141
B
>60 years old
<60 years old
% pDCs
PRE
1D
2D
C
% β-7+ CD1c+ mDCs
TEMRA CD4+ T-cells
TEMRA CD8+ T-cells
% IFN-γ+ T-cells 2D
p= 0.004**
r= -0.5612
p= 0.001**
r= -0.6106
% β-7+ CD1c+ mDCs 2D
D
% IDO+ CD16 mDCs
p= 0.0601
r= 0.3893
% Ki67+ Memory CD4+ T cells
% IDO+ CD1c mDCs
p= 0.0544
r= 0.4062
% Ki67+ Memory CD4+ T cells
E
% CD141+ mDCs
F
% CD86+CD40+ CD141+ mDCs
NS P:IC
% CD83+ CD141+ mDCs
G
% CD86+CD40+ CD1c+ mDCs
% CD83+ CD1c+ mDCs
H
% CD86+CD40+ CD16+ mDCs
% CD83+ CD16+ mDCs

Figure S5

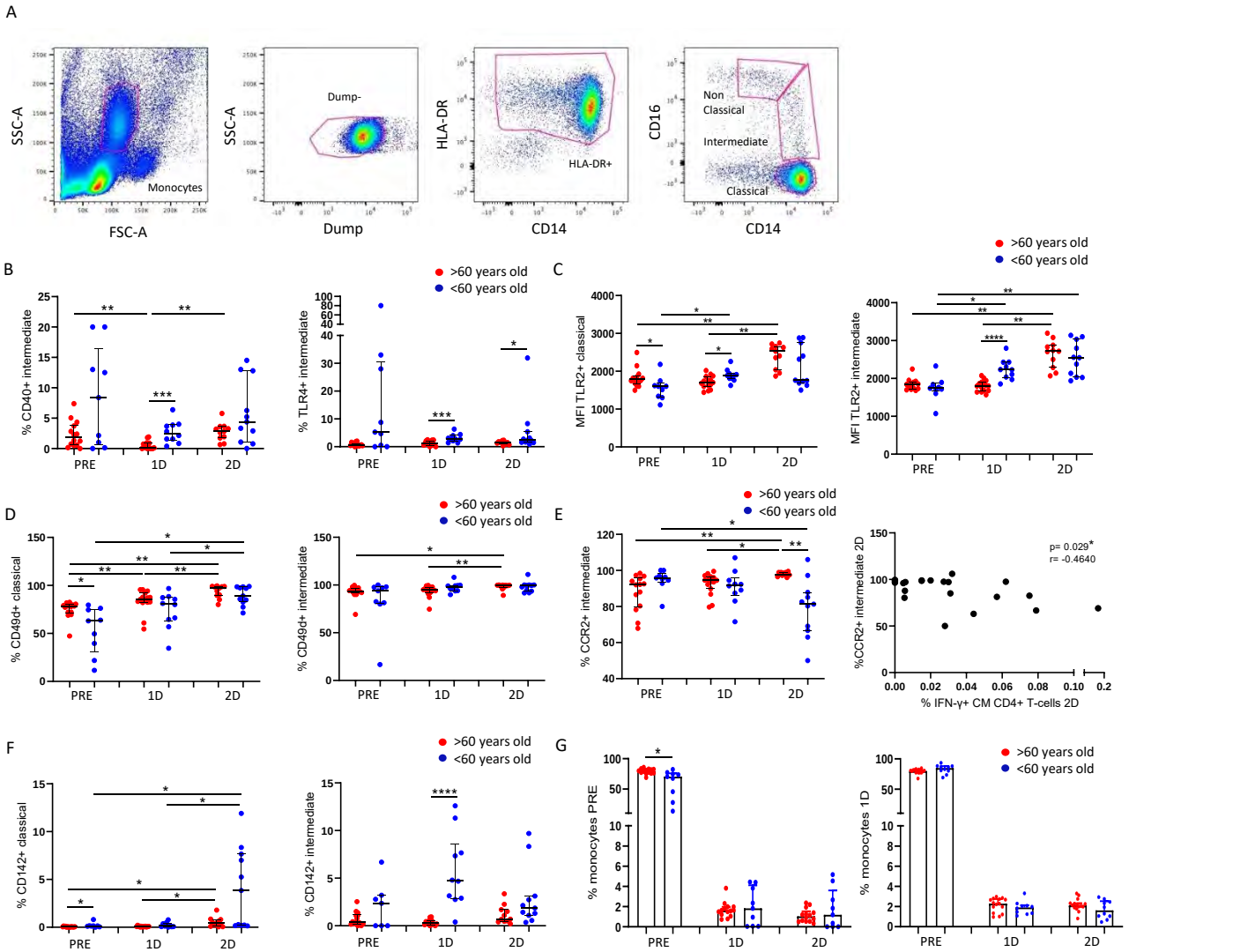

A
SSC-A
FSC-A
Monocytes
Dump-
Dump
HLA-DR
HLA-DR+
CD14
CD16
Non Classical
Intermediate
Classical
B
% CD40+ intermediate
% TLR4+ intermediate
C
MFI TLR2+ classical
MFI TLR2+ intermediate
D
% CD49d+ classical
% CD49d+ intermediate
E
% CCR2+ intermediate
%CCR2+ intermediate 2D
p= 0.029*
r= -0.4640
% IFN-γ+ CM CD4+ T-cells 2D
F
% CD142+ classical
% CD142+ intermediate
G
% monocytes PRE
% monocytes 1D
>60 years old
<60 years old
PRE
1D
2D

Figure S1. Sampling and clinical data of study participants and anti-IFN-α IgG plasma levels

(A) Aged and young donors were vaccinated with two doses of BNT162b2 mRNA vaccine, receiving the second dose three weeks after the first one (top). For this study, peripheral blood samples were extracted from all participants just before SARS-CoV-2 vaccination (PRE), three weeks after the first dose and just before the second one (1D), and two months after the second dose (2D) (top). The table describes the age, sex and comorbidities of studied donors. Variables are expressed as number (n) and percentages (%), and continuous variables are expressed as median with interquartile ranges [IQR]. **(B)** Dot plots showing the optical density (OD) representing anti-IFN-α IgG levels in plasma from >60 years old (red) and <60 years old (blue) participants before SARS-CoV-2 vaccination (PRE), three weeks after the first dose (1D) and two months after the second dose (2D) of vaccination. Eight non-vaccinated healthy donors and three severe COVID-19 patients were used as negative and positive controls, respectively. Mann-Whitney U and Wilcoxon tests were used (n= 54).

Figure S2. SARS-CoV-2 S-specific T cell response in aged and young people

(A) Gating strategy of T cells is shown. Lymphocytes were firstly identified according to their size and complexity (FSC-A and SSC-A) and cells negative for dump channel (viability, CD14, CD19, and CD56) were gated. Then, CD8+ (CD3+ CD8+) and CD4 (CD3+ CD8-) T cells were selected and T cell subsets were identified based on the expression of CD45RA and CD27: naïve (CD45RA+ CD27-) central memory (CM) (CD45RA- CD27+), effector memory (EM) (CD45RA- CD27-) and terminal differentiated effector memory (TEMRA) (CD45RA+ CD27+) cells. Total memory cells (Memory) correspond to the sum of CM, EM and TEMRA T cells. **(B and C)** Bar graphs showing the percentages of CM, EM and TEMRA CD4+ T cells expressing CD107a, IFN-γ, IL-2, PRF and TNF-α after S-specific SARS-CoV-2 stimulation, comparing >60 years old (red) and <60 years old (blue) subjects three weeks after the first dose (B) and two months after the second dose (C) of SARS-CoV-2 vaccine. **(D)** Pie charts representing SARS-CoV-2 S-specific EM, CM and TEMRA CD4+ T cell polyfunctionality. Each sector represents the proportion of S-specific CD4+ T cells producing two (green) or one (blue) functions. Arcs represents the type of function

(CD107a, IFN-γ, IL-2, PRF and TNF-α) expressed in each sector. **(E)** Bar graphs showing the percentage of EM and CM CD4+ T cells expressing different combinations of studied functions (CD107a, IFN-γ, IL-2, PRF and TNF-α) comparing >60 years old (red) and <60 years old (blue) subjects after the first (1D) and the second (2D) dose. Mann-Whitney U, Wilcoxon and Permutation tests were used (n= 41).

Figure S3. T cell homeostasis parameters and its association with SARS-CoV-2 specific T cell response in aged and young people

(A and B) Bar graphs representing the percentage of naïve (left), EM (middle) and TEMRA (right) CD4+ T cells expressing HLA-DR (A) and Ki67 (B) in >60 years old (red) and <60 years old (blue)participants before SARS-CoV-2 vaccination (PRE), three weeks after the first dose (1D) and two months after the second dose (2D) of vaccination. **(C)** Correlation matrix representing associations of TRECs/10^6 cells with the percentages of HLA-DR+ and Ki67+ T cells prior vaccination. **(D-G)** Dot plots representing the percentage of memory CD4+ and CD8+ T cells expressing the immune check points LAG-3 (D), PD-1 (E) and TIGIT (F) in >60 years old (red) and <60 years old (blue)participants at the three follow up time points. **(G)** Correlation matrix representing associations of the percentage of PD-1+ T cells with SARS-CoV-2 S-specific CD4+ and CD8+ T cells expressing IFN-γ and cytotoxicity markers. Mann-Whitney U, Wilcoxon and Spearman tests were used (n= 32).

Figure S4. Dendritic cells phenotype and function before and after SARS-CoV-2 vaccination in aged and young people

(A) Gating strategy of DCs. First live cells were selected and DCs were identified by gating HLA-DR+ and Lineage-2- cells. Then mDCs (CD11c+) and pDCs (CD123+) were selected and mDC subsets were identified according to the surface expression of CD1c, CD16 and

CD141. **(B)** Bar graphs representing the percentages of pDCs in >60 years old (red) and <60 years old (blue)participants before SARS-CoV-2 vaccination (PRE), three weeks after the first dose (1D) and two months after the second dose (2D) of vaccination. **(C)** Dot plots showing the percentage of integrin-β7+ CD1c+ mDCs in old and young participants at the three time points (left) and correlation plot between the percentage of integrin-β7+ CD1c+ mDCs and the percentage of S-specific IFN-γ+ TEMRA CD4+ and CD8+ T cells two months after the second dose (right). **(D)** Correlations between the percentages of IDO+ CD16+ and CD1c+ mDCs with Ki67+ Memory CD4+ T cells in all participants prior vaccination. **(E)** Bar graphs representing the percentages of CD141+ mDCs in >60 years old (red) and <60 years old (blue)participants at the three time points. **(F-H)** Before and after graphs showing the percentages of CD83+ and CD86+CD40+ within CD141+ (F), CD1c+ (G) and CD16+ (H) mDCs without stimulation (NS) or after TLR-3 stimulation with Poly I:C (P:IC) in >60 years old (red) and <60 years old (blue)participants at the three time points. Mann-Whitney U, Wilcoxon and Spearman tests were used (n= 32).

Figure S5. Monocyte phenotype before and after SARS-CoV-2 vaccination in aged and young people

(A) Gating strategy of monocytes. Monocytes were firstly identified according to their size and complexity (FSC-A and SSC-A) and cells negative for dump channel (viability, CD3, CD19, CD20 and CD56) were gated. Then, HLA-DR+ cells were selected and monocyte subsets were identified according to the expression of CD14 and CD16: classical (CD14++ CD16-), intermediate (CD14++ CD16+) and non-classical (CD14+ CD16+). **(B-F)** Dot plots showing the percentages of CD40+ (B, left) and TLR-4+ (B, right) (B), the median fluorescence intensity of TLR-2 (C) and the percentages of CD49d+ (D), CCR2+ (E) and CD142+ (F) monocytes in >60 years old (red) and <60 years old (blue)participants before SARS-CoV-2 vaccination (PRE), three weeks after the first dose (1D) and two months after the second dose (2D) of vaccination. Correlation of the percentage of CCR2+ monocytes with the percentage of IFN-γ+ CM CD4+ T cells after the second dose (E, right). **(G)** Bar graphs representing the percentage of classical, intermediate and non-classical monocytes in >60 years old (red) and <60 years old

(blue)participants before vaccination (left) and after the second dose (right). Mann-Whitney U, Wilcoxon and Spearman tests were used (n= 32).

DISCUSIÓN GLOBAL

En los trabajos desarrollados durante esta tesis doctoral se estudiaron factores asociados a la respuesta inmunitaria innata y a la adaptativa específica frente al SARS-CoV-2 tanto en infección aguda como siete meses tras la infección; además de la asociación existente entre la respuesta a la infección por el SARS-CoV-2 y otros coronavirus endémicos. Adicionalmente, se determinaron factores inmunológicos asociados a una menor respuesta vacunal frente a la COVID-19 en personas de edad avanzada. Estos trabajos se realizaron en un periodo de tiempo en el que los conocimientos acerca de la respuesta inmunitaria hacia SARS-CoV-2 y las características de las respuestas vacunales eran escasos.

Para combatir de forma efectiva la infección por el SARS-CoV-2 y por lo tanto prevenir una sintomatología grave, al igual que en otras infecciones virales, se requiere una respuesta adecuada tanto del sistema inmunitario innato (106) como el adaptativo (49,107–110).

Uno de los factores clave para combatir la infección viral, incluyendo la del SARS-CoV-2, es la producción de IFN-I por parte de las CDp (111). Esta citoquina activa distintos mecanismos inmunológicos y distintos tipos celulares, induciendo "*interferon related genes*" (112). Algunos estudios describieron que la infección por SARS-CoV-2 disminuía la producción de IFN-I por parte de las CDp, afectando a la regulación de la respuesta específica de los linfocitos T (113). Nosotros también observamos un déficit en los niveles de IFN-α en individuos que cursaron la COVID-19 y que se asociaba a gravedad. Esto podría estar relacionado, junto con su efecto intrínseco antiviral, con una peor calidad de la respuesta de los linfocitos T frente al virus, lo cual se traduciría en una menor polifuncionalidad de éstas como hemos observado en nuestros resultados y sucede en otras infecciones como en el VIH (114). Por otro lado, vemos que esta disminución de los niveles de IFN-α es más pronunciada en aquellos individuos más graves, es decir, se asocia a la gravedad de la enfermedad, tal y como ya se había observado en estudios previos (115,116). En estos estudios, también se investigaron la dinámica de las respuestas de las células T durante la COVID-19 y observaron que la gravedad de la enfermedad se correlacionaba con la disminución del número de células T, en particular de células T CD8+ (115), hallazgo que describimos nosotros comparando los individuos que cursaban la infección aguda en comparación con individuos sanos. Resultados como estos sugirieron que el déficit en la producción de IFN-α por las CDp en pacientes con COVID-19 grave puede contribuir a la disminución del número y la función de las células T (22).

Sin embargo, no solo la alteración en los niveles de IFN-α es importante en la COVID-19, sino también la capacidad de las CD para co-estimular la respuesta de los linfocitos T (44,117). Los resultados de esta tesis mostraron que marcadores de co-estimulacion estaban también alterados, además del número y marcadores de activación y tolerogénesis de las CD, lo que podría inducir una respuesta T específica del SARS-CoV-2 deficiente y, por lo tanto, asociarse con la gravedad, tal y como otros autores habían descrito anteriormente (47,118).

Aparte de la deficiencia de IFN-I y otras alteraciones encontradas en CD, uno de los rasgos distintivos de la COVID-19 aguda son los niveles descompensados de citoquinas proinflamatorias solubles (119). Infecciones como la de SARS-CoV-2 pueden desencadenar un síndrome de liberación de citoquinas, responsable de la consecuente inmunopatología. Nosotros observamos diferentes asociaciones entre la respuesta inmunitaria innata y adaptativa con niveles alterados de citoquinas presentes en plasma, relacionados con la gravedad de la enfermedad. Hallazgos descritos en otros trabajos (120,121), donde han demostrado que las concentraciones plasmáticas iniciales de moléculas proinflamatorias en los pacientes con SARS-CoV-2 se muestran alteradas respecto a las de los controles sanos y que varían en pacientes COVID-19 dependiendo de si han ingresado en UCI. Observaron que los pacientes con COVID-19 grave mostraban una respuesta hiperinflamatoria (121), estando asociada a distintos factores inmunológicos innatos y adaptativos, como sugieren nuestros resultados. De igual forma sucede en poblaciones de monocitos, protagonistas del proceso de inflamación, como se ha observado en el estudio de Trujillo-Rodríguez et al (2022) (122), donde utilizaron datos obtenidos de la misma cohorte que se emplearon en los primeros dos artículos de la presente tesis doctoral. Los resultados reflejaron niveles más altos de los marcadores de activación, maduración y migración de monocitos, en pacientes graves/críticos en comparación con pacientes leves (122).

Respecto a la inmunidad adaptativa frente al SARS-CoV-2, hemos identificado distintos perfiles de respuesta específica de los linfocitos T. Encontramos que la respuesta T SARS-CoV-2 específica que se caracterizaba por combinaciones de citoquinas con únicamente IFN-γ+ se asociaron a un peor pronóstico. Una potencial justificación puede ser que el aumento de IFN-γ promueve la apoptosis de las células epiteliales alveolares pudiendo ocasionar lesiones pulmonares a causa de una fibrosis (123). No obstante, los pacientes leves presentaban una respuesta T más polifuncional, cuyas combinaciones que presentaron IL-2 se asociaron con un mejor curso de la infección aguda, tal y como sucede en otras infecciones virales como al del VIH (124). Es interesante que este perfil se asociara con un pronóstico más favorable, ya que

recientemente Kundu et al. (125) sugirieron que la frecuencia de células T que responden con IL-2 con reactividad cruzada frente a SARS-CoV-2 y coronavirus endémicos en humanos (hCoV) está asociada a la protección frente a la infección en los contactos COVID-19 (125). De hecho, los resultados de esta tesis mostraron una asociación entre la respuesta al SARS-CoV-2 y a coronavirus endémicos, principalmente mediada por la expresión de la IL-2, en participantes pre-COVID. Es importante destacar que estos resultados sugieren que la respuesta al SARS-CoV-2 podría estar influenciada por el contacto con otros coronavirus endémicos previo a la pandemia COVID-19 que causan el resfriado común y esta respuesta inmunitaria cruzada preexistente puede influir en el curso de la enfermedad, en algunos casos fomentando la protección contra otras infecciones (126). Resultados similares se han publicado en distintos países, como el de Grifoni et al. en Estados Unidos (56), Braun et al. en Alemania (53), Le Bert et al. en Singapur (4) y Weiskopf et al. en Holanda (Paises Bajos) (57). En otros estudios (54), sin embargo, no observaron reactividad cruzada, lo que podría reflejar variaciones geográficas y temporales. Un mayor conocimiento de las respuestas inmunitarias reactivas cruzadas al SARS-CoV-2 puede contribuir a una mejor comprensión de la heterogeneidad de las manifestaciones clínicas de la enfermedad y de la respuesta a la vacunación frente a la COVID-19, además de permitirnos identificar epítopos inmunitarios que se conservan entre coronavirus genéticamente diversos y que tienen más probabilidades de estar funcionalmente limitados.

En resumen, nuestros resultados sugieren que alteraciones en la activación y función de las CD y linfocitos T contribuyen a un perfil más pro-inflamatorio y se asocian a la gravedad de la enfermedad en infección aguda. Además, una buena caracterización de la calidad de la respuesta T específica del SARS-CoV-2 y su relación con la respuesta a coronavirus endémicos podrían proporcionar información relevante para el desarrollo estrategias terapéuticas que puedan proteger contra las actuales y futuras variantes emergentes de SARS-CoV-2.

Algo que aún se sigue estudiando es el efecto a largo plazo de haber sufrido la COVID-19. Nosotros analizamos de una manera muy detallada la memoria inmunitaria frente al SARS-CoV-2 y las posibles alteraciones inmunitarias persistentes en dos grupos de sujetos con diferente evolución de la enfermedad siete meses después de la infección: pacientes que superaron la enfermedad sin necesidad de hospitalización y pacientes previamente hospitalizados. Observamos que el sistema inmunitario continuaba respondiendo a nivel humoral y celular siete meses después de la infección por el SARS-CoV-2. De hecho, más adelante se publicaron trabajos que describían que

hasta un año después continuaba habiendo respuestas inmunitarias humorales (127–133) y mediadas por células (52,54–56,65,134,135), con diferente magnitud dependiendo de la gravedad con la que se cursó la enfermedad (52,55,136–138). Que esta respuesta permaneciera presente siete meses después, podría atribuirse simplemente a la persistencia a largo plazo de células T de memoria específicas del SARS-CoV-2, pero también podría indicar la persistencia de SARS-CoV-2 a nivel tisular que provoca una respuesta inmunitaria en curso que se mantiene hasta los siete meses tras la infección. De hecho, datos preliminares de nuestro grupo mostraron la presencia del SARS-CoV-2 en el intestino varios meses después de la infección (datos no mostrados), en línea con resultados publicados en otros estudios (139). Además, los sujetos previamente hospitalizados presentaban un mayor agotamiento en células T, lo que concuerda con una continua estimulación con antígenos víricos debido a la presencia de virus en tejido. Este agotamiento que podría tener implicaciones para la gravedad de la enfermedad y la recuperación a largo plazo ante un posible caso de reinfección y las potenciales secuelas clínicas (140–144).

A pesar de seguir manteniendo una respuesta humoral y T específica de SARS-CoV-2, la disfunción de las CDp (producción de IFN- α) y la alteración en los niveles de marcadores de activación y co-estimulación T (CD86+ y CD4+) de las CD no se recuperaron siete meses tras la infección en pacientes previamente hospitalizados, frente a los que no requirieron ser hospitalizados. Esta disfunción puede ocasionar, que ante una reinfección por SARS-CoV-2 o cualquier otra infección viral, pueda haber un control temprano ineficaz contra ella, promoviendo la progresión de la enfermedad (145). En un estudio de Arunachalam et al. (2021), también descubrieron que los pacientes con COVID-19 hospitalizados presentaban niveles persistentemente bajos de expresión de marcadores de co-estimulación siete meses después de la infección, fundamentales para impulsar las respuestas de las células T (47). De este modo, nuestros datos nos hicieron hipotetizar que los déficits en las CD pueden afectar a la co-estimulación T, y por lo tanto a la activación y función de los linfocitos T a largo plazo**.** Adicionalmente, los bajos niveles de CD (CDm CD1c+ y CDp) en sangre periférica, especialmente las que expresaban marcadores de migración a mucosa intestinal (integrina β7), se mantuvieron siete meses tras la infección tanto en pacientes previamente hospitalizados como no hospitalizados, indicando que podíamos seguir observando migración de CD a tejido (146), probablemente por la presencia de virus en tejido varios meses tras la infección (139) . Además, otra de las hipótesis que nos planteamos fue que los defectos en células dendríticas también pudieron estar potencialmente ocasionadas por

infecciones contra otros virus (por ejemplo, EBV reactivado u otros virus herpes) o auto-antígenos (147).

Durante décadas se ha investigado acerca de infecciones víricas que se han vuelto crónicas como las ocasionadas por el VIH o el citomegalovirus (CMV). Estos trabajos demuestran que la persistencia del virus en tejido y la inflamación y activación inmunitaria continuas pueden provocar una mayor morbilidad en múltiples sistemas, lo cual aumenta el riesgo de enfermedad cardiovascular a través de la desregulación inmunitaria crónica (148–155). Aunque el SARS-CoV-2 se considera predominantemente una enfermedad respiratoria, los receptores víricos, como ECA2, pueden encontrarse en diversos tejidos del organismo, incluido el tejido endovascular y muchos órganos y sistemas (156–158). Estos hallazgos destacan la gran relevancia de los resultados de esta tesis en relación a las secuelas inmunológicas a largo plazo tras haber superado la COVID-19.

Fue a finales de 2020 cuando a España llegaron las primeras vacunas contra la COVID-19. Debido a la rapidez y seguridad con la que se desarrollaron las vacunas contra la COVID-19, no se conocía la respuesta a estas vacunas en la población general y poblaciones de riesgo, entre ellas, las personas de mayor edad. El tercer artículo se centró en investigar los defectos inmunológicos que podrían estar asociados con una menor respuesta específica de SARS-CoV-2 después de la vacunación en personas mayores. Se encontraron diferentes alteraciones a nivel de inmunidad innata y adaptativa relacionados con la edad, que se asociaron a una menor respuesta a la vacunación contra el SARS-CoV-2.

En esta tesis doctoral se ha asociado una disfunción tímica en ancianos con un desequilibrio de poblaciones de células T, influyendo en la respuesta virus-específica, al igual que se ha observado en otras infecciones como el VIH (159). Estos defectos, atribuidos a la inmunosenescencia, no solo afecta a la respuesta vacunal, sino que también afecta a la respuesta T específica inducida por el propio virus en infección aguda (160–162). Al mismo tiempo, una menor respuesta T tras la vacunación puede estar influenciada por una menor función y co-estimulación de CD, como es la respuesta mediada por IFN-I por parte de las CDp o la co-estimulación por parte de las CDm. Como hemos descrito anteriormente, la COVID-19 grave también se ha correlacionado con respuestas reducidas de IFN-I y una notable reducción del número total de CDp, un defecto que puede verse afectado con la edad, tal y como se ha descrito anteriormente (43,163), ya que los CDp envejecidas también tienen una capacidad reducida para producir IFN-I. Por otro lado, la inmunosenescencia también provoca defectos en la

función de las CDm CD1c+ y CD141+ (164–167), dando lugar a una respuesta inmunitaria adaptativa deficiente y que puede inducir la progresión de la enfermedad o disminuir la respuesta vacunal.

De forma adicional, la inmunosenescencia también se caracteriza por una inflamación crónica de bajo grado, provocado entre otros por la presencia de virus como el CMV (168,169). Esta inflamación es mediada por monocitos y otros tipos celulares, y hace que el sistema inmunitario este más activado en general, explicando la activación de células T que observamos en nuestros resultados en sujetos de mayor edad previo a la vacunación frente al SARS-CoV-2. Mayores niveles de inflamación, no solo se asocian a una sintomatología grave de la COVID-19, como se ha descrito anteriormente, si no también provoca una menor respuesta a la vacuna (170–172) asociada en este caso a la inmunosenescencia. A largo plazo y en comparación con nuestros resultados, podríamos hipotetizar que también podría estar sucediendo algo similar a lo que ocurre en individuos convalecientes, donde siete meses tras la infección del SARS-CoV-2 continuaba existiendo un proceso de hiperinflamación, probablemente ocasionado por la persistencia viral a largo plazo y la consecuente mayor activación del sistema inmunitario.

Por lo tanto, la inmunosenescencia conlleva una predisposición del sistema inmunitario de personas ancianas a mantener ciertas deficiencias en la función y co-estimulación por parte de células del sistema inmunitario innato y en la homeostasis y función de células T, provocando que los adultos de mayor edad sean especialmente susceptibles a la infección y un peor pronóstico de la enfermedad debido a respuestas inmunitarias adaptativas inadecuadas y a su vez estos factores son responsables de su reducida respuesta vacunal frente al SARS-CoV-2.

Los presentes trabajos tienen algunas limitaciones ya descritas en cada una de las discusiones individuales. Sin embargo, en la actualidad se han planteado algunas otras que son interesantes de mencionar. El primer desafío ha sido la aparición de distintas VOC del SARS-CoV-2, que podrían influir en los factores inmunológicos que hemos estudiado durante esta tesis doctoral. Sin embargo, postulamos que, a pesar de las distintas variantes, los defectos inmunológicos descritos se mantendrían, al igual que ocurre en el caso de otras infecciones virales. Otras de las limitaciones fue la falta de acceso a muestras de tejidos, lo que podría contribuir a una mejor comprensión de las secuelas inmunológicas de la COVID-19. Dados los datos que apuntan a una posible persistencia vírica en los tejidos intestinales, muchos de los posibles factores desencadenantes del SARS-CoV-2 podrían requerir una investigación tisular incluyendo

tejidos del endotelio humano nasofaríngeo, respiratorio, de los ganglios linfáticos, intestinal y vascular para comprender plenamente las implicaciones inmunitarias e inflamatorias a largo plazo de COVID-19. Sin embargo, en la actualidad, seguimos trabajando en el estudio de las alteraciones inmunitarias a largo plazo y hemos podido tener acceso a muestras de tejido intestinal de individuos incluidos en nuestra cohorte de los primeros dos artículos de esta tesis. Por otro lado, otra limitación es que los pacientes incluidos en su mayoría eran de edad avanzada, por lo que estos defectos durante la infección aguda y su persistencia podrían haberse visto alteradas por la propia inmunosenescencia de los participantes de esta cohorte.

En general, los resultados obtenidos en los tres trabajos que conforman esta tesis doctoral nos permiten comprender mejor la desregulación inmunitaria en pacientes con COVID-19 y sugieren posibles dianas terapéuticas para mejorar la respuesta de las células T y evitar alteraciones en células del sistema inmunitario innato, con el fin de controlar la propagación del virus. Además, estos resultados sugieren que la respuesta inmunológica al SARS-CoV-2 y a las vacunas y la duración de la protección inmunológica contra este virus puede ser compleja y estar influenciada por múltiples factores, incluyendo la gravedad de la enfermedad, la exposición previa a otros virus y/o la edad avanzada de los individuos. Por otro lado, estos estudios destacan la importancia de un seguimiento a largo plazo de la respuesta inmunitaria de los pacientes con COVID-19 y de la población vacunada para comprender mejor los efectos a largo plazo de la infección y la vacunación.

En conjunto, estos hallazgos pueden ser útiles para desarrollar estrategias de vacunación más eficaces y para identificar pacientes que pueden estar en mayor riesgo de enfermedad grave o complicaciones a largo plazo debido a deficiencias en la respuesta inmunitaria. Finalmente, estos resultados resaltan la necesidad de continuar investigando la respuesta inmunitaria al SARS-CoV-2 y sus variantes emergentes para garantizar una respuesta inmunológica efectiva y sostenida en la población mundial.

CONCLUSIONES

1. Los pacientes infectados por SARS-CoV-2 en fase aguda muestran un déficit en CDp, asociados a una menor producción de IFN-α, un patrón alterado de marcadores inflamatorios y variaciones en los marcadores de homing y activación de CD.

2. Siete meses tras la infección por SARCO-CoV-2, defectos asociados a marcadores de activación en CD no se recuperan en pacientes previamente hospitalizados, mientras que los niveles de CD y defectos asociados a marcadores de migración a mucosa intestinal y tolerogénesis, no se restablecían independientemente de la hospitalización previa.

3. En la respuesta T virus-específica durante la infección aguda de SARS-CoV-2, las combinaciones de citoquinas que incluían únicamente IFN-γ eran deletéreas, mientras que aquellas combinaciones que presentaban IL-2 se asociaban con un mejor curso de la infección aguda, mostrando a su vez una respuesta T más polifuncional.

4. Siete meses tras haber cursado la infección continúa existiendo respuesta celular y humoral, observándose una calidad de la respuesta T similar a la observada en infección aguda, aunque los sujetos previamente hospitalizados presentan un mayor agotamiento en células T.

5. La respuesta T específica frente a SARS-CoV-2 se asociaba a la respuesta a coronavirus endémicos, mediada principalmente por la expresión de IL-2.

6. La disfunción tímica y la consecuente alteración de la homeostasis de células T observadas en personas de mayor edad se asociaba con una menor respuesta T virus-específica tras la vacunación con la vacuna SARS-CoV-2 de ARNm BNT162b2.

7. Déficits en el número, migración y función de las CD, además de un perfil proinflamatorio inducido por monocitos, se asociaba a una menor respuesta vacunal frente al SARS-CoV-2 en personas de mayor edad.

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Anexo I. Otros trabajos desarrollados durante el periodo de la tesis doctoral

Otros artículos científicos

1. Real LM, Saez ME, Corma Gómez A, González-Pérez A, Thorball A, Ruiz R, Jiménez-León MR, González-Serna A, Gasca-Capote C, Bravo MJ, Royo JL, **Pérez-Gómez A**, Gutiérrez-Valencia A, Vidal F, Fellay J, Lichterfeld M, Ruiz-Mateos E. A meta genome-wide association study of HIV-disease progression in HIV-controllers. (2023, en revisión)
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Comunicaciones a congresos

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disease progression in HIV controllers". CROI 2021 (08-11 de marzo de 2021), Boston (Estados Unidos). Tipo de comunicación: póster. Código de póster: ID 1613.

16. Muñoz Muela E, Trujillo Rodríguez M, Serna-Gallego A, Gasca Capote C, **Pérez-Gómez A**, Gutiérrez Valencia A, López Cortés LF. "Anti-CD4+ T cells antibodies as a potencial cause of the poor recovery and depletion of CD4+ T cells in immunodiscordant HIV-infected patients". IV Congreso Nacional Jóvenes Investigadores en Biomedicina (04-06 de noviembre de 2020), Granada (España). Tipo de comunicación: póster. Código de póster: P01-32.
17. Gasca Capote C, Jimenez-León MR, Tarancón-Diez L, **Pérez-Gómez A**, Muñoz Muela E, Trujillo-Rodríguez M, Gutiérrez-Valencia A, Roca C, Espinosa N, Viciana P, López-Cortés LF, Ruiz-Mateos E. "Metabolic Gene Expression Profile of Plasmacitoid Dendritic Cells in Response to Agonists of TLR7/9". IV Congreso Nacional Jóvenes Investigadores en Biomedicina (04-06 de noviembre de 2020), Granada (España). Tipo de comunicación: póster. Código de póster: P01-12.
18. **Pérez-Gómez A**, Martínez-Hernández R, Serrano-Somavilla A, Sampedro-Nuñez M, Muñoz De Nova JL, Marazuela M. "Implementation of a new pipeline for the analysis of miRNoma using RNA-Seq in Hashimoto's Thyrohyditis and Graves' disease". IV Congreso Nacional Jóvenes Investigadores en Biomedicina (04-06 de noviembre de 2020), Granada (España). Tipo de comunicación: póster. Código de póster: P07-12.

Seminarios impartidos

1. **METABOLOMIC AND IMMUNOLOGICAL PROFILES ASSOCIATED WITH CARDIOVASCULAR EVENTS IN PLWHIV.** Fecha: 01 de julio de 2022. Evento: VII HIBIC, 2022 (organizado por Gilead Sciences, S.L.) en Madrid, España. Duración: 1 hora. Idioma: Inglés.
2. **INNATE AND ADAPTIE IMMUNE RESPONSES TO SARS-CoV-2.** Fecha: 24 de marzo de 2022. Evento: XVIII Foro de Investigadores (IBiS, CSIC, Junta de Andalucía) (organiza IBiS) en Sevilla, España. Duración: 1 hora. Idioma: Inglés.
3. **INNATE AND ADAPTIVE INMUNE DEFECTS ASSOCIATED WITH LOWER SARS-CoV-2 BNT162B2 MRNA VACCINE RESPONSE IN ELDERLY PEOPLE.** Fecha: 08 de febrero de 2022. Evento: Seminario del Departamento de Enfermedades Infecciosas y del Sistema Inmunitario (organizado por IBiS) en Sevilla, España. Duración: 0,5 horas. Idioma: Español.
4. **DECIPHERING THE QUALITY OF SARS-CoV-2 SPECIFIC T-CELL RESPONSE ASSOCIATED WITH DISEASE SEVERITY, IMMUNE MEMORY AND HETEROLOGOUS RESPONSE.** Fecha: 16 de noviembre de 2021. Evento:

Seminario del Departamento de Enfermedades Infecciosas y Sistema Inmunitario (organizado por IBiS) en Sevilla, España. Duración: 0,5 horas. Idioma: Español.

Actividades de divulgación

1. **FEBS-IUBMB-ENABLE 2022 INTERNACIONAL CONFERENCE.** Fecha: 16 al 18 de noviembre de 2022. Organizadores: Instituto de Investigación Biomédica (IRB Barcelona); el Radboud Institute for Molecular Life Sciences (RIMLS), el Novo Nordisk Foundation Center for Protein Research (CPR), Intituto de Biomedicina de Sevilla (IBiS), Federación Europea de Sociedades Bioquímicas (FEBS) y International Union of Biochemistry and Molecular Biology (UIBMB). Lugar de celebración: Sevilla. Grupo de trabajo: Sponsors. Idioma: Inglés. Participación como organizador.
2. **VII CONGRESO NACIONAL DE INVESTIGADORES EN BIOMEDICINA (2022).** Fecha: 05 al 07 de octubre de 2022. Organizadores: Comité Organizador CONBIOPREVAL y Universidad Católica de Valencia (UCV). Lugar de celebración: Valencia. Grupo de trabajo: Directiva, Sponsors y Redes. Sociales. Idioma: Español. Participación como coordinador y organizador.
3. **WEBINAR SOBRE EMPRESAS E INVESTIGACIÓN.** Fecha: 16 de junio de 2022. Organizador: Agrupación Extremeña de Jóvenes Investigadores). Lugar de celebración: online. Duración: 2 horas. Idioma: Español. Participación como ponente.

Anexo II. Comités éticos

CEI de los Hospitales Universitarios Virgen Macarena y Virgen del Rocío

D. Carlos García Pérez
Secretario del CEI de los Hospitales Universitarios Virgen Macarena y Virgen del Rocío

CERTIFICA

1º. Que el CEI de los Hospitales Universitarios Virgen Macarena y Virgen del Rocío en su reunión del día 15/04/2021, acta CEI VM-VR_06/2021 ha evaluado la propuesta del promotor para que se realice la **Modificación relevante nº 1 de fecha 13/04/2021** en el estudio:

Título: Papel de la respuesta T e interferón de tipo I mediada por las células plasmacitoides dendríticas en la infección por SARS-CoV-2. Valor pronóstico y opciones terapéuticas para la COVID-19

Código Promotor: pDCOVID **Código Interno:** 0896-N-20
Promotor: Fundación Pública Andaluza Gestión de la Investigación en Salud de Sevilla (FISEVI)
Versión Protocolo Evaluada: Versión 2.0 de 12 de abril de 2021
Versión Hoja Información al Paciente Evaluada: HIP-CI / 1.0 de fecha 07/04/2020

2º. La modificación **Modificación relevante nº 1 de fecha 13/04/2021**, de tipo **Relevante**, con fecha de registro **14/04/2021** solicita:

Mod. Documentación: Protocolo	
Versión protocolo	Versión 2.0 de 12 de abril de 2021

3º. Considera que

- Se respetan los principios éticos básicos y es adecuado el procedimiento para obtener el consentimiento informado
- Se cumple la legislación aplicable.

4º. Por lo que este CEI emite un **DICTAMEN FAVORABLE A LA MODIFICACIÓN**.

Lo que firmo en Sevilla, a 21 de abril de 2021

Fdo:

D. Carlos García Pérez
Secretario del CEI de los Hospitales Universitarios Virgen Macarena y Virgen del Rocío

CEI de los Hospitales Universitarios Virgen Macarena y Virgen del Rocío

D. Carlos García Pérez
Secretario del CEI de los Hospitales Universitarios Virgen Macarena y Virgen del Rocío

CERTIFICA

1º. Que el CEI de los Hospitales Universitarios Virgen Macarena y Virgen del Rocío en su reunión del día 30/03/2021, acta CEI VM-VR_05/2021 ha evaluado la propuesta del promotor referida al estudio:

Título: Humoral and cellular immune response in SARS-CoV-2-infected with different sequel conditions and in vaccinated subjects using a immunophenotyping and multi-omic approach

Código Promotor: COVIMARATON_2020 **Código Interno:** 0156-N-21
Promotor: Investigador
Versión Protocolo Evaluada: 1.1 de fecha 15/03/2021
Versión Hoja Información al Paciente Evaluada: HIP-CI / 1.1 de fecha 15/03/2021

1º. Considera que

- El estudio se plantea siguiendo los requisitos de la Ley 14/2007, de 3 de julio, de Investigación Biomédica y su realización es pertinente.
- Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y están justificados los riesgos y molestias previsibles para el sujeto.
- Son adecuados tanto el procedimiento para obtener el consentimiento informado como la compensación prevista para los sujetos por daños que pudieran derivarse de su participación en el estudio.
- El alcance de las compensaciones económicas previstas no interfiere con el respeto a los postulados éticos.
- La capacidad de los Investigadores y los medios disponibles son apropiados para llevar a cabo el estudio.

2º. Por lo que este CEI emite un **DICTAMEN FAVORABLE**.

3º. Este CEI acepta que dicho estudio sea realizado en los siguientes CEI/Centros por los Investigadores:

CEI de los Hospitales Universitarios Virgen Macarena y Virgen del Rocío	Ezequiel RUIZ MATEOS CARMONA *(Microbiología y Enfermedades Infecciosas) Hospital Universitario Virgen del Rocío*

Lo que firmo en Sevilla, en la fecha reseñada en la firma electrónica.

Fdo:

D. Carlos García Pérez
Secretario del CEI de los Hospitales Universitarios Virgen Macarena y Virgen del Rocío