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TESIS DOCTORAL

FACTORES INMUNOGENÉTICOS Y RESPUESTA INMUNOLÓGICA EN PACIENTES COVID-19

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A mi familia,

A mis amigos,

A mi mujer,

A mi hijo

INDICE

RESUMEN

La infección por el nuevo coronavirus SARS-CoV-2 ha generado muchas incógnitas del papel del sistema inmunitario en la patología y resolución de la infección. La COVID-19 produce un desequilibrio inmunológico caracterizado por linfopenia, así como elevación de marcadores bioquímicos de inflamación y citoquinas, pudiendo desembocar en tormenta de citoquinas y enfermedad más grave.
Por otro lado, el sistema inmunitario está formado por multitud de células y proteínas que intervienen en el transcurso de una infección viral, participando tanto en la inmunidad innata como adaptativa.

Todos estos factores intervienen en la evolución de la infección, siendo determinantes en la gravedad de esta.

Objetivos

Se buscó determinar el perfil inmunológico e inmunogenético de los pacientes COVID-19 y su relación con la gravedad de la enfermedad:

- En una primera parte se estudió y caracterizó el perfil linfocitario de pacientes con COVID-19, determinando la frecuencia de las subpoblaciones linfocitarias en pacientes hospitalizados. Además, se analizó el perfil funcional (activación y agotamiento) de estas subpoblaciones linfocitarias. Todo ello se relacionó con parámetros bioquímicos y clínicos de pacientes con distinto grado de severidad por COVID-19.

- Posteriormente, nos centramos en el papel de la inmunogenética. Estudiamos los sistemas polimórficos HLA y MICA. Buscamos alelos que puedan explicar la susceptibilidad o protección frente a la infección y la gravedad de la COVID-19.
- Por último, junto con el desarrollo de las vacunas para combatir la COVID-19, estudiamos la respuesta inmunitaria humoral y celular en individuos vacunados con la vacuna mRNA-1273 de Moderna. Además, analizamos el papel de las moléculas HLA de clase II en la respuesta humoral en vacunados con mRNA-1273.

Materiales y Métodos

Para el estudio de las subpoblaciones linfocitarias se utilizó una población de 144 pacientes ingresados por COVID-19. Se les clasificó en función de gravedad y se realizó un análisis de subpoblaciones linfocitarias mediante citometría de flujo multiparamétrica, determinando las subpoblaciones afectadas y su estado funcional. Además, se creó una base de datos con los distintos marcadores de inflamación y de gravedad. Todos estos parámetros fueron analizados y relacionados entre sí para determinar marcadores de pronóstico.

En el estudio de inmunogenética incluimos 483 pacientes de COVID-19 con distintos grados de severidad. Se realizó le tipaje de alta resolución para los alelos HLA y determinación de los alelos STR de MICA. Posteriormente comparamos las frecuencias alélicas entre los distintos grupos de gravedad y grupo control.

Para finalizar, obtuvimos 601 muestras de individuos vacunados con mRNA-1273 y que no pasaron previamente la infección por SARS-CoV-2. Se determinó la respuesta humoral y celular y, se clasificaron en tres grupos en función del grado de respuesta. Por

último, se seleccionaron 30 individuos de cada grupo y se les realizó el tipaje HLA de clase II.

Resultados

El análisis de la linfopenia mostró una disminución de las subpoblaciones linfocitarias T CD4+ y CD8+. El estudio de las subpoblaciones T helper mostró una afectación de la mayoría de ellas con una marcada disminución de linfocitos Th1, un aumento de linfocitos Th0 y bajo grado de activación linfocitaria. Los pacientes más graves presentaron mayor grado de linfopenia y neutrofilia junto a una mayor expresión de marcadores de activación y agotamiento en linfocitos T.

En lo referente a la inmunogenética, los resultados del tipaje HLA no mostraron ninguna asociación con el riesgo, protección o gravedad en la infección por SARS-CoV-2. En el análisis de las moléculas MICA se determinó que el alelo MICA*A9 está asociado a infección y a enfermedad moderada.

Finalmente, en el estudio de la respuesta inmunitaria en individuos vacunados con mRNA-1273, hubo una respuesta humoral general en la que destaca una gran amplitud. El estudio celular arrojó una respuesta del 86% que se relacionó positivamente con la magnitud de respuesta humoral. Por último, el alelo HLA-DRB1*07:01 y haplotipo HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 se relacionaron con una respuesta humoral más intensa.

Conclusiones

- El mal control de la infección por SARS-CoV-2 se caracteriza por linfopenia con disminución marcada de linfocitos Th1, aumento de linfocitos Th0 y un bajo grado de activación linfocitaria.
- Los alelos y haplotipos HLA no se relacionan con la susceptibilidad o gravedad en la infección por SARS-CoV-2.
- El alelo MICA*A9 un factor de riesgo de infección y gravedad en la infección por SARS-CoV-2.
- Hay respuesta humoral general en vacunados con mRNA-1273, similar a la observada en pacientes convaleciente de COVID-19. La respuesta celular se relaciona positivamente con el grado de respuesta humoral.
- El alelo HLA-DRB1*07:01 y su haplotipo asociado, se relacionan positivamente con la generación de una respuesta humoral de más intensidad en vacunados con mRNA-1273.

SUMMARY

Infection with the new SARS-CoV-2 coronavirus has generated many unknowns about the role of the immune system in the pathology and resolution of the infection. COVID-19 produces an immune imbalance characterized by lymphopenia, elevated biochemical markers of inflammation and cytokines, which can lead to a cytokine storm and more severe disease. On the other hand, the immune system is made up of a multitude of cells and proteins that intervene in the course of a viral infection, participating in both innate and adaptive immunity.

All these factors intervene in the evolution of the infection, being determinant in its severity.

Objectives

We set out to determine the immunologic and immunogenetic profile of patients with COVID-19 and its relationship to disease severity:

- In the first part, the lymphocyte profile of patients with COVID-19 was studied and characterized, determining the frequency and functional profile (activation and exhaustion) of lymphocyte subpopulations in hospitalized patients. In addition, the lymphocyte subpopulations functional profile (activation and depletion) was analyzed. These data were related to biochemical and clinical parameters of patients with different degrees of COVID-19 severity.

- Subsequently, we focused on the role of immunogenetics, studying the HLA and MICA polymorphic systems. We searched for alleles that could explain susceptibility or protection against infection and severity of COVID-19.
- Finally, in conjunction with the development of COVID-19 vaccines, we studied the humoral and cellular immune response in individuals vaccinated with the Moderna mRNA-1273 SARS-CoV-2 vaccine. In addition, we analyzed the role of HLA class II molecules in the humoral response of those vaccinated with mRNA-1273.

Materials and Methods

A population of 144 patients admitted for COVID-19 was used to study lymphocyte subpopulations. They were classified according to their severity. Analysis of lymphocyte subpopulations was performed by multiparametric flow cytometry, determining the affected subpopulations and their functional status. In addition, a database was created with different markers of inflammation and disease severity. All these parameters were analyzed and related to each other to determine prognostic markers.

In the immunogenetics study we included 483 COVID-19 patients with different degrees of severity. High resolution typing for HLA alleles and determination of MICA STR alleles were performed. Next, allele frequencies were compared between the different severity groups and the control group.

Finally, we obtained 601 samples from individuals vaccinated with mRNA-1273 and not previously infected with SARS-CoV-2. Humoral and cellular responses were determined and classified into three groups according to the degree of response. In the end, 30 individuals were selected from each group and HLA class II typing was performed.

Results

Analysis of lymphopenia showed a decrease in CD4+ and CD8+ T cells. The study of T helper (Th) revealed alterations in most of them, with a marked decrease of Th1 cells. An increase in Th0 lymphocytes and a low degree of lymphocyte activation were also observed. More severe patients were characterized by a higher degree of lymphopenia and neutrophilia along with an increased expression of markers of activation and exhaustion in T lymphocytes.

Regarding immunogenetics, HLA typing results showed no association with risk, protection, or severity of SARS-CoV-2 infection. In the analysis of MICA molecules, the MICA*A9 allele was associated with infection and moderate disease.

Finally, in the study of the immune response in individuals vaccinated with mRNA-1273, a general humoral response of great amplitude was observed. The cellular study yielded a response of 86% which was positively related to the magnitude of humoral response. In the end, the HLA-DRB1*07:01 allele and HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 haplotype were associated with a more intense humoral response.

Conclusions

- Poor control of SARS-CoV-2 infection is characterized by lymphopenia with a marked decrease in Th1 lymphocytes, an increase in Th0 lymphocytes and a low degree of lymphocyte activation.
- HLA alleles and haplotypes are not associated with severity and susceptibility to SARS-CoV-2 infection.
- MICA*A9 allele is a risk factor for SARS-CoV-2 infection and severity.

- There is a general humoral response in mRNA-1273 vaccinees, similar to that observed in convalescent COVID-19 patients. The cellular response was positively related to the degree of humoral response.
- The HLA-DRB1*07:01 allele and its associated haplotype are positively related to the generation of a more intense humoral response in those vaccinated with mRNA-1273.

INTRODUCCIÓN

En diciembre de 2019, la Organización Mundial de la Salud (OMS) anunció un brote de neumonía en Wuhan (China) producto de un nuevo coronavirus (1). El 30 de enero de 2020, la OMS declaró la emergencia sanitaria internacional. El 11 de febrero de 2020, la OMS nombró oficialmente al brote como Enfermedad por Coronavirus-2019 (COVID-19) (2) y el Comité Internacional de Taxonomía de Virus nombró al virus como SARS-CoV-2 (3). Junto al SARS-CoV en 2002-2003 y el MERS-CoV en 2012, este era el tercer coronavirus zoonótico que causa epidemia en humanos en el siglo XXI (4).

Los coronavirus son virus envueltos de RNA (ácido ribonucleico) de cadena simple, sentido positivo y no segmentado, que infectan principalmente a hospedadores vertebrados (5). Se tratan de los virus de RNA que poseen el genoma de mayor tamaño. Entre sus huéspedes podemos encontrar aves, ganado y humanos, por lo que se tratan de virus que tienen interés tanto para la salud pública como veterinaria (6). Presentan vías de transmisión principalmente aéreas o fecal-oral, y las infecciones suelen presentarse como enfermedades respiratorias y/o entéricas (7).

En lo referente al origen del SARS-CoV-2, presenta un 79,5% de identidad de secuencia con el SARS-CoV, mientras que comparte un 96.2% con el virus RatG13 de murciélago (8). Respecto a la secuencia de la proteína Spike (S), hay una similitud del 76% con SARS-CoV, pero se mantienen los residuos esenciales para la interacción con el receptor ACE2 (enzima convertidora de angiotensina 2) (9). Sin embargo, en el caso de la similitud de la proteína S con RatG13, solamente se mantiene uno de los seis residuos que interfieren en la unión a ACE2 (8). Estos datos sugieren que la alta capacidad de recombinación de los coronavirus relacionados con el síndrome respiratorio agudo severo que coexisten en los murciélagos, pudieron generar el SARS-CoV-2 (10). Además, los puntos de recombinación estimados de la proteína S la dividen en tres. La parte central,

que engloba los aminoácidos del 1030 al 1651 incluye el dominio de unión al receptor (RBD) del SARS-CoV-2 y, es más similar a la del SARS-CoV y los coronavirus WIV1 y RsSHC014 relacionados con el síndrome respiratorio agudo severo de murciélago, los cuales usan también el receptor ACE2 (6). Las regiones laterales carboxiterminal y aminoterminal (1-1029 y 1652-3804, respectivamente) son más similares a los coronavirus ZC45 y ZXC21 relacionados con el síndrome respiratorio agudo severo (6). Estos datos muestran el potencial de los coronavirus para crear nuevas especies gracias a la recombinación, lo que debe traducirse en un seguimiento estrecho de los coronavirus en murciélagos, para estudiar las potenciales nuevas especies que puedan infectar a los humanos.

Los murciélagos parecen ser el reservorio natural del SARS-CoV-2 (8,11). Mientras que se piensa que los pangolines pueden tratarse de los hospedadores intermedios (12). Estas sospechas se basan en la similitud genética entre el SARS-CoV-2 y el coronavirus del pangolín, que en términos de genes E, M, N y S, presenta un 100, 98.6, 97.8 y 90.7% de similitud respectivamente (12).

1. SARS-CoV-2

1.1 Taxonomía

El virus SARS-CoV-2 pertenece al subgénero Sarbecovirus, género Betacoronavirus, subfamilia Orthocoronavirinae, familia Coronaviridae, Suborden Cornidovirineae, orden Nidovirales, reino Riboviria (13).

1.2 Estructura

El genoma del SARS-CoV-2 está compuesto por una molécula de RNA monocatenaria de sentido positivo (RNAss+) (14). Posee una cola de poliadenina en la región 3' no traducida (3'UTR) y caperuza en el extremo 5' (5'-Cap) (4). El tamaño del genoma es de unas 29.9Kb (15) y presentan 14 marcos de lectura (4). Codifica para proteínas estructurales , proteínas no estructurales que participan en la replicación y ensamblaje del virus, y proteínas accesorias (4).

El SARS-CoV-2 consta de cuatro proteínas estructurales que incluyen la proteína espiga (S), proteína de envoltura (E), proteína de membrana (M) y proteína de la nucleocápside (N); además de 16 proteínas no estructurales llamadas nsp (nsp1-16) (12). La proteína S es una proteína transmembrana que interactúa con ACE2 en la célula diana. Se compone de la subunidad de unión al receptor (S1) y la subunidad de fusión de la membrana celular (S2) (16). La proteína N se une al genoma del virus e interviene en la replicación, formación de los viriones y la evasión de la respuesta inmunológica (17). La proteína M participa en el ensamblaje y gemación de partículas virales a través de la interacción con N y proteínas accesorias como 3a y 7a (18). Finalmente, la proteína E facilita la producción, maduración y liberación de los viriones (19).

La proteína S forma homotrímetros que sobresalen de la superficie viral y median la entrada en la célula huésped mediante la interacción con ACE2. Consta de dos

subunidades: S1 y S2. Por un lado, S1 contiene el extremo N-terminal y el dominio RBD. Su función es la de reconocer y unirse al receptor de las células huésped. Por otro lado, S2 contiene el extremo C-terminal con el péptido de fusión (FP), repetición de heptada 1 y 2 (HR1 y HR2), dominio transmembrana (TM) y tallo citoplasmático (CT) (20). Se encarga de la fusión de la membrana del virus con la membrana de la célula huésped.

El dominio RBD de la proteína S es considerado unos de los más complejos del genoma de los coronavirus (8). Son seis aminoácidos del dominio RBD los implicados en la unión con ACE2. En el caso del SARS-CoV-2 son leucina 455 (Leu455), fenilanalina 486 (Phe486), glutamina 493 (Gln493), serina 494 (Ser494), asparagina 501 (Asn501) y tirosina 505 (Tyr505), diferenciándose en cinco de los seis con el SARS-CoV, el cual también tiene como diana en las células huésped a ACE2 (21).

Las Nsp desempeñan numerosas funciones en los procesos de replicación y ensamblaje del virus. Participan en la patogénesis viral al modular la regulación de la transcripción temprana, actividad helicasa, inmunomodulación, transactivación de genes y contrarrestar la respuesta antiviral (Tabla 1) (22,23).

Tabla 1. Funciones de las proteínas Nsp.

Proteína Nsp	Función
Nsp1	Inhibidor de la traducción del huésped. Mediación del procesamiento y replicación del RNA. Degradación de RNA mensajero. (24)
Nsp2	Modulación de la vía de señalización de supervivencia de la célula huésped. (25)
Nsp3	Proteasa que fragmenta la poliproteína producida en las distintas proteínas. (26)
Nsp4	Posible función en el anclaje del complejo de replicación-transcripcional viral a las membranas del retículo endoplasmático. (27)
Nsp5	Procesamiento de las poliproteínas virales en la replicación. (28)
Nsp6	Inducción inicial del autofagosoma del retículo endoplasmático.
Nsp7 y Nsp8	Forman un complejo con estructura cilíndrica hueca que participa en la replicación. (29,30)
Nsp9	Proteína de unión a RNA de cadena sencilla que participa en la replicación. (31)
Nsp10	Metilación del 5'Cap de los RNAm virales. (32)
Nsp11	Función desconocida.
Nsp12	Replicación y transcripción del genoma viral. (33)
Nsp13	Helicasa. Participa en replicación y transcripción. (34)
Nsp14	Actividad exoribonucleasa 3'-5'. Actividad N7-guanina metiltransferasa. (35,36)
Nsp15	Actividad endoribonucleasa dependiente de manganeso. (35)
Nsp16	Metiltransferasa. Media la metilación del 5'Cap de los RNAm virales. (37)

1.3 Ciclo de replicación

La vía de infección principal del SARS-CoV-2 es entre personas durante contactos estrechos a través de fómites y gotitas (20). Además, se han notificado los aerosoles como vía de transmisión del virus, siendo esta posiblemente la más importante (20). No obstante, aunque la vía de transmisión predominante es la aérea, se puede encontrar transmisión fecal-oral, especialmente en pacientes pediátricos. Esto demuestra la capacidad del virus para su replicación y propagación en el trasto gastrointestinal (38). Las principales fuentes de infección son las personas contagiadas con síntomas o sin ellos, teniendo una gran importancia estos últimos ya que no se detecta la infección y por tanto no se mantienen las condiciones higiénicas necesarias para evitar la propagación (20).

Una vez penetra el virus al interior del cuerpo se une a los receptores del huésped y entra dentro de las células a través de la fusión de membranas. El receptor del SARS-CoV-2 es ACE-2 que se expresa altamente en las células epiteliales pulmonares (39). La proteína S, formada por las subunidades S1 y S2, reconoce a ACE-2 y se activa a través de una escisión de proteasa en dos pasos: primero entre las subunidades S1/S2, y una segunda escisión dentro de la subunidad S2 llevada a cabo por la proteasa transmembrana de serina 2 (TMPRSS2) de la células huésped (40). Esto conduce a la activación de la proteína S y un cambio conformacional que tiene como resultado la fusión de las membrana de la célula huésped y el virus (41). Tras la fusión, el virus libera su genoma de RNA en el citoplasma celular. Este RNA es traducido por las proteínas celulares para dar lugar a las proteínas efectoras no estructurales necesarias para la formación del complejo de replicación. Una RNA polimerasa dependiente de RNA genera una copia RNA de sentido negativo del genoma viral, que servirá como molde para producir nuevas copias de RNA positivo (42). La proteína N se une al nuevo RNA generado y junto a la proteína M forman una nucleocápsula que facilita la entrada al retículo endoplasmático. Los nuevos viriones

se ensamblan en el aparato de Golgi tras el transporte de las proteínas estructurales y accesorias desde el retículo endoplasmático (43). Finalmente, los nuevos virus maduros son liberados desde el aparato de Golgi mediante exocitosis al espacio extracelular listos para repetir el ciclo de infección (44).

1.4 COVID-19

La enfermedad por coronavirus 2019 (COVID-19) puede cursar con una serie de manifestaciones clínicas que van desde la ausencia de síntomas hasta enfermedad crítica. En general, se pueden clasificar a los pacientes en categorías de gravedad, pudiendo solaparse algunas de ellas o variar el estado clínico del paciente. Las categorías son:

- Infección asintomática o presintomática: individuos que están infectados por el virus pero que no presentan síntomas compatibles con COVID-19 (45).

- Enfermedad leve: individuos que presentan cualquiera de los diversos signos y síntomas de COVID-19 (fiebre, tos, dolor de garganta, malestar general, dolor de cabeza, dolor muscular, náuseas, vómitos, diarrea, pérdida del gusto y olfato) pero que no presentan dificultad respiratoria, disnea o imágenes torácicas anormales (45).

- Enfermedad moderada: individuos que muestran evidencias de enfermedad respiratoria durante la evaluación clínica o por imagen y que tienen una saturación de oxígeno en sangre (SpO2) ≥ 94% en aire ambiente y al nivel del mar (45).

- Enfermedad grave: individuos que tengan una SpO2 < 94% en aire ambiente a nivel del mar, una relación < 300 mm Hg entre la presión parcial arterial de oxígeno y la fracción de oxígeno inspirado, una frecuencia respiratoria > 30 respiraciones por minuto, o infiltrados pulmonares mayores del 50% (45).

- Enfermedad crítica: individuos con fallo respiratorio, shock séptico, y/o fallo multiorgánico (45).

En lo referente a hallazgos de laboratorio entre los pacientes hospitalizados por COVID-19 encontramos linfopenia, niveles elevados de transaminasas, lactato deshidrogenasa (LDH) y dímero D, y aumento de marcadores inflamatorios como ferritina, proteína C reactiva (CRP), procalcitonina, troponina I, péptido cerebral natriurético (BNP), interleucina 6 (IL-6), factor de necrosis tumoral □ (TNF-□□, interleucina 1 (IL-1), etc. (46). Además, se detectan anormalidades en los test de coagulación.

2. Vacunas mRNA

Pese a las numerosas medidas llevadas a cabo para evitar la propagación del virus, el desarrollo de vacunas para la generación de inmunidad era necesario para vencer a la pandemia (47). Entre las vacunas desarrolladas y aceptadas para uso en humanos encontramos vacunas de RNA mensajero (mRNA) (BNT16b2 y mRNA-1273), de vectores virales (AZD1222, Sputnik V, Sputnik V Light, Ad5-nCoV, Ad26.COV2.S), inactivadas (CoronaVac, BBIBP-CorV, vacuna inactivada de Wuham Sinopharm,

Covaxin, KoviVac, COVIran Barekat), y basadas en proteínas (EpiVacCorona, ZF2001, Abdala) (48).

Las vacunas de mRNA frente al SARS-CoV-2 han sido las primeras con este tipo de tecnología aprobadas para su uso en humanos. La eficacia y seguridad de las vacunas de mRNA ha sido demostrada (49), observándose una efectividad del 90% en la prevención de la COVID-19 (50).

Las vacunas basadas en la tecnología de mRNA han demostrado muchas ventajas que no poseen las vacunas convencionales. En primer lugar, el mRNA tiene el potencial para poder codificar y expresar todo tipo de proteínas (51). En segundo lugar, el mRNA tiene propiedades autoadyuvantes que activan respuestas adaptativas fuertes y duraderas a través del TNF-□, interferón □ (IFN-□□□ y otras citoquinas, mientras que las vacunas proteicas y de polisacáridos requieren adyuvantes adicionales (52). En tercer lugar, la expresión del mRNA evita la contaminación derivada de proteínas y virus, ya que el mRNA solo es activo de forma transitoria, lo que facilita su descomposición por completo a través de las vías metabólicas fisiológicas (53). Y, en cuarto lugar, en comparación a las vacunas de ácido desoxirribonucleico (DNA), las de mRNA pueden expresar proteínas de forma más eficaz debido a que se expresan en citoplasma sin necesidad de entrar al núcleo (54). Además, hay menos posibilidades de que el mRNA se inserte en el genoma del huésped (54).

Las vacunas de mRNA se desarrollan evitando el problema de la inmunogenicidad que tienen las moléculas de mRNA. Estás moléculas exógenas son reconocidas por receptores celulares como el gen I inducible por ácido retinoico (RIG-I), antígeno 5 de la diferenciación del melanoma (MDA5) y receptores tipo Toll (TLR-3, TLR-7 y TLR-8) lo que generaría la activación de la respuesta inmune innata (55,56). Para salvar esto, el mRNA es modificado mediante la adición de la cola poli (A), modificación química de

nucleótidos (como la eliminación de uridinas por su cambio con 5-metiluridina) (57) y la optimización de la secuencia generando regiones ricas en pares guanina-citosina (GC) (58). Además, la purificación del mRNA es esencial para eliminar la inmunogenicidad (59).

Por otra parte, el mRNA es vulnerable a la degradación. Por ello, existen mecanismos que estabilizan al mRNA para garantizar su expresión. Por ejemplo, el reemplazo de codones por otros más raros aumenta la estabilidad del mRNA. Del mismo modo, las regiones 5'UTR y 3'UTR, la cola poli (A) y la modificación de la tapa en 5', son sitios optimizables para fortalecer la estabilidad del mRNA (58). Sin embargo, estos cambios podrán influir en el tipo, intensidad y especificidad de las respuestas inmunológicas, además de en la precisión de la traducción y el plegamiento proteico (60,61).

La vacuna mRNA-1273 (desarrollada por investigadores del NIAID Vaccine Research Center y Moderna en Cambridge) codifica una versión estabilizada del trímero de glicoproteína de la proteína S de longitud completa que ha sido modificada para incluir dos sustituciones de prolina en la parte superior de la hélice central de la subunidad S2 (62). El mRNA se encapsula en nanopartículas lipídicas a una concentración de 0,5mg por mililitro (62). Su administración es vía intramuscular y requiere dos dosis de 100 µg separadas 28 días.

Las nanopartículas lipídicas que recubren al mRNA protegen de la degradación y permite su internalización en las células huésped. Una vez dentro, el mRNA se traduce en la proteína S del SARS-CoV-2 que es expresada en la superficie de las células huésped (63). La expresión de la proteína en las células huésped es reconocida por el sistema inmunológico que genera una respuesta inmune específica hacia la proteína S.

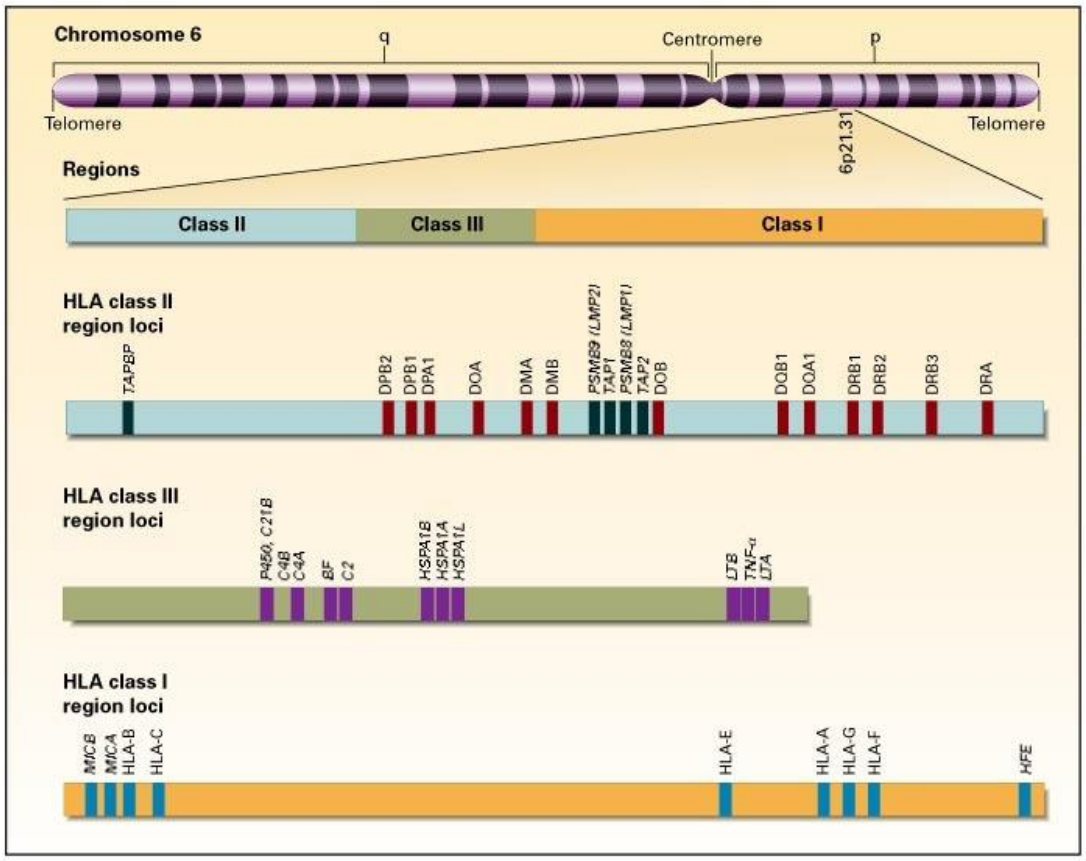
3. Antígeno Leucocitario Humano (HLA)

El Complejo Mayor de Histocompatibilidad (MHC) fue descubiertos a principios del siglo XX cuando se estudiaba el trasplante de órganos en ratones (64). Años después, se descubrió que juegan un papel fundamental en la respuesta frente a antígenos proteicos en todos los mamíferos (64). En humanos, se conoce como Antígeno Leucocitario Humano (HLA), ya que estas moléculas se identificaron y caracterizaron por primera vez utilizando aloanticuerpos contra los leucocitos (65). Es muy conocido su papel en el rechazo de injertos, pero el principal rol de las moléculas HLA es la presentación de péptidos a los linfocitos T para modular la respuesta inmunitaria (66).

3.1 Estructura de la región MHC

La región MHC está localizada en el brazo corto del cromosoma 6 (6p21) y tiene un tamaño aproximado de 3.6 Megabases (67). En total hay 224 loci identificados (68). En humanos se divide en tres regiones: región de clase I, clase II y clase III (Figura 1). En la región de clase I encontramos los genes que codifican para las cadenas α de las moléculas de clase I HLA-A, -B y -C. En la región de clase II se encuentran los loci de las cadenas α y β de las moléculas de clase II, HLA-DR, -DQ y -DQ. Por último, en la región III no se encuentran genes HLA pero si otros genes que forman parte del sistema inmunológico como son los componentes del complemento C2 y C4, y el TNF (67).

Figura 1. Estructura del MHC humano. Tomado de J Klein et al (69).

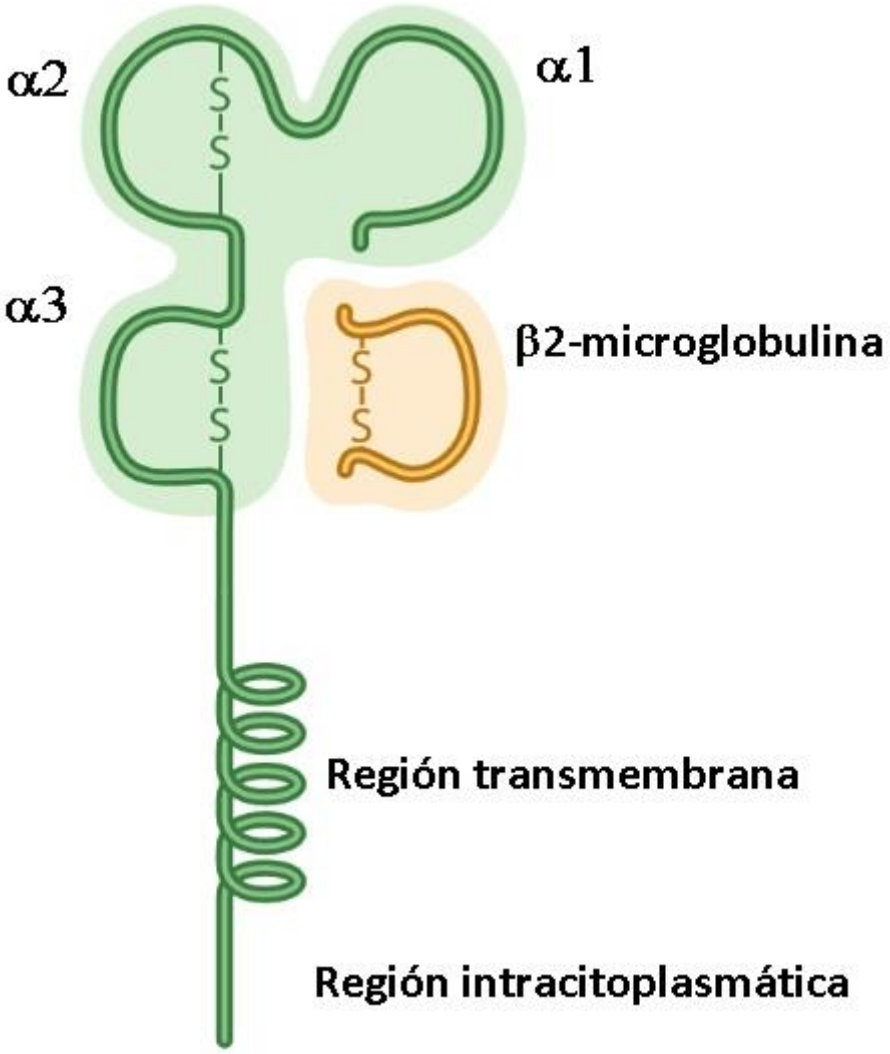


3.2 Estructura de las moléculas HLA

Las moléculas HLA de clase I son un heterodímero formadas por una cadena α polimórfica, codificada en la región de clase I del MHC, y una moléculas de β2-microglobulina, codificada fuera del MHC en el cromosoma 15 (70). La cadena α presenta tres dominios extracelulares (α1, α2 y α3), una región transmembrana y un dominio intracitoplasmático (Figura 2). Los dominios α1 y α2 forman la zona de unión al péptido de las moléculas HLA de clase I. Esta región es altamente polimórfica, lo que va a condicionar que tipo de péptidos son presentados, determinando las especificidades antigénicas de las moléculas HLA. El dominio α3 y la molécula β2-microglobulina

forman dominios similares a los de inmunoglobulinas (71). Las moléculas HLA de clase I pueden presentar péptidos de 8 a 10 aminoácidos de longitud (69).

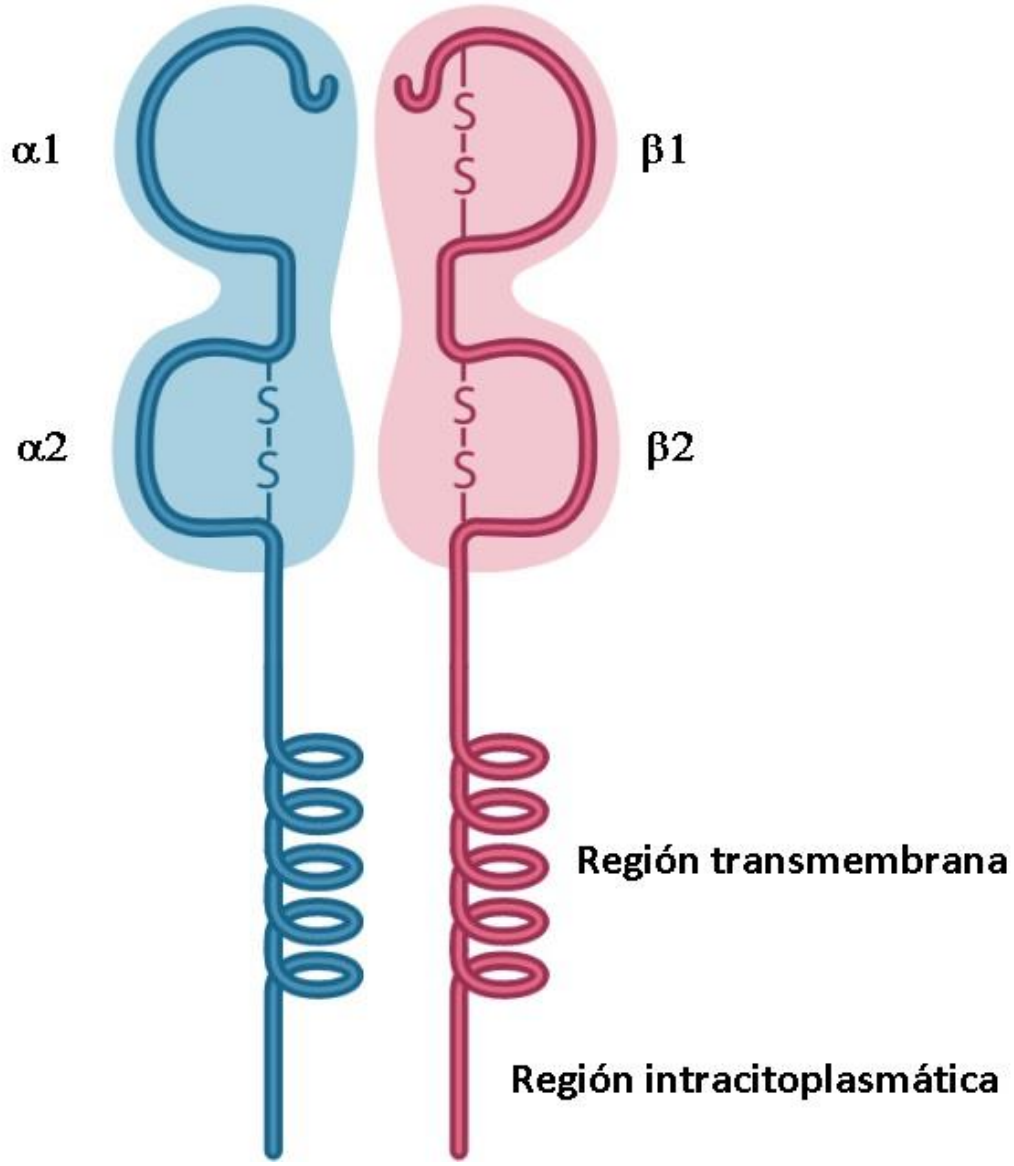
Figura 2. Estructura molécula HLA-I.



Las moléculas HLA de clase II también son un heterodímero formado por una cadena α y una cadena β. Al contrario que en las moléculas HLA de clase I, las dos cadenas están codificadas dentro del MHC (72). Ambas cadenas presentan dos dominios que forman la parte extracelular de la moléculas (α1 y α2; β1 y β2), una región transmembrana y un

tallo intracitoplasmático (Figura 3) (72) . Los dominios α1 y β1 forman la hendidura de unión al péptido y son las zonas más polimórficas de la molécula (73). Debido a una configuración más abierta de la hendidura de unión al péptido, las moléculas HLA de clase II presentan péptidos de mayor tamaño (12 a 28 aminoácidos) (73). Los dominios α2 y β2 presentan dominios tipo inmunoglobulina (73).

Figura 3. Estructura molécula HLA-II.



3.3 Características de las moléculas HLA

3.3.1 Poligénico

El sistema HLA es poligénico ya que presentan varios genes que realizan la misma función. Los genes HLA-A, HLA-B y HLA-C forman las moléculas HLA de clase I (74). Los genes HLA-DR, HLA-DP y HLA-DQ forman las moléculas HLA de clase II (74).

3.3.2 Polimórfico

El sistema HLA es el sistema más polimórfico conocido en humanos. Hasta la fecha, se han descrito más de 25 mil alelos HLA de clase I y más de 10 mil alelos HLA de clase II (75). Este polimorfismo no se extiende a toda la molécula HLA, sino que está presente en las zonas de unión al péptido ($\alpha1$ y $\alpha3$ para clase I; $\alpha1$ y $\beta1$ para clase II) (70). La variación de los aminoácidos que forman el surco de unión conduce a variaciones en sus propiedades físico-químicas que aumentan o disminuyen su capacidad para unirse y presentar ciertos péptidos. Por ello, un alelo puede ser un buen o mal presentador de péptidos derivados de un patógeno.

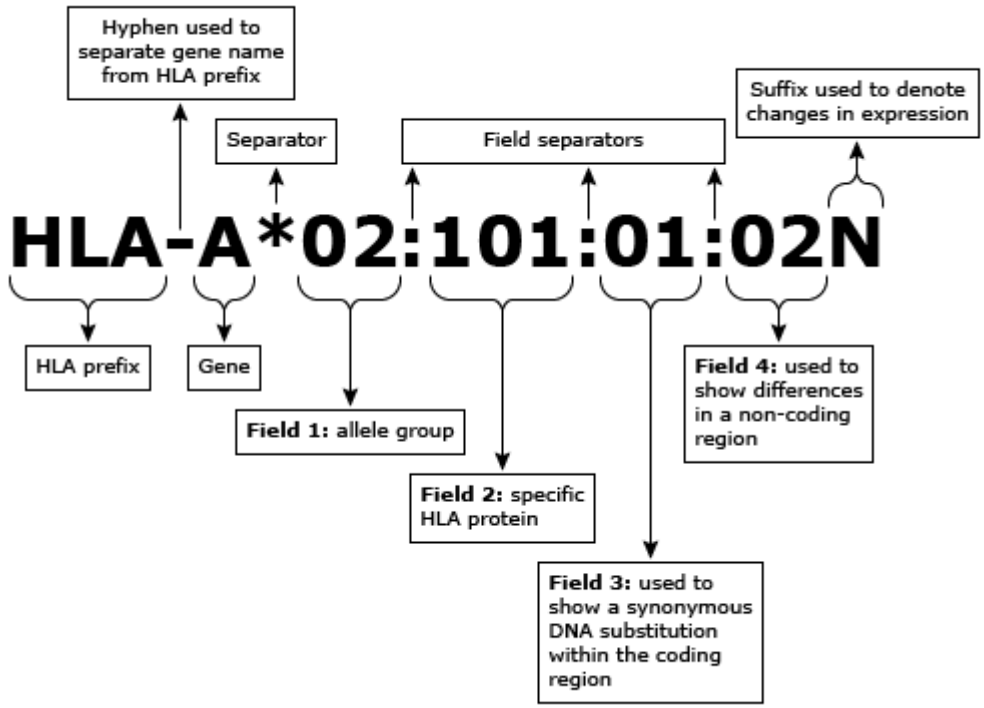
3.3.3 Expresión

Las moléculas HLA presentan expresión codominante, expresándose todos los alelos. No obstante, la expresión de las moléculas HLA de clase I está en todas las células nucleadas, salvo algunos excepciones, como son las plaquetas que expresan HLA de clase I o las células del trofoblasto que no lo expresan (74). En cambio, las moléculas de clase II presentan una expresión más restringida a las células presentadoras de antígenos (células dendríticas, linfocitos B y macrófagos), aunque en condiciones de activación también podemos encontrar su expresión en linfocitos T (74).

3.3.4 Nomenclatura

Desde 2010 se le adjudica un identificador único a cada alelo HLA (76). Este identificador comienza con las siglas HLA, seguido de un guion y el nombre del gen (A, B, C, DR, DP o DQ), un asterisco (*) y hasta un máximo de cuatro conjuntos de dos dígitos separados por dos puntos (:) (HLA-B*XX:XX:XX:XX) (Figura 4). El número de conjuntos de dos dígitos que se da depende del grado de resolución obtenida en el tipaje del alelo HLA, añadiéndose más campos cuanto mayor sea la resolución. El primer conjunto de dos dígitos corresponde al grupo alélico. El segundo conjunto se refiere a una proteína específica, por lo que cualquier alelo nombrado con los dos primeros conjuntos de dígitos determina inequívocamente una proteína específica. El tercer conjunto identifica secuencias sinónimas, mientras que el cuarto campo se refiere a diferencias en regiones no codificantes (76).

Figura 4. Nomenclatura HLA. *Tomado de J Robison et al* (75).



3.3.5 Funciones de las moléculas HLA

Las moléculas HLA se encargan de presentar péptidos a los linfocitos T, encontrándose diferencias entre las moléculas HLA de clase I y II (77). Las moléculas HLA de clase I presentan péptidos endógenos derivados del metabolismo celular o de microorganismos intracelulares, como virus y microbacterias. Este complejo de moléculas HLA de clase I, junto con el péptido endógeno, es reconocido por los linfocitos T CD8+, también conocidos como linfocitos T citotóxicos (78).

Las moléculas HLA de clase II presentan péptidos de origen exógeno, los cuales son captados, procesados y unidos a las moléculas HLA-II por vía endocítica. Estas moléculas de HLA-II son reconocidas por los linfocitos T CD+4 o T helper (79).

Los linfocitos T reconocen las moléculas HLA mediante su receptor TCR, el cual debe ser específico para el péptido presentado (80). De este modo, el reconocimiento del péptido por parte de las células T activarán el desarrollo de una respuesta inmunológica adaptativa celular y/o humoral.

Las células natural killer (NK) tienen la capacidad de detectar una disminución en la presencia de moléculas HLA-I en la superficie celular. A diferencias de los linfocitos T, las células NK no tienen TCR, por lo que no están restringidos a las moléculas HLA. Frecuentemente se observa una disminución en la expresión de moléculas HLA-I tanto en procesos tumorales como en infecciones intracelulares, en ambos casos como un intento de eludir la respuesta de los linfocitos T citotóxicos. Esta disminución de la expresión es detectada por las células NK y el resultado llevaría a la destrucción de las células infectadas o tumorales (81). Las células NK expresan una serie de receptores inhibidores y activadores que reconocen varias moléculas, incluido las moléculas HLA-I, que promueven sus capacidades antivirales, al tiempo que mantienen la autotolerancia

(94,95). Entre estos receptores encontramos los receptores KIR (receptores tipo inmunoglobulinas de las células NK), receptores de la familia CD94/NKG2A, NKG2D (receptor D miembro del grupo 2 de NK), receptores NCR (receptores de citotoxicidad natural) y receptores LIR (receptores leucocitarios tipo inmunoglobulina) (82). Las moléculas KIR y NCR presentan función activadora e inhibitoria, mientras que otras, como NKG2D inducen únicamente activación. El resultado de señales inhibitorias y de activación determinan el estado funcional de las células NK. En condiciones de reposo, donde no hay infección, proceso canceroso o daño tisular, las células NK se encuentran inactivas debido a señales inhibitorias que predominan sobre las señales de activación (83). En cambio, en un proceso inflamatorio, las señalas de activación sobrepasan las de inhibición y activa las funciones efectores de las células NK (83).

4. Principal complejo de histocompatibilidad de clase I relacionado con la cadena α (MICA)

Las moléculas MICA son proteínas MHC no clásicas que se encuentran codificadas dentro de la región MHC (Figura 2). Se expresan en la superficie celular de células epiteliales, fibroblastos, queratinocitos, células endoteliales y monocitos en condiciones de estrés, como infección viral o transformación tumoral (84,85). Tiene una estructura similar a las moléculas HLA-I pero carece de β2-microglobulina asociada (86). Presenta una porción extracelular formados por tres dominios (α1, α2 y α3), codificados por los exones 2, 3 y 4, respectivamente, junto a una región transmembrana codificada por el exón 5 (86).

Las proteínas MICA son muy polimórficas, encontrándose hasta 529 alelos que codifican para 280 proteínas (75). Algunos de estos polimorfismos se encuentran en la región

transmembrana, donde encontramos una repetición corta en tándem (STR) del trinucleótido guanina-citosina-timina (GCT), que codifica para residuos de alanina (86). El número de repeticiones GTC (4, 5, 6, 7, 8, 9 o 10) da lugar a los alelos MICA*A4, *A5, *A6, *A7, *A8, *A9 y *A10, respectivamente (87). Además, encontramos el alelo MICA*5.1 el cual no posee región transmembrana debido a la inserción de una guanina en el exón 5, lo que produce un codón de parada y una proteína truncada (87). Los alelos con región transmembrana presentan una expresión basolateral, mientras que el alelo MICA*A5.1 lo hace a nivel apical mediante su unión a glicosil-fosfatidil-inositol (GPI) (88,89). Debido a la unión del alelo MICA*A5.1 a la molécula GPI y su localización apical es más fácil su liberación, por lo que los niveles de MICA soluble (sMICA) han sido encontrados en aquellos individuos portadores de este alelo (90).

Las moléculas MICA son expresadas en momentos de estrés celular y son reconocidas por el receptor celular NKG2D, expresado en células NK, linfocitos T CD4+, linfocitos T CD8+ αβ, células T γδ y células iNKT (células natural killer invariantes) (91,92). NKG2D es capaz de inducir directamente la función citotóxica y la producción de interferón gamma (IFN-γ) de las células NK, mientras que actúa como coestimulador de la actividad citotóxica en las células T CD8+ αβ (91,92).

5. Respuesta inmunitaria y patogénesis en la infección por coronavirus

5.1 Respuesta inmunitaria innata

El virus SARS-CoV-2 es reconocido por el sistema inmune innato mediante receptores de reconocimiento de patógenos (PRR). Estos receptores reconocen patrones moleculares asociados a patógenos (PAMP). Entre ellos encontramos receptores tipo NOD (NLR), receptores tipo RIG-I (RLR), TLR y receptores tipo lectina C (97,98). El virus SARS-CoV-2 es un virus de RNA que es reconocido por los TLR (TLR3, TLR7 y TLR8) a nivel endosomal y RIG-I, MDA-5 y LGP2 a nivel citosólico (99,100). La detección del RNA viral lleva a la activación de los factores de transcripción NF-κB (factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas) y el factor regulador del interferón 3 (IRF3), que al translocarse al núcleo inducen la expresión de citoquinas proinflamatorias, quimiocinas e INF de tipo I (101).

Los INF de tipo I (α, β, ε, κ, ω, etc) son las citoquinas más importantes en la defensa antiviral. Son producidas tanto por células infectadas como por células de la inmunidad innata y tiene distintas funciones. Las células infectadas, como células endoteliales y fibroblastos, producen predominantemente IFN-β, mientras que las células de la inmunidad innata, como células dendríticas (DC) y macrófagos, producen IFN-α y IFN-β (102). Las DC plasmocitoides, importantes en las infecciones virales, son las mayores productores de IFN-α (102). En lo referente a las funciones del INF de tipo I destacan la inducción de mecanismos de defensa en la célula infectada para limitar la propagación viral, la modulación de las respuestas inmunitarias innatas favoreciendo la presentación de antígenos y las funciones de los linfocitos NK, y por último, la participación en la

activación del sistema inmunitario adaptativo, promoviendo el desarrollo de linfocitos T y B específicos del virus (102).

El IFN-I se une a los receptores IFN α/β que disparan la cascada de traducción de señales que termina con la inducción a la transcripción de los genes estimulados por IFN (ISG) (103). Entre ellos, encontramos varios que intervienen en la degradación de RNA viral como la oligoadenilato sintetasas (OAS), ZAP (proteína asociada a ζ) y el gen 20 estimulado por interferón (ISG20) (104). Sin embargo, los coronavirus presentan mecanismos que regulan a la baja la expresión de IFN de tipo I favoreciendo el escape inmunitario. Esto ayuda a la rápida expansión del virus y aumenta el daño en los tejidos que es respondido por parte de las células innatas mediante la producción de citoquinas proinflamatorias como IL-1, IL-6 y TNF (105). Estas citoquinas, las cuales se encuentran aumentada en los pacientes más graves por COVID-19, inducen la producción de más citoquinas proinflamatorias por parte de los linfocitos T y NK, incluidas interleucina 2 (IL-2), factor estimulante de colonias de granulocitos y macrófagos (GM-CSF) e IFN-γ (106). Los niveles tan elevados de citoquinas promueven la movilización de neutrófilos, macrófagos y células T al sitio de infección, aumentando el daño de, en este caso, el tejido alveolar, capilar, de la barrera vascular e incluso de múltiples órganos, lo que puede conducir a la muerte (107). Por ello, la COVID-19 es considerada una enfermedad mediada por una tormenta de citoquina (108).

Por último, las células NK representan un componente importante de la inmunidad innata que participa en la defensa contra las infecciones virales a través de la producción de perforina y granzimas para eliminar a las células infectadas (84); inducción de apoptosis a través de vías dependientes e independientes de caspasas (84); promoción de las respuestas innatas y adaptativas a través de la producción de citocinas y quimiocinas (93); producción de citotoxicidad dependiente de anticuerpos a través de CD16 (84) y

producción de IFN-γ, el cual estimula a las células T CD8+ citotóxicas y favorece la diferenciación de las células T CD4+ en células T-helper (Th) 1, importantes en las infecciones virales y un puente entre la inmunidad innata y el desarrollo de la inmunidad adaptativa (93).

5.2 Respuesta inmunitaria adaptativa

Para el control definitivo de cualquier tipo de infección, es necesario el desarrollo de la inmunidad adaptativa con la formación de linfocitos T, CD4+ y CD8+ específicos del patógeno, así como de células plasmáticas secretoras de anticuerpos. Una vez superada la inmunidad innata, el virus se enfrenta a las moléculas HLA. Las moléculas HLA de clase I presentan péptidos derivados del virus a los linfocitos T CD8+ específicos, los cuales producirán la lisis de las células infectadas (109).

Por otro lado, las células presentadoras de antígenos a través de sus moléculas HLA de clase II, intervienen en la activación, proliferación y diferenciación de los linfocitos B y linfocitos T CD4+ (110). El puente necesario para la generación de los linfocitos T CD8+ y B específicos, lo generan las subpoblaciones Th, también conocidas como Th subset. El conjunto de subpoblaciones que forman el Th subset se diferencian en el patrón de citoquinas que producen y en las funciones efectoras que presentan, pudiendo diferenciarse entre Th0 (naïve), Th1, Th2, Th9, Th17, Th1/Th17, reguladoras (Treg) y foliculares (Tfh) (111,112). Los linfocitos Th1, a través de la producción de las citoquinas IFN-γ e IL-2, participan en la activación, proliferación y diferenciación de los linfocitos T citotóxicos (CTL) y en la inducción de citotoxicidad celular de las células infectadas por virus (113).

Por otro lado, los linfocitos Tfh interaccionan con los linfocitos B foliculares en los centros germinales, estimulándolas a proliferar, a realizar cambio de isotipo e hipermutación somática (114). Con esta interacción se seleccionan los linfocitos B específicos del virus y se diferenciarán en células plasmáticas productoras de anticuerpos y linfocitos B memoria del centro germinal, que reaccionarán rápidamente en un nuevo contacto con el antígeno (115).

En el caso de la infección por SARS-CoV-2 se observa una clara linfopenia en pacientes más graves. Esto favorecería la supervivencia y propagación del virus (115). El mal control de la enfermedad por parte de la inmunidad adaptativa conduciría a una mayor activación de la inmunidad innata que empeoraría la tormenta de citoquinas y la patogenia de la COVID-19 (116).

HIPÓTESIS Y OBJETIVOS

1. HIPÓTESIS

El diferente curso clínico que siguen los pacientes tras infección por SARS-CoV-2, necesariamente debe de estar condicionado por la potencia, eficacia y adecuación de la respuesta inmunitaria. En este sentido, el sistema inmunitario parece jugar un carácter dual. Por una parte, intentando controlar la infección de manera adecuada y eficiente antes de que aparezcan o se desarrollen mecanismos de evasión. Pero por otra, es conocido que la mayoría de las complicaciones en los pacientes surgen también de respuestas inmunitarias excesivas. Como frecuentemente ocurre en otras patologías particularmente en el cáncer, el sistema inmunitario actúa en la prevención y progresión de la COVID-19.

2. OBJETIVOS

1. Investigar factores inmunitarios que expliquen la diferente evolución clínica en la infección por SARS-CoV-2. Para ello, proponemos analizar la respuesta celular mediante la caracterización inmunofenotípica de las principales poblaciones linfocitarias, su estado de activación y diferenciación funcional.

2. Investigar factores inmunogenéticos asociados al control de respuestas inmunitarias innatas y adaptativas: MICA y HLA. Pretendemos establecer marcadores inmunitarios que puedan ser predictores de pronóstico o de fallecimiento, así como de susceptibilidad o protección a la infección.

3. Investigar la respuesta celular y humoral a la vacunación frente al SARS-CoV-2 mediante la vacuna mRNA-1273. Se estudiará la cinética en la producción de anticuerpos y factores inmunogenéticos que puedan condicionar los niveles de

respuesta. Pretendemos así mismo determinar el papel de los alelos HLA y de los haplotipos relacionados con los distintos grados de respuesta.

MATERIALES, MÉTODOS Y RESULTADOS

Los materiales, métodos y resultados se encuentran recogidos en los siguientes artículos científicos:

- "Negative Clinical Evolution in COVID-19 Patients Is Frequently Accompanied With an Increased Proportion of Undifferentiated Th Cells and a Strong Underrepresentation of the Th1 Subset"
- "Study of HLA-A, -B, -C, -DRB1 and -DQB1 polymorphisms in COVID-19 patients"
- "Major Histocompatibility Complex Class I Chain-Related α (MICA) STR Polymorphisms in COVID-19 Patients"
- "Study of humoral and cellular immunity in vaccinated with mRNA-1273"
- "HLA Class II Polymorphism and Humoral Immunity Induced by the SARS-CoV-2 mRNA-1273 Vaccine"

Negative clinical evolution in COVID-19 patients is frequently accompanied with an increased proportion of undifferentiated Th cells and a strong underrepresentation of the Th1 subset.

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Key Words. Severe coronavirus disease 2019 (COVID-19), severe acute respiratory syndrome coronavirus 2 (SARS- CoV-2), T cell response, Th lymphocytes, T-CD8 lymphocytes flow cytometric immunophenotyping.

ABSTRACT

The severity of SARS-CoV-2 infection has been related to uncontrolled inflammatory innate responses and impaired adaptive immune responses mostly due to exhausted T lymphocytes and lymphopenia. In this work we have characterized the nature of the lymphopenia and demonstrate a set of factors that hinder the effective control of virus infection and the activation and arming of effector cytotoxic T CD8 cells and showing signatures defining a high-risk population. We performed immune profiling of the T helper (Th) CD4+ and T CD8+ cell compartments in peripheral blood of 144 COVID-19 patients using multiparametric flow cytometry analysis. On the one hand, there was a consistent lymphopenia with an overrepresentation of non-functional T cells, with an increased percentage of naive Th cells (CD45RA+, CXCR3-, CCR4-, CCR6-, CCR10-) and persistently low frequency of markers associated with Th1, Th17, and Th1/Th17 memory-effector T cells compared to healthy donors. On the other hand, the most profound alteration affected the Th1 subset, which may explain the poor T cells responses and the persistent blood virus load. Finally, the decrease in Th1 cells may also explain the low frequency of CD4+ and CD8+ T cells that express the HLA-DR and CD38 activation markers observed in numerous patients who showed minimal or no lymphocyte activation response. We also identified the percentage of HLA-DR+CD4+ T cells, PD-1+CD+4/CD8+ T cells in blood, and the neutrophil/lymphocyte ratio as useful factors for predicting critical illness and fatal outcome in patients with confirmed COVID-19.

INTRODUCTION

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of the coronavirus disease 2019 (COVID-19) pandemic that emerged in Wuhan (China) in early December 2019 (1). On the 30th of January 2020, the world health organization declared the SARS-CoV-2 outbreak an international health emergency and 9 months later more than 41,000,000 infected have been reported worldwide, with more than 1,125,000 deaths (2).

SARS-CoV-2 belongs to the betacoronavirus (β-CoVs) genus, as do the SARS-CoV and MERS-CoV (3). It is a zoonotic virus whose possible reservoirs are bats and/or pangolins (4). Phylogenetic analysis showed that SARS-CoV-2 is closely related to a bat coronavirus but also has sequence identity to SARS-CoV and MERS-CoV (5). Like SARS-CoV, SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) as a receptor for entry into the cells, infecting type II pneumocytes of the lung epithelium (6, 7). ACE2 is also expressed in the upper epithelium of the esophagus, ileum and colon enterocytes, myocardial cells, cells of the proximal kidney tubule, bladder urothelium, and the oral mucosa (8).

The SARS-CoV-2 infection is characterized by cough, fever, dyspnea, myalgia, rhinorrhea, diarrhea, and conjunctivitis (9, 10). Most cases (80–90%) are mild or asymptomatic, while 10% can develop severe disease (9). The most severe cases suffer unilateral or bilateral pneumonia and acute respiratory distress syndrome (ARDS), shock and multi-organ failure which might result in death (9). The mortality rate of the disease is around 3% (11). There are several factors that influence the risk of intensive care unit (ICU) admission or death, including advanced age, previous pathologies, and overweight (12).

Regarding clinical and biochemical parameters, COVID-19 patients present an elevation of proinflammatory cytokines like interleukin-1 (IL-1), IL-6, tumor necrosis factor alpha (TNF- α), interferon gamma (IFN-γ), C-X-C motif chemokine ligand 10 (CXCL10), and monocyte chemoattractant protein-1 (MCP-1), which can lead in certain cases to a cytokine storm (11, 12). The cytokine storm is one of the main reasons for the development of ARDS and multi-organ failure (13). In addition to cytokines, there are several inflammation and coagulation parameters that are elevated in these patients, such as C-reactive protein (CRP), ferritin, lactate dehydrogenase (LDH) D-dimer, and fibrinogen (12). Furthermore, lymphopenia has been observed in 85% of hospitalized patients (14) resulting in a worse prognosis (15).

Lymphopenia as an effect produced by SARS-CoV-2 infection has been reported in numerous works (16). However, no studies have specifically investigated functional Th subtypes or which specific subpopulation is affected by the lymphopenia. This information is valuable because it will clarify the nature of the compromised immunology response, and could provide a rationale for immune restorative treatments. Likewise, an answer to the cause of lymphopenia has not been found, although possible reasons have been described (17).

Our work is based on the clinical data of 145 COVID-19 patients admitted to the University Hospital Virgen de las Nieves (Granada), Spain. We have compared the immunological profile in peripheral blood between three groups of patients: asymptomatic, hospitalized, and patients admitted to the ICU. We performed a flow cytometric analysis of the different subpopulations of T lymphocytes [CD4, CD8, Th1, Th2, Th17, Th22, regulatory T cells (Treg), and T follicular helper cells (TFH)] at the time of admission, as well as activation and exhaustion markers [HLA-DR, CD38, CD39, programmed death 1 (PD-1) and T cell immunoreceptor with Ig and ITIM domains

(TIGIT)], looking for which subpopulations are affected by the lymphopenia and their relationship with the different clinical, biochemical and hospital stay parameters, as well as the different effects that SARS-CoV-2 infection can generate on the immune response.

In this work, we show for the first time a profound effect of a SARS-CoV-2 infection on the Th1 component. The fact that these cells are important in the control of the response mediated by CD8+ T cells through the production of IL-2 and IFN-γ (18), makes us believe that our findings are relevant, since they can generally explain the poor T cell response and the prolonged viremia in COVID-19 patients.

METHODS

Samples. Patients (N=144) diagnosed with COVID-19 admitted to University Hospital Virgen de las Nieves, Granada, Spain, were prospectively included in our study between March 2020 and June 2020 in order to conduct an observational study. The patients are distributed as follows.

A first cohort, "non-ICU hospitalized patients" was composed by one hundred patients recruited within 24 h of hospital admission. Peripheral blood was collected at enrolment. In this group the median age was 74.5 years and 51.0% were females. This cohort was composed of elderly patients, with a high incidence of cardiovascular diseases, with high blood pressure being the most common comorbidity, affecting more than half of the patients. Fourteen percent of our patients had a history of cerebrovascular disease, and 12% had a previous myocardial infarction. Approximately a quarter of patients were diabetic and 15% suffered from chronic kidney disease. Most of the patients had no other pre-existing pulmonary condition. Mean follow-up time for hospitalized patients was 12.5 (9.8–15.3) days. During the hospital stay, twenty patients (19.6%) from this group died and four (3.9%) needed transfer to the intensive care unit (ICU). From seven patients of this group, a second sample was collected 70 days after hospital admission for the realization of a longitudinal study.

A second cohort, "ICU hospitalized patients" was composed by an independent group of 17 ICU COVID-19 patients, composed mostly of men (76.5%) with a median age of 69 years. This group showed a high incidence of cardiovascular diseases, being hypertension the most common comorbidity, affecting 53% of the ICU patients. Diabetes mellitus was the second most common comorbidity among ICU patients, affecting approximately one

third of them. The median length of admission in this group exceeded 2 months. Mortality in this group was 11.8%.

The third cohort “asymptomatic recovered donors” was composed by a group of 27 hospital staff members with no previous symptoms that tested positive for IgG against SARS-CoV-2. This group was composed of 81.5% (22) females and 18.5% (5) males, with a median age of 43 (34.0–58.0) years.

The control group comprised 42 healthy blood donors recruited among hospital staff tested negative for SARS-CoV-2, with a median age of 61 years (55–62), 90.5% (38) females and 9.5% (4) males.

Peripheral blood was collected from all subjects. We followed the patients until discharge or death, collecting data about clinical manifestations, laboratory data, and demographic data. Sequential Organ Failure Assessment Score (SOFA score), that integrates data from cardiovascular, respiratory, hepatic, coagulation, neurological, and renal systems (19), was calculated by trained physicians at admission. Supplementary Table 1 provides a summary of the demographic and clinical features of patients. All individuals were natives from the Granada area.

All patient samples were collected according to the local medical ethics regulations, after informed consent was obtained by the subjects, their legal representatives, or both, according to the Declaration of Helsinki. The study was approved by the local ethics committee (Cod. 0766-N-20)..

Statistical analysis. Categorical data were described as percentages, and non-categorical data were expressed as median and quartile intervals. The parametric Student’s t test was used to compare groups when the distribution was normal (as checked by the

Kolmogorov-Smirnov test) and the non-parametric Mann–Whitney U test when it was not. Spearman analysis was used to evaluate correlations between quantitative variables. Fisher's exact test was used to determine if there were associations between two categorical variables. The R function ggcorrplot was used to calculate and visualize correlations between variables, displaying the positive correlations in red and negative correlations in blue. Correlations with P-values > 0.05 were considered as insignificant and left blank. To evaluate the capacity to predict mortality using cytometry and biochemical parameters measured at hospital admission, we plotted the receiver operating characteristics (ROC) curve, and calculated the area under the receiver operating characteristic (AUC).

SPSS statistical software (Windows version 20, IBM, Armonk, NY, USA) was used for statistical analysis. To compute and visualize the correlation matrix R package ggcorrplot was used. The P-values are not corrected and it was considered a type 1 error (α) of 0.05 to reject the hypothesis testing.

Immune Characterization by Cytometry. Whole peripheral blood (PB) samples were stained for cell surface markers using a direct immunofluorescence technique. Eight-color combinations of monoclonal antibodies (Mab) were used to identify the different T cell subsets. CD3 cell subpopulations were determined by the selection of CD45+, CD3+ cells in the lymphocyte gate. The Th1, Th2, Th1/Th17, Th17, Th22, and TFH subpopulations were detected in the CD4+ plot, based on the expression levels of CXC chemokine receptor 3 (CXCR3), CXC chemokine receptor 5 (CXCR5), CD194, CCR4, CD196, and chemokine receptor 6 (CCR6) (Figure 1A). A human regulatory T cell cocktail was used to identify Tregs, defined as CD127low-CD25bright-CD4+. Naïve CD4+ T cells were detected by bright expression of CD45RA and negative expression for all other chemokine receptors (Figure 1A). The amount of Tregs was expressed as a percentage of

total CD4+ cells. All Mabs were purchased from BD Biosciences, San Diego, CA. CD4+ T cell subsets were defined by chemokine receptors expression according to Supplementary Table 3.

The Mab combinations for the detection of the different Th subsets were based on the panels used by EuroFlow-IMM TCD4 (20). T cell activation-associated markers (HLA-DR, and CD38) and T cell exhaustion markers (TIGIT and PD-1) were also examined (Figure 1B). The specificity and fluorochromes of each reagent used are listed in Supplementary Table 2. Stained cell suspensions were analyzed on a FACSCanto II flow cytometer (BD Biosciences, San José, CA, USA). An average of 300,000 events per tube corresponding to the whole PB cellularity was acquired. The InfinicytTM22.0 software was employed for multiparametric analysis. For instrument set up, BD one flow set up standard operating procedures were used. T cells were selected in a SSC versus CD3 bivariate dot plot histogram after exclusion of debris.

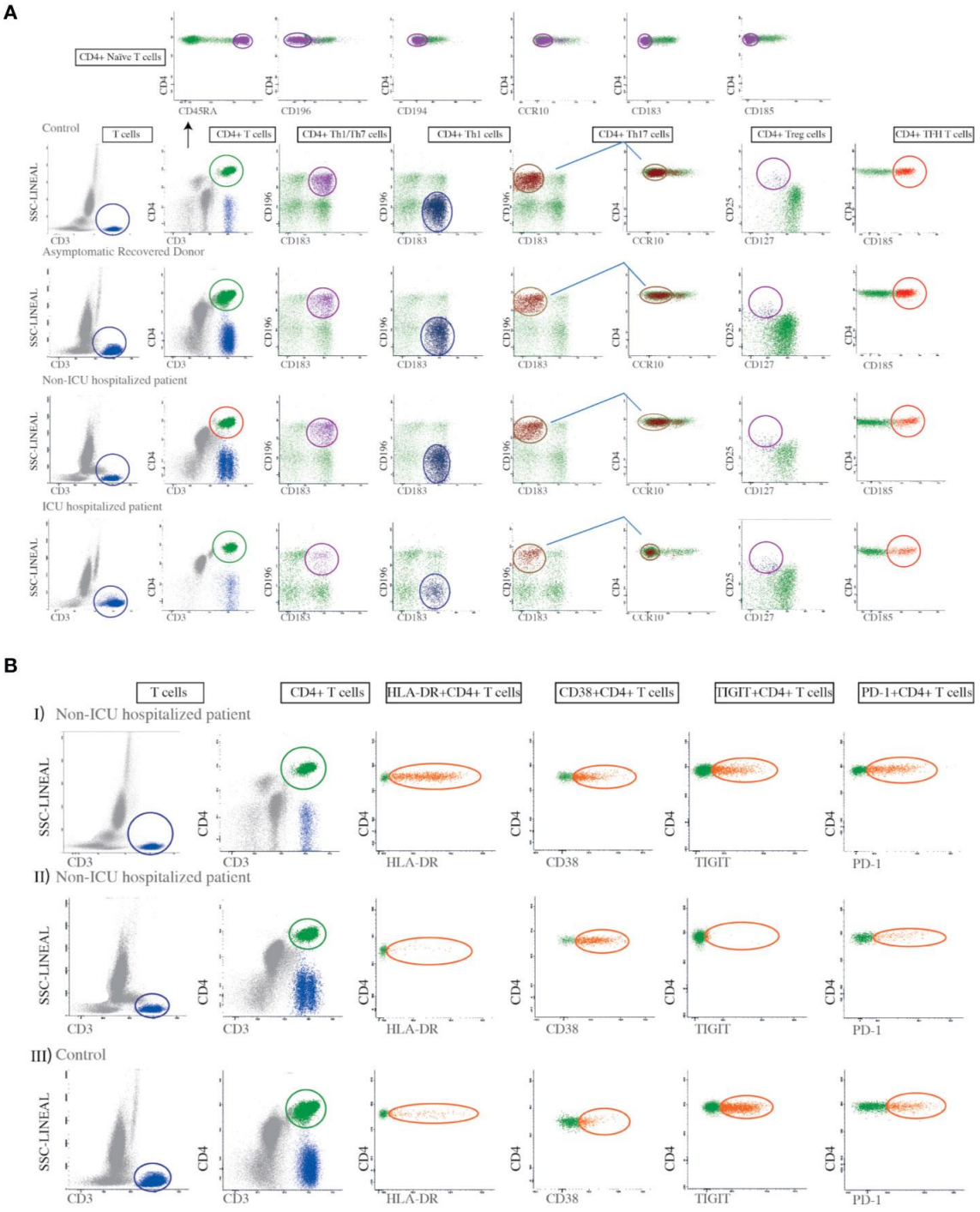


Figure 1. Gating strategies for the identification of Th subsets and the evaluation of T cell activation and exhaustion markers. (A) First a lymphocyte gate was defined based on high CD3 expression and low side-scatter (SSC) complexity. A minimum of 200,000 events/sample were collected in this gate. Second, the cells in the lymphocyte gate were divided into CD4+ and CD8- lymphocytes. Third, we analyzed the CD4+ lymphocytes for the expression of CD183, CD196, and CCR10 in order to identify Th1 (CD4+CD183+CD196-), Th1/Th17 (CD4+CD196+CD183+), and Th17 cells (CD4+CD183-CD196+CCR10-). A human regulatory T cell (Tregs) cocktail was used to identify regulatory T cell subsets defined as CD4+CD25brightCD127low. This gating strategy was applied to all controls, asymptomatic recovered donors, non-ICU hospitalized patients, and ICU hospitalized patients, as indicated in the figure. For the identification of naïve CD4+ T cells we used antibodies against CD45RA,

CD183, CD185, CD186, CD194, CD196, and CCR10, selecting those that were bright for CD45RA but negative for the rest of the mentioned markers, as shown in the top panel. The amount of each T cell subset was expressed as a percentage of total CD4+ cells. (B) Dot plots showing the expression of the HLA-DR, CD38, TIGIT, and PD-1 markers on CD4+ T cells from two patients with different degrees of HLA-DR expression, in addition to a control. Panel I shows a non-ICU hospitalized patient with high expression of HLA-DR on CD4+ T cells while II shows another non-ICU hospitalized patient with low expression of HLA-DR on CD4 + T cells. Panel III shows a control.

RESULTS

Clinical Inflammatory Syndrome in COVID-19 patients. Most COVID-19 patients presented at the time of hospital admission a clinical inflammatory syndrome, characterized by the elevation of several biochemical inflammatory markers.

In 93% of the non-ICU hospitalized patients, fibrinogen was raised. The levels of C reactive protein (CRP), lactate dehydrogenase (LDH), and D-dimer (a fibrin degradation product) were above normal range in 89, 85, and 75% of the non-ICU hospitalized patients, respectively. Two thirds of the non-ICU hospitalized patients showed an elevation of ferritin whereas troponin I was above normal range in 57%. Brain natriuretic peptide (BNP) was elevated in 53% of tested non-ICU hospitalized patients and procalcitonin was raised in 32%.

A total of 41 patients, were tested for IL-6 (30 non-ICU hospitalized patients and eleven ICU hospitalized patients). Twenty-three non-ICU hospitalized patients and all except one ICU hospitalized patients showed increased levels for IL-6 in serum. Additional analysis showed that IL-6 is positively correlated with Th17 ($P=0.014$), CRP ($P<0.001$), and fibrinogen ($P=0.008$).

All the ICU hospitalized patients had above normal levels of CRP, LDH, ferritin, troponin I, D-dimer, and fibrinogen, whereas procalcitonin was elevated in the 88% and BNP in 41%.

Changes in Leukocyte Populations in Peripheral Blood From COVID-19 Patients. Non-ICU hospitalized patients were observed to have an increased leukocyte count compared to controls. This was also observed in non-ICU hospitalized patients (Table 1). Though most patients had white blood cell counts (WBC) in the normal range, 23% of non-ICU hospitalized patients and 23.5% of ICU hospitalized patients showed clinical

leukocytosis. There were no significant intra-group differences associated with sex in leukocyte populations.

Increased neutrophil numbers were observed in non-ICU hospitalized patients and ICU hospitalized patients compared to healthy controls (Table 1). Furthermore, 25% of the non-ICU hospitalized patients and 17% of the ICU hospitalized patients had severe neutrophilia.

We also observed a significantly lower lymphocyte count in COVID-19 patients compared to healthy controls (Table 1). Besides, 49% of non-ICU hospitalized patients, and 65% of ICU hospitalized patients exhibited lymphopenia.

To assess the impact of acute SARS-CoV2 infection on T cell populations, we performed an in depth immunophenotypic analysis of the activation status and differentiation of functional T cell populations in peripheral blood of healthy donors, non-ICU hospitalized patients, ICU hospitalized patients, and asymptomatic recovered donors using flow cytometry. We could not detect significant intra-groups differences between sexes. A decrease in cell count was observed for CD4+ and CD8+ T cells in non-ICU hospitalized patients and in ICU hospitalized patients. The reduction in CD4+ and CD8+ T cells was equally pronounced. Indeed, there were no significant differences in the CD4:CD8 ratios between groups (Table 1).

Table 1 - Absolute count and percentages of the different cell populations in peripheral blood samples of healthy controls and COVID-19 patients.

	Controls (n=42)	Non-ICU Hospitalized patients (n=100)	ICU hospitalized patients (n=17)	Asymptomatic Recovered Donors (n=27)	P_1	P_2	P_3	P_4	P_5
WBC ($\times 10^3$/mL)	5970 (5200-5970)	6680 (4810-9960)	7250 (4010-10140)	6520 (5000-8610)	n.s	n.s	n.s	n.s	n.s
Lymphocyte pool ($\times 10^3$/mL)	2080 (1740-2430)	1120 (760-1580)	990 (680-1610)	2150 (1700-2510)	**$4.2x10^{-11}$**	**$1.28x10^{-04}$**	n.s	n.s	**$2.60x10^{-04}$**
T cells ($\times 10^3$/mL)	1216 (1006-1707)	859 (549-1248)	630 (500-876)	1482 (1075-1689)	**$1.22x10^{-05}$**	**$7.23x10^{-06}$**	n.s	n.s	**$3.91x10^{-06}$**
T-CD4+	773 (616-1075)	387 (258-573)	313 (262-467)	853 (627-1044)	**$2.28x10^{-11}$**	**$3.22x10^{-07}$**	n.s	n.s	**$1.05x10^{-06}$**
T-CD8+	330 (213-469)	172 (101-303)	167 (123-334)	337 (274-614)	**$2.27x10^{-07}$**	**0.007**	n.s	n.s	**0.001**
T lymphocytes (%)									
CD3	20.34 (16.34-23.34)	8.60 (4.72-12.96)	8.90 (4.03-15.72)	22.62 (16.84-24.93)	**$8.61x10^{-14}$**	**$5.73x10^{-06}$**	n.s	n.s	**$9.73x10^{-06}$**
CD4	12.46 (10.88-16.00)	5.41 (3.00-8.76)	4.32 (2.68-9.78)	11.93 (10.26-15.72)	**$2.62x10^{-14}$**	**$1.74x10^{-06}$**	n.s	n.s	**$5.43x10^{-05}$**
CD8	5.44 (3.89-7.64)	2.24 (1.40-4.38)	3.01 (1.59-4.75)	6.51 (4.92-8.91)	**$1.58x10^{-08}$**	**$1.68x10^{-03}$**	n.s	n.s	**$6.53x10^{-04}$**
Ratio T cells									
CD4/CD8	2.40 (1.85-3.40)	2.41 (1.50-3.53)	1.96 (1.04-3.03)	2.06 (1.48-2.82)	n.s	n.s	n.s	n.s	n.s
CD4 T lymphocytes (%)									
Th1	33.00 (27.49-38.10)	24.73 (17.32-31.50)	27.88 (20.72-36.19)	28.33 (24.82-34.62)	**$1.53x10^{-06}$***	n.s	n.s	n.s	n.s
Th17	10.52 (8.64-13.06)	9.91 (7.59-13.53)	11.64 (9.48-14.71)	9.62 (6.98-11.31)	n.s	n.s	n.s	**0.40***	**0.26***
Th1/Th17	7.93 (6.56-10.58)	5.62 (3.84-7.60)	6.93 (5.49-10.23)	7.96 (6.10-10.15)	**$7.84x10^{-07}$***	n.s	n.s	n.s	n.s
Treg	7.23 (5.99-8.73)	6.20 (4.94-7.90)	7.49 (6.26-9.47)	5.90 (7.16-8.39)	n.s	n.s	n.s	**0.24**	n.s
Th2	5.93 (4.19-8.60)	7.47(5.72-8.79)	8.51 (6.25-9.89)	7.06 (5.57-8.66)	n.s	**0.23**	n.s	n.s	n.s
Th22	0.70 (0.42-1.18)	1.17 (0.72-1.78)	0.54 (0.41-0.93)	1.59 (0.50-6.12)	**$8.18x10^{-05}$***	**0.001***	**0.005**	**$2.69x10^{-04}$**	**0.15**
TFH	22.99 (21.05-27.80)	18.52 (13.04-25.40)	18.52 (14.60-21.88)	24.53 (18.45-30.42)	**0.001**	**0.001***	n.s	n.s	**0.004**
PD-1+CD4+	32.54 (23.62-40.07)	26.45 (18.20-39.80)	35.76 (27.27-42.24)	25.40 (11.05-39.36)	**0.33**	n.s	n.s	n.s	n.s
TIGIT+CD4+	20.28 (14.32-26.10)	13.55 (8.57-18.10)	18.81 (14.22-24.75)	16.12 (10.69-23.55)	**$5.52x10^{-04}$***	n.s	n.s	**0.009**	n.s
HLA-DR+CD4+	11.73 (9.65-15.58)	14.38 (9.46-20.10)	14.75 (10.73-23.11)	9.46 (7.84-12.38)	n.s	n.s	**0.17**	n.s	**0.004**
CD39+CD4+	4.41 (1.56-6.48)	4.72 (1.93-8.02)	ND	ND	n.s	NA	NA	n.s	n.s
CD38+CD4+	5.78 (4.35-6.93)	4.66 (3.50-6.71)	6.32 (2.44-9.81)	4.97 (4.21-5.92)	**0.28**	n.s	n.s	n.s	n.s
CD4+ naive T cells	25.60 (19.40-33.70)	33.37 (20.91-43.59)	36.94 (32.15-50.21)	ND	**0.22***	**0.001***	NA	n.s	n.s
CD8 T lymphocytes (%)									
PD-1+CD8+	38.44 (27.95-46.19)	32.50 (19.24-42.80)	45.83 (29.91-55.99)	27.31 (16.55-34.61)	**0.043***	ns	**0.006**	**0.004**	**0.002**
TIGIT+CD8+	40.57 (26.36-55.72)	28.81 (14.54-39.64)	30.28 (21.58-46.90)	31.25 (20.69-39.91)	**0.001***	ns	**0.14***	ns	ns

Results are expressed as median (Q1-Q3). Mann–Whitney U test are displayed. P values indicated in bold are statistically significant. P1 value: statistic comparison between non-ICU Hospitalized patients and the control group. P2 value: statistic comparison between ICU patients and the control group. P3 value: statistic comparison between Asymtomatic Recovered Donors and the control group. P4 value: statistic comparison between ICU patients and non-ICU Hospitalized patients. P5 value: statistic comparison between ICU patients and Asymtomatic Recovered Donors. *Statistical analysis was evaluated by Student's t test. WBC: white blood cells, NA: not analyzed, n.s: not significant.

Interestingly, we found that the percentages of Th1, Th1/Th17, and TFH cells were significantly reduced in non-ICU hospitalized patients with respect to healthy donors (Table 1). The reduction of Th1 cells was especially pronounced, decreasing by almost 10% in non-ICU hospitalized patients. In contrast, the percentage of Th22 cells in non-ICU hospitalized patients was slightly elevated compared to healthy donors (Table 1). The decrease in Th17 and Tregs cells, although not statistically significant when comparing percentages, was significant when comparing cell counts. Conversely, naïve CD4+ T cells were greatly overrepresented in non-ICU hospitalized patients and ICU hospitalized patients (Table 1).

In ICU hospitalized patients, albeit the T lymphocyte pool was more depleted compared to the other group of patients, only the TFH population was significantly reduced, whereas the reduction in Th1, Th17, and Th1/Th17 percentages was less pronounced, not reaching significance. In contrast to the non-ICU hospitalized patients, the Th22 population was significantly reduced in ICU hospitalized patients compared to healthy controls (Table 1). However, absolute cell counts of Th1, Th17, Th1/Th17, TFH, and Treg cells were significantly reduced compared to healthy donors. Furthermore, naïve CD4+ T cells were also notably elevated in this group (Table 1).

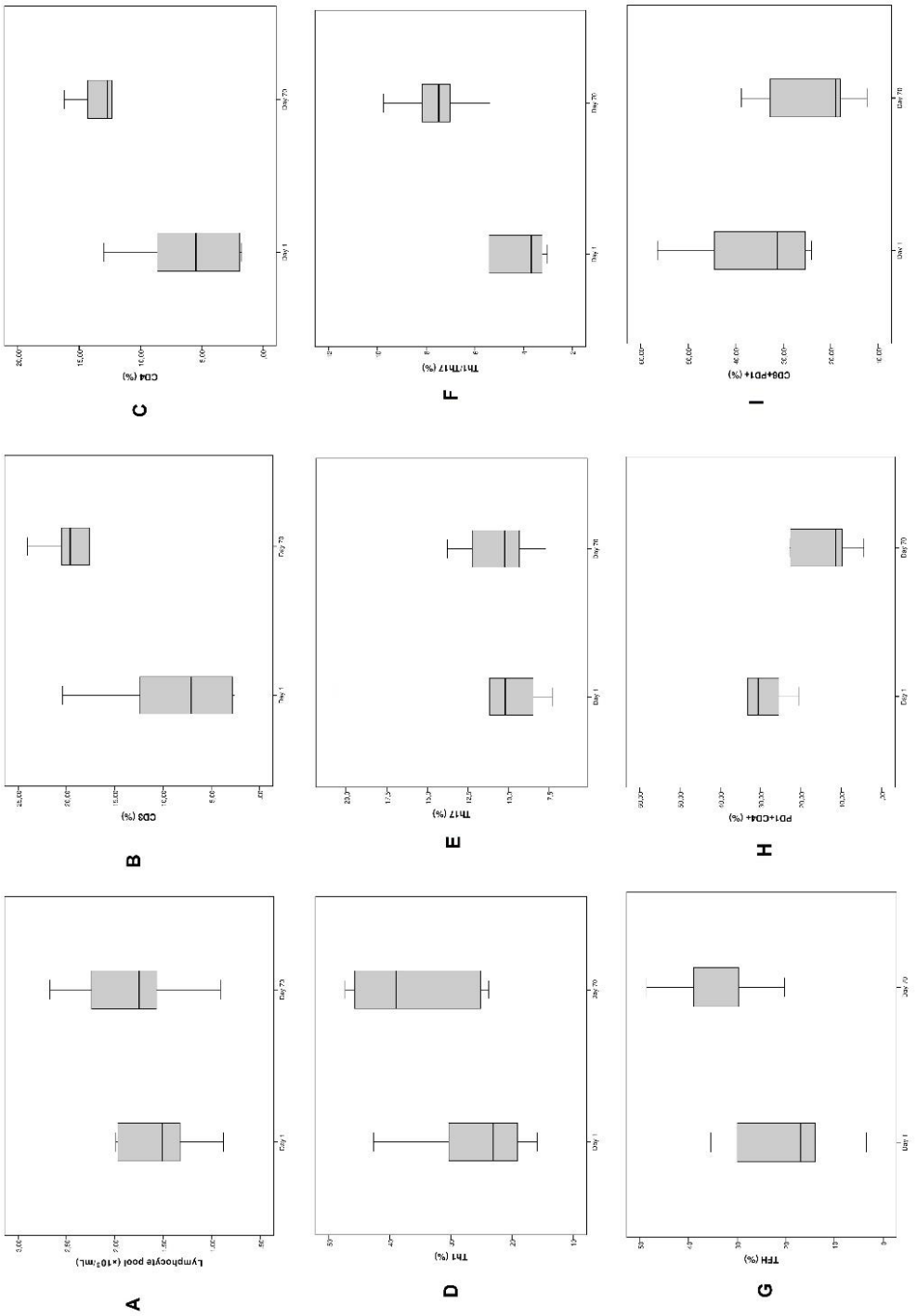
Asymptomatic recovered donors showed normal values in CD4+ T cells subpopulations, being only Th22 cells slightly elevated with respect to healthy donors.

Concerning T cell exhaustion markers, non-ICU hospitalized patients presented a significant decrease in PD-1+CD4+, and TIGIT+CD4+, PD-1+CD8+, and TIGIT+CD8+ populations. Noticeably, asymptomatic recovered donors still showed decreased levels of PD-1+CD8+ and TIGIT+CD8+ cell populations (Table 1).

Changes in lymphocyte subpopulations after recovery. To evaluate the long-term impact SARS-CoV2 infection on T cell populations, we tested seven COVID-19 patients 10 weeks after hospital admission using flow cytometry. Notably, when compared to day 1, we observed a significative reduction in PD-1+CD4+T cells and an increase of TFH. Although not statistically significant due to the low number of cases tested, we observed a noticeable recovery of T cells, CD4+ T cells, Th1, and Th1/Th17 cells (Figure 2).

We also compared the levels of lymphocyte subpopulations on day 70, with those of healthy donors. Recovered patients showed significantly lower levels of PD-1+CD4+ T cells, TIGIT+CD4+ T cells, and PD-1+CD8+ T cells, while on the other hand, the levels of Tfh cells were significantly higher (data not shown).

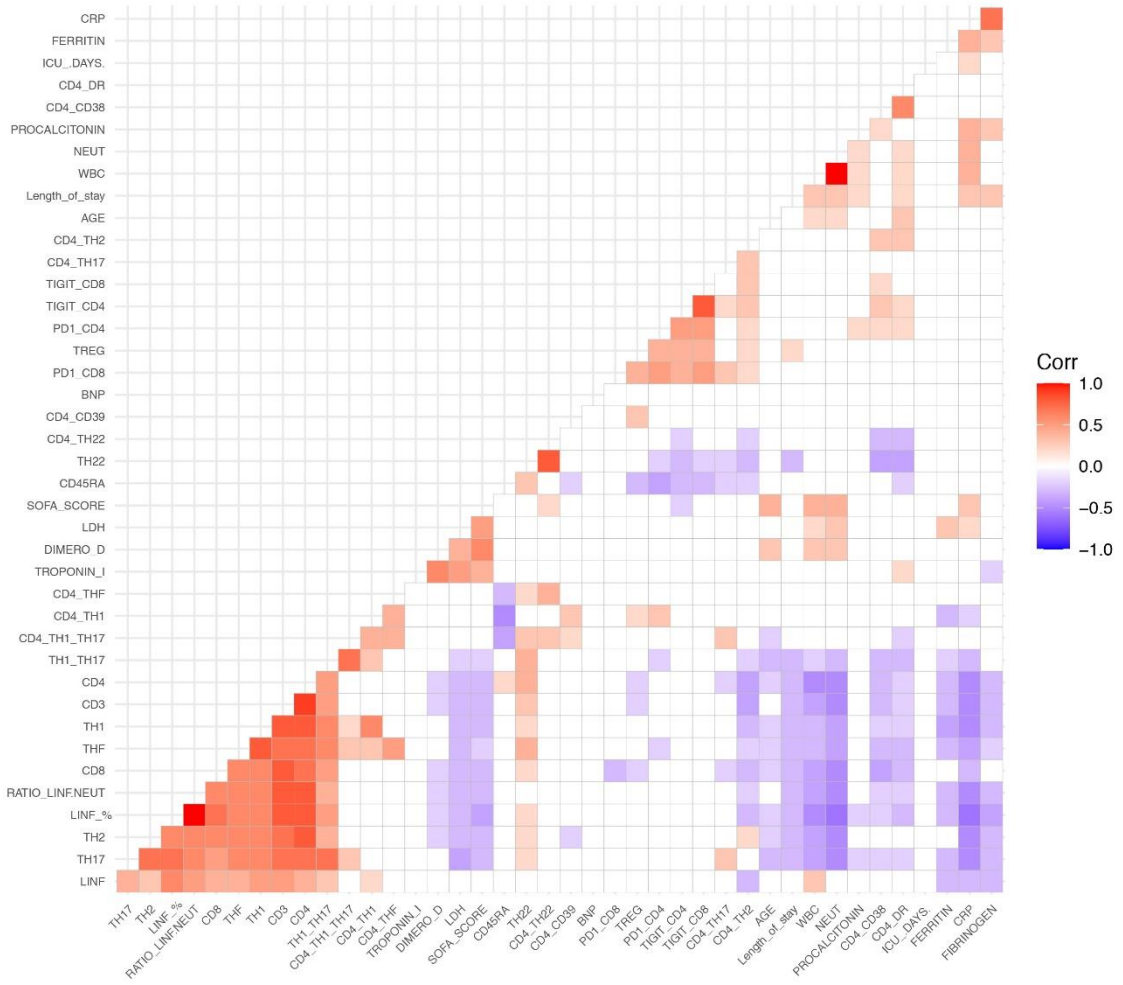
Figure 2. Longitudinal study of the fraction of Th populations in peripheral blood of non-intensive care unit (ICU) hospitalized patients. The line within the box represents the median, the top and the bottom of the box represents the 75th and 25th percentiles, respectively. (A–C) Boxplots for [left] lymphocyte pool (×10−3/ml), [middle] CD3 (%) and [right] CD4 (%). (D–F) Boxplots for [left] Th1 (%), [middle] Th17 (%), and [right] Th1/Th17 (%). (G–I) Boxplots for [left] TFH (%), [middle] CD4+PD-1+ T cells, and [right] CD8+PD-1+ T cells.



COVID-19 Infection Is Associated With Changes in Biochemical and Immunological Parameters. We next performed a correlation mapping to evaluate potential associations between clinical features, biochemical parameters, and leukocyte populations (Figure 3). We observed a negative correlation between biochemical inflammatory parameters (ferritin, fibrinogen, CRP, D-dimer, LDH) with the percentage of lymphocytes as well as with CD3, CD4, Th1, Th17, Th1/Th17, and THF. In contrast, the above inflammatory parameters showed a positive correlation with the neutrophils counts.

Interestingly, we found an inverse correlation between lymphocytes and neutrophils. Age exhibited a negative correlation with lymphocyte counts CD4, CD8, Th1, Th17, Th1/Th17, TFH; and a positive correlation with whole blood count and neutrophils.

Figure 3. Spearman correlation heatmap of all measured cell populations, biochemical parameters and clinical parameters. Red indicates a positive correlation and blue represent a negative correlation. Non-significant correlations are left blank.



The expression of the activation markers HLA-DR and CD38 correlated negatively with the percentage of CD3, CD4, CD8, Th17, Th1/Th17, TFH, and Th22 cells, while they correlated positively with CD4+TIGIT+ and CD4+PD-1+ cells. HLA-DR+CD4+ cells was positively correlated with length of hospitalization and with neutrophil counts.

The naïve T cell marker CD45RA had a strong negative correlation with Th1 cells and Tregs, as well as with exhaustion markers (CD4+TIGIT+, CD4+PD-1+, CD8+TIGIT+, CD8+PD-1+, and CD4+CD38+).

Length of stay was negatively correlated with CD3, CD4, CD8, Th17, Th1/Th17, and Th22. On the other hand, length of stay showed a positive correlation with neutrophils, Tregs, HLA-DR+CD4+, fibrinogen, CRP, and procalcitonin.

The SOFA score had a negative correlation with lymphocytes and T cells subpopulations, and a positive correlation with age, length of hospitalization, neutrophil counts, and biochemical inflammatory parameters (LDH, D-dimer, troponin I, and CRP).

In a further step, we evaluated the impact of neutrophilia and lymphopenia on the clinical outcome. We compared several biochemical and immunological parameters across patients with neutrophilia (n=25), and those with neutrophils in the normal range (n=75); besides across patients with lymphopenia (n=49), and those with lymphocytes in the normal range (n=51). Our results demonstrate that neutrophilic patients show an increase in the percentage of Tregs, CD38+CD4+ T cells, and HLA-DR+CD4+ T cells. Furthermore, patients with neutrophilia had increased levels of CRP, LDH, fibrinogen, D-dimer, procalcitonin, and troponin I, and reduced concentration of ferritin and BNP. The SOFA score was significantly elevated in neutrophilic patients (Table 2). In regard to the impact of lymphopenia, lymphopenic patients had increased Th2, CD38+CD4+ T cells, HLA-DR+CD4+ T cells, and Tregs (Table 3). In addition, patients with lymphopenia had increased mean levels of ferritin, CRP, fibrinogen, D dimer, procalcitonin, and troponin I (Table 3).

Table 2. Differences in diverse markers between patients with neutrophilia and no neutrophilia among non-intensive care unit (ICU) hospitalized patients.

	No Neutrophilia (n=75)		Neutrophilia (n=25)		
	Median	**(Q_1-Q_3)**	**Median**	**(Q_1-Q_3)**	**P**
WBC ($\times10^{-3}$/mL)	6010	(4540-7160)	14000	(11460-20090)	**$1.73x10^{-13}$***
Neutrophil pool ($\times10^{-3}$/mL)	4080	(3203-5660)	11490	(9440-17590)	**$8.46x10^{-14}$***
Lymphocyte pool ($\times10^{-3}$/mL)	1130	(760-1550)	1120	(850-1820)	n.s
T cells ($\times10^{-3}$/mL)	858	(546-1153)	889	(684-1341)	n.s
T-CD4+	385	(280-562)	398	(206-598)	n.s
T-CD8+	172	(101-319)	181	(102-282)	n.s
CD4 T lymphocytes (%)					
Th1	23.77	(17.17-31.63)	26.38	(18.49-30.34)	n.s
Th17	10	(7-13)	10	(9-14)	n.s
Th1/Th17	5.36	(3.77-7.42)	6.38	(4.8-8.55)	n.s
Th22	1.13	(0.77-1.739)	1.41	(0.64-2.12)	n.s
Th2	7.25	(5.71-8.77)	7.69	(6.54-8.92)	n.s
TFH	18.37	(13.09-25)	18.72	(11.7-25.53)	n.s
CD39+ CD4+	4.91	(1.81-7.5)	3.67	(2.47-13.01)	n.s
CD38+ CD4+	4.52	(3.28-6.2)	5.66	(4.06-8.11)	**0.16**
HLA-DR+CD4+	12.09	(9-18.31)	19.74	(12.08-24.03)	**0.004***
Tregs	5.67	(4.72-7.64)	7.46	(5.59-9.31)	**0.26**
CD45RA+	33.37	(18.11-43.77)	34.44	(22.62-40.95)	n.s
PD-1+ CD4+	25.44	(17.6-37.05)	31.82	(20.7-40.6)	n.s
TIGIT+CD4+	12.75	(7.37-17.15)	14.55	(12.5-20.8)	n.s
CD8 T lymphocytes (%)					
PD-1+ CD8+	32.38	(19.24-41.4)	34.43	(20.26-44.4)	n.s
TIGIT+ CD8+	25.60	(15.03-34.2)	35.30	(14-46.4)	n.s
Biochemical parameters					
Ferritin (ng/mL)	509.40	(276.3-908)	479.60	(306.6-689.8)	**$4.14x10^{-4}$**
CRP mg/dL	50.70	(13.6-116.5)	129.50	(88-180.2)	**0.001***
LDH (U/L)	322.00	(272-391)	381.50	(303.5-620.5)	n.s
Fibrinogen (mg/dL)	553	(465-709)	723	(589-773)	**0.14***
D dimer (mg/L)	0.87	(0.47-1.51)	2.44	(1.39-5.72)	**$1.66x10^{-4}$***
Procalcitonin (ng/mL)	0.13	(0.06-0.33)	0.75	(0.42-3.83)	**0.14***
Troponin I(pg/mL)	13.40	(5.7-30.8)	93.90	(20.5-376.9)	**0.003***
BNP (pg/mL)	181.10	(45.6-276.3)	65.50	(39-170.4)	n.s
SOFA score	1	(0-3)	3	(1.5-6.5)	**0.001***

P-value was considered significant only when it was smaller than 0.05. Q1. Percentile 25; Q3. Percentile 75. *Statistical analysis was evaluated by Mann–Whitney U test. WBC: white blood cells, n.s: not significant.

Table 3. Differences in diverse markers between patients with and without lymphopenia non-intensive care unit (ICU) hospitalized patients.

	No Lymphopenia (n=51)		Lymphopenia (n=49)		
	Median	**(Q_1-Q_3)**	**Median**	**(Q_1-Q_3)**	**P**
WBC ($\times 10^{-3}$/mL)	7160.00	(5540-11000)	6150.00	(4420-9160)	**0.23**
Neutrophil pool ($\times 10^{-3}$/mL)	4930.00	(3440-7910)	4730.00	(3340-7270)	n.s
Lymphocyte pool ($\times 10^{-3}$/mL)	1570.00	(1330-1970)	760.00	(580-900)	**6.92×10^{-18}***
T cells ($\times 10^{-3}$/mL)	1153	(1018-1478)	555	(372-725)	**2.14×10^{-13}***
T-CD4+	531	(418-692)	284	(177-358)	**1.46×10^{-10}***
T-CD8+	274.75	(169-380)	112.55	(74-173)	**7.96×10^{-08}***
CD4 T lymphocytes (%)					
Th1	26	(17.94-32.24)	22	(17.17-30.71)	n.s
Th17	10	(8-14)	10	(8-13)	
Th1/Th17	6	(4.09-8.55)	6	(3.8-6.81)	n.s
Th22	1	(0.77-1.76)	1	(0.68-1.78)	n.s
Th2	7	(5.17-8.21)	8	(6.02-9.68)	**0.40**
TFH	20	(13.98-26.81)	17	(11.7-24.89)	n.s
CD39+ CD4+	5.32	(1.98-7.47)	3.74	(1.77-10.23)	n.s
CD38+ CD4+	3.95	(3.24-5.47)	5.66	(4.06-7.48)	**0.003***
HLA-DR+ CD4+	11.36	(8.92-18.31)	14.83	(10.12-21.89)	**0.046**
Tregs	5.59	(4.72-6.71)	7.08	(5.22-8.88)	**0.037**
CD45RA+	36.76	(21.73-42.41)	28.59	(18.11-43.59)	n.s
PD-1+ CD4+	25.44	(15.3-36.8)	28.20	(20.6-40.6)	n.s
TIGIT+CD4+	12.80	(9.7-17.15)	13.85	(8.5-18.4)	n.s
CD8 T lymphocytes (%)					
PD-1+ CD8+	32.61	(21.8-42.9)	32.37	(17.4-41.5)	n.s
TIGIT+CD8+	30.80	(22.9-44.9)	24.00	(12.8-33.01)	n.s
Biochemical parameters					
Ferritin (ng/mL)	509.40	(219.6-767.9)	504.70	(288.4-1089.8)	**4.14×10^{-4}**
CPR mg/dL	40.6	(8-116.2)	97.1	(30.5-161.2)	**0.008***
LDH (U/L)	305.00	(266-381)	370.00	(294-429)	n.s
Fibrinogen (mg/dL)	558.00	(452-671)	649.00	(515-790)	**0.14***
D dimer (mg/L)	0.87	(0.47-1.755)	1.39	(0.66-2.46)	**1.66×10^{-4}***
Procalcitonin (ng/mL)	0.38	(0.11-0.745)	.27	(0.085-2.16)	**0.14***
Troponin I(pg/mL)	12.45	(5.55-25.8)	21.80	(7.7-117.3)	**0.003***
BNP (pg/mL)	96.900	(39-200)	174.200	(53.4-389.35)	n.s
SOFA score	1	(0-3)	1	(0.5-3)	**0.001***

P-value was considered significant only when it was smaller than 0.05.
Q1. Percentile 25; Q3. Percentile 75
*Statistical analysis was evaluated by Mann–Whitney U test
n.s: not significant

Differences in Biochemical Parameters and Lymphocyte Subpopulations Between Survivors and Non-Survivors. Among the 100 non-ICU hospitalized patients diagnosed with COVID-19, 21 died in the hospital (21%). As shown in Table 3, non-survivors showed at admission higher serum levels of troponin I, CRP, D-dimer, LDH, and BNP compared to survivors.

In regard to lymphocytes, the percentages of lymphocytes, T cells, CD4+ T cells, and CD8+ T cells were significantly higher in surviving patients, and the ratio of neutrophils:lymphocytes was considerably elevated in non-survivors (Table 4). Moreover, the percentage of HLA-DR+CD4+, PD-1+CD4+ and PD-1+CD8+ T cells was notably raised in the group of patients that died (Table 4).

With respect to comorbidities, heart failure is significantly more frequent in non survivors (Table 4). Although the differences are not statistically significant, hypertension, diabetes mellitus, chronic kidney disease, and cerebrovascular disease are also more frequent in non-survivors.

Table 4. Comorbidities and differences in several markers between survivors and deceased among non-intensive care unit (ICU) hospitalized patients.

	Deceased (n=21)	Survivors (n=79)	P
Age	87(78-90)	70(56-85))	**$1.65x10^{-4}$**
Male	11 (52.4%)	38((48.1%)	n.s
Female	10 (47.6%)	41 (51.9%)	n.s
WBC ($\times10^{-3}$/mL)	7840(6220-10760)	6430(4770-9450)	n.s
Neutrophil pool ($\times10^{-3}$/mL)	6480(4530-9440)	4620(3350-7290)	n.s
Lymphocyte pool ($\times10^{-3}$/mL)	760(510-1290)	1240(840-1680)	**0.007**
Neutrophils:Lymphocytes	7.22 (3.9-16.4)	4.08 (2-68-6.65)	**0.006**
T cells ($\times10^{-3}$/mL)	635(361-1086)	889(625-1248)	n.s
T-CD4+	312(204-467)	397(284-584)	n.s
T-CD8+	173(74-257)	172(104-315)	n.s
CD3 (%)	6.83 (2.35-10.33)	9.17 (5.54-13.97)	0.020
CD4 (%)	4.30 (1.68-7.18)	5.81 (3.46-9.17)	0.030
CD8 (%)	1.50 (0.74-2.92)	2.46 (1.38-4.42)	0.043
CD4 T lymphocytes (%)			
Th1	24.92(20-28.96)	24.58(17-32.7)	n.s
Th17	10.02(8.84-14.04)	9.85(7.48-13.41)	n.s
Th1/Th17	5.32(3.28-7)	5.79(3.97-7.77)	n.s
Th22	1.37(0.68-2.02)	1.13(0.76-1.77)	n.s
Th2	6.54(4.66-9.68)	7.47(5.75-8.64)	n.s
TFH	13.7(10.4-23.42)	20.09(13.68-25.53)	n.s
CD39+ CD4+	4.74(2.57-13.88)	4.72(1.81-7.47)	n.s
CD38+ CD4+	5.64(4.06-7.97)	4.54(3.48-6.2)	n.s
HLA-DR+ CD4+	19.74 (13.21-25.02)	12.30 (9.04-18.18)	**0.005**
Tregs	5.7(5.05-9.03)	6.21(4.9-7.89)	n.s
CD45RA+	28.6(15.41-38.25)	34.87(21.47-44.05)	n.s
PD-1+ CD4+	40.40 (25.42-45.05)	23.50 (16.20-36.30)	**0.004**
TIGIT+CD4+	12.4(5.9-21.5)	13.85(9.7-18)	n.s
CD8 T lymphocytes (%)			
PD-1+ CD8+	40.30 (25.05-51.97)	30.15 (18.75-41.03)	**0.0040***
TIGIT+CD8+	30.3(15.03-42.7)	28.7(14.38-38.8)	n.s
Biochemical parameters			
Ferritin (ng/mL)	677.2(311.2-1186.8)	494.1(239.6-871.9)	n.s
CRP mg/dL	108.80 (40.85-180.65)	57.60 (13.60-126.10))	**0.022***
LDH (U/L)	388 (325-544)	313 (267-392)	**0.006**
Fibrinogen (mg/dL)	649(540-809)	581.5(492-709)	
D dimer (mg/L)	1.50 (0.97-5.76)	0.87 (0.47-2.12))	**0.029**
Procalcitonin (ng/mL)	0.39(0.09-3.83)	0.3(0.06-0.63)	
Troponin I(pg/mL)	85(17.05-320.13)	12.9(5.1-30.9)	**0.001**
BNP (pg/mL)	845.00 (161.40-1575.20)	81.2(34.7-200)	
SOFA score	3(1.5-6.5)	81.20 (34.53-202.60)	**0.007**

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Comorbidities	Deceased (n=21)	Survivors (n=79)	P
Hypertension	43 (54.4%)	16 (76,2%)	n.s**
DM	17 (21.5%)	6 (28,6%)	n.s**
CKD	10 (12.7%)	6 (28,6%)	n.s**
CVD	9 (11.4%)	5 (23,8%)	n.s**
Smoker	12 (15.2%)	2 (9.5%)	n.s**
Overweight/Obesity	5 (6.3%)	1 (4.8%)	n.s**
MI	8 (10.1%)	4 (19.0%)	n.s**
HF	3 (3.8%)	7 (33.3%)	**0.001****
COPD	7 (8.9%)	2 (9.5%)	n.s**
Asthma	5 (6.3%)	1 (4.8%)	n.s**
PAD	5 (6.3%)	1 (4.8%)	n.s**

P-value was considered significant only when it was smaller than 0.05.

*Statistical analysis was evaluated by Mann–Whitney U test. ** Statistical analysis was evaluated by Fisher exact test. n.s: not significant

COPD, Chronic obstructive pulmonary disease; CKD, Chronic Kidney Disease; CVD, Cerebrovascular Disease; DM, Diabetes Mellitus; ET, endotracheal tube; HF; Heart Failure; ICU, Intensive Care Unit; NIMV, Noninvasive mechanical ventilation; MI, Myocardial infarction; PAD, Peripheral Artery Disease; Peptic Ulcer Disease.

Receiver Operating Characteristic Analysis. To examine the diagnostic usefulness of the measured parameters for the prediction of death, we compared sensitivities and specificities at optimal cut-off values determined by ROC analysis.

According to the AUC results obtained, an optimal cut-off value of <13.7% lymphocytes showed a 71% sensibility and a 68% specificity for the prediction of a fatal outcome. HLA-DR+CD4+ (cut-off value >16.2%), and PD-1+CD4+ (cut-off value >24.85%) could also predict death with a sensibility of 71 and 86%, respectively; and a specificity of 68 and 54%, respectively.

Death of non-ICU hospitalized patients could be predicted by measuring serum concentrations at hospital admission of troponin I, CRP, D-dimer, and LDH. Our results also indicate that an elevated SOFA score at hospital admission is associated with an unfavorable prognosis. The curves are shown in Figure 4. The AUC and optimal thresholds of each risk or protection factor can be found in Table 5.

Figure 4. Receiver operating characteristic (ROC) curve showing the accuracy of measuring lymphocytes, PD-1+CD4+ T cells, HLA-DR+CD4+ T cells, troponin I, CRP, D-dimer, lactate dehydrogenase (LDH), and the score Sequential Organ Failure Assessment Score (SOFA) at hospital admission for the prediction of a fatal outcome. The true positive rate (sensitivity) is plotted in function of the false positive rate (1-specificity). The area under the ROC curve (AUC) is a measure of the predictive efficiency of the analyzed parameters to distinguish between the different outcomes. (A–C) ROC curves performed for [left] lymphocytes (%), [middle] lymphocyte pool (×10−3/ml), and [right] Troponin I. (D–F) ROC curves performed for [left] CRP, [middle] D dimer, and [right] LDH. (G–I) ROC curves performed for [left] CD4+PD-1+ T cells, [middle] HLA-DR+CD4+ T cells, and [right] the SOFA score.

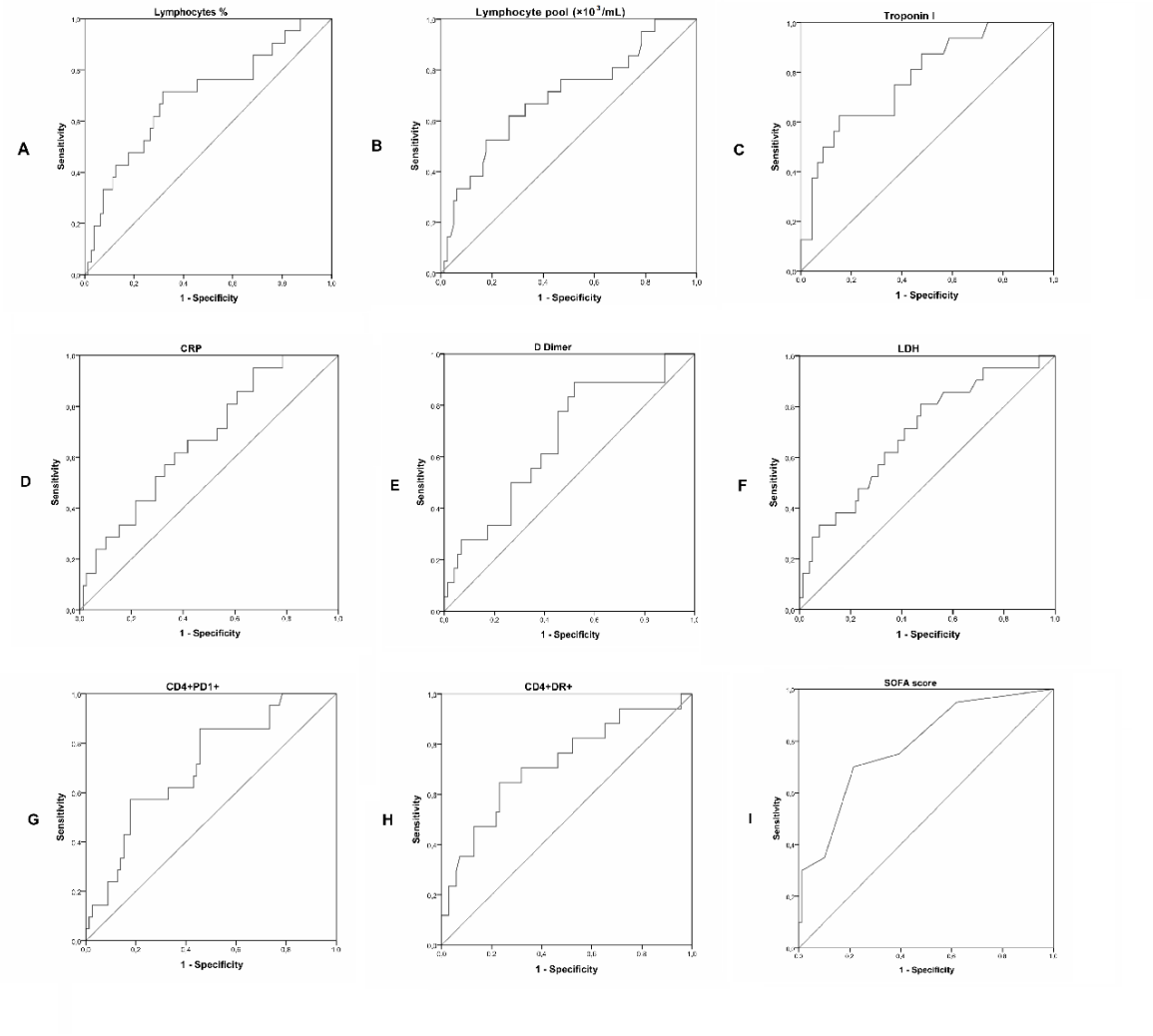

Table 5. Receiver operating characteristic (ROC) curves of biochemical and cytometry markers measured at hospital admission for prediction of death.

Predictive marker and cutoff value	AUC	Sensibility (%)	Specificity (%)
Lymphocytes (<13.7%)	0.69	71	68
Lymphocytes (<930×10^{-3}/mL)	0.69	67	67
Troponin I (>19.15 pg/mL)	0.78	75	67
CRP (>83.3 mg/L)	0.67	67	57
D-Dimer (>0.85 mg/L)	0.67	89	48
LDH (>342 U/L)	0.70	67	62
PD-1+CD4+ (>24.85%)	0.71	86	54
HLA-DR+CD4+ (>16.2%)	0.72	71	68
SOFA score (>3)	0.78	70	79

AUC=Area under the curve

DISCUSSION

SARS-CoV-2 infection produces an immune disorder promoted by lymphopenia that mainly affects T lymphocytes. So far, the lymphopenia has been the main finding that may explain the inadequate immune response to SARS-CoV-2. Recently, variations have been described in populations of memory, naïve and effector T lymphocytes, as well as different markers of activation and exhaustion, both on CD4 and CD8 T lymphocytes (21). In our study, we have performed a comprehensive analysis of the Th cell component and CD8 T cells. In addition, we have analyzed the activation status and the appearance of T cell exhaustion markers. Our data reveal a profound impairment of T cell immunity, which can be explained by four different reasons: 1—important lymphopenia. 2—The highest proportion of cells in a non-functional effector state (naïve T lymphocytes), suggesting a persistent hypoactivation of the immune system. 3—An important reduction in the proportion of Th1 cells. 4—An insufficient activation measured by the expression of HLA-DR and CD38, which in many cases was similar to that observed in the control group.

Lymphopenia affects T lymphocytes, showing significant differences between non-ICU hospitalized patients and ICU hospitalized patients, compared to the asymptomatic recovered donors and healthy controls, with the lowest percentage of T lymphocytes in the ICU group. Lymphopenia affected mainly the populations of CD4 T lymphocytes, with a reduction in absolute numbers of the subpopulations Th1, Th2, Th17, Th1/Th17, Th22, TFH cells, and Tregs, highlighting an approximate 10% reduction in the proportion of Th1 cells. This decrease in the percentages of effector T lymphocytes can be explained by the higher frequency of naïve cells, Th0 (CD45RA +, CXCR3-, CCR6-, CCR10-, CCR4-). This increase in naïve T cells is observed in both the hospitalized patients and

ICU patients, and has been referred to in other studies (22). We believe that it can be explained by a regenerative process in response to lymphopenia. Alternatively, it could correspond to a block in the complete stimulation carried out by dendritic cells. In this sense, a dysfunctional activation of dendritic cells has recently been observed, which would result in apoptosis and depletion of T lymphocytes (23).

Lymphopenia has been revealed in numerous studies on this disease, but the causes that produce it are not yet fully clarified and may be due to direct infection of lymphocytes or suppression of bone marrow by the antiviral response. The studies have been postulated from cytopathic effects by the virus (24), which have been questioned (25), to metabolic disorders (26). From an immunological point of view, the lymphopenia could depend on the possible dysfunctional activation of dendritic cells already mentioned (23) and the high concentration of cytokines such as TNF-α, IL-6, and IL-10, which act as negative regulators of the proliferation and survival of T lymphocytes (27). In this sense, we detected an inverse correlation between lymphocyte and neutrophil counts in COVID-19 patients. It is therefore very possible that this lymphopenia is caused by factors triggered during the exacerbation of the innate response. Hence the close association found between lymphopenia and the biochemical parameters analyzed (Table 3).

The low frequency of the cellular component Th1 is the main finding of this study and can negatively affect in the immune response against SARS-CoV-2 at various levels. Th1 cells are of vital importance in the elimination of intracellular microorganisms such as mycobacteria and viruses. Its reduction, as we see in COVID-19 patients, can have serious consequences for the control of the SARS-CoV-2 infection and its resolution. Th1 lymphocytes, through the production of the cytokines IL-2 and IFN-γ, participate in the activation, proliferation and differentiation of cytotoxic T lymphocytes (CTLs) and induction of cellular cytotoxicity of virus-infected cells (28). Furthermore, unlike SARS

patients, patients with COVID-19 also have elevated levels of Th2 cell-secreted cytokines (such as IL-4 and IL-10), which inhibit the inflammatory Th1 responses (13). The Th1 deficiency would lead to a decrease in the number of active CTLs and therefore a poor immune response to the viral infection. Other studies, some published during the evaluation of our work, show similar findings (29–31). However, these studies are based on fewer cases and focus on IFN-γ-producing cells, or naïve/effector-memory cells. Furthermore, these studies, although interesting, do not distinguish between Th1 and Th1/Th17 cells and do not include a comprehensive analysis of additional functional Th-subtypes. Interestingly, a reduced Th1-type specific immune response (i.e., a lower proportion of IFN-γ secreting cells) against different SARS-CoV-2 antigens was observed and was related with age/comorbidity (32). Finally, data by Roncati and colleagues, suggest that the "moonlighting protein" CD26/DPP4 could explain the Th1 immune lockdown observed in severe cases of COVID-19 (33).

We show that there is a low activation (HLA-DR, CD38) of CD8 cells, which is probably also related to the deficiency in the Th1 population. Finally, the decrease in Th1 lymphocytes can affect the development of antibodies (34). In this sense, it is known that human coronavirus infections occasionally fails to generate protective immunity due to a poor adaptive immune response (35–38). This may be due to an insufficient durability or magnitude of the T cell response as the production of neutralizing antibodies is dependent on the T cell response (39, 40). In agreement, Grifoni and colleagues recently showed that the antibody response to SARS-CoV-2 correlated positively with the magnitude of the Th1 response (41).

To further investigate the relationship between immune responses and COVID-19 disease severity, we used death as a marker of severity. In regard to lymphocytes, the percentage of lymphocytes, T cells, CD4+ T cells, and CD8+ T cells was significantly elevated in

survivors (Table 5). Moreover, the percentages of HLA-DR+CD4+, PD-1+CD4+, and PD-1+CD8+ T cells were notably raised in the group of patients that died (Table 5). PD-1 is a marker of exhausted T cells and is induced in response to continuous stimulation as occurs in chronic infections and cancer (42). In the case of SARS-CoV-2, the virus could be persistently stimulating T lymphocytes, inducing the exhausted state (27).

With regard to the expression of HLA-DR on CD4 and CD8 lymphocytes, it should be noted that in the large majority of patients there was either no or only a minimally significant increase in this marker which is in agreement with Mathew et al. (21). These authors show that the immune response is quite diverse and thus, while a subgroup of patients had T cell activation characteristic of acute viral infection another subgroup had lymphocyte activation comparable to uninfected subjects. This lack of activation contrasts with that observed in the course of other viral infections, which is very prolonged and stable over time (43–45). However, it should be noted that patients with a significant increase in HLA-DR exhibited a more aggressive disease course (Figure 1B). Interestingly, we found that COVID-19 patients with neutrophilia, lymphopenia and a more pronounced alteration in the biochemical parameters associated to inflammation had a higher percentage of CD4+ T cells with expression of activation markers HLA-DR and CD38. It is possible that the appearance of HLA-DR in patients with more intense lymphopenia could be due to an expression induced by inflammatory cytokines and is therefore not indicative of antigen-specific activation. It is therefore possible that the expression of HLA-DR is a consequence of dysregulated inflammation and the recruitment of inflammatory myeloid cells (21) and, hence its observation in the context of neutrophilia (Table 2). The increased inflammatory state induces the generation of neutrophils through the production of G-CSF, the regulation of the expression of chemoattractants and the activity of neutrophils (46). The positive relationship found

between neutrophils and acute phase proteins can be explained too by the inflammatory state (47). The production of acute phase proteins such as ferritin and CRP, in addition to affecting the balance between the pro and anticoagulative pathways (increasing the D-dimer) (48, 49), can induce apoptosis in lymphocytes (13, 50). The study of ROC curves showed that troponin I, CRP, D-dimer, and LDH concentrations at hospital admission can be used as predictors of death. Measuring lymphocytes, HLA-DR+CD4+ T cells, or PD-1+CD4+ T cells could also be useful for the prediction of a fatal outcome. Our results also indicate that an elevated SOFA score at hospital admission is associated with an unfavourable prognosis.

Our study has some limitations. Firstly, we have not included a group of PCR-positive patients (asymptomatic or with mild symptoms) that did not require hospitalization. This group could have clarified the apparent contradictory relevance of HLA-DR/CD38 expression on CD4+ T cells. Secondly, considering the obtained results, it would have been informative to measure the levels of IFN-γ in the serum of the patients in order to compare it to the levels of Th1 cells. Furthermore, it would have been interesting to compare the changes in Th1 cells with the levels of viremia.

In conclusion, the decrease of Th1 cells in COVID-19 infection, especially in older patients, is related to the clinical course of disease. Assuming that virus-induced IFN-γ production is essential for the anti-viral response, a profound decrease in the proportion of Th1 cells in combination with the highest levels of cells in a non-functional effector state, represent an unfavourable scenario for COVID-19 patients, especially for those with a strong lymphopenia. Another important finding of this study are the description of parameters associated with a fatal outcome to COVID-19, including a high neutrophil/lymphocyte ratio, expression of PD-1 on CD4+ and CD8+ lymphocytes, and the expression of HLA-DR on CD4+ T cells in patients with a marked lymphopenia.

These changes occur in the context of biochemical indicators associated with a clinical inflammatory syndrome.

Data Availability Statement. The raw data supporting the conclusiions of this article will be made available by the authors, without undue reservation.

Ethics Statement. The studies involving human participants were reviewed and approved by Portal de Ética de la Investigación Biomédica. Junta de Andalucía (Cod. 0766-N-20). The patients/participants provided their written informed consent to participate in this study.

Author Contributions. FR-C and PJ contributed to the design of the study. JG-B, PJ, FR-C, and AR-N performed the flow cytometry. JG-B and AR-N performed the analysis of the data obtained by cytometry. JG-B and AR-C built the clinical database. AR-N performed the statistical analysis of the data. ML-R and JG-B collected samples from non-ICU hospitalized patients, UCI hospitalized patients, asymptomatic recovered donors, and healthy donors. FR-C, AR-N, JG-B, PJ, and PA wrote the manuscript. AR-C and ML-R contributed to the clinical follow-up of patients. All authors contributed to the article and approved the submitted version.

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

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Study of *HLA-A, -B, -C, -DRB1* and *-DQB1* polymorphisms in COVID-19 patients

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ABSTRACT

Background: Human leukocyte antigen (HLA) plays an important role in immune responses to infections, especially in the development of acquired immunity. Given the high degree of polymorphisms that HLA molecules present, some will be more or less effective in controlling SARS-CoV-2 infection. We wanted to analyze whether certain polymorphisms may be involved in the protection or susceptibility to COVID-19.

Methods: We studied the polymorphisms in HLA class I (HLA-A, -B and -C) and II (HLA-DRB1 and HLA-DQB1) molecules in 450 patients who required hospitalization for COVID-19, creating one of the largest HLA-typed patient cohort to date.

Results: Our results show that there is no relationship between HLA polymorphisms or haplotypes and susceptibility or protection to COVID-19.

INTRODUCTION

COVID-19 (Coronavirus Disease 2019) is a respiratory tract infection, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), that can progress to pneumonia, acute respiratory distress syndrome (ARDS), cytokine storm, multiple organ failure, and death.[1] The severity of COVID-19 depends on increased age, and comorbidities such as obesity, arterial hypertension, diabetes, heart disease and respiratory disease.[2,3] There is a great need to study factors that play a relevant role in host defense, with the objective of identifying variables of susceptibility and severity. Of special interest are the human leukocyte antigens (HLA), which are highly polymorphic proteins that play a crucial role in the function of adaptive immunity. HLA presents pathogen-derived peptides on the surface of the infected cell, which are then recognized by specific T lymphocytes, inducing an immune response against the pathogen.[4, 5, 6, 7] The high level of polymorphism found in both HLA class I (HLA-A, -B and –C) and class II (HLA-DP, -DQ, -DR) molecules increases the variety of peptides that can be presented and recognized by the immune system. This is due to variations in their physical/chemical properties that increases or decreases their ability to bind and present certain peptides. Because of this, an allele can be a good or poor presenter of peptides derived from a pathogen. There is evidence of HLA alleles that can play a protective or susceptible role in infections caused by human immunodeficiency virus (HIV), hepatitis C virus (HCV), influenza virus and plasmodium.[8,9]

Each person has a different combination of HLA alleles (haplotypes) that determines their ability to respond to certain pathogens. These haplotypes have been selected throughout evolution due to the selective pressure carried out by pathogens.[10] It has been suggested that the differences observed in the number of cases and severity of COVID-19 between

different regions of the world, may in part be due to a skewed distribution of HLA alleles involved in protective immunity against SARS-CoV-2.[11,12]

Interestingly, there are studies that link HLA alleles to SARS-CoV-1, a coronavirus closely related to SARS-CoV-2. The HLA-B∗46:01 allele has been linked to disease severity in SARS-CoV-1 patients, while the HLA-B∗07:03 and HLA-DRB1∗03:01 alleles are related to susceptibility to SARS-CoV-1.[13,14]

Therefore, we hypothesize that HLA typing of patients infected by SARS-CoV-2 can help us find alleles that are involved in susceptibility, protection, and poor prognosis to COVID-19. To this end, we have performed a high-resolution HLA typing of 450 confirmed SARS-CoV-2 patients who required hospitalization, comparing their allele and haplotype frequencies with a group of 959 representative controls. We believe that such an analysis can generate data that could greatly aid the development of personalized treatments, diagnosis and vaccination.[15]

MATERIALS AND METHODS

Patients

The current study was performed on 450 hospitalized COVID-19 patients at the Hospital Universitario Virgen de las Nieves, Granada, Spain. The samples were collected from April 2020 to January 2021. The inclusion criteria were the need for hospitalization due to pneumonia or respiratory distress due to COVID-19, diagnosed by a positive SARS-CoV-2 PCR or the presence of SARS-CoV-2-specific IgG antibodies in blood as described below. The characteristics and comorbidities of the patient group can be seen in Table 1.

Table 1. Description of the hospitalized COVID-19 patients and comorbidities.

Features	Mean(range) or n(%)
Age	62 (25-98)
Female	220 (45.5%)
Male	263 (54.5%)
Diagnosis by PCR	363 (75.2%)
Diagnosis by antibodies	120 (24.8%)
UCI	126 (26.1%)
No UCI	324 (73.9%)
Mechanic Ventilation	101 (20.9%)
No Mechanic Ventilation	349 (79.1%)
Deceased	70 (14.5%)
Survivors	380 (85.5%)
Comorbidities	n(%)
Hypertension	196 (40.6%)
DM	101 (20.9%)
CKD	33 (6.8%)
CVD	22 (4.6%)
Overweight/Obesity	72 (14.9%)
MI	26 (5.4%)
HF	25 (5.2%)
COPD	32 (6.6%)
Asthma	26 (6%)
PAD	11 (2.3%)

ICU: Intensive Care Unit; DM: Diabetes Mellitus; CKD: Chronic Kidney Disease; CVD: Cerebrovascular Disease MI: Myocardial infarction; HF: Heart Failure; COPD: Chronic obstructive pulmonary disease; PAD: Peripheral Artery Disease.

The patients had pneumonia or respiratory distress and were classified according to severity of the disease in need to enter intensive care unit (ICU)/No ICU; need for Mechanical Ventilation/No Mechanical Ventilation; Deceased/Survivors (Table 1).

The control group (n = 959) is made up of healthy blood donors, representative of the Granada area, who were not infected at the time of donation. The average age of the group is 45 ± 5 years and 51% of the members are women.

The study was reviewed and approved by the Portal de Ética de la Investigación Biomédica. Junta de Andalucía (Cod. 0766-N-20). The patients/participants provided their written informed consent to participate in this study.

PCR and serological diagnosis of SARS-CoV-2 infection

For PCR diagnosis we use the cobas® SARS-CoV-2 assay on a cobas® 6800 system (Roche Molecular Systems, Pleasanton, California, USA). This is a single-well, double-target assay that enables both the specific detection of SARS-CoV-2 and the detection of pan-Sarbecovirus of the Sarbecovirus subgenus family, which includes SARS-CoV-2. The test detects the genetic signature (RNA) of the SARS-CoV-2 virus in nasal, nasopharyngeal and oropharyngeal swab samples from patients who meet COVID-19 clinical and/or epidemiological criteria for testing.

The ARCHITECT i System platform (Abbott Laboratories, Chicago, Illinois, USA), was used with the SARS-CoV-2 IgG reagent (Abbott Laboratories, REF. 6R86-22), which is a chemiluminescent microparticle immunoassay (CMIA), used for the qualitative detection of IgG antibodies against the SARS-CoV-2 virus. The results were considered positive when a value ≥ 1.4 URL was obtained and negative when it was <1.4 URL.

DNA extraction and determination of HLA class I and II genotypes

Venous blood was obtained from each patient and DNA was extracted using the QIAMP DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. High-resolution genotyping of HLA class I (A, B and C) and II (DRB1, DQB1 and DPB1) loci was performed using the LABType sequence-specific oligonucleotide typing test (One Lambda, Canoga Park, California, USA). Target DNA was amplified by PCR using sequence-specific primers, followed by hybridization with allele-specific oligodeoxynucleotides coupled with fluorescent phycoerythrin-labelled microspheres. Fluorescence intensity was determined using a LABScan 100 system (Luminex xMAP, Austin, Texas, USA). HLA alleles were assigned using the HLA-Fusion software (One Lambda).

Statistical analysis

Statistical tests for alleles, genotypes and haplotypes were performed using the R (R software; R Foundation for Statistical Computing) package BIGDAWG.16 Hardy–Weinberg equilibrium (HWE) tests were calculated using PyPop software ver. 0.7.0 (http://www.pypop.org). Frequencies of individual HLA alleles in patients and controls were compared using the $\chi 2$-test. Variants with expected counts less than five were combined into a common class (binned) prior to computing the $\chi 2$-test.

The R package BIGDAWG was also used for amino acid analysis. The software uses the collection of alleles in the input dataset, to retrieve a list of aligned amino acid sequences from the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/align.html) and to run case–control association tests on individual amino acid positions within exon 2 and exon 3 (HLA class I) or exon 2 (HLA class II).

Significance levels were corrected by Bonferroni correction for multiplicity of testing by the number of comparisons. A corrected P value of <0.05 was considered statistically significant for all statistical tests.

RESULTS

HLA-allele and haplotype analysis

Genotype frequencies of the HLA-A, -B, –C, -DRB1 and -DQB1 loci did not deviate from Hardy-Weinberg expectations (Supplementary Table 1).

The comparison of the HLA allele frequencies between the control group and hospitalized COVID-19 patients identified several alleles with significantly different frequencies. However, the significances were lost after Bonferroni correction (Supplementary Table 2).

The HLA-A∗25:01 and DRB1∗11:01 alleles had a significantly higher frequency in the control group without correction (Table 2). These alleles could represent possible protective alleles to COVID-19.

Table 2. Alleles with the P values closest to significance.

Alleles	Control group frequencies	Hospitalized COVID-19 frequencies	P	Pc
HLA-A*25:01	**0.0175**	0.0044	0.005	n.s
HLA-DRB1*11:01	**0.0695**	0.0464	0.017	n.s
HLA-A*66:01	0.0058	**0.0133**	0.042	n.s
HLA-B*40:02	0.0096	**0.0199**	0.024	n.s
HLA-B*55:01	0.0074	**0.0166**	0.026	n.s
HLA-DRB1*14:04	0.0032	**0.0121**	0.005	n.s
HLA-DQB1*05:03	0.0287	**0.0453**	0.024	n.s

The highest frequency for each allele is marked in bold. Pc: P corrected by Bonferroni. n.s: not significant.

The alleles HLA-A∗66:01, B∗40:02, B∗55:01, DRB1∗14:04 and DQB1∗05:03 also presented significant values without correction, being overrepresented in hospitalized COVID-19 patients (Table 2). These alleles could be considered as susceptibility alleles to COVID-19.

The haplotype study did not show significant results either, but the extended haplotype HLA-A∗29:02, B∗44:03, C∗16:01, DRB1∗07:01 and DQB1∗02:02 is of interest, which without correction had a p-value of 0.013 (Table 3).

Table 3. Frequencies of most represented haplotypes in COVID-19 hospitalized patients and controls.

A ~ B ~ C ~ DRB1 ~ DQB1	Controls	Patients	OR	P	Pc
01:01~08:01~07:01~03:01~02:01	0.0180	0.0132	0.73	0.3510	
02:01~07:02~07:02~01:03~05:01	0.0042	0.0099	2.35	0.0707	
02:01~07:02~07:02~15:01~06:02	0.0149	0.0132	0.89	0.7365	
02:01~18:01~05:01~03:01~02:01	0.0111	0.0077	0.69	0.3960	
03:01~07:02~07:02~15:01~06:02	0.0106	0.0132	1.25	0.5414	
11:01~27:05~01:02~01:01~05:01	0.0064	0.0066	1.04	0.9377	
11:01~35:01~04:01~01:01~05:01	0.0069	0.0044	0.64	0.4296	
23:01~44:03~04:01~07:01~02:02	0.0069	0.0077	1.12	0.8086	
29:02~44:03~16:01~07:01~02:02	0.0281	0.0464	1.68	**0.0129**	n.s
30:02~18:01~05:01~03:01~02:01	0.0191	0.0254	1.34	0.2805	
33:01~14:02~08:02~01:02~05:01	0.0111	0.0077	0.69	0.3960	

P values written in bold are significant. Variants with expected counts less than five were not computed in the $\chi 2$-test. OR: odds ratio. Pc: P corrected by Bonferroni. n.s: not significant.

To address the relationship between HLA and disease severity, we compared the allelic and haplotypic frequencies between the following groups: patients admitted to ICU versus patients that no admitted to ICU; patients that needed mechanical ventilation versus patients that did not require mechanical ventilation; deceased patients versus surviving patients; and between the aforementioned groups and controls. These comparisons did not result in any significant differences after Bonferroni correction (data not shown).

HLA supertype and amino acid analysis

Most of the polymorphism in HLA molecules is located in the peptide-binding region. Despite being extremely polymorphic, HLA-A and HLA-B alleles can be clustered into

supertypes, representing families of molecules that share an overlapping peptide binding specificity.[17]

To investigate the clinical relevance of peptide binding specificity, we examined the effects of HLA-A and HLA-B supertypes on COVID-19 risk (Table 4). The comparison of the HLA-A and HLA-B allele supertype frequencies between the control group and COVID-19 patients did not show any significant differences.

Table 4. HLA supertypes in COVID-19 patients and controls.

	Controls	Patients	OR	P
HLA A-supertypes				
A01	22.65	23.03	1.03	0.7872
A01 A03	2.07	1.36	0.66	0.2068
A01 A24	8.98	8.86	0.99	0.9326
A02	27.01	27.52	1.03	0.7362
A24	13.53	13.22	0.98	0.8428
A03	25.43	25.48	1.01	0.9392
Unclassified	0.12	0.00	0	0.3591
HLA B-supertypes				
B27	14.47	15.26	1.06	0.6056
B44	34.91	34.06	0.96	0.5992
B58	4.92	4.50	0.91	0.6086
B62	5.24	4.36	0.82	0.3071
B07	28.80	31.34	1.12	0.1836
B08	5.05	5.04	0.99	0.9696
Unclassified	5.65	4.91	0.86	0.4003

OR: odds ratio, P: p-value

In a further step, we performed an amino acid analysis of the HLA alleles identified in patients and controls. We examined individual amino acid positions within exon 2 and exon 3 (class I) or exon 2 (class II). These exons include the peptide binding groove and are the most polymorphic. After Bonferroni correction no specific amino acids in the HLA loci A, B, C, DRB1 or DQB1 were found to provide any significant contribution to COVID-19 risk or protection (Supplementary Table 3).

The amino acid analysis was also performed between the different severity groups: patients admitted to ICU versus patients that no admitted to ICU; patients that needed mechanical ventilation versus patients that did not require mechanical ventilation; deceased patients versus surviving patients; and between the aforementioned groups and controls, not finding significant values after Bonferroni correction (data not shown).

DISCUSSION

Our results suggests that there is no significant correlation between particular HLA alleles/haplotypes and susceptibility or protection against COVID-19, which is in agreement with a previous study.[18] However, numerous studies have reported alleles of protection or susceptibility to COVID-19. A study conducted with 82 Chinese patients found that the HLA-C∗07:29 and HLA-B∗15:27 alleles were more frequently detected in the COVID-19 group than in the control population.[19] Novelli et al., found that the HLA-B∗27:07, DQB1∗06:02 and -DRB1∗15:01 alleles were significantly increased in a group of 99 COVID-19 Italian patients compared to the control group.[20] In addition, the alleles HLA-B∗44 and C∗01 were positively and individually associated with COVID-19 in the Italian population.[21] The HLA-A∗02:01 allele has a possible positive association with the risk of COVID-19.[22] Another study, with 190 Chinese patients, found a positive correlation between the HLA-B22 serotype and COVID-19 susceptibility.[23] These studies were carried out with a smaller number of patients compared to our study, which could be one of the reasons as to why we could not detect the above associations. Furthermore, Correale et al.[21] and Tomita et al.,[22] did not perform HLA typing, but instead used the allelic frequencies in certain defined geographical regions and the incidence of COVID-

19 in these regions. Importantly, our work contains one of the largest HLA-typed COVID-19 patient cohort to date.

Studies of the relationship between HLA alleles and the development of COVID-19 have yielded both similar and contrasting results. The alleles HLA-A∗11:01, C∗04:01 and DQA1∗01:02 have been associated with a worse evolution of the disease.[24] Furthermore, the HLA-A∗11, C∗01, DQB1∗04[25,26], and DQB1∗08[27] alleles have all been associated with a higher mortality among COVID-19 patients. Interestingly, the HLA-C∗05 allele has been associated with risk of death,[28] while the work done by Poulton et al., speaks of a protective role of this allele.[29] It is interesting to note the discrepancies between different studies with regard to protection, risk or development of COVID-19, the ethnicity of the patients/control individuals may play a role.

In silico studies have identified alleles that are better or worse presenters of conserved SARS-CoV-2 peptides. The HLA-A∗02:02, B∗15:03 and C∗12:03 alleles are considered the best presenters of conserved peptides of SARS-CoV-2 while the HLA-A∗25:01, B∗46:01 and C∗01:02 alleles are poor presenters.[30] These data were confirmed by La Porta et al., who in addition identified HLA-A∗11:01 as a good presenter.[31] These results were corroborated by the study carried out by Barquera et al., which also showed that the HLA class II alleles DRB1∗01:01, DRB1∗10:01, DRB1∗11:02 and DRB1∗13:01 present more SARS-CoV-2 peptides whereas the HLA-DRB1∗03:02, DRB1∗03:03 and DQA1∗01:02/DQB1∗06 alleles were identified as the worst presenters of SARS-CoV-2-derived peptides.[32]

It is logical to think that patients requiring hospitalization have HLA alleles that do not bind strongly immunogenic SARS-CoV-2 peptides, resulting in an ineffective control of the disease by the immune system. This has been described by Iturrieta-Zuazo et al., who

determined that patients with mild disease have HLA alleles with a greater theoretical binding capacity to SARS-CoV-2 peptides.[33]

In contrast, our data showed that the poor presenting HLA-A∗25:01 allele was underrepresented in hospitalized COVID-19 patients, compared to the control group (Table 2). We only found three patients with the HLA-A∗25:01 allele in our cohort of seriously ill patients who required hospitalization. These results are in agreement with the study by Wang et al., who reported that the HLA-B∗46:01 allele, the worst presenter of peptides derived from SARS-CoV-2, is increased in patients with milder disease compared to those with serious illness.[34] However, we were not able to analyze the HLA-B∗46:01 allele since it was absent in both the control and COVID-19 hospitalized group.

In summary, contrary to our expectations, we observed that critically ill patients had a lower frequency of HLA alleles with poor virus presenting capacity (HLA-A∗25:01). These results, together with those of Wang et al.,[34] lead us to think of a possible hypothesis, in which the alleles with the lowest ability to present peptides derived from SARS-CoV-2 may be related with a protective role.

The clinical manifestations in severe COVID-19 patients are characterized by unilateral or bilateral pneumonia, with a state of hyperinflammation due to the activation of the IL-1 or IL-6 pathways that can evolve into a cytokine storm.[35] An increase in pro-inflammatory cytokines (IL-1β, IL-6, IL-12, TNF-α and IFN-γ),[36] as well as lymphopenia and an increase in the activation of the few remaining lymphocytes have been described in the most severe cases.[37] Likewise, the increased activation of CD8+ T lymphocytes,[38] possibly due to the high amount of cytokines, can cause great damage to the lung epithelium, resulting in to hypoxemia, hypotension and even shock.[35] We hypothesize that an efficient presentation of SARS-CoV-2-derived would increase the cytotoxic CD8+

T cell response, augmenting tissue damage in the lungs and elevating the risk of death in severe COVID-19 patients. In addition, it would produce an increase in the activation of T helper lymphocytes (Th), further promoting the inflammatory state. Patients with more severe disease have been shown to have broader and stronger T cell responses,[38] which may be due to a high viral load due to poor viral control by innate immunity,[38] an insufficient early T cell response or to a better recognition of the virus by lymphocytes.

However, our results suggest that there is no relationship between COVID-19 and HLA polymorphisms, also supported by supertypes and amino acid analysis, indicating an irrelevant role of HLA in the risk of COVID-19 infection, which is in agreement with recent genome-wide association studies (GWAS).[39] In fact, the GWAS identified a polymorphism in IFNAR2 (Interferon alpha and beta receptor subunit 2) related to COVID-19.[39] Given this, we believe that innate immunity is crucial for viral infection control, with interferon playing an important role.[40] The virus has numerous mechanisms to bypass the innate immune system and block an effective interferon response.[41] A poor response in the initial stages of infection, would lead to an increase in viral load and a poor prognosis of the disease.[42] The role of HLA in COVID-19 may be to generate robust T cell responses in cases where innate immunity has managed to efficiently control infection. However, in the most severe patients, where it is very possible that the viral load is high and a state of hyperinflammation is present, the HLA proteins that best present viral peptides could worsen the patient's condition by inducing a greater activation of Th and CD8 + cytotoxic lymphocytes, increasing the inflammatory state and tissue damage.

Finally, our data do not show significant values for haplotypes. In contrast, an Italian study found a positive significant correlation between the HLA-A∗01:01, B∗08:01, C∗07:01 and DRB1∗03:01 haplotype and the incidence and death from COVID-19

(haplotype of susceptibility).[43] In addition, the haplotype HLA-A∗02:01, B∗18:01, C∗07:01 and DRB1∗11:04 was inversely related to the incidence and death by COVID-19 (haplotype of protection).[43] Another study carried out in Sardinia identified a protective role of the haplotype HLA-A∗02:05, B∗58:01, C∗07:01, DRB1∗03:01.[44] Finally, the study carried out by Wang et al., showed that the HLA-A∗11:01, B∗51:01 and C∗14:02 haplotype was associated with more severe COVID-19.[34] The HLA-A∗29:02, B∗44:03, C∗16:01, DRB1∗07:01 and DQB1∗02:02 haplotype, which was close to significance in our study, presented almost a 2-fold higher frequency in the COVID-19 hospitalized group versus control group (Table 3). Interestingly, the frequency of this haplotype was significantly (P = 0.006 without correction) higher (more that 2-fold) in the ICU patients versus control group. These data may suggest that this haplotype is related to severity and admission to the ICU. The frequency of this haplotype is 6.2% in the Spanish population,[45] and may be one of the explanations to the high incidence of severity and mortality that the country has suffered.

Based on the results of our study, without taking into account the possibility that certain HLA alleles might modify the clinical course of the disease, we can conclude that HLA polymorphism is not a determining factor in the risk of COVID-19 infection. Furthermore, the allelic-level comparisons made by Ellinghaus et al. with a Spanish and Italian population, further reinforce our results.[46] Moreover, our results agree with the study by Shachar et al., published during the revision of our manuscript, where they found no relationship between HLA polymorphisms and haplotypes in the Israeli population.[47] In addition, the impact of HLA polymorphisms on the outcome (risk/protection) of COVID-19 might by partially masked by the current improvements in patient care and the possible emergence of antivirals, and might be appreciated with greater clarity in the long term. Further studies are needed to answer these important questions.

Supplementary Materials: https://ars.els-cdn.com/content/image/1-s2.0-S1684118221001833-mmc1.doc ; https://ars.els-cdn.com/content/image/1-s2.0-S1684118221001833-mmc2.docx ; https://ars.els-cdn.com/content/image/1-s2.0-S1684118221001833-mmc3.docx

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Major Histocompatibility Complex Class I Chain-Related α STR Polymorphisms in COVID-19 Patients

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Abstract: The SARS-CoV-2 disease presents different phenotypes of severity. Comorbidities, age, and being overweight are well established risk factors for severe disease. However, innate immunity plays a key role in the early control of viral infections and may condition the gravity of COVID-19. Natural Killer (NK) cells are part of innate immunity and are important in the control of virus infection by killing infected cells and participating in the development of adaptive immunity. Therefore, we studied the short tandem repeat (STR) transmembrane polymorphisms of the major histocompatibility complex class I chain-related A (MICA), an NKG2D ligand that induces activation of NK cells, among other cells. We compared the alleles and genotypes of MICA in COVID-19 patients versus healthy controls and analyzed their relation to disease severity. Our results indicate that the MICA*A9 allele is related to infection as well as to symptomatic disease but not to severe disease. The MICA*A9 allele may be a risk factor for SARS-CoV-2 infection and symptomatic disease.

Keywords: MICA STR polymorphisms, MICA, SARS-CoV-2, NK cells, Innate Immunity.

INTRODUCTION

The SARS-CoV-2 infection induces an early immune response in which various elements of innate immunity participate. Natural killer (NK) cells represent an important component of innate immunity participating in the defense against viral infections through their (i) production of perforin and granzymes, which eliminate infected cells [1]; (ii) induction of apoptosis through caspase-dependent and independent pathways [1]; (iii) promotion of the innate and adaptive responses through the production of cytokines and chemokines [2]; (iv) production of gamma interferon (IFN-γ) that stimulates cytotoxic CD8+ T cells and favors the differentiation of CD4+ T cells into T-helper (Th) 1 cells, which are important in viral infections [3]; and (v) production of antibody-dependent cytotoxicity via CD16 [1].

NK cells express a number of inhibitory and activating receptors that recognize various molecules, including major histocompatibility complex class I (MHC-I), that promote their antiviral capacities, while maintaining self-tolerance [4,5]. The NKG2D receptor is a potent activator of NK cells [6], which is also present on CD4+ T cells, CD8+ αβ T cells, γδ T cells, and iNKT cells [7]. NKG2D can directly induce the cytotoxic function and IFN-γ production of NK cells, while it acts as a costimulator of the cytotoxic activity in CD8+ αβ T cells [6,7].

The major histocompatibility complex class I chain-related A (MICA) is one of the NKG2D ligands [8]. These are highly polymorphic nonclassical MHC proteins, of which 108 alleles have been detected [7]. MICA is expressed on the cell surface of the epithelial cells, fibroblasts, keratinocytes, endothelial cells, and monocytes under stress conditions, such as a virus infection [1,9]. The structure of MICA is similar to that of MHC-I

molecules, but it is not associated with β2-microglobulin [8]. It has three extracellular domains (α1, α2, and α3) encoded by exons 2, 3, and 4, respectively, together with a transmembrane region encoded by exon 5 [8]. This transmembrane region has a short tandem repeat (STR) of GCT triplets, which codify alanine residues [8]. The different number of GTC repetitions (4, 5, 6, 7, 8, 9, or 10) gives rise to the alleles A4, A5, A6, A7, A8, A9, and A10, respectively [10]. In addition, there is an extra allele (A5.1) that has an insertion of a G in exon 5, which produces a stop codon and therefore a truncated protein that does not have the transmembrane region [10]. The transmembrane region is involved in the physiological localization of the MICA molecule. The alleles that have intact transmembrane regions present a basolateral expression, while the A5.1 allele presents an apical localization [11]. The A5.1 allele is expressed on the membrane via a glycosyl-phosphatidyl-inositol (GPI) anchor [12], with the highest levels of soluble MICA (sMICA) found in carriers of this allele [13].

Decreased NK cells numbers and their reduced ability to kill infected cells have been reported in coronavirus disease 2019 (COVID-19) patients [14]. The ability of the NK cell to bind to the target cell is altered, reducing its cytotoxic capacity [14]. Therefore, the presence of certain MICA alleles may favor NK cell cytotoxicity and allow better control of the disease. In contrast, other alleles can be related to a worse prognosis, as evidenced by a study linking an increase in sMICA with symptomatic COVID-19 [15]. Thus, allelic and biomechanical differences of MICA may be important factors for its interaction with NKG2D, thus modulating the function of NK cells and the innate immune response.

Previously, we have performed a study of the classic MHC molecules and their relationship with COVID-19 [16]. Currently, we have studied the association of COVID-19 with STR in exon 5 of the MICA gene, HLA-B genes, and HLA-B/MICA haplotypes

and in asymptomatic, moderate, and severe COVID-19 patients. In addition, we have compared the presence of these alleles with biochemical parameters, days of hospital admission, and other factors, attempting to clarify the role of MICA in COVID-19 infection.

Our hypothesis is that MICA molecules are very important for the activation of NK and T cells via NKG2D in infectious processes, such as COVID-19. The different MICA alleles may vary in the activation of these cells and produce different degrees of activation and control of the infection.

RESULTS

In the study of STR polymorphisms of MICA, the alleles A4, A5, A5.1, A6, and A9 were detected (Table 1). The allele with the highest representation in the population reference group and COVID-19 patients was MICA*A6, while the one with the lowest frequency was MICA*A5. The comparison of the allelic and genotypic frequencies between COVID-19 cases and the population reference group are presented in Table 2. However, we made comparisons between the different COVID-19 patients based on disease severity, grouped as asymptomatic, symptomatic, moderate, and severe patients, with each other and with the population reference group.

The result showed a statistical difference with a higher allele frequency of MICA*A9 in COVID-19 patients versus the population reference group ($p = 0.004$, $Pc = 0.025$, odds ratio = 1.399, 95% confidence interval = 1.110–1.762) (Table 1). When we compared asymptomatic and symptomatic patients versus the population reference group, only the symptomatic patients had a statistically higher frequency of the MICA*A9 allele. In addition, these differences were maintained when the comparison was performed between moderate patients and the population reference group. The rest of the comparisons among the different groups did not show a significant value.

The genotype comparisons did not show any statistical difference among the groups (Table 1). The genotype MICA*A9 homozygous showed a higher frequency in moderate patients versus the population reference group ($p = 0.041$), but the significance was lost after Bonferroni correction (Table 1).

Table 1. MICA alleles and genotypic frequencies.

Allele	Controls (2n=1234) n (%)	COVID-19 Patients (2n=892) n (%)	Asymptomatic Patients (2n=66) n (%)	Moderate Patients (2n=686) n (%)	Severe Patients (2n=206) n (%)	P1 (Pc)	P2 (Pc)	P3 (Pc)	P4 (Pc)	P5 (Pc)	P6 (Pc)
*MICA*A4*	192 (15.6)	119 (13.3)	11 (16.7)	95 (13.8)	24 (11.7)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A5*	140 (11.3)	102 (11.4)	6 (9.1)	81 (11.8)	21 (10.2)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A5.1*	310 (25.1)	196 (22.0)	19 (28.8)	158 (23)	38 (18.4)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A6*	423 (34.3)	309 (34.6)	22 (33.3)	226 (32.9)	83 (40.3)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A9*	169 (13.7)	166 (18.6)	8 (12.1)	126 (18.4)	40 (19.4)	**0.004 (0.025)**	n.s	**0.007 (0.035)**	n.s	n.s	n.s
Genotype	**Controls (n=617) n (%)**	**COVID-19 Patients (n=446) n (%)**	**Asymptomatic Patients (n=33) n (%)**	**Moderate Patients (n=343) n (%)**	**Severe Patients (n=103) n (%)**	**P1 (Pc)**	**P2 (Pc)**	**P3 (Pc)**	**P4 (Pc)**	**P5 (Pc)**	**P6 (Pc)**
*MICA*A4, *A4*	12 (1.9)	4 (0.9)	1 (3)	4 (1.2)	0 (0)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A4, *A5*	18 (2.9)	16 (3.6)	3 (9.1)	14 (4.1)	2 (1.9)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A4, *A5.1*	58 (9.4)	33 (7.4)	2 (6.1)	28 (8.2)	5 (4.9)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A4, *A6*	67 (10.8)	43 (9.6)	3 (9.1)	29 (8.5)	14 (13.6)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A4, *A9*	26 (4.2)	19 (4.3)	1 (3)	16 (4.7)	3 (2.9)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A5, *A5*	16 (2.6)	9 (2.0)	0 (0)	7 (2)	2 (1.9)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A5, *A5.1*	22 (3.6)	20 (4.5)	0 (0)	18 (5.2)	2 (1.9)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A5, *A6*	43 (7)	29 (6.5)	2 (6.1)	21 (6.1)	8 (7.8)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A5, *A9*	23 (3.7)	19 (4.3)	1 (3)	14 (4.1)	5 (4.9)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A5.1, *A5.1*	41 (6.6)	20 (4.5)	3 (9.1)	17 (5)	3 (2.9)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A5.1, *A6*	107 (17.3)	64 (14.3)	9 (27.3)	50 (14.6)	14 (13.6)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A5.1, *A9*	41 (6.6)	39 (8.7)	2 (6.1)	28 (8.2)	11 (10.7)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A6, *A6*	77 (12.5)	63 (14.1)	3 (9.1)	45 (13.1)	18 (17.5)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A6, *A9*	52 (8.4)	47 (10.5)	2 (6.1)	36 (10.5)	11 (10.7)	n.s	n.s	n.s	n.s	n.s	n.s
*MICA*A9, *A9*	14 (2.3)	21 (4.7)	1 (3)	16 (4.7)	5 (4.9)	n.s	n.s	**0.041 (n.s)**	n.s	n.s	n.s

Comparison of allele and genotype frequencies between COVID-19 patients and controls. P1: Controls vs COVID-19 patients; P2: Controls vs Asymptomatic Patients; P3: Controls vs Moderate Patients; P4: Controls vs Severe Patients; P5: Asymptomatic vs Moderate Patients; P6: Asymptomatic vs Severe Patients; Pc = P-value corrected by the Bonferroni test ; n.s: not significance.

Table 2. HLA-B/MICA haplotype frequencies.

	Controls		Asymptomatic		Moderate Patients		Severe Patients		COVID-19 Patients	
	F	%	F	%	F	%	F	%	F	%
*HLA-B*07/MICA*A51*	105	8.5	9	13.6	58	8.5	16	7.8	83	8.8
*HLA-B*41/MICA*A6*	14	1.1	2	3	10	1.5	3	1.5	15	1.6
*HLA-B*14/MICA*A5*	14	1.1	2	3	8	1.2	1	0.5	11	1.2
*HLA-B*35/MICA*A9*	56	4.5	2	3	38	5.5	12	5.8	51	5.4
*HLA-B*50/MICA*A6*	38	3.1	1	1.5	14	2	10	4.9	25	2.6
*HLA-B*40/MICA*A5*	20	1.6	1	1.5	20	2.9	3	1.5	24	2.5
*HLA-B*27/MICA*A4*	41	3.3	3	4.5	14	2	8	3.9	25	2.6
*HLA-B*18/MICA*A4*	125	10.1	5	7.6	58	8.5	13	6.3	75	7.9
*HLA-B*57/MICA*A9*	25	2	1	1.5	22	3.2	11	5.3	34	3.6
*HLA-B*44/MICA*A51*	57	4.6	4	6.1	29	4.2	10	4.9	43	4.6
*HLA-B*51/MICA*A6*	102	8.3	3	4.5	39	5.7	17	8.3	58	6.1
*HLA-B*15/MICA*A5*	33	2.7	3	4.5	19	2.8	9	4.4	30	3.2
*HLA-B*44/MICA*A6*	124	10	8	12.1	78	11.4	29	14.1	114	12.1
*HLA-B*35/MICA*A6*	20	1.6	2	3	9	1.3	2	1	13	1.4
*HLA-B*40/MICA*A51*	17	1.4	2	3	8	1.2	2	1	12	1.3
*HLA-B*08/MICA*A51*	53	4.3	3	4.5	36	5.2	4	1.9	42	4.4
*HLA-B*14/MICA*A6*	50	4.1	2	3	37	5.4	10	4.9	49	5.2
*HLA-B*39/MICA*A9*	15	1.2	1	1.5	12	1.7	2	1	16	1.7
*HLA-B*38/MICA*A9*	28	2.3	1	1.5	22	3.2	10	4.9	33	3.5
*HLA-B*53/MICA*A9*	15	1.2	1	1.5	15	2.2	3	1.5	19	2
*HLA-B*55/MICA*A4*	9	0.7	1	1.5	13	1.9	2	1	16	1.7
*HLA-B*13/MICA*A51*	19	1.5	1	1.5	9	1.3	3	1.5	13	1.4
*HLA-B*49/MICA*A6*	31	2.5	1	1.5	20	2.9	7	3.4	28	3
*HLA-B*52/MICA*A6*	14	1.1	1	1.5	10	1.5	5	2.4	16	1.7
*HLA-B*35/MICA*A5*	41	3.3	0	0	27	3.9	6	2.9	32	3.4
*HLA-B*58/MICA*A9*	10	1,06	0	0	8	1.2	2	1	16	1.3

Comparison of HLA-B/MICA haplotype frequencies between COVID-19 patients stratified by disease severity and controls. No significant differences were found.

The association between MICA and coinfection or sepsis development in COVID-19 patients was not possible to study due to the low number of cases in our studied population.

Finally, due to the age difference between cases and the population reference group, we studied a possible association between age and MICA STR alleles. We did a regression model that did not show any relation between age and MICA STR alleles (R Square "R2" = 0.004).

Additionally, we made the comparison between homozygous versus heterozygous genotypes in all COVID-19 groups, with each other and versus the population reference group, but did not find significant results (data not shown).

Due to the proximity of the HLA-B and MICA loci, we studied the HLA-B frequency and the HLA-B/MICA haplotypes.

The frequencies of the HLA-B alleles are presented in Supplementary Table S2. Alleles with a frequency lower than 1% were excluded from the study. There were no statistical differences between the groups and the population reference group. Only HLA-B*50 showed a higher frequency ($p = 0.027$, Pc= n.s) in severe (4.9%) compared to moderate patients (2%).

We next carried out the construction of the HLA-B/MICA haplotypes, studying those that presented a frequency > 1% (Table 3). The comparisons showed that the HLA-B*57/MICA*A9 haplotype was more frequent in COVID-19 cases than in the population reference group but significance was lost upon Bonferroni correction. We then analyzed the MICA*A9 haplotypes together obtaining a statistical difference ($Pc = 0.01$) between COVID-19 patients versus the population reference group. This result was due to MICA*A9. In the intragroup comparison, the HLA-B*50/MICA*A6 haplotype was more

represented in severe (4.9%) than in moderate (2%) or asymptomatic patients, although significance was lost after Bonferroni correction.

Table 3. Characteristics and comorbidities of the study population.

CHARACTERISTICS	
Age	**62 (25-98)**
Female	**220 (45.5%)**
Male	**263 (54.5%)**
Diagnosis by PCR	**363 (75.2%)**
Diagnosis by antibodies	**120 (24.8%)**
UCI	**126 (26.1%)**
No UCI	**324 (73.9%)**
Mechanic Ventilation	**101 (20.9%)**
No Mechanic Ventilation	**349 (79.1%)**
Deceased	**70 (14.5%)**
Survivors	**380 (85.5%)**
COMORBIDITIES	
Hypertension	**196 (40.6%)**
DM	**101 (20.9%)**
CKD	**33 (6.8%)**
CVD	**22 (4.6%)**
Overweight/Obesity	**72 (14.9%)**
MI	**26 (5.4%)**
HF	**25 (5.2%)**
COPD	**32 (6.6%)**
Asthma	**26 (6%)**
PAD	**11 (2.3%)**

ICU: Intensive Care Unit; DM: Diabetes Mellitus; CKD: Chronic Kidney Disease; CVD: Cerebrovascular Disease MI: Myocardial infarction; HF: Heart Failure; COPD: Chronic obstructive pulmonary disease; PAD: Peripheral Artery Disease.

Finally, we compared HLA-B and MICA with different biochemical parameters (lactate dehydrogenase, IL-6, platelets, PO2, bilirubin, creatinine, D-dimer, C reactive protein, ferritin, troponin, natriuretic peptide, procalcitonin, and fibrinogen), days of hospital stay or stay in the ICU, age at diagnosis, and the need for respiratory support (conventional oxygen therapy, noninvasive ventilation, or intubation). We did not detect any significant associations among the above parameters, HLA-B, MICA, or the HLA*B/MICA haplotypes (data not shown).

DISCUSSION

The SARS-CoV-2 infection has shown different response phenotypes (asymptomatic, moderate and severe disease, or death). These differences among individuals may be due to innate immunity. Therefore, we have tried to identify a possible role of NK cells by studying the different MICA alleles. NK cells may present variations in their response to MICA due to polymorphisms in its transmembrane region [17]. STR polymorphisms in the transmembrane region have been related to susceptibility or protection to various infectious agents [10,18].

We found a higher frequency of MICA*A9 in COVID-19 patients versus PRG. In addition, MICA*A9 was higher in symptomatic versus asymptomatic patients, with the highest frequency found in patients with moderate disease severity. Moreover, we did not find significance in severe patients due to the small population studied. MICA*A9 associations is independent from HLA-B locus.

The extracellular domain of MICA protein has a polymorphism at the amino acid position 129 that is important for the NKG2D interaction. The variant MICA V129 (valine in 129) is associated with reduced affinity for NKG2D, whereas M129 (methionine in 129)

confers high-binding affinity to NKG2D [19] (Figure 1). All MICA*A9 alleles shared M129, forming a haplogroup, presenting a high affinity for the NKG2D receptor [20].

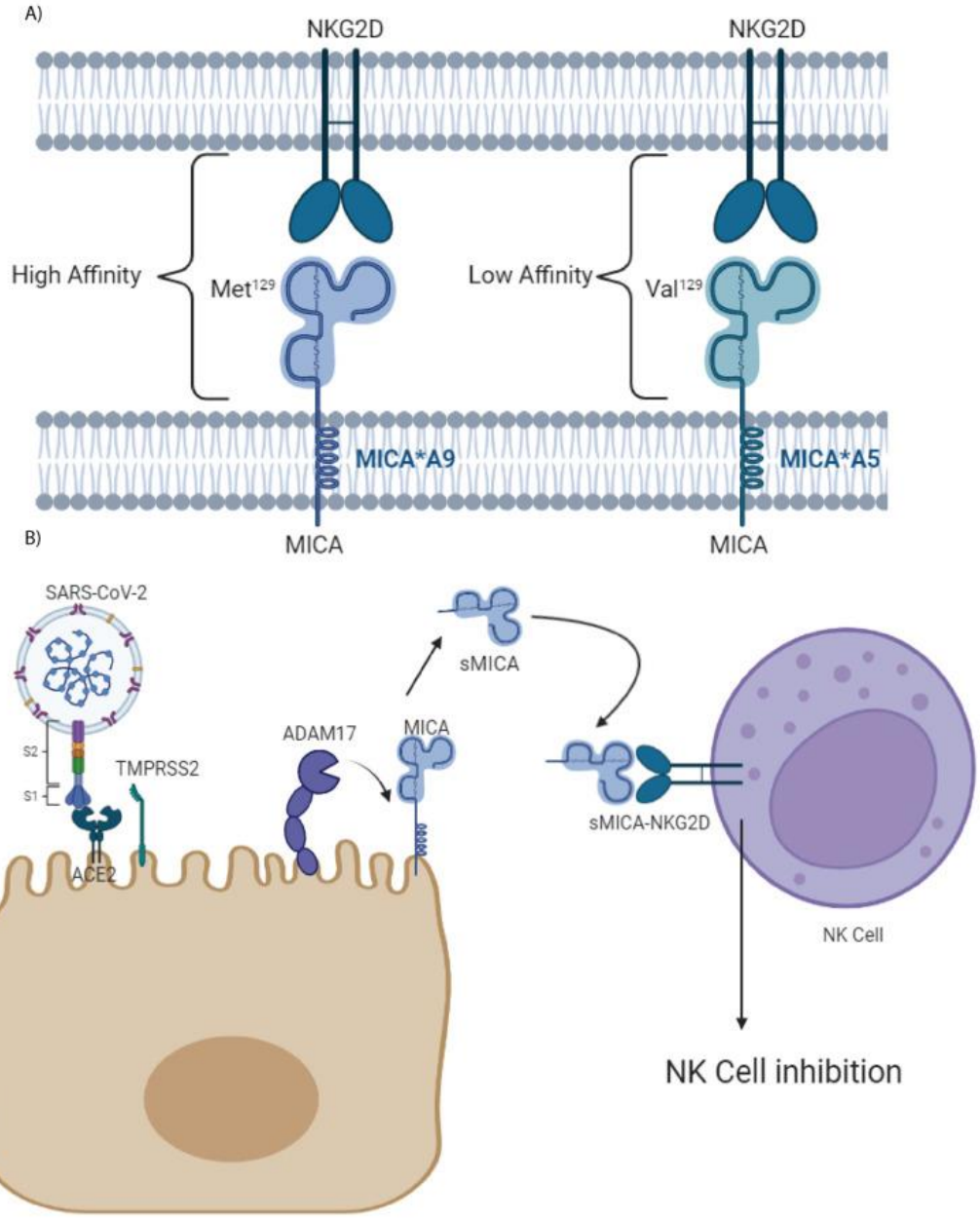

Figure 1. Graphic representation of the MICA-NKG2D interaction depending on the Met/Val polymorphism at 129 and the production of sMICA by ADAM17: (A) The Met/Val polymorphism at 129 of the MICA molecules influences binding to NKG2D. Alleles with Met129 have high affinity for the ligand, while those with Val129 have low affinity. The MICA*A4 and *A9 alleles have Met129, MICA*A5 present Val129, and the MICA*5.1 and *A6 alleles can have both polymorphisms [20]. (B) In COVID-19 infection, the metalloprotease ADAM17 hydrolyzes MICA generating sMICA, which binds to NKG2D causing inhibition. In the case of the MICA alleles with the Met129 variant, the inhibition will be greater, and there will be a worse control of the infection by the NK and T cells. Abbreviations—ACE2: angiotensin-converting enzyme 2; S1: S1 subunit of spike protein; S2: S2 subunit of spike protein; TMPRSS2: transmembrane protease serine 2; ADAM17: ADAM metallopeptidase domain 17.

SARS-CoV-2 infection generates more sMICA by ADAM17 metalloproteinase overexpression after ACE2 and spike protein interaction that decrease the activation of NK cells [21] (Figure 1). This immune evasion will be more effective in individuals with MICA alleles with high affinity for NKG2D, such as MICA*A9, inducing a stronger inhibition to NK and T cells and allowing an increased replication and dispersion of the virus.

Castelli et al. reported an association of MICA rs2596541 variants with symptomatic patients [15]. These variants increase mRNA levels and may favor the increase in sMICA. Furthermore, they observed that MICA*008 and MICA*019, which are in linkage disequilibrium with rs2596541, were increased in symptomatic patients [15]. The MICA*008 allele is associated with the STR MICA*A5.1 polymorphism, while the MICA*019 is associated with MICA*A5 [22]. Our results did not show significant results associated with symptoms or severity in any of these STR polymorphisms. However, it should be noted that each of the STR polymorphisms has more than one associated MICA allele [22].

Otherwise, the MICA expression level might be related to host MICA gene polymorphisms [23]. Nevertheless, virus infections can interfere with MICA expression. For example, in HCMV infection some MICA alleles are downregulated while MICA*008 is not. MICA*008 is in linkage disequilibrium with MICA*A5.1. Therefore, the cytotoxic effects of NK cells against HCMV-infected cells were stronger in individuals carrying MICA*008/MICA*A5.1 [23]. However, UL142-HCMV protein downregulates the expression of MICA proteins that have a large transmembrane region but not those with a short transmembrane region [12]. Moreover, SARS-CoV-2 acts in a similar manner, which makes individuals with MICA*A9 more at risk of infection and symptomatic diseases.

The allele MICA*A9 has been associated with a diversity of diseases. A lower frequency of MICA*A9 has been reported in syncytial virus infection (RSV) than in controls [19]. The presence of MICA*A9 in heterozygosity can protect against Chlamydia trachomatis infection [20]. Furthermore, MICA*A9 has a relation with autoimmune disease [21,22,23,24].

The studies in COVID-19 patients showed increased levels of exhausted NK cells. These NK cells exhibit a high level of programmed death 1 (PD-1) and NK group 2 member A receptor (NKG2A) expression [24,25]. NKG2A is one of the most important inhibitor-receptors of NK cells [26]. Also, other NK cells polymorphisms are related to COVID-19. The killer cell immunoglobulin-like receptors (KIRs) are a family of highly polymorphic transmembrane glycoproteins that induced inhibitory or activating signals to NK and T cells upon recognition of their ligands [27]. Studies showed that KIR2DS4 is related to severe COVID-19 infection [28,29].

MICA, similar to the other mentioned factors, is not only important for the correct function of NK cells but also for adaptative immunity. NK cells intervene in adaptive immunity due to their production of interferon-γ (IFN- γ) and IL-2, which induce the activation of CD4+ T cells and favor their differentiation into Th1 cells [30,31]. This is one reason for the low Th1 levels of COVID-19 patients [3].

It is known that the MICA-NKG2D interaction is important for the activation of NK and T cells for the elimination of cells stressed by tumors or infections [32]. Recent studies in the area of cancer have directed efforts to create a vaccine that prevents the production of sMICA due to its role in the immune escape, producing the inhibition of NK and T cells [33].

In conclusion, this study tries to better explain the different factors that model the NK cells and their impact on SARS-CoV-2 infection. The identification of MICA polymorphisms and susceptibilities to different diseases could promote earlier diagnosis and preventive measures. Our results indicate that the STR polymorphisms in MICA*A9 has an impact on the risk of contagion and disease severity. MICA*A9 is a possible factor of innate immunity that could help explain the risk of infection and the different responses observed in SARS-CoV-2-infected individuals. However, although there seems to be no relationship between age and the frequency of MICA STR alleles, a possible limitation of the study is the age difference between the population reference group and the cases. Therefore, our conclusions need to be further verified in other populations.

MATERIALS AND METHODS

Samples

The study population consisted of 483 individuals diagnosed with COVID-19 at the University Hospital Virgen de las Nieves. All the individuals tested positive in polymerase chain reaction (PCR) for SARS-CoV-2. These individuals were classified according to the severity of the disease:

1. Asymptomatic: 33 patients (23 women and 10 men); mean age of 42.6 years (26–63).

2. Symptomatic:

    a. Moderate patients: 344 patients (158 women and 186 men); mean age of 63.6 years (25–98).

    b. Severe patients: 106 patients (38 women and 67 men); mean age of 61.8 years (26–86).

The severity of the disease was determined following the guidelines of the National Institutes of Health [33]:

(a) Asymptomatic: Individuals who test positive for SARS-CoV-2 using a virologic test but who have no symptoms that are consistent with COVID-19 [34].

(b) Moderate Illness: Individuals who show evidence of lower respiratory disease during clinical assessment or imaging and who have an oxygen saturation (SpO2) ≥ 94% on room air at sea level [34].

(c) Severe Illness: Individuals who have SpO2 < 94% on room air at sea level, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen

(PaO2/FiO2) < 300 mm Hg, a respiratory rate > 30 breaths/min, or lung infiltrates > 50% [34].

The patients were recruited between April 2020 and January 2021. The variants of SARS-CoV-2 prevalent at this time in Spain were Wuhan and Alfa [35]. In addition, there were no vaccines available at this time.

All patient samples were collected according to the local medical ethics regulation after informed consent was obtained by the subjects, their legal representatives, or both, according to the Declaration of Helsinki. The studies involving human participants were reviewed and approved by the ethics committee via the Portal de Ética de la Investigación Biomédica de Andalucía (PIEBA) of the Andalusian Government (Code: 0766-N-20).

Population Reference Group

The population reference group (PRG) consisted of 617 blood samples from donors from the regional blood bank of Granada. The PRG was recruited from 2005 to 2015. Healthy individuals who did not present any disease of autoimmune etiology or immunodeficiencies were selected. The average age of the group is 45 years and 325 (51%) are women. This group was used as a representation of the allele and genotype frequencies in our population.

DNA extraction

DNA was extracted from peripheral blood of each of the patients using the QIAMP DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

HLA-B and MICA genotyping

HLA-B typing was performed at high resolution using the LABType sequence-specific oligonucleotide typing test (One Lambda, Canoga Park, CA, USA). Target DNA was

amplified by PCR using sequence-specific primers, followed by hybridization with allele-specific oligodeoxynucleotides coupled with fluorescent phycoerythrin-labeled microspheres. Fluorescence intensity was determined using a LABScan 100 system (Luminex xMAP, Austin, TX, USA). HLA alleles were assigned using the HLA-Fusion software (version 4.6.013925) (One Lambda).

The study of the STR polymorphism of exon 5 of MICA was performed by polymerase chain reaction (PCR) following the method of Ota et al. (https://pubmed.ncbi.nlm.nih.gov/9174136/) (accessed on 10 February 2022).

The amplified products were analyzed by electrophoretic separation using the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and their sizes were analyzed using the GenMapper version 4.0 software (Applied Biosystems).

PCR diagnosis of SARS-CoV-2 infection

PCR diagnosis was performed using the cobas® SARS-CoV-2 assay on a cobas® 6800 system (Roche Molecular Systems, Pleasanton, CA, USA). This is a single-well, double-target assay that enables both the specific detection of SARS-CoV-2 and the detection of pan-Sarbecovirus of the Sarbecovirus subgenus family, which includes SARS-CoV-2. The test detects the genetic signature (RNA) of the SARS-CoV-2 virus in nasal, nasopharyngeal, and oropharyngeal swab samples from patients who meet COVID-19 clinical and/or epidemiological criteria for testing.

Statistical analysis

Statistical analysis was performed to compare allelic, genotypic, and haplotypic distributions among patients and PRG using the X2 test or the two-tailed Fisher's exact test, when necessary, with contingency tables. Significance levels were corrected by Bonferroni correction for a multiplicity of testing by the number of comparisons. The

risk estimation was determined by calculating the odds ratio (OR) with a confidence interval (CI) of 95%. The Kruskal–Wallis and U Mann–Whitney were used to compare groups when the distribution was not normal (as checked by the Kolmogorov–Smirnov test). The software used was SPSS statistical software (Windows version 26, IBM, Armonk, NY, USA).

We applied a lineal regression using R Square "R2" to estimate the association between age and MICA STR allele.

The estimation of haplotype frequencies, LD, and haplotypically based hypothesis tests were calculated using Arlequin version 3.1 (Supplementary Table S1), the software package for population genetics.

A corrected p-value of <0.05 was considered statistically significant.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/ijms23136979/s1.

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HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 haplotype is related to a higher production of antibodies in vaccinated with Moderna

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Abstract: The vaccines designed against the SARS-CoV-2 coronavirus are based on the spike (S) protein. Processing of the S protein by antigen-presenting cells (APC) and its subsequent presentation to T cells is an essential part of the development of a humoral response. HLA-class II alleles are considered immune response genes because their codified molecules, expressed on the surface of APCs (macrophages, dendritic, and B cells) present antigenic peptides to T cell via their T cell receptor (TCR). The HLA-class II genes are highly polymorphic, regulating what specific peptides induce follicular helper T cells (TFH) and promote B lymphocyte differentiation into plasma or memory B cells. This work hypothesizes that the presence of certain HLA-class II alleles could be associated with the intensity of the humoral response (amount, length) to the SARS-CoV2 mRNA 1273 vaccine. We have studied the relationship between the HLA-class II typing of 87 health workers and the level of antibodies produced 30 days after vaccination. We show a possible association between the HLA-DRB1* 07:01 allele and the HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 haplotype to a higher production of antibodies 30 days after the administration of the second dose of mRNA-1273.

INTRODUCTION

Since December 2019, the rapid expansion of the SARS-CoV-2 coronavirus has resulted in a severe pandemic affecting the entire planet [1]. The countermeasures carried out, such as confinement, face masks, use of disinfectant gels, etc. have been of great help to combat the spread of the virus [2]. However, the development of vaccines against the virus is vitally important to avoid serious illness and death [3,4]. The mRNA-1273 vaccine employs messenger RNA (mRNA) technology and encodes a stabilized version of the SARS-CoV-2 full-length spike glycoprotein trimer [3]. The administration guideline requires two doses of 100 μg separated by 28 days [3].

Studies that monitor the cellular and humoral response of vaccinated people show different degrees of response depending on the vaccine [5,6]. Regarding antibody production, a good general response is observed at the beginning [7]. Subsequently, a decrease in the circulating level of anti-Spike (anti-S) antibodies is observed [8].

Differences in the antibody levels have been observed between individuals who received the same SARS-CoV-2 vaccine. Factors such as age, health, and immune system status intervene in the humoral response. In addition, immunogenetics can play an important role. The human leukocyte antigen class II (HLA-class II) molecules are part of the immunogenetic and perform antigen presentation during the generation of an immune response [9]. In the case of people vaccinated against SARS-CoV-2 (similar to other vaccines and infections), the spike (S) protein of SARS-CoV-2 generated artificially by the vaccine mRNA translation is taken up by dendritic cells and processed into peptides by the endosomal route. Those peptides are presented by HLA-class II molecules on dendritic cells to the T cell receptor (TCR) of T naive cells (Th0) to induce their differentiation to T follicular helper cells (Tfh) [10]. In the lymphoid follicle, B

lymphocytes capture the S protein through their B-cell receptor (BCR), process it into peptides, and, as antigen-presenting cells (APC), present them in the context of HLA-class II molecules to the TCR of CD4 Tfh lymphocytes, forming the immunological synapse [10]. This results in the formation the germinal centers and the differentiation of B lymphocytes into SARS-CoV-2 Spike-specific plasma and memory B cells with isotype switch and affinity maturation via somatic hypermutations [11].

The HLA class II alleles that code for the HLA-DR, HLA-DQ, and HLA-DP molecules have a high degree of polymorphism. This polymorphism resides in the exons that code the cleft where the antigenic peptide is bound (β1 domain of HLA-DR and the α1 and β1 domains for HLA-DQ and HLA-DP molecules) [12]. Due to these polymorphisms, each allele can only present peptides that have binding motifs compatible to its specific cleft. For that reason, each allele presents different peptides derived from the same antigenic molecule. The presence of certain alleles will make the antigenic presentation more efficient, leading to a better stimulation of B cells that will mature into plasma cells. Likewise, the presence of HLA-class II alleles capable of presenting more peptides efficiently will form more specific clones of different epitopes of protein S, thus favoring a higher circulating antibody titer and probably a higher neutralizing and protective capacity. Therefore, the different HLA-class II alleles may explain the differences in antibody production observed between individuals.

In our work, we have studied the relationship between HLA class II polymorphism and humoral immunity generated by the SARS-CoV-2 mRNA-1273 vaccine in a cohort of 87 health workers from University Hospital Virgen de las Nieves. The comparison was made depending on the level of circulating IgG antibodies against protein S at 30 days after the second dose.

MATERIALS AND METHODS

Population Studied. The study was carried out with a population of 87 workers made up of 42 women and 45 men, belonging to the Hospital Universitario Virgen de las Nieves complex, who were vaccinated with Moderna's mRNA-1273 vaccine. The mean general age was 48 years (23–65).

Control groups.

- HLA control group: 637 healthy blood donors, representative of the Granada area, recollected between 2015 and 2021. The average age of the group is 45 years and 325 (51%) of the members are women. This group was used for compared the HLA-class II allelic frequencies in our region.

-Vaccinated control group: 601 workers made up of 398 women and 203 men, belonging to the University Hospital Virgen de las Nieves complex, who were vaccinated with Moderna's mRNA-1273 vaccine. The mean general age was 48 years.

All patient samples were collected according to the local medical ethics regulation, after informed consent was obtained by the subjects, their legal representatives, or both, according to the Declaration of Helsinki. The studies involving human participants were reviewed and approved by Portal de Ética de la Investigación Biomédica. Junta de Andalucía (Cod. 0297-N-21). The patients/participants provided their written informed consent to participate in this study.

Measurement of antibodies against SARS-CoV-2. Participants underwent blood extraction, 30 days after inoculation of the second dose. A quantitative determination of immunoglobulin G (IgG) was performed against the S protein. The quantification of IgG was carried out by the chemiluminescent COVID-19 IgG Assay (Alinity, Abbott, IL,

USA) following the manufacturer's instructions. The results were expressed in BAU/mL (binding antibody units per milliliter). The cutoff for positivity was set at >7.5 BAU/mL.

Statistical Analysis. Frequencies of individual HLA alleles and haplotypes were compared using the χ2-test. Variants with expected counts less than five were compared using Fisher's exact test. Variants with an expected count of less than two were combined into a common class (binned) before computing the χ2-test. The software used was SPSS statistical software (Windows version 26, IBM, Armonk, NY, USA). Significance levels were corrected by Bonferroni correction for a multiplicity of testing by the number of comparisons.

The Mann–Whitney U test was used to compare groups when the distribution was not normal (as checked by the Kolmogorov–Smirnov test).

A corrected p-value of <0.05 was considered statistically significant.

HLA typing by Next Generation Sequencing (NGS). To amplify DNA target regions, we used the AllType FASTplex NGS 11 Loci Kit (One Lambda). For the HLA-DRB1/3/4/5 and HLA-DQB1 loci, the region between exon 2 and the 3'UTR region was amplified. The entire gene for the HLA-DQA1 locus was amplified. The technique was performed following the manufacturer's recommendations. To load the chip, we used the Ion Chef (Thermo Fisher Scientific, Waltham, MA USA) and for sequencing the Ion GeneStudio S5 Plus System (Thermo Fisher Scientific). For data analysis, we used the TypeStream Visual NGS Analysis Software One Lamda (Thermo Fisher Scientific).

RESULTS

Quantification of anti-S protein antibody titers after SARS-CoV2 mRNA-1273 vaccination. All the individuals studied presented specific anti-S IgG antibodies, 30 days after administration of the second dose of mRNA-1273 vaccine with a wide range: 65 to 10505 BAU/mL (Supplementary Table S1). The 87 cases were distributed in three groups. We based our classification on the distribution of anti-S antibodies levels in a cohort of 601 vaccinated individuals (Vaccinated control group). We use the mean value (2700 BAU/mL) +/- one standard deviation (SD) (1700 BAU/mL) to determine the groups (Supplementary Figure S1). The 87 cases for the HLA class II typification were selected to achieve a similar number, sex frequency, and age in each group. The resulting humoral response groups were:

- G1 (Low responders) (<1000 BAU/mL): 28 individuals (13 women and 15 men), with an average age of 46.8 years (26–65).

-G2 (Middle responders) (1000–4400 BAU/mL): 29 individuals (14 women and 12 men), with an average age of 49.3 years (28–65).

- G3 (High responders) (>4400 BAU/mL): 30 individuals (14 women and 16 men), with an average age of 48 years (23–65).

HLA-class II allelic frequencies. The HLA-class II alleles frequencies in the 87 cases were compared with an HLA control group of 637 blood donors, representative of the Granada area, without significant differences (Supplementary Table S2). HLA-DRB1 and HLA-DQB1 alleles frequencies in humoral response groups G1, G2, and G3 are represented in Table 1.

Table 1. Allele frequencies in the responder's group.

LOCUS	ALLELE	FREQUENCY		
		G1	G2	G3
HLA-DRB1	01:01	0.125	0.052	0.017
HLA-DRB1	01:02	0.107	0.034	0
HLA-DRB1	01:03	0.018	0	0.033
HLA-DRB1	03:01	0.071	0.172	0.067
HLA-DRB1	04:01	0	0.034	0.050
HLA-DRB1	04:02	0	0.034	0
HLA-DRB1	04:03	0.036	0	0.017
HLA-DRB1	04:04	0.054	0.052	0.017
HLA-DRB1	04:05	0.054	0	0.050
HLA-DRB1	04:06	0	0	0.017
HLA-DRB1	04:07	0.036	0	0
HLA-DRB1	07:01	0.018	0.172	0.250
HLA-DRB1	07:02	0	0	0.017
HLA-DRB1	08:01	0.018	0	0
HLA-DRB1	09:01	0.018	0.017	0
HLA-DRB1	10:01	0	0.017	0.017
HLA-DRB1	11:01	0.054	0.052	0.033
HLA-DRB1	11:02	0.018	0.034	0
HLA-DRB1	11:03	0.036	0	0
HLA-DRB1	11:04	0	0.052	0.050
HLA-DRB1	12:01	0.054	0	0
HLA-DRB1	13:01	0.071	0.069	0.100
HLA-DRB1	13:02	0.036	0.034	0.050
HLA-DRB1	13:03	0.018	0.017	0.017
HLA-DRB1	14:01	0.018	0	0
HLA-DRB1	14:54	0.018	0.034	0.017
HLA-DRB1	15:01	0.071	0.103	0.167
HLA-DRB1	16:01	0.054	0.017	0.017
HLA-DQB1	02:01	0.071	0.155	0.083
HLA-DQB1	02:02	0.036	0.121	0.200
HLA-DQB1	02:05	0	0.017	0
HLA-DQB1	02:10	0	0.017	0
HLA-DQB1	03:01	0.196	0.155	0.117
HLA-DQB1	03:02	0.179	0.103	0.117
HLA-DQB1	03:03	0	0.052	0.050
HLA-DQB1	03:19	0.018	0.034	0
HLA-DQB1	04:02	0.018	0	0.017
HLA-DQB1	05:01	0.232	0.086	0.067
HLA-DQB1	05:02	0.054	0.017	0.017
HLA-DQB1	05:03	0.036	0.034	0.017
HLA-DQB1	06:01	0	0.034	0
HLA-DQB1	06:02	0.071	0.069	0.150
HLA-DQB1	06:03	0.054	0.086	0.117
HLA-DQB1	06:04	0.018	0.017	0.017
HLA-DQB1	06:09	0.018	0	0.033

HLA-DRB1*07:01 and HLA-DQB1*02:02 alleles were more frequent in G3 vs G1 (high responders vs low responders). The p-value of HLA-DRB1*07:01 was potent and passed the Bonferroni correction (Pc = 0.0031), whereas HLA-DQB1*02:02 did not pass the Bonferroni correction (Table 2). HLA-DRB1*01:01 and HLA-DQB1*05:01 had a higher frequency in G1 but the p-value did not pass the Bonferroni correction. All the comparations with G2 for HLA-class II allelic frequencies were not significant.

Table 2. Comparison of allele frequencies.

ALLELE	FREQUENCY		OR	P	Pc
	LOW RESPONDERS	HIGH RESPONDERS			
HLA-DRB1*01:01	**0.125**	0.017	0.119	0.028	n.s
HLA-DQB1*05:01	**0.232**	0.067	0.236	0.016	n.s
HLA-DRB1*07:01	0.018	**0.250**	18.333	**2.3E-4**	**3.1E-3**
HLA-DQB1*02:02	0.036	**0.200**	6.750	0.008	n.s

The highest frequency for each allele is marked in bold. OR: odds ratio. Pc: P corrected by Bonferroni. n.s: not significant.

Due to the previous result, we added the study of class II haplotypes. We detected 13 haplotypes with more than 2% of frequency (Supplementary Table S3). The HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 and HLA-DRB1*15:01~DQA1*01:02~DQB1*06:02 haplotypes were more frequent in G3, whereas HLA-DRB1*01:01~DQA1*01:01~DQB1*05:01 was more frequent in G1.

When the frequencies of HLA-class II haplotypes were compared between G1 and G3, HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 was statistically significant after correction with a higher frequency in G3 (Table 3). The frequencies of HLA-DRB1*01:01~DQA1*01:01~DQB1*05:01 and HLA-DRB1*15:01~DQA1*01:02~DQB1*06:02 did not show significance after Bonferroni correction (Table 3).

Table 3. Comparison of haplotype frequencies.

HAPLOTYPE	FREQUENCY IN LOW RESPONDERS	FREQUENCY IN HIGH RESPONDERS	OR	P	Pc
HLA-DRB1*01:01~DQA1*01:01~DQB1*05:01	0.107	0.017	0.141	n.s	n.s
HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02	0.018	0.200	13.750	2.1E-3	**0.028**
HLA-DRB1*15:01~DQA1*01:02~DQB1*06:02	0.054	0.133	2.718	n.s	n.s

The highest frequency for each allele is marked in bold. OR: odds ratio. Pc: P corrected by Bonferroni. n.s: not significant

This result was in line with the allelic comparison and gives more value to HLA-DRB1*07:01.

Finally, we compared the mean anti-S antibody titers with to the presence or absence of HLA-DRB1*07:01, HLA-DRB1*01:01, and HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 in the volunteers studied (Figure 1). The presence of HLA-DRB1*07:01 was related to higher average levels of anti-S antibodies ($p = 0.002$), whereas the presence of HLA-DRB1*01:01 did not show significant differences. The comparison between cases with HLA-DRB1*07:01 versus cases with HLA-DRB1*01:01 showed a significant increase in anti-S anti-bodies in HLA-DRB1*07:01 carriers ($p= 0.004$). In addition, in cases with HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 we observed a higher anti-S antibody production versus subjects lacking this haplotype ($p = 0.004$) (Figure 1).

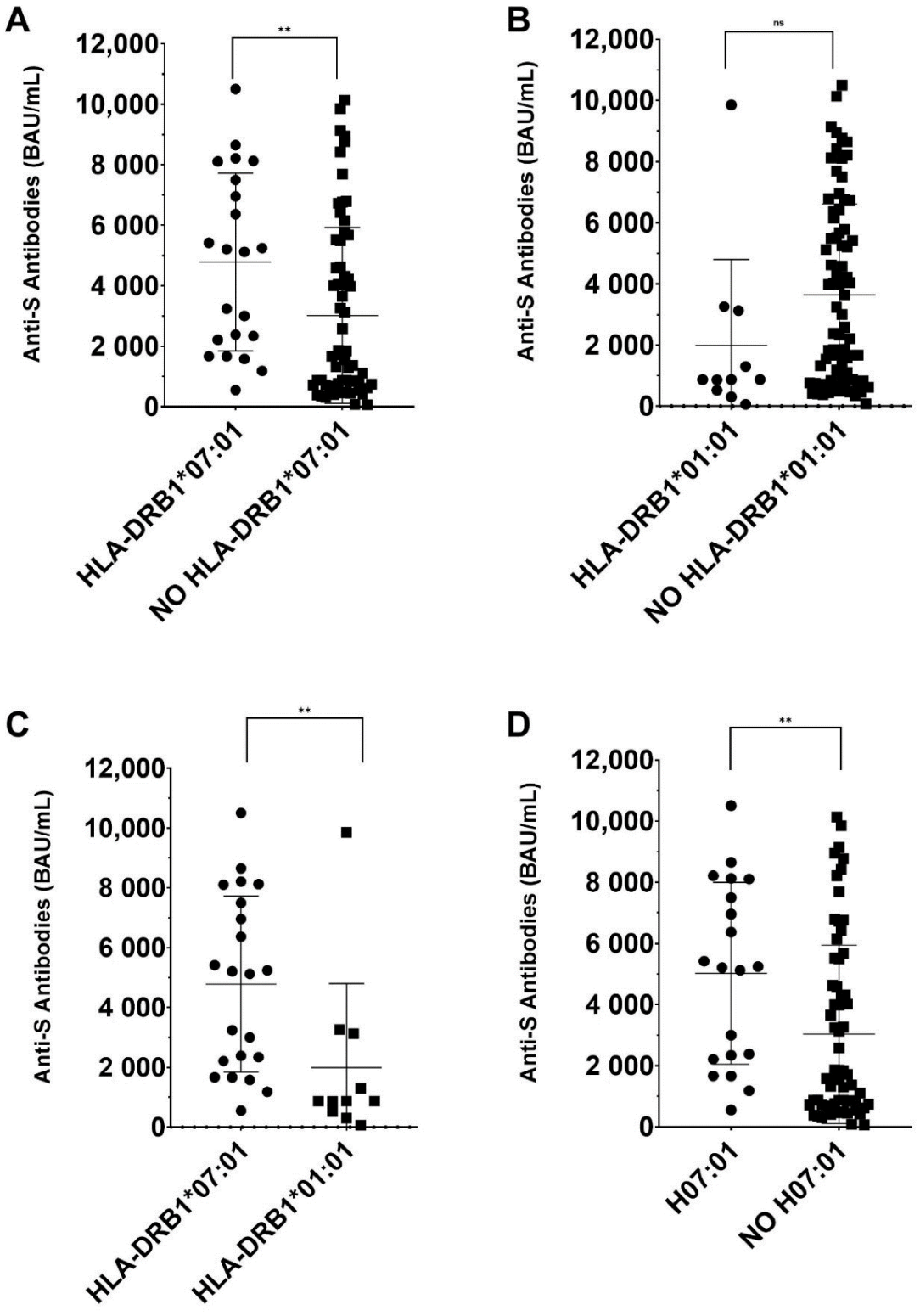

Figure 1. Anti-S antibodies mean comparations. Comparison of the mean production of anti-S antibodies depending on the presence or absence of certain alleles and haplotypes. (A) Cases with HLA-DRB1*07:01 versus cases without HLA-DRB1*07:01. (B) Cases with HLA-DRB1*01:01 versus cases without HLA-DRB1*01:01. (C) Cases with HLA-DRB1*07:01 versus cases with HLA-DRB1*01:01. (D) Cases with HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 haplotype (H07:01) versus cases without HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 haplotype (NO H07:01). ** = $p < 0.01$. ns= no significant.

We performed the comparison between HLA-DRB1*07:01 and HLA-DRB1*01:01 because of the results of the previous comparisons and because they are the alleles with the greatest frequency difference between groups.

DISCUSSION

The different production of antibodies against the S protein SARS-CoV-2 protein S in vaccinated people may be due to immunogenetic factors. Our results show that the HLA-DRB1*07:01 allele is significantly increased in the high responder's group, whereas the HLA-DRB1*01:01 allele is close to significantly increased in the low responder's group (Table 2). The HLA-DQB1*02:02 allele is close to being significant, being increased in high responders. This may be due to the haplotype that it forms together with the HLA-DRB1*07:01 allele. The haplotype study showed a significant increase for the HLA-DRB1 *07:01~DQA1*02:01~DQB1*02:02 haplotype in high responders. In addition, there is a significant difference in the mean production of anti-S antibodies in volunteers that are carriers of HLA-DRB1*07:01 and the haplotype HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02, with a higher level of anti-S antibodies (Figure 1). HLA-DRB1*01:01 does not influence the anti-S antibody production, but the subjects with HLA-DRB1*01:01 have lower anti-S antibody titers than volunteers with HLA-DRB1*07:01 (Figure 1).

The different ability of distinct HLA-class II alleles to present peptides derived from the S protein is a possible explanation for the wide range of anti-S antibody production observed in our study. The HLA-DRB1*01:01 allele has a lower ability to strongly bind S protein-derived peptides compared to the HLA-DRB1*07:01 allele [13]. HLA-DRB1*01:01 presents only five peptides with high affinity, whereas HLA-DRB1*07:01

presents 16 [13]. In addition, the HLA-DRB1*01:01 allele lacks an associated HLA-DRB3, DRB4, or DRB5 molecule, having one less HLA-class II presenting molecule [14]. HLA-DRB1*07:01 presents an HLA-DRB4 molecule associated with the haplotype [14]. Hence, the HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02, which is associated with a better response, may be able to activate a greater number of clones of distinct T cell clones resulting in a stronger humoral response, including higher production of antibodies against SARS-CoV-2 [9,15]. In contrast, the HLA-DRB1*01:01 allele and the HLA-DRB1*01:01~DQA1*01:01~DQB1*05:01 haplotype, have alleles with lower presentation capacity together with the absence of an HLA-DRB3, DRB4, or DRB5 molecule. This would induce a low stimulation of T cells, and therefore a lesser activation of B cells, leading to lower antibody production. In addition, HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 binds 30–34 peptides with high affinity, whereas HLA-DRB1*01:01~DQA1*01:01~DQB1*05:01 only binds to 16 peptides with high affinity. [13]. Moreover, HLA-DRB1*15:01 is the allele that presents more peptides with high affinity (26) [13] and it can be observed that its maximum frequency is found in high responders (Table 1). Moreover, HLA-DRB1*15:01~DQA1*01:02~DQB1*06:02, more frequent in high responders, binds to between 47 and 62 peptides with high affinity [13].

On the other hand, it is possible that the HLA-DRB1*01:01 allele is capable of generating a regulatory T cell response (Treg) that negatively regulates the immune response and stops further expansion and high antibody production [16]. However, this would be contrary to what is observed in patients with rheumatoid arthritis, where the presence of this allele poses a risk to the development of the disease with a dysregulation of the immune system [17]. From this, it can be thought that the HLA allele is as important as

the peptide that is presented, observing differences in the induction and regulation of an immune response depending on it.

A factor that may explain why the HLA-DRB1*07:01 allele induces greater production of antibodies is that it presents an immunodominant peptide of protein S, which induces a greater differentiation of Tfh and high cooperation of B and T cells [18]. Related to this, it has been suggested that the HLA-DRB1*07:01 allele presents exogenous peptides that may favor the development of monoclonal TCR-Vβ13.1 + /CD4 + /NKa + /CD8−/+ dim T-LGL lymphocytosis by chronic stimulation [19].

The HLA-DRB1*07:01 allele has been linked as a risk factor for systemic lupus erythematosus (SLE) in the Malaysian population [20], whereas in the Chilean population, HLA-DRB1*07:01 had a protective role in anti-citrullinated protein antibodies-positive rheumatoid arthritis [21]. Moreover, HLA-DRB1*07:01 and the HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 haplotype are related to a higher risk of asparaginase hypersensitivity [22,23].

Finally, the presence of other possible polymorphisms in cytokines, receptors, etc., associated with the haplotype in the MHC region, may play an important role in the induction of the immune response and should be studied.

The results published by Ragone et al. showed that there is no relationship between the level of antibodies in the short or medium term with the number of peptides bound with high affinity in each individual vaccinated with BNT162b2 (BioNTech/Pfizer) [13]. They concluded that the level of antibodies is unrelated to HLA-class II molecules. In contrast, our results show a possible association of the HLA-DRB1*07:01 allele, and HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 haplotype, with a higher production of antibodies. The differences between both works are because they carry out a quantitative

and theoretical study of the number of peptides that each allele presents with high affinity, whereas our study is based on traditional analysis of allele frequencies. However, it is possible that this different result is due to the vaccine studied but there are other reasonable explanations. For example, the excipients are different in the vaccines and the mRNA-1273 dose has a higher concentration of mRNA [24]. In addition, the mRNA-1273 vaccine induces a higher antibodies response than the BNT162b2 vaccine [24]. Finally, perhaps the mRNA-1273 vaccine induces a better stimulation of the immune system and higher antibody production that makes it possible to observe differences in the HLA-class II frequency.

In conclusion, our results show the first possible association between the circulating levels of anti-S antibodies induced by mRNA-1273 and HLA-class II alleles. However, complementary studies, a larger population will be necessary to confirm our results. In addition, it will be interesting to relate the neutralizing potential of the antibodies to the different HLA-class II alleles. Furthermore, will be interesting to compare the HLA-class II frequency between high and low responders in individuals vaccinated with mRNA-1273 versus individuals vaccinated with other vaccines. Finally, the study of the different peptides derived from protein S will be of great interest to clarify their immunological potential and the possible selection of immunodominant peptides.

Limitations of our work include the small population studied, not performing the neutralization study, and not comparing between vaccines.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vaccines10030402/s1 , Supplementary Figure S1, Supplementary Table S1, Supplementary Table S2, and Supplementary Table S3.

Author Contributions: M.Á.L.-N. and A.S. contributed to the design of the study. J.F.G.-B. performed the HLA typing. J.F.G.-B. performed the analysis of the data obtained by NGS. E.G.-V. and A.S. build the clinical database. J.F.G.-B. performed the statistical analysis of the data. M.Á.L.-N., A.S., J.F.G.-B., F.C. and E.G.-V. coordinated the collection of samples and the signing of the informed consent. A.S., J.A.R., and E.G.-V. participated in checking the clinical history of the participants. J.R.-G. and E.G.-V. performed the determination of antibodies. J.F.G.-B., M.Á.L.-N., F.R.-C. and A.S. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All patient samples were collected according to the local medical ethics regulation, after informed consent was obtained by the subjects, their legal representatives, or both, according to the Declaration of Helsinki. The study was approved by the local ethics committee (CEIM/CEI Provincial de Granada).

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DISCUSIÓN

1. Estudio funcional de las subpoblaciones linfocitarias en COVID-19

La infección por SARS-CoV-2 produce un trastorno inmunitario que afecta principalmente a los linfocitos T. La linfopenia es uno de los factores que explica la inadecuada respuesta del sistema inmunológico frente al SARS-CoV-2. Se han descrito variaciones en las poblaciones de linfocitos T vírgenes, memoria y efectores, así como de expresión de marcadores de activación o agotamiento en linfocitos T CD4 y CD8 (117). En nuestro estudio, hemos realizado un análisis exhaustivo del subconjunto de linfocitos Th y células CD8. Además, analizamos el estado de activación y aparición de marcadores de agotamiento en linfocitos T. Nuestros datos muestran un profundo deterioro de la inmunidad de las células T que puede ser explicado por 4 razones: I) El grado de linfopenia; II) Una mayor proporción de células en estado efector no funcional (Th0), lo que indica una hipoactivación del sistema inmunitario; III) Reducción importante de los linfocitos Th1; IV) Activación insuficiente determinada mediante la expresión de HLA-DR y CD38, que en muchos casos es similar a la observada en el grupo control.

La linfopenia afecta a linfocitos T, mostrando diferencias significativas entre los pacientes hospitalizados no ICU (unidad de cuidados intensivos) y los pacientes hospitalizados ICU frente al grupo control y los donantes recuperados asintomáticos. El menor porcentaje de linfocitos T lo encontramos en los pacientes de ICU. La linfopenia afectaba principalmente a las poblaciones de linfocitos T CD4+, con reducción en número absoluto de las subpoblaciones Th1, Th2, Th17, Th1/Th17, Th22, células Tfh y Tregs, destacando una reducción aproximada del 10% en la proporción de células Th1. Esta disminución en linfocitos efectores puede explicarse por un aumento en la frecuencia de linfocitos vírgenes Th0. Este aumento de linfocitos T vírgenes se observó tanto en

pacientes hospitalizados no UCI como en hospitalizados UCI, y ha sido referido en otros estudios (118). Una posible explicación al aumento de los linfocitos T vírgenes, es un proceso regenerativo en respuesta a la linfopenia, con un aumento de la salida de linfocito Th0 del timo. De otro modo, podría corresponder a un bloqueo en la estimulación necesaria para su diferenciación por parte de las células dendríticas. En este sentido, se ha descrito una activación disfuncional de las células dendríticas en infectados por SARS-CoV-2, lo que resultaría en la no diferenciación de linfocitos Th0, apoptosis y depleción de linfocitos T (119).

La linfopenia ha sido puesta de manifiesto en numerosos estudios sobre la infección por SARS-CoV-2, pero las causas que la producen aún no están del todo claras y pueden deberse a la infección directa de los linfocitos o a la supresión de la médula ósea por la respuesta antiviral. Se han postulado desde efectos citopáticos causados por el virus (120), que han sido cuestionadas (8), hasta posibles trastornos metabólicos (121). Desde un punto de vista inmunológico, la linfopenia podría deberse a la activación disfuncional de las células dendríticas (119), junto a la alta concentración de citoquinas como TNF-α, IL-6 e IL-10, que actúan como reguladores negativos de la proliferación y supervivencia de los linfocitos T (122). En este sentido, detectamos una correlación inversa entre el recuento de linfocitos y el de neutrófilos. Es muy posible que esta linfopenia esté provocada por factores desencadenados durante la exacerbación de la respuesta inmune innata, con la producción de las citoquinas anteriormente citadas y la elevación de los parámetros inflamatorios.

Como principal hallazgo del estudio de subpoblaciones linfocitarias, destacamos la baja frecuencia del componente celular Th1, que puede afectar negativamente a las infecciones virales a varios niveles, como es el caso del SARS-CoV-2. Los linfocitos Th1 son de vital

importancia en la eliminación de microorganismos intracelulares como micobacterias y virus. Su disminución puede tener graves consecuencias en el control de la infección por SARS-CoV-2 y la resolución de ésta. Los linfocitos Th1, a través de la producción de las citoquinas IL-2 e IFN-γ, participan en la activación, proliferación y diferenciación de los CTL y en la inducción de citotoxicidad celular de las células infectadas (113). La deficiencia en Th1 conduciría a una disminución en el número de CTL activos y, por lo tanto, una respuesta inmune adaptativa deficiente a la infección viral. Además, a diferencia de los pacientes de SARS-CoV, los pacientes COVID-19 también presentan niveles elevados de citoquinas Th2, como IL-4 e IL-10, que inhiben las repuestas inflamatorias Th1 (123). Otros estudios muestran hallazgos similares (124–126). Sin embargo, estos estudios se basan en una población menor y se centran en células productoras de IFN-γ, o en las células memoria efectoras o vírgenes. Además, estos estudios no distinguen entre las poblaciones de células Th1 y Th1/Th17, y no realizan un análisis completo de los subconjuntos Th.

Se ha observado una respuesta inmunitaria Th1 específica reducida (menor proporción de células productoras de IFN-γ) contra distintos antígenos del virus y se relacionó con la edad y las comorbilidades (127). Una posible explicación a esta disminución en el componente Th1, es la proteína pluriempleo CD26/DPP4 que se sobre expresa en linfocitos Th1, especialmente en población envejecida. SARS-CoV-2 interaccionaría con dicha proteína produciendo el bloqueo en Th1 y llevando a una disminución de estas células y de las CTL, lo que conduciría a una respuesta de tipo Th2, que no sería capaz de controlar la infección viral (128).

En relación a los niveles de activación, demostramos una disminución de marcadores de activación (HLA-DR y CD38) en linfocitos T CD8, lo cual puede estar relacionado con la deficiencia en la población Th1. Al igual que ocurre con la activación de las células T

CD8, los linfocitos Th1 intervienen en la desarrollo de anticuerpos, especialmente en el cambio de isotipo a inmunoglobulina G (IgG) (129). En este sentido, se ha descrito que las infecciones humanas por coronavirus en ocasiones no logran generar inmunidad protectora debido a una pobre respuesta inmune adaptativa (130–132). Este déficit de inmunidad puede estar generado por una respuesta de células T de corta duración o de baja intensidad, interviniendo negativamente en la generación de anticuerpos neutralizantes (133,134). Por ello, Grifoni y colaboradores, demostraron que la respuesta de anticuerpos al SARS-CoV-2 se correlacionó positivamente con la magnitud de la respuesta Th1 (135).

Utilizando el fallecimiento en infectados por SARS-CoV-2, investigamos la relación entre la respuesta inmunitaria y la gravedad de la enfermedad. Los supervivientes presentaban significativamente mayores niveles de linfocitos, linfocitos T, linfocitos T CD4+ y linfocitos T CD8+. Además, los pacientes fallecidos presentaban niveles de marcadores de activación HLA-DR y de agotamiento PD-1 (ligando 1 de muerte programada), aumentados en las poblaciones de linfocitos T CD4+ y, linfocitos T CD4+ y CD8+, respectivamente. El marcador PD-1 es un marcador de agotamiento característico de linfocitos T y se encuentra elevado en situaciones en la que encontramos una estimulación continua, como pueden ser infecciones crónicas o enfermedades neoplásicas (136). En nuestro caso, puede que el SARS-CoV-2 esté estimulando de manera continua a los linfocitos T, aumentando la inducción del estado de agotamiento en estas células (122).

La expresión del marcador de activación HLA-DR en linfocitos T CD4+ y T CD8+ en pacientes fue nula o mínimamente significativa. Datos que están en consonancia con los publicados por Mathew y colaboradores (117). Estos autores muestran que la respuesta inmune es bastante diversa y, por lo tanto, mientras un subgrupo de pacientes tenía una

activación de células T característica de la infección viral aguda, otro subgrupo tenía una activación de linfocitos comparable a los sujetos no infectados. Esta falta de activación contrasta con la observada en el curso de otras infecciones virales, que es muy prolongada y estable en el tiempo (137–139). Sin embargo, observamos que aquellos pacientes que presentaban niveles de activación más elevados sufrían un curso de la enfermedad más agresivo. Encontramos que los pacientes con neutrofilia, linfopenia y alteración más significativa de los parámetros bioquímicos asociados a inflamación, presentaban una mayor proporción de linfocitos T CD4+ con expresión de los marcadores de activación HLA-DR y CD38. Es posible que la presencia de estos marcadores de activación en los pacientes con linfopenia más intensa se deba a una expresión inducida por citoquinas inflamatorias y, por tanto, no se trate de una activación antígeno específica, sino más bien como una consecuencias de la inflamación desregulada y el reclutamiento de células mieloides (117). De ahí, su observación en el contexto de neutrofilia. El aumento del estado inflamatorio induce la producción del factor de crecimiento de colonias de granulocitos (G-CSF) junto a la expresión de quimiocinas y un aumento en la actividad de los neutrófilos (140). Por ello, la relación positiva que describimos entre los neutrófilos y las proteínas de la fase aguda puede explicarse por este aumento del estado inflamatorio (141). Además, la producción de las proteínas de fase aguda como la ferritina y la CRP, pueden afectar a las vías pro y anticoagulante (aumentando el dímero D) (142,143) e inducir la apoptosis de los linfocitos (123,144).

En lo referente al estudio de las curvas ROC, se encontró que las concentraciones de troponina I, CRP, dímero D y LDH en el momento del ingreso hospitalario pueden utilizarse como predictores de muerte. La determinación de linfocitos T HLA-DR+CD4+ o PD-1+CD4+ también son útiles para predecir una enfermedad más grave o riesgo de fallecimiento. Por último, nuestros resultados también indican que una puntuación SOFA

(evaluación de insuficiencia orgánica relacionada con sepsis) elevada en el momento del ingreso se asocia con un pronóstico desfavorable.

2. *Factores inmunogenéticos en la respuesta inmunitaria frente a SARS-CoV-2: HLA y MICA*

El sistema inmunitario está formado por multitud de células y moléculas. La inmunogenética es uno de ellos y puede intervenir en la respuesta a la infección por SARS-CoV-2. Dentro de la inmunogenética nosotros hemos centrado nuestros estudios en las moléculas HLA y MICA, moléculas altamente polimórficas que intervienen en la inmunidad adaptativa e innata, respectivamente.

En relación a las moléculas HLA, nuestros resultados sugieren que no existe una correlación significativa entre los alelos y haplotipos HLA con la susceptibilidad o protección contra la COVID-19, lo cual está en consonancia con otros estudios (145). No obstante, numerosos estudios han informado de alelos de protección o susceptibilidad al COVID-19. Un estudio realizado con 82 pacientes chinos encontró que los alelos HLA-C*07:29 y HLA-B*15:27 se detectaron con mayor frecuencia en el grupo COVID-19 que en la población de control (146). Novelli y colaboradores, encontraron que los alelos HLA-B*27:07, -DQB1*06:02 y -DRB1*15:01 estaban significativamente aumentados en un grupo de 99 pacientes italianos con COVID-19 en comparación con el grupo control (147). Además, los alelos HLA-B*44 y -C*01 fueron asociados positiva e individualmente con COVID-19 en la población italiana (148). Por otro lado, el alelo HLA-A*02:01 fue informado como posible asociación positiva con el riesgo de sufrir COVID-19 (149). Otro estudio, con una población de 190 pacientes chinos, encontró una correlación positiva entre el serotipo HLA-B22 y la susceptibilidad al COVID-19 (150). Todos estos estudios mencionados, fueron realizados con un número menor de pacientes en comparación a

nuestro trabajo, lo que podría ser una de las principales razones por las que no detectamos en nuestra población estas asociaciones anteriores. Además, algunos de estos trabajos, como los de Correale y colaboradores (148) y, Tomita y colaboradores (149), no realizaron el tipaje HLA de la población estudiada, sino que utilizaron las frecuencias alélicas en ciertas regiones geográficas junto a la incidencia de COVID-19 en estos territorios. Un punto importante a favor a nuestro estudio, es que nuestra cohorte de pacientes fue una de las mayores en el momento de la publicación.

En la bibliografía se pueden encontrar distintos estudios de la relación entre los alelos HLA y el desarrollo de COVID-19, con resultados similares o contradictorios. Los alelos HLA-A*11:01, -C*04:01 y -DQA1*01:02 se han asociado con peor evolución de la enfermedad (151). Por otro lado, los alelos HLA-A*11, -C*01, -DQB1*04 (152,153) y -DQB1*08 (154) han sido asociados con una mayor mortalidad entre los pacientes con COVID-19. En el caso del alelo HLA-C*05, ha sido asociado con riesgo de muerte (155) o con un papel protector (156) en distintos trabajos. Es interesante notar las discrepancias entre los distintos estudios con respecto a la protección, riesgo o el desarrollo de COVID-19, pudiendo ser el origen étnico de los pacientes e individuos del grupo control lo que esté causando estas discrepancias.

Los estudios in silico han identificado alelos que presentan de formas más o menos eficiente péptidos conservados del SARS-CoV-2. Los alelos HLA-A*02:02, B*15:03 y C*12:03 son considerados como los mejores presentadores de péptidos conservados de SARS-CoV-2, mientras que los alelos HLA-A*25:01, B*46:01 y -C*01:02 son malos presentadores (157). Estos datos fueron confirmados por La Porta y colaboradores, quienes además, identificaron a HLA-A*11:01 como un buen presentador (158). El estudio realizado por Barquera y colaboradores, corroboró los resultados anteriores y

además añadió los alelos HLA de clase II que eran buenos presentadores (HLA-DRB1*01:01, -DRB1*10:01, DRB1*11:02 Y DRB1*13:01) y malos presentadores (HLA-DRB1*-03:02, -DRB1*03:03 y DQA1*01:02/DQB1*06) de péptidos derivados del SARS-CoV-2 (159).

Es lógico pensar que los pacientes que requieren hospitalización deben tener alelos HLA que no unen fuertemente péptidos inmunogénicos del SARS-CoV-2, lo que resultaría en un control ineficaz de la infección por parte del sistema inmunológico. Así ha sido descrito por Iturreta-Zuazo y colaboradores, quienes determinaron que los pacientes con enfermedad leve tienen alelos HLA con mayor capacidad de unión teórica a los péptidos del SARS-CoV-2 (160).

Sin embargo, nuestros resultados mostraron que el alelo HLA-A*25:01, que presenta péptidos de SARS-CoV-2 de forma deficiente, estaba poco representado en los pacientes críticos. Estos resultados, junto a los publicados por Wang y colaboradores, nos hicieron postular una hipótesis, en la que los alelos con menor capacidad para presentar péptidos derivados del SARS-CoV-2 podrían estar relacionados con un papel protector.

Las manifestaciones clínicas en pacientes graves por COVID-19 se caracterizan por neumonía unilateral o bilateral, con un estado de hiperinflamación por activación de las vías IL-1 o IL-6 que puede evolucionar a una tormenta de citoquinas (161). Como se ha comentado anteriormente, en los casos más severos encontramos un aumento de citoquinas proinflamatorias (IL-1β, IL-6, IL-12, TNF-α e IFN-γ) (107), linfopenia, y aumento de la activación linfocitaria. Así mismo, el aumento de la activación de linfocitos T CD8+ (162), posiblemente en un contexto de elevación de citoquinas, puede causar un gran daño en el epitelio pulmonar, dando lugar a hipoxemia, hipotensión e incluso shock (161). Por ello nuestra hipótesis es que una presentación eficiente de las proteínas

derivadas del SARS-CoV-2 aumentaría la respuesta de las células T CD8+ citotóxicas, aumentando el daño tisular en los pulmones y elevando el riesgo de muerte en los pacientes graves por COVID-19. Además, se produciría un aumento de los linfocitos Th, favoreciendo aún más el estado inflamatorio. Todo esto enlaza con la presencia de respuestas de células T más amplias y fuertes en pacientes con enfermedad graves (161), lo que puede deberse a una carga viral elevada por un mal control de la inmunidad innata en las fases tempranas de infección (161), una respuesta de células T temprana insuficiente o a un mejor reconocimiento del virus por parte de los linfocitos.

No obstante, nuestro resultados sugieren que no existe relación entre COVID-19 y los polimorfismos HLA, respaldados por el estudio de supertipos y el análisis de aminoácidos, lo que indicaría un papel irrelevante de las moléculas HLA en el riesgo de infección por COVID-19, lo que está en consonancia con un estudio de asociación de genoma completo (GWAS) (163). Este GWAS identificó un polimorfismo en IFNAR2 (subunidad 2 del receptor de interferón α y β) relacionado con COVID-19 (163). Por ello, creemos que la inmunidad innata es crucial para el control de la infección viral, y el interferón juega un papel muy importante (164). El virus posee numerosos mecanismos que le ayudan a evitar el sistema inmunitario innato y bloquear una respuesta eficaz por parte del interferón (165). Una mala respuesta en los estadios iniciales de la infección conllevaría un aumento de la carga viral y un mal pronóstico de la enfermedad (166). El papel de las moléculas HLA en COVID-19 sería el de generar respuestas robustas de células T en casos donde la inmunidad innata ha logrado controlar la infección de manera eficiente. Sin embargo, en los pacientes más graves, donde es muy posible que la carga viral sea alta y se presente un estado hiperinflamatorio, las proteínas HLA que mejor presentan los péptidos virales podrían empeorar el estado del paciente al inducir una

mayor activación de linfocitos Th y CD8+ citotóxicos, contribuyendo al aumento del estado inflamatorio y del daño tisular.

Por último, nuestros datos no muestran valores significativos para el estudio de los haplotipos. Sin embargo, un estudio italiano encontró una correlación significativa positiva entre el haplotipo HLA-A*01:01, -B*08:01, -C*07:01 y DRB1*03:01 y la incidencia y muerte por COVID-19 (haplotipo de susceptibilidad) (167). Además, identificaron al haplotipo HLA-A*02:01, -B*18:01, C*07:01 y DRB1*11:04 como haplotipo protector al estar relacionado inversamente con la incidencia y muerte por COVID-19 (167). Otro estudio realizado en Cerdeña identificó al haplotipo HLA-A*25:01, -B*58:01, -C*07:01 y DRB1*03:01 como protector (168). Finalmente, el estudio realizado por Wang y colaboradores, mostró que el haplotipo HLA-A*11:01, -B*51:01 y C*14:02 se asociaba a enfermedad más grave por SARS-CoV-2 (169). En nuestro estudio, el haplotipo HLA-A*29:02, B*44:03, C*16:01, DRB1*07:01 y DQB1*02:02 estuvo cerca de la significancia, presentando casi dos veces más frecuencia en el grupo de hospitalizados por COVID-19 que el grupo control. Curiosamente, la frecuencia de este haplotipo fue significativamente mayor (sin corrección) en los pacientes de la ICU que en el grupo control. Estos datos pueden sugerir que este haplotipo está relacionado con la gravedad y el ingreso a ICU. La frecuencia de este haplotipo es de un 6,2% en la población española (170), lo que puede ayudar a explicar la alta incidencia de gravedad y mortalidad que ha sufrido el país.

En base a los resultados de nuestro estudio, sin tener en cuenta la posibilidad de que ciertos alelos puedan modificar el curso clínico de la enfermedad, podemos concluir que el polimorfismo HLA no es un factor determinante en el riego de infección por COVID-19. Además, las comparaciones a nivel alélico realizadas por Ellinghaus y colaboradores con población italiana y española, refuerzan aún más nuestros hallazgos (171). Por

último, nuestros resultados concuerdan con los publicados por Shachar y colaboradores en población iraní, donde no encontraron relación entre los polimorfismos HLA ni haplotipos con la infección por SARS-CoV-2 (172).

Siguiendo con el papel de la inmunogenética, estudiamos las moléculas MICA y su relación con la infección por SARS-CoV-2. La COVID-19 ha mostrado distintos fenotipos clínicos, los cuales se pueden clasificar en asintomático, enfermedad moderada, grave o muerte (45). Estas diferencias que observamos entre individuos pueden deberse a factores de la inmunidad innata. Dentro de la inmunidad innata frente a virus, tienen un papel importante las células NK. Por ello, hemos intentado identificar un posible papel de las células NK estudiando los distintos alelos MICA. Las células NK pueden presentar variaciones en su respuesta a MICA debido a los polimorfismos en su región transmembrana (173). Por tal motivo, los polimorfismos STR de la región transmembrana de MICA se han relacionado son la susceptibilidad o protección frente a diversos agentes infecciosos (85,174).

Nuestros resultados muestran una mayor frecuencia del alelo MICA*A9 en pacientes con COVID-19 frente al grupo control. Además, MICA*A9 fue mayor en pacientes sintomáticos que en asintomáticos, y la frecuencia más alta se encontró en pacientes con enfermedad moderada. Sin embargo, no encontramos significación en el grupo de pacientes graves, posiblemente, por el bajo número de casos estudiados. Adicionalmente, realizamos la comprobación de que la asociación de MICA era independiente de HLA-B por desequilibrio de ligamiento, demostrándose que esta asociación de MICA era independiente de HLA-B.

El dominio extracelular de la proteína MICA presenta un polimorfismo en la posición 129, la cual es importante en la interacción con su receptor NKG2D. La variante MICA

V129 (valina en 129) está asociada con una afinidad reducida por NKG2D, mientras que M129 (metionina en 129) confiere una alta afinidad de unión a NKG2D (175). Todos los alelos MICA*A9 presentan el polimorfismo M129, formando un halogrupo que presenta alta afinidad por el receptor NKG2D (176).

La infección por SARS-CoV-2 genera más sMICA por la sobreexpresión de la metaloproteinasa ADAM17 tras la interacción de la proteína S con ACE2, lo que disminuye la activación de las células NK y por lo tanto una disminución de la respuesta innata (177). Esta evasión inmune será más efectiva en individuos con alelos MICA que tengan alta afinidad por NKG2D, como MICA*A9, induciendo una inhibición mayor de las células NK y linfocitos T, permitiendo una mayor replicación y dispersión del virus.

El trabajo presentado por Castelli y colaboradores, informó de la asociación de la variante MICA rs2596541 con pacientes sintomáticos (178). Esta variante aumenta los niveles de mRNA y pueden favorecer el aumento de sMICA. Además, observaron que los alelos MICA*008 y MICA*019, que están en desequilibrio de ligamiento con rs2596541, aumentaron en pacientes sintomáticos (178). El alelo MICA*008 está asociado con el polimorfismo STR MICA*A5.1, mientras que el alelo MICA*019 lo está con MICA*A5 (179). Nuestros resultados no mostraron resultados significativos asociados con síntomas o gravedad en ninguno de estos polimorfismos STR. Sin embargo, cabe señalar que cada uno de los polimorfismos STR de MICA, presentan más de un alelo MICA asociado (179).

Por otra parte, el grado de expresión de MICA puede estar relacionado con el polimorfismo del gen MICA del huésped (180). Sin embargo, las infecciones por virus pueden interferir con la expresión de MICA. Por ejemplo, en la infección por citomegalovirus (HCMV), algunos alelos MICA están regulados a la baja mientras que

MICA*008 no lo está. MICA*008 está en desequilibrio de ligamiento con MICA*A5.1. Por lo tanto, los efectos citotóxicos de las células NK contra las células infectadas con HCMV fueron más fuertes en individuos portadores de MICA*A008/MICA*A5.1 (180). Además, la proteína UL142-HCMVregula a la baja la expresión de proteínas MICA que tienen una gran región transmembrana, pero no aquellas que tienen región transmembrana corta (89). El SARS-CoV-2 actúa de manera similar, lo que lleva a que los portadores de MICA*A9 tengan mayor riesgo de infección y enfermedades sintomáticas.

El alelo MICA*A9 se ha asociado con una gran diversidad de enfermedades. Se ha informado de una menor frecuencia de MICA*A9 en los infectados por el virus sincitial respiratorio (RSV) que en controles (175). La presencia de MICA*A9 en heterogosidad parece proteger contra la infección por Chlamydia trachomatis (176). De igual manera, se ha asociado al alelo MICA*A9 con enfermedades de etiología autoinmune (177,179–181).

Respecto a las células NK, los estudios en pacientes con COVID-19 mostraron niveles elevados de células NK agotadas. Estas células NK expresan niveles elevados de PD-1 y del receptor A miembro del grupo 2 de NK (NKG2A) (181,182). El receptor NKG2A se trata de una de las moléculas inhibidoras más importantes de las células NK (183). Por otra parte, otros polimorfismos de células NK están relacionados con COVID-19. Los receptores KIR son una familia de glicoproteínas transmembranas altamente polimórficas que inducen señales inhibitorias o activadoras a las células NK y linfocitos T tras el reconocimiento de sus correspondientes ligandos (184). Diversos estudios han demostrado que la molécula KIR2DS4 está relacionada con infección grave en pacientes con COVID-19 (185,186).

MICA, al igual que los otros factores ya mencionados, no solo es importante para el correcto funcionamiento de la inmunidad innata, sino también en la inmunidad adaptativa. Esto es debido a que las células NK modulan las respuestas adaptativas gracias a la producción de IFN-γ e IL-12, las cuales inducen la activación de las células T CD4+ y su diferenciación a células Th1 (187,188). Esto refuerza los bajos niveles de linfocitos Th1 encontrados en los pacientes COVID-19 en el estudio de las subpoblaciones linfocitarias de este estudio (189).

Nuestro grupo participó con el consorcio español "Spanish Coalition to Unlock Research on Host Genetics on COVID-19" (SCOOURGE). Este grupo encontró mediante GWAS, loci relacionados con la inmunidad innata y la gravedad de la enfermedad (190). El estudio reprodujo las asociaciones previamente descritas en 3p21.31 (cerca *de SLC6A20* y *LZTFL1-FYCO1*) y en 21q22.11 (en *IFNAR2*) (190). Como novedad, se encontraron señales significativas en las regiones 19p13.12 (intergénica con *UPK1A* y *ZBTB32*, también vinculada a la región transcripcional de *ARHGAP33*), y otra en el cromosoma 9p13.3 (intergénica con *AQP7* y *AQP3*) (190). En la región 19p13.12, los marcadores más plausibles son *ARHGAP33* y *ZBTB32*. *ARHGAP33* está regulado transcripcionalmente por el factor regulador de interferón 1 (*IRF1*), un importante efector antiviral y un gen estimulador por IFN. Por el lado de *ZBTB32*, se ha demostrado que altera la respuesta inmunitaria antiviral al intervenir en la producción de citoquinas, proliferación de células T y NK, además de la generación de células memoria (191). En lo referente al locus 9p13.3, los datos postulan al gen *AQP3* como el más probable e impulsa la asociación con la hospitalización por COVID-19 (190). El gen *AQP3* se expresa en pulmón entre otras localizaciones y codifica poros de transporte de agua entre las vías respiratorias y los capilares pulmonares (190). Por ello, *AQP3* puede influir en el aumento del volumen líquido en los espacios aéreos de los pulmones por un aumento en

el flujo alveolo-capilar, facilitando el desarrollo de SDRA y enfermedad grave (190). Además, de este estudio se determinó que las personas con un nivel más alto de homocigosis se asocian con un mayor riesgo de ser hospitalizados y desarrollar COVID-19 grave (190).

La asociación con la gravedad de la COVID-19 de los genes *ARHGAP33*, *ZBTB32* e *IFNAR2* refuerzan la importancia de una buena respuesta innata antiviral para mantener a raya al SARS-CoV-2 y evitar el desarrollo de enfermedad grave e ingreso hospitalario.

3. Factores inmunitarios e inmunogenéticos de la respuesta a la vacunación

Para controlar la pandemia ha sido necesario el desarrollo de vacunas para potenciar al sistema inmunitario frente al SARS-CoV-2. Realizamos una evaluación de la respuesta humoral y celular a la vacunación frente a SARS-CoV-2 con la vacuna mRNA-1273 y del papel de las moléculas HLA en la respuesta humoral. Nuestros resultados mostraron una respuesta humoral con la producción de anticuerpos IgG anti-S en el 100% de la población estudiada hasta 3 meses después de la administración de la segunda dosis de la vacuna. Estos resultados están en consonancia con los publicados por Doria-Rose y colaboradores, donde encuentran niveles de anticuerpos mantenidos hasta 6 meses después de la vacunación en un grupo de 33 pacientes (192). Del mismo modo, otros estudios mostraron una respuesta de anticuerpos en el 99,9% de los vacunados con mRNA-1273 (193). Los resultados de la vacunación muestran mejores niveles de respuesta humoral que los reportados en casos de infección donde la seroconversión no alcanzó el 100% (194). En comparación con otras vacunas, se ha visto una respuesta del 92% con la vacuna BNT162b2 y una duración de hasta 8 meses en los niveles de anticuerpos en vacunados con Ad26.COV.2.S, lo que parece indicar una buena respuesta con las distintas vacunas.

Nuestros resultados muestran una disminución media del 64% en los niveles circulantes de anticuerpos entre el primer y tercer mes tras la pauta completa de vacunación. El nivel medio de anticuerpos baja a 992 BAU/ml a los 90 días, que está cerca de los respondedores normales (1000-3000 BAU/ml). Estos descensos pueden indicar que no es necesaria la presencia de un título tan elevado para brindar protección. Los niveles tan elevados encontrados en la primera determinación pueden deberse a la reciente estimulación antigénica tras la segunda dosis de la vacuna y a la alta concentración de mRNA que se inocula con la vacuna de Moderna mRNA-1273 (100 μg). Esta puede ser una de las razones que explique los niveles más altos de anticuerpos en población vacunada con mRNA-1273 que los encontrados en vacunados con BNT162b2 (195).

Los niveles de IgG anti-S aumentados que encontramos en la primera determinación, pueden corresponder al pico de producción de anticuerpos que se detecta entre las 5 y 7 semanas tras la infección (196,197). Del mismo modo, la diminución detectada a los 3 meses correspondería a una etapa de contracción del nivel de anticuerpos que comprendería entre las 6 y 14 semanas post infección (197). Esta cinética de anticuerpos sigue la descrita en personas que pasaron la infección, con un patrón que comienza con un pico, seguido de una fase de meseta y una posterior persistencia a niveles bajos, con una rápida disminución inicial seguida de un descenso posterior más lento (198,199).

La cinética observada es muy similar a las respuestas de anticuerpos contra otros virus, con una actividad máxima pocas semanas después de la infección, seguida de una fase de contracción durante varias semanas (197). La meseta y posterior fase de mantenimiento de anticuerpo continuaron hasta 26 semanas después de la infección (197). Aún es pronto para saber si estas vacunas de mRNA son capaces de inducir una inmunidad duradera, pero los primeros resultados muestran anticuerpos persistentes y funcionales hasta 6

meses después de la segunda dosis de mRNA-1273 (200). Se ha descrito la presencia de anticuerpos neutralizantes contra el SARS-CoV hasta 17 años después de la infección, lo que demostraría la capacidad del sistema inmunitario de generar inmunidad de larga duración contra especies de coronavirus (199).

En lo referente a la reducción del título de anticuerpos entre la primera y segunda determinación, puede deberse a la disminución de plasmablastos (201). Es sabido que la vida media de la IgG es de tres semanas. Por tanto, es necesaria su producción continua por parte de las células plasmáticas, siendo esto lo que permite detectar durante décadas anticuerpos circulantes frente a los distintos patógenos (202). En pacientes convalecientes de SARS-CoV-2 se ha detectado la presencia de células plasmáticas de larga vida en médula ósea. Estas células pueden jugar un papel fundamental en la respuesta rápida a un nuevo contacto con el virus y en el mantenimiento de niveles de anticuerpos circulantes (201). Probablemente, este tipo de células plasmáticas también esté presente en las personas vacunadas. Lo que si se ha observado, es que las vacunas de mRNA inducen fuertes respuestas de linfocitos T CD4+, linfocitos B del centro germinal y células plasmáticas de larga duración con respuestas de anticuerpos neutralizantes en ratones (203,204).

Por otra parte, aunque no se detectaran anticuerpos circulantes, la memoria inmunológica formada por linfocitos T y B específicos sería capaz de responder rápidamente a una nuevo contacto antigénico (205). Es de esperar que esta memoria inmunológica también esté presente en los pacientes vacunados. Los primeros resultados indicaron que la frecuencia de células B memoria generadas por vacunación es aproximadamente la misma que las inducidas por personas con COVID-19 grave (206). Esto explicaría los resultados obtenidos en dos participantes incluidos en el estudio que, tras una nueva exposición al virus, han presentado un aumento entorno al 200% en los niveles de

anticuerpos circulantes. La nueva estimulación antigénica habría provocado la activación de los linfocitos B memoria, que se habrían diferenciado en plasmablastos y células plasmáticas productoras de anticuerpos, produciendo un aumento de estos (201). Se ha informado de la presencia de linfocitos B memoria contra la proteína S en personas convalecientes de COVID-19 hasta 8 meses después de la infección (207), datos que se esperan poder reproducir en personas vacunas.

El nivel de IgG contra la proteína S se correlaciona con la actividad neutralizante de estos (197). Sin embargo, las funciones efectoras mediadas por la fracción constante (Fc) de las inmunoglobulinas pueden desempeñar un papel importante (208). Por ejemplo, los resultados de Tauzin y colaboradores, mostraron una fuerte citotoxicidad celular dependiente de anticuerpos (ADCC) pero una capacidad de neutralización débil a las tres semanas después de la vacunación (209). Por ello, pese a la gran cantidad de estudios que hablan sobre el papel neutralizante de los anticuerpos, debemos valorar las otras funciones efectoras de los anticuerpos y la inmunidad celular. De hecho, deberíamos evaluar el potencia de la vacuna no solo por su potencial neutralizante sino también por su capacidad para evitar el ingreso hospitalario, la enfermedad grave y la muerte (210).

Nuestros resultados, en consonancia con los de Pegu y colaboradores, no encuentran diferencias del nivel de anticuerpos entre las distintas franjas de edad, ni en la cinética de disminución de estos (200). Sin embargo, se ha sugerido que numerosos factores pueden influir en la generación de inmunidad por parte de las vacunas, como son el género, la nutrición, el microbioma, la genética y el medio ambiente (211). Las mujeres parecen tener una mayor respuesta de anticuerpos a las vacunas frente al dengue, hepatitis A y B, y la viruela, mientras que los hombres tienen una mejor respuesta frente a las vacunas contra la difteria y el tétanos entre otras (212). De una forma general, se dice que las mujeres presenta mayores respuestas de anticuerpos y que esto las hace más resistentes a

enfermedades infecciosas (211). Nosotros no detectamos diferencias entre sexos, lo que puede deberse a la nueva tecnología de mRNA utilizada en esta vacuna.

Como hemos venido comentando, para un correcto control de una infección viral, se requiere de una respuesta inmunitaria robusta con participación anticuerpos y células T efectoras específicos (213). El papel de la inmunidad celular es de vital importancia en las infecciones virales (214). Nuestros resultados muestran una respuesta celular a los 90 días post vacunación del 86%. Curiosamente, en los individuos sin respuesta celular, encontramos una media de nivel de anticuerpos circulantes mucho más baja que entre los individuos con respuesta celular positiva. Esto puede deberse a la afinidad del péptido a los distintos alelos HLA de clase II, que podrían inducir la diferenciación a Tfh, pero no a linfocitos Th1 productores de IFN. Sin embargo, se ha observado una producción preferencial de citoquinas Th1 (IFN-γ, IL-2, TNF-α) en vacunados con mRNA-1273 (62). Los bajos niveles de anticuerpos IgG pueden estar relacionados con la falta de producción de IFN-γ que induce el cambio de isotipo hacia IgG (215). Quizás, en los individuos con respuesta celular negativa podemos encontrar otro isotipo de inmunoglobulina predominante. Por otro lado, algunos estudios recientes han demostrado que ambas dosis de la vacuna son necesarias para detectar inmunidad celular en el 95% de los vacunados (216). Esto puede indicar que sea necesaria una revacunación con el tiempo. Sin embargo, la literatura reporta casos de linfocitos T memoria que duran hasta 11 años en pacientes convalecientes de SARS-CoV (217). Igualmente, se han detectado linfocitos T de memoria de larga duración en vacunados de fiebre amarilla y viruela (218,219). Será necesario el paso de tiempo para poder determinar la presencia de linfocitos T memoria en individuos vacunados frente al SARS-CoV-2.

Como hemos comentado, la respuesta celular y humoral pueden verse afectadas por la afinidad de las moléculas HLA de clase II con los distintos péptidos derivados de la proteína S. Por ello, realizamos un estudio de la frecuencia de los distintos alelos HLA entre individuos con diferente grado de respuesta humoral. Nuestros resultados mostraron que el alelo HLA-DRB1*07:01 está significativamente más representado con el grupo de altos respondedores. En el caso del alelo HLA-DRB1*01:01, está próximo a la significancia con una frecuencia aumentada en el grupo de bajo respondedores. Por otra parte, el alelo HLA-DQB1*02:02 se acerca a la significancia, incrementándose en el grupo de altos respondedores, lo que puede deberse al haplotipo formado con el alelo HLA-DRB1*07:01. En lo referente a haplotipos, HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 se correlaciona con altos respondedores. Además, esta asociación se ve reforzada por la existencia de diferencias significativas en la producción media de IgG anti-S en portadores del alelo HLA-DRB1*0701 y el haplotipo correspondiente. HLA-DRB1*01:01 no influye en la producción de IgG anti-S, pero los portadores presentan títulos más bajos que los portadores del alelo HLA-DRB1*07:01.

La distinta capacidad de los alelos HLA de clase II para presentar péptidos derivados de la proteína S es una posible explicación del amplio rango de producción de anticuerpos detectado. El alelo HLA-DRB1*01:01 presenta una menor capacidad para unirse con fuerza a los péptidos derivados de la proteína S en comparación con el alelo HLA-DRB1*07:01 (220). HLA-DRB1*01:01 presenta cinco péptidos derivados de la proteína S con alta afinidad, mientras que HLA-DRB1*07:01 presenta 16 (220). Además, el alelo HLA-DRB1*07:01 tiene una molécula HLA-DRB4 asociada al haplotipo, mientras que HLA-DRB1*01:01 carece de una molécula HLA-DRB3, DRB4 o DRB5 asociada (221). Por lo tanto, el haplotipo HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02, asociado a

una mejor respuesta, puede activar una mayor variedad de clones de células T, lo que da como resultado una respuesta humoral más fuerte, con una mayor producción de anticuerpos frente al SARS-CoV-2 (222,223). En cambio, el alelo HLA-DRB1*01:01, junto con su haplotipo HLA-DRB1*01:01~DQA1*01:01~DQB1*05:01, tienen alelos con menor capacidad de presentación que junto a la ausencia de una molécula HLA-DRB3, DRB4 o DRB5 inducirá una menor estimulación de células T y, por tanto, una menor activación de linfocitos B y producción de anticuerpos. Además, el haplotipo HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 se une a unos 30-34 péptidos derivados de la proteína S con alta afinidad, mientras que HLA-DRB1*01:01~DQA1*01:01~DQB1*05:01 solo lo hace a 16 (220).

Es posible que el alelo HLA-DRB1*01:01 sea capaz de generar una respuesta de células Treg que regulan negativamente la respuesta inmune y detienen una mayor expansión y una producción alta de anticuerpos (224). Sin embargo, esto es contrario a lo que se observa en pacientes con artritis reumatoide, donde la presencia de este alelo supone un riesgo para el desarrollo de la enfermedad con la desregulación del sistema inmunitario (225). A partir de esto, se puede pensar que el alelo HLA es tan importante como el péptido que se presenta, observándose diferencias en la inducción y regulación de una respuesta inmune en función del mismo.

Un factor que puede explicar por qué el alelo HLA-DRB1*07:01 induce una mayor producción de anticuerpos es que presenta péptidos inmunodominantes de la proteína S, que induce una mayor diferenciación de Tfh y alta cooperación de linfocitos B y T (226). Relacionado con esto, se ha sugerido que el alelo HLA-DRB1*07:01 presenta péptidos exógenos que pueden favorecer el desarrollo de linfocitosis monoclonal TCR-Vβ13.1+/CD4+/NKa+/CD8−/+ dim T-LGL por estimulación crónica (227).

El alelo HLA-DRB1*07:01 se ha relacionado como un factor de riesgo para el lupus eritematoso sistémico (LES) en población de Malasia (228), mientras que en población chilena presenta un papel protector frente a artritis reumatoide con anticuerpos anti proteínas citrulinadas (229). Además, HLA-DRB1*07:01 y el haplotipo HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 están relacionados con un mayor riesgo de hipersensibilidad a la asparaginasa (230,231).

Los resultados publicados por Ragone y colaboradores, demostraron que no existe relación entre el nivel de anticuerpos a corto o medio plazo con el número de péptidos unidos con alta afinidad en individuos vacunados con BNT162b2 (220). Llegaron a la conclusión de que el nivel de anticuerpos no está relacionado con las moléculas HLA de clase II. Por el contrario, nuestros resultados muestran una posible asociación del alelo HLA-DRB1*07:01 y el haplotipo HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02, con una mayor producción de anticuerpos. Las diferencias entre ambos trabajos se deben a que ellos realizan un estudio cuantitativo y teórico del número de péptidos que presenta cada alelo con alta afinidad, mientras que nuestro estudio se basa en el análisis tradicional de frecuencias alélicas. Sin embargo, es posible que esta diferencia de resultados se deba a la vacuna estudiada. Por ejemplo, los excipientes son diferentes en ambas vacunas y la dosis de mRNA-1273 tiene una mayor concentración de mRNA (195). Además, la vacuna mRNA-1273 induce una mayor respuesta de anticuerpos que la vacuna BNT162b2 (195). Quizás la vacuna mRNA-1273 al inducir una mejor estimulación del sistema inmunológico y una mayor producción de anticuerpos, permita observar diferencias en las frecuencias alélicas de HLA de clase II.

Finalmente, la presencia de otros posibles polimorfismos en citoquinas, receptores, etc., asociados al haplotipo en la región MHC, pueden jugar un papel importante en la inducción de la respuesta inmune y deberían ser estudiados.

En conclusión, la infección por SARS-CoV-2 presenta cuadros clínicos diversos donde, entre otros factores, el sistema inmunitario tiene un papel fundamental. Un mal control de la infección por parte de la inmunidad innata puede condicionar la evolución del paciente y el correcto desarrollo de la inmunidad adaptativa. La linfopenia característica de los pacientes más graves está marcada por el descenso de los linfocitos Th1 y el aumento de linfocitos indiferenciados, presentándose un estado de respuesta inmunitaria insuficiente que conduce a estados clínicos más graves. Además, en estos pacientes hay mayor grado de agotamiento de linfocitos junto a niveles de activación más bajos. Las moléculas HLA no tienen un papel determinante en el riesgo de infección o gravedad de esta, mientras que el alelo MICA*A9 resulta ser un factor de riesgo a la infección y a enfermedad moderada.

La respuesta humoral inducida por la vacuna mRNA-1273 es general en la población estudiada, observándose distintos grados de intensidad. Las moléculas HLA-II intervienen en el desarrollo de inmunidad humoral, siendo el alelo HLA-DRB1*07:01 junto a su haplotipo, las moléculas relacionadas con una alta producción de IgG anti-S. A nivel celular no sé encontró una respuesta generalizada, pero una mayor respuesta humoral se relaciona con el desarrollo de respuesta celular.

CONCLUSIONES

1) Los pacientes ingresados por COVID-19 presentan un control deficiente de la infección debido a varias razones: linfopenia que afecta a las células T CD4+ y CD8+, marcada disminución de los linfocitos Th1, aumento de linfocitos Th0 y bajo grado de activación linfocitaria. La profunda disminución de Th1 junto a la alta tasa de linfocitos en estado indiferenciado, presenta un escenario desfavorable para luchar frente al SARS-CoV-2, debido principalmente a la disminución en la producción de IFN-γ e IL-2 necesarios para la diferenciación, proliferación y supervivencia de CTLs. Junto a esto, la presencia de una relación elevada de neutrófilos/linfocitos, aumento de la expresión de PD-1 en linfocitos T CD4+ y CD8+, y el incremento en la expresión de HLA-DR en linfocitos T CD4+, son otras características asociadas a desenlace fatal.

2) En lo referente a la inmunogenética, los alelos y haplotipos HLA no tienen relación con el riesgo de infección ni con la gravedad de ésta. Existe una variedad de resultados contradictorios que pueden ser explicados por los siguientes factores: a) tamaño muestral usado en los distintos; b) las distintas poblaciones étnicas estudiadas, que son determinantes en la frecuencia alélica en cada población; b) el posible enmascaramiento del impacto de los polimorfismos HLA en el riesgo o protección de la COVID-19 por las mejoras actuales en el tratamiento del paciente, pudiendo apreciarse con mayor claridad a largo plazo.

3) Nuestros resultados indican que el polimorfismo STR MICA*A9 tiene un impacto en el riesgo de contagio y la gravedad de la enfermedad. MICA*A9 es un factor de la inmunidad innata que podría condicionar el riesgo de infección y las diferentes respuestas observadas en individuos infectados por SARS-CoV-2.

4) Existe una respuesta humoral generalizada en los individuos vacunados frente a SARS-CoV-2. La cinética en estos individuos es similar a la observadas en los pacientes convalecientes por COVID-19. Por otro lado, la respuesta celular fue buena en la mayoría de los individuos con una relación directa entre la respuesta humoral y celular. En el caso de individuos con baja respuesta humoral y/o celular, faltan por definir los defectos en el sistema inmunitario que los justifiquen.

5) Se ha definido la primera asociación entre los niveles de anticuerpos IgG anti-S inducidos por la vacuna mRNA-1273 y el genotipo HLA de clase II. El alelo HLA-DRB1*07:01 y haplotipo HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 se asocian con un mayor nivel de anticuerpos IgG anti-S en individuos vacunados con mRNA-1273. De nuestros resultados se podría derivar un potencial uso en la selección de péptidos inmunodominantes para la generación de vacunas personalizadas. Para ello, faltaría por comparar el potencial neutralizante de los anticuerpos con los distintos alelos HLA de clase II, y analizar los diferentes péptidos derivados de la proteína S.

CONCLUSIONS

1. Patients admitted for COVID-19 present poor infection control due to several reasons, including: lymphopenia affecting CD4+ and CD8+ T lymphocytes, marked decrease in Th1 lymphocytes, increase in Th0 lymphocytes and low degree of lymphocyte activation, which is mainly due to decreased production of IFN-γ and IL-2 necessary for the differentiation, proliferation and survival of CTLs. In addition, the presence of an elevated neutrophil/lymphocyte ratio, increased PD-1 expression on CD4+ and CD8+ T cells, and increased HLA-DR expression on CD4+ T cells are other features associated with a fatal outcome.

2. In terms of immunogenetics, HLA alleles and haplotypes are not related to risk or severity of infection or severity of infection. The existing contradictory data can be explained by: i) the different sample sizes used; ii) the different ethnic populations studied, which are determinants of the allelic frequency in each population; iii) the possible masking of the impact of HLA polymorphisms on COVID-19 risk or protection by current improvements in patient management, which may be more clearly observed in the long term.

3. Our results indicate that the STR MICA*A9 polymorphism influences the risk of transmission and severity of COVID-19. MICA*A9 is a factor of innate immunity that could condition the risk of infection and the different responses observed in SARS-CoV-2 infected individuals.

4. There is a generalized humoral response in people vaccinated against SARS-CoV-2, with a similar kinetics to that observed in convalescent COVID-19 patients. In addition, the cellular response was good in most individuals, with a direct relationship between the humoral and cellular response. In low responders, the underlying defects of the immune system remain to be defined.

5. We have defined the first association between mRNA-1273 vaccine-induced anti-S IgG antibody levels and HLA class II genotype. The HLA-DRB1*07:01 allele and the HLA-DRB1*07:01~DQA1*02:01~DQB1*02:02 haplotype are associated with a higher level of anti-S IgG antibodies in individuals vaccinated with mRNA-1273. Potential utility in the selection of immunodominant peptides for the generation of personalized vaccines could be derived from our results. For this, it would be necessary to compare the neutralizing potential of the antibodies with the different HLA class II alleles, and to analyze the different peptides derived from the S protein.

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Abreviaturas

5'Cap: caperuza

ACE2: enzima convertidora de angiotensina 2

ADAM17: dominio 17 de metalopeptidasas ADAM

ADCC: citotoxicidad celular dependiente de anticuerpos

Asn: asparragina

AQP3: Acuaporina 3

AQP7: Acuaporina 7

ARHGAP33: Proteína 33 activadora de Rho GTPasa

BNP: péptido natriurético cerebral

COVID-19: enfermedad por coronavirus 2019

CRP: proteína C reactiva

CT: tallo citoplasmático

CTL: linfocito T citotóxico

DC: células dendríticas

DNA: ácido desoxirribonucleico

FP: péptido de fusión

FYCO1: Adaptador de autofagia de dominio FYVE y Coiled-Coil 1

GC: pares guanina-citosina

G-CSF: factor de crecimiento de colonias de granulocitos

GCT: trinucleótido guanina-citosina-timina

Gln: glutamina

GM-CSF: factor estimulante de colonias de granulocitos y macrófagos

GPI: glicosil-fosfatidil-inositol

GWAS: estudio de asociación de genoma completo

HCMV: citomegalovirus

Hg: mercurio

HLA: antígeno leucocitario humano

HR1,-2: repetición de heptada 1 y 2

ICU: unidad de cuidados intensivos

IFN-α: interferón alfa

IFNAR2: subunidad 2 del receptor de interferón alfa y beta

IFN-γ: interferón gamma

IgG: inmunoglobulina de isotipo G

IL-1: interleucina 1

IL-1β: interleucina 1 beta

IL-2: interleucina 2

IL-6: interleucina 6

iNKT: célula natural killer invariante

IRF1: factor regulador del interferón 1

IRF3: factor regulador del interferón 3

ISG: genes estimulados por interferón

ISG20: gen 20 estimulado por interferón

KIR: receptores tipo inmunoglobulina de las células NK

LDH: lactato deshidrogenasa

LES: lupus eritematoso sistémico

Leu: leucina

LIR: receptores leucocitarios tipo inmunoglobulina

LZTFLA1: factor de transcripción de cremallera de leucina 1

M129: metionina en posición 129

MDA5: antígeno 5 de la diferenciación del melanoma

MHC: complejo mayor de histocompatibilidad

MICA: complejo de histocompatibilidad de clase I relacionado con la cadena α

mRNA: ácido ribonucleico mensajero

NCR: receptores de citotoxicidad natural

NF-κB: factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas

NK: natural killer

NKG2A: receptor A miembro del grupo 2 de NK

NKG2D: receptor D miembro del grupo 2 de NK

NLR: receptores tipo NOD

NSP: proteínas no estructurales

OAS: oligoadenilato sintetasa

OMS: organización mundial de la salud

PAMP: patrones moleculares asociados a patógenos

PD-1: ligando 1 de muerte programada

Phe: fenilalanina

Proteína E: proteína de envuelta

Proteína M: proteína de membrana

Proteína N: proteína de la nucleocápside

Proteína S: proteína Spike

PRR: receptores de reconocimiento de patógenos

RBD: dominio de unión al receptor

RIG-I: gen I inducible por ácido retinoico

RLR: receptores tipo RIG-I

RNA: ácido ribonucleico

RNAss+: ácido ribonucleico de cadena sencilla de sentido positivo

RSV: virus respiratorio sincitial

S1: subunidad de unión al receptor

S2: subunidad de fusión de la membrana celular

SCOOURGE: Spanish Coalition to Unlock Research on Host Genetics on COVID-19

Ser: serina

SLC6A20 : Miembro 20 de la familia de transportadores de solutos 6

sMICA: MICA soluble

SOFA: evaluación de insuficiencia orgánica relacionada con sepsis

SpO2: saturación de oxígeno en sangre

STR: repetición corta en tandem

TCR: receptor de células T

Tfh: linfocito T folicular

Th: linfocito T helper

TLR: receptores tipo Toll

TM: dominio transmembrana

TMPRSS2: proteasa transmembrana de serina 2

TNF-α: factor de necrosis tumoral alfa

Treg: linfocito T regulador

Tyr: tirosina

UPK1A: uroplaquina 1

UTR: región no traducida

V129: valina en posición 129

ZAP: proteína asociada a ζ

ZBTB32: proteína 32 que contiene el dedo de zinc y el dominio BTB