MECANISMOS INMUNOREGULADORES IMPLICADOS EN LA DISMINUCIÓN DE RESPUESTA VACUNAL EN DISTINTOS ESCENARIOS MARCADOS POR LA INMUNOSENESCENCIA: SUJETOS ANCIANOS Y SUJETOS CON INFECCIÓN POR VIH

Tesis Doctoral

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CERTIFICAN:

Que el trabajo titulado "Mecanismos inmunoreguladores implicados en la disminución de respuesta vacunal en distintos escenarios marcados por la inmunosenescencia: sujetos ancianos y sujetos con infección por VIH", presentado por la Graduada en Biología **D.ª Inés Herrero Fernández**, ha sido realizado bajo nuestra dirección y asesoramiento en el Laboratorio de Inmunovirología del Instituto de Biomedicina de Sevilla (IBiS). Concluido el trabajo experimental y bibliográfico, autorizamos la presentación y la defensa de esta Tesis Doctoral, para que sea juzgada por el tribunal correspondiente.

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ABREVIATURAS

APC: Células presentadoras de antígenos

CCR5: Receptor de quimiocinas CCR5

CD4: Linfocitos T CD4

CD8: Linfocitos T CD8

CMV: Citomegalovirus

DC: Células dendríticas

ENOS: Eventos no definitorios de SIDA

mDC: Células dendríticas mieloides

MVC: Maraviroc

OMS: Organización Mundial de la Salud

RTE: Células recientemente emigradas del timo

SIDA: Síndrome de Iinmunodeficiencia adquirida

TARc: Tratamiento antirretroviral combinado

TCR: Receptores de células T

Treg: Células T reguladoras

usPCR: Proteína C reactiva ultrasensible

VHB: Virus de la Hepatitis B

VHC: Virus de la Hepatitis C

VIH: Virus de la Inmunodeficiencia Humana

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1. RESUMEN

RESUMEN

En la actualidad se está produciendo un cambio demográfico debido al envejecimiento de la población. Al aumentar la edad, el sistema inmunitario humano experimenta cambios característicos, conocidos como inmunosenescencia, que generan respuestas inmunológicas subóptimas. La inmunosenescencia natural o "inmunosenescencia cronológica" se refiere al deterioro progresivo del sistema inmunológico asociado a la edad.

Debido a este proceso se produce una alteración homeostática del sistema inmunológico y se generan deficiencias en la función celular B y T. En las células T se originan cambios relevantes, como la reducción de células naïve, debido principalmente a la menor timopoyesis que tiene lugar durante el envejecimiento. Además, se produce por compensación homeostática una acumulación de células T efectoras y memoria con escasa diversidad de sus receptores, con una elevada proliferación, activación, senescencia y resistencia a la apoptosis. Debido a estas alteraciones homeostáticas también se produce una expansión de las células T reguladoras (Treg), células supresoras de la respuesta inmunitaria, que contribuye a la desregulación del sistema. En la población anciana se presenta asimismo una inflamación sistémica característica del envejecimiento, proceso conocido como "*inflammaging*", que también dificulta la función inmunitaria. Estas alteraciones conducen a que se generen respuestas inmunológicas subóptimas, provocando, entre otras complicaciones, baja efectividad de las vacunas, lo que contribuye a un aumento de la morbimortalidad de los ancianos.

El virus de la gripe, o virus de la influenza, causa una elevada morbimortalidad en la población anciana. La vacuna frente a este virus es el método más efectivo frente a la infección y las complicaciones relacionadas con la enfermedad, sin embargo, los ancianos presentan una baja eficacia de la vacuna debido a las alteraciones de su sistema inmunológico.

El objetivo de la presente Tesis Doctoral fue explorar la asociación entre distintos parámetros homeostáticos en la población anciana (función tímica, proliferación de las

células T, frecuencia y fenotipo de las Treg y biomarcadores de inflamación) con la respuesta a la vacuna frente a influenza. Este estudio se desarrolló en el trabajo "**T-cell proliferative homeostatic alterations are negatively associated with the vaccine response against influenza in elderly people" (en revisión)**. Observamos que las alteraciones homeostáticas proliferativas que se desarrollan en las subpoblaciones de maduración de las células T CD4 y CD8, incluyendo las Treg, se asocian a una menor respuesta a la vacuna frente a influenza. Estas alteraciones homeostáticas podrían estar íntimamente relacionadas con el aumento de inflamación sistémica y con la disminución de la función tímica asociadas al envejecimiento.

Un espectro similar de alteraciones inmunológicas se produce en personas infectadas por el virus de la inmunodeficiencia humana (VIH), incluso las que están recibiendo un tratamiento antirretroviral combinado (TARc) supresor, proceso conocido como "inmunosenescencia prematura", un proceso prematuro y acentuado de la "inmunosenescencia cronológica". Estos procesos comparten bastantes rasgos, pero no todos. Debido a la exposición al VIH y sus antígenos se produce una depleción masiva de linfocitos T CD4 y un gran desgaste inmunológico, con expansiones oligoclonales. Además, se produce una reducción de la función del timo y, por tanto, de células naïve, debido posiblemente al timotropismo del VIH. Por otro lado, se produce un aumento en los niveles de marcadores de inflamación sistémica. Además, en este escenario también se ha observado una expansión de células Treg y de células efectoras.

Los sujetos infectados por VIH tienen un riesgo elevado de complicaciones hepáticas, puesto que hay una elevada tasa de coinfección por el Virus de la Hepatitis B (VHB) y por el Virus de la Hepatitis C (VHC). La coinfección por el VHB o el VHC aumenta notablemente la morbimortalidad hepática en estos pacientes VIH. La vacunación frente a VHB es la manera más importante de prevenir la coinfección, pero los individuos VIH muestran una baja respuesta a la vacuna. Además de la baja tasa de respuesta a la vacuna, en aquellos pacientes que responden a ella, la magnitud de los anticuerpos protectores es baja y, por consiguiente, se pierden rápidamente.

Estudios previos sugieren que Maraviroc (MVC), fármaco antirretroviral, posee propiedades inmunomoduladoras que pueden impactar de manera favorable en la respuesta inmunitaria y por tanto podrían favorecer la respuesta vacunal frente a VHB. Por ello, uno de los objetivos de la presente tesis doctoral fue analizar si un tratamiento antirretroviral combinado (TARc) que contenía MVC se asociaba a una mejor respuesta a la vacuna del VHB en pacientes infectados por VIH. Este objetivo se abordó en el trabajo **"Association between a suppressive combined antiretroviral therapy containing maraviroc and the hepatitis B virus vaccine response" Antimicrob Agents Chemother 2017; 62:e02050-17**. Observamos que un TARc que incluía MVC se asociaba con una mayor magnitud de la respuesta a la vacuna frente a VHB en sujetos VIH menores de 50 años. Por tanto, un régimen conteniendo MVC durante el protocolo de inmunización podría ser una estrategia terapéutica adecuada para mejorar la respuesta vacunal en estos sujetos.

Tras este trabajo se extendió su análisis, incluyendo variables inmunológicas para conocer los posibles factores, relacionados con la inmunosenescencia, que pudieran mediar en el impacto de MVC sobre la respuesta a la vacuna frente al VHB. Por tanto, exploramos el potencial efecto de un TARc que incluía MVC en diferentes parámetros relacionados con la inflamación, la función de las células T, incluidas las Treg, y de células dendríticas, que pudieran estar relacionados con la efectividad de la vacuna en la población VIH. Este análisis se desarrolló en el trabajo **"Improved CD4 T-cell profile in HIV-infected subjects on maraviroc-containing therapy is associated with better responsiveness to HBV vaccination" J Transl Med 2018; 16:238.** Observamos que los pacientes tratados con un TARc incluyendo MVC mostraron un perfil inmunológico menos activado, con mayor contribución de las células T recientemente emigradas del timo y una menor frecuencia de células Treg, perfil que se asocia con una mejor respuesta a la vacuna frente al VHB. Estos datos nos llevan a especular que MVC podría tener un efecto regenerador sobre el sistema inmunitario, promoviendo la función tímica y disminuyendo la proliferación homeostática compensatoria, lo que le permitiría repercutir también en un menor estado proinflamatorio.

2. INTRODUCCIÓN

INTRODUCCIÓN

En la actualidad se está produciendo un cambio demográfico en el primer mundo debido al envejecimiento de la población [1]. Por ello, el interés social y científico en el conocimiento de las bases fisiológicas del envejecimiento y la mejora de la calidad de vida en personas mayores está creciendo.

Al aumentar la edad, el sistema inmunitario humano experimenta cambios característicos, conocidos como inmunosenescencia natural, asociada al envejecimiento o inmunosenescencia cronológica, que generan respuestas inmunitarias subóptimas [2]. Dicha inmunosenescencia se refiere al deterioro progresivo del sistema inmunológico asociado a la edad, afectándose tanto el componente innato como el adaptativo [3,4]. Debido a este proceso se produce una alteración homeostática del sistema inmunológico y se producen deficiencias en la función celular B y T [5,6]. En las células T se originan cambios relevantes, como la reducción de células naïve, debido principalmente a la menor timopoyesis que se produce durante el envejecimiento [7]. Como consecuencia se desarrolla un mecanismo de compesación homeoestática, generando acumulación de células T efectoras y memoria con escasa diversidad de sus receptores de células T (TCR), con una elevada proliferación, activación, senescencia y resistencia a la apoptosis [8-11]. Debido a estas alteraciones homeostáticas también se produce una expansión de las células T reguladoras (Treg) [12-17], células supresoras del sistema inmunológico [18,19], expansión que contribuye a la desregulación del sistema. Las expansiones oligoclonales de células T pueden ser debidas a reactivaciones de infecciones silentes de virus tipo herpes como el citomegalovirus (CMV), que contribuyen al deterioro y agotamiento del sistema inmunológico [20]. En la población anciana se presenta asimismo una inflamación sistémica asociada al envejecimiento, proceso conocido como "*inflammaging*", que también afecta negativamente a la respuesta inmunitaria [20,21].

Las alteraciones que se desarrollan con el envejecimiento llevan, por tanto, a respuestas inmunitarias ineficientes, produciendo un mayor desarrollo de patologías autoinmunes, cardiovasculares y cáncer [22-28], así como una mayor prevalencia y

severidad de las enfermedades infecciosas [30], así como a una baja efectividad de las vacunas [31,32]. Todo ello incide, como consecuencia, en un aumento de la morbimortalidad de esta población.

EL virus de la gripe, o virus de la influenza, causa una elevada morbi-mortalidad en la población anciana [33]. La Organización Mundial de la Salud (OMS) estima que al año se producen entre 3 y 5 millones de casos graves de influenza y unas 650.000 muertes debidas a enfermedades respiratorias relacionadas con la gripe [34], siendo la población anciana la de mayor tasa de mortalidad [35]. La vacuna frente al virus de la gripe es el método más efectivo para prevenir la infección y sus complicaciones; debido a los cambios inmunitarios que se producen en el envejecimiento, los ancianos tienen una respuesta vacunal baja [36,37].

Las alteraciones del sistema inmunológico citadas anteriormente, junto a las alteraciones en los receptores tipo Toll [38], reducción de la actividad de la telomerasa [39,40] y deficiencias en la función celular B y T [41,42], se han relacionado específicamente con una menor respuesta a la vacuna frente a influenza. Sin embargo, las alteraciones relacionadas con la homeostasis de células T, como la menor timopoyesis y la proliferación periférica compensatoria, no se han explorado en este escenario. Además, el estado de inflamación ha sido relacionado con respuestas ineficientes a las vacunas, entre ellas la de la gripe [43-45], pero su papel en el escenario de ancianos no ha sido suficientemente explorado.

Por otro lado, las Treg suprimen la secreción de citoquinas de los linfocitos T CD4 y CD8, así como a las células presentadoras de antígenos (APC) y las células B [46]. De hecho, las Treg suprimen el cambio de clase de inmunoglobulinas en los centros germinales de los tejidos linfoides [47]. De esta manera, las Treg podrían limitar la respuesta vacunal en este escenario. Las Treg han sido estudiadas en modelos de respuesta vacunal animales [48,49] y humanos [50,51]. Además, se conoce que se expanden durante el envejecimiento [15] y tras la vacuna frente a influenza [52,53]. Sin embargo, su frecuencia y su papel antes de la vacuna frente a influenza en humanos han sido prácticamente inexplorados.

Por todo ello, uno de los objetivos de esta Tesis Doctoral ha sido explorar la asociación entre distintos parámetros homeostáticos en la población anciana, como la función tímica, proliferación de las células T, frecuencia y fenotipo de las Treg y varios biomarcadores de inflamación, con la respuesta a la vacuna frente a influenza. Este estudio se desarrolló en el trabajo "**T-cell proliferative homeostatic alterations are negatively associated with the vaccine response against influenza in elderly people" (en revisión)**.

Un espectro similar de alteraciones inmunológicas se produce en personas infectadas por el virus de la inmunodeficiencia humana (VIH), que desarrollan una "inmunosenescencia prematura", un proceso prematuro y acentuado de la "inmunosenescencia cronológica" [54,55]. Esta "inmunosenescencia prematura" comparte algunos rasgos con la "inmunosenescencia cronológica", pero no todos [56-58]. Debido a la exposición al VIH y sus antígenos se produce una depleción masiva de linfocitos T CD4 y un gran desgaste inmunológico, con expansiones oligoclonales. Además, se produce una reducción de la función del timo y, por tanto, de células naïve, posiblemente debido al timotropismo del VIH [59]. Por otro lado, se produce un aumento en los niveles de biomarcadoress de inflamación sistémica [60,61], lo que parece estar relacionado con una excesiva activación inmunitaria, no solo derivada de antígenos VIH, sino también de otras infecciones latentes y del fenómeno de traslocación bacteriana [62]. En este sentido, se ha sugerido que el aumento de proliferación de células T CD4 en este escenario puede estar muy relacionado con la mayor exposición a citoquinas proinflamatorias [63]. Además, en este escenario también se ha observado una expansión de células Treg [64].

Sin tratamiento la infección por VIH evoluciona a síndrome de inmunodeficiencia adquirida (SIDA). Esta fase de la enfermedad se caracteriza por la presencia de infecciones oportunistas y/o neoplasias que pueden ocasionar la muerte del paciente En la actualidad, el tratamiento antirretroviral combinado (TARc) consigue suprimir la viremia VIH permitiendo el aumento del recuento de linfocitos T CD4 y una recuperación inmunológica variable [65], reduciéndose la morbimortalidad asociada a SIDA [66,67]. Sin embargo, esta situación favorable que ocurre principalmente en los

países desarrollados en los que los pacientes VIH tienen acceso al tratamiento, ha hecho emerger otra realidad clínica; a consecuencia de la "inmunosenescencia prematura", la inflamación crónica y la toxicidad acumulada por la exposición a fármacos durante décadas, los sujetos infectados por VIH desarrollan eventos no definitorios de SIDA (ENOS), que incluyen un número de complicaciones clínicas, entre ellas procesos cardiovasculares, renales, óseos, hepáticos, metabólicos, neuropsiquiátricos y neoplasias [68,69], que en ocasiones resultan en muerte y elevan por tanto la morbimortalidad asociada a la infección crónica por VIH [70].

Además, los sujetos infectados por VIH tienen un riesgo elevado de complicaciones hepáticas, puesto que hay una elevada tasa de coinfección por el Virus de la Hepatitis B (VHB) y por el Virus de la Hepatitis C (VHC) [71,72]. La coinfección por el VHB o el VHC aumenta notablemente la morbimortalidad hepática en estos pacientes. El VIH acelera el daño hepático causado por el VHB y el VHC, la cirrosis prematura y el carcinoma hepático [73,74]. La vacunación frente a VHB es la manera más importante de prevenir la coinfección, pero los individuos VIH muestran una baja respuesta a la vacuna [75]. Además de la baja tasa de respuesta a la vacuna, en los sujetos respondedores, la magnitud de los anticuerpos protectores es baja y, por consiguiente, se pierden rápidamente [76].

El recuento de linfocitos T CD4 juega un papel fundamental en la respuesta a la vacuna frente a VHB [75,77,78]) y es conocido que el agotamiento del sistema inmunitario relacionado a la infección por VIH ocasiona el fallo de la respuesta vacunal [79]. El número de dosis y el protocolo de inmunización parecen también importantes para una mejor respuesta a la vacuna [80-82]. Además, el tratamiento antirretroviral mejora la respuesta antígeno-específica, recupera el repertorio de células T [83] y disminuye la carga viral [84], mejorando con ello dicha respuesta. Sin embargo, no se ha estudiado suficiente la influencia del tipo de TARc en la respuesta vacunal.

Estudios previos sugieren que MVC, tiene propiedades inmunomoduladoras que pueden impactar de manera favorable en la respuesta inmunitaria. MVC es un antagonista del receptor de quimiocinas CCR5. Dicho receptor es, además, utilizado por el virus del VIH para completar el proceso de entrada a la célula de las cepas virales

con tropismo R5 [85]. Por tanto, el uso clínico de MRV está actualmente indicado en pacientes virémicos, infectados por VIH con tropismo viral CCR5 [86] y ha demostrado eficacia inmunovirológica [87].

CCR5 se expresa tanto en células del sistema inmunológico innato (macrófagos, monocitos, células dendríticas, natural killers), como el adaptativo (células T) y tiene un papel importante en la respuesta inmunitaria adaptativa y en promover la migración de las células que expresan CCR5 a sitios de inflamación [88]. El bloqueo de CCR5 por MVC tiene pues implicaciones en la respuesta inmunitaria, confiriendo al fármaco propiedades inmunomoduladoras.

En apoyo de esta hipótesis, MVC se ha relacionado con el aumento de las células T CD4 [89,90], con reducción de la activación de las células T CD4 y CD8 [91] y de marcadores de inmunosenescencia de las células T [92], así como con la reducción de niveles de marcadores de inflamación [93]. Además, MVC mostró la capacidad de reducir las células Treg en sujetos VIH sin exposición previa a tratamiento antirretroviral (naïves) [94], células cuya elevada frecuencia se asoció a una menor respuesta a la vacuna frente a VHB [95]. Conjuntamente, MVC mejoró la distribución de las subpoblaciones de las células Treg [96].

Por todo ello, era razonable especular que los efectos inmunomoduladores del MVC pudieran impactar de manera favorable en la respuesta vacunal, mejorando tanto la respuesta humoral como la celular. De hecho, MVC había demostrado optimizar la neoinmunización meningocócica y mejorar la respuesta a los antígenos de recuerdo, como el toxoide tetánico [97]. Posiblemente, MVC, gracias a su efecto inmunomodulador, podría mejorar diferentes funciones del sistema inmunológico requeridas para ejercer una respuesta adecuada también tras la vacunación frente a VHB, incluyendo presentación de antígenos, funciones de las células T, regulación de la supresión de células T y funciones de las células B [98,99]. Por tanto, uno de los objetivos de la presente Tesis Doctoral fue analizar si un TARc que contenía MVC se asociaba a una mejor respuesta a la vacuna del VHB. Este objetivo se abordó en el trabajo **“Association between a suppressive combined antiretroviral therapy**

containing maraviroc and the hepatitis B virus vaccine response" Antimicrob Agents Chemother 2017; 62:e02050-17.

Tras este trabajo se extendió su análisis, incluyendo variables inmunológicas para conocer los posibles factores, relacionados con la inmunosenescencia, que pudieran mediar en el impacto de MVC sobre la respuesta a la vacuna frente al VHB. Por tanto, se exploró el potencial efecto de un TARc que incluía MVC en diferentes parámetros relacionados con la inflamación, la función de las células T, incluidas las Treg, y de células dendríticas (DC), que pudieran estar relacionados con la efectividad de la vacuna en esta población. Este análisis se desarrolló en el trabajo **"Improved CD4 T-cell profile in HIV-infected subjects on maraviroc-containing therapy is associated with better responsiveness to HBV vaccination" J Transl Med 2018; 16:238.**

3. OBJETIVOS

OBJETIVOS

- **Objetivo 1:** Explorar potenciales asociaciones entre distintos parámetros relacionados con la homeostasis de células T y la respuesta a la vacuna frente a influenza en sujetos ancianos. Con este fin, se consideraron principalmente los siguientes parámetros: función tímica, proliferación de las células T, frecuencia y fenotipo de las Treg y marcadores de inflamación. Este objetivo se abordó en el trabajo: "**T-cell proliferative homeostatic alterations are negatively associated with the vaccine response against influenza in elderly people"** (en revisión).

- **Objetivo 2:** Determinar si un TARc incluyendo MVC mejora la respuesta a la vacuna frente a VHB. Este objetivo se abordó en el trabajo: **"Association between a suppressive sombined antiretroviral therapy containing maraviroc and the hepatitis B virus vaccine response"** Antimicrob Agents Chemother 2017; 62:e02050-17.

- **Objetivo 3:** Explorar el potencial efecto de un TARc incluyendo MVC sobre diferentes parámetros relacionados con la homeostasis de células T, potencialmente implicados en la respuesta vacunal frente a VHB Con este fin, se consideraron principalmente los siguientes parámetros: función tímica, proliferación de las células T y frecuencia y fenotipo de las Treg, marcadores de inflamación y fenotipo de células dendríticas. Este objetivo se abordó en el trabajo: **"Improved CD4 T-cell profile in HIV-infected subjects on maraviroc-containing therapy is associated with better responsiveness to HBV vaccination"** J Transl Med 2018; 16:238.

4. MATERIAL, MÉTODOS Y RESULTADOS

4.1. T-cell proliferative homeostatic alterations are negatively associated with the vaccine response against influenza in elderly people

Herrero-Fernández I, Rosado-Sánchez I, Álvarez-Ríos AI, Galvá MI, De Luna-Romero M, Sanbonmatsu-Gámez S, Pérez-Ruiz M, Navarro-Marí JM, Carrillo-Vico A, Sánchez B, Ramos R, Cañizares J, Leal M, Pacheco YM.

(En revisión)

Title: Homeostatic T-cell proliferation was negatively associated with the vaccine response against influenza in a cohort of elderly people.

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ABSTRACT

Background: Seasonal influenza virus infection is a significant cause of morbimortality in the elderly. However, there is poor vaccine efficacy in this population due to immunosenescence. We aimed to explore several homeostatic parameters in the elderly that could impact influenza vaccine responsiveness.

Methods: Subjects (>60 years old) who were vaccinated against influenza virus were included, and the vaccine response was measured by a haemagglutination inhibition (HAI) test. At baseline, peripheral CD4 and CD8 T-cells were phenotypically characterized. Thymic function and the levels of different inflammation-related biomarkers, including Lipopolysaccharide Binding Protein (LBP) and anti-cytomegalovirus (CMV) IgG antibodies, were also measured.

Results: Influenza vaccine non-responders showed a higher frequency of regulatory T-cells (Tregs) before vaccination than responders (1.49 [1.08-1.85] *vs.* 1.12 [0.94-1.63], respectively, p=0.061), as well as higher expression of the proliferation marker Ki67 in Tregs and different CD4 and CD8 T-cell maturational subsets. The levels of inflammation-related biomarkers correlated with the frequencies of different proliferating T-cell subsets and with thymic function (e.g., thymic function with D-dimers, r=-0.442, p=0.001).

Conclusions: Age-related homeostatic dysregulation involving the proliferation of CD4 and CD8 T-cell subsets, including Tregs, was related to a limited responsiveness to influenza vaccination and a higher inflammatory status in a cohort of elderly people.

Keywords: Treg, Ki67, inflammation, thymic function, TREC

BACKGROUND

The seasonal influenza virus (flu) is a significant cause of morbidity and mortality in older adults [1]. The World Health Organization (WHO) estimates that 3-5 million cases of severe influenza illness and up to 650,000 deaths related to respiratory diseases are linked to seasonal flu each year [2], with the highest mortality rates occurring in the elderly [3]. Despite vaccination remaining the most effective approach for the prevention of influenza infection and influenza-related complications, there is poor vaccine efficacy in the elderly [4], which is due to the age-associated dysregulation of immune function known as immunosenescence [5,6].

Immunosenescence affects both the innate and adaptive branches of the immune system. Thus, age-related alterations, such as those affecting Toll-like receptors [7], reduced telomerase activity [8] and deficiencies in B and T-cell functions [9,10], have been associated with influenza vaccine responsiveness. Moreover, elderly people exhibit a chronic inflammatory status, called inflammaging, with increased levels of circulating inflammatory mediators such as pro-inflammatory cytokines and acute phase proteins, e.g., interleukin-6 (IL-6) and C-reactive protein (CRP), respectively, that disturb vaccine responses [11], specifically, the influenza vaccine response [12,13]. Several factors, mainly persistent stressors such as translocated microbial products (LPS) and cytomegalovirus coinfection (CMV) but also the age-related increasing activation of the coagulation system (D-dimers), have been proposed to contribute to this age-related inflammation [14]. Interestingly, regarding the adaptive immune system, naïve T-cells retain their proliferative capacity in both aged mice and humans [15] and naïve T-cell proliferation can even be enhanced in the elderly because of the age-dependent loss of thymic output [16-18]. This immunosenescence-related homeostatic dysregulation (mainly the thymic output – compensatory peripheral T-cell proliferation axis) could

also be related to inflammaging and even affect immune competence in the elderly. However, the potential role of this homeostatic dysregulation in vaccine responsiveness is mostly unexplored.

The proliferation and cytokine secretion of CD4 and CD8 T-cells is regulated by regulatory T-cells (Tregs), which are also involved in the suppression of antigen presenting cells (APC) and B cells [19]. In fact, Tregs suppress the B cell immunoglobulin class switching within germinal centres of human lymphoid tissue [20]. Because of that observation, Tregs have been explored in several immunization models in animals [21,22] and in humans [23-24]. Particularly, in the context of influenza vaccination, despite Tregs being known to expand after vaccination, which possibly attenuates the production of anti-influenza antibodies [26,27], the role of baseline Tregs remains mostly unexplored. However, this is an interesting question since Treg frequency increases with age [28], probably as another consequence of age-dependent homeostatic dysregulation [29,30].

In the present work, we aimed to further explore the potential association between several immune homeostatic parameters in the elderly, such as thymic function, T-cell proliferation, Tregs and several inflammation- and coagulation-related markers, and influenza vaccine responsiveness.

RESULTS

Rates of vaccine response

Sixty subjects were included in this study. The demographic and clinical characteristics of these subjects are summarized in Table 1. Briefly, 24/60 (40%) of the subjects were men, and the median age was 79 [70-87] years. Before vaccination, the subjects showed a median CD4/CD8 T-cell ratio of 1.8 [1.2-2.3] and a broad range of thymic function

measured as an sj/β-TREC ratio of 32 [0-50]. Seroprotection was present in 48/60 (80%) of the subjects before vaccination and in 59/60 (98%) of the subjects after vaccination. A vaccine response was observed in 27/60 (45%) of the subjects. The baseline and post-vaccination HAI titres for the whole group, as well as the titres for the responder and non-responder groups, are shown in Figure S1. The timing of the post-vaccination sampling did not affect the HAI titres or the seroconversion-fold data (data not shown).

Levels of inflammation-related markers according to vaccine responsiveness

The non-responders did not differ from the responders in terms of age, sex, CD4/CD8 ratio, sj/β-TREC ratio or anti-cytomegalovirus (CMV) titre (Table 1). Interestingly, several nonsignificant differences in inflammation-related markers were observed. Specifically, the non-responders showed higher levels of D-dimers (875 [445-1425] *vs* 620 [438-918], respectively; p=0.082), a higher % neutrophils (62.8 [54.3-66.9] *vs* 58.0 [52.4-62.5], respectively; p=0.089) and a higher neutrophil to lymphocyte ratio (NLR) (2.6 [1.7-3.0] *vs* 1.9 [1.5-2.4], respectively; p=0.056) than the responders. However, the non-responders showed lower % lymphocytes (24.9 [22.4-31.9] *vs* 29.0 [25.0-34.1], respectively; p=0.063) and % eosinophils (3.15 [1.73-4.45] *vs* 3.70 [3.20-4.20], respectively; p=0.094).

Frequency of Tregs and Tregs expressing Ki67 according to vaccine responsiveness

We explored the frequencies of total-Tregs and Treg subsets and the expression of different activation, proliferation and suppression markers in relation to influenza vaccine responsiveness (Figure 1 and Table S1). We observed a higher frequency of total-Tregs in the non-responders compared with the responders; however, the

difference was not significant (1.49 [1.08-1.85] *vs* 1.12 [0.94-1.63], respectively; p=0.061). Moreover, the two groups were split according to the total-Treg frequency by using the overall median value (1.38) as a cutoff, and 22/33 (67%) of the non-responders but only 8/27 (30%) of the responders showed a Treg frequency above the median (p=0.004). No differences were observed regarding the frequencies of Treg subsets. Nevertheless, the non-responders presented higher frequencies of both naïve-Tregs (nTregs) (38.9 [19.2-42.8] *vs* 19.5 [16.1-35.6], respectively; p=0.025) and non-Tregs (38.5 [24.9-44.1] *vs* 27.7 [17.9-40.4], respectively; p=0.053) expressing the proliferation marker Ki67; however, statistical significance was reached with only the nTregs.

Frequencies of CD4 and CD8 T-cell maturational subsets expressing Ki67 according to vaccine responsiveness

We examined CD4 and CD8 T-cell maturational subsets and the expression of different markers of activation (HLA-DR), apoptosis susceptibility (CD95), senescence (CD57), proliferation (Ki67) and suppression (CTLA-4) in CD4 and CD8 T-cell pools. We found no differences between the vaccine responsiveness groups in either the distribution of the maturational subsets or the expression of the abovementioned cellular markers (Table S2). We also analysed the expression of Ki67 specifically on the different CD4 and CD8 maturational subsets to study the proliferation of these subsets. We observed higher expression of Ki67 in both the CD4 and CD8 maturational subsets from the non-responders compared to those from the responders, and these differences in expression reached statistical significance for all comparisons (Figure 2).

Associations among inflammation-related biomarkers, the expression of Ki67 in T-cells and thymic function

We explored the potential associations of the analysed inflammation-related biomarkers and haematological parameters with the expression of Ki67 in different Treg subsets (naïve Tregs –nTregs– and effector Tregs –eTregs–) as well as in different CD4 and CD8 T-cell maturational subsets. Interestingly, we observed positive associations between Ki67 expression in several T-cell subsets and Lipopolysaccharide Binding Protein (LBP) levels, high sensitivity C reactive protein (hsCRP) levels (e.g., with %eTreg-Ki67$^+$; r=0.301, p=0.020), β2-microglobulin levels (e.g., with %CD4$^+$Ki67$^+$; r=0.322, p=0.010), D-dimer levels (e.g., with %CD4$^+$Ki67$^+$; r=0.446, p=0.001), the % monocytes, the % neutrophils (e.g., with %CD4$^+$EM Ki67$^+$; r=0.307, p=0.017), the % basophils, the platelet to lymphocyte ratio (PLR) and the NLR, while there were negative associations between the Ki67 expression in several T-cell subsets and the % lymphocytes (e.g., with %CD4$^+$EM Ki67$^+$; r=-0.310, p=0.016), % eosinophils, Mean Corpuscular Volume (MCV) (e.g., with CD8+TemRA Ki67+; r=-0.303, p=0.019) and Mean Platelet Volume (MPV) (e.g., with %nTreg Ki67+; r=-0.550, p<0.001) (Table S3). We also observed associations between thymic function and the levels of inflammation-related biomarkers (D-dimers, Erythrocyte Sedimentation Rate (ESR) and the PLR) as well as a trend with hsCRP levels (Figure 3). Moreover, two of the inflammation-related biomarkers (the ESR and LBP levels) also correlated with the anti-CMV titre (r=0.313, p=0.021 and r=0.263, p=0.057; respectively).

Remarkably, we found higher levels of inflammation-related biomarkers and expression of Ki67$^+$ in T-cell subsets and lower thymic function in the subjects who died during a year-long follow-up (Table S4). Six subjects (one responder and five non-responders) died during this follow-up period as a consequence of cardiovascular events, and despite acknowledging the low number of events, we explored the baseline immune characteristics of these subjects. As expected, the subjects who died showed higher

baseline levels of hsCRP than those who survived (5.10 [3.70-7.58] *vs* 2.60 [1.25-4.30], respectively; p=0.012). However, interestingly, we also found higher frequencies of T-cell subsets, including Tregs, expressing Ki67 in the subjects who died. Furthermore, the six subjects who died during the follow-up year had lower thymic function (0 [0-0] *vs.* 34 [10-51], respectively; p=0.001), and all of these subjects showed a failure in thymic function.

DISCUSSION

We report that the influenza vaccine responsiveness of an aged population was associated with age-related homeostatic dysregulation involving T-cell proliferation in CD4 and CD8 T-cell maturational subsets. Moreover, higher frequencies of not only CD4 Tregs but also proliferating Treg subsets were associated with vaccine non-response. Overall, this homeostatic dysregulation was directly correlated with the inflammatory status in this context.

Tregs are involved in the suppression of the immune system and prevent a proper antibody response to vaccination [19,20]. Herein, we studied the frequencies and several functional markers of Treg subsets in relation to the influenza vaccine response of elderly people. Interestingly, we observed a higher baseline frequency of total-Tregs in non-responders than responders. Our results are in line with those of van de Geest *et al*. [31], although those authors specifically found higher frequencies of the effector-Treg subset. As far as we know, no other previous studies focusing on baseline Tregs in the elderly in relation to vaccine responsiveness have been reported. Nevertheless, we previously showed that human immunodeficiency virus (HIV)-infected subjects who did not respond to a hepatitis B virus (HBV) vaccine had a higher baseline frequency of Tregs than those who did respond [24,25]. The fact that high baseline Treg frequencies

impaired the response to vaccination in the elderly and HIV-infected subjects reinforces Treg increases in the steady states of both scenarios as a common feature of immunosenescence [28], which could compromise the ability of the immune system to mount a proper immune response [21-27]. Further research about the underlying mechanism of these immunosenescence-related Treg increases is needed to develop novel approaches aimed to improve vaccine responsiveness in these scenarios.

In addition, we observed higher frequencies of Treg subsets expressing the proliferation marker Ki67 in non-responders. Heightened Treg proliferation has been shown in subjects exposed to infectious agents, in individuals with systemic autoimmunity and within tumours [32]. Along the same line, proliferating Tregs are associated with hyperactivation and disease progression in chronic HIV infection [33]. Furthermore, we found higher frequencies of all the CD4 and CD8 maturational subsets expressing the proliferation marker Ki67 in the non-responders to the influenza vaccine than the responders. In a recent work, we observed an inverse association between the magnitude of the HBV vaccine response and the frequency of proliferating CD4 T-cells in a cohort of HIV-infected patients [25]. As far as we know, no other previous evidence has associated a poor response to vaccination with conventional T-cell proliferation. However, Stervbo *et al*. reported an age-dependent association between influenza vaccine responsiveness and the proliferation of γδ T-cells [34].

The inflammatory status has been consistently shown to disturb vaccine responsiveness in the elderly [12,13]. Moreover, hsCRP levels predict herpes zoster vaccine responses in elderly nursing home residents [35]. Although we failed to observe higher hsCRP levels in the non-responders, we observed a tendency towards higher levels of other inflammation-related markers such as D-dimers, neutrophils or the NLR in the non-responders. The fact that the anti-CMV titre was not associated with the response to the

vaccine deserves a special mention, since CMV seropositivity has been previously associated with a negative effect on influenza vaccine responses [36,37]. Nevertheless, as expected, we observed an association between the anti-CMV titre and several inflammation-related biomarkers (the ESR and LBP levels).

Interestingly, the limited CD4 T-cell repertoire diversity in aged individuals, probably a consequence of reduced thymic function, has been associated with a poor response to influenza vaccination in a mouse model [38]. However, in our cohort, we failed to observe lower thymic function in the non-responders compared with the responders. Nevertheless, we observed a higher frequency of ki67+ naïve T-cells, which is a surrogate marker of T-cell activation and proliferation, in the non-responders. Along this line, Sauce *et al.* [16] showed an association between increased naive T-cell turnover and decreased thymic function in elderly subjects, young adults thymectomised during early childhood and HIV-infected subjects. Importantly, despite 30% of our cohort showing thymic failure, this cohort could globally have a partially preserved thymic function, which can be appreciated when comparing the thymic function of our cohort with that of a different elderly population with a similar age range and thymic function values quantified by the same technique [39]. Alternatively, the higher Ki67 expression in the T-cell subsets of the non-responders could better reflect their inflammatory status, since we report consistent associations between Ki67 expression and several soluble inflammation-related parameters. Along this line, previous data link the inflammatory environment of HIV infection with increased memory CD4 T-cell cycling [40]. Thus, reasonably, this age-dependent homeostatic dysregulation involving T-cell proliferation could contribute to inflammaging. Interestingly, we also observed negative associations between thymic function and the levels of different inflammation-related biomarkers (mainly D-dimers but also the ESR, PLR and hsCRP levels), and a

relationship between thymic involution and chronic inflammation has also been previously described [41]. Thus, one can speculate that in the ageing context, thymic function and inflammation could be inversely interrelated. Accordingly, again in comparison with the cohort of Ferrando-Martínez *et al.* [39], our cohort shows a trend towards a lower inflammatory status while showing higher levels of thymic function.

Our study is mainly descriptive and has several limitations. First of all, the results must be considered with caution because the statistical power might be compromised due to the multiple testing approach. In our case, the normally assumed 5% error rate (type I error, α=0.05) could not be restrictive enough to reject the null hypothesis. Second, no data are available about possible concomitant anti-inflammatory treatments (such as statins or aspirin), which could interfere with the levels of inflammation-related biomarkers. Additionally, although it was not an objective of our study, we show here data related to a low number of deaths recorded during a year of follow-up in our cohort. We acknowledge important limitations of this sub-analysis related to the cause of death, such as having such a low number of events or having not considered potential confounding factors for the levels of the biomarkers assessed. However, it is worth mentioning that the six subjects who died during the follow-up year showed lower thymic function but higher proliferation in the T-cell subsets, including the Treg subsets, as well as higher levels of inflammation-related biomarkers than those who survived during this follow-up period. In this sense, hsCRP levels have been previously associated with time to death in the elderly, and the risk of death is further elevated when high hsCRP levels are present in addition to CMV seropositivity [42] or low thymic function, as we previously reported [39]. Although larger studies need to be performed to corroborate our results, these findings suggest that both thymic function and age-dependent homeostatic dysregulation involving T-cell proliferation (probably

as a compensatory mechanism) are relevant to the underlying mechanisms of progression to death in elderly people.

CONCLUSIONS

In summary, age-dependent homeostatic dysregulation involving the proliferation of CD4 and CD8 T-cell subsets, including Tregs, was related to a reduced responsiveness to influenza vaccination as well as to a higher inflammatory status in an elderly population. Despite the statistical weakness of our results, our data support and extend previous observations from ageing studies of other human T-cell subsets and suggest that further research on the mechanisms underlying such relationships in the elderly could help to find better strategies to produce a proper vaccine response against influenza in this compromised population. Deepening this knowledge will also be useful to further understand how immunosenescence limits immune capacities.

LIST OF ABBREVIATIONS

APC: Antigen Presenting Cells

CM: Central Memory

CMV: Cytomegalovirus

ELISA: Enzyme-Linked Immunosorbent Assays

EM: Effector Memory

ESR: Erythrocyte Sedimentation Rate

eTreg: Effector Treg

HA: Haemagglutinin

HAI: Haemagglutination Inhibition

HBV: Hepatitis B Virus

HIV: Human Immunodeficiency Virus

hsCRP: High Sensitivity C Reactive Protein

IL-6: Interleukin-6

IQR: Interquartile Ranges

LBP: Lipopolysaccharide Binding Protein

MCV: Mean Corpuscular Volume

MPV: Mean Platelet Volume

NLR: Neutrophil to Lymphocyte Ratio

nTreg: Naïve Treg

PBMCs: Peripheral Blood Mononuclear Cells

PLR: Platelet to Lymphocyte Ratio

RBCs: Red Blood Cells

RDE: Receptor Destroying Enzyme

RTEs: Recent Thymic Emigrants

sCD163: Soluble CD163

sj/β-TREC: Signal Joint/β T-Cell Receptor Excision Circles

TemRA: Terminally Differentiated Effector Memory

Treg: Regulatory T-cell

WHO: World Health Organization

METHODS

Study design

We included elderly subjects from the Heliopolis Nursing Home, Seville, who were going to be vaccinated against influenza virus during November 2015 (the 2015-2016 campaign). Among these subjects, those older than 60 years, without cognitive impairment and able to sign the informed consent were included in this study. Subjects treated with antitumour therapy or any treatment that could influence their immune

status (mainly corticosteroids) during the preceding 6 months were excluded. The vaccination protocol (Figure S2) consisted of one intradermal dose of the trivalent influenza vaccine for the Northern Hemisphere (Intanza 15 µg, Sanofi Pasteur MSD, Lyon, France) with split and inactivated viruses of the strains: A/California/7/2009 H1N1pdm09, A/Switzerland/9715293/2013 H3N2 and B/Phuket/30731/2013 Yamagata lineage. Blood samples were collected pre-vaccination (from 29 to 0 days before the administration of the vaccine) and post-vaccination (from 12 to 33 days after vaccination) and processed at the Institute of Biomedicine of Seville, Virgen del Rocío University Hospital. Deaths occurring within one year after vaccination were recorded, except for one subject who was lost to follow-up due to a residency change. The study was approved by the Ethics Committee of the Virgen del Rocío University Hospital.

Haemagglutination inhibition (HAI) test

Influenza vaccine responses were measured at the Microbiology Service of the Virgen de las Nieves University Hospital, Granada through an HAI test analysis. Pre-vaccination and post-vaccination sera were tested for HAI titres. The standardized antigen for the HAI test was prepared using the 2015-2016 trivalent influenza vaccine for the Northern Hemisphere (Influvac, Mylan Pharmaceuticals, Barcelona, Spain). The standardized antigen contained 4 haemagglutinin (HA) units per 25 µl of each of the following inactivated strains: A/California/7/2009 H1N1pdm09, A/Switzerland/9715293/2013 H3N2 and B/Phuket/30731/2013 Yamagata lineage. The HAI tests were performed with chicken red blood cells (RBCs) according to the WHO standard procedures [31]. Briefly, serum samples were pre-treated with Receptor Destroying Enzyme (RDE II Seiken, Denka Seiken Co Ltd, Tokyo, Japan) in order to inactivate non-specific haemagglutination inhibitors according to the manufacturer's instructions. The RDE-treated sera were diluted 1:10 and then 25 µl was diluted 2-fold

in PBS and incubated at room temperature for 15 minutes with 25 µl of standardized antigen. Then, 50 µL of standardized RBCs were added to each well and incubated for 30 minutes at room temperature. The HAI titre was the last dilution at which haemagglutination was inhibited. Seroprotection was defined as an HAI titre ≥40. A positive response was defined as a 4-fold or greater increase in the HAI titre between the pre- and post-vaccination serum samples [43].

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) collected pre-vaccination were isolated from fresh blood and cryopreserved until analysis. The characterization of peripheral CD4 and CD8 T-cells was performed according to the distribution of their maturational subsets [naïve ($CD27^+CD45RA^+$), central memory ($CD27^+CD45RA^-$), effector memory ($CD27^-CD45RA^-$), terminally differentiated effector memory (TemRA) ($CD27^-CD45RA^+$) and recent thymic emigrants (RTEs; naïve $CD31^+$)]. Our gating strategy is shown in Figure S3. We measured the expression of an activation marker (HLA-DR), a senescence marker (CD57), an apoptosis susceptibility marker (CD95), a proliferation marker (Ki67) and a suppression marker (CTLA-4). Representative FACS plots for Ki67 staining are shown in Figure S4. We also identified total-Tregs ($CD25^{hi}FoxP3^+$), naïve-Tregs (nTregs, $CD45RA^+FoxP3^{lo}$), effector-Tregs (eTregs, $CD45RA^-FoxP3^{hi}$) and non-Tregs ($CD45RA^-FoxP3^{lo}$) as previously described by Miyara *et al*. [44]. We studied the expression of the abovementioned activation, proliferation and suppression markers and a functional marker (CD39) on these Treg subsets.

For immunophenotyping, PBMCs were thawed and stained with the following surface antibodies: anti-CD31 PE-CF594, anti-CD56 BV510, anti-CD25 BV605, anti-CD45RA BV650, anti-CD4 BV786, anti-CD3 APC-H7 (BD Biosciences, USA), anti-CD39 FITC, anti-CD57 PE-Cy7, anti-HLA-DR BV570, anti-CD95 BV711 and anti-CD27

AF700 (BioLegend, USA). For intracellular staining, the cells were then fixed and permeabilized according to the manufacturer's instructions (FoxP3/Transcription Factor Staining Buffer, eBioscience, USA) and intracellularly stained [anti-Ki67 PerCP-Cy5.5, anti-FoxP3 PE and anti-CTLA4 APC antibodies (BD Biosciences, USA)]. In each experiment, isotype controls for the antibodies specific for CD39, CD31, CD25, CD95, Ki67, FoxP3 and CTLA4 were included. The identification of viable cells was performed using LIVE/DEAD fixable Aqua Blue Dead Cell Stain (Life Technologies, USA). One million cells from each sample were stained, and a minimum of 100,000 total lymphocyte events were acquired. Flow cytometry was performed on an LSR Fortessa (BD Biosciences, USA). Analyses were performed using FlowJo version 9.3 (TreeStar).

sj/β-TREC ratio quantification

Thymic function was determined with pre-vaccination PBMC DNA by quantifying the sj/β-TREC ratio with a technique previously optimized by our group [43], with minor modifications. A schematic representation of the sj/β-TREC ratio quantification protocol is shown in the original report [45]. Briefly, in the same PCR reaction tube, the six dβJβ-TREC from cluster one were amplified, whereas the sj-TREC was amplified in a different PCR reaction tube. Twenty amplification rounds were performed to guarantee an accurate quantification at the real-time PCR step. All amplicons, dβJβ and sj-TREC, were then amplified together in a second round of PCR using the LightCycler® 480 System (Roche, Mannheim, Germany). We defined an sj/β-TREC ratio value lower than 10 as thymic function failure, since we previously found that this cutoff could forecast survival in a cohort of elderly people [39] as well as other clinical endpoints such as cytomegalovirus disease after solid organ transplantation [46] or HIV disease progression [47].

411 **Laboratory measurements and assaying soluble biomarkers**

412 All determinations were performed with pre-vaccination samples. Absolute numbers of
413 $CD4^+$ and $CD8^+$ T-cells and percentages of lymphocytes, monocytes, neutrophils,
414 basophils, eosinophils and platelets were determined with an Epics XL-MCL flow
415 cytometer (Beckman-Coulter, Brea, California). The high sensitivity C-reactive protein
416 (hsCRP) and β2-microglobulin levels were determined with an immunoturbidimetric
417 sera assay using Cobas 701 (Roche Diagnostics, Mannheim, Germany). The D-dimer
418 levels were measured with an automated latex enhanced immunoassay using plasma
419 samples (HemosIL D-Dimer HS 500, Instrumentation Laboratory, Bedford,
420 Massachusetts). The mean corpuscular volume (MCV), mean platelet volume (MPV)
421 and erythrocyte sedimentation rate (ESR) were determined with a Sysmex XN-200
422 analyser (Sysmex, Kobe, Japan). The platelet to lymphocytes ratio (PLR) and neutrophil
423 to lymphocyte ratio (NLR) were calculated as inflammatory indices.

424 Serum and plasma samples were aliquoted and stored at -20°C until subsequent analysis
425 of the levels of Interleukin-6 (IL-6), soluble CD163 (sCD163), and Lipopolysaccharide
426 Binding Protein (LBP) as well as anti-CMV IgG antibody titres by colorimetric
427 enzyme-linked immunosorbent assays (ELISA) according to manufacturer's
428 instructions. Specifically, the following kits were used: IL-6 (Quantikine® HS ELISA,
429 R&D Systems, Minneapolis, Minnesota), sCD163 (MacroCD163™, IQProducts,
430 Groningen, The Netherlands), LBP (Human ELISA kit, Hycult Biotech, Uden, The
431 Netherlands), and anti-CMV IgG (Cytomegalovirus IgG ELISA Kit, Abnova, Taiwan,
432 China).

433 **Statistical analysis**

434 Continuous variables were recorded as medians and interquartile ranges [IQR], and
435 categorical variables were recorded as the number of cases and percentages.

Comparisons among groups were made using the nonparametric Mann–Whitney *U*-test for continuous variables and the $\chi 2$ or Fisher exact test for categorical variables. Correlations were assessed using Spearman's rho correlation coefficient. A p value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS software (version 22; IBM SPSS, Chicago, USA), and graphs were generated using Prism (version 5, GraphPad Software, Inc.).

DECLARATIONS

- ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of the Virgen del Rocío University Hospital, and all subjects included in the study signed an informed consent form to participate.

- CONSENT FOR PUBLICATION

Not applicable

- AVAILABILITY OF DATA AND MATERIAL

The corresponding author has taken custody of all data generated during this work. The FlowJo files generated during flow cytometry experiments as well as the clinical analytics and the database generated for statistical analyses are available upon request, respecting individual data protection.

- COMPETING INTERESTS

The authors declare that they have no competing interests.

- FUNDING

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- AUTHORS' CONTRIBUTIONS

I. H-F was globally responsible for sample handling, performed flow cytometry and ELISA determinations, analysed data and wrote the draft. I. R-S performed flow cytometry and ELISA determinations and helped with data analysis. A. A-R, A C and B S performed haematological determinations. MI G, R R and J C assisted participants during the vaccination protocol and collected samples. M De L-R performed thymic function determinations. S S-G, M P-R and JM N-M performed vaccine response titre determinations. M L and YM P designed and supervised the study. YM P conceived the study and advised the data interpretation and writing. All authors critically reviewed the final manuscript.

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480 - **AUTHORS' INFORMATION (OPTIONAL)**

481 Not applicable

482 **REFERENCES**

483 1. Gasparini R, Amicizia D, Lai PL, Panatto D. Clinical and socioeconomic impact of
484 seasonal and pandemic influenza in adults and the elderly. Hum Vaccin
485 Immunother. 2012;8:21-28.

486 2. World Health Organization: Influenza seasonal. http://www.who.int/en/news-
487 room/fact-sheets/detail/influenza-(seasonal). Accessed 27 August 2018.

488 3. Iuliano AD, Roguski KM, Chang HH, Muscatello DJ, Palekar R, Tempia S *et al*.
489 Lancet. Estimates of global seasonal influenza-associated respiratory mortality: a
490 modelling study. 2018;391:1285-1300.

491 4. Goodwin K, Viboud C, Simonsen L. Antibody response to influenza vaccination in
492 the elderly: a quantitative review. Vaccine. 2006;24:1159-1169.

493 5. Haq K, McElhaney JE. Immunosenescence: influenza vaccination and the elderly.
494 Curr Opin Immunol. 2014;29C:38-42.

495 6. Goronzy JJ, Weyand CM. Understanding immunosenescence to improve responses to
496 vaccines. Nat Immunol. 2013;14:428-436.

497 7. Panda A, Qian F, Mohanty S, van Duin D, Newman FK, Zhang L *et al*. Age-
498 associated decrease in TLR function in primary human dendritic cells predicts influenza
499 vaccine response. J Immunol. 2010;184:2518-2527.

500 8. Najarro K, Nguyen H, Chen G, Xu M, Alcorta S, Yao X *et al*. Telomere length as an
501 indicator of the robustness of B- and T-cell response to influenza in older adults. J
502 Infect Dis. 2015;212:1261-1269.

503 9. Frasca D, Blomberg BB. B cell function and influenza vaccine responses in healthy
504 aging and disease. Curr Opin Immunol. 2014;29:112–118

505 10. Saurwein-Teissl M, Lung TL, Marx F, Gschösser C, Asch E, Blasko I *et al.* Lack of
506 antibody production following immunization in old age: association with
507 CD8(+)CD28(-) T-cell clonal expansions and an imbalance in the production of Th1
508 and Th2 cytokines. J Immunol. 2002;168:5893-5899.

509 11. Pinti M, Appay V, Campisi J, Frasca D, Fülöp T, Sauce D *et al.* Aging of the
510 immune system – focus on inflammation and vaccination. Eur J
511 Immunol. 2016;46:2286-2301

512 12. Trzonkowski P, Myśliwska J, Szmit E, Wieckiewicz J, Lukaszuk K, Brydak LB *et*
513 *al.* Association between cytomegalovirus infection, enhanced proinflammatory response
514 and low level of anti-hemagglutinins during the anti-influenza vaccination--an impact of
515 immunosenescence. Vaccine. 2003;21:3826-3836.

516 13. Parmigiani A, Alcaide ML, Freguja R Pallikkuth S, Frasca D, Fischl MA, Pahwa S.
517 Impaired Antibody Response to Influenza Vaccine in HIV-Infected and Uninfected
518 Aging Women Is Associated with Immune Activation and Inflammation. PloS one.
519 2013;8:e79816.

520 14. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential
521 contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci. 2014;69:S4-9.

522 15. Quinn KM, Fox A, Harland KL, Russ BE, Li J, Nguyen THO *et al*. Age-Related
523 Decline in Primary CD8+ T Cell Responses Is Associated with the Development of
524 Senescence in Virtual Memory CD8+ T Cells. Cell Rep. 2018;23:3512-3524.

525 16. Sauce D, Larsen M, Fastenackels S, Roux A, Gorochov G, Katlama C *et al.*
526 Lymphopenia-driven homeostatic regulation of naive T cells in elderly and
527 thymectomized young adults. J Immunol. 2012;189:5541-5548.

528 17. Ferrando-Martínez S, Ruiz-Mateos E, Hernández A, Gutiérrez E, Rodríguez-
529 Méndez MM, Ordoñez A, Leal M. Age-related dysregulation of naive T cell
530 homeostasis in elderly humans. AGE. 2011;33:197–207.

531 18. Hakim FT, Memon SA, Cepeda R, Jones EC, Chow CK, Kasten-Sportes C *et al.*
532 Age-dependent incidence, time course, and consequences of thymic renewal in adults. J
533 Clin Invest. 2005;115:930-939.

534 19. Plitas G, Rudensky AY. Regulatory T Cells: Differentiation and Function. Cancer
535 Immunol Res. 2016;4:721-725.

536 20. Lim HW, Hillsamer P, Kim CH. Regulatory T cells can migrate to follicles upon T
537 cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. J Clin
538 Invest. 2004;114:1640-1649.

539 21. Toka F, Suvas S, Rouse BT. CD4+ CD25+ T cells regulate vaccine-generated
540 primary and memory CD8+ T-cell responses against herpes simplex virus type 1. J
541 Virol. 2004;78:13082-13089.

542 22. Espinoza-Mora MR, Steeg C, Tartz S, Heussler V, Sparwasser T, Link A *et al.*
543 Depletion of regulatory T cells augments a vaccine-induced T effector cell response

against the liver-stage of malaria but fails to increase memory. PLoS One. 2014;12;9:e104627.

23. YangZ, WangL, NiuW, WuY, ZhangJ, Meng G. Increased CD4+CD25+FoxP3+regulatory T cells in the blood of nonresponders after standard hepatitis B surface antigen vaccine immunization. Clin Immunol. 2008;127:265-266.

24. Pozo-Balado MM, Leal M, Méndez-Lagares G, Mata RC, López-Cortés LF, Viciana P, Pacheco YM. Increased regulatory T cell counts in HIV-infected nonresponders to hepatitis B virus vaccine. J Infect Dis. 2010;202:362-369.

25. Herrero-Fernandez I, Rosado-Sánchez I, Genebat M, Tarancón-Díez L, Rodríguez-Méndez MM, Pozo-Balado MM et al. Improved CD4 T-cell profile in HIV-infected subjects on maraviroc-containing therapy is associated with better responsiveness to HBV vaccination. J Transl Med. 2018;16:238. doi: 10.1186/s12967-018-1617-1

26. Lin PH, Wong WI, Wang YL, Hsieh MP, Lu CW, Liang CY *et al.* Vaccine-induced antigen-specific regulatory T cells attenuate the antiviral immunity against acute influenza virus infection. Mucosal Immunol. 2018;11:1239-1253.

27. Wang SM, Tsai MH, Lei HY, Wang JR, Liu CC. The regulatory T cells in anti-influenza antibody response post influenza vaccination. Hum Vaccine Immunother. 2012;8:1243–1249.

28. Raynor J, Lages CS, Shehata H, Hildeman DA, Chougnet CA. Homeostasis and function of regulatory T cells in aging. Curr Opin Immunol. 2012;24:482-487.

29. Vianna PH, Canto FB, Nogueira JS, Nunes CF, Bonomo AC, Fucs R. Critical influence of the thymus on peripheral T cell homeostasis. Immun Inflamm Dis. 2016;4:474-486.

30. Fessler J, Ficjan A, Duftner C, Dejaco C. The impact of aging on regulatory T-cells. Front Immunol. 2013;4:231.

31. van der Geest KS, Abdulahad WH, Tete SM, Lorencetti PG, Horst G, Bos NA *et al*. Aging disturbs the balance between effector and regulatory CD4+ T cells. Exp Gerontol. 2014;60:190-196.

32. Attridge K, Walker LS. Homeostasis and function of regulatory T cells (Tregs) in vivo: lessons from TCR-transgenic Tregs. Immunol Rev. 2014;259:23–39.

33. Xing S, Fu J, Zhang Z, Gao Y, Jiao Y, Kang F *et al.* Increased Turnover of FoxP3high Regulatory T Cells Is Associated With Hyperactivation and Disease Progression of Chronic HIV-1 Infection. J Acquir Immune Defic Syndr. 2010;54:455–462.

34. Stervbo U, Pohlmann D, Baron U, Bozzetti C, Jürchott K, Mälzer JN *et al*. Age dependent differences in the kinetics of γδ T cells after influenza vaccination. PLoS One. 2017;12:e0181161.

35. Verschoor CP, Lelic A, Parsons R, Evelegh C, Bramson JL, Johnstone J *et al.* Serum C-Reactive Protein and Congestive Heart Failure as Significant Predictors of Herpes Zoster Vaccine Response in Elderly Nursing Home Residents. J Infect Dis. 2017;216:191-197.

36. Frasca D, Diaz A, Romero M, Landin AM, Blomberg BB. Cytomegalovirus (CMV) seropositivity decreases B cell responses to the influenza vaccine. Vaccine. 2015;33:1433-1439.

588 37. Merani S, Pawelec G, Kuchel GA, McElhaney JE. Impact of Aging and
589 Cytomegalovirus on Immunological Response to Influenza Vaccination and Infection.
590 Front Immunol. 2017;8:784.

591 38. Lanzer KG, Johnson LL, Woodland DL, Blackman MA. Impact of ageing on the
592 response and repertoire of influenza virus-specific CD4 T cells. Immun
593 Ageing. 2014;11:9.

594 39. Ferrando-Martínez S, Romero-Sánchez MC, Solana R, Delgado J, de la Rosa
595 R, Muñoz-Fernández MA *et al.* Thymic function failure and C-reactive protein levels
596 are independent predictors of all-cause mortality in healthy elderly humans. AGE
597 2013;35:251–259.

598 40. Jiang W, Younes SA, Funderburg NT, Mudd JC, Espinosa E, Davenport MP *et al*.
599 Cycling memory CD4+ T cells in HIV disease have a diverse T cell receptor repertoire
600 and a phenotype consistent with bystander activation. J Virol. 2014;88:5369-5380.

601 41. Coder BD, Wang H, Ruan L, Su DM. Thymic involution perturbs negative selection
602 leading to autoreactive T cells that induce chronic inflammation. J
603 Immunol. 201;194:5825-5837.

604 42. Simanek AM, Dowd JB, Pawelec G, Melzer D, Dutta A, Aiello AE. Seropositivity
605 to Cytomegalovirus, Inflammation, All-Cause and Cardiovascular Disease-Related
606 Mortality in the United States. PLoS One. 2011;6:e16103.

607 43. World Health Organization (WHO) (2011) Manual for the laboratory diagnosis and
608 virological surveillance of influenza.

http://www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza/en/. Accessed 27 August 2018.

44. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A *et al.* Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity. 2009;30(6):899-911.

45. Ferrando-Martínez S, Franco JM, Ruiz-Mateos E, Hernández A, Ordoñez A, Gutierrez E, Leal M. A reliable and simplified sj/beta-TREC ratio quantification method for human thymic output measurement. J Immunol Methods. 2010;352(1-2):111-117.

46. Gracia-Ahufinger I, Ferrando-Martínez S, Montejo M, Muñoz-Villanueva MC, Cantisán S, Rivero A *et al*. Pre-transplant thymic function is associated with the risk of cytomegalovirus disease after solid organ transplantation. Clin Microbiol Infect. 2015;21:511.e1-7.

47. Ferrando-Martinez S, De Pablo-Bernal RS, De Luna-Romero M, De Ory SJ, Genebat M, Pacheco YM et al. Thymic Function Failure Is Associated With Human Immunodeficiency Virus Disease Progression. Clin Infect Dis. 2017;64:1191-1197.

TABLES

Table 1. Characterization of the study population. Comparisons between groups regarding the vaccine response to the influenza vaccine.

Variable	TOTAL N=60	Non-Responders N=33	Responders N=27	p
Age (years)	79 [70-87]	80 [67-88]	77 [71-86]	0.806

Male sex, n (%)	24 (40)	15 (46)	9 (33)	0.340
$CD4^+$ T-cell count ($cells/mm^3$)	799 [614-1103]	715 [533-1236]	825 [749-1008]	0.281
$CD8^+$ T-cell count ($cells/mm^3$)	473 [279-685]	439 [159-671]	486 [384-698]	0.185
$CD4^+/CD8^+$ ratio	1.8 [1.2-2.3]	1.9 [1.2-2.3]	1.6 [1.1-2.7]	0.704
sj/β TREC ratio	32 [0-50]	31 [0-55]	34 [7-50]	0.615
Thymic failure (#)	19 (32)	12 (36)	7 (27)	0.441
CMV titre (AU/µL)	25.4 [13.3-40.3]	25.4 [12.4-39.0]	24.6 [13.8-43.0]	0.973
LBP (ng/µL)	12.7 [10.2-14.1]	13.0 [10.1-15.3]	12.7 [11.4-13.5]	0.564
hsCRP (mg/L)	2.8 [1.6-5.0]	2.3 [1.2-4.8]	3.1 [2.2-5.0]	0.330
B2M (µg/mL)	2.5 [2.1-3.5]	2.7 [2.4-3.7]	2.3 [2.0-3.3]	0.156
D-dimers (µg/L)	705 [438-1183]	875 [445-1425]	620 [438-918]	*0.082*
IL-6 (pg/mL)	3.5 [2.4-4.6]	3.4 [2.3-4.9]	3.6 [2.6-4.5]	0.691
sCD163 (ng/L)	1034 [844-1293]	1034 [792-1224]	1084 [878-1477]	0.222
% Lymphocytes	26.2 [23.2-33.1]	24.9 [22.4-32.0]	29.0 [25.0-34.1]	*0.063*
% Monocytes	6.5 [5.4-7.6]	6.6 [5.4-7.9]	6.5 [5.4-7.5]	0.894
% Neutrophils	59.6 [54.1-65.7]	62.8 [54.4-66.9]	58.0 [52.4-62.5]	*0.089*
% Basophils	0.2 [0.2-0.4]	0.3 [0.2-0.4]	0.2 [0.1-0.3]	0.258
% Eosinophils	3.4 [2.2-4.2]	3.2 [1.7-4.5]	3.7 [3.2-4.2]	*0.094*
Platelets (x10e9/L)	223 [180-294]	233 [181-294]	197 [170-296]	0.650
MCV (fL)	90.2 [86.1-93.6]	90.6 [85.5-93.5]	90.0 [86.6-95.1]	0.876
MPV (fL)	9.4 [7.9-10.1]	9.3 [7.8-10.3]	9.40 [8.1-10.0]	0.716
ESR (mm/h)	12 [6-22]	14 [6-21]	10 [6-24]	0.921
PLR	117 [87-164]	134 [88-166]	113 [82-144]	0.377
NLR	2.3 [1.6-2.9]	2.6 [1.7-3.0]	1.9 [1.5-2.4]	*0.056*

Continuous variables are expressed as median values [IQR], and categorical variables are expressed as the number of cases (%). Comparisons between the groups were made using the nonparametric Mann–Whitney *U* test for continuous variables and the $\chi 2$ or Fisher exact test for categorical variables. Variables with a *p* value <0.1 are shown in *italics*. Variables with a *p* value <0.05 were considered statistically significant and are shown in bold. Note: CMV, cytomegalovirus; LBP, Lipopolysaccharide Binding

Protein; hsCRP, high sensitivity C-Reactive Protein; B2M, β2-microglobulin; sCD163, soluble CD163; MCV, mean corpuscular volume; MPV, mean platelet volume; ESR, erythrocyte sedimentation rate; PLR, platelet to lymphocyte ratio; and NLR, neutrophil to lymphocyte ratio. (#) Thymic failure is defined as an sj/β TREC ratio<10.

FIGURE LEGENDS

Figure 1. Characterization of Treg subsets in relation to the response to the influenza vaccine. a-d) Frequencies of Treg subsets. **e-h)** Frequencies of Treg subsets expressing the proliferation marker $Ki67^+$. A total of 60 subjects, with a median age of 79 [70-87] years old (median [IQR]). Comparisons between the groups of vaccine non-responders (n=33) and responders (n=27) were made using the nonparametric Mann–Whitney *U* test. Variables with a *p* value <0.05 were considered statistically significant and are shown in bold. Note: nTreg, naïve-Treg; and eTreg, effector-Treg.

Figure 2. Maturational subsets of $CD4^+$ and $CD8^+$ T-cells expressing $Ki67^+$. a-d) Frequencies of $CD4^+$ maturational subsets expressing the proliferation marker $Ki67^+$. **e-h)** Frequencies of $CD8^+$ maturational subsets expressing the proliferation marker $Ki67^+$. Comparisons between the groups of vaccine non-responders (n=33) and responders (n=27) were made using the nonparametric Mann–Whitney *U* test. Variables with a *p* value <0.05 were considered statistically significant and are shown in bold. Note: CM, central memory; EM, effector memory; and TemRA, terminally differentiated effector memory.

Figure 3. Correlations between the sj/β TREC ratio and inflammation-related biomarkers. a) Correlation between sj/β TREC and D-dimers. **b)** Correlation between sj/β TREC and the ESR. **c)** Correlation between sj/β TREC and the PLR. **d)** Correlation

between sj/β TREC and hsCRP. A total of 60 subjects. Correlations were assessed using Spearman's rho correlation coefficient. Variables with a *p* value <0.1 are shown in *italics*. Variables with a *p* value <0.05 were considered statistically significant and are shown in bold. Note: ESR, erythrocyte sedimentation rate; PLR, platelets to lymphocyte ratio; and hsCRP, high sensitivity C-Reactive Protein**.**

ADDITIONAL MATERIAL

Table S1. Characterization of Treg subsets in relation to the response to the influenza vaccine.

Parameter	**Non-Responders N=33**	**Responders N=27**	***p***
total-Treg	1.49 [1.08-1.85]	1.12 [0.94-1.63]	*0.061*
HLADR$^+$	12.30 [7.51-17.60]	11.30 [9.48-14.50]	0.888
Ki67$^+$	16.91 [11.58-19.95]	14.75 [11.55-16.88]	0.204
CTLA4$^+$	60.56 [49.41-69.89]	56.14 [49.29-60.96]	0.262
CD39$^+$	76.81 [43.54-85.85]	65.90 [45.46-79.59]	0.547
nTreg	6.42 [4.31-8.57]	5.54 [3.55-8.30]	0.336
HLADR$^+$	0.80 [0.48-1.11]	0.87 [0.47-1.04]	0.795
Ki67$^+$	38.90 [19.20-42.85]	19.50 [16.10-35.60]	**0.025**
CTLA4$^+$	6.57 [5.85-8.00]	6.57 [6.06-7.92]	0.797
CD39$^+$	27.90 [12.60-42.35]	35.30 [12.90-42.20]	0.733
eTreg	2.86 [1.84-4.33]	2.87 [2.12-4.16]	0.873
HLADR$^+$	9.270 [4.470-14.60]	6.81 [4.59-11.30]	0.705
Ki67$^+$	10.90 [6.46-19.00]	10.00 [7.52-12.90]	0.683
CTLA4$^+$	48.20 [33.65-56.25]	37.00 [31.20-48.20]	0.152
CD39$^+$	48.00 [31.40-61.90]	43.00 [24.60-60.70]	0.499
nonTreg	20.29 [14.84-26.52]	21.18 [17.83-30.95]	0.353
HLADR$^+$	2.06 [1.19-3.56]	1.59 [1.35-3.24]	0.688
Ki67$^+$	38.50 [24.95-44.10]	27.70 [17.90-40.40]	*0.053*
CTLA4$^+$	10.60 [7.65-15.70]	9.20 [7.93-12.30]	0.365
CD39$^+$	33.20 [21.85-39.85]	24.00 [17.00-42.90]	0.305

Percentage of cells expressing each marker among the indicated subset. Continuous variables are expressed as median values [IQR]. Comparisons between the groups were made using the nonparametric Mann–Whitney *U* test. Variables with a *p* value <0.1 are shown in *italics*. Variables with a *p* value <0.05 were considered statistically significant and are shown in bold. Note: nTreg, naïve-Treg; and eTreg, effector-Treg.

Table S2. Comparison of CD4 and CD8 T-cell subsets in groups defined by the vaccine response to the influenza vaccine.

Parameter	Non-Responders N=33	Responders N=27	*p*
CD4			
CD4 RTE	52.20 [39.95-58.65]	53.00 [43.30-66.00]	0.199
CD4 naïve	28.50 [18.45-43.75]	22.70 [14.40-43.60]	0.385
CD4 CM	32.60 [22.25-41.35]	34.00 [29.50-45.20]	0.256
CD4 EM	32.00 [16.75-39.20]	27.70 [18.90-43.10]	0.761
CD4 TemRA	2.59 [1.45-5.83]	1.94 [1.00-4.85]	0.418
CD4 HLADR$^+$	1.24 [0.76-2.52]	1.19 [0.74-2.18]	0.923
CD4 Ki67$^+$	2.30 [1.85-2.80]	2.02 [1.56-2.68]	0.237
CD4 CD57$^+$	10.49 [5.88-21.22]	9.67 [4.27-18.04]	0.377
CD4 CD95$^+$	66.20 [49.15-80.50]	70.80 [53.40-86.20]	0.645
CD4 CTLA4$^+$	8.035 [6.38-10.68]	7.98 [6.90-9.58]	0.700
CD8			
CD8 RTE	96.20 [93.85-97.85]	95.50 [90.00-98.70]	0.824
CD8 naïve	6.985 [4.80-11.40]	6.08 [3.50-8.82]	0.216
CD8 CM	15.70 [9.455-23.15]	19.20 [12.90-27.30]	0.373
CD8 EM	25.60 [18.65-36.05]	24.30 [17.40-35.30]	0.778
CD8 TemRA	33.90 [26.75-55.55]	35.30 [23.20-52.90]	0.677
CD8 HLADR$^+$	2.53 [1.47-4.07]	3.14 [1.76-4.21]	0.325
CD8 Ki67$^+$	9.48 [7.94-12.05]	9.66 [6.55-12.35]	0.325
CD8 CD57$^+$	55.00 [40.50-70.10]	52.70 [39.80-66.00]	0.744
CD8 CD95$^+$	97.80 [96.35-98.30]	97.90 [96.18-98.83]	0.609

Frequencies of the maturational subsets and the percentage of cells expressing each marker among the CD4 and CD8 T-cell subsets. Continuous variables are expressed as median values [IQR]. Comparisons between the groups were made using the nonparametric Mann–Whitney *U* test. Variables with a p value <0.05 were considered statistically significant and are shown in bold. Note: RTE, recent thymic emigrants;

679 CM, central memory; EM, effector memory; and TemRA, terminally differentiated
680 effector memory.

Table S3. Associations among different inflammation-related and haematological parameters and the expression of Ki67 in T-cell subsets.

	LBP (ng/mL)	hsCRP (mg/L)	B2M (µg/mL)	DD (µg/L)	% Lymph	% Mono	% Neutro	% Baso	% Eosino	Platelets (x10e9/L)	MCV (fL)	MPV (fL)	PLR	NLR
% total-Treg				**0.329** **0.013**				0.235 0.071			-0.241 0.064	-0.221 0.089		
% nTreg Ki67$^+$				0.254 0.059	-0.217 0.095			**0.430** **0.001**			-0.223 0.087	**-0.550** **<0.001**		
% eTreg Ki67$^+$		**0.301** **0.020**		0.254 0.059				**0.269** **0.038**				**-0.242** **0.062**		
% nonTreg Ki67$^+$					-0.227 0.081			**0.401** **0.001**	-0.243 0.063			**-0.411** **0.001**		0.220 0.091
% CD4 Ki67$^+$			**0.332** **0.010**	**0.446** **0.001**		**0.264** **0.041**							**0.278** **0.031**	
% CD4 Naïve Ki67$^+$					**-0.266** **0.040**			**0.494** **<0.001**		**0.291** **0.024**	-0.223 0.087	**-0.510** **<0.001**		0.247 0.057
% CD4 CM Ki67$^+$					-0.233 0.073		0.225 0.084	**0.424** **0.001**	-0.249 0.057	0.239 0.066		**-0.415** **0.001**		0.225 0.083
% CD4 EM Ki67$^+$					**-0.310** **0.016**		**0.307** **0.017**	**0.425** **0.001**	-0.233 0.076	0.249 0.055		**-0.470** **<0.001**	0.239 0.066	**0.309** **0.016**
% CD4 TemRA Ki67$^+$	**0.295** **0.029**							**0.360** **0.005**	-0.224 0.090	0.231 0.079		**-0.469** **<0.001**		
% CD8 Ki67$^+$	**0.277** **0.041**	-0.219 0.096												
% CD8 Naïve Ki67$^+$				**0.269** **0.045**				**0.397** **0.002**		0.217 0.095	**-0.297** **0.021**	**-0.517** **<0.001**	0.242 0.062	

% CD8		**0.406**	0.235	**-0.298**	**-0.531**	0.231
CM Ki67$^+$		**0.001**	0.071	**0.021**	**<0.001**	0.076
% CD8	0.224	**0.408**	**0.267**	**-0.327**	**-0.551**	0.217
EM Ki67$^+$	0.088	**0.001**	**0.039**	**0.011**	**<0.001**	0.096
% CD8		**0.434**	0.247	**-0.303**	**-0.550**	
TemRA		**0.001**	0.057	**0.019**	**<0.001**	
Ki67$^+$						

Correlations were assessed using Spearman's rho correlation coefficient. Variables with a *p* value <0.1 are shown in *italics*. Variables with a *p* value <0.05 were considered statistically significant and are shown in bold. N=60. Note: LBP, Lipopolysaccharide Binding Protein; hsCRP, high sensitivity C-reactive protein; B2M, β2-microglobulin; DD, D-Dimers; Lymph, lymphocytes; Mono, monocytes; Neutro, neutrophils; Baso, basophils; Eosino, eosinophils; MCV, mean corpuscular volume; MPV, mean platelet volume; PLR, platelet to lymphocyte ratio; NLR, neutrophils to lymphocyte ratio; nTreg, naïve-Treg; eTreg, effector-Treg; CM, central memory; EM, effector memory; and TemRA, terminally differentiated effector memory.

Table S4. Inflammation-related biomarkers and Ki67 expression in the T-cells of the subjects who died during the follow-up-year.

	Death (YES) N=6	**Death (NO) N=53**	***p***
sj/β TREC ratio	0 [0-0]	34 [10-51]	**0.001**
Thymic failure (#)	6 (100)	13 (25)	**<0.001**
hsCRP (mg/L)	5.10 [3.70-7.58]	2.60 [1.25-4.30]	**0.012**
PLR	152 [152-229]	116 [87-166]	0.318
NLR	2.75 [2.30-3.57]	2.12 [1.63-2.85]	0.153
β2-microglobulin (µg/mL)	3.50 [2.45-5.40]	2.50 [2.03-3.30]	*0.076*
% nTreg Ki67$^+$	49.65 [34.78-51.15]	29.60 [16.70-39.65]	**0.007**
% eTreg Ki67$^+$	18.75 [11.49-23.23]	9.96 [6.50-13.95]	**0.034**
% nonTreg Ki67$^+$	45.05 [34.93-51.60]	36.10 [20.65-41.20]	**0.025**
% CD4 Ki67$^+$	2.65 [2.28-3.20]	2.08 [1.72-2.69]	*0.096*
% CD4 N Ki67$^+$	32.65 [20.39-42.28]	21.70 [12.00-30.35]	0.160
% CD4 CM Ki67$^+$	39.30 [29.70-48.78]	28.20 [19.15-36.90]	*0.068*
% CD4 EM Ki67$^+$	39.30 [30.80-44.05]	28.50 [19.75-35.60]	**0.041**
% CD4 TemRA Ki67$^+$	39.60 [30.93-42.73]	25.50 [18.75-33.30]	**0.033**
% CD8 Ki67$^+$	7.60 [4.20-12.35]	9.86 [7.79-12.18]	0.242
% CD8 N Ki67$^+$	47.55 [32.12-55.28]	15.20 [8.52-42.45]	**0.039**
% CD8 CM Ki67$^+$	38.15 [26.11-49.18]	17.70 [14.45-34.10]	*0.057*
% CD8 EM Ki67$^+$	42.15 [30.74-48.70]	20.40 [15.75-36.60]	**0.020**
% CD8 TemRA Ki67$^+$	41.05 [24.07-41.95]	18.70 [12.30-32.55]	**0.032**

Comparisons between the groups were made using the nonparametric Mann–Whitney *U* test. Variables with a *p* value <0.1 are shown in *italics*. Variables with a *p* value <0.05 were considered statistically significant and are shown in bold. Note: hsCRP, high sensitivity C-Reactive Protein; PLR, platelets to lymphocyte ratio; NLR, neutrophils to lymphocyte ratio; nTreg, naïve-Treg; eTreg, effector-Treg; N, naïve; CM, central memory; EM, effector memory; and TemRA, terminally differentiated effector memory. (#) Thymic failure is defined as an sj/β TREC ratio<10.

ADDITIONAL FIGURE LEGENDS

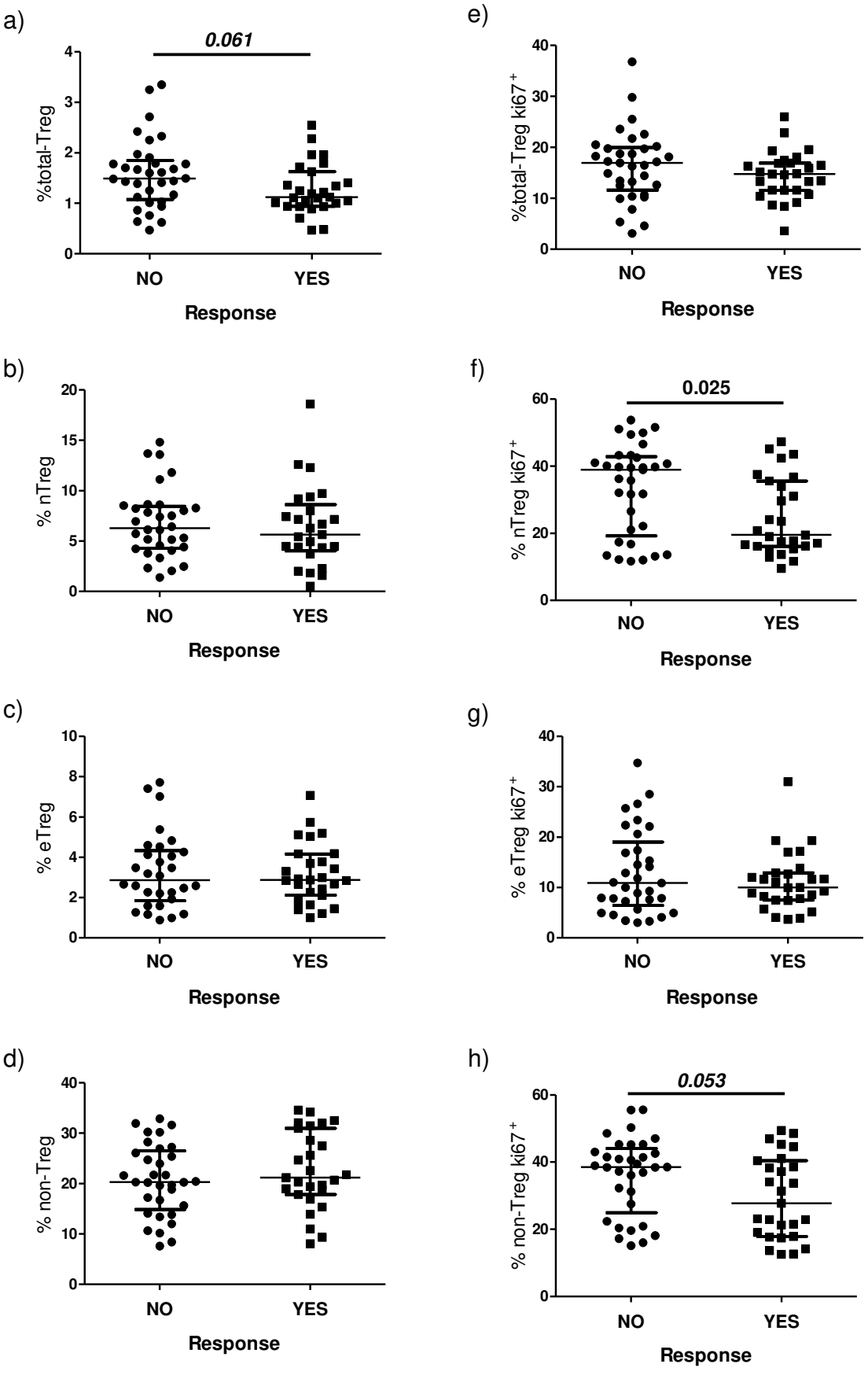
Figure S1. Baseline and post-vaccination HAI titres. Data from the Haemagglutination Inhibition (HAI) test, which was performed at baseline (circles) and post-vaccination (squares), are shown as data for the whole population (n=60) and the groups of influenza vaccine non-responders (n=33) and responders (n=27). Median [IQR] values are included in the data cells below each case.

Figure S2. Protocol. Subjects were vaccinated with one intradermal dose of the trivalent influenza vaccine Intanza (15 µg). Blood samples were collected pre-vaccination (from 29 to 0 days before the administration of the vaccine) and post-vaccination (from 12 to 33 days after vaccination). HAI titres were measured in the pre-vaccination and post-vaccination samples. T-cell immunophenotypes and soluble biomarkers were measured in the pre-vaccination samples. Deaths occurring within one year after vaccination were recorded.

Figure S3. Gating strategy for the T-cell subsets. The gating strategy for the different CD4 T-cell subsets (naïve, central memory, effector memory and TemRA) depending on their expression of CD27 and CD45RA is represented.

Figure S4. Representative FACS plots of ki67 staining. Treg subsets (naïve and effector Treg) and the non-Treg subsets were gated on CD4 T-cells depending on their expression of CD45RA and FoxP3. Then, the percentage of Ki67+ T-cells from each subset was quantified by using isotype control as it is shown in representative histograms.

Figure 1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Response
b)
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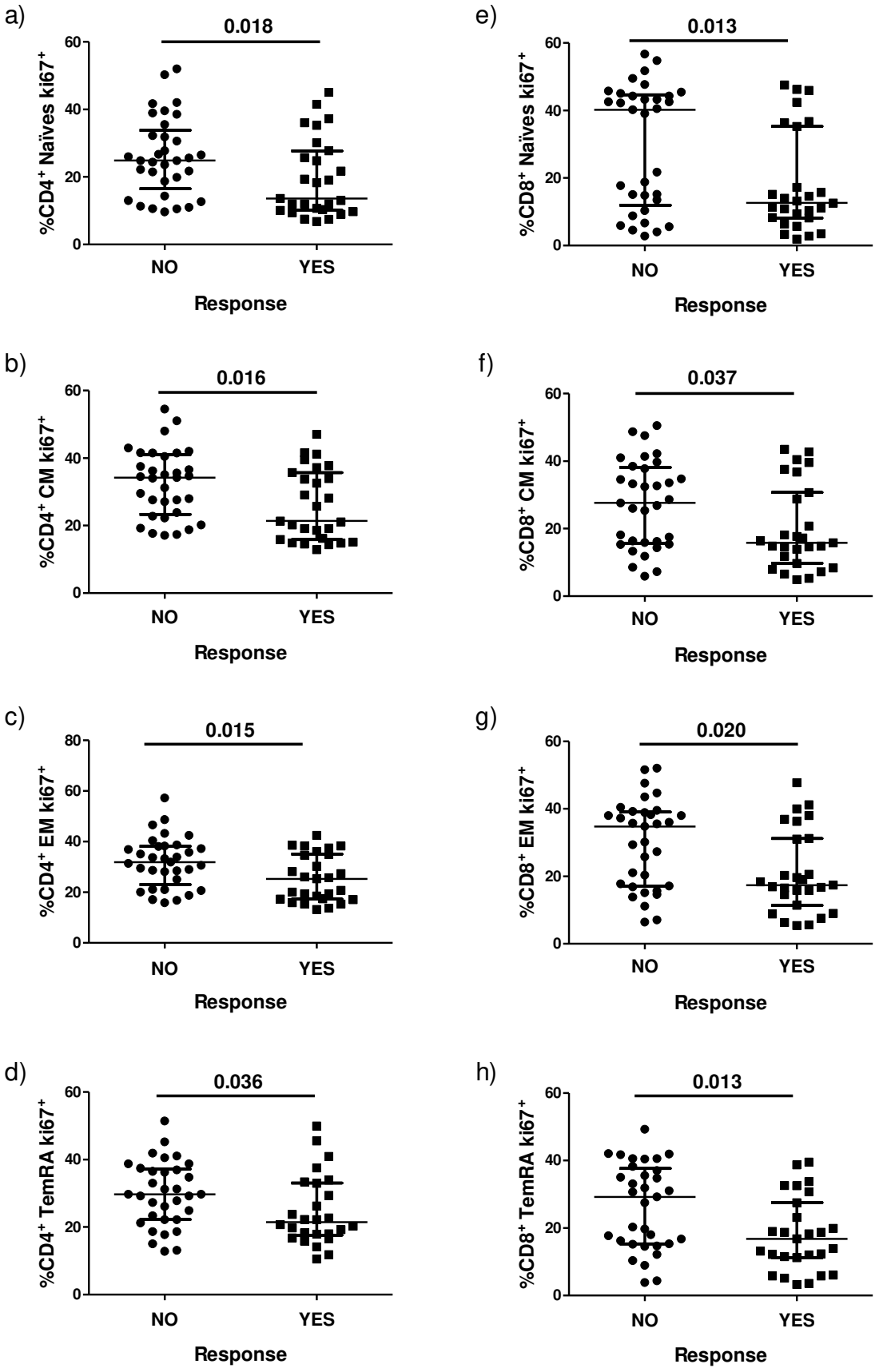
NO
YES
Response
c)
% eTreg
NO
YES
Response
d)
% non-Treg
NO
YES
Response
e)
%total-Treg ki67+
NO
YES
Response
f)
0.025
% nTreg ki67+
NO
YES
Response
g)
% eTreg ki67+
NO
YES
Response
h)
0.053
% non-Treg ki67+
NO
YES
Response

Figure 2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YES
Response
e)
0.013
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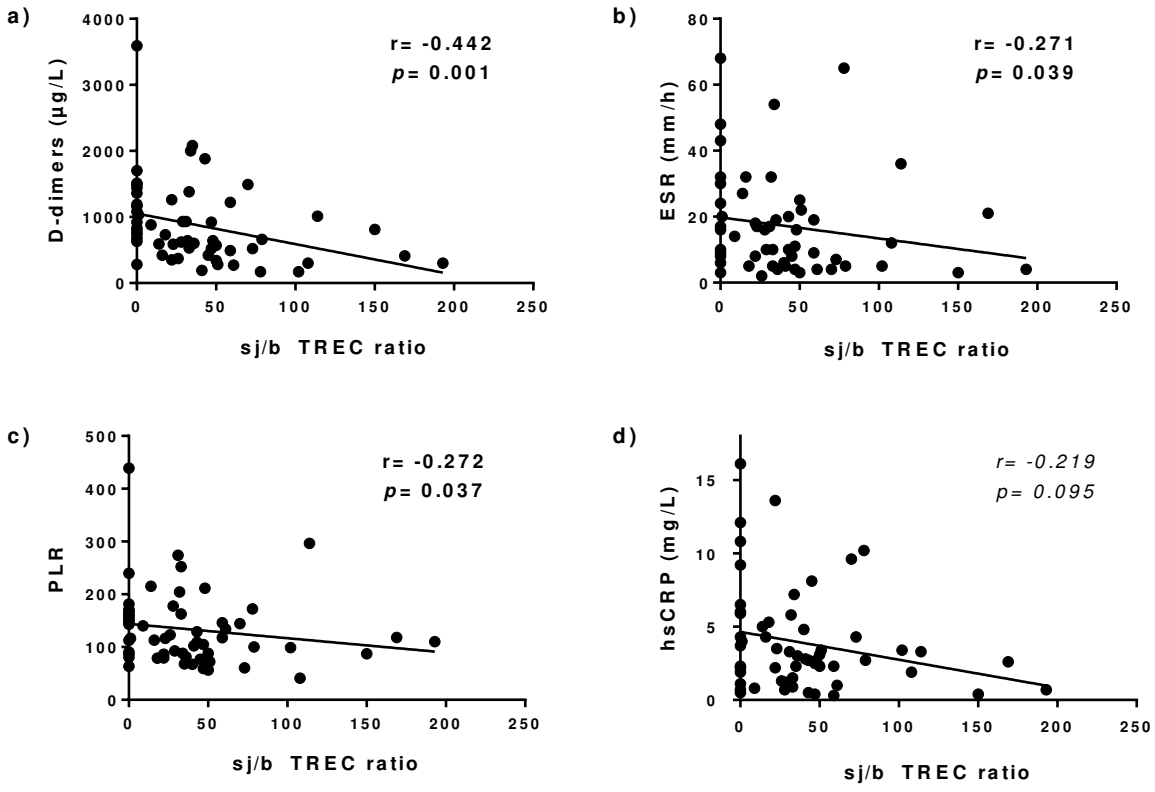
YES
Response
b)
0.016
%CD4+ CM ki67+
NO
YES
Response
f)
0.037
%CD8+ CM ki67+
NO
YES
Response
c)
0.015
%CD4+ EM ki67+
NO
YES
Response
g)
0.020
%CD8+ EM ki67+
NO
YES
Response
d)
0.036
%CD4+ TemRA ki67+
NO
YES
Response
h)
0.013
%CD8+ TemRA ki67+
NO
YES
Response

Figure 3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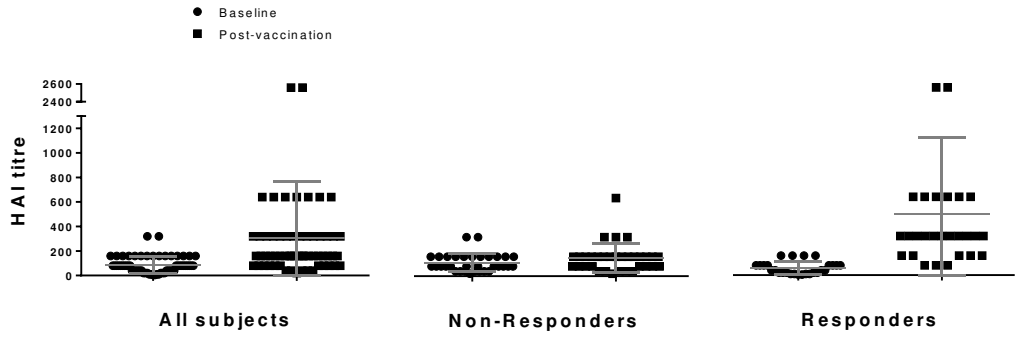
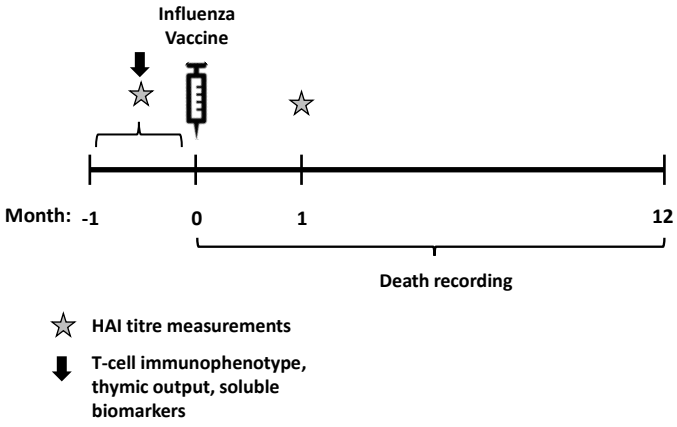
p= 0.001
sj/b TREC ratio
b)
ESR (mm/h)
r= -0.271
p= 0.039
sj/b TREC ratio
c)
PLR
r= -0.272
p= 0.037
sj/b TREC ratio
d)
hsCRP (mg/L)
r= -0.219
p= 0.095
sj/b TREC ratio

Supplementary Figure 1

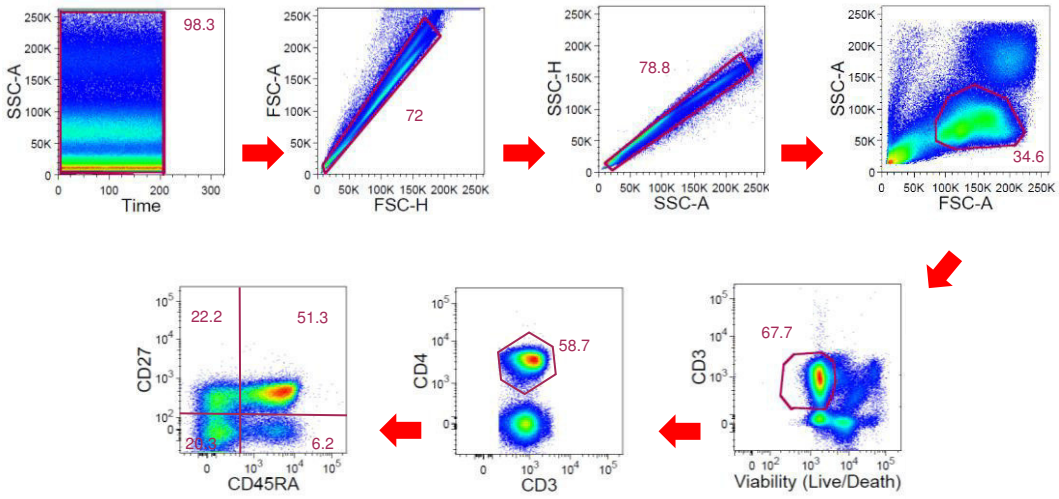


HAI titres	80 [40-160]	160 [80-320]	80 [60-160]	160 [80-160]	40 [20-80]	320 [160-640]

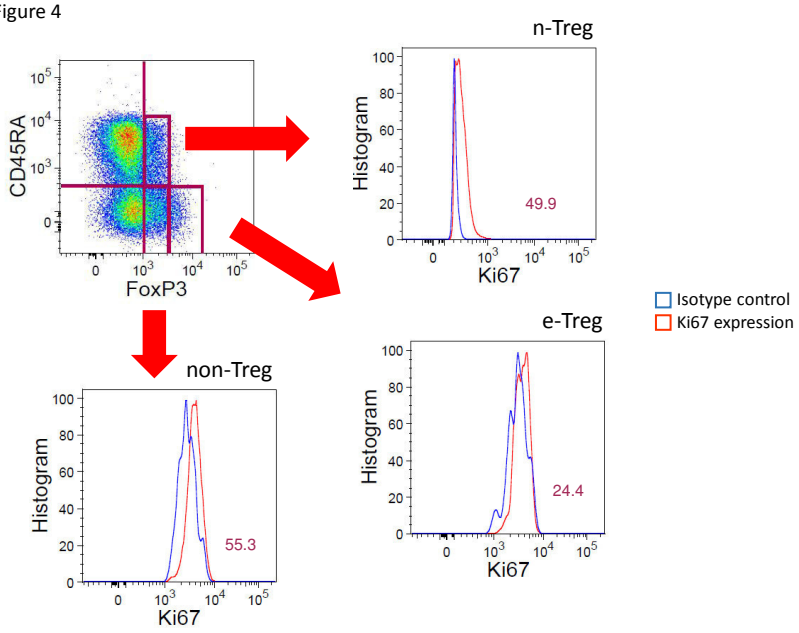
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



4.2. Association between a suppressive combined antiretroviral therapy containing maraviroc and the hepatitis B virus vaccine response

Herrero-Fernández I, Pacheco YM, Genebat M, Rodriguez-Méndez MDM, Lozano MDC, Polaino MJ, Rosado-Sánchez I, Tarancón-Diez L, Muñoz-Fernández MÁ, Ruiz-Mateos E, Leal M.

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ANTIVIRAL AGENTS

Association between a Suppressive Combined Antiretroviral Therapy Containing Maraviroc and the Hepatitis B Virus Vaccine Response

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ABSTRACT The response to the HBV vaccine in HIV-infected patients is deficient. Our aim was to analyze whether a suppressive combined antiretroviral treatment (cART) containing maraviroc (MVC-cART) was associated with a better response to HBV vaccine. Fifty-seven patients on suppressor cART were administered the HBV vaccine. The final response, the early response, and the maintenance of the response were assessed. An anti-HBs titer of >10 mIU/ml was considered a positive response. A subgroup of subjects was simultaneously vaccinated against hepatitis A virus (HAV). Lineal regression analyses were performed to determine demographic, clinical, and immunological factors associated with the anti-HBs titer. Vaccine response was achieved in 90% of the subjects. After 1 year, 81% maintained protective titers. Only simultaneous HAV vaccination was independently associated with the magnitude of the response in anti-HBs titers, with a *P* value of 0.045 and a regression coefficient (B) [95% confident interval (CI)] of 236 [5 to 468]. In subjects ≤50 years old ($n = 42$), MVC-cART was independently associated with the magnitude of the response ($P = 0.009$; B [95% CI], 297 [79 to 516]) together with previous vaccination and simultaneous HAV vaccination. High rates of HBV vaccine response can be achieved by revaccination, simultaneous HAV vaccination, and administration of cARTs including MVC. MVC may be considered for future vaccination protocols in patients on suppressive cART.

KEYWORDS maraviroc, vaccine, HIV infection, combined antiretroviral treatment, hepatitis A virus, hepatitis B virus

HIV accelerates viral hepatitis-related liver damage, early cirrhosis, and end-stage liver disease (1, 2). Direct antiviral agents for hepatitis C virus (HCV) infection are paving the way for HCV eradication in HIV-coinfected patients. For hepatitis B virus (HBV) and hepatitis A virus (HAV), the vaccine is the most effective method to prevent the morbimortality associated with these infections, specially in HIV-infected populations. However, the response to HBV vaccine following the classic vaccination schedule in HIV-infected patients is deficient (3). In addition to the insufficient number of patients responding to these vaccines, in those who respond, the magnitude of protective antibody titers is low and is subsequently lost quickly, which probably restricts the protection of these patients in the long term (4). For this reason, interventions to improve the response to HBV vaccine in HIV-infected patients are urgently needed.

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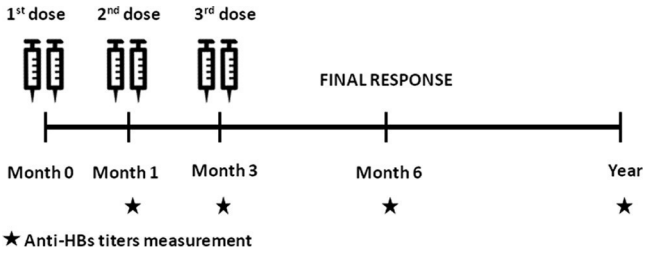

FIG 1 Vaccination protocol. Subjects received 3 intramuscular (deltoid region) double doses (40 μg) of the recombinant Engerix-B vaccine (GlaxoSmithKline, Brentford, United Kingdom) at 0, 1, and 3 months. The vaccine response was measured 6 months after the first dose. We measured the early response at months 1 and 3 after the first dose and the maintenance of the response 1 year after the first vaccination dose.

Apart from controlling for traditional clinical factors associated with lower rates of response such as CD4 T-cell counts and viral load levels (5), the number of doses and the immunization schedule seem to be important too (6, 7). However, no attention has been paid regarding the type of antiretroviral treatment in relation to the rate and magnitude of response. We hypothesize that a combined antiretroviral treatment (cART) containing the CCR5 antagonist maraviroc (MVC) might increase the rates of response to HBV vaccine in HIV-infected patients on suppressive therapy (cART). In support of this hypothesis, we have observed that a switch from a suppressive MVC-free cART to an MVC-containing cART decreased the levels of inflammatory biomarkers (8). In addition, a beneficial profile in HIV disease progression markers such as soluble CD14 (sCD14) and T-cell immunosenescence has been observed in a cART including MVC (9). Finally, we have shown that MVC is able to reduce the frequency of regulatory T-cell (Tregs) (10) and also that a higher frequency of Tregs was associated with a lower rate and magnitude of response to HBV vaccine in HIV-infected patients (11). Altogether, these immunomodulatory effects of MVC may allow synchronized and coordinated innate and adaptive immune responses, which may facilitate a better response to vaccines. In fact, MVC has been shown to enhance meningococcal neoimmunization and improve the response to recall antigens, such as tetanus toxoid (12). Thus, the aim of the present work was to analyze whether a suppressive MVC-containing cART was associated with a better response to HBV vaccine in HIV-infected patients.

RESULTS

Characteristics of the study subjects. Fifty-seven HIV-infected subjects were included in the study, whose vaccination protocol is summarized in Fig. 1; three subjects were lost between month 6 and the year of follow-up. Demographic, clinical, and immunological characteristics of the subjects are summarized in Table 1. Briefly, most were men (79%) with a median age of 44 years. During the vaccination, 46% of the subjects were receiving a nucleoside-reverse transcriptase inhibitor (NRTI)-containing cART (NRTI-cART) consisting of two NRTIs and one protease inhibitor (PI) or two NRTIs and one non-NRTI. On the other hand, nearly one-half of the subjects were receiving a maraviroc-containing cART (MVC-cART) consisting of MVC and a boosted PI or MVC and two NRTIs. All subjects on an MVC-cART had been previously tested for CCR5-tropic virus. Approximately one-third of the cohort had been previously vaccinated against HBV, and one-third received simultaneous HBV and HAV vaccinations.

Rates of vaccine response and antibody titers. The vaccine response (antiHBs $>$10 mIU/ml at month 6) was observed in 51/57 (90%) subjects. The mean of anti-HB titers at month 6 in this cohort was 644 (101 to 1,000). Early responses were observed in 20/57 (35%) subjects after the first dose (month 1) and in 42/57 (74%) of the subjects after the second dose (month 3) (Fig. 2). Vaccine response was maintained in 44/54 (81%) subjects after 1 year of follow-up.

Maraviroc-containing cART, previous vaccination, and simultaneous HAV vaccination were independently associated with the magnitude of the vaccine response in subjects younger than 50 years. The associations of different demo-

TABLE 1 Demographic, clinical, and immunological characteristics of the studied subjects

Characteristic, unit (n = 57)	n (%) or median value (IQR)
Male sex, *n*	45 (79)
Age, yr	44 (36–50)
Nadir $CD4^+$ T-cell count, cells/mm^3	257 (152–396)
$CD4^+$ T-cell count, cells/mm^3	696 (552–887)
$CD8^+$ T-cell count, cells/mm^3	647 (494–972)
$CD4^+/CD8^+$ ratio	1.02 (0.79–1.48)
Time since diagnosis, mo	95 (42–217)
Sexual transmission, *n*	51 (90)
Previous AIDS, *n*	4 (7)
Previous HCV coinfection, *n*	7 (12)
NRTI-containing cART, *n*	26 (46)
MVC-containing cART, *n*	28 (49)
Previous HBV vaccination, n^a	19 (33)
Simultaneous HAV vaccination, *n*	19 (33)
CCR5 WT/Δ32, *n*	10 (17)
hsCRP, mg/liter	1 (0.6–2)

[a]Previous HBV vaccination data were available for only 56 subjects.

graphic, clinical, and immunological characteristics with the magnitude of the final response (anti-HBs titer) are shown in Table 2. In the unadjusted model, female sex and simultaneous HAV vaccination were associated with the antibody titer; in addition, MVC-cART was positively associated with the magnitude of the response with borderline significance. In fact, the antibody titer was significantly higher in the group of subjects receiving MVC-cART (1,000 [320 to 1,000] versus 704 [36 to 1,000] mIU/ml; P = 0.048); these groups did not differ in the parameters of the study with the exception of age (42 [31 to 48] versus 47 [41 to 54] years; P = 0.012) and NRTI-containing cART (5 [18] versus 21 [72] subjects; P = <0.001) (see Table S1 in the supplemental material). Only simultaneous HAV vaccination was independently associated with the magnitude of the response in anti-HBs titers (B), with a P value of 0.045 and a regression coefficient (B) [95% confidence interval (CI)] of 236 [5 to 468], after adjusting for sex, CD4 T-cell counts, previous vaccination, and MVC-cART.

Interestingly, we observed that the effect of maraviroc was enhanced in subjects younger than 50 years (n = 42). After adjusting for sex, previous AIDS, previous vaccination, and simultaneous HAV vaccination, MVC-cART was independently

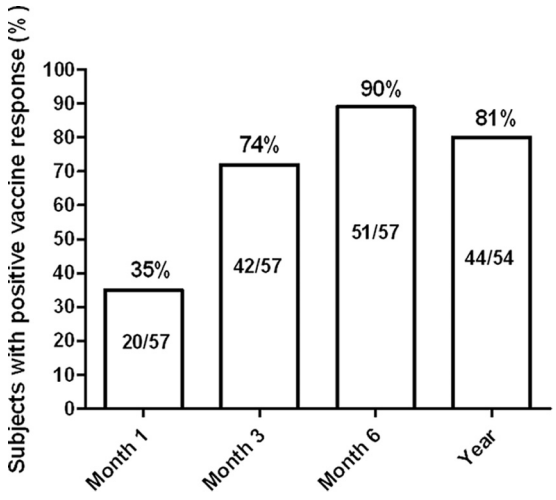

FIG 2 Rates of vaccine response. Percentage of subjects responding at month 1 (early first-dose response), month 3 (early second-dose response), and month 6 (final-vaccination response) and maintaining response at 1 year after the first dose. The numbers inside the bars quantify the subjects with positive vaccine response among the subjects with the vaccine response data recorded. The vaccine response was considered positive when the anti-HBs titer was ≥10 mIU/ml.

TABLE 2 Multivariate analysis of factors associated with the magnitude of HBV vaccine response[a]

Characteristic, unit (n = 57)	Unadjusted P value; B (95% CI)	Adjusted P value; B (95% CI)
Male sex, n (%)	**0.013; −344 (−615–−74)**	0.085; −237 (−509–33)
Age, yr	0.347; −5 (−17–6)	
Nadir CD4$^+$ T-cell count, cells/mm^3	0.685; 0.15 (−0.60–0.90)	
CD4$^+$ T-cell count, cells/mm^3	**0.122; 0.37 (−0.10–0.85)**	0.343; 0.21 (−0.24–0.67)
CD8$^+$ T-cell count, cells/mm^3	0.569; −0.09 (−0.42–0.23)	
CD4$^+$/CD8$^+$ ratio	0.265; 107 (−83–299)	
Time since diagnosis, mo	0.438; 0.46 (−0.73–1.66)	
Sexual transmission, n	0.906; 22 (−357–402)	
Previous AIDS, n	0.745; −74 (−529–381)	
Previous HCV coinfection, n	0.831; 38 (−317–393)	
NRTI containing cART, n	0.193; 152 (−79–385)	
MVC containing cART, n	**0.060; 217 (−9–444)**	0.107; 172 (−38–383)
Previous HBV vaccination, n[b] (%)	**0.119; 191 (−50–434)**	0.070; 205 (−17–428)
Simultaneous HAV vaccination, n (%)	**0.022; 282 (43–521)**	**0.045; 236 (5–468)**
CCR5 WT/Δ32, n (%)	0.630; 73 (−232–379)	
hsCRP, mg/liter	0.354; 3 (−4–11)	

[a]Different factors were associated with the anti-HBs titer. All demographic, clinical, and immunological variables with a P value of <0.15 in the unadjusted model were included in the adjusted model and are shown in bold. Male sex, CD4$^+$ T-cell count, MVC-containing cART, previous HBV vaccination, and simultaneous HAV vaccination were included in the multivariate model. Variables with a P value of <0.05 in the adjusted model were considered statistically significant and are shown in bold.

[b]Previous HBV vaccination data were available for 56 of 57 subjects.

associated with the magnitude of the response (P = 0.009; B [95% CI], 297 [79 to 516]) (Table 3).

The early response was favored mainly by previous vaccination, and an additional effect of maraviroc was also observed in subjects younger than 50 years. We also explored the potential associations of study variables with the early response after the first dose (month 1) (see Table S2 in the supplemental material) and after the second dose (month 3) (see Table S3 in the supplemental material). At both time points, to be previously vaccinated against HBV was independently associated with the antibody titer (P = <0.001; B [95% CI], 363 [216 to 510]) and (P = <0.001; B [95% CI], 483 [295 to 670]), respectively. At month 3, the sex was also independently associated with the antibody titer (P = 0.039; B [95% CI], −228 [−444 to −11]).

When we restricted these analyses to subjects with age ≤50 years (n = 42), in the early response to the second dose (month 3), we observed an independent association with the MVC-cART (P = 0.041; B [95% CI], 206 [8 to 403]), together with that of the

TABLE 3 Multivariate analysis of factors associated with the magnitude of vaccine response in subjects younger than 50 years[a]

Characteristic (n = 42)	Unadjusted P value; B (95% CI)	Adjusted P value; B (95% CI)
Male sex, n (%)	**0.055; −294 (−595–7)**	0.132; −196 (−454–61)
Age, yr	0.583; −4 (−22–12)	
Nadir CD4$^+$ T-cell count, cells/mm^3	0.349; 0.39 (−0.45–1.24)	
CD4$^+$ T-cell count, cells/mm^3	0.195; 0.36 (−0.19–0.93)	
CD8$^+$ T-cell count, cells/mm^3	0.448; −0.14 (−0.54–0.24)	
CD4$^+$/CD8$^+$ ratio	0.206; 138 (−79–356)	
Time since diagnosis, mo	0.251; 0.79 (−0.58–2.16)	
Sexual transmission, n (%)	0.579; 114 (−298–527)	
Previous AIDS, n (%)	**0.074; −548 (−1152–56)**	0.103; −421 (−933–90)
Previous HCV coinfection, n (%)	0.453; 154 (−257–566)	
NRTI containing cART, n (%)	0.351; 127 (−145–399)	
MVC containing cART, n (%)	**0.008; 344 (96–593)**	**0.009; 297 (79–516)**
Previous HBV vaccination, n (%)[b]	**0.082; 247 (−32–527)**	**0.047; 239 (3–474)**
Simultaneous HAV vaccination, n (%)	**0.029; 287 (30–544)**	**0.037; 238 (15–462)**
CCR5 WT/Δ32, n (%)	0.398; 137 (−187–462)	
hsCRP, mg/liter	0.643; 2 (−6–10)	

[a]Different factors were associated with the anti-HBs titer in subjects less than 50 years. All demographic, clinical, and immunological variables with a P value of <0.15 in the unadjusted model were included in the adjusted model and are shown in bold. Male sex, previous AIDS, MVC-containing cART, previous HBV vaccination, and simultaneous HAV vaccination were included in the multivariate model. Variables with a P value of <0.05 in the adjusted model were considered statistically significant and are shown in bold.

[b]Previous HBV vaccination data were available for 41 of 42 subjects.

TABLE 4 Analysis of factors associated with titer maintenance at 1 year[a]

Characteristic (n = 54)	Unadjusted P value; B (95% CI)	Adjusted P value; B (95% CI)
Male sex, n (%)	**0.003; −396 (−647–−144)**	**0.013; −304 (−543–−66)**
Age, yr	0.431; −4 (−16–7)	
Nadir $CD4^+$ T-cell count, cells/mm^3	0.354; −0.32 (−1.02–0.37)	
$CD4^+$ T-cell count, cells/mm^3	0.307; 0.23 (−0.22–0.68)	
$CD8^+$ T-cell count, cells/mm^3	0.714; −0.05 (−0.36–0.25)	
Ratio $CD4^+/CD8^+$	0.690; 36 (−145–218)	
Time since diagnosis, mo	0.228; 0.68 (−0.44–1.81)	
Sexual transmission, n (%)	0.919; 17 (−333–369)	
Previous AIDS, n (%)	0.897; −27 (−449–394)	
Previous HCV coinfection, n (%)	0.842; −33 (−362–296)	
NRTI containing cART, n (%)	0.327; 109 (−112–331)	
MVC containing cART, n (%)	0.325; 109 (−111–330)	
Previous HBV vaccination, n (%)[b]	**0.073; 204 (−20–430)**	**0.027; 221 (26–415)**
Simultaneous HAV vaccination, n (%)	**0.003; 350 (128–572)**	**0.008; 292 (80–503)**
CCR5 WT/Δ32, n (%)	0.838; −29 (−313–255)	
hsCRP, mg/liter	0.256; 4 (−3–11)	

[a]Factors associated with the anti-HBs maintenance 1 year after the first dose. All demographic, clinical, and immunological variables with a P value of <0.15 in the unadjusted model were included in the adjusted model and are shown in bold. Male sex, previous HBV vaccination, and simultaneous HAV vaccination were included in the multivariate model. Variables with a P value of <0.05 in the adjusted model were considered statistically significant and are shown in bold.
[b]Previous HBV vaccination data were available for 53 of 54 subjects.

previous vaccination (P = <0.001; B [95% CI], 539 [328 to 751]) (see Table S4 in the supplemental material).

Female sex, previous vaccination, and simultaneous vaccination were associated with the maintenance of the antibody titer 1 year after the vaccination. We additionally analyzed the factors associated with the antibody titer maintenance after 1 year after the first dose (Table 4). Adjusted analyses revealed that female sex (P = 0.013; B [95% CI], −304 [−543 to −66]), the previous vaccination (P = 0.027; B [95% CI], 221 [26 to 415]), and the simultaneous HAV vaccination (P = 0.008; B [95% CI], 292 [80 to 503]) were independently associated with the antibody titer.

At this time point, the restriction to subjects with age ≤50 (n = 42) did not show association of MVC-cART with the antibody titer maintenance (P = 0.236; B [95% CI], 126 [−86 to 340]), whereas only the sex P = 0.036; B (95% CI), −267 (−518 to −17) and the simultaneous HAV vaccination were independently associated (P = 0.012; B [95% CI], 287 [67 to 506]).

DISCUSSION

In this work, we show that the type of cART, specifically those combinations including the CCR5 antagonist MVC, was associated with the magnitude of the high rate of response (90%) to the HBV vaccine in subjects younger than 50 years old. Additionally, we observed that simultaneous HAV vaccination, previous HBV vaccination, and female sex were also associated with the magnitude of response in the overall population of HIV-infected patients on suppressive cART.

The association of the type of cART with the magnitude of the response only in subjects under 50 years old is especially important since the HIV-infected population is becoming older, with increased morbidity and mortality rates in people over 50 years old (13). The results presented here point to an early HBV vaccination in people under 50 years old, for which an additional strategy using a MVC-cART can be adopted to increase the magnitude of the response. The increased damage of immune function with age, also known as immunosenescence, decreases response to vaccines among other functions (13) and is accentuated or presents unique features in HIV infection scenario (14, 15). This age-related immune deterioration, in people older than 50, may compromise the beneficial immune profile exerted by MVC. The reasons why an MVC-cART was able to increase the early and final magnitudes of the HBV vaccine response are unknown but may reside in the very diverse immunomodulatory properties of this CCR5 antagonist. For instance, MVC has been shown to ameliorate graft-versus-host disease in the allogeneic hematopoietic stem cell transplantation

through inhibition of lymphocyte trafficking (16, 17). In addition, MVC suppressed tumor growth and induced cell apoptosis in the setting of acute lymphoblastic leukemia cells (18). In the scenario of HIV infection, in two clinical trials, MVC has been associated with improved duodenal immunity and reduction of bone loss in antiretroviral-naive HIV-infected patients (19, 20). Specifically, MVC may exert a better anti-inflammatory profile that may enhance the immune response after vaccination, as we have previously shown a decrease in levels of β2-microglobulin, soluble CD40L, and sCD14 after switching from an MVC-free cART to an MVC-cART (8). These data, together with the effect on reducing the Treg frequency (10, 21), which have been negatively associated with the rate of HBV response in patients on cART (11), support our results. Additionally, these results are in accordance with the beneficial effects of MVC in other settings of vaccination, such as the improved response to meningococcal neoimmunizations and the accelerated lymphoproliferation to tetanus toxoid boost (12). The absence of a CCR5 WT/Δ32 variant (see Materials and Methods) association with the magnitude of the HBV vaccine response suggests that this effect is drug specific or that the population size is too small to derive a definitive conclusion. Further research is encouraged to explore potential immunological mechanisms subjacent to the effect of MVC.

In this study, we observed that double doses (40 μg) on a rapid schedule (0, 1, and 3 months) of HBV vaccination are effective in reaching a high rate of response in HIV-infected patients on cART. This is in accordance to previous studies with all patients on cART that have shown improved response using a double dose of intramuscular vaccine compared to a single dose (20 μg) (7, 22). Despite the fact that most of the patients were male, the characteristics of the included subjects, who had relatively high CD4 T-cell counts ($>$300 CD4 T-cells/mm^3) and undetectable viral loads because of cART, are in accordance with previous works (23–25) as determinants to reach a high rate of response in HIV-infected people.

Regarding the factors associated with the magnitude of the response, simultaneous vaccination against HAV was associated not only with the final response but also with the maintenance of the magnitude after 1 year. There are limited data about this synergistic effect that suggest an increased rate of response in HIV-infected patients vaccinated with the combined HAV/HBV vaccine (26, 27). The reasons for the improved response, in our case after simultaneous vaccinations, are unknown at the moment. We can speculate about the increased amount of adjuvant, aluminum hydroxide, which was present in both vaccines and may favor innate and adaptive immune responses (28), and/or, alternatively, the increased immune response due to cross-reactivity of both types of viral antigens. In addition to this added value, HAV vaccination itself is very important now due to the recent outbreaks of HAV infection in men who have sex with men in HIV-infected populations (29).

Globally, two other important factors associated with the magnitude of the response were revaccination and female sex. The revaccination has been effective in several cohorts of nonresponders (26, 27, 30, 31). There is no global recommendation about which schedule to use. Although we do not know if the 33% of patients with antiHBs titers of $<$10 mUI/ml were nonresponders to the first vaccine or if these titers were lost during the follow-up, revaccination yielded a high response in these subjects. These results point out the presence of an anamnestic response favoring a higher magnitude of response in individuals revaccinated, which raises the question of whether these subjects are actually protected even with antiHBs levels of $<$10 mIU/ml (32). In any case, these data support a periodical supervision of antiHBs titers to determine when new boosters are needed. The influence of sex in the magnitude of the response is in accordance with previous works involving HIV-infected patients (5, 7, 30); however, the mechanisms behind a high response in females are poorly understood, but the association of sex steroids with an improved innate and adaptive immunity in women may explain it (33).

This study has some limitations. We were not able to analyze factors associated with the rate of response due to the fact that 90% of the subjects responded to the vaccine.

In addition, a deleterious effect has been attributed to NRTIs favoring a short telomere length in T cells (34), which has been associated with a paucity in vaccine response (35). Ideally, an MVC-cART with no NRTIs may be the best regimen for reaching the highest magnitude of response; however, that combination was not represented well enough to make any statistically significant contrast. In addition, we had to restrict the cohort to patients younger than 50 years old, and a larger cohort is needed to confirm our observations.

In summary, an early and high magnitude of response after HBV vaccination can be achieved with revaccination, simultaneous HAV vaccine, and the administration of a suppressive cART containing MVC. This CCR5 antagonist may act as an immune booster beyond its antiviral effect, enabling a favorable immune milieu to increase the magnitude of the HBV vaccine response. The inclusion of this drug in further HBV vaccination protocols in HIV-infected patients on suppressive cART should be considered.

MATERIALS AND METHODS

Study participants. In this observational study, 57 HIV-infected subjects were included from the Assistance Vaccination Program from the Virgen del Rocío University Hospital, Seville, Spain, between April 2013 and July 2015. Inclusion criteria were (i) being on suppressive cART (undetectable viral load) during at least 6 months, (ii) having CD4 T-cell populations of >300 cell counts/μl, (iii) having negative serology for HBsAg and anti-HBc, and (iv) having anti-HBs titers of <10 mIU/ml. Written informed consent was obtained from all study participants. The study was approved by the ethics review board from the Virgen del Rocío University Hospital.

Vaccination protocol. All subjects received 3 intramuscular (deltoid region) double doses (40 μg) of the recombinant Engerix-B vaccine (GlaxoSmithKline, Brentford, United Kingdom) at 0, 1, and 3 months (Fig. 1). The vaccine response was measured 6 months after the first dose. Additionally, we measured the early response at months 1 and 3 after the first dose and the maintenance of the response 1 year after the first dose of vaccination. The vaccine response was considered positive when the anti-HBs titer was ≥10 mIU/ml. A group of subjects was simultaneously vaccinated at 0 and 6 months against HAV (simultaneous HAV vaccination) with two intramuscular doses of the vaccine Havrix-1440 (GlaxoSmithKline, Brentford, United Kingdom). This subgroup of subjects had negative serology for HAV.

Clinical data and laboratory measurements. Demographic, clinical, and immunological data, including the presence of a 32-bp deletion in the CCR5 gene (CCR5Δ32) and high-sensitivity C-reactive protein (hsCRP) levels, were recorded in a database. Absolute CD4 and CD8 T cell counts were determined with an Epics XL-MCL flow cytometer (Beckman-Coulter, Brea, CA), according to the manufacturer's instructions. Plasma HIV-1 RNA levels were measured using quantitative PCR (Cobas Ampliprep/Cobas TaqMan HIV-1 test; Roche Molecular Systems, Basel, Switzerland), according to the manufacturer's protocol. The detection limit was 20 HIV-RNA copies/ml. Plasma samples were tested for HBV-related markers (HBsAg, anti-HBs, and anti-HBc) using an HBV enzyme-linked immunosorbent assay (ELISA; Siemens Healthcare Diagnosis, Malvern, PA). Qualitative PCR amplification was used for plasma HCV amplification (Cobas Amplicor; Roche Diagnosis); the test had a detection limit of 15 IU/ml. The hsCRP levels were determined with an immunoturbidimetric serum assay, using Cobas 701 (Roche Diagnostics, Mannheim, Germany). CCR5Δ32 deletion was assayed in DNA samples obtained from peripheral blood mononuclear cells (PBMCs) using the QIAamp blood kit (Qiagen) by PCR as previously described (36). Two fragments were generated, the wild-type (WT) fragment (185 bp) and the deleted fragment (153 bp), detected in 2% agarose gels. Heterozygosity (CCR5 WT/Δ32) was considered when the two fragments were present.

Statistical analyses. Continuous variables were expressed as medians and interquartile ranges [IQR] and categorical variables as numbers and percentages. Comparisons between groups were made using the nonparametric Mann-Whitney *U* test. Lineal regression analyses were performed to determine factors associated with the magnitude of response (absolute anti-HBs titer). Among covariates, we analyzed the type of cART as NRTI- or MVC-containing cART during the vaccination protocol. As a *post hoc* analysis, we restricted the study population to subjects younger than 50 years old. All demographic, clinical, and immunological variables with a *P* value of <0.15 in the unadjusted model were included in the adjusted model. Variables with a *P* value of <0.05 in the adjusted model were considered statistically significant. Statistical analyses were performed using SPSS software (version 22; IBM SPSS, Chicago, IL), and graphs were generated using Prism (version 5, GraphPad Software, Inc.).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02050-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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REFERENCES

1. Soto B, Sánchez-Quijano A, Rodrigo L, del Olmo JA, García-Bengoechea M, Hernández-Quero J, Rey C, Abad MA, Rodríguez M, Sales Gilabert M, González F, Mirón P, Caruz A, Relimpio F, Torronteras R, Leal M, Lissen E. 1997. Human immunodeficiency virus infection modifies the natural history of chronic parenterally-acquired hepatitis C with an unusually rapid progression to cirrhosis. J Hepatol 26:1–5.
2. Thio CL, Seaberg EC, Skolasky R, Jr, Phair J, Visscher B, Muñoz A, Thomas DL, Multicenter AIDS Cohort Study. 2002. HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicenter Cohort Study (MACS). Lancet 360:1921–1926. https://doi.org/10.1016/S0140-6736(02)11913-1.
3. Whitaker JA, Rouphael NG, Edupuganti S, Lai L, Mulligan MJ. 2012. Strategies to increase responsiveness to hepatitis B vaccination in adults with HIV-1. Lancet Infect Dis 12:966–976. https://doi.org/10.1016/S1473-3099(12)70243-8.
4. Kernéis S, Launay O, Turbelin C, Batteux F, Hanslik T, Boëlle PY. 2014. Long-term immune responses to vaccination in HIV-infected patients: a systematic review and meta-analysis. Clin Infect Dis 58:1130–1139. https://doi.org/10.1093/cid/cit937.
5. Overton ET, Sungkanuparph S, Powderly WG, Seyfried W, Groger RK, Aberg JA. 2005. Undetectable plasma HIV RNA load predicts success after hepatitis B vaccination in HIV-infected persons. Clin Infect Dis 41:1045–1048. https://doi.org/10.1086/433180.
6. Fonseca MO, Pang LW, de Paula Cavalheiro N, Barone AA, Heloisa Lopes M. 2005. Randomized trial of recombinant hepatitis B vaccine in HIV-infected adult patients comparing a standard dose to a double dose. Vaccine 23:2902–2908. https://doi.org/10.1016/j.vaccine.2004.11.057.
7. Launay O, van der Vliet D, Rosenberg AR, Michel ML, Piroth L, Rey D, Colin de Verdière N, Slama L, Martin K, Lortholary O, Carrat F, ANRS HB03 VIHVAC-B Trial. 2011. Safety and immunogenicity of 4 intramuscular double doses and 4 intradermal low doses vs standard hepatitis B vaccine regimen in adults with HIV-1: a randomized controlled trial. JAMA 305:1–9. https://doi.org/10.1001/jama.2011.351.
8. Romero-Sánchez MC, Alvarez-Ríos AI, Bernal-Morell E, Genebat M, Vera F, Benhnia MR, Bravo-Urbieta J, Galera-Peñaranda C, de Pablo-Bernal RS, Abad-Carrillo MA, Leal M, Ruiz-Mateos E, VIH-GEMUVIH Región de Murcia Study Group. 2014. Maintenance of virologic efficacy and decrease in levels of β2-microglobulin, soluble CD40L and soluble CD14 after switching previously treated HIV-infected patients to an NRTI-sparing dual therapy. Antiviral Res 111:26–32. https://doi.org/10.1016/j.antiviral.2014.08.011.
9. Romero-Sánchez MC, Machmach K, Gonzalez-Serna A, Genebat M, Pulido I, García-García M, Alvarez-Ríos AI, Ferrando-Martinez S, Ruiz-Mateos E, Leal M. 2012. Effect of maraviroc on HIV disease progression-related biomarkers. Antimicrob Agents Chemother 56:5858–5864. https://doi.org/10.1128/AAC.01406-12.
10. Pozo-Balado MM, Martínez-Bonet M, Rosado I, Ruiz-Mateos E, Méndez-Lagares G, Rodríguez-Méndez MM, Vidal F, Muñoz-Fernández MA, Pacheco YM, Leal M. 2014. Maraviroc reduces the regulatory T-cell frequency in antiretroviral-naive HIV-infected subjects. J Infect Dis 210:890–898. https://doi.org/10.1093/infdis/jiu180.
11. Pozo Balado MM, Leal M, Méndez Lagares G, Mata RC, López-Cortés LF, Viciana P, Pacheco YM. 2010. Increased regulatory T cell counts in HIV-infected nonresponders to hepatitis B virus vaccine. J Infect Dis 202:362–369. https://doi.org/10.1086/653707.
12. Westrop SJ, Moyle G, Jackson A, Nelson M, Mandalia S, Imami N. 2012. CCR5 antagonism impacts vaccination response and immune profile in HIV-1 infection. Mol Med 18:1240–1248. https://doi.org/10.2119/molmed.2012.00206.
13. Deeks SG. 2011. HIV infection, inflammation, immunosenescence, and aging. Annu Rev Med 62:141–155. https://doi.org/10.1146/annurev-med-042909-093756.
14. Ferrando-Martínez S, Ruiz-Mateos E, Romero-Sánchez MC, Muñoz-Fernández MÁ, Viciana P, Genebat M, Leal M. 2011. HIV infection-related premature immunosenescence: high rates of immune exhaustion after short time of infection. Curr HIV Res 9:289–294. https://doi.org/10.2174/157016211797636008.
15. De Pablo-Bernal RS, Ramos R, Genebat M, Cañizares J, Rafii-El-Idrissi Benhnia M, Muñoz-Fernández MA, Pacheco YM, Galvá MI, Leal M, Ruiz-Mateos E. 2016. Phenotype and polyfunctional deregulation involving interleukin 6 (IL-6)- and IL-10-producing monocytes in HIV-infected patients receiving combination antiretroviral therapy differ from those in healthy older individuals. J Infect Dis 213:999–1007. https://doi.org/10.1093/infdis/jiv520.
16. Reshef R, Luger SM, Hexner EO, Loren AW, Frey NV, Nasta SD, Goldstein SC, Stadtmauer EA, Smith J, Bailey S, Mick R, Heitjan DF, Emerson SG, Hoxie JA, Vonderheide RH, Porter DL. 2012. Blockade of lymphocyte chemotaxis in visceral graft-versus-host disease. N Engl J Med 367:135–145. https://doi.org/10.1056/NEJMoa1201248.
17. Moy RH, Huffman AP, Richman LP, Crisalli L, Wang XK, Hoxie JA, Mick R, Emerson SG, Zhang Y, Vonderheide RH, Porter DL, Reshef R. 2017. Clinical and immunologic impact of CCR5 blockade in graft-versus-host disease prophylaxis. Blood 129:906–916. https://doi.org/10.1182/blood-2016-08-735076.
18. Zi J, Yuan S, Qiao J, Zhao K, Xu L, Qi K, Xu K, Zeng L. 2017. Treatment with the C-C chemokine receptor type 5 (CCR5)-inhibitor maraviroc suppresses growth and induces apoptosis of acute lymphoblastic leukemia cells. Am J Cancer Res 7:869–880.
19. Serrano-Villar S, Sainz T, Ma ZM, Utay NS, Wook-Chun T, Mann S, Kashuba AD, Siewe B, Albanese A, Troia-Cancio P, Sinclair E, Somasunderam A, Yotter T, Deeks SG, Landay A, Pollard RB, Miller CJ, Moreno S, Asmuth DM. 2016. Effects of combined CCR5/integrase inhibitors-based regimen on mucosal immunity in HIV-infected patients naïve to antiret-

roviral therapy: a pilot randomized trial. PLoS Pathog 12:e1005540. https://doi.org/10.1371/journal.ppat.1005540.
20. Taiwo BO, Chan ES, Fichtenbaum CJ, Ribaudo H, Tsibris A, Klingman KL, Eron JJ, Berzins B, Robertson K, Landay A, Ofotokun I, Brown T, AIDS Clinical Trials Group A5303 Study Team. 2015. Less bone loss with maraviroc-versus tenofovir-containing antiretroviral therapy in the AIDS Clinical Trials Group A5303 Study. Clin Infect Dis 61:1179–1188. https://doi.org/10.1093/cid/civ455.
21. Pozo-Balado MM, Rosado-Sánchez I, Méndez-Lagares G, Rodríguez-Méndez MM, Ruiz-Mateos E, Benhnia MR, Muñoz-Fernández MA, Leal M, Pacheco YM. 2016. Maraviroc contributes to the restoration of the homeostasis of regulatory T-cell subsets in antiretroviral-naive HIV-infected subjects. Clin Microbiol Infect 22:461.e1–5. https://doi.org/10.1016/j.cmi.2015.12.025.
22. Chaiklang K, Wipasa J, Chaiwarith R, Praparattanapan J, Supparatpinyo K. 2013. Comparison of immunogenicity and safety of four doses and four double doses vs. standard doses of hepatitis B vaccination in HIV-infected adults: a randomized, controlled trial. PLoS One https://doi.org/10.1371/journal.pone.0080409.
23. Potsch DV, Camacho LA, Tuboi S, Villar LM, Miguel JC, Ginuíno C, Silva EF, Mendonça RM, Moreira RB, Barroso PF. 2012. Vaccination against hepatitis B with 4-double doses increases response rates and antibodies titers in HIV-infected adults. Vaccine 30:5973–5977. https://doi.org/10.1016/j.vaccine.2012.07.028.
24. Potsch DV, Oliveira ML, Ginuíno C, Miguel JC, Oliveira SA, Silva EF, Moreira RB, Cruz GV, Oliveira AL, Camacho LA, Barroso PF. 2010. High rates of serological response to a modified hepatitis B vaccination schedule in HIV-infected adult subjects. Vaccine 28:1447–1450. https://doi.org/10.1016/j.vaccine.2009.11.066.
25. Cruciani M, Mengoli C, Serpelloni G, Lanza A, Gomma M, Nardi S, Rimondo C, Bricolo F, Consolaro S, Trevisan M, Bosco O. 2009. Serologic response to hepatitis B vaccine with high dose and increasing number of injections in HIV infected adult patients. Vaccine 27:17–22. https://doi.org/10.1016/j.vaccine.2008.10.040.
26. Cardell K, Akerlind B, Sällberg M, Frydén A. 2008. Excellent response rate to a double dose of the combined hepatitis A and B vaccine in previous nonresponders to hepatitis B vaccine. J Infect Dis 198:299–304. https://doi.org/10.1086/589722.
27. Pettit NN, DePestel DD, Malani PN, Riddell J, IV. 2010. Factors associated with seroconversion after standard dose hepatitis B vaccination and high-dose revaccination among HIV-infected patients. HIV Clin Trials 11:332–339. https://doi.org/10.1310/hct1105-332.
28. He P, Zou Y, Hu Z. 2015. Advances in aluminum hydroxide-based adjuvant research and its mechanism. Hum Vaccin Immunother 11:477–488. https://doi.org/10.1080/21645515.2014.1004026.
29. Chen GJ, Lin KY, Sun HY, Sheng WH, Hsieh SM, Huang YC, Cheng A, Liu WC, Hung CC, Chang SC. 2017. Incidence of acute hepatitis A among HIV-positive patients during an outbreak among MSM in Taiwan: impact of HAV vaccination. Liver Int https://doi.org/10.1111/liv.13468.
30. De Vries-Sluijs TEMS, Hansen BE, van Doornum GJJ, Springeling T, Evertsz NM, de Man RA, van der Ende ME. 2008. A prospective open study of the efficacy of high-dose recombinant hepatitis B rechallenge vaccination in HIV-infected patients. J Infect Dis 197:292–294. https://doi.org/10.1086/524690.
31. Rowley K, Payne B, Schmid M. 2014. Determinants of response to repeat hepatitis B vaccination in HIV-infected prior non-responders. J Infect 69:98–99. https://doi.org/10.1016/j.jinf.2014.02.014.
32. Jarrosson L, Kolopp-Sarda MN, Aguilar P, Béné MC, Lepori ML, Vignaud MC, Faure GC, Kohler C. 2004. Most humoral non-responders to hepatitis B vaccines develop HBV-specific cellular immune responses. Vaccine 22:3789–3796. https://doi.org/10.1016/j.vaccine.2004.02.046.
33. Klein SL, Jedlicka A, Pekosz A. 2010. The Xs and Y of immune responses to viral vaccines. Lancet Infect Dis 10:338–349. https://doi.org/10.1016/S1473-3099(10)70049-9.
34. Leeansyah E, Cameron PU, Solomon A, Tennakoon S, Velayudham P, Gouillou M, Spelman T, Hearps A, Fairley C, Smit de V, Pierce AB, Armishaw J, Crowe SM, Cooper DA, Koelsch KK, Liu JP, Chuah J, Lewin SR. 2013. Inhibition of telomerase activity by human immunodeficiency virus (HIV) nucleos(t)ide reverse transcriptase inhibitors: a potential factor contributing to HIV-associated accelerated aging. J Infect Dis 207:1157–1165. https://doi.org/10.1093/infdis/jit006.
35. Najarro K, Nguyen H, Chen G, Xu M, Alcorta S, Yao X, Zukley L, Metter EJ, Truong T, Lin Y, Li H, Oelke M, Xu X, Ling SM, Longo DL, Schneck J, Leng S, Ferrucci L, Weng NP. 2015. Telomere length as an indicator of the robustness of B- and T-cell response to influenza in older adults. J Infect Dis 212:1261–1269. https://doi.org/10.1093/infdis/jiv202.
36. Vallejo A, Ruiz-Mateos E, Molina-Pinelo S, Soriano-Sarabia N, de Felipe B, Gutierrez S, Sánchez-Quijano A, Lissen E, Leal M. 2006. Immunovirologic characteristics of human immunodeficiency virus-infected patients consisting mainly of injecting drug users on highly active antiretroviral treatment with prolonged virologic failure. Viral Immunol 19:759–767. https://doi.org/10.1089/vim.2006.19.759.

Suppelemtary Table 1. Demographic, clinical and immunological characteristics of the subjects receiving or not a MVC-containing cART.

	MVC-containing cART (N=28)	MVC-sparing cART (N=29)	*p*
Male sex, n (%)	21 (75)	24 (83)	0.473
Age (years)	42 [31-48]	47 [41-54]	**0.012**
Nadir CD4+ T-cell count (cells/mm3)	289 [147-416]	246 [156-342]	0.436
$CD4^+$ T-cell count (cells/mm^3)	718 [593-872]	691 [510-908]	0.690
$CD8^+$ T-cell count (cells/mm^3)	821 [525-1051]	598 [419-885]	0.107
Ratio $CD4^+/CD8^+$	0.9 [0.7-1.2]	1.2 [0.9-1.8]	0.091
Time since diagnosis (months)	66 [37-234]	123 [69-211]	0.222
Sexual transmission, n (%)	24 (86)	27 (93)	0.363
Previous AIDS, n (%)	2 (7)	2 (7)	0.971
Previous HCV coinfection, n (%)	5 (18)	2 (7)	0.208
NRTI containing cART, n (%)	5 (18)	21 (72)	**<0.001**
Previous HBV vaccination, n (%)*	9 (32)	10 (35)	0.778
Simultaneous HAV vaccination, n (%)	11 (39)	8 (28)	0.349
CCR5 WT/Δ32, n (%)	6 (21)	4 (14)	0.449
hsCRP (mg/L)	1.0 [0.6-1.4]	1.3 [0.7-2.5]	0.206
Anti-HBs titer (mIU/mL)	1000 [320-1000]	704 [36-1000]	**0.048**

Continuous variables are expressed as median values [IQR] and categorical variables are expressed as number of cases (%). *Data of previous HBV vaccination only available in 56 subjects. Comparisons between groups were made using the nonparametric Mann-Whitney *U* test. Variables with a $p < 0.05$ were considered statistically significant are showed in bold.

Supplementary Table 2. Analysis of factors associated with early response to the first dose (month 1).

Characteristic (N=57)	Unadjusted P value; B (95% CI)
Male sex, n (%)	0.736; 34 [-169-238]
Age (years)	0.861; -0.74 [-9-7]
Nadir $CD4^+$ T-cell count (cells/mm^3)	0.598; 0.14 [-0.39-0.67]
$CD4^+$ T-cell count (cells/mm^3)	0.772; 0.05 [-0.29-0.39]
$CD8^+$ T-cell count (cells/mm^3)	0.646; 0.05 [-0.18-0.29]
Ratio $CD4^+/CD8^+$	0.448; -52 [-190-85]
Time since diagnosis (months)	0.833; 0.09 [-0.77-0.95]
Sexual transmission, n (%)	0.509; -89 [-359-180]
Previous AIDS, n (%)	0.851; -30 [-356-295]
Previous HCV coinfection, n (%)	0.341; -120 [-372-131]
NRTI containing cART, n (%)	0.375; 74 [-92-242]
MVC containing cART, n (%)	0.759; 25 [-141-193]
Previous HBV vaccination, n (%)*	**<0.001; 363 [216-510]**
Simultaneous HAV vaccination, n (%)	0.434; 70 [-108-248]
CCR5 WT/Δ32, n (%)	0.472; 78 [-139-296]
hsCRP (mg/L)	0.972; 0.09 [-5-5]

Analysis of factors associated with the anti-HBs at early response (month 1). Variables with a p <0.05 were considered statistically significant and are showed in bold. *Data of previous HBV vaccination were available in 56 subjects out of 57.

Supplementary Table 3. Analysis of factors associated with early response to the second dose (month 3).

Characteristic (N=57)	Unadjusted *p* value; B (95% CI)	Adjusted *p* value; B (95% CI)
Male sex, n (%)	**0.092; -224 [-487-38]**	**0.039; -228 [-444- -11]**
Age (years)	0.519; -3 [-14-7]	
Nadir $CD4^+$ T-cell count (cells/mm^3)	0.536; 0.22 [-0.48-0.92]	
$CD4^+$ T-cell count (cells/mm^3)	0.216; 0.28 [-0.17-0.73]	
$CD8^+$ T-cell count (cells/mm^3)	0.730; 0.05 [-0.25-0.36	
Ratio $CD4^+/CD8^+$	0.739; -30 [-212-151]	
Time since diagnosis (months)	0.301; 0.58 [-0.54-1.71]	
Sexual transmission, n (%)	0.314; 179 [-174-534]	
Previous AIDS, n (%)	0.582; -118 [-547-310]	
Previous HCV coinfection, n (%)	0.923; -16 [-351-319]	
NRTI containing cART, n (%)	0.527; 70 [-151-292]	
MVC containing cART, n (%)	0.264; 123 [-95-342]	
Previous HBV vaccination, n (%)*	**<0.001; 481 [288-675]**	**<0.001; 483 [295-670]**
Simultaneous HAV vaccination, n (%)	0.577; 66 [-170-302]	
CCR5 WT/Δ32, n (%)	0.999 -0.16 [-289-288]	
hsCRP (mg/L)	0.982; 0.08 [-7-7]	

Analysis of factors associated with the anti-HBs titer at early response (month 3). All demographic, clinical and immunological variables with a *p* value <0.15 in the unadjusted model were included in the adjusted model are showed in bold, thus, male sex and previous HBV vaccination were included in the multivariate model. Variables with a *p* <0.05 in the adjusted model were considered statistically significant are showed in bold. *Data of previous HBV vaccination were available in 56 subjects out of 57.

Supplementary Table 4. Analysis of demographic factors associated with early response to the second dose (month 3) in subjects younger than fifty years.

Characteristic (N=42)	Unadjusted *p* value; B (95% CI)	Adjusted *p* value; B (95% CI)
Male sex, n (%)	0.189; -197 [-495-101]	
Age (years)	0.982; -0.19 [-17-17]	
Nadir $CD4^+$ T-cell count (cells/mm^3)	0.648; 0.18 [-0.64-1.01]	
$CD4^+$ T-cell count (cells/mm^3)	0.629; 0.134 [-0.42-0.69]	
$CD8^+$ T-cell count (cells/mm^3)	0.676; -0.07 [-0.46-0.30]	
Ratio $CD4^+/CD8^+$	0.928; 9 [-205-224]	
Time since diagnosis (months)	0.208; 0.83 [-0.48-2.16]	
Sexual transmission, n (%)	0.184; 261 [-129-653]	
Previous AIDS, n (%)	0.158; -422 [-1014-170]	
Previous HCV coinfection, n (%)	0.668; 85 [-314-485]	
NRTI containing cART, n (%)	0.617; 66 [-199-331]	
MVC containing cART, n (%)	**0.106; 207 [-46-461]**	**0.041; 206 [8-403]**
Previous HBV vaccination, n (%)*	**<0.001; 540 [319-760]**	**<0.001; 539 [328-751]**
Simultaneous HAV vaccination, n (%)	0.887; 18 [-245-282]	
CCR5 WT/Δ32, n (%)	0.958; 8 [-308-324]	
hsCRP (mg/L)	0.443; -3 [-11-5]	

Analysis of factors associated with the anti-HBs titer at early response (month 3) in subjects less than fifty years. All demographic, clinical and immunological variables with a *p* value <0.15 in the unadjusted model were included in the adjusted model are showed in bold, thus, MVC-containing cART and previous HBV vaccination were included in the multivariate model. Variables with a *p* <0.05 in the adjusted model were considered statistically significant are showed in bold. *Data of previous HBV vaccination were available in 41 subjects out of 42.

4.3. Improved CD4 T-cell profile in HIV-infected subjects on maraviroc-containing therapy is associated with better responsiveness to HBV vaccination

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Improved CD4 T cell profile in HIV-infected subjects on maraviroc-containing therapy is associated with better responsiveness to HBV vaccination

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Abstract

Background: Maraviroc-containing combined antiretroviral therapy (MVC-cART) improved the response to the hepatitis B virus (HBV) vaccine in HIV-infected subjects younger than 50 years old. We aimed here to explore the effect of this antiretroviral therapy on different immunological parameters that could account for this effect.

Methods: We analysed baseline samples of vaccinated subjects under 50 years old (n = 41). We characterized the maturational subsets and the expression of activation, senescence and prone-to-apoptosis markers on CD4 T-cells; we also quantified T-regulatory cells (Treg) and dendritic cell (DC) subsets. We used binary logistic regression to evaluate the immunological impact of MVC-cART, correlation with MVC exposure and linear regression for association with the magnitude of the HBV vaccine response.

Results: HIV-infected subjects on MVC-cART prior to vaccination showed increased recent thymic emigrants levels and reduced myeloid-DC levels. A longer exposure to MVC-cART was associated with lower frequencies of Tregs and activated and proliferating CD4 T-cells. Furthermore, the frequencies of activated and proliferating CD4 T-cells were inversely associated with the magnitude of the HBV vaccine response.

Conclusion: The beneficial effect of MVC-cART in the HBV vaccine response in subjects below 50 years old could be partially mediated by its reducing effect on the frequencies of activated and proliferating CD4 T-cells prior to vaccination.

Keywords: Maraviroc **(**MVC), CD4 T-cell, Ki67, Activation, Treg, hsCRP, Inflammation, HBV vaccine, Dendritic cells (DC), Recent thymic emigrants (RTE)

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Background

Human immunodeficiency virus (HIV)-infected subjects are at high risk for hepatitis B virus (HBV) infection and progression of severe, life-threatening hepatic complications, such as cirrhosis and hepatocellular carcinoma [1, 2]. To prevent the associated morbimortality, worldwide current guidelines recommend vaccination against HBV in all HIV-infected subjects susceptible to be coinfected by HBV, but the response rates are lower than in HIV-uninfected subjects [reviewed in 3].

The best-known predictors of vaccine efficacy are an undetectable viral load and CD4 T-cell counts above 350 cells/mm^3 [3]. Thus, it is well assumed that successful combined antiretroviral therapy (cART) favours the vaccine response; however, the influence of the type of antiretroviral treatment has been scarcely explored until now. In this line, it was first described that maraviroc (MVC), a CCR5 antagonist, enhanced meningococcal neo-immunization and accelerated the response to tetanus boost [4]. More recently, we also reported that MVC-containing cART (MVC-cART) was associated with a better response against the HBV vaccine, at least in subjects younger than 50 years old [5]. Nevertheless, the potential underlying mechanisms were unaddressed.

Different antiretroviral combinations including MVC have comparatively proved their beneficial effects on the levels of inflammatory biomarkers [6, 7] and the T-cell immunophenotype [8]. In two clinical trials, an improvement of duodenal immunity and a reduction in bone loss has been associated with such combinations [9, 10]. Furthermore, MVC monotherapy also reduced the frequency of regulatory T-cells (Treg) in antiretroviral-naïve subjects [11], even improving the distribution of Treg subsets [12]. This could be relevant since we observed that Treg cells negatively impacted the HBV vaccine responsiveness in a previous cohort [13]. It is possible that MVC could enhance different functions required to mount an effective response following HBV vaccination, including antigen-presentation, T-cell help, regulatory T-cell suppression and B cell functions [14, 15].

In the present study, we aimed to explore the potential effect of MVC-cART in different parameters related to inflammation, T-cell function and dendritic cell subsets that could account for its effect on the HBV vaccine response; to this aim, we studied the same cohort of vaccinated subjects that had revealed a positive effect of such MVC-cART.

Methods

Study design, patients and samples

The vaccination protocol has been reported elsewhere [5]. Briefly, HIV-infected subjects from the Virgen del Rocío University Hospital were consecutively vaccinated against HBV. These subjects (a) were on suppressive cART (at least in the last 6 months), (b) had CD4 T-cell counts of >300 cells/μl, (c) had negative serology for HBsAg and anti-HBc and (d) had anti-HBs titers of ≤ 10 mIU/ml. The vaccination protocol consisted of 3 intramuscular double doses (40 μg) of the recombinant Engerix-B vaccine (GlaxoSmithKline, Brentford, United Kingdom) at 0, 1, and 3 months. The vaccine response was measured 6 months after the first dose. A group of subjects was simultaneously vaccinated at 0 and 6 months against hepatitis A virus (HAV) (simultaneous HAV vaccination) with two intramuscular doses of the vaccine Havrix-1440 (GlaxoSmithKline, Brentford, United Kingdom). This subgroup of subjects had a previous negative serology for HAV. Fresh blood samples were collected at baseline, just before the administration of the first vaccine dose. All patients gave informed consent to enter the study, which was approved by the Ethic Committee of our Hospital. We restricted the present analyses to subjects younger than 50 years old ($n=41$) from the total vaccinated population because the beneficial effect of MVC-cART on the vaccine response was observed in this population [5].

Laboratory measurements

Absolute numbers of CD4 and CD8 T cells were determined with an Epics XL-MCL flow cytometer (Beckman-Coulter). Plasma HIV-1 RNA levels were measured using quantitative PCR (Cobas Ampliprep/Cobas TaqMan HIV-1 test; Roche Molecular Systems, Basel, Switzerland) with a detection limit of 20 HIV-RNA copies/ml. Plasma samples were tested for HBV-related markers (HBsAg, anti-HBs, and anti-HBc) using an HBV enzyme-linked immunosorbent assay (ELISA; Siemens Healthcare Diagnosis, Malvern, PA). Qualitative PCR amplification was used for plasma hepatitis C virus (HCV) amplification (Cobas Amplicor; Roche Diagnosis, Mannheim, Germany) with a detection limit of 15 IU/ml. The highly sensitive C-reactive protein (hsCRP) levels were determined with an immunoturbidimetric serum assay using a Cobas 701 (Roche Diagnostics, Mannheim, Germany).

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood before the first dose of vaccine and cryopreserved. For the immunophenotyping of cellular subsets, PBMCs were thawed and immediately stained with the following surface antibodies: anti-CD31 PE-CF594, anti-CD56 BV510, anti-CD25 BV605, anti-CD45RA BV650, anti-CD4 BV786, anti-CD3 APC-H7, Lin2 FITC (anti-CD3, anti-CD19, anti-CD20, anti-CD14 and anti-CD56), anti-CD11c BV650, and anti-HLA-DR BV711 (BD Biosciences, USA);

anti-CD39 FITC, anti-CD57 PE-Cy7, anti-HLA-DR BV570, anti-CD95 BV711, and anti-CD27 AF700 (BioLegend, USA); and anti-CD123 AF700 (R&D, San Diego CA, USA). When necessary for intracellular staining, cells were fixed and permeabilized according to the manufacturer's instructions (FoxP3/Transcription Factor Staining Buffer, Ebioscience, USA) and stained with the following intracellular antibodies: anti-Ki67 PerCP-Cy5.5, anti-FoxP3 PE and anti-CTLA-4 APC (BD Biosciences, USA). Isotype controls for CD39, CD31, CD25, CD95, Ki67, FoxP3 and CTLA4 were included in each experiment.

We characterized peripheral CD4 T-cells according to the distribution of their maturational subsets [naïve ($CD27^+CD45RA^+$), central memory ($CD27^+CD45RA^-$), effector memory ($CD27^-CD45RA^-$) and TemRA ($CD27^-CD45RA^+$)], also including recent thymic emigrants (RTEs; naïve-$CD31^+$) and the expression of activation (HLA-DR), cell-cycle entry (Ki67), senescence (CD57) and prone-to-apoptosis (CD95) markers. We also identified Tregs with classical markers ($CD25^{hi}FoxP3^+$) and their expression of the mentioned activation markers but also of functional markers (CD39, CTLA-4). We immunophenotyped myeloid dendritic cells (mDCs) as $Lin2^-HLA\text{-}DR^+CD123^-CD11c^+$ and plasmacytoid dendritic cells (pDCs) as $Lin2^-HLA\text{-}DR^+CD11c^-CD123^+$.Viable cells were identified using LIVE/DEAD fixable Aqua Blue Dead Cell Stain (Life Technologies, USA). One million cells of each sample were stained, and a minimum of 100,000 events of total lymphocytes and 150,000 dendritic cells were acquired. Flow cytometry was performed on an LSR Fortessa (BD Biosciences, USA). Analysis was performed using FlowJo version 9.3 (TreeStar).

Statistical analysis

Continuous variables were expressed as medians and interquartile ranges [IQRs] and categorical variables as the number of cases and percentages. Binary logistic regression was used to analyse the potential effect of MVC-cART on the clinical and immunological parameters. Variables with a p value < 0.1 in the univariate analysis were considered in multivariable models. Linear regression analyses were performed to determine factors associated with the magnitude of response (absolute anti-HBs titre). Correlations were assessed using the Spearman's rho correlation coefficient. A p value of < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (version 22; IBM SPSS, Chicago, USA), and graphs were generated using Prism (version 5, GraphPad Software, Inc.).

Results

Demographic, clinical and immunological variables associated with MVC-containing cART

Around half of the population (51%) received MVC-cART, consisting of MVC and a boosted protease inhibitor (PI) or MVC and two nucleoside-reverse transcriptase inhibitors (NRTIs). We compared the demographic, clinical and immunological variables between patients receiving MVC-containing cART or MVC-lacking cART at the moment of vaccination (Table 1). The age, $CD4^+/CD8^+$ ratio, time on HIVtreatment, $\%CD4^+$RTE and %mDCs had p values < 0.1 in the univariate analyses and were therefore included in the multivariate analysis. Notably, 19% of the subjects treated with MVC-cART were also receiving NRTIs, whereas 70% of the subjects on MVC-lacking cART were receiving NRTIs. Thus, the absence of NRTIs was highly collinear with the presence of MVC and was not included for adjustment in the multivariate analysis. As is shown, the $CD4^+/CD8^+$ ratio ($p = 0.086$; OR [95% CI], 0.19 [0.03–1.26]) showed a trend toward independent association; however, $\%CD4^+$RTE ($p = 0.024$; OR [95% CI], 1.20 [1.02–1.41]) and %mDCs ($p = 0.048$; OR [95% CI], 0.16 [0.02–0.98]) were independently associated with MVC-cART.

Relationship between the time of exposure to MVC-containing cART and immunological variables

Since we observed a high degree of variability in the time of exposure to MVC-cART prior to vaccination (median [IQR], 16 [5–38] months), we explored whether this fact could have affected the immunological variables of the study. This analysis was logically restricted to the MVC-cART group (N = 21) (Additional file 1: Table S1). We found significant negative correlations between the time of exposure to MVC-cART and the $\%CD4^+Ki67^+$, the $\%CD4^+HLA\text{-}DR^+$ and the $\%CD4^+CD25^{hi}FoxP3^+$ (Fig. 1).

On the other hand, since we expected to find a direct association between MVC-cART and the inflammation-related marker hsCRP, we also explored potential correlations between the five immunological variables affected by MVC-cART in both a direct or a time-dependent way and the levels of hsCRP. hsCRP was only correlated with $\%CD4^+$RTE ($r = -0.326$; $p = 0.049$) and with $\%CD4^+HLA\text{-}DR^+$ with borderline significance ($r = 0.316$; $p = 0.057$) (Additional file 2: Figure S1).

Impact of immunological variables targeted by MVC-cART on the magnitude of the HBV vaccine response

To explore to what extent each of the immunological factors that were affected by MVC-cART could affect the magnitude of the response, we tested potential associations between these five variables and the magnitude of the HBV response. When analysed in the entire cohort

Table 1 Demographic, clinical and immunological variables associated with MVC-containing cART

Demographic, clinical and immunological variables (n = 41)	MVC-containing cART (N = 21)	MVC-lacking cART (N = 20)	Unadjusted *p* value; OR [95% CI]	Adjusted *p* value; OR [95% CI]
Male sex, n (%)	15 (71)	16 (80)	0.525; 0.625 [0.147–2.659]	
Age (years)	36 [31–44]	44 [39–48]	***0.024; 0.892 [0.800–0.985]***	0.878; 1.01 [0.87–1.18]
Nadir $CD4^+$ T-cell count (cells/mm^3)	294 [184–412]	264 [206–379]	0.539; 1.001 [0.997–1.005]	
$CD4^+$ T-cell count (cells/mm^3)	703 [565–869]	725 [529–911]	0.775; 1.000 [0.997–1.002]	
$CD8^+$ T-cell count (cells/mm^3)	781 [538–961]	596 [491–829]	0.109; 1.002 [1.000–1.004]	
$CD4^+/CD8^+$ ratio	0.9 [0.6–1.2]	1.1 [0.9–1.6]	***0.084; 0.309 [0.082–1.170]***	*0.086; 0.20 [0.03–1.26]*
Time since diagnosis (months)	65 [32–212]	132 [67–235]	0.158; 0.995 [0.989–1.002]	
Time on HIV-treatment (months)	46 [33–147]	120 [64–201]	***0.057; 0.992 [0.984–1.000]***	0.105; 0.99 [0.97–1.00]
Sexual transmission, n (%)	18 (86)	18 (90)	0.677; 1.500 [0.223–10.077]	
Previous AIDS, n (%)	1 (5)	1 (5)	0.972; 0.950 [0.055–16.293]	
Previous HCV coinfection, n (%)	4 (19)	1 (5)	0.199; 4.471 [0.454–44.011]	
NRTI containing cART, n (%)*	4 (19)	14 (70)	***0.002; 0.101 [0.024–0.430]***	
hsCRP (mg/l)	0.8 [0.5–1.1]	0.9 [0.6–1.5]	0.324; 0.612 [0.231–1.623]	
% $CD4^+$ naive	44.1 [34.5–55.6]	44.9 [33.7–50.1]	0.829; 0.995 [0.954–1.039]	
% $CD4^+$ RTE	78.0 [69.9–82.5]	69.3 [64.3–76.4]	***0.016; 1.116 [1.021–1.220]***	***0.024; 1.20 [1.03–1.41]***
% $CD4^+$ central memory	29.4 [23.3–33.2]	28.3 [22.9–42.5]	0.752; 0.989 [0.926–1.057]	
% $CD4^+$ effector memory	20.0 [18.0–27.5]	22.0 [12.9–28.5]	0.968; 0.999 [0.936–1.065]	
% $CD4^+$ TemRA	2.4 [1.1–4.3]	1.6 [0.8–4.4]	0.873; 1.017 [0.831–1.243]	
% $CD4^+$ $HLA\text{-}DR^+$	1.8 [1.0–2.0]	1.6 [1.1–2.3]	0.880; 1.066 [0.464–2.451]	
% $CD4^+$ $Ki67^+$	2.4 [2.0–3.2]	2.2 [2.1–2.6]	0.219; 1.629 [0.748–3.547]	
% $CD4^+$ $CD57^+$	4.8 [3.5–8.0]	4.47 [2.1–10.8]	0.879; 0.990 [0.873–1.124]	
% $CD4^+$ $CD95^+$	55.0 [40.7–66.0]	54.4 [44.1–63.9]	0.590; 1.011 [0.971–1.052]	
% $CD4^+$ $CD25^{hi}FoxP3^+$	1.2 [0.9–2.0]	1.5 [1.1–1.7]	0.992; 0.995 [0.358–2.765]	
% $CD4^+$ $CD25^{hi}FoxP3^+HLA\text{-}DR^+$	13.9 [9.9–21.6]	16.1 [12.2–22.1]	0.543; 0.973 [0.889–1.064]	
% $CD4^+$ $CD25^{hi}FoxP3^+ki67^+$	18.5 [13.5–25.2]	20.1 [17.6–25.0]	0.216; 0.930 [0.830–1.043]	
% $CD4^+$ $CD25^{hi}FoxP3^+CD39^+$	82.0 [41.7–88.2]	83.7 [81.2–85.1]	0.716; 1.005 [0.980–1.029]	
% $CD4^+$ $CD25^{hi}FoxP3^+CTLA4^+$	59.0 [46.2–65.2]	57.7 [47.8–69.2]	0.545; 0.986 [0.941–1.032]	
% mDCs	0.5 [0.3–0.8]	0.8 [0.6–1.4]	***0.042; 0.226 [0.054–0.949]***	***0.048; 0.16 [0.03–0.98]***
% pDCs	0.2 [0.1–0.2]	0.2 [0.1–0.3]	0.529; 0.109 [0.000–108.297]	

Continuous variables are expressed as median values [IQR], and categorical variables are expressed as the number of cases (%). All demographic, clinical and immunological variables with *p* values of < 0.1 in the unadjusted model, except NRTI-containing cART*, were included in the adjusted model and are shown in bolditalics. Hence, age, $CD4^+/CD8^+$ ratio, time on HIV treatment, $\%CD4^+$RTE and %mDCs were included in the multivariate model (n = 38). Variables with *p* values of < 0.1 are shown in *italics*. Variables with *p* values of < 0.05 in the adjusted model were considered statistically significant and are shown in bolditalic. * The absence of NRTIs was collinear with the presence of MVC

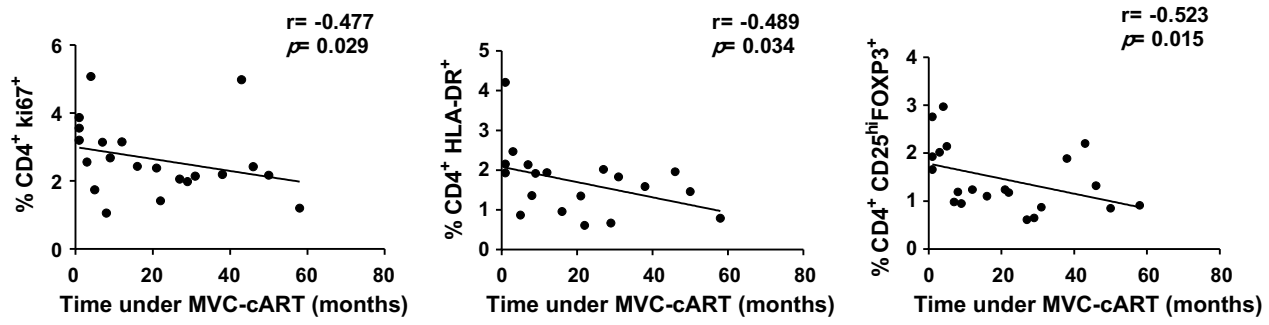

Fig. 1 Relationship between the time of exposure to MVC-cART and immunological variables. Only significant correlations between the time of exposure to MVC-cART and immunological variables are represented

(n = 41) (Additional file 3: Table S2), only the %CD4Ki67$^+$ showed a negative association with the magnitude of the response, with borderline significance ($p = 0.053$; B [95% CI], −199.5 [−401.4 to 2.5]). However, when restricting to the population treated with MVC-cART (n = 21) (Table 2), both the %CD4$^+$Ki67$^+$ ($p = 0.027$; B [95% CI], −241.7 [−452.8 to 30.5]) and the %CD4$^+$HLA-DR$^+$ ($p = 0.038$; B [95% CI], −211.2 [−409.6 to 12.8]) showed significant associations with the anti-HBs titres.

Discussion

We recently observed a beneficial effect of MVC-cART in the HBV vaccine response in a cohort of HIV-infected subjects younger than 50 years old [5]. We report now that HIV-infected subjects on MVC-cART have increased RTE but reduced mDC frequencies prior to vaccination. In addition, a longer time of exposure to MVC-cART was associated with lower frequencies of Tregs and activated and proliferating CD4 T-cells, with proliferating CD4 T-cells being inversely associated with the magnitude of the HBV vaccine response.

In the response to the HBV vaccine, a peptide antigen administered intramuscularly, helper CD4 T-cell function plays a major role [14, 15], and it is well assumed that T-cell exhaustion and senescence related to HIV infection may result in response failure [16]. Antiretroviral treatment improves antigen-specific T-cell responses and recovers the T-cell repertoire [17]. In fact, the duration of cART was associated with the HBV vaccine response [18]. However, the specific effects of different antiretroviral families have been less studied. It is reasonable to expect a negative impact of NRTIs because they favour cellular senescence through inducing accelerated shortening of telomeres in peripheral T-cells [19]. In fact, telomere length has been associated with the response to influenza vaccine in elderly non-HIV-infected subjects [20]. Moreover, we have recently found a better profile in T-cells in subjects on NRTI-lacking regimens regarding cell survival and replicative senescence [21]. On the other hand, there is some controversy about the potential immunological effects of MVC-cART. While some authors have described no effects on inflammatory biomarkers [10, 22], others have comparatively demonstrated their beneficial effects on these markers [6] and on the T-cell immunophenotype [8].

We have now explored the immunological profile associated with MVC-cART in the context of the HBV vaccine response, finding a less activated and proliferative phenotype, a higher contribution of RTEs and a lower frequency of mDC and Treg cells. Notably, HBV vaccine responsiveness has been associated with most of these factors in other cohorts, including decreased activation of T-cells [23], a higher frequency of CD34$^+$ precursors [24, 25] and a lower frequency of Tregs [13]. Dendritic cells are being targeted for improvement of HBV vaccine responsiveness [26]. As far as we know, no previous data link proliferative CD4 T-cells with vaccine response in this context. However, it is known that HIV-infected subjects have increased memory CD4 T-cell cycling, which has been proposed to be consequence of the inflammatory environment of HIV infection [27], and a compromised thymic output [28].

Since we found an increase of the RTE frequency along with a reduction of the frequency of proliferating CD4 T-cells associated with MVC-cART, we speculate a potential regenerative capacity for this regimen that requires further research. Indeed, proliferating CD4 T-cells in the absence of thymic output may show limited immunocompetence due to the constriction of TCR diversity [29]. Thus, this regimen could contribute to a potential enrichment of TCR diversity, which would favour the response against vaccine antigens. Along these lines, MVC positively impacted the response to different vaccine antigens in HIV-infected subjects [4, 5].

The effect on Treg cells also deserves discussion. We previously showed a net effect of MVC monotherapy in reducing Tregs in antiretroviral-naïve subjects [11]. In the present cohort of cART-experienced subjects, this reducing effect was dependent on the time of exposure. Notably, both cohorts differed very much, not only because of the presence/absence of treatment but also in age or time from diagnosis, among other factors. Thus, our current results strengthen the hypothesis that MVC exerts immunomodulatory effects through reducing Treg cells. Treg cells are being studied in several immunization models [30–32] because they suppress the proliferation and cytokine secretion of CD4 and CD8 T cells as well

Table 2 Relationship between variables affected by MCV-containing cART and the magnitude of the HBV vaccine response

Immunological variables	MVC-cART (n = 21)	Unadjusted p value; B (95% CI)
% CD4$^+$ RTE	78.0 [67.0–82.5]	0.599; 6.2 [− 18.0–30.3]
% CD4$^+$ HLA-DR$^+$	1.8 [1.0–2.0]	***0.038; − 211.2 [− 409.6–12.8]***
% CD4$^+$ ki67$^+$	2.4 [2.0–3.1]	***0.027; − 241.7 [− 452.8–30.5]***
% CD4$^+$ CD25^{hi}FoxP3$^+$	1.2 [0.9–2.0]	0.889; − 17.0 [− 267.7–233.78]
% mDCs	0.5 [0.3–0.8]	0.272; 196.1 [− 166.3–558.5]

Continuous variables are expressed as median values [IQR]. Linear regression analyses were performed to determine variables associated with the magnitude of response (absolute anti-HBs titre). Variables with *p* values of < 0.1 are shown in *italics*. Variables with *p* values of < 0.05 were considered statistically significant and are shown in bolditalic

as monocytes, dendritic cells and B cells [33, 34]. In fact, Treg cells were found within germinal centres of human lymphoid tissues, suppressing the B cell immunoglobulin class switching needed to mount a proper antibody response [35]. In our cohort, the frequencies of activated and proliferating Treg cells, which could be highly suppressive, were inversely associated with the magnitude of the vaccine response (data not shown).

Importantly, plasma levels of soluble inflammatory markers before vaccination negatively predicted responses to HAV, HBV, and tetanus vaccines in HCV and HIV infection [36]. Moreover, hsCRP levels were a significant predictor of herpes zoster vaccine response in elderly nursing home residents [37]. hsCRP levels were also inversely associated with the magnitude of the vaccine response in our cohort (data not shown), but we failed to observe a direct association between hsCRP levels and MVC-containing cART. However, we cannot exclude a potential effect of MCV-cART on other inflammatory cytokines, as previously reported [6]. Moreover, hsCRP was inversely associated with the frequency of RTEs and positively associated with activated CD4 T-cells, both of which were impacted by MVC-cART, suggesting a potential indirect effect of MVC-cART on the inflammatory state.

As a limitation, the size of our cohort was restricted to the vaccinated population younger than 50 years old, where the effect of MVC-cART on the magnitude of vaccine responsiveness was clear [5]. It is well-known that age limits HBV vaccine responsiveness [38]. Thus, it is reasonable to speculate that the added age-associated immunodeficiency could limit or mask the potential benefits of such antiretroviral therapy on the immunological profile. In this sense, aged people have lower thymic output concomitant with higher peripheral T-cell proliferation [27]. Interestingly, the group on MVC-cART had lower CD4/CD8 ratios, which have been reported to negatively impact the vaccine response [39]. This could be due to the shorter period of treatment in this group, which is critical for CD4/CD8 T-cell ratio normalization [40]. In any case, despite the lower CD4/CD8 ratio, the group on MVC-cART showed better vaccine responsiveness and improved CD4 T-cell profiles. Finally, we cannot discriminate among the particular effects due to the presence of MVC or to the absence of NRTIs in the cART, and thus, we can only draw conclusions about the beneficial effects of such combined therapy. Similar combined therapies are being explored in the clinical setting in an attempt to reduce toxicities and to improve immune reconstitution [41].

Conclusion

The beneficial effect of MVC-cART in the HBV vaccine response in subjects below 50 years old could be mediated at least partially by its reducing effect on the frequencies of activated and proliferating CD4 T-cells prior to vaccination. This fact could be related with a potential regenerative capacity of such therapy and deserves further research due to its relevance in the search for novel therapeutic targets that could improve immune function and vaccine responsiveness in HIV-infected subjects.

Additional files

Additional file 1: Table S1. Relationship between the time of exposure to MVC-containing cART and immunological variables.

Additional file 2: Figure S1. Associations between hsCRP and T-cell immunological variables affected by MVC-cART. Only significant correlations between hsCRP and T-cell immunological variables are represented.

Additional file 3: Table S2. Relationship between variables modified by MCV-containing cART and the magnitude of the HBV vaccine response in the whole cohort.

Abbreviations

cART: combined antiretroviral therapy; DC: dendritic cell; ELISA: enzyme-linked immunosorbent assay; HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HIV: human immunodeficiency virus; hsCRP: highly sensitive C-reactive protein; mDC: myeloid-DC; MVC: maraviroc; MVC-cART : maraviroc-containing combined antiretroviral therapy; NRTI: nucleoside reverse-transcriptase inhibitors; PBMCs: peripheral blood mononuclear cells; pDC: plamacytoid dendritic cells; PI: protease inhibitor; RTE: recent thymic emigrants; Treg: regulatory T-cells.

Authors' contributions

IH-F performed experiments, data analysis and interpretation and wrote the manuscript; IR-S, LT-D and ER-M performed experiments; MG and ML were the clinicians involved in the vaccination protocol and clinical assistance of the patients; MMR-M and MMP-B assisted in the vaccination protocol and sampling; CL performed anti-HBs titres; ML and YMP coordinated the study; YMP designed the study and participated in data analysis and interpretation and writing of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable.

Consent for publication
Not applicable.

Ethics approval and consent to participate
All participants signed informed consent forms at study entry. The study protocol was approved by the Comité de Ética de la Investigación (CEI) de los hospitales universitarios Vírgen Macarena-Virgen del Rocío.

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References

1. Thio CL. Hepatitis B and human immunodeficiency virus coinfection. Hepatology. 2009;49(5 suppl):S138–45.
2. Clifford GM, Rickenbach M, Polesel J, Dal Maso L, Steffen I, Ledergerber B, et al. Influence of HIV-related immunodeficiency on the risk of hepatocellular carcinoma. AIDS. 2008;22(16):2135–41.
3. Whitaker JA, Rouphael NG, Edupuganti S, Lai L, Mulligan MJ. Strategies to increase responsiveness to hepatitis B vaccination in adults with HIV-1. Lancet Infect Dis. 2012;12:966–76.
4. Westrop SJ, Moyle G, Jackson A, Nelson M, Mandalia S, Imami N. CCR5 antagonism impacts vaccination response and immune profile in HIV-1 infection. Mol Med. 2012;18:1240–8.
5. Herrero-Fernández I, Pacheco YM, Genebat M, Rodriguez-Méndez MDM, Lozano MDC, Polaino MJ, et al. Association between a suppressive combined antiretroviral therapy containing maraviroc and the hepatitis B virus vaccine response. Antimicrob Agents Chemother. 2017;62(1):e02050–117.
6. Romero-Sánchez MC, Alvarez-Ríos AI, Bernal-Morell E, Genebat M, Vera F, Benhnia MR, et al. Maintenance of virologic efficacy and decrease in levels of β2-microglobulin, soluble CD40L and soluble CD14 after switching previously treated HIV-infected patients to an NRTI-sparing dual therapy. Antiviral Res. 2014;111:26–32.
7. Funderburg N, Kalinowska M, Eason J, Goodrich J, Heera J, Mayer H, et al. Effects of maraviroc and efavirenz on markers of immune activation and inflammation and associations with CD4+ cell rises in HIV-infected patients. PLoS ONE. 2010;5(10):e13188.
8. Romero-Sánchez MC, Machmach K, Gonzalez-Serna A, Genebat M, Pulido I, García-García M, et al. Effect of maraviroc on HIV disease progression-related biomarkers. Antimicrob Agents Chemother. 2012;56:5858–64.
9. Serrano-Villar S, Sainz T, Ma ZM, Utay NS, Wook-Chun T, Mann S, et al. Effects of combined CCR5/integrase inhibitors-based regimen on mucosal immunity in HIV-infected patients naïve to antiretroviral therapy: a pilot randomized trial. PLoS Pathog. 2016;12:e1005540.
10. Taiwo BO, Chan ES, Fichtenbaum CJ, Ribaudo H, Tsibris A, Klingman KL, et al. Less bone loss with maraviroc-versus tenofovir-containing antiretroviral therapy in the AIDS Clinical Trials Group A5303 Study. Clin Infect Dis. 2015;61:1179–88.
11. Pozo-Balado MM, Martínez-Bonet M, Rosado I, Ruiz-Mateos E, Méndez-Lagares G, Rodríguez-Méndez MM, et al. Maraviroc reduces the regulatory T-cell frequency in antiretroviral-naive HIV-infected subjects. J Infect Dis. 2014;210:890–8.
12. Pozo-Balado MM, Rosado-Sánchez I, Méndez-Lagares G, Rodríguez-Méndez MM, Ruiz-Mateos E, Benhnia MR, et al. Maraviroc contributes to the restoration of the homeostasis of regulatory T-cell subsets in antiretroviral-naive HIV-infected subjects. Clin Microbiol Infect. 2016;22:461.
13. Pozo-Balado MM, Leal M, Méndez Lagares G, Mata RC, López-Cortés LF, Viciana P, et al. Increased regulatory T cell counts in HIV-infected nonresponders to hepatitis B virus vaccine. J Infect Dis. 2010;202:362–9.
14. Egea E, Iglesias A, Salazar M, Morimoto C, Kruskall MS, Awdeh Z, et al. The cellular basis for lack of antibody response to hepatitis B vaccine in humans. J Exp Med. 1991;173:531–8.
15. Filion LG, Saginur R, Szczerbak N. Humoral and cellular immune responses by normal individuals to hepatitis B surface antigen vaccination. J Exp Immunol. 1988;71:405–9.
16. Yao ZQ, Moorman JP. Immune exhaustion and immune senescence: two distinct pathways for HBV vaccine failure during HCV and/or HIV infection. Arch Immunol Ther Exp. 2013;61:193–201.
17. Vrisekoop N, van Gent R, de Boer AB, Otto SA, Borleffs JC, Steingrover R, et al. Restoration of the CD4 T cell compartment after long-term highly active antiretroviral therapy without phenotypical signs of accelerated immunological aging. J Immunol. 2008;181:1573–81.
18. Overton ET, Sungkanuparph S, Powderly WG, Seyfried W, Groger RK, Aberg JA. Undetectable plasma HIV RNA load predicts success after hepatitis B vaccination in HIV-infected persons. Clin Infect Dis. 2005;41:1045–8.
19. Leeansyah E, Cameron PU, Solomon A, Tennakoon S, Velayudham P, Gouillou M, et al. Inhibition of telomerase activity by human immunodeficiency virus (HIV) nucleos(t)ide reverse transcriptase inhibitors: a potential factor contributing to HIV-associated accelerated aging. J Infect Dis. 2013;207:1157–65.
20. Najarro K, Nguyen H, Chen G, Xu M, Alcorta S, Yao X, et al. Telomere length as an indicator of the robustness of B- and T-cell response to influenza in older adults. J Infect Dis. 2015;212(8):1261–9.
21. Gonzalez-Serna A, Ferrando-Martinez S, Tarancon-Diez L, De Pablo-Bernal RS, Dominguez-Molina B, Jiménez JL, et al. Increased $CD127^+$ and decreased $CD57^+$ T cell expression levels in HIV-infected patients on NRTI-sparing regimens. J Transl Med. 2017;15(1):259.
22. Pulido I, Genebat M, Alvarez-Rios AI, De Pablo-Bernal RS, Rafii-El-Idrissi Benhnia M, Pacheco YM, et al. Immunovirological efficacy of once-daily maraviroc plus ritonavir-boosted atazanavir After 48 weeks in naive HIV-infected patients. Viral Immunol. 2016;29(8):471–7.
23. Wilson CM, Ellenberg JH, Sawyer MK, Belzer M, Crowley-Nowick PA, Puga A, et al. Adolescent Medicine HIV/AIDS Research Network. Serologic response to hepatitis B vaccine in HIV infected and high-risk HIV uninfected adolescents in the REACH cohort. Reaching for excellence in adolescent care and health. J Adolesc Health. 2001;29:123–9.
24. Anthony DD, Umbleja T, Aberg JA, Kang M, Medvik K, Lederman MM, et al. Lower peripheral blood CD14+ monocyte frequency and higher CD34+ progenitor cell frequency are associated with HBV vaccine induced response in HIV infected individuals. Vaccine. 2011;29:3558–63.
25. Veiga AP, Casseb J, Duarte AJ. Humoral response to hepatitis B vaccination and its relationship with T CD45RA+ (naive) and CD45RO+ (memory) subsets in HIV-1-infected subjects. Vaccine. 2006;4:7124–8.
26. Jing M, Wang J, Zhu S, Ao F, Wang L, Han T, et al. Development of a more efficient hepatitis B virus vaccine by targeting hepatitis B virus preS to dendritic cells. Vaccine. 2016;34(4):516–22.
27. Jiang W, Younes SA, Funderburg NT, Mudd JC, Espinosa E, Davenport MP, et al. Cycling memory CD4+ T cells in HIV disease have a diverse T cell receptor repertoire and a phenotype consistent with bystander activation. J Virol. 2014;88(10):5369–80.
28. Sauce D, Larsen M, Fastenackels S, Roux A, Gorochov G, Katlama C, et al. Lymphopenia-driven homeostatic regulation of naive T cells in elderly and thymectomized young adults. J Immunol. 2012;189(12):5541–8.
29. Kohler S, Thiel A. Life after the thymus: CD31+ and CD31− human naïve CD4+ T-cell subsets. Blood. 2009;113:769–74.
30. Toka FN, Suvas S, Rouse BT. $CD4^+$ $CD25^+$ T cells regulate vaccine-generated primary and memory $CD8^+$ T-cell responses against herpes simplex virus type 1. J Virol. 2004;78(23):13082–9.

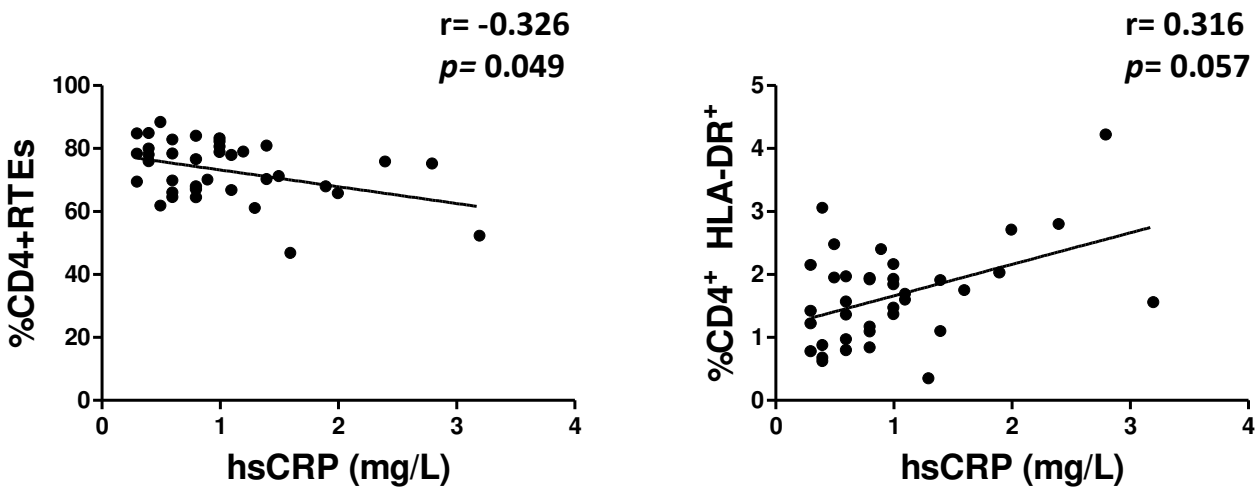
31. Espinoza-Mora MR, Steeg C, Tartz S, Heussler V, Sparwasser T, Link A, et al. Depletion of regulatory T cells augments a vaccine-induced T effector cell response against the liver-stage of malaria but fails to increase memory. PLoS ONE. 2014;9(8):e104627.
32. Lin PH, Wong WI, Wang YL, Hsieh MP, Lu CW, Liang CY, et al. Vaccine-induced antigen-specific regulatory T cells attenuate the antiviral immunity against acute influenza virus infection. Mucosal Immunol. 2018. https://doi.org/10.1038/s41385-018-0004-9.
33. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. Trends Mol Med. 2007;13:108–16.
34. Tiemessen MM, Jagger AL, Evans HG, van Herwijnen MJ, John S, Taams LS. $CD4^+CD25^+Foxp3^+$regulatory T cells induce alternative activation of human monocytes/macrophages. Proc Natl Acad Sci USA. 2007;104:19446–51.
35. Lim HW, Hillsamer P, Banham AH. KimCH. Cutting edge: direct suppression of B cells by CD4+CD25+ regulatory T cells. J Immunol. 2005;175:4180–3.
36. Shive CL, Judge CJ, Clagett B, Kalayjian RC, Osborn M, Sherman KE, et al. Pre-vaccine plasma levels of soluble inflammatory indices negatively predict responses to HAV, HBV, and tetanus vaccines in HCV and HIV infection. Vaccine. 2018;36(4):453–60.
37. Verschoor CP, Lelic A, Parsons R, Evelegh C, Bramson JL, Johnstone J, et al. Serum C-reactive protein and congestive heart failure as significant predictors of herpes zoster vaccine response in elderly nursing home residents. J Infect Dis. 2017;216(2):191–7.
38. Fisman DN, Agrawal D, Leder K. The effect of age on immunologic response to recombinant hepatitis B vaccine: a meta-analysis. Clin Infect Dis. 2002;35:1368–75.
39. Fuster F, Vargas JI, Jensen D, Sarmiento V, Acuña P, Peirano F, et al. CD4/CD8 ratio as a predictor of the response to HBV vaccination in HIV-positive patients: a prospective cohort study. Vaccine. 2016;34:1889–95.
40. Caby F, Guihot A, Lambert-Niclot S, Guiguet M, Boutolleau D, Agher R, et al. Determinants of a low CD4/CD ratio in HIV-1 infected individuals despite long term viral suppression. Clin Infect Dis. 2016;62:1297–303.
41. Nozza S, Svicher V, Saracino A, d'Ettorre G, De Luca A, Maggiolo F, et al. State of the art of dual therapy in 2015. AIDS Rev. 2015;17:127–34.

Supplementary Table 1. Relationship between the time of exposure to MVC-containing cART and immunological variables.

Immunological variables (n=21)	r	p
hsCRP (mg/L)	0.003	0.989
% $CD4^+$ Naive	0.185	0.434
% $CD4^+$ RTE	-0.161	0.497
% $CD4^+$ Central Memory	-0.151	0.526
% $CD4^+$ Effector Memory	-0.085	0.723
% $CD4^+$ TemRA	-0.087	0.714
% $CD4^+$ $HLA\text{-}DR^+$	-0.489	**0.034**
% $CD4^+$ $Ki67^+$	-0.477	**0.029**
% $CD4^+$ $CD57^+$	0.110	0.663
% $CD4^+$ $CD95^+$	-0.170	0.460
% $CD4^+$ $CD25^{hi}FoxP3^+$	-0.523	**0.015**
% $CD4^+$ $CD25^{hi}FoxP3^+HLA\text{-}DR^+$	-0.126	0.586
% $CD4^+$ $CD25^{hi}FoxP3^+ki67^+$	-0.318	0.172
% $CD4^+$ $CD25^{hi}FoxP3^+CD39^+$	0.116	0.617
% $CD4^+$ $CD25^{hi}FoxP3^+CTLA4^+$	0.226	0.324
% mDCs	-0.133	0.567
% pDCs	0.105	0.650

Correlations were assessed using Spearman's rho correlation coefficient. Variables with *p* values of <0.05 were considered statistically significant and are shown in bold.

Supplementary Figure 1.



Supplementary Table 2. Relationship between variables modified by MCV-containing cART and the magnitude of the HBV vaccine response in the whole cohort.

Immunological variables	All cohort (n=41)	Unadjusted P value; B (95% CI)
% $CD4^+$ RTE	75.6 [66.5-79.8]	0.204; 9.2 [-5.2-23.7]
% $CD4^+$ $HLA\text{-}DR^+$	1.7 [1.1-2.0]	0.161; -128.1 [-309.6-53.4]
% $CD4^+$ $ki67^+$	2.3 [2.1-2.7]	*0.053;* -199.5 [-401.4-2.5]
% $CD4^+$ $CD25^{hi}FoxP3^+$	1.3 [1.0-1.8]	0.565; -64.7 [-290.3-160.9]
% mDCs	0.7 [0.4-1.1]	0.477; -85.4 [-325.9-155.1]

Continuous variables are expressed as median values [IQR]. Linear regression analyses were performed to determine variables associated with the magnitude of response (absolute anti-HBs titre). Variables with *p* values of <0.1 are shown in *italics*.

ANEXOS

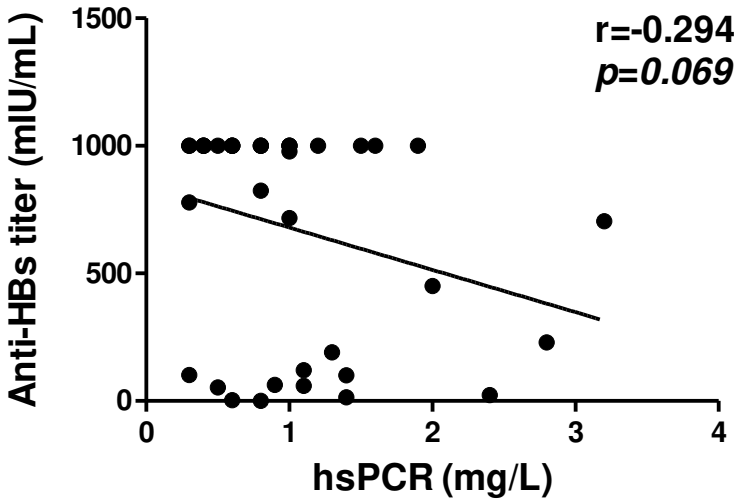
Anexo 1. Demographic, clinical and immunological variables associated with HBV vaccine responsiveness.

Clinical and immunological variables	n=41	Unadjusted P value; B (95% CI)	Adjusted P value; B (95% CI)
Male sex, n (%)	31 (76)	**0.055; -294.2 [-595.5-7.1]**	
Age (years)	41.0 [33.5-46.5]	0.717; -3.3 [-21.3-14.8]	
Nadir $CD4^+$ T-cell count (cells/mm^3)	287.0 [206.3-402.0]	0.288; 0.46 [-0.40-1.32]	
$CD4^+$ T-cell count (cells/mm^3)	703.0 [553.0-887.5]	0.302; 0.30 [-0.28-0.88]	
$CD8^+$ T-cell count (cells/mm^3)	643.0 [501.0-927.0]	0.344; -0.19 [-0.58-0.21]	
Ratio $CD4^+/CD8^+$	0.9 [0.8-1.4]	0.205; 139.1 [-79.0-357.1]	
Time since diagnosis (months)	75.0 [37.5-212.0]	0.200; 0.89 [-0.49-2.27]	
Sexual transmission, n (%)	36 (88)	0.579; 114.4 [-298.7-527.4]	
Previous AIDS, n (%)	2 (5)	**0.074; -548.2 [-1152.6-56.3]**	
Previous HCV coinfection, n (%)	5 (12)	0.453; 154.4 [-257.3-566.0]	
NRTI containing cART, n (%)	18 (44)	0.496; 92.4 [-179.4-364.2]	
MVC containing cART, n (%)	21 (51)	**0.017; 310.8 [58.7-563.0]**	**0.016; 293.3 [58.1-5280.5]**
Previous HBV vaccination, n (%)*	13 (32)	**0.067; 262.6 [-19.5-544.8]**	
Simultaneous HAV vaccination, n (%)	18 (44)	**0.029; 287.9 [31.0-545.0]**	
Time on HIV-treatment (months)	69.5 [38.0-178.8]	0.376; 0.74 [-0.93-2.42]	
hsCRP (mg/L)	0.8 [0.5-1.3]	**0.096; -165.8 [-362.4-30.9]**	
% Lymphocytes	35.6 [26.1-39.2]	0.555; 4.9 [-11.7-21.4]	
% Monocytes	5.6 [5.3-6.1]	0.393; 66.5 [-89.5-222.4]	
% Neutrophils	53.6 [49.3-63.3]	0.593; -3.7 [-17.8-10.3]	
% $CD4^+$ Naives	44.6 [34.2-51.0]	0.496; 3.2 [-6.3-12.7]	
% $CD4^+$ RTE	75.6 [66.5-79.8]	0.204; 9.2 [-5.2-23.7]	
% $CD4^+$ Central Memory	28.3 [23.2-39.5]	0.791; -1.9 [-16. 7-12. 8]	
% $CD4^+$ Effector Memory	20.7 [14.9-27.8]	0.400; -6.1 [-20.5-8.4]	

% $CD4^+$ TemRA	1.8 [1.1-4.3]	0.154; -31.4 [-75.2-12.3]	
% $CD4^+$ HLA-DR^+	1.7 [1.1-2.0]	0.161; -128.1 [-309.6-53.4]	
% $CD4^+$ $ki67^+$	2.3 [2.1-2.7]	**0.053; -199.5 [-401.4-2.5]**	
% $CD4^+$ $CD57^+$	4.7 [3.1-8.0]	0.825; -3.1 [-31.3-25.2]	
% $CD4^+$ $CD95^+$	55.0 [43.1-65.0]	0.394; -3.7 [-12.3-5.0]	
% $CD4^+$ $CD25^{hi}$$FoxP3^+$	1.3 [1.0-1.8]	0.565; -64.7 [-290.3-160.9]	
% $CD4^+$ $CD25^{hi}$$FoxP3^+$HLA-$DR^+$	15.1 [11.0-21.6]	**0.039; -19.7 [-38.3--1.0]**	
% $CD4^+$ $CD25^{hi}$$FoxP3^+$$ki67^+$	19.8 [16.9-25.0]	**0.011; -28.9 [-50.7--7.1]**	**0.005; -29.6 [-49.6--9.5]**
% $CD4^+$ $CD25^{hi}$$FoxP3^+$$CD39^+$	81.2 [41.7-87.0]	0.274; -2.9 [-8.2-2.4]	
% $CD4^+$ $CD25^{hi}$$FoxP3^+$$CTLA4^+$	58.7 [46.9-68.3]	0.232; -5.9 [-15.8-4.0]	
% mDCs	0.7 [0.4-1.1]	0.477; -85.4 [-325.9-155.1]	
% pDCs	0.2 [0.18-0.24]	**0.072; 1323.5 [-122.5-2769.6]**	**0.009; 1961.2 [530.4-3392.0]**

Continuous variables are expressed as median values [IQR] and categorical variables are expressed as number of cases (%). All demographic, clinical and immunological variables with a *p* value of <0.1 in the unadjusted model were included in the adjusted model and are shown in bold. Hence, male sex, previous AIDS, MVC containing cART, previous HBV vaccination, simultaneous HAV vaccination, hsCRP, %$CD4^+ki67^+$, %$CD4^+CD25^{hi}FoxP3^+$HLA-DR^+, %$CD4^+CD25^{hi}FoxP3^+ki67^+$, %pDCs were included in the stepwise forward multivariate model. As shown, the resulting model only retained the variables MVC containing cART, %$CD4^+CD25^{hi}FoxP3^+ki67^+$ and %pDCs. Variables with a *p* value of <0.05 in the adjusted model were considered statistically significant and are shown in bold.

Anexo 2. Association between hsPCR and anti-HBs titer.



Correlations were assessed using the Spearman's rho correlation coefficient. Variables with a *p* value of <0.1 are shown in *italics*.

5. RESUMEN GLOBAL DE LOS RESULTADOS

RESUMEN GLOBAL DE LOS RESULTADOS

En el primer artículo de la presente Tesis Doctoral "**T-cell proliferative homeostatic alterations are negatively associated with the vaccine response against influenza in elderly people" (en revisión)**, encontramos que los ancianos no respondedores a la vacuna frente a influenza mostraron mayor frecuencia de células Treg antes de la vacuna, así como una mayor expresión del marcador de proliferación ki67 en las Treg y en diferentes subpoblaciones de maduración de células T CD4 y CD8. Además, los biomarcadores de inflamación estudiados correlacionaron con las frecuencias de diferentes subpoblaciones de células T proliferativas y con la función tímica.

Con respecto al segundo artículo de la presente Tesis Doctoral, **"Association between a suppressive combined antiretroviral therapy containing maraviroc and the hepatitis B virus vaccine response" Antimicrob Agents Chemother 2017; 62:e02050-17**, observamos que, en pacientes infectados por VIH menores de 50 años, un TARc que incluía MVC se asociaba con una mayor magnitud de la respuesta a la vacuna frente a VHB. Además, la vacunación simultánea frente al Virus de la Hepatitis A (VHA), la vacuna previa frente a VHB y el sexo femenino se asociaron positivamente con la magnitud de la respuesta en la población global de pacientes VIH bajo TARc supresor.

En el tercer artículo de la presente Tesis Doctoral **"Improved CD4 T-cell profile in HIV-infected subjects on maraviroc-containing therapy is associated with better responsiveness to HBV vaccination" J Transl Med 2018; 16:238**, observamos que pacientes VIH menores de 50 años tratados con un TARc incluyendo MVC presentaban mayor contribución de las células recientemente emigradas del timo (RTE) y menor frecuencia de células dendríticas mieloides (mDC). Una mayor exposición a un TARc con MVC se asoció con menores frecuencias de Treg y células T CD4 activadas y proliferativas. Además, las frecuencias de células T CD4 activadas y proliferativas se asociaron inversamente con la magnitud de la respuesta a la vacuna frente a VHB.

6. DISCUSIÓN GLOBAL

DISCUSIÓN GLOBAL

La presente Tesis Doctoral profundiza en el conocimiento del proceso de inmunosenescencia en el contexto de la respuesta vacunal. Por un lado, estudiamos la respuesta vacunal frente a la gripe en sujetos ancianos y, por otro, la respuesta a la vacuna frente a VHB en sujetos VIH, siendo ambos escenarios de inmunosenescencia cronológica y prematura, respectivamente.

En el primer trabajo, "*T-cell proliferative homeostatic alterations are negatively associated with the vaccine response against influenza in elderly people*", encontramos que la respuesta a la vacuna frente a influenza en ancianos se asoció con las alteraciones homeostáticas que se producen en el sistema inmunitario durante el envejecimiento, y que afectan a la proliferación de las distintas subpoblaciones de las células T, tanto de CD4 como CD8.

Sauce y *cols.* [11] observaron una asociación inversa entre la función tímica y la proliferación en células T naïves en sujetos ancianos, en sujetos timectomizados durante la infancia y también en sujetos infectados por VIH. Sin embargo, aunque en nuestra cohorte de ancianos observamos una mayor proliferación de células T naïves en el grupo de los no-respondedores a la vacuna, esto no se asoció aparentemente con una menor función tímica. Quizá esto podría explicarse por el hecho de que, aparentemente, nuestra cohorte mostró una función tímica relativamente preservada, en comparación con los valores de otra cohorte en la que la función tímica se determinó utilizando la misma técnica [100]. Lo que sí observamos fue una asociación entre la elevada proliferación de las subpoblaciones de células T estudiadas y diversos marcadores de inflamación, pudiendo, por tanto, esta proliferación ser un reflejo más directo del estado de inflamación. En esta línea, trabajos previos mostraron también una relación entre la inflamación en sujetos VIH y una mayor proliferación de las células T memoria [63]. Por tanto, es razonable especular que el incremento de la proliferación homeostática que se observa durante el envejecimiento pueda contribuir al *"inflammaging".* Por otro lado, nosotros observamos una relación inversa entre los niveles de los marcadores de inflamación y la función tímica, y otros autores también

han descrito previamente una relación entre la involución del timo y la inflamación crónica [101]. De esta manera, en el escenario del envejecimiento, la función tímica y la inflamación parecen estar inversamente relacionadas.

Además, el hecho de que los ancianos que fallecen en nuestra cohorte, durante un año de seguimiento posterior a la vacunación, presenten conjuntamente fallo tímico, elevada proliferación de las células T y mayor inflamación, refuerza la hipótesis de que estos parámetros estén estrechamente interrelacionados.

Con relación a la respuesta vacunal en ancianos, además de la asociación con la mencionada proliferación de células T, observamos también una tendencia a la asociación con marcadores de inflamación. El papel deletéreo de la inflamación en este escenario es consistente con lo descrito previamente [43,44]. Por otro lado, la infección por CMV, que es uno de los principales factores asociados a *inflammaging*, más concretamente, el título de anticuerpos frente a CMV, se ha asociado con la respuesta vacunal en otras cohortes de ancianos [102,103]. En nuestra cohorte, no hemos observado esta asociación, pese a que sí hemos encontrado una asociación entre el título de anticuerpos anti-CMV y los niveles de marcadores de inflamación. De nuevo, quizá el hecho de que en nuestra cohorte los niveles de los marcadores de inflamación estén relativamente preservados (en comparación con otras cohortes de ancianos), puede explicar la aparente contradicción en nuestros resultados. Tampoco podemos descartar que exista asociación con marcadores de inflamación diferentes de los analizados en este estudio.

En relación con el escenario de la infección por VIH, los niveles de marcadores de inflamación han sido también previamente asociados con la escasa respuesta vacunal frente a VHA, VHB y tétanos, en sujetos coinfectados por VHC y VIH [104]. Además, la proteína C reactiva ultrasensible (usPCR) se ha propuesto como predictor de la respuesta a la vacuna del herpes zoster en ancianos [105]. En nuestra cohorte de sujetos VIH hemos observado también que los niveles de usPCR tendían a asociarse inversamente con la magnitud de la respuesta a la vacuna frente a VHB (Anexo 2). Por otro lado, los niveles de usPCR se asociaron inversamente con la frecuencia de células RTE y directamente con la frecuencia de células T CD4 activadas. Dado que la

frecuencia de RTE depende directamente de la función tímica, esto sugiere que, en este escenario, también pueda existir una relación entre la inflamación y la función tímica, en la que es interesante profundizar. En este sentido, nuestro grupo ha descrito recientemente que la función tímica impacta en la progresión clínica de los sujetos VIH [106,107]. Es interesante destacar que, actualmente, se asume que la progresión clínica de los pacientes VIH tratados depende del estado inflamatorio crónico que persiste a pesar de la supresión virológica, y de la concomitante (parcial) reconstitución inmunitaria. Esta relación indirecta (timo-progresión + progresión-inflamación) también refuerza la hipótesis del papel del timo en la inflamación crónica de pacientes VIH.

En estos escenarios de inmunosenescencia, también hemos explorado el papel de las Treg en la respuesta vacunal, puesto que las Treg están involucradas en la supresión del sistema inmunitario, impidiendo una respuesta apropiada frente a las vacunas [46,47], entre otras funciones. Además, en ambos escenarios la frecuencia de Treg se ve aumentada [15,64]. Hemos observado una frecuencia basal de Treg más elevada en los ancianos no-respondedores a la vacuna, resultados que están en línea con los de Geest *et al.* [108] y con los hallados previamente en nuestro grupo en sujetos infectados por VIH [51]. Asimismo, encontramos una mayor proliferación de las Treg en los ancianos que no respondían a la vacuna, resultado que concuerda con lo observado en el escenario VIH, en el que la respuesta a la vacuna de VHB se asoció inversamente con las células Treg proliferativas (Anexo 1 del tercer trabajo de la presente Tesis Doctoral, "*Improved CD4 T-cell profile in HIV-infected subjects on maraviroc-containing therapy is associated with better responsiveness to HBV vaccination*"). El hecho de que una mayor frecuencia de Treg pudiera impedir una adecuada respuesta a la vacuna tanto en sujetos ancianos, como en sujetos infectados por VIH, refuerza el concepto de que este incremento de las Treg sea una característica común de inmunosenescencia en ambos escenarios [15], que puede comprometer la capacidad del sistema inmunitario para responder de manera apropiada [48-53]. Podemos especular que la expansión y elevada proliferación de las Treg en estos escenarios estén relacionadas con la proliferación homeostática que trata de compensar la menor función tímica en estos pacientes, como se ha observado

previamente en modelos animales [109] y, más recientemente en nuestro grupo, en un modelo humano de proliferación homeostática [110]. Un análisis en profundidad del papel de la inmunosenescencia en las Treg y viceversa, así como del papel de las Treg en la respuesta vacunal, podría contribuir a la búsqueda de estrategias terapéuticas que mejoren la respuesta vacunal en estos escenarios de inmunosenescencia.

En esta Tesis Doctora, hemos explorado una potencial estrategia terapéutica para mejorar la respuesta vacunal atendiendo a la expansión de Treg en estos escenarios de inmunosenescencia y a su efecto deletéreo en dicha respuesta. Dado que en estudios previos, nuestro grupo había mostrado que MVC podía reducir la frecuencia de Treg en sujetos con infección por VIH [94], nos propusimos estudiar si el tratamiento con un TARc que incluyese MVC podría mejorar la respuesta a la vacuna frente a VHB, mediante un efecto reductor de la frecuencia de las Treg. Pero, además, un potencial efecto beneficioso de MVC en este escenario, también podría implicar otros mecanismos de acción como una reducción de la inflamación o una mejora de la presentación antigénica. De hecho, MVC ha presentado numerosas propiedades inmunomoduladoras, como mejorar la enfermedad de injerto frente a huésped en trasplante de células madre hematopoyéticas alogénicas [111,112], suprimir el crecimiento tumoral e inducir apoptosis en células de leucemia linfoblástica aguda [113]. En el escenario de la infección por VIH, MVC se ha asociado a una mejora de la inmunidad de la mucosa duodenal y a una reducción de la pérdida ósea [114,115]. También, parece ejercer un perfil antiinflamatorio, disminuyendo marcadores de inflamación como la β2-microglobulina, el CD40L soluble y el CD14 soluble [93], lo que podría mejorar la respuesta inmunitaria a las vacunas. De hecho, se había descrito previamente que MVC ejercía un efecto beneficioso en otros escenarios distintos de respuesta vacunal, como era la respuesta a la inmunización frente a tétanos, meningoco o cólera [97].

Por todo ello, en el segundo trabajo de la presente Tesis Doctoral, "*Association between a suppressive combined antiretroviral therapy containing maraviroc and the hepatitis B virus vaccine response. Antimicrob Agents Chemother*", quisimos explorar el posible efecto beneficioso de un TARc que incluyese MVC en la respuesta vacunal

frente al VHB. Observamos que este tratamiento efectivamente se asociaba con una mayor magnitud de la respuesta a la vacuna, aunque principalmente en sujetos VIH menores de 50 años. Estos resultados llevan a recomendar una estrategia de vacunación temprana, en sujetos menores de 50 años, y acompañada de un TARc que incluya MVC. Con la edad, el sistema inmunitario se altera debido a la inmunosenescencia y la respuesta a las vacunas disminuye [59]. En los individuos con infección por VIH, este proceso está acentuado además por la inmunosenescencia prematura que propicia el propio virus [56,57]. Es probable que esta inmunosenescencia prematura esté interfiriendo con el efecto beneficioso de MVC en los sujetos mayores de 50 años.

Nos propusimos profundizar también en los posibles mecanismos inmunológicos implicados en este efecto beneficioso de un TARc que incluye MVC, concretamente, en el tercer trabajo de la presente Tesis Doctoral, "*Improved CD4 T-cell profile in HIV-infected subjects on maraviroc-containing therapy is associated with better responsiveness to HBV vaccination".* Así, hemos explorado el potencial efecto de un TARc que incluía MVC en diferentes parámetros asociados con la inmunosenescencia, la inflamación, la función de las células T (incluidas las Treg), y células presentadoras de antígenos (incluidas las DC). Lo hemos analizado en el contexto de la eficacia de la vacuna VHB en la misma población VIH menor de 50 años estudiada en el trabajo anterior. Hemos encontrado que los pacientes tratados con un TARc incluyendo MVC mostraban un perfil inmunológico menos activado, con mayor contribución de las RTE y una menor frecuencia de mDC y de Treg. Estos factores habían sido previamente asociados con la respuesta vacunal en otras cohortes [116-118].

La elevada frecuencia de RTE en sujetos tratados con un TARc que incluía MVC, junto con la reducción de sus células T CD4 proliferativas, hace pensar que dicho régimen posee capacidad regenerativa del sistema inmune. La involución tímica que sufren los sujetos VIH [11] promueve la proliferación de células T por compensación homeostática, limitando la competencia inmunitaria al reducir el repertorio de los TCR [119]. Por tanto, si MVC mejora la función tímica, uno de los mecanismos por los cuales MVC podría estar mejorando la respuesta a la vacuna, sería aumentando la diversidad de los TCR. Por otro lado, un potencial efecto sobre la función tímica podría

repercutir a su vez en el grado de inflamación sistémica asociada a la infección por VIH. Además, el tiempo de exposición al TARc que incluía MVC también se asoció a una reducción en la frecuencia de células T CD4 activadas. Al respecto, resulta interesante que la menor frecuencia de células T CD4 proliferativas y células T CD4 activadas, correlaciona inversamente con la magnitud de la respuesta a la vacuna de VHB. En esta línea, como ya se ha comentado anteriormente, en el escenario de ancianos pudimos observar que, tanto las Treg proliferativas, como las subpoblaciones de maduración CD4 y CD8 proliferativas, correlacionaban inversamente con la respuesta a la vacuna frente a influenza.

Respecto al posible efecto inmunomodulador de un TARc que incluye MVC sobre la inflamación en la cohorte de sujetos con infección por VIH, no observamos un efecto directo de dicho tratamiento sobre el marcador de inflamación usPCR. Sin embargo, la usPCR, como hemos comentado previamente, se asoció inversamente con la frecuencia de RTE y directamente con la frecuencia de células T CD4 activadas, variables que sí se vieron impactadas por el TARc con MVC. Esto sugiere un posible efecto indirecto del TARc con MVC en el estado inflamatorio. Además, no podemos descartar que el TARc con MVC ejerza su efecto en otras citoquinas inflamatorias, como ya ha sido descrito [93].

Curiosamente, tampoco observamos una asociación directa entre estar bajo un TARc incluyendo MVC y una reducción de Treg. Esta aparente discrepancia con nuestro trabajo previo podría explicarse en base a las notables diferencias en las cohortes estudiadas, sobre todo en relación con el hecho de ser pacientes no tratados previamente con antirretrovirales, pero también en cuanto al periodo de infección calculado desde la fecha del diagnóstico [94]. Sin embargo, el tiempo de exposición a dicho TARc con MVC sí se asoció con menores frecuencias de Treg. Lo que indica que, probablemente en esta cohorte, al haber experimentado los pacientes una mayor progresión clínica, sea necesaria una prolongada exposición a MVC para que su efecto reductor sobre la frecuencia delas Treg sea patente. Creemos, por tanto, que nuestros resultados refuerzan la hipótesis de que el efecto inmunomodulador que ejerce MVC sobre las Treg puede ser otro mecanismo por el cual MVC mejora la respuesta vacunal.

En resumen, el efecto beneficioso de un TARc que incluye MVC en la respuesta a la vacuna en sujetos VIH menores de 50 años, se debe en parte a la reducción que ejerce sobre la proliferación y la activación de las células T CD4, al efecto reductor de la frecuencia de células Treg, incluso a su potencial regeneración del sistema inmunitario y todo ello además parece impactar positivamente en el grado de inflamación. Por tanto, MVC, gracias a su efecto inmunomodulador, mejora diferentes funciones del sistema inmunológico necesarias para ejercer una respuesta adecuada tras la vacunación frente a VHB.

REFLEXIÓN FINAL

La originalidad de esta Tesis Doctoral recae en que es la primera vez que se asocia la respuesta vacunal con alteraciones homeostáticas proliferativas propias de la inmunosenescencia. Además, hemos encontrado asociaciones interesantes entre la función tímica, la proliferación homeostática, la inflamación y la respuesta vacunal, en las que sería interesante profundizar en el futuro, puesto que una mejor comprensión de estas podría ayudar a entender mejor cómo la inmunosenescencia limita las capacidades del sistema inmunitario de manera global. Además, ayudaría a encontrar mejores estrategias para mejorar la respuesta vacunal en estas poblaciones inmunosenescentes. En este sentido, los resultados obtenidos en los sujetos infectados por VIH son de gran relevancia clínica y terapéutica y nos llevan a recomendar que sea considerada la prescripción de un régimen antirretroviral incluyendo MVC en los protocolos de vacunación frente a VHB.

Asimismo, dado que la pérdida de la función tímica asociada al envejecimiento podría desempeñar un papel en el origen de la inflamación y de las alteraciones proliferativas de células T, pensamos que alternativas terapéuticas encaminadas a mejorar la función tímica también podrían tener repercusión en mejorar la respuesta vacunal en estos escenarios. Por ello, sería interesante desarrollar ensayos clínicos en este contexto, como los realizados con la hormona del crecimiento en sujetos infectados por VIH, en los que se muestra una mejora de la respuesta inmunitaria frente al VIH, debido a su efecto beneficioso sobre la función tímica [120,121].

7. CONCLUSIONES

CONCLUSIONES

1. Las alteraciones homeostáticas proliferativas que se dan en las subpoblaciones de maduración de las células T CD4 y CD8, incluyendo las Treg, asociadas al fenómeno de inmunosenescencia, repercuten en una menor respuesta a la vacuna frente a influenza en ancianos. Estas alteraciones homeostáticas podrían estar íntimamente relacionadas con el aumento de inflamación sistémica y con la disminución de la función tímica que se producen durante el envejecimiento.

2. Un tratamiento antirretroviral incluyendo Maraviroc se asocia con una mayor magnitud de la respuesta a la vacuna frente a Virus de la Hepatitis B en sujetos con infección por VIH menores de 50 años. Por tanto, un régimen conteniendo este fármaco durante el protocolo de inmunización podría ser una estrategia terapéutica adecuada para mejorar la respuesta vacunal en estos sujetos.

3. Los pacientes tratados con un tratamiento antirretroviral incluyendo Maraviroc muestran un perfil inmunológico menos activado, con mayor contribución de las células T recientemente emigradas del timo y una menor frecuencia de células T reguladoras, perfil que se asocia con una mejor respuesta a la vacuna frente al Virus de la Hepatitis B. Así, Maraviroc podría tener un efecto regenerador sobre el sistema inmunitario, promoviendo la función tímica y disminuyendo la proliferación homeostática compensatoria, lo que puede repercutir también en un menor estado proinflamatorio.

8. REFERENCIAS

REFERENCIAS

1. United Nations. Department of Economic and Social Affairs.

[http://www.un.org/en/development/desa/population/theme/ageing/index.shtml]

2. Gruver AL, Hudson LL, Sempowski GD. Immunosenescence of ageing. J Pathol. 2007; 211:144–56.

3. Pfister G, Savino W. Can the immune system still be efficient in the elderly? An immunological and immunoendocrine therapeutic perspective. Neuroimmunomodulation. 2008; 15:351–64.

4. Shaw AC, Goldstein DR, Montgomery RR. Age-dependent dysregulation of innate immunity. Nat Rev Immunol. 2013; 13:875–87.

5. Frasca D, Blomberg BB. Aging affects human B cell responses. J Clin Immunol. 2011; 31:430–5.

6. Czesnikiewicz-Guzik M, Lee WW, Cui D, Hiruma Y, Lamar DL, Yang ZZ, Ouslander JG, Weyand CM, Goronzy JJ.. T cell subset-specific susceptibility to aging. Clin Immunol. 2008; 127:107–18.

7. Haynes BF, Markert ML, Sempowski GD, Patel DD, Hale LP. The role of the thymus in immune reconstitution in aging, bone marrow transplantation, and HIV-1 infection. Ann Rev Immunol. 2000; 18:529–560.

8. Pawelec G, Barnett Y, Forsey R, Frasca D, Globerson A, McLeod J, Caruso C, Franceschi C, Fülöp T, Gupta S, Mariani E, Mocchegiani E, Solana R. T cells and ageing, January 2002 update. Front Biosci. 7:d1056–d1183.

9. Hakim FT, Memon SA, Cepeda R, Jones EC, Chow CK, Kasten-Sportes C, Odom J, Vance BA, Christensen BL, Mackall CL, Gress RE. Age-dependent incidence, time course, and consequences of thymic renewal in adults. J Clin Invest. 2005; 115:930-939.

10. Ferrando-Martínez S, Ruiz-Mateos E, Hernández A, Gutiérrez E, Rodríguez-Méndez MM, Ordoñez A, Leal M. Age-related deregulation of naive T cell homeostasis in elderly humans. AGE. 2011; 33:197–207.

11. Sauce D, Larsen M, Fastenackels S, Roux A, Gorochov G, Katlama C, Sidi D, Sibony-Prat J, Appay V. Lymphopenia-driven homeostatic regulation of naive T cells in elderly and thymectomized young adults. J Immunol. 2012; 189:5541-5548.

12. Gregg R, Smith CM, Clark FJ, Dunnion D, Khan N, Chakraverty R, Nayak L, Moss PA. The number of human peripheral blood CD4+ CD25high regulatory T cells increases with age. Clin Exp Immunol. 2005; 140:540–546.

13. Vukmanovic-Stejic M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, Masters JE, Rustin MH, Taams LS, Beverley PC, Macallan DC, Akbar AN. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. J Clin Invest. 2006; 116:2423–2433.

14. Lages CS, Suffia I, Velilla PA, Huang B, Warshaw G, Hildeman DA, Belkaid Y, Chougnet C. Functional regulatory T cells accumulate in aged hosts and promote chronic infectious disease reactivation. J Immunol. 2008; 181:1835–1848.

15. Raynor J, Lages CS, Shehata H, Hildeman DA, Chougnet CA. Homeostasis and function of regulatory T cells in aging. Curr Opin Immunol. 2012; 24:482-487.

16. Fessler J, Ficjan A, Duftner C, Dejaco C. The impact of aging on regulatory T-cells. Front Immunol. 2013; 4:231.

17. Vianna PH, Canto FB, Nogueira JS, Nunes CF, Bonomo AC, Fucs R. Critical influence of the thymus on peripheral T cell homeostasis. Immun Inflamm Dis. 2016; 4:474-486.

18. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol. 2004; 22:531-62.

19. Jiang H, Chess L. Regulation of immune responses by T cells. N Engl J Med. 2006; 354:1166-76.

20. Pawelec G, Derhovanessian E. Role of CMV in immune senescence. Virus Res. 2011; 157:175–179.

21. Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, De Benedictis G. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci. 2000; 908:244-254.

22. Singh T, Newman AB. Inflammatory markers in population studies of aging. Ageing Res Rev 2011; 10:319-29.

23. Walston J, McBurnie MA, Newman A, Tracy RP, Kop WJ, Hirsch CH, Gottdiener J, Fried LP; Cardiovascular Health Study. Frailty and activation of the inflammation and coagulation systems with and without clinical comorbidities: results from the Cardiovascular Health Study. Arch. Intern. Med. 2002; 162:2333–41.

24. Cesari M, Pennix BW, Newman AB, Kritchevsky SB, Nicklas BJ, Sutton-Tyrrell K, Rubin SM, Ding J, Simonsick EM, Harris TB, Pahor M. Inflammatory markers and onset of cardiovascular events: results from the Health ABC study. Circulation. 2003; 108:2317–22.

25. Shlipak MG, Fried LF, Crump C, Bleyer AJ, Manolio TA, Tracy RP, Furberg CD, Psaty BM. Elevations of inflammatory and procoagulant biomarkers in elderly persons with renal insufficiency. Circulation. 2003; 107:87–92.

26. Wikby A, Ferguson F, Forsey R, Thompson J, Strindhall J, Löfgren S, Nilsson BO, Ernerudh J, Pawelec G, Johansson B. An immune risk phenotype, cognitive impairment, and survival in very late life: impact of allostatic load in Swedish octogenarian and nonagenarian humans. J Gerontol A Biol Sci Med Sci. 2005; 60:556-65.

27. Aiello AE, Haan M, Blythe L, Moore K, Gonzalez JM, Jagust W. The influence of latent viral infection on rate of cognitive decline over 4 years. J Am Geriatr Soc. 2006; 54:1046-54.

28. Holmes C, Cunningham C, Zotova, Woolford J, Dean C, Kerr S, Culliford D, Perry VH. Systemic inflammation and disease progression in Alzheimer disease. Neurology. 2010; 73:768-74.

29. Roberts ET, Hann MN, Dowd JB, Allison EA. Cytomegalovirus antibody levels, inflammation, and mortality among elderly Latinos over 9 years of follow-up. Am J Epidemiol. 2010; 172:363-71.

30. Stowe RP, Kozlova EV, Yetman DL, Walling DM, Goodwin JS, Glaser R. Chronic herpesvirus reactivation occurs in aging. Exp Gerontol. 2007; 42:563-70.

31. Goronzy JJ, Weyand CM. Understanding immunosenescence to improve responses to vaccines. Nat Immunol. 2013; 14:428-436.

32. Pinti M, Appay V, Campisi J, Frasca D, Fülöp T, Sauce D, Larbi A, Weinberger B, Cossarizza A. Aging of the immune system – focus on inflammation and vaccination. Eur J Immunol. 2016; 46:2286-2301.

33. Gasparini R, Amicizia D, Lai PL, Panatto D. Clinical and socioeconomic impact of seasonal and pandemic influenza in adults and the elderly. Hum Vaccin Immunother. 2012; 8:21-28.

34. World Health Organization: Influenza seasonal.

[http://www.who.int/mediacentre/factsheets/fs211/en/]

35. Iuliano AD, Roguski KM, Chang HH, Muscatello DJ, Palekar R, Tempia S, Cohen C, Gran JM, Schanzer D, Cowling BJ, Wu P, Kyncl J, Ang LW, Park M, Redlberger-Fritz M, Yu H, Espenhain L, Krishnan A, Emukule G, van Asten L, Pereira da Silva S, Aungkulanon S, Buchholz U, Widdowson MA, Bresee JS; Global Seasonal Influenza-associated Mortality Collaborator Network.Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. Lancet. 2018; 391:1285-1300.

36. Goodwin K, Viboud C, Simonsen L. Antibody response to influenza vaccination in the elderly: a quantitative review. Vaccine. 2006; 24:1159-1169.

37. Haq K, McElhaney JE. Immunosenescence: influenza vaccination and the elderly. Curr Opin Immunol. 2014; 29C:38-42.

38. Panda A, Qian F, Mohanty S, van Duin D, Newman FK, Zhang L, Chen S, Towle V, Belshe RB, Fikrig E, Allore HG, Montgomery RR, Shaw AC. Age-associated decrease in TLR function in primary human dendritic cells predicts influenza vaccine response. J Immunol. 2010; 184:2518-2527.

39. Weng NP. Telomere and adaptive immunity. Mech Ageing Dev. 2008; 129:60–6.

40. Najarro K, Nguyen H, Chen G, Xu M, Alcorta S, Yao X, Zukley L, Metter EJ, Truong T, Lin Y, Li H, Oelke M, Xu X, Ling SM, Longo DL, Schneck J, Leng S, Ferrucci L, Weng NP. Telomere length as an indicator of the robustness of B- and T-cell response to influenza in older adults. J Infect Dis. 2015; 212:1261-1269.

41. Frasca D, Blomberg BB. B cell function and influenza vaccine responses in healthy aging and disease. Curr Opin Immunol. 2014; 29C:112–118.

42. Saurwein-Teissl M, Lung TL, Marx F, Gschösser C, Asch E, Blasko I, Parson W, Böck G, Schönitzer D, Trannoy E, Grubeck-Loebenstein B. Lack of antibody production following immunization in old age: association with CD8(+)CD28(-) T-cell clonal expansions and an imbalance in the production of Th1 and Th2 cytokines. J Immunol. 2002; 168:5893-5899.

43. Trzonkowski P, Myśliwska J, Szmit E, Wieckiewicz J, Lukaszuk K, Brydak LB, Machała M, Myśliwski A. Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination--an impact of immunosenescence. Vaccine. 2003; 21:3826-3836.

44. Parmigiani A, Alcaide ML, Freguja R, Pallikkuth S, Frasca D, Fischl MA, Pahwa S. Impaired Antibody Response to Influenza Vaccine in HIV-Infected and Uninfected Aging Women Is Associated with Immune Activation and Inflammation. PloS one. 2013; 8:e79816.

45. Pinti M, Appay V, Campisi J, Frasca D, Fülöp T, Sauce D, Larbi A, Weinberger B, Cossarizza A. Aging of the immune system – focus on inflammation and vaccination. Eur J Immunol. 2016; 46:2286-2301.

46. Plitas G, Rudensky AY. Regulatory T Cells: Differentiation and Function. Cancer Immunol Res. 2016; 4:721-725.

47. Lim HW, Hillsamer P, Kim CH. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. J Clin Invest. 2004; 114:1640-1649.

48. Toka F, Suvas S, Rouse BT. CD4+ CD25+ T cells regulate vaccine-generated primary and memory CD8+ T-cell responses against herpes simplex virus type 1. J Virol. 2004; 78:13082-13089.

49. Espinoza-Mora MR, Steeg C, Tartz, S, Heussler V, Sparwasser T, Link A, Fleischer B, Jacobs T. Depletion of regulatory T cells augments a vaccine-induced T effector cell response against the liver-stage of malaria but fails to increase memory. PLoS One. 2014; 12;9:e104627.

50. YangZ, WangL, NiuW, WuY, ZhangJ, Meng G. Increased CD4+CD25+FoxP3+regulatory T cells in the blood of nonresponders after standard hepatitis B surface antigen vaccine immunization. Clin Immunol. 2008; 127:265-266.

51. Pozo-Balado MM, Leal M, Méndez-Lagares G, Mata RC, López-Cortés LF, Viciana P, Pacheco YM. Increased regulatory T cell counts in HIV-infected nonresponders to hepatitis B virus vaccine. J Infect Dis. 2010; 202:362-369.

52. Wang SM, Tsai MH, Lei HY, Wang JR, Liu CC. The regulatory T cells in anti-influenza antibody response post influenza vaccination. Hum Vaccine Immunother. 2012; 8:1243–1249.

53. Lin PH, Wong WI, Wang YL, Hsieh MP, Lu CW, Liang CY, Jui SH, Wu FY, Chen PJ, Yang HC. Vaccine-induced antigen-specific regulatory T cells attenuate the antiviral immunity against acute influenza virus infection. Mucosal Immunol. 2018; 11:1239-1253.

54. Franceschi C, Capri M, Monti D, Giunta S, Olivieri F, Sevini F, Panourgia MP, Invidia L, Celani L, Scurti M, Cevenini E, Castellani GC, Salvioli S. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. Mech Ageing Dev. 2007; 128:92-105.

55. Almeida JR, Price DA, Papagano L, Arkoub ZA, Sauce D, Bornstein E, Asher TE, Samri A, Schnuriger A, Theodorou I, Costagliola D, Rouzioux C, Agut H, Marcelin AG, Douek D, Autran B, Appay V. Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. J Exp Med. 2007; 204:2473-85.

56. Ferrando-Martínez S, Ruiz-Mateos E, Romero-Sánchez MC, Muñoz-Fernández MÁ, Viciana P, Genebat M, Leal M. HIV infection-related premature immunosenescence: high rates of immune exhaustion after short time of infection. Curr HIV Res. 2011; 9:289-294.

57. De Pablo-Bernal RS, Ramos R, Genebat M, Cañizares J, Rafii-El-Idrissi Benhnia M, Muñoz-Fernández MA, Pacheco YM, Galvá MI, Leal M, Ruiz-Mateos E. Phenotype and Polyfunctional Deregulation Involving Interleukin 6 (IL-6)- and IL-10-Producing Monocytes in HIV-Infected Patients Receiving Combination Antiretroviral Therapy Differ From Those in Healthy Older Individuals. J Infect Dis. 2016; 213:999-1007.

58. De Pablo-Bernal RS, Ruiz-Mateos E, Rosado I, Dominguez-Molina B, Alvarez-Ríos AI, Carrillo-Vico A, De La Rosa R, Delgado J, Muñoz-Fernández MA, Leal M, Ferrando-Martínez S. TNF-α levels in HIV-infected patients after long-term suppressive cART persist as high as in elderly, HIV-uninfected subjects. J Antimicrob Chemother. 2014; 69:3041-6.

59. Deeks SG. HIV infection, inflammation, immunosenescence, and aging. Annu. Rev. Med. 2011; 62:141-55.

60. Haissman JM, Vestergaard LS, Sembuche S, Erikstrup C, Mmbando B, Mtullu S, Lemnge MM, Gerstoft J, Ullum H. Plasma cytokine levels in Tanzanian HIV-1- infected adults and the effect of antiretroviral treatment. J Acquir Immune Defic Syndr. 2009; 52:493-7.

61. Kuller LH, Tracy R, Belloso W, De Wit S, Drummond F, Lane HC, Ledergerber B, Lundgren J, Neuhaus J, Nixon D, Paton NI, Neaton JD; INSIGHT SMART Study Group. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. PlosMed. 2008; 5:e203.

62. Wang H1, Kotler DP. HIV enteropathy and aging: gastrointestinal immunity, mucosal epithelial barrier, and microbial translocation. Curr Opin HIV AIDS. 2014; 9:309-16.

63. Jiang W, Younes SA, Funderburg NT, Mudd JC, Espinosa E, Davenport MP, Babineau DC, Sieg SF, Lederman MM. Cycling memory CD4+ T cells in HIV disease have a diverse T cell receptor repertoire and a phenotype consistent with bystander activation. J Virol. 2014; 88:5369-5380.

64. Chevalier MF, Weiss L. The split personality of regulatory T cells in HIV infection. Blood. 2013; 519 121:29-37.

65. Guihot A, Bourgarit A, Carcelain G, Autran B. Immune reconstitution after a decade of combined antiretroviral therapies for human immunodeficiency virus. Trends Immunol. 2011; 32:131-7.

66. Brodt HR, Kamps BS, Gute P, Knupp B, Staszewski S, Helm EB. Changing incidence of AIDS-defining illnesses in the era of antiretroviral combination therapy. AIDS. 1997; 15:1731-8.

67. Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD. Declining Morbidity and Mortality among Patients with Advanced Human Immunodeficiency Virus Infection. N Engl J Med. 1998; 339:405-6.

68. Guaraldi G, Orlando G, Zona S, Menozzi M, Carli F, Garlassi E, Berti A, Rossi E, Roverato A, Palella F. Premature Age-Related Comorbidities Among HIV-Infected Persons Compared With the General Population. Clin Infect Dis. 2011; 53:1120-6.

69. Deeks SG, Lewin SR, Havlir DV. The End of AIDS: HIV Infection as a Chronic Disease. Lancet. 2013; 382:1525-1533.

70. Mocroft A, Brettle R, Kirk O, Blaxhult A, Parkin JM, Antunes F, Francioli P, D'Arminio Monforte A, Fox Z, Lundgren JD; EuroSIDA study group. Changes in the cause of death among HIV positive subjects across Europe: results from the EuroSIDA study. AIDS. 2002; 16:1663-71.

71. Thio CL. Hepatitis B in the human immunodeficiency virus-infected patient: epidemiology, natural history, and treatment. Semin Liver Dis. 2003; 23:125-36.

72. Alejos B, Hernando V, Iribarren J, Gonzalez-García J, Hernando A, Santos J, Asensi V, Gomez-Berrocal A, Del Amo J, Jarrin I; CoRIS (Cohort of the Spanish Network on HIVAIDS Research). Overall and cause-specific excess mortality in HIV-positive persons compared with the general population: Role of HCV coinfection. Medicine (Baltimore). 2016; 95:e4727.

73. Soto B, Sanchez-Quijano A, Rodrigo L, del Olmo JA, Garcia-Bengoechea M, Hernandez-Quero J, Rey C, Abad MA, Rodriguez M, Sales Gilabert M, Gonzalez F, Miron P, Caruz A, Relimpio F, Torronteras R, Leal M, Lissen E. Human immunodeficiency virus infection modifies the natural history of chronic parenterally-acquired hepatitis C with an unusually rapid progression to cirrhosis. J Hepatol. 1997; 26:1-5.

74. Thio CL, Seaberg EC, Skolasky R, Jr, Phair J, Visscher B, Munoz A, Thomas DL, Multicenter AIDS Cohort Study. HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicenter Cohort Study (MACS). Lancet. 2002; 360:1921-1926.

75. Whitaker JA, Rouphael NG, Edupuganti S, Lai L, Mulligan MJ. Strategies to increase responsiveness to hepatitis B vaccination in adults with HIV-1. Lancet Infect Dis. 2012; 12:966-976.

76. Kerneis S, Launay O, Turbelin C, Batteux F, Hanslik T, Boelle PY. Long-term immune responses to vaccination in HIV-infected patients: a systematic review and meta-analysis. Clin Infect Dis. 2014; 58:1130 –1139.

77. Filion LG, Saginur R, Szczerbak N. Humoral and cellular immune responses by normal individuals to hepatitis B surface antigen vaccination. J Exp Immunol. 1988; 71:405-409.

78. Egea E, Iglesias A, Salazar M, Morimoto C, Kruskall MS, Awdeh Z, Schlossman SF, Alper CA, Yunis EJ. The cellular basis for lack of antibody response to hepatitis B vaccine in humans. J Exp Med. 1991; 173:531-538.

79. Yao ZQ, Moorman JP. Immune Exhaustion and Immune Senescence: Two Distinct Pathways for HBV Vaccine Failure During HCV and/or HIV Infection. Arch Immunol Ther Exp. 2013; 61:193-201.

80. Fonseca MO, Pang LW, de Paula Cavalheiro N, Barone AA, Heloisa Lopes M. Randomized trial of recombinant hepatitis B vaccine in HIVinfected adult patients comparing a standard dose to a double dose. Vaccine. 2005; 23:2902–2908.

81. Launay O, van der Vliet D, Rosenberg AR, Michel ML, Piroth L, Rey D, Colin de Verdiere N, Slama L, Martin K, Lortholary O, Carrat F, ANRS HB03 VIHVAC-B Trial. Safety and immunogenicity of 4 intramuscular double doses and 4 intradermal low doses vs standard hepatitis B vaccine regimen in adults with HIV-1: a randomized controlled trial. JAMA. 2011; 305:1–9.

82. Chaiklang K, Wipasa J, Chaiwarith R, Praparattanapan J, Supparatpinyo K. Comparison of immunogenicity and safety of four doses and four double doses vs. standard doses of hepatitis B vaccination in HIVinfected adults: a randomized, controlled trial. PLoS One. 2013; 12:e80409.

83. Vrisekoop N, van Gent R, de Boer AB, Otto SA, Borleffs JC, Steingrover R, Prins JM, Kuijpers TW, Wolfs TF, Geelen SP, Vulto I, Lansdorp P, Tesselaar K, Borghans JA, Miedema F. Restoration of the CD4 T cell compartment after long-term highly active antiretroviral therapy without phenotypical signs of accelerated immunological aging. J Immunol. 2008; 181:1573-1581.

84. Overton ET, Sungkanuparph S, Powderly WG, Seyfried W, Groger RK, Aberg JA. Undetectable plasma HIV RNA load predicts success after hepatitis B vaccination in HIV-infected persons. Clin Infect Dis. 2005; 41:1045-1048.

85. Berger EA, Murphy PM, Farber JM. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism and disease. Annu Rev Immunol. 1999; 17:657-700.

86. Hammer SM, Eron JJ Jr, Reiss P, Schooley RT, Thompson MA, Walmsley S, Cahn P, Fischl MA, Gatell JM, Hirsch MS, Jacobsen DM, Montaner JS, Richman DD, Yeni PG, Volberding PA; InternationalAIDS Society-USA. Antiretroviral treatment of adult HIV infection: 2008 recommendations of the International AIDS Society-USA panel. JAMA. 2008; 300:555-70.

87. Hardy WD, Gulick RM, Mayer H, Fätkenheuer G, Nelson M, Heera J, Rajicic N, Goodrich J. Two-year safety and virologic efficacy of maraviroc in treatment-experienced patients with CCR5-tropic HIV-1 infection: 96-week combined analysis of MOTIVATE 1 and 2. J Acquir Immune Defic Syndr. 2010;

88. Lederman MM, Penn-Nicholson A, Cho M, Mosier D. Biology of CCR5 and its role in HIV infection and treatment. JAMA. 2006; 296:815-26.

89. Saag M, Goodrich J, Fätkenheuer G, Clotet B, Clumeck N, Sullivan J, Westby M, van der Ryst E, Mayer H; A4001029 Study Group. A double-blind, placebo-controlled trial of maraviroc in treatment-experienced patients infected with non-R5 HIV-1; A4001029 Study Group. J Infect Dis. 2009; 199:1638-47.

90. Asmuth DM, Goodrich J, Cooper DA, Haubrich R, Rajicic N, Hirschel B, Mayer H, Valdez H. CD4+ T-cell restoration after 48 weeks in the maraviroc treatment-experienced trials MOTIVATE 1 and 2. J Acquir Immune Defic Syndr. 2010; 54:394-7.

91. Funderburg N, Kalinowska M, Eason J, Goodrich J, Heera J, Mayer H, Rajicic N, Valdez H, Lederman MM. Effects of maraviroc and efavirenz on markers of immune activation and inflammation and associations with CD4+ cell rises in HIV-infected patients. PLoS One. 2010; 5:e13188.

92. Romero-Sánchez MC, Machmach K, Gonzalez-Serna A, Genebat M, Pulido I, García-García M, Alvarez-Ríos AI, Ferrando-Martinez S, Ruiz-Mateos E, Leal M. Effect of maraviroc on HIV disease progression-related biomarkers. Antimicrob Agents Chemother. 2012; 56:5858-64.

93. Romero-Sanchez MC, Alvarez-Rios AI, Bernal-Morell E, Genebat M, Vera F, Benhnia MR, Bravo-Urbieta J, Galera-Penaranda C, de Pablo-Bernal RS, Abad-Carrillo MA, Leal M, Ruiz-Mateos E, VIH-GEMUVIH Region de Murcia Study Group. Maintenance of virologic efficacy and decrease in levels of b2-microglobulin, soluble CD40L and soluble CD14 after switching

previously treated HIV-infected patients to an NRTI-sparing dual therapy. Antiviral Res. 2014; 111:26-32.

94. Pozo-Balado MM, Martinez-Bonet M, Rosado I, Ruiz-Mateos E, Mendez-Lagares G, Rodriguez-Mendez MM, Vidal F, Munoz-Fernandez MA, Pacheco YM, Leal M. Maraviroc reduces the regulatory T-cell frequency in antiretroviral-naive HIV-infected subjects. J Infect Dis. 2014.; 210:890-898.

95. Pozo Balado MM, Leal M, Mendez Lagares G, Mata RC, Lopez-Cortes LF, Viciana P, Pacheco YM. Increased regulatory T cell counts in HIV-infected nonresponders to hepatitis B virus vaccine. J Infect Dis. 2010; 202:362-369.

96. Pozo-Balado MM, Rosado-Sánchez I, Méndez-Lagares G, Rodríguez-Méndez MM, Ruiz-Mateos E, Benhnia MR, Muñoz-Fernández MA, Leal M, Pacheco YM. Maraviroc contributes to the restoration of the homeostasis of regulatory T-cell subsets in antiretroviral-naive HIV-infected subjects. Clin Microbiol Infect. 2016; 22:461.e1-5.

97. Westrop SJ, Moyle G, Jackson A, Nelson M, Mandalia S, Imami N. CCR5 antagonism impacts vaccination response and immune profile in HIV-1 infection. Mol Med. 2012; 18:1240-8.

98. Egea E, Iglesias A, Salazar M, Morimoto C, Kruskall MS, Awdeh Z Schlossman SF, Alper CA, Yunis EJ. The cellular basis for lack of antibody response to hepatitis B vaccine in humans. J Exp Med. 1991; 173:531-538.

99. Filion LG, Saginur R, Szczerbak N. Humoral and cellular immune responses by normal individuals to hepatitis B surface antigen vaccination. J Exp Immunol. 1988; 71:405-409.

100. Ferrando-Martínez S, Romero-Sánchez MC, Solana R, Delgado J, de la Rosa R, Muñoz-Fernández MA, Ruiz-Mateos E, Leal M. Thymic function failure and C-reactive protein levels are independent predictors of all-cause mortality in healthy elderly humans. AGE. 2013; 35:251-259.

101. Coder BD, Wang H, Ruan L, Su DM. Thymic involution perturbs negative selection leading to autoreactive T cells that induce chronic inflammation. J Immunol. 2015; 194:5825-5837.

102. Frasca D, Diaz A, Romero M, Landin AM, Blomberg BB. Cytomegalovirus (CMV) seropositivity decreases B cell responses to the influenza vaccine. Vaccine. 2015; 33:1433-1439.

103. Merani S, Pawelec G, Kuchel GA, McElhaney JE. Impact of Aging and Cytomegalovirus on Immunological Response to Influenza Vaccination and Infection. Front Immunol. 2017; 8:784.

104. Shive CL, Judge CJ, Clagett B, Kalayjian RC, Osborn M, Sherman KE, Fichtenbaum C, Gandhi RT, Kang M, Popkin DL, Sieg SF, Lederman MM, Rodriguez B, Anthony DD. Pre-vaccine plasma levels of soluble inflammatory indices negatively predict responses to HAV, HBV, and tetanus vaccines in HCV and HIV infection. Vaccine. 2018; 36:453-60.

105. Verschoor CP, Lelic A, Parsons R, Evelegh C, Bramson JL, Johnstone J, Loeb MB, Bowdish DME. Serum C-reactive protein and congestive heart failure as significant predictors of herpes zoster vaccine response in elderly nursing home residents. J Infect Dis. 2017; 216:191–7.

106. Rosado-Sánchez I, Herrero-Fernández I, Genebat M, Ruiz-Mateos E, Leal M, Pacheco YM. Thymic Function Impacts the Peripheral CD4/CD8 Ratio of HIV-Infected Subjects. Clin Infect Dis. 2017; 64:152-158.

107. Ferrando-Martinez S, De Pablo-Bernal RS, De Luna-Romero M, De Ory SJ, Genebat M, Pacheco YM, Parras FJ, Montero M, Blanco JR, Gutierrez F, Santos J, Vidal F, Koup RA, Muñoz-Fernández MÁ, Leal M, Ruiz-Mateos E. Thymic Function Failure Is Associated With Human Immunodeficiency Virus Disease Progression. Clin Infect Dis. 2017; 64:1191-1197.

108. van der Geest KS, Abdulahad WH, Tete SM, Lorencetti PG, Horst G, Bos NA Kroesen BJ, Brouwer E, Boots AM. Aging disturbs the balance between effector and regulatory CD4+ T cells. Exp Gerontol. 2014;60:190-196.

109. Curotto De Lafaille MA, Lino AC, Kutchukhidze N, Lafaille JJ. CD25- T cells generate CD25+Foxp3+regulatory T cells by peripheral expansion. J Immunol. 2004; 173:7259-68.

110. Rosado-Sánchez I, González-Magaña A, Pozo-Balado MM, Herrero-Fernández I, Polaino MJ, Rodríguez-Méndez MM, González-Escribano MF, Leal M, Pacheco YM. An in vitro system of autologous lymphocytes culture that allows the study of homeostatic proliferation mechanisms in human naive CD4 T-cells. Lab Invest. 2018; 98:500-511.

111. Reshef R, Luger SM, Hexner EO, Loren AW, Frey NV, Nasta SD, Goldstein SC, Stadtmauer EA, Smith J, Bailey S, Mick R, Heitjan DF, Emerson SG, Hoxie JA, Vonderheide RH, Porter DL. Blockade of lymphocyte chemotaxis in visceral graft-versus-host disease. N Engl J Med. 2012; 367:135-145.

112. Moy RH, Huffman AP, Richman LP, Crisalli L, Wang XK, Hoxie JA, Mick R, Emerson SG, Zhang Y, Vonderheide RH, Porter DL, Reshef R. Clinical and immunologic impact of CCR5 blockade in graft-versus-host disease prophylaxis. Blood. 2017; 129:906-916.

113. Zi J, Yuan S, Qiao J, Zhao K, Xu L, Qi K, Xu K, Zeng L. Treatment with the C-C chemokine receptor type 5 (CCR5)-inhibitor maraviroc suppresses growth and induces apoptosis of acute lymphoblastic leukemia cells. Am J Cancer Res. 2017; 7:869-880.

114. Serrano-Villar S, Sainz T, Ma ZM, Utay NS, Wook-Chun T, Mann S, Kashuba AD, Siewe B, Albanese A, Troia-Cancio P, Sinclair E, Somasunderam A, Yotter T, Deeks SG, Landay A, Pollard RB, Miller CJ, Moreno S, Asmuth DM. Effects of combined CCR5/integrase inhibitors-based regimen on mucosal immunity in HIV-infected patients naive to antiretroviral therapy: a pilot randomized trial. PLoS Pathog. 2016; 12:e1005540.

115. Taiwo BO, Chan ES, Fichtenbaum CJ, Ribaudo H, Tsibris A, Klingman KL, Eron JJ, Berzins B, Robertson K, Landay A, Ofotokun I, Brown T, AIDS Clinical Trials Group A5303 Study Team. Less bone loss with maraviroc-versus tenofovir containing antiretroviral therapy in the AIDS Clinical Trials Group A5303 Study. Clin Infect Dis. 2015; 61:1179-1188.

116. Wilson CM, Ellenberg JH, Sawyer MK, Belzer M, Crowley-Nowick PA, Puga A, Futterman DC, Peralta L; Adolescent Medicine HIV/AIDS Research Network. Adolescent Medicine HIV/AIDS Research Network. Serologic response to hepatitis B vaccine in HIV infected and high-risk HIV uninfected adolescents in the REACH cohort. Reaching for excellence in adolescent care and health. J Adolesc Health. 2001; 29:123-9.

117. Veiga AP, Casseb J, Duarte AJ. Humoral response to hepatitis B vaccination and its relationship with T CD45RA+ (naive) and CD45RO+ (memory) subsets in HIV-1-infected subjects. Vaccine. 2006; 4:7124–8.

118. Anthony DD, Umbleja T, Aberg JA, Kang M, Medvik K, Lederman MM, Peters MG, Koziel MJ, Overton ET. Lower peripheral blood CD14+ monocyte frequency and higher CD34+ progenitor cell frequency are associated with HBV vaccine induced response in HIV infected individuals. Vaccine. 2011; 29:3558–63.

119. Kohler S, Thiel A. Life after the thymus: CD31+ and CD31− human naïve CD4+ T-cell subsets. Blood. 2009; 113:769-74.

120. Plana M, Garcia F, Darwich L, Romeu J, López A, Cabrera C, Massanella M, Canto E, Ruiz-Hernandez R, Blanco J, Sánchez M, Gatell JM, Clotet B, Ruiz L, Bofill M; Red de Investigación en Sida (RIS). The reconstitution of the thymus in immunosuppressed individuals restores CD4-specific cellular and humoral immune responses. Immunology. 2011; 133(3):318-28.

121. Herasimtschuk AA, Hansen BR, Langkilde A, Moyle GJ, Andersen O, Imami N. Low-dose growth hormone for 40 weeks induces HIV-1-specific T cell responses in patients on effective combination anti-retroviral therapy. Clin Exp Immunol. 2013; 173:444-53.

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10. OTRAS PUBLICACIONES

OTRAS PUBLICACIONES

- Rosado-Sánchez I, Herrero-Fernández I, Sobrino S, Carvajal AE, Genebat M, Tarancón-Díez L, Fontillón-Alberdi M, de Pablo RM, Muñoz-Fernández MA, Leal M, Pacheco YM. **Thymic function impacts the colonic mucosal integrity of successfully-treated HIV-infected subjects.** (En revisión)

- Rosado-Sánchez I, Herrero-Fernández I, Genebat M, Del Romero J, Riera M, Podzamczer D, Olalla J, Vidal F, Muñoz-Fernández MA, Leal M, Pacheco YM. **HIV-Infected Subjects With Poor CD4 T-Cell Recovery Despite Effective Therapy Express High Levels of OX40 and α4β7 on CD4 T-Cells Prior Therapy Initiation.** Front Immunol. 2018; 9:1673.

- Rosado-Sánchez I, González-Magaña A, Pozo-Balado MM, Herrero-Fernández I, Polaino MJ, Rodríguez-Méndez MM, González-Escribano MF, Leal M, Pacheco YM**. An in vitro system of autologous lymphocytes culture that allows the study of homeostatic proliferation mechanisms in human naive CD4 T-cells.** Lab Invest. 2018; 98:500-511.

- Rosado-Sánchez I, Herrero-Fernández I, Tarancon-Diez L, Moreno S, Iribarren JA, Dalmau D, Vera-Méndez F, Leal M, Pacheco YM. **Increased frequencies of Th17 cells and IL17a-producing regulatory T-cells preceding the immunodiscordant response to antiretroviral treatment.** J Infect. 2018; 76:86-92.

- Rosado-Sánchez I, Herrero-Fernández I, Álvarez-Ríos AI, Genebat M, Abad-Carrillo MA, Ruiz-Mateos E, Pulido F, González-García J, Montero M, Bernal-Morell E, Vidal F, Leal M, Pacheco YM. **A Lower Baseline CD4/CD8 T-Cell Ratio Is Independently Associated with Immunodiscordant Response to Antiretroviral Therapy in HIV-Infected Subjects.** Antimicrob Agents Chemother. 2017; 61:e00605-17.

- Rosado-Sánchez I, Jarrín I, Pozo-Balado MM, de Pablo-Bernal RS, Herrero-Fernández I, Alvarez-Ríos AI, Rodríguez-Gallego E, Genebat M, Vera M,

Berenguer J, Martín ML, Bernal E, Vidal F, Blanco J, Leal M, Pacheco YM. **Higher levels of IL-6, CD4 turnover and Treg frequency are already present before cART in HIV-infected subjects with later low CD4 recovery.** Antiviral Res. 2017; 142:76-82.

- Rosado-Sánchez I, Herrero-Fernández I, Genebat M, Ruiz-Mateos E, Leal M, Pacheco YM. **Thymic Function Impacts the Peripheral CD4/CD8 Ratio of HIV-Infected Subjects.** Clin Infect Dis. 2017; 64:152-158.