UNIVERSIDAD DE OVIEDO

DEPARTAMENTO DE BIOLOGÍA FUNCIONAL

MODIFICACIONES FENOTÍPICAS Y FUNCIONALES DE LA RESPUESTA CELULAR EN EL PROCESO DE INMUNOSENESCENCIA

TESIS DOCTORAL

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OVIEDO, 2015

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: MODIFICACIONES FENOTÍPICAS Y FUNCIONALES DE LA RESPUESTA CELULAR EN EL PROCESO DE INMUNOSENESCENCIA	Inglés: PHENOTYPIC AND FUNCTIONAL CHANGES IN CELLULAR RESPONSE IN THE PROCESS OF IMMUNOSENESCENCE

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FOR-MAT-VOA-010-BIS

RESUMEN (en español)

El campo de investigación de esta tesis es el envejecimiento del sistema inmunológico y sus efectos sobre el estado de salud y la calidad de vida de las personas de edad avanzada. Se sabe que el envejecimiento humano se caracteriza por cambios en el sistema inmune con un profundo impacto en el compartimiento de las células T, así como en la mayoría de células del sistema inmune innato e immunomediadores. Este deterioro progresivo conduce a una mayor incidencia o reactivación de las enfermedades infecciosas, así como al desarrollo de enfermedades autoinmunes y el cáncer. Esta respuesta inmune defectuosa también se manifiesta en una disminución de la capacidad de inducir memoria inmunológica a vacunas e infecciones, lo cual tiene un impacto clínico profundo.

La senescencia del sistema inmune se puede producir no sólo por el envejecimiento fisiológico, sino también por diferentes enfermedades crónicas e infecciosas, como artritis reumatoide, aterosclerosis, deterioro cognitivo, infecciones por CMV o VIH... Intervenir para frenar el deterioro de la respuesta del sistema inmune podría mejorar la calidad de vida de los ancianos y de los jóvenes con patologías caracterizadas por inmunosenescencia. En este sentido, estrategias tales como el aumento de la actividad física y la ingesta de probióticos inmunomoduladores han sido evaluadas en nuestro laboratorio. De los numerosos resultados obtenidos a lo largo de estos años realizando la tesis doctoral podemos destacar:

- La expresión de la molécula NKG2D en los linfocitos T CD4+ se encuentra significativamente elevada en los individuos ancianos respecto a los adultos jóvenes. Esta población celular presenta el fenotipo y las funciones típicas de las células altamente diferenciadas que han sufrido un gran número de divisiones.

- La citocina IL-15 posee un efecto muy notorio sobre las células T CD4+, con gran capacidad de activar células $CD28^{null}$, mucho más que sobre la población CD28+. La IL-15 aumenta la proliferación y la frecuencia de la población $CD4+CD28^{null}$ frente a la población CD4+CD28+. Además, la activación en las células $CD4+CD28^{null}$ está claramente inducida por la IL-15, como se puede comprobar por la expresión de diversos marcadores de activación y por el aumento de sus propiedades citotóxicas. También encontramos un aumento de la respuesta antígeno-específica tras el tratamiento con IL-15.

- Existe una relación entre el envejecimiento, la seropositividad a CMV y los niveles de anticuerpos frente a CMV, que están claramente asociados con la frecuencia de las células T CD4+ específicas frente a CMV en ancianos. Además, tanto la seropositividad a CMV como el título de anticuerpos se relacionan con el grado de diferenciación de las células T CD4+ y con el perfil de riesgo inmunológico en ancianos. La capacidad in vivo en ancianos de responder a nuevos antígenos también se encuentra influenciada por la infección por CMV.

- El envejecimiento del sistema inmune se relaciona con el declive funcional en

ancianos. Existen diferencias significativas en la distribución y en el estado de diferenciación de las distintas poblaciones celulares, en la respuesta celular in vitro y a su capacidad de responder in vivo a la inmunización. Además, los ancianos con una peor capacidad funcional tienen los títulos de anticuerpos frente a CMV y la respuesta T específica frente al virus más altos que los individuos con una mejor capacidad funcional.
- Los pacientes con insuficiencia cardiaca crónica (ICC) muestran un envejecimiento de su respuesta inmune adaptativa que se correlaciona con el estadio de la patología. Las concentraciones séricas de IL-6, una de las citocinas pro-inflamatorias más importantes, se relaciona con el desarrollo del proceso de inmunosenescencia en los pacientes con ICC.
- El consumo de Lactobacillus delbrueckii subsp. bulgaricus 8481 se relaciona con una mejora en el estado del sistema inmune en ancianos. El consumo de este probiótico podría favorecer el mantenimiento de una adecuada respuesta inmune, ralentizando el envejecimiento de las subpoblaciones de linfocitos T y aumentando el número de células inmaduras potencialmente respondedoras frente a nuevos antígenos.
- Existe una asociación clara entre altos niveles de actividad física mantenidos a lo largo de toda la vida y el envejecimiento del sistema inmune, contrariamente a lo que cabría esperar. Los altos niveles de actividad física mantenidos a lo largo de muchos años se correlacionan con el estado de la respuesta inmune adaptativa. Esto puede afectar a la eficacia de la respuesta inmune en atletas, a su estado de salud y a su rendimiento deportivo, de gran importancia para estos individuos.

RESUMEN (en Inglés)

The main purpose of this thesis is to study the aging of the immune system and its effects on health status and quality of life in older people. It is known that human aging is characterized by changes in the immune system with a profound effect on the T cell compartment, as well as in most cells of the innate immune system and immunomediators. This progressive deterioration leads to increased incidence or reactivation of infectious diseases, the development of autoimmune diseases and cancer. This defective immune response is manifested in a decrease in the ability to induce immunological memory to infections or vaccines, which has a profound clinical impact.
The senescence of the immune system can occur not only by physiological aging, but also by various infectious and chronic diseases, such as rheumatoid arthritis, atherosclerosis, cognitive impairment, HIV or CMV infections... Intervention to halt the deterioration of the response of the immune system could improve the quality of life in old people and young people with several disease characterized by immunesenescence. Strategies such as increasing physical activity and intake of immunomodulatory probiotics have been evaluated in our laboratory. Among the numerous results obtained throughout the years of realization of the thesis we can highlight:
- We have demonstrated that expression of NKG2D in CD4+ T-cells is significantly increased in elderly individuals with respect to young adults. This cell subset exhibits the typical phenotype and function of end-differentiated cells which have undergone a high number of cell divisions.
- We have observed that IL-15 displays a striking effect on CD4+ T cells, with greater capacity for activating $CD28^{null}$ cells than CD28+ cells. IL-15 increases the proliferation and frequency of $CD4+CD28^{null}$ T cells, compared to CD4+CD28+ T cells. Moreover, activation is clearly induced by IL-15 on $CD4+CD28^{null}$ T cells as shown by both expression of activation markers and an enhancing effect on their cytolytic properties.
- Our results suggest a relationship between aging, CMV seropositivity and the levels of anti-CMV antibodies, which are clearly associated with CMV-specific CD4+ T cells in elderly individuals. Moreover, both CMV seropositivity and antibody titers are related to the differentiation degree of CD4+ T cells and to the IRP parameters in elderly people. The in vivo ability of elderly individuals to respond to new antigens is also influenced by CMV infection.

- We have demonstrated a clear association between physical decline of elderly individuals and aging of their immune system. We found significant differences in the distribution and differentiation state of cell subpopulations, in the cellular response in vitro, and in the in vivo ability for immunization. Furthermore, the elderly with worse functional capacity had the higher anti-CMV titer and T cell response to CMV than the elderly with better functional status.
- We have shown that patients with chronic heart failure (CHF) develop aging of the adaptive immune response that correlates with the stage of the pathology. We also demonstrated that IL-6 concentration, one of the most important pro-inflammatory cytokines, is closely related to the acceleration of the immunosenescence process in CHF patients.
- We have demonstrated a clear association between Lactobacillus delbrueckii subsp. bulgaricus 8481 consumption and a great benefit on the immune system of elderly people. We can assert that consumption of the probiotic L. delbrueckii subsp. bulgaricus 8481 could promote the maintenance of an adequate immune response, slowing the aging of the subpopulations of T lymphocytes and increasing the number of immature cells potentially responders.
- We have found a clear association between high levels of physical activity sustained throughout life and the aging of the immune system. We have revealed a relationship between a high level of physical activity maintained over many years and the condition of the adaptive immune response. This may affect the efficacy of the immune response of athletes, and therefore their health and, which is of great importance to these individuals, their sporting performance.

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AGRADECIMIENTOS

A lo largo de estos más de seis años que me ha llevado la confección de mi tesis doctoral he recibido la ayuda y el apoyo de un montón de gente a la cual querría agradecer de todo corazón el haber estado ahí:

¿Quién me iba a decir a mí que acabaría leyendo una tesis doctoral? Cuando llegué a este laboratorio después de varios años trabajando como Bioquímico Clínico, la verdad, que mi interés por trabajar como investigador era bastante reducido. Pero una persona cuando empecé me dijo "esto engancha", y la verdad, vaya que si engancha. Esta persona es la Dra. Rebeca Alonso Arias, mi co-directora de tesis, pero sobre todo una de mis mejores amigas y una de las personas que más ha luchado y me ha ayudado para que siga en este mundo tan adictivo.

Gracias al Dr. Carlos López Larrea, co-director de tesis, por haberme dado la oportunidad de formar parte de su laboratorio y así poder conocer las satisfacciones que conlleva el trabajar como investigador. Gracias al Dr. Segundo González por aceptar ser el tutor de este trabajo.

Gracias a todos los compañeros del Laboratorio de Inmunología del HUCA: Dr. Antonio Vázquez, Dr. Jesús Martínez, Dra. Lourdes Mozo, Dra. Lourdes Tricas, Sonia, María Jesús, Eladio, Ramona, Juan Carlos, Lici, Dulce, Esther, Eva, Reyes, Aurora, pero sobre todo a las técnicos más marchosas y rumberas, Anadí y Vicky, con las que me he echado unas buenas carcajadas esperando para poder utilizar nuestro querido citómetro. Muchas gracias al Dr. Pablo Martínez Clambor alias "el estadístico" por hacer parecer fácil lo que es muy difícil y sobre todo por sus amenas conversaciones entre chi cuadrado y chi cuadrado.

Mi agradecimiento más grande es para la "peña del café". ¿Qué hubiera sido de mi tesis sin las tertulias científicas que compartimos durante el mejor momento del día? Aroa, Alba, Jose, Isa, Ainara, María, Rober, Catu, espero no olvidarme de nadie, porque la verdad que durante estos años ha pasado mucha gente maravillosa, con muchos de los cuales me une una amistad más allá de las puertas del laboratorio.

Gracias a todos los pacientes y voluntarios que desinteresadamente han apoyado mi investigación, sin ellos nada sería posible. Gracias también a todos los facultativos e

investigadores del HUCA y otros hospitales que han contribuido con su ayuda y entusiasmo a la elaboración de esta tesis y a todos los artículos publicados: Dr. Benjamín Fernández, Dr. Francisco Manuel Suárez, Dr. Juan José Jaurrieta, Dr. César Morís, Dr. Pablo Avanzas, Dr. Fernando López y finalmente a la eficiente Esther.

Gracias a mi familia y mis amigos por su apoyo y comprensión durante este periodo. Sé que estáis muy orgullosos de mí, pero os puedo asegurar que yo lo estoy mucho más de vosotros, os quiero.

Finalmente, gracias Lundi, lo mejor que me ha pasado en la vida.

ABREVIATURAS

ABVD: Actividades básicas de la vida diaria
ACC/AHA: "American College of Cardiology-American Heart Association"
ADHF: Insuficiencia cardiaca crónica descompensada
AICC: Ancianos insuficiencia cardiaca crónica
APC: "Allophycocyanin" (Aloficocianina)
AR: Artritis reumatoide
AS: Ancianos sanos
BSA: "Bovine serum albumin" (Albúmina sérica bovina)
CD: Célula dendríticas
CFSE: "Carboxy-(5,6)-fluorescein diacetate succinimidyl ester"
CM : Célula T de memoria central
CMV: Citomegalovirus
DO: Densidad óptica
E: Célula T efectora terminal
ELISA: "Enzyme-Linked ImmunoSorbent Assay" (Ensayo por inmunoabsorción ligado a enzimas)
ELISPOT: "Enzyme-Linked ImmunoSpot"
EM: Célula T de memoria efectora
EMR: Emigrantes recientes del timo
EMRA: Célula T de memoria efectora terminal
EEM: Error estándar de la media
FEVI: Fracción de eyección ventrículo izquierdo
FITC: "Fluorescein isotiocyanate" (isotiocianato de fluoresceína)
GALT: Tejido linfoide asociado a mucosa
G-CSF: Factor estimulante de colonias de granulocitos
GH: Hormona de crecimiento
GM-CSF: Factor estimulante de colonias de granulocitos y macrófagos
Grb2: "Growth factor receptor bound protein 2"
hBD-2: β2-defensina humana
HDACs: Histona deacetilasas
hHSP60: Proteína de choque térmico 60 humana

HLA: “Human leukocyte antigen” (Antígenos leucocitarios humanos)

HTLV: Human T-lymphotropic virus Type I (Virus linfotrópico humano tipo 1)

IB: Índice de Barthel

IC: Intervalo de confianza

ICC: Insuficiencia cardiaca crónica

IFN: Interferón

IGF-1: Factor de crecimiento insulínico tipo 1

IL-1Ra: Antagonista del receptor de la IL-1

IRP: Immune risk profile (Perfil de riesgo inmunológico)

JICC: Jóvenes insuficiencia cardiaca crónica

JS: Jóvenes sanos

KGF: Factor de crecimiento de queratinocitos

KIR: “Killer cell Ig-like receptor” (Receptor tipo Ig de las células NK)

LHRH: Hormona liberadora de gonadotrofina

LPX: Lipoxinas

LT: Leucotrienos

MBP: Proteína básica de la mielina

mCDs: Células dendríticas mieloides

MCP-1: Proteína quimioatrayente de monocitos I

MHC: “Major histocompatibility complex” (Complejo mayor de histocompatibilidad)

MICA: “MHC class-I-related chain A”

MICB: “MHC class-I-related chain B”

MOG: Glicoproteína oligodendroglial de la mielina

NF-Kb: “Nuclear factor κB” (Factor nuclear κB)

NK: “Natural killer” (Célula asesina natural)

NKG2D: “Natural-killer Group 2, member D”

NKT: “Natural killer T cells” (Células asesinas naturales de tipo T)

NKT-like: “Natrual killer T cells-like”

NYHA: Escala “New York heart association”

OR: Odds ratio

PBMC: “Peripheral Blood Mononuclear Cell” (Células mononucleares de sangre periférica)

PBS: “Phosphate buffered saline” (Tampón fosfato)

pCDs: Células dendrícas plasmacitoides

PCR: Polymerase chain reaction (Reacción en cadena de la polimerasa)

PCR: Proteína C reactiva

PE: “Phycoeritrin” (Ficoeritrina)

pE1: Células pre-efectoras de tipo 1

pE2: Células pre-efectoras de tipo 2

PerCP: “Peridinin chlorophyll-A proteína”

PG: Prostaglandinas

PI3K: “Class I Phosphoinositide 3-kinase” (3-fosfoinositol quinasa)

RI: Rango intercuartílico

TBS: “Tris Buffered Saline” (Tampón Tris)

TCR: “T cell receptor” (Receptor de células T)

TGF: “Transforming growth factor” (Factor de crecimiento transformante)

TH: “T helper lymphocyte” (Linfocito T colaborador)

TLR: “Toll-like receptor” (Receptore tipo toll)

TNF: “Tumor necrosis factor” (Factor de necrosis tumoral)

TREC: “T cell receptor excision DNA circle” (Círculos de escisión del receptor de células T)

TSLP: Linfopoyetina estromal tímica

UA: Unidades arbitrarias

UFP: Unidades formadoras de placa

ULBPs: UL16-binding proteins (Proteínas de unión a UL-16)

VEB: Virus de Epstein-Barr

VHC: Virus de la hepatitis C humana

VHS: Virus herpes simple

VIH: Virus de la inmunodeficiencia humana

VO_{2max}: Consumo máximo de oxígeno

VVZ: Virus varicela-zoster

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INTRODUCCIÓN

I. INTRODUCCIÓN

I.1. INMUNOSENESCENCIA

El envejecimiento del sistema inmune implica una compleja serie de cambios que colectivamente reciben el nombre de inmunosenescencia (Miller 1996; Cambier 2005). Estos cambios afectan tanto al sistema inmune innato como adaptativo, dando como resultado en muchas ocasiones un estado, no completamente entendido, de inmunodeficiencia. Las principales manifestaciones de la senescencia del sistema inmune incluyen una escasa capacidad de respuesta frente a patógenos nuevos o evolucionados, frente al desarrollo de tumores y una reducida protección inducida por vacunación frente a diversas enfermedades (Gardner 1980; Lang *et al.* 2011). Por el contrario, la memoria inmunológica en respuesta a patógenos ya conocidos se encuentra mucho menos afectada (Haynes *et al.* 2003; Nikolich-Zugich & Rudd 2010). La baja capacidad de activación, proliferación y secreción de citocinas en respuesta a antígenos es consecuencia de una respuesta alterada de linfocitos B y linfocitos T efectores (Ginaldi *et al.* 1999; Haynes *et al.* 1999; Frasca *et al.* 2011). Los defectos en el sistema inmune de los ancianos que afectan al procesamiento y presentación del antígeno y la producción de citocinas tienen el potencial de afectar a la respuesta inmune, así como al mantenimiento de las poblaciones linfoides (Pamer 1999; Ku *et al.* 2000). Clínicamente, estos defectos se correlacionan con una mayor morbilidad y mortalidad de los ancianos a causa de enfermedades infecciosas.

El envejecimiento del sistema inmune abarca dos tipos de cambios: alteraciones primarias, que son consecuencia directa del envejecimiento y alteraciones secundarias, que representan una reacción o respuesta a los cambios primarios, y que, por sí mismos, también pueden verse afectados por el proceso del envejecimiento. El envejecimiento celular sería la principal alteración primaria, y es resultado de varios procesos que se superponen. Éstos incluyen daños en el ADN; estrés oxidativo (peroxidación de lípidos, proteínas y azúcares); cambios epigenéticos; reducida biosíntesis, degradación y reciclado de macromoléculas… (Kenyon 2010; Cannizzo *et al.* 2011; Ben-Avraham *et al.* 2012). La acumulación de estas alteraciones primarias se produce debido a que los mecanismos de reparación están actuando de forma defectuosa, ya sea porque la tasa de reparación es menor, la tasa de daño mayor o ambas cosas. La consecuencia es que la homeostasis celular y tisular ponen en marcha las alteraciones secundarias. Estas alteraciones incluyen cambios compensatorios que se producen en las células como la apoptosis, la incapacidad para proliferar y la aparición del fenotipo senescente. El resultado final de las alteraciones primarias y secundarias consiste en un deterioro y/o alteración de la función de la célula afectada. Todos estos cambios son

especialmente importantes en la población de linfocitos T. Las alteraciones primarias que afectan a los linfocitos T derivan de la involución tímica y la consiguiente reducción en la producción de células T (Kohler & Thiel 2009; Sauce & Appay 2011). Las alteraciones secundarias se manifiestan mediante una expansión homeostática compensatoria de las células T maduras que intentan mantener intacta la proporción de células T (Surh & Sprent 2002; Moro-Garcia *et al.* 2012) (Figura 1). Otro tipo de cambios secundarios se deben a la naturaleza de la respuesta de las células T a patógenos, con ciclos repetidos de intensa proliferación y apoptosis y el mantenimiento continuado de estos linfocitos de memoria generados. Para frenar la aparición de estos procesos o tratar de revertirlos se están comenzando a plantear una amplia variedad de estrategias basadas fundamentalmente en moléculas con actividad antioxidante.

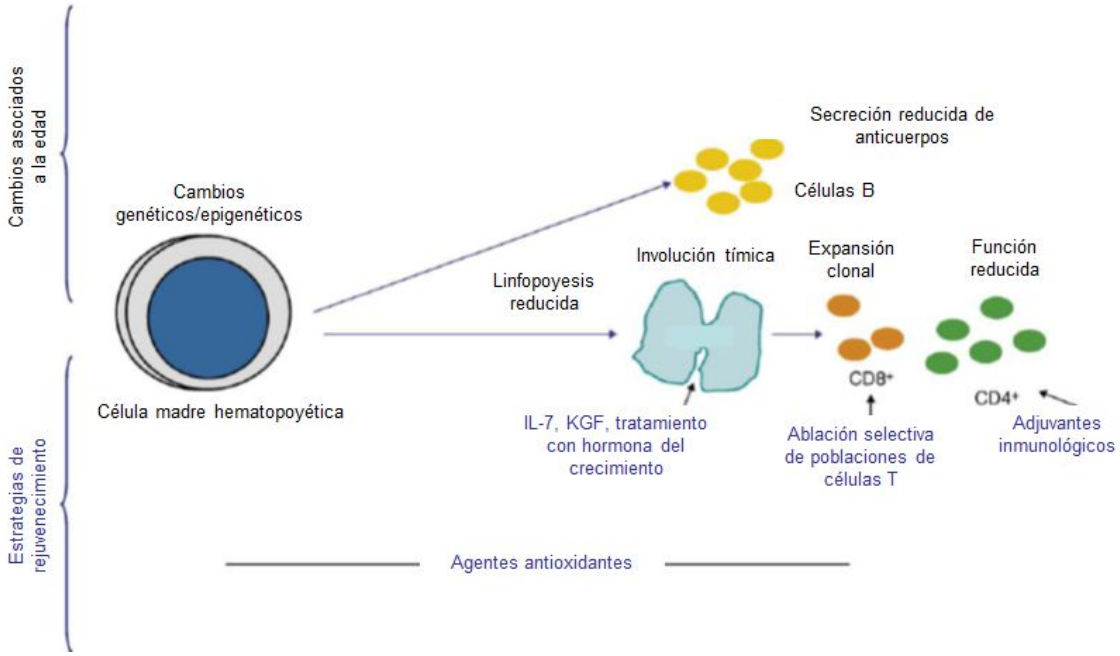

Figura 1. Efectos del envejecimiento en la producción y función de los linfocitos T y varias estrategias propuestas para revertir este proceso (Adaptado de *Dorshkind et al., 2009, Curr Opin Immunol).*

El impacto sobre la expansión o contracción de la población de células T y la generación de la memoria inmunológica difiere dependiendo de si los patógenos dan lugar a una infección aguda o persistente. A diferencia de los virus que producen las infecciones agudas, los patógenos persistentes que inducen las infecciones latentes (citomegalovirus (CMV), virus de Epstein-Barr (VEB), virus de la varicela zoster (VVZ), virus del herpes simple (VHS) y agentes patógenos que provocan las infecciones crónicas, incluidos los virus de la hepatitis C (VHC) y el virus de la inmunodeficiencia humana (VIH)) han establecido un equilibrio evolutivo con el

huésped y su estimulación repetida sobre el sistema inmune produce un gran aumento de las poblaciones de linfocitos T memoria. A largo plazo, la repetida estimulación del sistema inmunológico, especialmente de las células T, por estos virus se cree que tiene un papel primordial en el envejecimiento inmunológico (Pawelec *et al.* 2004; Simpson 2011). Los efectos de la interacción entre las fuerzas homeostáticas y los virus persistentes pueden ser los factores clave que impulsan el envejecimiento del sistema inmune en humanos.

I.1.1. INMUNIDAD INNATA E INMUNOSENESCENCIA

El sistema inmune innato está compuesto por un conjunto de células que median las primeras interacciones con patógenos e incluyen a neutrófilos, células "natural killer" (NK y NKT), monocitos/macrófagos y células dendríticas. Se han detectado defectos asociados con la edad en la activación de todos estos tipos celulares, vinculados sobre todo a vías de transducción comprometidas, incluyendo los receptores Toll-like (TLR), así como cambios en el número de ciertas poblaciones celulares en sangre periférica. Por otra parte, el proceso de envejecimiento se caracteriza por un entorno constitutivo pro-inflamatorio ("inflamm-aging") que provoca una activación crónica continuada del sistema inmune innato que puede provocar un aumento del daño tisular causado por infecciones en personas de edad avanzada. Por lo tanto, el envejecimiento del sistema inmune innato parece reflejar más una desregulación que un deterioro de su función (Shaw *et al.* 2010; Solana *et al.* 2012).

Las células NK forman parte del sistema inmune innato y median citotoxicidad independiente de MHC frente a infecciones virales y algunos tumores malignos. Los números absolutos de células NK aumentan con la edad, así como las células $CD56^{dim}$, subpoblación madura altamente citotóxica. Sin embargo, la capacidad citotóxica de las células NK está disminuida y los niveles de citocinas y quimiocinas tales como RANTES, MIP1α, e IL-8 producidas tras la activación de las células NK se encuentran también reducidos (Mocchegiani *et al.* 2009). En consonancia con todo esto, el riesgo de infección y la mortalidad en personas de edad avanzada parecen estar fuertemente correlacionados con la actividad de las células NK (Ogata *et al.* 2001).

Los monocitos representan unos componentes altamente móviles del sistema inmune innato localizados tanto en sangre periférica como en bazo. Estas células responden a la inflamación diferenciándose a células presentadoras de antígenos como macrófagos y células dendríticas. Los números absolutos de monocitos aumentan con la edad (Della Bella *et al.* 2007). Este aumento en el número de monocitos es contrarrestado con la disminución en la función de

los macrófagos, sobre todo en la actividad mediada por TLR. Estudios en humanos relacionados con la función de los TLR en monocitos han revelado una disminución en la producción de citocinas con la edad (van Duin *et al.* 2007b). La capacidad de aumentar la expresión de la molécula coestimuladora CD80 tras activación de TLR, confirma la relación con la inmunidad adaptativa (van Duin *et al.* 2007a). Así, los TLR juegan un papel crucial en la vinculación entre la respuesta inmune innata y humoral.

Los neutrófilos constituyen la primera barrera de defensa contra bacterias, levaduras y hongos infecciosos, mediante mecanismos microbicidas tales como especies reactivas de oxígeno y nitrógeno y la liberación de enzimas proteolíticos y péptidos microbicidas contenidos en los gránulos citoplasmáticos. Los neutrófilos también pueden atacar y matar a los microorganismos extracelulares mediante la liberación de las llamadas trampas extracelulares de neutrófilos, "neutrophil extracellular traps" (NETs) (Brinkmann *et al.* 2004).

Las células del sistema inmune derivan de las células madre, "stem cells" hematopoyéticas (HSCs) comprometidas en el linaje linfoide o mieloide. Con el envejecimiento se produce un sesgo hacía la línea mieloide a expensas de los progenitores linfoides, por lo tanto, no hay una disminución en el número de neutrófilos con la edad (Beerman *et al.* 2010). Por el contrario, los neutrófilos en ancianos presentan una funcionalidad reducida, con gran parte de sus capacidades microbicidas afectadas. Estas células también presentan una reducida capacidad quimiotáctica observada en los neutrófilos envejecidos, que podría afectar el tiempo que éstos necesitan para llegar al lugar de infección, permitiendo a los microorganismos patógenos establecer un foco de infección potente (Wenisch *et al.* 2000). Esta quimiotaxis alterada también podría aumentar el daño tisular producido por la proteasas secretadas por los neutrófilos como las elastasas, que ayudan a estas células a moverse a través de los tejidos (Nomellini *et al.* 2008). El retorno de estos neutrófilos al final de su vida activa a médula ósea también se encuentra mediado por quimiotaxis, al estar alterada se podría producir una acumulación de células envejecidas en periferia, con la consiguiente pérdida de funcionalidad (Weisel *et al.* 2009).

I.1.1.1. Inflamación

Se ha observado que existe un estado inflamatorio crónico en individuos de edad avanzada, con niveles más altos de citocinas como el TNF-α, IL-6 e IL-1β, entre otros, y de marcadores pro-inflamatorios como la proteína C reactiva y factores de coagulación. El aumento significativo relacionado con la edad en los niveles circulantes de citocinas inflamatorias, dando

lugar a niveles plasmáticos de dos a tres veces mayores en ancianos que en jóvenes, probablemente esté reflejando el efecto acumulativo de la producción de citocinas en el linaje de los monocitos, así como en células envejecidas del estroma, como los fibroblastos. Esta condición recibe el nombre de "envejecimiento inflamatorio" (Fagiolo *et al.* 1993; Franceschi *et al.* 2007). De hecho, el envejecimiento se asocia con una activación tanto de la rama innata como de la adaptativa del sistema inmune. Simplificando mucho las cosas, podemos decir que en el sistema inmune innato se produce un estado inflamatorio crónico, de bajo grado; mientras en el sistema inmune adaptativo se produce un agotamiento de la respuesta. Entre las características más importantes del "envejecimiento inflamatorio" se encuentran:

- Un aumento en los niveles séricos de citoquinas pro-inflamatorias (IL-6, IL-8, IL-15).
- Un papel muy importante de las infecciones subclínicas por CMV y VEB. De hecho, los clones de células memoria que aparecen en personas de edad avanzada parecen estar directamente relacionados con las reactivaciones de este tipo de virus.
- Presencia de un componente genético pro-inflamatorio, como se puede desprender de varios estudios en diversos de alelos y genotipos que codifican para moléculas pro-inflamatorias, como citoquinas y factores de coagulación (Cipriano *et al.* 2005; Franceschi *et al.* 2005).

En ancianos centenarios se encuentra una mezcla compleja y muy peculiar de características pro y anti-inflamatorias, tanto fenotípicas como genéticas. El que estos individuos hayan llegado a ser centenarios nos indica que son capaces de montar una respuesta inflamatoria correcta. Pero por otra parte, esta capacidad inflamatoria que se relaciona con un riesgo aumentado de fragilidad y patologías, está compensado por el desarrollo de una respuesta anti-inflamatoria robusta y efectiva y con el aumento de marcadores genéticos asociados con una reducida capacidad pro-inflamatoria (Figura 2).

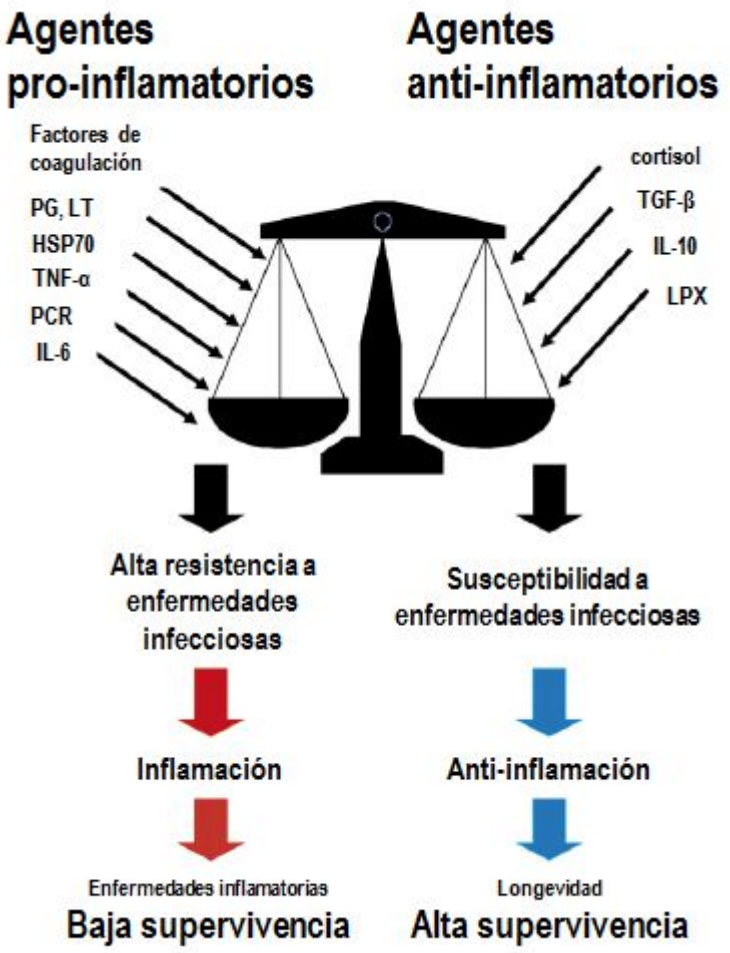

Figura 2. Balance entre los agentes pro y anti-inflamatorios y su efecto sobre la supervivencia en ancianos.

Los niveles aumentados de citocinas pro-inflamatorias están fuertemente asociados con aumento de la discapacidad en ancianos (Barbieri *et al.* 2003) y con el síndrome de fragilidad inmunológica, que ha sido validado como un predictor de discapacidad, caídas, hospitalización y mortalidad (Fried *et al.* 2001).

El efecto de este estado inflamatorio crónico sobre ciertas patologías asociadas a la edad está comenzando a tenerse muy en cuenta, con un papel claro en ateroesclerosis, Alzheimer, sarcopenia, cáncer y síndrome metabólico (Licastro *et al.* 2005; Vasto *et al.* 2007). La aterosclerosis, hasta fechas recientes considerada una enfermedad debida al acúmulo de lípidos, cursa con una respuesta inflamatoria severa. Se ha establecido un papel fundamental tanto de la inmunidad innata como adaptativa desde el inicio, a la progresión e incluso en las complicaciones trombóticas. El aumento de marcadores de inflamación puede predecir el pronóstico del paciente, independientemente del daño miocárdico. De hecho, los niveles de PCR, IL-6 e IL-1β han sido sugeridos como factores de riesgo predictivos para futuros eventos

cardiovasculares y se han asociado con insuficiencia cardiaca congestiva e hipertensión (Ridker *et al.* 2000; Hansson *et al.* 2002).

La inflamación asociada a la enfermedad de Alzheimer aparece principalmente en las zonas patológicamente vulnerables del cerebro de estos pacientes. En la periferia de estas zonas, el tejido degenerado y la deposición de sustancias altamente insolubles son estímulos para la inflamación. Por otra parte, se ha visto que factores inflamatorios como citocinas, quimiocinas, componentes del complemento y proteínas de fase aguda se co-localizan en placas seniles o están aumentadas en los cerebros de pacientes con enfermedad de Alzheimer (McGeer & McGeer 1998).

La sarcopenia tiene un papel principal, como es obvio, en la fragilidad asociada a la edad. En los últimos años el papel de la inflamación en la sarcopenia ha empezado a cobrar importancia, la inflamación crónica de baja intensidad se ha asociado con la pérdida de masa muscular, así como con la discapacidad funcional en ancianos (Roubenoff 2003) (Krabbe *et al.* 2004).

La inflamación crónica inducida tanto por lesiones biológicas, químicas, mecánicas o físicas se ha asociado con la incidencia de cáncer en diferentes tejidos humanos (Thun *et al.* 2004). Por ejemplo, la enfermedad inflamatoria intestinal, la colitis ulcerosa y la enfermedad de Crohn son patologías que predisponen a padecer ciertos tipos de cáncer en intestino grueso o íleon terminal. Además se sabe que la susceptibilidad a padecer algún tipo de cáncer, así como su severidad, se pueden asocian con polimorfismos funcionales de ciertas citocinas implicadas en el proceso inflamatorio (Macarthur *et al.* 2004; Taniguchi & Karin 2014).

Desde hace varios años se ha sugerido que la inflamación de baja intensidad está muy asociada al síndrome metabólico, así como se ha asociado el aumento en la PCR, IL-6 y TNF-α con la adiposidad visceral (Das 2004; Donath 2014). Los adipocitos expresan de forma constitutiva la molécula pro-inflamatoria TNF-α, que disminuye tras la pérdida de peso. También se sabe que en estos pacientes existen altas concentraciones plasmáticas de PCR, IL-6 y del inhibidor del plasminógeno 1 (Krabbe *et al.* 2004).

I.1.2. IMMUNIDAD ADAPTATIVA EN ANCIANOS

La respuesta inmune adaptativa sufre profundas alteraciones dependientes de la edad (Haynes & Maue 2009). La alteración de la respuesta en la inmunidad adaptativa en ancianos es una causa importante de morbilidad y mortalidad (Weng 2006). Al ser tan dinámico, el sistema inmune está cambiando constantemente, incluso en ausencia de infecciones o antígenos

exógenos (Goldrath & Bevan 1999). El envejecimiento afecta a la respuesta inmune humoral cuantitativa y cualitativamente, ya que tanto la especificidad como el tipo de anticuerpos generados se encuentran alterados (Linton & Dorshkind 2004; Frasca & Blomberg 2009). En cuanto a las células T, dos de las principales características que adquieren a medida que envejecen son la pérdida de la capacidad proliferativa y la adquisición de marcadores típicos de células NK. Estas dos alteraciones pueden ser causados por: un acortamiento de los telómeros, cambios en las señales de transducción de las células T, alteraciones en la interacción entre la respuesta innata y adaptativa, errores en la reparación del ADN, alteración de los mecanismos antioxidantes, cambios epigenéticos, y una persistente estimulación antigénica (Weiskopf *et al.* 2009; Fasth *et al.* 2010).

Las células T naïve tienen una vida media de 6 a 12 meses y las células T de memoria de 15 a 45 días (Macallan *et al.* 2004). Las células progenitoras de timocitos van a timo y se diferencian en células T naïve CD4+ o CD8+, que luego salen a periferia. Este proceso está regulado por citoquinas y hormonas, así como por células epiteliales, dendríticas, macrófagos y fibroblastos situados en el estroma tímico. En los seres humanos, se sabe muy poco sobre el recuento absoluto de "emigrantes recientes del timo" (EMR), aunque se observa una disminución dramática de ellos en el envejecimiento (McFarland *et al.* 2000). Una de las posibles causas de esta disminución podría ser que el timo sufre un proceso de involución a medida que envejecemos y puede no llegar a satisfacer la demanda de reposición de células T en edad adulta (Douek *et al.* 1998). A partir de los 40-50 años de edad, prácticamente todo el suministro de células T se genera a partir de células naïve existentes y de células T de memoria (Hakim *et al.* 2005). En estas circunstancias, los mecanismos de homeostasis pueden volverse insuficientes y esto puede conducir a una disminución en el repertorio de células T necesarias para combatir nuevos patógenos (Goronzy *et al.* 2007). Además, el estrés relacionado con la replicación continua, descrita previamente, puede inducir senescencia celular y cambios fenotípicos que afecten a la competencia del sistema inmune adaptativo (Vallejo *et al.* 2004; Akbar & Fletcher 2005).

En cuanto a las alteraciones de las principales células presentadoras de antígenos de clase II, las células dendríticas, no existe ningún estudio donde se demuestre de forma fehaciente si con la edad se producen modificaciones en su número. De manera general, se puede decir que el envejecimiento produce una disminución en la función de las células dendríticas, pero no está del todo demostrado, ya que muchos de los parámetros estudiados permanecen inalterados y muchos incluso parecen estar potenciados con la edad (Jing *et al.* 2009) (Panda *et al.* 2010) (Castle *et al.* 1999; Pietschmann *et al.* 2000).

I.1.2.1. Efecto de la edad sobre las células B

Los cambios en la respuesta humoral en ancianos contribuyen significativamente al aumento en la susceptibilidad de éstos a las enfermedades infecciosas y a la reducida protección que las vacunas les proporcionan (McElhaney & Effros 2009). Además de reducirse la producción de anticuerpos se reduce el tiempo durante el cual éstos son eficaces (Steger *et al.* 1996).

La reducida habilidad de los individuos de edad avanzada de producir respuestas con anticuerpos de alta afinidad frente a patógenos puede explicarse en parte a defectos en los linfocitos T, necesarios para la producción de una respuesta humoral T dependiente. Por otro lado, existe controversia en cuanto a si el número absoluto de precursores de células B en la médula ósea disminuye moderadamente (McKenna *et al.* 2001) o permanece inalterado (Rossi *et al.* 2003) con la edad. Lo que sí está claro es que el número de células B maduras disminuye significativamente con la edad (Chong *et al.* 2005; Shi *et al.* 2005). La disminución de esta población y el aumento de la población naïve parece sugerir la presencia de un fallo en la capacidad de las células B para realizar la recombinación de cambio de clase (Chong *et al.* 2005; Shi *et al.* 2005).

A pesar de que la funcionalidad de los linfocitos B pueda verse afectada por los defectos en la población de linfocitos T, las células B también presentan defectos intrínsecos que tienen un importante impacto en la producción de anticuerpos. Entre estos cambios intrínsecos podemos nombrar la disminución en la expresión del factor de transcripción E47, debido a una pérdida de la estabilidad de su mRNA (Lai *et al.* 2006); reducción en la activación de la citidina deaminasa (Sayegh *et al.* 2003) y por lo tanto disminución en la recombinación de cambio de clase de las inmunoglobulinas tanto en respuesta a estímulos policlonales como a estímulos específicos como el virus de la gripe (Frasca *et al.* 2010), tal y como comentamos anteriormente.

Finalmente, el envejecimiento comporta un aumento en las concentraciones de anticuerpos reactivos contra antígenos propios en suero (Prelog 2006). Dado que estos anticuerpos presentan una baja afinidad por el antígeno y que se encuentran en el 80% de personas ancianas y animales viejos, no parecen tener consecuencias patológicas relevantes.

I.1.2.2. Diferenciación y homeostasis de linfocitos T

La mayoría de los linfocitos T con receptor αβ (TCRαβ) se diferencian en el timo a partir de precursores de médula ósea y luego se exportan a los órganos linfoides secundarios. La tasa de producción de estos EMR es proporcional a la masa total del timo, y asciende a

aproximadamente el 1% del contenido total de células del timo por día (Scollay *et al.* 1980; Dixit 2010). La supervivencia de las células T naïve requiere de la presencia de IL-7, miembro de la familia de citocinas del receptor de la cadena γ común (γ_c). La frecuencia y diversidad de las células T naïve es mantenida por el reemplazo regular de las células T naïve por EMR (Berzins *et al.* 1998; Sprent & Surh 2011). El mantenimiento de la diversidad de las células T es necesario para garantizar una respuesta óptima a nuevos patógenos, con los que no se ha estado previamente en contacto. En condiciones normales *in vivo*, las células T naïve presentan un nivel muy bajo de proliferación espontánea (Figura 3A). Sin embargo, la proliferación homeostática se ve en gran medida aumentada en condiciones de linfopenia. Bajo estas circunstancias las células T perciben el "exceso de espacio" y tratan de llenar el hueco existente por medio de una proliferación homeostática antígeno-independiente (Fry & Mackall 2005).

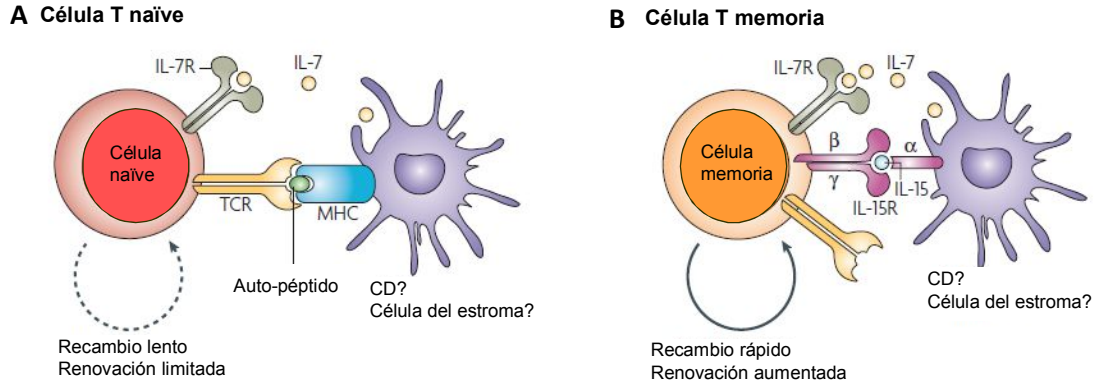

Figura 3. Homeostasis de las células T. Representación esquemática de los factores claves para la supervivencia y mantenimiento de las células naïve (A) y memoria (B) *(*Adaptado de *Nikolich-Zugich, 2008, Nat Rev Immunol).*

Tras el encuentro con el antígeno, las células T naïve primero generan células T efectoras, que son las que se enfrentarán al patógeno directamente y posteriormente aparecen las células T de memoria, que se diferencian en células T de memoria central y las células T memoria efectoras. Las células T de memoria son menos dependientes de contacto con el complejo MHC-péptido para su supervivencia (Caserta & Zamoyska 2007) (Figura 3B). Además la auto-renovación en vivo es de tres a cuatro veces más rápida que en linfocitos T naïve y presentan una alta tasa de proliferación en condiciones de linfopenia (Surh & Sprent 2002).

Existen diversos marcadores cuya expresión permite distinguir el estado de diferenciación de los linfocitos T. Dependiendo de la expresión de la isoforma CD45RA del antígeno leucocitario común CD45 y del receptor de quimiocinas CCR7, los linfocitos T se pueden dividir en: naïve (NAIVE; CD45RA+CCR7+), memoria central (CM; CD54RA-CCR7+),

memoria efectora (EM; CD45RA-CCR7-) y memoria efectora diferenciada de forma terminal (EMRA; CD45RA+CCR7-) (Figura 4) (Sallusto *et al.* 1999).

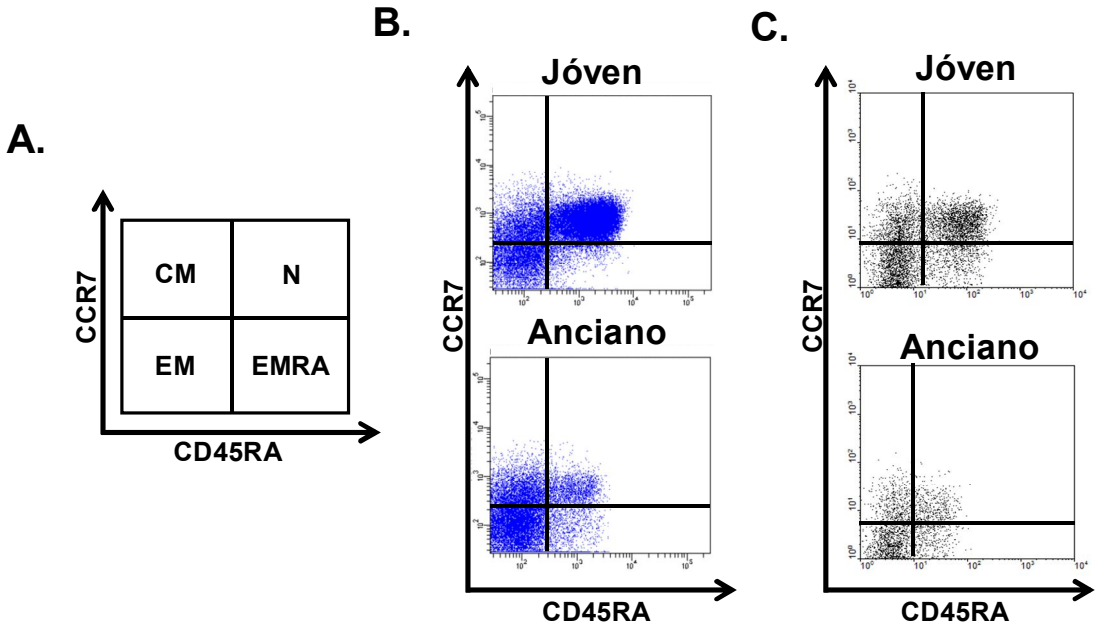

Figura 4. Distribución de los linfocitos T en las subpoblaciones naïve, CM, EM y EMRA en función de la expresión de CD45RA y CCR7. (A) Modelo esquemático de las distintas subpoblaciones. Dot-plots representativos de las distintas poblaciones en jóvenes y ancianos en linfocitos T CD4+ (B) y CD8+ (C).

EM y EMRA son poblaciones altamente heterogéneas, y la expresión de CD27, de la familia del receptor del TNF, y CD28, de la familia del receptor de B7, han demostrado ser útiles en la identificación de células más ($CD27^{null}CD28^{null}$) o menos (CD27+ y/o CD28+) diferenciadas (Romero *et al.* 2007; Koch *et al.* 2008) (Figura 5). Las células T EM se pueden dividir en EM1 (CD27+CD28+), EM2 ($CD27+CD28^{null}$, esta población solo aparece en células T CD8+), EM3 ($CD27^{null}CD28^{null}$) y EM4 ($CD27^{null}$CD28+). Funcionalmente, EM1 y EM4 son muy similares y exhiben propiedades de memoria. EM2 tiene una parcial función efectora, mientras que EM3 muestra propiedades de célula efectora. Del mismo modo, las células T EMRA se puede dividir en pE1 (CD27+CD28+) y pE2 ($CD27+CD28^{null}$, solo aparece en células T CD8+), que son dos subpoblaciones menos diferenciadas y muy similares entre sí, y E ($CD27^{null}CD28^{null}$), la subpoblación más diferenciada del conjunto de células T.

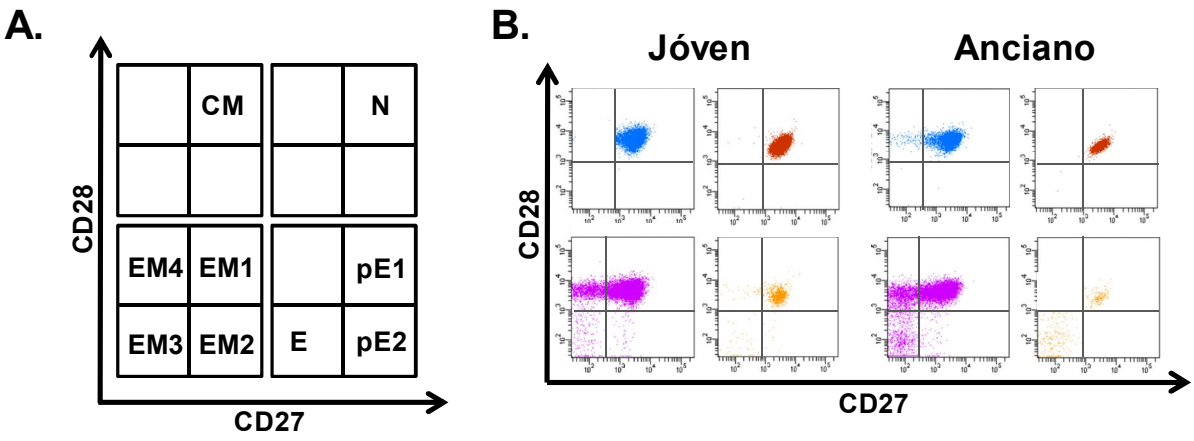

Figura 5. Distribución de EM y EMRA en linfocitos T CD4+ de acuerdo a la expresión de CD27 y CD28. (A) Modelo esquemático de las distintas poblaciones. (B) Dot-plots representativos de las distintas subpoblaciones en jóvenes y ancianos en linfocitos T.

I.1.2.3. Senescencia de linfocitos T

Aunque el proceso de inmunosenescencia afecta muchos aspectos de la inmunidad innata y adaptativa, los efectos en la inmunidad mediada por células T son los más acusados y mejor documentados. De hecho, en los individuos de edad avanzada, la restauración del equilibrio y el número en las distintas poblaciones de células T a menudo se correlaciona con una mejoría en la respuesta a antígenos o patógenos (Haynes *et al.* 2005). Los defectos de las células en el proceso de senescencia abarcan fallos en la señalización a través del TCR y de los receptores coestimuladores (Miller *et al.* 2005; Larbi *et al.* 2011) (Figura 6A). Estas alteraciones son el resultado de cuatro eventos relacionados entre sí: la involución del timo, la disminución del número de células T naïve, la reducción en la diversidad del repertorio de células T y la acumulación de la células T memoria específicas para patógenos persistentes (Figura 6B).

Todos estos fenómenos tienen el potencial de reducir la reserva de células T naïve necesarias para la protección contra nuevos agentes patógenos. La involución tímica y su manifestación principal, la reducción en la producción de células T naïve, comienzan en una etapa tan temprana como 1 año después del nacimiento. La mayor parte de los estudios recientes han demostrado que defectos de las células precursoras hematopoyéticas del linaje linfoide y la migración de estos precursores linfoides a timo están implicados en el proceso de involución tímica (Min *et al.* 2004; Zook *et al.* 2011).

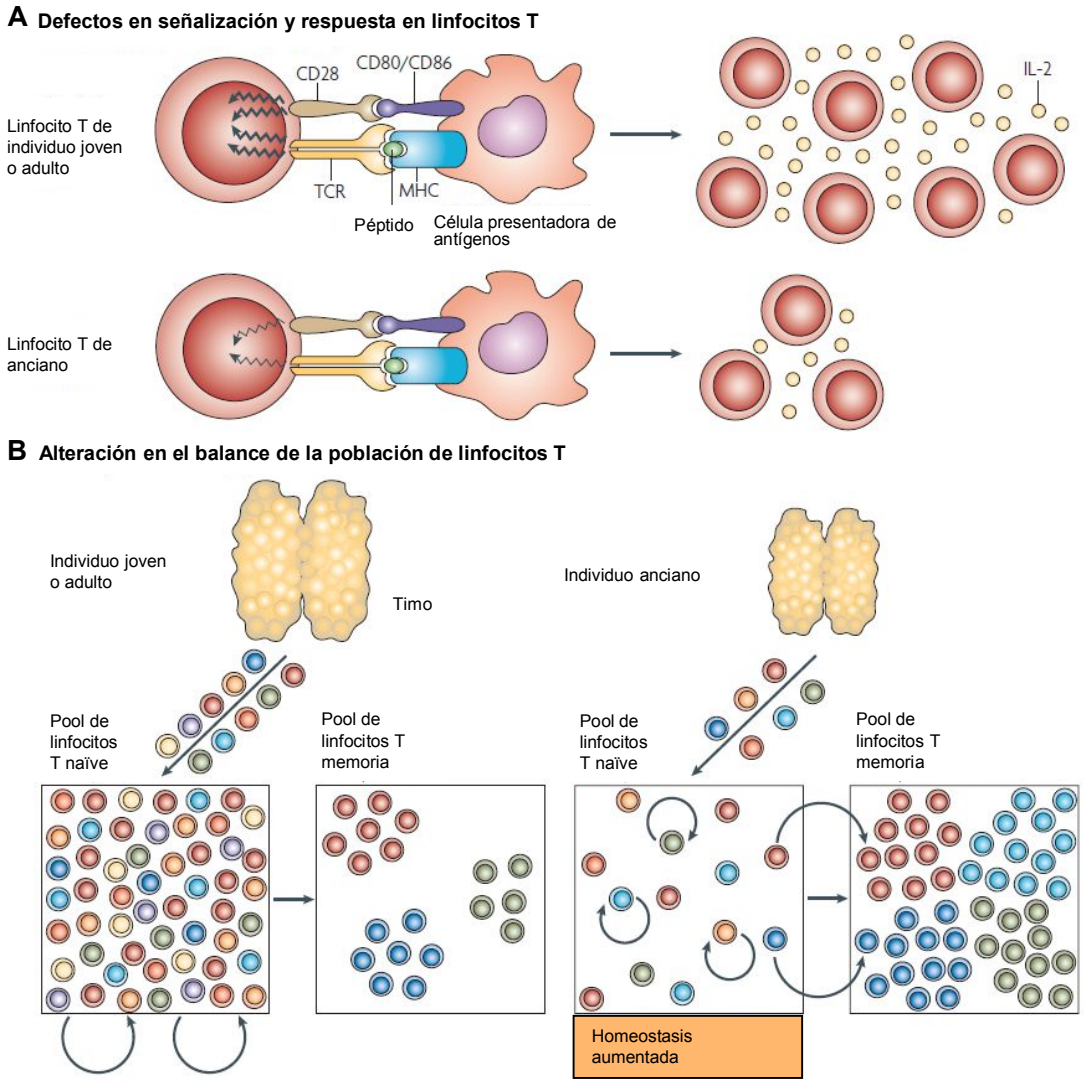

Figura 6. Cambios celulares y poblacionales en linfocitos T en senescencia *(*Adaptado de *Nikolich-Zugich, 2008, Nat Rev Immunol).*

En paralelo con la involución del timo, existe una marcada disminución en el número de EMR. El timo parece producir EMRs en proporción a su celularidad global, incluso en la senescencia (Gruver *et al.* 2007). Así, a medida que disminuye la celularidad del timo, disminuye la salida de células T naïve a periferia. La involución tímica y el consecuente declive progresivo de la producción de células T naïve ejercen presión sobre los mecanismos homeostáticos que se necesitan para mantener el compartimento de células T naïve durante gran parte de la edad adulta y durante la senescencia.

La funcionalidad tímica puede ser cuantificada indirectamente mediante el recuento de las células T naïve en la periferia sanguínea o por tomografía computarizada midiendo el volumen del timo. El primer método no puede discriminar las células T naïve de larga duración, y

la segunda se basa en la suposición de que el volumen de tejido del timo se correlaciona completamente con su funcionalidad. En 1998 Douek y colaboradores cuantificaron por primera vez los círculos de escisión del receptor TCR (TRECs), subproductos de los reordenamientos que se producen durante la formación del TCR. Utilizaron los TRECs para estudiar los cambios en la frecuencia de EMR con la edad y en pacientes con VIH (Douek *et al.* 1998). Los TRECs son moléculas circulares de ADN episomal presentes en el núcleo, generados durante el reordenamiento intratímico de los loci de las cadenas α- (Figura 7) y β- (Figura 8) del TCR.

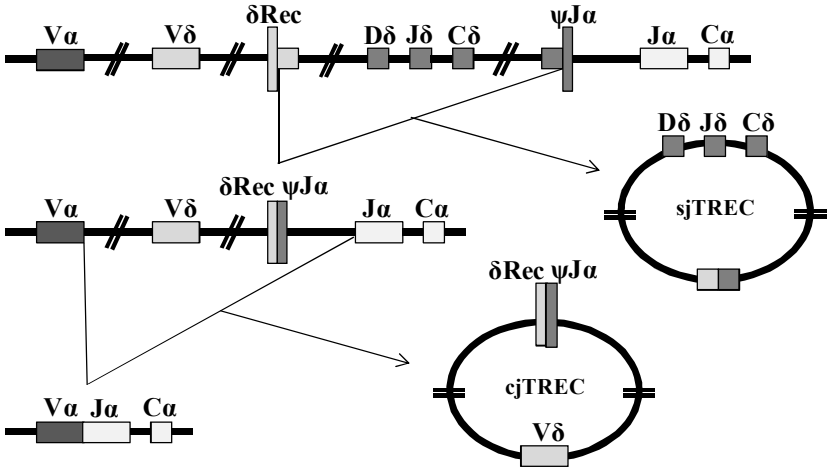

Figura 7. Formación de TRECs en el reordenamiento del locus de la cadena α del receptor TCR en los linfocitos T.

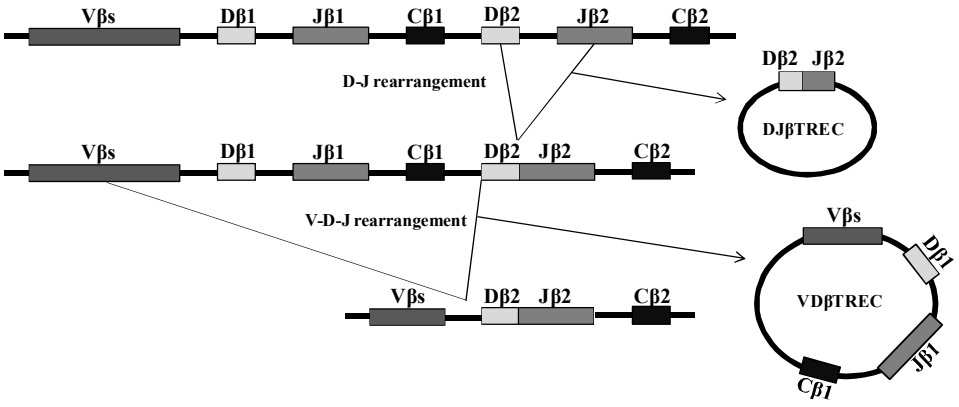

Figura 8. Diagrama esquemático del reordenamiento secuencial del locus de la cadena β del receptor TCR en los linfocitos T.

La escisión de los genes de las cadenas de TCR no implica su eliminación, estos fragmentos de ADN permanecen en el núcleo como moléculas circulares que no se pueden replicar. Así, cuando una célula se divide, los TRECs pasan sólo a una de las dos células hijas.

Durante los ciclos celulares siguientes, los TRECs se diluyen en la población que se origina a partir de la primera célula (Ye & Kirschner 2002; d'Adda di Fagagna *et al.* 2003). Los niveles de estas moléculas de ADN en linfocitos periféricos son un reflejo de la actividad del timo y actualmente son los mejores marcadores de la función tímica (Arellano *et al.* 2006). El nivel de TRECs en las células T de ancianos es mucho menor que en personas jóvenes, debido tanto a la división que sufren los linfocitos T periféricos y a la reducción de la actividad del timo asociada con la involución tímica. La cuantificación de TRECs ha demostrado que estas moléculas disminuyen en los linfocitos T de manera proporcional a la edad (Ou *et al.* 2011). La cuantificación de TRECs no sólo es útil en el estudio de las células T durante el envejecimiento fisiológico, sino también en otros procesos relacionados con la senescencia inmunológica, tales como la infección por VIH y en patologías autoinmunes. Varios estudios han demostrado que las células T de pacientes infectados por el VIH tienen un menor contenido en TRECs que individuos no infectados de la misma edad (Bains *et al.* 2009). En autoinmunidad, las células T responden a la estimulación antigénica con divisiones clonales, por lo tanto, se produce una reducción de los niveles periféricos de TRECs (Lorenzi *et al.* 2009). Se han encontrado niveles reducidos de TRECs en sangre periférica de pacientes con lupus eritematoso sistémico activo, pero no en aquellos con la forma quiescente (Vieira *et al.* 2008).

En las personas de edad avanzada, los pocos EMR que alcanzan la periferia se enfrentan a condiciones que difieren a las encontradas en individuos más jóvenes: hay menos células T naïve, y su supervivencia y mantenimiento se ve alterada. Estos EMR, en particular los de fenotipo CD4+, no parecen sobrevivir bien en la periferia (Hale *et al.* 2006), como consecuencia de distintos procesos. En primer lugar, el pool de linfocitos T naïve en la periferia se encuentra disminuido por muerte celular y/o por el paso de estas células naïve a células memoria. En segundo lugar, las células T naïve podrían verse afectadas indirectamente por la competencia con un creciente número de células T memoria. Por último, si el agotamiento de las células T naïve se perpetúa en el tiempo, las condiciones linfopénicas que se generan podrían producir un exceso de citocinas de supervivencia y mantenimiento, lo que desataría la expansión homeostática proliferativa de linfocitos T naïve. Esto podría conducir a la conversión de muchas células T naïve hacia un fenotipo de memoria, como se ha demostrado en varios estudios (Goldrath *et al.* 2000; Hamilton *et al.* 2006; Surh *et al.* 2006) (Figura 9A). Todos estos mecanismos: la reducción en el número de linfocitos T naïve, el aumento en la conversión de células T naïve en células de memoria y la acumulación de células clonales de memoria debido a encuentros con patógenos, conducen a una reducción en la diversidad de los receptores TCR en personas de edad avanzada (Callahan *et al.* 1993; Naylor *et al.* 2005) (Figura 9B).

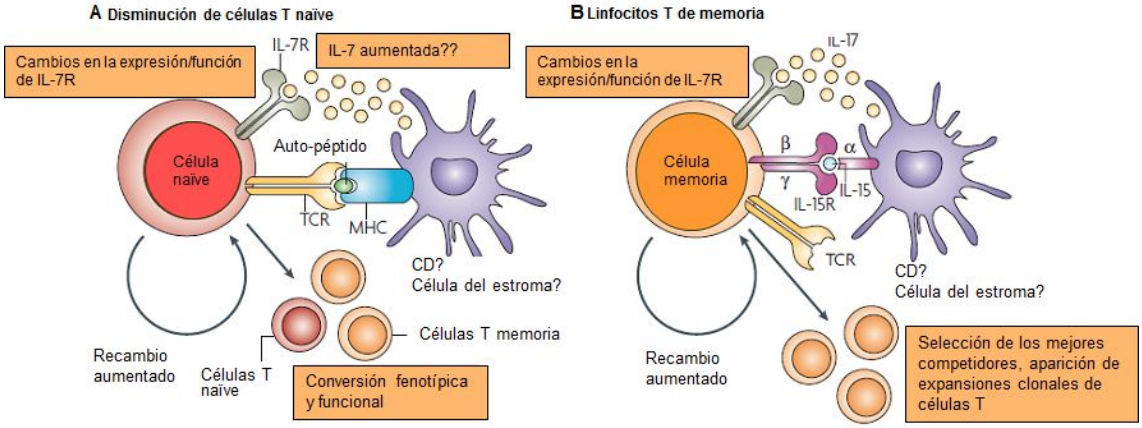

Figura 9. Posibles causas de la alteración de la homeostasis en linfocitos T *(*Adaptado de *Nikolich-Zugich, 2008, Nat Rev Immunol).*

Todos estos cambios se producen en paralelo, y es probable que estén muy influidos por los cambios en el perfil de citocinas. Es bien conocido que la producción IL-2 disminuye con la edad (Thoman & Weigle 1981). Por el contrario, está menos claro si existen alteraciones relacionadas con la edad en los niveles de IL-7 o IL-15 (Kim *et al.* 2006; Herndler-Brandstetter *et al.* 2012) o en la expresión y la función de sus receptores en diferentes subpoblaciones de células T (Messaoudi *et al.* 2006), aunque parece que en modelos animales, al menos la IL-15, disminuye con la edad (Quinn *et al.* 2010).

En resumen, el envejecimiento está asociado con un dramático aumento en la proporción de células T de memoria en sangre y con una disminución concomitante y equivalente en la proporción de células T naïve circulantes. En la figura 10 podemos ver las principales alteraciones celulares que aparecen en el proceso de inmunosenescencia.

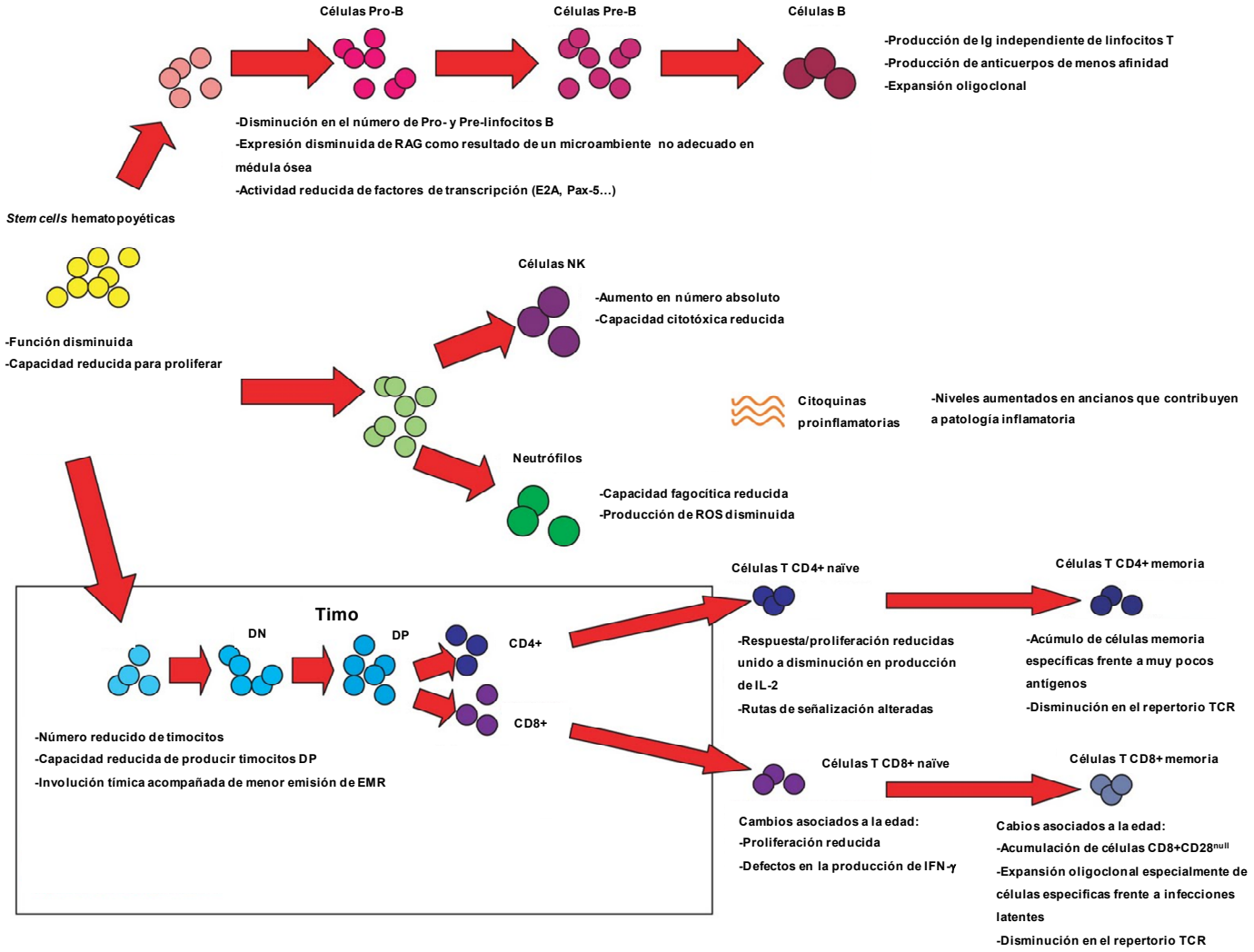

Figura 10. Efectos de la edad en la respuesta inmune innata y adaptativa.

I.1.3. RESPUESTA INMUNOLÓGICA EN ANCIANOS

En las personas de edad avanzada, el proceso de inmunosenescencia provoca un aumento de la susceptibilidad a las infecciones, que conlleva una mayor morbilidad y mortalidad en comparación con los adultos más jóvenes. La disminución de la respuesta inmune en ancianos hace que esta población sea cada vez más susceptible a enfermedades infecciosas como la gripe y la neumonía y conduce a la reaparición de infecciones latentes como el herpes zoster, así como a la infección por patógenos oportunistas como son las bacterias de los géneros *Clostridium* y *Staphylococcus*. Estas infecciones contribuyen de manera significativa a la morbilidad en este grupo de edad y con frecuencia conducen a una fragilidad irreversible y dependiente (Boraschi *et al.* 2010). Uno de los principales problemas de las infecciones en personas mayores es que con frecuencia aparecen con signos y síntomas inespecíficos y se pueden confundir con condiciones crónicas subyacentes (Ginaldi *et al.* 2001). La alta susceptibilidad a las infecciones en ancianos, además de depender del estado de inmunosenescencia, puede deberse también a diversos factores epidemiológicos, desnutrición, así como múltiples cambios fisiológicos y anatómicos asociados a la edad (Gavazzi & Krause 2002). Con el envejecimiento, la repuesta inmune frente a antígenos conocidos podría estar conservada, pero la capacidad para la inmunización frente a nuevos antígenos disminuye notablemente, lo que podría explicar la mayor predisposición a infectarse con nuevos patógenos. La evidencia epidemiológica revela que los individuos de mayor edad son a menudo los primeros en ser afectados por patógenos nuevos o emergentes como son el virus del Nilo Occidental, el virus del síndrome respiratorio agudo severo y las distintas pandemias de gripe (Haynes & Maue 2009; Ongradi & Kovesdi 2010). La epidemia del 2002 de virus del Nilo Occidental en Estados Unidos causó 284 muertes con una media de edad de los fallecidos de 78 años.

También se sabe que el proceso de inmunosenescencia está asociado con el desarrollo de tumores en personas de edad avanzada. El sistema inmune, interactuando íntimamente con las células cancerosas en un tumor, puede combatir o favorecer el desarrollo y/o la progresión del cáncer. Uno de los factores de riesgo más importantes para el desarrollo de cánceres sólidos es la edad. Los cambios más importantes que pueden disminuir la eficacia de la respuesta inmune son los cambios en las funciones y los fenotipos de las células T, concomitantes con la presencia de una inflamación de bajo grado. Un consenso está emergiendo en oncología que postula que no sólo las propias células de cáncer se deben estudiar, sino también sus macro y microambientes. En este contexto, el estudio de la interrelación de la respuesta inmune y el

tumor en varias etapas es esencial para mejorar nuestra capacidad de intervención en pacientes de edad avanzada con cáncer (Pawelec *et al.* 2010).

El envejecimiento afecta a todos los compartimentos de la respuesta inmune y tiene un impacto importante en el resultado del trasplante. Aunque los ensayos clínicos en la población anciana trasplantada siguen siendo escasos, nuestra comprensión actual de la inmunosenescencia nos proporciona una base para poder realizar una inmunosupresión y una asignación de órganos adaptada a la edad con el objetivo de optimizar la utilización y mejorar los resultados en los receptores de mayor edad (Heinbokel *et al.* 2013).

La vacunación exitosa contra agentes infecciosos de primer orden en las personas de edad avanzada representa una estrategia preventiva muy a tener en cuenta. Por desgracia, la inmunosenescencia no sólo deteriora la capacidad defensiva frente a las infecciones, sino también la capacidad para responder a la vacunación (Lang *et al.* 2011). La vacunación antigripal de adultos jóvenes proporciona un 65-80% de protección contra la enfermedad, mientras que la vacunación de las personas de edad avanzada garantiza solamente un 30-50% de protección (Nichol *et al.* 2007). Las personas de edad avanzada cuya capacidad de producir anticuerpos o desarrollar una buena respuesta celular frente a la vacunación está muy disminuida presentan un riesgo de infección elevado, pero incluso aquellos que responden a la vacunación muestran un título de anticuerpos y una respuesta celular reducida en comparación con los individuos jóvenes.

Los cambios del sistema inmune que se asocian con la inmunosenescencia y puede influir en la respuesta deficiente a la vacunación en ancianos son: una disminución en el número y funcionalidad de los linfocitos B; una función alterada de los linfocitos T CD4+; la reducción en el número de células T naïve como consecuencia de la involución tímica; el aumento de citocinas pro-inflamatorias; cambios cualitativos en los anticuerpos y una función de las células dendríticas alterada. Por otra parte, tres estudios realizados de forma independiente, indican que una disminuida capacidad de respuesta a la vacunación frente al virus de la gripe en ancianos se asocia significativamente con la presencia de una alta proporción de linfocitos T CD8+CD28^{null} (Goronzy *et al.* 2001; Saurwein-Teissl *et al.* 2002; Trzonkowski *et al.* 2003). De hecho, en muchas personas de edad avanzada, más del 50% de los linfocitos T CD8+ periféricos carecen de la molécula CD28 en comparación con <20% de los linfocitos T CD4+ que carecen de ella (Morley *et al.* 1995). Uno de los factores generadores de esta población CD28^{null}, con un fenotipo de memoria muy diferenciado, parecen ser los herpesvirus. Éstos al establecer infecciones latentes y persistir a lo largo de muchas décadas, requieren una inmunovigilancia continua, lo que conlleva la expansión clonal de linfocitos T específicos (Pawelec *et al.* 2004).

Es de destacar que tanto la falta de expresión de la molécula $CD28^{null}$ como la infección por CMV forman parte de un conjunto de parámetros inmunológicos que conforman el denominado perfil de riesgo inmunológico (IRP). El IRP se asocia con una mayor mortalidad a corto plazo en estudios longitudinales llevados a cabo en personas mayores (Ouyang *et al.* 2002). En principio este parámetro se definió a través de los estudios OCTO y NONA, dos estudios inmunológicos longitudinales que se realizaron en población sueca de octo y nonagenarios para establecer factores predictivos en longevidad en individuos que presentan diversos grados de discapacidad funcional, dicha discapacidad también se incluye en estos estudios (Wikby *et al.* 1998). El IRP deducido a través del estudio OCTO incluye altos niveles de células T CD8+, niveles disminuidos de células T CD4+ y de células B CD19+, cociente invertido CD4/CD8, y respuesta proliferativa disminuida a la concanavalina A (Ferguson *et al.* 1995). En el posterior estudio NONA, al IRP se añadieron características de inmunosenescencia, como un mayor número de células T $CD8+CD28^{null}$ efectoras y de memoria y una disminución en el número de células T naïve (Wikby *et al.* 2005). Posteriores estudios revelaron que el IRP se halla asociado con la infección por CMV que parece tener un papel incluso más importante de lo que se creía en la modulación del sistema inmunológico y también en comparación con otros herpesvirus, también examinados en estos estudios (Olsson *et al.* 2000) (Figura 11).

La acumulación de células T CD8+ específicas para CMV (Wikby *et al.* 2002), así como el hallazgo de que la mayoría de las expansiones clonales en las personas muy mayores son también específicas para el CMV, ha proporcionado apoyo adicional para la hipótesis de que el CMV contribuye notablemente al desarrollo del IRP y que por lo tanto constituye un buen biomarcador de inmunosenescencia en ancianos. Actualmente el IRP se caracteriza por una tasa de infección por CMV del 100% (en oposición a un 80% en ancianos con un IRP negativo) y una proporción CD4:CD8 inferior a uno, debido sobre todo, a la acumulación de células T CD8+ altamente diferenciadas, en particular $CD27^{null}$, $CD28^{null}$ y CD57+. Además, muchos de estos linfocitos T diferenciados han demostrado ser específicos para CMV (Ouyang *et al.* 2003a; Hadrup *et al.* 2006).

El estudio inmunitario NONA también evaluó el estado de inflamación crónico presente en los individuos de edad avanzada. Se encontró que tanto el IRP como la inflamación crónica eran buenos predictores de supervivencia y que este valor predictivo no se veía afectado por el estado de salud de los ancianos. Estos resultados sugieren que los procesos de inmunosenescecia que ocurren durante el envejecimiento y que afectan a los linfocitos T y producen un estado de inflamación crónico tienen una importancia crucial en la supervivencia en personas de edad muy avanzada (Wikby *et al.* 2006).

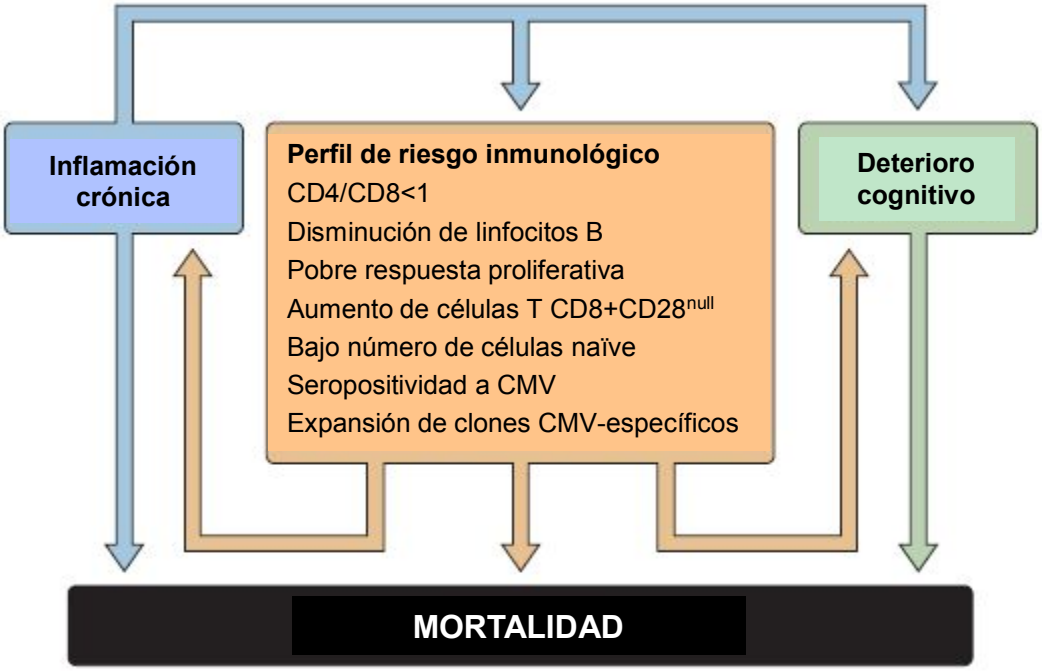

Figura 11. Interacción entre el IRP, inflamación crónica y deterioro cognitivo y su relación con la mortalidad *(*Adaptado de *Larbi et al., 2008, Physiology).*

Se postulan una serie de pasos que podrían comenzar a una edad temprana con la infección por CMV, seguido por los cambios homeostáticos que se producen en las células T, generación de grandes expansiones de células efectoras y de memoria $CD28^{null}$ y una disminución en el número de linfocitos T CD4+, y finalmente, el desarrollo del IRP. Esto va acompañado de una inflamación crónica, proceso que se produce en etapas muy avanzadas de la vida. Todo esto apoya la hipótesis de que el proceso de inmunosenescencia está impulsado por la presencia de antígenos crónicos, como podrían ser los portados por el CMV.

I.2. MARCADORES DE INMUNOSENESCENCIA

El continuo contacto de los linfocitos T con los patógenos a lo largo de la vida lleva a una disminución en la pool de linfocitos T naïve y a un aumento en el número de linfocitos T de memoria con un repertorio de TCR muy disminuido, producido por un aumento exponencial de los linfocitos T oligoclonales. Una de las principales características de estas células oligoclonales es la pérdida de la molécula coestimuladora CD28. A pesar de esta pérdida, estas células senescentes y oligoclonales son funcionalmente activas (Vallejo 2005) no son ni anérgicas ni

propensas a la apoptosis (Vallejo *et al.* 2000). Sin embargo, su función parece depender cada vez menos de la estimulación a través de TCR (Scheuring *et al.* 2002). Una explicación de por qué estas células son capaces de ser activadas, a pesar de su falta de molécula CD28, es la expresión de *novo* de varias moléculas propias de las célula NK, los llamados receptores relacionados con las células NK (NKRs) (Abedin *et al.* 2005; Vallejo *et al.* 2011). Entre los más estudiados se encuentran los receptores CD16, CD56, CD94, KLRG1, varios miembros de las moléculas de membrana de tipo 2 (NKG2) y los receptores de NK tipo inmunoglobulina (KIR) (Figura 12).

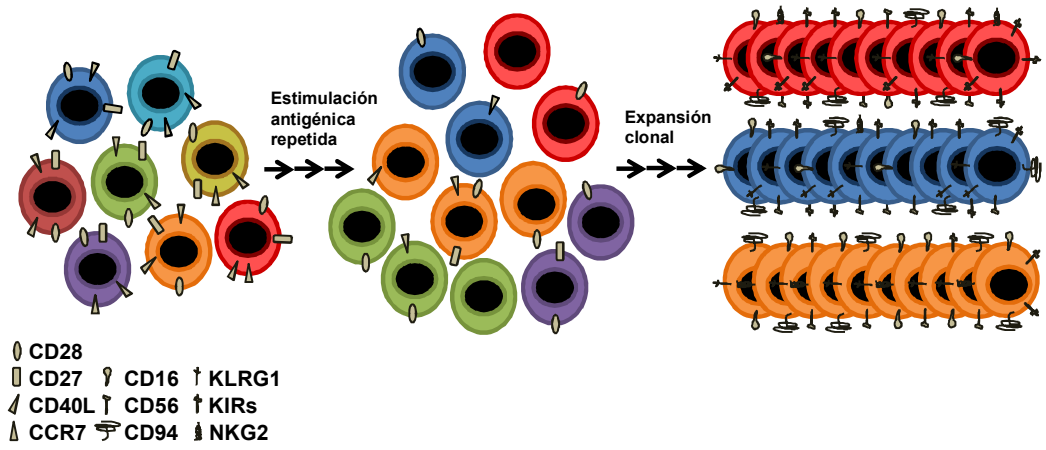

Figura 12. Las células T en personas mayores presentan un repertorio de receptores diferente de la de los individuos jóvenes. Las células T de los individuos de edad avanzada adquieren nuevas funciones relacionadas con la expresión de NKR como son los receptores KIR, CD94, CD16, CD56, NKG2 y KLRG1.

La expresión de estas moléculas se relaciona con un aumento en la capacidad citotóxica de las células, con aumentos en la expresión intracitoplasmática de perforina y granzima, pero con una capacidad disminuida de proliferar y de producir IL-2 (Tarazona *et al.* 2000; Brown *et al.* 2012). La expresión de estos receptores en los linfocitos T probablemente regule la citotoxicidad de estas células e incluso citocinas implicadas en la activación de las células NK, como la IL-15, son capaces de aumentar su capacidad citotóxica. La expansión de estas células no solo aparece en ancianos, sino también en algunas alteraciones clínicas que producen una activación permanente del sistema inmune, como las infecciones virales, enfermedades autoinmunes y reumáticas, algunos tumores y enfermedades de tipo cardiovascular (Thewissen *et al.* 2007; Zal *et al.* 2008; Alonso-Arias *et al.* 2009) (Figura 13).

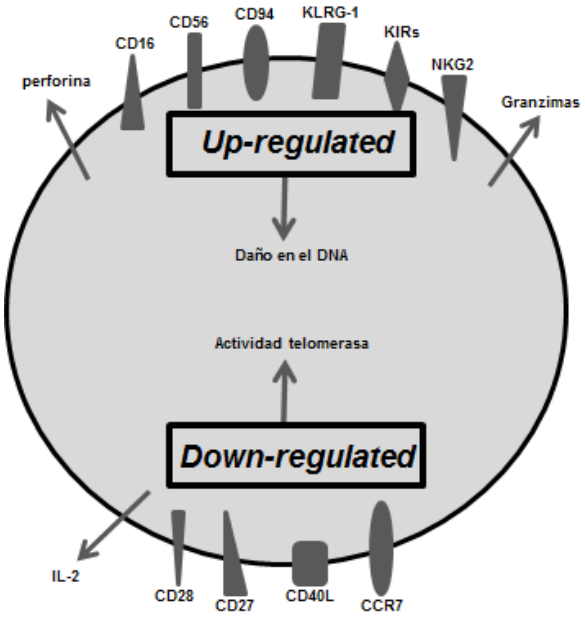

Figura 13. Resumen de los cambios que se producen en las céluas T exhaustas. La edad se asocia con varios cambios en el sistema inmune, especialmente en el fenotipo de las células T. Estas células pierden la capacidad de ir a órganos linfoides secundarios, aumentan la producción de citoquinas pro-inflamatorias y presentan una alta capacidad citotóxica.

NKG2D es un receptor activador que se expresa en células NK, células T CD8+ y en linfocitos T γδ (Bauer *et al.* 1999; Lopez-Larrea *et al.* 2008). Es una glicoproteína transmembrana, formada por dos dímeros unidos por puentes disulfuro que no presenta motivos de señalización en su región intracelular. Para suplir esta carencia, NKG2D tiene asociada de forma no covalente la proteína adaptadora DAP10 (Wu *et al.* 1999). Los ligandos de NKG2D pertenecen a una familia de glicoproteínas de superficie celular con homología estructural con las proteínas MHC de clase I (Raulet 2003; Gonzalez *et al.* 2008). En humanos, los ligandos de NKG2D son las cadenas asociadas a MHC de clase I (MICA y MICB) (Bauer *et al.* 1999) y la familia de proteinas de unión a UL-16 (ULBP, también llamadas RAET1) (Bacon *et al.* 2004; Lopez-Soto *et al.* 2006). Los ligandos de NKG2D MICA/B se sabe que son moléculas inducibles por estrés (Groh *et al.* 2001). Las principales actividades que se le atribuyen al receptor NKG2D son: respuesta frente a virus, implicación en inmunidad tumoral y papel en procesos autoinmunes. Dependiendo de la situación, el desarrollo de estrategias para bloquear o para aumentar la interacción entre el receptor NKG2D y sus ligandos pueden tener implicaciones importantes en diversos procesos patológicos.

El receptor NKG2D fue identificado inicialmente en las células NK, linfocitos T CD8+ y linfocitos T γδ, pero no en linfocitos T CD4+. Los linfocitos T CD4+NKG2D+ son un grupo particular de células T CD4+ y se han descrito en algunos pacientes con cáncer, en

enfermedades crónicas autoinmunes como la AR, en algún tipo de mielopatias, infecciones por retrovirus como HTLV y VIH y en la granulomatosis de Wegener (Groh *et al.* 2006; Capraru *et al.* 2008; Alonso-Arias *et al.* 2009; Matusali *et al.* 2013). Varios estudios han demostrado que la IL-15, una citocina secretada por multitud de células e inducida en infecciones virales e inflamación, aumenta y/o mantiene la expresión y la función de la molécula NKG2D en linfocitos T (Roberts *et al.* 2001). La IL-15 funciona como un regulador clave en la activación y función de los linfocitos T citotóxicos, entre ellos la población CD4+NKG2D+, permitiendo la expresión de esta molécula bajo condiciones inflamatorias. Se ha relacionado a la población linfocitaria CD4+NKG2D+ y la expresión de IL-15 con diversas patologías, como la AR (Groh *et al.* 2003).También se ha visto que la exposición de células mononucleares de sangre periférica a extractos de CMV in vitro induce la expresión de NKG2D+ en linfocitos T CD4+ (Saez-Borderias *et al.* 2006). Estos datos apoyan la hipótesis de que NKG2D funciona como un receptor coestimulador en un subgrupo de linfocitos T CD4+ CMV específicos y por lo tanto puede tener un papel en la respuesta contra las células infectadas que expresan HLA de clase II y ligandos de NKG2D (Jensen *et al.* 2012).

I.2.1. EL RECEPTOR CD28

La activación de las células T es un proceso que incluye varias etapas. En primer lugar, el receptor polimórfico TCR se une junto a los receptores CD4/CD8 con el complejo mayor de histocompatibilidad (MHC) de tipo I (CD8) o de tipo II (CD4) que llevará unido su antígeno específico y que se presentará en la superficie de las células presentadoras de antígeno (APC). Este contacto es la primera señal imprescindible para que se produzca la activación de los linfocitos (Mazza & Malissen 2007; Smeets *et al.* 2012). El complejo TCR presenta largas colas intracitoplasmáticas que transmiten la señal a otras moléculas a través de los motivos ITAM. Después de la unión del TCR con el MHC, muchos motivos ITAM se fosforilan y esto hace que un gran número de quinasas, moléculas adaptadoras y mediadores intermediarios sean reclutados (Guy & Vignali 2009). El reconocimiento antigénico no es suficiente para desencadenar una respuesta inmune, es necesaria una segunda señal para la activación linfocitaria. Estas señales son producidas por moléculas coestimuladoras, la mejor conocida de todas es el receptor CD28.

El receptor CD28 es una glicoproteína de membrana de tipo 1 que se expresa de manera constitutiva como un homodímero unido por puentes disulfuro en los linfocitos T CD4+ y CD8+ (Gross *et al.* 1990; Vallejo 2005). La proteína precursora del receptor CD28 presenta 220 aminoácidos y la proteína madura 202 debido a la escisión de una cola aminoterminal. Además,

la molécula CD28 posee una cola intracitoplasmática de 41 aminoácidos que es crítica para la transducción de señales y la posterior activación celular (Figura 14).

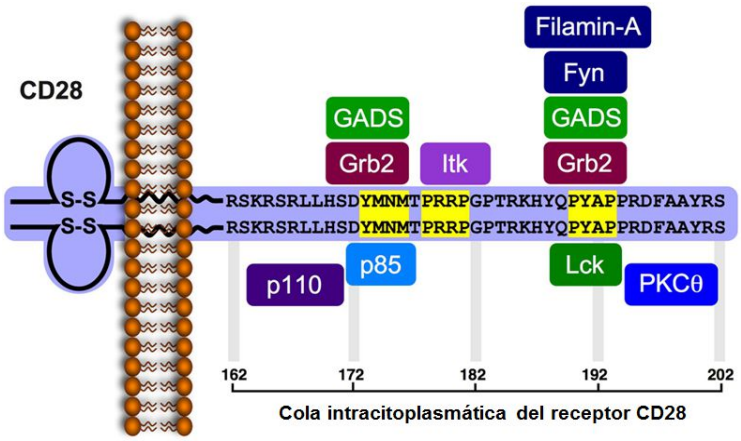

Figura 14. Representación esquemática y motivos activadores del receptor CD28 *(*Adaptado de *Isakov et al., 2012, Frontiers in Immunology).*

La unión del receptor CD28 con sus ligandos (B7-1, CD80; B7-2, CD86) hace que el umbral necesario para que se produzca la activación de los linfocitos T disminuya y produce un efecto amplificador de la primera señal producida por la unión del TCR con MHC. Así la unión TCR-MHC y CD28-B7 produce la activación de los linfocitos T (Appleman *et al.* 2002) (Figura 15).

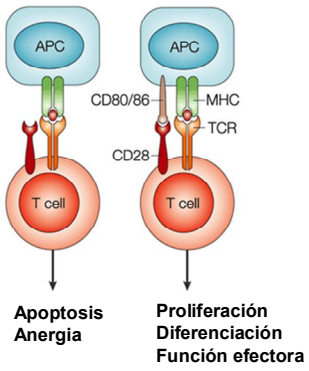

Figura 15. Unión TCR-MHC/CD28-CD80 en la sinapsis inmunológica *(*Adaptado de *Alegre et al., 2001, Nature Immunology Reviews).*

I.2.1.1. Pérdida del receptor CD28 en el proceso de inmunosenescencia

La pérdida de la principal molécula coestimuladora de los linfocitos T (CD28) a medida que los individuos envejecen está muy bien documentada tanto en CD4+ (Weyand *et al.* 1998) como en CD8+ (Nociari *et al.* 1999) (Figura 16). La pérdida de esta molécula se ha asociado con el proceso de inmunosenescencia, y por tanto, con una reducción de la respuesta inmune en ancianos. Se sabe que estas células son menos capaces de proliferar que las células CD28+, presentan un repertorio de reconocimiento antigénico muy disminuido y adquieren una gran capacidad citotóxica (Scheuring *et al.* 2002; Bryl & Witkowski 2004).

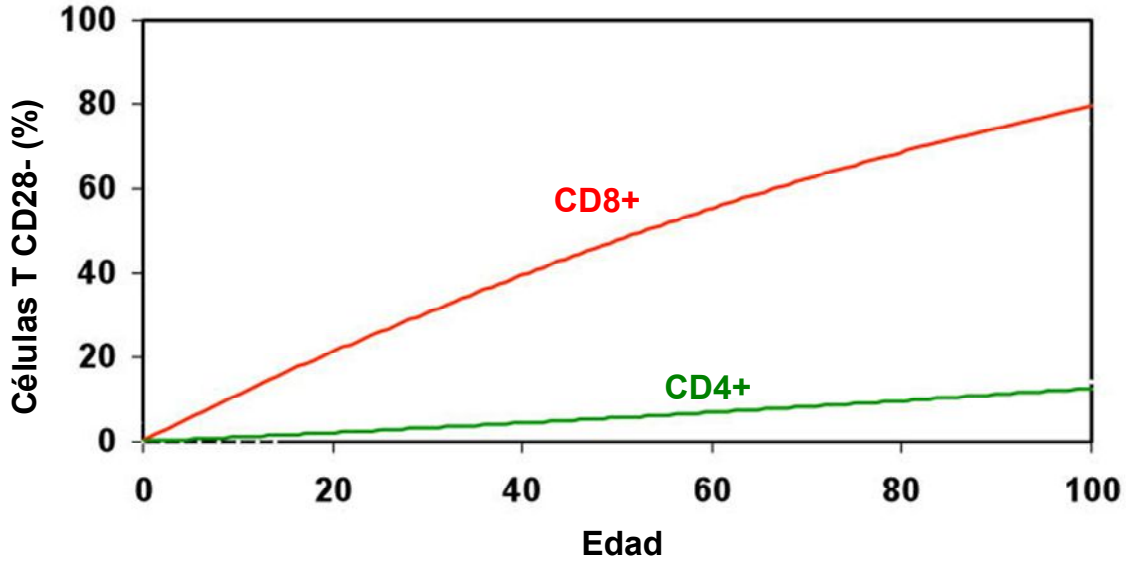

Figura 16. Aumento de la población $CD28^{null}$ con la edad *(*Adaptado de *Weng et al., 2009, Trends in Immunology).*

La pérdida de esta molécula se produce tras una persistente estimulación antigénica y tras cada ciclo de estimulación/proliferación la expresión de CD28 disminuye en la superficie de las células T, produciéndose un aumento de linfocitos T muy diferenciados. Esto se ve corroborado ya que las células que han perdido el receptor CD28 presentan telómeros más cortos que las células T CD28+, incluso cuando se estudian en la misma población clonal (Posnett *et al.* 1999). Esta disminución en la expresión de CD28 implica una regulación transcripcional y un aumento en la recaptación de esta molécula de la superficie celular mediante un mecanismo de feedback (Swigut *et al.* 2001).

Cuando las células T reconocen un antígeno, la expresión de CD28 decrece rápidamente, pero inmediatamente vuelve a valores normales. Sin embargo, cuando la estimulación antigénica persiste, la expresión disminuye y puede llegar a perderse. Inicialmente

se puede inducir la re-expresión de CD28 por IL-12 (Warrington *et al.* 2003) o con el tratamiento con agentes anti-TNF-α (Rizzello *et al.* 2006), pero una vez que la pérdida de CD28 está establecida, la re-expresión no es posible, por lo que se cree que se produce un silenciamiento a nivel transcripcional. Varios trabajos que utilizaron el promotor mínimo de CD28 demostraron que la activación transcripcional estaba alterada en las células $CD28^{null}$. Este defecto se ha relacionado con un único iniciador transcripcional (INR) en el promotor de CD28, que está formado por dos motivos (α y β) que unen diferentes proteínas, pero que funcionan como una unidad. La transcripción basal del gen CD28 está regulada por estas dos secuencias situadas "downstream" de una caja TATA atípica. La mutación o deleción de cualquiera de estos motivos es suficiente para inactivar el promotor CD28. En las células $CD28^{null}$, el αβ-INR se encuentra inoperativo por la falta de varios sitios de unión para factores de transcripción tanto del motivo α como β. Estos incluyen factores de transcripción de tipo general como los factores II-I y los componentes del factor de transcripción IID o proteínas reguladoras como YY1 y USF (Vallejo *et al.* 1998; Vallejo *et al.* 2001). De todas formas, el αβ-INR de CD28 no presenta homología con otros INRs, por lo tanto, existen moléculas específicas de unión a estos motivos como la nucleolina y la isoforma A de la ribonucleoproteína heterogénea (hnRNP-D0A), dos proteínas ubicuas en mamíferos (Vallejo *et al.* 2002). Los complejos de unión a DNA que se forman se pierden muy rápidamente en los linfocitos T CD8+ que están proliferando, mientras que son mucho más persistentes en las células T CD4+, lo que podría explicar la resistencia de las células T CD4+ a perder el receptor CD28 a medida que envejecemos.

I.2.1.2. Implicación patogénica de los linfocitos T $CD4+CD28^{null}$

Varios estudios han descrito la expansión de las células $CD4+CD28^{null}$ en la circulación periférica de pacientes con diversos trastornos inmunes, incluyendo enfermedades autoinmunes (Capraru *et al.* 2008), enfermedades crónicas inflamatorias (Allez *et al.* 2007; Alber *et al.* 2009) e inmunodeficiencias (Fernandez *et al.* 2011). Los pacientes que sufren estos trastornos a menudo muestran una expansión no propia de su edad de los linfocitos T $CD4+CD28^{null}$, indicando el envejecimiento prematuro de su sistema inmune (Fasth *et al.* 2009). Recientemente, se ha visto que las células $CD4+CD28^{null}$ son menos susceptibles a la supresión por células T reguladoras $CD4+CD25^{high}$ que las células T CD4+ convencionales (Thewissen *et al.* 2007). La capacidad citotóxica, su resistencia a apoptosis, y la infiltración en tejidos por parte de los linfocitos $CD4+CD28^{null}$ sugirieron que estas células podrían jugar un papel principal en el desarrollo y/o evolución de varias patologías. Donde primero se demostró esta participación fue en la AR, la

expansión de las células CD4+CD28^{null} se correlacionó positivamente con manifestaciones extra-articulares (Martens *et al.* 1997). Posteriormente la expansión de esta población de linfocitos se describió en la esclerosis múltiple, donde estas células se encontraron infiltradas en cerebro y médula espinal (Scholz *et al.* 1998).

Tabla 1. Características de las células CD4+CD28^{null} en diferentes desórdenes inmunológicos *(Bieke Broux et al., 2012, Trends in Molecular Medicine).*

Patología	Características	Correlación con la enfermedad
Artritis reumatoide	- Oligoclonales - Citotóxicas - Resistentes a apoptosis - Infiltradas en tejido - Autorreactivas (antígeno desconocido) - Pro-inflamatorias (IFN-γ, IL-2) - Diversidad TCR restringida - Expresión NKG2D	- Manifestaciones extra-articulares - Disminución tras tratamiento con anti-TNF
Esclerosis múltiple	-Oligoclonales - Citotóxicas - Infiltradas en tejido - Autorreactivas (MBP, MOG) - Pro-inflamatorias (IFN-γ) - Diversidad TCR restringida	- ND
Granulomatosis con poliangitis	- Infiltrados tisulares	- ND
Enfermedad de Graves	- Pro-inflamatorias (IFN-γ)	- Oftalmopatía de Graves - Anticuerpos anti-receptor de tirotropina
Lupus sistémico eritematoso	- Activacion vía NKG2D	- ND
Miopatías autoinmunes	- Pro-inflamatorias (TNF, IFN-γ) - Citotóxicas - Infiltrados tisulares - Diversidad TCR restringida	- % de células T disminuye con el aumento de la duración de la patología
Síndrome coronario agudo	- Pro-inflamatorias (IFN-γ) - Oligoclonales - Diversidad TCR restringida - Citotóxicas - Autorreactivas (hHSP60)	- Aterosclerosis severa - Disminuyen tras tratamiento con estatinas
Enfermedad intestinal inflamatoria	- Infiltrados tisulares - Citotóxicas - Pro-inflamatorias (TNF, IFN-γ, IL-2) - Activación vía NKG2D	- Fenotipo penetrante - % de células T disminuyen tras operación
Hepatitis B crónica	- Citotóxicas	- Alta carga viral - Niveles elevados de aminotransferasas
Virus inmunodeficiencia humana	- Pro-inflamatorias (IFN-γ)	- ND

ND, no determinada; TCR, receptor de células T; MBP, proteína básica de la mielina; MOG, glicoproteína oligodendroglial de la mielina; hHSP60, proteína de choque término 60 humana.

Posteriormente estas células fueron identificadas en granulomatosis con poliangitis (antiguamente granulomatosis de Wegener), enfermedad de Graves, lupus sistémico eritematoso y en miopatías autoinmunes, entre otros. Recientemente las células CD4+CD28^{null} se han descrito en patología cardiovascular (síndromes coronarios agudos, anginas estables, aterosclerosis), enfermedad inflamatoria intestinal (enfermedad de Crohn y colitis ulcerosa) y en infecciones virales (VIH, hepatitis B). Un resumen de las características de la población CD4+CD28^{null} en estas patología se muestra en la Tabla 1.

Aunque ya se han realizado algunas investigaciones respecto a la modulación de esta población de células, no todos los enfoques terapéuticos funcionan igual en los diferentes trastornos inmunológicos. Por ejemplo, la inhibición del TNF-α es un tratamiento estándar para los pacientes con AR, pero puede tener consecuencias perjudiciales para pacientes con esclerosis múltiple (van Oosten *et al.* 1996). Por lo tanto, se debe seguir buscando un agente modulador potente y ampliamente utilizable capaz de dirigirse específicamente a las células CD4+CD28^{null} en una amplia gama de trastornos del sistema inmune.

I.3. INTERLEUCINA 15 (IL-15)

La IL-15 es una citoquina pleiotrópica perteneciente a la familia de citocinas cuyos receptores comparten la cadena gamma como transductora de señales. A esta familia también pertenecen la IL-2, IL-4, IL-7, IL-9 e IL-21, pero también factores de crecimiento como el factor estimulante de colonias de granulocitos y macrófagos (GM-CSF), factor estimulante de colonias de granulocitos (G-CSF), eritropoyetina y hormonas clásicas, incluyendo la hormona de crecimiento humana y la prolactina (Bazan 1990) (Figura 17). El mRNA de la IL-15 es expresado constitutivamente por monocitos/macrófagos, CD, queratinocitos, células epiteliales, fibroblastos, células nerviosas, riñones, placenta, pulmones, corazón y músculo esquelético (Budagian *et al.* 2006). La IL-15 no solamente utiliza las subunidades β (IL-2/IL-15Rβ) y γ del complejo del receptor de la IL-2 para ejercer sus funciones, sino que también se une a un componente único, el IL-15Rα, que existe unido a membrana y en forma soluble. El complejo IL-15R se expresa en monocitos, CD, células NK, linfocitos T y fibroblastos. Una característica única de la IL-15 y del IL-15R es la capacidad que tienen de estimular células vecinas a través de un mecanismo conocido como trans-presentación (Stonier & Schluns 2010). Este mecanismo consiste en que, al contrario de otras citocinas que son secretadas, la IL-15 se encuentra unida al receptor de alta afinidad IL-15Rα. Cuando el complejo IL-15/IL-15Rα es enviado a la superficie celular, puede estimular otras células que presenten el complejo β/γ_C del receptor de la IL-15.

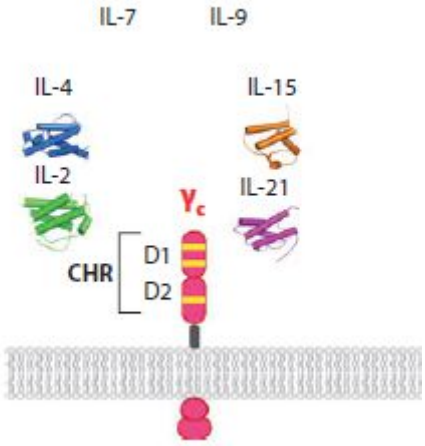

Figura 17. Receptor de citoquinas compartido γ_c. (Adaptado de *Wang et al., 2009, Annu. Rev. Immunol).*

La IL-15 ejerce profundos efectos en la proliferación, la supervivencia y la diferenciación de diferentes tipos de células. La IL-15 regula la activación y proliferación de células T y NK y favorece la aparición de señales de supervivencia que mantienen vivas a las células T de memoria en ausencia de antígeno. Esta citocina también está implicada en el desarrollo de las células NK. En los linfocitos de roedores, la IL-15 previene la apoptosis mediante la inducción de un inhibidor de la apoptosis, BCL2L1/BCL-x(L) (Oh *et al.* 2008). En los seres humanos la IL-15 suprime la apoptosis en los linfocitos T mediante la inducción de Bcl-2 y/o Bcl-xL, como por ejemplo ocurre en enfermedad celíaca (Malamut *et al.* 2010). La IL-15 aumenta la citotoxicidad de las células NK, así como la producción de las citocinas derivadas de NK, como el IFN-γ, GM-CSF y TNF-α (Fehniger & Caligiuri 2001) y es muy importante en la maduración funcional de los macrófagos y CD en respuesta a infecciones microbianas. También aumenta la capacidad fagocítica de monocitos y macrófagos e induce la producción de factores pro-inflamatorios como la IL-8 y la proteína quimioatrayente de monocitos (MCP-1) (Carroll *et al.* 2008). La tabla 2 resume los diferentes efectos de la IL-15 sobre las células del sistema inmune.

Tabla 2. Principales efectos biológicos de la IL-15 sobre las células inmunes (Di Sabatino et al., 2011, Cytokine & Growth Factor Reviews).

Célula diana	Efecto funcional IL-15
Células NK	- Induce desarrollo, crecimiento y homeostasis - Aumenta la citotoxicidad y la producción de citocinas - Regula la interacción de las células NK con CD y macrófagos
Monocitos/Macrófagos	- Aumento de la actividad fagocítica - Inducción de la producción de factores pro-inflamatorios
Células dendríticas	- Protección frente a apoptosis - Aumento de la expresión de moléculas coestimuladoras - Aumento de la producción de IFN-γ - Aumento de la capacidad de las CD de estimular a las células T
Neutrófilos	- Induce cambios morfológicos - Aumenta la fagocitosis - Inhibe la apoptosis - Promueve la síntesis y liberación de IL-8
Eosinófilos	- Inhibe la apoptosis - Estimula la producción de GM-CSF
Mastocitos	- Aumenta la proliferación - Inhibe la apoptosis
Células NK-T	- Induce la expansión y controla la homeostasis
Linfocitos intraepiteliales	- Regula el desarrollo y homeostasis - Inhibe apoptosis
Linfocitos T CD8+	- Regula la generación y mantenimiento de células memoria - Aumenta la capacidad citotóxica y la producción de citocinas - Inhibe la apoptosis
Linfocitos T CD4+	- Apoya la supervivencia junto a IL-7
Linfocitos B	- Induce proliferación - Inhibe la apoptosis - Aumenta la producción de citocinas
Linfocitos T_{reg}	- Induce diferenciación *in vitro*
Linfocitos Th17	- Aumenta la proliferación dependiente de TCR

I.3.1. IL-15 E INMUNOSENESCENCIA

Está ampliamente aceptado que la señalización mediante el receptor de la IL-7 (IL-7R) es esencial para prolongar la supervivencia y la proliferación de los linfocitos T naïve y memoria.

Las células naïve dependen para su supervivencia de señales producidas mediante el contacto con MHC propios más la IL-7. Por otra parte, las células T memoria son típicamente independientes de contacto con MHC y su supervivencia y proliferación homeostática dependen de IL-7 e IL-15 (Boyman *et al.* 2012) (Figura 16).

Ambas citoquinas parecen igualmente esenciales para permitir a estas células llevar a cabo una proliferación homeostática, pero la IL-15 parece tener un papel menos importante en las células T CD4+ memoria que en los linfocitos T CD8+ memoria y células NK (Surh & Sprent 2008). Las células memoria CD4+ compiten menos eficientemente por la IL-15 que los linfocitos CD8+ y las células NK ya que los linfocitos T CD4+ presentan unos niveles mucho menores del receptor IL-15R (Lenz *et al.* 2004). La proliferación homeostática de los linfocitos T puede ser una de las principales causas de la pérdida de la molécula CD28 asociada a la edad, ya que las células T CD8+ memoria, en presencia de IL-15, sin estimulación vía TCR, pierden la molécula CD28 y siguen proliferando con una tasa igual que las células CD8+CD28+ (Chiu *et al.* 2006). Estudios recientes han demostrado que la IL-15 juega un papel muy importante en la proliferación de las células T CD4+ bajo condiciones fisiológicas y después de estimulación *in vitro*, sobre todo en la población $CD28^{null}$ (Lenz *et al.* 2004; Alonso-Arias *et al.* 2011). Las células T CD4+ memoria utilizan el STAT5, el factor de transcripción utilizado por la IL-15, mucho más que las células efectoras (Tripathi *et al.* 2010). Aunque la actividad citotóxica de los linfocitos T CD4+ aún no se entiende completamente, el aumento de esta capacidad por la IL-15 puede tener un gran impacto, ya que las células $CD4+CD28^{null}$ son principalmente específicas frente a antígenos crónicos, como el CMV y el VIH. Además la IL-15 juega un papel primordial en la respuesta inmune temprana a las infecciones y en la inflamación crónica ampliando los efectos pro-inflamatorios y aumentando la respuesta antígeno especifica (Smeltz 2007; Alonso-Arias *et al.* 2011).

I.4. IMPACTO DE LAS INFECCIONES LATENTES EN INMUNOSENESCENCIA

Cuando se produce una invasión por patógenos, las células T específicas para los epítopos del patógeno sufren una marcada expansión clonal, de manera que una única célula T naïve puede generar hasta 10^5 células T efectoras. Una vez el patógeno es eliminado, más del 90% de estas células se eliminan por apoptosis, y las que quedan son reclutadas para engrosar la población de células T memoria (Figura 18A). Si el patógeno no se elimina, es decir, si se trata de un patógeno persistente se puede establecer una infección crónica o latente. Estos dos tipos de infección difieren en la manera en que estimulan al sistema inmunológico. La replicación de

los patógenos que provocan una infección crónica nunca es verdaderamente controlada por el sistema inmune, aunque el nivel de patógenos en sangre probablemente sea inferior a la medida en el pico de la infección aguda, estos agentes patógenos se replican constantemente y mantienen una carga viral más o menos constante en el huésped. Por lo tanto, las células inmunológicas, incluidas las células T, están de forma constante y sistemática, estimuladas por estos patógenos. En muchos, sino la mayoría de los casos, estos patógenos causan enfermedades crónicas que duran años y tienen a menudo un mal pronóstico, en parte porque se agotan las células T que intervienen en su control (Figura 18B).

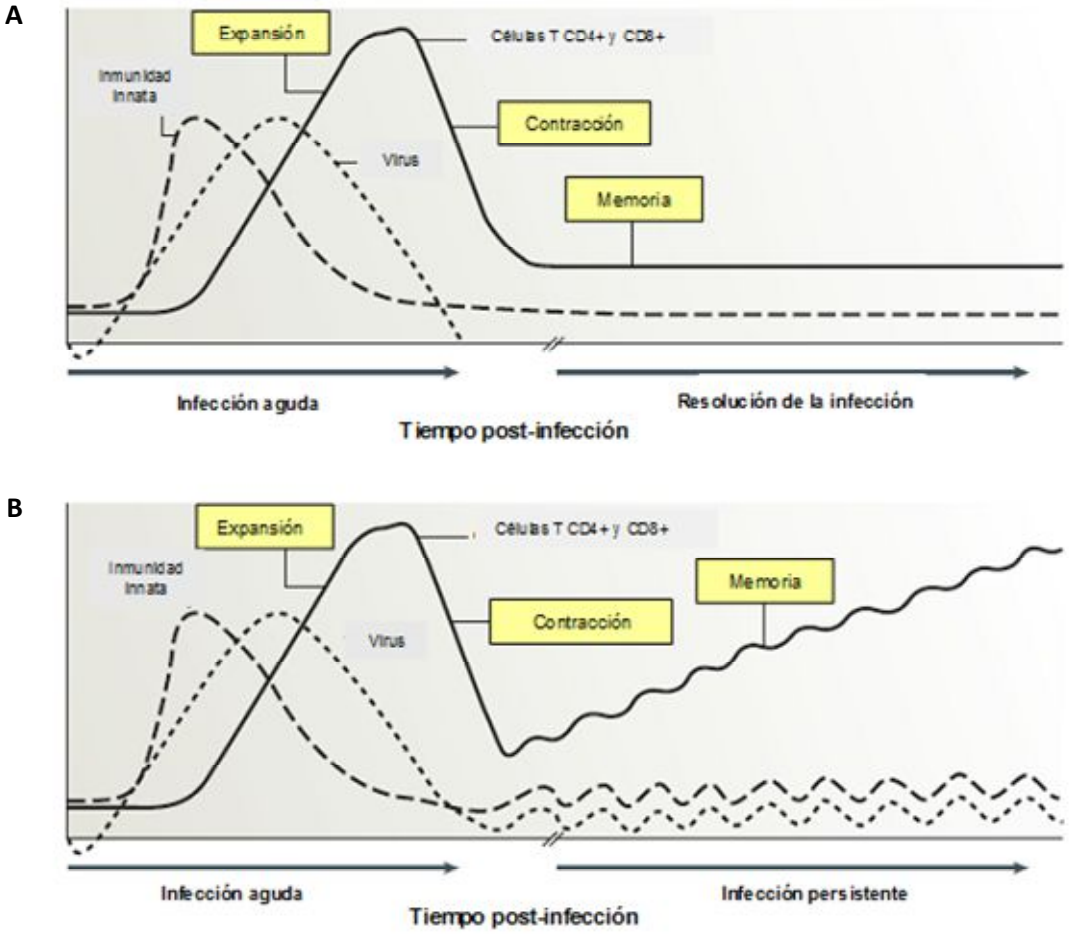

Figura 18. Impacto de los agentes patógenos sobre el sistema inmune, (A) infección aguda resuelta, (B) infección persistente *(*Adaptado de *Nikolich-Zugich, 2008, Nat Rev Immunol).*

En contraste con las infecciones crónicas, en las infecciones latentes el patógeno es controlado sistemáticamente y por lo tanto no es detectable en sangre en todo momento (Figura 19). En ocasiones, el patógeno en latencia se reactivará, momento en el que puede ser detectado a nivel sistémico o local. Esto, puede estimular las células T, principalmente linfocitos T de memoria, lo que obligará a los patógenos a pasar a su estado latente. Sin embargo, esta

estimulación intermitente puede conducir a la expansión de células T específicas frente a este patógeno. Aunque la estimulación de las células T en las infecciones latentes se repite, no es un proceso continuo y por lo tanto no está asociado con un gran agotamiento y/o eliminación de las células T, como ocurre en las infecciones crónicas. De hecho, es raro que se produzca una pérdida del control inmunológico sobre la infección latente, incluso a edades avanzadas, a menos que se produzca una inmunosupresión independiente de la edad. Esta vinculación entre infección latente y expansión de células T se ha visto corroborada por evidencias experimentales y clínicas (Olsson *et al.* 2000; Pawelec *et al.* 2004; Akbar & Fletcher 2005; Almanzar *et al.* 2005)

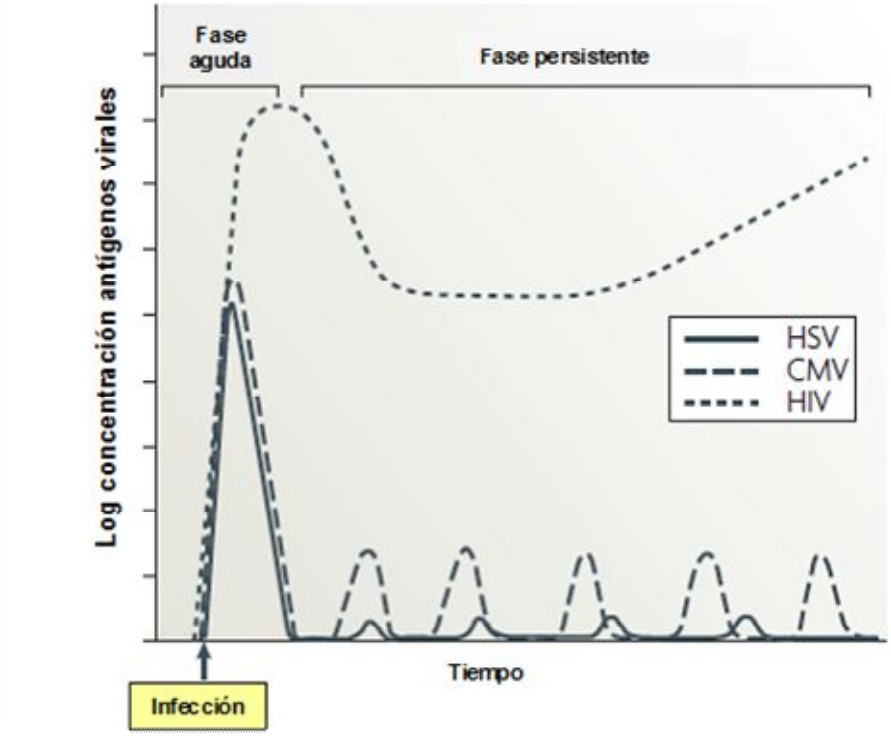

Figura 19. Virus persistentes y su carga antigénica a lo largo del tiempo. Citomegalovirus (CMV), virus del herpes simple (VHS), virus de la inmunodeficiencia humana (VIH) *(*Adaptado de *Nikolich-Zugich, 2008, Nat Rev Immunol).*

I.4.1. INMUNOSENESCENCIA Y HERPESVIRUS

Los patógenos candidatos a ejercer el máximo impacto sobre el sistema inmunológico en el proceso de envejecimiento deben estar muy adaptados al huésped de manera que no generen enfermedad. Deben ser patógenos ubicuos, y capaces de infectar a la mayoría de la población poco tiempo después del nacimiento. Aunque hay numerosos agentes patógenos humanos que persisten después de producirse la infección primaria, incluyendo micobacterias y helmintos, la familia de patógenos que mejor se corresponde con la descripción anterior es el grupo de los Herpesviridae (Lodoen *et al.* 2003).

Los herpesvirus normalmente establecen latencia en determinados tipos de células o tejidos y se sirven de múltiples mecanismos para evadir la inmunidad innata y adaptativa (Johnson & Hill 1998; Novak & Peng 2005). VHS, VEB y CMV son probablemente los herpesvirus que están más vinculados a la expansión de las células T en personas de edad avanzada (Pawelec *et al.* 2004). Entre ellos, el CMV se destaca porque tipifica las características clave de la familia de los herpesvirus que influyen en el envejecimiento de las células T. En primer lugar, la prevalencia del CMV varía entre el 60% en los países industrializados y cerca del 100% en países en desarrollo, con una prevalencia muy superior en ancianos, hasta el 90%, incluso en los países industrializados. En segundo lugar, la respuesta de las células T CD8+ contra el CMV es extraordinariamente amplia, dirigido frente a numerosos péptidos virales (Sylwester *et al.* 2005; Munks *et al.* 2006). Esta respuesta también muestra una característica inusual y es la producción de manera progresiva, de una expansión a largo plazo de las células T CD8+ de memoria específicas frente a antígenos de CMV (Ouyang *et al.* 2004). De hecho, la respuesta de las células T CD8+ a epítopos individuales de este virus puede llegar hasta 20% del total de células de memoria y la respuesta a todos sus epítopos se estima que puede llegar al 50% o más de las células T CD8+ de memoria. En cuanto a los linfocitos T CD4+, tradicionalmente no se han relacionado con la citotoxicidad específica frente a CMV en la misma medida que las células T CD8+, a pesar de que la existencia de células CD4+ citotóxicas frente a células infectadas por CMV ya se había descrito en 1986 (Lindsley *et al.* 1986). La citotoxicidad en las células T CD4+ se había descubierto incluso antes (Krensky *et al.* 1982). Appay et al. Confirmaron en 2002 que las células CD4+CD28^{null} podían lisar células diana (Appay *et al.* 2002). Sin embargo, la verdadera implicación de las células T CD4+ en la respuesta inmune a CMV no estuvo clara hasta que en 2005 se publicó un trabajo (Sylwester *et al.* 2005) que cambió la manera de pensar sobre la respuesta de los linfocitos T CD4+ y CD8+ a CMV. Con este trabajó se vio que los linfocitos T de una población de 33 individuos reconocían al menos 151 proteínas de CMV, los linfocitos T CD4+ reconocían 125 proteínas y los CD8+ 107 y además, 87 proteínas eran reconocidas por las dos poblaciones simultáneamente.

Esta respuesta progresiva y tan pronunciada se ha asociado con una acumulación de células T disfuncionales específicas para el CMV, así como con una disminución en la esperanza de vida en gente de edad avanzada (Ouyang *et al.* 2003b; Wikby *et al.* 2005), aunque la naturaleza de la relación entre estas asociaciones no esté muy clara en la actualidad. La seropositividad al CMV y el título de anticuerpos anti-CMV están relacionados con el grado de diferenciación de los linfocitos T y a los parámetros del IRP en ancianos (Olsson *et al.* 2000; Alonso Arias *et al.* 2013). A pesar de todas las evidencias que sugieren que el CMV induce el

envejecimiento de los linfocitos T, la reactivación más frecuente y/o las reactivaciones intensas en los ancianos pueden ser una consecuencia más que la causa de la inmunosenescencia observada.

I.5. ESTRATEGÍAS PARA REVERTIR EL PROCESO DE INMUNOSENESCENCIA

El envejecimiento reduce el número y el potencial hematopoyético de los precursores de las células T, además la involución tímica lo hace incapaz de realizar la diferenciación de los linfocitos T con normalidad. Por todo esto, la edad compromete la capacidad funcional de los linfocitos, haciendo que la población de linfocitos T en ancianos tenga un repertorio de especificidad antígenica muy reducido (Akbar & Fletcher 2005; Colonna-Romano *et al.* 2007). Varios agentes parecen tener capacidad de revertir este proceso en personas con un proceso de inmunosenescencia avanzado. Se han realizado varios estudios sobre los efectos de distintas sustancias inmunomoduladoras (Figura 20), el efecto del consumo de probióticos y la realización de ejercicio moderado en la reconstitución del repertorio de linfocitos T y en el efecto anti-inflamatorio que estos agentes pueden tener sobre los individuos de edad avanzada.

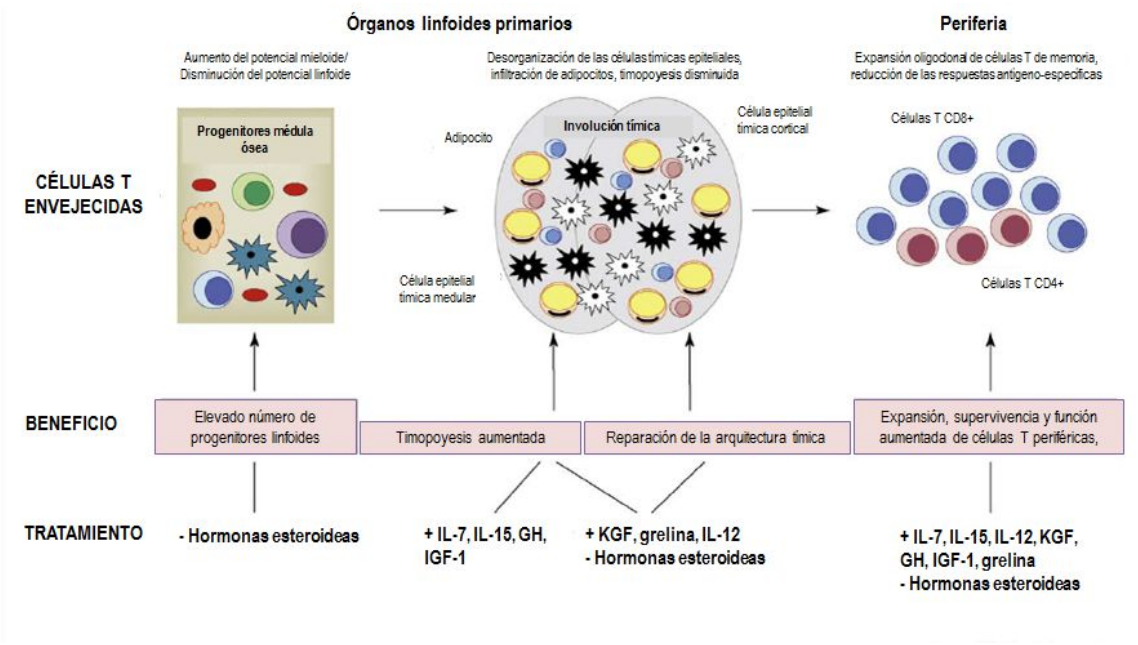

Figura 20. Distintas estrategias para potenciar la función inmune mejorando el desarrollo, expansión y función de las células T *(*Adaptado de *Amanda M Holland, 2009, Current Opinion in Immunolgy).*

Los probióticos pueden modular la función de las células del sistema inmune y regular el estado inflamatorio en las personas de edad avanzada. La interacción entre las células inmunes y el probiótico puede producirse de varias formas y desencadenar así sus efectos

inmunomoduladores (Figura 21). Uno de los procesos que más beneficios podría acarrearles a las personas de edad avanzada sería la anti-inflamación que diversas cepas de probióticos pueden producir. La infección de la línea celular humana Caco-2 por *Shigella flexneri* normalmente induce una respuesta pro-inflamatoria, pero la preincubación de las células Caco-2 con el probiótico *Lactobacillus casei* DN-114 001 reduce la respuesta inducida por *S. flexneri* a través del bloqueo de la activación de NF-κβ (Tien *et al.* 2006). El TNF induce la apoptosis de células epiteliales de colón en ratones, pero esto se puede inhibir mediante el aporte de *Lactobacillus rhamnosus* GG que activa la proteína anti-apoptótica Akt (Yan & Polk 2002).

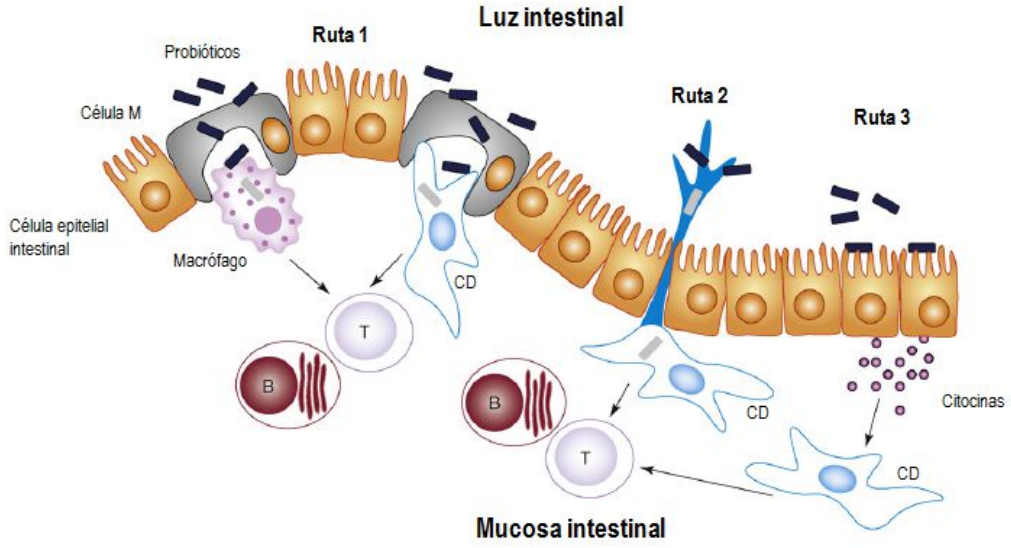

Figura 21. Posibles vías de contacto entre el sistema inmune y lo probióticos T (Adaptado de *Kan Shida et al., 2008, Trends in Immunology).*

Otro de los mecanismos por los que los probióticos podrían ejercer su efecto anti-inflamatorio es la inducción de la producción de IL-10. La adición de algunas cepas de *Lactobacillus* a altas dosis en cultivos de macrófagos o CD de ratón aumenta los niveles de producción de IL-10 y disminuye los de la citocina pro-inflamatoria IL-12. (Christensen *et al.* 2002; Shida *et al.* 2006). También se ve aumentada la producción de IL-10 por células Tr1 tras ponerlas en contacto con algunas cepas de *Bifidobacterium* (Jeon *et al.* 2012). Los probióticos también podrían ejercer su acción anti-inflamatoria inhibiendo la secreción de citocinas inflamatorias como la IL-6 y la IL-8. El complejo de polisacáridos y peptidoglicano de *L. casei* Shirota inhibe la producción de IL-6 en PBMCs estimuladas con lipopolisacárido bacteriano en pacientes con colitis ulcerosa (Matsumoto *et al.* 2005). Células Caco-2 pretratadas con *L. rhamnosus* GG vivo y muerto por calor secretaron concentraciones mucho menores de IL-8 tras estimulación con TNF que células no pretratadas con probiótico (Zhang *et al.* 2005). Aunque los

mecanismos exactos de estos efectos anti-inflamatorios no se conocen se ha propuesto que los probióticos suprimen la activación de la vía del NFκβ inhibiendo la degradación de IκB en las células epiteliales intestinales, inhibiendo así la respuesta inflamatoria en la mucosa intestinal (Jijon *et al.* 2004).

Cada vez hay más evidencias que muestran que el consumo de probióticos aumenta la inmunidad innata, incluyendo la capacidad fagocítica de los neutrófilos y la capacidad citotóxica de las células NK. Tras la suplementación dietética con *L. rhamnosus* HN001 se ha observado un aumento del número de células NK (Gill *et al.* 2001) y además *L. casei* Shirota aumenta la citotoxicidad de estas células frente a las células K562 (Takeda *et al.* 2006).

Por otra parte, los efectos de los probióticos también producen cambios en las poblaciones linfocitarias, pudiendo modular el balance Th1:Th2 hacía Th1 indirectamente a través del estímulo de monocitos, macrófagos y CD. También se ha propuesto que los probiótiocos son capaces de inducir la formación de células Treg a través de la estimulación de las células epiteliales intestinales. De hecho, ciertas cepas de *Lactobacillus* pueden estimular a células Caco-2 a secretar TGF-β, así como linfopoyetina estromal tímica (TSLP) y estos dos factores juntos promueven la diferenciación de CD inmaduras a CD reguladoras, que a su vez inducen la formación de células Treg productoras de TGF-β (Zeuthen *et al.* 2008; Smelt *et al.* 2012).

Por lo tanto, los probióticos tienen el potencial de inducir tanto la formación de células pro-inflamatorias Th1, así como células anti-inflamatorias como las Treg. Esta dicotomía podría ser explicada por las diferencias que encontramos entre distintas cepas de probióticos. Alternativamente, diferentes rutas de administración del probiótico pueden producir distintas respuestas inmunes. Parece ser que la administración parenteral o adición a cultivos in vitro de los probióticos parece inducir una respuesta Th1, la administración oral lleva a efectos inmunomoduladores promoviendo el desarrollo de células Treg en el microambiente que encontramos en el tejido linfoide asociado a mucosas (GALT).

La población de células altamente pro-inflamatorias Th17 es abundante en intestino y se ha visto que la administración oral de probióticos podría afectar el desarrollo de estas células y mejorar los síntomas clínicos en las patologías en que esta población está involucrada (Tanabe 2013). En resumen, los probióticos podrían tener un papel en el control de muchas de las respuestas inmunitarias con su papel inmunoregulador en el intestino (Figura 22).

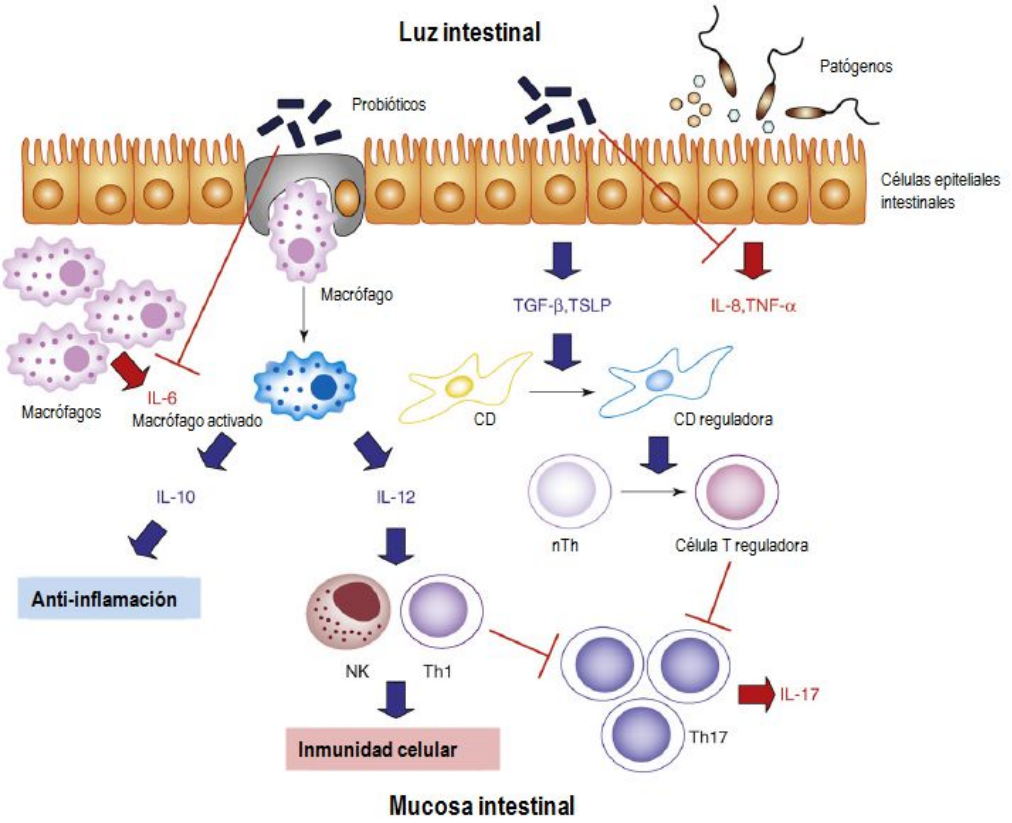

Figura 22. Circuitos inmunes en el intestino modulados por probióticos *(*Adaptado de *Kan Shida et al., 2008, Trends in Immunology).*

El ejercicio físico regular se está postulando como una estrategia alternativa económica y segura para combatir los efectos detrimentales de la inmunosenescencia que se inducen en la vejez y con las reactivaciones virales (Simpson & Guy 2009). A pesar de los beneficios conocidos del ejercicio habitual en varios aspectos de la inmunidad relacionados con la inmunosenescencia, los mecanismos subyacentes no se conocen completamente. Una sesión de ejercicio agudo produce una movilización preferencial de células T altamente diferenciadas y senescentes (muchas de las cuales son específicas para CMV) desde los tejidos periféricos a la sangre antes de ser rápidamente eliminadas del torrente circulatorio (Simpson 2011). Así, sesiones frecuentes de ejercicio agudo podría servir para "crear espacio" inmunológico para células T naïve y así aumentar el repertorio antigénico de esta población celular.

A pesar de que está ampliamente aceptado que la realización de ejercicio de moderada intensidad de manera habitual puede ayudar a prevenir el declive funcional del sistema inmune en los ancianos, no está tan claro si el ejercicio también podría ayudar a restaurar la funcionalidad perdida (Simpson & Guy 2010).

La realización habitual de ejercicio ejerce sus efectos beneficiosos sobre la inmunidad en ancianos mediante dos mecanismos, uno preventivo y otro restaurativo o de tratamiento.

Desde el punto de vista preventivo, el ejercicio regular podría ayudar a restringir las oportunidades que tienen los virus latentes para reactivarse y así atenuar sus efectos perjudiciales a lo largo de la vida. Esto puede estar causado por un efecto indirecto (se sabe que el ejercicio afecta a otros factores que están implicados en las reactivaciones virales como el estrés) y/o de manera directa mediante mecanismos que aún han de ser investigados. Al restringir el potencial para las reactivaciones del CMV con el ejercicio podría evitar la acumulación de células T altamente diferenciadas que ocupan espacio inmune que podría ser rellenado con células T naïve. Desde el segundo punto de vista, el de usar el ejercicio como tratamiento, el ejercicio podría movilizar a las células senescentes resistentes a la apoptosis de los tejidos donde se encuentran a otros donde sí sufren apoptosis, dejando espacio libre para nuevas células T naïve (Hoffman-Goetz & Quadrilatero 2003; Kruger *et al.* 2008; Kruger *et al.* 2011). Para que esto ocurra deben de producirse tres fases: primero una movilización selectiva de las células T senescentes de los tejidos periféricos a sangre durante el ejercicio; a continuación debe producirse una extravasación de células T senescentes desde la sangre y su posterior apoptosis en tejido periféricos durante la recuperación del ejercicio; por último, una regeneración de células T naïve para reemplazar las células T senescentes (Figura 23).

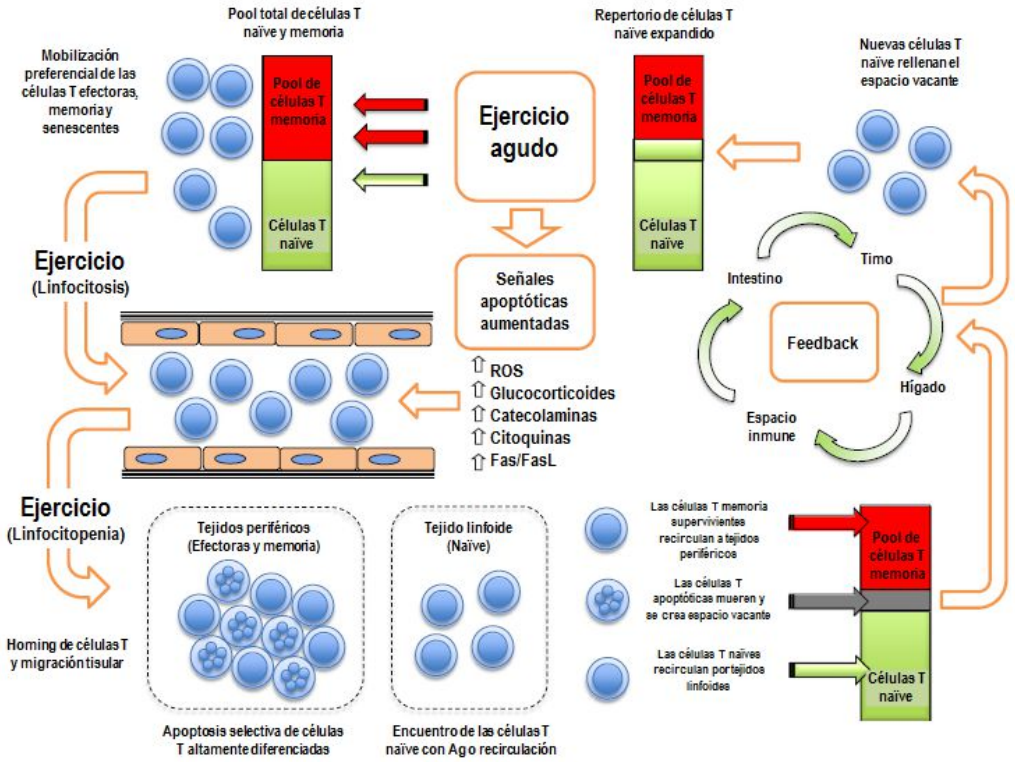

Figura 23. Modelo hipotético del efecto del ejercicio agudo en el espacio inmunológico *(*Adaptado de *Simpson RJ et al., 2008, Exercise and Sport Sciences Reviews).*

Por otra parte, el ejercicio posee efectos muy potentes y de gran alcance en el sistema inmunológico, siendo uno de los principales su capacidad anti-inflamatoria. Aunque en un primer momento se pensó que el ejercicio producía mediadores inflamatorios tales como el TNF, IL-1β y PCR, más tarde se vio que esto solo ocurría después de realizar un ejercicio extremo como pueden ser las maratones o las pruebas de triatlón, donde hay daño muscular e incluso endotoxemia sistémica (Jeukendrup *et al.* 2000). Sin embargo, en la mayoría de los casos el ejercicio posee efectos anti-inflamatorios, el perfil de citocinas inducidas por el ejercicio es básicamente anti-inflamatorio, se producen marcados aumentos de potentes anti-inflamatorios: IL-10, antagonista del receptor de la IL-1 (IL-1Ra) e IL-6. La prevención o atenuación de la inflamación es probablemente uno de los mecanismos más importantes mediante el cual el ejercicio protege frente al desarrollo de enfermedades metabólicas crónicas (Lancaster & Febbraio 2014).

II. OBJETIVOS

OBJETIVOS

El envejecimiento del sistema inmune implica una compleja serie de cambios que colectivamente reciben el nombre de inmunosenescencia. No sólo el envejecimiento fisiológico es el responsable de la inmunosenescencia, determinadas situaciones, como la inflamación de bajo grado continuada, situaciones de estrés prolongado, infecciones crónicas e incluso el ejercicio de alto rendimiento se asocian también frecuentemente a estos estados de inmunosupresión.

Dentro de los factores que condicionan de forma fundamental estos procesos se encuentran los cambios que se producen en el compartimento de los linfocitos T. Todos estos mecanismos han sido ampliamente descritos en los linfocitos T CD8+, sin embargo son muy escasos los estudios llevados a cabo en los linfocitos T CD4+. Por este motivo, el trabajo de investigación planteado se centra fundamentalmente en el estudio de los linfocitos T, y más exhaustivamente en el de los linfocitos T CD4+, en distintas situaciones relacionadas con la inmunosenescencia. Los objetivos de este trabajo fueron los siguientes:

1. Estudio de las características fenotípicas y funcionales de los linfocitos T CD4+ en individuos de edad avanzada y su respuesta al tratamiento con la citocina homeostática IL-15.

2. Asociación entre la infección crónica por citomegalovirus y el proceso de diferenciación de los linfocitos T CD4+.

3. Análisis del estado de diferenciación del sistema inmunológico en individuos de edad avanzada con distinto grado de capacidad funcional y en pacientes con insuficiencia cardiaca crónica como modelo de patología inflamatoria crónica.

4. Estudio del efecto modulador de los probióticos y el ejercicio físico intenso sobre el envejecimiento del sistema inmune.

III. RESULTADOS

III.1. EXPRESIÓN DE LA MOLÉCULA NKG2D EN LINFOCITOS T CD4+ EN PERSONAS DE EDAD AVANZADA

El proceso de envejecimiento se caracteriza por una serie de cambios en el sistema inmune. En los linfocitos T los cambios se producen fundamentalmente en el compartimento CD8+, mientras que no se dispone de marcadores de senescencia bien definidos para los linfocitos T CD4+. La expresión "aberrante" de la molécula NKG2D en células T CD4+ se ha descrito en determinadas patologías autoinmunes (Dai *et al.* 2009) e infecciosas (Alonso-Arias *et al.* 2009) caracterizadas por alto grado de inmunosenescencia. El objetivo de este estudio fue analizar la expresión de la molécula NKG2D en las células T CD4+ de los individuos de edad avanzada y su utilidad como marcador de envejecimiento del sistema inmune.

ARTÍCULO 1:

Rebeca Alonso Arias, **Marco Antonio Moro García,** Antonio López Vázquez, Luis Rodrigo, José Baltar, Francisco Manuel Suárez García, Juan José Solano Jaurrieta, Carlos López Larrea. *"NKG2D expression in CD4+ T lymphocytes as a marker of the global T-cell senescence in the aged immune system".*

Age (Dordr). 2011 Dec; 33(4):591-605.

Para la realización de este estudio se analizó por citometría de flujo la expresión de NKG2D en un grupo de 100 ancianos y 50 individuos jóvenes. Se efectuó una caracterización fenotípica (marcadores de activación y diferenciación), funcional (respuesta a antígenos, producción de citocinas) y ontogénica (cuantificación de TRECs por PCR a tiempo real) de la subpoblación CD4+NKG2D+.

La mediana del porcentaje de CD4+NKG2D+ en ancianos fue significativamente mayor que en individuos jóvenes (5,3% [IR: 8,74%] vs 1,4% [IR: 1,7%], $p=0,3x10^{-10}$). Mediante la expresión de CD28 pudimos distinguir dos subpoblaciones de células CD4+NKG2D+ claramente diferenciadas. Las células CD4+CD28+NKG2D+ presentaron características de células muy inmaduras, con alto porcentaje de co-expresión de CD45RA y CD31. Las células $CD4+CD28^{null}NKG2D+$, mayoritarias en los individuos de edad avanzada, presentaron características fenotípicas de células CD4+ muy diferenciadas, con bajos niveles de CD25 y altos de HLA-DR, perforina y granzima B. La respuesta específica de estas células

$CD28^{null}$NKG2D+ frente a CMV no presentó diferencias con respecto al resto de las células CD4+, sin embargo fueron significativamente menores frente a un antígeno de contacto reciente como la vacuna de la gripe estacional. Estas células presentan una mayor producción de IFN-γ en respuesta a la activación con anti-CD3, un menor umbral de activación y un menor contenido en TRECs.

Como conclusión podemos decir que la expresión de NKG2D en linfocitos T $CD4+CD28^{null}$ define una subpoblación de células CD4+ con un alto grado de diferenciación que caracteriza la senescencia del sistema inmunológico.

Aportación personal al trabajo:

En este trabajo, mi labor se centró principalmente en recolectar y procesar las muestras del estudio, realizar y/o supervisar los distintos experimentos y analizar los resultados obtenidos. También participé en el diseño del manuscrito.

AGE
DOI 10.1007/s11357-010-9200-6

NKG2D expression in CD4+ T lymphocytes as a marker of senescence in the aged immune system

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Received: 29 July 2010 / Accepted: 6 December 2010
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Abstract Human aging is characterized by changes in the immune system which have a profound impact on the T-cell compartment. These changes are more frequently found in CD8+ T cells, and there are not well-defined markers of differentiation in the CD4+ subset. Typical features of cell immunosenescence are characteristics of pathologies in which the aberrant expression of NKG2D in CD4+ T cells has been described. To evaluate a possible age-related expression of NKG2D in CD4+ T cells, we compared their percentage in peripheral blood from 100 elderly and 50 young adults. The median percentage of CD4+ NKG2D+ in elders was 5.3% (interquartile range (IR): 8.74%) versus 1.4% (IR: 1.7%) in young subjects ($p<0.3\times10^{-10}$). CD28 expression distinguished two subsets of CD4+ NKG2D+ cells with distinct functional properties and differentiation status. CD28+ cells showed an immature phenotype associated with high frequencies of CD45RA and CD31. However, most of the NKG2D+ cells belonged to the $CD28^{null}$ compartment and shared their phenotypical properties. NKG2D+ cells represented a more advanced stage of maturation and exhibited greater response to CMV (5.3±3.1% versus 3.4±2%, $p=0.037$), higher production of IFN-γ (40.56±13.7% versus 24±8.8%, $p=0.015$), lower activation threshold and reduced TREC content. Moreover, the frequency of the CD4+ NKG2D+ subset was clearly related to the status of the T cells. Higher frequencies of the NKG2D+ subset were accompanied with a gradual decrease of NAIVE and central memory cells, but also with a higher level of more differentiated subsets of CD4+ T cells. In conclusion, CD4+ NKG2D+ represent a subset of highly differentiated

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Published online: 06 January 2011

T cells which characterizes the senescence of the immune system.

Keywords Immunosenescence · NKG2D · CD4+ · T lymphocytes · Differentiation · CMV

Introduction

The human immune system progressively deteriorates with age, which leads to a greater incidence or reactivation of infectious diseases, as well as to the development of autoimmune disorders and cancer (DelaRosa et al. 2006; Prelog 2006). These defective immune responses are also manifested in a reduced capacity to induce immunological memory to vaccines and infections. In fact, the incidence of acute transplantation rejections is significantly lower in elderly transplant patients (Bradley 2002; Deng et al. 2004; Trzonkowski et al. 2010). Thus, aging is characterized by a reduction in adaptive responses, with a profound impact on the T-cell compartment. Elders retain a relatively intact innate immunity (Pawelec et al. 1998). It is well known that the size of the thymus diminishes with age (Taub and Longo 2005). Thymic involution implies a decreased output of naïve T cells, which is evident in peripheral blood and the lymph nodes (Fagnoni et al. 2000; Sauce et al. 2009; Appay et al. 2010). In contrast, elderly donors display a marked increase in the proportion of highly differentiated effector and memory T cells due to a lifetime of exposure to a variety of pathogens. In the elderly, a significant proportion of T cells loses the expression of costimulatory molecules and acquires inhibitory receptors. T cells from elders exhibit reduced responses to mitogen activation, such as decreased proliferation and interleukin (IL)-2 production, and also present a short telomere length, a common property of end-stage differentiation (Effros et al. 2005; DelaRosa et al. 2006). Accumulation of these highly differentiated T cells is partially explained by their reduced susceptibility to apoptosis and their oligoclonal expansions against CMV and other chronic antigens (Vescovini et al. 2004; Almanzar et al. 2005; Vasto et al. 2007; Derhovanessian et al. 2009). The accumulation of clonal cells with pro-inflammatory capabilities and an impaired ability to distinguish "self" and "foreign" antigens may be contributing to the development of autoimmune diseases (Prelog 2006). Interestingly, the most dramatic differences between aged and young individuals have been found in the CD8+ T cell subset, whereas CD4+ T cells were more resistant to age-related phenotypic and functional changes (Olsson et al. 2000; Wikby et al. 2002; Weinberger et al. 2007; Czesnikiewicz-Guzik et al. 2008). Oligoclonal expansions within the CD4 compartment are rare, preferentially found in patients with autoimmune diseases (Schmidt et al. 1996; Thewissen et al. 2007a, b). In fact, markers of differentiation are not as well defined in CD4+ T cells as in CD8+ T cells. The diminished expression of CD28, which is frequently encountered in CD8 T cells with age, is inconsistently observed in CD4+ T cells (Goronzy et al. 2007; Czesnikiewicz-Guzik et al. 2008).

Cellular immunosenescence is characteristic of some pathologies in which immune activation and inflammation become generalized. In this way, some autoimmune diseases, such as rheumatoid arthritis (Thewissen et al. 2005), and some infectious diseases, such as HIV infection (Appay et al. 2007), are characterized by an accelerated immune senescent phenotype, which includes higher frequencies of the CD4+ $CD28^{null}$ subset. These CD4+ $CD28^{null}$ T cells exhibit increased effector functions and appear to amplify autoimmune and inflammatory responses. Another common property described in CD4+ T cells from patients with these pathologies is the expression of NKG2D (Groh et al. 2003; Alonso-Arias et al. 2009), a killer lectin-like receptor originally identified on NK cells, TcR$\gamma\delta$+ cells and TcR$\alpha\beta$+ CD8+ T lymphocytes, but not on normal CD4 T cells. CD4+ NKG2D+ T cells comprise a particular subset of CD4+ T cells which have only been described in patients with cancer, chronic autoimmune diseases, or persistent infection (Duftner et al. 2003; Azimi et al. 2006; Groh et al. 2006; Allez et al. 2007; Capraru et al. 2008; Alonso-Arias et al. 2009). Cytomegalovirus (CMV) infection, a model of antigen persistence with transient reactivations, has been associated with NKG2D expression in CD4+ T cells (Saez-Borderias et al. 2006). Mechanistically, repeated exposures to the same antigens appear to lead to NKG2D expression in CD4+ T cells and favour the differentiation of CD4+ T cells.

The typical features of immunosenescence and the possible association with CMV infection shared by patients with rheumatoid arthritis and patients with HIV infection prompted us to postulate a possible rise in

the frequency of CD4+ T cells expressing NKG2D during aging. In this study, we describe for the first time an increased frequency of CD4+ NKG2D+ T cells in older people and this cellular subset is strongly associated with a senescent phenotype in CD4+. These NKG2D+ cells present a more advanced replicative status with respect to CD4+ $CD28^{null}$ T cells, and NKG2D+ could be a useful marker of cellular differentiation.

Materials and methods

Study population Blood samples were obtained from 100 healthy elderly donors (75 females/25 males) in the "El Cristo" Health Centre, the Hospital Universitario Central de Asturias and the Monte Naranco Hospital (Oviedo, Spain). The volunteers were not rigorously selected according to their health status in order to study a representative sample from the population. However, those volunteers with serious diseases such as cancer, chronic diseases (diabetes, autoimmune diseases), or congestive heart failure, and those receiving ongoing treatment with immunosuppressive drugs were excluded from participation. All subjects were vaccinated with a trivalent influenza vaccine (Solvay Biologicals BV, Olst, Holland). Samples from 50 young healthy controls (20 females/30 males) were obtained from the Centro de Transfusiones del Principado de Asturias (Oviedo, Spain). Inclusion criteria were a minimum age of 65 years for the elderly group and a maximum age of 55 for the young group. The mean age of the elderly individuals was 84.3± 7.7 years (range: 68–105 years) and that of the young controls was 38.8±9.4 years (range: 18–55 years).

Informed consent was obtained from patients and controls prior to participating in the study. The study was approved by the Hospital Central de Asturias Ethics Committee.

CD4+ T cell phenotyping The percentage of CD4+ NKG2D+ T cells was determined in peripheral blood from both the young and elderly participants by staining with anti-CD45, anti-CD3, anti-CD4 (BD Bioscience, San Jose, CA, USA) and anti-NKG2D (eBioscience). Whole blood samples (100 μL) were stained with different combinations of labelled monoclonal antibodies for 30 min at room temperature. Subsequently, samples were treated with FACS Lysing Solution (BD Bioscience) to lyse red blood cells, washed in phosphate buffered saline (PBS), and analyzed with a FACSCalibur Cytometer and CellQuest software. CaliBRITE Beads (BD Biosciences, San Jose, CA, USA) were used to adjust instrument settings, set fluorescence compensation and check instrument sensitivity. BD Multicheck Control and Multicheck CD4 Low Control were used as quality controls.

Phenotypical characteristics of CD4+ NKG2D+ T subsets were also determined by flow cytometry. CD4+ T cells (isolated as described below) were stained with anti-NKG2D (PE), anti-CD45RA (FITC), anti-CD45RO (PE), anti-HLA-DR (FITC), anti-CD25 (FITC), anti-CD28 (FITC or PE), and anti-CD31 (APC; BD Bioscience). Frequencies of cells with intracytoplasmic stores of granzyme B and perforin in CD4+ NKG2D+ T cells were measured. Cells were surface stained for 20 min at room temperature, lysed and fixed with FACS Lysing Solution, permeabilized with BD FACS Permeabilizing Solution 2 (Perm II; BD Bioscience), and stained with anti-granzyme A-FITC or anti-perforin-FITC for 30 min at room temperature. Cells were washed and resuspended in 1% paraformaldehyde until FACS analysis.

To analyze the differentiation status of CD4+ T cells, peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll–Hypaque gradients (Lymphoprep; Nycomed, Oslo, Norway). CD4+ T cells from young (n=20) and elderly subjects with different frequencies of CD4+ NKG2D+ (<5%, 5–20% and >20%, n=20 in each group) were isolated (Myltenyi Biotec GmbH, Bergisch Gladbach, Germany) and stained with anti-CD45RA (FITC; Immunostep, Salamanca, Spain), anti-CCR7 (Alexa Fluor 647; BD Bioscience), anti-CD27 (PE; Immunostep) and anti-CD28 (PerCP; BD Bioscience).

Quantification of telomerase activity PBMC were separated from the blood of 20 elderly individuals with different levels of NKG2D expression. CD4+ T cells isolated by negative selection (Miltenyi Biotec GmbH) were lysed and the protein concentration was measured in the extracts. The number of telomeric repeat sequences (TTAGGG) added to the 3′-end of an oligonucleotide substrate was quantified using real-time PCR (ABI 7500 Real-Time PCR Systems,

AppliedBiosystems, Carlsbad, CA, USA) using TeloExpress Quantitative Telomerase Detection Kit (XpressBio, Thurmont, MD, USA).

Antigen stimulation of whole blood cultures CMV-infected cell lysate was prepared by infecting human embryonic lung fibroblasts with the AD169 strain of CMV. Viral titers in the supernatant were determined by standard plaque assays. The virus was inactivated by repeated freeze–thaw cycles. Heparinized venous blood samples were stimulated with CMV (10^4 PFU/mL) or a 1/100 dilution of the influenza vaccine (Solvay Biologicals BV) in 15-mL conical polypropylene tubes. The blood cultures were incubated in humidified 37°C incubator for 18 h. Activation was assessed by surface staining with anti-CD69 (eBioscience). The cells were also stained with anti-CD4, anti-CD28 and anti-NKG2D. Samples were red blood cell lysed with FACS Lysing Solution, washed in PBS, and analyzed with CellQuest software.

Stimulation for cytokine assays PBMC (4×10^6 cells/mL) were stimulated with soluble anti-CD3 ($1–10^3$ ng/mL; eBioscience) for 6 h. Activation was assessed by intracellular staining with anti-IFN-γ (BD Biosciences), anti-IL-2 and anti-IL-17 (eBioscience). Cultures for the detection of intracytoplasmic cytokines were treated after the first 2 h with the secretion inhibitor Brefeldin A (10 μg/mL; Calbiochem, Darmstadt, Germany). After four additional hours, cells were treated with 2 mM EDTA for 15 min at room temperature, washed, and stained with antibodies against T cell surface molecules at 4°C. Intracellular staining was performed as previously described for granzyme B and perforin.

Virological testing Determination of antibodies to CMV in sera from elderly individuals was performed using the VIR-ELISA test ANTI-CMV-IgG (Viro-Immun Labor-Diagnostica GmbH, Oberursel, Germany).

T cell receptor excision circle quantification PBMC were separated from the blood of three elderly individuals with >20% CD4+ NKG2D+. CD4+ T cells isolated by negative selection (Miltenyi Biotec GmbH) were stained with anti-CD28-PE, and CD28+ cells were isolated with anti-PE magnetic microbeads. The negative fraction was then stained with anti-NKG2D-PE and CD4+ $CD28^{null}$ T cells were separated into NKG2D positive and negative subsets with the aforementioned microbeads. The DNA of the isolated subsets (purity >90%) was extracted using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Quantification of signal-joint (sj) T cell receptor excision circle (TREC) was performed by using SYBR Green real-time quantitative PCR and an iCycler thermocycler (Bio-Rad; Life Science Research Group, Hercules, CA, USA). The sequences of the utilized primers were the following: forward primer 5′-CCATGCTGACACCTCTGGTT-3′, reverse primer 5′-TCGTGAGAACG GTGAATGAAG-3′. As an internal control measurement to normalize for input DNA, the Cα constant region that remains present on TCR genes despite the rearrangement processes was amplified in every sample tested (forward primer 5′-CCTGATCCTCTTGTCCCACAG-3′, reverse primer 5′-GGATTT AGAGTCTCTCAGCTGGTACA-3′). Thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Experimental samples were run in duplicate and the replicate average value was taken as the sample result.

Statistical analysis Comparisons between groups were performed with the non-parametric Mann–Whitney *U* method, Kruskal–Wallis test or with the Wilcoxon signed-rank test when data were not normally distributed; or with Student's *t* test for independent or paired data. The outlier value of the CD4+ NKG2D+ T cells in normal individuals was calculated by adding 1.5 times the interquartile range (IR) to the 75th percentile. The χ^2 test was used to compare dichotomous variables. Analyses were performed using the SPSS 15.0 statistical software package program (SPSS Inc. Chicago, IL, USA). *P* values of 0.05 or less were considered significant.

Results

NKG2D expression in CD4+ T cells from elderly and young individuals

To evaluate a possible age-associated expression of NKG2D in $CD4^+$ T cells, we assessed the NKG2D status on CD4+ T cells in peripheral blood cells from

100 elderly volunteers and 50 young donors (Fig. 1a). The median percentage of CD4+ T cells expressing NKG2D was almost fourfold higher in elderly volunteers (5.3% [IR: 8.74%]) than in young subjects (1.4% [IR: 1.7%]) (Mann–Whitney U test, $p=0.3\times10^{-10}$; Fig. 1b). The size of the CD4+ NKG2D+ pool was significantly different between the age groups. Approximately 83% of the elderly individuals displayed CD4+ NKG2D+ T cell levels higher than 2.3%, the 75th percentile in control donors ($\chi2$ test, $p=2\times10^{-12}$; OR: 15.5 [CI: 6.7–35.5]). Despite the decline of the absolute numbers of CD4+ T cells with age, elderly individuals also showed higher CD4+ NKG2D+ count than young subjects, with 30.7 (IR: 56.7) and 19.4 (IR: 18.4) cells per μL, respectively (Mann–Whitney U test, $p=0.005$). NKG2D-expressing CD4+ T cells were clearly more abundant in the elderly volunteers when compared with young adults.

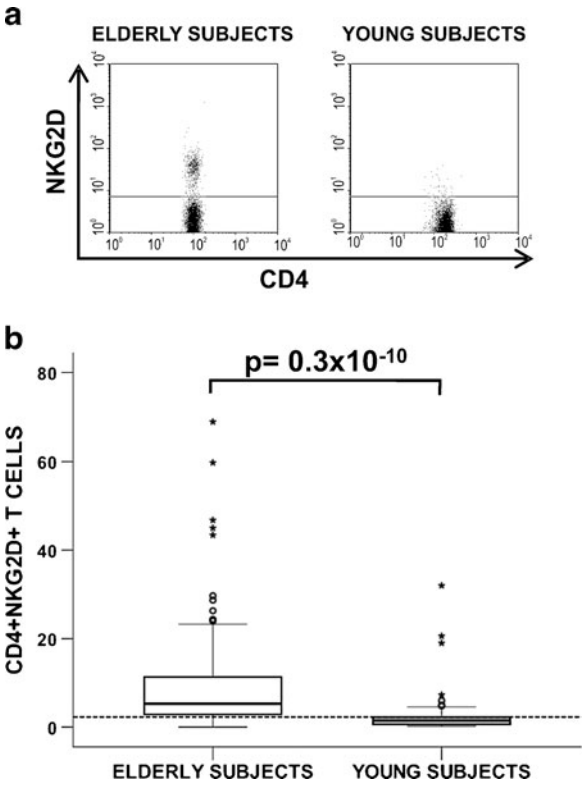

Fig. 1 CD4+ NKG2D+ T cells in peripheral blood from young and elderly individuals. (**a**) Representative dot plots showing the frequency of NKG2D expression in CD4+ T cells in elderly and young individuals. (**b**) Percentages of CD4+ T cells expressing NKG2D in a group of 100 elderly subjects compared with a group of 50 young subjects. Whole blood was stained with CD45-FITC/NKG2D-PE/CD3-PerCP/CD4-APC and 10^5 cells were acquired in each experiment. Frequencies of NKG2D+ cells in gated CD45+ CD3+ CD4+ lymphocytes were analyzed. Outlier values are represented by circles and extreme values by stars, and were calculated by adding 1.5 and 3 times the IR to the 75th percentile, respectively. The horizontal dotted line illustrates the 75th percentile in young donors (2.3%). The non-parametric Mann-Whitney U method was used to compare frequencies between groups

CD4+ T cell differentiation subsets in young and elderly people related to NKG2D expression

Our studies have demonstrated that NKG2D expression in CD4+ T cells was clearly associated with aging; thus, NKG2D expression might represent a biomarker for differentiation and/or immunosenescence. We postulated that factors that lead to NKG2D expression can be affecting the global memory phenotype in CD4+ T lymphocytes. We studied the state of differentiation of CD4 T cells in three groups of elder individuals with different frequencies of CD4+ NKG2D+ (<5%, 5–20%, and >20%; $n=20$ in each group), and in a subset of young individuals ($n=20$). CD4+ T cells were isolated from peripheral blood, stained with CD45RA, CCR7, CD28, and CD27 and analyzed by flow cytometry. Depending on the CD45RA and CCR7 expression, T lymphocytes were divided into naïve (NAIVE; CD45RA+CCR7+), central memory (CM; CD54RA$^-$CCR7+), effector memory (EM; CD45RA$^-$CCR7$^-$), and effector memory RA (EMRA; CD45RA+CCR7$^-$; Sallusto et al. 1999; Fig. 2a). A comparison between young and elderly subjects revealed a significant decrease in the frequency of NAIVE cells in the CD4+ T cell in all the NKG2D groups of elderly subjects. Moreover, an age-associated increase was found in CD4+ EM EMRA subset. The CD4+ CM subset was only significantly decreased in the >20% NKG2D group and no differences in the EMRA subsets were found (Fig. 2b and c). The increasing NKG2D expression in the elderly was directly associated with the diminished frequencies of NAIVE and CM cells, as well as the increased EM subsets in CD4+ T cells.

EM and EMRA are heterogeneous populations, and the staining of two additional markers, CD27 and CD28, has proven useful in identifying less (CD27+ and/or CD28+) or more (CD27^{null}CD28^{null}) differentiated cells (Romero et al. 2007; Koch et al. 2008). In CD4+ T cells, EM T cells were divided into EM1

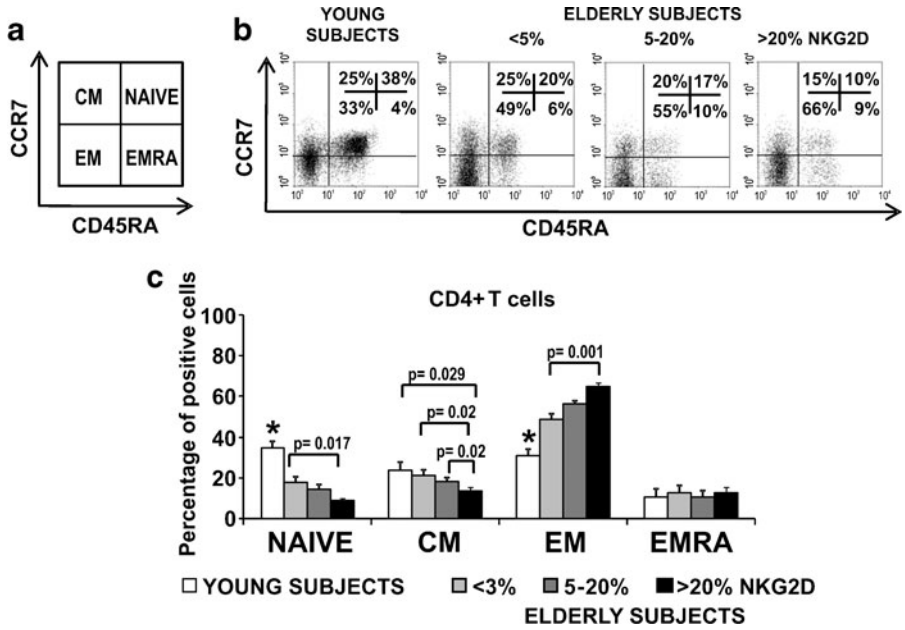

Fig. 2 Distribution of CD4+ and CD8+ T cells into naïve, central memory, effector memory, and effector memory RA. CD45RA and CCR7 expression was analysed by flow cytometry in isolated CD4+ T cells from young people (n=20) and elderly subjects with different frequencies of NKG2D (<5%, 5–20%, and >20%; n=20 in each group). **a** Schematic model of the T cell differentiation subsets according to CD45RA and CCR7 expression. **b** Representative dot plots of the subsets defined by CD45RA and CCR7 expression for individuals in each group. **c** Histograms depict cell percentage in each subset (mean±SEM) in young (*white bars*) and elderly subjects according to NKG2D expression (*light grey bars* <5%, *dark grey bars* 5–20%, *black bars* >20%). Significant differences between subsets are indicated (Student's t test or non-parametric Mann–Whitney method). *Asterisks* represent significant differences with all the groups (p<0.05)

(CD27+ CD28+), EM3 ($CD27^{null}CD28^{null}$), and EM4 ($CD27^{null}$CD28+; Fig. 3a). Functionally, EM1 and EM4 are very similar and exhibit memory-like properties, whereas EM3 displayed effector-like properties. Similarly, EMRA can be divided into less differentiated pE1 (CD27+ CD28+), and the E ($CD27^{null}CD28^{null}$), the most differentiated T cell subset. Differentiating CD4+ T cells first lose expression of CD27 and subsequently in a later phase, they lose CD28 (Amyes et al. 2003; van Leeuwen et al. 2004). In contrast, CD8+ T cells first lose expression of CD28 and then CD27 (Gamadia et al. 2003).

We compared the frequencies of these subsets of CD4+ T cells in young subjects and the NKG2D-based groups in elderly subjects. We found that the more differentiated subsets (EM3 and E) were increased in elderly individuals (Fig. 3b). Summarized p values of these differences clearly showed that the frequency of the subsets was associated with age and CD4+ NKG2D+ cell frequency (Fig. 3c). Elderly individuals in the <5% NKG2D group displayed only minor differences in the frequency of the subsets compared with young individuals. The frequencies of the subsets in the NKG2D 5–20% and >20% elderly groups became increasingly different than those of the young adult group (Fig. 3c). The gradual changes in the frequencies of differentiated populations in the three NKG2D-based groups of elderly subjects in CD4+ are presented in pie charts in Fig. 3c. Significant increases in the more differentiated subsets, EM3 and E, were observed, but decreases in the less differentiated EM1 and pE1 subsets were detected in the elderly groups. Significant differences were mainly detected between the <5% and >20% NKG2D groups.

To corroborate if these highly differentiated cells also display features of immunosenescence, we quantified the telomerase activity in CD4+ T cells from the three groups of elderly subjects. We found a progressive and significant reduction of the telomerase activity with the increased levels of NKG2D, with a median of 2.4×10^{-5} amol (IR: 2.7×10^{-5}amol) in <5%, 1×10^{-5}amol (IR: 0.27×10^{-5}amol) in >5–20%, and 0.26×10^{-5}amol (IR: 0.12×10^{-5}amol) in >20% NKG2D group (Kruskal–Wallis test, p=0.008; Fig. 3d).

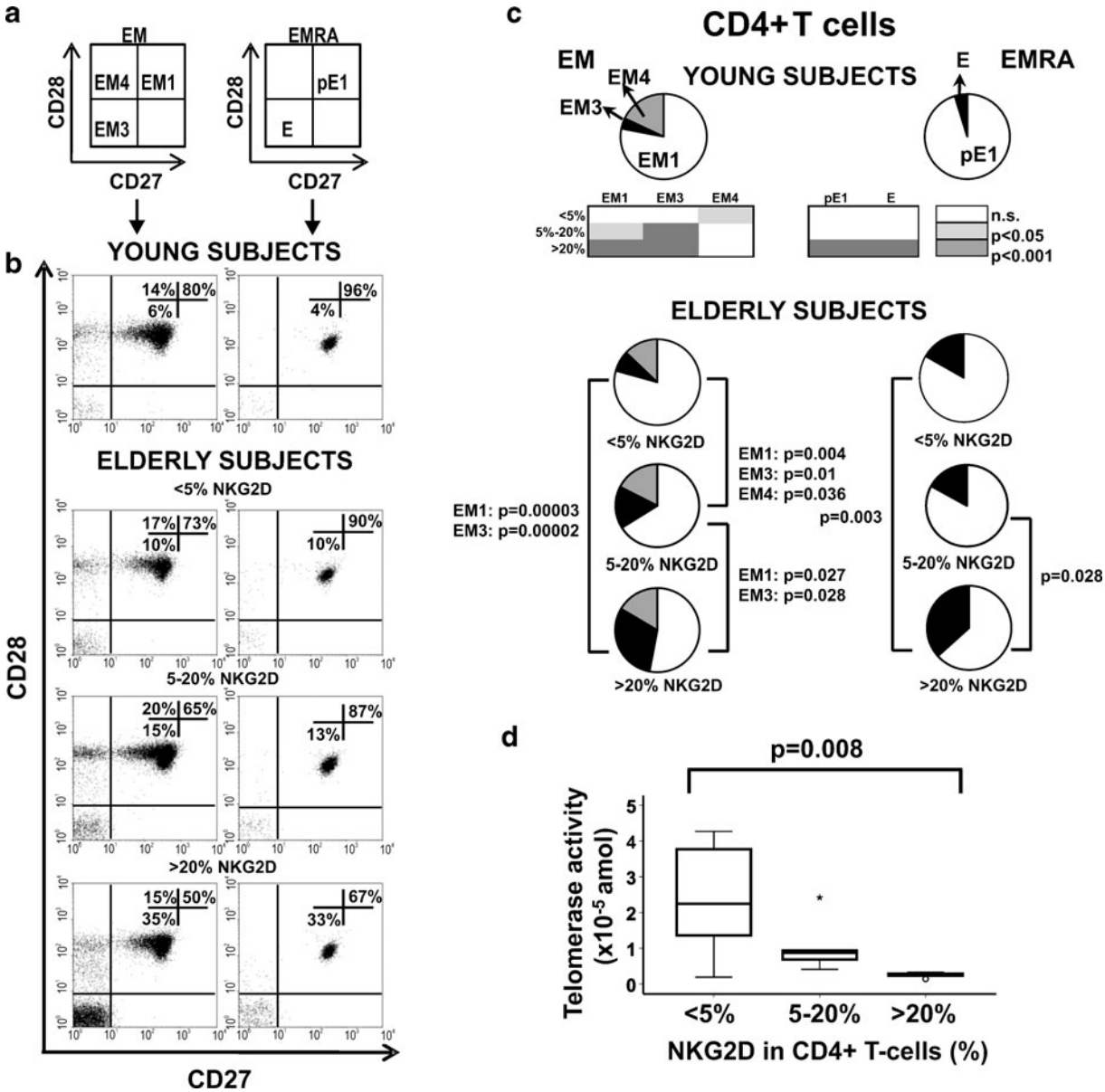

Fig. 3 Distribution of EM and EMRA CD4+ T cells into subsets defined by CD28 and CD27 expression. **a** Schematic model of the EM and EMRA differentiation subsets according to CD28 and CD27 expression. **b** Representative dot plots of the subsets defined by CD27 and CD28 expression for individuals in each group. **c** Individual segments of the pie charts represent the proportions of cells with each combination of CD28 and CD27 in the EM and EMRA CD4 T cell subsets in young donors ($n=20$) and in the three NKG2D groups (<5%, 5–20%, and >20% NKG2D, $n=20$ in each group) of elderly individuals. *P* values of differences between young and elderly people in each NKG2D group are shown in the rectangles below the pie charts (Student's *t* test or non-parametric Mann–Whitney method). No significant differences were represented by *white*; $p<0.05$ by *light grey*; and $p>0.001$ by *dark grey rectangles*. **d** Quantification of telomerase activity. CD4+ T cells were isolated from eight individuals in <5%, seven in 5–20%, and five in >20% NKG2D groups. The telomerase repeat sequence product added to the oligo substrate was determined in triplicate in the cellular extracts and the results were corrected by the total protein content. Outlier values were represented by circles and extreme values by stars, and calculated by adding 1.5 and 3 times the IR to the 75th percentile, respectively. The non-parametric Kruskal–Wallis test was used to compare frequencies between the three groups

In short, the frequency of the CD4+ NKG2D+ subset is clearly related to the differentiated status and the immunosenescence of the CD4+ T cell populations in the elderly. Levels of CD4+ $CD28^{null}$ T cells did not distinguish those elders belonging to <5% and 5–20% NKG2D groups in spite of the differences between both groups (data not shown). Thus, differences between young and elderly people are also directly related to the expression of the NKG2D marker.

Characterization of CD4+ NKG2D+ T cells

Normally, cells found in the CD4+ NKG2D+ subset-related pathologies had poor or no expression of CD28. Our analysis of the presence of this costimulatory receptor in elderly people revealed that most of the CD4+ NKG2D+ T cells belonged to the $CD28^{null}$ compartment, with a low proportion in the CD28 positive cells (Fig. 4a). Differences between CD4+ NKG2D+ T cell frequencies in elderly and young people were mainly due to the $CD28^{null}$ compartment (Fig. 4a). Remarkably, the frequency of NKG2D expression in CD4+ $CD28^{null}$ T cells was very variable, from 1.25% to 100%.

CD28 expression distinguished two subsets of CD4+ T cells with different functional properties and differentiation status (a in Fig. 4b). The majority of CD4+ $CD28^{null}$NKG2D+ T cells had a memory phenotype previously described in CD4+ $CD28^{null}$ cells. These cells exhibited CD45RO and HLA-DR expression, but a diminished expression of activation marker CD25. Moreover, these CD4+ $CD28^{null}$ cells showed cytotoxic properties and had intracytoplasmic stores of granzyme B and perforin (b and d in Fig. 4b). In contrast, the CD4+ CD28+ T cell subset showed CD25 expression but diminished HLA-DR expression and an almost absence of granzyme B and perforin (c and d Fig. 4b). This CD4+ CD28+ T cell subset that expressed the NKG2D molecule displayed a naïve phenotype, with significantly higher frequencies of CD45RA (55.7±26.6%) than the CD4+ CD28+ $NKG2D^-$ cells (29±7.8%; Student's t test, $p=0.04$). Moreover, CD4+ CD28+ NKG2D+ displayed an increased expression of CD31 in the CD45RA+ compartment (74.5±11.7% versus 42.4±23%, respectively; Student's t test, $p=0.005$; Fig. 4c).

In conclusion, CD28 expression distinguished two different subsets of CD4+ NKG2D+ T cells in elderly people. CD28 expressing cells predominantly showed a very immature phenotype, whereas $CD28^{null}$ T cells mainly represented a highly differentiated, memory subset.

Activation in response to antigens

CD4+ $CD28^{null}$ T lymphocytes have been described as antigen-specific cells against chronic viral antigens, and have been observed mainly in some autoimmune diseases (Thewissen et al. 2007a). In order to investigate this point in aging individuals, the response to chronic and to recent contact antigens was tested. We compared the induction of CD69 expression in response to CMV antigens and to influenza vaccine in CD4+ T cells with and without NKG2D expression from a group of 11 elderly subjects (Fig. 5). These elders were seropositive for CMV and had been recently vaccinated against the influenza virus. Their response to influenza vaccine did not show differences in the mean value of CD69 positive cells between the NKG2D+ subset at 0.6± 0.5% and the $NKG2D^-$ subset at 0.9±1.2%. However, there were statistically significant differences in response to CMV (paired t test, $p=0.037$), with 5.3± 3.1% of NKG2D+ cells expressing CD69 but only 3.4±2% of $NKG2D^-$ cells. Within the CD4+ CD28+ T cells, NKG2D expression did not affect the response to both CMV and influenza vaccine (data not shown). Since CMV increased NKG2D expression in CD4+ T cells (Saez-Borderias et al. 2006; Alonso-Arias et al. 2009), we analyzed the evidence of CMV infection (seropositivity) in the three groups of elder individuals with different frequencies of CD4+ NKG2D+ previously described (<5%, 5–20%, and >20%). Sixteen out of the 20 individuals belonging to the <5% NKG2D group were CMV seropositive. Furthermore, 19 of 20 volunteers in the 5–20% and >20% groups had evidence of CMV infection. However, one seronegative individual belonged to the 5–20% group and another seronegative participant was in the >20% NKG2D+ group. Therefore, other factors in addition to CMV infection must be implicated in the upregulation of NKG2D expression.

These results show that CD4+ $CD28^{null}$NKG2D+ T cells displayed phenotypic properties and antigen responses typical of highly differentiated cells. Furthermore, the expression of NKG2D seemed to be partially explained by CMV infection.

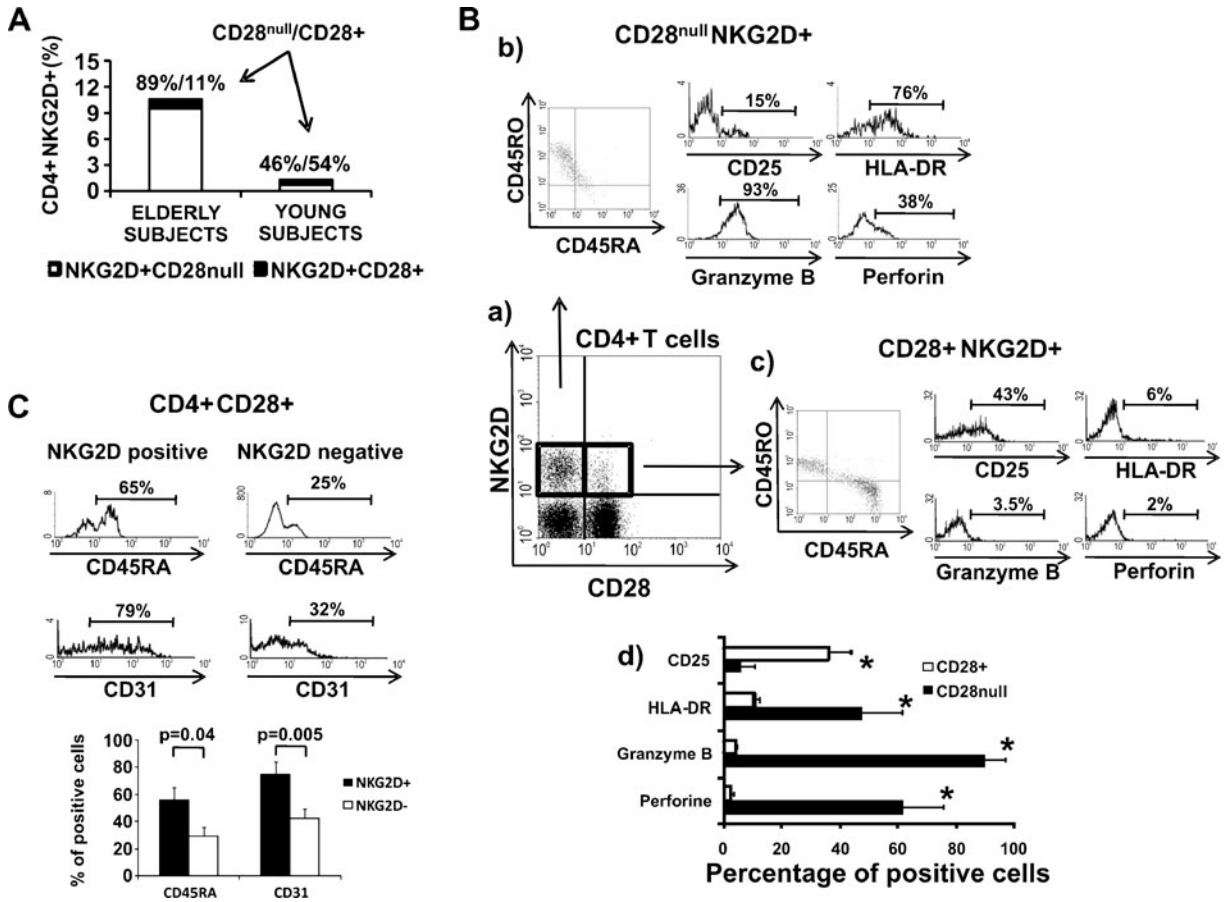

Fig. 4 Phenotypic characterization of CD4+ NKG2D+ T cells. **a** Expression of CD28 was analyzed in CD4+ NKG2D+ T cells from 20 elderly and 20 young individuals. Whole blood was stained with CD28-FITC/NKG2D-PE/CD3-PerCP/CD4-APC and 10^5 cells were acquired in each experiment. Histograms depict the percentage of CD28+ NKG2D+ (*black bar*) and $CD28^{null}$NKG2D+ (*white bar*) cells in gated CD3+ CD4+ lymphocytes. Percentages of $CD28^{null}$ and CD28+ cells within NKG2D+ subset in elderly and young individuals are summarized. **b** (*a*) Characterization of CD4+ NKG2D+ T cells related to expression of the CD28 marker. (*b*) CD4+ T cells from five aged donors were isolated and the expression of CD45RA-FITC, CD45RO-PercP, CD25-FITC, and HLA-DR-PerCP was analyzed in $CD28^{null}$NKG2D+ (Bb) and in CD28+ NKGD+ cells (*c*). Intracellular staining of granzyme B and perforin expression. The percentages of positive cells in the indicated cell populations in this representative experiment were expressed in each histogram plot. (*d*) Histograms summarize the percentage of positive cells for each marker in $CD28^{null}$NKG2D+ and in CD28+ NKG2D+ cells (mean± SEM). *Asterisks* significant differences between the groups ($p < 0.05$). (Student's *t* test or non-parametric Mann–Whitney method). (**c**) CD4+ T cells from the same donors were stained with CD45RA-FITC/NKG2D-PE/CD28-PercP/CD31-APC. The frequencies of CD45RA+ and CD31+ expression in the CD45RA subset were analyzed in CD28+ NKG2D+ and in CD28+ $NKG2D^-$ subsets. The percentage of positive cells in the indicated cell populations in this representative experiment were depicted in each histogram plot. Histograms summarize the percentage of positive cells to each marker (mean±SEM) in CD28+ NKG2D+ (*black bars*) and in CD28+ $NKG2D^-$ cells (*white bars*) from five elderly donors. Student's *t* test was used to compare means between groups

Differentiation status of elderly CD4+ $CD28^{null}$ T cells expressing NKG2D

IL-2-expressing cells are enriched in the early differentiated subsets of CD4+ T cells, whereas IFN-γ expression is seen at all stages of differentiation, but are predominantly enriched in responses to persistent antigens and late-differentiated cells (Harari et al. 2005). Following anti-CD3 stimulation for 6 h, CD4+ CD28+ T cells produced low levels of IL-2. IL-2-producing cells were not detected in the CD4+ $CD28^{null}$ subset (Fig. 6a). However, IFN-γ expression was induced in a greater proportion of CD4+ $CD28^{null}$ (mean: 28.7±9%) than in CD4+ CD28+ T

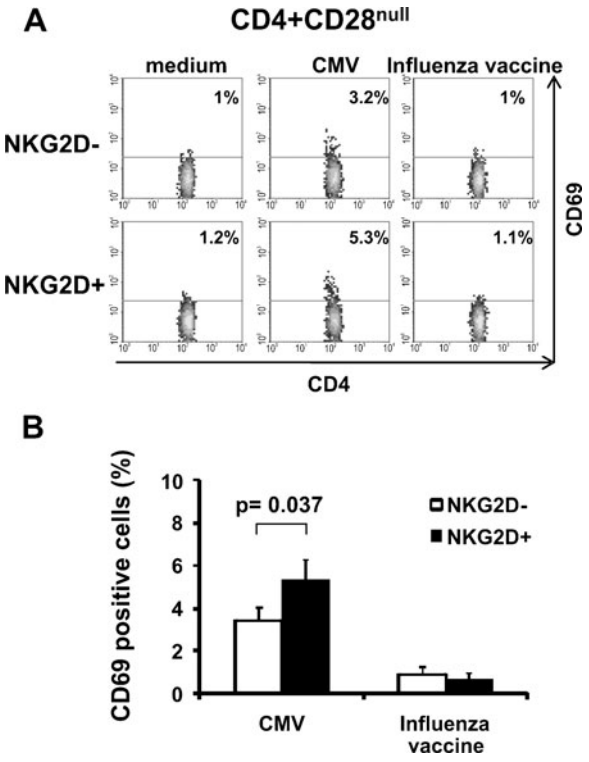

Fig. 5 CD69 expression in response to CMV antigens and to the influenza vaccine. PBMCs from elderly individuals were stimulated for 18 h and CD69 expression was evaluated by flow cytometry. **a** Representative dot plots of the frequency of CMV- and influenza vaccine-specific CD4 T cells with and without NKG2D expression. Percentages of positive cells in this representative experiment are expressed in dot plots. **b** Histograms summarize the percentage of CD69-cells positive for CMV and influenza vaccine in the CD4+ CD28^{null}NKG2D+ (*black bars*) and CD4+ CD28^{null}NKG2D- subsets (*white bars*) obtained from 11 elderly donors. Paired *t* test was used to compare paired frequencies

cells (mean: 2.2±1.3%; Fig. 6b). IFN-γ levels showed no significant differences in CD4+ CD28+ T cells based in NKG2D expression. However, IFN-γ production by CD4+ CD28^{null} T cells were significantly different in NKG2D negative (24±8.8%) and NKG2D positive cells (40.56±13.7%; paired *t* test, $p=0.015$) of elderly participants (Fig. 6b).

CD4+ CD28^{null} T cells presented a low activation threshold which could be implicated in its predisposition to the breakage of self-tolerance (Yung et al. 1996). In order to determine whether NKG2D status affected response threshold of CD4+ CD28^{null} T cells in elderly subjects, we measured the production of IFN-γ by CD4+ CD28^{null} and CD4+ CD28+ T cells following stimulation with 1, 10, 100 and 1000 ng/mL of anti-CD3 (Fig. 6c). The dose–response curve indicated that the enhanced IFN-γ production was elicited at concentrations as low as 1 ng/mL. Anti-CD3 at 10 ng/mL stimulated maximal IFN-γ production, while higher concentrations induced less IFN-γ. CD28^{null}NKG2D+ cells produced higher IFN-γ levels than CD28^{null} NKG2D^{-} cells at all anti-CD3 concentrations tested. However, an activator effect through NKG2D stimulation was not detected. In fact, we performed antibody-dependent cytotoxicity assays with CD4+ NKG2D+ T cells isolated from peripheral blood, but ligation of NKG2D neither induced redirected lysis of P815 cells, nor enhanced lysis induced by anti-CD3 (data not shown).

Responses to low activation doses of anti-CD3 could be related to autoimmune processes frequently described in the elderly. However, we did not detect IL-17 production in CD4+ CD28^{null} T cells, regardless of NKG2D status (data not shown). Moreover, an analysis of the presence of autoantibodies, detected by indirect immunofluorescence, revealed no significant differences between individuals with or without NKG2D expression (data not shown).

Since NKG2D-expressing cells produce higher levels of IFN-γ, this subset may represent a more mature stage in the CD4+ CD28^{null} T cells. To test differences in their replicative history, TREC content was then quantified in CD4+ CD28+, CD4+ CD28^{null}NKG2D^{-} and CD4+ CD28^{null} NKG2D+ T cell subsets isolated from three elderly individuals. TREC are episomal DNA products generated during T cell receptor rearrangement and not duplicated during mitosis (Hazenberg et al. 2001). Therefore, the lowest TREC content should be observed in those cells that have experienced the highest number of cell divisions. CD4+ CD28+ T cells displayed a higher TREC content than CD4+ CD28^{null} cells (Fig. 6d). CD4+ CD28^{null}NKG2D+ cells had the lowest TREC content. Of note, the expression of CD57, which characterizes highly differentiated memory CD4 and CD8 T cells (Brenchley et al. 2003), displayed no significant differences in frequency between both NKG2D subsets of CD4+ CD28^{null} T cells (data not shown). Taken together, these results indicated that NKG2D expression in CD4+ CD28^{null} T cells was associated with high levels of IFN-γ production and a low activation threshold. Based on TREC, the CD4+ CD28^{null}NKG2D+ subset in the elderly has under-

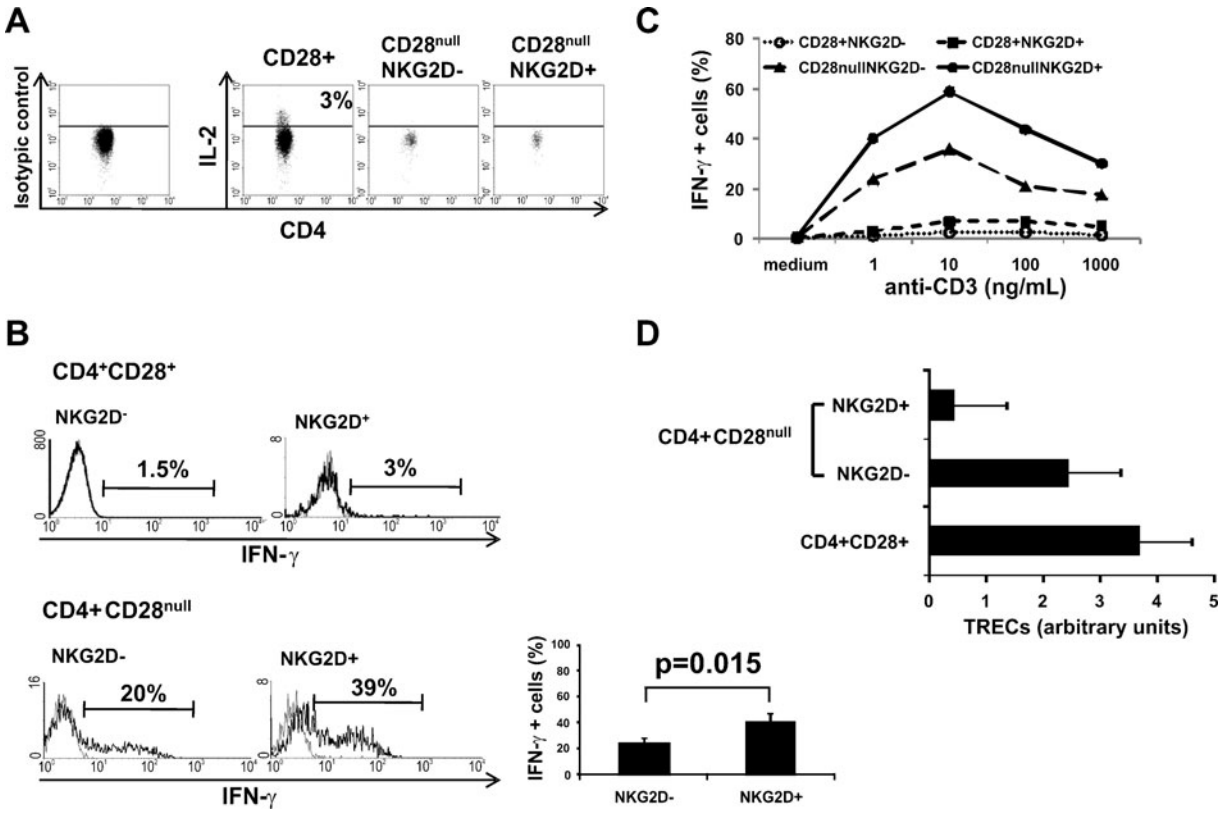

Fig. 6 Differentiated status of CD4+ subsets defined by IL-2/IFN-γ production and TREC content. **a** PBMCs were stimulated for 6 h with soluble anti-CD3 (1 μg/mL). The responder cells were analyzed for intracellular IL-2-APC staining in the indicated populations. **b** Cells were treated as described previously and IFN-γ expression was analyzed by intracellular staining. Percentages of positive cells in the indicated populations in this representative experiment are expressed on the histogram-plots. Histograms summarize the percentage of IFN-γ-positive cells in CD4+ $CD28^{null}$ and in CD4+ CD28+ lymphocytes NKG2D− and $NKG2D^{+}$ (mean±SEM) from the six elderly donors tested. A paired *t* test was used to compare paired means. **c** Dose-response curves of the IFN-γ production by CD4 subsets defined by CD28 and NKG2D expression in response to anti-CD3. The cells were cultured for 6 h in medium alone or with increasing concentrations of anti-CD3 (1–1,000 ng/mL). A fluorescence analysis was carried out as previously described. One representative experiment of three is shown. **d** Quantification of TREC copy number. CD28+, $CD28^{null}NKG2D^{-}$, and $CD28^{null}$NKG2D+ populations were isolated by sorting (magnetic bead separation) and the TREC copy number was determined by real-time PCR. Experiments were conducted in duplicate and bars represented results from three aging donors (mean±SEM)

gone more past episodes of activation and cell cycling than CD4+ $CD28^{null}NKG2D^{-}$ and was in the late stages of differentiation.

Discussion

We have demonstrated in this study that expression of NKG2D in CD4+ T cells is significantly increased in elderly individuals with respect to young adults. This cell subset exhibits the typical phenotype and function of end-differentiated cells which have undergone a high number of cell divisions. Furthermore, immune systems of donors with expansions of CD4+ NKG2D+ showed a mature phenotype in CD4+ T cells, supporting the hypothesis that NKG2D may be a useful marker for the evaluation of the degree of differentiation of the immune system.

Extensive research on the effect of age on immune responses clearly indicates its profound impact on the T-cell compartment (Pawelec et al. 1998, 2010). Thus, the phenotypic and functional analysis of T cells in elderly donors shows a significant decrease in naïve T cells and a marked increase in memory and effector/

memory T cells (Fagnoni et al. 2000; Effros et al. 2005; Taub and Longo 2005; Koch et al. 2008; Appay et al. 2010). These changes are less frequently described in $CD4^+$ than in $CD8^+$ T cells and there were no well-defined markers of differentiation in the $CD4^+$ subset. Towards this goal, an increased expression of NKG2D, which is almost absent in healthy young people, can be used as a marker for the aging of the immune system; this can be explained in part by the accumulation of end-differentiated effector CD4+ T cells. In pathologies such as rheumatoid arthritis (Groh et al. 2003) and HIV infection (Alonso-Arias et al. 2009), the expansion of the CD4+ NKG2D+ T cells has been associated with chronic immune activation and progressive differentiation. The extensive differentiation can limit the naïve T cell repertoire and reduce the ability to elicit protective immunological responses (Fasth et al. 2007; Thewissen et al. 2007a). Interestingly, the T cell alterations observed in rheumatoid arthritis and HIV-1 infected donors are "age-inappropriate" and similar to those observed in elderly individuals (Weyand et al. 2003; Appay et al. 2007). In both pathologies, T cell immune senescence occurs prematurely, probably due to the inability to generate sufficient numbers of naïve T cells. In addition, inflammation and immune activation become generalized and may lead to the accumulation of highly differentiated cells over time (Weyand et al. 2003; Appay and Sauce 2008). Among these, CD4+ $CD28^{null}$ T cells are identified as a consequence of chronic immune stimulation by a viral or an autoantigen, and may constitute an important amplification mechanism.

CD28 expression distinguished two subsets of CD4+ NKG2D+ T cells with different properties and differentiation status. CD28-positive cells found in young and elderly people showed a very immature phenotype, which correlated with high frequencies of CD45RA and CD31 coexpression. In accordance with their phenotypic proximity to thymocytes, the frequency of CD4+ CD31+ cells among CD45RA decreases with age (Kohler and Thiel 2009). This subset of CD4+ CD28+ T cells may represent the "normally occurring" population of CD4+ NKG2D+ T cells, recently defined by Dai et al. (2009). This CD4+ CD28+ subset is characterized by autoreactivity, an immunosuppressive function, regulatory properties, and a lack of conventional markers of antigen exposure. These results may be in line with those indicating that CD8 thymocytes upregulate NKG2D as they complete their developmental program in the thymic medulla before seeding the periphery (Hue et al. 2003). They identified NKG2D as a potential regulator of the developmental processes in T cells which are essential for immune homeostasis. Of note, differences between elderly and young people were mainly due to the $CD28^{null}$ compartment. The $CD28^{null}$ T cells expressed a similar pattern of phenotypical markers and intracytoplasmic granzyme B and perforin stores regardless of NKG2D expression. Both subsets of $CD28^{null}$ showed responder cells against CMV antigens, with higher frequencies in NKG2D+ cells, but no response against recent contact antigens. NKG2D expression can constitute an end-stage maturation marker, since we have demonstrated that these cells display a more terminally differentiated phenotype. CD57 expression, which decorates highly differentiated memory CD4 and CD8 T cells (Appay et al. 2007, 2008), did not differ in the NKG2D subsets of CD4+ $CD28^{null}$ T cells. However, the reduced TREC levels and the higher frequency of responder cells to CMV among NKG2D+ cells with respect to CD4+ $CD28^{null}NKG2D^-$ T cells may point to an increased proliferation against specific antigens and to a more differentiated phenotype.

This hypothesis is also supported by the enhanced ability of NKG2D+ cells to produce IFN-γ in response to TcR stimulation. IFN-γ expression is present at all stages of differentiation, but is mostly improved in late-differentiated cells that lack IL-2-production (Yue et al. 2004; Harari et al. 2005). The dominant IFN-γ CD4+ T cell response was associated with models of antigen persistence and high antigen levels. These data suggest the acquisition of NKG2D expression is induced by repeated exposure to the same antigens and leads to a more differentiated subset of cells (Appay et al. 2002; Snyder et al. 2004; Goronzy and Weyand 2005). For example, CMV infection represents a model of antigen persistence with transient reactivations, mainly in immunosuppressed individuals. CMV was previously associated with NKG2D expression in CD4+ T cells (Saez-Borderias et al. 2006; Alonso-Arias et al. 2009). Although CMV infection may account for the high number of CD4+ NKG2D+ T cells in many of the CMV-infected elderly subjects, it does not explain the high frequencies of CD4+ NKG2D+ in individuals who were seronegative for CMV. These data suggest

that other persistent viral infections and/or the pro-inflammatory cytokines produced during some infectious processes may drive their differentiation. Another possible explanation is the corroborated fact that advanced age is accompanied by a low-grade, chronic upregulation of inflammatory responses evidenced by increased serum levels of proinflammatory cytokines (IL-6, IL-15, IL-8), coagulation factors, and reactive oxygen species (Mari et al. 1995; Forsey et al. 2003; Zanni et al. 2003; Ferrucci et al. 2005; Wikby et al. 2006; Giunta et al. 2008).

Our most important finding related to NKG2D expression in CD4+ T cells is its association with a more senescent and differentiated phenotype in CD4+ T cells. Thus, the phenotypic analysis of T cells in elderly donors showed a significant decrease in naïve T cells and a marked increase in those with a memory or effector/memory phenotype. Correlations between CD4+ and CD8+ effector cells had been demonstrated in latent CMV infection in the elderly, but to our knowledge, this is the first time that NKG2D has been demonstrated as a marker of differentiation in the CD4+ T-cell compartment. We applied models that categorize the T cell differentiation states based on CD45RA, CCR7, CD28, and CD27 expression (Sallusto et al. 1999; Rufer et al. 2003; Romero et al. 2007). Although differences between these T cell populations in young and elderly people have already been reported, we have demonstrated that levels of NKG2D expression define the state of differentiation in CD4+ T cells in the elderly. Aging donors with high frequencies of CD4+ NKG2D+ exhibited a more differentiated CD4+ T-cell compartment. These more differentiated CD4+ T cells can be the result of a non-specific effect of the pro-inflammatory environment on the differentiation state of other T cells which leads to higher frequencies of differentiated cells in individuals with higher levels of CD4+ NKG2D+. Moreover, NKG2D is correlated with the senescence status of CD4+ T cells, since telomerase activity was reduced progressively with the increase of NKG2D expression. When cells lack telomerase activity, telomeres shorten and are no longer protective and the cell enter this non-replicative state (Campisi 1997).

In conclusion, NKG2D expression in CD4+ T cells can be a part of the differentiation process. This phenomenon is a consequence of the successive rounds of activation throughout a lifetime the elderly have gone through, and can be accelerated by the chronic activation induced by persistent viral infections or auto-antigens.

Acknowledgments We thank Isabel Cuevas Pérez, Sonia García Baragaño, and María Jesús Redondo García for their excellent technical assistance.

This work was supported by Red de Investigación Renal (REDinREN), FIS PI080566 from Instituto "Carlos III", and PEST 08-05 from FICYT.

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III.2. ACCIÓN PREFERENCIAL DE LA CITOCINA IL-15 SOBRE LA POBLACIÓN LINFOCITARIA CD4+CD28^{null} RESPECTO A LA POBLACIÓN CD4+CD28+

Uno de los cambios más significativos observados en el sistema inmune durante el envejecimiento en humanos es la acumulación de linfocitos T CD4+ sin expresión de la molécula coestimuladora CD28. La potenciación de las propiedades funcionales de estas células podría tener relevancia en los ancianos ya que suponen una defensa específica frente a patógenos crónicos. Puesto que se ha demostrado la implicación de la interleuquina-15 (IL-15) en la generación y proliferación de los linfocitos T CD8+CD28^{null} (Chiu *et al.* 2006), nos planteamos analizar el posible efecto activador de esta citocina sobre la subpoblación CD4+CD28^{null} en un grupo de individuos de edad avanzada.

ARTÍCULO 2:

Rebeca Alonso Arias, **Marco Antonio Moro García**, José Ramón Vidal Castiñeira, Juan José Solano Jaurrieta, Francisco Manuel Suárez García, Eliecer Coto, Carlos López Larrea. *"IL-15 preferentially enhances functional properties and antigen-specific responses of CD4+CD28^{null} compared to CD4+CD28+ T cells".*

Aging Cell. 2011 Oct;10(5):844-52.

En este estudio se obtuvieron muestras de sangre periférica de 30 individuos de edad avanzada (>70 años) y se estudió la frecuencia y capacidad funcional (proliferación, activación, actividad citotóxica, producción de IFN-γ) de las células T CD4+CD28^{null} en respuesta al tratamiento con IL-15. Al analizar los datos obtenidos encontramos un incremento significativo de células CD4+CD28^{null} en presencia de IL-15 con respecto a células cultivadas en medio (15,8% ± 5,1% vs 8,6% ± 3,3%, p=0,00006), consecuencia de una mayor proliferación. El tratamiento con IL-15 no indujo un incremento en la expresión de su receptor, sin embargo, si se observó una activación del factor de transcripción STAT5 respecto a las células CD28+ (p<0,05). El cultivo a tiempos cortos con IL-15 indujo en esta subpoblación celular un incremento significativo en la expresión, tanto a nivel de mRNA como de proteína, de granzima B y perforina. La degranulación inducida por anti-CD3 estuvo también significativamente incrementada en presencia de IL-15 (p<0,05), así como la lisis redirigida frente a la línea celular P815 recubierta de anti-CD3. Por

último, el precultivo de las células CD4+CD28^{null} en presencia de esta citocina produjo un efecto activador en la producción antígeno-específica de IFN-γ inducida por CMV.

Como conclusión podemos afirmar que la IL-15 ejerce un efecto activador sobre los linfocitos T CD4+CD28^{null} en los individuos de edad avanzada, potenciando su capacidad efectora frente a sus antígenos específicos.

Aportación personal al trabajo:

En este estudio, mi labor se centró principalmente en recolectar y procesar las muestras del estudio, realizar y/o supervisar los distintos experimentos y analizar los resultados obtenidos. También participé en el diseño del manuscrito.

Aging Cell (2011) **10,** pp844–852 Doi: 10.1111/j.1474-9726.2011.00725.x

IL-15 preferentially enhances functional properties and antigen-specific responses of CD4+CD28^{null} compared to CD4+CD28+ T cells

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Summary

One of the most prominent changes during T-cell aging in humans is the accumulation of CD28^{null} T cells, mainly CD8+ and also CD4+ T cells. Enhancing the functional properties of these cells may be important as they provide an antigen-specific defense against chronic infections. Recent studies have shown that IL-15 does in fact play an appreciable role in CD4 memory T cells under physiological conditions. We found that treatment with IL-15 increased the frequency of elderly CD4+CD28^{null} T cells by the preferential proliferation of these cells compared to CD4+CD28+ T cells. IL-15 induced an activated phenotype in CD4+CD28^{null} T cells. Although the surface expression of IL-15R α-chain was not increased, the transcription factor STAT-5 was preferentially activated. IL-15 augmented the cytotoxic properties of CD4+CD28^{null} T cells by increasing both the mRNA transcription and storage of granzyme B and perforin for the cytolytic effector functions. Moreover, pretreatment of CD4+CD28^{null} T cells with IL-15 displayed a synergistic effect on the IFN-γ production in CMV-specific responses, which was not observed in CD4+CD28+ T cells. IL-15 could play a role enhancing the effector response of CD4+CD28^{null} T cells against their specific chronic antigens.

Key words: CD4+CD28^{null} T cells; chronic viral antigens; cytotoxicity; IL-15; Immunosenescence; memory T cells.

Introduction

Aging is characterized by a reduction in adaptive responses and has a profound impact on the T-cell compartment. Thymic involution implies a decreased output of naïve T cells, which is evident in peripheral blood and the lymph nodes (Sauce *et al.*, 2009; Appay *et al.*, 2010). In contrast, elderly donors display a marked increase in the proportion of highly differentiated effector and memory T cells owing to a lifetime of exposure to a variety of pathogens. One of the most prominent changes during T-cell aging in humans is the accumulation of CD28^{null} T-cells. Their accumulation is partially explained by their reduced susceptibility to apoptosis and their oligoclonal expansions against CMV and other chronic antigens (Vescovini *et al.*, 2004; Almanzar *et al.*, 2005; Derhovanessian *et al.*, 2009). Loss of CD28 expression is a hallmark of the age-associated decline of T-cell function. Because CD28 provides a pivotal role during T-cell activation, such as inducing cytokine production (IL-2) and promoting cell proliferation, lack of this costimulatory signal during activation results in a partial activation or even an anergic state of T cells (Godlove *et al.*, 2007). In this way, the accumulation of CD28^{null} T cells, particularly within the CD8 subset, is associated with a reduced overall immune response to pathogens and vaccines in the elderly (Saurwein-Teissl *et al.*, 2002). Despite CD4+ T cells are more resistant to age-related phenotypic and functional changes (Wikby *et al.*, 2002; Weinberger *et al.*, 2007), a progressive increase in the percentage of CD4+ T cells that lack CD28 expression is common with increasing age in healthy individuals (Goronzy *et al.*, 2007; Czesnikiewicz-Guzik *et al.*, 2008) and in patients with chronic infections and autoimmune diseases(Komocsi *et al.*, 2002; Fletcher *et al.*, 2005; Thewissen *et al.*, 2007). Thereby, CD4+CD28^{null} T cells can comprise up to 50% of the total CD4+ T-cell compartment in some individuals older than 65 years(Vallejo *et al.*, 2000). CD4+CD28^{null} T cells are functionally distinct from CD4+CD28+ T cells but exhibit similarities with CD8+CD28^{null} T cells. CD4+CD28^{null} T cells acquire expression of several receptors commonly associated with NK cells, secrete large amounts of IFN-γ, and express perforin and granzyme B, which convey cytotoxic capability to the cells (Appay *et al.*, 2002; van Leeuwen *et al.*, 2004).

Generation of long-term memory CD4+ and CD8+ T cells is dependent upon antigenic stimulation, but their survival is antigen-independent and requires peripherally produced cytokines, particularly those that use the common γ-chain for signaling, such as IL-15(Ku *et al.*, 2000). The importance of IL-15 in memory T cells was established by the characterization of IL-15 and IL-15Rα knockout mice which showed a striking lack of memory phenotype CD8+ T cells (Zhang *et al.*, 1998; Kennedy *et al.*, 2000). Furthermore, memory CD8+ T cells were increased in an IL-15-overexpressing mouse model (Fehniger *et al.*, 2001). Homeostatic proliferation of T cells can be one cause for the age-associated loss of CD28 expression, because CD8+ memory T cells in the presence of IL-15 alone, without TCR stimulation, lose CD28 expression and proliferate at a similar rate to CD8+CD28+ T cells (Chiu *et al.*, 2006). IL-15 not only potently induces the proliferation of memory CD8+ T cells but also augments their effector function by the induction of perforin expression and cytotoxic properties (White *et al.*, 2007). Because antigen-specific memory CD4+ T cells have distinct requirements for homeostatic regulation compared with CD8+ T cells, the role of IL-15 in the maintenance of antigen-specific memory CD4 T cells was missed by analyses of memory phenotype cells in early studies of IL-15$^{-/-}$ mouse models. Recent studies have shown that IL-15 does in fact play an appreciable role in CD4+ memory T-cell proliferation under physiological conditions (Purton *et al.*, 2007). IL-15 treatment promotes the proliferation of human memory CD4+ T cells *in vitro* and mouse Ag-specific CD4+ memory T cells *in vivo* (Geginat *et al.*, 2001; Lenz *et al.*, 2004). These findings demonstrate that in a normal environment, memory CD4+ T cells closely resemble memory CD8+ cells in their dependency on IL-15 for their homeostasis (Geginat *et al.*, 2001; Lenz *et al.*, 2004).

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Accepted for publication *26 May 2011*

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As CD8+CD28^{null} T cells are highly influenced by IL-15, it is of great interest to determine the responsiveness of CD4+CD28^{null} T cells to this homeostatic cytokine, mainly because it has been postulated as a treatment of T-cell reconstitution in the elderly. We have demonstrated in this study that IL-15 may not only improve CD4+CD28^{null} T-cell expansion but also promote both their functional properties and their antigen-specific responses.

Results

Effect of IL-15 on CD28 expression in CD4+ T cells

We evaluated the possible effect of IL-15 on the expression of CD28 in CD4+ T cells from 10 elderly volunteers with percentages of CD28^{null} cells higher than 5% of total CD4+ T cells. Peripheral blood mononuclear cells (PBMC) were cultured in the presence and absence of the IL-15 for 7 days. Frequency of CD4+CD28^{null} T cells showed a significant increase in response to IL-15 treatment compared with cells cultured in medium alone, with a mean percentage of 15.8 ± 5.1% and 8.6 ± 3.3%, respectively (paired *T* test, $P = 0.00006$) (Fig. 1A). In contrast to that described in CD8+ T cells (Chiu *et al.*, 2006), IL-15 did not induce loss of CD28 expression in CD4+CD28+ T cells in individuals without CD4+CD28^{null} T-cell subset (data not shown). In spite of their defects in the proliferative ability (Appay *et al.*, 2002), CD4+CD28^{null} T cells may have increased owing to an enhanced proliferative response to IL-15 compared with that of CD28+. To analyze the effect of IL-15 on the proliferation of CD4+CD28^{null} T cells, PBMC were stained with CFSE and cultured in the presence of IL-15 for 7 and 14 days. When we compared the growth kinetics of both populations in response to the cytokine, we found that CD4+CD28^{null} cells effectively underwent cell division and expansion faster than did CD4+CD28+ cells. Figure 1B shows a representative experiment in which around 40% of the CD28^{null} cells have divided at least once after a 7-day culture with IL-15, whereas only 3% of CD28+ cells did. Differences in frequency of proliferating cells were maintained in the samples cultured for 14 days, but the number of division cycles was higher in CD28+ cells.

Differentiating CD4+ T cells first lose expression of CD27, and subsequently in a later phase, they lose CD28 (Amyes *et al.*, 2003; van Leeuwen *et al.*, 2004). To characterize the differentiation degree of the IL-15-generated CD28^{null} T cells, we analyzed the expression of CD27. All the CD4+CD28^{null} T cells present in the PBMC cultures both in medium alone and in the presence of IL-15 were also negative for CD27 (Fig. 1C). Moreover, depending on CD45RA and CCR7 expression, these CD27^{null}CD28^{null} cells can be divided into effector memory (EM; CD45RA−CCR7−) and the most differentiated effector memory RA (EMRA; CD45RA+CCR7−) subsets (Sallusto *et al.*, 1999). The increase induced by IL-15 was even more relevant in the EMRA (12.2% ± 8.4% in medium and 24.9% ± 12.5% with IL-15; paired T cells, $P = 0.005$) than in the EM cells (10.8% ± 4.4% in medium and 17.1% ± 8.3% with IL-15; paired T cells, $P = 0.005$) (Fig. 1D).

In conclusion, IL-15 increased the frequency of CD4+CD28^{null} T cells mainly in the highly differentiated subset EMRA by the preferential proliferation of these cells with respect to those expressing CD28.

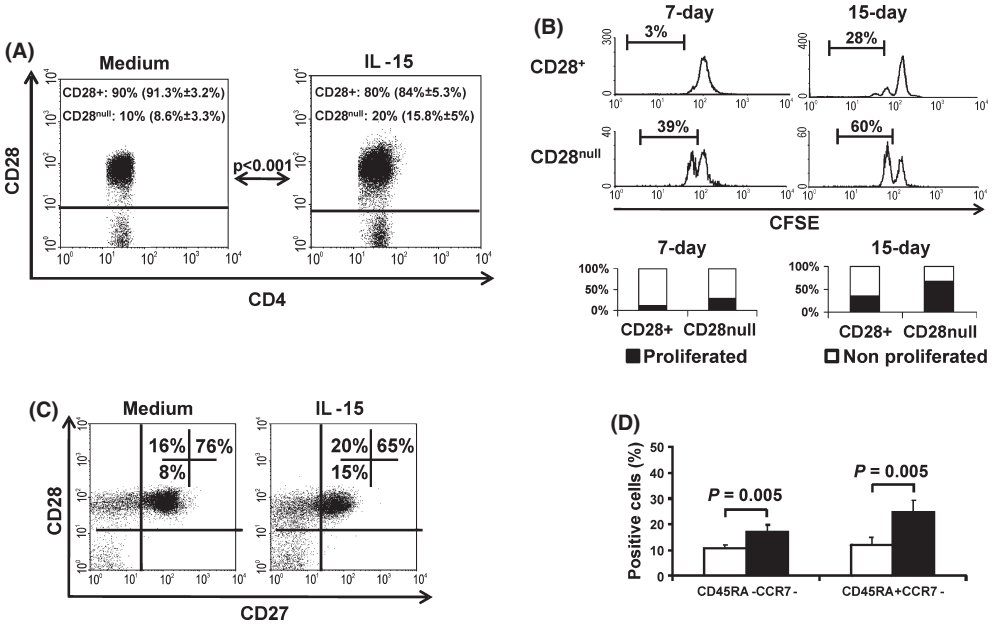

Fig. 1 Effect of IL-15 on the growth of CD4+CD28^{null} T cells in elderly individuals. (A) Frequency of CD4+CD28^{null} T cells in response to treatment with IL-15. Peripheral blood mononuclear cells (PBMC) from 10 elderly subjects were cultured in medium or in the presence of IL-15 (50 ng mL^{-1}) for 7 days and analyzed by flow cytometry. Cells were stained with CD3-FITC/CD28-PE/CD4-PerCP/CD45-APC, and 5×10^4 cells were acquired in each experiment. Dot plots show the frequencies of CD28+ and CD28^{null} cells in gated CD4+ T cells in this representative experiment, and the frequencies were averaged from all donors (mean ± SD). (B) Proliferative capacity of CD4+CD28+ and CD4+CD28^{null} T cells in response to IL-15. PBMC were labeled with CFSE (1.5μM) and cultured in the presence of IL-15 (50 ng mL^{-1}) for 7 and 15 days. CD4+CD28+ and CD4+CD28^{null} T cells were gated. Percentages of dividing cells were analyzed on days 7 and 15 of culture. Representative histogram plots of cells from one of five experiments are shown. Histograms depict the mean of the percentages of proliferated (black bar) and nonproliferated (white bar) cells in gated CD4+CD28+ and CD4+CD28^{null} T lymphocytes in the five experiments. (C) PBMC treated as described above were stained with CD3-FITC/CD28-PE/CD4-PerCP/CD27-APC. Dot plots show the expression of CD27 and CD28 in gated CD4+ T cells in this representative experiment. (D) Cells were stained with CD45RA-FITC/CD28-PE/CD4-PerCP/CCR7-APC and frequency of CD28^{null} cells in the EM (CD45RA-CCR7−) and EMRA (CD45RA+CCR7−) CD4+ T-cell subsets was analyzed. Histograms summarize the percentage of CD28^{null} cells in the indicated cell populations (mean ± SEM) after cells were cultured in medium (white bars) and in the presence of IL-15 (black bars). Paired *T* test was used to compare paired frequencies.

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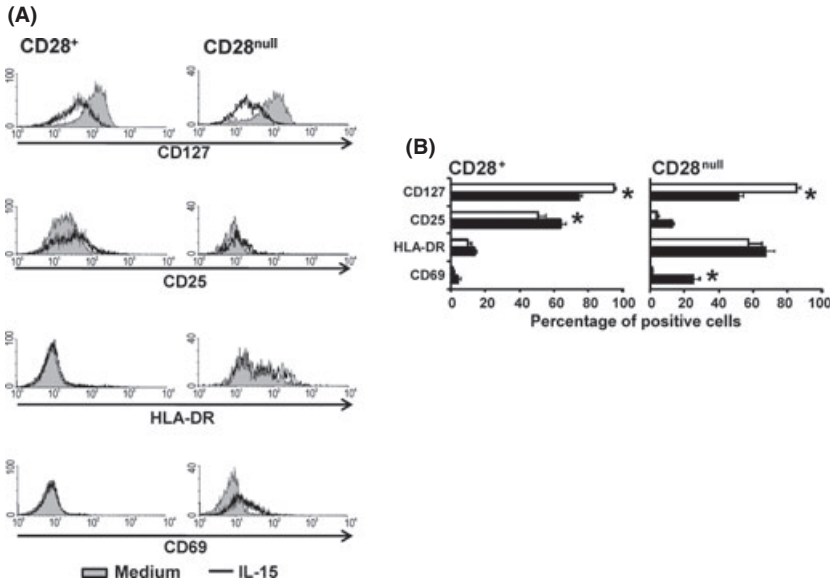

Fig. 2 Effect of IL-15 on the expression of activation markers. Results were representative for five elderly donors. Peripheral blood mononuclear cells were cultured for 18 h in the presence or absence of IL-15. Expression of CD127-PE, CD25-FITC, HLA-DR-FITC, and CD69-FITC was analyzed in CD4+CD28+ and CD4+CD28^{null} T cells by flow cytometry. (A) Histogram plots show a representative experiment of 5 performed. (B) Histograms represent percentage of positive cells (mean ± SEM) in CD28+ and CD28^{null} cells cultured in medium (white bars) and in the presence of IL-15 (black bars) cells. Paired *T* test was used to compare differences; significant differences were indicated (*$P < 0.05$).

Activation induced by IL-15

To further evaluate other differential effects of IL-15 on CD4+CD28^{null} T cells compared with CD28+, we studied the expression of several activation markers in response to the cytokine. Expression patterns of CD127, CD25, HLA-DR, and CD69 in CD4+CD28+ and CD4+CD28^{null} T cells cultured for 24 h in medium alone and in the presence of IL-15 are represented in Fig. 2A. IL-15 activation reduced CD127 expression in both CD28+ and CD28^{null} cells with respect to cells cultured in medium alone ($P = 0.005$ and $P = 0.002$, respectively) (Fig. 2B). CD25 was significantly increased only in CD28+ cells, whereas CD28^{null} cells showed low CD25 expression both basally and in response to IL-15. On the contrary, IL-15 induced higher expression of HLA-DR and mainly CD69 in CD28^{null} cells ($P = 0.027$) with no significant effect on CD28+ cells (Fig. 2B).

We then analyzed the expression of the specific IL-15R α-chain in CD4+CD28+ and CD4+CD28^{null} T cells to evaluate a possible mechanism for the different responsiveness of both subsets to IL-15. This receptor was barely expressed in resting CD4+ T cells and stimulation for 24 h with IL-15 did not induce up-regulation in CD4+CD28^{null} nor in CD4+CD28+ T cells (Fig. 3A). To investigate whether there was a difference in intracellular signaling after IL-15 interaction with its receptor, we analyzed the STAT-5 activation. As expected, phosphorylation of this signal factor was strongly induced by IL-15 in both subsets, but CD4+CD28^{null} showed significantly higher levels of pSTAT-5 with respect to CD4+CD28+ T cells (mean: 82.5% ± 6.5% vs. 69.6% ± 7.8%, paired *T* test, $P = 0.036$) (Fig. 3B).

IL-15 induced an activated phenotype in CD4+CD28^{null} T cells, with enhanced expression of CD69 but diminished CD127 expression. Moreover, the surface expression of IL-15R α-chain was not increased, but STAT-5 was preferentially activated.

IL-15 up-regulates granzyme B and perforin expression and cytolytic properties in CD4+CD28^{null} T cells

CD4+ T cells have not been classically considered as cytotoxic cells, although intracytoplasmic stores of granzyme B and perforin have been previously described in CD4+CD28^{null} T cells (Appay *et al.*, 2002). Because IL-15 is known to induce cytotoxic properties in NK and CD8+ T cells, we analyzed its influence on granzyme B and perforin expression in CD4+CD28^{null} T cells. After 5 h of culture with IL-15, expression of both proteins was up-regulated in CD28^{null} cells (Fig. 4A). Percentages and mean fluorescence intensity (MFI) of perforin-positive cells showed

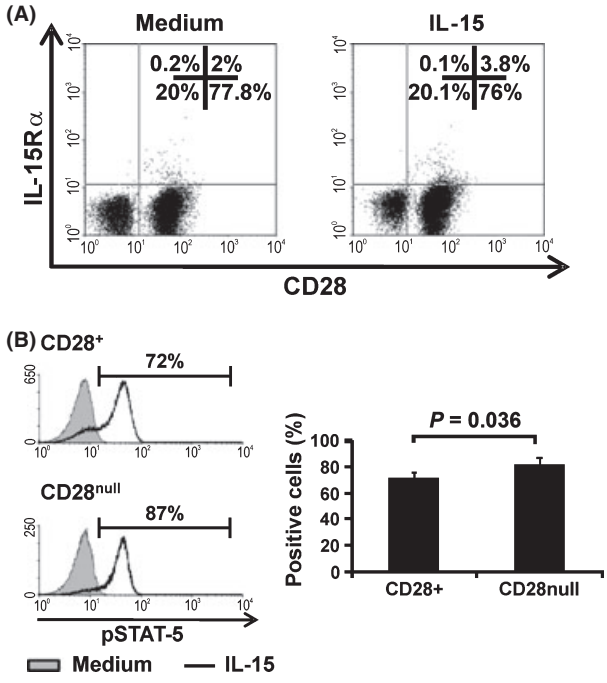

Fig. 3 Expression of IL-15Rα chain and activation of STAT-5 in response to IL-15. (A) Peripheral blood mononuclear cells from three elderly donors were cultured in medium or in the presence of IL-15 (50 ng mL^{-1}) for 24 h. Cells were stained with CD28-FITC/IL-15Rα-PE/CD4-PerCP, and 5×10^4 cells were acquired in each experiment. Dot plots show the frequencies of expression of IL-15Rα in CD4+ T cells related to CD28 expression in this representative experiment. (B) CD4+ T cells from 5 elderly subjects were isolated and stimulated with IL-15 for 15 min at 37 °C. Cells were surface-stained with CD28-PE and intracellularly stained with pSTAT5-AlexaFluor® 488. The percentages of pSTAT5-positive cells in CD4+CD28+ and CD4+CD28^{null} T cells in this representative experiment are expressed in each histogram plot. Histograms summarize the percentage of positive cells as mean ± SEM after the culture with IL-15 in the five experiments performed. Paired *T* test was used to compare paired frequencies.

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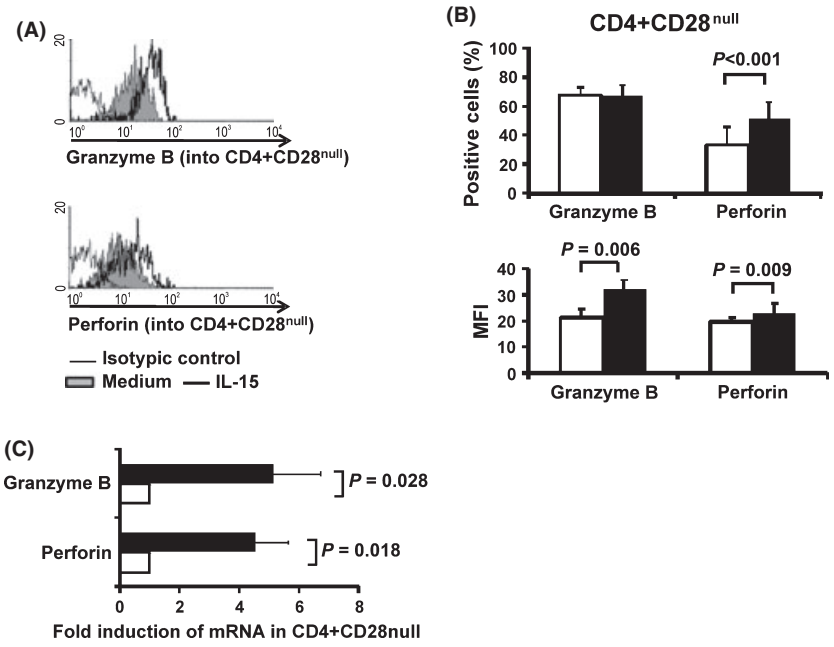

Fig. 4 IL-15 up-regulates granzyme B and perforin expression in CD4+CD28^{null} T cells. Granzyme B and perforin expression was studied by intracellular staining in 9 and 7 elderly donors, respectively, after 5 h of culture in medium in the absence or presence of IL-15 (50 ng mL^{-1}). (A) Histogram plots show a representative experiment. (B) Histograms represent the percentage and the MFI of positive cells (mean ± SEM) in CD28^{null} cells cultured in medium (white bars) and in the presence of IL-15 (black bars). (C) Granzyme B and perforin mRNA expression was determined by quantitative RT–PCR in isolated CD4+ T cells from six donors cultured for 5 h in medium (white bars) and in the presence of IL-15 (black bars) cells. Results were normalized with respect to mRNA expression in medium. Nonparametric Wilcoxon signed-ranks test (granzyme B) and paired *T* test (perforin) were used to compare differences.

significant differences in CD28^{null} cells treated with IL-15 compared with cells cultured in medium alone (P = 0.00028 and P = 0.009, respectively) (Fig. 4B). Similar results were found in the levels of expression (P = 0.006), but not in the percentage of cells expressing granzyme B (Fig. 4B). Quantification of granzyme B and perforin mRNA expression by RT–PCR in purified CD4+ T lymphocytes, with more than 15% of CD28^{null} cells, also showed higher expression following 5 h of culture with IL-15 (paired *T* test, P = 0.028 and P = 0.018, respectively) (Fig. 4C).

Lytic granules consisting of granzymes and perforin are contained in membrane-bound lysosomes coated with lysosome-associated membrane proteins (LAMPs). LAMPs are not usually present on the surface of T cells but are exposed only during active degranulation. CD107a (LAMP-1) expression has been described as a good candidate marker for the cytotoxic cellular activity (Aktas *et al.*, 2009). Isolated PBMC were cultured in medium or stimulated for 5 h with anti-CD3, IL-15, or both, and levels of surface CD107a were assessed in CD4+CD28^{null} T cells. Culture in medium alone or in the presence of IL-15 displayed no effect on degranulation (Fig. 5A). However, anti-CD3 stimulation induced expression of CD107a (mean CD107a+ 17.4% ± 4.9%), which was significantly increased with the addition of IL-15 (mean 24.5% ± 4.9%) (P = 0.015).

The effector function of CD4+CD28^{null} T cells (≥ 15% CD28^{null}) was examined in a redirected cytotoxicity assay against P815 cells previously coated with anti-CD3 mAbs. CD4+ T cells were isolated and cultured for 6 h with P815 cells in medium with or without IL-15. Cells treated with IL-15 showed cytotoxicity that was greater than that of medium-cultured cells at all target–effector cell ratios tested (from 1:1 up to 1:30) (Fig. 5B).

IL-15 showed an increasing effect on cytolytic properties of CD4+CD28^{null} T cells, both in the expression and storage of involved proteins and in the cytolytic effector functions.

IL-15 enhanced production of IFN-γ by CD4+CD28^{null} T cells in response to specific antigens

CD4+CD28^{null} T lymphocytes have been described as antigen-specific cells against chronic viral antigens, mainly in some autoimmune diseases (The-

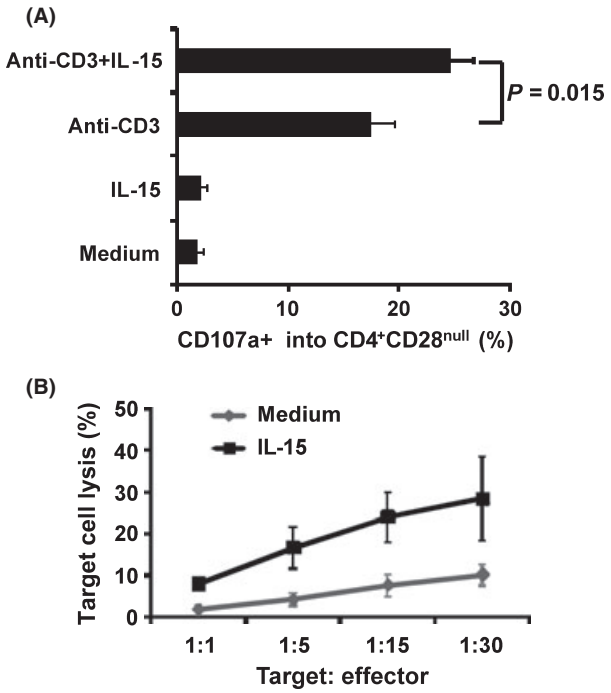

Fig. 5 Degranulation and redirected cytotoxicity induced by anti-CD3 and IL-15. (A) Peripheral blood mononuclear cells from six elderly donors were stimulated with anti-CD3 (1 μg mL^{-1}), IL-15 (50 ng mL^{-1}), and a combination of both for 5 h. Surface CD107a expression was analyzed in CD4+CD28^{null} T cells by flow cytometry. Histograms represent percentage of positive cells (mean ± SEM). Paired *T* tests were used to compare differences between medium and Il-15 and between anti-CD3 and anti-CD3 plus IL-15. (B) Cytotoxicity of purified CD4+CD28^{null} T cells from three elderly individuals in a redirected cytotoxicity assay was performed as described in Experimental procedures. Purified CD4+CD28^{null} T cells were cultured in medium or stimulated with IL-15 for 18 h, and cytotoxicity was measured against P815 cell line previously labeled with CFSE and coated with anti-CD3 (10 μg mL^{-1}). Data are shown as the percent lysis that was determined as [(%7-AAD staining sample – % 7-AAD staining of negative control) / (100 × %7-AAD staining of negative control)] × 100 (mean ± SEM).

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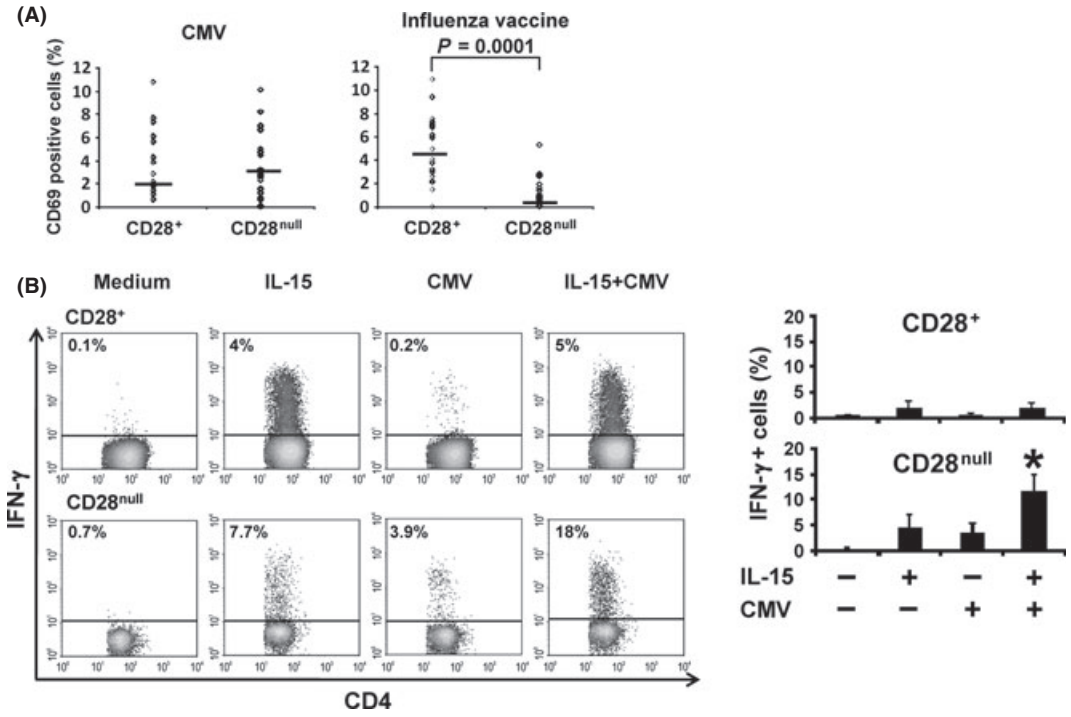

Fig. 6 CD4+CD28+ and CD4+CD28^{null} T-cell antigen-specific response to chronic and recent contact antigens. (A) CD69 expression in response to CMV antigens and to the influenza vaccine. Whole blood from elderly individuals were stimulated for 18 h with CMV (10^4 PFU mL^{-1}) or influenza vaccine (dilution 1/100), and CD69 expression was evaluated by flow cytometry. Dispersion plots summarize the percentage of CD69+ cells for CMV and influenza vaccine in the CD4+CD28+ and CD4+CD28^{null} subsets obtained from 20 elderly donors. Paired *T* test was used to compare paired frequencies. (B) Peripheral blood mononuclear cells were precultured for 18 h in medium or in the presence of IL-15 (50 ng mL^{-1}), washed, and cultured for 6 h in medium or with CMV (10^4 PFU mL^{-1}). The responder cells in the CD4+CD28+ and CD4+CD28^{null} T-cell subsets were analyzed for intracellular IFN-γ staining. Percentages of IFN-γ-positive cells in the indicated populations in this representative experiment are expressed on the dot plots. Histograms summarize the percentage of IFN-γ-positive cells in both subsets (mean ± SEM) from the five elderly donors tested. Paired *T* test was used to compare paired means (*represent $P < 0.05$ with respect all other conditions).

wissen *et al.*, 2007). In order to investigate this point in aging individuals, the response to chronic and to recent contact antigens was tested. We compared the induction of CD69 expression in response to CMV antigens and to influenza vaccine in CD4+ T cells with and without CD28 expression from a group of 20 elderly subjects (Fig. 6A). These elders were seropositive for CMV and had been recently vaccinated against the influenza virus. Their response to CMV did not induce differences in the median value of CD69-positive cells between the CD4+CD28+ subset at 2% (IR: 4.1%) and the CD4+CD28^{null} subset at 3% (IR: 3.65%) (Fig. 6A). However, there were statistically significant differences in response to influenza vaccine (Mann–Whitney *U* test, $P = 0.00013$), with 4.5% (IR: 10.9%) of CD4+CD28+ T cells expressing CD69 and only 0.67% (IR: 1.47%) of CD4+CD28^{null} T cells (Fig. 6A). To evaluate the effect of IL-15 on the antigen-specific response to chronic CMV infection, the cells were precultured for 18 h with IL-15 or in medium alone and then stimulated with an extract of CMV for 18 h. Both CD4+CD28+ and CD4+CD28^{null} T cells produced measurable levels of IFN-γ in response to IL-15 and to CMV; nevertheless, differences with cells cultured in medium alone were not statistically significant (Fig. 6B). CD4+CD28^{null} T cells pretreated with IL-15 and stimulated with CMV displayed the highest frequencies of IFN-γ-producing cells ($P < 0.05$, compared with all other stimulation conditions), showing a greater than additive effect and potentially a synergistic effect which was not observed in CD4+CD28+ T cells (Fig. 6B).

CD4+CD28^{null} T cells were mainly specific against chronic contact antigens, and pretreatment with IL-15 enhanced their antigen-specific responses.

Discussion

The effect of IL-15 was thought to be more pronounced in the CD8+ than in the CD4+ T-cell compartment. Nevertheless, to our knowledge, this is the first study demonstrating that IL-15 displays a striking effect on CD4+ T cells, with greater capacity for activating CD28^{null} cells than CD28+ cells. IL-15 increases the proliferation and frequency of CD4+CD28^{null} T cells, compared with CD4+CD28+ T cells. Moreover, activation is clearly induced by IL-15 on CD4+CD28^{null} T cells as shown by both expression of activation markers and an enhancing effect on their cytolytic properties. Pretreatment with IL-15 also enhances the IFN-γ production in the antigen-specific responses of CD28^{null} T cells with minimal effects on CD28+ cells. These effects of IL-15 may be important because the accumulation of CD28^{null} T cells is associated with reduced overall immune responses to pathogens and vaccines in the elderly(Saurwein-Teissl *et al.*, 2002) and IL-15 could potentially be useful in enhancing the antigen-specific response of CD4+CD28^{null} T cells.

A progressive increase in the percentage of T cells that lack CD28 expression is common with increasing age in healthy individuals (Goronzy *et al.*, 2007; Czesnikiewicz-Guzik *et al.*, 2008) and in patients with chronic infections and autoimmune diseases (Komocsi *et al.*, 2002; Effros *et al.*, 2005; Fletcher *et al.*, 2005; Thewissen *et al.*, 2007; Alonso-Arias *et al.*, 2009). These cells have a memory phenotype, are long-lived *in vivo*, display reduced diversity of the T-cell receptor (TCR), and have reduced division potential (Vallejo *et al.*, 1999, 2000; Appay *et al.*, 2002). At the same time, they express perforin and granzyme B, which confer cytotoxic

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ability and the capability to produce large amounts of IFN-γ (Appay *et al.*, 2002; van Leeuwen *et al.*, 2004; Alonso-Arias *et al.*, 2011). The data suggest that the loss of CD28 expression is induced by repeated exposure to the same antigens such as CMV or other persistent viral infections; chronic CMV represents a model of antigen persistence with transient reactivations mainly in immunosuppressed individuals (Appay *et al.*, 2002; Goronzy & Weyand, 2005). The cause of loss of CD28 expression in T cells with age has been attributed to repeated antigenic stimulation (Valenzuela & Effros, 2002; Vallejo, 2005), and it is now accepted that CD28^{null} T cells have experienced past episodes of activation and cell cycling. However, the loss of CD28 can result not only from repeated TCR activation but also from homeostatic proliferation. IL-15-mediated proliferation, without antigenic stimulation, results in a stable loss of CD28 expression in CD8+ memory T cells in part through the induction of TNF-α secretion (Chiu *et al.*, 2006). In this study, we failed to demonstrate a similar effect of IL-15 on CD4+ T cells because IL-15 did not induce loss of CD28 expression in CD4+CD28+ T cells in our culture conditions. Despite this, we found a significant increase in the proportion of CD4+ T cells without CD28 expression, which was attributable to an enhanced proliferative response to IL-15 of the CD4+CD28^{null} T cells with respect to CD4+CD28+ T cells. IL-15 might be able to counteract the widely described defects in the proliferative ability of CD28^{null} T cells (Appay *et al.*, 2002), even in the most differentiated EMRA cells. The possible role of this cytokine in the maintenance of CD28^{null} T cells *in vivo* was previously suggested because CD8+CD28^{null} T cells also exhibited normal growth under IL-15 stimulation *in vitro* (Chiu *et al.*, 2006).

There are no differences in the serum levels of IL-15 between old and young controls (Gangemi *et al.*, 2005), and PBMC from aged donors produced IL-15 *in vitro* at levels similar to those found in the young (Tortorella *et al.*, 2002). However, IL-15 is overproduced in some inflammatory and autoimmune diseases, and it has been speculated that chronic exposure to this cytokine is likely the mechanism of the expansion of CD4+CD28^{null} T cell *in vivo* (Yamada *et al.*, 2007). In the case of rheumatoid arthritis, the best characterized disease in which IL-15 is of substantial clinical importance, the levels of the cytokine are elevated in the patients correlating with the disease severity (McInnes *et al.*, 1997). Simultaneously, the progression of the pathology is thought to be accompanied by the recruitment and rise of oligoclonal, autoreactive CD4+CD28^{null} T cells, suggesting the potential relevance of IL-15 in this expansion.

Nevertheless, the CD4+CD28^{null} population express activation markers with little or no evidence of proliferation (Appay *et al.*, 2002) and present a low activation threshold in response to TCR stimulation, which could be implicated in its predisposition to the breakdown of self-tolerance (Yung *et al.*, 1996). Similarly, we corroborated the activating effect of IL-15 on CD4+ T cells in short-term cultures. An activated phenotype was induced in both subsets of CD4+ T cells, with the most notable differences in the expression of CD69 and CD127 in the CD28^{null} cells. CD69 displayed a clear up-regulation, whereas the percentage of cells expressing CD127, the IL-7 receptor which is down-regulated on all human T cells after activation, was decreased by nearly half. Whereas IL-15 has minimal effect on the expression of other γ-chain cytokine receptors in CD28^{null} cells, such as IL-2 and IL-15 (Alves *et al.*, 2005), the expression of CD127 in CD8+ T cells is decreased after stimulation with IL-15. IL-15 binds to a trimeric receptor composed of the γ_c chain, the IL-2/IL-15 β-chain (CD122), and the unique IL-15R α-chain. IL-15Rα is barely expressed in naïve CD8+ T cells and completely down-regulated in cultures where IL-15 is present (Alves *et al.*, 2003). It has been shown that the IL-15R α-chain is dispensable on CD8+ T cells for IL-15-mediated proliferation (Dubois *et al.*, 2002). In CD4+ T cells, we found that an increase in receptor expression is not necessary to induce the activation of STAT5, the downstream signaling molecule used by IL-15. It has been postulated that CD4+ memory T cells rely on STAT5 considerably more than do effector CD4+ T cells (Purton *et al.*, 2007; Tripathi *et al.*, 2010). Accordingly, we have found the activating effect of IL-15 on STAT5 was more prominent on CD28^{null} than on CD28+ CD4+ T cells. IL-15 increased the cytolytic properties of CD4+CD28^{null} T cells and enhanced their antigen-specific responses. Granzyme B and perforin expression in CD4+ T cells is closely associated with the loss of CD28 on the cell surface. These CD4+CD28^{null} T cells resembled cytotoxic CD8+ T cells, because their cytotoxic capacity is mediated by TCR stimulation. In addition, they lack costimulatory molecule requirements (Appay *et al.*, 2002). Similar to our findings in CD4+CD28^{null} T cells, in CD8 T cells, IL-15 may regulate perforin expression at the level of transcription through STAT5 activation, and it augments anti-CD3-induced degranulation, in agreement with the induction of cytotoxic activity (Weng *et al.*, 2002). Although the role of CD4+ T cells as cytotoxic effector cells is not well known, the enhancing effector activity of IL-15 may have much impact, because CD4+CD28^{null} T cells were mainly specific against chronic contact antigens. IL-15 plays a critical role in the immune responses to early infection and chronic inflammation by amplifying the effects of proinflammatory cytokines on IFN-γ secretion (Smeltz, 2007). In this way, pretreatment with IL-15 enhanced the antigen-specific responses of CD4+CD28^{null}, increasing the frequencies of IFN-γ-producing cells, but exerted no synergistic effect on CD4+CD28+ T cells. IFN-γ production from CD4+CD28^{null} T cells is induced at much lower doses of anti-CD3 than in their CD28+ counterparts and is also independent of costimulation (Alonso-Arias *et al.*, 2011).

It has been hypothesized that CD4+CD28^{null} T cells might be playing a role in containing viral infections tropic for HLA class II cells, such as EBV in B cells, HIV-1 in activated CD4+ T cells, monocytes and dendritic cells, and CMV in endothelial cells, although the mechanism of this antigen presentation is unknown at this time. In the case of CMV infection, endothelial cells are poor antigen-presenting cells under normal conditions in a classical immune response because they lack costimulatory molecules. But because the CD28^{null} T cells do not require costimulation and present a low activation threshold, antigen presentation could be effective by nonprofessional cells such as endothelial cells. This hypothesis is supported by the fact that the class II pathway may be preferentially targeted because both EBV and CMV prevent normal MHC class I expression as part of their strategies of immune evasion (Alcami & Koszinowski, 2000).

In summary, our data show that IL-15 plays a role in enhancing activation, proliferation, and the effector response of CD4+CD28^{null} T cells against their specific antigens. This may be mainly relevant in the elderly immune system characterized by lower adaptive responses, and treatment with IL-15 has been proposed as a strategy to alleviate the effects of T-cell deficiencies and to improve the immune function.

Experimental procedures

Donors

Blood samples were obtained from elderly donors in the Hospital Universitario Central de Asturias and the Monte Naranco Hospital (Oviedo, Spain). The volunteers were not rigorously selected according to their health status in order to study a representative sample from the population. However, those volunteers with serious diseases such as cancer, chronic diseases (diabetes, autoimmune diseases), or congestive heart failure and those receiving ongoing treatment with immunosuppressive drugs were excluded from participation. Samples from 45 elderly donors (≥ 65 years, mean age: 76 ± 6 years) were analyzed in the study to determine whether they met the inclusion criteria of the presence of

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CD4+CD28^{null} T cells at > 5% of total CD4+ T cells (n = 30). Informed consent was obtained from donors prior to participation in the study. The study was approved by the Hospital Central de Asturias (Oviedo, Spain) ethics committee.

Quantification of CD4+CD28^{null} T cells

The percentage of CD4+CD28^{null} T cells was determined in peripheral blood from the elderly participants by staining with anti-CD3 (FITC), anti-CD28 (PE) (eBioscience, San Diego, CA, USA), anti-CD4 (PerCP), and anti-CD45 (APC) (Immunostep, Salamanca, Spain). One hundred microliters of whole blood from elderly was stained with the combination of labeled monoclonal antibodies for 30 min at room temperature. Samples were red blood lysed with FACS Lysing Solution (BD Biosciences, San José, CA, USA), washed in PBS, and analyzed with CellQuest software in the FACSCalibur Cytometer (BD Biosciences). Appropriate isotype control monoclonal (mAbs) were used for marker settings. CaliBRITE Beads (BD Biosciences) were used to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity. BD Multicheck Control and Multicheck CD4 Low Control were used as quality controls.

Isolation of PBMC and cell cultures

Peripheral blood mononuclear cells were isolated from peripheral blood that had been anticoagulated with EDTA by centrifugation on Ficoll-Hypaque gradients (Lymphoprep; Nycomed, Oslo, Norway). Cultures were performed in RPMI 1640 medium containing 2×10^{-3}M L-glutamine and Hepes (BioWhitaker, Verviers, Belgium) and supplemented with 10% FCS (ICN Flow; Costa Mesa, CA, USA) and antibiotics. Cells were incubated at 37 °C and 5% carbon dioxide.

Stimulation with IL-15

Peripheral blood mononuclear cells (4×10^6 cells mL^{-1}) were cultured in the presence and absence of IL-15 (50 ng mL^{-1}) (Peprotech INC, Rockyhill, NJ, USA) for different times. Frequencies of CD4+CD28^{null} T cells were quantified after 7 days of culture with the same combination of labeled antibodies described earlier. Additional staining was performed with anti-CD27 (APC) (eBioscience), anti-CCR7 (Alexa Fluor 647) (BD Bioscience), and CD45RA (FITC) (Immunostep). To analyze an activated phenotype, cells were cultured for 18 h and then stained with anti-CD3 (APC), anti-CD28 (FITC or PE), anti-CD69 (FITC), (eBioscience), anti-CD4 (PerCP), anti-CD127 (PE), anti-IL-15R α-chain (eBioscience), anti-HLA-DR (FITC), and anti-CD25 (PE) (BD Bioscience). Frequencies of cells with intracytoplasmic stores of granzyme B and perforin in CD4+CD28^{null} T cells after 6 h of culture with IL-15 were measured. Cells were surface-stained for 30 min at room temperature, lysed and fixed with FACS lysing solution, permeabilized with BD FACS Permeabilizing Solution 2 (Perm II) (BD Bioscience), and stained with anti-granzyme B (FITC) or anti-perforin (FITC) (BD Bioscience) for 30 min at room temperature. Cells were washed and resuspended in 1% paraformaldehyde until FACS analysis.

Proliferation assays

Peripheral blood mononuclear cells were resuspended in PBS at a final concentration of 5–10 $\times$ 10^6 cells mL^{-1} and incubated with 1.5 μM CFSE (Invitrogen, Paisley, Scotland, UK) for 10 min at 37 °C, washed with RPMI 1640 medium containing 2×10^{-3}M L-glutamine and Hepes twice, and cultured at 2×10^6 cells mL^{-1} in the presence of human recombinant IL-15 (50 ng mL^{-1}). The proliferative responses of CD4+ and CD8+ T cells were analyzed on days 7 and 14 by FACSCalibur after staining with anti-CD4 (PerCP) and anti-CD28 (PE).

mRNA quantification

CD4+ T cells from individuals with more than 15% CD4+CD28^{null} T cells were isolated (Myltenyi Biotec GmbH, Bergisch Gladbach, Germany). mRNA was extracted using a Total RNA Isolation (Macherey-Nagel GmbH & CoKG, Düren, Germany) according to the manufacturer's instructions. Reverse transcription of mRNA isolated from each sample was carried out in a 20-μL final volume with the iScript cDNA Synthesis Kit (Bio-Rad, Life Science Research Group, Hercules, CA, USA) following manufacturer's instructions. The mixture was incubated at 25ºC for 5 min, at 42 °C for 30 min, and at 85 °C for 5 min and stored at –80 °C until required for PCR.

Quantification of perforin and granzyme B expression was performed using SYBR Green real-time quantitative PCR and an iCycler thermocycler (Bio-Rad) with the previously described primers (Morissette *et al.*, 2007). Briefly, a calibration curve was generated with serial dilutions of granzyme B and perforin and GAPDH external standards that allowed the quantification of cDNA samples, leading to the determination of mRNA relative units. Reactions without cDNA were always included as a negative control, and cDNA samples were quantified in duplicate. External granzyme B and perforin standards were serial dilutions of the cDNA obtained from CD8+ T cells stimulated for 3 h with anti-CD3 (1 μg mL^{-1}). Thermal cycling conditions began with 50 °C for 2 min and 94 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 58 °C for 30 s, and 72 °C for 1 min. Experimental samples were run in duplicate, and the replicate average value was recorded as the sample result.

STAT5 quantification

Isolated CD4+ T cells were stimulated with IL-15 recombinant protein (50 ng mL^{-1}) for 15 min at 37°°C. Cells were fixed using BD Cytofix Fixation Buffer (10 min at 37°°C), stained on the surface with anti-CD28 (PE), and permeabilized in BD Phosflow Perm Buffer III (30 min on ice). Cells were then washed twice in BD Pharmingen Stain Buffer and stained with 20 μL Alexa Fluor 488-conjugated phospho-Stat5 antibody for 1 h at room temperature. The cells were analyzed on a BD FACSCalibur flow cytometer.

Lysosomal degranulation assay

CD107a lysosome-associated membrane protein-1 (LAMP-1) expression was used to measure CD4+ T-cell degranulation. PBMC were incubated for 5 h in medium alone, IL-15 (50 ng mL^{-1}), anti-CD3 (1 μg mL^{-1}) (eBioscience), and IL-15 plus anti-CD3. After the first hour of culture, monensin (2 μM) (BD Biosciences), a protector of the cell degranulation, was added. Cells were then stained with anti-CD28 (FITC), anti-CD4 (PerCP), and CD107a antibody (Alexa Fluor 647) (eBioscience). All samples were analyzed with CellQuest software in the FACSCalibur Cytometer.

Redirected cytotoxicity assay

Peripheral blood mononuclear cells were separated from the blood of three elderly individuals with > 20% CD4+CD28^{null} T cells. Isolated CD4+ T cells (Miltenyi Biotec GmbH) were stained with anti-CD28-PE, and CD28^{null} cells were also isolated by negative selection with anti-PE

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magnetic microbeads (Miltenyi Biotec GmbH). Cytotoxicity of T-cell subsets was determined as previously described (Lecoeur *et al.*, 2001). Briefly, purified CD4+CD28^{null} T cells from donors were cultured in medium with or without IL-15 for 18 h as a source of effector cells and resuspended at appropriate concentrations for the desired effector–target cell (E/T) ratio. Target cells (P815) were labeled with CFSE and washed three times in complete medium. The P815 cells were then incubated for 30 min on ice with 10 μg mL^{-1} of anti-CD3 mAb, resuspended in medium, and adjusted to 10^5 cells mL^{-1}. Effector cells were added to 100 μL target cells in round-bottom polystyrene tubes (BD Biosciences) to yield E/T ratios of 1:1, 1:5, 1:15, and 1:30, mixed by gentle tapping, incubated at 37 °C for 4 h, and stained with 5 μL 7-ADD (Immunostep) for 20 min before data acquisition. Percentage of lysis was determined as [(%7-AAD+ cells in test sample – % 7-ADD+ cells in negative control)/(100 × %7-ADD+ cells in negative control)] × 100.

Antigen-specific stimulation

Antigen-specific responses were analyzed in PBMC from CMV-seropositive individuals vaccinated with a trivalent influenza vaccine (Solvay Biologicals BV, Olst, Holland). CMV-infected cell lysate was prepared by infecting human embryonic lung fibroblasts with the AD169 strain of CMV. Viral titers in the supernatant were determined by standard plaque assays. The virus was inactivated by repeated freeze–thaw cycles. Heparinized venous blood samples were stimulated with CMV (10^4 PFU mL^{-1}) or a 1/100 dilution of the influenza vaccine (Solvay Biologicals BV) in 15-mL conical polypropylene tubes in a humidified 37 °C incubator for 18 h. Activation was assessed by surface staining with anti-CD69. The cells were also stained with anti-CD3, anti-CD4, and anti-CD28. Samples were red blood cell lysed with FACS lysing solution, washed in PBS, and analyzed with CellQuest software.

To quantify IFN-γ production, PBMC (4×10^6 cells mL^{-1}) were precultured for 18 h in medium or in the presence of IL-15 (50 ng mL^{-1}), washed, and cultured again in medium or with CMV (10^4 PFU mL^{-1}) for 6 h. Cultures for the detection of intracytoplasmic cytokines were treated after the first 2 h with the secretion inhibitor Brefeldin A (10 μg mL^{-1}) (Calbiochem, Darmstadt, Germany). After four additional hours, cells were treated with 2 mM EDTA for 15 min at room temperature, washed, and stained with antibodies against CD4 and CD28 at 4 °C. Intracellular staining of anti-IFN-γ (BD Biosciences) was performed as previously described for granzyme B and perforin.

Virological testing

Immunoglobulin G levels of CMV-specific antibodies were determined by enzyme-linked immunosorbent assay Vir-ELISA Anti-CMV-IgG (Viro-Immun Labor-Diagnostika GmbH, Oberursel, Germany) according to the manufacturer's specifications. Patient samples were quantified and interpreted by means of the calculation of the ratio (Cutoff Index = OD value of sample/cutoff value), whereby a ratio of 1.0 is equivalent to the cutoff value. Cutoff indexes > 1.1 were considered positive.

Statistical analysis

Comparisons between groups were performed with the nonparametric Wilcoxon signed-rank test when data were not normally distributed or with Student's *t* test for paired data. Analyses were performed using the SPSS 15.0 statistical software package program (SPSS Inc., Chicago, IL, USA). *P*-values of 0.05 or less were considered significant.

Acknowledgments

This work was supported by Red de Investigación Renal (REDinREN), Spanish grant FIS PI080566 from Instituto ''Carlos III'' (European FEDER founds).

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III.3. RELACIÓN EXISTENTE ENTRE LA RESPUESTA HUMORAL AL CMV Y EL ESTADO FENOTÍPICO Y FUNCIONAL DEL SISTEMA INMUNE

La seropositividad a CMV se asocia con un envejecimiento de las subpoblaciones celulares responsables de la inmunidad adaptativa, causa, a su vez, de una respuesta inmunológica defectuosa en los ancianos (Nikolich-Zugich 2008). El tiempo transcurrido desde la infección y/o la intensidad y frecuencia de las reactivaciones virales podrían estar condicionando la respuesta específica frente al virus e influyendo de diferente modo sobre el sistema inmune. Por todo esto, el título de anticuerpos frente a CMV podría reflejar la historia de la infección. El objetivo de este estudio fue analizar el título de anticuerpos frente a CMV en ancianos y su correlación con la respuesta celular frente al virus y el envejecimiento de su sistema inmunológico.

ARTÍCULO 3:

Rebeca Alonso Arias, **Marco Antonio Moro García**, Ainara Echeverría, Juan José Solano-Jaurrieta, Francisco Manuel Suárez-García, Carlos López-Larrea. *"Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system".*

J Virol. 2013 Apr;87(8):4486-95.

Para estudiar el impacto de los títulos de anticuerpos anti-CMV en el sistema inmune comparamos las características fenotípicas y funcionales de los linfocitos T de 92 ancianos sanos y 70 controles jóvenes sanos. Estudiamos las subpoblaciones linfocitarias por citometría de flujo y medimos la proliferación y la activación celular (CD69) frente a CMV. También estudiamos el porcentaje de emigrantes recientes del timo (EMR) mediante cuantificación de TRECs por PCR a tiempo real. Los anticuerpos específicos frente a CMV se determinaron mediante ELISA. Los títulos de anticuerpos anti-CMV fueron significativamente mayores en los ancianos que en los individuos jóvenes y se correlacionaron positivamente con la respuesta específica frente a CMV en linfocitos T CD4+. En ancianos, los anticuerpos se asociaron con el grado de diferenciación y con el contenido en TREC en los linfocitos T CD4+, se correlacionaron con diversos parámetros del IRP y presentaron una gran asociación sobre la capacidad de respuesta frente a la inmunización in vivo. La falta de correlación en los individuos jóvenes

podría ser debido a la menor carga de anticuerpos frente a CMV que presentan. El aumento del número de células altamente diferenciadas en los individuos con altos niveles de anticuerpos frente a CMV puede estar compensado con la reducción en el número absoluto de células T CD4+ para así evitar el aumento del número total de linfocitos T.

En resumen, nuestros datos muestran que los títulos de anticuerpos anti-CMV y no solamente la seropositividad a CMV influyen en el estado de diferenciación y en la funcionalidad del sistema inmune en ancianos, siendo por tanto, el control de la infección una posible diana para intentar mejorar la respuesta inmune en ancianos.

Aportación personal al trabajo:

En este trabajo, mi labor se centró principalmente en recolectar y procesar las muestras del estudio, realizar y/o supervisar los distintos experimentos y analizar los resultados obtenidos. También participé en el diseño del manuscrito.

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Intensity of the Humoral Response to Cytomegalovirus Is Associated with the Phenotypic and Functional Status of the Immune System

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Cytomegalovirus (CMV) infection exerts an enormous effect on human immunity, as it is associated with an immune-impaired response, a variety of chronic diseases, and overall survival in elderly individuals. Levels of anti-CMV antibodies may be associated with the differentiation degree of T cell subsets. Titers are significantly higher in the elderly and positively correlated with specific CD4$^+$ T cell responses to CMV. In the elderly, antibody titers are associated with the degree of differentiation and the T cell receptor excision circle (TREC) content in CD4$^+$ T cells, with other features of the immune risk profile, and with a reduced ability to respond to immunization *in vivo*. Associations may be absent in young subjects because their anti-CMV antibody titers are lower than those of the elderly. However, comparing young and elderly individuals with similar antibody levels reveals differences in their highly differentiated and naïve T cells. These are more marked in individuals with high titers. In parallel with the increase in anti-CMV antibodies, the elderly experience a significant reduction in absolute counts of naïve CD4$^+$ T cells, which may be a strategy to compensate for the expansion of differentiated cells and to avoid an increase in total T cells. In summary, our results show that titers of anti-CMV antibodies, and not only CMV seropositivity, are related to differentiation status and immunocompetence in the elderly, making this as an important prognostic marker of the status of immune system function.

The betaherpesvirus cytomegalovirus (CMV) is a persistent activating virus that primarily resides in the myeloid cell compartment but also spreads to other cell types. Clinically, infection is considered essentially asymptomatic in immunocompetent hosts. However, immunosuppressed individuals may suffer serious consequences as a result of viral reemergence. After infection, the virus establishes lifelong latency within the host and periodically reactivates. Reactivation from latency is a key step in the pathogenesis of the infection and can be detected in response to inflammation, infection, stress, or immunosuppression (1, 2). Activation of protein kinase C and NF-κB by tumor necrosis factor alpha (TNF-α) and increasing concentrations of cyclic AMP by stress hormones and prostaglandins promote viral reactivation and replication. Reactivation of CMV is more frequent in the elderly, as demonstrated by the direct detection of CMV DNA in the urine. Although reactivation is frequent in the elderly, it is subclinical in nature (3).

Recently, CMV has been linked to a variety of chronic diseases with an inflammatory component, including cardiovascular diseases, cancer, and cognitive and functional impairment (4–7). The specific mechanisms responsible for these associations have not been fully determined but are likely to have an inflammatory and immune component. Reactivation may result in increased levels of proinflammatory molecules such as interleukin 6 (IL-6), TNF-α, and C-reactive protein (CRP). In fact, CMV has been associated with a significantly greater risk of all-cause mortality and cardiovascular disease-related mortality in subjects with high CRP levels (8). Longitudinal aging studies suggest that CMV seropositivity is associated with a cluster of immune parameters, the "immune risk profile" (IRP), which predicts 2-year mortality in a population-based sample of Swedish octogenarians (9). Moreover, CMV seropositivity is also associated with the phenomenon of immunosenescence, which is characterized by clonal expansion of T cells, mainly CD8$^+$ T cells, with a highly differentiated cell surface phenotype and with the acquisition of new functional features. Moreover, the hallmark of human immunosenescence, irrespective of its association with CMV, is low numbers of naïve T cells, particularly CD8$^+$ T cells, and a corresponding lower diversity of the T cell receptor (TCR) repertoire (10, 11). This has been proposed as an explanation for much of the decreased ability of the elderly to resist new infections and to respond effectively to reinfection and persistent infections (12). Indeed, despite the evidence suggesting that CMV induces aging of T lymphocytes, more frequent and/or intensive reactivations in the elderly may be a consequence rather than the cause of immunosenescence. A general result of these alterations is that adaptive immunity seems to weaken with age, whereas innate immunity is maintained or even enhanced. This may be one of several explanations for the commonly reported increase in proinflammatory status in the elderly, which itself is associated with frailty and mortality (13).

The frequency of CMV seropositivity in the elderly in developed countries is above 80%. In spite of the enormous impact of CMV infection on human immunity, the degree of immunosenescence varies greatly, as is apparent even when only CMV-infected individuals are studied (14). We postulate that the humoral response to CMV may be a consequence of deleterious changes in the immune system and may be related to other changes induced in the immune response, rather than merely infection *per se*. To investigate this, we studied the association between anti-CMV antibody titers and various characteristics that define the degree of differentiation and the functional abilities of the T cell fraction.

Received 6 September 2012 Accepted 29 January 2013
Published ahead of print 6 February 2013
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doi:10.1128/JVI.02425-12

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MATERIALS AND METHODS

Study population. Blood samples for hematological and immunological analyses were obtained from 92 healthy elderly donors (70 females and 22 males) living in the Santa Teresa nursing home (Oviedo, Spain). To ensure that a representative sample of the population was studied, volunteers were not rigorously selected with respect to their health status. Exclusion criteria were conditions with a possible influence on the immune system, such as recent or current infection, inflammation, autoimmune or malignant disease, malnutrition, abnormal laboratory data, and pharmacological interference (steroids, nonsteroidal anti-inflammatory agents, and immunosuppressive drugs). Samples from 70 young healthy controls (24 females and 46 males) were obtained from the Centro de Transfusiones del Principado de Asturias (Oviedo, Spain). Inclusion criteria were a minimum age of 65 years for the elderly group and a maximum age of 50 for the young group. The mean age of the elderly individuals was 85.1 ± 6.3 years (range, 68 to 97 years), and that of the young controls was 38.7 ± 8.4 years (range, 20 to 50 years).

Informed consent was obtained from patients and controls before they participated in the study. The Hospital Universitario Central de Asturias Ethics Committee (Oviedo, Spain) gave ethical approval for the study.

CMV and influenza virus serology. Levels of CMV-specific immunoglobulin G (IgG) antibodies were determined by the enzyme-linked immunosorbent Vir-ELISA anti-CMV IgG assay (Viro-Immun Labor-Diagnostika GmbH, Oberursel, Germany), carried out in accordance with the manufacturer's specifications.

Anti-influenza virus antibodies in serum obtained from elderly individuals were measured by enzyme-linked immunosorbent assay (ELISA) as previously described (15). The optical density (OD) values of individual samples were compared against a calibration curve comprising the OD values of serial dilutions of the same internal control serum from a healthy vaccinated young person throughout the experiments. Results are expressed as arbitrary units. The antibody titer decreased significantly with time since vaccination, so these values were normalized by adjusting the titer by the time elapsed since immunization.

Hematological analysis and immunological phenotyping. Hematological parameters were determined with a Sysmex XT-2000i analyzer (Sysmex, Hamburg-Norderstedt, Germany). The results of the cytometric studies were analyzed using a FACSCalibur cytometer with CellQuest software (BD Biosciences, San Jose, CA). EDTA-treated peripheral blood was surface stained with the following antibodies (antibody labels are in parentheses): anti-CD8 (phycoerythrin [PE]), anti-CD45RA (fluorescein isothiocyanate [FITC]) (Immunostep, Salamanca, Spain), anti-CD4 (peridinin chlorophyll protein [PerCP]), anti-CCR7 (Alexa Fluor 647), anti-CD3 (FITC) (BD Biosciences), and anti-CD28 (PE) (eBioscience, San Diego, CA). One hundred microliters of whole blood from elderly individuals was stained with different combinations of labeled monoclonal antibodies (MAbs) for 20 min at room temperature. Samples were red-blood lysed with FACS lysing solution (BD Biosciences), washed in phosphate-buffered saline (PBS), and analyzed with CellQuest software in the FACSCalibur cytometer. Appropriate isotype control MAbs were used for marker settings. In the study of the parameters associated with immunosenescence, staining was performed with anti-CD3 (FITC), anti-CD28 (PE), anti-CD4 (allophycocyanin [APC]) (eBioscience), and anti-CD8 (PerCP). Frequencies of $CD4^+$ and $CD8^+$ T cells were quantified into gated $CD3^+$ T cells, and those of $CD8^+$ $CD28^{null}$ and $CD4^+$ $CD28^{null}$ cells were gated into $CD3^+$ $CD8^+$ and $CD3^+$ $CD4^+$ T cells, respectively.

Activation studies. CMV-infected cell lysate was prepared by infecting human embryonic lung fibroblasts with the AD169 strain of CMV, and viral titers in the supernatant were determined by standard plaque assays (viral stock, 10^5 PFU/ml). The virus was inactivated by five freeze-thaw cycles.

Activation of T lymphocytes from heparinized whole blood by anti-CD3 or CMV extract was assessed by surface staining with anti-CD69 (eBioscience). Briefly, heparinized whole blood (250 μl) was stimulated with soluble anti-CD3 (10 ng/ml) (eBioscience) or with the CMV supernatant (dilution, 10^4 PFU/ml) in 15-ml conical polypropylene tubes for 18 h at 37°C in an atmosphere of 5% carbon dioxide. After stimulation, cells were stained with anti-CD69 and anti-CD4 monoclonal antibodies.

Proliferation cultures. To determine the proliferation status of $CD4^+$ T cells, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Ficoll-Hypaque gradients (Lymphoprep; Nycomed, Oslo, Norway). PBMCs were resuspended in PBS at a final concentration of 5×10^6 to 10×10^6 cells/ml and incubated with 1.5 μM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Paisley, United Kingdom) for 10 min at 37°C, washed with RPMI 1640 medium containing 2×10^3 M L-glutamine and HEPES (BioWhittaker, Verviers, Belgium) twice, and cultured at 1×10^6 cells/ml in the presence of soluble anti-CD3 (10 ng/ml) and CMV extract (10^4 PFU/ml). The proliferative responses of $CD4^+$ T cells were analyzed on day 7 using a FACSCalibur after staining with anti-CD4.

TREC quantification. DNA was extracted from isolated $CD4^+$ T cells (>90% purity) from PBMCs using a QIAamp DNA minikit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. To isolate $CD4^+$ T cells, PBMCs were isolated by centrifugation on Ficoll-Hypaque gradients (Lymphoprep) after 20 min of incubation with RosetteSep human $CD4^+$ T cell enrichment cocktail (StemCell Technologies, Grenoble, France). Signal-joint TREC was quantified by SYBR green real-time quantitative PCR and an iCycler thermocycler (Bio-Rad; Life Science Research Group, Hercules, CA), as previously described (16). Experimental samples were run in duplicate, and the replicate average was used as the sample result.

HLA-DRB1 typing. HLA-DRB1 alleles were typed, in accordance with the manufacturer's protocols, using Lifecodes HLA-SSO typing kits (Tepnel Lifecodes Corporation, Stamford, CT) based on the Luminex Corporation's X-Map technology.

TNF-α promoter polymorphism genotyping. Single nucleotide polymorphisms (SNPs) at position −308 of the TNF-α gene were determined by analyzing the melting temperature (T_m) of the probe/target duplex after PCR amplification and hybridization with fluorescently labeled probes matched with one sequence variant (LightCycler; Roche Diagnostics, Mannheim, Germany). The primers used were CCTGCATC CTGTCTGGAAGTTA and CTGCACCTTCTGTCTCGGTTT. The hybridization probes (designed by TIB Molbiol, Berlin, Germany) used were AACCCCGTCCCCATGCCCC-F and LC Red 640-CCAAACCTATTGC CTCCATTTCTTTTGGGGAC.

Cytokine quantification. Cytokine levels were measured in the sera of elderly subjects using X-Map technology with a Milliplex map kit (Millipore, Billerica, MA), according to the manufacturer's specifications. This bead-based analyte detection system was used to quantify human TNF-α and IL-15 using the Luminex Corporation's X-Map technology.

Statistical analysis. Groups were compared by the nonparametric Mann-Whitney U and Kruskal-Wallis tests (for non-normally distributed data) or by Student's independent-samples *t* test (for normally distributed data). Correlations between variables were assessed by the nonparametric Spearman rho test. The χ^2 test was used to compare dichotomous variables, and multiple linear regression was used to consider variables simultaneously. Analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL). Significance was concluded for P values of <0.05.

RESULTS

CMV seropositivity and antibody titers in young and elderly people. The characteristics of the individuals enrolled in the study are shown in Table 1. We quantified levels of anti-CMV antibodies in the sera of the 70 young and 92 elderly donors. The frequencies of seropositivity were 52% and 91%, respectively (Fig. 1A) (χ^2 test; odds ratio [OR], 9.64 to 22.8; $P < 0.001$). Levels of anti-CMV antibodies in seropositive individuals were significantly higher in elderly than in young individuals, with medians of 1,625 VIRO units (VU)/ml (interquartile range [IR], 586 VU/ml) and 1,150

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TABLE 1 Characteristics of the study subjects

Parameter[a]	Elderly donors ($n = 92$)	Young donors ($n = 70$)
Demographic data		
Age (yrs)		
Mean ± SE	85.1 ± 6.3	38.7 ± 8.4
Range	68–97	20–50
No. of subjects investigated		
Women	70	24
Men	22	46
Hematology values (means ± SDs)		
RBCs (10^6/μl)	4.28 ± 0.63	5.19 ± 0.71
Hemoglobin (g/dl)	13.0 ± 1.89	14.76 ± 1.17
Hematocrit (%)	39.19 ± 5.6	44.06 ± 2.87
MCV (fl)	91.78 ± 6.47	90.6 ± 3.7
Platelets (10^3/μl)	221.65 ± 71.08	312.56 ± 62.44
WBCs (10^3/μl)	6.30 ± 1.80	7.54 ± 1.53
Neutrophils (10^3/μl)	3.67 ± 1.32	4.18 ± 0.98
Monocytes (10^3/μl)	0.53 ± 0.36	0.46 ± 0.25
Lymphocytes (10^3/μl)	1.84 ± 0.68	3.0 ± 0.76

[a] RBCs, red blood cells; MCV, mean corpuscular volume; WBCs, white blood cells.

VU/ml (IR, 535.5 VU/ml), respectively (Mann-Whitney U test; $P < 0.001$) (Fig. 1B).

Aging was associated not only with the percentage of CMV seropositivity but also with the levels of anti-CMV antibodies.

Correlation between anti-CMV-specific T cells and antibody titer. To analyze whether individuals with higher anti-CMV antibody titers also have stronger CMV-specific T cell responses, the $CD4^+$ T cell response was measured by stimulating whole-blood cultures with CMV antigens and with anti-CD3. CD69 expression in response to CMV extracts and to anti-CD3 was analyzed in $CD4^+$ T cells. The magnitude of the $CD4^+$ T cell immune responses to CMV was positively correlated with anti-CMV antibody titers in the elderly (Spearman test; rho = 0.490 and $P = 0.002$) (Fig. 1C) but not in young donors (data not shown). No correlations were found between antibody titers and activation in response to anti-CD3 in $CD4^+$ T cells in elderly subjects (Fig. 1C). Similarly, when proliferative responses were quantified in PBMC cultures, there was a significant correlation with $CD4^+$ T cell proliferation only in the elderly group in response to CMV (Spearman test; rho = 0.516 and $P = 0.01$) but not in response to anti-CD3 (Fig. 1D). No correlations were found between activation or proliferation in $CD4^+$ T cells with anti-CMV antibody titers in young donors (data not shown).

Levels of anti-CMV antibodies and CMV-specific $CD4^+$ T cells were clearly related in elderly individuals.

T cell differentiation subsets and anti-CMV antibody titer. It is widely accepted that the progressive deterioration of the T cell compartment with advancing age is related to CMV seropositivity. T cells can be separated into functionally distinct populations using combinations of cell surface markers such as CD45RA and CCR7. We used these markers to classify the T cells into naïve ($CD45RA^+$ $CCR7^+$), central memory (CM; $CD45RA^-$ $CCR7^+$), effector memory (EM; $CD45RA^-$ $CCR7^-$), and effector memory RA (EMRA; $CD45RA^+$ $CCR7^-$) groups (17). We wanted to verify

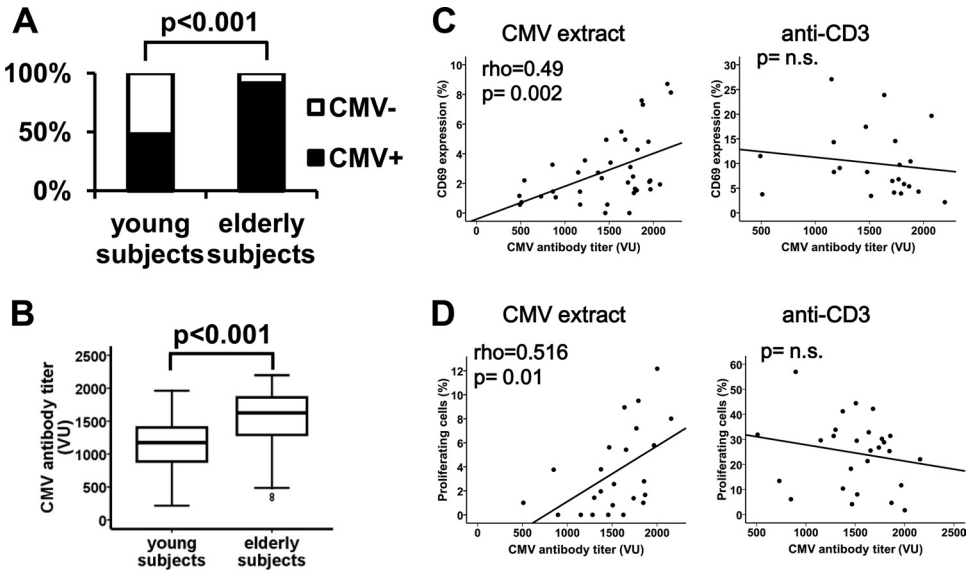

FIG 1 Frequencies of CMV infection and titers of anti-CMV antibodies in young and elderly subjects and response of $CD4^+$ T cells from elderly subjects to CMV and anti-CD3. Immunoglobulin G levels of CMV-specific antibodies were determined by ELISA and a semiquantitative diagram was used to calculate the CMV titer. (A) Histograms show the percentages of CMV-seropositive (black bars) and CMV-seronegative (white bars) young ($n = 70$) and elderly ($n = 92$) subjects. The calculated titers of the patient samples are indicated as VIRO units (VU). Percentages of CMV-infected individuals were compared using the χ^2 test. (B) Titers of anti-CMV antibodies in infected young ($n = 37$) and elderly ($n = 82$) subjects are illustrated in the box plots. Significant group differences, assessed with the Mann-Whitney U test, are indicated. (C and D) CD69 expression and proliferative capacity of $CD4^+$ T cells from elderly subjects in response to CMV *in vitro* stimulation were analyzed. (C) Correlation between anti-CMV antibody titers and the frequency of CD69 expression in $CD4^+$ subsets from elderly individuals in response to a CMV supernatant (10^4 PFU/ml) ($n = 37$) and to anti-CD3 (10 ng/ml) ($n = 22$). Whole blood from CMV-seropositive elderly people was stimulated for 18 h. (D) Proliferative capacity of $CD4^+$ T cell subsets in response to CMV stimulation ($n = 28$) and to anti-CD3 ($n = 27$). PBMCs were labeled with CFSE (1.5 μM) and cultured in the presence of CMV supernatant or anti-CD3 for 5 days. The percentage of dividing $CD4^+$ T cells is represented. Spearman correlation coefficients and corresponding *P* values are presented in the upper left-hand corner. n.s., not significant.

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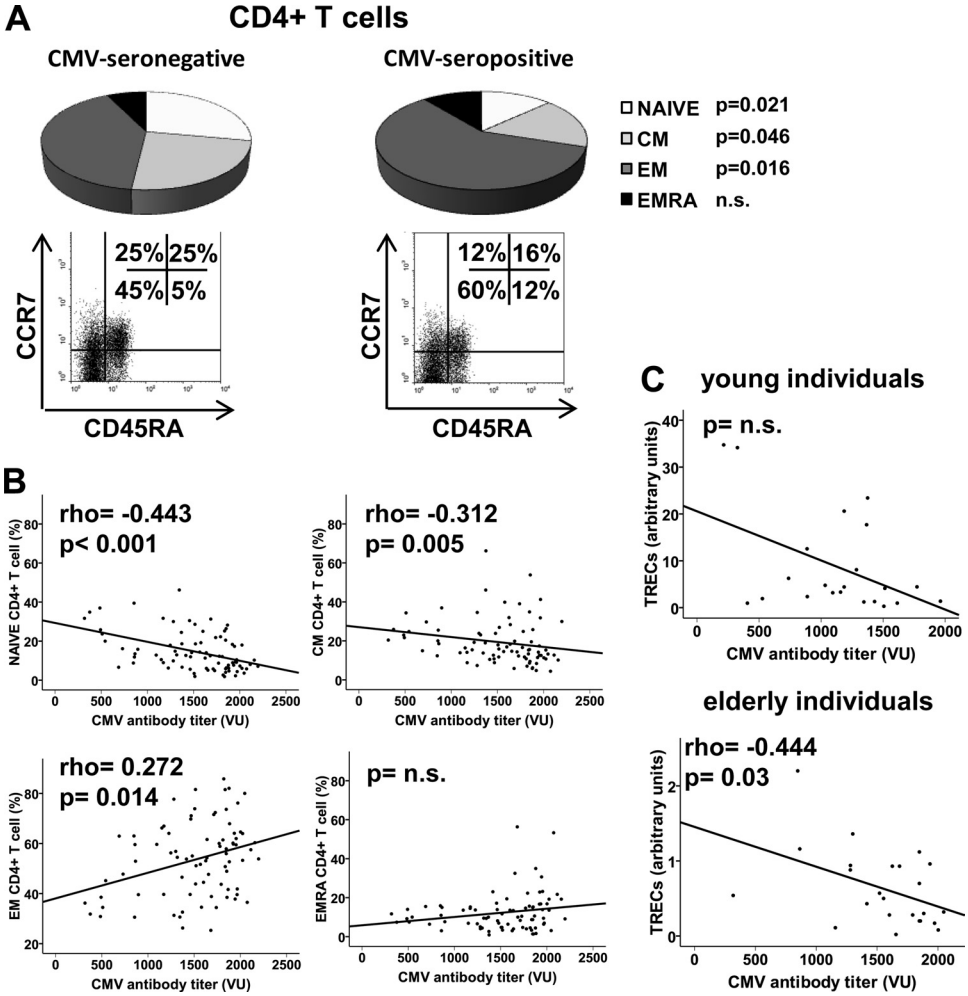

FIG 2 Distribution of $CD4^+$ T cells into naïve ($CD45RA^+$ $CCR7^+$), central memory ($CD45RA^-$ $CCR7^+$), effector memory ($CD45RA^-$ $CCR7^-$), and effector memory RA ($CD45RA^+$ $CCR7^-$) related to CMV seropositivity and anti-CMV antibody titers in elderly subjects. CD45RA and CCR7 expression was analyzed by flow cytometry in gated $CD3^+$ $CD4^+$ T cells. (A) Individual segments of the pie charts show the proportions of each subset in CMV-seronegative and CMV-seropositive elderly subjects. Representative dot plots of the subsets defined by CD45RA and CCR7 expression for individuals in each group show the percentages of positive cells. Student's *t* test or the Mann-Whitney U test was used to examine differences between the groups, and *P* values are represented. (B) Relationship between anti-CMV antibody titers and frequency of the $CD4^+$ T cell populations defined by CD45RA and CCR7 expression. (C) TREC content was measured in $CD4^+$ T cells from young ($n = 25$) and elderly ($n = 30$) CMV-seropositive subjects. The $CD4^+$ population was isolated by magnetic bead separation and the TREC copy number was determined by real-time PCR. Experiments were conducted in duplicate, and the results are presented relative to anti-CMV antibody titers in each subject. Spearman correlation coefficients and corresponding *P* values are shown in the upper left-hand corner.

the association between CMV seropositivity and the degree of differentiation of T cell subsets in young and elderly individuals. First, we compared the distributions of the T cell subpopulations in seropositive and seronegative individuals and found that CMV seropositivity was related to the reduced frequency of undifferentiated subsets, naïve and CM, only in the $CD4^+$ T cells of elderly individuals (Fig. 2A). No differences were found in the $CD8^+$ T cells from elderly people. Most $CD8^+$ T cells belonged to the EM and EMRA subsets, which are the final stages of differentiation (data not shown). Moreover, the frequencies of the four populations were similar in young seropositive and seronegative subjects in $CD4^+$ and $CD8^+$ T cells (data not shown).

We then calculated the correlation between the anti-CMV antibody titer and the frequency of these T cell subsets. We were unable to demonstrate any associations in young people, but the effect was evident in $CD4^+$ T cells in elderly individuals. In fact, the frequency of naïve and CM $CD4^+$ T cells showed a significant negative correlation with antibody titers (Spearman test; rho $= -0.443$ and $P < 0.001$ and rho $= -0.312$ and $P = 0.005$, respectively) (Fig. 2B). On the other hand, the more differentiated EM subset showed a significant and progressive increase (Spearman test; rho $= 0.272$ and $P = 0.014$), and no effect on the proportions of EMRA was found (Fig. 2B). Again, frequencies of $CD8^+$ T cell subsets were independent of the antibody titers (data not shown).

To examine the differences in the differentiated status of $CD4^+$ subsets in CMV-seropositive individuals further, we assessed the replicative history of these cells by quantifying the content of T cell receptor excision circles (TREC) in $CD4^+$ T cells. TREC constitute a traceable molecular marker produced in newly naïve T cells; the content of TREC in peripheral T cells is an indicator of the number of divisions that the cell has undergone (18). The analysis

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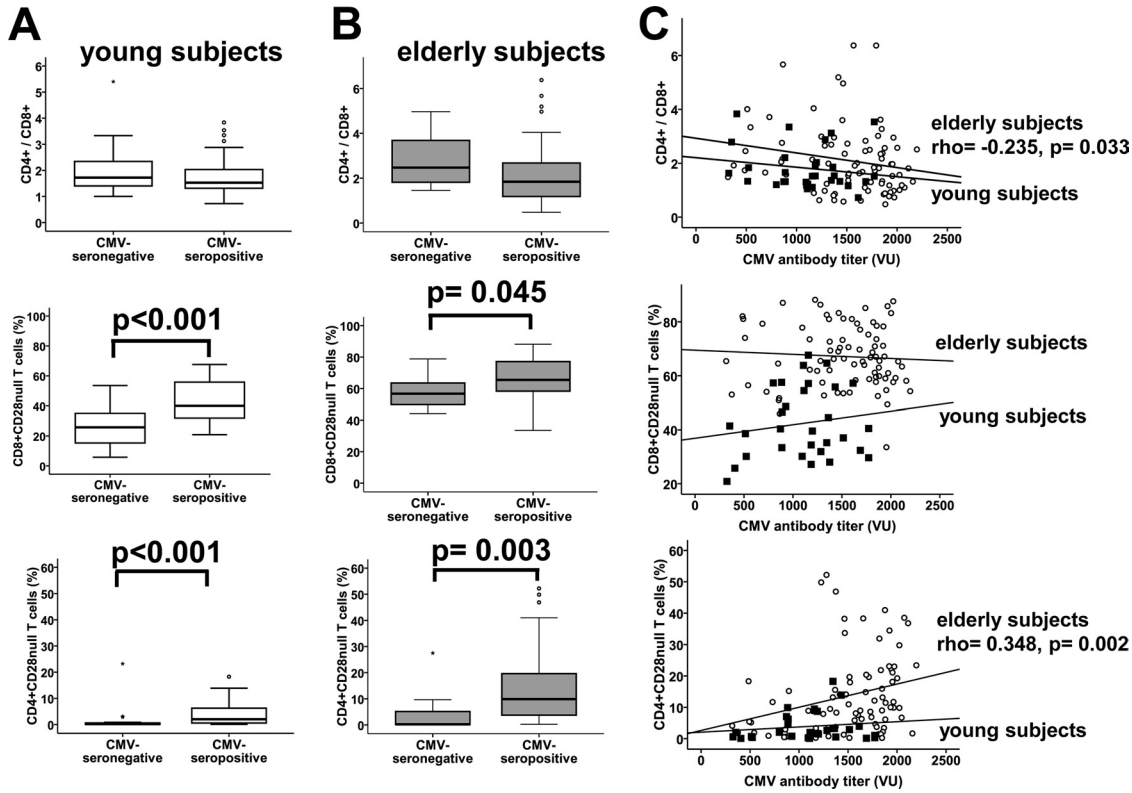

FIG 3 Relationship between CMV seropositivity and anti-CMV antibody titer with CD4/CD8 ratio and percentages of $CD8^+$ $CD28^{null}$ and $CD4^+$ $CD28^{null}$ T cells in young and elderly subjects. CD4/CD8 ratio and percentages of $CD8^+$ $CD28^{null}$ and $CD4^+$ $CD28^{null}$ T cells were compared between CMV-seropositive and CMV-seronegative young (A) and elderly (B) subjects. Significant differences are indicated (Student's *t* test or Mann-Whitney U test). (C) Correlation of anti-CMV antibody titers and CD4/CD8 ratio and percentages of $CD8^+$ $CD28^{null}$ and $CD4^+$ $CD28^{null}$ T cells in young (black squares) and elderly (white circles) subjects are represented in the dot plots. Spearman correlation coefficients and corresponding *P* values are listed on the right-hand side.

revealed significant differences in TREC content depending on the levels of anti-CMV antibodies in elderly patients (Spearman test; rho = −0.444 and $P = 0.03$) and a nonsignificant trend in young individuals (Fig. 2C).

In conclusion, both CMV seropositivity and antibody titers influenced the degree of $CD4^+$ T cell differentiation in the elderly, without affecting their $CD8^+$ T cells, and the degree of differentiation in $CD4^+$ and $CD8^+$ T cells of young people.

IRP parameters. CMV infection is considered a predictor of survival in very elderly people, but we wanted to evaluate the association of CMV serological status with the other parameters that define the IRP, the CD4/CD8 ratio and the frequency of $CD8^+$ $CD28^{null}$ T cells (9, 19). The CD4/CD8 ratio was slightly lower in seropositive than in seronegative young (Fig. 3A) and elderly (Fig. 3B) individuals, although neither was statistically significant. However, a significant negative association between anti-CMV antibody titers and the CD4/CD8 ratio was found in elderly people (Spearman test; rho = −0.235 and $P = 0.033$) (Fig. 3C). Conversely, the proportion of $CD8^+$ $CD28^{null}$ T cells was higher in CMV-seropositive than CMV-seronegative individuals among both the young and elderly subjects (Fig. 3A and B) (Student's *t* test; $P < 0.001$ and $P = 0.045$, respectively) but was not correlated with the levels of antibodies (Fig. 3C).

Since we had demonstrated that differences in the differentiation status of $CD4^+$ T cells are related to CMV infection, we proceeded to analyze the relationship between the virus and the abundance of $CD4^+$ $CD28^{null}$ T cells. As expected, young and elderly CMV-seropositive people had the highest percentages of $CD4^+$ $CD28^{null}$ T cells (Fig. 3A and B) (Mann-Whitney U test; $P < 0.001$ and $P = 0.003$, respectively). Furthermore, this subset was significantly increased with higher levels of anti-CMV antibodies in the elderly (Spearman test; rho = 0.348 and $P = 0.002$), but not in the young (Fig. 3C).

Anti-CMV antibody titers were related to other parameters of IRP and to $CD4^+$ $CD28^{null}$ T cells in the elderly but not in young individuals.

Comparisons between young and elderly individuals with similar anti-CMV antibody titers. The lack of correlation between anti-CMV antibodies and other aspects of immunosenescence in the young but not in elderly people may be because the antibody levels in the elderly are much higher than in young individuals. To check whether the profile of cell differentiation found in these groups was due solely to these differences in the antibody titer, we compared the degrees of cell differentiation between young and elderly individuals with similar anti-CMV antibody titers. Individuals were classified into groups defined according to their CMV serological status and the antibody levels in young people, as shown in Fig. 4A (group 0, seronegative; group 1, ≤50th percentile in young individuals; group 2, between 50th and 95th percentiles in young people; group 3, >95th percentile in young people).

As there were too few young individuals (only three) in group

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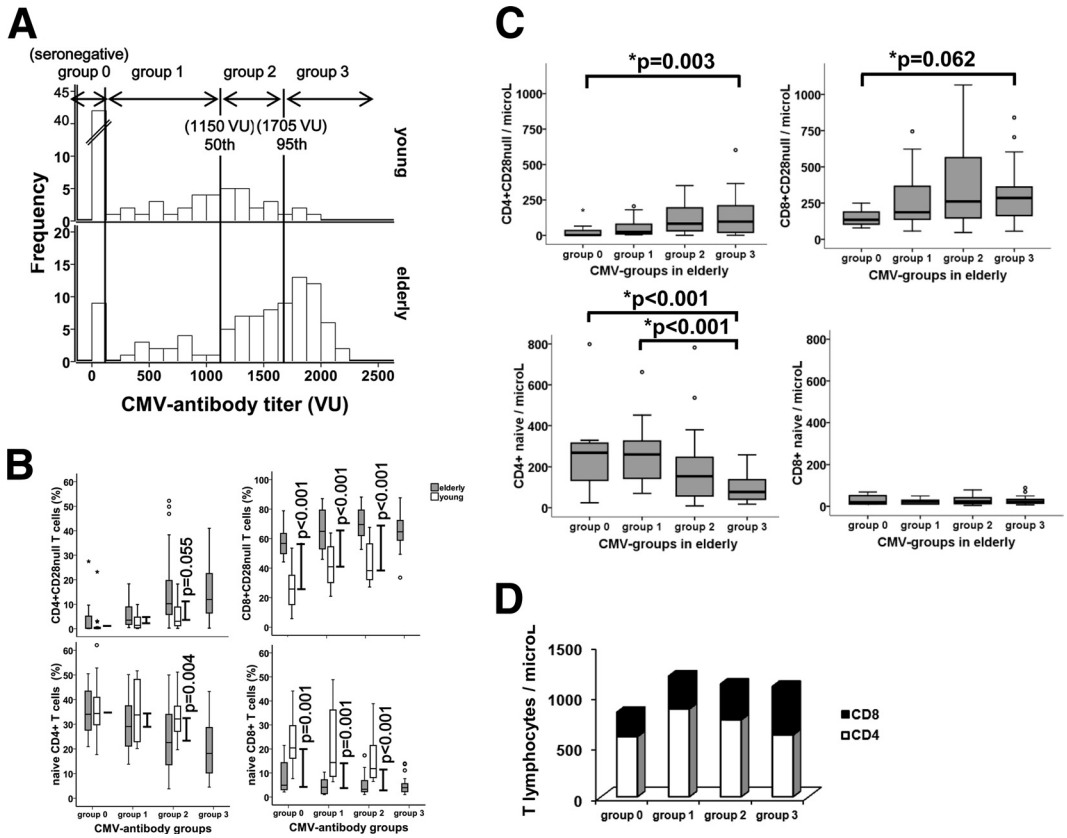

FIG 4 Grouping of young and elderly subjects by anti-CMV antibody titer. (A) Distribution of frequencies of anti-CMV antibody titers in young and elderly subjects and classification into groups defined according to the antibody levels in young people (50th and 95th percentiles). (B) Comparisons of naïve and CD28^{null} T cells (CD4$^+$ and CD8$^+$) between young and elderly individuals with similar anti-CMV antibody titers. Differences in the median frequencies between the young and the elderly in each group are represented by lines. Significant differences between subsets, assessed by Student's *t* test or the Mann-Whitney U test, are indicated. (C) Absolute counts of naïve and CD28^{null} T cells (CD4$^+$ and CD8$^+$) in elderly subjects. The Kruskal-Wallis test was used to identify significant differences in frequencies between groups. "*p" indicates a significant difference in comparisons of all groups; "**p" indicates a significant difference when only groups of CMV-seropositive subjects were considered. (D) Absolute counts of CD3$^+$ T cells, distributed into CD4$^+$ (white bars) and CD8$^+$ (black bars), belonging to each group in elderly subjects.

3 to carry out any meaningful statistical analysis, they were excluded, so only the elderly individuals in the group were considered. The median anti-CMV antibody titer in each group did not differ between elderly and young people. The mean ages of the elderly individuals were 82.5 ± 8.5 years in group 0, 86 ± 5.5 years in group 1, 85.5 ± 6.3 years in group 2, and 87 ± 5.6 years in group 3. For the young individuals, the mean ages were 38 ± 7.2 years in group 0, 41 ± 10 years in group 1, and 42 ± 9 years in group 2.

Frequencies of CD4$^+$ CD28^{null} and naïve CD4$^+$ T cells in elderly and young CMV-seronegative individuals (group 0) were very similar, but the differences were progressively greater in the groups with higher antibody levels. This pattern arose mainly because of the variations experienced by these cellular subsets in the elderly groups (Fig. 4B). On the other hand, in the case of CD8$^+$ T cells, differences were observed between young and elderly individuals in all groups (Fig. 4B). There were no changes in the differences in the frequency of CD28^{null} subsets, while differences in naïve subsets diminished in the groups with higher anti-CMV antibody titers. The reason for this in the latter case was the reduction in the number of naïve cells in young people, since their frequency in the elderly was very low in all groups.

The frequency of naïve CD4$^+$ T cells was related to CMV serological status and to anti-CMV antibody titers in elderly people but showed no association in young individuals. We checked whether this effect merely reflects the increase in the number of more differentiated cells, a consequence of which is a reduction in the frequency of naïve cells in the CD4$^+$ T cell compartment. We compared the absolute counts of CD28^{null} and naïve subsets in CD4$^+$ and CD8$^+$ T cells in the elderly groups (Fig. 4C). As expected, levels of highly differentiated cells increased significantly in the groups with higher antibody titers, but the differences were not statistically significant when only CMV-seropositive individuals were compared. Surprisingly, a significant reduction in the absolute numbers of CD4$^+$ naïve T cells and an almost complete absence of CD8$^+$ naïve T cells were found in comparisons of all elderly individuals and those who were CMV seropositive. This may be due to some homeostatic mechanisms that limit the generation of new naïve T cells in order to maintain the levels of total T lymphocytes. In fact, in a comparison of the counts of T lymphocytes, those of the three groups of CMV-infected individuals were very similar and higher than those of CMV-seronegative individuals (Fig. 4D), despite the notable increase in CD28^{null} cells. We also found a gradual reduction in CD4$^+$ and an increase in CD8$^+$ T cells as the antibody titers rose.

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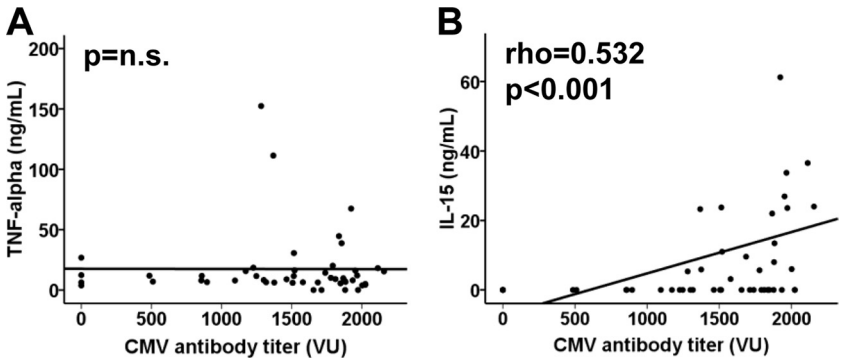

FIG 5 Levels of TNF-α and IL-15 related to anti-CMV antibody titers. Spearman correlation coefficients and corresponding *P* values are shown in the upper left-hand corner.

Overall, differences between elderly and young individuals in highly differentiated and naïve $CD4^+$ T cells increased with their anti-CMV antibody titers. The reduction in naïve cells may be a strategy to compensate for the expansion of differentiated cells and to avoid an increase in the total number of T cells.

Genetic features of CMV-infected elderly people. To identify factors favoring CMV reactivation or the poor control of the infection, which may be responsible for the higher antibody titers, we analyzed some genetic characteristics of elderly individuals. Although HLA-DRB1 alleles are associated with increased susceptibility to CMV pathologies in HIV-infected and transplant patients, we were unable to find any associations between levels of anti-CMV antibody and the HLA-DRB1 profile of the elderly donors (data not shown).

Conversely, TNF-α is a key mediator in the reactivation of CMV, as well as a differentiation factor associated with the loss of expression of CD28 in T lymphocytes (20). Mutations in this gene leading to over- or underexpression of the cytokine may be significant in reactivation and the immune response against the virus. We studied the SNP at position −308 (G/A), which has functional effects on gene transcriptional activity, whereby ~308A* is a stronger transcriptional activator than ~308G* after *in vitro* lymphocyte stimulation (21). Sixty (65.2%) elderly individuals had the GG, 27 (30%) the GA, and 5 (5.5%) the AA genotype. These are frequencies similar to those described for our population. There were no overall differences in the frequency of CMV seropositivity or the anti-CMV antibody titers between the TNF-α genotype groups.

We nevertheless tested the levels of TNF-α and IL-15, a cytokine that could have a significant role in the proliferation of $CD28^{null}$ T cells, in the sera of the CMV-seropositive elderly individuals. Again, no association was found between CMV antibody titers and TNF-α levels (Fig. 5A). However, IL-15 levels were positively correlated with antibody titers (Spearman test; rho = 0.532 and $P < 0.001$), although they were undetectable in 60.4% of the elderly individuals analyzed (Fig. 5B).

Our results suggest that anti-CMV antibody titers are not related to either HLA-DRB1 alleles or the −308 TNF-α polymorphism in elderly individuals but are associated with serum IL-15 levels.

Response to vaccination and CMV infection. To determine whether CMV serological status influences the ability to respond to immunization *in vivo*, we measured the specific antibodies produced against influenza virus vaccination in the four groups of elderly people. The production of specific antibodies to the vaccine diminished gradually and significantly in the groups of CMV-seropositive individuals and was related to levels of anti-CMV antibodies (comparisons within all groups, Kruskal-Wallis test; $P < 0.001$; within CMV-seropositive groups, $P = 0.002$) (Fig. 6). The association between $CD8^+$ $CD28^{null}$ T cells and the response to vaccination has been demonstrated previously (22). To verify the influence of CMV seropositivity or the titer of anti-CMV antibodies on the response to vaccination, we performed a multivariate linear regression, including age and levels of $CD8^+$ $CD28^{null}$ T cells in elderly individuals as predictor variables (Table 2). It should be noted that in both models, including CMV seropositivity and anti-CMV antibody titer, only CMV status and age emerged as independent predictors of the magnitude of the humoral response to influenza virus vaccination. The influence of levels of $CD8^+$ $CD28^{null}$ T cells could not be determined in the individuals studied. However, when the titer of anti-CMV antibodies in all elderly individuals (CMV seronegative and CMV seropositive) was included in the model, $CD8^+$ $CD28^{null}$ T cells were also significantly associated (data not shown).

The *in vivo* ability to respond to new antigens is influenced by both CMV infection and antibody titers in elderly individuals.

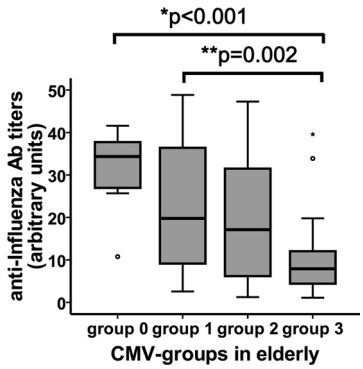

FIG 6 Response to influenza virus vaccination and its correlation with anti-CMV antibody (Ab) titer. Anti-influenza virus antibody titers were quantified by ELISA in the sera of elderly individuals after vaccination. The Kruskal-Wallis test was used to compare the influenza antibody titer between CMV groups. "*p" indicates a significant difference in comparisons between all groups; "**p" indicates a significant difference when only groups of CMV-seropositive subjects were considered.

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TABLE 2 Effect of CMV infection on response to influenza virus vaccination in elderly individuals, corrected by age and absolute counts of $CD8^+$ $CD28^{null}$ cells[a]

Variable	Regression coefficient	SE	95% confidence interval	*P* value
CMV seropositivity	−15.24	5.083	−25.3 to −5.14	0.004
Age	−0.437	0.233	−0.9 to 0.027	0.064
$CD8^+$ $CD28^{null}$ (cells/μl)	0.006	0.005	−0.003 to 0.16	0.191
Anti-CMV antibody titer	−0.011	0.003	−0.017 to −0.005	<0.001
Age	−0.485	0.242	−0.967 to −0.002	0.049
$CD8^+$ $CD28^{null}$ (cells/μl)	0.016	0.012	−0.007 to 0.04	0.178

[a] Data from two regression models are given.

DISCUSSION

This study examined whether anti-CMV antibody titers in the serum of young and elderly individuals are related to the phenotypic and functional status of their immune system. Our results suggest a relationship between aging and CMV seropositivity frequency and the levels of anti-CMV antibodies, which are clearly associated with CMV-specific $CD4^+$ T cells in elderly individuals. Moreover, both CMV seropositivity and antibody titers are related to the degree of differentiation of $CD4^+$ T cells and to the IRP parameters of elderly people. Differences between elderly and young individuals in highly differentiated and naïve $CD4^+$ T cells increase depending on their anti-CMV antibody titers, and the reduction in the frequency of naïve cells may be a strategy to compensate for the expansion of differentiated cells and to avoid an increase in the total number of T cells. Our results also suggest that anti-CMV antibody titers are not related to HLA-DRB1 alleles or to the TNF-α polymorphism −308 in elderly individuals but are correlated with serum IL-15 levels. The *in vivo* ability of elderly individuals to respond to new antigens is also influenced by CMV infection.

The wide-ranging means of transmission of CMV lead to very high seroprevalence, estimated to be between 30% and 90% in developed countries, where it also increases with age (23). After primary infection, the virus persists for the rest of an individual's life, most commonly in a latent form in a variety of tissues but particularly in precursor cells of the monocytic lineage (24). Reactivations from latency are likely to occur routinely in healthy virus carriers and chronically in elderly individuals (3). The relationship between anti-CMV antibodies and the evolution of the infection has been poorly understood. We found that elderly people have higher levels of antibodies than young individuals. One possible explanation for this is that titers of anti-CMV antibodies are indicative of the history of infection, but this hypothesis could be tested by analyzing other characteristics, such as time since infection and the severity or frequency of reactivations. In this way, while individuals with detectable CMV DNA in monocytes have significantly higher percentages of anti-CMV-specific $CD8^+$ T cells, it has not been possible to determine the association with anti-CMV antibody titers. However, the detection of CMV DNA in monocytes could be a marker of current CMV reactivation, and the associated cellular response may reflect the expansion of memory T cells to control the infection (25). In the present study, we found that activation and proliferation of anti-CMV $CD4^+$ T cells in the elderly was associated with levels of specific antibodies. These results are consistent with a recent paper (26) reporting a correlated increase in humoral and $CD4^+$ T cell responses to CMV antigens of extracellular origin in very old individuals. Furthermore, we demonstrated a clear association between higher levels of antibodies and a greater degree of $CD4^+$ T cell differentiation in the elderly. The process of human immunosenescence, irrespective of its association with CMV, induces important changes in the T cell compartment. Although the two populations of $CD8^+$ and $CD4^+$ T cells undergo the same principal phenotypic shifts, the rates at which they occur or accumulate with age are very different. $CD4^+$ T cells are more resistant than $CD8^+$ T cells to phenotypic and functional changes with aging, probably because they have important regulatory roles and need to be subjected to strict control mechanisms (27). In this way, there was an association between titers of anti-CMV antibodies and the $CD4^+$ T cell phenotype in elderly but not in young individuals. This might be explained by the different levels of antibodies in the two age groups, but when we compared elderly and young individuals with equal levels of anti-CMV antibodies, we found that, as expected, the differences were not explained solely by anti-CMV antibody titers. Nevertheless, the changes were more pronounced in $CD4^+$ than in $CD8^+$ T cells in the elderly, and there were substantial differences with young individuals with increasing antibody titers. The accumulation of highly differentiated cells may not be the only reason for the changes in T cell phenotype, and the impaired ability to replenish the pool of naïve T cells in elderly individuals also contributes to this. In humans, a dramatic drop in the absolute cell count of recent thymic emigrants with age is evident (28). Despite this thymic degeneration, clinical lymphopenia is rare in elderly people and the number of circulating T cells is maintained over the life span, probably due to the increase in highly differentiated memory cells. Accordingly, we found that higher anti-CMV antibody titers were correlated with lower absolute counts of naïve and more highly differentiated T cells. However, there was no correlation with the total T lymphocyte count. Experienced T lymphocytes may fill the immunological space and homeostatic mechanisms block the generation of new naïve cells to maintain the numbers of peripheral T lymphocytes. These mechanisms make it difficult to preserve the T cell repertoire diversity that combats new pathogens as well as the host's ability to mount vigorous recall responses to recurrent infections (11). Indeed, the loss of functionality with increasing differentiation and the reduction in naïve cells may be responsible for the suppression of immunity to other viruses and the impaired response to influenza vaccination reported for elderly donors with CMV infection (29). In this way, an insufficient antibody response following influenza vaccination in elderly individuals has also been linked to a high frequency of $CD8^+$ $CD28^{null}$ T cells (22). In our study, both CMV seropositivity and the titer of anti-CMV antibodies corrected by age and $CD8^+$ $CD28^{null}$ T cells were independently as-

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sociated with the antibody response to influenza virus vaccination. The impaired response to new antigens may be considered a marker of CMV infection-related immunosuppression, which has other possible consequences. CMV seropositivity is strongly associated with an IRP that is known to predict 2-year mortality in a population-based sample of Swedish octogenarians (9). The IRP may be summarized by the presence of expanded populations of $CD8^+$ $CD28^{null}$ memory T cells and an inverted CD4/CD8 ratio (30). Recently, two studies have reported that not only the CMV infection but also the titer of CMV-specific antibodies is crucial for determining the risk of increased mortality (31, 32). Differences related to CMV seropositivity in our study were more striking in $CD28^{null}$ T lymphocytes from elderly and young individuals, but only the CD4/CD8 ratio and the frequency of $CD4^+$ $CD28^{null}$ T cells were correlated with antibody titers, and again not in the case of $CD8^+$ $CD28^{null}$ T cells of elderly individuals.

The magnitude of CMV infection may be influenced by genetic factors in the host. In HIV infection and renal transplantation, the presence of HLA-DR7 has been correlated with an increased risk of infection and CMV disease and with poor immune responses to CMV (33, 34). It has been postulated that defective control of infection in HLA-DR7 patients could lead to frequent virus reactivations, contributing to the clonal expansion of specific subsets of $CD4^+$ T cells restricted against a limited number of CMV antigens (35). In our study, we found no association between titers of anti-CMV antibodies and the HLA-DR alleles of the elderly individuals. Moreover, we found no association with their TNF-α polymorphisms, even though TNF-α is considered to be a key mediator of CMV reactivation. In turn, TNF-α, which is augmented by aging and CMV infection, induces CD28 expression loss and T cell differentiation (36). Despite the high levels of anti-CMV antibodies and the high frequencies of $CD28^{null}$ T cells, we found no association between CMV infection and TNF-α levels in the sera of elderly individuals. There was a significant correlation with the level of IL-15, another cytokine implicated in the loss of CD28 expression in $CD8^+$ T cells and an inducer of preferential proliferation and functional capabilities of $CD4^+$ $CD28^{null}$ T cells (20, 37).

In summary, our data show that levels of anti-CMV antibodies and CMV seropositivity are related to differentiation status and immunocompetence in the elderly. Elevated anti-CMV antibody titers could be a consequence rather than the cause of immunosenescence, but they may have value as a prognostic marker of the deterioration of immunological status and the risk of age-related diseases.

ACKNOWLEDGMENTS

This work was supported by the Red de Investigación Renal (REDinREN) and FIS-Fondos FEDER European Union PI080566 and PI1202527 from the Instituto Carlos III.

We thank Isabel Cuevas Pérez and José María Díaz Pérez for their excellent technical assistance.

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AUTHOR'S CORRECTION

Intensity of the Humoral Response to Cytomegalovirus Is Associated with the Phenotypic and Functional Status of the Immune System

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Volume 87, no. 8, p. 4486–4495, 2013. Page 4486: The byline should appear as shown above.

Page 4494, Acknowledgments, paragraph 1, lines 2 and 3**:** "PI1202527 from the Instituto Carlos III" should read "PI1202587 from the Instituto Carlos III (SPAIN)."

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doi:10.1128/JVI.01378-13

III.4. RELACIÓN ENTRE LA CAPACIDAD FUNCIONAL EN ANCIANOS, SITEMA INMUNE E INTENSIDAD DE RESPUESTA AL CMV

El deterioro del sistema inmune se asocia con una menor supervivencia en ancianos, habiéndose definido incluso un perfil de riesgo inmunológico que incluye la inversión del cociente CD4/CD8 y la seropositividad a CMV (Hadrup *et al.* 2006). La capacidad funcional de los individuos también se relaciona con la longevidad y existen determinados índices, como el de Barthel (IB), que permiten su clasificación. El objetivo de este trabajo fue estudiar la posible asociación entre el deterioro funcional e inmunológico en individuos de edad avanzada.

ARTÍCULO 4:

Marco Antonio Moro Garcia, Rebeca Alonso Arias, Antonio López Vazquez, Francisco Manuel Suárez García, Juan José Solano Jaurrieta, José Baltar, Carlos López-Larrea. *"Relationship between functional ability of older people, immune system status and intensity of response to CMV".*

Age (Dordr). 2012 Apr;34(2):479-95.

En este trabajo se estudiaron 100 ancianos procedentes de la residencia Santa Teresa de Oviedo, que fueron divididos en cuatro grupos, homogéneos en edad, según su IB. De mayor a menor capacidad funcional: grupo 0 (n=24), grupo 1 (n=26), grupo 2 (n=27) y grupo 3 (n=23). Se realizó una caracterización de las subpoblaciones celulares por citometría de flujo, cuantificación de TRECs mediante PCR a tiempo real y se midió la respuesta proliferativa y de activación (CD69, IFN-γ por ELISPOT) de células T frente a anti-CD3 y CMV. También se realizó la cuantificación por ELISA del título de anticuerpos específicos frente al virus de la gripe generados tras la vacunación y CMV. Los individuos con peor estado funcional presentaron niveles significativamente aumentados de células NK y disminuidos de linfocitos B, no encontrándose diferencias en otras poblaciones leucocitarias como células polimorfonucleares, monocitos y linfocitos T. Estos individuos también presentaban una proporción significativamente menor de células CD4+ y mayor de CD8+, con un cociente CD4/CD8 disminuido. Los niveles de TRECs en linfocitos T CD4+ fueron significativamente menores en los individuos de los grupos 2 y 3, correlacionándose con una menor frecuencia de las subpoblaciones de células naïve (CD45RA+CCR7+) y mayor de células efectoras (CD45RA-CCR7-). La respuesta celular frente a

anti-CD3 disminuyó gradualmente a medida que empeoraba el estado funcional y aumentaba la respuesta de células específicas a CMV y el título de anticuerpos frente a este virus. Inversamente, la respuesta funcional in vivo, valorada por el título de anticuerpos generados tras la vacunación frente al virus de la gripe fue mayor en los individuos con mejor estado funcional.

En resumen, podemos decir que existe una clara asociación entre el deterioro funcional de los individuos de edad avanzada y el envejecimiento de su sistema inmune, estando ambos parámetros directamente relacionados con la intensidad de la respuesta a CMV.

Aportación personal al trabajo:

En este trabajo, mi labor se centró en recolectar y procesar las muestras del estudio, realizar y/o supervisar los distintos experimentos y analizar los resultados obtenidos. Finalmente, participé en el diseño y en la escritura del presente manuscrito.

AGE (2012) 34:479–495
DOI 10.1007/s11357-011-9240-6

Relationship between functional ability in older people, immune system status, and intensity of response to CMV

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Received: 19 October 2010 / Accepted: 18 March 2011 / Published online: 13 April 2011
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Abstract Shorter survival in the elderly has been associated with deterioration of the immune system and also with functional disability. To analyze the relationship between functional and immune impairment in older individuals, we studied 100 elderly who lived in a nursing home, were age matched, and grouped according to their functional status. We characterized cell subpopulations by flow cytometry, quantified TREC by RT–PCR, and measured the T-cell proliferation and activation response (IFN-γ by ELISPOT, CD69) against anti-CD3 and CMV. Specific antibody titers against influenza virus and CMV were determined by ELISA. Individuals with worse functional status had significantly higher levels of NK cells and fewer B cells. These poorly functioning elders also had a significantly lower proportion of CD4+ T cells, increased CD8+ T cells, and a decreased CD4/CD8 ratio. TREC levels in CD4+ T cells were significantly lower in individuals with a high disability. Lower TREC levels correlated with a lower frequency of naïve T-cell subpopulations (CD45RA+CCR7+) and higher percentages of effector cells (CD45RA−CCR7−). The functionally impaired group had lower anti-CD3 responses, but gradually increased responses against CMV. Similarly, the higher CMV titers were found in elderly with worse functional status. On the contrary, the functional response in vivo, and the titer of antibodies generated after vaccination against influenza virus, was higher in individuals with better performance status. In summary, we concluded that the functional decline of elderly individuals was clearly associated with the aging of their immune system, and the intensity of the response to CMV.

Keywords Immunosenescence · Functional status · T lymphocytes · Differentiation · CMV

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Introduction

Older people suffer from age-associated changes in the immune system, including decreased immune

function, increased incidence and severity of infections, development of autoimmune phenomena, and cancer (DelaRosa et al. 2006; Prelog 2006). These defective immune responses are also manifested in a reduced ability of vaccines and infections to induce immunological memory, and a lower incidence of acute rejection in elderly transplant patients (Bradley et al. 2001; Weinberger et al. 2008). The aging process seems to alter both branches of the immune system, innate and adaptive in different ways: the innate immunity seems to be better preserved globally (Dace and Apte 2008; Le Garff-Tavernier et al. 2010), while the adaptive immune response exhibits profound age-dependent modifications (Haynes and Maue 2009). Lower T-cell counts can be partially explained by thymic involution which decreases output of naïve T cells and reduces the numbers of T cells in peripheral blood and lymphoid tissues (Aspinall and Andrew 2000; Linton et al. 2005). In support, studies characterizing the T-cell receptor excision circles (TREC) (Ribeiro and Perelson 2007) showed that the frequency of the TREC declines exponentially with age (Naylor et al. 2005). The elderly accumulate highly differentiated T cells. Mature T cells have a reduced susceptibility to apoptosis, and oligoclonal expansions against CMV and other chronical antigens are evident (Clambey et al. 2005; Cao et al. 2009). Since CMV can reactivate promptly after periods of immunosuppression, a substantial proportion of the immune repertoire may be required to control its replication. Studies have associated the changes in the number of lymphocytes expressing activation markers both with age and with CMV antibody titer (Looney et al. 1999). The expansion of these functional T cells may contribute to anti-CMV surveillance, but these T cells may also exert pathogenic effects upon cells and tissues in close proximity via recently suggested molecular mechanisms (Bolovan-Fritts et al. 2007; Qiu et al. 2008). Moreover, they may contribute to the inflammation of unknown origin that occurs during aging (Ferrucci et al. 2005), as well as the pathogenesis and progression of inflammatory diseases (Soderberg-Naucler 2006).

In agreement, an immune risk profile (IRP) was initially identified in the Swedish OCTO immune longitudinal study using a cluster analysis approach (Ferguson et al. 1995). A higher 2-year mortality occurred in a population of very old Swedish individuals who had a high frequency of CD8 T cells, a low frequency of CD4 cells, and a poor proliferative response to Con A. The inverted CD4/CD8 ratio was the sole marker significantly associated with the IRP (Wikby et al. 1998). Subsequently, cytomegalovirus (CMV) infection has been shown to exert a major impact on the immunosenescence process (Hadrup et al. 2006).

Functional disability is an important health indicator in the elderly, and jeopardizes quality of life, causes heavy social impact with long-term institutionalization, and increases use of medical care (Guralnik et al. 1996). The main risk factors for functional disability in the elderly were low sociocultural level, chronic diseases, immune disorders, body mass index above 25, cognitive impairment, depression, and sedentary lifestyle. The Barthel index (BI) was developed to assess disability in patients with neuromuscular and musculoskeletal conditions that required inpatient rehabilitation (Mahoney and Barthel 1965; Sainsbury et al. 2005). Extensive literature has evaluated the predictors of functional decline in samples of elderly people (age, cognitive status, etc.), but few studies have tried to find a physiological cause for this deterioration (Ishizaki et al. 2004).

Both the impairment of functional capacity (Wilkinson and Sainsbury 1998) and the deterioration of the immune system (Wayne et al. 1990) have been associated with increased morbidity and mortality. To further explore this relationship, the BI was used to group our elders, who had similar ages and diseases, into four groups with different functional capabilities. We conducted an observational cross-sectional study that evaluated and compared the state of the immune system at both phenotypic and functional levels in the groups. We described, for the first time, a direct relationship between the functional status of the elderly and their immune system. We observed a poorer functional status in elderly groups with reduced immune capabilities and with increased humoral and cellular response to CMV.

Materials and methods

Study population

One hundred elderly (76 women and 24 men) living at the Santa Teresa nursing home (Oviedo, Spain)

were enrolled in the study. Blood for the hematological and immunological analyses were drawn from the 100 individuals. All subjects received a physical examination and answered standardized questionnaires to assess clinical history, current disease, and medication. Inclusion criteria were elders who were older than 69 years and who were classified according to the Barthel index as a measure of their functional status. Exclusion criteria were conditions with possible influence on the immune system such as recent or current infection, inflammation, autoimmune or malignant disease, malnutrition, abnormal laboratory data (hemoglobin <12 mg/dL, leukopenia <3,500 cells/μL, neutropenia <1,500 cells/μL, leukocytosis >15,000 cells/μL, platelets $<10^5$ cells/μL, and PCR >5 mg/dL), and pharmacological interference (steroids, nonsteroidal anti-inflammatory agents, and immunosuppressive drugs). Informed consent was obtained from the elders prior to participation in the study. The study was approved by the Hospital Central de Asturias (Oviedo, Spain) ethics committee.

The functional abilities of the subjects were assessed by using the Barthel Index of Activities of Daily Living (BI) (Mahoney and Barthel 1965). Each person was evaluated at 10 tasks that measured daily functioning for various activities of daily living and mobility. The highest BI score (100) meant that the person needed no assistance with any part of the tasks. The BI scores were used to divide the elderly into four groups from total independence to total dependence: group 0=100 (n=25), group 1=95–80 (n=25), group 2=75–40 (n=27), and group 3=35–0 (n=23), as previously described (Saxena et al. 2006; Supervia et al. 2008).

Hematological analysis and immunological phenotyping

The hematological parameters were determined by a Sysmex XT-2000i (Syxmex, Hamburg-Norderstedt, Germany). Cytometric studies were acquired and analyzed in the FACSCalibur Cytometer using CellQuest software (BD Biosciences, San José, CA, USA). CaliBRITE Beads (BD Biosciences) were used to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity. Surface staining of EDTA peripheral blood was performed with Multiset CD3-FITC/CD16+56-PE/CD45-PerCP/CD19-APC Reagent, anti-CD4 (APC), anti-CD8 (PE), anti-CD8 (PerCP), anti-CD45RA (FITC), anti-CD27 (PE) (Immunostep, Salamanca, Spain), anti-CD4 (PerCP), anti-CD28 (PerCP), anti-CCR7 (Alexa Fluor 647), anti-CD3 (FITC), anti-CD45RO (FITC), anti-CD25 (APC) (BD Biosciences), anti-NKG2D (PE), and anti-CD127 (PE) (eBioscience, San Diego, CA, USA). One hundred microliters of whole blood from elderly were stained with different combinations of labeled monoclonal antibodies for 20 min at room temperature. Samples were red blood lysed with FACS Lysing Solution (BD Biosciences), washed in PBS, and analyzed with CellQuest software in the FACSCalibur Cytometer. Appropriate isotype control mAbs were used for marker settings.

To analyze the proliferation status of CD4+ and CD8+ T cells, peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Hypaque gradients (Lymphoprep; Nycomed, Oslo, Norway). CD4+ and CD8+ T cells from elderly were isolated with magnetic beads (Myltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were lysed and fixed with Fixation/Permeabilization Solution (eBioscience), permeabilized with Permeabilizing Buffer (eBioscience), and stained with anti-Ki-67-PE (eBioscience) for 30 min at room temperature. Cells were washed and resuspended in 1% paraformaldehyde until FACS analysis. Frequencies of positive cells for Ki-67 from groups 0 and 1 (n=10) and groups 2 and 3 (n=10) were compared.

TRECs quantification

The DNA of isolated CD4+ (purity>90%) from PBMCs was extracted using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Quantification of signal-joint (sj) TREC was performed by using SYBR Green real-time quantitative PCR and an iCycler thermocycler (Bio-Rad; Life Science Research Group, Hercules, CA, USA). The sequences of the utilized primers were the following: forward primer 5′-CCATGCTGACACCTCTGGTT-3′, reverse primer 5′-TCGTGAGAACG GTGAATGAAG-3′. As an internal control measurement to normalize for input DNA, the Cα constant region that remains present on TCR genes despite rearrangement processes was amplified in every sample tested (forward primer 5′-CCTGATCCTCTTGTCCCACAG-3′, reverse primer 5′-GGATTT AGAGTCTCTCAGCTGGTACA-3′). Thermal cycling conditions began with 50°C for

2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Experimental samples were run in duplicate and the replicate average value recorded as the sample result.

Cytomegalovirus (CMV) and influenza virus serology

Immunoglobulin G levels of CMV-specific antibodies were determined by enzyme-linked immunosorbent assay Vir-ELISA Anti-CMV-IgG (Viro-Immun Labor-Diagnostika GmbH, Oberursel, Germany) according to the manufacturer's specifications. Patient samples were quantified and interpreted by means of the calculation of the ratio (cutoff index=OD value of sample/cut-off value), whereby a ratio of 1.0 is equivalent to the cut-off value. Cutoff indexes >1.1 were considered positive, and the result of this ratio is a semi-quantitative titer.

Anti-influenza virus antibodies in serum obtained from elderly individuals were measured by ELISA as previously described (Ohishi et al. 2002), with some modifications (Kang et al. 2004). The OD values of individual samples were compared against a calibration curve made by the OD values of serial dilutions of the same internal control serum from a healthy vaccinated young person throughout the experiments. Because antibody titer decreased significantly with time since vaccination, we normalized this value by dividing the titer by the elapsed time since immunization.

Activation studies

The activation of heparinized whole blood by anti-CD3 or CMV antigens was assessed by surface staining with anti-CD69 (eBioscience). Briefly, heparinized whole blood (250 μL) was stimulated with soluble anti-CD3 (10 ng/mL) (eBioscience) or with a CMV supernatant (10^4 PFU/mL) in 15 mL conical polypropylene tubes for 18 h at 37°C and 5% carbon dioxide. CMV-infected cell lysate was prepared by infecting human embryonic lung fibroblasts with the AD169 CMV strain, and viral titers in the supernatant were determined by standard plaque assays. The virus was inactivated by five repeated freeze–thaw cycles. The cells were also stained with anti-CD4 and anti-CD8 mononuclear antibodies.

Proliferation cultures

PBMCs were resuspended in PBS at a final concentration of $5–10\times10^6$ cells/mL and incubated with 1.5 μM CFSE (Invitrogen, Paisley, Scotland, UK) for 10 min at 37°C, washed with RPMI 1640 medium containing 2×10^3 M L-glutamine and Hepes (Bio-Whitaker, Verviers, Belgium) twice, and cultured at 1×10^6 cells/mL in the presence of soluble anti-CD3 (10 ng/mL) and CMV extract (10^4 PFU/mL). The proliferative responses of CD4+ and CD8+ T cells were analyzed on day 7 by FACSCalibur after staining with anti-CD4 and anti-CD8.

ELISPOT assay

PBMCs were resuspended to 2×10^6/mL in RPMI 1640 medium containing 2×10^{-3} M L-glutamine and Hepes and supplemented with 10% FCS (ICN Flow, Costa Mesa, CA, USA) and antibiotics. PBMC were stimulated in triplicate with anti-CD3 (10 ng/mL) and with the same extract of CMV used in the activation and proliferation cultures. PBMC (100 μL, 1.5×10^5/well) were placed into each well of a 96-well filter plate (Millipore, Billerica, MA, USA) that was coated with anti-IFN-γ Ab (BD Biosciences), and the cells were cultured for 20 h. IFN-γ captured by the plate-bound Ab was detected by biotinylated anti-IFN-γ Ab (BD Biosciences), followed by streptavidin-conjugated alkaline phosphatase (BD Biosciences). Spots were developed using the red color-forming substrate 3-amino-9-ethylcarbazole (BD Biosciences). Spots were counted with the ELISPOT reader system ELR02 (Autoimmun Diagnostika GmbH, Straβberg, Germany). A sample of cultured cells was stained with anti-CD3 (FITC) and this percentage of $CD3^+$ cells was used to calculate the number of T cells in each well of the assay.

Statistical analysis

Two groups were compared with the Mann–Whitney *U* non-parametric method for data that were not normally distributed, or with the Student's *t* test. More than two groups were compared by using the non-parametric Kruskal–Wallis test or by ANOVA analysis for data that were normally distributed. Results were expressed as median and range or mean and standard deviation. In some graphs, mean and standard error of the mean were displayed. Correlations between variables were assessed by the non-parametric Spearman test (ρ). The χ^2 test was used to compare dichotomous variables and multiple linear

regression was used in multivariate analysis. Analyses were performed using the SPSS 15.0 statistical software package program (SPSS Inc. Chicago, IL, USA) and p values of 0.05 or less were considered significant.

Results

Demographic and hematologic characteristics of the study population

The characteristics of the 100 individuals enrolled in the study are shown in Table 1. People were placed into four groups according to their BI. The female/male ratio in each group was 2.1:1 in group 0, 4.0:1 in group 1, 2.7:1 in group 2, and 2.8:1 in group 3. There were no significant differences in the age of donors belonging to the four groups. The average number of drugs taken by members included in the study and the most frequent pathologies associated with each group were listed.

Blood cell counts and an immune phenotype of majority populations were performed in all individuals included in the study. The groups did not show significant differences in absolute numbers or percentages in the blood cell counts (Table 2). In contrast, the groups with worse functional status showed a gradual higher percentage of NK cells (CD16+56+) and reduction of B cells (CD19+) (Fig. 1a). Although the differences in T lymphocytes between the four groups were not significant, the populations of CD4+ and CD8+ T lymphocytes were significantly different between groups (ANOVA test, $p=0.02$ and $p=0.027$, respectively) (Fig. 1b). Elders in group 3 had the lowest percentage of CD4+ and the highest percentage of CD8+ T cells. The four groups presented similar frequencies of CD4+ regulatory T cells (data not shown).

Loss of CD28 is a typical feature of senescence in T cells (Borthwick et al. 1996). CD28 expression on CD8+ T cells did not differ significantly between groups, whereas the CD4+CD28− T-cell population showed a tendency to increase with worse functional status (data not shown). The double positive CD4+CD8+ and CD4+NKG2D+ are two other subsets of T cells that have been related to aging (Pawelec 1995; Colombatti et al. 1998; Alonso-Arias et al. 2009). The proportion of CD4+CD8+ and CD4+NKG2D+ in the peripheral blood increased in elders with worse

Table 1 Characteristics of the subjects participating in the study according to their BI

	Group 0 (n=24)	Group 1 (n=26)	Group 2 (n=27)	Group 3 (n=23)
Age (years)				
Mean±SE	85.7±1.2	86.0±0.9	87.0±1.1	88.2±1.3
Range	(75–97)	(77–94)	(74–97)	(69–96)
No. of subjects investigated				
Women	16	22	20	17
Men	8	4	7	6
Mean number of drugs	4.2	6.3	6.9	5.9
Pathologies				
Cognitive impairment	0	1	7	9
Dementia	1	2	2	4
Osteoporosis	4	5	8	2
Arterial hypertension	9	13	11	5
COPD	3	2	2	3
Osteoarthritis	7	3	8	3
Depression	4	4	4	3
Heart failure	1	1	2	2
Ischemic heart disease	1	3	2	0
Dyslipidemia	3	4	7	2
Diabetes	2	5	3	6

COPD chronic obstructive pulmonary disease

Table 2 Hematology values of studied subjects

	Group 0 (n=24)	Group 1 (n=26)	Group 2 (n=27)	Group 3 (n=23)	p value between groups[a]
RBCs (10^6/μL)	4.4 (3.5–5.2)	4.4 (3.1–5.4)	4.3 (2.2–5.7)	4.2 (2.0–4.8)	ns
Hemoglobin (g/dL)	13.4 (9.9–16.4)	13.0 (10.4–17.3)	13.2 (9.5–17.3)	12.3 (7.9–16.2)	ns
Hematocrit (%)	40.6 (31.1–48.9)	39.3 (29.1–48.1)	40.2 (18.0–51.4)	38.0 (23.5–46.9)	ns
MCV (fL)	90.7 (86.2–108.5)	90.1 (73.8–101.5)	92.2 (85.5–103.8)	92.7 (69.6–119.3)	ns
Platelets (10^3/μL)	200.5 (129.0–270.0)	229.5 (132.0–571.0)	218.3 (130.0–334.0)	226.1 (140.0–402.0)	ns
WBCs (10^3/μL)	5.9 (3.1–8.2)	6.7 (3.8–11.9)	6.1 (3.5–11.8)	6.5 (3.2–9.5)	ns
Neutrophils (10^3/μL)	3.4 (1.8–5.3)	4.0 (1.4–7.7)	3.6 (1.9–6.7)	3.8 (1.6–7.2)	ns
Monocytes (10^3/μL)	0.48 (0.29–0.77)	0.49 (0.24–3.5)	0.49 (0.28–1.0)	0.44 (0.05–0.86)	ns
Lymphocytes (10^3/μL)	1.77 (0.87–2.81)	1.77 (0.85–3.37)	1.82 (0.88–5.01)	1.94 (0.65–3.44)	ns

[a] Calculated using the Mann–Whitney U non-parametric test among the four BI groups

functional status (Fig. 1c). Donors in groups 2 and 3 had significantly higher levels than those in group 0. In summary, these results demonstrated that many of the age-related changes in immune parameters were dependent on the functional status of the elderly.

IRP parameters and functional status

Parameters that define the IRP are the inverted ratio CD4/CD8 (ratio<1.0) and CMV infection (Olsson et al. 2000; Hadrup et al. 2006). Low CD4+ numbers and elevated CD8+ T cells were associated with a worse functional status. Accordingly, the CD4/CD8 ratio were significantly different between groups (Kruskal–Wallis non-parametric test, p=0.022) (Fig. 2a). The proportion of elders with an inverted ratio also showed a gradual and significant increase with reduced functional status (χ^2 test, p=0.041) (Table 3).

The relationship between CMV infection and functional status was also analyzed. Although the largest percentage of CMV-seronegative elders was in group 0 (16%), no significant differences between groups were observed (Table 3). However, we detected a gradual increase in CMV antibody titer concomitant with deteriorating functional status (Fig. 2b).

We have also examined the relationship between the median BI score and CMV serological status. The median BI score was not significantly related to CMV serological status: the median in seronegative individuals was 95 points (IR=53.75 points) and in CMV seropositive was 75 points (IR=50 points). However, comparison of the CMV antibody titer with the BI score across all individuals revealed a negative correlation (Spearman Rho test; rho −0.346; p= 0.0005). Only CMV serological status was independently associated with the BI score in a multivariate analysis (multiple linear regression, p<0.001) with age and gender as confounding factors.

Comparison between the BI score and CMV titer separately in men and women showed that the correlation remained significant among women (Spearman Rho test; rho −0.379; p=0.001) (Fig. 2c) but not among men (Fig. 2d). The lack of correlation in men may be due to the low number of male volunteers in the study. In fact, there is a clear trend, which likely would be significant with a higher number of volunteers.

T-cell differentiation subsets

One of the most widely accepted models in immunosenescence is that the T-cell compartment is progressively deteriorating with advancing age. T cells can be separated into functionally different populations using combinations of cell surface markers such as the tyrosine phosphatase isoform CD45RA and the chemokine receptor CCR7. With these markers, we subdivided the T cells into naïve (NAÏVE; CD45RA+CCR7+), central memory (CM; CD45RA−CCR7+), effector memory (EM; CD45RA−CCR7−), and effector memory RA (EMRA; CD45RA+CCR7−) (Sallusto et al. 1999). To detect an association between functional status and the differentiation degree of T-cell subsets, we compared the distribution of the distinct T-cell subpopulations in

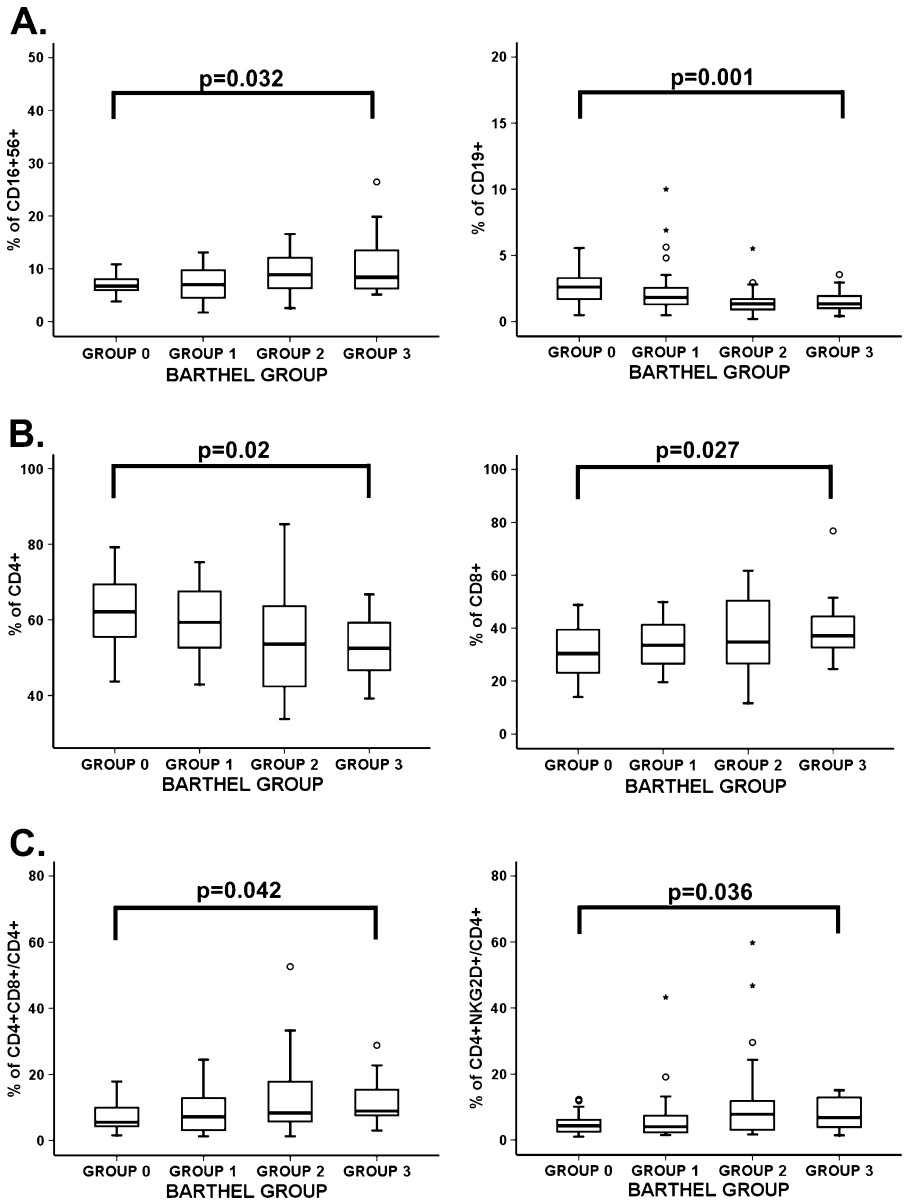

Fig. 1 Immune phenotype in peripheral blood from elderly according to their BI group. Elders were stratified according to their BI (group 0 BI=100, group 1 BI=95–80, group 2 BI=75–40, and group 3 BI=35–0). The number of donors in each group was group 0=24, group 1=26, group 2=27, and group 3=23. Whole blood from elderly individuals was stained with different antibody combinations and analyzed by flow cytometry (10^5 cells acquired in each experiment). Outlier values were represented by *circles* and extreme values by *stars*, calculated by adding 1.5 and 3 times the IR to the 75th percentile, respectively. The ANOVA test (when data were normally distributed) and Kruskal–Wallis non-parametric methods (when data were not normally distributed) were used to compare frequencies between groups. *p* values are depicted in the panels. **a** Percentages of CD16+56+ and CD19+ cells with respect to the total CD45+ cells were compared between the four groups of elderly. Staining was performed with "Multiset CD3-FITC/CD16+56-PE/CD45-PerCP/CD19-APC" and frequencies of CD16+56 and CD19+ cells in gated CD45+ subsets were analyzed. **b** Percentages of CD4+ and CD8+ cells were analyzed with respect to the total CD45+CD3+ and were compared between the four groups. Staining was performed with anti-CD3-FITC, anti-CD4-APC, and anti-CD8-PerCP to gate CD4+ and CD8+ T cells. **c** Percentages of CD4+ T cells expressing CD8 and expressing NKG2D in peripheral blood from elderly. Whole blood was stained with anti-CD3-FITC, anti-NKG2D-PE, anti-CD8-PerCP, and anti-CD4-APC. Frequencies of NKG2D+ cells in gated CD3+CD4+ lymphocytes were quantified

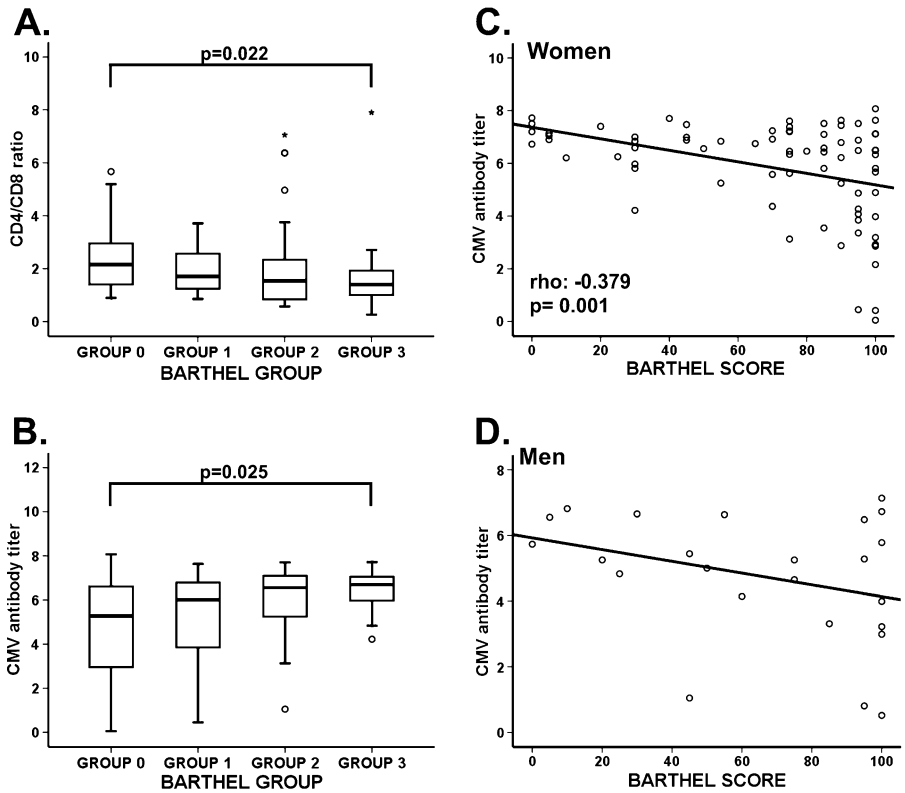

Fig. 2 CD4/CD8 ratio, anti-CMV antibody titer from elderly of the four BI groups, and correlation between the CMV antibody titer and the BI score in women and men. The two most important parameters that define the immune risk profile (IRP) are the inverted CD4/CD8 ratio and CMV infection. **a** CD4/CD8 ratios were analyzed and compared between the four groups. Staining was performed with anti-CD3-FITC, anti-CD4-APC, and anti-CD8-PerCP to gate CD4+ and CD8+ T cells. CD4/CD8 ratio less than 1.0 was used to identify individuals with an IRP. **b** Serum anti-CMV antibody titer was measured by ELISA and compared between the BI groups. Patient samples are quantified and interpreted by means of the calculation of the ratio (cut-off index=OD value of sample/cut-off value), whereby a ratio of 1.0 is equivalent to the cut-off value. Cut-off index >1.1 were considered positive and the result of this ratio is a semi-quantitative titer. Outlier and extreme values are represented by *circles* and extreme values by *stars*, calculated by adding 1.5 and 3 times the IR to the 75th percentile, respectively. The Kruskal–Wallis non-parametric method was used to compare frequencies between groups and *p* values are depicted in the panels. **c** Correlation between CMV antibody titer and the BI score in women. **d** Relationship between CMV antibody titer and BI score in men. A non-parametric Spearman test was applied to calculate the correlations, *p* value, and coefficient of correlation which are listed in the *lower left hand corner*

young and elderly individuals, as shown by representative samples (Fig. 3a). The analysis of these subsets of CD4+ T cells revealed that the functional deterioration in elderly was related to the reduced population of undifferentiated subsets (Fig. 3b). In fact, the frequency of both the NAÏVE CD4+ cells and the CM popula-

Table 3 IRP parameters in the BI groups

	Group 0 (n=24)	Group 1 (n=26)	Group 2 (n=27)	Group 3 (n=23)	p value between groups
Inverted CD4/CD8 ratio					
Ratio<1.0	2	4	10	8	
(%)	(8.3%)	(15.4%)	(37.0)	(34.8%)	0.041[a]
CMV serology					
Negative	4	1	2	1	
(%)	(16%)	(4%)	(7.4%)	(4.4%)	ns[a]

[a] Calculated using χ^2 test between all groups

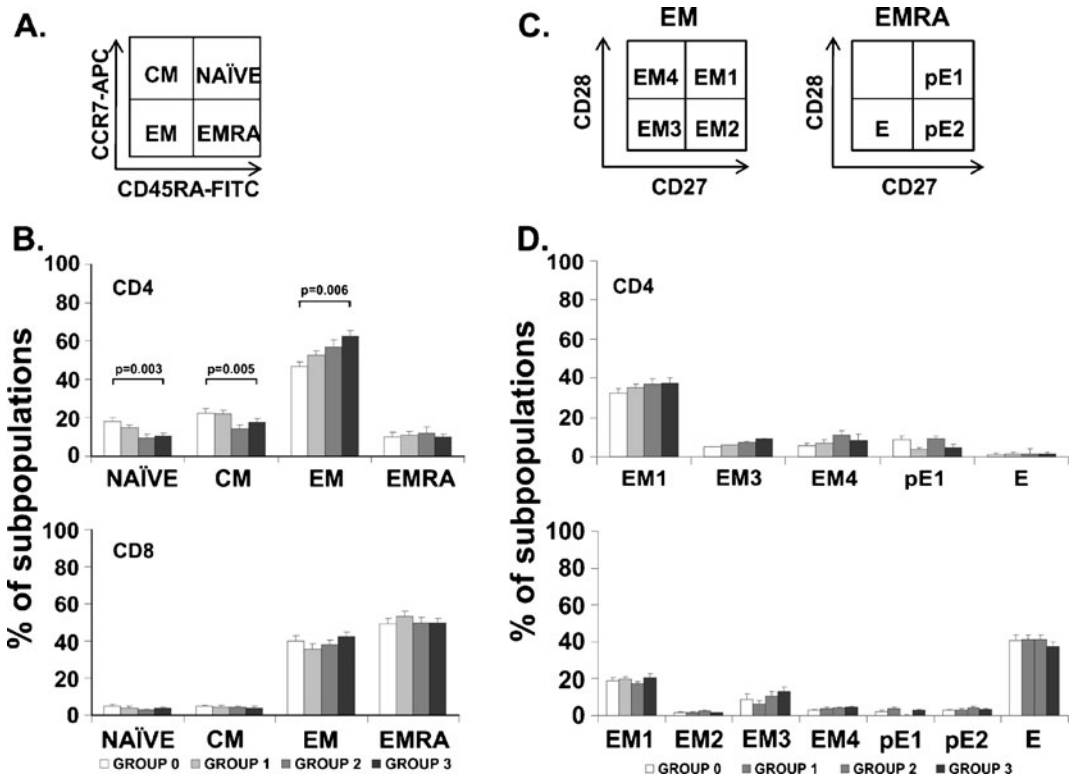

Fig. 3 Distribution of CD4+ and CD8+ T cells into naïve, central memory, effector memory and effector memory RA, and distribution of EM and EMRA in CD4+ and CD8+ T cells into subsets defined by CD28 and CD27 expression. Expression of CD45RA, CCR7, CD27, and CD28 was analyzed by flow cytometry in isolated CD4+ and CD8+ T cells from the four BI groups of elders. **a** Schematic model of the T-cell differentiation subsets accordingly to CD45RA and CCR7 expression. Whole blood was stained with anti-CD45RA-FICT, anti-CD8-PE, anti-CD4-PerCP, and anti-CCR7-APC, and 10^5 cells were acquired in each experiment. **b** Histograms represent percentage of cells in each subset (NAÏVE, CM, EM, and EMRA) in the four groups of elderly accordingly to their functional status (group 0—*white bars*, group 1—*light gray bars*, group 2—*dark gray bars*, group 3—*black bars*). Significant differences between subsets are indicated (ANOVA or Kruskal–Wallis non-parametric method). Each *bar* in the histograms represented the mean±SEM. **c** Representative dot plots of the subsets defined by CD27 and CD28 expression for individuals in each group. EM T cells can be divided into EM1 (CD27+CD28+), EM2 (CD27+CD28null, only in CD8+ T cells), EM3 (CD27nullCD28null), and EM4 (CD27nullCD28+). Similarly, EMRA can be divided into pE1 (CD27+CD28+) and pE2 (CD27+CD28null, only in CD8 T cells) and E (CD27nullCD28null). **d** Percentage of cells in each subset in the four groups of elderly accordingly to their functional status (group 0—*white bars*, group 1—*light gray bars*, group 2—*dark gray bars*, group 3—*black bars*). *Bars* in the histograms represented the mean±SEM

tions in elders from groups 2 and 3 were significantly decreased compared to those from groups 0 and 1. On the contrary, the more differentiated subset EM had a significant and progressive increase from group 0 to group 3. Although the proportions of EMRA cells increased with age, they were not significantly different between the groups (Fig. 3b). CD8+ T cells also did not differ significantly among groups: the level of the four populations was similar in all groups. Most CD8+ T cells were in the EM and EMRA subsets, which were the last stages of differentiation (Fig. 3b).

EM and EMRA are heterogeneous populations, and the staining of two additional markers, CD27 and CD28, has proven useful in identifying less differentiated (CD27+ and/or CD28+) or more differentiated ($CD27^{null}CD28^{null}$) cells (Koch et al. 2008) (Fig. 3c). Differentiating CD4+ T cells lose expression of CD27 first and subsequently lose CD28 in a later phase (Amyes et al. 2003; van Leeuwen et al. 2004). In

contrast, CD8+ T cells lose expression of CD28 first and then CD27 (Gamadia et al. 2003). Despite the existence of a different degree of differentiation defined by the expression of CCR7 and CD45RA markers, we found no significant differences in CD27 and CD28 expression between groups (Fig. 3d).

Taken together, these results indicated that a higher differentiation degree of CD4+ T cells, but not CD8+ T cells, was related to worse functional status. Thus, the maturation stage and number of past episodes of activation and cell cycling of CD4+ T cells differentiated elders with different motor abilities.

TREC quantification and basal proliferation

To further examine the differences found in the differentiated status of CD4+ subsets, we assessed the replicative history of these cells by quantifying the content of TREC in CD4+ T cells belonging to the four elder groups. TREC is a traceable molecular marker produced in newly naïve T cells; the content of TREC in peripheral T cells is an indicator of the number of divisions that the cell has undergone (Douek et al. 1998). Since sample volumes were insufficient to perform CD4+ T-cell isolation in all donors and differences were not found between groups 0 and 1 and between groups 2 and 3, we grouped samples: group 0+1 and group 2+3. The analysis of the groups displayed significant differences in TREC content. The TREC was lower in CD4+ T cells from elders with worse functional status and more differentiated subsets (Mann–Whitney U test, $p=0.016$) (Fig. 4a).

Next, to determine whether the lower TREC number observed in elders with worse functional capabilities correlated to a lower frequency of naïve CD4+ T cells, we compared the TREC content to levels of naïve CD4+ T cells and detected a positive correlation (Spearman Rho test; rho 0.421; $p=0.041$) (Fig. 4b). The elders with a lower TREC content had a worse functional status and lower frequency of naïve CD4+ T cells. Thus, cells from the elderly groups 2 and 3 have undergone more cell divisions since they migrated from the thymus than cells from groups 0 and 1.

To further examine the proliferative capacity, we measured the level of the cellular marker for proliferation Ki-67 on CD4+ and CD8+ populations isolated from 10 elderly in groups 0 and 1 and from 10 volunteers from groups 2 and 3 (Fig. 4c). No significant differences in Ki-67 levels on both cell populations between the two groups of elderly were detected, and thus the basal proliferation did not significantly differ between the groups (Fig. 4d).

Functional immunoresponse in vitro

To evaluate whether phenotypic changes that were observed with the deteriorating functional ability were also associated with the reduced immune responsiveness associated with aging in previous reports, we measured the activation capability by comparing CD69 expression in CD4+ and CD8+ T cells after anti-CD3 stimulation. Elders with better functional status (group 0+1) showed a higher CD69 expression level in both CD4+ and CD8+ T cells than group 2+3 did (Student's t test, $p=0.025$ and $p=0.015$, respectively) (Fig. 5a). Group 0+1 also exhibited a higher anti-CD3 proliferative response in the CD4+ and CD8+ T-cell subsets than group 2+3 (Student's t test, $p=0.0005$ and $p=0.037$, respectively) (Fig. 5b).

CMV antibody titer may reflect the number of previous CMV reactivations. Since elders who had the highest levels of CMV antibodies also mounted the worse response to anti-CD3, we postulated that episodes of viral activation could reflect this deficient cellular immune response or, by the contrary, could boost CMV-specific T-cell responses. The cellular response against CMV was measured by stimulating whole-blood cultures with CMV antigens. The magnitude of the CD4+ T-cell immune response to CMV was significantly higher in the elders with the worst functional status, as detected by CD69 expression (Mann–Whitney U test, $p=0.025$) (Fig. 5c) and by specific proliferation (Mann–Whitney U test, $p=0.001$) (Fig. 5d). Differences in CD8+ T-cell activation were not found (Fig. 5c), and proliferative responses in both groups were very limited (Fig. 5d).

Accordingly to the above results, the frequency of IFN-γ production by anti-CD3 stimulated T cells in group 0+1 was 123.8 (IR=85.6) and in group 2+3 was 85.8 (IR=61.1) (Fig. 6; Mann–Whitney U test, $p=0.013$). The median frequency of CMV stimulated group 0+1 T cells was 2.9 (IR=9.2) and that from group 2+3 was 11.3 (IR=17.05) (Mann–Whitney U test, $p=0.005$). These results indicated that despite an impaired cellular immune response, elders with worse functional status presented the highest activation,

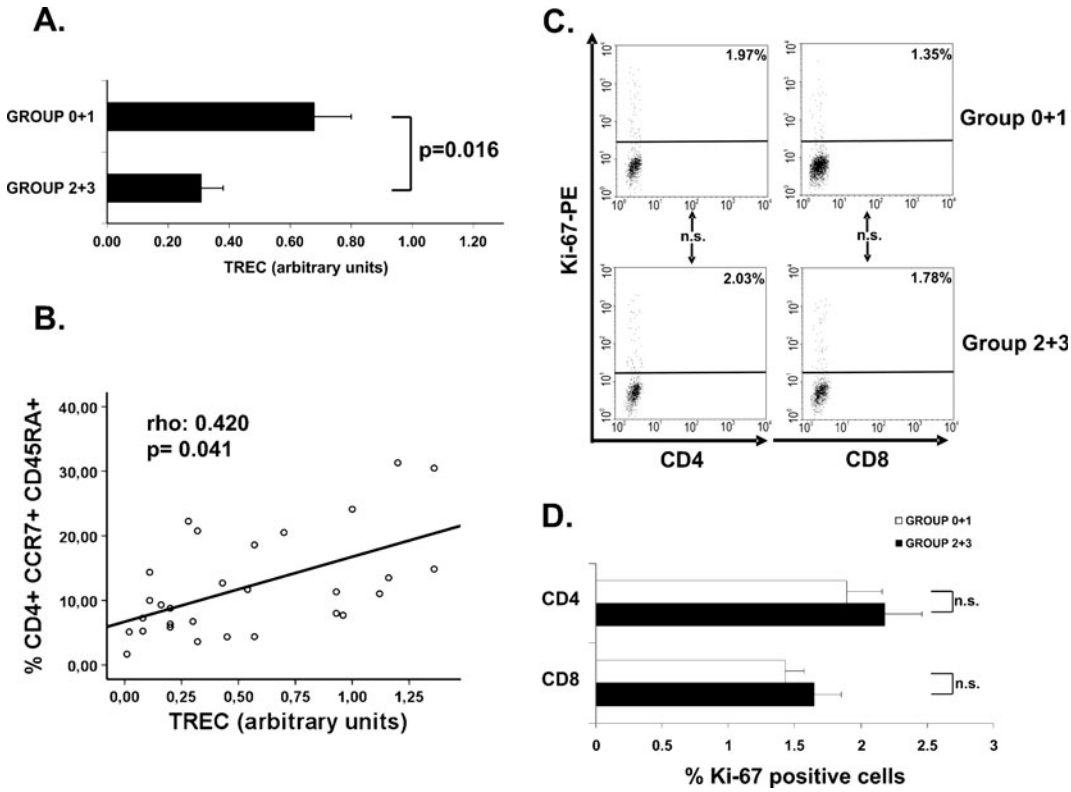

Fig. 4 TREC content in CD4+ T cells, its correlation with the NAÏVE subset and Ki-67 quantification. **a** The TREC content was measured in CD4+ T cells from elders belonging to group 0+1 (n=14) and group 2+3 (n=15). CD4+ population was isolated by magnetic bead separation and the TREC copy number was determined by real-time PCR. Experiments were conducted in duplicate and *bars* represented results from the grouped elders (mean±SEM). **b** Relationship between TREC content and NAÏVE (CD4+CCR7+CD45RA+) subset in the four groups of elderly was analyzed. The correlations, p value, and coefficient of correlation were calculated by using the non-parametric Spearman test and are listed in the *upper left hand corner*. **c** The quantification of Ki-67 was performed in CD4+ and CD8+ T cells from elders belonging to group 0+1 (n=10) and group 2+3 (n=10). CD4+ and CD8+ populations were isolated by magnetic bead separation and the Ki-67 quantification was determined by intracellular staining and flow cytometry. Representative dot plots show the frequency of Ki-67 expression in CD4+ and CD8+ subsets from elderly with different functional status. Percentage of positive cells in each subpopulation in these representative experiments are expressed in the *upper right corner*. Appropriate isotype control mAbs were used for marker settings. **d** Histograms summarize the percentage of positive cells for Ki-67 (mean±SEM). The Student'st test method was used to compare frequencies between groups

proliferation, and IFN-γ production against CMV antigens.

Response to vaccination and functional capacity

Next, to determine whether the lower immune response observed in vitro in elders with worse functional capabilities correlated to poor ability to respond to immunization in vivo, we measured the specific antibodies produced against influenza virus vaccination in the four groups of elders. The production of specific antibodies to vaccine was significantly lower in the group 3 than in the other groups (Fig. 7a). Comparison of the CMV antibody titer with influenza vaccination response revealed a negative correlation (Spearman Rho test; rho −0.303; p=0.002) (Fig. 7b).

Therefore, we concluded that those elders with a higher CMV antibody titer had worse functional status and may indicate a causal effect between CMV reactivation and reduced immune responses.

Discussion

In this study, we have demonstrated for the first time a clear association between functional decline of elderly

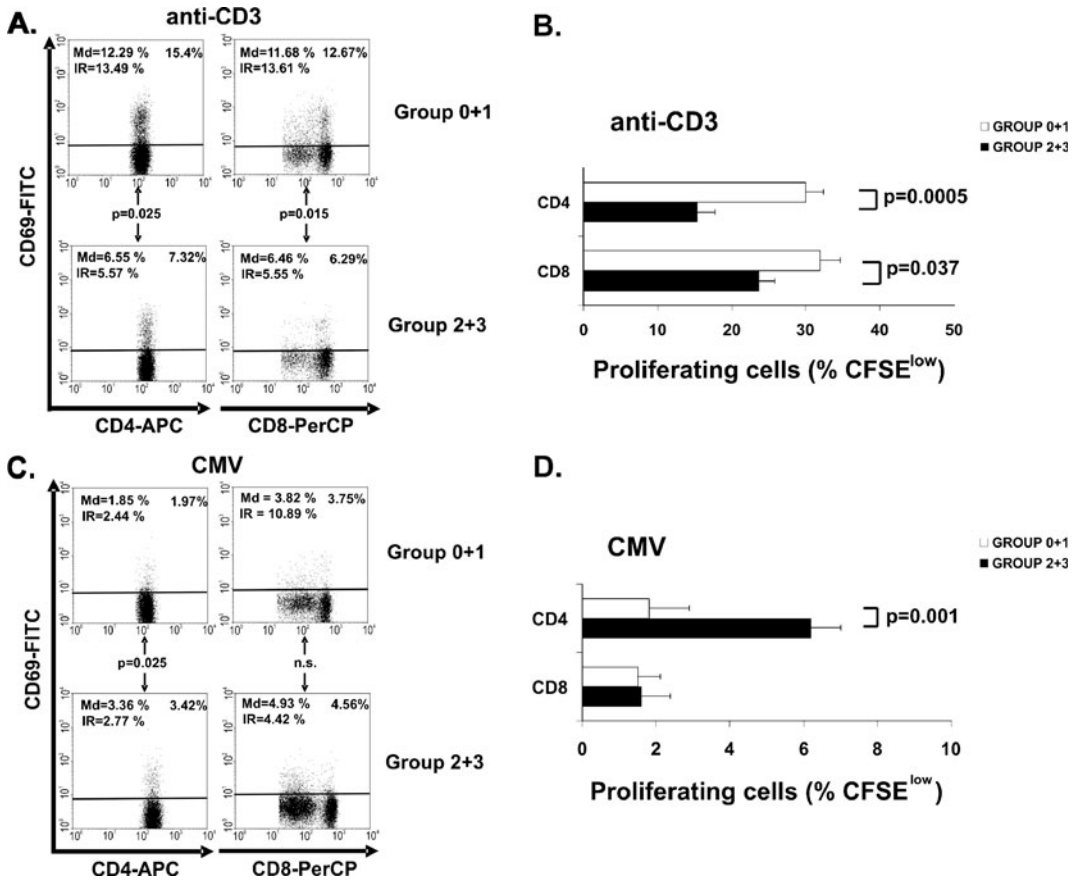

Fig. 5 CD69 expression and proliferative capacity of CD4+ and CD8+ depending on the functional capacity. Whole blood from the BI groups of elders (group 0+1, $n=12$ and group 2+3, $n=14$) was stimulated for 18 h and expression of CD69 in CD4+ and CD8+ T cells was evaluated by flow cytometry. Proliferative capacity of CD4+ and CD8+ T cells subsets was also evaluated in the two groups (group 0+1, $n=19$ and group 2+3, $n=16$) by labeling the PBMC with CFSE. Cells were stained and 1×10^5 cells were acquired per experiment. **a** Representative dot plots showing the frequency of CD69 expression in CD4+ and CD8+ subset from elderly with different functional status. Cells were stimulated using anti-CD3 (10 ng/mL). Percentage of positive cells in each subpopulation in this representative experiment is expressed in the *upper right corner* and summarized results from all donors (median and IR) were also expressed in dot plots. **b** Proliferative capacity of CD4+ and CD8+ T cells subsets in response to anti-CD3. PBMC were labeled with CFSE (1.5 μM) and cultured in presence of anti-CD3 (10 ng/mL) for 5 days. Percentage of dividing CD4+ and CD8+ T cells is represented. *Bars* represent results from the grouped elders (mean±SEM). **c** Expression of CD69 into de CD4+ and CD8+ T cell subset was analyzed in the same way as in Fig. 5a in response to a CMV supernatant (10^4 PFU/mL). **d** Proliferative capacity of CD4+ and CD8+ T-cell subsets in response to the CMV supernatant. *Bars* represent resulted from the grouped elders (mean±SEM). The Student's*t* test (when data were normally distributed) and Mann–Whitney non-parametric (when data were not normally distributed) methods were used to compare frequencies between groups. *p* values are depicted in the panels

individuals and aging of their immune system. We found significant differences in the distribution and differentiation state of cell subpopulations, in the cellular response in vitro, and in the in vivo ability for immunization. Furthermore, the elderly with worse functional capacity had the higher anti-CMV titer and T-cell response to CMV than the elderly with better functional status. In summary, we demonstrated a relationship between the intensity of the response to CMV, the immune system status, and the functional ability of older people.

Although several aspects of the innate immune response are affected by normal human aging, we only found significant changes in NK cells. As is well

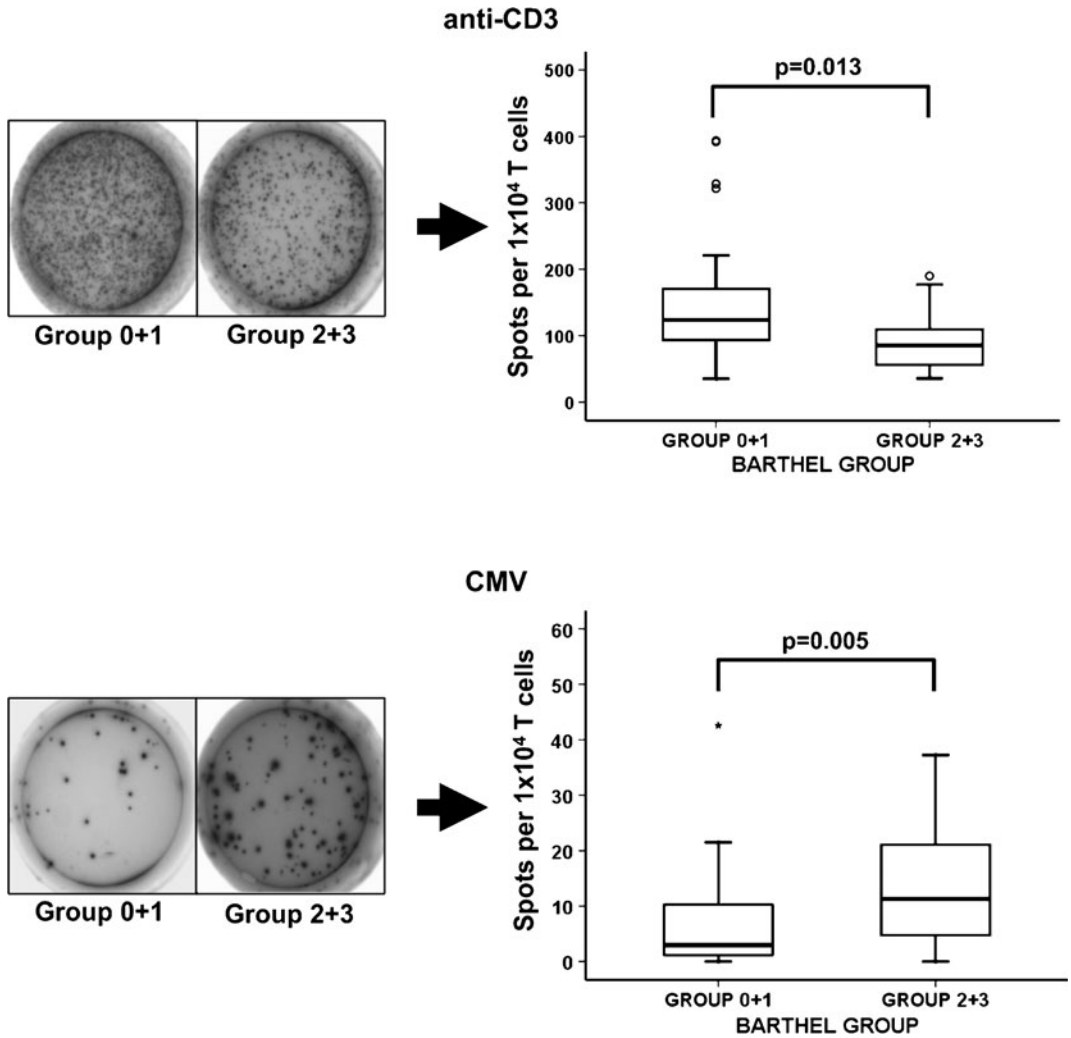

Fig. 6 IFN-γ production in response to anti-CD3 and to CMV antigens. Production of IFN-γ was measured in the two groups of elders (group 0+1, n=28 and group 2+3, n=21) by ELISPOT assay. PBMCs from elderly were stimulated with anti-CD3 (10 ng/mL) or CMV extracts (10^4 PFU/mL) for 20 h at 37°C/5% CO_2. An example of the spots generated in response to anti-CD3 and to CMV is represented for both groups. The mean numbers of antigen-specific spot forming cells after background subtraction of control wells with no antigen were plotted. Experiments were conducted in triplicate. Outlier values are represented by *circles* and extreme values by *stars*, calculated by adding 1.5 and 3 times the IR to the 75th percentile, respectively. The Student's*t* test (when data were normally distributed) and Mann–Whitney non-parametric (when data were not normally distributed) methods were used to compare frequencies between groups and p values are depicted in the panels

known, the number of NK cells increase with age (Le Garff-Tavernier et al. 2010). Elderly donors with worse functional status have a higher NK cell percentage than elderly with better status. NK cells play a role in the recognition and regulation of virally infected cells (Bottino et al. 2006) and they are particularly important in immunosurveillance against CMV (Lopez-Botet et al. 2004). In agreement, we found that older people with worse functional status had a higher antibody titer against CMV. Possibly, NK cells are increased in order to fight against this herpesvirus.

The factors that influenced the IRP include the CD4/CD8 ratio and CMV seropositivity (Olsson et al. 2000; Hadrup et al. 2006). However, CMV antibody titer may also be important factor in the time of survival since a high CMV antibody titer is related to a reduced survival time (Strandberg et al. 2009; Roberts et al. 2010). Elders in our study with poor

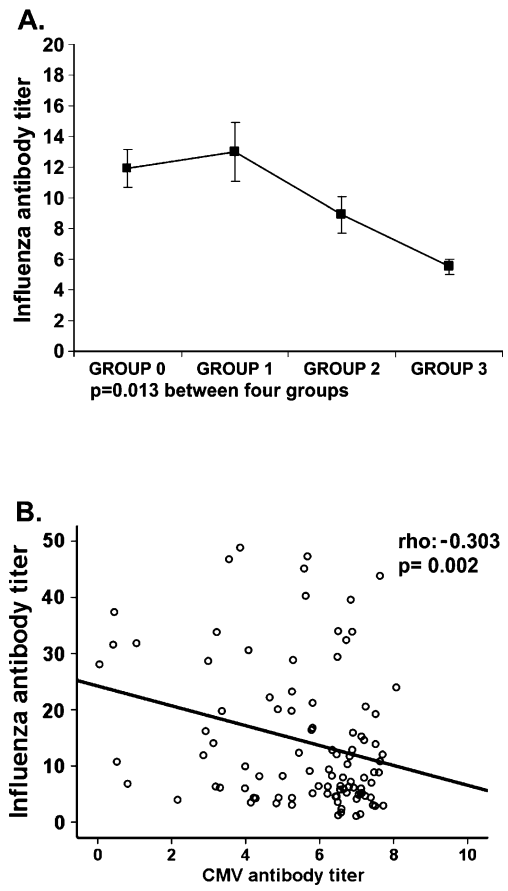

Fig. 7 Response to influenza virus vaccination and its correlation to CMV titer. Influenza antibodies titer was quantified by ELISA in the serum of the elders after vaccination. **a** The Kruskal–Wallis non-parametric method was used to compare the influenza antibody titer between the four groups. **b** Relationship between influenza and CMV antibody titer in the four groups of elderly was analyzed. A non-parametric Spearman test was applied to calculate the correlations, *p* value and coefficient of correlation are listed in the *upper right hand corner*

functional status had a lower CD4/CD8 ratio and had a higher CMV antibody titer compared to the other groups. CMV infection caused a significant decrease in the CD4/CD8 ratio in elderly individuals, because it increased CD8+ T-cell count and reduced CD4+ T-cell numbers (Chidrawar et al. 2009). Here, subjects with the worst IRP occurred in groups with worse functional status, but it did not associate with individuals' age.

As people age, differentiated cells (EM and EMRA) of the immune system accumulate and the less-differentiated immune cells (Naïve and CM) decline in frequency. These changes could be due to several mechanisms, but a factor with more influence is CMV infection. Elders in the BI groups with the highest CMV antibody titers also had more differentiated subpopulations of CD4+ T cells, but no differences were detected in the CD8+ T cells. The two subsets undergo the same principal phenotypic shifts, but the rate at which they occur or accumulate with age was different. CD4+ T cells were more resistant to phenotypic and functional changes with aging than CD8+ T cells (Czesnikiewicz-Guzik et al. 2008).

One of these phenotypic changes which appeared in old age is the lack of the costimulatory molecule CD28 (Fagnoni et al. 1996). The population of CD8+CD28^{null} is a majority, and we found an increasing trend in the population of CD4+CD28^{null} among the elderly with poorer functional status. We have recently found that expression of NKG2D+ in the CD4+CD28^{null} T cells may be a better marker of cellular senescence (Alonso-Arias et al. 2011). In fact, the proportion of CD4+NKG2D+ significantly increased in the groups of elders with worse functional status. Similarly, double-positive CD4+CD8+ T cells (Pawelec 1995), which generally contain a high proportion of memory and differentiated cells, were also increased in groups with worse functional status. CD4+NKG2D+ and CD4+CD8+ subsets have been associated with CMV infection; there was a significant correlation between their expansion and CMV infection and viral reactivation (Saez-Borderias et al. 2006; Alonso-Arias et al. 2009).

Naïve T cells, which are needed to protect against new pathogens, are reduced in the elderly by several interrelated events: involution of the thymus, decline of naïve T cells, reduction in T-cell repertoire diversity, and accumulation of memory T cells that are specific for persisting pathogens (Nikolich-Zugich 2008). One way to verify the differentiation status of T cells is to measure TREC. We observed that CD4+ T cells in elders in worse functional status had a lower TREC content. This corroborates the findings that more differentiated cells have undergone more division cycles, and therefore they have a low TREC content. The lack of significant differences in the Ki-67 quantitation may occur because the cells of the subjects in this study have a very low turnover due to their advanced age and this very low cellular turnover may limit the number of cells that would show differences between the groups.

The ability to activate via TCR stimulation was significantly lower in groups with worse functional status in both CD4+ and CD8+ T-cell subsets. Highly differentiated T cells lose their ability to proliferate in response to stimulation (Appay et al. 2002). In contrast, CD4-specific response against CMV was increased in our groups with worse functional capacity. In an analogous manner, Vescovini et al. recently reported that groups of elderly individuals with cognitive impairment and poor functional capabilities had significantly higher anti-CMV IgG titers and higher CD4+ T cells specific for CMV, although they used different criteria to score their patients and their subjects (Vescovini et al. 2010) and we have also analyzed a larger number of variables which have never been correlated before to functional status in elderly. The cell repertoire limitation and the high degree of differentiation caused by CMV infection could play a major role in the reduced anti-CD3 response of T cells in the elderly with worse functional status. The importance of the CMV-specific CD4+ T-cell response in elders has been less studied than the constriction of the CD8+ T-cell repertoire. We found differences in the response to CMV in the CD4+ T-cell subset, but not in CD8+ T cells. CMV-specific CD4+ T cells typically display a memory phenotype (Fletcher et al. 2005), and this subset is indeed increased in elderly CMV-seropositive donors at the expense of the less-differentiated populations. The individuals with worse functional status presented a greater number of CMV-specific cells, and the high frequency of anti-CMV may occur from a large number of virus reactivations or an enhanced response against the virus.

The most profound clinical impact of age on the immune system concerned the response of the elderly to vaccination. In our study, the percentage of B cells is significantly decreased in the groups of elderly with the worse functional capacity. This may partly explain the poorer response to vaccination against influenza virus in the BI groups 2 and 3. CD4+CD28^{null} are deficient in providing help to B cells, and their accumulation of CD4+CD28^{null} T cells could provide one mechanism for impaired humoral responses in the elderly. Despite the aforementioned, we believe that the increase in the CMV titer and therefore the increase in the percentage of cells involved in the response against this herpesvirus is, maybe, the greatest limitation in the response to vaccination. In fact, we found a negative correlation between the CMV titer and the response to the vaccine influenza virus: the worst responses to vaccination were observed in the groups with the more compromised functional capacity.

Since the deterioration of the physical and functional status in elders may be directly related to the state of the immune system, "slowing" the deterioration of the immune system could possibly improve the quality of life of elders. Findings from cross-sectional studies mostly show enhanced immunity in physically active elders compared to sedentary older adults (Simpson and Guy 2009). Another field of action would be the regeneration of the T-cell population since in principle it is one of the most affected subsets by the aging process.

To our knowledge, this is the first time that a study correlates a poorer motor ability in old age and compromised immune system, both in the cellular response in vitro and response to immunization in vivo. The functional status in older people may be influenced by the state of their immune system or vice versa. All immunological parameters we studied are more impaired in the elderly with worse functional status, but these elders had a higher antibody titer and a higher response to CMV infection. Therefore, we plan to study these parameters in more detail using more subjects in each group in the future. Further studies will help us to develop new strategies to slow or reverse age-associated immune dysfunction.

Acknowledgments We thank Isabel Cuevas Pérez, José María Díaz Pérez, and Ramona Julia Fernández García for their excellent technical assistance. This work was supported by Red de Investigación Renal (REDinREN), Spanish grant FIS PI080566 from Instituto “Carlos III” (European FEDER founds) and PEST 08-05 from FICYT.

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III.5. LA INMUNOSENESCENCIA Y LA INFLAMACIÓN CARACTERIZAN A LOS PACIENTES CON INSUFICIENCIA CARDIACA CRÓNICA (ICC) AVANZADA

La ICC es una enfermedad compleja con una alta mortalidad, en la que el fallo mecánico del ventrículo lleva a multitud de fallos sistémicos, incluyendo una activación neurohormonal e inmunológica. La activación del sistema inmune lleva consigo la producción y liberación de citocinas proinflamatorias (IL-1, IL-2, IL-6 y TNF), la activación del sistema de complemento y la producción de autoanticuerpos (Haynes *et al.* 2004). Nuestra hipótesis se basó en el hecho de que la inflamación producida en estos pacientes podría llevar a una inmunosenescencia acelerada y que esto podría estar asociado a un peor estadio de la enfermedad.

ARTÍCULO 5:

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Int J Cardiol. 2014 Jul 1;174(3):590-9.

Para estudiar el estado de inmunosenescencia en los pacientes con ICC comparamos las características inmunológicas de 58 ancianos con ICC (AICC), 40 jóvenes con ICC (JICC), 60 ancianos sanos (AS) y 40 jóvenes sanos (JS). Caracterizamos las poblaciones leucocitarias y linfocitarias por citometría de flujo y las concentraciones de IL-6 por ELISA. La gravedad de la ICC se clasificó mediante criterios funcionales y/o morfológicos: escala NYHA (New York Heart Association), estadios de ICC (ACC/AHA guidelines, American College of Cardiology–American Heart Association), fracción de eyección del ventrículo izquierdo (FEVI) e hipertrofia del ventrículo izquierdo. Los pacientes con ICC mostraron un número elevado de leucocitos, neutrófilos y monocitos, pero un número disminuido de linfocitos. Estos pacientes presentaron niveles significativamente disminuidos de linfocitos B y linfocitos T CD4+. En los pacientes JICC vimos un aumento en las células NK, mientras que en los pacientes AICC observamos un aumento de los linfocitos T CD8+. Por otro lado, la ICC se relacionó con un aumento en la diferenciación de las poblaciones linfocitarias T CD4+ y CD8+. Tanto el aumento de

diferenciación de las subpoblaciones de linfocitos T como las concentraciones elevadas de IL-6 se relacionaron con un peor estado clínico. La IL-6 también se correlacionó positivamente con el número de linfocitos T altamente diferenciados y con su envejecimiento acelerado.

Podemos concluir que los pacientes con ICC muestran un mayor grado de inmunosenescencia que los controles pareados por edad. La diferenciación linfocitaria y los niveles de IL-6 se encuentran elevados en los pacientes con enfermedad más avanzada y esto puede contribuir al empeoramiento de la enfermedad a través de una respuesta inmune innata incrementada y una respuesta adaptativa comprometida debido al envejecimiento acelerado de su sistema inmunológico.

Aportación personal al trabajo:

En este trabajo, mi labor se centró en recolectar y procesar las muestras del estudio, realizar y/o supervisar los distintos experimentos y analizar los resultados obtenidos. Finalmente, participé en el diseño y en la escritura del presente manuscrito.

International Journal of Cardiology 174 (2014) 590–599

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Immunosenescence and inflammation characterize chronic heart failure patients with more advanced disease ☆

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ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form 15 March 2014
Accepted 12 April 2014
Available online 21 April 2014

Keywords:
Aging
Immune system
Lymphocytes
Inflammation
Interleukins

ABSTRACT

Background: Chronic heart failure (CHF) is characterized by an inflammatory status with high levels of cytokines such as IL-6. We hypothesized that patients with CHF may develop immunosenescence due to inflammation and that this may be associated with a worse stage of the disease.
Methods and results: We compared the immunological features of 58 elderly CHF patients (ECHF), 40 young CHF patients (YCHF), 60 healthy elderly controls (HEC) and 40 healthy young controls (HYC). We characterized leukocyte and lymphocyte subpopulations by flow cytometry, and IL-6 concentration by ELISA. The extent of CHF was classified according to functional and/or morphological criteria: New York Heart Association functional class, AHA/ACC heart failure stages, left ventricular ejection fraction, and left ventricular hypertrophy. CHF patients showed an increased number of leukocytes, neutrophils and monocytes, but a decreased number of lymphocytes. CHF patients had significantly lower levels of B-cells and CD4+ T-cells, increased NK-cells in YCHF, and increased CD8+ T-cells only in ECHF. CHF was associated with high differentiation in CD4+ and CD8+ T-lymphocyte subsets. Aging of T-lymphocyte subpopulations and high IL-6 levels were associated with a worse clinical status. IL-6 also correlated positively with the number of highly differentiated T-lymphocytes and with their accelerated aging.
Conclusions: We conclude that CHF patients show a higher degree of immunosenescence than age-matched healthy controls. T-lymphocyte differentiation and IL-6 levels are increased in patients with an advanced clinical status and may contribute to disease impairment through a compromised adaptive immune response due to accelerated aging of their immune system.

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1. Introduction

Chronic heart failure (CHF) is characterized by inflammation, insulin resistance, and progressive catabolism. CHF is a complex illness with high mortality, in which mechanical failure of the ventricle leads to a multitude of systemic effects, including neurohormonal and immunologic activation. Following reports that serum tumor necrosis factor (TNF) concentrations were elevated in patients with CHF [1], considerable effort has been made to describe the role of the immune system in this disease. Activation of the immune system occurs in CHF, which results in the production and release of proinflammatory cytokines (IL-1, IL-2, IL-6, and TNF), activation of the complement system, and the production of autoantibodies [2]. Cytokines are essential for the propagation and magnification of the immune response; they are involved in recruiting cells to the area of inflammation, stimulating cell division, proliferation, and differentiation. The excessive production of pro-inflammatory cytokines, such as TNF and IL-6, contributes to the pathogenesis of CHF. The activation of protein kinase C and NF-κB by TNF and IL-6 activates a chronic inflammatory response that results in

☆ Acknowledgement of grant support: This work was supported by the Asturcor Foundation, Spain, and FIS FIS-Fondos FEDER European Union RD06/0013/0028 (Red Temática de Investigación Cooperativa en Envejecimiento y Fragilidad) from the Instituto Carlos III (Spain).
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[1] These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

http://dx.doi.org/10.1016/j.ijcard.2014.04.128
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Table 1
Characteristics of the study subjects.

	Elderly chronic heart failure patients (ECHF) ($n = 58$)	Healthy elderly controls (HEC) ($n = 60$)	p value between groups	Young chronic heart failure patients (YCHF) ($n = 40$)	Healthy young controls (HYC) ($n = 40$)	p value between groups
Age ± SD (years)	84.6 ± 4.9	83.98 ± 6.4	NS	55.5 ± 6.9	53.6 ± 7.1	NS
Male (%)	34 (58.6)	16 (26.7)	0.001	32 (80.0)	29 (72.5)	NS
BMI ± SD (kg/m^2)	27.2 ± 6.6	26.4 ± 6.7	NS	24.1 ± 3.5	24.7 ± 4.2	NS
Smoking status, current (%)	6 (10.3)	8 (13.3)	NS	10 (25)	9 (22.5)	NS
Hypertension (%)	27 (46.5)	19 (31.6)	0.045	14 (35.0)	0 (0)	<0.001
% LVEF ± SD	48.7 ± 7.4	>60	NA	36.4 ± 12.2	>60	NA
Diabetes mellitus (%)	10 (17.24)	8 (13.4)	NS	9 (22.5)	0 (0)	<0.001
Cholesterol ± SD (mg/dL)	159.3 ± 31.3	148.6 ± 26.5	NS	167.1 ± 36.5	ND	NA
NT-proBNP, pg/mL (IQR)	4485 (5938)	ND	NA	4529 (4137)	ND	NA
CRP, mg/dL (IQR)	2.0 (2.7)	ND	NA	1.6 (1.9)	ND	NA
IL-6, pg/mL (IQR)	5.13 (11.37)	ND	NA	1.27 (1.9)	ND	NA
CHF etiology						
Coronary artery disease (%)	24 (41.4)	NA		15 (37.7)	NA	
Idiopathic dilated cardiomyopathy (%)	26 (44.8)	NA		16 (40.0)	NA	
Others (%)	8 (13.8)	NA		9 (22.5)	NA	
Hematological variables (mean and SD)						
WBCs (10^3/μL)	7.9 ± 2.61	6.41 ± 1.98	<0.001	8.05 ± 1.9	7.54 ± 1.62	0.04
Neutrophils (10^3/μL)	6.51 ± 2.55	3.77 ± 1.43	<0.001	5.25 ± 1.8	4.38 ± 0.9	0.006
Neutrophils (%)	75.2 ± 9.3	57.8 ± 8.4	<0.001	64.2 ± 8.3	56.6 ± 7.2	0.001
Lymphocytes (10^3/μL)	1.2 ± 0.55	1.86 ± 0.6	<0.001	1.8 ± 0.64	3.21 ± 0.74	<0.001
Lymphocytes (%)	15.0 ± 7.4	29.92 ± 8.25	<0.001	23.9 ± 7.7	39.12 ± 4.4	<0.001
Monocytes (10^3/μL)	0.63 ± 0.2	0.54 ± 0.44	NS	0.65 ± 0.19	0.54 ± 0.65	0.008
Monocytes (%)	7.72 ± 2.46	8.27 ± 3.73	NS	8.4 ± 2.5	6.61 ± 3.5	0.009

CAD indicates coronary artery disease; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal protype B natriuretic peptide; BMI, body mass index; CRP, C-reactive protein; WBCs, white blood cells; SD, standard deviation; IQR, interquartile range. NA, not applicable. ND, not done. NS, not significant.

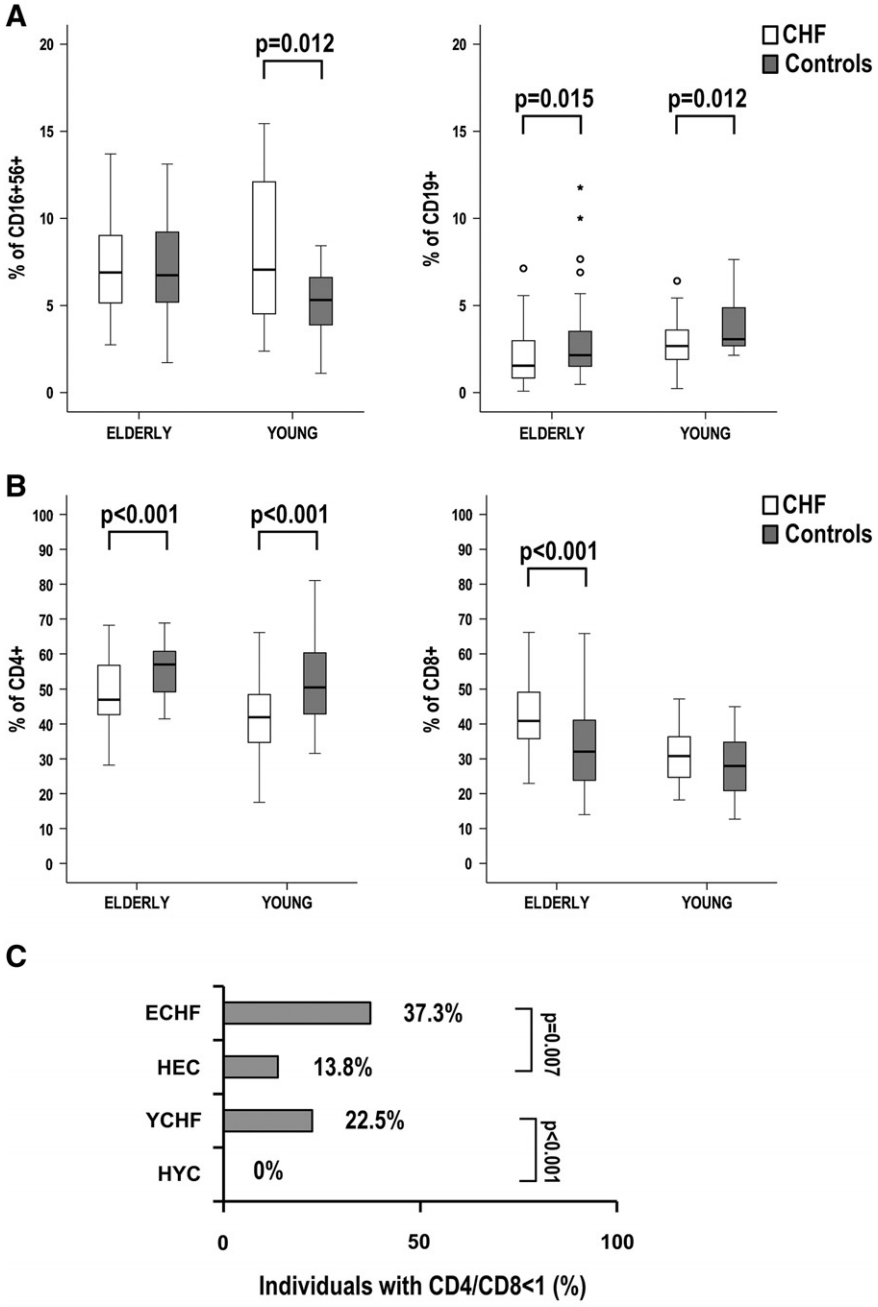

Fig. 1. Immune phenotype in peripheral blood. The number of donors in each group was as follows: ECHF = 58, HEC = 60, YCHF = 40 and HYC = 40. (A) Percentages of CD16+ 56+ and CD19+ cells with respect to the total number of CD45+ cells in the different groups were compared. Cells were stained with "Multiset CD3-FITC/CD16+ 56-PE/CD45-PerCP/CD19-APC" and the frequencies of CD16+ 56 and CD19+ cells in gated CD45+ subsets were analyzed. (B) Percentages of CD3+ CD4+ and CD3+ CD8+ T-cells were determined with respect to the total number of CD45+ cells, and compared between groups. Cells were stained with anti-CD3-FITC, anti-CD45-PE, anti-CD8-PerCP, and anti-CD4-APC to gate CD4+ and CD8+ T-cells. Outlier values are represented by circles and extreme values by stars, calculated by adding 1.5 and 3 times the IR to the 75th percentile, respectively. (C) CD4/CD8 ratios were calculated and the histogram represents the frequencies of individuals with an inverted ratio (<1) in the groups. Mann–Whitney *U* method was used to compare frequencies between groups. *P*-values are depicted in the panels.

hypertrophy [3] and the cardiotoxic activation of cell death pathways [4]. All these events are involved in cardiac depression and in the progression of heart failure. Inflammation may also be directly related to the impairment of T-cell responses. Progressive T-cell differentiation and low-grade inflammation are two processes that occur simultaneously in healthy aging and/or enhance each other. Highly differentiated cells increase the levels of proinflammatory cytokines, whereas inflammatory mediators are thought to be involved in the development of differentiated T-cell phenotypes. Moreover, as a result of continual antigenic stress throughout life, both processes increase, and this is considered to be a major contributor to age-associated frailty, morbidity and mortality [5]. Older people suffer from age-associated changes in the immune system, including a decline in immune function, which is responsible for the increased incidence and severity of infections, development of autoimmune phenomena, and cancer [6,7]. These defective immune responses are also manifested in the reduced ability of vaccines and infections to induce immunological memory [8,9]. Elderly donors display a decline in the numbers of naïve T-cells in peripheral blood and lymphoid tissues [10]; in contrast, they have a marked increase in the proportion of highly differentiated effector and memory T-cells like the $CD28^{null}$ T-cells [11,12]. An

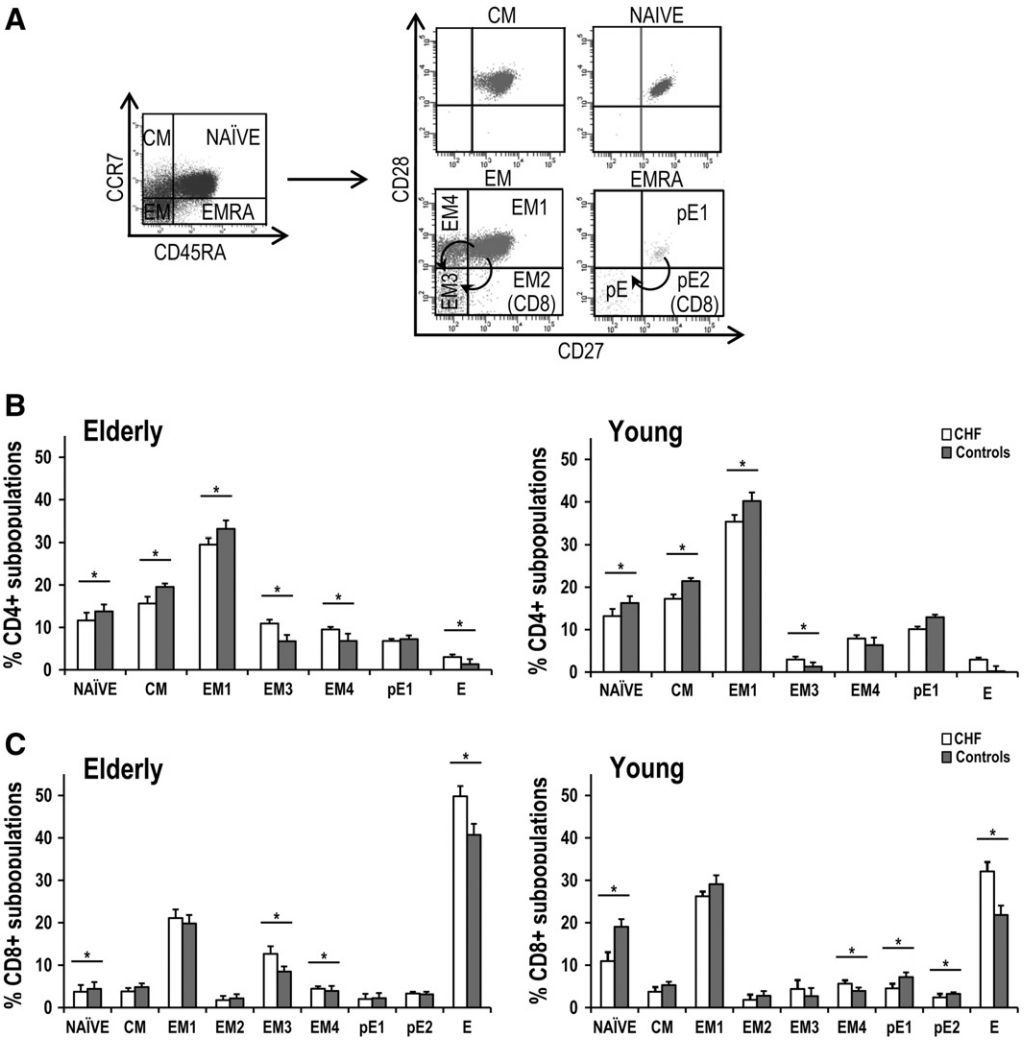

Fig. 2. Distribution of CD4+ and CD8+ T-cells among naïve, central memory (CM), effector memory (EM) and effector memory RA (EMRA) and distribution of EM and EMRA in CD4+ and CD8+ T-cells among subsets defined by CD28 and CD27 expression. Expression of CD45RA, CCR7, CD27, and CD28 was analyzed by flow cytometry in isolated CD4+ and CD8+ T-cells from the studied groups. (A) Schematic model of the T-cell differentiation subsets according to CD45RA and CCR7 expression and EM and EMRA T-cell differentiation subsets according to CD27 and CD28 expression. Histograms show the percentage in each subset of (B) CD4+ and (C) CD8+ T-cells in the groups (ECHF and YCHF: white bars; HEC and HYC: grey bars). Student's *t* test was used to compare groups. Data are mean ± SEM. $^{*}p < 0.05$.

Immune Risk Profile (IRP) was defined using a cluster analysis approach [13]. In agreement with a higher IRP, a higher 2-year mortality was observed in a population of very old Swedish individuals who had an inverted CD4/CD8 ratio, an accumulation of CD8+ $CD28^{null}$ T-cells, and CMV infection [14]. In summary, considerable evidence suggests a clear association between immune function and the longevity of individuals [15]. All these findings prompted us to study whether the high inflammatory status produced in CHF leads to a greater degree of immunosenescence and therefore to higher morbidity and mortality. We conducted an observational study that evaluated the state of the immune system in CHF. We report, for the first time, a relationship between the degree of inflammation and the aging of the immune system in patients with CHF.

2. Methods

2.1. Study population

One hundred and ninety-eight volunteers (87 women and 111 men) were recruited to the study. Individuals in the study were divided into four groups: healthy young controls (HYC) ($n = 40$), healthy elderly controls (HEC) ($n = 60$), young with CHF (YCHF) ($n = 40$) and elderly with CHF (ECHF) ($n = 58$). The HYC group was recruited from the Centro de Transfusiones del Principado de Asturias (Oviedo, Spain). For the HEC group, we selected people living at the Santa Teresa nursing home (Oviedo, Spain) and who were judged to be physically fit. CHF patients with symptomatic HF (New York Heart Association class I to III), a left ventricular ejection fraction ≥20%, and at least one hospitalization for HF during the previous 6 months were eligible for enrollment. Patients were recruited at the Hospital Universitario Central de Asturias and the Monte Naranco Hospital (Oviedo, Spain). All subjects underwent a physical examination and answered a standardized questionnaire to assess their medical history, current illnesses, and any medication they were taking. Exclusion criteria included all conditions that might influence the immune system, such as a recent or current infection, autoimmune disease or tumor, malnutrition, abnormal laboratory data (hemoglobin <12 g/dL, leucopenia <3500 cells/μL, neutropenia <1500 cells/μL, leukocytosis >15,000 cells/μL and platelets $<10^5$ cells/μL), and pharmacological interference. Informed consent was obtained from all volunteers before participation in the study. The study was approved by the ethics committee of the Hospital Central de Asturias (Oviedo, Spain).

Peripheral blood samples were drawn from all subjects for hematological and immunological analyses. Samples were taken from 26 ECHF patients twice during the study: time 0 (baseline, immediately before the study began) and time 6 m (6 months later).

CHF patients were classified according to the New York Heart Association functional class (NYHA), AHA/ACC heart failure stages (HFS), left ventricular ejection fraction (LVEF), and the degree of left ventricular hypertrophy (LVH).

2.2. Hematological analysis and immunological phenotyping

The hematological parameters were determined using a Sysmex XT-2000i (Syxmex, Hamburg-Norderstedt, Germany) and the biochemistry values using a Cobas c 711 analyzer series (Roche Diagnostics, Indianapolis, USA). For flow cytometry analysis peripheral blood cells were surface-stained with Multiset CD3-FITC/CD16+ 56-PE/CD45-PerCP/CD19-APC Reagent (BD Biosciences, San Jose, CA, USA), anti-CD4 (APC), anti-CD8 (PE or PerCP), anti-CD31 (PE), anti-CD45 (PE), anti-CD45RA (FITC) (Immunostep, Salamanca, Spain), anti-CD4 (PerCP), anti-CD28 (PE or PerCP) (eBioscience, San Diego, CA, USA), anti-CCR7

Table 2
Functional/morphological classifications of CHF patients.

	Elderly chronic heart failure patients ($n = 58$)	Young chronic heart failure patients ($n = 40$)
NYHA functional class (%)		
Class I	11 (19)	6 (15)
Class II	21 (36)	18 (45)
Class III	26 (45)	16 (40)
Class IV	0 (0)	0 (0)
AHA/ACC heart failure stages (%)		
Stage A	14 (24)	6 (15)
Stage B	24 (41)	15 (38)
Stage C	20 (35)	19 (47)
Stage D	0 (0)	0 (0)
LVEF (%)		
LVEF > 50%	26 (45)	13 (33)
LVEF ≤ 50%	32 (55)	27 (67)
LVH (%)		
Mild	27 (46)	19 (48)
Moderate	21 (36)	13 (32)
Severe	10 (18)	8 (20)

NYHA indicates New York Heart Association; AHA/ACC, American College of Cardiology–American Heart Association; LVEF, left ventricular ejection fraction; LVH, left ventricular hypertrophy.

(Alexa Fluor 647), and anti-CD3 (FITC) (BD Biosciences). One hundred microliters of whole blood from volunteers was stained with different combinations of labeled monoclonal antibodies for 20 min at room temperature. Samples were red-blood lysed with FACS Lysing Solution (BD Biosciences), washed in PBS, and analyzed using CellQuest software in a FACSCalibur Cytometer (BD Biosciences). Appropriate isotype control mAbs were used for marker settings.

2.3. IL-6 ELISA

Blood was collected by venipuncture and sera were stored at −80 °C until use. The secretion of IL-6 was monitored at time 0 in ECHF and YCHF and time 6 m in ECHF. IL-6 concentrations were determined by enzyme-linked immunosorbent assay for IL-6 (eBioscience) according to the manufacturer's specifications.

2.4. Statistical analysis

The results are expressed as the median and interquartile range (IR) or the mean and standard deviation, or the standard error of the mean in some graphs. Groups were compared using the nonparametric Mann–Whitney *U* test (for non-normally distributed data) or Student's *t* test (for normally distributed data). More than two groups were compared using the non-parametric Kruskal–Wallis test or by ANOVA analysis for data that were normally distributed. Paired analyses were performed using the Wilcoxon non-parametric method when data were not normally distributed, or with Student's *t* test for paired samples. The outlier and the extreme values were calculated by adding 1.5 and 3 times the interquartile range (IR) to the 75th percentile, respectively. Correlations between variables were assessed using the nonparametric Spearman test (ρ). Analyses were performed using the SPSS 15.0 statistical software package program (SPSS Inc. Chicago, IL) and *p*-values of 0.05 or less were considered significant.

3. Results

3.1. Demographic and hematological characteristics of the study population

The characteristics of the 198 individuals enrolled in the study are shown in Table 1. Age was comparable across the ECHF and the HEC groups, and across the YCHF and the HYC groups. The female/male ratio differed between groups; however we found no significant differences between men and women in any of the parameters examined in the study. All patients with CHF and controls were white. IL-6 and hematological variables were measured one week after hospital admission. Patients with CHF differed from the healthy control groups in the following measures: hypertension, diabetes mellitus (YCHF vs HYC), history of coronary artery disease, and left ventricular ejection fraction.

Blood cell counts and an immune phenotype were determined in all individuals included in the study. Comparison of the groups of individuals revealed significant differences between CHF patients and healthy controls in the absolute numbers of leukocytes and, in the absolute numbers and percentages of neutrophils and lymphocytes (Student's *t* test) (Table 1). Monocytes were significantly increased in YCHF with respect to HYC (Student's *t* test) (Table 1). Examination of the immunological phenotype of the lymphocyte subpopulations revealed that the YCHF group had a higher percentage of NK cells (CD16+56+) with respect to the HYC group (Student's *t* test: $p < 0.05$) whereas the percentage of B cells (CD19+) showed significant decreases in CHF groups (Student's *t* test: $p < 0.05$, in all cases) (Fig. 1A). Regarding T-cell subsets, we found a decrease in the percentage of CD4+ T-cells and an increase in CD8+ T-cells only in ECHF patients with respect to HEC (Student's *t* test: $p < 0.001$, in all cases) (Fig. 1B). When we compared the absolute values of the lymphocyte populations we found the same results (data not shown).

One of the parameters defining the IRP is the inverted CD4/CD8 ratio (ratio < 1.0). A significantly higher percentage of ECHF patients (37.3%)

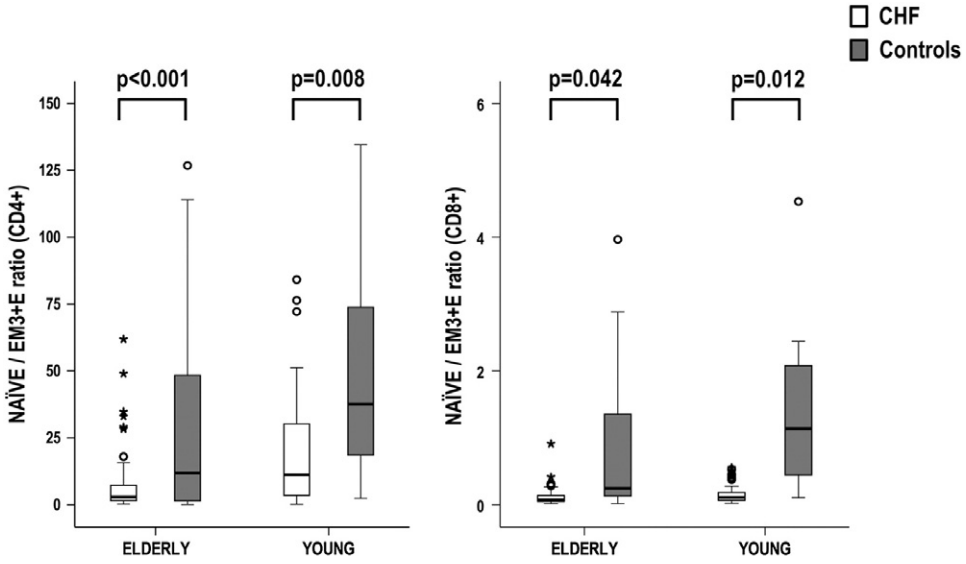

Fig. 3. Degree of differentiation in CD4+ and CD8+ T-lymphocyte subsets using the naïve/EM3+E ratio. (A) Naïve/EM3+E ratio in peripheral blood from the ECHF, HEC, YCHF and HYC groups. Outlier values are represented by circles and extreme values by stars, calculated by adding 1.5 and 3 times the IR to the 75th percentile, respectively. Mann–Whitney *U* method was used to compare the CHF patient and healthy control groups. *P*-values are depicted in the panels.

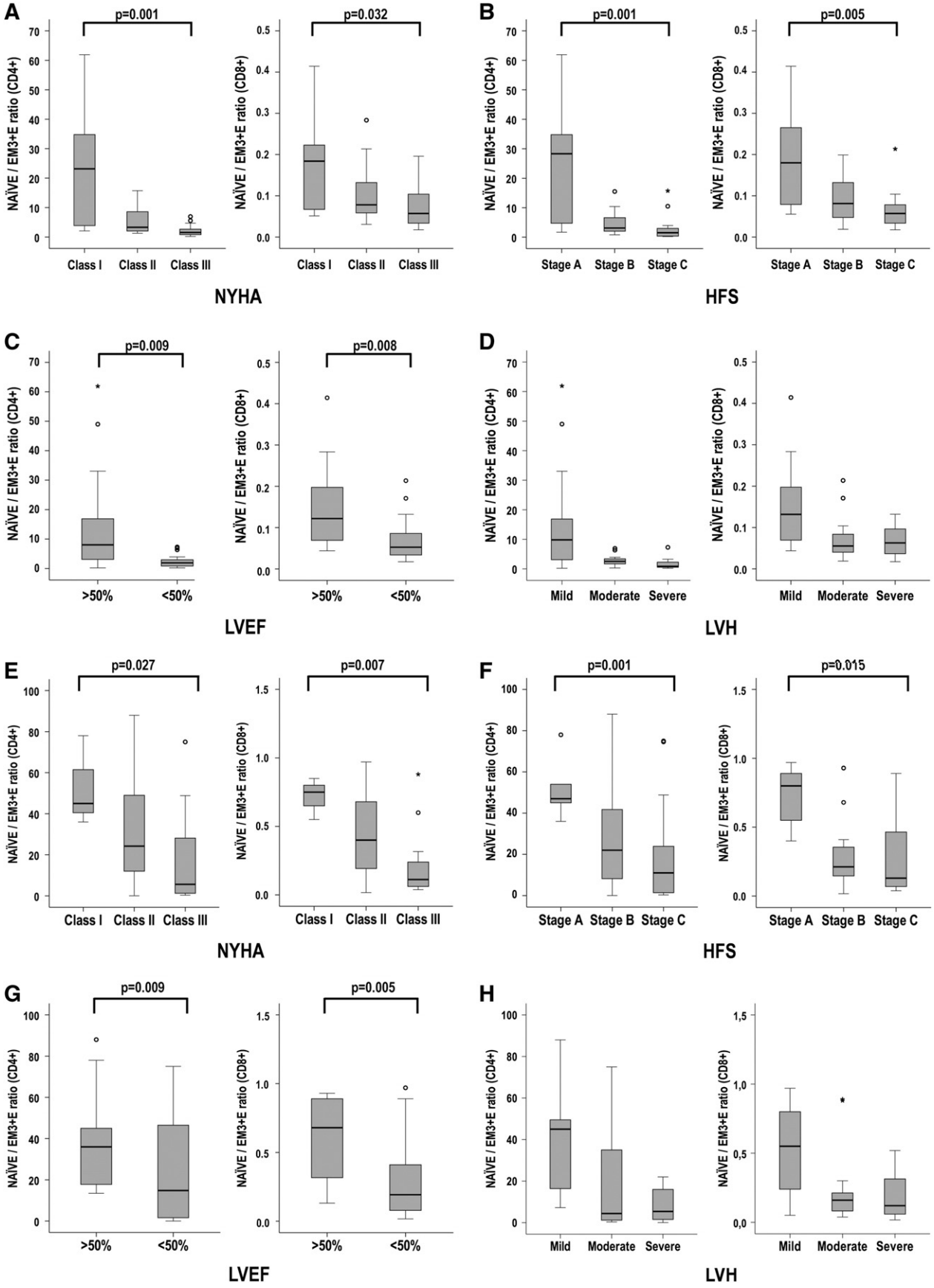

Fig. 4. Naïve/EM3 + E ratio of CD4 + and CD8 + T-cells were analyzed in ECHF (A–D) and in YCHF (E–H) with respect to the different degrees of HF classified according to functional and/or morphological criteria: NYHA (New York Heart Association), HFS (AHA/ACC heart failure stages), LVEF (left ventricular ejection fraction), and LVH (left ventricular hypertrophy). Outlier values are represented by circles and extreme values by stars, calculated by adding 1.5 and 3 times the IR to the 75th percentile, respectively. The Kruskal–Wallis or Mann–Whitney *U* method non-parametric methods were used to compare the variables defining status disease in the CHF groups. *P*-values are depicted in the panels.

displayed an inverted CD4/CD8 ratio, with respect to the HEC group (13.8%) and of YCHF patients (22.5%) with respect to the HYC group (0%) (χ^2 test, $p = 0.007$ and $p < 0.001$, respectively).

In summary, these results demonstrate that patients with CHF exhibit a more senescent profile in leukocyte and lymphocyte subpopulations than healthy individuals matched by age.

3.2. T-lymphocyte differentiation

One of the most widely accepted models in immunosenescence is that the T-cell compartment progressively deteriorates with advancing age. T-cells can be separated into functionally different populations using combinations of cell surface markers such as the tyrosine phosphatase isoform CD45RA and the chemokine receptor CCR7. Using these markers, we subdivided the T-cells into naïve (NAÏVE), central memory (CM), effector memory (EM), and effector memory RA (EMRA) from less to more differentiated stages [16] (Fig. 2A). EM and EMRA are heterogeneous populations, and staining with two additional markers, CD27 and CD28, can distinguish between less differentiated (CD27+ and/or CD28+) and more differentiated ($CD27^{null}CD28^{null}$) cells [17] (Fig. 2A). To detect any possible association between CHF and the degree of differentiation of T-cell subsets, we compared their distribution in the ECHF, HEC, YCHF and HYC groups. The analysis of these subsets of T-cells revealed that CHF was related to reduced populations of undifferentiated subsets and increased populations of highly differentiated subsets. In terms of CD4+ T-cells, CHF patients differed significantly from the two groups of healthy controls in NAÏVE, CM, EM1 and EM3; whereas ECHF and HEC also differed in EM4 and E (Fig. 2B). In terms of CD8+ T-cells, the main difference between CHF patients and healthy controls was in E subset (Fig. 2C).

Taken together, these results indicate that CHF patients show a high degree of differentiation with respect to healthy controls in terms of both CD4+ and CD8+ T-cells.

3.3. Association between functional/morphological classification of CHF and status of the adaptive immune response

The extent of HF can be classified according to functional and/or morphological criteria. The distribution of the 58 ECHF and the 40 YCHF patients enrolled in the study is shown in Table 2. We calculated the ratio between the frequencies of immature cells (NAÏVE) and the frequencies of more differentiated cells (EM3+ E) in CD4+ and CD8+ T-cell compartments, as a measure of the aging of the adaptive immune response. As expected, when we analyzed the distribution of these ratios in the four groups studied, we found that the highest ratios (less aging populations) for both CD4+ and CD8+ T-cells were found in the healthy control groups. On the other hand, the lowest ratios (more aging populations) were found in the groups of patients with CHF (Mann–Whitney *U* test: $p < 0.05$ in all cases) (Fig. 3). We subsequently analyzed the distribution of these ratios in ECHF (Fig. 4A–D) and YCHF patients (Fig. 4E–H) in relation to functional/morphological variables. We found that patients in the groups with a worse functional or morphological status showed a more aged profile in both CD4+ and CD8+ T-cells: NYHA (Kruskal–Wallis test: $p < 0.05$ in all cases) (Fig. 4A and E), HFS (Kruskal–Wallis test: $p < 0.05$ in all cases) (Fig. 4B and F), LVEF (Kruskal–Wallis test: $p < 0.05$ in all cases) (Fig. 4C and G), and LVH (only mild with respect to moderate and severe; Mann–Whitney *U* test: $p < 0.05$ in all cases) (Fig. 4D and H).

With these results we can conclude that the aging of the adaptive immune response is not only associated with CHF but also with a worse disease status.

3.4. Inflammation and aging of adaptive immune responses in CHF

Immunosenescence is compounded by the presence in the elderly of chronic low-grade inflammation characterized by increased proinflammatory cytokines, such as IL-6. Moreover, in patients with CHF, elevated serum levels of IL-6 independently predict decreased heart function and poor survival [18]. When we studied the distribution of IL-6 in our CHF patients classified using the NYHA and HFS, we found that the highest concentrations of this cytokine were found in the most affected patients (Kruskal–Wallis test: $p < 0.05$ in all cases) (Fig. 5A and B). Similarly, the other markers of functional or morphological status analyzed (LVEF and LVH) showed significant associations with IL-6 levels (data not shown).

Since a pro-inflammatory environment is one of the main factors involved in immunosenescence, we investigated the relationship between the serum concentration of IL-6 and the degree of immunosenescence in the adaptive immune response. We found a negative correlation between IL-6 concentration and the NAÏVE/EM3+ E ratio, described above, in ECHF CD4+ and CD8+ T-cells (Spearman Rho test; rho: −0.530; $p < 0.001$ and rho: −0.507; $p < 0.001$, respectively) (Fig. 5C) and in YCHF (Spearman Rho test; rho: −0.454; $p = 0.03$ and rho: −0.500; $p = 0.001$, respectively) (Fig. 5D). The association between IL-6 levels and T-lymphocyte aging was independent of the NYHA or HFS of the patients (data not shown).

In summary, in patients with CHF, high concentrations of IL-6 are closely associated with a poor functional status and HF prognosis and with the aging of the adaptive immune response.

3.5. Concentration of IL-6 over time is related to the status of immunosenescence in CHF

In order to investigate the influence of the inflammatory status of CHF patients on the progressive aging of T-cell subsets, an additional analysis was performed in 27 ECHF patients 6 months after recruitment to the study. IL-6 levels showed no significant differences between samples taken on two occasions (Wilcoxon test), with a median value of 7.5 pg/mL at baseline time (time 0), and 8.7 pg/mL at 6 months (time 6 m). Subsequently, we divided individuals into two groups: low IL-6 and high IL-6, using the median basal IL-6 as the cutoff. First, we observed that, as found above, the state of all parameters studied were characteristic of an aged immune system in individuals with high IL-6 levels: lower CD4/CD8 ratio, higher NK cell percentage, lower percentage of naïve cells and higher percentage of more differentiated populations of EM3 and E (Fig. 6). Pairwise comparisons between time 0 and time 6 m revealed that in patients with a low concentration of IL-6 there were no significant differences in the studied parameters; however, there were significant differences in patients with high IL-6 levels. We found a decrease in the CD4/CD8 ratio and an increase in NK cells (paired *t*-test, $p = 0.026$ and $p = 0.006$, respectively) (Fig. 6A). Moreover, we observed a decrease in naïve CD4+ T-cells (paired *t*-test, $p = 0.041$) but not in CD8+ T-cells (Fig. 6B). Among the most differentiated EM cells (EM3), we found an increase in CD4+ and CD8+ T-cells (paired *t*-test, $p = 0.02$ and $p = 0.025$, respectively) (Fig. 6C). Finally, the most differentiated T-cell subset (E) was increased in CD4+ and CD8+ T-cells in CHF patients with high IL-6 (paired *t*-test, $p = 0.008$ and $p = 0.047$, respectively) (Fig. 6D).

In summary, CHF patients with high levels of IL-6 suffer an accelerated deterioration of the immune response, with high concentrations of this cytokine correlating with aging of the immune system.

4. Discussion

CHF has traditionally been characterized by hemodynamic and neurohormonal perturbations. Recent studies suggest that immune activation and inflammation play a role in the progression of this disorder [19,20]. The present study shows for the first time that patients with CHF develop aging of the adaptive immune response that correlates with the stadium of the pathology. We also demonstrated that IL-6 concentration, one of the most important pro-inflammatory cytokines, is closely related to the acceleration of the immunosenescence process

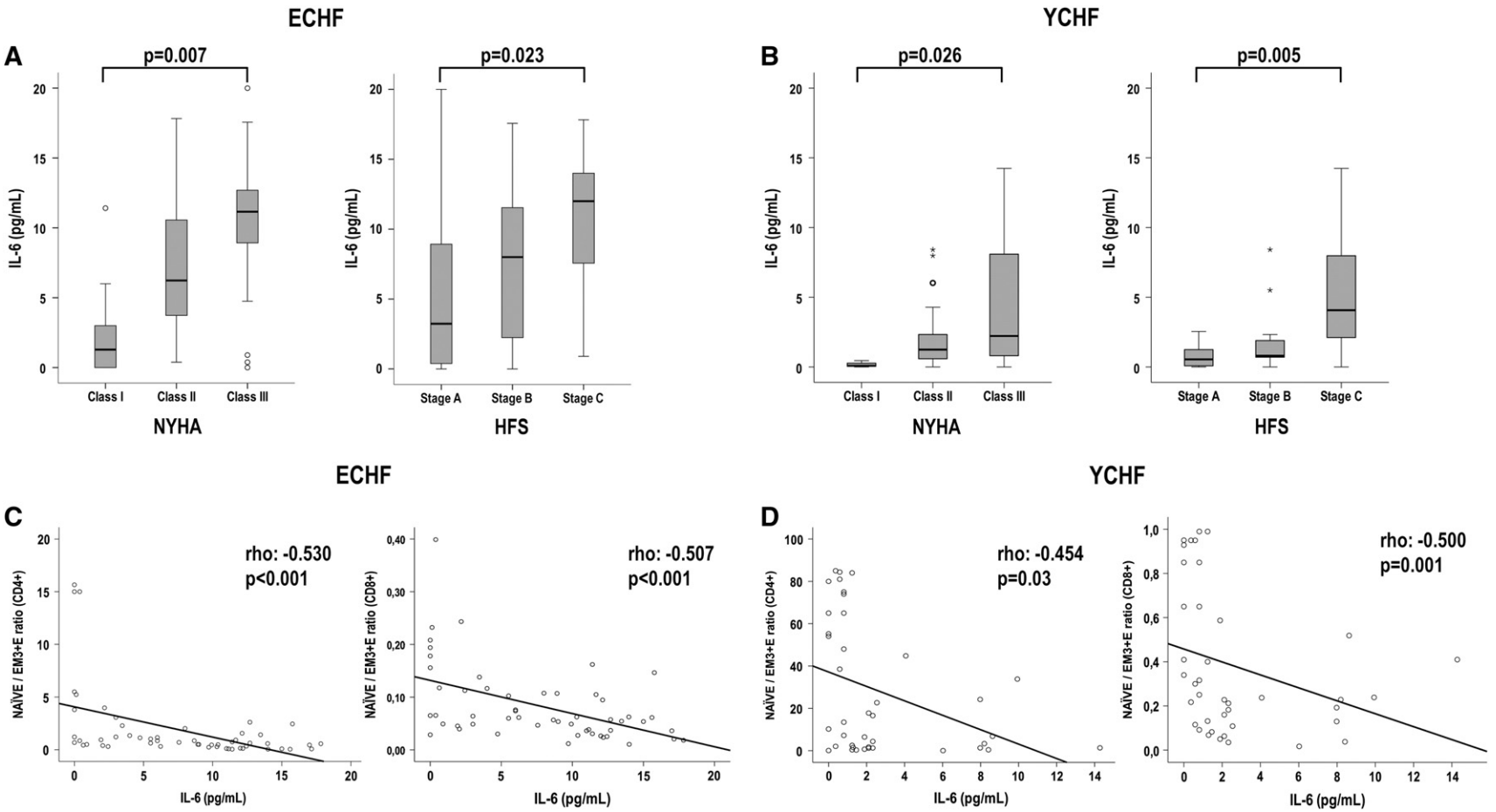

Fig. 5. Levels of IL-6 related to disease status and T-lymphocyte populations. Levels of IL-6 (pg/mL) in ECHF (A) and YCHF patients (B) classified according to NYHA and HFS. Comparisons were made using the Kruskal–Wallis non-parametric method. *P*-values are depicted in the panels. (C, D) Correlation between IL-6 levels and naïve/EM3 + E ratio in CD4 + and CD8 + −T-cells. Spearman's test was applied to calculate the correlations; the *p*-value and coefficient of correlation are listed in the bottom right hand corner.

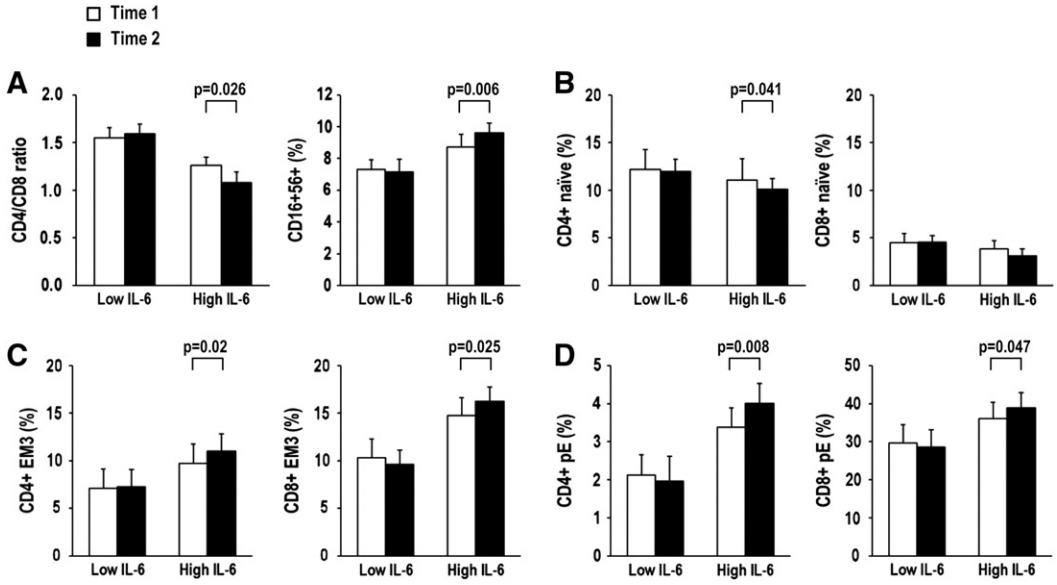

Fig. 6. Changes in CD4/CD8 ratio, and percentage of NK cells, naïve, EM3 and E cell subsets at time 0 and time 6 m, in ECHF patients. We divided individuals into two groups: low IL-6 and high IL-6, using the median IL-6 as the cutoff (7.5 pg/mL). Measurements were made in 27 ECHF patients at baseline and 6 months after the start of the study. (A) CD4/CD8 ratio and percentages of CD16 + 56 + cells with respect to the total CD45 + cells. (B–D) Distribution of CD4 + and CD8 + T-cells into naïve, EM3 and E at time 0 and time 6 m in the grouped individuals according to IL-6 levels. Bars in the histograms represent the mean ± SEM. Student's *t* test for paired samples or the non-parametric Wilcoxon method was used to compare frequencies between times. *P*-values are depicted in the panels.

in CHF patients. Based on these results, we propose a model in which CHF, inflammation and immunosenescence increase following a feedback mechanism (Fig. 7).

We found that patients with advanced CHF stages display immunosenescence and a high inflammatory status. An increase in leukocyte numbers is a classical marker of acute or chronic systemic inflammation, and a risk marker for cardiovascular disease. Some studies of the general population showed that moderately increased leukocyte concentrations are associated with an increased incidence of hospitalizations due to HF [21,22]. In our study, leukocytes were markedly increased in CHF patients with respect to the healthy control groups, and this was clearly due to the large increase in neutrophils, both in percentage terms and absolute numbers. Neutrophilia has been associated with an increased incidence of decompensated heart failure (ADHF) in patients admitted with acute myocardial infarctions [23], and even the neutrophil to lymphocyte ratio has been reported to predict long-term outcomes in ADHF [24]. The most interesting aspect from the point of view of immunosenescence is the ability of neutrophils to produce the cytokine IL-6 [25] and the response that neutrophils have to this inflammatory cytokine, all of this contributing to the inflammation present in CHF and also to the chronic low-grade inflammation seen in the elderly. Inflammation is a physiological process that repairs tissues in response to endogenous or exogenous aggressors; however a chronic state of inflammation may have detrimental consequences. Aging and inflammatory chronic diseases are associated with increased levels of circulating cytokines and pro-inflammatory markers. Age-related changes in the immune system, and increased secretion of cytokines by adipose tissue, represent the major causes of chronic inflammation, a phenomenon known as "inflamm-aging" [26]. High levels of interleukin IL-6, IL-1, TNF, and CPR are associated in the older subject with an increased risk of morbidity and mortality [27]. In particular, cohort studies have indicated that TNF-α and IL-6 levels are markers of frailty [28]. In CHF this state of inflammation is even more pronounced; several mechanisms for this immune activation, which are not mutually exclusive, have been suggested, including neurohormonal activation, hemodynamic overload, and activation of the innate immune system secondary to cardiac stress [29,30]. Events involved in cardiac depression and in the progression of heart failure, such as hypertrophy [3] and cardiotoxic activation of cell death pathways [4], may be a consequence of the chronic inflammatory response. On the other hand, inflammation maintained over time causes the continuous activation and proliferation of T-lymphocytes, leading to clear lymphocyte aging. In this paper we have confirmed that high levels of IL-6 over time are associated with more rapid and extensive deterioration in lymphocyte subsets. These highly differentiated cells, in turn, produce important levels of pro-inflammatory cytokines and display a reduced ability to generate potent immune responses against antigens. Therefore, measurement of this cytokine in patients with CHF may be a good approach to detecting and halting the degree of immunosenescence and, therefore, reducing their susceptibility to comorbidities and infections. In terms of the differentiation status of T-lymphocyte subsets in patients with CHF, we observed clear aging of these populations not only in ECHF but also in younger patients. We found differences between the groups of CHF patients and healthy controls in many T-cell subsets, but mainly in the most differentiated population of CD8 + T-lymphocytes. This could be due in part to the better and stricter homeostasis to which CD4 + T-cells are subjected [12]; however, even this strong homeostasis may not prevent the immune-aging, because CD4 + T-cells are in an inflammatory environment that causes their division and differentiation over time. Moreover, the poor functional and/or morphological heart status of these patients, according to different indicators, is related to a more aged lymphocyte population, as demonstrated in this study. All this allows us to associate the high degree of immunosenescence in these patients with the high comorbidity present in CHF [31–34]. The inflammatory condition that produces an aging adaptive immune response makes this population of elderly patients with CHF more susceptible and less responsive to certain infections in comparison with the general elderly population [35,36]. At the same time, this

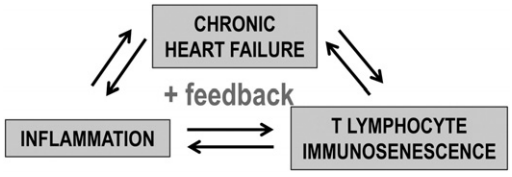

Fig. 7. Model of feed-back mechanism in CHF.

immunodepression favors repeated contact with antigens, and the reactivation of chronic pathogens or recent infections, increasing the degree of immunosenescence and thus immunodepression and disease-associated comorbidities.

In summary, our results suggest that CHF and the high levels of IL-6 maintained over time are associated with a compromised adaptive immune response with a high degree of differentiation, and, in fact, with less functional responsiveness. Both IL-6 levels and highly differentiated lymphocyte populations are associated with a worse disease status, and thus, the monitoring of this state of immunosenescence may be indicated to prevent pathologies and infections related to an impaired immune response.

Acknowledgments

We thank Isabel Cuevas Pérez and José María Díaz Pérez for their excellent technical assistance.

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III.6. POTENCIACIÓN DE LA RESPUESTA INMUNE EN INDIVIDUOS DE EDAD AVANZADA TRAS LA SUPLEMENTACIÓN ORAL CON *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* 8481

Los probióticos son microorganismos no patógenos, que cuando se ingieren ejercen una influencia positiva sobre la salud o la fisiología del huésped. Entre las propiedades beneficiosas de estos microorganismos se encuentra su actividad inmunomoduladora. En este proyecto se evaluaron las posibles propiedades beneficiosas sobre la respuesta inmune en individuos ancianos de una cepa seleccionada in vitro de *L. delbrueckii* subsp. *bulgaricus*. La supervivencia de determinadas cepas de esta especie bacteriana al tránsito intestinal ha sido demostrada, así como su gran capacidad de resistir condiciones ambientales adversas. Estas características, junto con el origen de las bacterias, aisladas en una región de Bulgaria donde la población presenta características inusuales de longevidad nos llevaron a postular sus posibles efectos favorables sobre el sistema inmune.

ARTÍCULO 6:

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Age (Dordr). 2013 Aug;35(4):1311-26.

En este trabajo se incluyeron 47 sujetos con edad superior a 65 años sin una patología inmunológica de base y atendidos en el área sanitaria VIII del Principado de Asturias. Los voluntarios se dividieron en dos grupos (grupo probiótico, n=26; grupo placebo, n=21). El diseño fue idéntico a un ensayo clínico: prospectivo, doble ciego, controlado con placebo. A los individuos en estudio se les administraron 3 cápsulas al día, tanto de probiótico como de placebo, durante un periodo de 6 meses. Se realizó un fenotipado de las subpoblaciones celulares por citometría de flujo, cuantificación de citocinas, cuantificación de TRECs por PCR a tiempo real y cuantificación por ELISA de la β-2 defensina (hBD-2), IL-8 y anticuerpos frente a CMV. Todas estas determinaciones se realizaron a tiempo 0, 3 y 6 meses. También se

obtuvieron muestras de algunos individuos (n=9) 6 meses después de dejar de tomar el probiótico. Los ancianos respondieron a la toma del probiótico con un aumento en el porcentaje de células NK, una mejora de los parámetros que definen el IRP y un aumento de las poblaciones de linfocitos T menos diferenciadas. Los ancianos del grupo probiótico también mostraron una disminución en la concentración de la citocina proinflamatoria IL-8 y un aumento del péptido antimicrobiano hBD-2. Todos estos efectos desaparecieron después de 6 meses sin tomar el probiótico.

Como conclusión podemos afirmar que la inmunomodulación producina por el probiótico podría favorer el mantenimiento de una adecuada respuesta inmune, principalmente disminuyento el envejecimiento de las subpoblaciones de linfocitos T y aumentando el número de células T inmaduras, potenciales respondedores frente a nuevos antígenos.

Aportación personal al trabajo:

En este estudio, mi labor se centró principalmente en el seguimiento de los individuos seleccionados para el estudio, recolectar y procesar las distintas muestras, realizar y/o supervisar los distintos experimentos y analizar los resultados obtenidos. Finalmente, participé en el diseño y en la escritura del presente manuscrito.

AGE
DOI 10.1007/s11357-012-9434-6

Oral supplementation with *Lactobacillus delbrueckii* subsp. *bulgaricus* 8481 enhances systemic immunity in elderly subjects

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Received: 10 February 2012 / Accepted: 15 May 2012
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Abstract Throughout life, there is an aging of the immune system that causes impairment of its defense capability. Prevention or delay of this deterioration is considered crucial to maintain general health and increase longevity. We evaluated whether dietary supplementation with *Lactobacillus delbrueckii* subsp. *bulgaricus* 8481 could enhance the immune response in the elderly. This multi-center, double-blind, and placebo controlled study enrolled 61 elderly volunteers who were randomly assigned to receive either placebo or probiotics. Each capsule of probiotics contained at least 3×10^7 *L. delbrueckii* subsp. *bulgaricus* 8481. Individuals in the study were administered three capsules per day for 6 months. Blood samples were obtained at baseline (time 0), end of month 3, and month 6. We characterized cell subpopulations, measured cytokines by flow cytometry, quantified T cell receptor excision circle (TREC) by real-time PCR (RT-PCR), and determined human β-defensin-2 (hBD-2) concentrations and human cytomegalovirus (CMV) titers by enzyme-linked immunosorbent assay (ELISA). Elderly responded to the intake of probiotic

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Published online: 30 May 2012

with an increase in the percentage of NK cells, an improvement in the parameters defining the immune risk profile (IRP), and an increase in the T cell subsets that are less differentiated. The probiotic group also showed decreased concentrations of the pro-inflammatory cytokine IL-8 but increased antimicrobial peptide hBD-2. These effects disappeared within 6 months of stopping the probiotic intake. Immunomodulation induced by *L. delbrueckii* subsp. *bulgaricus* 8481 could favor the maintenance of an adequate immune response, mainly by slowing the aging of the T cell subpopulations and increasing the number of immature T cells which are potential responders to new antigens.

Keywords Immunosenescence · Immunomodulation · T lymphocytes · Differentiation · *Lactobacillus delbrueckii*

Introduction

Numerous organisms meet the criteria established by the World Health Organization (WHO) for a probiotic: "a live organism, which provides a benefit to the host when provided in adequate quantities." Probiotics impact metabolism, endocrinology, proper gut development, and regulation of the immune system (Shida and Nanno 2008; Cani and Delzenne 2009). By modifying the microbial community within the gut, we may be able to prevent or treat gut disorders, such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) as well as systemic disorders like eczema, allergies, asthma, and diabetes (Lorea Baroja et al. 2007; Strowski and Wiedenmann 2009; Boyle et al. 2010). Focusing on effects on the immune system, probiotics act on a wide variety of cells in the intestine to modulate immune cells towards a pro- or anti-inflammatory action, depending on the bacterial strain, setting, measured immunological parameters, and types of modulated immune cells (Diaz-Ropero et al. 2007; O'Flaherty et al. 2010).

Lactobillus delbrueckii subsp. *bulgaricus* has been used to ferment milk for many centuries. Many researchers have shown various beneficial effects for this bacterial species (Kano et al. 2002; Medici et al. 2005). The immunopotentiating activity of lactobacilli varies considerably between strains but little information on the immunopotentiating activity for *L. delbrueckii* subsp. *bulgaricus* is available. It was isolated from a region of Bulgaria where its population shows unusual longevity. Since *L. delbrueckii* subsp. *bulgaricus* is one of the most common bacteria used in the production of fermented milk in the world, knowledge of its immunopotentiating activity would be useful for the development of dairy products with a more beneficial effect on human health. However, several authors who evaluated the ability of the yogurt bacteria *L. delbrueckii* subsp. *bulgaricus* to survive and proliferate in the human intestine found contradictory results (del Campo et al. 2005; Garcia-Albiach et al. 2008). Although live probiotics clearly modulate gut immune and barrier function, studies have shown immunomodulatory effects of probiotic DNA, suggesting that isolated probiotic bacteria DNA is as efficacious in attenuating intestinal inflammation as is treatment with live bacteria (Jijon et al. 2004).

Beyond midlife, the immune system begins to age and cause impairment of its defensive capability, which is known as immunosenescence. Immunosenescence involves multiple changes in both the innate and adaptive responses. Innate immunity seems to be better preserved globally (Le Garff-Tavernier et al. 2010), while the adaptive immune response exhibits profound age-dependent modifications (Haynes and Maue 2009). Elderly donors display a decline in numbers of naïve T cells in peripheral blood and lymphoid tissues (Fagnoni et al. 2000; Sauce et al. 2009); in contrast, they have a marked increase in the proportion of highly differentiated effector and memory T cells like the $CD28^{null}$ T cells (Goronzy et al. 2007; Czesnikiewicz-Guzik et al. 2008). An IRP was defined using a cluster analysis approach (Ferguson et al. 1995). In agreement with a higher IRP, a higher 2-year mortality occurred in a population of very old Swedish individuals who had an inverted CD4/CD8 ratio, an accumulation of $CD8+CD28^{null}$ T cells, and CMV infection (Hadrup et al. 2006). In summary, considerable evidence suggests a clear association between immune function and longevity of individuals (Moro-Garcia et al. 2011), and immunosenescence correlates higher morbidity and mortality. In this sense, prevention or delay of the deterioration of the immune system is considered crucial for maintaining a better overall health and increasing longevity.

L. delbrueckii subsp. *bulgaricus* 8481 was isolated from a region of Bulgaria known for the longevity of its population (Dixon 2002). The ability of the lactobacilli to resist adverse environmental conditions

(Mater et al. 2005; Elli et al. 2006), together with its association with human longevity, led us to postulate the possible beneficial effects of this bacterial strain on the immune system. Thus, the purpose of this double-blind, placebo-controlled, and randomized study was to determine the potential beneficial properties of the selected strain of *L. delbrueckii* subsp. *bulgaricus* 8481 on the innate and acquired immune responses of elderly individuals.

Materials and methods

Study population and study design

Elderly volunteers were invited to enroll in this study via interview with their primary care physician and through the publication of a diptych and newspaper advertisement, from October 2008 to April 2011. Inclusion criteria were age greater than 65 years and treatment in the Health Centers of Barredos, Blimea, Laviana, and Sotrondio (Asturias, Spain). Exclusion criteria were conditions with possible influence on the immune system, such as recent or current infection, inflammation, autoimmune or malignant disease, malnutrition, abnormal laboratory data (hemoglobin <12 mg/dL, leukopenia <3,500 cells/μL, neutropenia <1,500 cells/μL, leukocytosis >15,000 cells/μL, platelets $<10^5$ cells/μL, and PCR >5 mg/dL), and pharmacological interference (steroids, non-steroidal anti-inflammatory agents, and immunosuppressive drugs). Informed consent was obtained from the elders prior to participation in the study. The study was approved by the Hospital Central de Asturias (Oviedo, Spain) ethics committee.

Patients who met the inclusion criteria were randomly assigned to one of two treatment groups: (1) placebo and (2) probiotics. Each capsule of probiotics contained at least 3×10^7 *L. delbrueckii* subsp. *bulgaricus* registered in the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC) under No. 8481 combined with a strain of *Streptococcus thermophillus* NBIMCC No. 8357 (European patent No. 2076139). A sample was taken for culture to ensure no pathogenic bacteria, yeasts, or fungus remained in the capsules. All volunteers assigned to the placebo group received capsules with cornstarch; the treatments were identical in appearance. The probiotic capsules were provided by the pharmacy Xalabarder following the necessary requirements for safe human consumption.

This was a multi-center, randomized, double-blind, and placebo-controlled study. Individuals in the study were administered three capsules per day (probiotic or placebo) for a period of 6 months. Peripheral blood samples were obtained from subjects by venipuncture at three time points: time 0 (baseline, immediately before the study began), at the end of month 3, and at the end of month 6. Blood samples were obtained at 6 months after stopping the capsules from nine volunteers who had taken the probiotic.

Throughout the study, the subjects' general health was assessed at each immune measurement time point via direct interview conducted by the health care provider. Volunteers were asked to confirm their compliance with, or deviation from, the dietary regimens.

Bacterial strains

This study included *L. delbrueckii* subsp. *bulgaricus* 8481 dairy strain isolated from the district of Stara Planina (Bulgaria) from a fresh cheese made of cow milk, fermented 5 days, with a subsequent selection. The bacterial culture was produced first in laboratory as follows: 1 g of the product was suspended in a 9-mL sterile physiological solution. Repeated dilutions were made, and a 10^7 dilution was sown onto a Man, Rogosa and Sharpe broth (MRS broth, LAB MTM, IDG Ltd., Lancashire, UK). Cultivation was carried out at 44–45°C for 48 h, and the strain was selected from the final single colonies. The selected strain was sown again in 10-mL sterile skimmed milk till coagulation at pH 4.8 for 6 h. From this culture, named "primary culture," a series of subcultures were made with the aim to increase the number of cells of the pure strain and to prepare "mother cultures," which were stored for later use as inoculants during the industrial preparation of bacterial culture of the strain *L. delbrueckii* subsp. *bulgaricus* 8481. For preparation of the starter culture, symbiotic culture of strain *L. delbrueckii* subsp. *bulgaricus* 8481 and strain *Streptococcus thermophilus* 8357 were repeatedly sown during 2.6 months until the symbiosis was stabilized. For preparation of dry symbiotic product, the coagulated milk with pH 4.9–5.0 was submitted to two steps of cooling: step one, coagulated milk at 25°C for 20–

25 min and step two at 6–7°C for 15–20 min. Within 24 h, it was dried at −38°C to −40°C until the product reached liquor contents of 4–6 % by using the lyophilization method. The lyophilized encapsulated strains were kindly provided from LB Lactis (Scientific-Applied Laboratory for Starter Cultures and Probiotic Products, Plovidv, Bulgaria) culture collection.

Blood sampling and immunological phenotyping

The hematological parameters were determined by using a Sysmex XT-2000i (Syxmex, Hamburg-Norderstedt, Germany) and the biochemistry values in a Cobas 6000 analyser series (Roche Diagnostics, Indianapolis, USA). Cytometric studies were acquired and analyzed in the FACSCalibur Cytometer using CellQuest software (BD Biosciences, San José, CA, USA). CaliBRITE Beads (BD Biosciences) were used to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity. Surface staining of EDTA peripheral blood was performed with Multiset CD3-FITC/CD16+56-PE/CD45-PerCP/CD19-APC Reagent, anti-CD4 (APC), anti-CD8 (PE), anti-CD8 (PerCP), anti-CD31 (PE) (Immunostep, Salamanca, Spain), anti-CD4 (PerCP), or anti-NKG2D (PE) (eBioscience, San Diego, CA, USA). One hundred μL of whole-blood from elderly were stained with different combinations of labeled monoclonal antibodies for 20 min at room temperature. Red blood cells in samples were lysed with FACS Lysing Solution (BD Biosciences), and samples were washed in PBS and analyzed with CellQuest software in the FACSCalibur Cytometer. Appropriate isotype control monoclonal antibodies (mAbs) were used for marker settings.

Isolation of CD4+ and CD8+ T cells

To analyze the differentiation status of CD4+ and CD8+ T cells, peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Hypaque gradients (Lymphoprep; Nycomed, Oslo, Norway). To achieve cell separation, the blood sample mixed with an equal part of phosphate buffered saline (Oxoid Limited, Hampshire, UK) was deposited on a fluid with a density of 1.077 g/mL (Lymphoprep) and separated by centrifugation (1,800 rpm, 30 min). CD4+ and CD8+ T-cells were isolated (Myltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, CD4+ or CD8+ T cells were magnetically labeled with CD4 or CD8 MicroBeads, and the cell suspension was loaded on a column which was placed in the magnetic field. The magnetically labeled cells were retained on the column. The unlabeled cells were not retained, and this cell fraction was depleted of CD4+ or CD8+ cells, respectively. After removal of the column from the magnetic field, the magnetically retained CD4+ or CD8+ cells were eluted as the positively selected cell fractions. Subsequently, cells were stained with anti-CD45RA (FITC) (Immunostep), anti-CCR7 (Alexa Fluor 647) (BD Bioscience), anti-CD27 (PE) (Immunostep), and anti-CD28 (PerCP) (BD Bioscience).

CMV serology

Immunoglobulin G (IgG) titers of CMV-specific antibodies were determined by enzyme-linked immunosorbent assay, Vir-ELISA Anti-CMV-IgG (Viro-Immun Labor-Diagnostika GmbH, Oberursel, Germany), according to the manufacturer's specifications. Patient samples were quantified and interpreted by calculating a ratio (Cutoff Index=OD value of sample/Cut-off value), whereby a ratio of 1.0 is equivalent to the cut-off value. Cutoff indexes >1.1 were considered positive, and the result of this ratio is a semi-quantitative titer.

TREC quantification

DNA from PBMC was extracted by using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Quantification of signal-joint (sj) TREC was performed by using SYBR Green RT-PCR and an iCycler thermocycler (Bio-Rad; Life Science Research Group, Hercules, CA, USA). The primer sequences for TREC were the following: forward primer 5'-CCAT GCTGACACCTCTGGTT-3', reverse primer 5'-TCGTGAGAACG GTGAATGAAG-3'. The Cα constant region, which was the internal control for quantity of input DNA because it remains present on TCR genes despite the rearrangement processes, was amplified in every sample tested (forward primer 5'-CCTGATCCTCTTGTCCCACAG-3', reverse primer 5'-GGATTT AGAGTCTCTCAGCTGGTACA-3'). Thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Experimental samples were

run in duplicate, and the replicate average value was presented as the sample result.

Immunoglobulin, complement, and cytokine quantification

IgG, IgA, IgM, and complement C3 and C4 were measured in the IMMAGE Immunochemistry System (Beckman Coulter, Minnesota, USA) at baseline (time=0) and 6 months after the beginning of the probiotic/placebo intake. This fully automated random access immunochemistry system featured high-performance detection technologies which included near-infrared particle immunoassay system (NIPIA) that increased the analytical sensitivity nearly a thousand-fold.

Quantification of cytokines in serum was assessed by Human Th1/Th2 11plex Ready-to-Use Kit Flow-Cytomix (eBioscience), a fluorescent bead immunoassay. Each type of bead was coated with antibodies specifically reacting with one of the cytokines to be detected in the multiplex system. The beads can be differentiated by their sizes and by their distinct spectral profiles. Coated beads for each relevant cytokine were mixed, and the bead mixtures were incubated with the samples or mixture of standard cytokine concentrations. A biotin-conjugated second antibody mixture was added, and the specific antibodies bound to their respective cytokines captured by the first antibody. After addition of streptavidin–phycoerythrin molecules, they bound to the biotin conjugates and emitted fluorescent signals. This bead-based Analyte Detection System was used to quantitate human IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and TNF-β by flow cytometry. Quantification of cytokines was performed at time 0 and 6 months.

hBD-2 ELISA

Blood was collected by venipuncture and allowed to clot. Serum was harvested by centrifugation at room temperature, and aliquots were stored at −80°C until use. The secretion of hBD-2 was monitored in serum of the elderly at baseline, 3 months, and 6 months. hBD-2 concentrations were determined by ELISA for Human Beta Defensin 2 (Alpha Diagnostic, San Antonio, TX, USA) according to the manufacturer's specifications. The results were calculated using an immunoassay software package with a four-parameter curve-fit.

Statistical analysis

Results are expressed as median and range, mean and standard deviation (SD), or in some graphs, mean and standard error of the mean (SEM), as indicated. Comparisons between groups were performed with the Wilcoxon non-parametric method when data were not normally distributed, or with Student's *t* test for paired samples. Analyses were performed using the SPSS 15.0 statistical software package program (SPSS Inc. Chicago, IL), and *p*-values of 0.05 or less were considered significant.

Results

Demographic and hematologic characteristics of the study population

Recruitment took place in four centers in Spain from April 2008 to April 2011. Sixty one participants were in the randomized arm (24 allocated to placebo and 28 to probiotic) (Fig. 1). Of the 61 subjects initially enrolled in the study, 6 from the placebo group and 3 from the probiotic group decided to leave the study before beginning the intervention. Moreover, three elderly from the placebo group and two from the probiotic group were lost to follow up in the study, due to illness not associated with the study. The characteristics of the 47 individuals enrolled in the study are listed in Table 1. The female/male ratio was 4.3:1 in the placebo group and 7.7:1 in the probiotic group. No significant differences in age, sex, hematology values, and biochemistry values at baseline were observed between the groups (Table 1).

Blood cell counts and an immune phenotype of majority populations were performed in all individuals included in the study. Between-group analyses indicated no significant overall differences in immune variables between the placebo and probiotic groups at baseline (Table 2). The placebo group showed no difference in the distribution of the measured immune populations at 3 or 6 months. In contrast, the probiotic group had significantly lower percentage of CD8+ T cells at 3 months (Paired *t*-test, $p=0.008$) and significantly higher percentile of NK cells (CD16+56+) at 3 and 6 months (Paired *t*-test, $p=0.012$ and $p=0.001$, respectively). The double positive CD4+CD8+ and CD4+ NKG2D+are two other subsets of T cells that have been related to aging (Alonso-Arias et al. 2009; Alonso-Arias

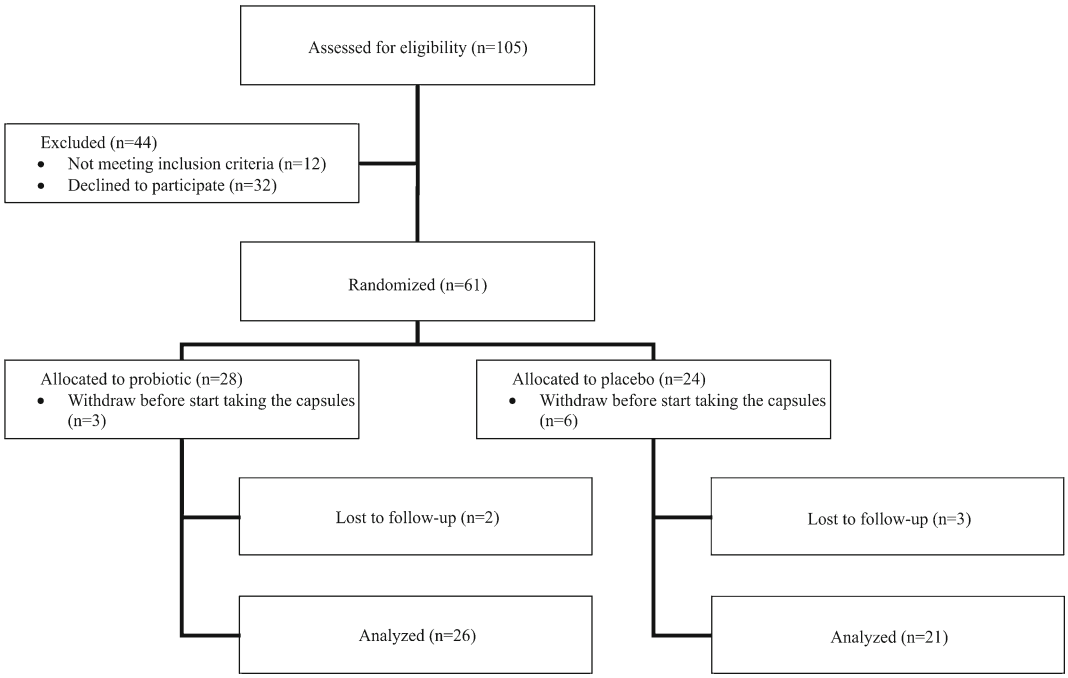

Fig. 1 Flowchart of participants through trial

et al. 2011). Percentages of these two subpopulations in the probiotic group did not differ significantly between groups at different times of analysis; however, these subpopulations showed a tendency to decrease at 3 and at 6 months (Table 2). In summary, these results demonstrated that consumption of probiotic modulates percentages of different immune populations.

IRP parameters and probiotic consumption

Parameters that define the IRP are the inverted ratio CD4/CD8 (ratio<1.0), increase of CD8+CD28^{null} T cells, and CMV infection (Hadrup et al. 2006). As aforementioned, percentages of CD4+ T cells showed no significant differences throughout the study; however, the percentage of CD8+ T cells significantly decreased within 3 months of consumption of the probiotic. Accordingly, the CD4/CD8 ratio was significantly different between 0 and 3 months (Wilcoxon test, p=0.008) (Fig. 2a). In aging, there is an accumulation of CD8+CD28^{null} T cells which are oligoclonal and show characteristics of cellular senescence. In the probiotic group, the percentage of CD8+CD28^{null} T cells significantly declined at both 3 and 6 months compared to that at baseline (Fig. 2b). As expected, for the placebo group, the CD4/CD8 ratio and the percentage of CD8+CD28^{null} T cells remained unaltered throughout the study (Fig. 2a, b).

The relationship between CMV infection and probiotic consumption was also analyzed by comparing titers of antibodies against CMV at the beginning of the study and at 6 months. The elderly group who took the placebo capsules showed a significant increase in the mean CMV titer at 6 months compared to that at the beginning of the study. In contrast, the probiotic group did not show significant differences in CMV titer between baseline and 6 months possibly because their immune system was able to better control the viral reactivations (Fig. 2c).

Therefore, we concluded that those elders who took the probiotic had an improvement in their IRP, an index that is related to increased 2-year mortality in elderly people.

T cell differentiation subsets

T cells appear to be more sensitive to the aging process than other types of immune cells, and significant changes in both functional and phenotypic profiles occur in elderly humans as a function of time. T cells can be separated into functionally different populations using combinations of cell surface markers such as the tyrosine phosphatase isoform CD45RA and the

Table 1 Characteristics of the study subjects at baseline

	Placebo group (n=21)	Probiotic group (n=26)	p value between groups[a]
Demographic data			
Age (years)			
Mean±SE	69.5±9.19	71.6±5.46	ns
Range	(65–90)	(65–82)	
No. of subjects investigated			
Women	17	23	
Men	4	3	
Hematology values (mean±SD)			
RBCs (10^6/μL)	4.63±0.18	4.59±0.3	ns
Hemoglobin (g/dL)	13.84±1.39	13.76±0.97	ns
Hematocrit (%)	41.67±3.34	41.06±2.67	ns
MCV (fL)	90.01±6.44	89.6±4.7	ns
Platelets (10^3/μL)	246.28±37.39	212.56±52.94	ns
WBCs (10^3/μL)	6.16±1.73	6.2±1.6	ns
Neutrophils (10^3/μL)	3.31±1.08	3.15±0.92	ns
Monocytes (10^3/μL)	0.44±0.14	0.49±0.19	ns
Lymphocytes (10^3/μL)	2.2±0.97	2.31±0.76	ns
Biochemistry values (mean ± SD)			
Glucose (mg/dL)	106.43±14.87	98.92±14.59	ns
Creatinine (mg/dL)	0.76±0.15	0.75±0.13	ns
AST (U/L)	19.43±6.78	20.62±5.82	ns
ALT (U/L)	16.14±6.44	18.29±4.93	ns
AP (U/L)	81.14±29.69	78.21±18.6	ns
Albumin (g/L)	46.14±3.53	43.58±2.46	ns
Immunological values (mean ± SD)			
CD3+/lymphocytes	25.08±3.8	27.44±5.95	ns
CD4+/CD3+	38.93±7.03	41.42±7.98	ns
CD8+/CD3+	30.72±3.4	33.13±9.17	ns
CD 16 + 56/CD45+	10.74±3.83	9.44±3.52	ns
CD 19+/CD45+	3.87±1.75	3.79±1.79	ns

RBCs red blood cells, *MCV* mean corpuscular volume, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *AP* alkaline phosphatase

[a] Calculated using the Student *t* test

chemokine receptor CCR7. With these markers, we subdivided the T cells into naïve (NAÏVE; CD45RA+ CCR7+), central memory (CM; CD45RA-CCR7+), effector memory (EM; CD45RA-CCR7-), and effector memory RA (EMRA; CD45RA+CCR7-) (Sallusto et al. 1999). The distribution of the distinct T cell subpopulations at 0, 3, and 6 months into the placebo and probiotic groups were compared. First, the elderly in the placebo group showed no significant changes in the percentage of any T cell subpopulations (data not shown). In contrast, probiotic intake in elderly was associated with an increased population of undifferentiated T cell subsets. In fact, the frequency of the NAÏVE CD4+ T cells in elders was significantly increased from baseline and from 3 to 6 months. The percentage of CM and the more differentiated subtype, EM CD4+ T cells declined (Fig. 3a). Similar to CD4+ T lymphocytes, CD8+ T cells showed a significant increase in the younger population, the NAÏVE cells from baseline to 3 and 6 months. CM cells showed no

Table 2 Immunological parameters (mean ± SD) measured at baseline, 3 months, and 6 months

	Placebo group			Probiotic group		
	Baseline	3 months	6 months	Baseline	3 months	6 months
CD3+/lymphocytes	25.08±3.8	25.62±5.43	27.0±6.6	27.44±5.95	27.44±5.95	26.81±5.52
CD4+/CD3+	38.93±7.03	40.62±8.42	41.87±8.75	41.42±7.98	41.73±8.75	41.08±7.27
CD8+/CD3+	30.72±3.4	29.18±7.14	28.39±7.01	33.13±9.17	30.45±8.0*	30.67±7.4
CD4+CD8+/CD4+	2.79±2.48	2.9±2.31	2.47±1.66	2.86±1.3	2.74±1.59	2.57±1.45
CD4+NKG2D+/CD4+	5.12±2.34	5.33±2.14	5.35±2.33	5.3±3.56	4.81±3.01	4.74±3.04
CD16+56/CD45+	10.74±3.83	10.35±4.18	11.98±4.85	9.44±3.52	11.18±4.81*	11.55±4.09*
CD 19+/CD45+	3.87±1.75	4.25±2.04	4.42±2.3	3.79±1.79	3.85±1.97	3.71±1.69

* Statistically significant difference (paired Student's t test, $p<0.05$) respect to time 0

differences, and the CD8+ EM subset, a more differentiated population, exhibited a significant decrease from baseline to 6 months (Fig. 3b).

Although it is assumed that EMRA cells are considered the more differentiated cells, the frequency of EMRA cells significantly increased from baseline to 6 months both in CD4+ as in CD8+ T cells. However, EMRA is a heterogeneous population, and the staining with two additional markers, CD27 and CD28, can distinguish between less differentiated (CD27+ and/or CD28+) or more differentiated ($CD27^{null}CD28^{null}$) cells (Koch et al. 2008). EMRA subset can be further subdivided into very poorly differentiated pE1 (CD27+CD28+), pE2 ($CD27+CD28^{null}$, only in CD8+ T cells), and most differentiated T-cell subset, E ($CD27^{null}CD28^{null}$). We compared the frequencies of these subsets of CD4+ and CD8+ T cells at 0, 3, and 6 months in the placebo and probiotic groups. The less differentiated subset (pE1) in the probiotic group was increased at 3 and 6 months from baseline. Conversely, the most differentiated subset (pE) decreased both in CD4+ T cells as in CD8+ T cells from baseline to 6 months (Fig. 3c, d). Thus, the increase observed in the EMRA population at 6 months in the CD4+ and CD8+ T cells is due to an increase in the less differentiated subset pE1. In contrast, the oldest population of T lymphocytes (pE) declined in the probiotic group. Taken together, these results indicated that the probiotic intake was related to an increase in the less differentiated subsets of both CD4+ and CD8+ T cells. These populations are necessary for the maintenance of an adequate immune response, slowing the aging of the T lymphocyte subpopulations and increasing the number of immature naïve T cells.

Proximity to the thymus of lymphocyte populations

Full thymic activity in young healthy individuals leads to continuous replenishment with naïve T cells with high TREC content. These recent thymic emigrants co-express CD45RA and CD31: This co-expression is high in CD45RA+ T cells from young subjects but decreases continually during ageing (Kimmig et al. 2002). First, we examined whether frequencies of CD45RA+ T cells co-expressing the CD31 molecule changed throughout the study. The CD31+CD45RA+subset was significantly higher in the elderly probiotic group, both in CD4 and CD8 T cells within 6 months (Fig. 4a). By contrast, no differences were observed in the placebo group (data not shown).

To further examine the differences in the differentiated status of CD4+ and CD8+ T subsets, we assessed the replicative history of these cells by quantifying the TREC content. TREC is a traceable molecular marker produced in newly naïve T cells; the content of TREC in peripheral T cells is a direct indicator of the number of divisions that the cell has undergone (Douek et al. 1998). TREC content was measured at baseline and 6 months in both groups. No significant differences were detected in the placebo group. In contrast, the TREC content significantly increased between baseline and 6 months in the probiotic group (Paired t-test, $p=0.002$) (Fig. 4b).

In summary, these results suggest that the strain of *L. delbrueckii* subsp. *bulgaricus* 8481 produces an increase in the generation of immature T cells and their output to peripheral blood needed to mount an adequate immune response.

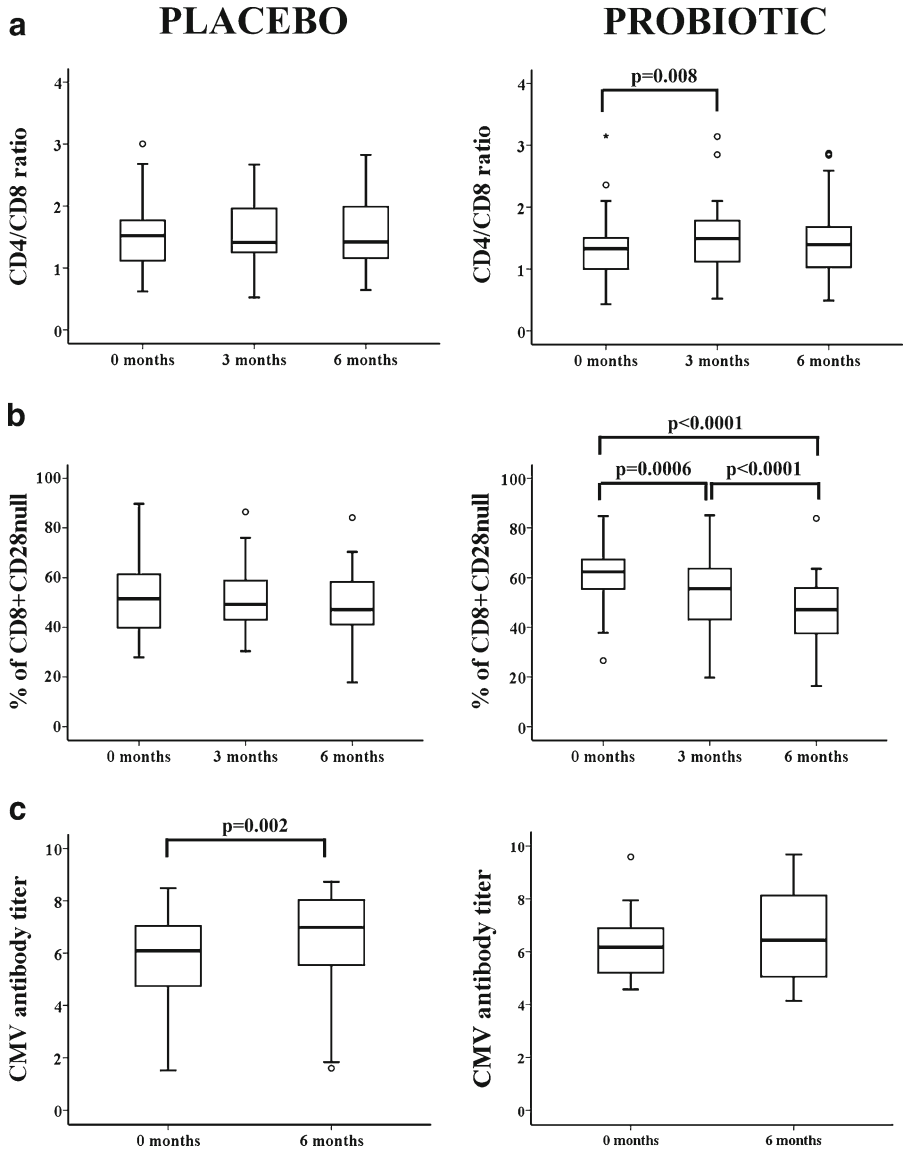

Fig. 2 CD4/CD8 ratio, percentage of CD8+CD28^{null}, and CMV antibody titer in elderly from the placebo and probiotic groups. **a** CD4/CD8 ratios were analyzed and compared between the two groups at 0, 3, and 6 months post-initiation of probiotic or placebo consumption. Staining was performed with anti-CD3-FITC, anti-CD4-APC, and anti-CD8-PerCP to gate CD4+ and CD8+ T cells. CD4/CD8 ratio less than 1.0 was used to identify individuals with an IRP. **b** Percentages of CD8+ T cells lacking CD28 expression in peripheral blood of elderly. Whole blood was stained with anti-CD3-FITC, anti-CD28-PE, and anti-CD8-PerCP. Frequencies of CD28^{null} cells in gated CD3+CD8+ lymphocytes were quantified. **c** Serum anti-CMV antibody titer was measured by ELISA at baseline and 6 months and was compared. Patient samples are quantified and interpreted by comparing the cut-off index ratio (Cutoff Index=OD value of sample/Cut-off value). A ratio of 1.0 is equivalent to the cut-off value. Cutoff index>1.1 was considered positive, and the result of this ratio is a semi-quantitative titer. Outlier and extreme values, which were calculated by adding 1.5 and 3 times the IR to the 75th percentile, were represented by *circles* and *stars*, respectively. Paired *t*-test was used to compare values between groups, and *p*-values are depicted in the panels

Changes in humoral immunity

After demonstrating that the consumption of our probiotic produced significant modifications in the cells of the elderly immune system, we decided to investigate if these modifications were reflected in humoral changes in serum. First, we assessed concentrations of immunoglobulin (IgG, IgM, and IgA) and complement molecules C3

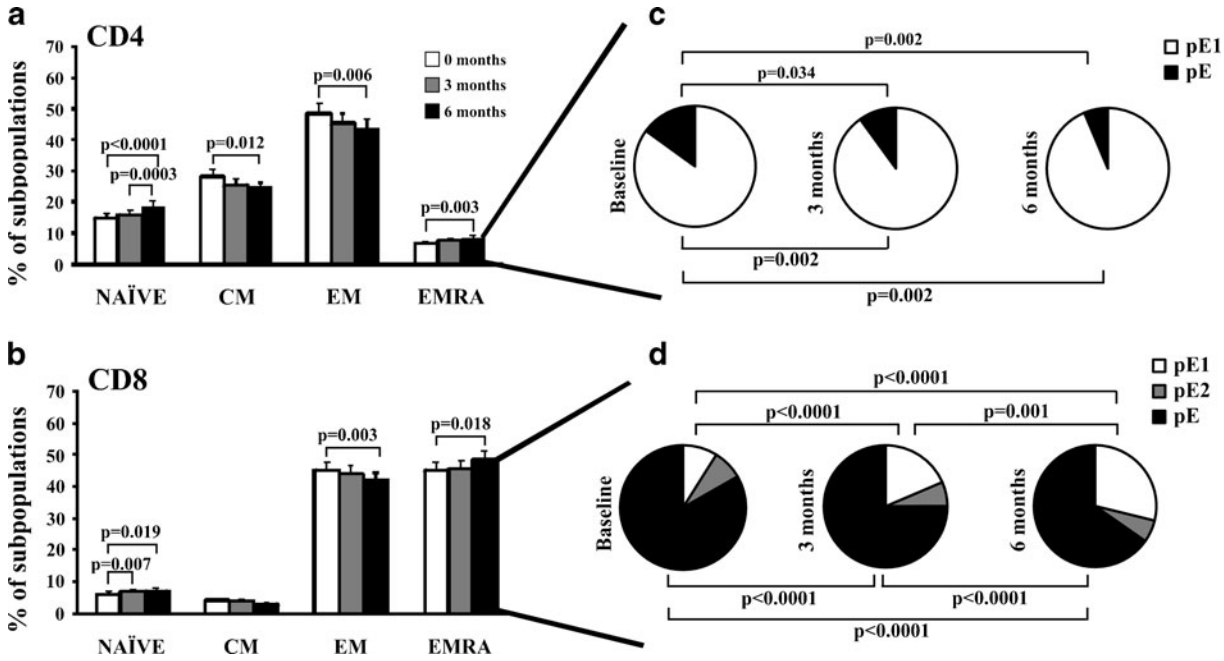

Fig. 3 Distribution of T cell subsets in the probiotics group. CD4+ and CD8+ T cells were categorized into NAÏVE, CM, EM, and EMRA cells. Distribution of EMRA in CD4+ and CD8+ T cells into subsets defined by CD28 and CD27 expression. Expression of CD45RA, CCR7, CD27, and CD28 was analyzed by flow cytometry in isolated CD4+ and CD8+ T cells from the two groups of elders at 0, 3, and 6 months. Histograms represent percentage of **a** CD4+ and **b** CD8+ T cells in each subset (NAÏVE, CM, EM, and EMRA) in the groups of elderly (time 0: *white bars*, time 3 months: *gray bars*, time 6 months: *black bars*). **c**, **d** Individual segments of the pie charts represent the proportions of cells with each combination of CD28 and CD27 in the EMRA CD4+ (**c**) and CD8+ (**d**) T cell subsets. EMRA can be divided into pE1 (CD27+ CD28+) and pE2 (CD27 + $CD28^{null}$, only in CD8 T cells) and E ($CD27^{null}CD28^{null}$). Significant differences between subsets related to total CD4+ and CD8+ T cells are indicated (Paired *t*-test). Each bar in the histograms represented the mean ± SEM

and C4 (Table 3). No significant differences were detected between values at baseline and 6 months for either group.

We also assessed the serum concentrations of major cytokines with pro- and anti-inflammatory activities for changes at the three time points by flow cytometry. Concentrations of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, TNF-α, and TNF-β did not significantly change during the study (data not shown). The concentration of IL-8, one of the major mediators of the inflammatory response, significantly decreased in the probiotic group, but not the placebo group, by 6 months (Fig. 5a).

It is known that specific probiotic strains including lactobacilli up-regulate expression of the hBD-2 (Wehkamp et al. 2004). As expected, the placebo group had no change in the concentration of hBD-2 during the study. In contrast, the hBD-2 concentration significantly increased in the serum of the elderly of the probiotics group at 3 (Wilcoxon test, $p=0.005$) and 6 months (Wilcoxon test, $p<0.0001$) (Fig. 5b).

In summary, we can affirm that the changes that we had observed at a cellular level are reflected at a humoral level with changes in some cytokine concentration and antimicrobial molecules.

Cessation of probiotic intake

After 6 months of study, we wanted to assess the stability of the changes in immunological variables by the 6-month probiotic intake. Thus, 6 months after stopping the probiotic intake, we managed to obtain blood samples from nine elders who had taken the probiotic capsules. The higher percentage of NK cells, the modulated T cell differentiation subsets, the higher percentage of very poorly differentiated cells CD45+CD31+, and the higher TREC content were not maintained for 6 months without probiotics. These

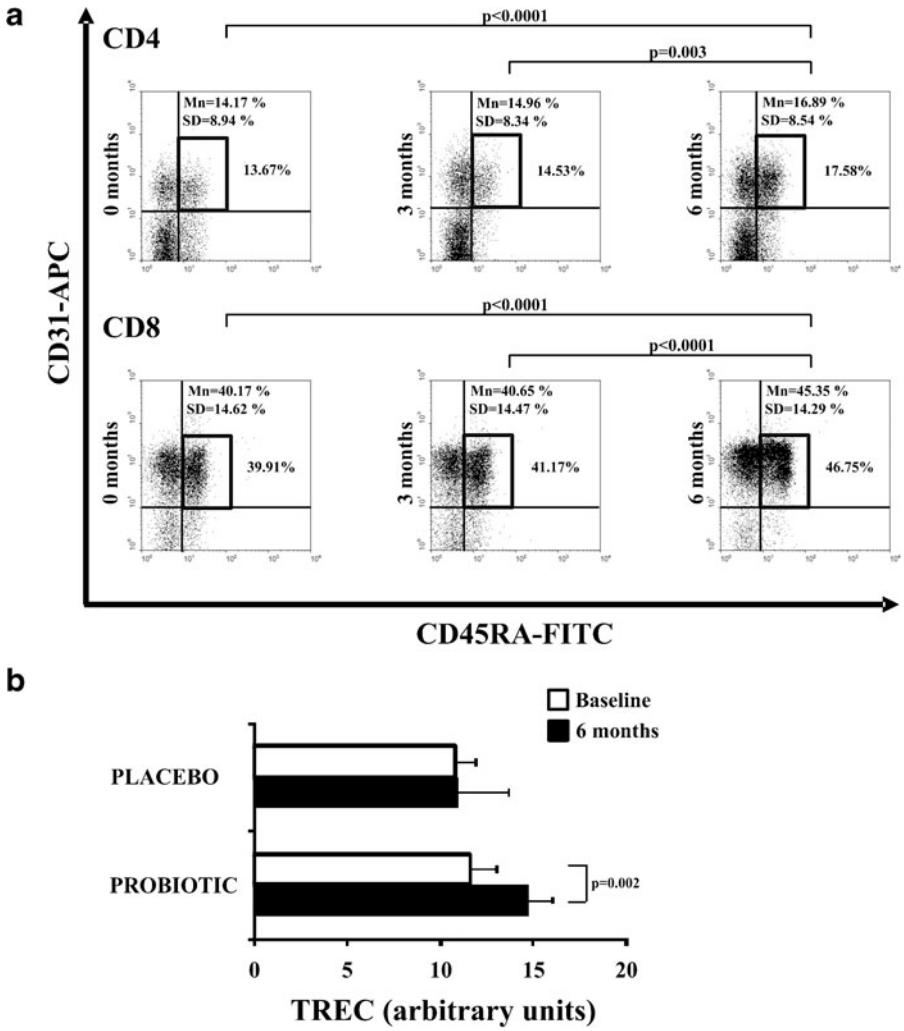

Fig. 4 Proximity to the thymus of the T cell subsets and TREC content. Whole blood from the elderly of the probiotic group was stained with anti-CD45RA-FITC, anti-CD31-PE, and these CD4+ and CD8+ T cell subsets were evaluated by flow cytometry. **a** Representative dot-plots showing the frequency of CD45RA+CD31+ in CD4+ and CD8+ T cells from elderly. Percentage of positive cells in each subpopulation in this representative experiment is expressed in the *right side*, and summarized results from all donors (mean and SD) were also expressed in dot-plots. **b** The TREC content was measured in PBLs cells from elders belonging to placebo/probiotic group. TREC copy number was determined by real-time PCR. Experiments were conducted in duplicate, and *bars* represented results from the grouped elders (mean ± SEM)

parameters returned to levels equal to or very similar to the baseline before probiotic intake (Fig. 6). Therefore, we concluded that maintenance of the beneficial effects produced by the probiotic requires ongoing probiotic intake.

Discussion

The present study used a double-blind, placebo-controlled, and randomized design to determine the effects of *L. delbrueckii* subsp. *bulgaricus* 8481 on the

Table 3 Immunoglobulin and complement quantification (g/L) (mean ± SD) at baseline, 3 months, and 6 months

	Placebo group				Probiotic group			
	Baseline	3 months	6 months	*p* value between groups[a]	Baseline	3 months	6 months	*p* value between groups[a]
IgG	9.86±1.47[a]	9.44±2.67	9.67±1.97	ns	10.89±1.9	10.56±1.89	9.89±1.77	ns
IgM	0.99±0.59	0.95±0.72	1.06±0.61	ns	1.75±0.95	1.65±0.86	1.48±0.7	ns
IgA	2.22±0.84	2.12±0.87	2.06±0.94	ns	3.39±1.38	3.3±1.3	3.18±1.64	ns
C3	1.95±0.49	1.95±0.51	1.74±0.38	ns	1.83±0.12	1.65±0.47	1.66±0.2	ns
C4	0.44±0.1	0.45±0.09	0.43±0.1	ns	0.48±0.08	0.45±0.08	0.46±0.06	ns

[a] Calculated using the paired Student's *t* test

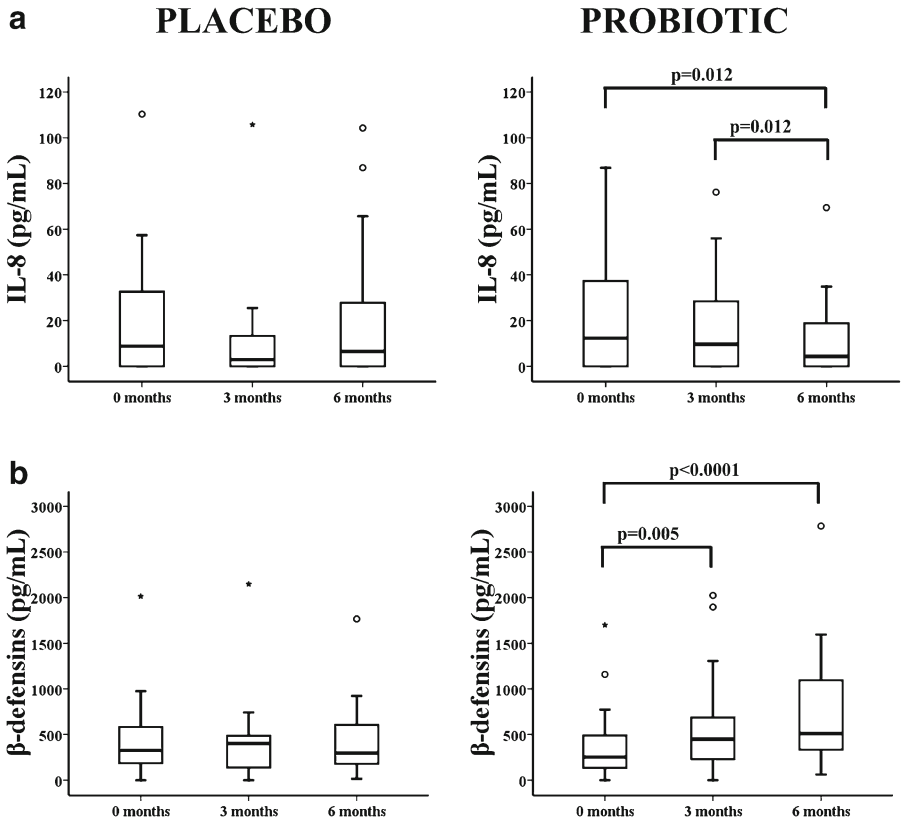

Fig. 5 Quantification of pro-inflammatory cytokine IL-8 and hBD-2 antimicrobial peptide in elderly in the placebo/probiotic groups at 0, 3, and 6 months. **a** Serum IL-8 concentrations into the placebo and probiotic groups were measured by flow cytometry. **b** Quantification of the hBD-2 peptide in serum from the elderly in the two groups by ELISA. Outlier values were represented by *circles* and extreme values by *stars*, calculated by adding 1.5 and 3 times the IR to the 75th percentile, respectively. The Wilcoxon non-parametric method was used to compare frequencies in the groups. *P*-values are depicted in the panels

elderly immune system. In this study, we have demonstrated a clear association between *L. delbrueckii* subsp. *bulgaricus* 8481 consumption and a great benefit on the immune system of elderly people. We can assert that consumption of the probiotic *L. delbrueckii* subsp. *bulgaricus* 8481 could promote the maintenance of an adequate immune response, slowing the aging of the subpopulations of T lymphocytes and increasing the number of immature cells potentially responders.

Older people suffer from age-associated changes in the immune system, including decreased immune function, increased incidence and severity of infections, development of autoimmune phenomena, and cancer (DelaRosa et al. 2006; Prelog 2006). NK cells play a critical role in immune surveillance against tumor development and viral infections, and intestinal microflora can modulate NK activity (Takeda and Okumura 2007). NK activity are depressed in the elderly in comparison to younger subjects (Hodes 1997). Therefore, our results strongly suggest that consumption of capsules containing *L. delbrueckii* subsp. *bulgaricus* 8481 significantly increased the NK cell percentage in elderly people, which may result in the maintenance of a healthy life and prevention of diseases.

Factors involved in IRP include the inverted CD4/CD8 ratio, an increase in the CD8+CD28^{null} T cell subset, and CMV seropositivity (Hadrup et al. 2006). Elders of the probiotic group had a significant decrease in the CD8+ T cell subset and a resulting increase in the CD4/CD8 ratio compared to the placebo group. Moreover, we observed a marked decline in the late differentiated memory and effector CD8+CD28^{null} T cell population throughout the study. Accumulation of CD8+CD28^{null} T cells is associated with a reduced overall immune response to pathogens and vaccines in the elderly (Riha and Rudd 2010). The cause of loss of CD28 expression in T cells with age has been attributed to repeated antigenic stimulation such as CMV or other persistent viral infections (Vallejo 2005). It is now accepted that CD28^{null} T cells have experienced past episodes of activation and cell cycling. Regards CMV infection, a high CMV antibody titer is associated with a reduced survival time (Caruso et al. 2009). In our study, elderly who took the placebo capsules had a higher CMV titer at 6 months than at baseline, while elderly who took the probiotic maintained the same CMV titer at 6 months and baseline. These results suggest that taking probiotic could prevent CMV

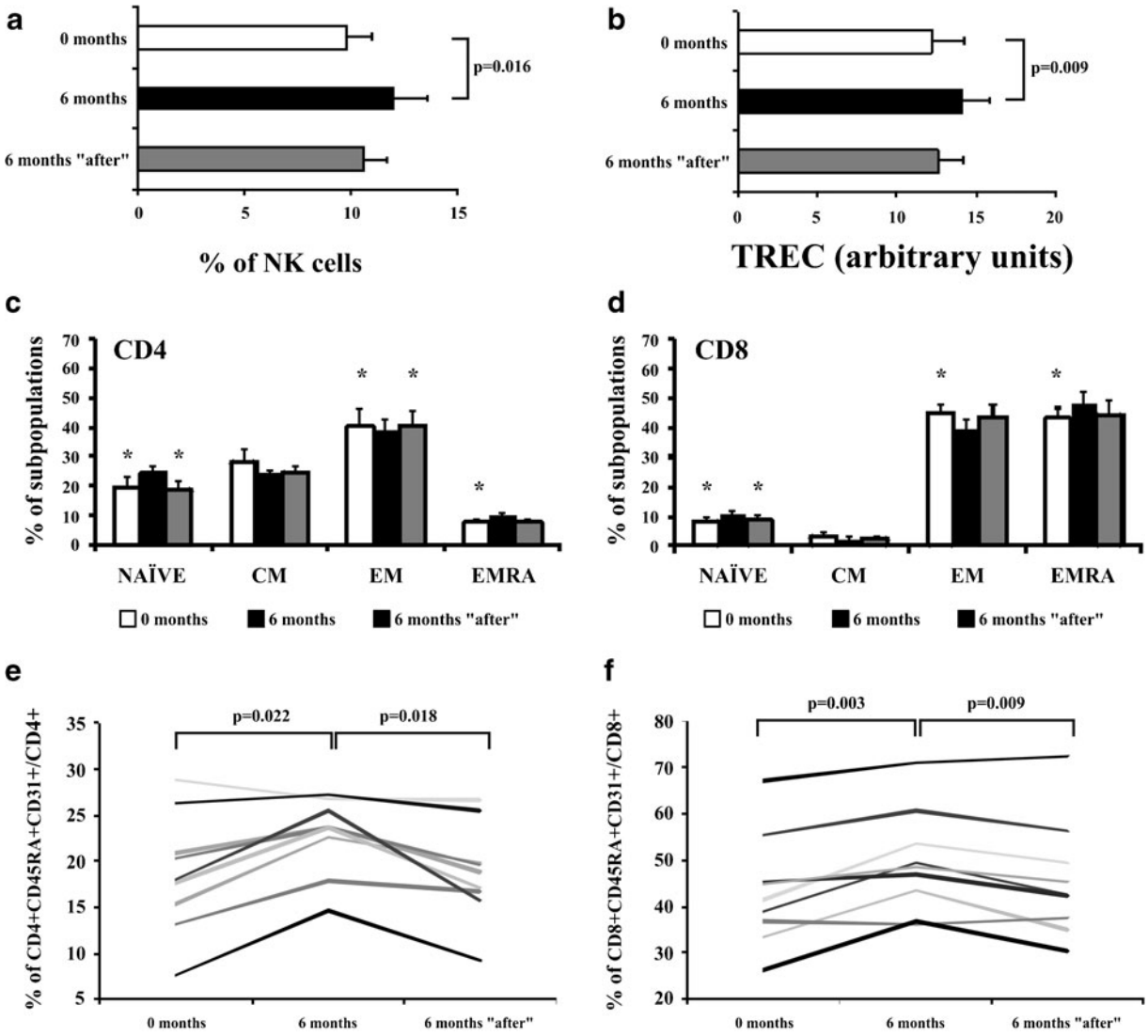

Fig. 6 Changes in percentage in NK cells, TREC content, T cell subsets distribution, and percentage of CD45RA+CD31+ into CD4+ and CD8+ T cells 6 months after stopping the probiotic intake. Measurements were made in nine elderly people at baseline, 6 months from the beginning of the study, and 6 months after stopping the probiotic intake. **a** Percentages of CD16+56+ cells with respect to the total CD45+ cells were compared throughout the study. Staining was performed with "Multiset CD3-FITC/CD16+56-PE/CD45-PerCP/CD19-APC," and frequencies of CD16+56 cells in gated CD45+ subsets were analyzed. **b** TREC content in elderly T cells. The TREC content was measured in T cells from elders, and TREC copy number was determined by real-time PCR. Experiments were conducted in duplicate. **c**, **d** Distribution of CD4+ and CD8+ T cells into NAÏVE, CM, EM, and EMRA. Expression of CD45RA and CCR7 was analyzed by flow cytometry in isolated CD4+ and CD8+ T cells. **e**, **f** Frequency of CD45RA+CD31+ in CD4+ and CD8+ T cells from elderly. Whole blood from the elderly of the probiotic group was stained with anti-CD45RA-FITC, anti-CD31-PE, and anti-CCR7-APC; and CD4+ and CD8+ T cells were evaluated by flow cytometry. *Bars* in the histograms represented the mean ± SEM. The Paired *t*-test (when data were normally distributed) and Wilcoxon non-parametric method (when data were not normally distributed) were used to compare frequencies between groups. *Significant differences with values obtained 6 months after beginning the probiotic intake ($p<0.05$)

replication by controlling their spread and avoiding the occurrence of further viral reactivation via unclear mechanisms.

As people age, differentiated T cells (EM and EMRA subsets) of the immune system accumulate, and the less differentiated immune cells (Naïve and

CM) decline in frequency (Appay et al. 2010). The differentiated cells, EM and EMRA, have a memory phenotype, display reduced diversity of the T-cell receptor (TCR), have reduced division potential (Vallejo et al. 2000; Appay et al. 2002), and are less able to respond to pathogens. Consumption of *L. delbrueckii* subsp. *bulgaricus* 8481 enhanced the proportions of less differentiated cells and decreased the more differentiated cells in the peripheral circulation. As an alternative mechanism, probiotic consumption stimulated production of naïve T cells and NK cells, which diluted the more differentiated cells and subsequently reduced the memory T cell compartment in the peripheral circulation via mechanisms of T cell homeostasis. These changes in T lymphocytes may be associated with health improvements in the elderly such as an enhanced antibody response to vaccination and markedly reduced duration of infection-related morbidity in elderly subjects.

To further investigate the composition of the T cell repertoire in our elderly, we examined the expression of CD31 and the TREC content (Kimmig et al. 2002; Kohler and Thiel 2009). These two cell markers indicate the time elapsed since these cells left the thymus; less differentiated naïve cells are essential to respond and develop immune responses against new antigens. The thymus gland is greatly reduced in elderly individuals and usually plays a very small role in their immune responses. The CD31+ marker in CD4+ and CD8+ T cells of CD45RA+ phenotype significantly increased in those individuals taking the probiotic. These data suggest that probiotic consumption may stimulate the thymus gland. We also observed that T cells in elders of the probiotic group had a higher mean TREC content, which corroborates the increase in poorly differentiated naïve cells after consumption of the probiotic.

One of the main factors that are postulated as triggers of the process of immunosenescence is a chronic inflammatory state (Ferrucci et al. 2005). Probiotics may also inhibit the production of pro-inflammatory cytokines (Lopez et al. 2008). Elderly who consumed the capsules with *L. delbrueckii* subsp. *bulgaricus* 8481 showed a decline over 6 months in the serum concentration of the chemokine IL-8. This chemokine is one of the major mediators of the inflammatory response, so its decline could be very beneficial for the elderly people.

The mucosal immune system consists of an integrated network of tissues, lymphoid and mucous membrane-associated cells, innate effector proteins (mucins and defensins), and previously induced antibody molecules. The gastrointestinal tract in the elderly is particularly susceptible to infectious diseases, in part due to changes in their intestinal microbiota but probably also because of dramatic changes to mucosal immunity itself (Schmucker et al. 1996; Fujihashi and Kiyono 2009). We wanted to determine whether *L. delbrueckii* subsp. *bulgaricus* 8481 was able to stimulate the production of hBD-2 and improve the immune innate response in the elderly, with a defective mucosal immune system. Consumption of the probiotic was associated with a very marked increase in hBD-2 antimicrobial molecule in serum, suggesting an enhancement in the innate immunity through defensin induction. Recently, 2 of 46 strains of *Lactobacillus fermentum* induced hBD-2 production from human intestinal Caco-2 cell line (Ghadimi et al. 2011). Pathogenic strains of *Salmonella* ssp. and *Helicobacter pylori* have also been reported to induce hBD-2 expression (Ogushi et al. 2001; Wehkamp et al. 2003); in contrast, lactobacilli and other probiotics appear to induce the intestinal barrier defense system without provoking inflammatory events (Santos Rocha et al. 2011). The increase in hBD-2 production and the increase in the number of NK cells suggest an improvement in innate immunity in individuals consuming *L. delbrueckii* subsp. *bulgaricus*. The role played by NK cells in innate immunity is analogous to that of cytotoxic T cells in the adaptive immune response. NK cells provide rapid responses to virally infected cells and respond to tumor formation. Human β-defensin-2 (hBD-2) code for genes which improve the function of the innate immune system (Hellgren and Sheldon 2011), and its production is enhanced by some strains of *Lactobacillus* (Schlee et al. 2008). These genes are responsible for production of antimicrobial polypeptides found in white blood cells such as macrophages, granulocytes and NK-cells, and antimicrobial β-defensins which are also found in epithelial cells.

In our study, we found that the beneficial effects attributed to probiotic disappeared after 6 months of stopping their consumption. Therefore, it is necessary to ingest these bacteria on a regular and ongoing basis to obtain significant and lasting results. We speculate that the bacteria only pass through the intestinal tract, without establishing residence in the intestinal tract. Whether colonization of part of the intestinal tract with *L. delbrueckii* subsp. *bulgaricus* 8481 could be enhanced by

altering the diet is unknown. Accordingly, once we stopped using the product, its effect disappeared.

The effects of probiotics seem promising. It is important to note that all parameters studied were evaluated systemically. Most previous immunological studies on the effects of probiotics have shown only local effects in the intestinal mucosa but did not examine their effects in the peripheral blood. Immunomodulation induced by our probiotic could favor the maintenance of an adequate immune response, slowing the aging of the subpopulations of T lymphocytes and increasing the number of immature cells which are potential responders to new antigens. The *L. delbrueckii* subsp. *bulgaricus* 8481 probiotic also has a powerful effect on the innate immune system. Therefore, taking the probiotic might help produce a better response to vaccination and greater resistance to contracting various infectious diseases. These results must be confirmed in a larger number of individuals subjected to a longer duration of probiotic consumption. Further studies will help us to determine whether *L. delbrueckii* subsp. *bulgaricus* 8481 can promote increased resistance to infection and disease in the elderly and other age groups.

Acknowledgments We would like to thank Isabel Cuevas Pérez for the excellent technical assistance. The authors are grateful to Mr. Miguel de las Heras, patent holder on the bacteria, and to Dr. Celina Quirós Díaz for their support of the research.

This work was supported by Red de Investigación Renal (REDinREN), Spanish grant FIS PI080566 from Instituto "Carlos III" (European FEDER founds), PEST 08-05 and CNCT-Leche from FICYT.

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III.7. LA REALIZACIÓN DE ENTRENAMIENTO DE ALTO VOLUMEN A LO LARGO DE LA VIDA SE ASOCIA A UNA RESPUESTA INMUNE ADAPTATIVA MÁS DIFERENCIADA

El ejercicio produce una serie de cambios en el estado del sistema inmune dependiendo del tipo, intensidad y duración de la actividad realizada. En general, el ejercicio altera la distribución, el tráfico y las capacidades funcionales de diferentes tipos de células del sistema inmune (Simpson *et al.* 2012). De hecho, se han descrito periodos transitorios de inmunosupresión después de entrenamiento intenso, así como, efectos beneficiosos anti-inflamatorios en diversas patologías y en el proceso de envejecimiento. El objetivo de este estudio fue analizar el efecto del entrenamiento de alto volumen durante largos periodos de tiempo a lo largo de la vida de los individuos sobre la respuesta funcional de los linfocitos T y las células NK.

ARTÍCULO 7:

Marco Antonio Moro García, Benjamín Fernández García, Ainara Echeverría, Manuel Rodriguez Alonso, Francisco Manuel Suárez García, Juan José Solano Jaurrieta, Carlos López-Larrea, Rebeca Alonso-Arias. *"Frequent participation in high volume exercise throughout life is associated with a more differentiated adaptive immune response".*

Brain Behav Immun. 2014 Jul;39:61-74.

Para estudiar el impacto del entrenamiento de alto volumen a lo largo de la vida de los individuos sobre diferentes aspectos inmunológicos, comparamos las características inmunológicas de 27 jóvenes y 12 ancianos deportistas con 30 jóvenes y 26 ancianos no deportistas estratificados según su sero-positivad o sero-negatividad frente al CMV. Caracterizamos las subpoblaciones leucocitarias y linfocitarias por citometría de flujo, cuantificamos el contenido en TREC, medimos la capacidad de activación y proliferación de los linfocitos T en respuesta a anti-CD3 y la actividad citotóxica de las células NKs. El entrenamiento de alta intensidad redujo el número total de leucocitos, neutrófilos y linfocitos circulantes. Cuando caracterizamos las subpoblaciones linfocitarias vimos que los atletas presentaban un aumento de las células NK y linfocitos T CD8+, mientras que los linfocitos T CD4+ estaban disminuidos en los atletas seropositivos para el CMV. Por otra parte, encontramos que los atletas que realizaban un entrenamiento de alto volumen presentaban unas poblaciones de linfocitos T CD4+ más

diferenciadas. Los linfocitos T CD8+ de los atletas jóvenes presentaron un contenido en TREC más reducido y menores frecuencias de EMR. Además, la capacidad funcional de los linfocitos T CD4+ y CD8+ estaba seriamente comprometida en los jóvenes atletas pero no en los atletas de mayor edad respecto a los individuos ancianos no deportistas. Esta capacidad funcional disminuida podría estar compensada en parte por el aumento observado en la activación y en la degranulación de las células NK de los atletas.

Como conclusión, el ejercicio de alto volumen realizado a lo largo de la vida de un individuo está asociado con un aumento en el envejecimiento del sistema inmune, que parece compensarse a medida que avanza el envejecimiento fisiológico.

Aportación personal al trabajo:

En este trabajo, mi labor se centró en recolectar y procesar las muestras del estudio, realizar y/o supervisar los distintos experimentos y analizar los resultados obtenidos. Finalmente, participé en el diseño y en la escritura del presente manuscrito.

Brain, Behavior, and Immunity 39 (2014) 61–74

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Frequent participation in high volume exercise throughout life is associated with a more differentiated adaptive immune response

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ARTICLE INFO

Article history:
Available online 31 December 2013

Keywords:
Exercise
Aging
T-lymphocytes
Lymphocyte differentiation
NK-cells

ABSTRACT

Exercise induces changes in the immune system depending on its intensity and duration. For example, transient states of immunodepression can be induced after acute intense physical activity whereas beneficial anti-inflammatory effects of moderate chronic exercise on many diseases and longevity have been described. To study the impact of high volume exercise over a lifetime on aspects of immunity we compared immunological features of 27 young and 12 elderly athletes with 30 young and 26 elderly non-athletes stratified by their CMV serostatus. We characterized blood leukocyte and lymphocyte subpopulations by flow cytometry, quantified TREC content, and measured activation and proliferation ability of T-lymphocytes in the presence of anti-CD3. NK-cells functionality was determined in response to K-562, 721.221 and 721.221-AEH cell-lines. High volume physical activity reduced the total number of circulating leukocytes, neutrophils, and lymphocytes. In the lymphocyte compartment, athletes had higher frequencies of NK-cells and CD8+ T-lymphocytes, whereas CD4+ T-lymphocytes were present at significantly lower levels in CMV-seropositive athletes. We found, in the high volume physical activity individuals, a higher degree of differentiation in CD4+ T-lymphocytes. CD8+ T-lymphocytes from young athletes had reduced TREC content and lower frequencies of recent thymic emigrants. Furthermore, the functional ability of CD4+ and CD8+ T-lymphocytes was significantly impaired in young but not in elderly athletes, and may be compensated for significantly higher activation and degranulation of NK-cells.

In conclusion, high volume exercise throughout life appears to be associated with increased levels of biomarkers that are associated with an aging immune system, which are partially reduced with physiological aging.

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1. Introduction

Exercise involves a series of changes in the status of an individual's immune system that vary with the type, intensity, and duration of the activity undertaken over a lifetime. In general, exercise alters the distribution, trafficking, and functional capabilities of different types of immune cell, as well as local and systemic levels of various soluble mediators (interleukine (IL)-2, IL-4, IL-6, CRP) (Romeo et al., 2008; Simpson et al., 2012; Walsh et al., 2011). These aspects clearly differ depending on whether the study is conducted after short-term high intensity exercise, after prolonged submaximal exercise, or at rest in trained individuals. In fact, transient states of immunodepression are induced after acute intense physical activity and beneficial anti-inflammatory effects of moderate chronic exercise on many diseases and longevity have been described (Woods et al., 1999b).

The relationship between exercise and susceptibility to infection has been modeled on a "J" curve (Nieman, 1994), suggesting that moderate activity increases immunity and reduces the risk of illness with respect to sedentary individuals (Matthews et al., 2002). Conversely, excessive volumes of strenuous endurance exercise may induce a reduction in the protective capacity of the

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0889-1591/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbi.2013.12.014

immune system, thereby increasing the risk of illness (Cox et al., 2008; Mackinnon and Hooper, 1996). The cause of this immunodepression is probably multifactorial, with hormonal, nutritional, and inflammatory factors interacting. Intense exercise induces transient inflammatory responses in the exercised muscles, which are involved in repair processes, hypertrophy, and muscular angiogenesis secondary to exercise. However, the repetition of these inflammatory reactions can cause intense and chronic local inflammatory conditions, and may even have systemic consequences (Peake et al., 2005a,b). This systemic effect results in an acute phase response to inflammation and, when intense and sustained over time, alters the immune capacity of athletes and can lead to situations of immunodepression (Helenius et al., 2005; Reid et al., 2004). Another cause of this immunodepression could be the anti-inflammatory effect that occurs during prolonged exercise, with elevations of IL-6, IL-10, IL-1Ra, and sTNFR (Gleeson et al., 2011; Petersen and Pedersen, 2005). These anti-inflammatory mediators may have opposite effects, causing transient immunodepression but long-term beneficial effects that decrease the low-grade chronic inflammation and the risk of chronic diseases. Numerous observational studies have demonstrated the salutary effects of exercise in patients with various diseases and even on comorbidity and survival in older individuals (Braith and Stewart, 2006; Manini et al., 2006; Thompson et al., 2003). Sedentary elderly individuals have a greater risk of mortality than those with intermediate or high levels of physical activity, whereas moderate levels of physical activity are associated with a reduced risk of coronary heart disease, neurodegeneration, cancer incidence, and disability (functional impairment) (Hambrecht et al., 2000; Simpson et al., 2012; Speelman et al., 2011).

There are certain parallels between the immune system of athletes undergoing intense training programs and elderly individuals. Sedentary elderly people also experience a chronic inflammatory state that is considered responsible for many of the changes that occur in the immune system as a result of aging (Franceschi et al., 2000; Simanek et al., 2011). Aging of the immune system is associated with increased susceptibility to infections and cancer, as well as increased morbidity and mortality (Simanek et al., 2011). Over a lifetime, as the immune system suffers repeated encounters with the same antigens and the degree of thymic atrophy increases, levels of highly differentiated cells increase. T-cells can be separated into functionally distinct populations using combinations of cell-surface markers such as the tyrosine phosphatase isoform CD45RA and the chemokine receptor CCR7. With these markers, T-lymphocytes are subdivided into naïve (NAÏVE; CD45RA+CCR7+), central memory (CM; CD45RA-CCR7+), effector memory (EM; CD45RA-CCR7−), and effector memory cells that re-express CD45RA (EMRA; CD45RA+CCR7−) (Sallusto et al., 1999). Meanwhile, EM and EMRA are heterogeneous populations, and the staining with two additional markers, CD27 and CD28, can distinguish between less-differentiated (CD27+ and/or CD28+) and more-differentiated ($CD27^{null}CD28^{null}$) cells (Koch et al., 2008). EM T-cells can be divided into EM1 (CD27+CD28+), EM2 ($CD27+CD28^{null}$, only in CD8+T-cells), EM3 ($CD27^{null}CD28^{null}$), and EM4 ($CD27^{null}$CD28+) cells. Functionally, EM1 and EM4 are very similar and exhibit memory-like properties and EM2 are intermediate, whereas EM3 display effector-like properties. Similarly, the EMRA subset can be subdivided into very poorly differentiated pre-effector-1 (pE1, CD27+CD28+), pre-effector-2 (pE2, $CD27+CD28^{null}$, only in CD8+T-cells), and the most differentiated T-cell subset, effector (E, $CD27^{null}CD28^{null}$). In these highly mature T-lymphocytes, mainly $CD28^{null}$ T-cells, oligoclonal expansions against CMV and other chronic antigens are evident (Clambey et al., 2005). Studies have associated the changes in the number of lymphocytes expressing activation and differentiation markers both with age and with CMV seropositive status and antibody titer against the virus (Looney et al., 1999; Roberts et al., 2012). Moreover, these cells are less effective in generating potent immune responses against antigens due to the restricted number of antigens that they can recognize and their reduced functional abilities (Lynch et al., 2009).

These findings prompted us to study whether self-reported scores of habitual exercise training are associated with an enhanced state of their immune system, providing evidence of the beneficial effect of physical activity or, conversely, if it contributes to more acute or premature differentiation degree of the immune system in athletes than in individuals who undertake moderate or low levels of physical activity.

2. Materials and methods

2.1. Study population

Ninety-five volunteers were recruited to the study. In all cases, at least 18 h had elapsed since the last training session before the blood sample was extracted, in a fasted state. Individuals in the study were divided into four groups: young non-athletes (30), young athletes (27), elderly non-athletes (26), and elderly athletes (12). The young athlete group consisted of rowers that performed a program of training specifically involving water rowing training, running and resistance training. The athletes performed 6.2 ± 1 days a week, an average of 125.3 ± 41.5 min a day, for 13 ± 4.2 years (mean ± SD). Exercise intensity in this group, based on the records of past season training, was 60% easy-moderate, 30% intense and 10% very intense exercise or competition. Training period preceding the blood drawn was a period of general adaptation or pre-season training and incremental exercise tests, performed using the blood lactate concentration, did not show performance decrements. All individuals included in the young non-athlete group were recruited from the Centro de Transfusiones del Principado de Asturias (Oviedo, Spain) and the selection criteria were not performing 150 min or more of moderate weekly exercise or 75 min or more of intense exercise. For the elderly athlete group, we selected people aged over 65 years who were involved in athletic activities such as endurance exercise, stretching and body core exercises. Also, elderly athletes performed some resistance training exercises less frequently, usually with low loads. To estimate physical activity in the senior athletes, we asked them about their physical sports activities through their lives by mean of a quantitative and qualitative semi-structured interview including the following questions: What is your main sports activity? What is your secondary sports activity? How many years have you been training routinely? How many days a week do you exercise? What is the average length of time that you train daily? What is the average intensity that you perform routinely? From this interview we determined that senior athletes trained 4.9 ± 1 days a week, an average of 79 ± 29.9 min per day, for 45.1 ± 11 years with an easy-moderate intensity (mean ± SD). The elderly non-athlete group consisted of older people living at the Santa Teresa nursing home (Oviedo, Spain) and who were judged to be physically fit. The functional abilities of the subjects were assessed using the Barthel Index of Activities of Daily Living (BI) (Mahoney and Barthel, 1965). Each person was evaluated at 10 tasks that measured daily functioning for various activities of daily living and mobility. The highest BI score (100) meant that the person needed no assistance in any part of the tasks. All the participants in the study had a BI of 90 or more. Each participant also provided his physical activity score using a questionnaire, which assigns numerical value (physical activity rating; PA-R) for infrequent (0–1), moderate (2–3) and vigorous (4–7) exercisers. Cardiorespiratory fitness was measured as the maximal oxygen uptake (VO_{2max}) that is the maximum capacity of an indi-

vidual's body to transport and use oxygen during incremental exercise, which reflects the physical fitness of the individual. Cardiorespiratory fitness was estimated from the questionnaire with the Jackson et al. equation: $VO_{2max} = 56.363 + 1.921(PAR) - 0.381(A) - 0.754(BMI) + 10.987(G)$, where PAR = physical activity rating, A = age (in years), BMI = body mass index and G = gender (0 for women, 1 for men) (Jackson et al., 1990).

To define the intensity of the physical activity, we used the increased heart rate that occurs with exercise. This method is expressed as a percentage of maximum heart rate (MHR), and levels of exercise, as measured by heart rate, are defined as follows: easy is about 40–54% MHR, moderate is 55–69% MHR, intense (or vigorous) is equal to or greater than 70% MHR, and very intense (or very vigorous) is greater than 90% MHR. All subjects underwent a physical examination and answered a standardized questionnaire to assess their medical history, current illnesses, and any medication they were taking. Exclusion criteria included all conditions that might influence the immune system, such as a recent or current infection (past 4 weeks), any inflammation, autoimmune disease or tumor, malnutrition, abnormal laboratory data (hemoglobin < 12 mg/dL, leukopenia < 3500 cells/μL, neutropenia < 1500 cells/μL, leukocytosis > 15,000 cells/μL, platelets < 10^5 cells/μL, and CRP > 5 mg/dL), and pharmacological interference (steroids, anti-inflammatory drugs, and immunosuppressive drugs). The study was approved by the ethics committee of the Hospital Central de Asturias (Oviedo, Spain). Informed consent was obtained from all volunteers before they participated in the study.

2.2. Hematological analysis and immune phenotyping

All hematological variables were measured using a Sysmex XT-2000*i* Automated Hematology Analyzer (Sysmex, Hamburg-Norderstedt, Germany). Cytometry was performed using a FACSCalibur cytometer and analyzed with CellQuest software (BD Biosciences, San Jose, CA, USA). CaliBRITE Beads (BD Biosciences) were used to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity. EDTA peripheral blood was surface-stained with Multiset CD3-FITC/CD16+56-PE/CD45-PerCP/CD19-APC Reagent, anti-CD4 (APC), anti-CD8 (PE or PerCP), anti-CD31 (PE), anti-CD45 (PE), anti-CD45RA (FITC) (Immunostep, Salamanca, Spain), anti-CD4 (PerCP), anti-CD28 (PE or PerCP) (eBioscience, San Diego, CA, USA), anti-CCR7 (Alexa Fluor 647), and anti-CD3 (FITC or PE) (BD Biosciences). One hundred microliter of whole blood from volunteers were stained with different combinations of labeled monoclonal antibodies for 20 min at room temperature. Samples were red-blood lysed with FACS Lysing Solution (BD Biosciences), washed in PBS, and analyzed with CellQuest software in the FACSCalibur Cytometer. Appropriate isotype control mAbs were used for marker settings.

2.3. T-cell receptor excision circle (TREC) quantification

DNA was extracted from isolated CD4+ and CD8+ T-cells (purity > 90%) from peripheral blood mononuclear cells (PBMCs) using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. To isolate CD4+ and CD8+ T-cells negatively, PBMCs were isolated by centrifugation on Ficoll-Hypaque gradients (Lymphoprep; Nycomed, Oslo, Norway) after 20 min of incubation with the RosetteSep Human CD8+ or CD4+ T-cell Enrichment Cocktail (StemCell Technologies, Grenoble, France). Signal-joint (sj) TREC and Cα constant region of TCR was quantified by SYBR Green real-time quantitative PCR and an iCycler thermocycler (Bio-Rad; Life Science Research Group, Hercules, CA, USA) as previously described (Alonso-Arias et al., 2011). Experimental samples were run in duplicate and the average value of the replicates was used as the sample result.

2.4. Cytomegalovirus (CMV) serology

Serum levels of CMV-specific antibodies was determined by enzyme-linked immunosorbent assay, Vir-ELISA Anti-CMV-IgG (Viro-Immun Labor-Diagnostika GmbH, Oberursel, Germany), according to the manufacturer's specifications. Results were interpreted by calculating the cut-off index (OD value of sample/cut-off value),

Table 1
Participant characteristics in relation to CMV serostatus (mean ± SD).

	Young CMV− (*n* = 30)		Linear regression	Young CMV+ (*n* = 27)		Elderly CMV+ (*n* = 38)		ANOVA		
	Non-athletes	Athletes	*p*	Non-athletes	Athletes	Non-athletes	Athletes	p_{age}	$p_{exercise}$	$p_{interaction}$
Demographic data										
Age (years)	37.3 ± 7.7	32.6 ± 9.9	NS	34.2 ± 10.4	36.5 ± 11.1	75.5 ± 4.2	73.2 ± 5.8		NA	
Number of subjects										
Women	3	0	NA	2	0	18	1		NA	
Men	15	12		10	15	8	11			
Mass (kg)	74.3 ± 5.8	78.8 ± 7.4	NS	78.9 ± 6.8	82.0 ± 5.1	73.4 ± 7.1	66.5 ± 8.5	0.003	NS	NS
Height (cm)	175.3 ± 4.6	183.3 ± 6.1	0.03	177.5 ± 5.9	187.4 ± 2.4	170.1 ± 6.3	167.9 ± 7.9	0.01	NS	NS
BMI (kg m^{-2})	24.3 ± 3.8	23.8 ± 1.2	NS	25.2 ± 4.3	23.4 ± 1.1	25.4 ± 6.7	23.7 ± 2.6	NS	0.04	NS
VO_{2max} (mL kg^{-1} min^{-1})[a]	38.6 ± 9.3	57.2 ± 8.5	0.002	39.1 ± 7.6	54.9 ± 7.2	22.2 ± 5.6	35.1 ± 7.1	<0.001	<0.001	NS
Smoking status, current (%)	3 (16.6)	0 (0)	NS	3 (25)	0 (0)	3 (11.5)	1 (8.3)	NS	NS	NS
CMV IgG antibody titer	–	–		3.0 ± 0.24	4.1 ± 1.7	4.2 ± 1.5	3.5 ± 1.2	NS	NS	NS
Hematological variables										
Hematocrit (%)	40.8 ± 7.0	42.0 ± 2.8	NS	42.6 ± 6.9	43.1 ± 2.1	41.4 ± 4.8	43.9 ± 2.2	NS	NS	NS
WBCs (10^3/μL)	7.6 ± 1.6	6.3 ± 1.5	0.001	8.3 ± 0.7	6.4 ± 2.1	6.6 ± 1.9	5.8 ± 0.9	NS	0.001	NS
Neutrophils (10^3/μL)	4.0 ± 6.9	3.5 ± 1.3	0.02	4.8 ± 0.8	3.1 ± 1.1	3.8 ± 1.3	3.2 ± 0.8	NS	<0.001	NS
Neutrophils (%)	53.9 ± 7.3	51.0 ± 8.1	0.035	57.6 ± 8.1	48.4 ± 6.0	56.7 ± 7.9	53.9 ± 7.3	NS	0.015	NS
Monocytes (10^3/μL)	0.36 ± 0.16	0.37 ± 0.1	NS	0.45 ± 0.3	0.4 ± 0.18	0.47 ± 0.2	0.55 ± 0.2	NS	NS	NS
Monocytes (%)	5.9 ± 2.5	5.9 ± 1.2	NS	6.9 ± 3.8	6.1 ± 0.9	7.1 ± 2.3	9.4 ± 3.2	0.001	NS	NS
Lymphocytes (10^3/μL)	3.0 ± 0.7	2.1 ± 0.4	0.003	3.2 ± 0.6	2.54 ± 0.8	2.13 ± 0.6	1.75 ± 0.4	0.002	0.004	NS
Lymphocytes (%)	40.7 ± 5.2	35.8 ± 7.0	0.038	38.4 ± 4.8	38.4 ± 6.4	32.6 ± 6.2	30.4 ± 7.9	<0.001	NS	NS

NA, not applicable.
NS, not significant.
[a] Using Jackson et al. (1990) equation.

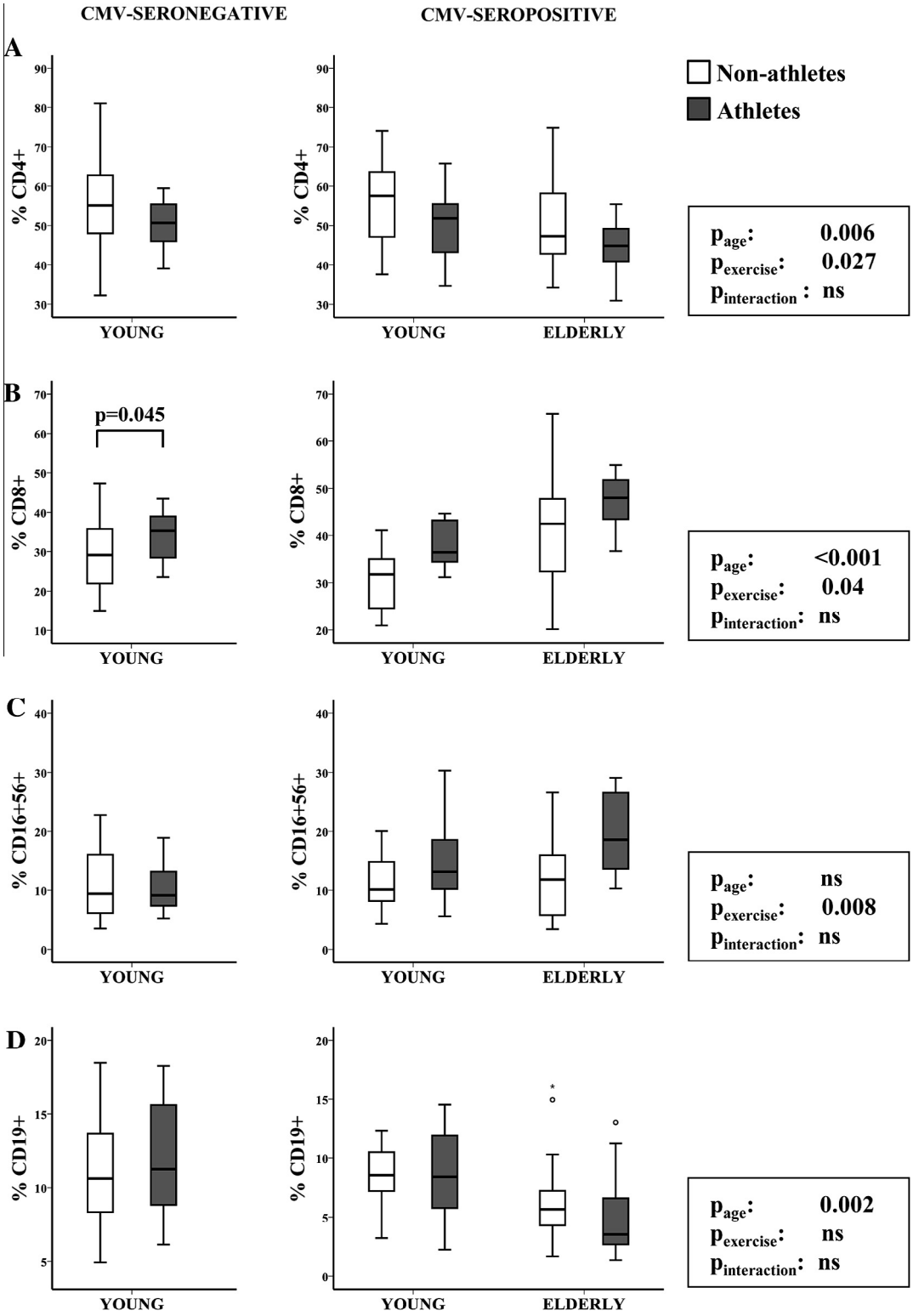

Fig. 1. Lymphocyte immune phenotype in peripheral blood. Donors were stratified by their CMV serostatus, level of physical activity and age (young non-athletes CMV−, $n = 18$; young athletes CMV−, $n = 12$; young non-athletes CMV+, $n = 12$; young athletes CMV+, $n = 15$; elderly non-athletes CMV+, $n = 26$; elderly athletes CMV+, $n = 12$). Outliers values are indicated by circles, calculated by adding 1.5 times the IR to the 75th percentile. The ANOVA test was used to examine differences between the groups adjusting for sex and CMV antibody titers; *p*-values are depicted in the panels (boxed text); the interaction is between age and exercise. (A, B) Percentages of CD3+CD4+ and CD3+CD8+ T-cells were determined with respect to the total number of lymphocytes, and group differences were compared. Cells were stained with anti-CD3-FITC, anti-CD45-PE, anti-CD8-PerCP, and anti-CD4-APC. (C, D) Percentages of CD16+56+ and CD19+ cells with respect to the total number of lymphocytes in the different groups were compared. Cells were stained with "Multiset CD3-FITC/CD16+56-PE/CD45-PerCP/CD19-APC" and the frequencies of CD16+56 and CD19+ cells into the lymphocyte subset were analyzed.

for which a ratio of 1.0 is equivalent to the cut-off value. Cut-off indices >1.1 were considered positive.

2.5. Activation and proliferation studies

Lymphocyte activation by anti-CD3 was assessed by surface staining with anti-CD69 (eBioscience, San Diego, CA, USA). Briefly, heparinized whole blood (250 μL) was cultured alone or stimulated with soluble anti-CD3 (10 ng/mL) (eBioscience) in 15 ml conical polypropylene tubes for 18 h at 37 °C and in an atmosphere of 5% carbon dioxide. Cells were then stained with anti-CD69 (FITC), anti-CD8 (PE), anti-CD4 (PerCP), and anti-CD3 (APC) mononuclear antibodies. For the proliferation studies, PBMCs were resuspended in PBS at a final concentration of 5–10 $\times$ 10^6 cells/mL and incubated with 1.5 μM CFSE (Invitrogen, Paisley, Scotland, UK) for 10 min at 37 °C, washed with cold RPMI 1640 medium containing 2 mM L-glutamine and Hepes (BioWhittaker, Verviers, Belgium) twice, and cultured at 1 $\times$ 10^6 cells/mL in medium or in the presence of soluble anti-CD3 (10 ng/mL). The proliferative responses of CD4+ and CD8+ T-cells were analyzed on day 7 by FACSCalibur after staining with anti-CD4, anti-CD8, and anti-CD3.

2.6. NK-cell functional studies

To determine the degree of expression of NKG2D and NKG2A molecules, heparinized whole blood was stained with CD16 (FITC), CD56 (FITC), NKG2D (PE) (eBioscience), CD3 (PerCP) (BD Biosciences), and NKG2A (APC) (R&D Systems, Minneapolis, USA). CD107a lysosome-associated membrane protein-1 (LAMP-1) expression was used to measure NK-cell degranulation and expression of CD69 to assess NK-cell activation, adapting the classical cytotoxicity test, which uses the K562 cell line as the target. Cell lines 721.221 and 721.221-AEH were used to test the inhibition of cytotoxicity in NK-cells mediated by NKG2A. The 721.221 (.221) is a HLA class I-deficient EBV-transformed B lymphoblastoid cell line. 721.221-AEH (.221-AEH) cells, kindly provided by Dr. D.E. Geraghty (Fred Hutchinson Cancer Research Centre, Seattle, WA), were generated by stable transfection of .221 cells with a construct in which the leader sequence of the HLAE*0101 allele was replaced by that of HLA-A2 (Lee et al., 1998); .221-AEH cells were cultured in complete medium supplemented with 300 μg/mL hygromycin B (Calbiochem, San Diego, CA). To perform the cytotoxic assays, 100 μL of whole blood was co-cultured with 2 $\times$ 10^5 cell lines for 1 h, then monensin, diluted at 1/1000, was added directly to the cultures, which were then incubated for another 3 h. Cells were stained with CD69 (FITC), CD56 (PE), CD-3 (PerCP) (BD Biosciences), and CD107a (APC) (eBioscience) before flow cytometry analysis.

2.7. Statistical analysis

Results are expressed as the median and interquartile range (IR) or the mean and standard deviation (or the standard error of the

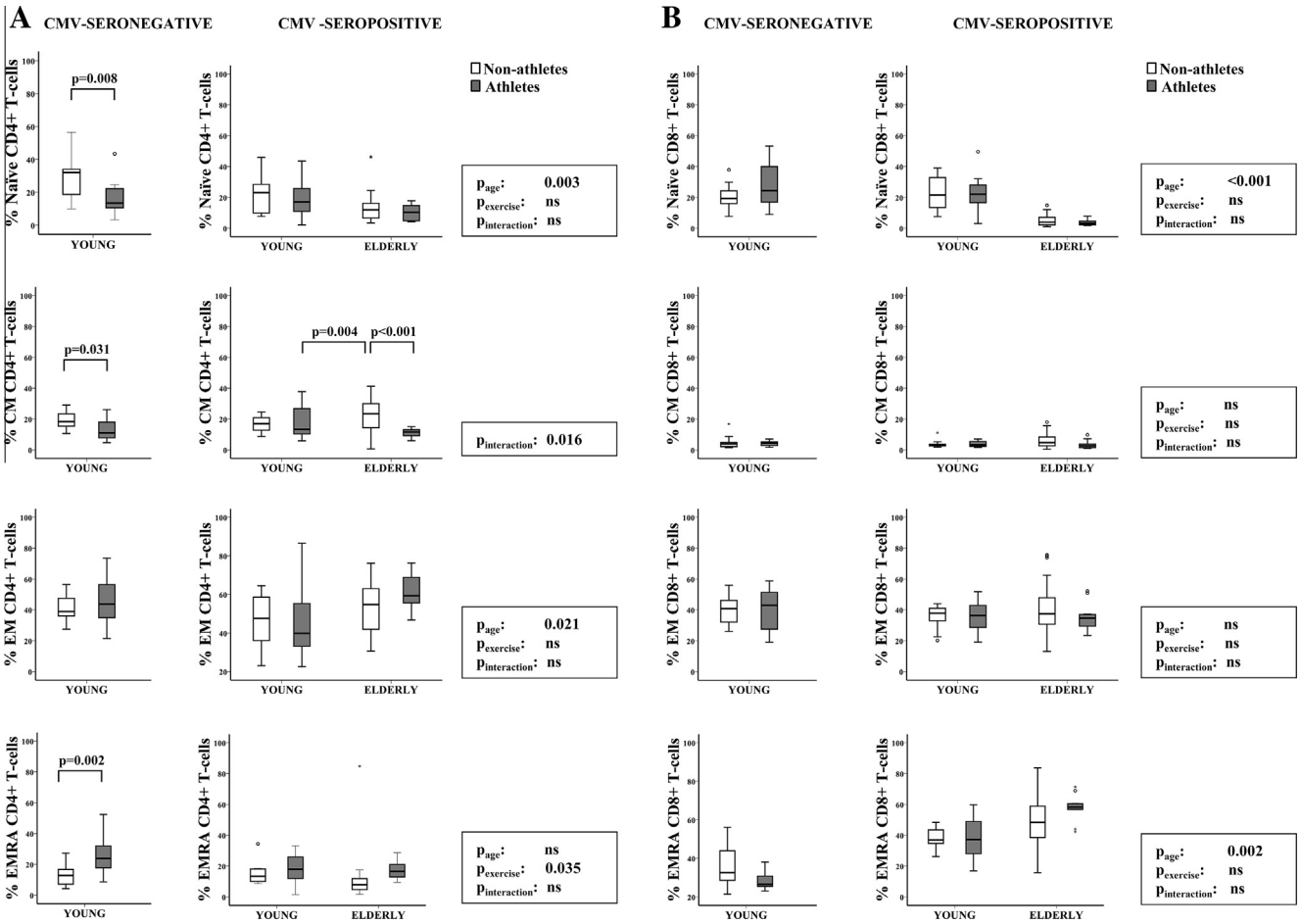

Fig. 2. Distribution of CD4+ and CD8+ T-cells in naïve, central memory, effector memory and effector memory RA subsets (NAIVE, CM, EM, and EMRA). Expression of CD45RA and CCR7 was analyzed by flow cytometry in CD3+ CD4+ and CD3+CD8+ T-cells from the four groups. Whole blood was stained with anti-CD45RA-FICT, anti-CD3-PE, anti-CD8-PerCP or anti-CD4-PerCP, and anti-CCR7-APC. 10^5 cells were acquired for each experiment. Outlier values are indicated by circles, calculated by adding 1.5 times the IR to the 75th percentile. The ANOVA test was used to examine differences between the groups adjusting for sex and CMV antibody titers; *p*-values are depicted in the boxed text; the interaction is between age and exercise. If significant interactions were observed, comparisons with a Bonferroni correlated post hoc test were performed and *p*-values are represented in the panels. Box-plots show the percentage of each subset in (A) CD4+ and (B) CD8+ T-cells in the four groups.

mean in some graphs). In CMV-seropositive individuals, quantitative variables were compared using the analysis of variance (ANOVA) analyzing the effect of age (young or elderly) and exercise (control or exercise training), and adjusting for sex and CMV antibody titers. If significant interactions were observed in any of these analyses, comparisons with a Bonferroni correlated post hoc test were performed. In CMV-seronegative individuals, quantitative variables were compared using linear regression adjusting for sex. In order to perform these analyses, non-parametric variables were normalized by logarithmic transformation. Comparisons between two groups were performed with the Student's *t* test. Analyses were performed using the SPSS 15.0 statistical software package program (SPSS Inc., Chicago, IL) and *p*-values of 0.05 or less were considered significant.

3. Results

3.1. Demographic and hematological characteristics of the study population

The characteristics of the 95 individuals included in the study are shown in Table 1. Individuals were grouped into four categories according to their age range and level of physical activity. In the young groups individuals were classified according to their CMV-serological status, whereas in elderly groups only CMV-seropositive individuals were included in the study. Due to the different distribution of gender and anti-CMV antibody titers in the elderly CMV-seropositive individuals, all statistical analyses in this study were performed adjusting for sex and antibody titers. There were no significant differences in age among either the groups of young or elderly individuals.

Blood cell counts and an immune phenotype were determined in all individuals included in the study. The comparison of groups with the ANOVA test revealed a significant increase in the percentage of monocytes and a significant decrease in the percentage and absolute number of lymphocytes due to age. Lifetime high volume physical activity significantly decreased number of leukocytes, and absolute number and percentage of neutrophils and lymphocytes (Table 1).

Examining the immunological phenotype of the lymphocyte subpopulations, we only found differences between young CMV-seronegative individuals in CD8+ T-cell subsets (Fig. 1). High volume physical activity in CMV-seropositive individuals generated a decrease in the percentage of CD4+ T-cells (Fig. 1A), and an increase in CD8+ T-cells and NK-cells (Fig. 1B and C). Finally, we found no significant differences in the percentage of B cells (CD19+) between the groups due to activity level (Fig. 1D). All variables studied except the percentage of NK-cells were affected by age; we found a decrease in the percentage of CD4+ T-cells and B-cells and an increased in the percentage of CD8+ T-cells due to age (Fig. 1).

In summary, these results demonstrate that high volume exercise throughout life induces changes in leukocyte populations that are manifested in young individuals and that are maintained in the elderly.

3.2. T-lymphocyte differentiation

To detect any association between maintained high levels of physical activity and the differentiation state of T-lymphocyte subpopulations, we compared the distribution of T-lymphocyte subpopulations in CMV-seronegative and CMV-seropositive groups (Fig. 2). In CD4+ T-cells, we observed an effect of high volume physical activity in CMV-seronegative young individuals in the naïve, CM, and EMRA subsets (Fig. 2A). In CMV-seropositive individuals, EMRA cells were increased in both young and elderly athletes but CM cells were only reduced in elderly athletes. Similarly, naïve and EM cells were also influenced by age. In the case of CD8+ T-cells, we found no differences due to activity level and

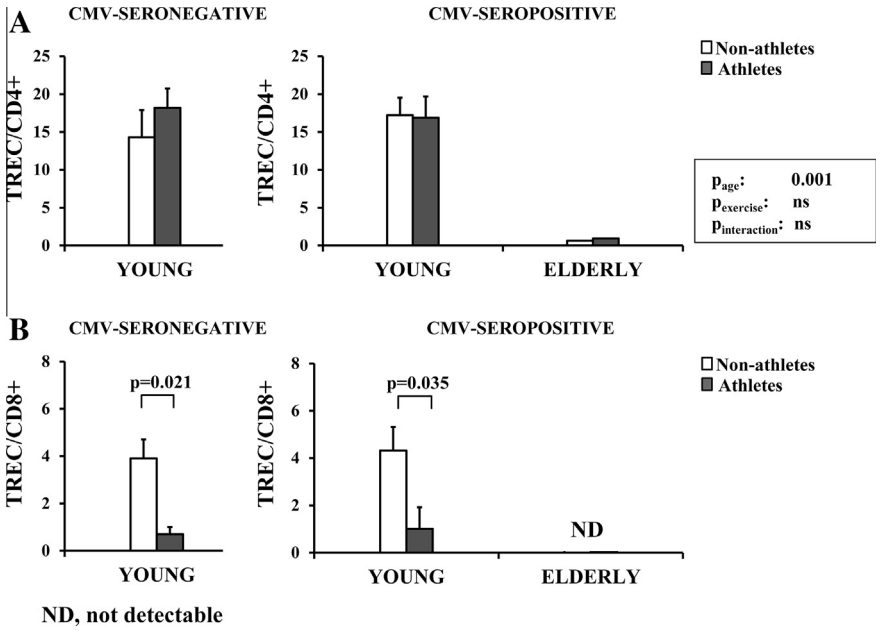

Fig. 3. TREC content in T-cells of young and elderly non-athletes and athlete donors stratified by their CMV serostatus. TREC content was measured in CD4+ (A) and CD8+ (B) T-cells from 27 young men (non-athletes, $n = 12$; athletes, $n = 15$). CD4+ and CD8+ populations were isolated by centrifugation with a human CD4+ or CD8+ T-cell enrichment cocktail on Ficoll-Hypaque gradients, and the TREC copy number was determined by real-time PCR. Experiments were conducted in duplicate. Bars represent results from the grouped donors (mean ± SEM). Groups were compared using the Student's *t* test and the ANOVA test, adjusting for sex and CMV antibody titers in the case of CMV seropositive CD4+ T-cells; *p*-values are depicted in the panels; the interaction is between age and exercise.

only in the naïve and EMRA subsets did we observe an influence of age in CMV-seropositive individuals (Fig. 2B).

Taken together, these results indicate that high levels of physical activity are associated with greater differentiation of the CD4+ T-lymphocyte populations.

3.3. Thymic function

To corroborate the differences found in the degree of differentiation of the subpopulations of T-cells, we assessed the replicative history of these cells by measuring their TREC content, an indicator of the number of divisions that the cell has undergone (Douek et al., 1998). The TREC content of older individuals was, as expected, lower than of younger individuals and did not differ significantly between the elderly groups (Fig. 3A and B). However, contrary to expectation, we found no difference in TREC levels in CD4+ T-cells between the groups of young individuals (Fig. 3A). In contrast, TREC levels in CD8+ T-lymphocytes were significantly higher in young non-athletes, whether CMV-seronegative or seropositive, and were undetectable in aged individuals (Fig. 3B).

To explain the discrepancy between the differentiation status of the CD4+ and CD8+ T-cells and their TREC content, we examined the composition of the naïve and the highly differentiated EM and EMRA subsets. We found no difference in the frequency of naïve T-cells expressing CD31, a marker of recent thymic emigrants (Kimmig et al., 2002), between the two elderly groups (data not shown). By contrast, this subset of naïve T-cells was significantly lower in the CD8+ T-cells of young athletes compared to young non-athletes, although no differences between groups were observed in the CD4 T-cells (Fig. 4).

The frequencies of the EM subsets did not differ in CD4+ or CD8+ T-cells (data not shown). When we considered the effect of lifetime high volume physical activity on EMRA CD4+ T-cells in CMV-seronegatives we found significantly higher levels of the less-differentiated pE1 (EMRA CD27+CD28+) subpopulation (Fig. 5A). In CMV-seropositives, pE1 (EMRA CD27+CD28+) was increased in young and elderly athletes, and the most differentiated E (EMRA $CD27^{null}$-$CD28^{null}$) subset only in the elderly athletes (Fig. 5A). With respect to age, in CMV-seropositives, E (EMRA $CD27^{null}CD28^{null}$) subset was increased in the elderly athletes compared to the young individuals (Fig. 5A). In CD8+ T-cells, pE1 (EMRA CD27+CD28+), in CMV-seronegatives and seropositives, and pE2 (EMRA $CD27+CD28^{null}$), only in CMV-seronegatives, were significantly decreased in young athletes (Fig. 5B). The highest differentiated E (EMRA $CD27^{null}CD28^{null}$) subset was significantly increased, in CMV-seropositives, in both groups of athletes. Only the young non-athletes, but not young athletes, presented differences with older individuals in the few differentiated subset pE1 (Fig. 5B).

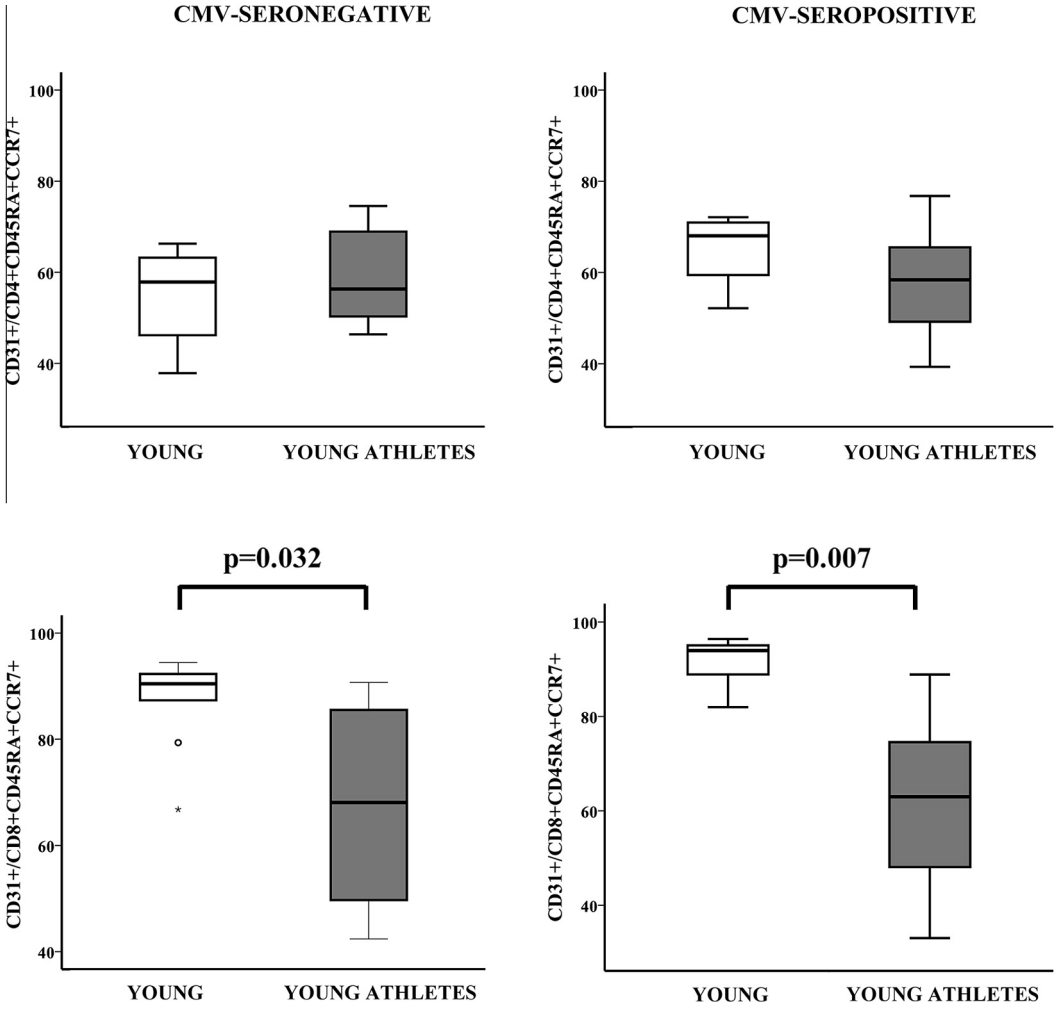

Fig. 4. Frequency of CD31+ cells in CD4+ and CD8+ naive T-cells from the groups of young individuals stratified by their CMV serostatus. Whole blood from the donors of each young group was stained with anti-CD45RA-FITC, anti-CD31-PE, anti-CCR7-APC, and CD4-PerCP or CD8-PerCP, and subsets were evaluated by flow cytometry. Student's *t* test was used to compare group values; *p*-values are depicted in the panels.

After analyzing the replicative history of T-cells, we found that young athletes had a lower TREC content in their CD8+ T-cells, but not in the CD4+ cells, than did young non-athletes. These differences can be partly explained by the reduced rate of output of CD8+ T-cells from the thymus and the different distribution of the subpopulations of the EMRA subset in CD4+ and CD8+ T-cells.

3.4. Functional in vitro immune response of T-cells

To evaluate whether phenotypic changes observed between the four groups were also associated with a different adaptive immune response, we measured the activation capability of T-cells by comparing CD69 basal expression (data not shown) and after anti-CD3 stimulation. We found that the young non-athlete group more frequently expressed CD69 in CD4+ (Fig. 6A) and CD8+ (Fig. 6B) T-cells than did the young athlete group independently of CMV-serological status, and no differences were found between elderly groups. With respect to age, we found differences between young non-athletes and both groups of elderly in CD4+ and CD8+ T-cells in CMV-seropositives.

We next studied the proliferative capacity of CD4+ and CD8+ T-lymphocytes in response to anti-CD3. As in the case of cell activation, we detected differences in cell proliferation between groups of young individuals, CMV-seronegatives and seropositives, with respect to their physical activity level in both subsets of T-lymphocytes. We also found differences with respect to high volume physical activity in CMV-seropositive elderly individuals in CD4+ T-cells. No differences were found between groups with respect to age (Fig. 7A and B).

These results indicate that the functional capacity of T-lymphocytes in the group of young athletes is lower than in young people who did not undertake high volume physical activity, although this difference in functional capacity was not observed in older individuals, it being similarly lower in the athlete and non-athlete groups.

3.5. Functional activity of NK-cells

After observing that the functional T-cell responses in young athletes were lower than in young non-athletes, we postulated that there might be some compensatory immune mechanisms to maintain an adequate immune response. We performed phenotypic and functional studies to determine the functional capacity of NK-cells. First, we quantified the expression of two typical molecules expressed in NK-cells: NKG2D, a stimulatory receptor, and NKG2A, an inhibitory receptor. We found no difference in the levels of expression of these molecules between the groups of young people (results not shown). As we found no differences in these NK receptors, we wanted to determine the basal cytotoxic capacity

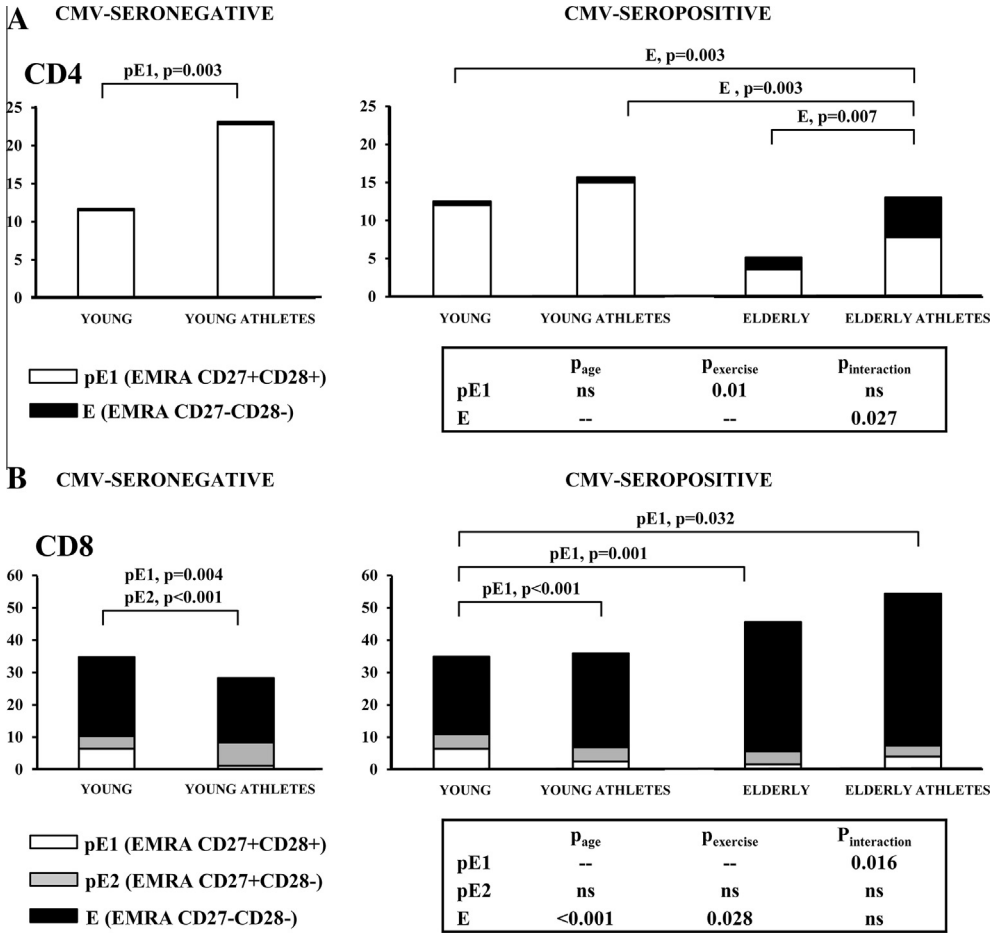

Fig. 5. Characterization of EMRA CD4+ and CD8+ T-cells. Distribution of EMRA CD4+ (A) and CD8+ T-cells in subsets defined by CD28 and CD27 expression. Expression of CD27 and CD28 was analyzed by flow cytometry in EMRA subsets from isolated CD4+ and CD8+ T-cells in the athlete and non-athlete groups considering their CMV serotatus. Individual segments of the histograms represent the proportions of cells with each combination of CD28 and CD27 in the EMRA CD4+ and CD8+ T-cell subsets. EMRA can be divided into pE1 (CD27+CD28+), pE2 (CD27+CD28^{null}, only in CD8 T-cells) and E (CD27^{null}CD28^{null}). The ANOVA test was used to examine differences between the groups adjusting for sex and CMV antibody titers; *p*-values are depicted in the boxed text; interaction is between age and exercise. If significant interactions were observed, comparisons with a Bonferroni correlated post hoc test were performed and *p*-values are represented in the panels.

with a conventional stimulation of NK cells (Dons'koi et al., 2011). We studied the activation ability of NK-cells by the expression of the CD69 molecule and their degranulation capacity by measuring the expression of the CD107a molecule. We analyzed these abilities of the NK-cells by challenging them with the cell line K562, classically used as a target in cytotoxicity assays. We quantified the percentage of cells expressing both these molecules (positive cells) as the number of molecules of CD107a present in each cell (median fluorescent intensity, MFI). At baseline, we found no differences in either the cell percentage of activated cells or the number of CD107a molecules per cell. However, after challenging NK-cells with the K562 cell line, we observed that the levels of activation and degranulation (positive cells and MFI) were significantly higher in the NK-cells of young athletes than in those of the young non-athletes (Fig. 8A and B).

Cell lines .221 and .221-AEH were used to test the inhibition of cytotoxicity in NK-cells mediated by NKG2A. After confronting NK-cells with the .221 cell line, we observed that the levels of

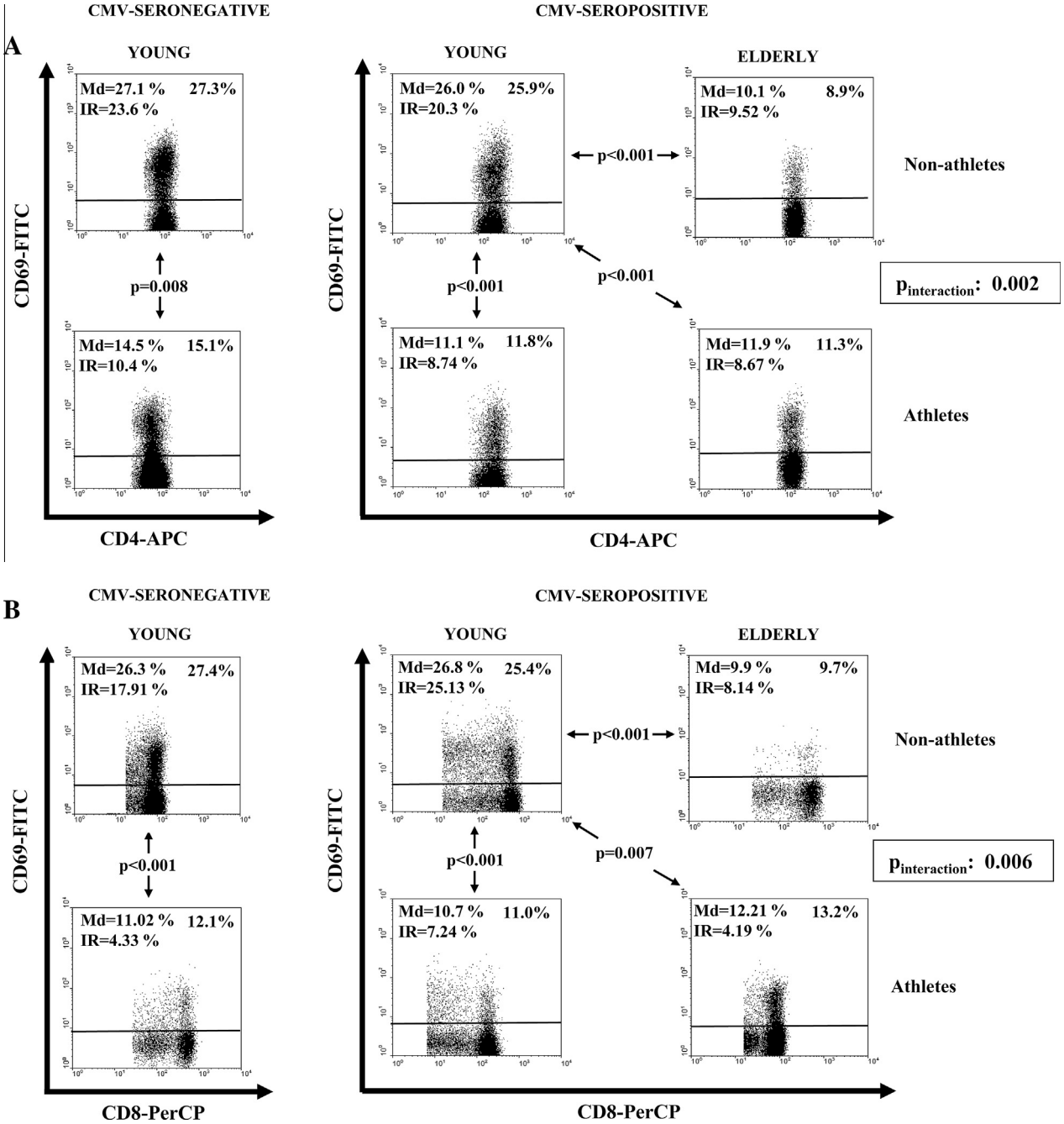

Fig. 6. CD69 expression of CD4+ and CD8+ T-cells by age, level of physical activity and CMV serostatus. Whole blood was stimulated for 18 h with anti-CD3 (10 ng/mL) and expression of CD69 in CD4+ and CD8+ T-cells was evaluated by flow cytometry (young non-athletes CMV−, $n = 11$; young athletes CMV−, $n = 12$; young non-athletes CMV+, $n = 8$; young athletes CMV+, $n = 15$; elderly non-athletes CMV+, $n = 19$; elderly athletes CMV+, $n = 12$). Cells were stained and 1×10^5 cells were acquired for each experiment. Representative dot-plots showing the frequency of CD69 expression from young donors and elderly donors, in CD4+ (A) and CD8+ (B) T-cell subsets with different levels of physical activity. Percentage of positive cells in each subpopulation in this representative experiment is expressed in the upper-right corner and summarized results from all donors (median and IR) are also represented by dot-plots. CMV− groups were compared using the Student's *t* test, and the ANOVA test was used to examine differences between the groups adjusting for sex and CMV antibody titers. As significant interactions were observed (boxed text), comparisons with a Bonferroni correlated post hoc test were performed, *p*-values are represented in the panels and interaction is between age and exercise.

degranulation (positive cells and MFI) were similar to those found with the K562 cell line (data not shown). When we used .221-AEH HLA-E transfected cells as target we found no differences in the inhibition of cytotoxicity between the non-athlete and athlete groups (Fig. 8C).

The increase in the percentage of NK-cells and in their functional capacity might represent a compensatory mechanism for the functional defects of the T-lymphocytes, summarized in Table 2.

4. Discussion

In this study, we have demonstrated for the first time an association between frequent participation in high volume exercise sustained throughout life and a highly differentiated profile of the immune system. We found a decrease in the number of leukocytes, neutrophils and lymphocytes in the studied athlete groups. Individuals with high volume physical activity had a decrease in the percentage of CD4+ and an increase in CD8+ and NK-cells. Moreover, individuals who had undertaken high-volume physical activity exhibited a more-differentiated status in CD4+ T-cell compartments, young and elderly donors, but not in the CD8+ T-cell subpopulations. However, when we quantified the TREC content of the CD4+ and CD8+ T-cells in the young groups, we found significant differences only in the CD8+ T-cells, in which the TREC content was higher in young non-athletes. This can be partly explained by the reduced rate of output of CD8+ T-cells from the thymus and the distribution of the different subpopulations of the EMRA subset in CD4+ and CD8+ T-cells. Finally we observed a diminished adaptive immune response in young athletes who might be offset by the increase in the cytotoxicity of NK-cells. This may affect the efficacy of the athlete's immune response against novel pathogens, and therefore their health and their sporting performance, which is of great importance to these individuals.

Moderate levels of physical activity are associated with an optimum overall health status and a reduced risk of coronary heart disease, neurodegeneration, cancer incidence, and disability (functional impairment) (Hambrecht et al., 2000; Simpson et al., 2012; Speelman et al., 2011). Moreover, sedentary elderly individuals have a greater risk of mortality than those with intermediate or high levels of physical activity. One effect of regular activity may be its preventive and/or rejuvenating properties on the aging immune system. In fact, aerobic fitness has been associated with a lower proportion of senescent and a higher proportion of naïve CD8+ T-cells (Spielmann et al., 2011). In contrast, frequent periods of intense/very intense exercise or a high volume of exercise may induce different effects. Accordingly, Cosgrove et al. have reported an accumulation of differentiated T-cells in response to a 6-month training regimen in a group of club level triathletes (Cosgrove et al., 2012). In our groups of athletes, who reported frequent participation in high volume exercise throughout life, we also found a more differentiated phenotype and reduced functional abilities in T-cell subsets. It is important to remark that the young athletes in our study showed higher values of VO_{2max} than those associated with a beneficial effect on differentiated T-cell status (Spielmann et al., 2011). It is acknowledged, however, that a longitudinal experimental design is necessary to validate this hypothesis.

In the present study, we found a decline in neutrophil and lymphocyte populations in young and elderly athlete groups in comparison with non-athletes. Although a decrease in the number of total white cells and neutrophils has also been described in individuals doing intense physical activity over a long period and in aerobic sports compared with team or skill-based sports (Gleeson and Bishop, 2005; Horn et al., 2010), the pattern for lymphocytes is less clear. The lower number of blood leukocyte subsets found in our study were not due to the hemodilution caused by plasma volume expansion, a known adaptation to exercise training, as hematocrit levels showed no significant differences between groups. In the case of NK-cells, as with other leukocyte populations, there may

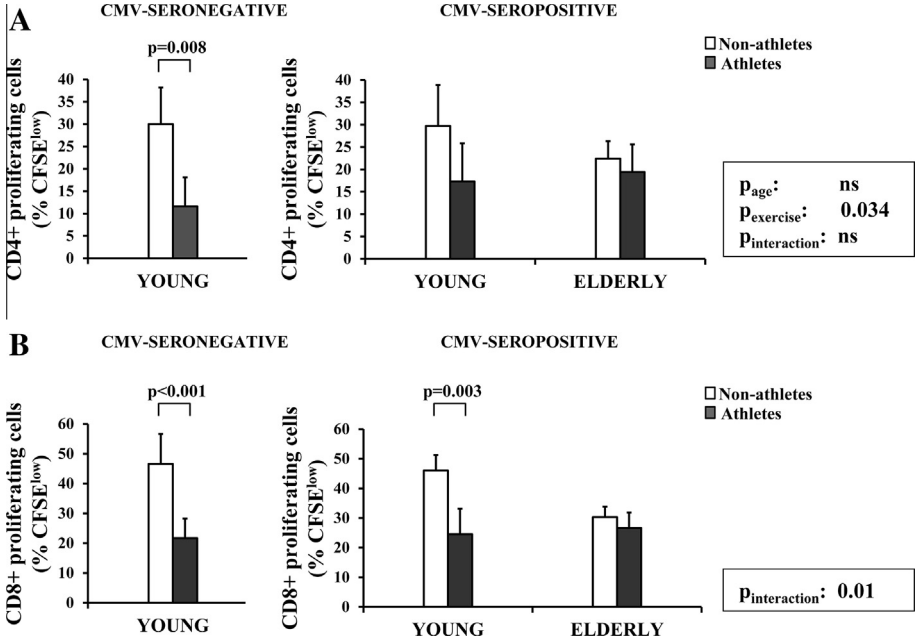

Fig. 7. Proliferative capacity of CD4+ and CD8+ T-cells by age, level of physical activity and CMV serostatus. Proliferative capacity of CD4+ (A) and CD8+ (B) T-cell subsets in response to anti-CD3 (10 ng/mL) were evaluated in the groups (young non-athletes CMV−, $n = 5$; young athletes CMV−, $n = 5$; young non-athletes CMV+, $n = 5$; young athletes CMV+, $n = 5$; elderly non-athletes CMV+, $n = 8$; elderly athletes CMV+, $n = 12$). PBMCs were isolated and labeled with CFSE (1.5 μM) and cultured in the presence of anti-CD3 for 5 days. Cells were stained and 1×10^5 cells were acquired for each experiment. Percentage of dividing CD4+ and CD8+ T-cells is shown. Bars represent results from the grouped donors (mean ± SEM). CMV− groups were compared using the Student's t test, and the ANOVA test was used to examine differences between the groups adjusting for sex and CMV antibody titers; p-values are depicted in the boxed text; interaction is between age and exercise. If significant interactions were observed, comparisons with a Bonferroni correlated post hoc test were performed and p-values are represented in the panels.

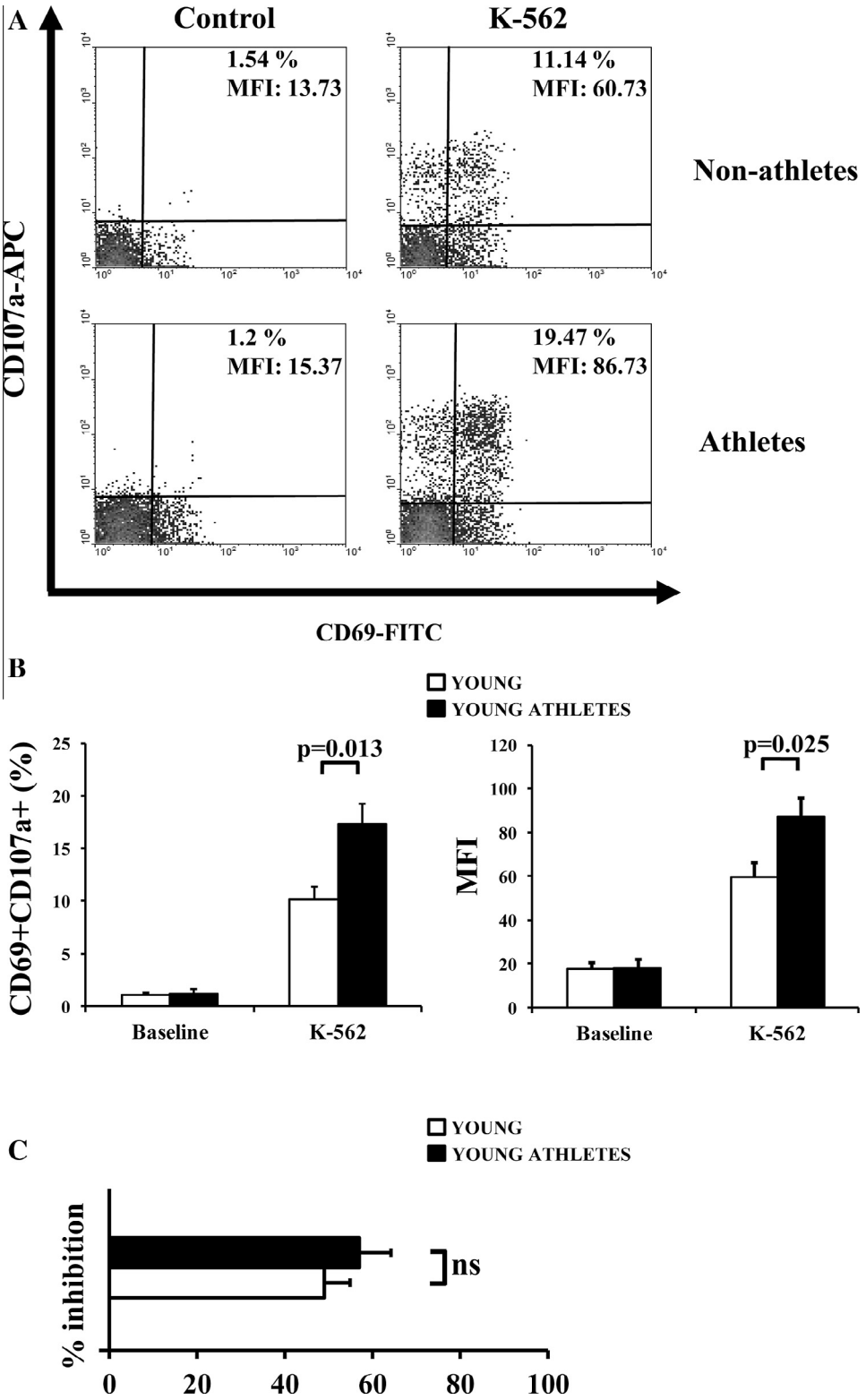

Fig. 8. Functional ability of NK-cells from young donors. CD69 and CD107a co-expression in NK-cells was studied by flow cytometry in 24 young men (young non-athletes, $n = 14$; young athletes, $n = 10$). Cell lines 721.221 and 721.221-AEH were used to test the inhibition of cytotoxicity in NK-cells mediated by NKG2A (young non-athletes, $n = 9$; young athletes, $n = 10$). (A) Representative dot-plots showing CD69 and CD107a expression in NK-cells from young non-athletes and young athletes. Percentage of positive cells and MFI in this representative experiment is expressed in the upper-right corner. (B) Histograms show the percentage and the MFI of positive cells (mean ± SEM) in NK-cells from young non-athlete donors (white bars) and young athlete donors (black bars). (C) Histograms show the percentage of cytotoxicity inhibition (mean ± SEM) in NK-cells from young non-athlete donors (white bars) and young athlete donors (black bars). The Student's *t* test was used to compare frequencies between groups; *p*-values are depicted in the panels.

be the mobilization to peripheral circulation primarily due to the release of catecholamines during intense exercise (Timmons and Cieslak, 2008). Moreover, several studies have reported an increase in these leukocyte populations after acute sessions of intense exercise (Peake, 2002; Shek et al., 1995). The response might be biphasic, with an increase in blood leukocyte numbers soon after the beginning of exercise, and later tissue leukocyte redistribution improving immune responses against pathogens in the skin, lungs, gastrointestinal and urinary-genital tracts, mucosal surfaces, and lymph nodes (Walsh et al., 2011; Yang et al., 2012). Despite the many studies of athletes who undergo a long-term training program, no data are available that would enable this matter to be addressed definitively (McFarlin et al., 2005; Woods et al., 1999a).

Another important difference we have noted in athletes is the respectively lower and higher levels of CD4+ and CD8+ T-cells. This reorganization of the T-lymphocyte compartment is common in aging immune systems, where the CD4:CD8 ratio may even be inverted. This last parameter is included in the Immune Risk Profile (IRP), which has been defined as an indicator to predict two-year mortality in a population-based sample of Swedish octogenarians (Strindhall et al., 2007; Wikby et al., 1998). Recently it has been shown that characteristics of the IRP may also be present in young people (Turner et al., 2013). IRP may be summarized by the presence of an inverted CD4/CD8 ratio, expanded populations of CD8+CD28^{null} memory T-cells, and CMV seropositivity (Olsson et al., 2000). Recently, it has been reported that not only the CMV infection but also the titer of CMV-specific antibodies is crucial for determining the higher T-cell differentiation degree and the risk of increased mortality Alonso-Arias et al., 2013; Roberts et al., 2012; Strandberg et al., 2009). In our study, differences due to activity level were found even after adjusting comparisons for CMV serological status. Moreover, it is well known that functional ability of elderly individuals markedly influences the degree of immunosenescence (Moro-Garcia et al., 2012; Simpson et al., 2012). At this point we should mention that our group of elderly non-athletes was in excellent physical condition and did sufficient physical activity for their age. None of the members of this group were dependent and all of them could perform all their daily activities without any difficulty.

Interestingly, in the initial study of the T-cell subsets, we found in high volume physical activity individual a more-differentiated status in CD4+ T-cell compartments in young and elderly donors. In contrast, we found no change in the CD8+ T-cell subpopulations. Previous studies have reported that the homeostasis of the CD4+ T-cell population is much stricter than that experienced by the population of CD8+ T-cells. In fact, the aging of lymphocyte populations was described primarily in CD8+ T-lymphocytes, where these changes occur more precociously (Czesnikiewicz-Guzik et al., 2008; van Lier et al., 2003). According to these results, the increase in the degree of differentiation in young athletes is not completely correlated with that produced as a consequence of physiological aging. However, when we quantified the TREC content of the CD4+ and CD8+ T-cells in the young groups, we found significant differences only in the CD8+ T-cells, in which the TREC content was higher in young non-athletes. This apparent discrepancy may have arisen because the distribution of recent thymic emigrants expressing CD31 in naïve cells and the proportion of pE1 (EMRA CD27+CD28+) and E (EMRA CD27^{null}CD28^{null}) in the EMRA subset. The frequency of cells co-expressing CD45RA and CD31 is a reflection of thymic activity. Our results suggest that levels of CD8+ naïve T-cells are maintained due to expansion of these cells in the periphery, which leads to a reduced frequency of CD31+ cells in young athletes. This division in the periphery would explain the reduced levels of TREC we found. In the case of CD4+ T-cells, young athletes present elevated levels of pE1 population (EMRA CD27+CD28+), which might compensate for the reduction in naïve CD4+T-cells. pE1 is a population of T-cells that is part of the EMRA subset, and possibly ontogenetically close to classic naïve cells but not to E (EMRA CD27^{null}CD28^{null}), which are the most differentiated cells. At least in CD8+ T-cells, the pE1 (EMRA CD27+CD28+) subset has phenotypic and functional features that are intermediate between naïve and effector cells, with measurable levels of TREC, with intermediate levels of granzyme B, perforin, and IFN-γ and with relatively potent cytolytic activity (Rufer et al., 2003).

All these phenotypic features of T-cells have consequences for functional abilities. Differences in lymphocyte subpopulations are marked in young and elderly athletes, but the main differences in the functional capacity of T-cells were also found among the groups of young volunteers. The cellular responses of young athletes were weaker and very similar to those of older individuals. This low activation and proliferation response has been described in previous studies (Baj et al., 1994; Lancaster et al., 2004), that reported that T-lymphocytes of athletes sustaining high levels of physical activity over a long period may have reduced functionality. A transient state of immunodepression may occur during periods of very intense exercise, and this may be responsible for the infections that athletes endure, such as viral respiratory infections and CMV reactivations (Gleeson and Bishop, 2005; Robson et al., 1999). High levels of stress-related hormones, particularly cortisol, and alterations in the ratio of pro-inflammatory:anti-inflammatory responses to exercise may be the cause. The repeated elevations of circulating cortisol with strenuous exercise sessions is related to thymic involution (Bauer et al., 2009), but, on the other hand, this highly differentiated profile could be due to the continuous encounter that athletes have with chronic antigen due to the immunodepression caused by strenuous training (Smithey et al., 2012). Specific cells for re-encountered antigens will proliferate and acquire a more differentiated phenotype simultaneously with a reduced ability to respond to pathogens. These processes might induce a change in the T-cell subsets profiles, similar to those found in the athlete groups. Moreover, this immunosuppressive effect could be used in the treatment of illnesses, such as autoimmune diseases, in which the functionality of the immune system is exacerbated.

In our study, we found that NK-cells had a much greater cytotoxic ability in the young group of athletes than in the young group of non-athletes. One possible explanation is that NK-cells may exert a compensatory mechanism that attempts to maintain adequate immunity. This solution has been described under other

Table 2
Exercise effects on immunological parameters.

	Young	Elderly
Immune phenotype		
CD4+	↓	↓
CD8+	↑	↑
NKs	↑	↑
B	=	=
CD4+ subsets		
Undifferentiated cells	↓	↓
Differentiated cells	↑	↑
CD8+ subsets		
Undifferentiated cells	=	=
Differentiated cells	=	=
Thymic emigrants		
CD4+	=	=
CD8+	↓	=
T-cell functionality		
CD4 + activation/proliferation	↓	=/↓
CD8 + activation/proliferation	↓	=
NK-cell functionality	↑	Not done

circumstances in which the T-cell response is diminished by a high degree of differentiation and possible immune dysfunction (Jadeski and Hoffman-Goetz, 1996; McFarlin et al., 2005; Wang and Weng, 2011).

In summary, our results suggest that frequent participation in high volume exercise throughout life is associated with phenotypic and functional changes in the adaptive immune response with a high degree of differentiation, and with less functional responsiveness. These differences are more pronounced in young people with prolonged high levels of physical activity, but they appear to be reduced as physiological aging occurs. This also suggests that there may be compensatory mechanisms that help maintain the immune health of athletes.

Funding

This work was supported by the Red de Investigación Renal (REDinREN), Spain, and FIS FIS-Fondos FEDER European Union PI1202587 from the Instituto Carlos III (Spain).

This work was awarded first prize in the Premio Nacional de Investigación en Medicina del Deporte (2011), Escuela de Medicina del Deporte, Universidad de Oviedo.

Acknowledgments

We thank Isabel Cuevas Pérez and José María Díaz Pérez for their excellent technical assistance and Pablo Martínez Camblor (CAIBER, Oficinade Investigación Biosanitaria (OIB), Asturias, Spain) for his contribution to statistical analysis. We would also like to acknowledge the participation of all the volunteers of the Santa Teresa nursing home (Oviedo, Spain), and all the rowers of R.D.S. Astillero (El Astillero, Spain).

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III.8. OTROS TRABAJOS RELACIONADOS CON LA TESIS DOCTORAL

Durante el desarrollo de esta Tesis Doctoral he tenido la oportunidad de participar en otras líneas de investigación. También he podido participar en la elaboración de dos revisiones sobre el envejecimiento del sistema inmune, especialmente centradas en las poblaciones de linfocitos T.

ARTÍCULO 8:

Rebeca Alonso Arias, Beatriz Suárez Alvarez, Antonio López Vázquez, **Marco Antonio Moro García**, José Baltar, Ernesto Gómez Huerta, Teresa Ortega, Francisco Ortega, Carlos López Larrea. *"CD127^{low} expression in CD4+CD25^{high} T-cells as immune biomarker of renal function in transplant patients".* Transplantation. 2009 Aug 15;88(3 Suppl):S85-93.

ARTÍCULO 9:

José Ramón Vidal Castiñeira, Antonio López Vázquez, Rebeca Alonso Arias, **Marco Antonio Moro García**, Pablo Martinez Camblor, Santiago Melón, Jesús Prieto, Rosario López Rodriguez, Paloma Sanz Cameno, Luis Rodrigo, Rosa Pérez López, Ramón Pérez Álvarez, Carlos López Larrea. *"A predictive model of treatment outcome in patients with chronic HCV infection using IL28B and PD-1 genotyping".* J Hepatol. 2012 Jun;56(6):1230-8.

ARTÍCULO 10:

Marco Antonio Moro García, Rebeca Alonso Arias, Carlos López Larrea. *"Molecular mechanisms involved in the aging of the T-cell immune response".* Curr Genomics. 2012 Dec;13(8):589-602.

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IV. DISCUSIÓN

IV.1. ENVEJECIMIENTO DEL SISTEMA INMUNE

El envejecimiento se asocia con una disminución en la función inmune, con una mayor susceptibilidad a infecciones, incidencia de cáncer y autoinmunidad (Linton & Dorshkind 2004; McElhaney & Effros 2009; Pawelec *et al.* 2010). El envejecimiento también se relaciona con una reducida respuesta a la vacunación, incluida la de la vacuna contra el virus de la gripe (Haynes & Swain 2006). En la actualidad, las personas pueden vivir hasta los 80-100 años, mucho más tiempo que nuestros antepasados. El sistema inmunológico humano tiene que hacer frente a lo largo de un periodo de tiempo mucho más largo a la exposición frente una variedad de antígenos "crónicos" que es la base de la inmunosenescencia, y para lo cual no hemos evolucionado, con el consiguiente aumento en la morbilidad y la mortalidad debido a infecciones y patologías relacionadas con la edad (Gavazzi & Krause 2002; Macaulay *et al.* 2012). Este proceso de immunosenescencia no se produce únicamente durante el envejecimiento fisiológico, sino también en algunas patologías en las que la activación inmune y la inflamación están muy generalizadas. De esta manera, algunas enfermedades autoinmunes, como la artritis reumatoide (Thewissen *et al.* 2005), y algunas enfermedades infecciosas, como la infección por el VIH (Appay *et al.* 2007), se caracterizan por un fenotipo inmunosenescente. Tanto el envejecimiento fisiológico, como las patologías asociadas a la inmunosenescencia se asocian con un estado de inflamación mantenido, que se cree que es uno de los procesos básicos que conducen al propio envejecimiento del sistema inmune (Zanni *et al.* 2003; Candore *et al.* 2010). El proceso de envejecimiento parece alterar las dos ramas del sistema inmunológico, la innata y la adaptativa, pero de diferente manera. Mientras que la respuesta inmune adaptativa sufre profundas modificaciones dependientes de la edad (Haynes & Maue 2009), la inmunidad innata parece mejor conservada a lo largo de la vida (Dace & Apte 2008; Le Garff-Tavernier *et al.* 2010). A pesar de esto, datos recientes muestran que el envejecimiento se asocia con una activación inmune innata crónica y con cambios significativos en la función de los monocitos (Hearps *et al.* 2012).

Dos características que los linfocitos T adquieren a medida que se diferencian son una pérdida de la capacidad proliferativa y la adquisición de marcadores típicos de células NK. Estos dos desórdenes pueden deberse a: acortamiento telomérico, cambios en la señalización celular, alteraciones en la interacción del sistema innato con el adquirido, reparación errónea del DNA y mecanismos oxidativos, cambios epigenéticos, estimulación antigénica persistente y factores anti-inflamatorios alterados.

Un campo de acción prometedor para intentar revertir los efectos que el envejecimiento produce sobre la respuesta inmune sería intentar mantener la funcionalidad del timo, mediante la cual podríamos evitar la pérdida de las células T naïve y ralentizar la acumulación de células T $CD28^{null}$ agotadas y envejecidas. Los estudios preclínicos y clínicos de los efectos de esteroides sexuales, factor de crecimiento de queratinocitos (KGF), hormona del crecimiento (GH), las citocinas IL-7, IL-12 e IL-15, factor de crecimiento de insulina-1 (IGF-1) y la grelina sobre la remodelación de los linfocitos T indican que estas estrategias se pueden utilizar para paliar los efectos de la inmunosenescencia sobre estas poblaciones linfocitarias en los ancianos. También sería interesante tratar de regular los mecanismos epigenéticos que se activan en el proceso de diferenciación de las células T, con fármacos que controlan estos mecanismos , tales como los inhibidores de deacetilasas (HDACs) de clase I y de clase II, evaluados especialmente en terapias contra el cáncer y enfermedades inflamatorias. Lo que parece evidente es que futuros estudios nos ayudarán a desarrollar nuevas estrategias para frenar o revertir la disfunción inmunológica asociada a la edad.

IV.2. LINFOCITOS T CD4+ E INMUNOSENESCENCIA. IMPLICACIONES FENOTIPICAS Y FUNCIONALES.

Los linfocitos T CD4+ a medida que se van diferenciando adquieren determinadas características propias de células senescentes como es la aparición de receptores celulares clásicos de células citotóxicas. Dentro de estas moléculas se encuentra el receptor celular NKG2D. Mediante diversos experimentos hemos demostrado que la expresión de NKG2D en las células T CD4+ es significativamente mayor en personas de edad avanzada que en adultos jóvenes. Esta subpoblación celular CD4+NKG2D+ exhibe un fenotipo y unas funciones típicas de células totalmente diferenciadas que han sido objeto de un elevado número de divisiones celulares. También encontramos que los linfocitos T de los individuos de edad avanzada con un elevado porcentaje de células CD4+NKG2D+ presentaban un fenotipo muy maduro, tanto en las células CD4+ como en las CD8+, apoyando la hipótesis de que el NKG2D puede ser un marcador útil para la evaluación del grado de envejecimiento global del sistema inmunológico.

Los cambios que afectan a la inmunidad adaptativa han sido mucho más estudiados en linfocitos T CD8+ que en CD4+ y hasta el momento no se había descrito ningún buen marcador de senescencia dentro de la población de linfocitos T CD4+. La expansión de esta población puede ser explicada por la acumulación de células T CD4+ efectoras muy diferenciadas que,

como consecuencia de un contacto muy persistente con el antígeno, adquieren nuevas propiedades, entre las que se podría encontrar la expresión de NKG2D.

Por otra parte, la expresión de la molécula coestimuladora CD28 distinguió dos subpoblaciones de células T CD4+NKG2D+ con diferentes propiedades y estado de diferenciación. Hay que destacar que las diferencias encontradas entre los ancianos y los jóvenes se deben principalmente a las células T CD4+CD28^{null}NKG2D+. Estas células expresaron un patrón de marcadores fenotípicos y acúmulos intracitoplasmáticos de granzima B y perforina, similar al que presentan las células CD4+CD28^{null}. También la especificidad antigénica fue similar, estando dirigida hacia antígenos crónicos. Estos resultados apoyarían el papel inductor de los antígenos crónicos en la expresión de NKG2D, pudiendo constituir un marcador de maduración celular en fase terminal ontogénicamente más diferenciado que la ausencia de CD28. El fenotipo terminal más diferenciado asociado a la expresión de NKG2D se corroboró por el menor contenido de TRECs que presentan estas células, probablemente como consecuencia de haber sufrido una proliferación mayor frente a antígenos específicos.

Esta hipótesis también es apoyada por la mayor capacidad de las células NKG2D+ para producir IFN-γ en respuesta a la estimulación del TCR. La producción de IFN-γ está presente en todas las etapas de diferenciación celular, pero está muy aumentada en estadios finales, en los que las células T pierden la capacidad de producir IL-2 (Yue *et al.* 2004; Harari *et al.* 2005). Según esto, la producción predominante de IFN-γ por las células T CD4+ se asocia con persistencia y niveles aumentados de antígenos, entre los que el principal candidato sería CMV. La infección por CMV representa un modelo de persistencia antigénica con reactivaciones transitorias, sobre todo en individuos inmunodeprimidos y se ha asociado con anterioridad a la expresión de NKG2D en células T CD4+ (Saez-Borderias *et al.* 2006; Alonso-Arias *et al.* 2009). Sin embargo, la expansión de la población CD4+NKG2D+ encontrada en un reducido número de ancianos seronegativos para CMV sugiere la existencia de otros factores implicados en la expresión de esta molécula. No se puede excluir que otras infecciones virales persistentes y/o la presencia de citocinas pro-inflamatorias producidas durante algunos procesos infecciosos puedan conducir a su aparición. Otra posible explicación es el hecho corroborado de que en edades avanzadas existe un aumento de las respuestas inflamatorias crónicas, evidenciado por el aumento de los niveles séricos de citocinas proinflamatorias (IL-6, IL-15, IL-8), factores de coagulación, y especies reactivas del oxígeno (Mari *et al.* 1995; Forsey *et al.* 2003; Zanni *et al.* 2003; Ferrucci *et al.* 2005; Giunta *et al.* 2008).

Nuestro hallazgo más importante relacionado con la expresión de NKG2D en las células T CD4+ es su asociación con un fenotipo global más senescente y diferenciado en la población

de linfocitos T CD4+. Así, el análisis fenotípico y funcional de las células T en donantes de edad avanzada mostró una disminución significativa de células T naïve y un marcado aumento de las células con un fenotipo efector o de memoria. La correlación entre la proporción de células efectoras CD4+ y la infección latente por CMV ha sido ya demostrada en ancianos, pero la expresión de NKG2D en linfocitos T CD4+ es el primer marcador que define una senescencia global en el compartimento de las células T. El diferente grado de diferenciación en las subpoblaciones de linfocitos T ya se había descrito entre jóvenes y ancianos, pero en este estudio hemos demostrado que los niveles de expresión de NKG2D definen el estado de diferenciación en células T CD4+ y T CD8+ mejor que la edad del individuo. Todo esto puede ser el resultado de un efecto no específico del ambiente pro-inflamatorio sobre el estado de diferenciación de las células T, que podría contribuir simultáneamente a la expansión de la población CD4+NKG2D+.

La homeostasis de estas células altamente diferenciadas, principalmente CD4+CD28^{null}, no depende tanto del contacto TCR con MHC-péptido, ni de la citocina IL-7, sin embargo, se sabe que entre los factores más importante en el mantenimiento y supervivencia de las células diferenciadas se encuentra la citocina IL-15. En células menos diferenciadas la citocina más importante implicada en homeostasis celular es la IL-7, pero a medida que las células van diferenciándose el papel de la IL-15 aumenta (Figura 18). Varios estudios han demostrado que la IL-15 aumenta y/o mantiene la expresión y la función de la molécula NKG2D en linfocitos T (Roberts *et al.* 2001; Groh *et al.* 2003). La IL-15 funciona como un regulador clave en la activación y función de los linfocitos T citotóxicos, entre ellos la población CD4+NKG2D+, permitiendo la expresión de esta molécula bajo condiciones inflamatorias.

La causa de la pérdida de CD28 en las células T en ancianos se ha atribuido a la repetida estimulación antigénica (Valenzuela & Effros 2002; Vallejo 2005), y ahora se acepta que las células CD28^{null} han experimentado episodios de activación y división celular. Sin embargo, la pérdida de CD28 puede deberse no solo a la activación repetida del TCR, sino también a proliferación homeostática. La IL-15 media este tipo de proliferación, sin activación antigénica, con la consiguiente pérdida de CD28 de manera estable en los linfocitos T memoria, en parte mediante la inducción de la secreción de TNF-α (Chiu *et al.* 2006). En nuestro laboratorio encontramos un aumento significativo en la proporción de células T sin expresión de CD28 en respuesta a IL-15, que se puede atribuir a un aumento en la proliferación de la población CD4+CD28^{null} respecto a la población CD4+CD28+. La IL-15 podría contrarrestar los defectos descritos en la capacidad proliferativa de las células T CD28^{null} (Appay *et al.* 2002b), incluso en las poblaciones EMRA más diferenciadas.

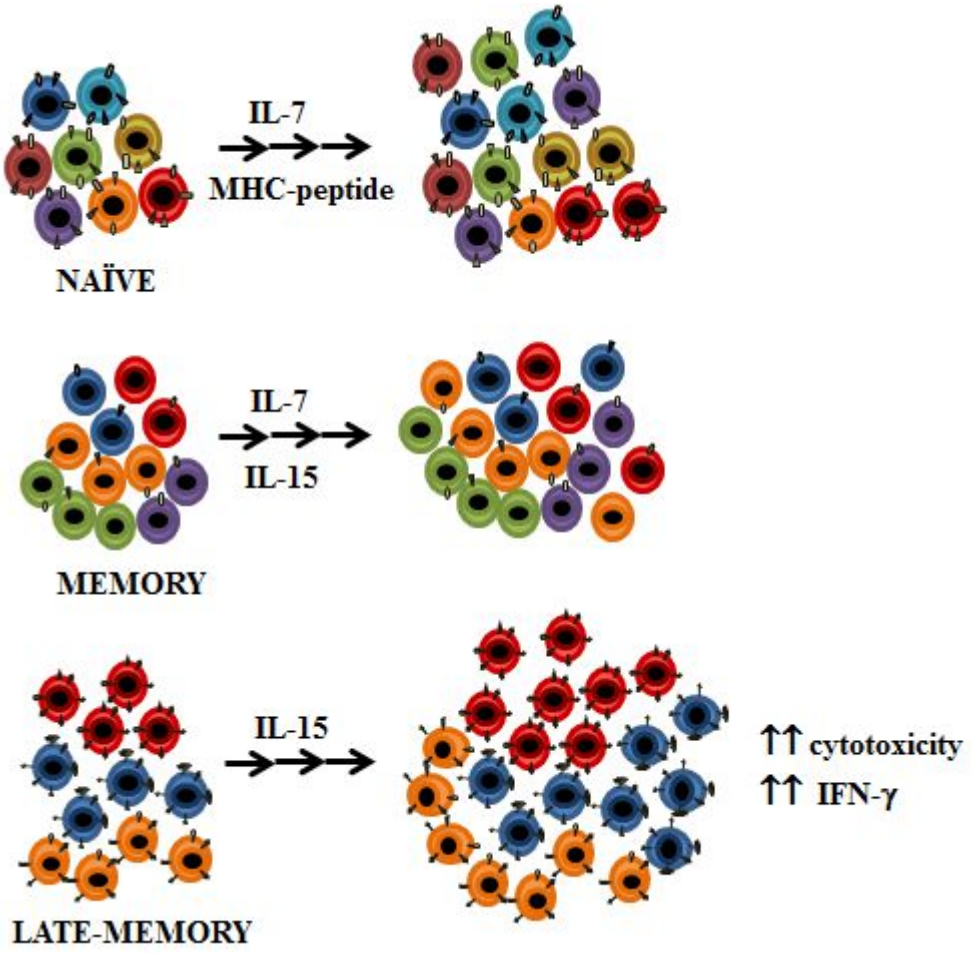

Figura 24. Efecto de las citocinas homeostáticas IL-7 e IL-15 sobre los linfocitos T.

La citocina IL-15 se encuentra a muy altos niveles en algunas patologías inflamatorias y autoinmunes, y se ha especulado que la exposición crónica a esta citocina podría ser uno de los mecanismos que producen la expansión de la población CD4+CD28^{null} in vivo (Yamada *et al.* 2007). Esta población expresa marcadores de activación con muy poca evidencia de proliferación (Appay *et al.* 2002b) y presenta un umbral de activación muy bajo a estimulación vía TCR, que podría estar implicado en la predisposición de estas células a romper la auto-tolerancia (Yung *et al.* 1996). En cuanto a la expresión del receptor para esta citocina, se ha visto que la cadena α del receptor de la IL-15 no es imprescindible en las células T CD8+ para inducir la proliferación mediada por esta citocina (Dubois *et al.* 2002). En las células CD4+, observamos que no es necesario un aumento en la expresión de este receptor para inducir la activación del STAT5, el mediador en la vía de activación de IL-15. Se ha postulado que las células memoria son más dependientes del STAT5 que las células efectoras (Purton *et al.* 2007; Tripathi *et al.*

2010), de acuerdo con esto, nosotros encontramos que el efecto activador de la IL-15 sobre el STAT5 es más pronunciado en las células T CD4+CD28^{null} que en las células T CD4+CD28+.

La IL-15 aumenta las propiedades citotóxicas de la población CD4+CD28^{null} y potencia sus respuestas antígeno específicas. La expresión de granzima B y perforina en las células T CD4+ se relaciona con la pérdida de la molécula CD28 en la superficie celular. Estas células CD4+CD28^{null} se parecen mucho a las células T CD8+ citotóxicas, ya que su capacidad citotóxica está mediada por estimulación vía TCR, además, no requieren de moléculas coestimuladoras clásicas (Appay *et al.* 2002b). A pesar de que el papel de las células CD4+ como células citotóxicas no es muy conocido, el efecto potenciador de su respuesta mediado por IL-15 puede tener gran impacto, debido a su especificidad frente a antígenos crónicos. La IL-15 juega un papel primordial en la respuesta inmune temprana y en inflamaciones crónicas, amplificando los efectos de las citocinas pro-inflamatorias en la secreción de IFN-γ (Smeltz 2007). Relacionado con esto, el pre-tratamiento con IL-15 potencia la respuesta antígeno específica de las células CD4+CD28^{null} frente al CMV, aumentando la frecuencia de las células productoras de IFN-γ, sin ejercer efecto sinérgico en la población CD4+CD28+. Todos estos efectos de la IL-15 sobre las poblaciones más diferenciadas del sistema inmune podrían ser de gran relevancia para el tratamiento de personas ancianas con respuestas adaptativas muy menguadas, donde esta citocina podría aliviar las deficiencias de la respuesta de los linfocitos T y así mejorar la función inmune.

Como hemos comentado anteriormente, la infección por CMV produce gran diferenciación celular y una acumulación de estas células altamente diferenciadas. Todos los datos sugieren que otra probable causa de la pérdida de expresión de la molécula CD28 sea la exposición repetida a los mismos antígenos como el propio CMV u otras infecciones virales persistentes. La infección crónica por CMV representa un modelo de persistencia antigénica con reactivaciones periódicas, sobre todo en pacientes inmunodeprimidos (Goronzy & Weyand 2005). En nuestro laboratorio hemos demostrado que el título de anticuerpos anti-CMV se relaciona con el fenotipo y el estado funcional de su sistema inmune en ancianos pero no en individuos jóvenes. La alta tasa de infección que presenta el virus de CMV hace que tenga una alta sero-prevalencia, que se ha estimado que está entre el 30% y el 90% en países desarrollados, donde aumenta con la edad (Staras *et al.* 2006). Las reactivaciones tras periodos de latencia ocurren rutinariamente en individuos sanos infectados y crónicamente en ancianos (Stowe *et al.* 2007). La relación entre los niveles de anticuerpos anti-CMV y la evolución de la infección no ha sido estudiada en profundidad y no se comprende completamente. En nuestro estudio encontramos que los individuos de mayor edad presentaban unos títulos

significativamente superiores que los jóvenes. Una posible explicación sería que los títulos de anticuerpos anti-CMV serían un reflejo de la historia de la infección. Sobre esto, podemos decir que los individuos con DNA de CMV detectable en monocitos tienen porcentajes significativamente mayores de células T CD8+ específicas frente a CMV, sin embargo, no ha sido posible determinar la asociación con el título de anticuerpo anti-CMV. La detección del DNA de CMV en monocitos podría ser un marcador de las reactivaciones actuales, mientras que la respuesta celular asociada podría reflejar la expansión de las células T memoria para controlar las sucesivas reactivaciones (Leng *et al.* 2011). Nosotros encontramos una asociación entre los títulos de anticuerpos anti-CMV y el fenotipo de las células T CD4+ en ancianos, pero no en jóvenes. Esto podría ser explicado por los diferentes niveles de anticuerpos en los dos grupos de edad, pero cuando comparamos el grado de diferenciación entre jóvenes y ancianos con igual título de anticuerpos frente a CMV, encontramos que, como cabía esperar, las diferencias no se pueden explicar solo por el título de anticuerpos anti-CMV. De todas formas, los cambios que encontramos en ancianos fueron más acusados en los linfocitos T CD4+ que en CD8+ y las diferencias con los individuos jóvenes fueron mayores a medida que aumentaba el título de anticuerpos. La acumulación de células T altamente diferenciadas podría no ser la única razón para los cambios en el fenotipo de los linfocitos T, y el deterioro en la capacidad de producir nuevas células T naïve en ancianos también contribuiría a este envejecimiento del fenotipo.

En humanos se produce una disminución muy importante en el número de EMR con el envejecimiento (McFarland *et al.* 2000). A pesar de esta degeneración del timo, la aparición de linfopenia es muy extraña en ancianos y el número de células T se mantiene a lo largo de la vida, probablemente debido al aumento de las células de memoria altamente diferenciadas. Estos linfocitos T altamente diferenciados podrían llenar el espacio inmunológico y distintos mecanismos homeostáticos bloquearían la generación de nuevas células naïve para mantener el número de linfocitos T periféricos. Estos mecanismos hacen difícil preservar la diversidad del repertorio de linfocitos T que combaten nuevos patógenos, así como la capacidad del huésped de montar respuestas frente a infecciones recurrentes (Nikolich-Zugich 2008). De hecho, la pérdida de funcionalidad con el aumento de la diferenciación y la reducción en el número de células naïve podrían ser los responsables de la deficiente respuesta frente a patógenos y de la pobre respuesta a la vacunación frente al virus de la gripe que hemos visto en los ancianos infectados por CMV (Trzonkowski *et al.* 2009). La respuesta defectuosa a nuevos antígenos podría ser consecuencia de la inmunosupresión relacionada con la infección por CMV.

La magnitud de la infección por CMV puede estar influenciada por factores genéticos del huésped. En pacientes VIH y en trasplantados renales, la presencia del HLA-DR7 se ha

correlacionado con un aumento del riesgo de infección por CMV y con una respuesta muy pobre frente a esta infección (Kraat *et al.* 1993; Schrier *et al.* 1995). Se ha postulado que el control insuficiente de la infección en pacientes HLA-DR7 puede llevar a una reactivación continuada del virus, contribuyendo a la expansión clonal de poblaciones específicas de linfocitos T CD4+ restringida frente a un número limitado de antígenos de CMV (Alonso-Arias *et al.* 2009). En nuestro estudio, en individuos sanos, nosotros no encontramos ninguna asociación entre el título de anticuerpos frente a CMV y los alelos HLA-DR que presentan los individuos. Además, tampoco encontramos asociación con distintos polimorfismos de TNF-α, aunque se considera que es un mediador en las reactivaciones del CMV. A su vez, el TNF-α, que se encuentra aumentado en ancianos e infecciones por CMV induce la pérdida de expresión de la molécula co-estimuladora CD28 y la diferenciación celular (Bryl *et al.* 2005). A pesar de los altos niveles de anticuerpos anti-CMV y de la alta frecuencia de la población $CD28^{null}$ en los linfocitos T, no encontramos ninguna asociación entre la infección por CMV y los niveles de TNF-α en el suero de los individuos en estudio. Sí encontramos una asociación significativa con los niveles de la citocina IL-15, implicada también en la pérdida de CD28 en linfocitos T CD8+ (Chiu *et al.* 2006). Clásicamente se ha aceptado que el efecto de la IL-15 era mucho más pronunciado en los linfocitos T CD8+ que en linfocitos T CD4+. Sin embargo, como vimos anteriormente, la citocina IL-15 tiene un efecto muy potente en las células T CD4+, con mucha mayor capacidad para activar células $CD28^{null}$ que CD28+.

En resumen, hemos visto que en individuos ancianos se produce un aumento de linfocitos T CD4+ altamente diferenciados que se caracterizan, entre otras cosas, por la adquisición de marcadores propios de células citotóxicas, principalmente células NK y por la pérdida de la molécula coestimuladora CD28. Estos fenómenos son consecuencia de las sucesivas rondas de activación que el sistema inmune de los individuos sufre a lo largo de toda su vida, y puede ser acelerado por la activación crónica inducida por la persistencia de determinadas infecciones virales u otro tipo de antígenos. La IL-15 juega un papel importante en la potenciación de la activación, proliferación y respuestas efectoras en estas poblaciones altamente diferenciadas de linfocitos T CD4+ (principalmente en las poblaciones que han perdido la expresión de la molécula $CD28^{null}$) contra sus antígenos específicos. Por último, los niveles de anticuerpos anti-CMV y la seropositividad frente al CMV se relacionan con el grado de diferenciación celular y con la inmunocompetencia en ancianos. Los títulos elevados de anticuerpos anti-CMV podrían ser tanto una consecuencia como una causa de la inmunosenescencia, estos niveles podrían ser utilizados como marcador pronóstico del deterioro del sistema inmune y del riesgo de desarrollar patologías asociadas a la edad.

IV.3. INMUNOSENESCENCIA Y CAPACIDAD FUNCIONAL EN ANCIANOS

El proceso de envejecimiento conduce a un estado denominado fragilidad que precede a la incapacidad y por tanto, es un factor de riesgo muy a tener en cuenta en los individuos de ead avanzada. Esta fragilidad se caracteriza por una reserva fisiológica disminuida con pérdida de resistencia, aumento de la vulnerabilidad y disminución de la capacidad del organismo para responder al estado de estrés que producen los procesos patológicos. Todos estos parámetros pueden estar a su vez asociados o condicionados por el grado de inmunosenescencia que presenta el individuo. De hecho, hemos demostrado que existe una clara asociación entre el descenso en la capacidad funcional en ancianos y la mayor diferenciación del sistema inmune. Cuando agrupamos a los ancianos por su capacidad funcional encontramos diferencias en la distribución y estado de diferenciación de sus poblaciones leucocitarias, así como en la respuesta celular tanto *in vitro* como *in vivo* cuando estudiamos la respuesta a la vacunación de la gripe. Por otra parte, vimos que los ancianos con un peor estado funcional presentaban niveles más elevados de anticuerpos frente a CMV y una mayor respuesta celular frente a este virus que los ancianos con un mejor estado funcional. Por lo tanto, demostramos que existe una relación entre la intensidad de la respuesta a CMV, es estado funcional del sistema inmune y la capacidad funcional en ancianos.

Aunque varios estudios han demostrado que múltiples aspectos de la respuesta inmune innata se ven afectados por el envejecimiento, en nuestro estudio solamente encontramos cambios significativos en los niveles de células NK. Como es bien sabido, el número de células NK aumenta con la edad (Sansoni *et al.* 1993; Miyaji *et al.* 2000; Le Garff-Tavernier *et al.* 2010). Este aumento fue más acusado en los ancianos conforme empeoraba su estado funcional, con un incremento gradual desde el grupo con una mejor capacidad funcional al grupo con peor estado funcional. Esto puede deberse, a que las células NK desempeñan un papel muy importante en la inmunovigilancia frente a CMV (Lopez-Botet *et al.* 2004; Guma *et al.* 2006), es posible que las células NK se incrementen en estos ancianos con el fin de controlar las reactivaciones de CMV, más frecuentes en los ancianos con peor estado funcional, como se deduce del título aumentado de anticuerpos frente a CMV que presentan.

Es bien sabido que con la edad, se produce una acumulación de células diferenciadas (EM y EMRA) y una disminución de las células inmaduras y poco diferenciadas (naïve y CM) (Zanni *et al.* 2003; Effros *et al.* 2005; Taub & Longo 2005; Koch *et al.* 2008; Appay *et al.* 2010). Cuando analizamos la distribución de estas poblaciones en nuestros grupos de ancianos no encontramos diferencias en el grado de diferenciación de los linfocitos T CD8+, posiblemente

debido a que se encuentran todos en los estadios finales de diferenciación. Sin embargo, en los linfocitos T CD4+ sí existió una asociación entre el grado de deterioro funcional y el grado de diferenciación celular. Además, el contenido en TREC de las células T CD4+ en ancianos con peor estado funcional estaba significativamente reducido, corroborando el mayor número de divisiones sufridas por las células más diferenciadas. El diferente comportamiento de los linfocitos CD8+ y CD4+ podría explicarse debido a que, en general, las poblaciones de linfocitos T CD4+ y CD8+ sufren los mismos cambios fenotípicos fundamentales pero la velocidad a la que estos cambios se producen o se acumulan con la edad es diferente. La aparición de células altamente diferenciadas en linfocitos T CD8+ se inicia antes que en las células T CD4+, posiblemente debido a que la homeostasis de los linfocitos T CD8+ es mucho menos estricta que la de las células T CD4+ (Czesnikiewicz-Guzik *et al.* 2008). Es decir, en nuestros grupos de ancianos es posible que el alto grado de diferenciación que existe en las subpoblaciones de linfocitos T CD8+ en todos los grupos estudiados nos impida ver la relación entre estas subpoblaciones y el estado funcional de los individuos.

La capacidad de activación a través de la estimulación del TCR con anti-CD3 fue significativamente menor en el grupo de ancianos con peor situación funcional tanto en linfocitos T CD4+ como en linfocitos T CD8+. Esta disminución de la respuesta puede deberse al envejecimiento funcional de las poblaciones celulares. De hecho, se sabe que las células altamente diferenciadas pierden su capacidad de proliferar en respuesta a estimulación (Appay *et al.* 2002a). En cambio, la respuesta específica de los linfocitos T CD4+ frente al CMV fue mayor en los grupos con peor capacidad funcional. La limitación del repertorio de células T y el alto grado de diferenciación provocado por la infección por CMV puede estar detrás de la disminución de la respuesta de células T frente a anti-CD3 en los ancianos con peor estado funcional. La importancia de la respuesta específica de los linfocitos T CD4+ frente a CMV en los ancianos ha sido menos estudiada que el efecto de este virus sobre las poblaciones de linfocitos T CD8+, sin embargo, en nuestro trabajo, encontramos diferencias en la respuesta a CMV en los linfocitos T CD4+, pero no en las células T CD8+. Este hecho puede tener relación con las diferencias en la homeostasis entre CD4+ y CD8+ mencionados previamente. La respuesta de las células T CD8+ se agotaría antes, mientras que la funcionalidad de las células T CD4+ podría mantenerse sin cambios por más tiempo.

El impacto clínico más profundo del envejecimiento del sistema inmune en personas de edad avanzada es la respuesta a la inmunización. La capacidad de los ancianos para responder a la vacuna antigripal trivalente es reducida y la edad se correlaciona negativamente con la producción de anticuerpos contra el virus de la gripe, lo que nos demuestra el papel crucial de la

inmunosenescencia en la capacidad de respuesta frente a la vacunación (Goronzy *et al.* 2001). En nuestro estudio la peor respuesta a la vacunación contra el virus de la gripe se encontró en los ancianos de los grupos con peor capacidad funcional. Esto podría explicarse, en parte, por un reducido porcentaje de células B que presentaban los grupos de ancianos con peor capacidad funcional. Por otra parte, se sabe que los linfocitos T CD4+CD28^{null} tienen alterada su capacidad para coestimular a los linfocitos B, lo que sugiere que la acumulación de linfocitos T CD4+CD28^{null} podría estar implicada en la peor respuesta humoral en los ancianos. No obstante, el aumento en el título de anticuerpos frente a CMV, correlacionado negativamente con el título de anticuerpos desarrollados frente a la vacuna de la gripe, y el consiguiente aumento de células implicadas en la respuesta frente a CMV puede suponer la mayor limitación en la respuesta a la vacunación por la reducción en los niveles de células T naïve.

Existen un gran número de patologías en ancianos que se relacionan con una capacidad funcional reducida e incluso con una incapacidad total para la realización de las actividades de la vida cotidiana. Una de estas patologías es la insuficiencia cardiaca crónica (ICC) que tradicionalmente se ha caracterizado por trastornos hemodinámicos y neurohormonales. Estudios recientes sugieren que la activación inmune y la inflamación juegan un papel muy importante en el avance de la patología (Dixon *et al.* 2011; Consoli *et al.* 2013). De hecho, cuando estudiamos un grupo de pacientes con ICC encontramos que los pacientes con ICC avanzada presentaban un alto grado de inmunosenescencia y un estado inflamatorio severo.

Estos pacientes presentaban un número de leucocitos aumentado respecto al grupo control, este aumento es un marcador clásico de inflamación aguda o crónica y un marcador de riesgo cardiovascular. La población leucocitaria que se encontraba más aumentada eran los neutrófilos, tanto en porcentaje como en número absoluto. La neutrofilia se ha relacionado con la ICC descompensada (ADHF) en pacientes con infartos agudos de miocardio (Engstrom *et al.* 2009), incluso el ratio neutrófilos:linfocitos se ha utilizado para el pronóstico en ADHF (Uthamalingam *et al.* 2011). Desde el punto de vista de la inmunosenescencia, lo más interesante de este aumento de neutrófilos es su capacidad para producir IL-6 (Melani *et al.* 1993) y la respuesta de los propios neutrófilos a esta citocina, contribuyendo a la inflamación que observamos en ICC, así como a la inflamación de bajo nivel encontrada en ancianos.

Los cambios asociados a la edad del sistema inmune y el aumento en la secreción de citocinas por el tejido adiposo, representan las principales causas de la inflamación crónica, conocida como “inflammaging” (Franceschi *et al.* 2000). Los altos niveles de las interleucinas IL-6, IL-1, TNF-α y proteína C reactiva (PCR) se asocian en ancianos con un aumento de la morbilidad y de la mortalidad (Michaud *et al.* 2013). En ICC este estado inflamatorio es incluso

más marcado. Se han sugerido varios mecanismos no excluyentes entre sí que pueden explicar esta activación inmune como son: la activación neurohormonal, sobrecarga hemodinámica y la activación del sistema inmune innato secundaria a estrés cardiaco (Picano *et al.* 2010; Wrigley *et al.* 2011). Varios mecanismos implicados en la depresión cardiaca y en la progresión de la ICC como son la hipertrofia (Purcell *et al.* 2001) y la activación cardiotóxica de vías de muerte celular (Hamid *et al.* 2011), podrían ser una consecuencia de la respuesta inflamatoria exacerbada en estos individuos.

Esta inflamación mantenida a lo largo del tiempo produce una activación y proliferación continuada de los linfocitos T, llevando al envejecimiento de estas poblaciones. De hecho, hemos visto que altos niveles de IL-6 mantenidos a lo largo del tiempo se asocian con un deterioro más rápido y extenso de las poblaciones de linfocitos T. A su vez, estas células altamente diferenciadas producen grandes cantidades de citocinas pro-inflamatorias y tienen una capacidad reducida para responder frente a nuevos antígenos. Por tanto, la medida de estas citocinas en pacientes con ICC puede ser un buen marcador para detectar e intentar detener la inmunosenescencia y por lo tanto, reducir la susceptibilidad de estos pacientes a comorbilidades e infecciones. Además, todos los indicadores estudiados que nos indican un peor estado funcional y/o morfológico en los pacientes con ICC se relacionaron con unas poblaciones de linfocitos T más diferenciadas. Esto nos permite asociar el alto grado de inmunosenescencia en estos pacientes con la gran comorbilidad presente en ICC (Lee *et al.* 2011; Ather *et al.* 2012; Holmstrom *et al.* 2012; Koller *et al.* 2013).

Como resumen podemos afirmar que existe una relación entre el deterioro de la capacidad motora en las personas de la tercera edad y un estado de inmunosupresión, tanto en la respuesta celular *in vitro*, como en la respuesta a la inmunización *in vivo* (Figura 19). El estado funcional en las personas mayores puede estar influido por la “edad” de su sistema inmunológico o viceversa. Todos los parámetros inmunológicos que estudiamos se encontraban más comprometidos en los ancianos con peor estado funcional, a diferencia de lo que se observa en respuesta a CMV, con mayor título de anticuerpos y una mayor respuesta celular.

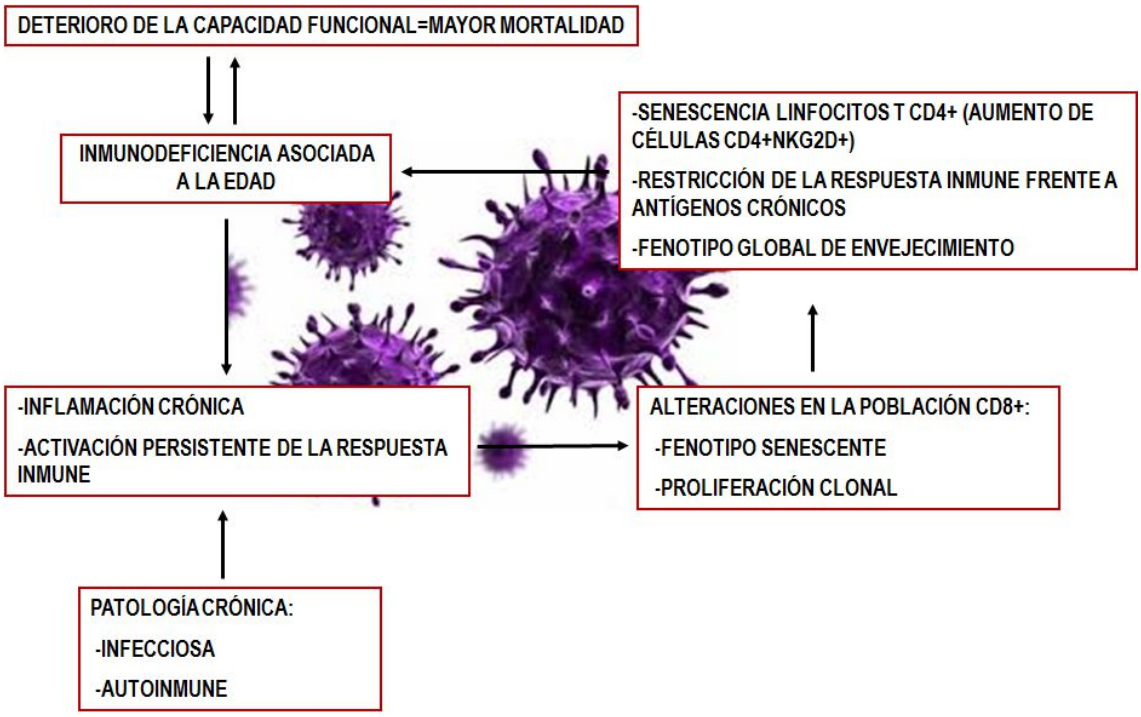

Figura 25. Posible cascada de acontecimientos en el proceso de inmunosenescencia. Las personas de edad avanzada suelen presentar un deterioro de su capacidad funcional, que está directamente relacionado con una mayor morbilidad y menor supervivencia. Este deterioro funcional puede ser causa o efecto de la inmunodeficiencia asociada a la edad, la cual implica una activación persistente del sistema inmune y un estado de inflamación crónico. A esta situación de inmunodeficiencia también se puede llegar por un proceso infeccioso (VIH) o por una enfermedad autoinmune (AR). A nivel celular las primeras manifestaciones que podemos apreciar en el proceso de inmunosenescencia son una respuesta alterada de los linfocitos T CD8+, así como su proliferación clonal en respuesta a ciertos antígenos persistentes. Por último, también se ve afectada la población de linfocitos T CD4+, con la aparición de la población CD4+NKG2D+, una respuesta muy dirigida frente a ciertos antígenos crónicos y en definitiva, la aparición de un fenotipo global de envejecimiento a nivel de linfocitos T. Todas estas características se relacionan con la inmunodeficiencia asociada a la edad. Uno de los principales desencadenantes de todos estos procesos sería la infección por CMV, aunque no el único.

También hemos demostrado que este empeoramiento del sistema inmune con la capacidad funcional se puede observar en patologías con un alto componente invalidante como es la ICC. El estadio de esta patología y los altos niveles de IL-6 mantenidos a lo largo del tiempo se asocian con una respuesta inmune adaptativa comprometida con un alto nivel de diferenciación celular, y con una menor respuesta funcional. Tanto los altos niveles de IL-6 como las poblaciones linfocitarias altamente diferenciadas se asocian con peor estado de la enfermedad. Basado en estos resultados, proponemos un modelo en el cual la ICC, inflamación e inmunosenescencia aumentan siguiendo un modelo de retroalimentación (Figura 20).

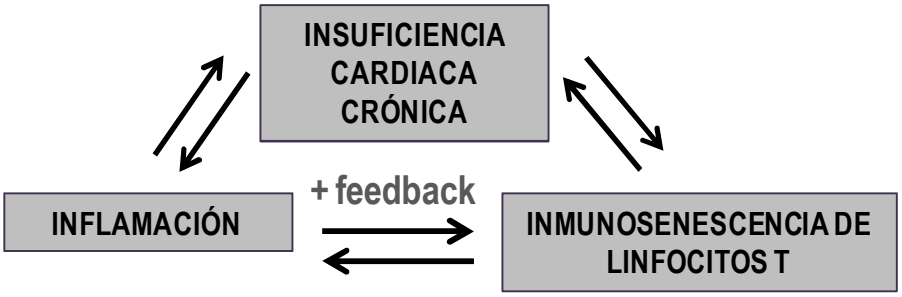

Figura 26. Modelo de los mecanismos de retroalimentación propuesto en ICC.

IV.4. MECANISMOS MODULADORES DE INMUNOSENESCENCIA

Debido a que el deterioro de la actividad física y funcional en ancianos puede estar directamente relacionado con el estado del sistema inmune, ralentizar o revertir el deterioro del sistema inmune podría mejorar la calidad de vida de los ancianos o de cualquier individuo con un sistema inmune comprometido. Una de las estrategias que más se están estudiando en los últimos años es el consumo de probióticos. Los probióticos son microorganismos vivos, bacterias o levaduras, que después de ser ingeridos transitan a través del intestino y pueden ejercer funciones específicas y beneficiosas en la salud humana. Los probióticos tienen un efecto inmunomodulador en el organismo, es decir, que contribuyen a regular el sistema inmune, defendiendo al organismo de una amplia variedad de agentes patógenos que pueden ocasionar diferentes enfermedades. Para estudiar los posibles efectos beneficiosos que el consumo de la cepa de *Lactobacillus delbrueckii* subsp. *bulgaricus* 8481 podría tener sobre el sistema inmune en personas de edad avanzada realizamos un estudio doble ciego, controlado con placebo y aleatorizado.

En nuestro estudio pudimos comprobar que tras el consumo del probiótico el porcentaje de células NK aumentó significativamente en los ancianos. Las células NK juegan un papel primordial en la inmunovigilancia frente a tumores y en las infecciones víricas, y se ha visto que la flora intestinal puede modular su actividad (Takeda & Okumura 2007). Por otra parte, los factores involucrados en el IRP incluyen el cociente invertido CD4/CD8, el aumento de las poblaciones más diferenciadas de linfocitos T y la seropositividad a CMV (Hadrup *et al.* 2006). Tras el consumo del probiótico el cociente CD4/CD8 aumentó y disminuyó el porcentaje de células T altamente diferenciadas ($CD28^{null}$), en cuanto al título de anticuerpos anti-CMV se mantuvieron sin cambios. En los individuos que consumieron el placebo no se observaron

cambios significativos a lo largo del estudio. El aumento de la población CD8+CD28^{null} se asocia con una respuesta inmune reducida frente a patógenos y frente a la vacunación en ancianos (Riha & Rudd 2010), por eso una disminución de esta población podría ayudar a prevenir patologías en ancianos.

Uno de los procesos más asociado al proceso de inmunosenescencia en un estado de inflamación crónica (Ferrucci *et al.* 2005). Los probióticos pueden inhibir la producción de citocinas pro-inflamatorias (Lopez *et al.* 2008). Los ancianos que consumieron las cápsulas con el probiótico durante seis meses presentaron una bajada en la concentración de IL-8 sérica. Esta citocina es uno de los mayores mediadores de la respuesta inflamatoria, por lo que su bajada podría ser muy beneficiosa para las personas de edad avanzada reduciendo la inflamación crónica.

El sistema inmune asociado a mucosas es una red integrada de tejidos, células linfoides, células mucosas asociadas a membrana, proteínas efectoras pertenecientes al sistema inmune innato (mucinas y defensinas), así como anticuerpos inducidos previamente. El consumo del probiótico se asoció con un aumento muy marcado de la concentración de la defensina hBD-2 en suero, sugiriendo una potenciación del sistema inmune innato a través de la inducción de la producción de esta molécula. Este aumento y el aumento en el número de células NK nos sugieren una mejora en el sistema inmune innato en los individuos que consumieron la cepa de *L. delbrueckii* subsp. *bulgaricus*.

En nuestro estudio vimos que los efectos beneficiosos que tenía el probiótico sobre el sistema inmune desaparecían tras seis meses sin consumirlo. Por lo tanto, sería necesario ingerir esta bacteria de manera regular para obtener resultados significativos y duraderos. Este dato sugiere que la bacteria solamente pasa a través del tracto digestivo, sin establecerse en el mismo. Es importante recalcar que todos los parámetros estudiados se evaluaron a nivel sistémico, en sangre periférica.

Otro mecanismo estudiado en nuestro laboratorio que en principio podría prevenir, ralentizar o revertir la diferenciación de las poblaciones celulares y por tanto de la respuesta inmune es la realización de ejercicio físico. Niveles moderados de actividad física se han asociado con un estado de salud óptimo y con un menor riesgo de padecer enfermedad coronaria, neurodegeneración, incidencia de cáncer y fragilidad (deterioro funcional) (Hambrecht *et al.* 2000; Speelman *et al.* 2011; Simpson *et al.* 2012). Además, los individuos ancianos sedentarios tienen un mayor riesgo de mortalidad que aquellos con un nivel moderado de actividad física. Uno de los efectos de la actividad regular puede ser sus propiedades preventivas y/o “rejuvenecedoras” sobre el envejecimiento del sistema inmune. De hecho, el fitness aeróbico

se ha asociado con una menor proporción de células senescentes y una mayor proporción de células T CD8+ naïve (Spielmann *et al.* 2011). Por el contrario, periodos frecuentes de ejercicio intenso o muy intenso o un volumen de ejercicio muy alto puede inducir efectos no deseados (Figura 21).

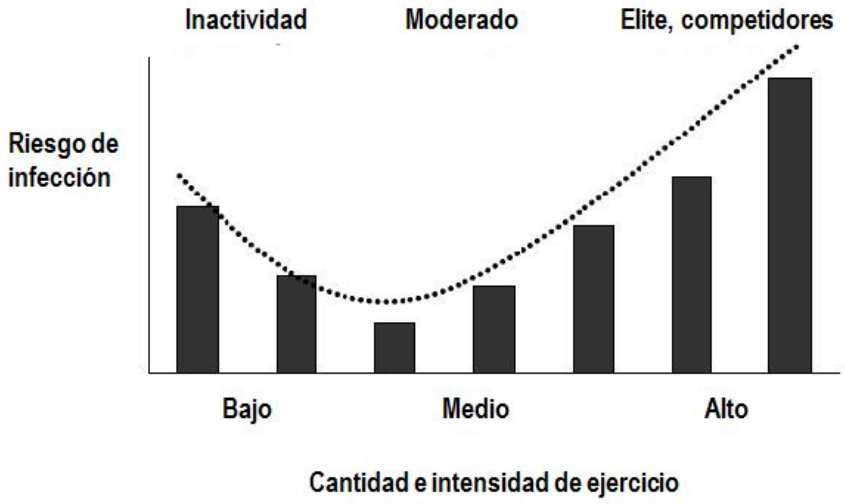

Figura 27. Modelo del grado de actividad del sistema inmunológico y el riesgo para adquirir enfermedades a medida que se aumenta el volumen y/o intensidad del ejercicio físico.

Toda persona que realice entrenamiento físico de alta intensidad, ya sea a nivel profesional o aficionado, puede experimentar una inmunosupresión cuando somete a su cuerpo a un gran estrés (volúmenes e intensidades superiores a los habituales de forma mantenida a lo largo del tiempo). A veces ocurre cuando se quiere ganar fuerza, volumen, velocidad o cualquier otro aspecto rápidamente, es lo que solemos denominar sobreentrenamiento.

De acuerdo con esto, el grupo de Cosgrove et al. han descrito un aumento de células altamente diferenciadas en respuesta a un periodo de alto entrenamiento de seis meses en un grupo de triatletas jóvenes (Cosgrove *et al.* 2012). En nuestro grupo de atletas, que realizaron ejercicio de alta intensidad y gran volumen a lo largo de su vida, también encontramos un fenotipo celular inmune más diferenciado y una capacidad funcional reducida en las poblaciones de linfocitos T. Esto podría afectar la eficacia de la respuesta inmune contra nuevos patógenos y por consiguiente su salud y sus resultados deportivos, que es de gran importancia para estos individuos. Es importante remarcar que los atletas jóvenes incluidos en nuestro estudio mostraban valores de consumo máximo de oxígeno (VO_{2max}) mucho mayores que los valores de

los individuos de los estudios donde se ve que la práctica del ejercicio aumenta el número de células indiferenciadas y disminuye el de las diferenciadas (Spielmann *et al.* 2011).

Encontramos que las células NK, como otras poblaciones leucocitarias, presentan una movilización hacia sangre periférica debido probablemente al aumento de la concentración de catecolaminas durante el ejercicio intenso (Timmons & Cieslak 2008). Esta movilización podría ser bifásica, con un aumento rápido en sangre periférica del número de leucocitos tras el comienzo del ejercicio, y una posterior redistribución de los mismos para mejorar la respuesta inmune en piel, pulmones, tractos gastrointestinal y urinario, superficies mucosas y nódulos linfáticos (Walsh *et al.* 2011; Yang *et al.* 2012). A pesar de los varios estudios en atletas con un alto grado de entrenamiento, no se han encontrado datos suficientes que confirmen lo anteriormente descrito (Woods *et al.* 1999; McFarlin *et al.* 2005).

Sorprendentemente, cuando analizamos el estado de diferenciación de las subpoblaciones de linfocitos T, encontramos que los atletas, tanto jóvenes como de edad avanzada, que habían realizado entrenamiento de alta volumen presentaban un estado de diferenciación mayor en sus linfocitos T CD4+. Por el contrario, no encontramos cambios en las subpoblaciones de linfocitos T CD8+. Como ya mencionamos anteriormente, la homeostasis en las células T CD4+ es mucho más estricta que en linfocitos T CD8+. De hecho, el envejecimiento de los linfocitos T CD8+ se describió principalmente en células T CD8+, donde los cambios ocurren más precozmente (van Lier *et al.* 2003; Czesnikiewicz-Guzik *et al.* 2008).

Todos estos cambios en el fenotipo de las células T tienen consecuencias en su capacidad funcional. La respuesta celular en los atletas jóvenes fue mucho menor que en los jóvenes que no realizaban ejercicio de alto nivel y estuvo a niveles de los individuos de más edad. Esta baja capacidad de activación y proliferación se ha descrito en trabajos anteriores (Baj *et al.* 1994; Lancaster *et al.* 2004), que describieron que los linfocitos T en atletas que llevaron a cabo ejercicio de alto nivel a lo largo de largos periodos de tiempo presentaban baja funcionalidad. Durante periodos de ejercicio muy intenso, pueden producirse estados transitorios de inmunodepresión y esto puede ser la causa de las frecuentes infecciones que sufren los atletas en estos periodos, como las infecciones respiratorias y las reactivaciones de CMV (Robson *et al.* 1999; Gleeson & Bishop 2005). Los altos niveles de hormonas relacionadas con periodos de estrés, particularmente el cortisol, y alteraciones en el ratio de citocinas pro-inflamatorias:anti-inflamatorias en respuesta al ejercicio podrían ser la causa. La elevación repetida de los niveles de cortisol después de la realización de ejercicio extenuante se ha relacionado con involución tímica (Bauer *et al.* 2009), pero por otra parte, este alto grado de diferenciación podría ser debido al continuo encuentro con antígenos crónicos que sufren los

atletas de alto nivel debido al estado de inmunosupresión causado por este alto nivel de entrenamiento (Smithey *et al.* 2012). Las células específicas frente a estos antígenos proliferarían y adquirirían un fenotipo más diferenciado, lo que conllevaría a una capacidad reducida para responder a nuevos antígenos. Estos procesos podrían inducir un cambio en el perfil de las poblaciones de linfocitos T, similares a los encontrados en los grupos de atletas.

Por último, en nuestro estudio vimos que las células NK presentaban una capacidad citotóxica mucho mayor en el grupo de jóvenes atletas que en el grupo de jóvenes no atletas. Esto podría ejercer un posible mecanismo compensatorio que podría mantener una adecuada respuesta inmune. Este proceso ha sido descrito en otras situaciones en las cuales la respuesta inmune adaptativa estaba disminuida debido a un alto grado de diferenciación y una posible disfunción inmune (Jadeski & Hoffman-Goetz 1996; McFarlin *et al.* 2005; Wang & Weng 2011).

Tanto el consumo de probióticos como la realización de ejercicio moderado podrían modificar los efectos del envejecimiento sobre el sistema inmune. La inmunomodulación inducida por el probiótico estudiado en nuestro laboratorio podría ayudar a mantener una respuesta inmune adecuada, aumentando el número de células inmaduras, potenciales respondedoras a nuevos antígenos. La toma del probiótico podría ayudar a producir una mejor respuesta a la vacunación y una mayor resistencia a contraer enfermedades infecciosas. Por otra parte, a pesar de que la realización de ejercicio moderado es beneficiosa para mantener una respuesta inmune adecuada, el entrenamiento de alto volumen está asociado con cambios fenotípicos y funcionales en la respuesta inmune adaptativa con un mayor grado de diferenciación celular, y con una menor respuesta celular. Estas diferencias son más pronunciadas en los individuos jóvenes que realizan un ejercicio de alto nivel, pero parecen reducirse a medida que el individuo envejece. Todo esto parece sugerir que podría existir un mecanismo compensatorio que ayudaría a mantener una respuesta inmune adecuada en estos individuos.

V. CONCLUSIONES

CONCLUSIONES

1. La expresión de la molécula NKG2D se encuentra incrementada en los linfocitos T CD4+ de los ancianos en comparación con los individuos jóvenes. La frecuencia de esta población es un marcador de senescencia de linfocitos T CD4+.

2. Dentro de la subpoblación de linfocitos T CD4+CD28^{null} la expresión de NKG2D caracteriza células funcionalmente más diferenciada y ontogénicamente más maduras.

3. La IL-15 juega un papel muy importante en la potenciación de la activación, proliferación y respuesta funcional frente a antígenos específicos de la población linfocitaria CD4+CD28^{null}.

4. La seropositividad a CMV y los niveles de anticuerpos anti-CMV se relacionan con el estado de diferenciación linfocitaria e inmunocompetencia en ancianos.

5. El estado funcional de los ancianos se correlaciona con modificaciones en la distribución de las poblaciones celulares en sangre periférica, así como en el grado de diferenciación y maduración de los linfocitos T CD4+.

6. Los ancianos con peor estado funcional se caracterizan por presentar una elevada respuesta celular y humoral frente a CMV, al mismo tiempo que un deterioro de su respuesta inmunológica in vitro y de su capacidad de inmunización in vivo.

7. La ICC y los altos niveles de IL-6 mantenidos a lo largo del tiempo se asocian con una respuesta inmune adaptativa comprometida, con un alto grado de diferenciación linfocitaria y con una capacidad funcional disminuida.

8. El consumo de la cepa probiótica de *Lactobacillus delbrueckii* subsp. *bulgaricus* 8481 promueve el aumento de las células T inmaduras y ralentiza el acúmulo de linfocitos T altamente envejecidos. Estos cambios se ven reflejados a nivel humoral con una disminución en la producción de la citocina pro-inflamatoria IL-8 y un aumento en la concentración de la molécula antimicrobiana hBD-2.

9. La realización de ejercicio de alto nivel durante largos periodos de tiempo se asocia con poblaciones linfocitarias con un alto grado de diferenciación y una menor respuesta funcional, principalmente en individuos jóvenes. El aumento en la capacidad citotóxica en las células NK podría compensar la defectiva respuesta inmune encontrada en atletas de alto nivel.

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