



**Programa de Doctorado en Biociencias
Moleculares**

**Papel de la lamina A/C en la
enfermedad cardiovascular asociada
al envejecimiento**

Alberto Del Monte Monge

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**Papel de la lamina A/C en la enfermedad
cardiovascular asociada al
envejecimiento**

Alberto Del Monte Monge

Licenciado en Biología

Director: Vicente Andrés

**F.S.P. Centro Nacional de Investigaciones
Cardiovasculares Carlos III (CNIC)**



Certificado del Director:

Vicente Andrés, Doctor por la Universidad de Barcelona:

Certifica:

Que D. Alberto Del Monte Monge, licenciado en Biología por la Universidad de Alcalá ha realizado bajo su dirección el trabajo de Tesis Doctoral que lleva por título:

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En Madrid, a 25 de febrero de 2020

Fdo.: Vicente Andrés

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RESUMEN

Las enfermedades cardiovasculares (ECVs) son la primera causa de mortalidad en el mundo. El envejecimiento y la hipercolesterolemia son dos factores de riesgo cardiovascular muy importantes. La lamina A/C es una proteína nuclear codificada por el gen *LMNA* que juega un papel estructural y regulador de diversas funciones celulares. El objetivo de este trabajo de Tesis Doctoral ha sido estudiar el papel del envejecimiento y la hipercolesterolemia sobre la expresión de lamina A/C y su posible efecto sobre la ECV asociada. Por un lado, observamos un descenso en los niveles de lamina A/C en el sistema inmune humano y de ratón durante el envejecimiento. Además, demostramos que la hipercolesterolemia está asociada a una disminución de la expresión de lamina A/C en leucocitos de ratón. Experimentos funcionales de trasplante de ratones deficientes en *Ldlr* (*Ldlr*^{-/-}, del inglés *Low-density lipoprotein receptor*) utilizando médula ósea (MO) *Lmna*^{-/-} y silvestre (WT: Wild-type) (MO *Lmna*^{-/-} → *Ldlr*^{-/-} y MO WT → *Ldlr*^{-/-}, respectivamente) demostraron un aumento en el desarrollo de arteriosclerosis en la arteria aorta y en arterias carótidas de los ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-} alimentados con dieta grasa (DG) en comparación con los controles MO WT → *Ldlr*^{-/-}, sin que los parámetros fisiológicos básicos estuvieran alterados. El aumento de arteriosclerosis en ratones hipercolesterolémicos carentes de lamina A/C en las células del sistema inmune estaba asociado a un aumento de la extravasación de leucocitos y a una menor velocidad de movimiento de los leucocitos extravasados. De acuerdo con los resultados anteriores, demostramos mediante experimentos *in vitro* que los monocitos proinflamatorios *Lmna*^{-/-} migraban con mayor facilidad a través de constricciones de pequeño tamaño, pero eran más lentos al desplazarse en ausencia de constricciones. Por otra parte, observamos un descenso en los niveles de lamina A/C durante el envejecimiento en tejido arterial humano y de ratón debido a una disminución de la expresión en células de músculo liso vascular (CMLVs) y células endoteliales. Para profundizar en el papel de lamina A/C en el desarrollo de ECV, desarrollamos el modelo de ratón *Lmna*^{flox/flox} *SM22αCre*, deficiente en lamina A/C en CMLVs, cardiomiocitos, y fibroblastos cardíacos. En comparación con los controles, los ratones *Lmna*^{flox/flox} *SM22αCre* morían prematuramente (vida media ≈ 5 semanas) y mostraban una pérdida grave de función cardíaca en ambos ventrículos, así como problemas de conducción eléctrica en el corazón. Además, los ratones *Lmna*^{flox/flox} *SM22αCre* mostraron fibrosis cardíaca asociada a la expresión exacerbada de marcadores de miofibroblastos (FSP-1 y SMA). En resumen, nuestros resultados sugieren que el descenso de la lamina A/C contribuye al desarrollo de ECVs asociadas al envejecimiento e hipercolesterolemia. Serán necesarios más estudios para determinar los mecanismos moleculares que subyacen a esta nueva función reguladora de la lamina A/C en el envejecimiento y la ECV

SUMMARY

Cardiovascular disease (CVD) is the main cause of mortality worldwide. Aging and hypercholesterolemia are critical cardiovascular risk factors. Lamin A/C, encoded by the *LMNA* gene, are nuclear proteins that play important structural roles and regulate a broad range of cellular function. In this Doctoral Thesis, we studied the regulation of lamin A/C by aging and hypercholesterolemia, and its possible role in CVD. We observed an age dependent decrease in lamin A/C expression in human and mouse immune cells. Likewise, hypercholesterolemia is associated with lower lamin A/C expression in mouse leukocytes. We next performed functional studies by reconstituting lethally-irradiated low-density lipoprotein receptor mice (*Ldlr*^{-/-}) with wild-type or *Lmna*-null (*Lmna*^{-/-}) bone marrow (BM-WT → *Ldlr*^{-/-}, BM-*Lmna*^{-/-} → *Ldlr*^{-/-}, respectively). We found higher atherosclerosis burden in aorta and carotid arteries of BM-*Lmna*^{-/-} → *Ldlr*^{-/-} mice fed high fat diet for 6 weeks compared with BM-WT → *Ldlr*^{-/-} controls, without significant changes in circulating blood cell populations, body weight and lipid profile. Augmented atherosclerosis in BM- *Lmna*^{-/-} → *Ldlr*^{-/-} mice was associated with increased leukocyte migration, although their migration velocity within the tissue was lower. Consistent with these findings, *Lmna*^{-/-} proinflammatory monocytes migrated *in vitro* more easily through small constrictions but exhibited slower velocity when passing through straight channels without constrictions. We also found a significant downregulation of lamin A/C in aged mouse and human arterial tissue, due to diminished expression in both smooth muscle cells (SMCs) and endothelial cells. To further investigate the role of A-type lamins in CVD, we generated a mouse model with genetic disruption of *Lmna* specifically in SMCs, cardiomyocytes and cardiac fibroblasts (*Lmna*^{flox/flox} *SM22αCre*). Compared with controls, *Lmna*^{flox/flox} *SM22αCre* mice died prematurely (average life span: ≈5 weeks) and exhibited cardiac alterations, including defective conduction and repolarization, severe left ventricle and right ventricle dysfunction. Remarkably, *Lmna*^{flox/flox} *SM22αCre* developed prominent interstitial cardiac fibrosis associated with augmented expression of FSP-1 and SMA. Collectively, our results suggest that lamin A/C downregulation contributes to CVD during aging and in the setting of hypercholesterolemia. Future studies will examine the molecular mechanisms underlying these new regulatory functions of lamin A/C in aging and CVD

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ABREVIATURAS

AA - Arco aórtico
AT - Arteria torácica
BSA - Albúmina de suero bovino (del inglés '*Bovine Serum Albumin*')
CMLVs - Células de músculo liso vascular
DG - Dieta grasa
DN - Dieta normal
ECG - Electrocardiograma
ECV - Enfermedad cardiovascular
FA - Fibrilación auricular
FBS - Suero fetal bovino (del inglés '*Fetal Bovine Serum*')
FCs - Fibroblastos cardíacos
FEVI - Fracción de eyección del ventrículo izquierdo
HE - Hematoxilina-eosina
HRP - Peroxidasa de rábano picante
IL-4 - Interleuquina 4
LDLs - Lipoproteínas de baja densidad (del inglés '*Low-density lipoprotein*')
Ldlr^{-/-} - Ratón deficiente en el receptor de LDLs
MDMO - Macrófagos derivados de médula ósea
MIF - Media de intensidad de fluorescencia
MM - Modo M
MNE - Membrana nuclear externa
MNI - Membrana nuclear interna
MO - Médula ósea
ORO - Oil Red O
PBS - Tampón fosfato salino (del inglés '*Phosphate Buffered Saline*')
PDMS - Polidimetilsiloxano
PFA - Paraformaldehído
PVDF - Polifluoruro de vinilo
Sel-E - Selectinas-E
Sel-L - Selectinas-L
Sel-P - Selectinas-P
SEM - Error estándar de la media (del inglés '*Standard Error of the Mean*')
SI - Sistema inmune
TAPSE - Excursión sistólica plana anular tricúspide (del inglés '*tricuspid annular plane systolic excursion*')
WB - Western blot
WT - Fenotipo Silvestre (del inglés '*Wild-type*')

INTRODUCCIÓN

1. Componentes del sistema cardiovascular

La mayoría de las especies animales han desarrollado mecanismos para el transporte de sustancias a través del cuerpo. Los mamíferos poseen un sistema cardiovascular capaz de transportar por la sangre el oxígeno y los nutrientes a todas las células, así como eliminar el dióxido de carbono y transportar proteínas y células del sistema inmune (SI). El sistema cardiovascular está formado por el corazón y un conjunto de vasos sanguíneos ramificados, compuesto de arterias, venas y capilares (Betts et al., 2017) (**Figura 1**).

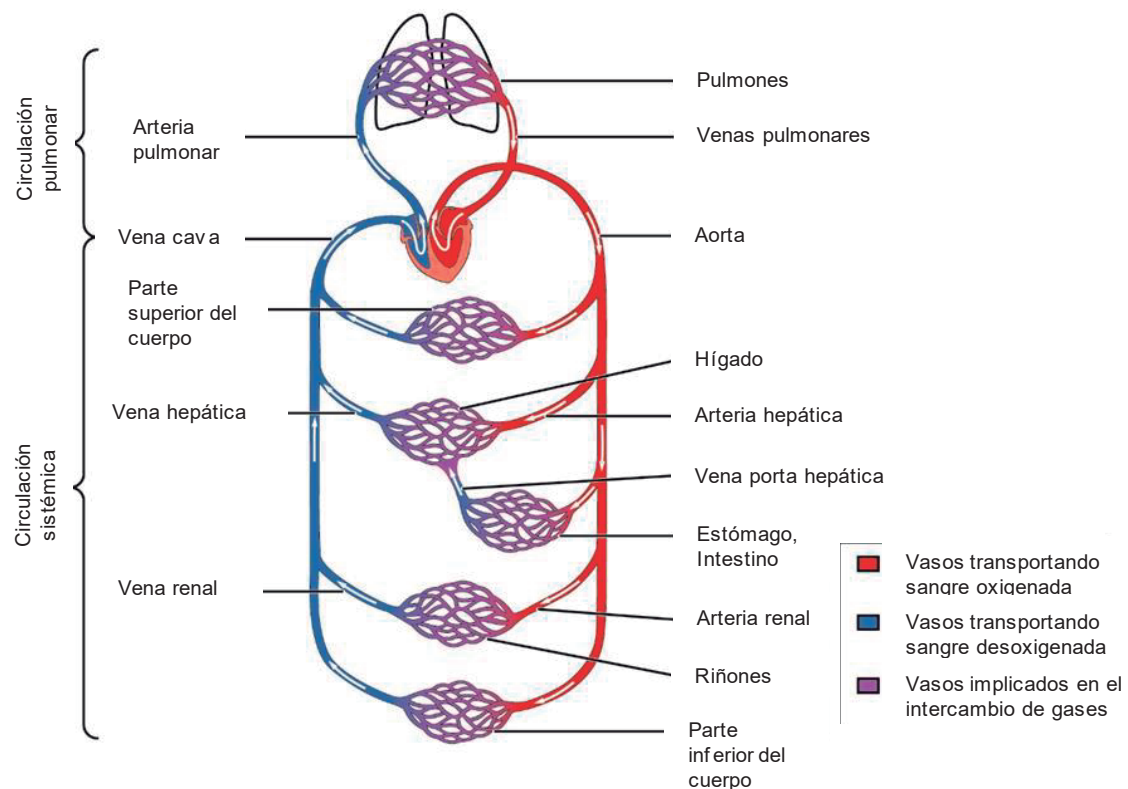


Figura 1. Esquema del sistema circulatorio. Circuito que sigue la sangre desde el corazón hacia los pulmones para su oxigenación y posterior distribución a los distintos órganos y tejidos. Figura adaptada de Betts et al., 2017.

El corazón es un órgano contráctil capaz de bombear sangre hacia las arterias. Se compone de cuatro cámaras: dos ventrículos, situados en la zona inferior, y dos aurículas, situadas en la parte superior. En el tejido cardíaco existen varios tipos de células, principalmente cardiomiocitos, fibroblastos cardíacos (FCs), células endoteliales y células perivasculares, además de células de músculo liso vascular (CMLVs), células marcapasos y células de Purkinje (Xin et al., 2013; Zhou & Pu, 2016) (**Figura 2**).

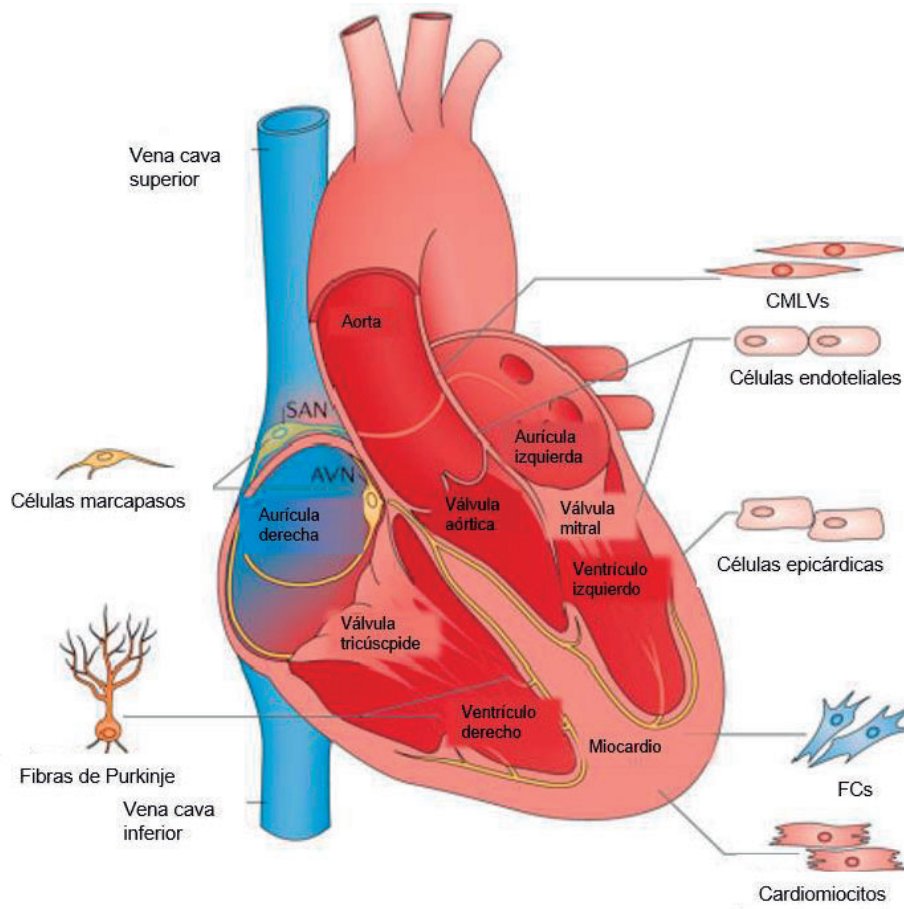


Figura 2. Composición celular del corazón. Esquema ilustrativo del corazón con sus diferentes tipos celulares. Figura adaptada de Xin, Olson and Bassel-Duby, 2013.

La sangre bombeada por el corazón pasa a través de la red de vasos sanguíneos formada por arterias, arteriolas, capilares, vénulas y venas. Las arterias se encargan de transportar la sangre oxigenada desde el corazón a los tejidos. Las venas llevan la sangre sin oxigenar de los tejidos al corazón, con la excepción de las venas pulmonares, encargadas de llevar la sangre oxigenada de los pulmones al corazón (**Figura 1**).

Las arterias están compuestas por tres capas celulares superpuestas: túnica íntima, túnica media y túnica adventicia (**Figura 3**). La túnica íntima es la primera barrera en contacto con la sangre y se compone de una sola capa de células endoteliales con una membrana basal subyacente. La túnica media confiere resistencia mecánica al vaso sanguíneo y contiene capas concéntricas de CMLVs fijadas a fibras elásticas. La túnica adventicia está compuesta por fibroblastos y fibras dispersas de colágeno.

La nutrición de estas capas se produce gracias a la irrigación del *vasa vasorum* y la innervación del *nervi vasorum*, que cruzan desde la túnica adventicia hasta la túnica media.

La principal arteria del cuerpo es la arteria aorta, dividida en tres partes fundamentales: arco aórtico (AA), aorta torácica (AT) y aorta abdominal (Betts et al., 2017).

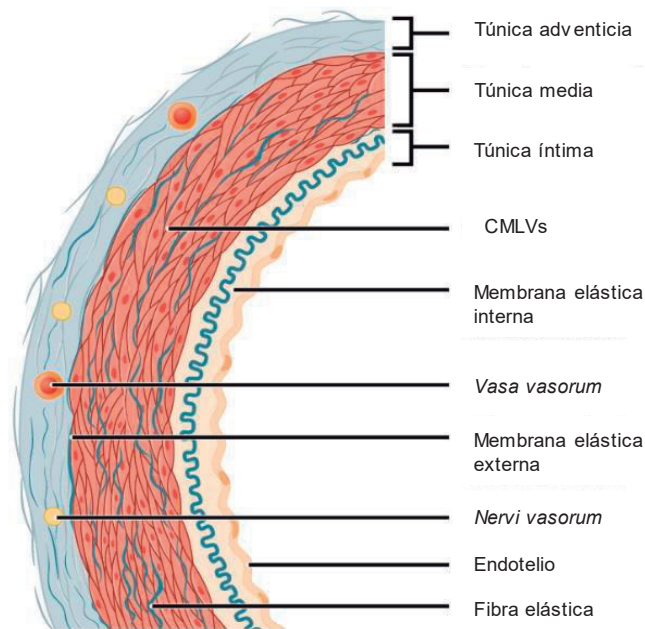


Figura 3. Estructura de la pared arterial. Esquema ilustrativo de las distintas capas de la pared arterial. Figura Adaptada de Betts et al. 2017

2. Envejecimiento y enfermedad cardiovascular (ECV)

Desde hace décadas, la población mundial está envejeciendo progresivamente debido principalmente al incremento de la longevidad y a un descenso de la natalidad. Se estima que en 2045-2050 la esperanza de vida global alcance los 75 años (www.who.int/cardiovascular_diseases). Este hecho tiene un gran impacto sanitario, social y económico, ya que el envejecimiento es el mayor factor de riesgo de sufrir ECVs, que continúan siendo la primera causa de muerte en el mundo. La asociación entre ECVs y envejecimiento puede deberse a un aumento de la exposición a factores de riesgo cardiovascular al prolongar el tiempo de vida, pueden ser la consecuencia de un proceso de envejecimiento que promueve mecanismos fisiopatológicos concretos, o una combinación de ambas (Lakatta & Levy, 2003).

Las ECVs asociadas al envejecimiento incluyen todo tipo de enfermedades arteriales (Savji et al., 2013), entre las que se encuentra la arteriosclerosis (Wang & Bennett, 2012). Por otra parte, se han relacionado varias alteraciones cardíacas con el envejecimiento, como fallo cardíaco y fibrilación auricular, asociadas a mecanismos

fisiológicos como la pérdida de función sistólica y/o eléctrica, y también a cambios estructurales y celulares como la fibrosis (Steenman & Lande, 2017).

2.1. Arteriosclerosis

La arteriosclerosis es una enfermedad vascular que se caracteriza por una acumulación de lípidos y elementos de origen fibroso en la pared de las arterias llamada placa de ateroma, que progresa dificultando la circulación del vaso afectado. En última instancia, la placa puede sufrir una ruptura, produciéndose un trombo que puede dar lugar a un infarto de miocardio o a un accidente cerebrovascular.

La arteriosclerosis comienza cuando las lipoproteínas de baja densidad (LDLs) atraviesan el endotelio de forma pasiva y se acumulan en la túnica íntima, en el espacio entre el endotelio y la membrana basal. Estas LDLs sufren un proceso de oxidación, lo que las convierte en un compuesto proinflamatorio que inicia una serie de procesos de activación endotelial (Skålén et al., 2002). El endotelio activado empieza a expresar moléculas de adhesión endotelial y quimiocinas que favorecen la interacción de leucocitos circulantes, principalmente monocitos, que atraviesan el endotelio y se depositan en la túnica íntima.

El reclutamiento leucocitario se produce en tres fases bien diferenciadas: rodamiento, adhesión y diapédesis (Braunersreuther & Mach, 2006). La fase de rodamiento es el primer contacto entre los leucocitos y el endotelio. Comienza después de la activación del endotelio dañado por la acción de diversos factores de riesgo cardiovascular. Este contacto leucocito-endotelio es dependiente de unas moléculas expresadas por ambos tipos celulares llamadas selectinas, de las que se conocen tres: selectinas-P (Sel-P), selectinas-L (Sel-L) y selectinas-E (Sel-E) (Kansas, 1996). El endotelio activado expresa Sel-E y Sel-P, mientras que los leucocitos expresan Sel-L y PSGL-1, principales ligandos de las selectinas endoteliales durante este proceso (Huo & Ley, 2001).

La adhesión firme se produce después del rodamiento y está mediada por la unión entre moléculas de adhesión de la familia de las integrinas y sus ligandos. Esta fase comienza cuando las quimiocinas producidas por las células endoteliales activan las integrinas de los leucocitos (Imhof & Aurrand-Lions, 2004). Las principales moléculas implicadas en el proceso de adhesión son VCAM-1, expresada por las células endoteliales, y su ligando VLA-4, expresado por los leucocitos (Alon et al., 1995).

La diapédesis o migración trans-endotelial es la última etapa del reclutamiento leucocitario y consiste en la migración de los leucocitos hacia la túnica íntima a través del endotelio. Este proceso ocurre principalmente a través de la unión entre células

endoteliales (Muller, 2003), pero también atravesando la propia célula endotelial, fenómeno conocido como migración trans-celular (Carman & Springer, 2008). La migración trans-endotelial está mediada por integrinas, como JAM-A (Schmitt et al., 2014), mientras que la migración trans-celular ocurre gracias a ICAM-1 y VCAM-1 (Carman & Springer, 2004) (**Figura 4**).

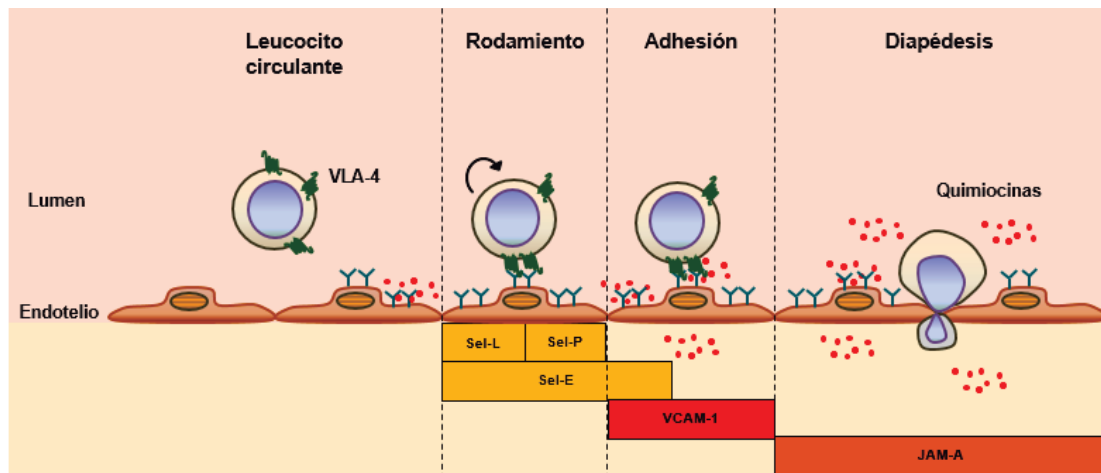


Figura 4. Esquema de las etapas del reclutamiento leucocitario. Se muestran las principales moléculas expresadas por leucocitos y células endoteliales en cada una de las fases. Figura adaptada de Braunersreuther and Mach 2006.

Los monocitos que han migrado a la túnica íntima se diferencian a macrófagos, que proliferan *in situ* (Hilgendorf et al., 2015) y expresan receptores “escoba”, como CD36, SR-A1 o SCARB1. Estos receptores permiten la captación de las lipoproteínas acumuladas en la túnica íntima (Kzhyshkowska et al., 2012), que son procesadas hasta producir colesterol libre, que se acumula en vesículas intracelulares (Maxfield & Tabas, 2005). Estos macrófagos cargados de colesterol, denominados ‘células espumosas’, secretan quimiocinas proinflamatorias que promueven el reclutamiento de más células inmunes (A. C. Li & Glass, 2002). Este proceso es el principal responsable de la formación de placas de ateroma de pequeño tamaño, llamadas “estrías grasas” (**Figura 5A**).

Durante la progresión de la placa de ateroma, los macrófagos continúan captando lípidos. Cuando no son capaces de metabolizarlos o de desprenderse del exceso de lípidos, las células espumosas mueren y se acumulan en los denominados “núcleos necróticos” ricos en cristales de colesterol (Martinet et al., 2011). Por otra parte, se produce una proliferación de las CMLVs que, además, migran desde la túnica media a la íntima debido a las quimiocinas producidas por los macrófagos disfuncionales de la placa de ateroma. Las CMLVs continúan proliferando y empiezan a sintetizar matriz

extracelular, formando una nueva estructura que se conoce como “capa fibrosa” (Bennett et al., 2016) (**Figura 5B**). Cuando la placa está muy avanzada aumenta el riesgo de sufrir una ruptura o erosión, que tiene como consecuencia la liberación de factores procoagulantes que acaban produciendo un trombo sanguíneo (Stefanadis et al., 2017). Las placas de ateroma son más vulnerables de sufrir ruptura y erosión cuanto mayor es su contenido en células inflamatorias y menos sólida es su capa fibrosa (Finn et al., 2010). También se ha asociado la vulnerabilidad de la placa de ateroma a un aumento de la vascularización intra-placa, proceso que favorece la llegada de nuevas células inflamatorias a través de los *vasa vasorum* y produce hemorragias y descelularización de la lesión aterosclerótica (Doyle & Caplice, 2007).

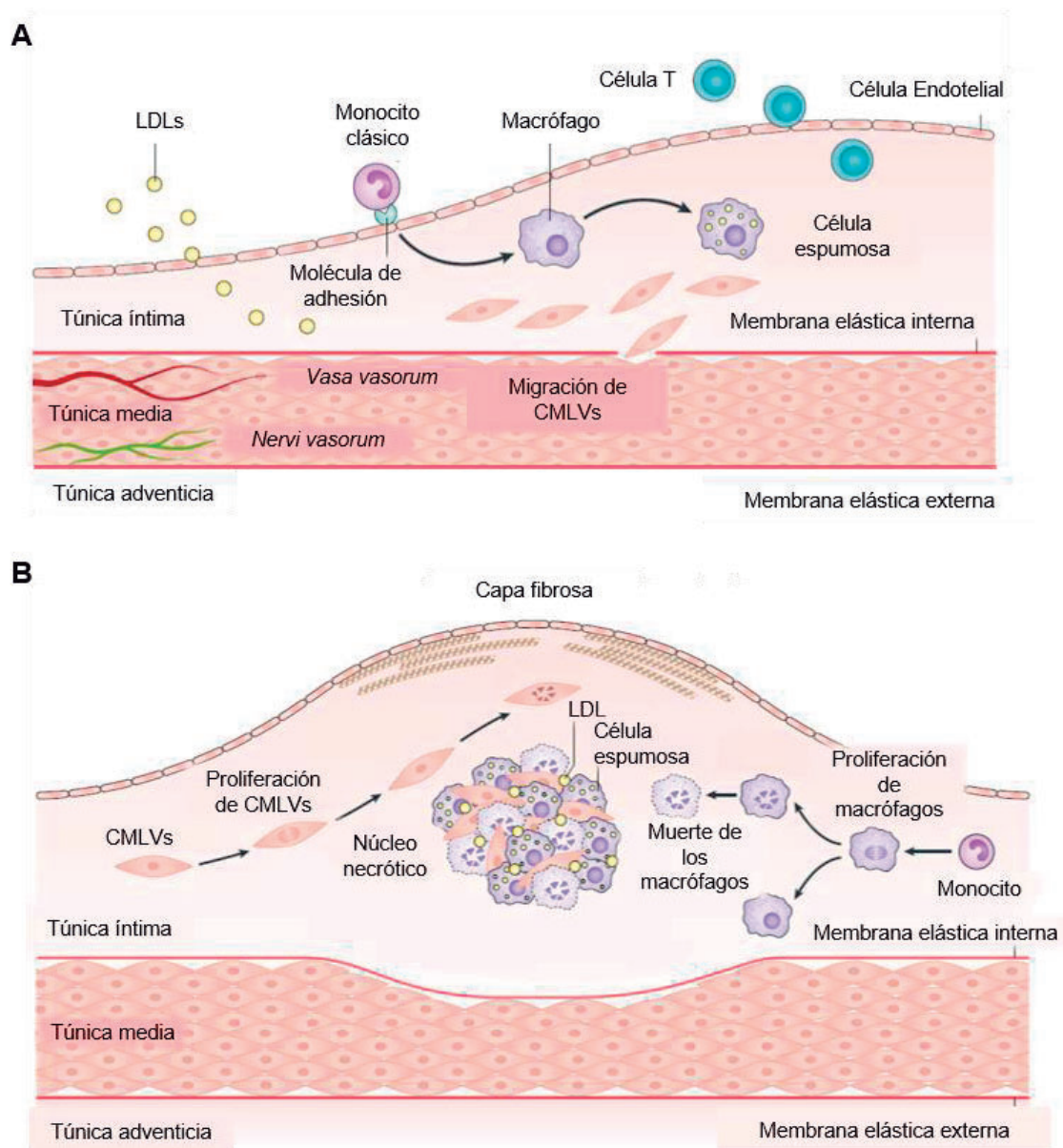


Figura 5. Inicio y progresión de la formación de la placa de ateroma. Esquema ilustrativo de los principales eventos implicados en la formación de la placa de ateroma (**A**) y su progresión a un estado más avanzado (**B**). Figura adaptadas de Libby et al., 2019.

El papel del sistema inmune (SI) en la arteriosclerosis ha sido ampliamente descrito, principalmente en el caso de los monocitos y los macrófagos (Hilgendorf et al., 2015; Moore et al., 2013), pero también para otras células inmunitarias, como neutrófilos y linfocitos. Como ya se ha comentado, los monocitos proinflamatorios (CD14⁺CD16⁻ en humanos, Ly6C^{hi} en ratones) son los principales precursores de los macrófagos residentes en la placa de ateroma (Moroni et al., 2019; Swirski et al., 2007; Tacke et al., 2007). Sin embargo, existe otra subpoblación de monocitos “patrulla” (CD14⁺CD16⁺ en humanos, Ly6C^{low} en ratones) que tienen una función anti-inflamatoria, aunque su implicación en el desarrollo de la placa de ateroma está aún bajo investigación (Hanna et al., 2012; Woollard & Geissmann, 2010). Los macrófagos son células con gran plasticidad que pueden diferenciarse a múltiples sub-tipos, entre ellos los macrófagos M1 y M2 (Murray, 2017; Shapouri-Moghaddam et al., 2018). Los macrófagos M1 secretan factores proinflamatorios como TNF α e IL-6, por lo que se les atribuye un papel proaterogénico. Por el contrario, los macrófagos M2 secretan citoquinas anti-inflamatorias, como IL-10, por lo que están relacionados con funciones “curativas” en la placa de ateroma (Colin et al., 2014). Además de proliferar dentro de la placa de ateroma favoreciendo su desarrollo (Sakai et al., 2000; Zotes et al., 2013), los macrófagos pueden sufrir apoptosis, favoreciendo la generación de núcleos necróticos y la consiguiente desestabilización de la placa (Andrés et al., 2012; Seimon & Tabas, 2009). Aunque debido a su corta vida media no ha sido fácil observar neutrófilos en lesiones ateroscleróticas, estas células juegan un papel importante en todas las fases del desarrollo de la enfermedad. Así, además de producir una serie de proteínas granulares que agravan la disfunción endotelial, los neutrófilos reclutados en la túnica íntima promueven el reclutamiento de monocitos, fomentan la diferenciación a macrófagos M1, oxidan LDLs y aumentan la vulnerabilidad de la placa de ateroma (Döring et al., 2015; Soehnlein, 2012).

En cuanto al SI adaptativo, tanto los linfocitos T como los B participan en el desarrollo de arteriosclerosis. Se acepta que los linfocitos T, mediante respuestas Th1, promueven la formación de la placa de ateroma y su vulnerabilidad, mientras que las respuestas T reguladoras tienen una actividad ateroprotectora. Por otra parte, se sabe que los linfocitos B producen anticuerpos anti-LDLs oxidadas y se han encontrado trazas de Inmunoglobulinas M y G en lesiones ateroscleróticas (Hansson et al., 2006; Lahoute et al., 2011; Tsiantoulas et al., 2014). Sin embargo, el papel de los linfocitos B en arteriosclerosis es todavía controvertido (Sage et al., 2019; Srikakulapu & McNamara, 2017).

2.1.1. El modelo de ratón *Ldlr*^{-/-} para el estudio de la arteriosclerosis

Existen diferentes modelos animales para estudiar la arteriosclerosis (Getz & Reardon, 2012). Entre ellos, el uso de los modelos de ratón se ha extendido principalmente por motivos económicos y de facilidad de manejo, y por la relativa facilidad para establecer modificaciones genéticas controladas. Sin embargo, existen limitaciones para el completo estudio de la arteriosclerosis humana en modelos murinos. Por un lado, los ratones son muy resistentes al desarrollo de arteriosclerosis incluso en condiciones de hipercolesterolemia y, por tanto, es necesario modificarlos genéticamente para aumentar su predisposición a sufrir la enfermedad. Por otro lado, las características de las placas de ateroma son distintas a las humanas, ya que los ratones desarrollan lesiones muy estables en comparación con las humanas, que son más vulnerables (Veseli et al., 2017).

Uno de los principales modelos animales es el ratón deficiente para el receptor de LDLs (*Ldlr*^{-/-}). Se trata de un receptor de membrana con un peso molecular de 160 kDa, implicado en la endocitosis de LDLs ricas en colesterol que juega un papel crítico en la regulación de los niveles plasmáticos de LDLs. También facilita la adquisición de apolipoproteínas B y E. Diversas mutaciones en el gen que codifica para el receptor de LDLs están relacionadas con hipercolesterolemia familiar en humanos (Defesche, 2004; Marais, 2004). En comparación con los controles de fenotipo silvestre (WT), los ratones *Ldlr*^{-/-} se caracterizan por presentar niveles moderadamente elevados de colesterol y LDLs en plasma, sin alteraciones en los niveles de HDL (S Ishibashi et al., 1993). Si bien no desarrollan o desarrollan levemente arteriosclerosis si son alimentados con dieta normal (DN) (Shun Ishibashi et al., 1994), los ratones *Ldlr*^{-/-} alimentados con una dieta grasa (DG) rica en colesterol desarrollan placas de ateroma con presencia de capa fibrosa y núcleo necrótico (Hartvigsen et al., 2007). La formación de lesiones ateroscleróticas depende del tiempo de alimentación con DG y ocurre primero en zonas arteriales de flujo turbulento (Knowles & Maeda, 2000). Este modelo de ratón es óptimo para el estudio de la arteriosclerosis, ya que el colesterol se transporta principalmente en partículas de LDLs. Además, la deficiencia del gen del receptor de LDLs en ratón tiene características similares a la hipercolesterolemia familiar observada en humanos.

2.2. Alteraciones cardíacas asociadas al envejecimiento y papel de la fibrosis cardíaca

El envejecimiento está asociado a diversas alteraciones cardíacas. Por un lado, la función diastólica disminuye con la edad, produciendo problemas de llenado de la válvula mitral (Strait & Lakatta, 2012). Por otro lado, aunque la función sistólica no está afectada por el envejecimiento en condiciones basales, sí se ve reducida durante el ejercicio (Lakatta & Levy, 2003). Ambas pérdidas de función cardíaca están relacionadas con el fallo cardíaco. También se han descrito alteraciones en la función eléctrica del corazón asociadas a la pérdida de células marcapasos y al aumento de la remodelación tisular del corazón con la edad. Estas alteraciones producen cambios en la despolarización y repolarización de las aurículas y los ventrículos, que puede dar lugar a arritmias, principalmente fibrilación auricular (FA) (Curtis et al., 2018; Jones, 2006; Strait & Lakatta, 2012). A su vez, la FA puede producir pérdida de función diastólica y, como consecuencia final, un fallo cardíaco (Keller & Howlett, 2016).

Uno de los procesos más estudiados durante el envejecimiento del corazón es la fibrosis cardíaca, caracterizada por una deposición exacerbada de matriz extracelular, principalmente colágeno producido por los FCs. La fibrosis cardíaca puede ser de tipo reactivo, sin pérdida de cardiomiocitos, o reparativa, en la que la matriz extracelular sustituye a los miocitos cardíacos. En el proceso de envejecimiento se produce principalmente fibrosis reactiva (Horn & Trafford, 2016). Después de un daño cardíaco, los FCs se activan y comienzan a expresar moléculas profibróticas, como FSP-1 y SMA, transformándose en miofibroblastos (Ma et al., 2018). Los miofibroblastos activados secretan citoquinas proinflamatorias que favorecen la infiltración de células inmunes y la proliferación de fibroblastos. Por otra parte, secretan metaloproteasas que degradan la matriz extracelular y facilitan la migración y el depósito de colágeno (Travers et al., 2016) (**Figura 6**). Cuando se cronifica la fibrosis cardíaca durante el envejecimiento, se puede producir pérdida de función diastólica y sistólica (Almehmadi et al., 2014; Jiang et al., 2018). También se ha observado que la fibrosis cardíaca afecta a la conducción eléctrica durante el envejecimiento (De Jong et al., 2011; Francis Stuart et al., 2018; Song et al., 1999) debido en parte a la dispersión de la señal eléctrica entre las fibras intersticiales de colágeno (De Bakker et al., 1993; Ten Tusscher & Panfilov, 2007), por acoplamiento de los miofibroblastos con los cardiomiocitos (Nguyen et al., 2012), o por la expresión de moléculas arritmogénicas por parte de los miofibroblastos (Pedrotty et al., 2009).

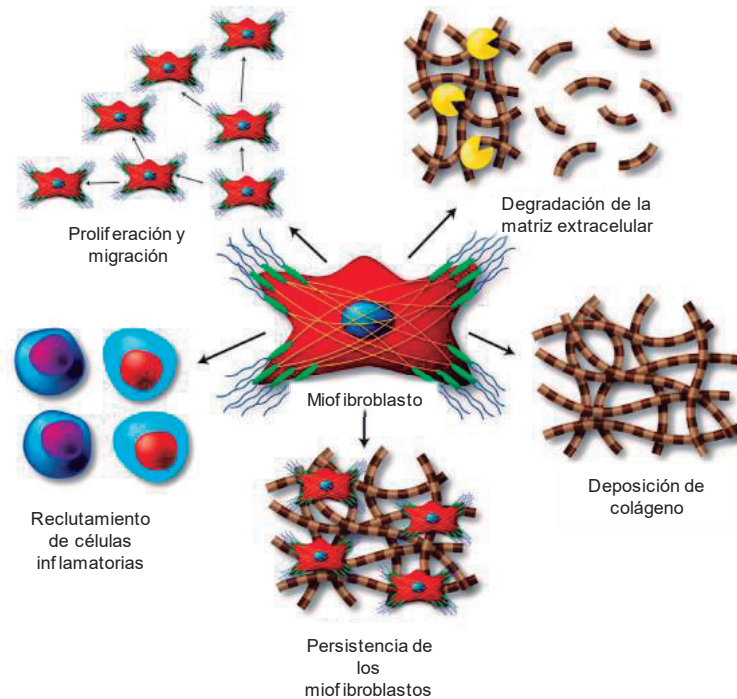


Figura 6. Fibrosis cardíaca. Esquema ilustrativo de los principales eventos implicados en el desarrollo de fibrosis cardíaca asociada al envejecimiento. Figura adaptada de Travers et al., 2016.

3. Lamina A/C

El núcleo de las células está separado del citoplasma por la envoltura nuclear, una estructura compleja formada por la membrana nuclear externa, la membrana nuclear interna, los poros nucleares y la lámina nuclear. La lámina nuclear es una red filamentosa que se sitúa por debajo de la membrana nuclear interna, formada por unas proteínas llamadas laminas (**Figura 7**). Las laminas son filamentos intermedios de tipo V que se dividen en dos grupos: las laminas de tipo A (principalmente lamina A y C), codificadas por el gen *LMNA*, y las laminas de tipo B (lamina B1, B2 y B3), codificadas por los genes *LMNB1* y *LMNB2* (Gerace & Burke, 1988; Stuurman et al., 1998). La estructura de las laminas consiste en un extremo amino-terminal globular seguido de 4 dominios de barra conectados por regiones conectoras, y un extremo carboxi-terminal globular, en el que se encuentra una secuencia de localización nuclear (Broers et al., 2006; Fisher et al., 1986). En esta tesis doctoral nos hemos centrado en el estudio de las laminas de tipo A.

El procesamiento (“splicing”) alternativo del ARNm precursor transcrito del gen *LMNA* da lugar a la forma precursora de la lamina A (pre-lamina A), además de la lamina C, y en menor cantidad las isoformas lamina $\Delta 10$ y lamina C2. La pre-lamina A sufre una

serie de modificaciones post-traduccionales que culminan con el corte de un residuo farnesilado de 15 aminoácidos para generar la lamina A madura, con un peso molecular de 72 kDa (Broers et al., 2006; Machiels et al., 1996).

La lamina A/C es tiene una función estructural que confiere resistencia mecánica al núcleo. Además, mediante sus conexiones con el citoesqueleto vía las proteínas SUN y KASH del complejo LINC (linker of the nucleoskeleton and cytoskeleton), las laminas de tipo A regulan la forma y polaridad de la célula (Hutchison & Worman, 2004) (**Figura 7** y sección **3.1**). Además, numerosos estudios han demostrado que la lamina A/C juega un papel fundamental en numerosos procesos celulares, como la regulación de la reparación del ADN y su replicación, organización de la cromatina, transcripción de genes, transducción de señal y proliferación, diferenciación y migración celular (Andrés & González, 2009; Dechat et al., 2008).

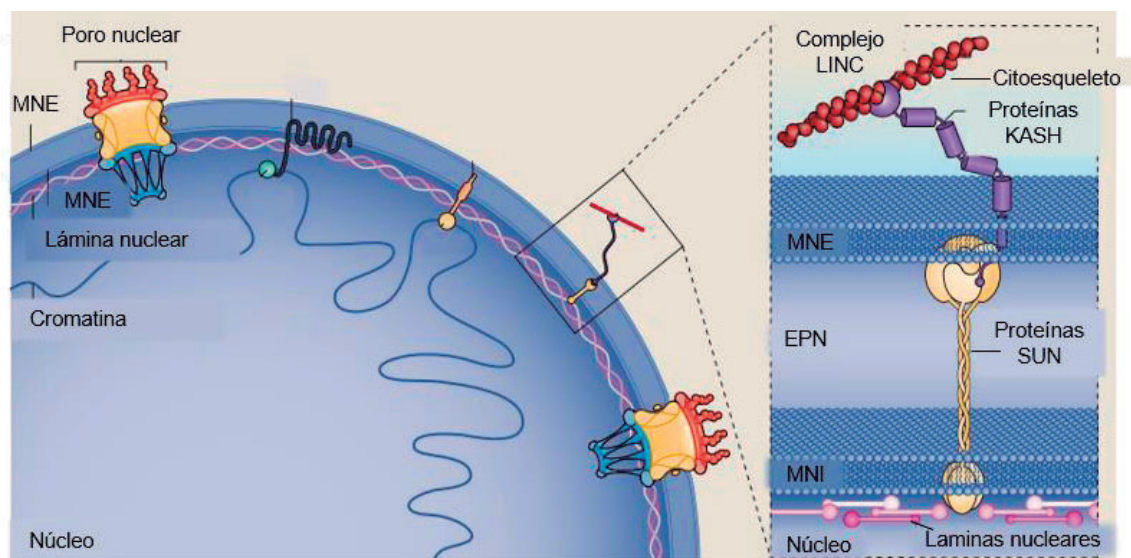


Figura 7. Envoltura nuclear y complejo LINC. Esquema ilustrativo de los principales componentes de la envoltura nuclear y detalle del complejo LINC con sus principales elementos. Figura adaptada de Burke and Stewart, 2013. MNE = Membrana nuclear externa, MNI = membrana nuclear interna, EPN = Espacio perinuclear.

La lamina A/C se expresa en casi todas las células adultas diferenciadas, aunque con niveles de expresión variables. En los tejidos con matriz extracelular más rígida, como el hueso y el corazón, la relación lamina A:B es más alta que en tejidos con una matriz más blanda, como el cerebro. Este hecho refuerza la idea de la importancia de la lamina A/C en el mantenimiento de la estructura celular y su entorno tisular. Si bien el SI se caracteriza por niveles de expresión de laminas de tipo A muy bajos (Swift et al., 2013), nuestro grupo ha demostrado un papel muy relevante de estas proteínas en diversas funciones celulares de los linfocitos T, entre las que se incluyen activación,

diferenciación y formación de la sinapsis inmune (González-Granado et al., 2014; Toribio-Fernández et al., 2018). Además, otros autores han descrito recientemente una asociación entre la ausencia de lamina A/C y un incremento en la diferenciación de granulocitos y aumento de la metástasis de células cancerosas (Ishii et al., 2020).

Una muestra más de la relevancia de la lamina A/C es el hecho de que se han descrito más de 400 mutaciones en el gen *LMNA* humano que provocan enfermedades llamadas laminopatías (<http://umd.be/LMNA/>). Estas enfermedades pueden afectar a tejidos específicos, como el músculo estriado, nervios periféricos y tejido adiposo, o de forma sistémica, como es el caso de los síndromes de envejecimiento prematuro o progeria (Broers et al., 2006) (**Tabla 1**).

Tabla 1. Descripción de las principales laminopatías humanas.

Laminopatía	Tejido afectado	Características	Bibliografía
Distrofia muscular de Emery-Dreifuss (DMED)	Músculo estriado	- Contracturas en las articulaciones - Cansancio y debilidad progresivos - Alteraciones cardíacas	(Helbling-leclerc et al., 2002)
Cardiomiopatía dilatada (CMD)	Músculo estriado	- Dilatación cardíaca - Función sistólica reducida - Problemas de conducción cardíaca - Fallo cardíaco	(McNally & Mestroni, 2017)
Síndrome de Charcot-Marie-Tooth	Nervios periféricos	- Conducción nerviosa reducida - Cansancio y debilidad - Arreflexia	(Bouhouche et al., 1999)
Lipodistrofia parcial familiar de Dunningan	Tejido adiposo	- Distribución anormal de la grasa corporal - Diabetes Mellitus	(Dunningan et al., 1974; Jackson et al., 1997)
Síndrome de progeria de Hutchinson-Gilford	Múltiples tejidos	- Alopecia - Anormalidades en la piel - Pérdida de peso corporal - Rigidez en las articulaciones - Osteoporosis - Aterosclerosis - Muerte prematura por infarto de miocardio, ictus o insuficiencia cardíaca (longevidad media: 14,6 años)	(Eriksson et al., 2003; Sandre-giovannoli et al., 2003; Ullrich & Gordon, 2015)

3.1. Papel de la lamina A/C en la migración celular

Las proteínas de dominio SUN y KASH son los componentes del complejo LINC que conectan la lámina nuclear y el citoesqueleto celular. Las proteínas SUN forman un trímero que se ancla a la membrana nuclear interna y se une a la lámina nuclear en su dominio intranuclear. Las proteínas KASH están formadas por un oligómero anclado a la membrana nuclear externa. Las proteínas SUN y KASH se unen entre sí en el espacio perinuclear, entre las dos membranas nucleares, mientras que las proteínas KASH se unen al citoesqueleto en su dominio extranuclear (Bouzid et al., 2019; Burke & Stewart, 2013) (**Figura 7**). El complejo LINC es determinante para la mecano-transducción entre el exterior celular y el núcleo, necesario para una correcta migración celular (Chambliss et al., 2013; Lee et al., 2007; Lombardi et al., 2011). En ausencia de lamina A/C se producen defectos en la estructura del complejo LINC y en la migración celular (D. H. Kim et al., 2014). Sin embargo, diversos estudios amplían el análisis de la implicación de la lamina A/C en la migración, poniendo el foco en la estructura y deformabilidad del núcleo celular.

El núcleo celular es una estructura limitante en la deformabilidad celular durante la migración a través de poros de pequeño tamaño (Wolf et al., 2013). Estudios *in vitro* demostraron que la sobreexpresión de lamina A dificulta la migración de neutrófilos a través de pequeñas constricciones (Rowat et al., 2013). Por el contrario, otro estudio ha demostrado que la ausencia de lamina A favorece la migración celular a través de constricciones, pero disminuye la supervivencia de las células, siendo el incremento de la migración celular una respuesta asociada a un aumento en la deformabilidad del núcleo (Harada et al., 2014).

Por otra parte, la deficiencia de lamina A/C durante la migración celular favorece la ruptura de la envoltura nuclear, un daño que es reparado por ESCRT III, que además limita el daño en el ADN provocado por el proceso de ruptura (Raab et al., 2016). Uno de los mecanismos moleculares que podrían explicar el papel de la lamina A/C en la migración celular a través de pequeñas constricciones es la nucleación de Arp 2/3 alrededor del núcleo, un proceso que promueve la disrupción de la lámina nuclear y facilita la migración de células dendríticas en estas condiciones. Al eliminar la lamina A/C, este mecanismo deja de ser necesario para la migración (Thiam et al., 2016). Además, se ha descrito otro mecanismo molecular mediado por la osteopontina, una glicoproteína que puede actuar como citoquina promigratoria. En células mesenquimales derivadas de médula ósea (MO), la osteopontina aumenta los niveles de FAK y ERK 1/2 en sus estados fosforilados y reduce los niveles de lamina A/C,

disminuyendo la rigidez nuclear y aumentando la migración de las células a través de pequeños poros (Liu et al., 2017).

En cuanto a su implicación fisiológica, se ha demostrado que la lamina A/C regula la migración celular desde la MO hasta la sangre durante la hematopoyesis (Shin et al., 2013). También se han detectado niveles bajos de lamina A en tumores humanos y se ha relacionado la ausencia de lamina A con la capacidad de migración de las células tumorales en procesos de invasión cancerígena (Bell & Lammerding, 2016; Belt et al., 2011; Saarinen et al., 2015; Willis et al., 2008).

3.2. Papel de la lamina A/C en cardiomiocitos y fibrosis

Como ya se ha comentado anteriormente, diversas mutaciones en el gen de la lamina A/C se asocian con cardiomiopatías (**Tabla 1**). Numerosos estudios han analizado la función de la lamina A/C en los cardiomiocitos con el fin de identificar mecanismos que expliquen la patología cardíaca asociada a alteraciones en la lamina A/C (Carmosino et al., 2014).

Una de las principales hipótesis es la incapacidad del cardiomiocito para soportar la tensión ejercida por los ciclos de contracción cardíaca debido a la fragilidad del núcleo celular que produciría la ruptura del núcleo y la muerte celular. De acuerdo con esta hipótesis, se ha observado un aumento de apoptosis y fragmentación del ADN y alteraciones en la estructura nuclear en cardiomiocitos de ratones carentes de lamina A/C (Nikolova et al., 2004). Otra teoría sugiere que las alteraciones en la expresión de la lamina A/C modifican vías de señalización importantes en el cardiomiocito, produciendo fibrosis cardíaca (Gerbino et al., 2018). Efectivamente, el ratón mutante *Lmna*^{H222P/H222P} presenta cardiomiopatía y fibrosis cardíaca exacerbada asociada a una translocación nuclear anormal de las proteínas Smad 2/3 fosforiladas, efectoras intracelulares de la ruta profibrótica de TGF- β (Arimura et al., 2005). Además, en este mismo modelo, ERK 1/2 actúa sobre el factor de transcripción CTGF/CCN2 promoviendo fibrosis miocárdica y la pérdida de función en el ventrículo izquierdo (Chatzifrangkeskou et al., 2016). Esta teoría se ve reforzada por el hallazgo de fibrosis cardíaca en pacientes con una delección del extremo 5' del gen *LMNA* (Van Tintelen et al., 2007). Sin embargo, el aumento de fibrosis no es exclusivo de los cardiomiocitos con alteraciones en el gen de la lamina A/C, pues estudios con fibroblastos embrionarios de ratón demuestran que la lamina A/C aumenta la producción de colágeno por un mecanismo mediado por la activación de la ruta TGF- β (Van Berlo et al., 2005)

3.3. Modelos de ratón deficientes de lamina A/C

Para estudiar la funcionalidad de la lamina A/C se han generado y caracterizado diversos modelos de ratón modificado genéticamente (Zhang et al., 2013).

El primer modelo que se generó es el ratón *Lmna*^{-/-}, deficiente para lamina A y lamina C debido a la delección del gen *Lmna* desde el exón 8 hasta la mitad del exón 11 (Sullivan, 1999). Los ratones *Lmna*^{-/-} homocigotos son normales al nacer, pero sufren problemas de crecimiento a partir de las 2-3 semanas, desarrollan distrofia muscular y alteraciones en la contractibilidad cardíaca, y mueren a las 5-8 semanas (Frock et al., 2012). El análisis del músculo cardíaco de los ratones *Lmna*^{-/-} revela polarización nuclear de la emerina y acumulación citoplasmática de desmina. Los ratones heterocigotos *Lmna*^{+/-} no presentan anomalías.

Otro modelo de ratón deficiente para lamina A/C es el *Lmna*^{GT-/-} (Kubben et al., 2011). En este modelo se insertó una construcción genética “trampa” en el exón 2 del gen *Lmna*, generando un alelo de fusión que produce la carencia de lamina A/C. Al igual que el modelo *Lmna*^{-/-}, estos ratones presentan una tasa de crecimiento disminuida, distrofia muscular, función cardíaca alterada y muerte prematura (esperanza de vida de 2-3 semanas), además de mostrar anomalías en el tejido adiposo y un ritmo cardíaco reducido.

Un tercer modelo de gran importancia para el estudio funcional de la lamina A/C es el ratón *Lmna*^{flox/flox}, en el que puede eliminarse el exón 2 del gen *Lmna* cuando se activa la recombinasa CRE (Y. Kim & Zheng, 2013). Gracias a este modelo es posible estudiar el papel de la lamina A/C en diferentes tejidos debido a la disponibilidad de modelos de ratón que expresan la recombinasa CRE bajo la acción de promotores de genes tejido-específicos. Además, es posible controlar el momento de la eliminación de la lamina A/C utilizando modelos de ratón con expresión de la recombinasa CRE de forma inducible (H. Kim et al., 2018).

Por otro lado, se ha generado un modelo de ratón que expresa lamina C y carece de lamina A al eliminar los últimos 150 nucleótidos del exón 11 y la totalidad del intrón 11, y que no desarrolla ningún fenotipo aparente (Fong et al., 2006). Del mismo modo, no se han observado anomalías en un modelo de ratón que expresa lamina A y carece de lamina C gracias a la eliminación del exón 10 del gen *Lmna* (Coffinier et al., 2010). El estudio de ambos modelos sugiere que sólo la eliminación conjunta de la lamina A y la lamina C es capaz de generar defectos en los animales de experimentación.

OBJETIVOS

Nuestro laboratorio ha estudiado ampliamente el papel de la lamina A/C y su forma mutada 'progerina' en contextos fisiológicos y patológicos. Sin embargo, aún no se ha analizado el papel que juega la lamina A/C en la ECV asociada al envejecimiento. Por este motivo, los principales objetivos de la presente tesis doctoral son:

1. Investigar la posible regulación de la expresión de lamina A/C durante el envejecimiento de las células inmunitarias y su implicación en la ECV.
2. Investigar la posible regulación de la expresión de lamina A/C durante el envejecimiento en células de la pared vascular y su implicación en la ECV.

MATERIALES Y MÉTODOS

1. Reactivos de uso común

- Tampón fosfato salino (PBS): NaCl 8 g/L, KCl 0,2 g/L, Na₂HPO₄ 1,44 g/L y KH₂PO₄ 0,24 g/L en H₂O destilada.
- T-PBS (Tween-20 PBS): PBS, 0,05% Tween 20.
- Tampón de lisis para la obtención de extractos de proteínas de ratón: Tris·Cl 50 mM, pH 7.2, Triton X-100 1% (9002-93-1, Sigma), SDS 0,1%, NaCl 500 mM y MgCl₂ 10 mM, suplementado con inhibidores de fosfatasa y proteasas (Complete®, Roche).
- Tampón de lisis para la obtención de extractos de proteínas de humano: EGTA 0,5 mM, NaCl 100 mM, glicerol-2-fosfato 100 mM, HEPES 50 mM, Glycerol 10 %, TWEEN-20 0,1 %.
- Tampón de lisis de eritrocitos: KH₄Cl 0,15 M, KHCO₃ 0,01 M, EDTA.N₂ 0,01 M, pH 7.4.
- Tampón citrato para exposición de antígenos en cortes histológicos: citrato sódico 10 mM, Tween 20 0,05%, ajustado a pH 6.0.
- Paraformaldehído (PFA): 4% en PBS.
- Tampón Tyrodes: NaCl 136 mM, KCl 2 mM, CaCl₂·2H₂O 1,3 mM, MgCl₂·6H₂O 0,5 mM, NaH₂PO₄·2H₂O 0,4 mM, NaHCO₃ 12 mM, D-Glucosa 5,5 mM, pH 7.4.
- Medio de diferenciación de macrófagos: RPMI 1640 suplementado con suero fetal bovino (FBS) inactivado 10%, medio LCM 20%, L-glutamina 2 mM, penicilina 100 U/mL y estreptomicina 100 µg/mL.
- Medio de polarización M1: RPMI 1640 suplementado con FBS inactivado 10%, medio LCM 20%, L-glutamina 2 mM, penicilina 100 U/mL, estreptomicina 100 µg/mL, LPS 100 ng/mL e interferón γ 10 ng/mL.
- Medio de polarización M2: RPMI 1640 suplementado con FBS inactivado 10%, medio LCM 20%, L-glutamina 2 mM, penicilina 100 U/mL, estreptomicina 100 µg/mL e interleuquina 4 (IL-4) 10 ng/mL.
- Tampón de digestión de arteria aorta: PBS 1X, CaCl₂ 1 mM, MgCl₂ 1mM, BSA 0,1%, 450 unidades de Colagenasa I (C0130-1G, Sigma-Aldrich), 125 unidades de Colagenasa XI (C7657-1G, Sigma-Aldrich), 60 unidades de Hialuronidasa (H3506-1G, Sigma-Aldrich) y 60 unidades de DNasa I (D5025-150KU-1G, Sigma-Aldrich).
- Medio de cultivo de monocitos: RPMI 1640 + Glutamax, GM-CSF 50 ng/mL y FBS 10%.
- Tampón de carga: SDS 0,1%, azul de bromofenol 0,2%, glicerol 20% y 2-mercaptoetanol 10%.
- Tampón de electroforesis: Tris 25 mM, glicina 192 mM y SDS 0,1%.
- Tampón de transferencia: Tris 25 mM pH 8.3, glicina 192 mM y metanol 20%.

2. Muestras humanas

Para analizar la expresión de lamina A/C en células hematopoyéticas humanas se extrajo sangre de individuos de entre 19 y 89 años en el Hospital Universitario 12 de Octubre según el proyecto CEI PI 44_2015-v3 aprobado por el Comité de Ética y de la Investigación y Bienestar Animal del Instituto de Salud Carlos III. Los individuos se separaron en dos grupos experimentales: de edades comprendidas entre 19-44 años y entre 67-89 años. Para analizar la expresión de lamina A/C en tejido arterial humano se extrajeron muestras de arterias coronarias humanas de individuos ≤ 30 años y ≥ 58 años del Hospital Sant Pau obtenidas según el proyecto 04/2016 aprobado por el Comité Ético de Investigación Clínica (CEIC) del Hospital de la Santa Creu i Sant Pau.

3. Líneas de ratón

Todos los procedimientos animales se hicieron conforme a la Directiva Europea 2010/63EU y la recomendación 2007/526/EC, impuesta bajo el Real Decreto 53/2013. Los protocolos animales fueron aprobados por el Comité Ético y el Área de Protección Animal de la Comunidad Autónoma de Madrid (PROEX135/14, PROEX134/14 y PROEX 354/15).

Todos los ratones usados tenían un fondo genético C57BL/6J. Todos los estudios se realizaron con animales machos y hembras distribuidos de manera equilibrada entre los grupos experimentales. En los experimentos de análisis de expresión de lamina A/C en células hematopoyéticas y vasculares se utilizaron ratones de WT de edades comprendidas entre 3 y 115 semanas. Para los estudios de desarrollo de arteriosclerosis se usaron ratones deficientes para el receptor LDLs con el marcador de membrana CD45.1 (*Ldlr*^{-/-}), (*Ldlr*^{tm1Her}, The Jackson Laboratory, núm. catálogo: 002207) y ratones carentes de lamina A/C de forma ubicua (*Lmna*^{-/-}) (Sullivan, 1999) o específica gracias a la utilización de ratones con el exón 2 del gen *Lmna* flanqueado por secuencias *loxP* (*Lmna*^{flox/flox}) (Y. Kim & Zheng, 2013) el cual, en presencia de una recombinasa CRE, genera una forma truncada de la lamina A/C. Estos ratones *Lmna*^{flox/flox} se cruzaron con ratones *CD4Cre* (The Jackson Laboratory, núm. catálogo: 017336) o *LysMCre* (Clausen et al., 1999) para generar ratones deficientes para lamina A/C específicamente en los linfocitos T (*Lmna*^{flox/flox} *CD4Cre*) (Toribio-Fernández et al., 2018) o en células mieloides (*Lmna*^{flox/flox} *LysMCre*). Para comprobar la eliminación de lamina A/C de las células de la línea mieloides se utilizó el ratón *Lmna*^{flox/flox} *LysMCre* que además, expresaba la proteína fluorescente tomato bajo la acción de la recombinasa CRE (The Jackson Laboratory, núm. catálogo: 007908). Para el estudio del papel de la lamina A/C de forma específica

en CMLVs se cruzaron ratones *SM22αCre* (*Tagln-Cre*, The Jackson Laboratory, num. catálogo: 017491) con *Lmna*^{flox/flox}.

4. Genotipado y sacrificio de los ratones

Los ratones se destetaron a las 3 semanas de edad y se estabularon por sexos. Se genotiparon mediante la técnica de PCR a partir de ADN genómico procedente de una muestra de la cola. El ADN se extrajo incubando las muestras a 95°C en 100 µl de NaOH durante 30 minutos. Tras la incubación se mezclaron las muestras aplicando vórtex. Después se añadieron 10 µl de TRIS y se centrifugaron las muestras 10 minutos a 16000 g. Con 1 µl del sobrenadante se realizaron las reacciones de PCR. Los cebadores empleados para las distintas reacciones de PCR, así como los fragmentos resultantes de la reacción están detallados en la **Tabla 2**. Los ratones fueron sacrificados por inhalación de dióxido de carbono o dislocación cervical a la edad indicada en cada experimento.

Tabla 2. Cebadores empleados en el genotipado de las distintas líneas de ratón.

Línea de ratón	Oligonucleótidos cebadores (5'-3')	Tamaño de las bandas
<i>Ldlr</i> ^{-/-}	TATGCATCCCCAGTCTTTGG CTACCCAACCAGCCCCTTAC ATAGATTGCGCCCTTGTGTCC	WT 351 pb Mutante 179 pb
<i>Lmna</i> ^{-/-}	AGCTGGAGTCTTCCGAGAGC TTGGCTACCCGTGATATTGC AGGCAGGGGAACAGCTTAAT	WT 448 pb Mutante 822 pb
<i>Lmna</i> ^{flox/flox}	AACCCAGCCTCAGAACTGGTGGATG GACAGCTCTCCTCTGAAGTGCTTGA	WT 470 pb Mutante 598 pb
<i>LysMCre</i>	CCCAGAAATGCCAGATTACG CTTGGGCTGCCAGAATTTCTC TTACAGTCGGCCAGGCTGAC	WT 350 pb Mutante 700 pb
<i>CD4Cre</i>	ATTCTCCACCGTCAGTAG CGTTTTCTGAGCATACCTGGA	Transgén 500 pb
<i>SM22αCre</i>	CTAGGCCACAGAATTGAAAGA GTAGGTGGAATTCTAGCATCATCC GCGGTCTGGCAGTAAAACTATC GTGAAACAGCATTGCTGTCACTT	Control interno 324 pb Transgén 100 pb

5. Estudios de longevidad

Desde las 4 semanas de edad, los ratones *Lmna^{flox/flox} SM22αCre* fueron pesados, monitorizándose su salud y supervivencia al menos una vez a la semana. Los animales sacrificados debido a hidrocefalia, maloclusión dental, agresiones entre machos u otras razones ajenas al fenotipo fueron excluidos del análisis.

6. Irradiación y trasplante

Para realizar los experimentos de inducción de arteriosclerosis en ratones con el SI deficiente de lamina A/C, los ratones *Ldlr^{-/-}* fueron irradiados con 2 dosis de 6,5 Gy de 10 minutos cada una en un irradiador JL 1-68A (Shepherd & Associates) con una fuente de 1000 Ci de Cs-137. Al día siguiente, se inyectaron en la vena de caudal de los animales 100 µl de células de MO (7×10^6 células diluidas en suero salino Braun) obtenidas de un grupo de 3 fémures y tibias de ratones *Lmna^{-/-}*, *Lmna^{flox/flox} CD4Cre*, *Lmna^{flox/flox} LysMCre* ó WT. Después de 4 semanas de recuperación, se comprobó la eficiencia del trasplante mediante la tinción de leucocitos frescos aislados de sangre periférica (*material y métodos 5*) con anticuerpos de membrana anti CD45.1 y CD45.2 (**Tabla 3**) y el análisis por citometría de flujo. Dado que los ratones receptores *Ldlr^{-/-}* presentan el marcador de membrana CD45.1 en sus células hematopoyéticas y los donantes CD45.2, la eficiencia de trasplante se representó como el porcentaje de células CD45.2⁺. Posteriormente, se alimentaron los ratones durante 6 semanas con DG o DN (*material y métodos 6*).

7. Experimentos con dieta grasa

Para los experimentos de inducción de aterosclerosis, los animales se alimentaron con DG que contenía 10,7% de grasa total y 0,75% de colesterol (S9167-E010, SSNIF) durante 6 o 12 semanas. Los animales control fueron alimentados con DN durante el mismo tiempo. Los animales fueron sacrificados al final de la alimentación con la dieta por inhalación de CO₂ (*métodos 4*).

8. Cuantificación del tamaño de las placas ateroscleróticas en ratón

Se extrajeron arterias aortas de ratones hipercolesterolémicos que fueron fijadas con PFA 4% durante al menos 24 horas. Se limpiaron de tejido graso y se tiñeron con Oil Red O (ORO) (O0625, Sigma). Las arterias aortas fueron abiertas longitudinalmente y

sujetadas con alfileres de disección con el lumen expuesto (en face) para un análisis planimétrico asistido. Las imágenes fueron obtenidas con una cámara (OLYMPUS UC30) montada en un microscopio estereoscópico (OLYMPUS SZX3). El porcentaje de área de lesión (teñido con ORO) fue cuantificado con el software SigmaScan Pro 5 (Systat Software Inc.).

9. Hematología y bioquímica

La hematología fue realizada por técnicos de la unidad de animalario del CNIC para conocer la concentración de distintas poblaciones de leucocitos en sangre. La sangre fresca extraída (*material y métodos 5*) fue analizada en el equipo PENTRA80 hematology analyzer (Horibo).

Para realizar los estudios bioquímicos se extrajo plasma centrifugando sangre de ratón a 5000 rpm durante 10 minutos a T^a ambiente. El perfil lipídico del plasma se analizó en el equipo Dimension RxL Max Integrated Chemistry System (Siemens Healthineers).

10. Microscopía intravital en músculo cremáster de ratón

Tanto la adhesión como la migración leucocitaria en el modelo de trasplante y desarrollo de aterosclerosis *in vivo* se analizó por microscopía intravital de las vénulas del cremáster de ratón.

Para preparar la correcta visualización del músculo cremáster se requiere una cirugía especializada. Primero, se anestesiaron los ratones con una inyección intraperitoneal de ketamina (Imalgene 1000, Merial) y medetomidina (Medeson, Urano) en una dosis de 50 y 0,5 mg/kg respectivamente. Posteriormente, se inmovilizaron en posición decúbito supino. Bajo la lupa, se diseccionó el músculo cremáster tratando de aislarlo lo máximo posible de los tejidos adyacentes. El músculo fue cortado de forma longitudinal sobre un pedestal cúbico transparente. A continuación, fue perfundido con tampón Tyrodes a 37°C durante todo el procedimiento para mantener unas condiciones fisiológicas óptimas. 10 minutos antes de la adquisición de las imágenes, se inyectaron los anticuerpos de membrana (**Tabla 3**) para marcar monocitos (Ly6C⁺) y neutrófilos (Ly6G⁺). La visualización de las vénulas se llevó a cabo mediante un microscopio intravital AXIO Examiner Z.1 work station (Zeiss) montado en una plataforma y equipado con una cámara CoolSnap HQ2 (Photometrics). Se usó un objetivo APO 20x NA 1.0 de inmersión acuosa (**Figura 8**). Fueron analizadas tres vénulas por ratón y se grabaron vídeos de 5 minutos de duración. Los leucocitos fueron considerados adherentes

cuando no se observó movimiento en la célula durante al menos 30 segundos. Por otra parte, la migración se analizó cuantificando el número de células migradas al músculo cremáster por campo de visión. La velocidad de las células migradas se calculó midiendo los μm recorridos por cada célula en un tiempo determinado.

11. Microscopía intravital en arteria carótida de ratón

Para el estudio del rodamiento de leucocitos sobre el endotelio se realizaron experimentos de microscopía intravital de arteria carótida de ratón. Los ratones *Ldlr*^{-/-} trasplantados con MO *Lmna*^{-/-} o WT alimentados con DG fueron anestesiados e inmovilizados de manera similar a *material y métodos 13*. Se realizó una pequeña incisión vertical en el cuello para exponer la arteria carótida del ratón. A continuación, se fijó la glándula salival y se separaron los músculos adyacentes a la arteria carótida, anudándolos con suturas. Después, se apartó el nervio vago derecho y se colocó una pieza metálica justo debajo de la arteria, que, a su vez, se sujetó a un soporte lateral. Posteriormente, para que el movimiento del pulso cardíaco no fuese excesivo, se colocó un cubreobjetos pulido sobre la arteria, que se fijó a la parte superior del soporte lateral con plastilina. La zona estabilizada se colocó bajo el objetivo acuoso del microscopio. La región de visualización fue perfundida con tampón Tyrodes a 37°C en todo momento para mantener unas condiciones fisiológicas óptimas (**Figura 8**). 10 minutos antes de la adquisición de las imágenes, se inyectaron los anticuerpos de membrana (**Tabla 3**) para marcar monocitos (Ly6C⁺) y neutrófilos (Ly6G⁺). La adquisición de las imágenes se llevó a cabo de la misma forma que en *material y métodos 13*. El rodamiento de los leucocitos se determinó contando el número de células que atravesaron una línea ficticia perpendicular a la arteria durante un tiempo determinado y se representó como el número de células por minuto.

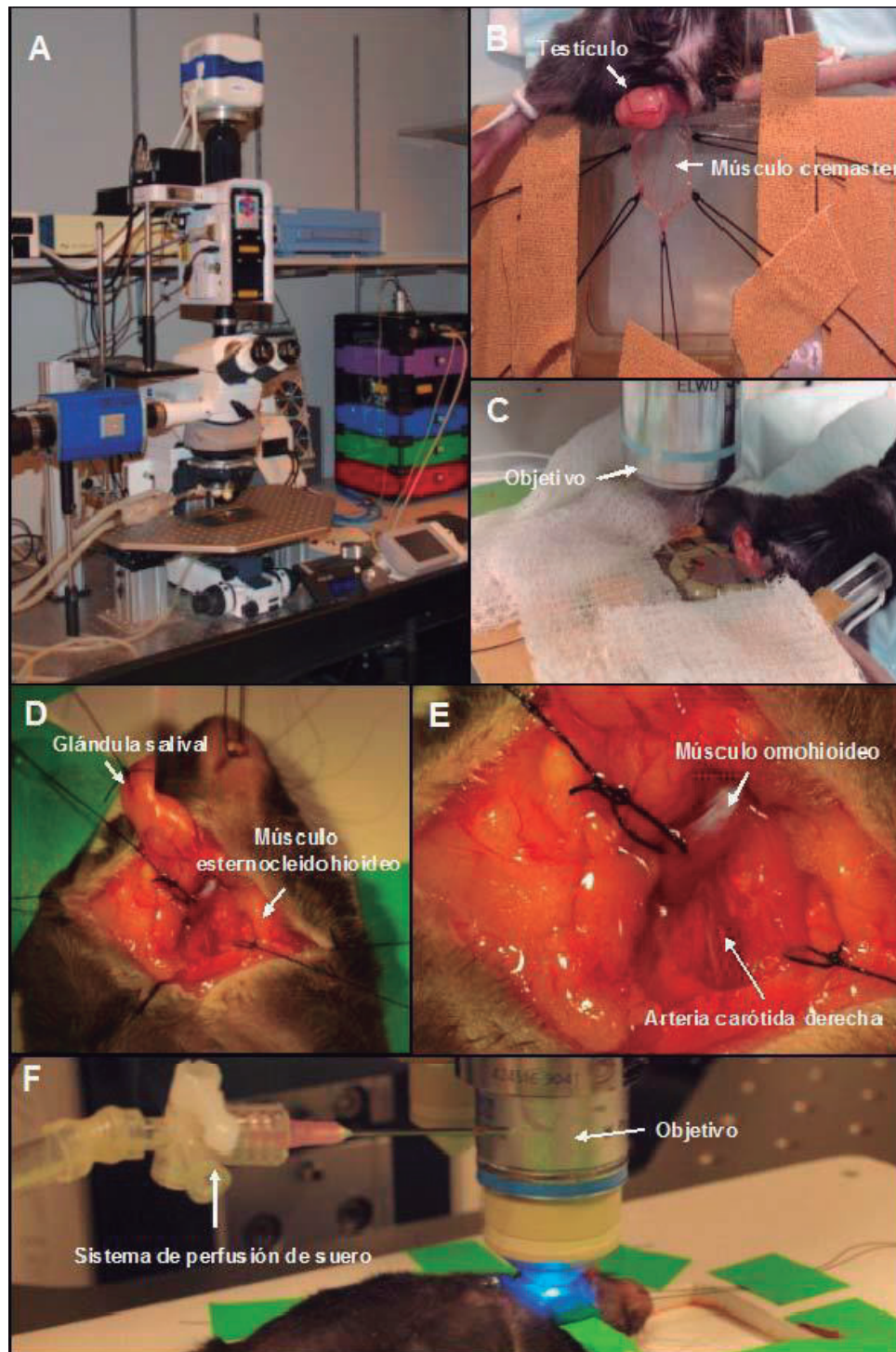


Figura 8. Microscopía intravital. (A) Microscopio de epifluorescencia adaptado para microscopía intravital. (B) Preparación del músculo cremáster de ratón para su correcta visualización. (C) Colocación del músculo cremáster bajo el objetivo. (D, E) Exposición de la arteria carótida para su visualización. (F) Colocación del animal bajo el objetivo para la observación de la arteria carótida. Figura adaptada de Andres & Dorado, 2015.

12. Digestión de aorta para citometría de flujo

Para analizar la proporción de las poblaciones leucocitarias en la arteria aorta durante el desarrollo de arteriosclerosis por citometría de flujo, se utilizaron dos arterias aortas de ratón. Se extrajeron las arterias aortas según *material y métodos 12* y se retiró el tejido adyacente, así como la grasa exterior de las mismas. Después, se añadió tampón de digestión de arteria aorta, se cortaron las arterias en pequeños fragmentos y se incubaron durante 45 minutos a 37°C. Se interrumpió la reacción de digestión con PBS 1X con de FBS 10% a 4°C. Posteriormente, se filtraron las arterias digeridas a través de filtros de 70 µm de diámetro de poro (Becton Dickinson). Se centrifugó la muestra a 1500 rpm a 4°C durante 5 minutos y se descartó el sobrenadante para obtener un sedimento de células aórticas. A continuación, se fijaron las células durante 10 minutos con PFA al 4% a Tª ambiente. Tras un lavado con PBS 1X, las células se incubaron durante 1 hora a Tª ambiente con anticuerpos de membrana para detectar diferentes subpoblaciones de leucocitos (**Tabla 3**). Las muestras se analizaron con el citómetro BD LSR Fortessa. Después de una primera selección de los leucocitos totales de la aorta (CD45⁺), se calculó el porcentaje de macrófagos (F4/80⁺) y neutrófilos (Ly6G⁺).

Para estudiar los niveles de lamina A/C en las células de la pared vascular durante el envejecimiento, las arterias aortas fueron procesadas como se ha descrito anteriormente. En este caso, a los 5 minutos de digestión se extrajo la túnica adventicia para ser digerida en paralelo. Por otra parte, las células fueron filtradas con filtros de 100 µm de diámetro de poro (Becton Dickinson). Después de la fijación, se permeabilizaron las células con Triton X-100 al 0,25% durante 15 minutos. Tras lavar la muestra, se incubó con anticuerpos de membrana para marcar los diferentes tipos de células vasculares (**Tabla 3**). Finalmente, las células se incubaron con el anticuerpo anti-lamina A/C-Alexa 488 (CST) durante 1 hora a Tª ambiente en PBS 1X. Las muestras se adquirieron en el citómetro BD LSR Fortessa. En el caso de las células de la pared vascular, se seleccionaron, por un lado, células de músculo liso (SMA⁺) y, por otro lado, células endoteliales (CD102⁺). En el caso de la túnica adventicia, se seleccionaron todas las células. Los niveles de lamina A/C de las poblaciones seleccionadas se analizaron como en *material y métodos 5*.

13. Análisis de la expresión de lamina A/C en leucocitos de sangre de ratón y humana mediante citometría de flujo

Para analizar los niveles de lamina A/C en leucocitos de sangre de ratón y humana se utilizaron técnicas de citometría de flujo. Se extrajo sangre periférica de ratón procedente del seno submaxilar en tubos Microvette 500 EDTA (Sarstedt). Posteriormente, la sangre se incubó con tampón de lisis de eritrocitos en una proporción 1:14 durante 10 minutos a 4°C. Se añadió PBS 1X para eliminar el exceso de tampón y se centrifugó 5 minutos a 4°C a 1500 rpm. Los leucocitos aislados se incubaron con PFA 4% durante 10 minutos a Tª ambiente para fijar las células y, después de un lavado con PBS 1X, se incubaron con Triton X-100 (9002-93-1, Sigma) 0,25% durante 15 minutos a Tª ambiente para permeabilizar las membranas celulares. Se realizó un lavado con PBS 1X y se incubaron con Fc blocker (101302, Biolegend) 1/200 en PBS 1X durante 30 minutos a Tª ambiente con el objetivo de bloquear uniones inespecíficas del anticuerpo. Las células se incubaron con marcadores de membrana (**Tabla 3**) durante 1 hora a Tª ambiente en PBS 1X para poder distinguir entre distintas poblaciones leucocitarias. Después de un lavado con PBS 1X, se incubaron las células con el anticuerpo anti-lamina A/C-Alexa 488 (CST) y estreptavidina durante 1 hora a Tª ambiente. Las muestras teñidas se analizaron en el citómetro BD LSR Fortessa (Becton Dickinson). Se analizaron tres tinciones distintas: por un lado, se seleccionaron las células de la línea mieloide (CD11b⁺), y, a partir de estas, se seleccionaron los monocitos proinflamatorios (Ly6C⁺), los monocitos patrulla (Ly6C⁻) y los neutrófilos (Ly6G⁺, Ly6C⁺); por otro lado, se seleccionaron los linfocitos CD4⁺ y CD8⁺; por último, se seleccionaron los linfocitos B (B220⁺). Los niveles de lamina A/C se analizaron cuantificando la media de intensidad de fluorescencia (MIF) de la señal emitida por el anticuerpo anti-lamina A/C unido a estos tipos celulares.

La sangre de origen humano se incubó con los marcadores de membrana (**Tabla 3**) durante 15 minutos a Tª ambiente. Posteriormente, se incubó 10 minutos a Tª ambiente con el tampón de fijación y lisis de eritrocitos BD FACS Lysing Solution 1X (Becton Dickinson). Después de un lavado, se lisaron las células con Triton X-100 0,25% durante 15 minutos a Tª ambiente. Las células se lavaron y se incubaron con el anticuerpo anti-lamina A/C-Alexa 488 (CST) durante 1 hora a Tª ambiente en PBS 1X. Las muestras se analizaron en el citómetro BD LSR Fortessa. Se analizaron tres tinciones diferentes: en una de ellas, se seleccionaron los monocitos clásicos (CD14⁺⁺, CD16⁻), monocitos intermedios (CD14⁺⁺, CD16⁺) y monocitos no clásicos (CD14⁺, CD16⁺⁺); en la siguiente, se seleccionaron los neutrófilos (CD66b⁺); por último, se seleccionaron linfocitos T (CD3⁺) y linfocitos B (CD19⁺). Se cuantificó la MIF del anticuerpo anti-lamina A/C unido a

todos estos tipos celulares. Para reducir la señal fluorescente inespecífica se restó a la MIF del anticuerpo anti-lamin A/C la MIF del control de isotipo Mouse (MOPC-21) mAb IgG1(97146, CST).

Tabla 3. Lista de anticuerpos utilizados. WB= Western-Blot, IHQ= Inmunohistoquímica, IF= Inmunofluorescencia, MI= Microscopía intravital. CF= Citometría de flujo.

Anticuerpo	Descripción	Concentración de uso	Técnica	Casa comercial y referencia
Anti-Lamin A/C- A488	Monoclonal de ratón Conjugado al fluorocromo Alexa 488	1/100	IF, CF	Biolegend- 302220
Anti-Ly6C-APC	Monoclonal de rata Conjugado al fluorocromo APC	1/200	IF, CF	Biolegend- 128016
Anti-CD11b-A647	Monoclonal de rata Conjugado al fluorocromo Alexa 647	1/200	IF	BD-557886
Anti-CD11b-biotinilado	Monoclonal de rata Conjugado a biotina	1/200	CF	BD-51 017 125
Anti-Ly6G-PE	Monoclonal de rata Conjugado al fluorocromo PE	1/200	CF	BD-551461
Anti-CD4-PE	Monoclonal de rata Conjugado al fluorocromo PE	1/200	CF	BD-553653
Anti-CD8-A647	Monoclonal de rata Conjugado al fluorocromo Alexa647	1/200	CF	BD-557886
Anti-B220-A647	Monoclonal de rata Conjugado al fluorocromo Alexa 647	1/200	CF	BD-557683
Anti-CD14-A647	Monoclonal de ratón Conjugado al fluorocromo Alexa 647	1/100	CF	Biolegend- 325612
Anti-CD16-PE	Monoclonal de ratón Conjugado al fluorocromo PE	1/100	CF	Biolegend- 302056
Anti-CD66b-A647	Monoclonal de ratón Conjugado al fluorocromo Alexa 647	1/100	CF	Biolegend- 305110

Anticuerpo	Descripción	Concentración de uso	Técnica	Casa comercial y referencia
Anti-CD3-PE	Monoclonal de ratón Conjugado al fluorocromo PE	1/100	CF	Biolegend-300308
Anti-CD19-A647	Monoclonal de ratón Conjugado al fluorocromo Alexa 647	1/100	CF	Biolegend-302220
Anti-SMA-Cy3	Monoclonal de ratón Conjugado al fluorocromo Cy3	1/100	CF	Sigma Aldrich-C6198
Anti-CD106 (VCAM-1)	Monoclonal de rata	1/100	CF	ebioscience-14-1061-82
Anti-IgG-Rata-A633	Monoclonal de cabra Conjugado al fluorocromo Alexa 633	1/400	CF	Invitrogen-A21094
Anti-CD45-V450	Monoclonal de rata Conjugado al fluorocromo V450	1/200	CF	BD-560501
Anti-F4/80-PE-Cy7	Monoclonal de rata Conjugado al fluorocromo PE/Cy7	1/200	CF	Biolegend-123114
Anti-Lamin A/C	Policlonal de cabra	1/500	WB	Santa Cruz-6215
Anti-GAPDH	Monoclonal de ratón	1/10000	WB	Millipore-MAB374
Anti-Ki67	Monoclonal de conejo	Solución lista para usar	IHQ	Master Diagnostica-MAD-000310QD
Anti-Lamp2 (Mac-3)	Monoclonal de rata	1/200	IHQ	Santa Cruz-19991
Anti-Caspase-3	Policlonal de conejo	1/200	IHQ	R y D Systems-AF835
Anti-FSP-1	Policlonal de conejo	1/200	IF	Dako- A5114
Anti-Lamin A/C	Monoclonal de ratón	1/100	IF	Santa Cruz-376248

14. Análisis de expresión de proteínas mediante western blot

Para analizar la expresión de lamina A/C en tejido arterial de ratón y humano durante el envejecimiento, se extrajeron las proteínas y se realizó un ensayo de western blot (WB).

En el caso de las arterias aortas de ratón, se añadieron 70-90 μ L de tampón de lisis de proteínas sobre 2 arcos aórticos o 2 aortas torácicas. Se disgregaron los fragmentos de arteria mediante el sistema TissueLyser (QUIAGEN), que rompe el tejido golpeándolo con pequeñas bolas metálicas a alta velocidad. La muestra homogenizada se centrifugó a 16000 rpm durante 30 minutos a 4°C para descartar los restos de tejido. Se recuperó el sobrenadante y se preservó a -80°C.

La extracción de proteínas en muestras de arteria coronaria humana se realizó pulverizando el tejido arterial congelado. Después, se añadieron 250 μ L de tampón de lisis de proteínas para muestras humanas a 30 μ g de tejido pulverizado. Posteriormente, se homogenizó la mezcla utilizando el homogenizador polytron homogenizer (PRO-Scientific). La muestra homogenizada se centrifugó a 12000 rpm durante 5 minutos a 4°C. Se recuperó el sobrenadante y se preservó a -80°C.

El siguiente paso fue medir la concentración de los extractos proteicos a través del método colorimétrico descrito por Bradford (Bradford, 1976). Se determinó la concentración de las muestras mediante la extrapolación a partir de una recta patrón que se realizó empleando soluciones de concentraciones conocidas de albúmina de suero bovino (BSA) (A7906-100G, SIGMA) entre 0 y 10 g/L. Este procedimiento se llevó a cabo añadiendo 1 mL de una solución 1/5 de Bio-Rad Protein Assay (500-0006-Bio-Rad) en una cubeta de espectrofotometría, además de 1 μ L de las muestras de la curva patrón, así como de cada una de las muestras de estudio. Las cubetas se agitaron y se incubaron durante 15 minutos a Tª ambiente. A continuación, se midió la absorbancia de las cubetas con el espectrofotómetro UV-1603 (Shimadzu) empleando una longitud de onda de 595 nm.

Se añadió tampón de carga a las muestras y se calentaron 5 minutos a 95°C para desnaturalizarlas. Posteriormente, se cargaron 10-25 μ g de cada muestra en geles de poliacrilamida al 10%, con el objetivo de separar las proteínas por su tamaño. Se sumergió el gel en tampón de electroforesis y se aplicó una corriente de 100 V hasta que el frente hubo recorrido todo el gel.

A continuación, se transfirieron las proteínas del gel a una membrana de polifluoruro de vinilo (PVDF) aplicando una corriente de 100 V durante 90 minutos cubierto con tampón de transferencia (Tris 25 mM pH 8.3, glicina 192 mM y metanol 20%). Una vez

transferidas a la membrana, se bloquearon las uniones inespecíficas incubando la membrana en leche desnatada al 4% en TBS-T durante 1 hora a T^a ambiente. Después, se incubaron con los anticuerpos primarios específicos (**Tabla 3**) en la solución de bloqueo durante 12 horas a 4°C en agitación suave. Las membranas se lavaron 3 veces durante 5 minutos con TBS-T. A continuación, se incubaron con anticuerpos secundarios correspondientes (**Tabla 3**) conjugados con HRP durante 1 hora a T^a ambiente. Tras 3 lavados de 5 minutos en TBS-T, se añadió el sustrato HRP Western Luminata Forte (WBLUF0100, Millipore) que genera una reacción luminiscente en contacto con la HRP. La señal se registró y cuantificó utilizando el sistema ImageQuant LAS 4000 mini (GE Health care). Los niveles de lamina A/C se representaron como la suma de la señal de las bandas de lamina A y lamina C, relativizada respecto de la señal de la banda del control de carga correspondiente.

15. Análisis de la expresión génica por PCR cuantitativa

15.1. Extracción de ARN

La expresión de los genes estudiados en este trabajo se realizó por PCR cuantitativa a tiempo real (qPCR) partiendo de ADNc obtenido mediante la retrotranscripción del ARNm.

La extracción se llevó a cabo tanto en leucocitos de sangre como en macrófagos derivados de MO. Los leucocitos de ratón aislados (*material y métodos 5*) fueron resuspendidos en 800 µl del reactivo TRIzol (15596-026, Life Technologies). La misma cantidad de TRIzol fue añadida sobre los macrófagos en cultivo (*material y métodos 9*) que se rasparon para facilitar la ruptura celular.

Para obtener el ARN de los homogenizados en TRIzol se añadieron 160 µl de cloroformo, se mezcló durante 30 segundos y se dejó reposar 10 minutos a T^a ambiente. Después, se centrifugó a 8000 rpm durante 10 min a T^a ambiente para separar las distintas fases. Se transfirió la fase acuosa a un tubo limpio y se añadió el mismo volumen de isopropanol. Después de mezclar, se dejó precipitar el ARN a -20°C durante un mínimo de 12 horas. Posteriormente, se centrifugó a 10000 g durante 20 minutos a 4°C, se descartó el sobrenadante y se lavó el ARN sedimentado 2 veces con etanol al 70%. Después de retirar el etanol, se dejó secar el ARN y se resuspendió en 10-30 µl de agua miliQ.

El ARNm obtenido se cuantificó por absorbancia espectrofotométrica mediante el sistema NanoDrop (Thermo Scientific). Se consideraron muestras con valores de pureza óptimos aquellas cuyo cociente de absorbancias a 260 y 280 nm fuesen cercanos a 2.

15.2. Retrotranscripción y qPCR

Se retrotranscribieron entre 0,5 y 2 µg de ARNm a ADNc utilizando el kit High-Capacity cDNA Reverse Transcription Kit (4368814, Applied biosystems).

La qPCR se realizó mediante el método SYBR Green PCR Master mix (4472908, Applied Biosystems). Por cada reacción se amplificaron 10 ng de ADNc. Los ensayos de qPCR se realizaron en el equipo 7900HT Fast Real-Time PCR System (Applied Biosystems). El análisis se llevó a cabo con los softwares SDS (Applied Biosystems) y Biogazelle qbase (Biogazelle). Dependiendo del tipo celular, los resultados obtenidos se normalizaron a la expresión de sus correspondientes genes control. Los cebadores utilizados para las reacciones de qPCR están incluidos en la **Tabla 4**.

Tabla 4. Cebadores empleados en las reacciones de qPCR.

Gen (Proteína)	Cebador en sentido (5' a 3')	Cebador en antisentido (5' a 3')
<i>Arg1</i> (ARG-1)	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC
<i>Nos2</i> (INOS-2)	GGATCTTCCCAGGCAACCA	TCCACAACCTCGCTCCAAGATT
<i>Cd36</i> (CD36)	GATGACGTGGCAAAGAACAG	TCCTCGGGGTCTGAGTTAT
<i>Scarb1</i> (SCARB-1)	TCTGGCGCTTTTCTATCGT	ACGGCCCATACCTCTAGCTT
<i>Lmna</i> (LAMINA A/C)	TGAGTACAACCTGCGCTCAC	TGACTAGTTGTCCCCGAAG
<i>Lmna</i> (LAMINA A)	GGTTGAGGACAATGAGGATGA	TGAGCGCAGGTTGTAATCAG
<i>Gapdh</i> (GAPDH)	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT
<i>Rn18s</i> (18S)	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCCG
<i>Hprt1</i> (HPRT)	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
<i>Actb</i> (β-actin)	CAGAAGGAGATTACTGCTCTGGCT	TACTCCTGCTTGCTGATCCACATC

16. Inmunofluorescencia de células hematopoyéticas

Para comprobar la ausencia de lamina A/C en las células hematopoyéticas de ratón, se colocaron gotas de una solución de leucocitos aislados en un portaobjetos y se dejó evaporar el líquido en una estufa a 37°C para fijar la muestra al portaobjetos. A continuación, se fijaron las células con PFA al 4% durante 10 minutos a Tª ambiente. Después de lavar la muestra, se permeabilizó con PBS 1X 0,5% Triton X100 durante 15 minutos a Tª ambiente. Tras lavar la muestra, se incubó con una solución bloqueante compuesta por PBS 1X y BSA 5% durante 1 hora a Tª ambiente. Las muestras se tiñeron

con anticuerpos de membrana (**Tabla 3**) diluidos en PBS 1X y BSA 2,5% durante 1 hora a T^a ambiente. Se lavó la muestra 3 veces y se incubó con el anticuerpo anti-lamin A/C-Alexa 488 (CST) durante 1 hora a T^a ambiente en la solución anterior. Tras lavar las muestras, se montaron con medio de montaje Slow fade Gold (S36937, Thermo Fisher).

17. Estudios histopatológicos e inmunohistoquímica

Para analizar la composición de la placa de ateroma se realizaron 3 cortes histológicos de 5 µm de grosor de 3 regiones diferentes de las válvulas aórticas procedentes de corazones fijados con PFA al 4% y embebidos en parafina de ratones *Ldlr*^{-/-} trasplantados con MO *Lmna*^{-/-} o WT alimentados con DG durante 6 semanas. Los cortes fueron desparafinados con lavados de xileno (3 lavados de 5 minutos). Posteriormente, fueron rehidratados mediante lavados de etanol al 95% (2 lavados de 5 minutos), etanol al 70% (2 lavados de 5 minutos) y etanol al 50% (2 lavados de 5 minutos). Finalmente se pasaron por agua miliQ durante 1 segundo.

Para estudiar la proporción de colágeno de la placa de ateroma se tiñeron las muestras con el kit de tinción Masson-Goldner (1.00485, Merck). Esta tinción se llevó a cabo preparando dos soluciones de trabajo: una solución A compuesta por hematoxilina de Weigert al 1% en etanol absoluto preparada al menos una semana antes de su uso y mantenida en oscuridad, y una solución B formada por 0,4% de cloruro férrico al 30% y 0,1% de ácido clorhídrico en agua destilada. Para realizar la tinción se mezclaron ambas soluciones vertiendo B sobre A. Los cortes desparafinados se sumergieron en la mezcla 4 minutos y, después, se lavaron con agua corriente 10 minutos. A continuación, se incubaron con fucsina al 0,1% y ácido acético al 10% en agua destilada. Seguidamente se incubaron en una solución de ácido fosfotúngstico al 10% durante 4 minutos. Posteriormente, los cortes se sumergieron en ácido acético al 10% en agua destilada durante 5 minutos para provocar la desdiferenciación de la muestra. Finalmente, los cortes fueron sumergidos en una solución de verde luz al 1% y ácido acético al 0,5% en agua destilada durante 50 segundos. Los cortes histológicos teñidos se lavaron en ácido acético al 10% durante 2 minutos y se deshidrataron sumergiéndolos 1 minuto en alcohol al 95%, 1 minuto en etanol absoluto y 1 minuto en xileno. Las muestras se montaron con Eukitt (O. Kindler GmbH & Co). Se tomaron las imágenes con un microscopio OLYMPUS BX41 a un aumento de 10X. Por medio de una deconvolución de colores “Feulgen Light Green” predeterminada por el software Image J (NIH) se cuantificó el porcentaje de colágeno en el área de lesión.

El estudio de la proporción de macrófagos, células proliferantes y área apoptótica se realizó mediante inmunohistoquímica. Después de la rehidratación, se desenmascararon los antígenos con tampón citrato durante 20 minutos en el microondas a máxima potencia. Una vez atemperadas, las muestras se bloquearon con H₂O₂ al 3% para eliminar la actividad de la enzima peroxidasa endógena. Tras lavar las muestras, se bloquearon durante 30 minutos con T-PBS con 10% de FBS. Después de un lavado, las muestras se incubaron durante 45 minutos en una solución de suero normal de caballo al 5% (S-2000, Vector Labs) en PBS con los anticuerpos primarios correspondientes: mac-3 (macrófagos), Ki67 (proliferación celular) y caspasa-3 (apoptosis) (**Tabla 3**). Se lavaron las muestras y se incubaron con anticuerpos secundarios conjugados con peroxidasa de rábano picante (HRP) durante 1 hora. Se volvieron a lavar las muestras y se añadió el compuesto DAB y su sustrato para poder generar una reacción cromogénica, produciendo un color parduzco. A continuación, se lavaron las muestras y se contratiñeron con hematoxilina de Mayer durante 5 minutos para marcar los núcleos en azul. Después, se montaron con Eukitt. Se tomaron las imágenes como se ha descrito anteriormente. Las imágenes se analizaron con el software Image J utilizando la deconvolución de colores predeterminada “H&E DAB”. Se calculó el porcentaje de área marcada con anticuerpo dentro del área de lesión.

Para el análisis histopatológico de los ratones *Lmna*^{flox/flox} *SM22αCre* se utilizaron cortes de diferentes órganos incluidos en parafina. Por un lado, se realizó la tinción y el análisis de la cantidad de colágeno mediante la tinción de Masson-Goldner. Por otra parte, se realizó un estudio complementario del contenido en colágeno del corazón utilizando la tinción de Sirius Red F3B (C.I.35782, SIGMA) que, principalmente, es usado para cuantificar la fibrosis intersticial. Se procedió de la misma manera que con la tinción de Masson-Goldner hasta el primer lavado con agua corriente. Después, se lavaron las muestras con agua destilada y se incubaron con Sirius red durante 1 hora. Se lavaron las muestras con una solución de ácido acético al 0,5%. Finalmente, se deshidrataron y montaron las muestras como en la tinción de Masson-Goldner. Las imágenes se tomaron como se ha descrito anteriormente. Mediante la deconvolución de colores predeterminada “Fastred-Fastblue” del software Image J se analizó el porcentaje de colágeno en cortes histológicos de corazón. Por último, en el caso de las muestras de arteria aorta, se realizó una tinción de hematoxilina-eosina (HE). Los cortes desparafinados se sumergieron en una solución de hematoxilina de Harris (Sigma) durante 30 segundos, tiñéndose los núcleos de las células con un color violeta intenso. Después, se hicieron lavados rápidos con una solución de diferenciación (0,25% de HCl en etanol al 70%). Posteriormente, se tiñeron los citoplasmas sumergiendo las muestras

en una solución hidro-alcohólica de eosina al 50% durante 20 segundos. Finalmente, se deshidrataron las secciones histológicas mediante concentraciones crecientes de etanol de manera inversa a lo expuesto anteriormente. Después se montaron con Eukitt. Se adquirieron las imágenes y, utilizando la deconvolución de colores predeterminada “H&E” del software Image J, se cuantificó el número de núcleos en el área seleccionada.

18. Inmunofluorescencia en tejidos

Para comprobar la delección de lamina A/C en diferentes tejidos y para analizar el corazón de los ratones *Lmna^{flox/flox} SM22αCre* se utilizaron órganos fijados e incluidos en parafina. Se realizaron 3 cortes de 5 µm de 3 zonas diferentes de cada órgano. Las secciones fueron desparafinadas y los antígenos desenmascarados como en *material y métodos 15*. Después, se bloquearon y permeabilizaron las secciones de tejido con PBS 1X, Triton X-100 0,3%, BSA 5% (A7906-100G, SIGMA) y suero normal de cabra 5% (005-000-001, Jackson ImmunoResearch) durante 1 hora a Tª ambiente. A continuación, las muestras se incubaron durante 12 horas a 4°C con los anticuerpos primarios para detectar CMLVs y miofibroblastos (SMA), células endoteliales (CD31), lamina A/C, fibroblastos activados (FSP-1) y membranas celulares (WGA) (**Tabla 3**). Tras lavar los anticuerpos primarios, se incubaron las muestras durante 2 horas a Tª ambiente con los anticuerpos secundarios correspondientes que llevaban moléculas fluorescentes conjugadas y con los marcadores nucleares DAPI o Hoechst 33342 (H3570, ThermoFischer). Después de los lavados, se montó la muestra usando el medio de montaje Fluoromount G (4958-02, Affymetrix eBioscience). Las imágenes se adquirieron con un microscopio confocal Zeiss LSM 700 (Zeiss) y fueron analizadas con el software Image J. En el caso de las muestras de corazón, se cuantificó en endocardio, miocardio y epicardio el porcentaje de área marcada con SMA, FSP-1 y WGA respecto al área de tejido realizando la media entre las 3 regiones mencionadas. En el análisis de SMA el área de tejido considerado no incluía las arteriolas del corazón. Por otra parte, se seleccionaron los cardiomiocitos por su morfología y los FCs por su tinción de FSP-1 y, posteriormente, se cuantificó el porcentaje de ambos tipos celulares que expresaban lamina A/C.

19. Aislamiento de células de médula ósea

Inicialmente, se extrajeron el fémur y la tibia de los ratones. Se retiró el pelo y la musculatura hasta limpiar completamente el hueso. Se cortaron las cabezas de los huesos y se introdujo PBS 1X a través de una jeringa por un extremo para poder extraer la médula. Una vez extraída, se disgregó hasta obtener una solución de células aisladas que se centrifugaron a 1350 rpm, 5 minutos a 4°C. Posteriormente, se incubaron con tampón de lisis de eritrocitos durante 5 minutos en hielo. Las células se lavaron y se centrifugaron a 1350 rpm, 5 minutos a 4°C.

20. Aislamiento y diferenciación de macrófagos derivados de médula ósea

Se aislaron y diferenciaron Macrófagos derivados de médula ósea (MDMO) de ratones WT y *Lmna*^{-/-} para analizar su expresión génica.

Primero, se extrajeron las células de MO según *material y métodos* 9. Las células progenitoras procedentes de 2 fémures y 2 tibias de cada animal se distribuyeron en 3 pocillos de una placa multi-pocillo P6 (Falcon) con medio de diferenciación de macrófagos a 37°C en una atmósfera con el 5% de CO₂ durante 7 días, cambiando el medio de cultivo cada 2 días. Después, los macrófagos se incubaron con medio de polarización M1 (expresan iNOS2), medio de polarización M2 (expresan arginasa-1) o medio de diferenciación de macrófagos (M₀) durante 24h a 37°C con 5% de CO₂. Los ensayos de expresión génica se llevaron a cabo según *métodos* 6.

21. Ensayos de migración en chips de polidimetilsiloxano

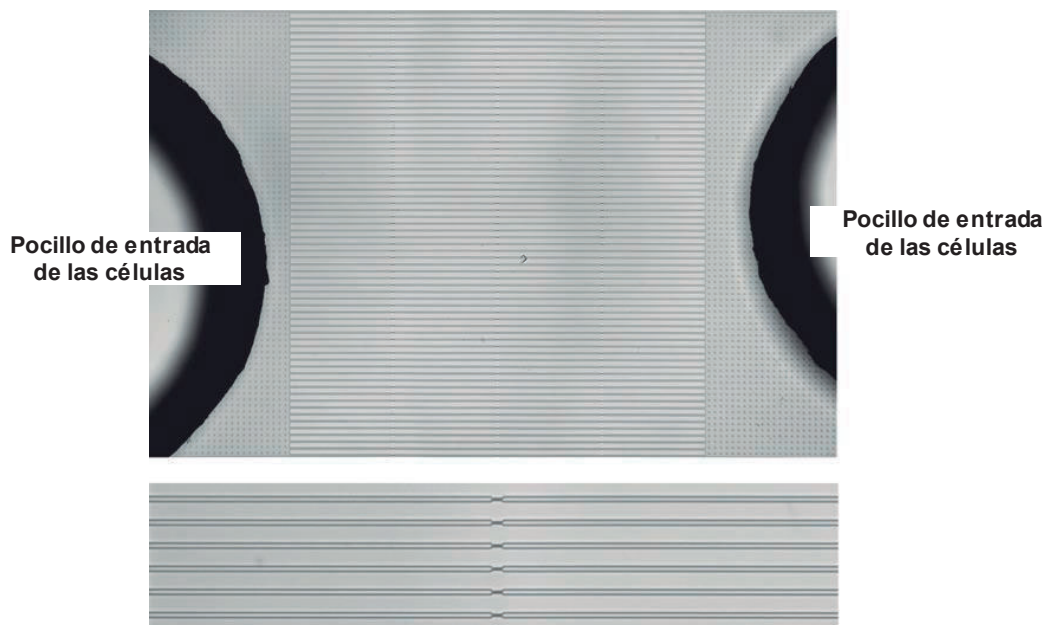
Para profundizar en el estudio del papel de las laminas A/C en la migración de células del SI se realizaron experimentos *in vitro* con monocitos proinflamatorios procedentes de MO en chips de polidimetilsiloxano (PDMS).

Se extrajo MO de ratones WT y *Lmna*^{-/-} según *material y métodos* 9. Las células aisladas de la MO se tiñeron durante 30 minutos a 4°C con los anticuerpos de membrana anti-CD11b, anti-Ly6C y anti-Ly6G (**Tabla 3**) y el marcador de viabilidad celular 4',6-diamidino-2-phenylindol (DAPI) (Thermo Fisher), que penetra en las células muertas facilitando el descarte de las mismas. Se utilizó el sorteador de células SY3200 cell sorter (Sony biotechnology) para seleccionar la población de leucocitos de interés. Primero, se descartaron las células muertas, teñidas con DAPI. Después, se seleccionó

la población CD11b⁺, Ly6C⁺, Ly6G⁻ que se corresponde con los monocitos proinflamatorios.

Las células se tiñeron con Hoechst 33342 para su seguimiento y se sembraron con medio de cultivo de monocitos en chips de PDMS que contenían canales tubulares de 5 x 2.8 μm y también en chips similares que presentaban constricciones de 1 μm a la mitad del recorrido del canal (Sáez et al., 2018). Después de 24 horas a 37°C y 5% de CO₂, las células comenzaron a migrar a través de los canales. En ese momento, se adquirieron vídeos durante 24 horas a través del microscopio invertido DMi8 (Leica) con un objetivo 10x (NA 0.40 phase), capaz de mantener las condiciones de cultivo celular (**Figura 9**). Se analizó la velocidad de los monocitos a través de los canales rectos y el tiempo que empleaban las células en migrar a través de las constricciones mediante el software image J.

A



B

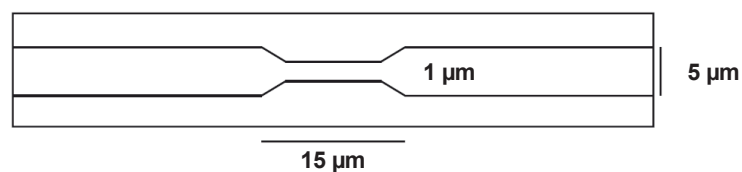


Figura 9. Chips microfabricados para estudiar la migración celular *in vitro*. (A) Imagen con un microscopio de contraste de un chip de PDMS con canales, mostrando en los extremos los pocillos de entrada de las células. La imagen inferior muestra un detalle ampliado de la región del chip con constricciones. (B) Esquema ilustrativo del tipo de constricción utilizada en el experimento. Figura adaptada de Sáez et al., 2018.

22. Ecocardiografía

Para analizar la función cardíaca de los ratones *Lmna^{flox/flox} SM22αCre* se realizaron estudios de imagen ecocardiográfica. Estos estudios fueron llevados a cabo por personal técnico experto del CNIC utilizando el sistema de ultrasonidos de alta frecuencia Vevo 2100 (Visualsonics Inc.) con una sonda linear de 40 MHz. Se realizó una ecografía bi-dimensional (2D) y en modo M (MM) por encima de 230 cuadros por segundo. Tras depilarlos, los animales fueron anestesiados ligeramente con 0,5-2% de isoflurano en oxígeno, inhalado a través de una máscara facial ajustando la dosis de isoflurano, intentando mantener el ritmo cardíaco en 450±50 pulsaciones por minuto. Los ratones se colocaron en posición decúbito supino utilizando una plataforma termostatzada y gel de ultrasonidos caliente para mantener las condiciones de Tª óptimas. Se monitorizó el electrocardiograma (ECG) en todo momento. Las imágenes fueron analizadas mediante el software Vevo 2100 Workstation (Visualsonics Inc.). El análisis de la función sistólica se llevó a cabo de la siguiente manera: para medir la función del ventrículo izquierdo se adquirieron imágenes 2D paraesternal estándar y MM y vistas de los ejes cortos y largos (vistas LAX y SAX, respectivamente). Con estas imágenes se calculó la fracción de eyección del ventrículo izquierdo (FEVI) y las dimensiones de la cámara. La función sistólica del ventrículo derecho se estimó indirectamente usando la excursión sistólica plana anular tricúspide (TAPSE), obtenida de una vista apical de cuatro cámaras en MM, midiendo el máximo movimiento anular lateral tricúspide. Por último, se midió el grosor de la pared del ventrículo izquierdo.

23. Estudios electrocardiográficos

Para estudiar la actividad eléctrica del corazón de los ratones *Lmna^{flox/flox} SM22αCre* se realizó un análisis electrocardiográfico. Los animales fueron anestesiados con 0,5-1% de isoflurano en oxígeno, inhalado a través de una máscara facial. Para evitar variaciones debidas a los ritmos circadianos, los ECGs se realizaron por la mañana. Los electrodos de ECG fueron insertados subcutáneamente en las cuatro extremidades. Se grabaron ECGs secuenciales a 2 KHz usando una estación de adquisición MP36R (Biopac Systems) y fueron exportados con el software AcqKnowledge (Biopac Systems). Posteriormente, se realizó un análisis automático con el programa R en el que, primero, se eliminó el ruido de fondo y las fluctuaciones de la línea base. Después, se detectaron los latidos cardíacos, los picos y las ondas. A continuación, se excluyeron los artefactos. Por último, se calculó el ritmo cardíaco, los intervalos del ECG (PQ, QRS, QT) y la pendiente de la onda T. Se seleccionó la segunda derivación para el estudio, ya que la

señal era más estable en los experimentos, lo que permitió una identificación de onda más robusta. El ritmo cardíaco fue calculado como la inversa de la diferencia entre 2 picos de onda R consecutivos (RR). Debido al potencial ruido de interferencia en la detección del inicio de las ondas P, el intervalo PQ (conducción aurículo-ventricular) se midió desde el pico de la onda P hasta el inicio de la onda Q. El final de la onda S (J) no es evidente en ratón porque el segmento ST está ausente en esta especie y es remplazado por una onda J correspondiente a un segmento positivo de la onda T. Por lo tanto, el complejo QRS (conducción ventricular) fue calculado desde Qs al mínimo de la onda S y el intervalo QT (conducción ventricular) desde Qs al pico de la onda T. Las alteraciones morfológicas de la onda T que definen la capacidad de repolarización fueron cuantificadas definiendo la pendiente de la onda T como un indicador del aplanamiento de la onda T que representa el valor absoluto de la pendiente (variación de voltaje en el tiempo) entre el pico de la onda T y T_{90} (**Figura 10**).

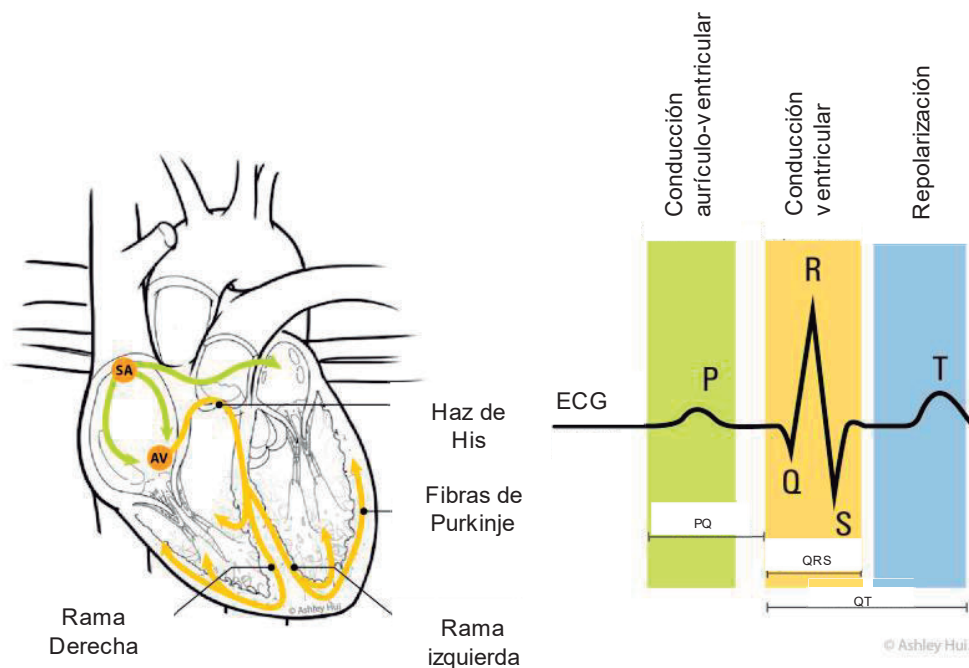


Figura 10. Análisis de un electrocardiograma. Esquema ilustrativo de un ECG con los principales parámetros analizados. Figura adaptada de ptexphys.utorontoit.com/cardiovascular-physiology/heart-function-and-physiology/.

24. Análisis estadístico

Los datos experimentales se representan como la media y las barras de error indican el error estándar de la media. En los experimentos en los que se comparan dos grupos, las diferencias significativas se analizaron mediante el test T de student no pareado de 2 colas. En el caso del análisis de la expresión de lamina A/C en arterias humanas, se aplicó un test de Mann-Whitney de 2 colas. Para comparar varios grupos entre sí, se utilizó el test One-way ANOVA y un post-test de Bonferroni. Las diferencias fueron consideradas significativas para valores $p < 0.05$. Todos los análisis estadísticos se llevaron a cabo con el software GraphPad Prism 5.

RESULTADOS

1. Regulación de la expresión de lamina A/C en el sistema inmune mediada por el envejecimiento y la hipercolesterolemia y estudio de su posible papel en el desarrollo de enfermedades cardiovasculares

1.1. Regulación de la expresión de lamina A/C en el sistema inmune mediada por el envejecimiento y la hipercolesterolemia

El envejecimiento y la hipercolesterolemia son los dos mayores factores de riesgo para el desarrollo de ECVs. Teniendo en cuenta que las células del SI juegan un papel clave en el desarrollo de ECVs (Hansson et al., 2006; Hansson & Libby, 2006) y que las laminas de tipo A regulan la activación y diferenciación de linfocitos T (González-Granado et al., 2014; Rocha-Perugini & González-Granado, 2014; Toribio-Fernández et al., 2018), quisimos comprobar si el envejecimiento y la hipercolesterolemia regulan la expresión de lamina A/C en el SI.

Inicialmente, estudiamos los niveles de lamina A/C durante el envejecimiento en leucocitos humanos. Para ello, obtuvimos leucocitos de muestras de sangre de individuos de edades comprendidas entre 19-44 años y entre 67-89 años. Mediante citometría de flujo demostramos que la expresión de lamina A/C disminuía significativamente en neutrófilos y linfocitos B de los individuos de mayor edad, observando una tendencia descendente en el resto de subpoblaciones leucocitarias (**Figura 11**). Estos resultados concuerdan con estudios previos que describen un descenso de la lamina A durante el envejecimiento en otros tipos de células humanas (Golubtsova, 2016).

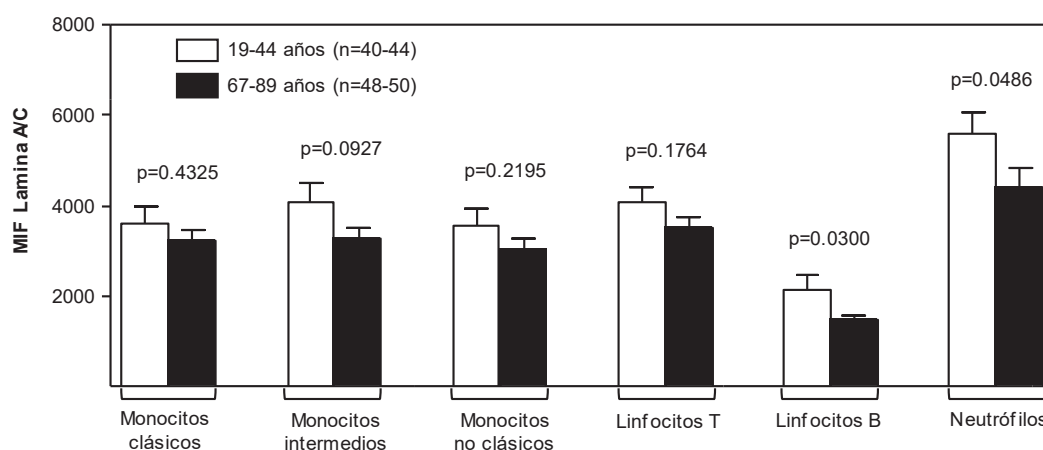
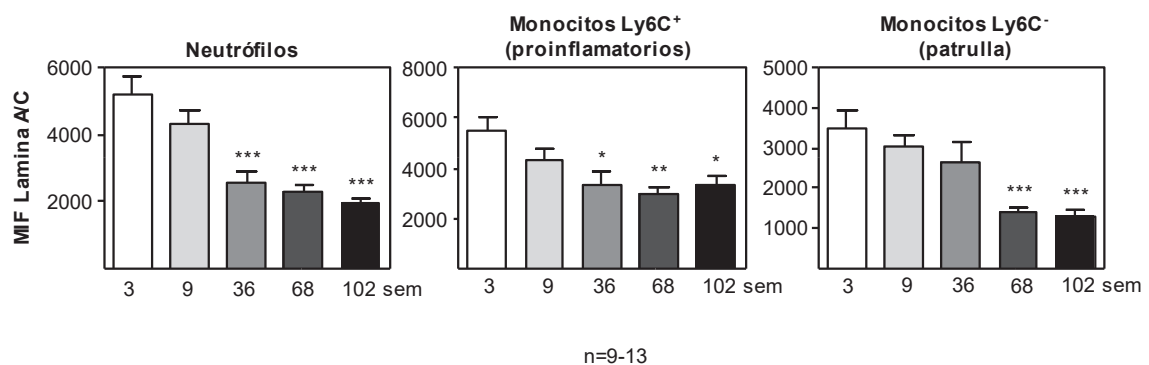


Figura 11. Los niveles de lamina A/C descienden durante el envejecimiento en distintas poblaciones de células del sistema inmune humano. Análisis por citometría de flujo de expresión de lamina A/C en distintas subpoblaciones leucocitarias procedentes de sangre de individuos de las edades indicadas. Las diferencias entre los dos grupos de edad en cada tipo celular se analizaron mediante test T de student.

A continuación, realizamos estudios similares en leucocitos de ratón, y observamos también un descenso en los niveles de lamina A/C durante el envejecimiento, tanto en subpoblaciones leucocitarias de origen mieloide (neutrófilos, monocitos proinflamatorios y monocitos patrulla) como en sub-poblaciones de origen linfoide (células T CD8⁺ y células T CD4⁺) (**Figura 12**). Estos resultados concuerdan con varios estudios que han demostrado una disminución de la expresión de lamina A/C durante el envejecimiento en otros tipos celulares de ratón (Afilalo et al., 2007; Duque & Rivas, 2006).

A



B

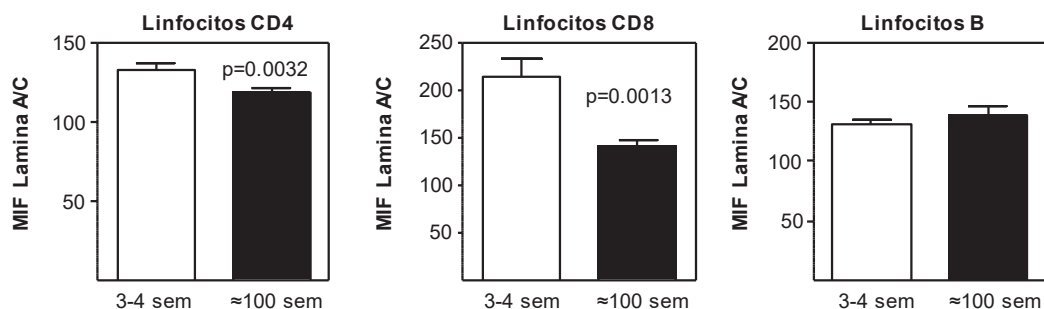


Figura 12. Los niveles de lamina A/C descienden durante el envejecimiento en diversas poblaciones del sistema inmune de ratón. (A) Análisis por citometría de flujo de la expresión de lamina A/C en distintas subpoblaciones leucocitarias de origen mieloide procedentes de sangre de ratones de edades comprendidas entre 3 y 102 semanas (sem). Los resultados se analizaron comparando el grupo de 3 semanas de edad con el resto usando el test estadístico one-way ANOVA y un post-test de Bonferroni. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **(B)** Análisis por citometría de flujo de poblaciones leucocitarias de origen linfoide procedentes de sangre de ratones jóvenes (3-4 semanas de edad) y viejos (≈100 semanas de edad). Los resultados se analizaron con el test T de student.

Para estudiar el papel de la hipercolesterolemia sobre la expresión de lamina A/C en leucocitos totales de sangre de ratón, realizamos un experimento en ratones *Ldlr*^{-/-} alimentados con DN o DG durante 12 semanas. Tal como era de esperar, los ratones alimentados con DG desarrollaron hipercolesterolemia (**Figura 13A**) y arteriosclerosis (**Figura 13B**). Mediante qPCR observamos una disminución de los niveles de ARNm de lamina A y lamina A/C en los ratones alimentados con DG (**Figura 13C**).

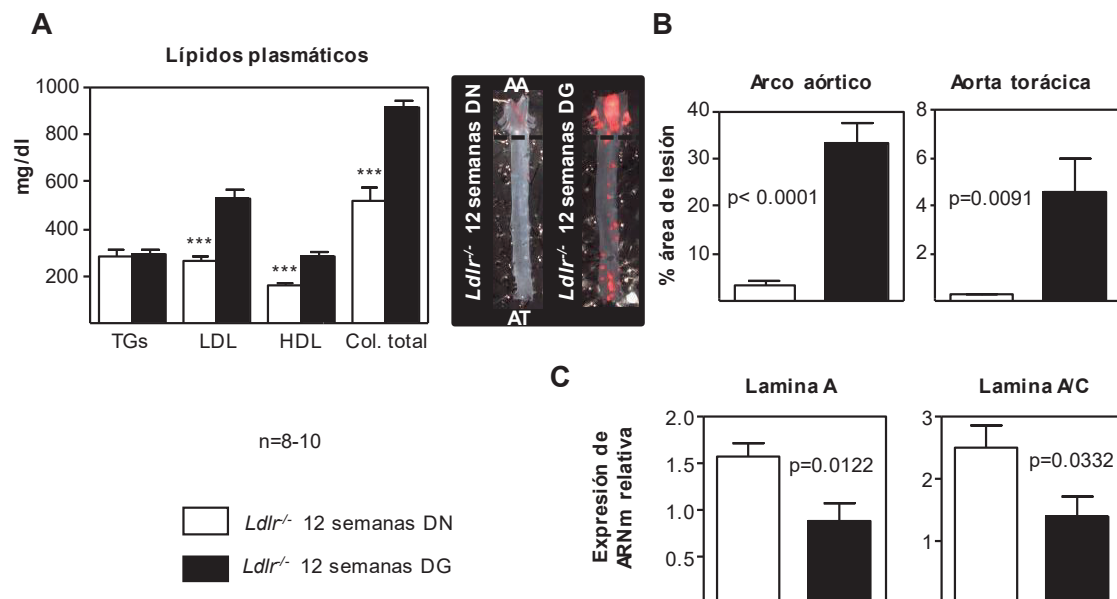


Figura 13. La hipercolesterolemia regula negativamente los niveles de ARNm de lamina A/C en leucocitos de ratón. Ratones *Ldlr*^{-/-} fueron alimentados con DN o DG durante 12 semanas. **(A)** Perfil lipídico en plasma. Para cada lípido, los resultados con DN y DG se analizaron usando un test T de student. ***, $p < 0.0001$. **(B)** Imágenes representativas de aortas teñidas con Oil-Red O (AA: arco aórtico; AT: aorta torácica). Las gráficas muestran la cuantificación de la placa de ateroma en el AA y la AT. Los datos se analizaron con el test T de student. **(C)** Análisis de los niveles de ARNm de lamina A y lamina A/C en leucocitos de sangre de ratón. Los datos están representados como la expresión relativa de cada gen con respecto a la expresión de los genes control HPRT y β -actina. Los datos se analizaron con el test T de student.

1.2. La ausencia de lamina A/C en células del sistema inmune aumenta el desarrollo de arteriosclerosis en ratones *Ldlr*^{-/-}

Los resultados anteriores demuestran que el envejecimiento y la hipercolesterolemia se asocian con una marcada reducción de los niveles de lamina A/C en el SI. Con el fin de determinar si los niveles de expresión de lamina A/C en el SI afecta al proceso de aterogénesis, realizamos experimentos de trasplante de MO de ratones *Lmna*^{-/-} o WT (CD45.2) en ratones receptores *Ldlr*^{-/-} (CD45.1) irradiados letalmente (MO *Lmna*^{-/-} $\rightarrow$ *Ldlr*^{-/-} y MO WT $\rightarrow$ *Ldlr*^{-/-}, respectivamente). Tras el trasplante, los ratones se alimentaron durante 6 semanas con DG (**Figura 14**). Los resultados mostraron una eficiencia de trasplante cercana al 100% en ambos grupos (**Figura 15**).

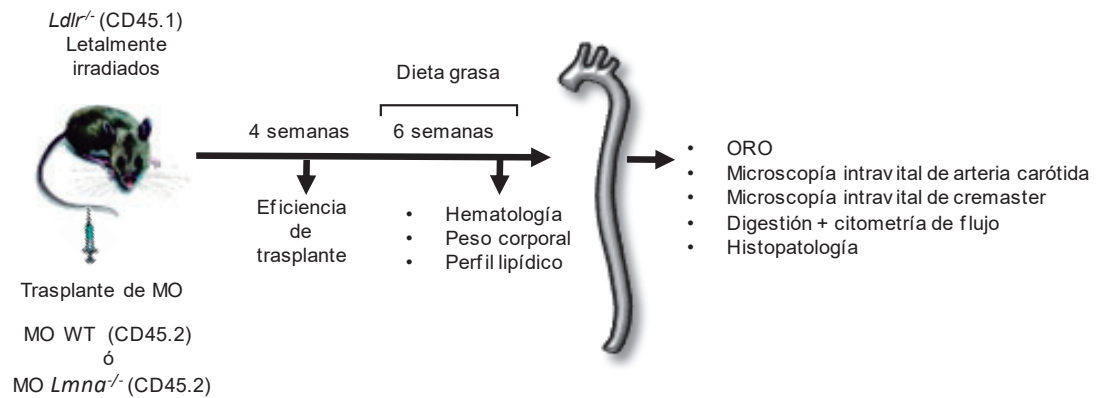


Figura 14. Esquema del procedimiento experimental del modelo de ratón *Ldlr*^{-/-} trasplantado y alimentado con DG.

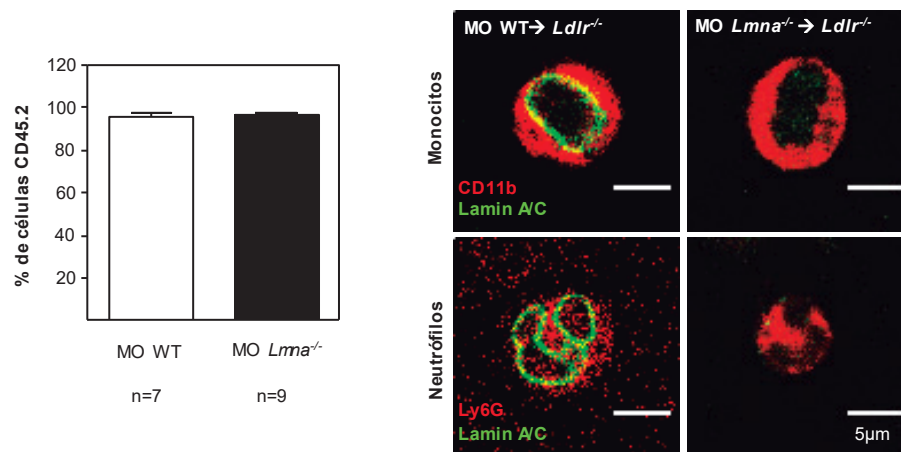


Figura 15. La ausencia de lamina A/C en el sistema inmune no afecta a la eficiencia de trasplante de médula ósea en ratones *Ldlr*^{-/-}. La gráfica muestra los resultados de análisis por citometría de flujo para cuantificar el porcentaje de células CD45.2⁺ en sangre extraída a ratones *Ldlr*^{-/-} (fenotipo CD45.1) 4 semanas tras ser trasplantados con MO WT o *Lmna*^{-/-} (CD45.2). Las imágenes muestran la expresión de lamina A/C (en verde) en monocitos (CD11b) y neutrófilos (Ly6G) (en rojo) procedentes de la sangre de los ratones trasplantados.

Después de 6 semanas con DG, se midieron parámetros fisiológicos básicos, incluyendo peso corporal (**Figura 16 A**), cantidad de monocitos, neutrófilos y linfocitos circulantes (**Figura 16 B**), y niveles de lípidos en plasma (**Figura 16C**), no observándose diferencias estadísticamente significativas en ninguno de estos parámetros al comparar ambos grupos de ratones.

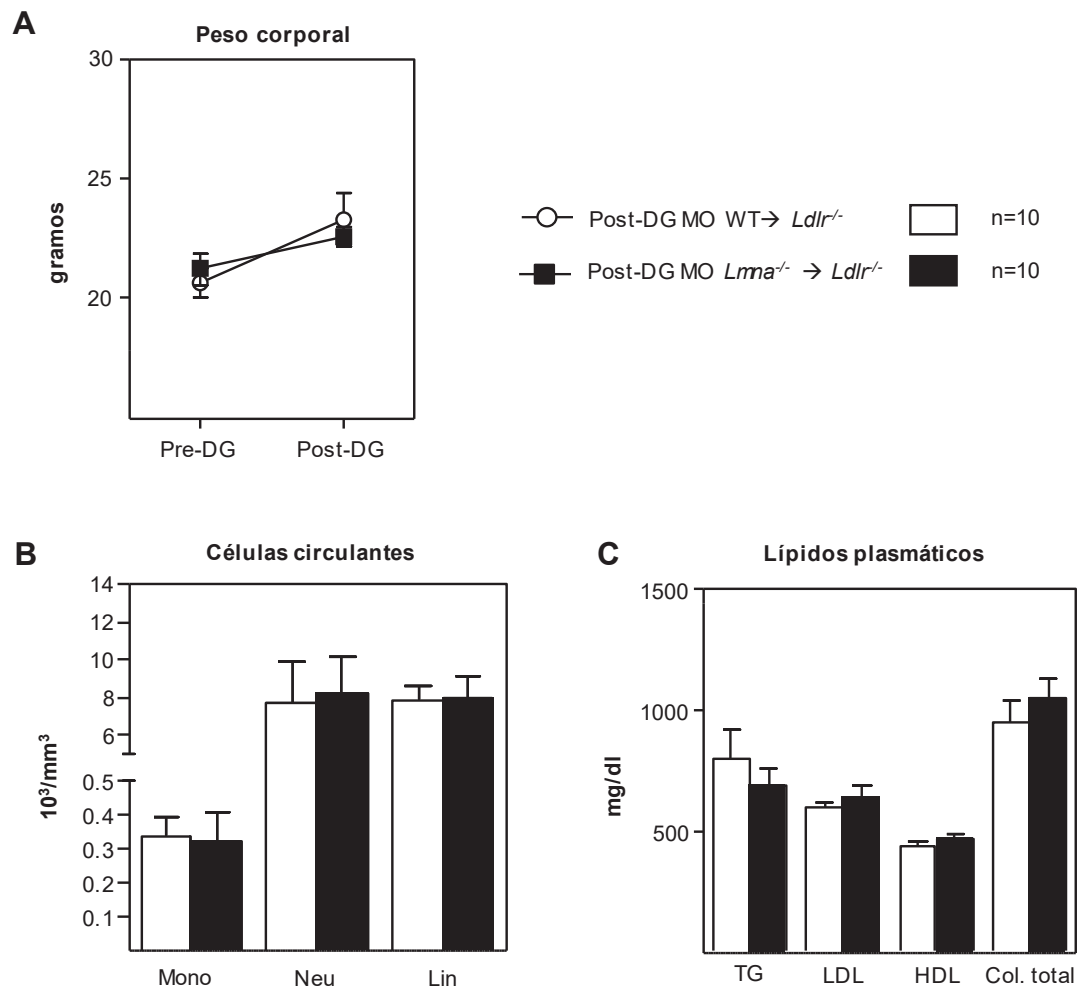


Figura 16. La ausencia de lamina A/C en el sistema inmune no afecta a parámetros fisiológicos básicos en ratones *Ldlr*^{-/-} alimentados con dieta grasa. Ratones *Ldlr*^{-/-} fueron trasplantados con MO *Lmna*^{-/-} o WT y alimentados con DG durante 6 semanas. **(A)** Peso corporal antes (Pre-DG) y después (post-DG) de la alimentación con DG. **(B)** Concentración de monocitos (Mono), neutrófilos (Neu) y linfocitos (Lin) después de la DG. **(C)** Perfil lipídico de los ratones al final de la DG.

La cuantificación del AA y las arterias carótidas teñidas en face con ORO mostró un aumento en el porcentaje de placa de ateroma en los ratones MO *Lmna*^{-/-} $\rightarrow$ *Ldlr*^{-/-} comparado con los controles (**Figura 17**). Estos resultados demuestran una relación causal entre la eliminación de la lamina A/C en el SI y el desarrollo de placas de ateroma en ratones *Ldlr*^{-/-}.

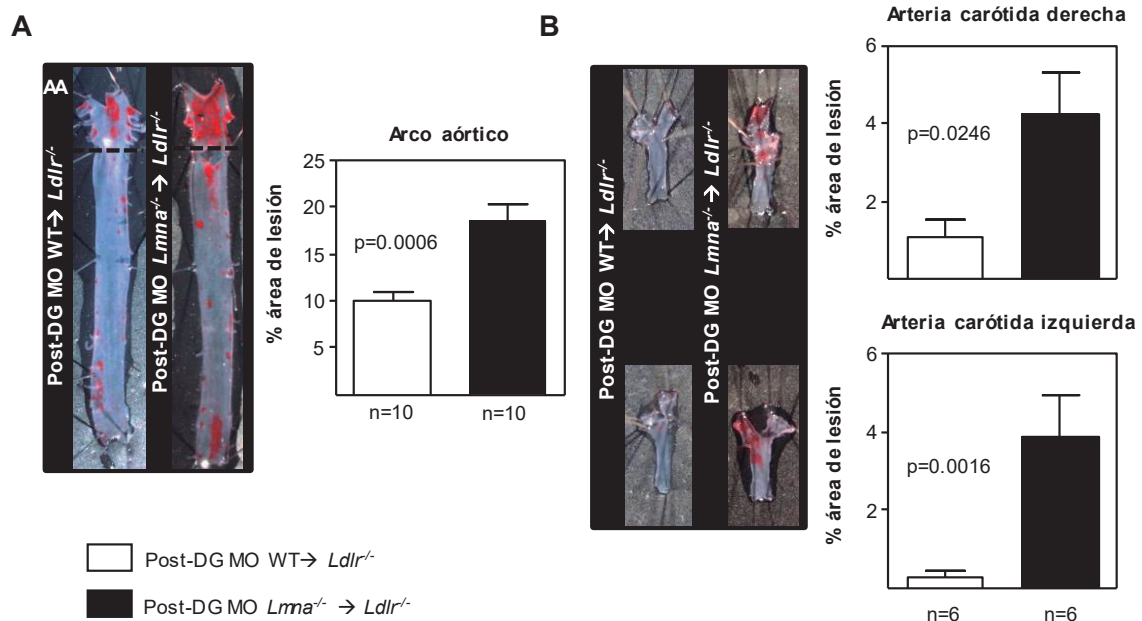


Figura 17. La ausencia de lamina A/C en el sistema inmune aumenta el desarrollo de aterosclerosis en ratones *Ldlr*^{-/-} alimentados con dieta grasa. Ratones *Ldlr*^{-/-} fueron trasplantados con MO *Lmna*^{-/-} o WT y alimentados con DG durante 6 semanas. Se extrajo la aorta y las arterias carótidas para realizar tinción con Oil-Red O para cuantificar la cantidad de arteriosclerosis. **(A)** Imágenes representativas de arterias aortas y cuantificación del porcentaje de placa de ateroma en el AA. **(B)** Imágenes representativas de arterias carótidas y cuantificación del porcentaje de placa de ateroma las arterias carótidas.

1.3. Efectos de la ausencia de lamina A/C en el sistema inmune sobre el reclutamiento leucocitario durante la arteriosclerosis

Con el objetivo de conocer los mecanismos celulares implicados en el aumento de aterosclerosis en los ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-}, decidimos estudiar las primeras etapas del desarrollo de la enfermedad, concretamente el reclutamiento leucocitario *in vivo*. Para ello realizamos estudios de microscopía intravital de la arteria carótida, que mostraron un flujo similar de rodamiento de monocitos y neutrófilos sobre el lumen arterial similar en los dos grupos experimentales (**Figura 18**).

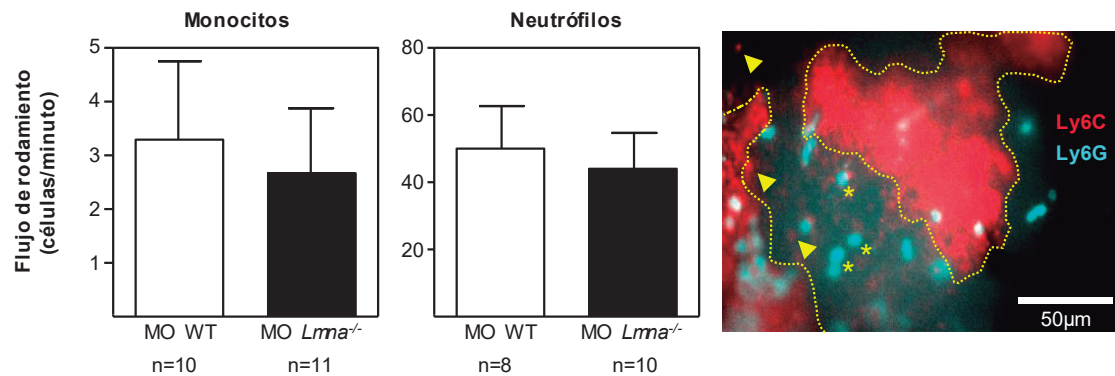


Figura 18. La ausencia de lamina A/C en el sistema inmune no afecta al rodamiento leucocitario sobre la arteria carótida durante la arteriosclerosis. Cuantificación del rodamiento de neutrófilos y monocitos en la arteria carótida de ratones *Ldlr*^{-/-} trasplantados con MO WT o *Lmna*^{-/-} alimentados con DG. La imagen muestra leucocitos rodando por el endotelio de la arteria carótida. Las flechas apuntan a los monocitos (Ly6C) (en rojo) y los asteriscos a los neutrófilos (Ly6G) (en cyan) durante el proceso de rodamiento. La línea discontinua marca placas de ateroma establecidas.

Después de descartar el rodamiento como causante del aumento de arteriosclerosis en los ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-}, decidimos analizar la adhesión de leucocitos al endotelio, que es la siguiente fase del reclutamiento leucocitario durante el desarrollo de la placa de ateroma. Para ello, realizamos estudios de microscopía intravital en vénulas del músculo cremáster de ratón, tejido que permite una mayor capacidad de visualización comparado con la microscopía intravital en arteria carótida. Estos experimentos no mostraron diferencias significativas en la adhesión de monocitos y neutrófilos al endotelio de las vénulas del músculo cremáster al comparar ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-} con sus controles (**Figura 19**).

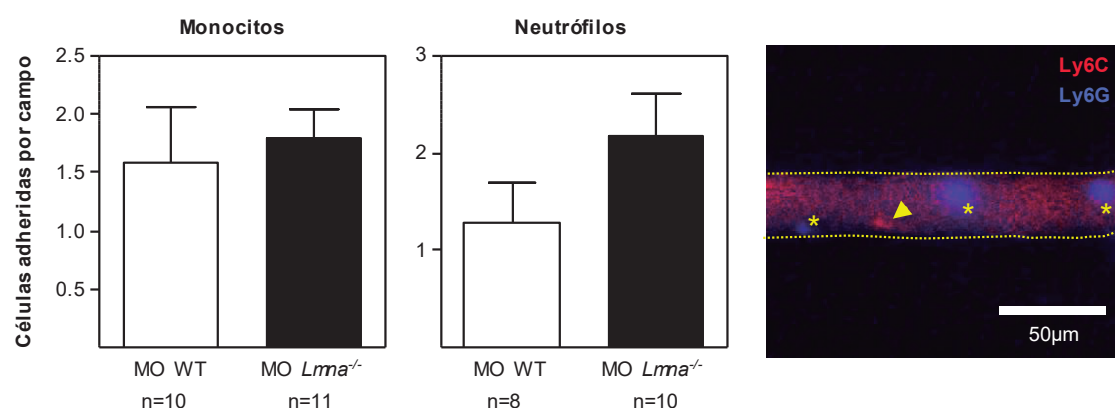


Figura 19. La ausencia de lamina A/C en el sistema inmune no afecta a la adhesión de leucocitos al endotelio de las vénulas del músculo cremáster. Cuantificación de la adhesión de neutrófilos y monocitos en vénulas del músculo cremáster de ratones *Ldlr*^{-/-} trasplantados con MO WT o *Lmna*^{-/-} alimentados con DG. La imagen muestra leucocitos adheridos a una vénula cremastérica. La flecha apunta a un monocito (Ly6C) (en rojo) y los asteriscos a neutrófilos (Ly6G) (en azul) adheridos. La línea discontinua muestra el contorno del vaso.

Al no observar diferencias en los procesos de rodamiento y adhesión, decidimos investigar si el aumento del desarrollo de arteriosclerosis en los ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-} podría explicarse por un incremento en la extravasación de los leucocitos tras su adhesión al endotelio. Mediante microscopía intravital en el músculo cremáster observamos que los animales MO *Lmna*^{-/-} → *Ldlr*^{-/-} alimentados con DG presentaban más leucocitos extravasados que el grupo control (**Figura 20A**), si bien se movían más lentamente una vez extravasados (**Figura 20B**). Los resultados obtenidos sugieren que la ausencia de lamina A/C en células inmunitarias aumenta el desarrollo de arteriosclerosis en un entorno hipercolesterolémico, al menos en parte debido a una mayor extravasación de leucocitos (que sin embargo son más lentos), sin que el rodamiento ni la adhesión leucocitaria se vean afectados.

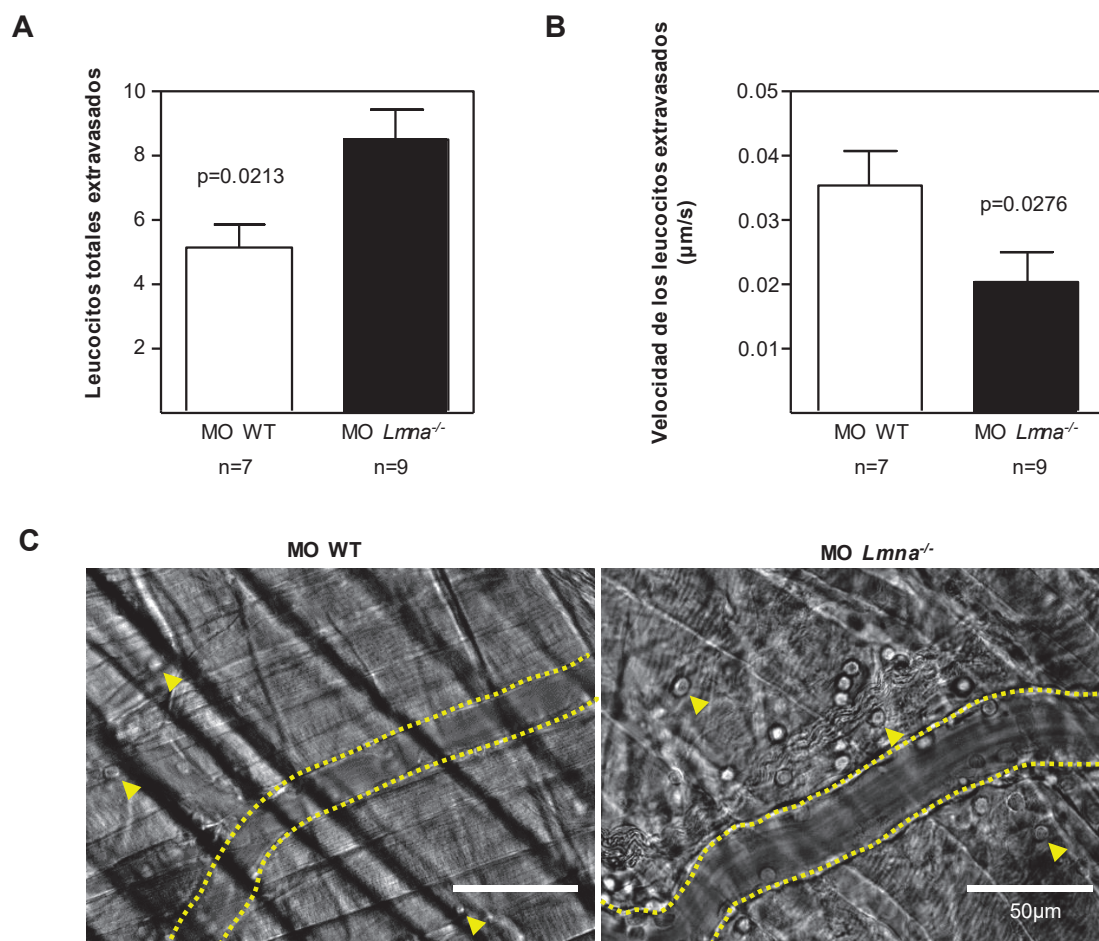


Figura 20. Efectos de la ausencia de lamina A/C en el sistema inmune sobre la extravasación leucocitaria al músculo cremáster y su posterior velocidad de migración. Ratones *Ldlr*^{-/-} fueron trasplantados con MO WT o *Lmna*^{-/-} y alimentados con DG. Se analizó mediante microscopía intravital la extravasación de leucocitos circulantes al músculo cremáster y su velocidad de migración. **(A)** Cuantificación de la extravasación de leucocitos. **(B)** Velocidad de migración de los leucocitos extravasados al músculo cremáster. **(C)** Imágenes representativas de leucocitos extravasados en ambos grupos experimentales. La línea discontinua muestra el contorno de una vénula cremastérica y las flechas apuntan a leucocitos extravasados.

1.4. Efectos de la ausencia de lamina A/C sobre la migración *in vitro* de monocitos proinflamatorios

Para profundizar en el estudio de la migración de los leucocitos deficientes en lamina A/C y su posible relación con el desarrollo de arteriosclerosis, realizamos experimentos *in vitro* con leucocitos, controlando las condiciones de migración en cámaras de cultivo con canales y/o constricciones de pequeño tamaño. Decidimos utilizar monocitos proinflamatorios, ya que son los que juegan un papel más importantes en las primeras etapas del desarrollo de arteriosclerosis (Moroni et al., 2019). En primer lugar, observamos que las células carentes de lamina A/C permanecían menos tiempo dentro de las constricciones (**Figura 21A**). Además, las células *Lmna*^{-/-} eran más lentas cuando cruzaban a través de canales rectos sin constricciones (**Figura 21B**). Estos resultados *in vitro* demuestran que los monocitos proinflamatorios deficientes en lamina A/C migran más fácilmente a través de constricciones de pequeño tamaño, pero son más lentos cuando migran en ausencia de las mismas, de acuerdo con nuestros resultados de microscopía intravital (**Figura 20**).

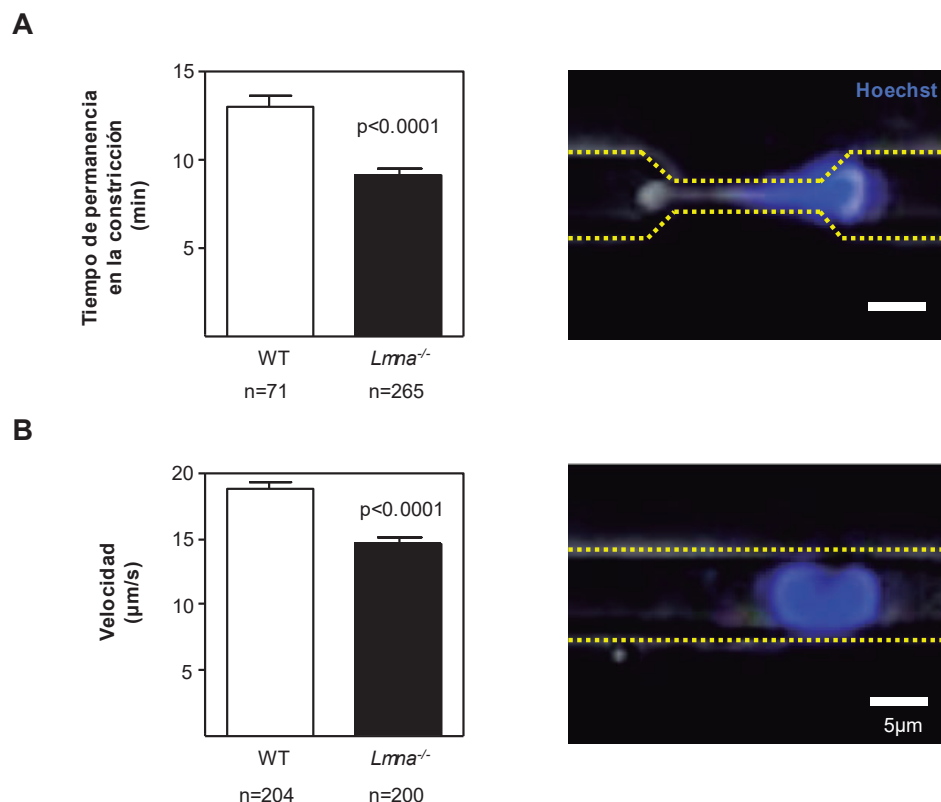


Figura 21. Efectos de la ausencia de lamina A/C sobre la migración *in vitro* de monocitos proinflamatorios. (A) Tiempo de permanencia de monocitos Ly6Chi⁺ en constricciones. La imagen muestra un monocito atravesando una constricción. (B) Velocidad de los monocitos Ly6Chi⁺ a través de canales de 5x2,8 μm. La imagen muestra un monocito migrando a través de un canal recto. En ambos estudios, las células utilizadas para cada experimento eran un “pool” de células procedentes de 3-4 ratones y el tamaño poblacional es la suma de 3 experimentos independientes.

1.5. La composición de la placa de ateroma y la proporción de leucocitos infiltrados no se afectan por la ausencia de lamina A/C en el sistema inmune

A continuación, estudiamos las poblaciones leucocitarias en la arteria aorta de ratones *Ldlr*^{-/-} trasplantados con MO WT o *Lmna*^{-/-} después de ser alimentados con DG. Para ello se digirió enzimáticamente el tejido y analizamos las suspensiones celulares mediante citometría de flujo. Estos estudios mostraron valores similares en los porcentajes de macrófagos (**Figura 22A**) y neutrófilos (**Figura 22B**) infiltrados en la aorta de ambos grupos experimentales.

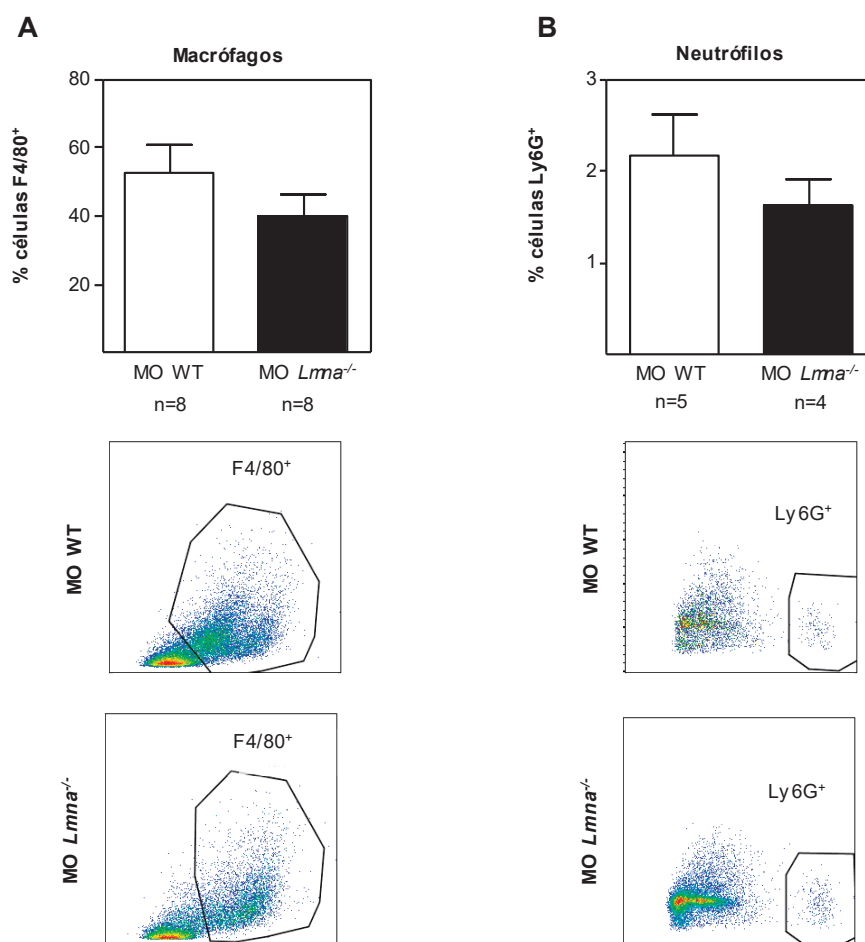


Figura 22. Proporción de macrófagos y neutrófilos en la arteria aorta durante la arteriosclerosis en ratones control y carentes de lamina A/C en el sistema inmune. Ratones *Ldlr*^{-/-} trasplantados con MO WT o *Lmna*^{-/-} se alimentaron con DG y se extrajo la arteria aorta para análisis por citometría de flujo. **(A)** Porcentaje de macrófagos. **(B)** Porcentaje de neutrófilos. Se muestran plots de citometría de flujo representativos.

La composición de la placa de ateroma determina su estabilidad y, por lo tanto, la posibilidad de sufrir un accidente aterotrombótico (Finn et al., 2010). Por este motivo, analizamos en placas ateromatosas de las válvulas aórticas el porcentaje de macrófagos y de colágeno mediante inmunohistoquímica de Mac-3 y tinción de tricrómico de Masson, respectivamente. Estos estudios mostraron unos porcentajes similares de macrófagos (**Figura 23A**) y colágeno (**Figura 23B**) en el interior de la placa de ateroma en ambos grupos experimentales. Globalmente, nuestros resultados de caracterización de la placa de ateroma sugieren que la ausencia de lamina A/C en el SI no altera ni la proporción de macrófagos y neutrófilos infiltrados ni la cantidad de colágeno depositado.

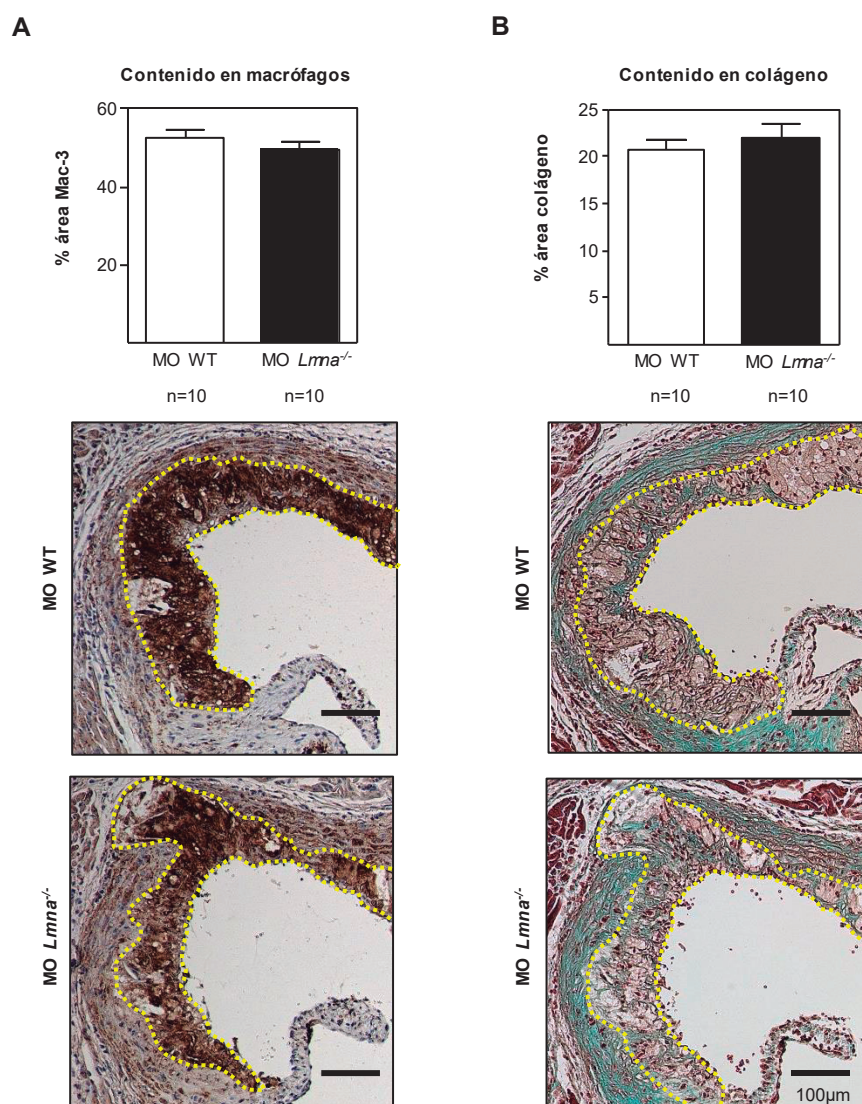


Figura 23. Composición de la placa de ateroma en ratones *Ldlr*^{-/-} control y carentes de lamina A/C en el sistema inmune. Ratones *Ldlr*^{-/-} trasplantados con MO WT o *Lmna*^{-/-} se alimentaron con DG y se extrajo la arteria aorta para análisis inmunohistoquímicos e histopatológicos en cortes a nivel de las válvulas aórticas. **(A)** Porcentaje de área de placa ateromatosa con macrófagos (células positivas para Mac-3). **(B)** Porcentaje de área de placa ateromatosa con depósitos de colágeno (tricrómico de Masson). Se muestran imágenes representativas. La línea discontinua marca el área de placa de ateroma.

1.6. Efectos de la ausencia de lamina A/C en el sistema inmune sobre la proliferación y la apoptosis en células de la placa de ateroma

Teniendo en cuenta la importancia de la proliferación celular y apoptosis en la arteriosclerosis, examinamos ambos procesos en placas de ateroma de las válvulas aórticas de ratones control y carentes de lamina A/C en el SI. Mediante estudios de marcaje inmunohistoquímico detectamos un menor porcentaje de células con expresión nuclear del marcador de proliferación Ki67 en la placa de ateroma de los ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-} (**Figura 24A**), mientras que la expresión del marcador de apoptosis caspasa-3 era similar en ambos grupos experimentales (**Figura 24B**).

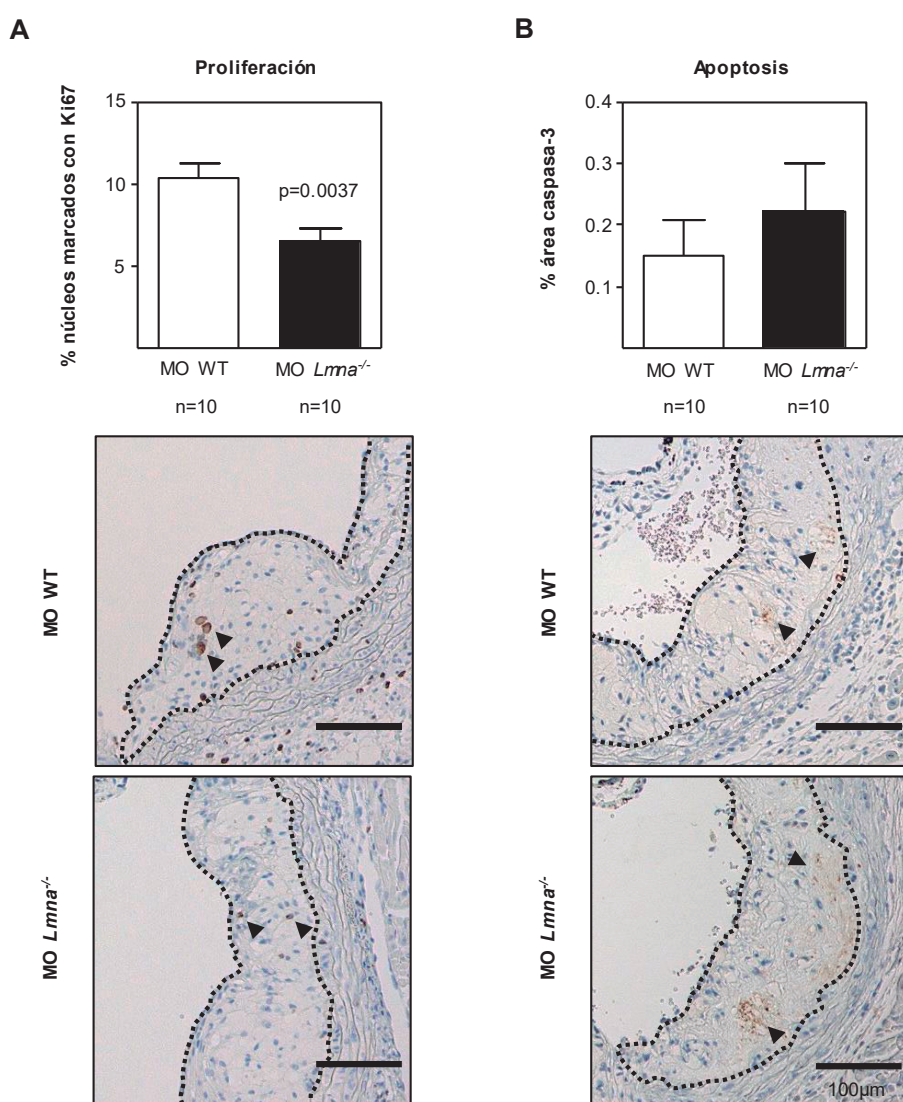


Figura 24. Efectos de la ausencia de lamina A/C en el sistema inmune sobre la proliferación y la apoptosis en células de la placa de ateroma. Ratones *Ldlr*^{-/-} trasplantados con MO WT o *Lmna*^{-/-} fueron alimentados con DG. Las gráficas muestran el porcentaje de células proliferantes (Ki67) (**A**) y de área apoptótica (Caspasa-3) (**B**). Las imágenes muestran imágenes representativas. La línea discontinua marca el área de placa de ateroma y las flechas apuntan a núcleos con expresión de Ki67 (**A**) o a zonas con expresión de caspasa-3 (**B**).

1.7. Efectos de la ausencia de lamina A/C sobre la polarización y funcionalidad de los macrófagos

Los macrófagos representan la población celular mayoritaria en las placas de ateroma (Moore et al., 2013). Son células con una gran plasticidad y heterogeneidad fenotípica, modulada por múltiples estímulos micro-ambientales que determinan su polarización y activación (Colin et al., 2014). El amplio espectro fenotípico de los macrófagos se caracteriza en sus extremos por los macrófagos M1 y M2, con funciones proinflamatorias/proaterogénicas y antiinflamatorias/antiaterogénicas, respectivamente (Colin et al., 2014).

Para analizar si la ausencia de lamina A/C afectaba a la diferenciación de los macrófagos realizamos ensayos *in vitro* induciendo la polarización de MDMO (M₀) a macrófagos M1 y M2. Mediante qPCR observamos que los macrófagos carentes de lamina A/C polarizados a M1 expresaban niveles de iNOS 2 ligeramente inferiores a los WT, sugiriendo una menor diferenciación a macrófagos proinflamatorios. Sin embargo, los análisis de expresión del marcador arginasa-1 demostraron que ambos genotipos presentaban una diferenciación similar hacia macrófagos M2 (**Figura 25A**).

La captación de lipoproteínas oxidadas por parte de los macrófagos es un evento importante en el desarrollo de la placa de ateroma, proceso en el que están implicados los receptores “escoba” (Moore et al., 2013). El análisis de los niveles de expresión de los receptores “escoba” SCARB1 y CD36 mostraron niveles similares en macrófagos M₀, M1 y M2 de ratones WT y *Lmna*^{-/-} (**Figura 25B**). Estos resultados, en su conjunto, sugieren que la ausencia de lamina A/C afecta ligeramente a la polarización de macrófagos M1, pero no tiene efecto sobre la expresión de receptores de lipoproteínas.

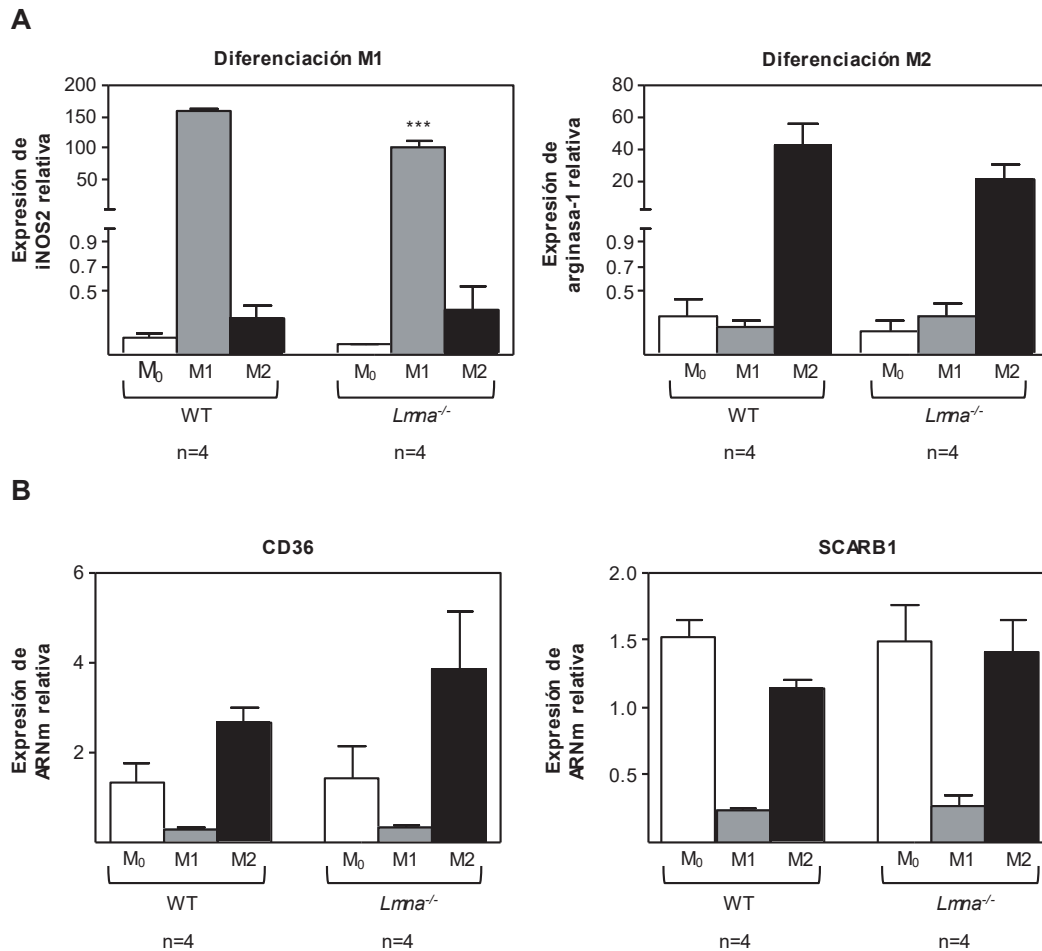


Figura 25. Efectos de la ausencia de lamina A/C sobre la polarización y funcionalidad de los macrófagos. Niveles de mRNA analizados por qPCR de MDMO procedentes de ratones WT o *Lma*^{-/-} sin diferenciar (M0) y polarizados a M1 o a M2. **(A)** Expresión de iNOS2 (M1) y arginasa-1 (M2). Los datos están representados como la expresión relativa de cada gen con respecto a la del gen control GAPDH. **(B)** Niveles de expresión de CD36 y SCARB1. Los datos están representados como la expresión relativa de cada gen con respecto a la de los genes control 18S y GAPDH. Los resultados se analizaron usando un test estadístico one-way ANOVA con un post-test de Bonferroni. ***, $p < 0.001$ versus M1 WT.

1.8. La ausencia de lamina A/C restringida a células de la línea mieloide o linfoide no afecta el desarrollo de arteriosclerosis en ratones *Ldlr*^{-/-}

Una vez demostrado que la ausencia de lamina A/C en todo el SI favorecía el desarrollo de arteriosclerosis, asociado a un incremento de la extravasación leucocitaria en ratones *Ldlr*^{-/-}, nuestro siguiente objetivo fue estudiar si el papel regulador de la lamina A/C en arteriosclerosis estaba mediado específicamente por alguna subpoblación leucocitaria. Empezamos analizando la ausencia de lamina A/C en poblaciones de origen mieloide. Para ello, se trasplantaron ratones *Ldlr*^{-/-} con MO procedente de ratones *Lmna*^{fllox/fllox} *LysMCre* o su control *Lmna*^{fllox/fllox} (MO *Lmna*^{fllox/fllox} *LysMCre* → *Ldlr*^{-/-} o MO *Lmna*^{fllox/fllox} → *Ldlr*^{-/-}, respectivamente) y se alimentaron durante 6 semanas con DG. El análisis de la

aorta mediante tinción de ORO evidenció una tendencia al aumento del desarrollo de arteriosclerosis en el AA de los ratones MO *Lmna*^{flax/flax} *LysMCre* → *Ldlr*^{-/-}, aunque las diferencias con el grupo control no alcanzaron significatividad estadística (**Figura 26A**).

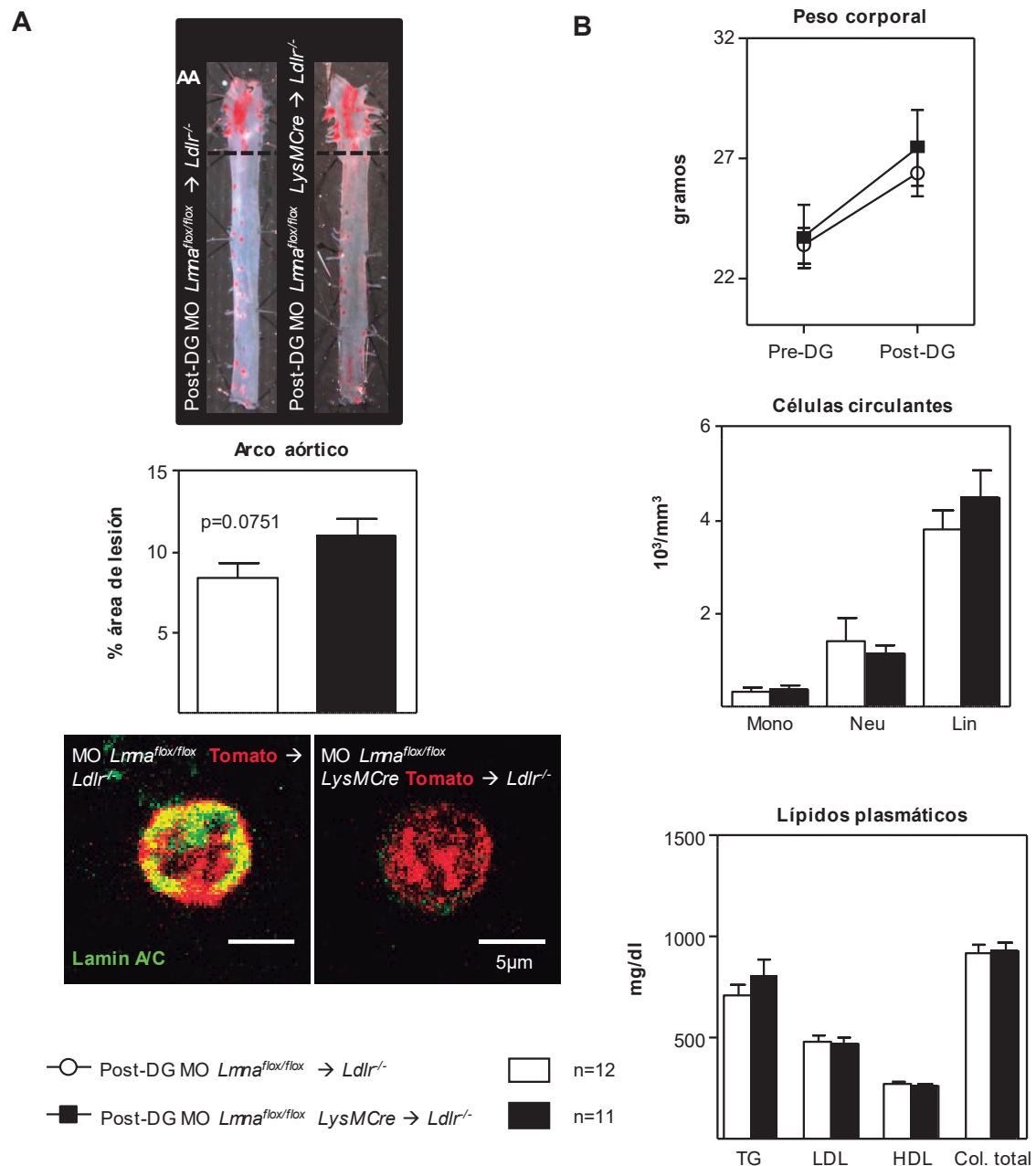


Figura 26. La ausencia de lamina A/C específicamente en células de la línea mieloide no aumenta el desarrollo de arteriosclerosis en ratones *Ldlr*^{-/-}. Ratones *Ldlr*^{-/-} fueron trasplantados con MO *Lmna*^{flax/flax} *LysMCre* o *Lmna*^{flax/flax} y alimentados con DG. **(A)** Imágenes representativas de arterias aortas teñidas con ORO. Las gráficas muestran la cuantificación de la placa de ateroma en el AA. Las imágenes de inmunofluorescencia muestran la expresión de lamina A/C (en verde) en células de origen mieloide (en rojo) procedentes de la sangre de ratones *Ldlr*^{-/-} trasplantados con MO *Lmna*^{flax/flax} *LysMCre* o *Lmna*^{flax/flax} que expresaban la proteína tomato bajo el control del promotor de *LysM*. **(B)** Peso corporal, concentración de distintas poblaciones de leucocitos y perfil lipídico de ratones al final de la DG.

A continuación, analizamos si la ausencia de lamina A/C en los linfocitos T era suficiente para aumentar el desarrollo de arteriosclerosis. Para ello usamos el modelo de ratón *Lmna*^{flox/flox} *CD4Cre* (previamente utilizado en Toribio-Fernández et al., 2018) y sus controles *Lmna*^{flox/flox} para trasplantar ratones *Ldlr*^{-/-} (MO *Lmna*^{flox/flox} *CD4Cre* → *Ldlr*^{-/-} o MO *Lmna*^{flox/flox} → *Ldlr*^{-/-}, respectivamente). Tras 6 semanas con DG, observamos niveles de arteriosclerosis similares en los ratones de ambos grupos (**Figura 27A**). Estos resultados indican que la ausencia de lamina A/C de manera específica en células de la línea mieloide o linfóide no es suficiente para aumentar la formación de placa de ateroma, como sí sucede cuando eliminamos la expresión de lamina A/C en todo el SI.

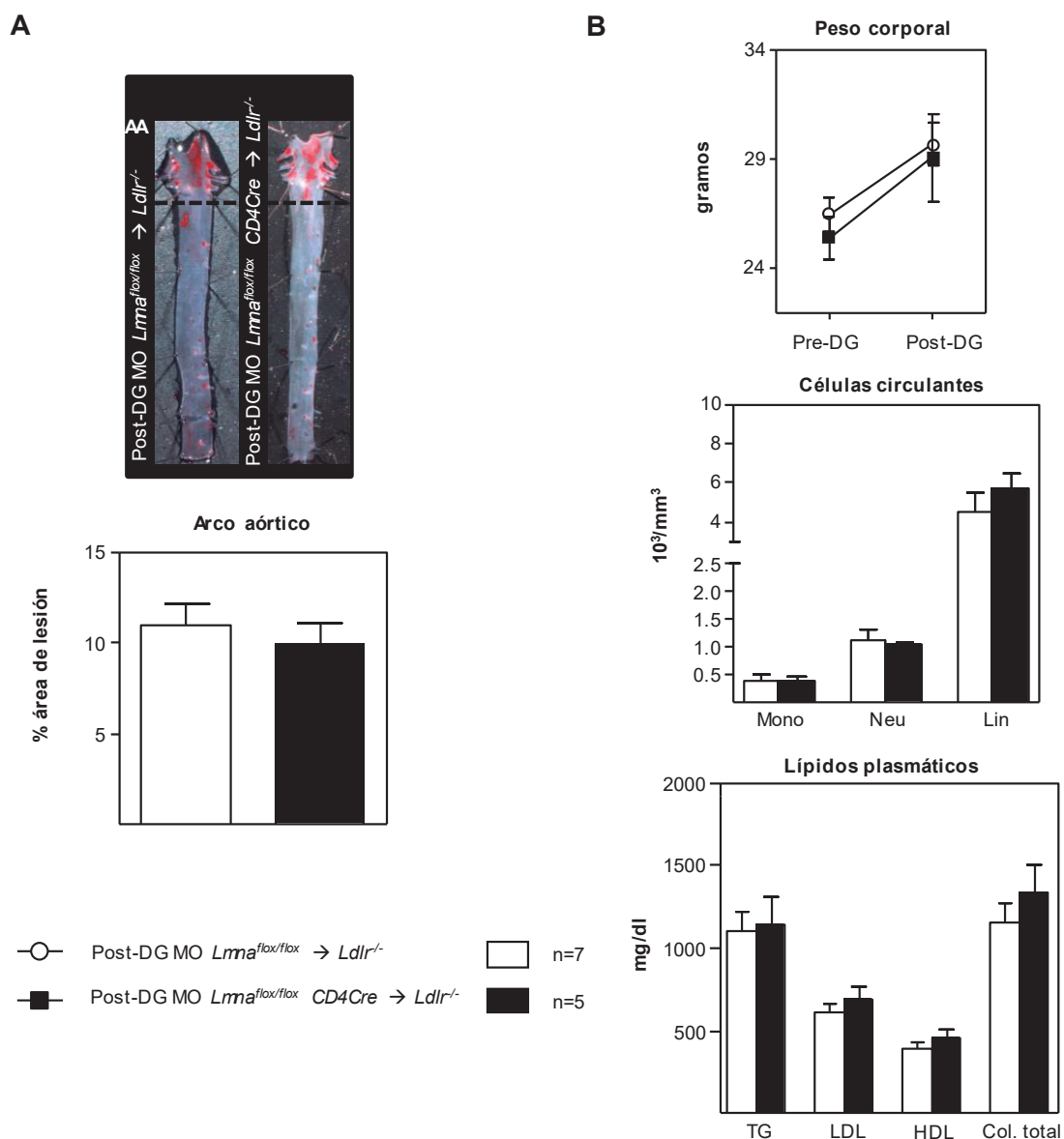


Figura 27. La ausencia de lamina A/C específicamente en células de la línea linfóide no aumenta el desarrollo de arteriosclerosis en ratones *Ldlr*^{-/-}. Ratones *Ldlr*^{-/-} fueron trasplantados con MO *Lmna*^{flox/flox} *CD4Cre* o *Lmna*^{flox/flox} y alimentados con DG. **(A)** Imágenes representativas de arterias aortas teñidas con ORO. Las gráficas muestran la cuantificación de la placa de ateroma en el AA. **(B)** Peso corporal, concentración de distintas poblaciones de leucocitos y perfil lipídico de ratones al final de la DG.

2. Regulación de la expresión de lamina A/C en la pared arterial durante el envejecimiento y estudio de su posible papel en el desarrollo de enfermedades cardiovasculares

2.1. Regulación de la expresión de lamina A/C en la pared arterial durante el envejecimiento

Basándonos en los resultados anteriores, decidimos estudiar el papel de la lamina A/C en las células vasculares, componentes de la pared arterial que juegan un papel clave en la ECV asociada al envejecimiento. En primer lugar, examinamos mediante WB los niveles de lamina A/C en arterias coronarias de personas de diferentes edades. Los resultados obtenidos demostraron niveles de lamina A/C inferiores en las arterias procedentes de individuos de mayor edad (**Figura 28**).

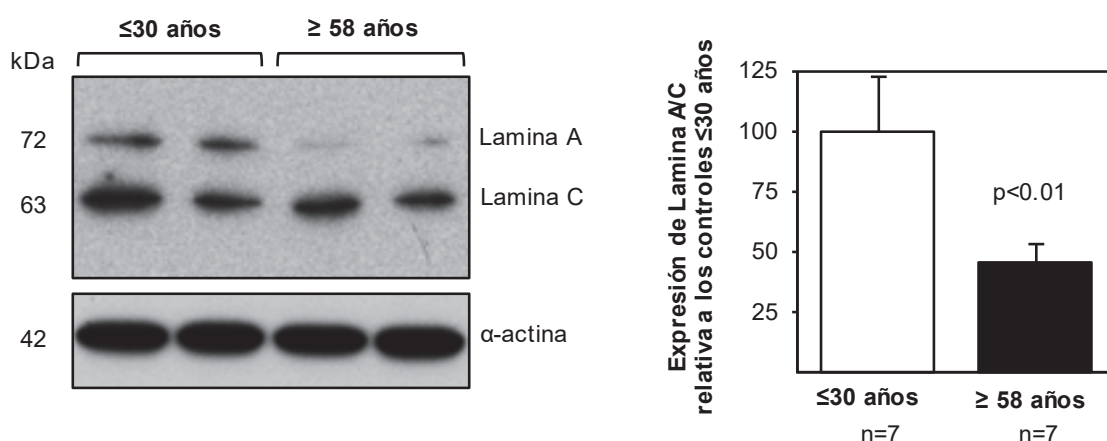


Figura 28. Los niveles de lamina A/C descenden durante el envejecimiento en tejido arterial humano. Imagen de un WB representativo y cuantificación de los niveles de lamina A/C en arterias coronarias de origen humano de individuos ≤30 años y ≥58 años.

A continuación, estudiamos tejido arterial de ratón mediante WB de distintas zonas de la arteria aorta (AA y AT) de machos y hembras de distintas edades. Los resultados demostraron una disminución generalizada de la lamina A/C durante el envejecimiento, siendo más acusada en el AA comparado con la AT (**Figura 29A**). Para determinar el tipo celular responsable de este descenso, realizamos análisis de citometría de flujo en la túnica adventicia (compuesta principalmente por fibroblastos) y en el resto de tejido arterial (compuesto principalmente por CMLVs y células endoteliales, identificadas con anticuerpos específicos). Los resultados mostraron una menor expresión de lamina A/C

tanto en CMLVs como en células endoteliales de los ratones de edad avanzada comparados con los jóvenes, sin observarse cambios en las células de la adventicia (**Figura 29B**). Los resultados obtenidos en arterias humanas y de ratón sugieren que la expresión de lamina A/C en las células vasculares disminuye durante el envejecimiento.

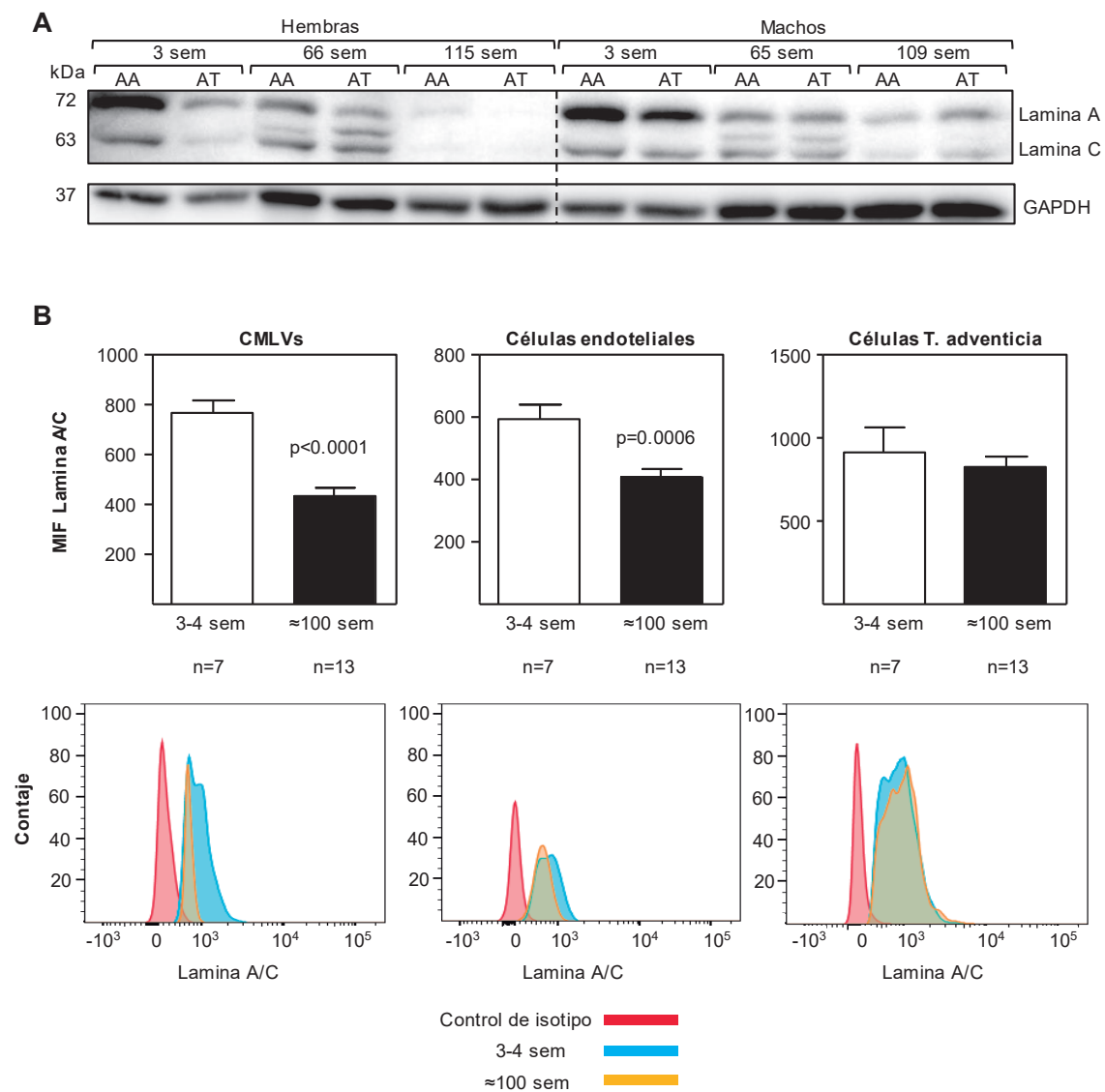


Figura 29. Los niveles de lamina A/C descienden durante el envejecimiento en arterias aortas de ratón. (A) Imagen de un WB representativo y cuantificación de los niveles de lamina A/C en distintas zonas de la arteria aorta de ratones machos y hembras de edades comprendidas entre las 3 y las 115 semanas (sem). Cada carril corresponde a un “pool” de 2 AAs ó 2 ATs. **(B)** Análisis por citometría de flujo de los niveles de lamina A/C en células de la túnica adventicia, CMLVs (identificadas con anticuerpos anti-SMA) y células endoteliales (identificadas con anticuerpos anti-CD106) de ratones jóvenes (3-4 semanas) y envejecidos (≈100 semanas). Se muestran histogramas representativos. Cada individuo corresponde a un “pool” de 2 arterias aortas.

2.2. Generación y caracterización del modelo de ratón *Lmna*^{flox/flox} *SM22α*Cre

Una vez averiguado que los niveles de lamina A/C disminuyen con el envejecimiento en células vasculares, nos preguntamos si la ausencia de lamina A/C específicamente en CMLVs o células endoteliales podía estar relacionada con la ECV. Teniendo en cuenta que representan el componente celular más abundante de la pared vascular, en esta Tesis Doctoral decidimos generar un modelo de ratón con deficiencia de lamina A/C en CMLVs. Para ello cruzamos ratones portadores del gen de la lamina A/C flanqueado por secuencias loxP (Y. Kim & Zheng, 2013) con ratones que expresan la recombinasa CRE bajo el control del promotor del gen *SM22α*, cuya expresión ocurre principalmente en CMLVs (Holtwick et al., 2002; Joseph & Eric, 1996; L. Li et al., 1996). En todos los experimentos, los ratones se sacrificaron a las 4 semanas de edad. La eficiencia en la delección de lamina A/C en las CMLVs se analizó mediante inmunofluorescencia en distintos órganos. La eliminación de la lamina A/C fue completa y específica en CMLVs de la arteria aorta, el hígado, el riñón y el pulmón (**Figura 30**). Sin embargo, en el corazón la lamina A/C estaba ausente no solo en CMLVs de las arterias coronarias, sino también en algunos cardiomiocitos y FCs (**Figura 31**).

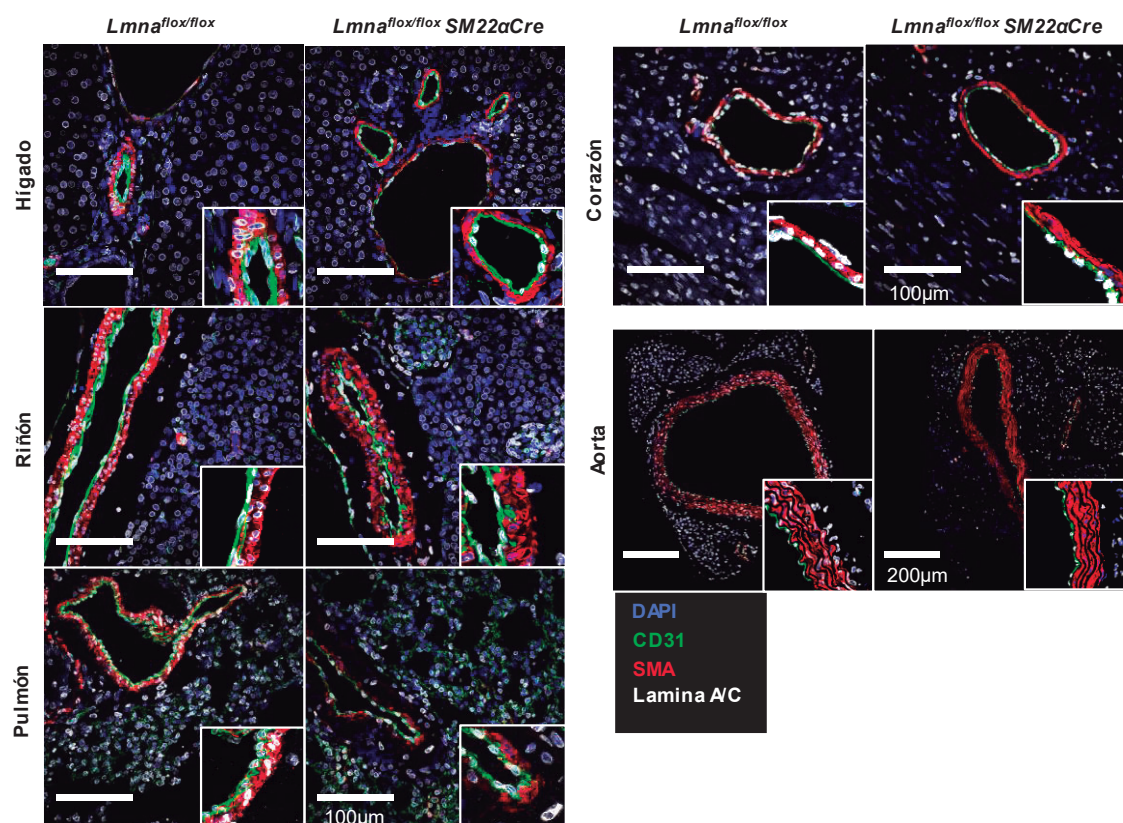


Figura 30. Deficiencia de lamina A/C en células de músculo liso vascular de ratones *Lmna*^{flox/flox} *SM22α*Cre. Imágenes representativas de cortes histológicos de distintos órganos con los núcleos marcados con DAPI (azul), las células endoteliales con anti-CD31 (verde), las CMLVs con anti-SMA (rojo) y las células positivas para lamina A/C con anti-Lamina A/C (blanco).

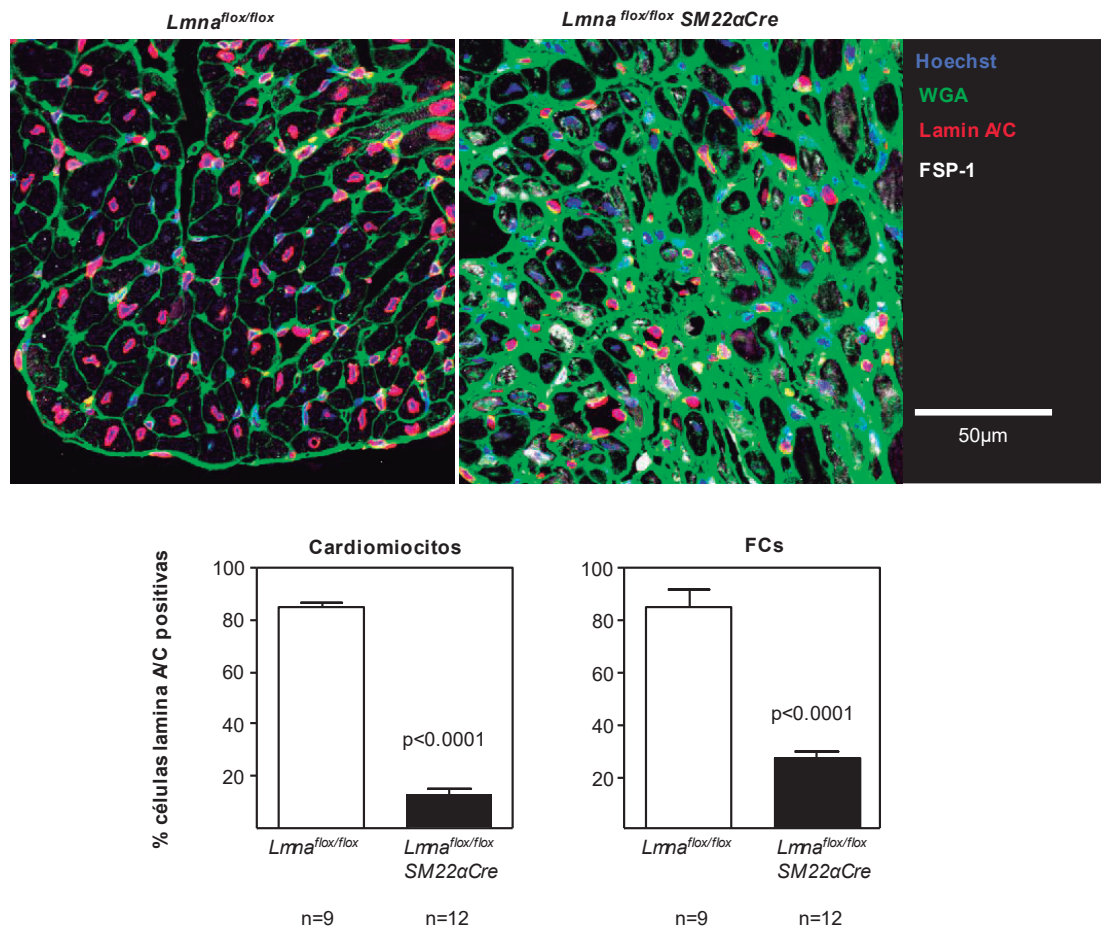


Figura 31. Deficiencia de lamina A/C en cardiomiocitos y fibroblastos cardíacos de ratones *Lmna*^{flox/flox} *SM22αCre*. Imágenes representativas de cortes histológicos de corazón con los núcleos marcados con Hoechst (azul), las membranas celulares con WGA (verde), las células positivas para lamina A/C con anti-Lamin A/C (rojo) y los FCs con anti-FSP-1 (blanco). Las gráficas muestran el porcentaje de cardiomiocitos y FCs positivos para lamina A/C.

La caracterización fenotípica de este nuevo modelo de ratón mostró un peso corporal ligeramente reducido y una esperanza de vida media dramáticamente reducida a aproximadamente 5 semanas en los ratones *Lmna*^{flox/flox} *SM22αCre* en comparación con los controles (**Figura 32**).

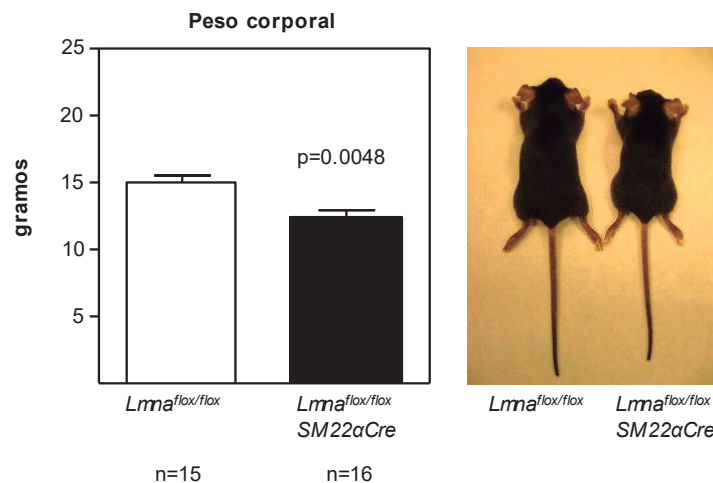
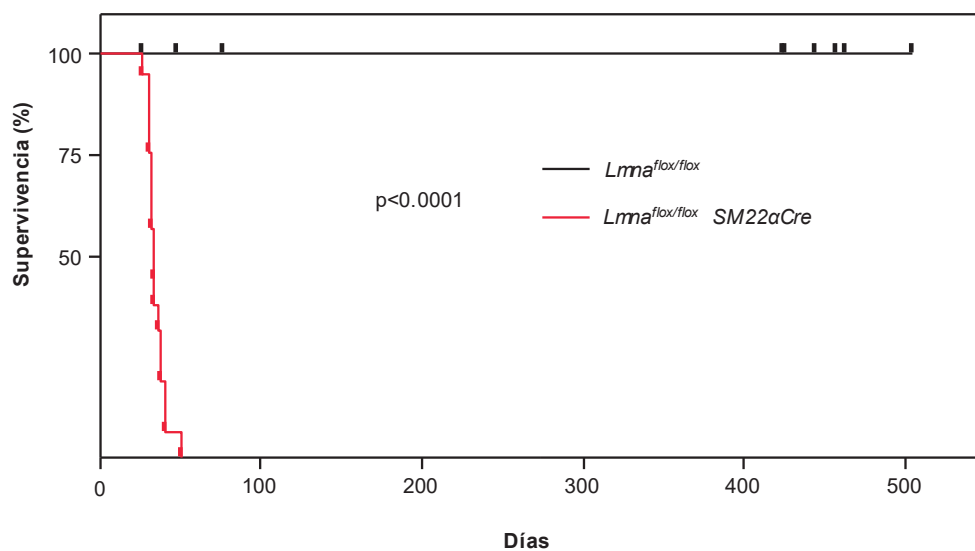
A**B**

Figura 32. Los ratones *Lmna^{flox/flox} SM22αCre* presentan menos peso corporal y una reducción muy significativa en su esperanza de vida media. Curvas de supervivencia de Kaplan-Meier (A) y peso corporal a las 4 semanas (B) de los ratones *Lmna^{flox/flox}* y *Lmna^{flox/flox} SM22αCre*. La imagen representativa en A muestra un ratón de cada genotipo.

A continuación, comprobamos si los ratones *Lmna^{flox/flox} SM22αCre* sufrían alguna alteración histopatológica en diferentes órganos. Como la disfunción de las CMLVs puede provocar la producción excesiva de matriz extracelular (Hinz et al., 2012), cuantificamos el porcentaje de colágeno en hígado, pulmones y riñón mediante tinción de tricrómico de Masson. Estos estudios no evidenciaron cambios en la estructura de los tejidos ni en la cantidad de colágeno en los ratones *Lmna^{flox/flox} SM22αCre* comparados con los controles (**Figura 33**).

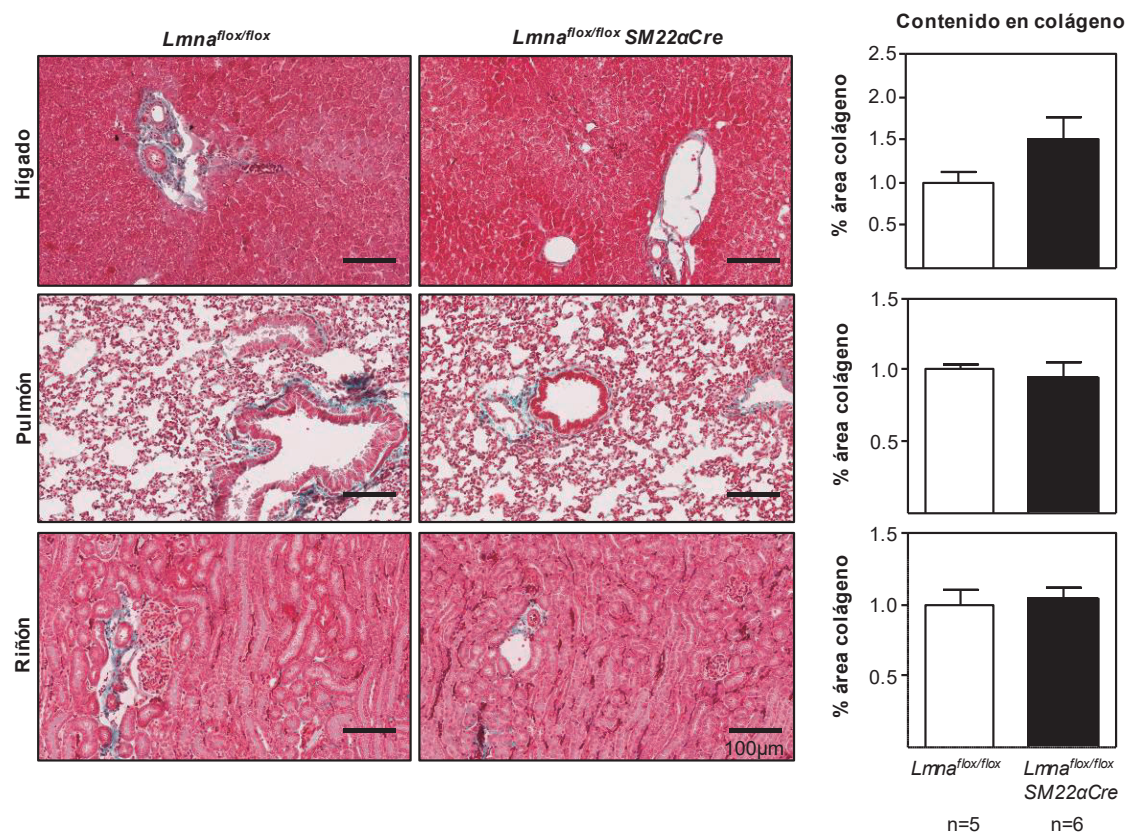


Figura 33. Los ratones *Lmna^{flox/flox} SM22 α Cre* no presentan alteraciones en hígado, riñón y pulmón. Imágenes representativas de cortes histopatológicos de hígado, pulmón y riñón teñidos con tricrómico de Masson y cuantificación del porcentaje de colágeno normalizado con respecto a ratones control *Lmna^{flox/flox}*.

El análisis de cortes histológicos teñidos con HE tampoco demostró diferencias significativas en el número de núcleos en células de la túnica media en el AA y la AT (**Figura 34A**), y tinciones de tricrómico de Masson revelaron una tendencia no significativa a un aumento en la acumulación de colágeno en la capa muscular en ambas regiones de la arteria aorta de los ratones *Lmna^{flox/flox} SM22 α Cre* (**Figura 34B**). En conclusión, la caracterización de este modelo de ratón indica que la eliminación de la lamina A/C en CMLVs y en una fracción de cardiomiocitos y FCs causa una mortalidad muy prematura.

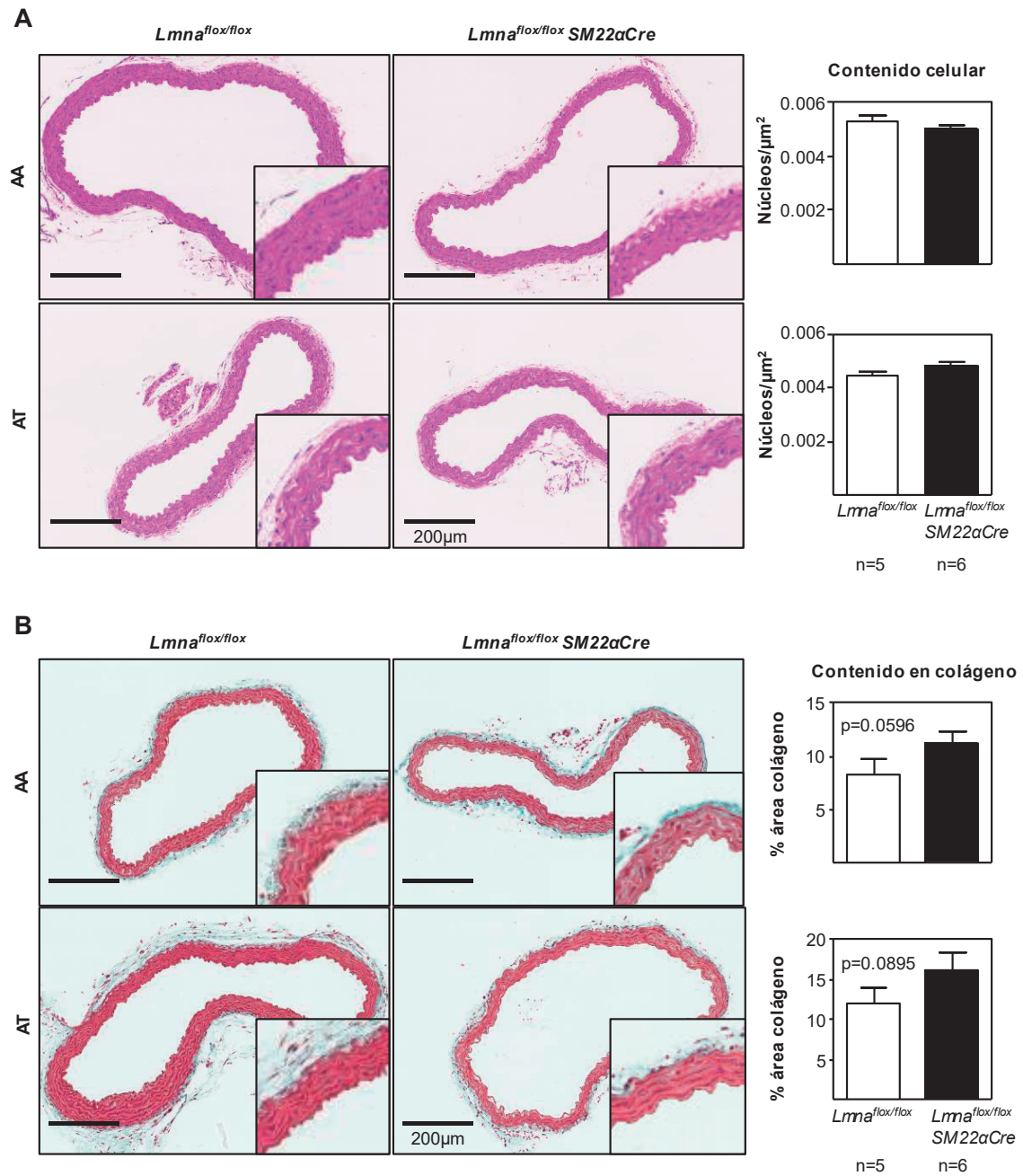


Figura 34. Los ratones *Lmna*^{flox/flox} SM22αCre no presentan alteraciones en la arteria aorta. (A) Imágenes representativas de tinciones con HE y cuantificación del número de núcleos celulares por área. **(B)** Imágenes representativas de tinciones con tricrómico de Masson y cuantificación del porcentaje de colágeno normalizado al control *Lmna*^{flox/flox}. En ambos paneles se usaron cortes histológicos de arcos AA y aortas torácicas AT de ratones *Lmna*^{flox/flox} y *Lmna*^{flox/flox} SM22αCre.

2.3. Los ratones *Lmna*^{flox/flox} SM22αCre desarrollan alteraciones cardíacas graves

Ninguno de los órganos analizados demostró un daño grave que explicara la mortalidad prematura de los ratones *Lmna*^{flox/flox} SM22αCre. Teniendo en cuenta que estos animales presentan ausencia de lamina A/C en las CMLVs y en un porcentaje de cardiomiocitos y FCs, decidimos examinar posibles alteraciones cardíacas. El análisis mediante

ecocardiografía demostró una drástica pérdida de función cardíaca en los ventrículos izquierdo y derecho de los ratones *Lmna^{flox/flox} SM22αCre* (Figura 35A), así como una ligera disminución en el grosor de la pared del ventrículo izquierdo (Figura 35B).

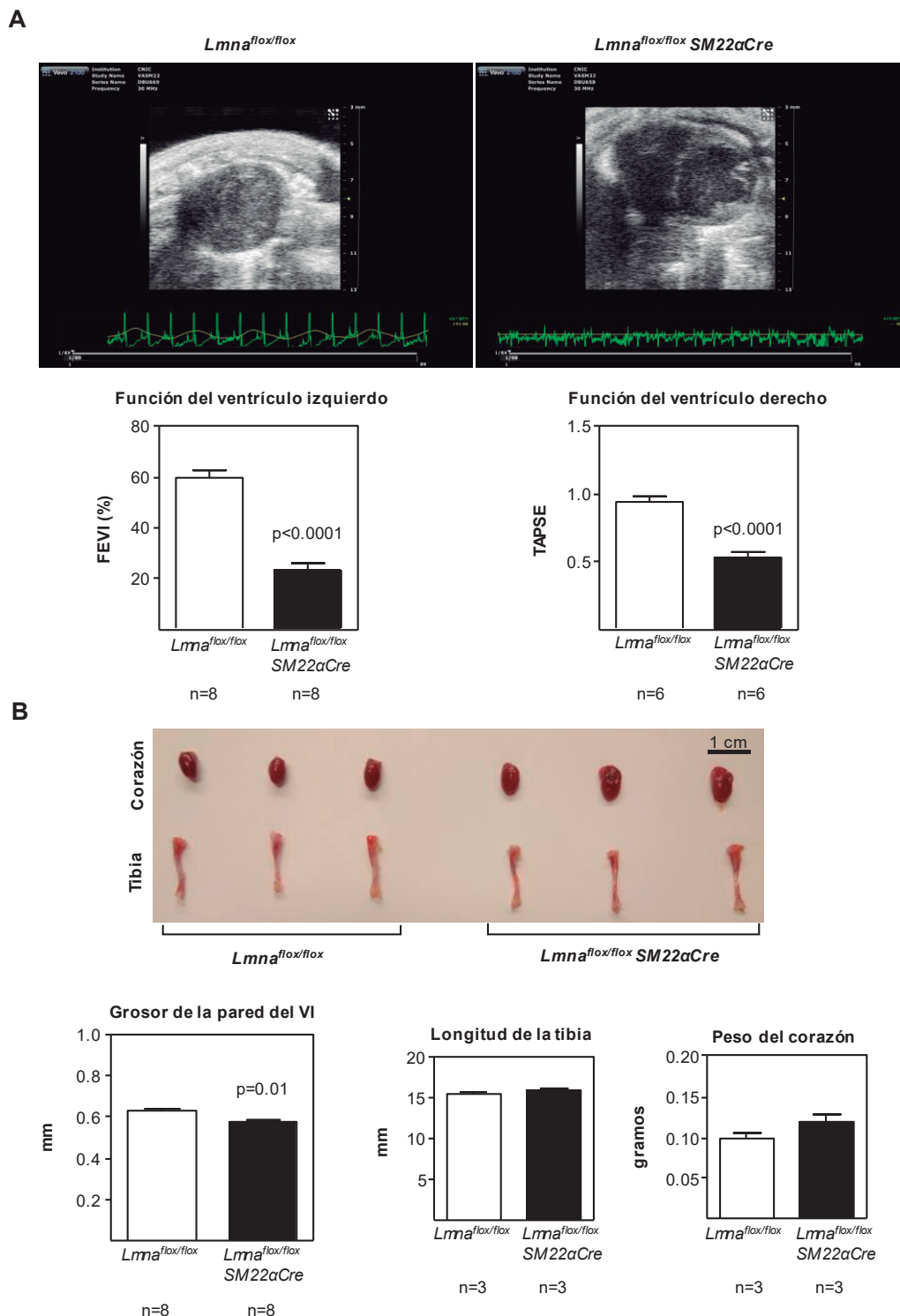


Figura 35. Los ratones *Lmna^{flox/flox} SM22αCre* presentan pérdida de función cardíaca en ambos ventrículos. Ratones *Lmna^{flox/flox}* y *Lmna^{flox/flox} SM22αCre* de 4 semanas de edad. **(A)** Imágenes representativas de las ecocardiografías y cuantificación de la función del ventrículo izquierdo (FEVI) y derecho (TAPSE). **(B)** Cuantificación del grosor de la pared del ventrículo izquierdo y medidas control de corazón y tibia con sus imágenes representativas.

Mediante análisis electrocardiográfico encontramos una prolongación de los intervalos QRS y QT (conducción ventricular) y menor tasa de repolarización en los ratones *Lmna*^{flox/flox} *SM22αCre* comparado con sus controles, sin observarse alteraciones significativas en la conducción aurículo-ventricular (intervalo PQ) y en el ritmo cardíaco (**Figura 36**). Estos resultados sugieren que la ausencia de lamina A/C en las CMLVs y otras células del corazón provoca una grave pérdida de la función cardíaca y alteraciones electrocardiográficas en los ventrículos que podrían estar relacionadas con la muerte prematura de los ratones *Lmna*^{flox/flox} *SM22αCre*.

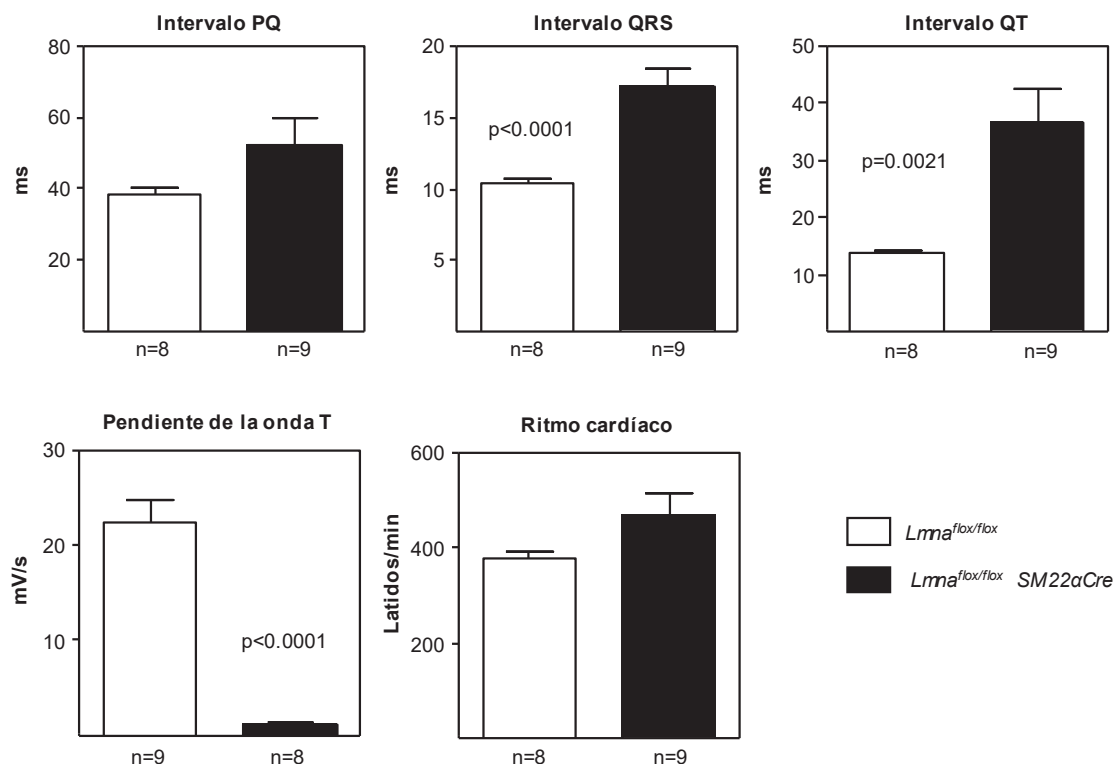


Figura 36. Los ratones *Lmna*^{flox/flox} *SM22αCre* presentan alteraciones electrocardiográficas. Medidas de los intervalos PQ, QRS, QT, pendiente de la onda T y ritmo cardíaco obtenidas mediante estudios electrocardiográficos en ratones *Lmna*^{flox/flox} y *Lmna*^{flox/flox} *SM22αCre* a las 4 semanas de edad.

2.4. Los ratones *Lmna*^{flox/flox} *SM22αCre* desarrollan fibrosis cardíaca

Con el objetivo de identificar los mecanismos responsables del fenotipo cardíaco en ratones *Lmna*^{flox/flox} *SM22αCre*, analizamos la deposición de colágeno como medida de la fibrosis cardíaca mediante tinciones de tricrómico de Masson y Sirius red. Estos estudios demostraron que los ratones *Lmna*^{flox/flox} *SM22αCre* presentaban fibrosis cardíaca intersticial generalizada (**Figura 37**).

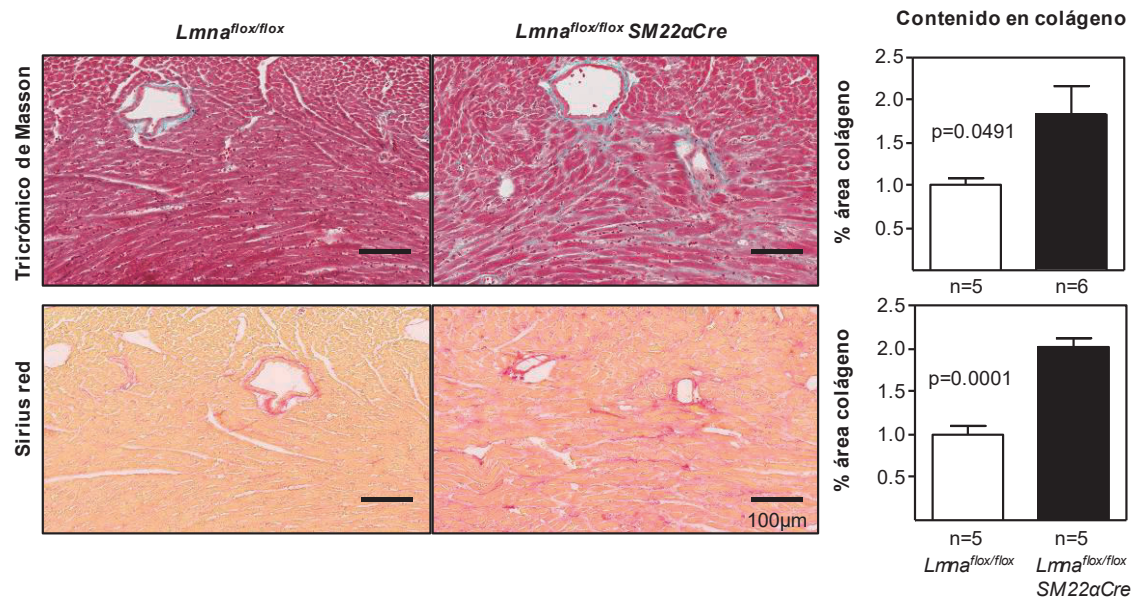


Figura 37. Los ratones *Lmna^{flox/flox} SM22αCre* presentan fibrosis cardíaca generalizada. Imágenes representativas de tinciones con tricrómico de Masson y Sirius red en cortes histológicos de corazón de ratones *Lmna^{flox/flox}* y *Lmna^{flox/flox} SM22αCre*. Las gráficas muestran la cuantificación del porcentaje de área de colágeno en ambas tinciones normalizado al control *Lmna^{flox/flox}*.

Para profundizar en los mecanismos celulares y moleculares responsables de la fibrosis cardíaca en ratones *Lmna^{flox/flox} SM22αCre*, examinamos la expresión de marcadores celulares que se han asociado con el desarrollo de fibrosis cardíaca: WGA como marcador general de fibrosis (Emde et al., 2014; Teekakirikul et al., 2010) y FSP-1 y SMA como marcadores de FCs profibróticos (Kong et al., 2013; Ma et al., 2018). Mediante estudios de inmunofluorescencia confocal observamos un aumento en el porcentaje de área de tejido cardíaco con expresión de WGA, FSP-1 y SMA en ratones *Lmna^{flox/flox} SM22αCre* (**Figura 38**). Estos resultados indican que la ausencia de lamina A/C en células del sistema cardiovascular provoca el desarrollo de fibrosis cardíaca asociada a la expresión de marcadores de FCs activados.

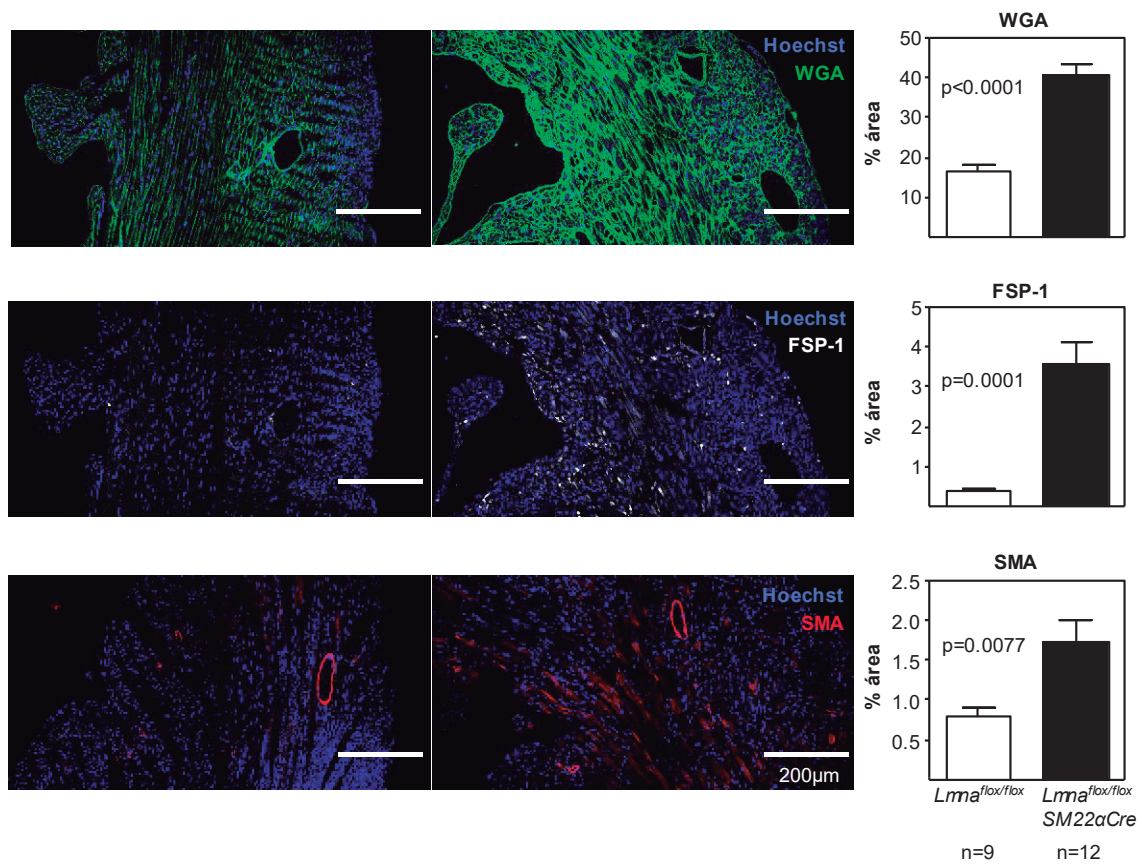


Figura 38. Los ratones *Lmna^{flox/flox} SM22αCre* presentan un aumento de expresión de marcadores profibróticos en tejido cardíaco. Porcentaje de área marcada con WGA (verde), FSP-1 (blanco) y SMA (rojo) en cortes de tejido cardíaco de ratones *Lmna^{flox/flox}* y *Lmna^{flox/flox} SM22αCre* de 4 semanas de edad. Imágenes de inmunofluorescencia representativas.

DISCUSIÓN

Está ampliamente descrito que el envejecimiento y la hipercolesterolemia son los principales factores de riesgo de sufrir ECVs (Burkhardt, 2015; Lakatta & Levy, 2003; Nelson, 2013; Savji et al., 2013; Steenman & Lande, 2017; Wang & Bennett, 2012). La lamina A/C, codificada por el gen *LMNA*, es una proteína nuclear con una función estructural que regula multitud de procesos celulares. Diversas mutaciones en el gen *LMNA* producen enfermedades humanas llamadas laminopatías (Broers et al., 2006). Además, modelos de ratón que no expresan la lamina A/C o expresan formas mutadas tienen fenotipos muy marcados y, en algunos casos, su supervivencia está gravemente comprometida (Zhang et al., 2013). Diversos estudios han demostrado un descenso en la expresión de lamina A/C durante el proceso de envejecimiento, tanto en células humanas (Golubtsova, 2016) como en varios tejidos de ratón (Afilalo et al., 2007; Duque & Rivas, 2006).

Con estos antecedentes, en el presente trabajo de Tesis Doctoral hemos estudiado si los niveles de lamina A/C se regulan durante el envejecimiento e hipercolesterolemia en tipos celulares implicados en ECVs, y si estas proteínas nucleares juegan un papel determinante en el desarrollo de arteriosclerosis y otras ECVs asociadas al envejecimiento.

Está bien establecido que el SI participa activamente en la fisiopatología de la arteriosclerosis (Hansson & Libby, 2006). Además, varios trabajos realizados en nuestro laboratorio han demostrado que la lamina A/C juega un papel importante en células inmunes, como los linfocitos T (González-Granado et al., 2014; Rocha-Perugini & González-Granado, 2014; Toribio-Fernández et al., 2018). Por ello, como primera aproximación estudiamos el posible papel de lamina A/C en el desarrollo de arteriosclerosis. En primer lugar, analizamos si dos factores de riesgo para el desarrollo de ECVs, como el envejecimiento y la hipercolesterolemia, regulaban los niveles de lamina A/C en células del SI. El análisis de muestras de sangre humana y de ratón demostró una menor expresión de lamina A/C en los leucocitos procedentes del grupo de individuos de más edad. Observamos también un descenso en los niveles de lamina A/C en leucocitos circulante de ratones hipercolesterolémicos *Ldlr*^{-/-} alimentados con DG comparado con controles alimentados con DN. Estos resultados demuestran por primera vez que tanto el envejecimiento como la hipercolesterolemia se asocian con menor expresión de lamina A/C en el SI. El envejecimiento se asocia con un proceso inflamatorio crónico (Ferrucci & Fabbri, 2018). Así mismo, la hipercolesterolemia favorece el desarrollo de aterosclerosis en parte debido a la activación de un proceso inflamatorio mediado por células del SI (Hansson et al., 2006; Hansson & Libby, 2006; Siasos et al., 2012). Estas características compartidas por ambos factores de riesgo

cardiovascular podrían contribuir a la reducción de los niveles de lamina A/C durante el envejecimiento y en condiciones de hipercolesterolemia. De acuerdo con esta hipótesis, se ha descrito que moléculas proinflamatorias, como IFN- β o TNF- α , disminuyen los niveles de lamina A/C (Alldridge et al., 1991; Guicciardi et al., 2000).

A continuación, estudiamos si los niveles de expresión de lamina A/C en el SI regulan el desarrollo de arteriosclerosis. Para ello utilizamos un protocolo de irradiación de ratones *Ldlr*^{-/-} y trasplante con MO de ratones WT o *Lmna*^{-/-} (MO WT $\rightarrow$ *Ldlr*^{-/-} y MO *Lmna*^{-/-} $\rightarrow$ *Ldlr*^{-/-}, respectivamente), con el fin de reconstituir su SI con células WT o deficientes en lamina A/C. Aunque se ha demostrado que la lamina A/C regula negativamente el tráfico de células desde la MO hacia la sangre durante la hematopoyesis (Shin et al., 2013), la recuperación del SI después del trasplante en los ratones MO *Lmna*^{-/-} $\rightarrow$ *Ldlr*^{-/-} fue tan alta como en los ratones control, lo que indica que la carencia de lamina A/C en las células hematopoyéticas no afecta a la repoblación celular de la MO y la sangre. Del mismo modo, el análisis hematológico de ratones de ambos grupos de ratones sometidos a un régimen de DG no mostró diferencias significativas ni en la concentración de poblaciones leucocitarias ni en los niveles de lípidos en plasma y en la ganancia de peso. Sin embargo, observamos un aumento en el desarrollo de arteriosclerosis en los ratones *Ldlr*^{-/-} carentes de lamina A/C en el SI con respecto a sus controles.

Ya que nuestro modelo de ratón sólo carecía de lamina A/C en el SI, decidimos centrarnos en el estudio de los procesos ateroscleróticos en los que están implicados los leucocitos. Después de la activación endotelial, la primera etapa de la formación de placa de ateroma es el reclutamiento leucocitario (Braunersreuther & Mach, 2006), que comienza con el rodamiento de los leucocitos por la pared arterial. Para investigar este primer proceso empleamos microscopía intravital de arteria carótida, una nueva técnica desarrollada en nuestro laboratorio que permite observar *in situ* el rodamiento de monocitos y neutrófilos sobre el endotelio de la arteria carótida (Chèvre et al., 2014). Los resultados no mostraron un aumento en el rodamiento leucocitario en los ratones MO *Lmna*^{-/-} $\rightarrow$ *Ldlr*^{-/-} alimentados con DG respecto a sus controles, descartando este mecanismo como causante del incremento de arteriosclerosis en los ratones con deficiencia en lamina A/C en su SI.

Dentro del contexto de reclutamiento leucocitario, el siguiente proceso que estudiamos fue la adhesión de los leucocitos a la pared arterial mediante microscopía intravital en el músculo cremáster. Nuestros experimentos mostraron que los leucocitos de los ratones MO *Lmna*^{-/-} $\rightarrow$ *Ldlr*^{-/-} alimentados con DG se adherían a la pared endotelial de vénulas

del músculo cremáster de manera similar a los controles, descartando también un aumento en la adhesión leucocitaria como mecanismo responsable del agravamiento de aterosclerosis asociado a la ausencia de lamina A/C en las células hematopoyéticas.

A continuación, analizamos la diapédesis, la última etapa del proceso de reclutamiento leucocitario durante la arteriosclerosis que culmina con la extravasación de las células del SI y su incorporación a la placa aterosclerótica (Muller, 2003). Utilizando microscopía intravital en el músculo cremáster, observamos mayor número de leucocitos extravasados en los ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-} en comparación con los controles. Este hallazgo indica que la ausencia de lamina A/C en las células del SI favorece su extravasación bajo el estímulo aterosclerótico de la DG. Estudios previos *in vitro* han demostrado que la ausencia de lamina A/C favorece el paso de leucocitos a través de pequeños poros gracias a presentar un núcleo más deformable (Harada et al., 2014). Por el contrario, experimentos *in vitro* han demostrado que la sobreexpresión de lamina A aumenta la rigidez nuclear y dificulta la migración a través de constricciones (Rowat et al., 2013). En un contexto vascular *in vivo*, los poros de pequeño tamaño corresponderían a los espacios intercelulares de las células endoteliales a través de los cuales migran los leucocitos en presencia de estímulos proaterogénicos, como los presentes a edad avanzada y en presencia de hipercolesterolemia. Nuestros resultados no solo confirman *in vivo* estos estudios previos, sino que también sugieren un nuevo mecanismo proaterosclerótico en el que la ausencia de lamina A/C aumenta la extravasación leucocitaria durante el desarrollo de la enfermedad. En el mismo ensayo, comprobamos que los leucocitos deficientes en lamina A/C en los ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-} eran más lentos que los controles en su movimiento tras la extravasación. Estos resultados *in vivo* están de acuerdo con hallazgos *in vitro*, en los que el defecto en migración celular se ha asociado a un incorrecto acoplamiento entre la lamina A/C y el citoesqueleto de actina (D. H. Kim et al., 2014). Sin embargo, su implicación en la aterogénesis necesita un análisis más extenso.

Los experimentos de microscopía intravital de cremáster no permiten diferenciar entre los subtipos de leucocitos extravasados. Para profundizar en el estudio del papel de la lamina A/C en la migración celular, realizamos estudios *in vitro* con monocitos proinflamatorios (Ly6G^{hi}), la principal subpoblación leucocitaria que se recluta durante las primeras etapas de la arteriosclerosis (Moroni et al., 2019). Al analizar el paso de estas células a través de constricciones de pequeño tamaño, observamos que los monocitos proinflamatorios *Lmna*^{-/-} permanecían menos tiempo en las constricciones que las células control. Estos datos sugieren que los monocitos proinflamatorios deficientes en lamina A/C pasan con mayor facilidad por estrechamientos, como los que

se forman entre las células endoteliales, confirmando nuestros resultados *in vivo* obtenidos mediante microscopía intravital. Por otra parte, pudimos observar que los monocitos proinflamatorios *Lmna*^{-/-} migraban a menor velocidad a través de canales rectos sin constricciones, resultados que también concuerdan con la menor velocidad que presentaban los leucocitos *Lmna*^{-/-} extravasados al músculo cremáster en nuestros experimentos *in vivo*, así como con la idea de que la ausencia de lamina A/C afecta a la motilidad de las células debido, al menos en parte, a una conexión defectuosa con el citoesqueleto de actina.

Una vez demostrado que el aumento de aterosclerosis en los ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-} alimentados con DG podía deberse en parte a un aumento en el reclutamiento leucocitario, decidimos estudiar etapas más avanzadas de la enfermedad para conocer si la ausencia de lamina A/C en los leucocitos tiene consecuencias en el desarrollo de la placa de ateroma a largo plazo. Por este motivo, estudiamos la proporción de diferentes poblaciones leucocitarias dentro de la arteria aorta. Ya que los monocitos se diferencian a macrófagos una vez infiltrados en la túnica íntima, examinamos este tipo celular (Hilgendorf et al., 2015). Además, analizamos la proporción de neutrófilos en la arteria aorta, ya que también se extravasan durante la arteriosclerosis (Döring et al., 2015). Mediante digestión enzimática y citometría de flujo observamos que los porcentajes de macrófagos y neutrófilos dentro de la arteria eran similares en ambos grupos experimentales. Si correlacionamos estos resultados con los datos obtenidos en los experimentos de microscopía intravital, podemos afirmar que monocitos y neutrófilos se extravasan de manera similar en ausencia de lamina A/C en el SI. Posteriormente, examinamos la composición de las placas de ateroma, ya que una placa de ateroma con un alto contenido en macrófagos y bajo en colágeno está asociado a una mayor vulnerabilidad de la misma (Finn et al., 2010). Mediante inmunohistoquímica comprobamos que la ausencia de lamina A/C en leucocitos no modificaba el porcentaje de macrófagos dentro de la placa de ateroma. Así mismo, comprobamos que el contenido en colágeno de las lesiones ateroscleróticas no estaba alterado. Por lo tanto, podemos afirmar que una deficiencia de lamina A/C en el SI no genera placas de ateroma más vulnerables.

Continuando con el estudio de la placa de ateroma, analizamos los procesos celulares de proliferación y apoptosis por su implicación en el desarrollo de arteriosclerosis (Andrés et al., 2012; Martinet et al., 2011; Tabas, 2010). Por un lado, observamos un descenso en la proliferación de células dentro de la placa de ateroma en los ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-}. Dado que un aumento del desarrollo de arteriosclerosis se asocia a una mayor proliferación de los macrófagos de la placa de ateroma (Sakai et al., 2000), en

los ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-} esperaríamos encontrar un aumento de la proliferación respecto de los controles. Además, de acuerdo con la bibliografía, las células deficientes de lamina A/C deberían proliferar más que los controles WT (Van Berlo et al., 2005). Sin embargo, nuestros datos muestran lo contrario. Este resultado podría indicar que los leucocitos que han migrado a través del endotelio hacia la túnica íntima no son capaces de proliferar correctamente, fenómeno que podría asociarse a la pérdida de estabilidad genómica que sufren las células deficientes de lamina A/C al pasar por constricciones de pequeño tamaño (Raab et al., 2016). Por otra parte, observamos que los niveles de expresión de la proteína proapoptótica caspasa-3 no variaban en las placas de ateroma de los ratones MO *Lmna*^{-/-} → *Ldlr*^{-/-}, descartando que la ausencia de lamin A/C aumente el desarrollo de arteriosclerosis mediante un incremento en la apoptosis de los macrófagos de la placa de ateroma. Sin embargo, sería necesario analizar los procesos de proliferación celular y apoptosis en distintas fases del proceso aterosclerótico para discernir con mayor detalle su posible papel en esta patología.

Teniendo en cuenta que los macrófagos son las células del SI con mayor presencia en la placa aterosclerótica, decidimos analizar en profundidad el papel de la lamina A/C en este tipo celular, utilizando MDMO cultivados *in vitro*. Una posible hipótesis es que los macrófagos aislados de ratones *Lmna*^{-/-} se diferenciase en mayor medida a fenotipos proinflamatorios (M1) y/o en menor medida a fenotipos anti-inflamatorios (M2) (Colin et al., 2014). Sin embargo, observamos que los macrófagos *Lmna*^{-/-} se diferenciaban menos a M1 y de igual manera a M2 en comparación con los controles, apuntando a que el mecanismo de polarización de macrófagos no está implicado en el incremento de lesión aterosclerótica observado *in vivo*. Esta disminución en la diferenciación a M1 por parte de los macrófagos *Lmna*^{-/-} ha sido observada anteriormente en el contexto de la diabetes (Y. Kim et al., 2018). En cuanto al proceso de captación de lipoproteínas oxidadas que tiene lugar durante la aterogénesis gracias a los receptores “escoba” expresados por los macrófagos, nuestros resultados muestran que la ausencia de lamina A/C no afecta a la expresión de estos receptores, sugiriendo que este mecanismo celular tampoco está alterado en ausencia de lamin A/C en el SI. Sin duda, estudios de análisis masivo de expresión serían de gran interés para caracterizar en profundidad las alteraciones que la ausencia de lamina A/C provoca en las células del SI, por ejemplo mediante técnicas de transcriptómica y proteómica.

Los datos anteriores constataron que la ausencia de lamina A/C en todas las células del SI juega un papel proaterogénico. Por esta razón, quisimos conocer qué subpoblaciones leucocitarias estaban implicadas. Los datos obtenidos demostraron que la ausencia de lamina A/C de manera específica en células de la línea mieloide no era suficiente para

generar un aumento en la arteriosclerosis. Tampoco observamos diferencias en el desarrollo de arteriosclerosis al eliminar de forma específica la lamina A/C en células de la línea linfóide. De estos experimentos concluimos que es necesaria la carencia de lamina A/C en diversos tipos celulares del SI para que se genere un incremento en la formación de placa de ateroma.

En su conjunto, estos resultados ponen de manifiesto que los niveles de lamina A/C disminuyen durante el envejecimiento en células del SI de humano y ratón, y que eliminar estas proteínas en los leucocitos aumenta el desarrollo de arteriosclerosis en ratones *Ldlr^{-/-}*, debido al menos en parte a un aumento de la extravasación leucocitaria. Proponemos que el descenso de los niveles de lamina A/C durante el proceso de envejecimiento y en condiciones de hipercolesterolemia disminuye la rigidez nuclear, lo cual facilita que los leucocitos atraviesen el endotelio vascular, acelerando así la formación de la placa de ateroma (**Figura 39**).

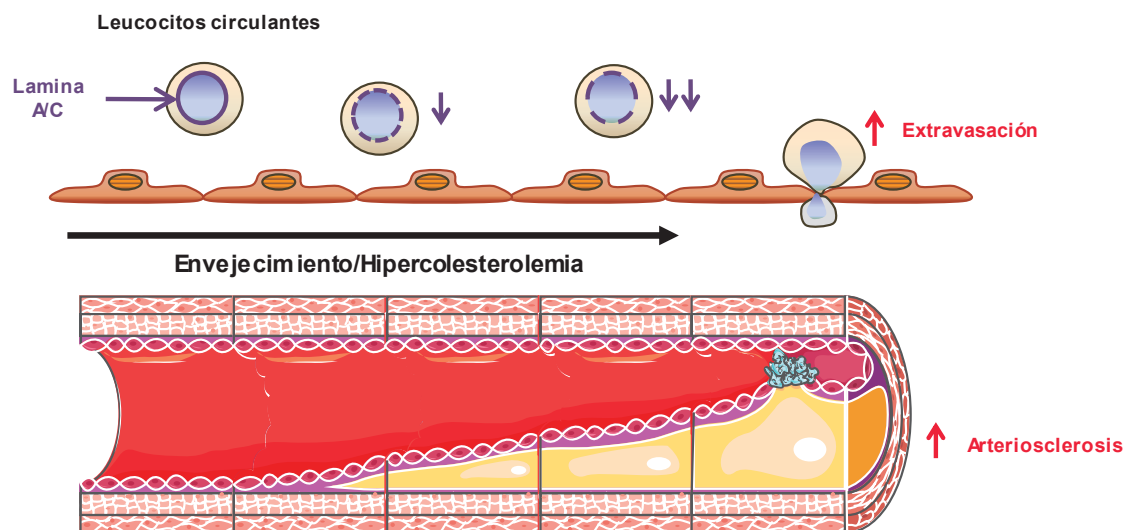


Figura 39. Mecanismo propuesto para el incremento de la arteriosclerosis asociada al descenso de lamina A/C en el sistema inmune durante el envejecimiento y la hipercolesterolemia. La línea perinuclear discontinua refleja el descenso en la expresión de lamina A/C que facilita la extravasación de leucocitos circulantes.

Teniendo en cuenta nuestros resultados, será de gran interés investigar los mecanismos moleculares que provocan el descenso de los niveles de lamina A/C en las células del SI durante el envejecimiento y la hipercolesterolemia. También es crucial seguir ahondando en los mecanismos moleculares que conducen al aumento de la extravasación celular en ausencia de lamina A/C y que producen un aumento del desarrollo de arteriosclerosis. Además, sabiendo que la ausencia de lamina A/C en los leucocitos agrava el proceso de arteriosclerosis, sería importante estudiar si la

sobreexpresión de lamina A/C en estas células tiene un efecto antiaterogénico, por ejemplo, mediante la generación y caracterización de un ratón transgénico. De ser así, aumentar los niveles de lamina A/C en el SI podría ser una terapia para reducir el desarrollo de la arteriosclerosis.

El segundo objetivo que nos planteamos en esta Tesis Doctoral fue investigar la posible regulación de la expresión de lamina A/C durante el envejecimiento en células de la pared vascular y su posible implicación en la ECV. Estudios previos de otros laboratorios han demostrado que la expresión de lamina A/C desciende con el envejecimiento en cardiomiocitos de ratón (Afilalo et al., 2007). Nuestros resultados confirman que el envejecimiento también se asocia con una disminución de los niveles de lamina A/C en arterias, tanto humanas como de ratón. Para estudiar si diferencias en los niveles de expresión de lamina A/C en la pared vascular afectan al desarrollo de ECVs, generamos un modelo de ratón carente de lamina A/C en CMLVs cruzando ratones transgénicos *SM22αCre* con ratones *Lmna^{flox/flox}*. El modelo de ratón *Lmna^{flox/flox} SM22αCre* resultó no ser específico para las CMLVs, ya que también observamos ausencia de lamina A/C en FCs y cardiomiocitos. A pesar de no conseguir generar un modelo de ratón deficiente de lamina A/C de manera completamente específica en CMLVs, la caracterización de los ratones *Lmna^{flox/flox} SM22αCre* nos aportó una visión más global de los efectos de la ausencia lamina A/C en el sistema cardiovascular.

Comenzamos observando un pronunciado descenso en la esperanza de vida de los ratones *Lmna^{flox/flox} SM22αCre*, con una vida media de solo 5 semanas, muy similar a la observada en el modelo de ratón con ausencia de lamina A/C en todas las células del organismo (Sullivan, 1999). Estos resultados sugerían que la mortalidad en el modelo ubicuo está originada por la ausencia de lamina A/C en las células del sistema cardiovascular. Los ratones *Lmna^{flox/flox} SM22αCre* a las 4 semanas de edad presentaban un peso ligeramente inferior comparado con sus controles, aunque deducimos que esta diferencia de peso no es lo suficientemente grave como para explicar un aumento de la mortalidad tan acusado. Debido a su corta esperanza de vida, este modelo de ratón no se pudo emplear para el estudio de ECV de largo desarrollo, como es el caso de la arteriosclerosis.

Nuestros estudios histopatológicos realizados a los ratones *Lmna^{flox/flox} SM22αCre* no revelaron alteraciones tisulares ni aumento de fibrosis en hígado, pulmón y riñón. Estos resultados parecen indicar que la eliminación de lamina A/C en las CMLVs de los vasos sanguíneos de estos órganos no es la causa de muerte prematura en los ratones *Lmna^{flox/flox} SM22αCre*.

Puesto que las arterias de gran calibre están formadas principalmente por CMLVs, analizamos si estaba afectada de una manera significativa la estructura histológica de la aorta de los ratones *Lmna^{flox/flox} SM22 α Cre*. Observamos en estos animales que la carencia de lamina A/C en las CMLVs producía un aumento casi significativo de la deposición de colágeno en la túnica media de la arteria aorta. Sin embargo, no encontramos otras afectaciones en el tejido. Es posible que sea necesario un tiempo más prolongado de vida para que los ratones *Lmna^{flox/flox} SM22 α Cre* desarrollasen una patología aórtica más grave.

La descripción del modelo *Lmna^{flox/flox} SM22 α Cre* realizada hasta el momento demostró un efecto nulo o reducido en hígado, pulmón, riñón y aorta a pesar de la ausencia de lamina A/C en las CMLVs de estos tejidos. Los experimentos de ecocardiografía, sin embargo, mostraron una pérdida de función cardíaca muy grave en ambos ventrículos, los cuales no eran capaces de contraerse con normalidad. En estudios anteriores utilizando modelos de ratón con deficiencia ubicua de lamina A/C se han descrito también este tipo de alteraciones cardíacas (Frock et al., 2012; Sullivan, 1999), lo que nos lleva a hipotetizar que la mortalidad prematura en estos modelos puede estar debida a la ausencia de lamina A/C en tipos celulares presentes en el corazón. Esta posibilidad se ve reforzada por los problemas de conducción y repolarización cardíaca que observamos mediante ECG en los ratones *Lmna^{flox/flox} SM22 α Cre*. Dado que la falta de lamina A/C produce fragilidad nuclear, los fallos en la función cardíaca observados podrían estar debidos a un aumento de la apoptosis de los cardiomiocitos carentes de lamina A/C, que serían incapaces de soportar el estrés mecánico ejercido en los ciclos de contracción-relajación del músculo cardíaco (Nikolova et al., 2004). Por otra parte, se ha demostrado que las fuerzas mecánicas celulares modulan la señalización por medio de la acción de canales iónicos en cardiomiocitos (J. C. Kim et al., 2017; Peyronnet et al., 2016). La ausencia de lamina A/C podría alterar la respuesta de los cardiomiocitos ante la contracción cardíaca debido a una conexión defectuosa con el citoesqueleto, que a su vez produciría anomalías en la conducción eléctrica mediante la alteración de canales iónicos.

Durante el envejecimiento aumenta la fibrosis en el corazón, una alteración que contribuye al desarrollo de problemas de función cardíaca y conducción eléctrica (Almehmadi et al., 2014; Jiang et al., 2018; Song et al., 1999). Nuestros resultados demostraron que los ratones *Lmna^{flox/flox} SM22 α Cre* presentaban una fibrosis generalizada en el tejido cardíaco. Debido a la corta esperanza de vida de estos ratones, es difícil asegurar que la pérdida de función y conducción cardíaca esté producida por la extensa fibrosis. No obstante, numerosos estudios describen la relación entre la

ausencia o mutaciones de la lamina A/C con el aumento de la fibrosis cardíaca (Arimura et al., 2005; Chatzifrangkeskou et al., 2016; Gerbino et al., 2018; Van Tintelen et al., 2007). Además, el hecho de que también se observe en células embrionarias un incremento en la fibrosis en ausencia de lamina A/C (Van Berlo et al., 2005), apunta a que en los ratones *Lmna*^{flox/flox} *SM22αCre* la fibrosis empieza a desarrollarse en etapas muy tempranas, por lo que podría considerarse como una de las causas contribuyentes al deterioro de la funcionalidad y conducción del corazón. El desarrollo de fibrosis cardíaca se caracteriza por un aumento en la expresión de moléculas profibróticas en FCs, como por ejemplo FSP-1 y SMA, marcadores que indican una conversión celular a miofibroblastos (Ma et al., 2018). Además de corroborar la generación de fibrosis cardíaca mediante la cuantificación del área de WGA, observamos en los ratones *Lmna*^{flox/flox} *SM22αCre* un incremento en la expresión de FSP-1 y SMA. Estos resultados nos llevan a plantear dos hipótesis. Por un lado, proponemos que la ausencia de lamina A/C en CMLVs, FCs y cardiomiocitos provoca la conversión de los FCs a miofibroblastos (células que expresan FSP-1 y SMA) (Desmouliere et al., 1993), promoviendo así la fibrosis cardíaca. Por otro lado, el desarrollo de fibrosis asociada a la expresión de FSP-1 y SMA podría ser secundario al daño cardíaco provocado por la falta de expresión de lamina A/C (Nikolova et al., 2004).

En resumen, la segunda parte de los resultados de este trabajo de Tesis Doctoral demuestran que los niveles de lamina A/C disminuyen durante el envejecimiento en arterias de ratón y humanas. Además, observamos que los ratones carentes de lamina A/C en CMLVs, cardiomiocitos y FC mueren prematuramente y presentan graves problemas cardíacos asociados a fibrosis en el corazón. Por ello proponemos que la disminución progresiva de los niveles de lamina A/C durante el envejecimiento en CMLVs, y también en células del corazón como los FCs y los cardiomiocitos, es un nuevo mecanismo que favorece fibrosis y pérdida de función y conducción cardíaca (**Figura 40**).

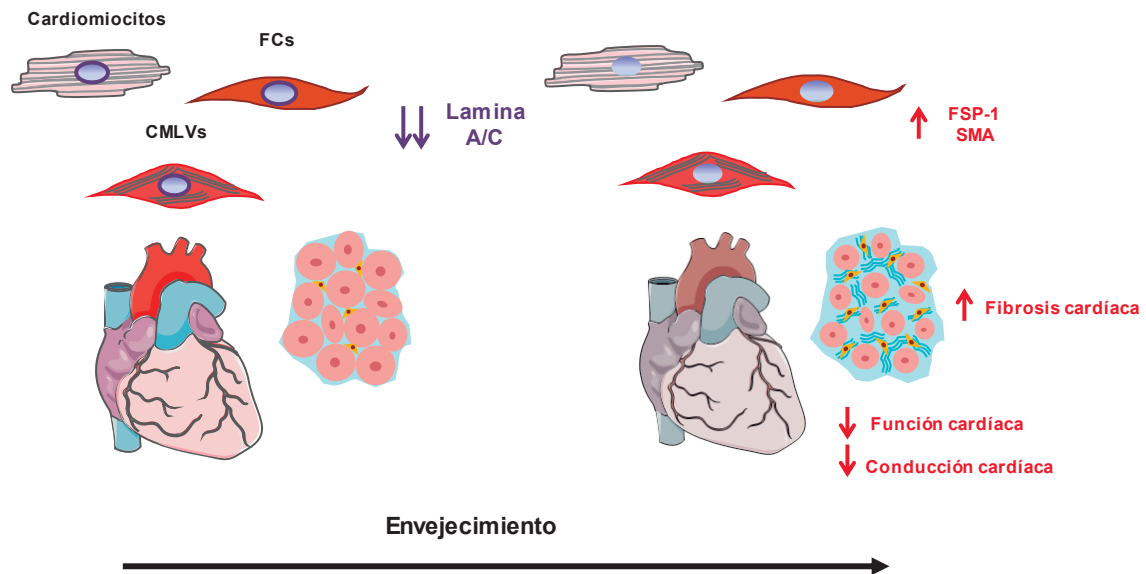


Figura 40. Mecanismo propuesto para el desarrollo de alteraciones cardíacas asociada al descenso de lamina A/C en células del sistema cardiovascular durante el envejecimiento.

Aunque parece claro que la falta de contracción de los ventrículos podría provocar la muerte de los ratones *Lmna^{flox/flox} SM22αCre*, es importante seguir investigando las causas finales de la mortalidad prematura en este modelo de ratón. Igualmente, se necesita estudiar con más profundidad los mecanismos celulares y moleculares que subyacen a la formación de fibrosis y a las alteraciones cardíacas en ausencia de lamina A/C específicamente en células del corazón y la pared arterial, con el objetivo final de identificar nuevas dianas terapéuticas potencialmente traslacionales al ser humano en el contexto del envejecimiento y la enfermedad cardíaca. Por último, debido a que observamos un descenso de los niveles de lamina A/C durante el envejecimiento en células endoteliales de ratón, sería muy interesante abordar el desarrollo de un modelo de ratón deficiente de lamina A/C en este tipo celular para estudiar su relación con las ECVs.

CONCLUSIONES

1. El envejecimiento se asocia con una reducción marcada de los niveles de lamina A/C en células del sistema inmune humano y de ratón.
2. La hipercolesterolemia se asocia con una reducción de los niveles de lamina A/C en células del sistema inmune de ratón.
3. La ausencia de lamina A/C en células del sistema inmune favorece el desarrollo de arteriosclerosis en ratones *Ldlr*^{-/-} alimentados con una dieta rica en grasa y colesterol.
4. La ausencia de lamina A/C en células del sistema inmune promueve la extravasación de leucocitos en ratones *Ldlr*^{-/-}, pero disminuye su velocidad de migración una vez extravasados.
5. La ausencia de lamina A/C facilita la migración *in vitro* de monocitos proinflamatorios a través de constricciones, pero disminuye su velocidad de movimiento.
6. El envejecimiento se asocia con una marcada reducción de los niveles de lamina A/C en tejido arterial humano y de ratón. El descenso en arterias de ratón se observa en células endoteliales y de músculo liso vascular de la íntima y túnica media, respectivamente, pero no en células de la túnica adventicia.
7. En ratón, la ausencia de lamina A/C en células de músculo liso vascular, cardiomiocitos y FCs:
 - 7.1. Reduce drásticamente la esperanza de vida.
 - 7.2. Provoca graves alteraciones en la función cardíaca, así como problemas de conducción eléctrica del corazón.
 - 7.3. Está asociada a un aumento de la fibrosis en el tejido cardíaco relacionada con la expresión de los marcadores FSP-1 y SMA.

BIBLIOGRAFÍA

- Afilalo, J., Sebag, I. A., Chalifour, L. E., Rivas, D., Akter, R., Sharma, K., & Duque, G. (2007). Age-related changes in lamin A/C expression in cardiomyocytes. *The American Journal of Physiology-Heart and Circulatory Physiology*, 293, 1451–1456.
- Alldridge, L. C., O'Farrell, M. K., & Dealtry, G. B. (1991). Down regulation of Lamin A and C by murine interferon β . *Experimental Cell Research*, 195, 546–550.
- Almehmadi, F., Joncas, S. X., Nevis, I., Zahrani, M., Bokhari, M., Stirrat, J., Fine, N. M., Yee, R., & White, J. A. (2014). Prevalence of myocardial fibrosis patterns in patients with systolic dysfunction: prognostic significance for the prediction of sudden cardiac arrest or appropriate implantable cardiac defibrillator therapy. *Circulation: Cardiovascular Imaging*, 7(4), 593–600.
- Alon, R., Kassner, P. D., Carr, M. W., Finger, E. B., Hemler, M. E., & Springer, T. A. (1995). The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *Journal of Cell Biology*, 128(6), 1243–1253.
- Andrés, V., & Dorado, B. (2015). Methods in Mouse Atherosclerosis. *Methods in Molecular Biology*, 1339, 349–366.
- Andrés, V., & González, J. M. (2009). Role of A-type lamins in signaling, transcription, and chromatin organization. *Journal of Cell Biology*, 187(7), 945–957.
- Andrés, V., Pello, O. M., & Silvestre-Roig, C. (2012). Macrophage proliferation and apoptosis in atherosclerosis. *Current Opinion in Lipidology*, 23(5), 429–438.
- Arimura, T., Helbling-Leclerc, A., Massart, C., Varnous, S., Niel, F., Lacène, E., Fromes, Y., Toussaint, M., Mura, A. M., Kelle, D. I., Amthor, H., Isnard, R., Malissen, M., Schwartz, K., & Bonne, G. (2005). Mouse model carrying H222P-Lmna mutation develops muscular dystrophy and dilated cardiomyopathy similar to human striated muscle laminopathies. *Human Molecular Genetics*, 14(1), 155–169.
- Bell, E. S., & Lammerding, J. (2016). Causes and consequences of nuclear envelope alterations in tumour progression. *European Journal of Cell Biology*, 95(11), 449–464.
- Belt, E. J. T., Fijneman, R. J. A., Van Den Berg, E. G., Bril, H., Delis-Van Diemen, P. M., Tijssen, M., Van Essen, H. F., De Lange-De Klerk, E. S. M., Beliën, J. A. M., Stockmann, H. B. A. C., Meijer, S., & Meijer, G. A. (2011). Loss of lamin A/C expression in stage II and III colon cancer is associated with disease recurrence. *European Journal of Cancer*, 47(12), 1837–1845.

- Bennett, M. R., Sinha, S., & Owens, G. K. (2016). Vascular smooth muscle cells in atherosclerosis. *Physiology & Behavior*, 118(4), 692–702.
- Betts, J. G., Desaix, P., Johnson, E., Johnson, J. E., Korol, O., Kruse, D., Poe, B., Wise, J. A., Womble, M., & Young, K. A. (2017). Anatomy and Physiology. *Openstax*, 823–958.
- Bouhouche, A., Benomar, A., Birouk, N., Mularoni, A., Meggouh, F., Tassin, J., Grid, D., Vandenberghe, A., Yahyaoui, M., Chkili, T., Brice, A., & LeGuern, E. (1999). A locus for an axonal form of autosomal recessive Charcot-Marie-Tooth disease maps to chromosome 1q21.2-q21.3. *American Journal of Human Genetics*, 65(3), 722–727.
- Bouزيد, T., Kim, E., Riehl, B. D., Esfahani, A. M., Rosenbohm, J., Yang, R., Duan, B., & Lim, J. Y. (2019). The LINC complex, mechanotransduction, and mesenchymal stem cell function and fate. *Journal of Biological Engineering*, 13(1), 1–12.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248–254.
- Braunersreuther, V., & Mach, F. (2006). Leukocyte recruitment in atherosclerosis: potential targets for therapeutic approaches? *Cellular and Molecular Life Sciences*, 63(18), 2079–2088.
- Broers, J. L. V., Ramaekers, F. C. S., Bonne, G., Ben Yaou, R., & Hutchison, C. J. (2006). Nuclear lamins: laminopathies and their role in premature ageing. *Physiological Reviews*, 86(3), 967–1008.
- Burke, B., & Stewart, C. L. (2013). The nuclear lamins: Flexibility in function. *Nature Reviews Molecular Cell Biology*, 14(1), 13–24.
- Burkhardt, R. (2015). Hyperlipidemia and cardiovascular disease: Reinforcement for “lower is better.” *Current Opinion in Lipidology*, 26(5), 468–469.
- Carman, C. V., & Springer, T. A. (2004). A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *Journal of Cell Biology*, 167(2), 377–388.
- Carman, C. V., & Springer, T. A. (2008). Trans-cellular migration: cell-cell contacts get intimate. *Current Opinion in Cell Biology*, 20(5), 533–540.

- Carmosino, M., Torretta, S., Procino, G., Gerbino, A., Forleo, C., Favale, S., & Svelto, M. (2014). Role of nuclear Lamin A/C in cardiomyocyte functions. *Biology of the Cell*, 106(10), 346–358.
- Chambliss, A. B., Khatau, S. B., Erdenberger, N., Robinson, D. K., Hodzic, D., Longmore, G. D., & Wirtz, D. (2013). The LINC-anchored actin cap connects the extracellular milieu to the nucleus for ultrafast mechanotransduction. *Scientific Reports*, 3, 1–9.
- Chatzifrangkeskou, M., Dour, C. Le, Wu, W., Morrow, J. P., Joseph, L. C., Beuvin, M., Sera, F., Homma, S., Vignier, N., Mougnot, N., Bonne, G., Lipson, K. E., Worman, H. J., & Muchir, A. (2016). ERK1/2 directly acts on CTGF/CCN2 expression to mediate myocardial fibrosis in cardiomyopathy caused by mutations in the lamin A/C gene. *Human Molecular Genetics*, 25(11), 2220–2233.
- Chèvre, R., González-Granado, J. M., Megens, R. T. A., Sreeramkumar, V., Silvestre-Roig, C., Molina-Sánchez, P., Weber, C., Soehnlein, O., Hidalgo, A., & Andrés, V. (2014). High-resolution imaging of intravascular atherogenic inflammation in live mice. *Circulation Research*, 114(5), 770–779.
- Clausen, B. E., Burkhardt, C., Reith, W., Renkawitz, R., & Förster, I. (1999). Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Research*, 8(4), 265–277.
- Coffinier, C., Jung, H. J., Li, Z., Nobumori, C., Yun, U. J., Farber, E. A., Davies, B. S., Weinstein, M. M., Yang, S. H., Lammerding, J., Farahani, J. N., Bentolila, L. A., Fong, L. G., & Young, S. G. (2010). Direct synthesis of lamin A, bypassing prelamin A processing, causes misshapen nuclei in fibroblasts but no detectable pathology in mice. *Journal of Biological Chemistry*, 285(27), 20818–20826.
- Colin, S., Chinetti-gbaguidi, G., & Staels, B. (2014). Macrophage phenotypes in atherosclerosis. *Immunological Reviews*, 262(1), 153–166.
- Curtis, A. B., Karki, R., Hattoum, A., & Sharma, U. C. (2018). Arrhythmias in Patients ≥80 Years of Age. In *Journal of the American College of Cardiology* (Vol. 71, Issue 18, pp. 2041–2057).
- De Bakker, J. M. T., Van Capelle, F. J. L., Janse, M. J., Tasseron, S., Vermeulen, J. T., De Jonge, N., & Lahpor, J. R. (1993). Slow conduction in the infarcted human heart: “Zigzag” course of activation. *Circulation*, 88(3), 915–926.

- De Jong, S., Van Veen, T. A. B., Van Rijen, H. V. M., & De Bakker, J. M. T. (2011). Fibrosis and cardiac arrhythmias. *Journal of Cardiovascular Pharmacology*, 57(6), 630–638.
- Dechat, T., Pflieger, K., Sengupta, K., Shimi, T., Shumaker, D. K., Solimando, L., & Goldman, R. D. (2008). Nuclear lamins: major factors in the structural organization and function of the nucleus and chromatin. *Genes and Development*, 22(7), 832–853.
- Defesche, J. C. (2004). Low-density lipoprotein receptor-its structure, function, and mutations. *Seminars in Vascular Medicine*, 4(1), 5–11.
- Desmouliere, A., Geinoz, A., Gabbiani, F., & Gabbiani, G. (1993). Transforming growth factor- β 1 induces α -smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *Journal of Cell Biology*, 122(1), 103–111.
- Döring, Y., Drechsler, M., Soehnlein, O., & Weber, C. (2015). Neutrophils in atherosclerosis: from mice to man. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 35(2), 288–295.
- Doyle, B., & Caplice, N. (2007). Plaque neovascularization and antiangiogenic therapy for atherosclerosis. *Journal of the American College of Cardiology*, 49(21), 2073–2080.
- Dunningan, M. G., Cochrane, M. A., Kelly, A., & Scott, J. W. (1974). Familial lipodistrophic diabetes with dominant transmission. *Quarterly Journal of Medicine*, 169, 33–48.
- Duque, G., & Rivas, D. (2006). Age-related changes in lamin A/C expression in the osteoarticular system: laminopathies as a potential new aging mechanism. *Mechanisms of Ageing and Development*, 127, 378–383.
- Emde, B., Heinen, A., Gödecke, A., & Bottermann, K. (2014). Wheat germ agglutinin staining as a suitable method for detection and quantification of fibrosis in cardiac tissue after myocardial infarction. *European Journal of Histochemistry*, 58(4), 315–319.

- Eriksson, M., Brown, W. T., Gordon, L. B., Glynn, M. W., Singer, J., Scott, L., Erdos, M. R., Robbins, C. M., Moses, T. Y., Berglund, P., Dutra, A., Pak, E., Durkin, S., Csoka, A. B., Boehnke, M., Glover, T. W., & Collins, F. S. (2003). Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome. *Nature*, 423(6937), 293–298.
- Ferrucci, L., & Fabbri, E. (2018). Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. *Nature Reviews Cardiology*, 15(9), 505–522.
- Finn, A. V., Nakano, M., Narula, J., Kolodgie, F. D., & Virmani, R. (2010). Concept of vulnerable/unstable plaque. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 30(7), 1282–1292.
- Fisher, D. Z., Chaudhary, N., & Blobel, G. (1986). cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 83(17), 6450–6454.
- Fong, L. G., Bergo, M. O., Young, S. G., Fong, L. G., Ng, J. K., Lammerding, J., Vickers, T. A., Meta, M., Côté, N., Gavino, B., Qiao, X., Chang, S. Y., Young, S. R., Yang, S. H., Stewart, C. L., Lee, R. T., Bennett, C. F., Bergo, M. O., & Young, S. G. (2006). Prelamin A and lamin A appear to be dispensable in the nuclear lamina. *The Journal of Clinical Investigation*, 116(3), 743–752.
- Francis Stuart, S. D., Wang, L., Woodard, W. R., Ng, G. A., Habecker, B. A., & Ripplinger, C. M. (2018). Age-related changes in cardiac electrophysiology and calcium handling in response to sympathetic nerve stimulation. *Journal of Physiology*, 596(17), 3977–3991.
- Frock, R. L., Chen, S. C., Da, D., Frett, E., Lau, C., Brown, C., Pak, D. N., Wang, Y., Muchir, A., Worman, H. J., Santana, L. F., Ladiges, W. C., Rabinovitch, P. S., & Kennedy, B. K. (2012). Cardiomyocyte-specific expression of lamin A improves cardiac function in Lmna^{-/-} mice. *PLoS ONE*, 7(8), 1–9.
- Gerace, L., & Burke, B. (1988). Functional organization of the nuclear envelope. *Annual Review of Cell Biology*, 4, 335–374.
- Gerbino, A., Procino, G., Svelto, M., & Carmosino, M. (2018). Role of Lamin A/C Gene Mutations in the signaling defects leading to cardiomyopathies. *Frontiers in Physiology*, 9(SEP), 1–7.

- Getz, G. S., & Reardon, C. A. (2012). Animal models of atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 32(5), 1104–1115.
- Golubtsova, N. N. (2016). Lamin A and lamin-associated polypeptide 2 (LAP-2) in human skin in the process of aging. *Advances in Gerontology*, 29(4), 577–585.
- González-Granado, J. M., Silvestre-Roig, C., Rocha-Perugini, V., Trigueros-Motos, L., Cibrián, D., Morlino, G., Blanco-Berrocal, M., Osorio, F. G., Freije, J. M. P., López-Otín, C., Sánchez-Madrid, F., & Andrés, V. (2014). Nuclear envelope lamin-A couples actin dynamics with immunological synapse architecture and T cell activation. *Science Signaling*, 7(322), 1–31.
- Guicciardi, M. E., Deussing, J., Miyoshi, H., Bronk, S. F., Svingen, P. A., Peters, C., Kaufmann, S. H., & Gores, G. J. (2000). *Cathepsin B contributes to TNF- α – mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c*. 106(9), 1127–1137.
- Hanna, R. N., Shaked, I., Hubbeling, H. G., Punt, J. A., Wu, R., Herrley, E., Zaugg, C., Pei, H., Geissmann, F., Ley, K., & Hedrick, C. C. (2012). NR4A1 (Nur77) deletion polarizes macrophages toward an inflammatory phenotype and increases atherosclerosis. *Circulation Research*, 110(3), 416–427.
- Hansson, G. K., & Libby, P. (2006). The immune response in atherosclerosis: a double-edged sword. *Nature Reviews Immunology*, 6(7), 508–519.
- Hansson, G. K., Robertson, A.-K. L., & Söderberg-Nauclér, C. (2006). Inflammation and atherosclerosis. *Annual Review of Pathology*, 1, 297–329.
- Harada, T., Swift, J., Irianto, J., Shin, J. W., Spinler, K. R., Athirasala, A., Diegmiller, R., Dingal, P. C. D. P., Ivanovska, I. L., & Discher, D. E. (2014). Nuclear lamin stiffness is a barrier to 3D migration, but softness can limit survival. *Journal of Cell Biology*, 204(5), 669–682.
- Hartvigsen, K., Binder, C. J., Hansen, L. F., Rafia, A., Juliano, J., Hörrkö, S., Steinberg, D., Palinski, W., Witztum, J. L., & Li, A. C. (2007). A diet-induced hypercholesterolemic murine model to study atherogenesis without obesity and metabolic syndrome. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 27(4), 878–885.
- Helbling-leclerc, A., Bonne, G., & Schwartz, K. (2002). Emery-Dreifuss muscular dystrophy. *European Journal of Human Genetics*, 10, 157–161.

- Hilgendorf, I., Swirski, F. K., & Robbins, C. S. (2015). Monocyte fate in atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 35(2), 272–279.
- Hinz, B., Phan, S. H., Thannickal, V. J., Prunotto, M., Desmouliere, A., Varga, J., De Wever, O., Mareel, M., & Gabbiani, G. (2012). Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *American Journal of Pathology*, 180(4), 1340–1355.
- Holtwick, R., Gotthardt, M., Skryabin, B., Steinmetz, M., Potthast, R., Zetsche, B., Hammer, R. E., Herz, J., & Kuhn, M. (2002). Smooth muscle-selective deletion of guanylyl cyclase-A prevents the acute but not chronic effects of ANP on blood pressure. *Proceedings of the National Academy of Sciences of the United States of America*, 99(10), 7142–7147.
- Horn, M. A., & Trafford, A. W. (2016). Aging and the cardiac collagen matrix: novel mediators of fibrotic remodelling. *Journal of Molecular and Cellular Cardiology*, 93, 175–185.
- Huo, Y., & Ley, K. (2001). Adhesion molecules and atherogenesis. *Acta Physiologica Scandinavica*, 173(1), 35–43.
- Hutchison, C. J., & Worman, H. J. (2004). A-type lamins: guardians of the soma? *Nat Cell Biol*, 6(11), 1062–1067.
- Imhof, B. A., & Aurrand-Lions, M. (2004). Adhesion mechanisms regulating the migration of monocytes. *Nature Reviews Immunology*, 4(6), 432–444.
- Ishibashi, S., Brown, M. S., Goldstein, J. L., Gerard, R. D., Hammer, R. E., & Herz, J. (1993). Hypercholesterolemia in LDL receptor Knock-Out mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.*, 92(2), 883–893.
- Ishibashi, S., Goldstein, J. L., Brown, M. S., Herz, J., & Burns, D. K. (1994). Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *Journal of Clinical Investigation*, 93(5), 1885–1893.
- Ishii, H., Park, W.-Y., So, J., Kuhn, S., Koparde, V. N., Pang, Y., Greten, T. F., Hollander, M. C., & Yang, L. (2020). Loss of myeloid-specific lamin AC drives lung metastasis through Gfi-1 and C/EBP ϵ -mediated granulocytic differentiation. *Molecular Carcinogenesis*, 2020, 1–12.

- Jackson, S. N. J., Howlett, T. A., McNally, P. G., O'Rahilly, S., & Trembath, R. C. (1997). Dunnigan-Kobberling syndrome: an autosomal dominant form of partial lipodystrophy. *QJM*, 90(1), 27–36.
- Jiang, M., Wang, Z., Su, X., Gong, X., Pu, J., Wu, L., Liu, C., Yao, Q., Kong, L., Xu, J., & He, B. (2018). The significance of interstitial fibrosis on left ventricular function in hypertensive versus hypertrophic cardiomyopathy. *Scientific Reports*, 8(1), 1–10.
- Jones, S. A. (2006). Ageing to arrhythmias: conundrums of connections in the ageing heart. *Journal of Pharmacy and Pharmacology*, 58(12), 1571–1576.
- Joseph, M., & Eric, N. (1996). SM22 alpha , a marker of adult smooth muscle , is expressed in multiple myogenic lineages during embryogenesis. *Circulation Research*, 78(February), 188–195.
- Kansas, G. S. (1996). Selectins and their ligands: current concepts and controversies. *Blood*, 88(9), 3259–3287.
- Keller, K. M., & Howlett, S. E. (2016). Sex differences in the biology and pathology of the aging heart. *Canadian Journal of Cardiology*, 32(9), 1065–1073.
- Kim, D. H., Cho, S., & Wirtz, D. (2014). Tight coupling between nucleus and cell migration through the perinuclear actin cap. *Journal of Cell Science*, 127(11), 2528–2541.
- Kim, H., Kim, M., Im, S.-K., & Fang, S. (2018). Mouse Cre-LoxP system: general principles to determine tissue-specific roles of target genes. *Laboratory Animal Research*, 34(4), 147–159.
- Kim, J. C., Son, M. J., Wang, J., & Woo, S. H. (2017). Regulation of cardiac Ca²⁺ and ion channels by shear mechanotransduction. *Archives of Pharmacal Research*, 40(7), 783–795.
- Kim, Y., Bayona, P. W., Kim, M., Chang, J., Hong, S., Park, Y., Budiman, A., Kim, Y. J., Choi, C. Y., Kim, W. S., Lee, J., & Cho, K. W. (2018). Macrophage lamin A/C regulates inflammation and the development of obesity-induced insulin resistance. *Frontiers in Immunology*, 9(APR), 1–14.
- Kim, Y., & Zheng, Y. (2013). Generation and characterization of a conditional deletion allele for Lmna in mice. *Biochemical and Biophysical Research Communications*, 440(1), 8–13.
- Knowles, J. W., & Maeda, N. (2000). Genetic modifiers of atherosclerosis in mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 20(11), 2336–2345.

- Kong, P., Christia, P., Saxena, A., Su, Y., & Frangogiannis, N. G. (2013). Lack of specificity of fibroblast-specific protein 1 in cardiac remodeling and fibrosis. *American Journal of Physiology of Heart and Circulatory Physiology*, 305(9), 1363–1372.
- Kubben, N., Voncken, J. W., Konings, G., van Weeghel, M., van den Hoogenhof, M. M. G., Gijbels, M., van Erk, A., Schoonderwoerd, K., van den Bosch, B., Dahlmans, V., Calis, C., Houten, S. M., Misteli, T., & Pinto, Y. M. (2011). Post-natal myogenic and adipogenic developmental: defects and metabolic impairment upon loss of a-type lamins. *Nucleus*, 2(3), 195–207.
- Kzhyshkowska, J., Neyen, C., & Gordon, S. (2012). Role of macrophage scavenger receptors in atherosclerosis. *Immunobiology*, 217(5), 492–502.
- Lahoute, C., Herbin, O., Mallat, Z., & Tedgui, A. (2011). Adaptive immunity in atherosclerosis: mechanisms and future therapeutic targets. *Nature Reviews Cardiology*, 8(6), 348–358.
- Lakatta, E. G., & Levy, D. (2003). Arterial and cardiac aging: Major shareholders in cardiovascular disease enterprises: Part II: The aging heart in health: Links to heart disease. *Circulation*, 107(2), 346–354.
- Lee, J. S. H., Hale, C. M., Panorchan, P., Khatau, S. B., George, J. P., Tseng, Y., Stewart, C. L., Hodzic, D., & Wirtz, D. (2007). Nuclear lamin A/C deficiency induces defects in cell mechanics, polarization, and migration. *Biophysical Journal*, 93(7), 2542–2552.
- Li, A. C., & Glass, C. K. (2002). The macrophage foam cell as a target for therapeutic intervention. *Nature Medicine*, 8(11), 1235–1242.
- Li, L., Miano, J. M., Mercer, B., & Olson, E. N. (1996). Expression of the SM22alpha promoter in transgenic mice provides evidences for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *The Journal of Cell Biology*, 132(5), 849–859.
- Libby, P., Buring, J. E., Badimon, L., Hansson, G. K., Deanfield, J., Bittencourt, M. S., Tokgözoğlu, L., & Lewis, E. F. (2019). Atherosclerosis. *Nature Reviews Disease Primers*, 5(1), 1–18.

- Liu, L., Luo, Q., Sun, J., Wang, A., Shi, Y., Ju, Y., Morita, Y., & Song, G. (2017). Decreased nuclear stiffness via FAK-ERK1/2 signaling is necessary for osteopontin-promoted migration of bone marrow-derived mesenchymal stem cells. *Experimental Cell Research*, 355(2), 172–181.
- Lombardi, M. L., Jaalouk, D. E., Shanahan, C. M., Burke, B., Roux, K. J., & Lammerding, J. (2011). The interaction between nesprins and sun proteins at the nuclear envelope is critical for force transmission between the nucleus and cytoskeleton. *Journal of Biological Chemistry*, 286(30), 26743–26753.
- Ma, Z., Yuan, Y., Wu, H., Zhang, X., & Tang, Q. (2018). Cardiac fibrosis : new insights into the pathogenesis. *International Journal of Biological Sciences*, 14(12), 1645–1657.
- Machiels, B. M., Zorenc, A. H. G., Endert, J. M., Kuijpers, H. J. H., Van Eys, G. J. J. M., Ramaekers, F. C. S., & Broers, J. L. V. (1996). An alternative splicing product of the lamin A/C gene lacks exon 10. *Journal of Biological Chemistry*, 271(16), 9249–9253.
- Marais, A. D. (2004). Familial hypercholesterolaemia. *Clinical Biochemical Review*, 25, 49–68.
- Martinet, W., Schrijvers, D. M., & De Meyer, G. R. Y. (2011). Necrotic cell death in atherosclerosis. *Basic Research in Cardiology*, 106(5), 749–760.
- Maxfield, F. R., & Tabas, I. (2005). Role of cholesterol and lipid organization in disease. *Nature*, 438, 612–621.
- McNally, E., & Mestroni, L. (2017). DCM: genetic determinants and mechanisms. *Circulation Research*, 121(7), 731–748.
- Moore, K. J., Sheedy, F. J., & Fisher, E. a. (2013). Macrophages in atherosclerosis: a dynamic balance. *Nature Reviews. Immunology*, 13(10), 709–721.
- Moroni, F., Ammirati, E., Norata, G. D., Magnoni, M., & Camici, P. G. (2019). The role of monocytes and macrophages in human atherosclerosis, plaque neoangiogenesis, and atherothrombosis. *Mediators of Inflammation*, 2019, 1–11.
- Muller, W. A. (2003). Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends in Immunology*, 24(6), 326–333.
- Murray, P. J. (2017). Macrophage polarization. *Annual Review of Physiology*, 79(1), 541–566.

- Nelson, R. H. (2013). Hyperlipidemia as a risk for cardiovascular disease. *Prim Care*, 40(1), 195–211.
- Nguyen, T. P., Xie, Y., Garfinkel, A., Qu, Z., & Weiss, J. N. (2012). Arrhythmogenic consequences of myofibroblastmyocyte coupling. *Cardiovascular Research*, 93(2), 242–251.
- Nikolova, V., Feneley, M. P., Fatkin, D., Nikolova, V., Leimena, C., McMahon, A. C., Tan, J. C., Chandar, S., Jogia, D., Kesteven, S. H., Michalicek, J., Otway, R., Verheyen, F., Rainer, S., Stewart, C. L., Martin, D., Feneley, M. P., & Fatkin, D. (2004). Defects in nuclear structure and function promote dilated cardiomyopathy in lamin A / C-deficient mice Find the latest version : and function promote dilated cardiomyopathy in lamin A / C – deficient mice. *The Journal of Clinical Investigation*, 113(3), 357–369.
- Pedrotty, D. M., Klinger, R. Y., Kirkton, R. D., & Bursac, N. (2009). Cardiac fibroblast paracrine factors alter impulse conduction and ion channel expression of neonatal rat cardiomyocytes. *Cardiovascular Research*, 83(4), 688–697.
- Peyronnet, R., Nerbonne, J. M., & Kohl, P. (2016). Cardiac mechano-gated ion channels and arrhythmias. *Circulation Research*, 118(2), 311–329.
- Raab, M., Gentili, M., Belly, H. De, Thiam, H., Vargas, P., Jimenez, A. J., Lautenschlaeger, F., Voituriez, R., Manel, N., & Piel, M. (2016). ESCRT III repairs nuclear envelope ruptures during cell migration to limit DNA damage and cell death. *Science*, 352(6283), 359–363.
- Rocha-Perugini, V., & González-Granado, J. M. (2014). Nuclear envelope lamin-A as a coordinator of T cell activation. *Nucleus*, 5(5), 396–401.
- Rowat, A. C., Jaalouk, D. E., Zwerger, M., Ung, W. L., Eydelnant, I. A., Olins, D. E., Olins, A. L., Herrmann, H., Weitz, D. A., & Lammerding, J. (2013). Nuclear envelope composition determines the ability of neutrophil-type cells to passage through micron-scale constrictions. *Journal of Biological Chemistry*, 288(12), 8610–8618.
- Saarinen, I., Mirtti, T., Seikkula, H., Boström, P. J., & Taimen, P. (2015). Differential predictive roles of A-and B-Type nuclear lamins in prostate cancer progression. *PLoS ONE*, 10(10), 1–16.

- Sáez, P. J., Barbier, L., Attia, R., Thiam, H.-R., Piel, M., & Vargas, and P. (2018). Leukocyte migration and deformation in collagen gels and microfabricated constrictions. *Cell Migration: Methods and Protocols, Methods in Molecular Biology*, 1749, 351–360.
- Sage, A. P., Tsiantoulas, D., Binder, C. J., & Mallat, Z. (2019). The role of B cells in atherosclerosis. *Nature Reviews Cardiology*, 16(3), 180–196.
- Sakai, M., Kobori, S., Miyazaki, A., & Horiuchi, S. (2000). Macrophage proliferation in atherosclerosis. *Current Opinion in Lipidology*, 11(5), 503–509.
- Sandre-giovannoli, A. De, Bernard, R., Cau, P., Navarro, C., Amiel, J., Boccaccio, I., Lyonnet, S., Stewart, C. L., Munnich, A., Merrer, M. Le, & Lévy, N. (2003). Lamin A truncation in Hutchinson-Gilford progeria. *Science*, 300, 2055.
- Savji, N., Rockman, C. B., Skolnick, A. H., Guo, Y., Adelman, M. A., Riles, T., & Berger, J. S. (2013). Association between advanced age and vascular disease in different arterial territories: a population database of over 3.6 million subjects. *Journal of the American College of Cardiology*, 61(16), 1736–1743.
- Schmitt, M. M. N., Fraemohs, L., Hackeng, T. M., Weber, C., & Koenen, R. R. (2014). Atherogenic mononuclear cell recruitment is facilitated by oxidized lipoprotein-induced endothelial junctional adhesion molecule-A redistribution. *Atherosclerosis*, 234(2), 254–264.
- Seimon, T., & Tabas, I. (2009). Mechanisms and consequences of macrophage apoptosis in atherosclerosis. *Journal of Lipid Research*, 50(SUPPL.), 382–387.
- Shapouri-Moghaddam, A., Mohammadian, S., Vazini, H., Taghadosi, M., Esmaeili, S. A., Mardani, F., Seifi, B., Mohammadi, A., Afshari, J. T., & Sahebkar, A. (2018). Macrophage plasticity, polarization, and function in health and disease. *Journal of Cellular Physiology*, 233(9), 6425–6440.
- Shin, J., Spinler, K. R., Swift, J., Chasis, J. a, Mohandas, N., & Discher, D. E. (2013). Lamins regulate cell trafficking and lineage maturation of adult human hematopoietic cells. *Proceedings of the National Academy of Sciences of the United States of America*, 110(47), 18892–18897.
- Siasos, G., Tousoulis, D., Oikonomou, E., Zaromitidou, M., Stefanadis, C., & G. Papavassiliou, A. (2012). Inflammatory Markers in Hyperlipidemia: From Experimental Models to Clinical Practice. *Current Pharmaceutical Design*, 17(37), 4132–4146.

- Skálén, K., Gustafsson, M., Knutsen Rydberg, E., Hultén, L. M., Wiklund, O., Innerarity, T. L., & Boren, J. (2002). Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. *Nature*, 417(6890), 750–754.
- Soehnlein, O. (2012). Multiple roles for neutrophils in atherosclerosis. *Circulation Research*, 110(6), 875–888.
- Song, Y., Yao, Q., Zhu, J., Luo, B., & Liang, S. (1999). Age-related variation in the interstitial tissues of the cardiac conduction system; and autopsy study of 230 Han Chinese. *Forensic Science International*, 104(2–3), 133–142.
- Srikakulapu, P., & McNamara, C. A. (2017). B cells and atherosclerosis. *American Journal of Physiology - Heart and Circulatory Physiology*, 312(5), 1060–1067.
- Steenman, M., & Lande, G. (2017). Cardiac aging and heart disease in humans. *Biophysical Reviews*, 9(2), 131–137.
- Stefanadis, C., Antoniou, C. K., Tsiachris, D., & Pietri, P. (2017). Coronary atherosclerotic vulnerable plaque: Current perspectives. *Journal of the American Heart Association*, 6(3), 1–18.
- Strait, J. B., & Lakatta, E. G. (2012). Aging-associated cardiovascular changes and their relationship to heart failure. *Heart Failure Clinics*, 8(1), 143–164.
- Stuurman, N., Heins, S., & Aeby, U. (1998). Nuclear lamins: their structure, assembly, and interactions. *Journal of Structural Biology*, 122(1–2), 42–66.
- Sullivan, T. (1999). Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. *The Journal of Cell Biology*, 147(5), 913–920.
- Swift, J., Ivanovska, I. L., Buxboim, A., Harada, T., Dingal, P. C. D. P., Pinter, J., Pajerowski, J. D., Spinler, K. R., Shin, J.-W., Tewari, M., Rehfeldt, F., Speicher, D. W., & Discher, D. E. (2013). Nuclear Lamin-A scales with tissue stiffness and enhances matrix-directed differentiation. *Science*, 341, 975–990.
- Swirski, F. K., Libby, P., Aikawa, E., Alcaide, P., Luscinskas, F. W., Weissleder, R., & Pittet, M. J. (2007). Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytes and give rise to macrophages in atheromata. *The Journal of Clinical Investigation*, 117(1), 195–205.
- Tabas, I. (2010). Macrophage death and defective inflammation resolution in atherosclerosis. *Nature Reviews Immunology*, 10(1), 36–46.

- Tacke, F., Alvarez, D., Kaplan, T. J., Jakubzick, C., Spanbroek, R., Llodra, J., Garin, A., Liu, J., Mack, M., Rooijen, N. Van, Lira, S. A., Habenicht, A. J., & Randolph, G. J. (2007). Monocyte subsets differentially employ CCR2, CCR5, and CXCR1 to accumulate within atherosclerotic plaques. *The Journal of Clinical Investigation*, 117(1), 185–194.
- Teekakirikul, P., Seidman, C. E., Jonathan, G., Teekakirikul, P., Eminaga, S., Toka, O., Alcalai, R., & Wang, L. (2010). Cardiac fibrosis in mice with hypertrophic cardiomyopathy is mediated by non-myocyte proliferation and requires Tgf- β . *Journal of Clinical Investigation*, 120(10), 3520–3529.
- Ten Tusscher, K. H. W. J., & Panfilov, A. V. (2007). Influence of diffuse fibrosis on wave propagation in human ventricular tissue. *Europace : European Pacing, Arrhythmias, and Cardiac Electrophysiology : Journal of the Working Groups on Cardiac Pacing, Arrhythmias, and Cardiac Cellular Electrophysiology of the European Society of Cardiology*, 9 Suppl 6, 38–45.
- Thiam, H., Vargas, P., Carpi, N., Crespo, C. L., Raab, M., Terriac, E., King, M. C., Jacobelli, J., Alberts, A. S., Stradal, T., & Piel, M. (2016). Perinuclear Arp2/3-driven actin polymerization enables nuclear deformation to facilitate cell migration through complex environments. *Nature Communications*, 7, 1–14.
- Toribio-Fernández, R., Zorita, V., Rocha-Perugini, V., Iborra, S., Martínez Del Hoyo, G., Chevre, R., Dorado, B., Sancho, D., Sanchez-Madrid, F., Andrés, V., & Gonzalez-Granado, J. M. (2018). Lamin A/C augments Th1 differentiation and response against vaccinia virus and Leishmania major article. *Cell Death and Disease*, 9(1), 1–15.
- Travers, J. G., Kamal, F. A., Robbins, J., Yutzey, K. E., & Blaxall, B. C. (2016). The Fibroblast Awakens. *Circulation Research*, 118, 1021–1040.
- Tsiantoulas, D., Diehl, C. J., Witztum, J. L., & Binder, C. J. (2014). B cells and humoral immunity in atherosclerosis. *Circulation Research*, 114(11), 1743–1756.
- Ullrich, N. J., & Gordon, L. B. (2015). Hutchinson-Gilford progeria syndrome. *Handbook of Clinical Neurology*, 132, 249–264.
- Van Berlo, J. H., Voncken, J. W., Kubben, N., Broers, J. L. V., Duisters, R., van Leeuwen, R. E. W., Crijns, H. J. G. M., Ramaekers, F. C. S., Hutchison, C. J., & Pinto, Y. M. (2005). A-type lamins are essential for TGF- β 1 induced PP2A to dephosphorylate transcription factors. *Human Molecular Genetics*, 14(19), 2839–2849.

- Van Tintelen, J. P., Tio, R. A., Kerstjens-Frederikse, W. S., van Berlo, J. H., Boven, L. G., Suurmeijer, A. J. H., White, S. J., den Dunnen, J. T., te Meerman, G. J., Vos, Y. J., van der Hout, A. H., Osinga, J., van den Berg, M. P., van Veldhuisen, D. J., Buys, C. H. C. M., Hofstra, R. M. W., & Pinto, Y. M. (2007). Severe myocardial fibrosis caused by a deletion of the 5' end of the lamin A/C gene. *Journal of the American College of Cardiology*, 49(25), 2430–2439.
- Veseli, B. E., Perrotta, P., De Meyer, G. R. A., Roth, L., Van Der Donckt, C., Martinet, W., & De Meyer, G. R. Y. (2017). Animal models of atherosclerosis. *European Journal of Pharmacology*, 816, 3–13.
- Wang, J. C., & Bennett, M. (2012). Aging and atherosclerosis: mechanisms, functional consequences, and potential therapeutics for cellular senescence. *Circulation Research*, 111(2), 245–259.
- Willis, N. D., Cox, T. R., Rahman-Casañs, S. F., Smits, K., Przyborski, S. A., van den Brandt, P., van Engeland, M., Weijnenberg, M., Wilson, R. G., de Bruïne, A., & Hutchison, C. J. (2008). Lamin A/C Is a Risk Biomarker in Colorectal Cancer. *PLoS ONE*, 3(8), e2988.
- Wolf, K., te Lindert, M., Krause, M., Alexander, S., te Riet, J., Willis, A. L., Hoffman, R. M., Figdor, C. G., Weiss, S. J., & Friedl, P. (2013). Physical limits of cell migration: control by ECM space and nuclear deformation and tuning by proteolysis and traction force. *Journal of Cell Biology*, 201(7), 1069–1084.
- Woollard, K. J., & Geissmann, F. (2010). Monocytes in atherosclerosis: subsets and functions. *Molecular and Cellular Biology*, 7(2), 77–86.
- Xin, M., Olson, E. N., & Bassel-Duby, R. (2013). Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. *Nature Reviews Molecular Cell Biology*, 14(8), 529–541.
- Zhang, H., Kieckhafer, J. E., & Cao, K. (2013). Mouse models of laminopathies. *Aging Cell*, 12(1), 2–10.
- Zhou, P., & Pu, W. T. (2016). Recounting cardiac cellular composition. *Circulation Research*, 118(3), 368–370.
- Zotes, T. M., Arias, C. F., Fuster, J. J., Spada, R., Pérez-Yagüe, S., Hirsch, E., Wymann, M., Carrera, A. C., Andrés, V., & Barber, D. F. (2013). PI3K p110 γ deletion attenuates murine atherosclerosis by reducing macrophage proliferation but not polarization or apoptosis in lesions. *PLoS ONE*, 8(8), 1–10.

ANEXO

Durante la realización de esta tesis doctoral se ha contribuido a la publicación de los siguientes artículos:

Del Monte A.*, Arroyo A.B.* , Andrés-Manzano M.J., García-Barberá N., Caleprico M.S., Vicente V., Roldán V., González-Conejero R., Martínez C., Andrés V. (2018). miR-146a deficiency in hematopoietic cells is not involved in the development of atherosclerosis. *PLoS One*. 13, 1-13. *, autores con contribución equivalente.

González-Ramos S., Paz-García M., Rius C. *, **Del Monte-Monge A. ***, Rodríguez C., Fernández-García V., Andrés V., Martínez-González J., Lasunción M.A., Martín-Sanz P., Soehnlein O., Boscá L. (2019). Endothelial NOD1 directs myeloid cell recruitment in atherosclerosis through VCAM-1. *FASEB J*. 33, 3912-3921. *, autores con contribución equivalente.

Vila-Caballer M., González-Granado J.M., Zorita V., Abu Nabah Y.N., Silvestre-Roig C., **Del Monte-Monge A.**, Molina-Sánchez P., Ait-Oufella H., Andrés-Manzano M.J., Sanz M.J., Weber C., Kremer L., Gutiérrez J., Mallat Z., Andrés V. (2019). Disruption of the CCL1-CCR8 axis inhibits vascular Treg recruitment and function and promotes atherosclerosis in mice. *Journal of Molecular and Cellular Cardiology*. 132, 154-163.

Ho Y.H., Del Toro R., Rivera-Torres J., Rak J., Korn C., García-García A., Macías D., González-Gómez C., **Del Monte A.**, Wittner M., Waller A.K., Foster H.R., López-Otín. C., Johnson R.S, Nerlov C., Ghevaert C., Vainchenker W., Louache F., Andrés V., Méndez-Ferrer S. (2019). Remodeling of Bone Marrow Hematopoietic Stem Cell Niches Promotes Myeloid Cell Expansion during Premature or Physiological Aging. *Cell Stem Cell*. 25, 407-418.

RESEARCH ARTICLE

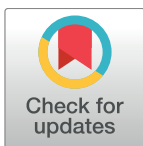
miR-146a deficiency in hematopoietic cells is not involved in the development of atherosclerosis

Alberto del Monte^{1,2}, Ana B. Arroyo³, María J. Andrés-Manzano^{1,2}, Nuria García-Barberá³, María S. Caleprico¹, Vicente Vicente³, Vanessa Roldán³, Rocío González-Conejero^{3*}, Constantino Martínez^{3*}, Vicente Andrés^{1,2*}

1 Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain, **2** CIBER de Enfermedades Cardiovasculares (CIBER-CV), Murcia, Spain, **3** Servicio de Hematología y Oncología Médica, Hospital Universitario Morales Meseguer, Centro Regional de Hemodonación, Universidad de Murcia, IMIB-Arrixaca, Murcia, Spain

☞ These authors contributed equally to this work.

* rocio.gonzalez@carm.es (RG-C); constant@um.es (CM); vandres@cnic.es (VA)



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Abstract

Background

Atherosclerosis involves activation of the IRAK1/TRAF6/NF-κB inflammatory cascade, which is negatively regulated by miR-146a. Previous studies showed that the TT genotype of rs2431697, located near the *miR-146a* gene, drives lower miR-146a transcription and predicts adverse cardiovascular events in anticoagulated atrial fibrillation patients. Moreover, systemic miR-146a administration protects mice from atherosclerosis. Here we evaluated the ability of miR-146a expression in the hematopoietic component to regulate atherosclerosis in low-density lipoprotein receptor-null mice (*Ldlr*^{-/-}).

Methods and results

Lethally-irradiated *Ldlr*^{-/-} mice transplanted with bone marrow from wild-type or *miR-146a*-null mice were fed an atherogenic diet for 8 and 20 weeks. *Irak1*, *Traf6* and *MIR146A* expression were quantified in thoracic aorta by qRT-PCR and Western blot. Aortic plaque size and composition were characterized by Oil-Red staining and immunohistochemistry and leukocyte recruitment by intravital microscopy. Blood cell counts were similar in fat-fed *Ldlr*^{-/-} mice with or without hematopoietic miR-146a expression. However, plasma cholesterol decreased in fat-fed *Ldlr*^{-/-} mice transplanted with bone marrow deficient for miR-146a. Finally, aortic atherosclerosis burden and recruitment of leukocytes into the vessel wall were undistinguishable between the two groups, despite higher levels of *Irak1* and *Traf6* mRNA and protein in the aorta of fat-fed mice lacking hematopoietic miR-146a expression.

Conclusions

miR-146a deficiency exclusively in hematopoietic cells modulates cholesterol levels in plasma and the expression of its targets in the artery wall of fat-fed *Ldlr*^{-/-} mice, but does not

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accelerate atherosclerosis. Atheroprotection upon systemic miR-146a administration may therefore be caused by specific effects on vascular cells.

Introduction

Atherosclerosis is a complex inflammatory process involving several factors and cell types that interact in response to different forms of injury [1]. Together with endothelium and smooth muscle cells, hematopoietic cells play a central role in atherogenesis [2]. Indeed, a key early event in the inflammatory response during atherosclerosis is the adhesion of neutrophils to the vascular endothelium and their recruitment into the injured artery wall. The secretion of neutrophil-derived myeloperoxidase seems to be an important event in vascular injury [3]. More recent research has implicated the production of neutrophil extracellular traps (NETs) in atherogenesis [4], although further research is required to fully define the underlying mechanisms [5]. Other important hemostatic factors in atherosclerosis are monocytes/macrophages, important inflammatory and invasive cells that regulate plaque formation and necrosis [2,6]. As they infiltrate the artery wall, monocytes polarize into different macrophage subsets that play distinct roles in atherosclerosis [7].

MicroRNAs (miRNAs) are small, non-coding RNAs that posttranscriptionally regulate gene expression by promoting mRNA degradation and/or inhibiting mRNA translation [8,9]. Since their discovery, miRNAs have been implicated as key modulators of numerous physiological and pathological processes. Their role in cardiovascular disease has been extensively studied in recent years [10,11]. In particular, miR-146a has an anti-inflammatory function [12] and its expression is regulated by the single-nucleotide polymorphism (SNP) rs2431697; homozygote TT individuals have lower levels of miR-146a than GG individuals [13]. We recently demonstrated that the levels of miR-146a in monocytes may play an important role in the development of cardiovascular adverse events in patients with atrial fibrillation (AF), with rs2431697-TT patients displaying higher risk of developing adverse cardiovascular events [14]. In turn, monocytes from rs2431697-TT individuals have an increased pro-inflammatory response when subjected to inflammatory stress.

A role for miR-146a in atherosclerosis is suggested by its ability to negatively regulate several pro-inflammatory factors that promote disease progression, including Toll-like receptor 4 (TLR4), IL-1 receptor-associated kinase 1 (IRAK1), and TNF receptor-associated protein factor 6 (TRAF6) [15,16]. Moreover, low miR-146a levels in neutrophils are associated with carotid intima-media thickening in patients with systemic lupus erythematosus [17]. Recent mouse studies reveal that apolipoprotein E (ApoE) enhances miR-146a expression in monocytes and macrophages, suppressing NF- κ B-mediated inflammation and atherosclerosis, and that systemic delivery of miR-146a mimetic attenuates monocyte/macrophage activation and atherosclerosis in the absence of plasma lipid reduction [18]. Given these recent studies and the relevance of leukocytes in all stages of atherosclerosis, we investigated whether miR-146a deficiency restricted to the hematopoietic compartment can aggravate the development of high-fat diet (HFD)-induced atherosclerosis in mice deficient for the low-density lipoprotein receptor (*Ldlr*^{-/-}) at early (8 weeks) and late stages (20 weeks) of disease progression.

Materials and methods

Mice and bone marrow transplantation

Animal welfare, and all experimental and other scientific procedures with animals conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC, enforced in Spanish law under Real Decreto 53/2013. In addition, the animal research included in this work was granted a formal ethics approval by the Committees in charge of animal welfare at Centro Nacional de Investigaciones Cardiovasculares Carlos III (OEBA-CNIC) and Universidad de Murcia (CEEA-UM), the Ethics Committee for Research from Universidad Autónoma de Madrid (OH-UAM), the committee from the Animal Protection Area of the Comunidad Autónoma de Madrid (CAM), and the Animal Health Service of the Department of Agriculture and Water from Región de Murcia (DAW-RM). The ethics approval numbers from the legally competent authorities are PROEX 134/14 (CAM) and A13150602 (DAW-RM). The animals were housed in cages in ventilated racks. Cages contained a bed of wood chips and nesting material, and objects for environmental enrichment. All the animals had ad libitum access to food and water. The maximum number of animals per cage was limited to 5. Mice were maintained under controlled environmental conditions (relative humidity: 45–65%; temperature: 20–24 °C) with light/darkness cycles of 12h/12h.

miR-146a^{-/-} mice (The Jackson laboratory, Bar Harbor, ME) were backcrossed for more than 8 generations in a C57BL/6J CD45.2 background. Irradiation and bone marrow (BM) transplantation was as described [19]. Briefly, *Ldlr*^{-/-} CD45.1 mice (8 to 10-week-old, Charles River) were irradiated with 2 doses of 6.5 Gy (10 minutes each, temperature: 37 °C) using a JL Shepherd & Associates 1-68A irradiator with a source of 1000 curies of Cs-137. Next day, the animals were injected in the tail vein with 100μl of BM cells (7 x 10⁶, in saline) obtained from a pool of 4 femurs and 4 tibias of *miR-146a*^{-/-} or wild-type (wt) mice (both C57BL/6J CD45.2; 8-week-old). After 4 weeks on a standard diet, transplanted mice were fed HFD for 8 or 20 weeks (10.7% total fat, 0.75% cholesterol, S9167-E010, SSNIFF, Germany). Transplant efficiency was assessed as the percentage of donor CD45.2-immunoreactive cells in the blood of BM recipient mice, assessed by CD45.2 and CD45.1 immunostaining and flow cytometry.

Animal were all euthanized at the end of every experiment by inhalation of carbon dioxide gas.

Quantification of atherosclerosis burden and immunohistopathology

All quantitative analyses were performed by an investigator blinded to genotype. Atherosclerosis burden was quantified as described [20]. Briefly, mice were euthanized in a CO₂ chamber and the heart and aorta were extracted after *in situ* perfusion with PBS. Tissues were fixed with 4% paraformaldehyde/PBS overnight at 4°C. Atherosclerosis burden was quantified by computer-assisted morphometric analysis (SigmaScan pro 5, Systat Software Inc., San Jose, CA) of the aortic arch stained with Oil Red O (O0625, Sigma, 0.2% Oil Red O in 80% MeOH) and of hematoxylin/eosin-stained cross-sections from the aortic root; for each mouse, results were the mean of 3 cross-sections. The area of the necrotic core in atheroma plaques was quantified by analyzing hematoxylin/eosin-stained aortic cross-sections (mean of 3 cross-sections per mouse).

Blood cell counting and biochemical parameters

Blood was extracted from the facial vein and was analyzed to quantify and identify circulating blood cell populations using the PENTRA 80 hematology platform (HORIBA Medical, Madrid, Spain). Plasma was isolated by centrifugation of whole blood (2000g, 15' at room

temperature), and lipid profile was obtained using DIMENSION RxL MAX (Siemens, Munich, Germany).

Intravital microscopy

Male *Ldlr*^{-/-} mice transplanted with BM wt or BM *miR-146a*^{-/-} fed HFD for 20 weeks after transplantation were used for cremaster muscle intravital microscopy as previously described [21]. Briefly, once exteriorised, the cremaster muscle was placed onto an optical clear viewing pedestal, cut longitudinally with a high temperature surgical cautery (Lifeline Medical) and held extended at the corners of the exposed tissue using surgical suture. Ten min before image acquisition, antibodies (0.5 µg) antiCD4 (Tonbo), anti-Ly6G (Biolegend) and anti-Ly6C (eBioscience) were injected to label myeloid cell subsets. To maintain the correct temperature and physiological conditions, exposed tissues were perfused continuously with 37°C pre-warmed Tyrode's buffer solution (139 mM NaCl, 3 mM KCl, 17 mM NaHCO₃, 12 mM Glucose, 3 mM CaCl₂ and 1 mM MgCl₂). The cremasteric microcirculation was visualized using an AXIO Examiner Z.1 work station (Zeiss) mounted on a 3-dimensional motorized stage (Sutter Instrument) and equipped with a CoolSnap HQ2 camera (Photometrics). An APO 20x NA 1.0 water-immersion objective was used. Slidebook software 5.0 (Intelligent Imaging Innovations) was employed for acquisition and image processing. Three randomly-selected arterioles and venules were analysed per mouse, and T cells/monocytes/neutrophils were considered adherent when no rolling was observed using a reference line perpendicular to blood flow for at least 30 seconds.

Quantitative real-time-PCR (qRT-PCR)

Total RNA was isolated from thoracic aortas using RNeasy reagent (Molecular Research Center Inc) and was retrotranscribed (100 ng) using the SuperScript[™] III First-Strand Synthesis System (ThermoFisher). MiR-146a cDNA was synthesized using individual miRNA-specific RT primers and the TaqMan[®] MicroRNA Reverse Transcription Kit (ThermoFisher). cDNA was amplified using TaqMan[®] probes (*Irak1*: Mm01193538_m1, *Traf6*: Mm00493836_m1) together with TaqMan[®] Universal PCR Master Mix, No AmpErase[®] UNG (ThermoFisher). The 2^{-ΔCt} method was used to calculate the relative abundance of mRNA compared with endogenous actin (*Actb*: Mm02619580_g1) mRNA and of miRNA compared with U6 for miR-146a (Ct = Threshold Cycle; ΔCt = Ct sample gene-Ct endogenous control).

Western blotting

Protein lysates were obtained from thoracic aortas of BM *miR-146a*^{-/-} and BM wt transplanted mice fed with a HFD for 20 weeks. Tissue homogenization was performed using TissueLyser (Qiagen). Normalization was based on the amount of protein loaded. Total protein (20 µg) was separated by SDS-PAGE (8%) in reducing conditions. Gels were transferred onto PVDF membranes (Amersham Hybond P 0.45, GE Healthcare, Barcelona Spain) and blocked for 1 hour in 5% w/v BSA-TBS. Membranes were incubated overnight at 4°C with primary antibodies against human Traf6 (#sc-8409, 1:1,000, Santa Cruz, Heidelberg, Germany), Irak1 (D51G7, 1:1,000, Cell Signalling Technology, Leiden, The Netherlands), and β-actin (AC-15 clone, 1:5,000, Sigma-Aldrich, Madrid, Spain). After washing, membranes were incubated with secondary antibodies labeled with peroxidase (1:10,000, GE Healthcare, Barcelona, Spain). ECL Prime Detection Kit and ImageQuant LAS 4000 Imager (GE Healthcare, Barcelona, Spain) were used for protein detection. Densitometric analyses were performed with ImageJ software (<http://rsb.info.nih.gov/ij/>).

Statistical analysis

Data are presented as mean $\pm$ SEM. In experiments with 2 groups, statistical significance was evaluated using a 2-tailed, unpaired Student's *t*-test. For experiments comparing more than one group, a 1 or 2-way ANOVA with the Bonferroni post hoc tests were performed (Graph-Pad Prism software, La Jolla, CA). Differences were considered statistically significant at $P < 0.05$.

Results

Genetic disruption of miR-146a in hematopoietic cells does not increase diet-induced atherosclerosis in *Ldlr*^{-/-} mice

To assess whether miR-146a deficiency restricted to the hematopoietic compartment affects atherosclerosis, *Ldlr*^{-/-} mice (CD45.1 background) were transplanted with BM from *miR-146a*^{-/-} (BM *miR-146a*^{-/-}) or wt (BM wt) mice (both CD45.2 background) (Fig 1A). After one month, *Ldlr*^{-/-} mice were examined to evaluate transplant efficiency by quantifying CD45.1 and CD45.2 expression in circulating blood cells. These studies revealed similar transplant efficiency in both experimental groups (Fig 1B). Transplanted mice were then fed a HFD. Analysis of BM *miR-146a*^{-/-} and BM wt mice fed normal chow (pre-diet) and HFD (post-diet) revealed no statistically significant between-group differences in body weight (Fig 1C). MiR-146a levels in blood leukocytes were significantly decreased in BM *miR-146a*^{-/-} transplanted mice before and after HFD (Fig 1D). Moreover, in agreement with previously published results [22], we found enlarged spleens and pale BM in *miR-146a*^{-/-} transplanted mice (Fig 1E).

Circulating blood cell counts and populations were similar in both groups of mice, except for platelets that were significantly reduced in BM *miR-146a*^{-/-} after 20 weeks (Fig 2A). Plasma levels of low-density lipoprotein cholesterol (LDL), total cholesterol, and triglycerides increased significantly in both groups of mice fed HFD for 8 weeks compared with their pre-diet values, but no statistically-significant between-group differences were observed (Fig 2B). However, after 20 weeks of HFD, plasma lipid levels significantly decreased in BM *miR-146a*^{-/-} mice compared with BM wt mice (Fig 2B). Thus, genetic disruption of miR-146a in hematopoietic cells did not affect body weight, nor blood cell counts and populations, but reduced plasma lipids after 20 weeks of HFD.

We next quantified atherosclerosis burden in fat-fed mice by planimetric analysis of whole-mount Oil Red-O-stained aortic arch (Fig 3A) and hematoxylin/eosin-stained cross-sections through the aortic root (Fig 3B) after 8 and 20 weeks of HFD. As expected, these studies revealed increased atherosclerosis burden in mice of both groups at 20 weeks of HFD compared with 8 weeks of HFD; however, atherosclerosis development at both times of HFD was similar in BM *miR-146a*^{-/-} and BM wt mice in all regions (Fig 3). Similarly, lack of miR-146a in hematopoietic cells did not affect the formation of necrotic cores in the atherosclerotic plaques of mice fed HFD for 8 and 20 weeks (Fig 3C).

Lack of miR-146a in hematopoietic cells does not augment adhesion of leukocytes in fat fed *Ldlr*^{-/-} mice

We next evaluated if miR-146a deficiency in the BM compartment may affect the recruitment and adhesion of leukocytes to the inflamed vessel wall of *Ldlr*^{-/-} mice fed HFD for 20 weeks. For this purpose, we analyzed by intravital microscopy the *in vivo* adhesion of labeled leukocytes (Ly6C⁺ monocytes, Ly6G⁺ neutrophils, and CD4⁺ T cells) to the endothelium of cremaster muscle venules. These studies revealed no differences in the adhesion of the different subtypes of leukocytes between BM *miR-146a*^{-/-} and BM wt fat-fed *Ldlr*^{-/-} mice (Fig 4).

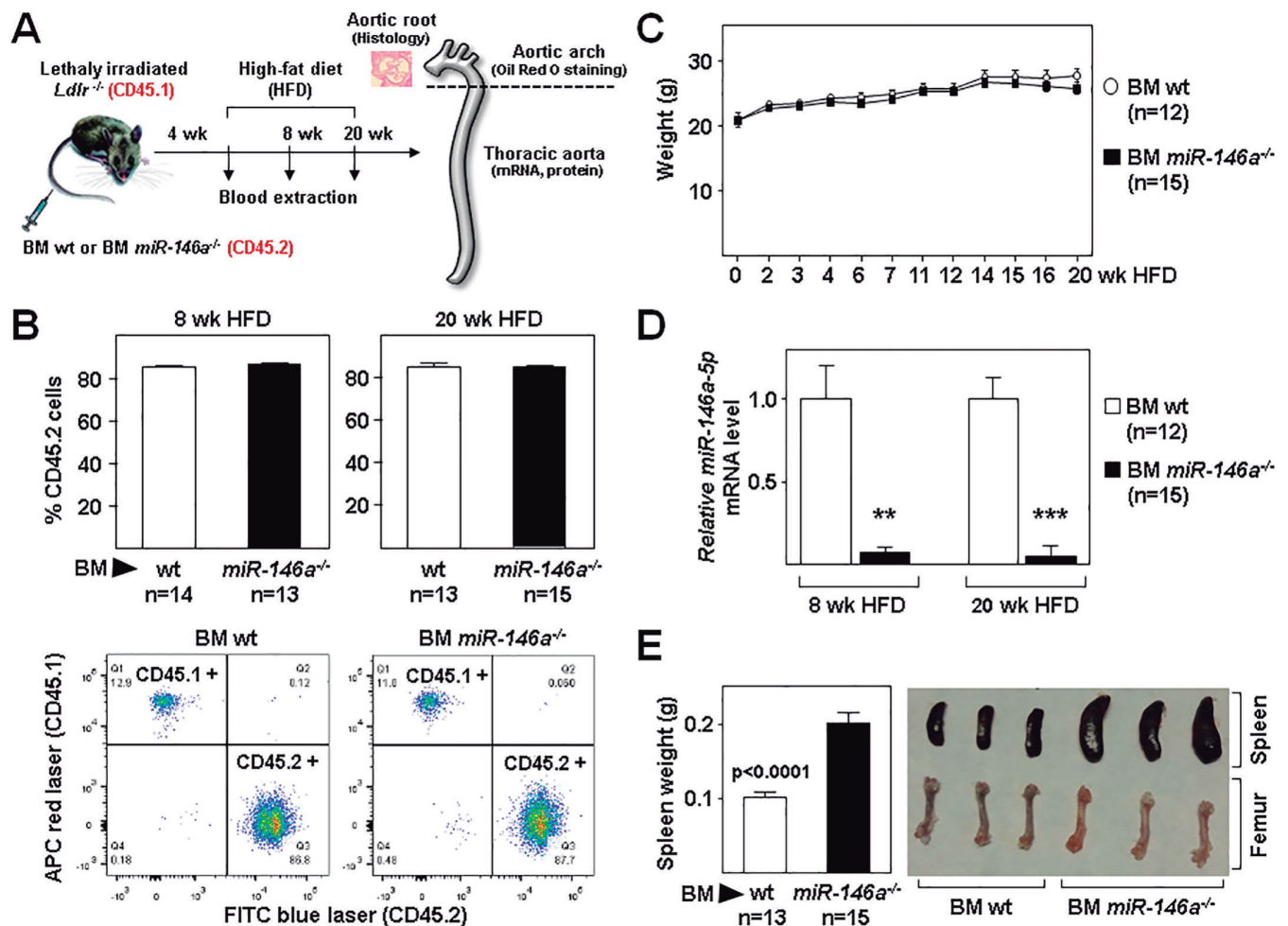


Fig 1. Study design and efficiency of bone marrow transplantation. (A) Lethally-irradiated *Ldlr*^{-/-} mice (CD45.1, 8 to 10-week-old) were transplanted with cells obtained from bone marrow (BM) of wt or *miR-146a*^{-/-} mice (both CD45.2). After 4 weeks of recovery, mice were challenged with a high-fat diet for 8 and 20 weeks. Blood and aorta were collected for biochemical and expression studies and to quantify atherosclerosis burden. (B) Quantification of transplant efficiency in both groups of mice as determined by flow cytometry of blood extracted 1 month after BM transplant. Donor and host cells are CD45.2- and CD45.1-immunoreactive, respectively. Representative flow cytometry profiles are shown below the chart. (C) Body weight at different times after the onset of HFD. (D) Pre and post diet *miR-146a* levels in blood leukocytes were measured by qRT-PCR. The $2^{-\Delta\Delta Ct}$ method was used to calculate *miRNA* abundance relative to *U6* (Ct = Threshold Cycle; ΔCt = Ct sample gene – Ct endogenous control). **: $p < 0.01$, ***: $p < 0.001$ BM wt vs BM *miR-146a*^{-/-} at both time points. (E) Spleen from BM wt or BM *miR-146a*^{-/-} mice were weighed after 20 weeks of HFD. Shown are representative images of spleens and femurs.

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Expression of *miR-146a* targets in fat-fed mice

Thoracic aortas were homogenized and RNA extracted. As expected, the level of *miR-146a* was lower in tissue from fat-fed BM *miR-146a*^{-/-} mice compared with BM wt mice at both times of HFD (Fig 5A). Accordingly, mRNA levels of the *miR-146a* targets *Traf6* and *Irak1* were significantly higher in thoracic aortas of BM *miR-146a*^{-/-} mice after 8 weeks of HFD (Fig 5B). Similarly, *Traf6* and *Irak1* protein levels were significantly higher in thoracic aortas of BM *miR-146a*^{-/-} mice after 20 weeks of HFD (Fig 5C).

Discussion

There is increasing evidence connecting *miRNAs* with the molecular pathophysiological processes leading to atherosclerosis (reviewed in [11,23]). Inflammation plays a pivotal role in all

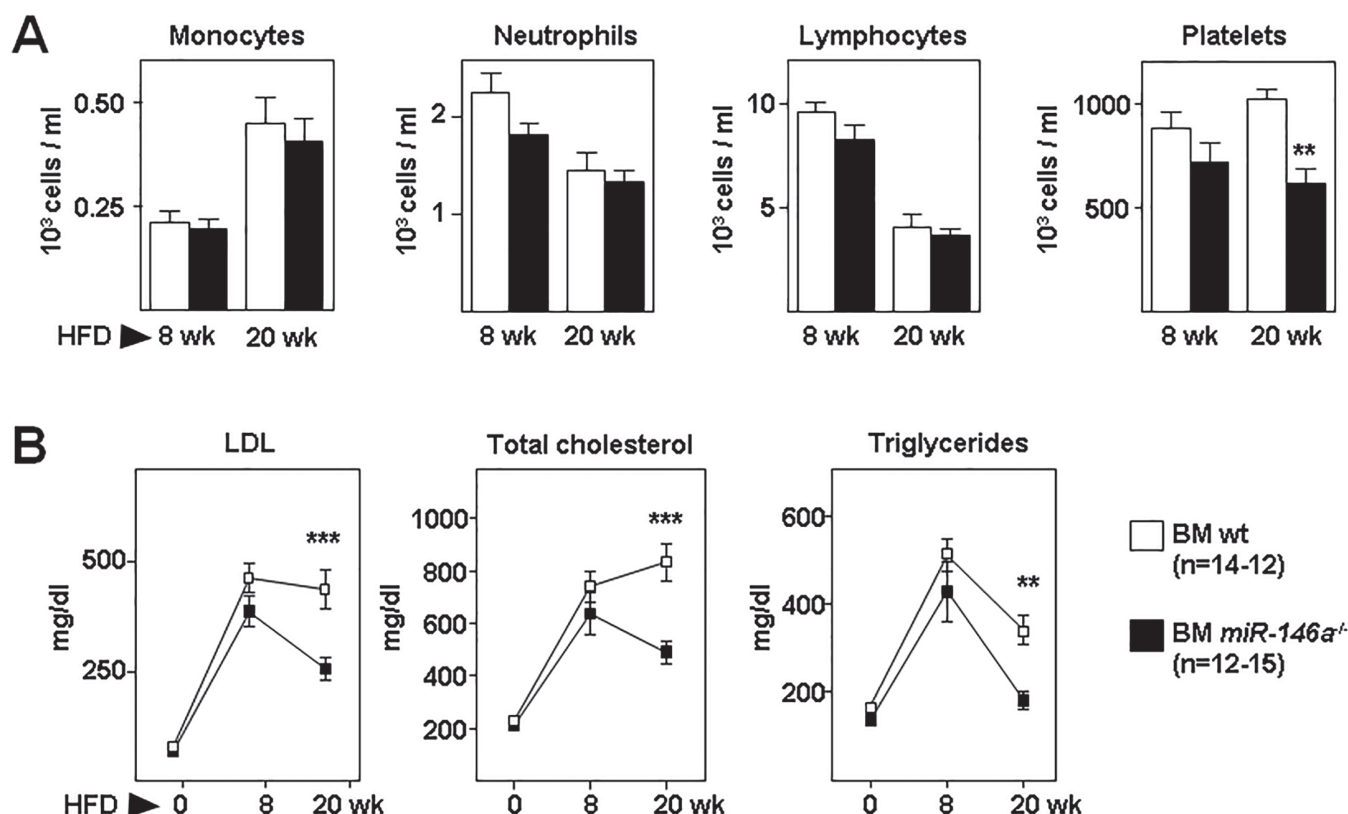


Fig 2. Hematology and plasma lipids in transplanted *Ldlr*^{-/-} mice. Lethally-irradiated *Ldlr*^{-/-} mice (CD45.1, 8 to 10-week-old) were transplanted with cells obtained from bone marrow (BM) of wt or *miR-146a*^{-/-} mice (both CD45.2). After 4 weeks of recovery, mice were challenged with a high-fat diet (HFD) for 8 and 20 weeks. (A) Blood cell populations from BM wt or BM *miR-146a*^{-/-} mice after HFD. (B) Plasma levels of LDL, total cholesterol, and triglycerides at 0, 8 and 20 weeks of HFD. **: $p < 0.01$, ***: $p < 0.001$ BM wt vs BM *miR-146a*^{-/-} at 20 weeks of HFD.

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stages of atherogenesis, from its initiation until plaque rupture [1,6]. MiR-146a was one of the first miRNAs implicated in inflammatory processes [12]. Through the regulation of different targets of the TLR/NF- κ B pathway, miR-146a acts as a negative feedback regulator of the innate immune response [24]. However, the implication of this miRNA in atherosclerosis has been addressed only recently, and several questions remain. Defining the cells in which miR-146a exerts its action will improve understanding of the role of this miRNA in atherogenesis. Recently, Li *et al.* reported that the anti-atherogenic action of ApoE, which is highly expressed in monocytes and macrophages, is mediated through increased expression of the transcription factor PU.1, resulting in enhanced miR-146a expression in monocytes/macrophages and suppression of NF- κ B-driven inflammation [18]. These authors also found that systemic delivery of miR-146a mimetic inhibits monocyte/macrophage activation and atherosclerosis development [18]. We observed that lack of miR-146a in the hematopoietic compartment does not affect atherosclerotic plaque formation in *Ldlr*^{-/-} mice at early and late stages of disease progression (8 and 20 weeks of HFD). This finding was unexpected because inhibition of the main miR-146a TLR/NF- κ B pathway targets in monocytes/macrophages seemed to be protective [18]. Moreover, inhibition of NF- κ B activation in macrophages increases atherosclerosis in *Ldlr*^{-/-} mice, although surprisingly the opposite occurs in endothelial cells [25]. This was further demonstrated in a model of NEMO/IKK γ deletion and upon endothelial cell-specific expression of a dominant-negative IkBa mutant [26]. Different roles of a TLR/NF- κ B

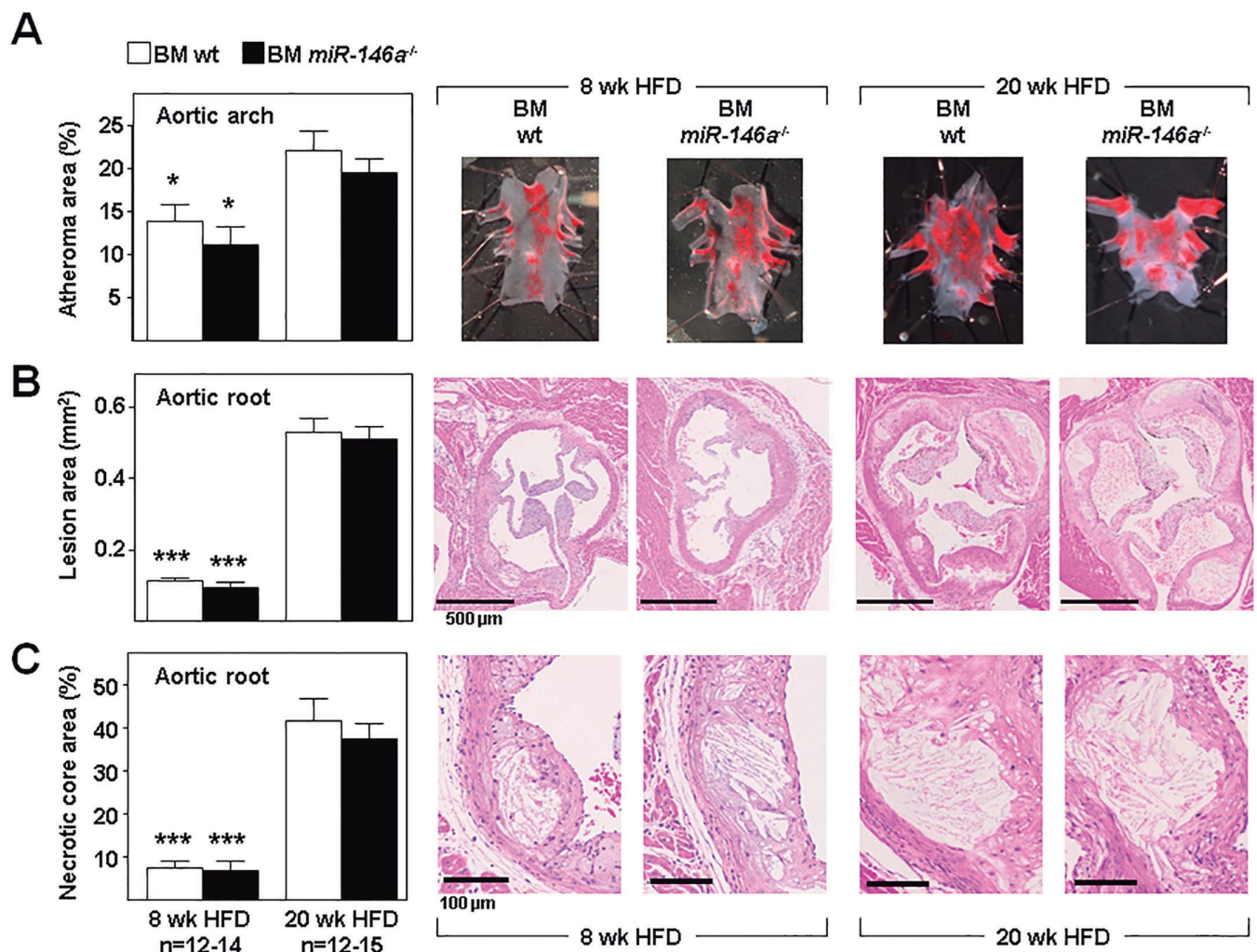


Fig 3. Ablation of miR-146a gene in hematopoietic cells does not affect diet-induced atherosclerosis in *Ldlr*^{-/-} mice. Lethally-irradiated *Ldlr*^{-/-} mice (CD45.1, 8 to 10-week-old) were transplanted with cells obtained from bone marrow (BM) of wt or *miR-146a*^{-/-} mice (both CD45.2). After 4 weeks of recovery, mice were challenged with a high-fat diet (HFD). Mice were euthanized after 8 or 20 weeks of HFD and tissues were harvested for immunohistopathological characterization of atherosclerotic lesions. Representative images are shown in each panel. (A) Percentage of aortic arch are occupied by atheroma visualized by *en face* Oil Red O staining. (B) Quantification of atheroma size in hematoxylin/eosin-stained sections of the aortic sinus. (C) Area of atherosclerotic lesions occupied by necrotic cores quantified in histological sections from the aortic sinus. *: $p < 0.05$, ***: $p < 0.001$ vs same BM genotype at 20 weeks of HFD.

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pathway component in endothelium vs. myeloid cells have also been described for TRAF6 by Polykratis *et al* [27]. These authors showed that endothelial-cell-expressed TRAF6 induces the expression of proinflammatory mediators that enhance the adhesion and recruitment of macrophages to the endothelium, whereas in myeloid cells the opposite occurs, with TRAF6 deficiency favoring the development of atherosclerosis by inhibiting the expression of IL-10 [27]. Our results demonstrate that decreasing the levels of miR-146a in the hematopoietic compartment does not increase atherosclerosis at both early (8 weeks HFD) and late (20 weeks HFD) stages of disease development. Of note, Cheng *et al.* recently showed no differences in atherosclerosis burden in the aortic arch of *Ldlr*^{-/-} mice transplanted with BM *miR-146a*^{-/-} and fed HFD for 4 weeks; however, they found a significant decrease in plaque burden in BM *miR-146a*^{-/-} *Ldlr*^{-/-} mice after 12 weeks of HFD [22]. Importantly, these authors observed unchanged

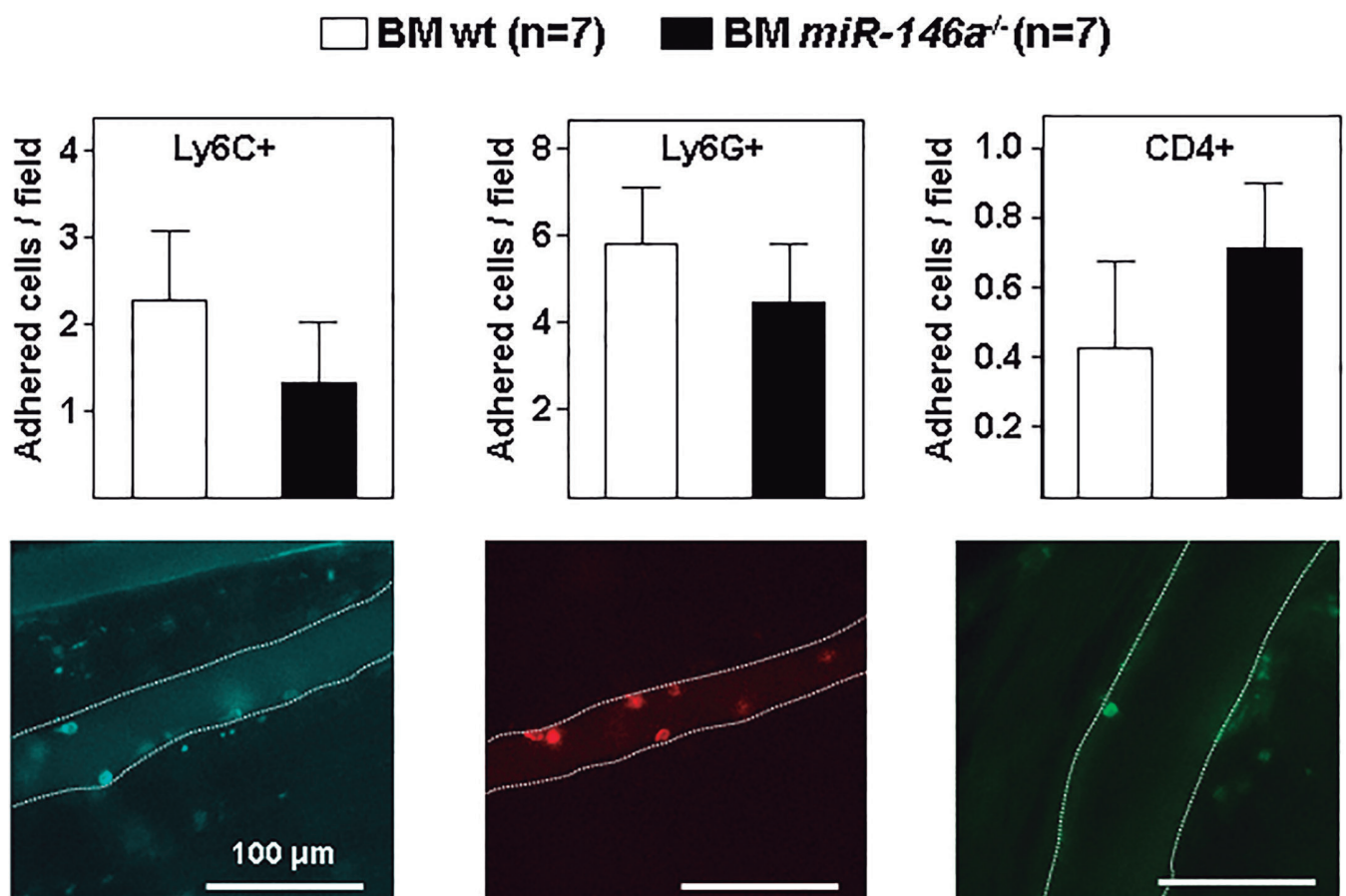


Fig 4. Deletion of miR-146a gene in hematopoietic cells does not increase adhesion in *Ldlr*^{-/-} mice fed HFD. Lethally-irradiated *Ldlr*^{-/-} mice (CD45.1, 8 to 10-week-old) were transplanted with cells obtained from bone marrow (BM) of wt or *miR-146a*^{-/-} mice (both CD45.2). After 4 weeks of recovery, mice were challenged with a high-fat diet (HFD) for 20 weeks and analyzed by intravital microscopy in the cremaster muscle. Images are representative for Ly6C⁺ monocytes, Ly6G⁺ neutrophils, and CD4⁺ T cells adhered to the wall of cremasteric venules. Dashed lines mark the boundaries of the venules.

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plaque burden in the descending thoracic aorta and aortic root at both time points with HFD. Among other reasons, Cheng *et al.* suggested that low levels of cholesterol after 12 weeks of HFD might explain this atheroprotective role in aortic arch provoked by a miR-146a deficiency in the hematopoietic compartment [22]. We also observed a significant decline in cholesterol after 20 weeks of HFD in BM *miR-146a*^{-/-}, but this was not sufficient to reduce plaque burden under our experimental conditions. Consistent with our results showing no differences in atherosclerosis development in fat-fed BM *miR-146a*^{-/-} *Ldlr*^{-/-} mice, our intravital microscopy studies revealed no differences in vascular leukocyte recruitment upon *miR-146a*^{-/-} deficiency in BM cells.

Paradoxically it has also been described that inhibiting miR-146a in endothelial cells may increase the expression of some of its targets, such as TRAF6, thus enhancing NF-κB activation and promoting atherogenesis [28]. Indeed, Cheng *et al.* also demonstrated that mice deficient for miR-146a except in the hematopoietic compartment have a significant increase in atherosclerotic plaque burden in comparison with mice lacking miR-146a in all cells [22]. In line with these results, endothelium-specific delivery of miR-146a-loaded E-selectin-targeting microparticles reduced atherosclerosis in *ApoE*^{-/-} mice [29]. In their study of the effect of systemically delivered miR-146a mimetics, Li *et al.* examined expression of endogenous miR-

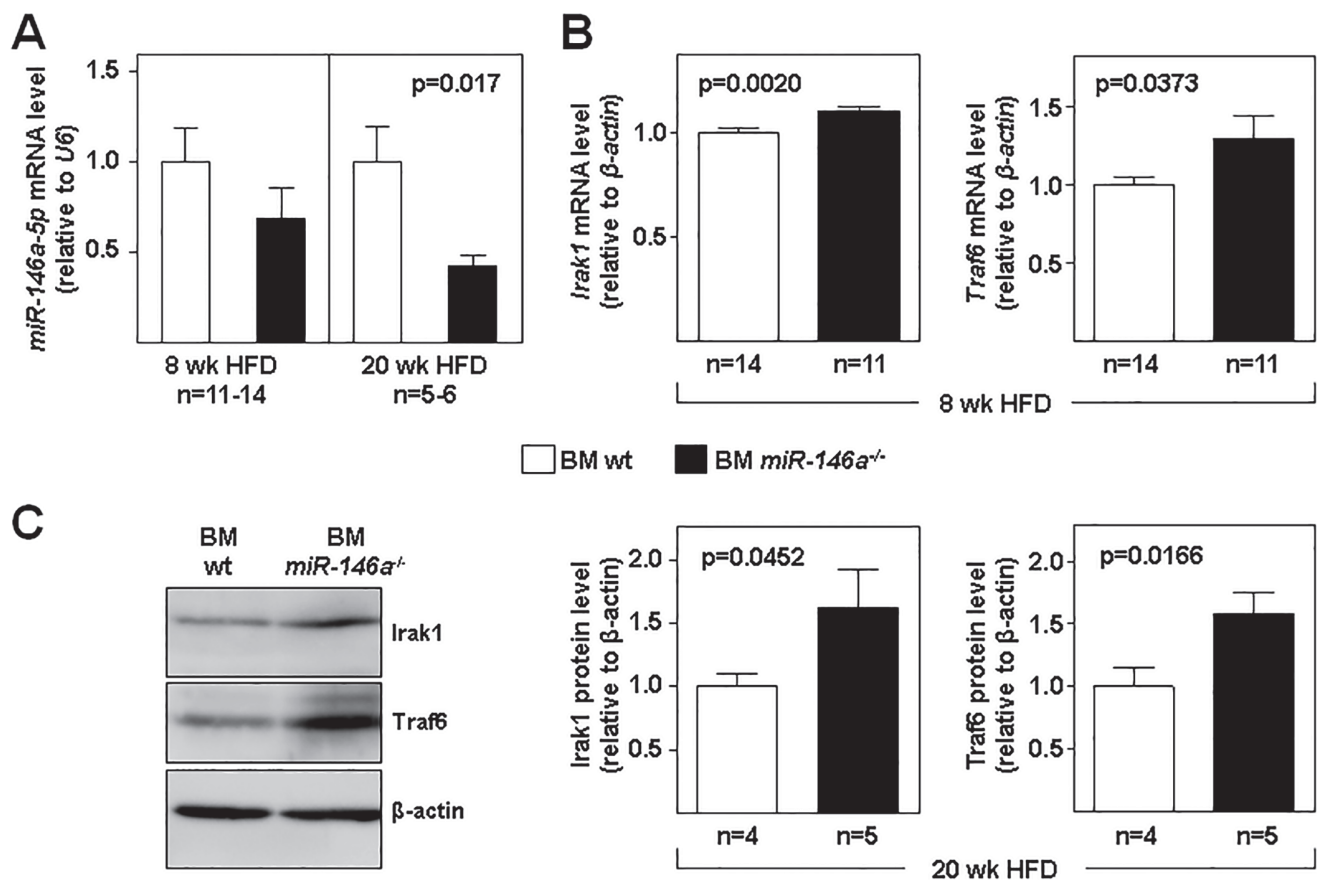


Fig 5. Expression of *miR-146a* targets in fat-fed mice. Lethally-irradiated *Ldlr*^{-/-} mice (CD45.1, 8 to 10-week-old) were transplanted with cells obtained from bone marrow (BM) of wt or *miR-146a*^{-/-} mice (both CD45.2). After 4 weeks of recovery, mice were challenged with a high-fat diet (HFD) for the indicated times. (A) *miR-146a* expression was measured by qRT-PCR in total RNA extracted from the thoracic aorta of mice fed high-fat diet (HFD) for 8 weeks (individual thoracic aortas) and 20 weeks (pools of 3 thoracic aortas per samples). (B) *miR-146a* targets *Irak1* and *Traf6* mRNA levels were measured by qRT-PCR after 8 weeks of HFD. The $2^{-\Delta Ct}$ method was used to calculate miRNA/mRNA abundance relative to endogenous control expression (U6 in A; β -actin in B) (Ct = Threshold Cycle; ΔCt = Ct sample gene – Ct endogenous control). (C) Representative western blot and quantification of *Irak1* and *Traf6* protein levels (normalized to β -actin) in thoracic aorta of mice receiving BM wt or BM *miR-146a*^{-/-} after 20 weeks of HFD (pools of 3 thoracic aortas per sample). *: $p < 0.05$, **: $p < 0.01$ BM wt vs BM *miR-146a*^{-/-} at both time points.

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146a in leukocytes, but not in the aorta [18]. It is therefore possible that this treatment increased *miR-146a* levels predominantly in endothelium, as reported for *in vivo* injection of miRNA mimetics in other studies [30,31]. Thus, the potential beneficial effect of *miR-146a* in atherosclerosis might be due in part to increased expression in endothelial cells. Thus, despite observing no differences in atherosclerosis burden, we found above-normal expression of specific targets of *miR-146a* such as *Traf6* and *Irak1* in the aorta of BM *miR-146a*^{-/-} mice, which exhibited low level of *miR-146a* expression. Since levels of *miR-146a* expression in non-hematopoietic cells are unaffected by BM transplantation, differences in the expression of *miR-146a* and its two targets *Traf6* and *Irak1* might be due to variations in infiltrated leukocytes. Since *miR-146a* deficiency does not increase leukocyte adhesion, leukocyte infiltration should be similar in BM *miR-146a*^{-/-} and BM wt mice. Thus, *miR-146a* deficiency in hematopoietic cells affects *miR-146a* targets in the inflamed aorta but not sufficiently to aggravate atherosclerosis development in *Ldlr*^{-/-} mice. Future studies using conditional *miR-146a*-deficient mice for endothelium or vascular smooth muscle cells may help to unequivocally establish this

hypothesis. Additionally, since miR-146a^{-/-} mice still express miR-146b, which shares most of the targets with miR-146a, we cannot discard that miR-146b has a compensatory effect in the setting of atherosclerosis model [32]. In conclusion, our results strongly suggest that miR-146a expressed in the hematopoietic compartment plays a negligible role in atherosclerosis development in *Ldlr*^{-/-} mice, although its ablation increases in the aorta the proinflammatory state promoted by an atherogenic diet. Identifying how expression of this miRNA limits atherosclerosis remains of high interest. It will therefore be important to develop in vivo models in which to investigate the cells, with special attention to endothelial cells, in which miR-146a exerts its anti-atherogenic function. Finally, investigating the effect of low levels of miR-146a in humans due for example to rs2431697 in atherosclerosis may be of interest to evaluate the clinical impact of a partial miR-146a deficiency.

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Author Contributions

Conceptualization: Rocío González-Conejero, Constantino Martínez, Vicente Andrés.

Data curation: Rocío González-Conejero, Constantino Martínez, Vicente Andrés.

Formal analysis: Alberto del Monte, Ana B. Arroyo.

Funding acquisition: Rocío González-Conejero, Constantino Martínez, Vicente Andrés.

Investigation: Alberto del Monte, Ana B. Arroyo, Rocío González-Conejero, Constantino Martínez, Vicente Andrés.

Methodology: Alberto del Monte, Ana B. Arroyo, María J. Andrés-Manzano, Nuria García-Barberá, María S. Caleprico, Rocío González-Conejero, Constantino Martínez, Vicente Andrés.

Project administration: Rocío González-Conejero, Constantino Martínez, Vicente Andrés.

Resources: Rocío González-Conejero, Constantino Martínez, Vicente Andrés.

Supervision: Rocío González-Conejero, Constantino Martínez, Vicente Andrés.

Writing – original draft: Rocío González-Conejero, Constantino Martínez, Vicente Andrés.

Writing – review & editing: Alberto del Monte, Ana B. Arroyo, Vicente Vicente, Vanessa Roldán, Rocío González-Conejero, Constantino Martínez, Vicente Andrés.

References

1. Lu H, Daugherty A (2015) Atherosclerosis. *Arterioscler Thromb Vasc Biol* 35: 485–491. <https://doi.org/10.1161/ATVBAHA.115.305380> PMID: 25717174
2. Murphy AJ, Tall AR (2016) Disordered haematopoiesis and athero-thrombosis. *Eur Heart J* 37: 1113–1121. <https://doi.org/10.1093/eurheartj/ehv718> PMID: 26869607
3. Tiyerili V, Camara B, Becher MU, Schrickel JW, Lutjohann D, Mollenhauer M, et al (2016) Neutrophil-derived myeloperoxidase promotes atherogenesis and neointima formation in mice. *Int J Cardiol* 204: 29–36. <https://doi.org/10.1016/j.ijcard.2015.11.128> PMID: 26655530
4. Warnatsch A, Ioannou M, Wang Q, Papayannopoulos V (2015) Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. *Science* 349: 316–320. PMID: 26185250
5. Soehnlein O, Ortega-Gomez A, Döring Y, Weber C (2015) Neutrophil-macrophage interplay in atherosclerosis: protease-mediated cytokine processing versus NET release. *Thromb Haemost* 114: 866–867. <https://doi.org/10.1160/TH15-08-0623> PMID: 26310276

6. Ross R (1995) Cell biology of atherosclerosis. *Annu Rev Physiol* 57: 791–804. <https://doi.org/10.1146/annurev.ph.57.030195.004043> PMID: 7778883
7. Pello OM, Silvestre C, De PM, Andres V (2011) A glimpse on the phenomenon of macrophage polarization during atherosclerosis. *Immunobiology* 216: 1172–1176. <https://doi.org/10.1016/j.imbio.2011.05.010> PMID: 21802768
8. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* 136: 215–233. <https://doi.org/10.1016/j.cell.2009.01.002> PMID: 19167326
9. Guo H, Ingolia NT, Weissman JS, Bartel DP (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* 466: 835–840. <https://doi.org/10.1038/nature09267> PMID: 20703300
10. McManus DD, Freedman JE (2015) MicroRNAs in platelet function and cardiovascular disease. *Nat Rev Cardiol* 12: 711–717. <https://doi.org/10.1038/nrcardio.2015.101> PMID: 26149483
11. Feinberg MW, Moore KJ (2016) MicroRNA Regulation of Atherosclerosis. *Circ Res* 118: 703–720. <https://doi.org/10.1161/CIRCRESAHA.115.306300> PMID: 26892968
12. Taganov KD, Boldin MP, Chang KJ, Baltimore D (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A* 103: 12481–12486. <https://doi.org/10.1073/pnas.0605298103> PMID: 16885212
13. Lofgren SE, Frostegard J, Truedsson L, Pons-Estel BA, D'Alfonso S, Witte T, et al (2012) Genetic association of miRNA-146a with systemic lupus erythematosus in Europeans through decreased expression of the gene. *Genes Immun* 13: 268–274. <https://doi.org/10.1038/gene.2011.84> PMID: 22218224
14. Roldan V, Arroyo AB, Salloum-Asfar S, Manzano-Fernandez S, Garcia-Barbera N, Marin F, et al (2014) Prognostic role of MIR146A polymorphisms for cardiovascular events in atrial fibrillation. *Thromb Haemost* 112: 781–788. <https://doi.org/10.1160/TH14-01-0092> PMID: 25008481
15. Tabas I, Garcia-Cardena G, Owens GK (2015) Recent insights into the cellular biology of atherosclerosis. *J Cell Biol* 209: 13–22. <https://doi.org/10.1083/jcb.201412052> PMID: 25869663
16. Guo L, Chen CH, Zhang LL, Cao XJ, Ma QL, Deng P, et al (2015) IRAK1 mediates TLR4-induced ABCA1 downregulation and lipid accumulation in VSMCs. *Cell Death Dis* 6: e1949. <https://doi.org/10.1038/cddis.2015.212> PMID: 26512959
17. Perez-Sanchez C, Aguirre MA, Ruiz-Limon P, Barbarroja N, Jimenez-Gomez Y, de la Rosa IA, et al (2016) 'Atherothrombosis-associated microRNAs in Antiphospholipid syndrome and Systemic Lupus Erythematosus patients'. *Sci Rep* 6: 31375. <https://doi.org/10.1038/srep31375> PMID: 27502756
18. Li K, Ching D, Luk FS, Raffai RL (2015) Apolipoprotein E enhances microRNA-146a in monocytes and macrophages to suppress nuclear factor-kappaB-driven inflammation and atherosclerosis. *Circ Res* 117: e1–e11. <https://doi.org/10.1161/CIRCRESAHA.117.305844> PMID: 25904598
19. Fuster JJ, Gonzalez-Navarro H, Vinue A, Molina-Sanchez P, Andres-Manzano MJ, Nakayama KI, et al (2011) Deficient p27 phosphorylation at serine 10 increases macrophage foam cell formation and aggravates atherosclerosis through a proliferation-independent mechanism. *Arterioscler Thromb Vasc Biol* 31: 2455–2463. <https://doi.org/10.1161/ATVBAHA.111.235580> PMID: 21885849
20. Diez-Juan A, Andres V (2001) The growth suppressor p27(Kip1) protects against diet-induced atherosclerosis. *FASEB J* 15: 1989–1995. <https://doi.org/10.1096/fj.01-0130com> PMID: 11532979
21. Rius C, Sanz MJ (2015) Intravital Microscopy in the Cremaster Muscle Microcirculation for Endothelial Dysfunction Studies. *Methods Mol Biol* 1339: 357–366. https://doi.org/10.1007/978-1-4939-2929-0_26 PMID: 26445803
22. Cheng HS, Besla R, Li A, Chen Z, Shikatani EA, Nazari-Jahanigh M, et al (2017) Paradoxical Suppression of Atherosclerosis in the Absence of microRNA-146a. *Circ Res* 121: 354–367. <https://doi.org/10.1161/CIRCRESAHA.116.310529> PMID: 28637783
23. Andreou I, Sun X, Stone PH, Edelman ER, Feinberg MW (2015) miRNAs in atherosclerotic plaque initiation, progression, and rupture. *Trends Mol Med* 21: 307–318. <https://doi.org/10.1016/j.molmed.2015.02.003> PMID: 25771097
24. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, et al (2011) miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med* 208: 1189–1201. <https://doi.org/10.1084/jem.20101823> PMID: 21555486
25. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, et al (2003) Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *J Clin Invest* 112: 1176–1185. <https://doi.org/10.1172/JCI18580> PMID: 14561702
26. Gareus R, Kotsaki E, Xanthouleas S, van dM I, Gijbels MJ, Kardakaris R, et al (2008) Endothelial cell-specific NF-kappaB inhibition protects mice from atherosclerosis. *Cell Metab* 8: 372–383. <https://doi.org/10.1016/j.cmet.2008.08.016> PMID: 19046569

27. Polykratis A, van LG, Xanthoulea S, Hellmich M, Pasparakis M (2012) Conditional targeting of tumor necrosis factor receptor-associated factor 6 reveals opposing functions of Toll-like receptor signaling in endothelial and myeloid cells in a mouse model of atherosclerosis. *Circulation* 126: 1739–1751. <https://doi.org/10.1161/CIRCULATIONAHA.112.100339> PMID: [22935568](#)
28. Cheng HS, Sivachandran N, Lau A, Boudreau E, Zhao JL, Baltimore D, et al (2013) MicroRNA-146 represses endothelial activation by inhibiting pro-inflammatory pathways. *EMBO Mol Med* 5: 949–966.
29. Ma S, Tian XY, Zhang Y, Mu C, Shen H, Bismuth J, et al (2016) E-selectin-targeting delivery of micro-RNAs by microparticles ameliorates endothelial inflammation and atherosclerosis. *Sci Rep* 6: 22910. <https://doi.org/10.1038/srep22910> PMID: [26956647](#)
30. Sun X, Icli B, Wara AK, Belkin N, He S, Kobzik L, et al (2012) MicroRNA-181b regulates NF-kappaB-mediated vascular inflammation. *J Clin Invest* 122: <https://doi.org/10.1172/JCI61495> PMID: [22622040](#)
31. Xiang Y, Cheng J, Wang D, Hu X, Xie Y, Stitham J, et al (2015) Hyperglycemia repression of miR-24 coordinately upregulates endothelial cell expression and secretion of von Willebrand factor. *Blood* 125: 3377–3387. <https://doi.org/10.1182/blood-2015-01-620278> PMID: [25814526](#)
32. Lin N, An Y (2017) Blockade of 146b-5p promotes inflammation in atherosclerosis-associated foam cell formation by targeting TRAF6. *Exp Ther Med* 14: 5087–5092. <https://doi.org/10.3892/etm.2017.5121> PMID: [29201219](#)

Endothelial NOD1 directs myeloid cell recruitment in atherosclerosis through VCAM-1

Silvia González-Ramos,^{*,†} Marta Paz-García,^{*,†} Cristina Rius,^{*,1} Alberto del Monte-Monge,^{†,‡,1} Cristina Rodríguez,^{†,§} Victoria Fernández-García,^{*,†} Vicente Andrés,^{†,‡} José Martínez-González,^{†,¶} Miguel A. Lasunción,^{||} Paloma Martín-Sanz,^{*,†} Oliver Soehnlein,^{#,**,††,2} and Lisardo Boscá^{*,†,3}

^{*}Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Madrid, Spain; [†]Centro de Investigación Biomédica en Red en Enfermedades Cardiovasculares (CIBER-CV), Madrid, Spain; [‡]Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain; [§]Institut de Recerca del Hospital de la Santa Creu i Sant Pau, Instituto Catalán de Ciencias Cardiovasculares (ICCC), and [¶]Instituto de Investigaciones Biomédicas de Barcelona (CSIC-IIBB), Sant Pau Biomedical Research Institute (IIB-Sant Pau), Barcelona, Spain; ^{||}Hospital Universitario Ramón y Cajal (IRyCIS), Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Madrid, Spain; [#]Institute for Cardiovascular Prevention, Ludwig-Maximilians-University (LMU), Munich, Germany; ^{**}Karolinska Institutet, Stockholm, Sweden; and ^{††}German Center for Cardiovascular research (DZHK), Partner Site Munich Heart Alliance (MHA), Berlin, Germany

ABSTRACT: Atherosclerosis is a chronic disease characterized by vascular lipid retention and inflammation, and pattern recognition receptors (PRRs) are important contributors in early stages of the disease. Given the implication of the intracellular PRR nucleotide-binding oligomerization domain 1 (NOD1) in cardiovascular diseases, we investigated its contribution to early atherosclerosis. We evidenced NOD1 induction in atherosclerotic human and mouse tissues, predominantly in vascular endothelial cells. Accordingly, NOD1 genetic inactivation in *Apoe*^{-/-} mice reduced not only atherosclerosis burden, but also monocyte and neutrophil accumulation in atheromata. Of note, in the presence of either peptidoglycan or oxidized LDLs, endothelial NOD1 triggered VCAM-1 up-regulation through the RIP2-NF- κ B axis in an autocrine manner, enhancing firm adhesion of both sets of myeloid cells to the inflamed micro- and macrovasculature *in vivo*. Our data define a major proatherogenic role for endothelial NOD1 in early leukocyte recruitment to the athero-prone vasculature, thus introducing NOD1 as an innovative therapeutic target and potential prognostic molecule.—González-Ramos, S., Paz-García, M., Rius, C., del Monte-Monge, A., Rodríguez, C., Fernández-García, V., Andrés, V., Martínez-González, J., Lasunción, M. A., Martín-Sanz, P., Soehnlein, O., Boscá, L. Endothelial NOD1 directs myeloid cell recruitment in atherosclerosis through VCAM-1. FASEB J. 33, 000–000 (2019). www.fasebj.org

KEY WORDS: cardiovascular disease • innate immunity • leukocyte

The response-to-injury theory poses that endothelial dysfunction as a consequence of disturbed flow, as well as exposure to noxious stimuli, represents the initial event in the pathogenesis of atherosclerosis (1). This endothelial dysfunction enhances endothelial permeability to atherogenic

lipoproteins (mainly apolipoprotein B-containing LDL) and activation of endothelial cell adhesion molecules (ECAMs) to promote infiltration of leukocytes to the subendothelial space. The most relevant ECAMs in leukocyte arrest belong to the Ig superfamily of adhesion molecules (Ig-CAMs). In this regard, endothelial VCAM 1 binds to the integrin cell-surface ligand very late antigen 4 (VLA-4) in monocytes and T cells preferably; meanwhile neutrophils use the endothelial intracellular adhesion molecule 1 (ICAM-1) to transiently bind to its ligand lymphocyte function-associated antigen 1 (2).

Although the best-known danger signal in human atherosclerosis is oxidized LDL (oxLDL) (3), the atherosclerotic lesion contains a mixture of PRR ligands also implicated in the inflammatory response in the vessel wall (4). The cytoplasmic PRR nucleotide-binding oligomerization domain (NOD)-1 receptor is a family member of the nucleotide-binding leucine-rich repeat-containing receptors. To date, 2 NOD-containing molecules, NOD1 (CARD4) and NOD2 (CARD15), have been identified.

ABBREVIATIONS: BM, bone marrow; EC, endothelial cell; ECAM, endothelial cell adhesion molecule; FDG, fluorodeoxyglucose; HE, hematoxylin-eosin; HFD, high-fat diet; iE-DAP, γ -D-glutamyl-meso-diaminopimelic acid; MLEC, murine lung endothelial cell; oxLDL, oxidized LDL; PGN, peptidoglycan; PRR, pattern-recognition receptor; RIP, receptor interacting protein-2; SUV, standardized uptake value; WT, wild type

¹ These authors contributed equally to this work.

² Correspondence: Institute for Cardiovascular Prevention, Ludwig-Maximilians-University Munich, Pettenkoferstr. 9, 80336 Munich, Germany. E-mail: oliver.soehnlein@gmail.com

³ Correspondence: Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM). Arturo Duperier 4. 28029 Madrid, Spain. E-mail: lbosca@iib.uam.es

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Although both receptors detect bacterial peptidoglycan (PGN) fragments, NOD1 senses a conserved region of PGN (γ -D-glutamyl-meso-diaminopimelic acid, iE-DAP) common to almost all gram-negative and some gram-positive bacteria (5). Upon recognition of PGN, NOD1 and NOD2 homodimerize and interact with the receptor interacting protein-2 (RIP2 or RICK). This essential adaptor molecule is a serine/threonine kinase that through the assembly to large multiprotein complexes (including the IKK complex) lately activates the canonical NF- κ B signaling pathway (6–8).

Here we determined the role of NOD1 in atherosclerosis-related endothelial dysfunction. Our results indicate that NOD1 in the presence of oxLDL or PGN induces an autocrine response in the vascular endothelium that promotes early atherosclerosis through VCAM-1 up-regulation and concomitant monocyte and neutrophil recruitment to the vasculature.

MATERIALS AND METHODS

Human samples

Human coronary arteries were collected from patients undergoing heart transplant at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). Atherosclerotic and nonatherosclerotic coronary arteries were from coronary artery disease and non-coronary artery disease patients, respectively. Written consent was obtained from all participating subjects. The studies were approved by the Ethics Committee and were conducted in accordance with the Helsinki Declaration.

Mouse models

Animal studies were approved by the local ethics committee, and all animal procedures conformed to EU Directive 2010/63 and Recommendation 2007/526/endothelial cell (EC) regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 53/2013.

C57BL/6 wild-type (WT) and *Apoe*^{-/-} mice (9, 10) were obtained from Charles River Laboratories (Wilmington, MA, USA) (JAX mice 000664 and 002052, respectively). The homozygous *Nod1*^{-/-} mice (mixed C57BL/6 and 129/C57BL/6 background) had been previously described (11) and were a kind gift from Dr. Gabriel Núñez (University of Michigan Health System, Ann Arbor, MI, USA).

Double-knockout *Apoe*^{-/-}*Nod1*^{-/-} mice were generated by crossing *Apoe*^{-/-} mice with *Nod1*^{-/-} mice. Heterozygous mice were intercrossed to generate homozygous *Apoe*^{-/-} bearing combinations of *Nod1*^{+/+} and *Nod1*^{-/-} mice at the expected mendelian ratio. All mice were genotyped by PCR from ear samples by using standard procedures. The absence of NOD1 expression in cells from *Apoe*^{-/-}*Nod1*^{-/-} mice was confirmed by RT-PCR analysis (data not shown).

All experiments compared male *Apoe*^{-/-} mice with male *Apoe*^{-/-}*Nod1*^{-/-} littermates. To accelerate the development of atherosclerotic lesions, at 12 wk of age, males were placed on high-fat diet (HFD; 10.7% total fat, 0.75% cholesterol; Ssniff, Soest, Germany) for 3 wk.

Mouse lung ECs isolation and culture

One of the greatest sources of ECs is the pulmonary endothelium, a continuous endothelial monolayer on the luminal surface of the

lung vasculature. Therefore, to boost the yield of primary ECs isolation, we isolated murine lung endothelial cells (MLECs) from male WT, *Nod1*^{-/-}, *Apoe*^{-/-}*Nod1*^{-/-}, and *Apoe*^{-/-} lungs by positive selection using mouse anti-CD102 antibody (BD Biosciences, San Jose, CA, USA) bound to magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (12).

Subconfluent primary cell cultures then underwent a second round of positive selection using mouse anti-CD31 antibody (BD Biosciences). The EC culture purity was further assessed by flow cytometry [FACSCanto II; Becton Dickinson (BD), San Diego, CA, USA] for EC surface markers (VCAM-1, R&D Systems, Minneapolis, MN, USA; CD54, BD Biosciences) using standard procedures. This procedure resulted in a $\geq 99\%$ pure population of ECs.

MLECs were grown on 0.1% gelatin (MilliporeSigma, Burlington, MA, USA) in low-glucose DMEM-F12 (MilliporeSigma) supplemented with 20% fetal bovine serum, EC growth supplement (50 μ g/ml; MilliporeSigma), and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin).

When indicated, MLECs were preincubated with 1 μ M Nodinitib-1 (Cayman Chemicals, Ann Arbor, MI, USA), 5 μ M BAY 11-7082 (Merck Millipore, Etobicoke, ON, Canada), 5 μ M GSK583 (Cayman Chemicals), or 1 μ g/ml LOX1 neutralizing antibody (Abcam, Cambridge, MA, USA) for 1 h and then treated with 50 μ g/ml LDL, 50 μ g/ml oxidized LDL (Biochemistry-Research Unit at Instituto Ramón y Cajal de Investigación Sanitaria, Spain), 10 ng/ml mouse TNF- α (Pepro-Tech, London, United Kingdom), 1 μ g/ml iE-Lys, or 1 μ g/ml C12-iE-DAP (Invivogen, San Diego, CA, USA) for 24 h. For co-culture assays, cell-free MLEC supernatants were collected after 24 h stimulation and directly transferred to nonstimulated MLEC subconfluent monolayers.

Quantitative RT-PCR

Total RNA was isolated by homogenization in Quiazol by a TissueLyser LT and eluted using MinElute columns (Qiagen, Germantown, MD, USA). RNA integrity was assessed by RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA). Two hundred and fifty nanograms of RNA were retrotranscribed by using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), and quantitative RT-PCR was conducted in 7900HT Fast Real-Time PCR System equipment using a Taqman Gene Expression Assay specific for mouse *Nod1* (1308180) and *18S* (4352930E) (Thermo Fisher Scientific). SYBR Green was used for quantitative RT-PCR detection of *Nod2* (5'-TGGACA-CAGTCTGGAACAAGG-3' and 5'-CAGGACCCATACAGTTCAAAGG-3'), *Lox1* (5'-CAAGATGAAGCCTGCGAATGA-3' and 5'-ACCTGGCGTAATTGTGTCCAC-3'), and *18S* (5'-CCAGTAAGTGCGGGTCATAAGC-3' and 5'-CCTCACTAAACCATCAATCGG-3').

Calculations were made from measurement of technical triplicates of each sample. The relative amount of mRNA was calculated with the comparative 2^{- $\Delta\Delta C_t$} method using mouse *18S* or *36b4* as endogenous control transcripts.

Western blot analysis

Human specimens and mouse aortic samples for Western blotting were snap-frozen in liquid nitrogen and stored at -80°C. Human protein extracts were obtained using ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% (w/v) Triton X-100, 150 mM NaCl, and 1 mM DTT, supplemented with phosphatase cocktail and protease inhibitors (MilliporeSigma) (13). Mouse tissue and MLEC whole protein extracts were obtained using ice-cold proprietary detergent in 25 mM bicine, 150 mM sodium chloride (pH 7.6) (T-PER Tissue Protein Extraction Reagent;

Thermo Fisher Scientific) supplemented with the same phosphatase and protease inhibitors.

Proteins were resolved on SDS-PAGE gels and then transferred to nitrocellulose membranes. Proteins were detected using rabbit pAb against NOD1 (1:500; Abcam), rabbit mAb against phospho-RIP2 (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit pAb against phospho-RIP2 (1:1000; Cell Signaling Technology), rabbit pAb against phospho-NF- κ B (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA), mouse mAb against GAPDH (1:10,000; Thermo Fisher Scientific), mouse mAb against β -actin (1:40,000; MilliporeSigma), mouse mAb against α -tubulin (1:10,000; MilliporeSigma), and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA).

Protein bands were visualized using a Luminata chemiluminescence detection system (Merck Millipore) and a ImageQuant LAS 500 imager (GE Healthcare Life Sciences, Freiburg, Germany) and were quantified using ImageJ [National Institutes of Health (NIH), Bethesda, MD, USA]. Intensities of the NOD1 and pRIP2 bands were expressed as a percentage of those of the GAPDH or β -actin bands as indicated.

Lesion quantification

After mouse cardiac perfusion with PBS supplemented with 5 mM of EDTA, mouse hearts were harvested and fixed in 4% paraformaldehyde for 24 h at 4°C, incubated 24 h in PBS supplemented with 30% sucrose, embedded in optimal cutting temperature, and cryopreserved at -70°C.

Cryocut cross sections (5 μ m) of aortic roots were evaluated for conventional hematoxylin-eosin (HE) staining. Images were captured with a Zeiss Axiophot microscope with a Plan-Neofluar $\times$ 10/0.3 objective (Carl Zeiss, Oberkochen, Germany) and a DP70 camera (Olympus, Tokyo, Japan). To avoid specific biases due to potential differences in lesion shape, cross sections of the entire lesion were analyzed and averaged.

To obtain the aortas for the analysis, after being fixed in paraformaldehyde overnight at 4°C, the aortas were whole-mount stained with 0.2% Oil Red O (MilliporeSigma) in 80% methanol, opened longitudinally, and pinned to black wax to expose the entire luminal surface. Images were acquired using a Leica MZ6 SZX10 stereomicroscope (Leica Microsystems, Buffalo Grove, IL, USA) coupled to a Leica DFC300 digital color camera (Leica Microsystems). The planimetric area of atherosclerotic plaques was measured in pixels using ImageJ (NIH).

Immunostaining

Human arteries were fixed overnight in 4% paraformaldehyde/0.1 M PBS (pH 7.4), embedded in paraffin, and sectioned into 5 μ m sections with a microtome (Jung RM2055; Leica Microsystems). Deparaffinized sections were rehydrated, subjected to antigen retrieval in 10 mM citrate buffer (pH 6.0), blocked, and incubated with a rabbit pAb against NOD1 (1:40; Abcam). After extensive washes, sections were incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA). Immunocomplexes were detected after incubation with Vectastain Elite ABC reagent (PK6100; Vector Laboratories) and DAB substrate (Roche, Basel, Switzerland). Images were acquired with an Olympus Vanox AHB3 microscope and digitalized by a Sony (Tokyo, Japan) camera (DXC-S500).

For immunostaining of cryo-section samples and ECs, slides were stained with antibodies specific for mouse CD31 (1:100; BD Biosciences), CD54 (1:100; BD Biosciences), CD68 (1:200; Abcam), Ly6G (1:100; BD Biosciences), NF- κ B p65 (1:100; Santa Cruz Biotechnology) and VCAM-1 (1:100; R&D Systems), followed by secondary staining using standard procedures. Secondary antibodies for immunofluorescence were Alexa Fluor 647-conjugated

anti-rabbit (Thermo Fisher Scientific), Alexa Fluor 594-conjugated anti-rat (Thermo Fisher Scientific), FITC-conjugated anti-mouse (MilliporeSigma), and Cy3-conjugated anti-Armenian hamster (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Nuclei were counterstained with DAPI (Thermo Fisher Scientific). Immunofluorescence staining of cryo-sections were mounted in Prolong Gold Antifade mounting medium (Thermo Fisher Scientific). Primary control panel was performed with an appropriate isotype control IgG, and secondary controls incubations were performed in the absence of primary antibody.

A LSM710 confocal microscope with a Plan-Apochromat $\times$ 25/0.8 oil immersion objective (Carl Zeiss, Oberkochen, Germany) was used to capture images from immunofluorescence staining. Images were analyzed using ImageJ and were processed for presentation with Zen2009 (Carl Zeiss) software.

[¹⁸F]-FDG PET/CT imaging

[¹⁸F]-FDG PET/CT images were acquired by using combined modality PET/CT as previously described (14). Briefly, [¹⁸F]-FDG (37 MBq/kg; 0.2 ml) was administered intraperitoneally in overnight starved animals. PET/CT imaging was performed in animals under general anesthesia (inhalant isoflurane; Abbott Laboratories, Chicago, IL, USA) in an INVEON microCT-microSPECT-microPET (Siemens, Munich, Germany) system for small animals.

The parameter for the relative measure of FDG uptake was the standardized uptake value (SUV). The target-to-background ratio was calculated as a quotient between the maximum SUV (SUV_{max}) of 5 regions of interest in the descending aorta corrected by mean SUV in the reference tissue (paraspinus muscle on the axial view).

Flow cytometry

To characterize leukocyte populations in *Apoe*^{-/-}*Nod1*^{-/-} and *Apoe*^{-/-} vasculature, mechanically digested adventitia-free aortas were immersed in Aorta Dissociation Enzyme Solution (395 U/ml type II collagenase, 0.744 U/ml elastase) for 30 min at 37°C. Single cell suspensions were prepared by passing the resulting solution through a 70 μ m cell strainer. After 400 g centrifugation for 5 min at 4°C, pellet was resuspended in HBSS (Thermo Fisher Scientific) supplemented with 10 mM Hepes and 0.5% bovine serum albumin (pH 7.4), and incubated for 30 min at 4°C with rat APC-Cy7-conjugated mAb against CD45 (1:200; BioLegend, San Diego, CA, USA), rat PE-conjugated mAb against CD115 (1:100; Thermo Fisher Scientific), rat PerCpCy5.5-conjugated mAb against Ly6G (1:100; BioLegend), rat FITC-conjugated mAb against Ly6C (1:100; BioLegend), and rat APC-conjugated mAb against F4/80 (1:100; BioLegend). For cell counting, absolute counting beads were used (CountBright; Thermo Fisher Scientific).

Flow cytometry was conducted in a FACSCanto II (Becton Dickinson), and leukocyte subsets were defined using FlowJo software (Treestar, Ashland, OR, USA): neutrophils (CD45⁺CD115⁻Ly6G⁺), inflammatory monocytes (CD45⁺CD115⁺Ly6C⁺), and tissue macrophages (CD45⁺CD115⁻F4/80⁺).

Bone marrow transplantation

Bone marrow (BM) transplantation was performed as previously described (15). Briefly, 8-wk-old male *Apoe*^{-/-} and *Apoe*^{-/-}*Nod1*^{-/-} mice were irradiated (2 same-day doses of 4.5 Gy) and transplanted by intravenous injection through the orbital vein under general anesthesia of the animal (inhalant isoflurane; Abbott Laboratories). Bone marrow-derived cells (10⁷) were obtained from tibias and femurs of euthanized donors *Apoe*^{-/-}

or $Apoe^{-/-}Nod1^{-/-}$ mice giving 4 groups: $Apoe^{-/-}$ BM $\rightarrow$ $Apoe^{-/-}$; $Apoe^{-/-}$ BM $\rightarrow$ $Apoe^{-/-}Nod1^{-/-}$; $Apoe^{-/-}Nod1^{-/-}$ BM $\rightarrow$ $Apoe^{-/-}Nod1^{-/-}$; $Apoe^{-/-}Nod1^{-/-}$ BM $\rightarrow$ $Apoe^{-/-}$. After 6 wk on chow diet, transplanted mice were placed on HFD for 3 wk. Successful engraftment was confirmed by PCR (data not shown).

Intravital microscopy

Leukocyte-endothelial interactions along the carotid artery and the cremaster microcirculation were analyzed in $Apoe^{-/-}Nod1^{-/-}$ and $Apoe^{-/-}$ mice that received HFD for 3 wk. For VCAM-1 blocking studies *in vivo*, and before recording, mice were pretreated with 100 μ g/mice C12-iE-DAP or iE-Lys (Invivogen) for 24 h and then treated with 1 mg/kg mice BIO 5192 (Tocris Bioscience, Bristol, United Kingdom) for other 24 h. The blood flow from live animals was recorded while the mice were under general anesthesia (ketamine/xylazine combination at 100 mg/kg mice and 10 mg/kg mice, respectively).

Cremaster surgical exposure was performed as previously described (16). Briefly, once exteriorized, it was placed onto an optical clear viewing pedestal, cut longitudinally with a high temperature surgical cautery (Lifeline Medical, Brooksville, FL, USA), and held extended at the corners of the exposed tissue using surgical suture. For intravital microscopy in the carotid artery, mice were placed in supine position, and the right jugular vein was cannulated with a catheter (PE10; Becton Dickinson) for antibody injection.

Ten minutes before recording the videos, antibodies (at 0.5 μ g) against Ly6C (BioLegend), Ly6G (BioLegend), and CD11b (Thermo Fisher Scientific) were injected to label myeloid cell subsets.

To maintain the correct temperature and physiologic conditions, the exposed tissues were perfused continuously with 37°C prewarmed Tyrode's buffer solution (139 mM NaCl, 3 mM KCl, 17 mM NaHCO_3 , 12 mM Glucose, 3 mM CaCl_2 , and 1 mM MgCl_2).

The cremasteric microcirculation was observed using an AXIO Examiner Z.1 work station (Carl Zeiss) mounted on a 3-dimensional motorized stage (Sutter Instrument, Novato, CA, USA) and equipped with a CoolSnap HQ2 camera (Photometrics, Tucson, AZ, USA). The objective used is an APO $\times 20$ NA 1.0 water-immersion objective. Slidebook software 5.0 (Intelligent Imaging Innovations, London, United Kingdom) was used for acquisition and image processing. Three randomly selected arterioles and venules were analyzed per mouse, and monocytes/neutrophils were considered adherent when no rolling was observed using a reference line perpendicular to blood flow for at least 30 s.

Carotid intravital microscopy was performed using a BX51 microscope (Olympus) equipped with a 9100-02 EMCCD camera (Hamamatsu Photonics, Shizuoka, Japan) and a $\times 10$ saline immersion objective. For image acquisition and analysis, Olympus Cell R software was used. Leukocyte adhesion was measured as previously described for the cremaster for 5 min.

Plasma lipoprotein analysis

Whole blood was extracted *postmortem* by cardiac puncture, and plasma was obtained by centrifugation at 2000 g during 10 min at 4°C. Plasma concentrations of total cholesterol, free cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides were measured enzymatically using Kinetic colorimetric kits (Spinreact, Girona, Spain) according to manufacturer's instructions. Mouse inflammatory mediators were quantified in plasma using the Milliplex Map Mouse Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore) including IL-1 β , 6 and TNF- α in a

Luminex (Austin, TX, USA) 100 IS system as per the manufacturer's specifications.

Quantification and statistical analysis

All values are expressed as means $\pm$ SEM. Statistical calculations were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). After normality was calculated by D'Agostino-Pearson omnibus test, either a nonparametric test (Mann-Whitney *U* test), or a normality test (unpaired Student's *t* test with Welch's correction, ordinary 1-way ANOVA) was used as appropriate. Statistical significance was deemed at values $P < 0.05$. Removal of outliers was assessed by the ROUT method. Statistical tests and *P* values are specified for each panel in the respective figure legends; *n* indicated in the figure legends refers to the number of individual animals for *in vivo* and *ex vivo* assays.

RESULTS

Endothelial NOD1 is upregulated in atherosclerotic human arteries, and its genetic deletion prevents early atherosclerosis development in $Apoe^{-/-}$ mice

To evaluate the importance of NOD1 activation in human atherosclerosis, we compared atherosclerotic coronary arteries with nonatherosclerotic arteries. NOD1 and pRIP2 protein expression was markedly higher in atherosclerotic vessels than in nonatherosclerotic coronary arteries, as revealed by Western blot analysis (Fig. 1A).

We also examined the upstream signaling cascade of NOD receptors in the aortic lesions of WT and $Apoe^{-/-}$ mice fed HFD for 3 wk. *Nod1* mRNA expression in $Apoe^{-/-}$ aortas showed 5-fold induction *vs.* nonatherosclerotic aortas (Fig. 1B); however, *Nod2* mRNA levels were unaffected (Supplemental Fig. S1). Differences in atherosclerotic condition regarding *Nod1* induction, but not *Nod2*, were accompanied by correspondingly higher levels of RIP2 phosphorylation in $Apoe^{-/-}$ mice (Fig. 1C).

To investigate the contribution of NOD1 to atheroma formation, we compared $Apoe^{-/-}$ *vs.* $Apoe^{-/-}Nod1^{-/-}$ mice after 3 wk on HFD. No significant differences between $Apoe^{-/-}$ and $Apoe^{-/-}Nod1^{-/-}$ mice were observed in body weights, proinflammatory cytokine profile (Supplemental Fig. S2), plasma concentrations of triglycerides, and total and free cholesterol, as well as HDL and LDL levels (Supplemental Table S1). However, HE staining of aortic sinus cross sections revealed a significantly smaller lesion area in $Apoe^{-/-}Nod1^{-/-}$ mice than in $Apoe^{-/-}$ mice (Fig. 1D). Accordingly, evaluation of [^{18}F]-FDG incorporation and Oil Red O-stained lesion area revealed a significant decrease on disease progression in the aorta of $Apoe^{-/-}Nod1^{-/-}$ mice compared with $Apoe^{-/-}$ mice (Fig. 1E, F). Similarly, decreased amounts of plaque leukocytes correlating with a decreased accumulation of tissue F4/80 $^{+}$ macrophages and Ly6G $^{+}$ neutrophils were found in $Apoe^{-/-}Nod1^{-/-}$ *vs.* $Apoe^{-/-}$ vasculature (Fig. 1G and Supplemental Fig. S3). Altogether, these results indicate that *Nod1* deficiency inhibits the onset of atherosclerosis by reducing the infiltration of myeloid cells to the early atheroma.

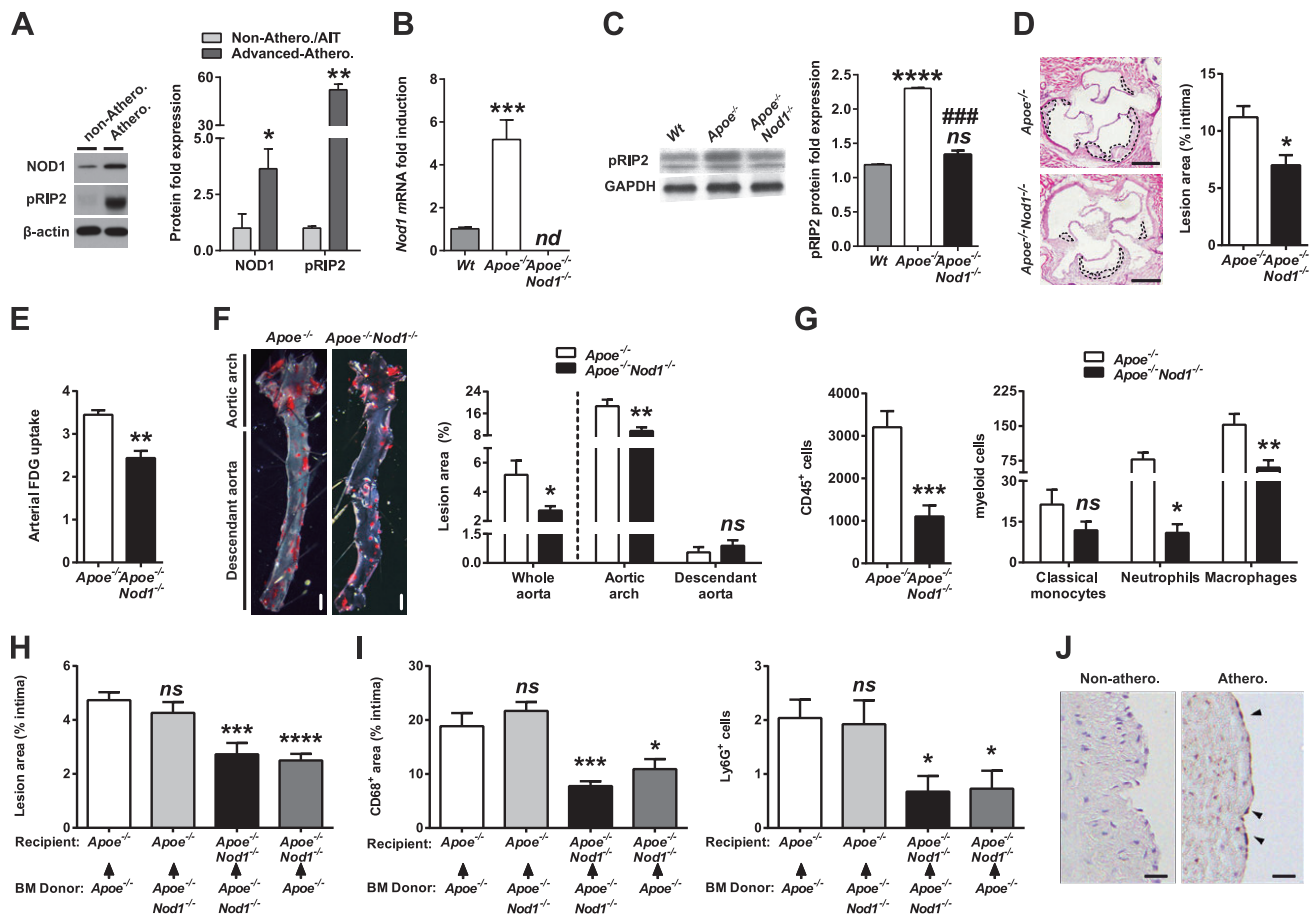


Figure 1. Endothelial NOD1 inflicts vascular inflammation linked to atherosclerosis. **A)** Immunoblot analysis and representative panel of NOD1 and pRIP2 in atherosclerotic (athero.) ($n = 6$) and nonatherosclerotic (non-athero.) or with arterial intimal thickening (AIT) ($n = 6$) human coronary arteries. Protein levels were normalized to β -actin. **B)** Quantitative RT-PCR (qRT-PCR) analysis of *Nod1* in aortic arches from C57BL/6 (WT; $n = 8$), *Apoe*^{-/-} ($n = 10$), and *Apoe*^{-/-}*Nod1*^{-/-} ($n = 8$) mice fed HFD for 3 wk. mRNA levels were normalized to *m18S*. **C)** Immunoblot analysis and representative panel of pRIP2 in the same cohort. Protein levels were normalized to GAPDH. **D)** Quantification of lesion area in the semilunar valve cups of *Apoe*^{-/-} ($n = 9$) and *Apoe*^{-/-}*Nod1*^{-/-} ($n = 8$) mice fed HFD for 3 wk. **E)** Target-to-background (TBR) ratio of arterial [¹⁸F]-FDG uptake in the same cohort. **F)** Quantification of positive Oil Red O (ORO) lesion area in the indicated aortic regions in the same cohort. Panels show representative *en face* ORO staining of aortas from these animals. **G)** Flow cytometry analysis of total number of leukocytes, macrophage, classic monocyte, and neutrophil subsets in *Apoe*^{-/-} ($n = 8$) and *Apoe*^{-/-}*Nod1*^{-/-} ($n = 8$) mice aortas after 3 wk on HFD. **H)** Quantification of lesion area in the aortic sinus of *Apoe*^{-/-} mice transplanted with BM from *Apoe*^{-/-} ($n = 17$) or *Apoe*^{-/-}*Nod1*^{-/-} ($n = 15$) mice and in *Apoe*^{-/-}*Nod1*^{-/-} mice that underwent BM reconstitution with *Apoe*^{-/-} ($n = 15$) or *Apoe*^{-/-}*Nod1*^{-/-} ($n = 16$) donors as indicated. All mice groups were fed HFD for 3 wk. **I)** Quantification of monocyte/macrophage and neutrophil neointimal content in lesions in the aortic sinus of the same cohort. **J)** Representative images for NOD1 immunohistochemistry in the intima of athero. and non-athero. or with arterial intimal thickening human coronary arteries. Arrows point out NOD1⁺ cells in the vascular endothelium. Data are represented as the mean $\pm$ SEM of the indicated number (n) of repeats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. nonathero by Mann-Whitney *U* test (A), vs. WT (B, C) mice by 1-way ANOVA, vs. *Apoe*^{-/-} (D–G) mice by Student's *t* test and vs. *Apoe*^{-/-} $\rightarrow$ *Apoe*^{-/-} by 1-way ANOVA (H, I); **** $P < 0.001$ vs. *Apoe*^{-/-} by Student's *t* test. Nd, not detected; ns, not significant. Scale bars, 100 μ m (D), 1 mm (F), 20 μ m (J).

To further examine the role of *Nod1* in atherosclerosis, we performed BM transplantation in *Apoe*^{-/-} and *Apoe*^{-/-}*Nod1*^{-/-} mice. HE staining revealed larger lesions in chimeric *Apoe*^{-/-} mice transplanted with *Apoe*^{-/-} or *Apoe*^{-/-}*Nod1*^{-/-} BM cells than in *Apoe*^{-/-}*Nod1*^{-/-} mice transplanted with either *Apoe*^{-/-} or *Apoe*^{-/-}*Nod1*^{-/-} BM, suggesting that NOD1 expression in BM-derived cells is not critical for atherosclerosis progression (Fig. 1H). Consistent with the importance of nonhematopoietic NOD1 in early atherosclerosis, the total content of CD68⁺ and Ly6G⁺ cells in the aortic sinus of *Apoe*^{-/-} mice was significantly reduced in *Apoe*^{-/-}*Nod1*^{-/-} mice transplanted with either *Apoe*^{-/-} or *Apoe*^{-/-}*Nod1*^{-/-} BM

cells (Fig. 1I). Of note, human atherosclerotic lesions showed marked NOD1 staining in the endothelial layer compared with nonatherosclerotic coronary arteries (Fig. 1J), suggesting that NOD1 may play a crucial role in the endothelial dysfunction that precedes the sequential recruitment of circulating platelets, neutrophils, and monocytes to the intima in early stages of atherogenesis (17).

NOD1 modulates endothelial VCAM-1 as an autocrine response to danger signals

Ig-CAMs expressed by ECs bind to leukocyte integrins and participate in leukocyte adhesion to the inflamed

vasculature. Immunohistochemical staining in the aortic valve revealed significantly reduced expression of the adhesion molecule VCAM-1, but not ICAM-1 nor PECAM-1, in the endothelium of fat-fed *Apoe*^{-/-} *Nod1*^{-/-} lesions compared with those on *Apoe*^{-/-} mice (Fig. 2A). These data suggest a role for endothelial NOD1 in promoting inflammatory cell infiltration by

sustaining the expression of VCAM-1 in the context of atherosclerosis.

The stimuli most commonly used to induce VCAM-1 expression in vascular ECs is TNF- α . However, further conceivably inflammatory stimuli that accentuate atherogenesis might not only be microbial agents (18), where NODs play a major role, but also lipid products (19), where

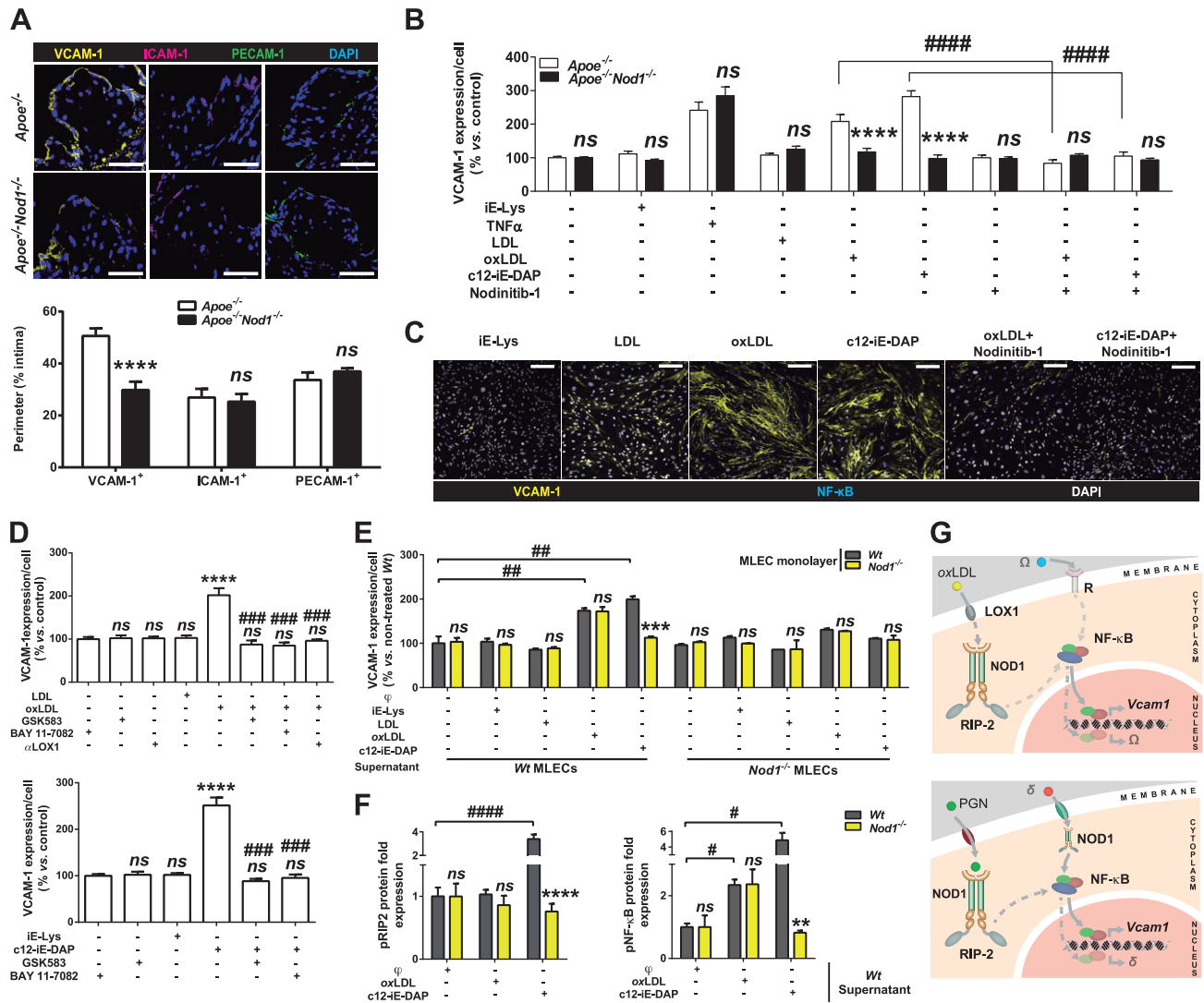


Figure 2. Bacterial PGN and oxLDL induce VCAM-1 expression in vascular ECs through NOD1 in an autocrine manner. A) Representative immunofluorescence images of VCAM-1, ICAM-1, and PECAM-1 staining and its corresponding perimeter quantification in lesions from the aortic sinus of *Apoe*^{-/-} ($n = 8-14$) and *Apoe*^{-/-} *Nod1*^{-/-} ($n = 8-16$) mice fed HFD for 3 wk. B-D) Primary ECs from *Apoe*^{-/-} ($n = 15$) and *Apoe*^{-/-} *Nod1*^{-/-} ($n = 8$) mice were preincubated with 1 μ M nodinitib-1, 5 μ M BAY 11-7082, 5 μ M GSK583 or 1 μ g/ml LOX1 neutralizing antibody for 1 h and then exposed to 50 μ g/ml oxLDL, 50 μ g/ml LDL, 10 ng/ml TNF- α , 1 μ g/ml iE-Lys, or 1 μ g/ml c12-iE-DAP for 24 h. E) Primary ECs from WT ($n = 5$) and *Nod1*^{-/-} ($n = 5$) mice were incubated with 50 μ g/ml oxLDL, 50 μ g/ml LDL, 1 μ g/ml iE-Lys, or 1 μ g/ml c12-iE-DAP for 24 h. Cell-free supernatants were transferred to a new batch of nontreated WT and *Nod1*^{-/-} MLECs for 24 h. Endothelial monolayers were immunostained for VCAM-1 (B-E) and NF- κ B (C). F) Immunoblot analysis of pRIP2 and pNF- κ B in primary ECs from WT ($n = 8$) and *Nod1*^{-/-} ($n = 10$) mice. Cell-free supernatants produced in WT MLECs for 24 h in presence of 50 μ g/ml oxLDL or 1 μ g/ml c12-iE-DAP were transferred to a new batch of nontreated WT and *Nod1*^{-/-} MLECs for 24 h. Protein levels were normalized to α -tubulin. G) In the presence of bacterial PGN and oxLDL, NOD1 activates RIP2 phosphorylation and secretion of yet unidentified metabolites (δ and Ω , respectively). Although metabolite Ω , upon binding to a nondefined receptor (R), induces VCAM1 up-regulation through NF- κ B, metabolite δ up-regulates VCAM1 expression in a NOD1-RIP2-NF- κ B-dependent manner. Blurry dotted arrows represent mechanistic hypotheses for NOD1-dependent autocrine signaling. Data are represented as means $\pm$ SEM of the indicated number (n) of repeats. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. *Apoe*^{-/-} (A, B), BAY 11-7082 control (D), or WT (E, F) by Student's t test (A, B, E) or 1-way ANOVA (D, F); # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ vs. oxLDL (B, D, upper panel), c12-iE-DAP (B, D, lower panel) or nontreated WT (E, F) by Student's t test (B, D) or 1-way ANOVA (E, F). Ns, not significant. Scale bars, 50 (A), and 20 (C) μ m.

the best-investigated effector signal is oxLDL. Therefore, through VCAM-1 immunofluorescence staining we tested straightforward the response of endothelial NOD1 to these danger signals.

Not surprisingly, MLECs from both *Apoe*^{-/-} and *Apoe*^{-/-}*Nod1*^{-/-} mice upregulated VCAM-1 expression when challenged with TNF- α compared with nontreated controls (Fig. 2B). This effect was maintained in *Apoe*^{-/-} MLECs after pharmacological activation of NOD1 with the selective ligand c12-iE-DAP, but not with the negative control iE-Lys (Fig. 2B). Interestingly, VCAM-1 up-regulation was prevented in both *Apoe*^{-/-}*Nod1*^{-/-} MLECs and in *Apoe*^{-/-} MLECs treated with nodinitib-1 to inhibit NOD1. These findings confirm c12-iE-DAP as a specific agonist for NOD1 and demonstrate a major role for NOD1 in the endothelial expression of VCAM-1.

oxLDLs are potent inducers of ECAM expression in early atherosclerosis (20, 21). We found increased VCAM-1 expression in *Apoe*^{-/-} MLECs incubated with oxLDL compared with *Apoe*^{-/-} and *Apoe*^{-/-}*Nod1*^{-/-} MLECs treated with native LDL (Fig. 2B). However, oxLDL-induced VCAM-1 expression was significantly reduced to control levels in *Apoe*^{-/-}*Nod1*^{-/-} or nodinitib-1 pretreated *Apoe*^{-/-} MLECs (Fig. 2B). Thus, NOD1 is required for oxLDL-triggered endothelial VCAM1 expression.

The NOD1 signaling pathway involves NF- κ B activation, which in turn promotes *Vcam1* gene expression. We confirmed that in the presence of PGN and oxLDL, NOD1 triggers translocation of NF- κ B to the nucleus (Fig. 2C), and hence the transcription of *Vcam1* is promoted (Fig. 2D). Similarly, pretreatment of *Apoe*^{-/-} MLECs with GSK583 to inhibit RIP2 activity prevented the expression of VCAM-1 following stimulation with both oxLDL and c12-iE-DAP (Fig. 2D), further demonstrating the importance of the NOD1-RIP2-NF- κ B signaling cascade that mediates VCAM-1 up-regulation in ECs in response to these danger signals. In ECs, the lectin-like oxLDL receptor-1 (LOX1) has been suggested to internalize oxLDL (22). Hence, we pretreated *Apoe*^{-/-} MLECs with a neutralizing antibody against LOX1 receptor (Fig. 2D, upper panel). Blockade of LOX1 prevented VCAM-1 expression in oxLDL-treated *Apoe*^{-/-} MLECs. These results were accompanied by corresponding decreases in RIP2 and NF- κ B activations (Supplemental Fig. S4). Given that there were no statistical differences in *Lox1* expression in the aortic arches of *Apoe*^{-/-}*Nod1*^{-/-} and *Apoe*^{-/-} mice (Supplemental Fig. S5), we suggest LOX1 as the endothelial receptor that allows oxLDL to transduce the signal to cytoplasmic NOD1.

To refine this cellular mechanistic link between NOD1 and VCAM-1, we performed cell coculture experiments to assess whether this interplay was dependent on intrinsic or extrinsic cell mediators. Interestingly, our results show that in the oxLDL-mediated response, only supernatants produced by *Nod1*^{+/+} MLECs induce VCAM-1 expression, regardless of the MLEC genotype. However, the iE-DAP response requires the presence of NOD1 in both stimulated and supernatant-producing MLECs to significantly increase VCAM-1 expression (Fig. 2E), highlighting anew the selectivity of the chemical agonist c12-iE-DAP in the NOD1-mediated specific response. To further confirm this

NOD1-autocrine signaling mechanism, we analyzed RIP2 and NF- κ B activations in WT and *Nod1*^{-/-} MLECs stimulated for 24 h with supernatant produced by WT MLECs in response to oxLDL and c12-iE-DAP (Fig. 2F). Interestingly enough, although oxLDL-intermediate metabolite (Ω , Fig. 2G) activates VCAM-1 protein expression through NF- κ B in a non-NOD1-dependent manner, NOD1 presence is mandatory in both the production and the signaling through PGN-derived metabolite (δ , Fig. 2G).

Taken together, our results unveil proatherogenic oxLDL and bacterial PGNs as paramount NOD1 activity regulators that mediate in an autocrine manner, the induction of VCAM-1 expression in ECs through the RIP2 and NF- κ B signaling cascade (Fig. 2G), promoting leukocyte recruitment into the vessel wall during atherosclerosis development.

Nod1 controls leukocyte recruitment to the micro- and macrocirculation

To assess the importance of NOD1 during leukocyte adhesion, we performed intravital microscopy in the cremaster muscle and the carotid artery of *Apoe*^{-/-} and *Apoe*^{-/-}*Nod1*^{-/-} mice fed HFD for 3 wk. We found reduced adhesion of both Ly6C- and Ly6G-labeled leukocytes in *Apoe*^{-/-}*Nod1*^{-/-} postcapillary venules when compared with *Apoe*^{-/-} mice (Fig. 3A). Although NOD1 activation upon treatment with c12-iE-DAP significantly increased the adhesion of both leukocytes subsets to the cremasteric vasculature in *Apoe*^{-/-} mice, treatment with BIO 5192 to selectively inhibit the integrin VLA-4 completely reversed the c12-iE-DAP effect on leukocyte adhesion (Fig. 3B), suggesting a relevant link between NOD1 and VCAM-1 in leukocyte recruitment to the microvasculature. In line with these results, the adhesion of Ly6C⁺ monocytes and Ly6G⁺ neutrophils to the carotid bifurcation in *Apoe*^{-/-}*Nod1*^{-/-} mice was also significantly diminished compared with *Apoe*^{-/-} mice (Fig. 3C). Furthermore, injection of an anti-CD11b antibody revealed that most of these adherent cells were inflammatory myeloid cells (Supplemental Fig. S6), highlighting the substantial importance of the genetic ablation of *Nod1* on impairing peripheral blood leukocyte recruitment to the circulatory system.

DISCUSSION

In the present study, we assessed the role of NOD1 in the early atherosclerosis development. Our results demonstrate that NOD1 expression is induced in murine and human atherosclerotic tissues and strongly suggest that NOD1 participates in the initial endothelial dysfunction associated to atherosclerosis. In addition, we have identified several mechanisms involving endothelial NOD1 activation. In particular, we have shown that NOD1 mediates monocyte and neutrophil infiltration into atherosclerotic lesions through the autocrine regulation of endothelial VCAM-1 expression. We here also demonstrate that similar to PGNs, oxLDLs up-regulate in an autocrine

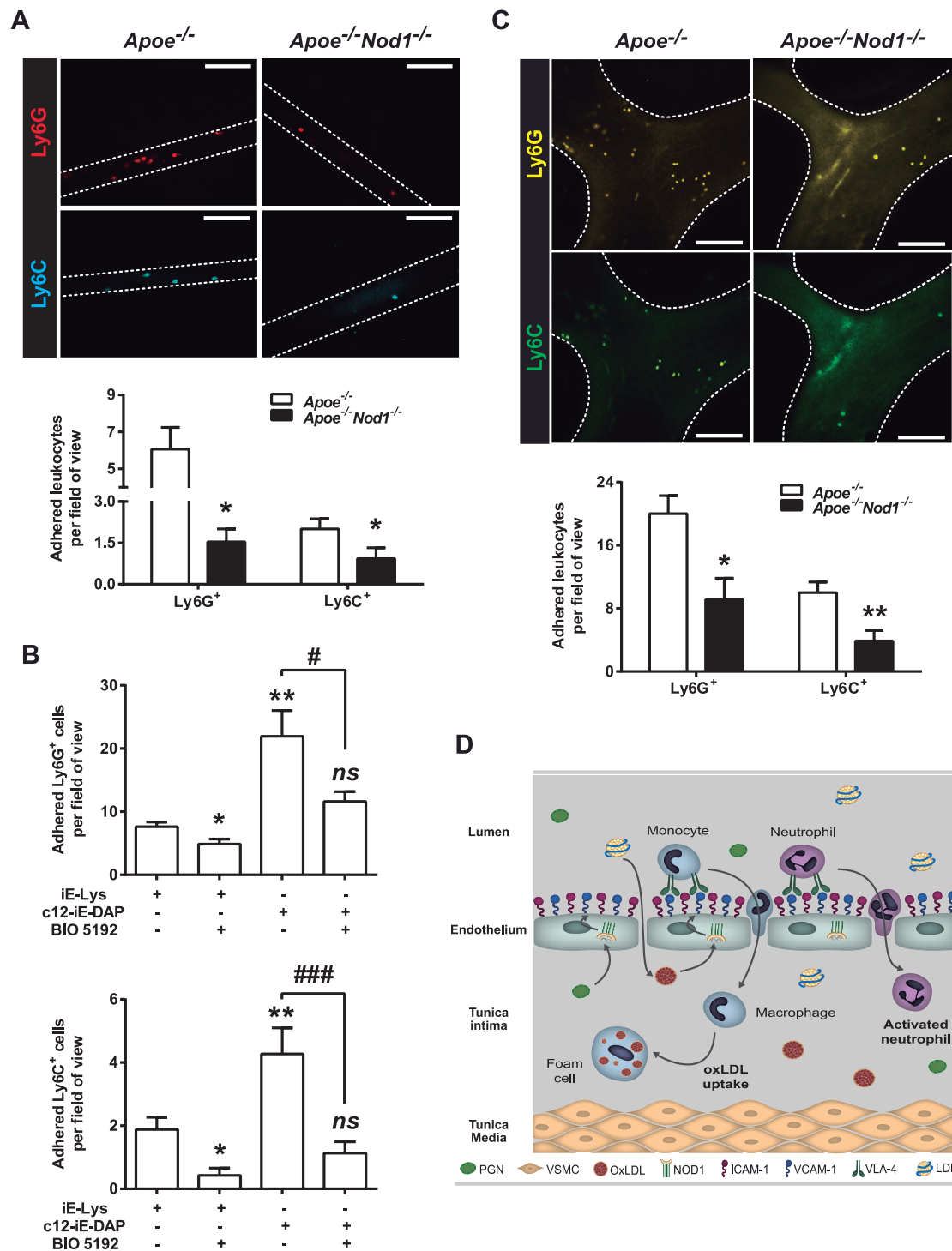


Figure 3. Neutrophils and monocytes have impaired adhesion to the *Apoe*^{-/-}*Nod1*^{-/-} micro- and macrocirculation. *A–C*) Intravital microscopy imaging in the cremaster muscle (*A*, *B*) and carotid bifurcation (*C*) to quantify leukocyte-endothelial wall interactions 3 wk after HFD are shown. Quantification and representative images of adhered Ly6C⁺ classic monocytes and Ly6G⁺ neutrophils to the cremasteric endothelium of *Apoe*^{-/-} (*n* = 5–8) and *Apoe*^{-/-}*Nod1*^{-/-} (*n* = 4–7) mice (*A*). *Apoe*^{-/-} mice were injected intravenously with 100 μg of either iE-Lys (*n* = 22) or c12-iE-DAP (*n* = 20) (*B*). The next day, and before the intravital microscopy recording, 1 mg/kg BIO 5192 was injected intravenously to half of each group of mice. Quantification of adhered Ly6G⁺ neutrophils and Ly6C⁺ monocytes to the endothelium of each set of pretreated *Apoe*^{-/-} mice is represented. Quantification and representative images of adhered Ly6C⁺ monocytes and Ly6G⁺ neutrophils to the carotid bifurcation endothelium of *Apoe*^{-/-} (*n* = 8) and *Apoe*^{-/-}*Nod1*^{-/-} (*n* = 8) mice (*C*). The dotted lines delimit the vascular wall. *D*) In early stages of the disease, once LDL undergoes oxidative modification in the subendothelial space, oxLDLs promote the expression of VCAM-1 in ECs through the cytoplasmic receptor NOD1, enabling monocyte and neutrophil recruitment to the vessel wall. Likewise, bacterial PGN-derived peptides upon recognition by endothelial NOD1 may induce the expression of VCAM-1 that promotes leukocyte infiltration to the subendothelial space and subsequent plaque progression. Data are represented as means ± SEM of the indicated number (*n*) of repeats. **P* < 0.05, ***P* < 0.01 vs. *Apoe*^{-/-} by Student's *t* test (*A*, *C*) or iE-Lys by 1-way ANOVA (*B*); [#]*P* < 0.05, ^{###}*P* < 0.001 vs. c12-iE-DAP (*B*). Ns, not significant. Scale bars, 100 μm (*A*, *C*).

manner the expression of VCAM-1 in ECs through the canonical NOD1 signaling pathway that includes RIP2 and NF- κ B proteins. This early activation of endothelial NOD1 contributes to the vascular dysfunction preceding atherosclerosis progression *in vivo* (Fig. 3D).

So far, only a limited number of studies *in vivo* have shown the effect of NOD1 in the cardiovascular field (23–26). Consistent with a previous report (27), we here establish that *Nod1* deficiency prevents early atherosclerosis development in *Apoe*^{-/-} mice. Although human and murine atherosclerosis differ in several aspects, critical features are shared. Our findings indicate that 1 such feature is NOD1 activation in the endothelium, suggesting prognostic value in atherosclerosis progression to the prompt detection of endothelial NOD1 activation (28–30). Endothelial cell injury during atherogenesis triggers leukocyte infiltration through up-regulation of ECAMs (31). In this regard, VCAM-1 expression has been shown to be stimulated in response to bacterial components in human ECs through the TLR2/4 signaling pathway (32–34), as well as through NOD1/2 in periodontal fibroblasts (35). In agreement with these observations, our findings provide evidence for endothelial NOD1 in directing the expression of VCAM-1 in the inflamed vessel wall. Even though endogenous NOD1/2 ligands are still unknown, here we also unveil new mediators of NOD1 activity. Upon oxLDL endothelial uptake through LOX1, NOD1 classic signaling pathway (36) is activated and modulates the expression of VCAM-1 in a cell extrinsic way alike PGNs. To date, the most studied autocrine signaling loop in vascular endothelium is mediated by the soluble VEGF (37), also known to activate the expression of VCAM-1 mainly through NF- κ B (38). However, no association between VEGF and NOD1 has been established yet. Thus, extensive research on VEGF and other mediators will be required to decipher the autocrine function of the vascular endothelium in the innate response mediated by NOD1.

Exposure to different shear stress forces makes arterial and microvascular ECs display singular patterns on cell surface components (39–41). However, our intravital microscopy imaging studies in both cremaster muscle and carotid artery bifurcation in *Apoe*^{-/-} mice yield consistent results concerning NOD1. The novel and imperative role of NOD1 in firm leukocyte adhesion to the inflamed endothelia extends the significance of the process identified in the present study to other arterial pathologies such as peripheral vascular disease, aneurysm formation, or arteritis (42–44).

Dysfunction of the arterial endothelium is important not only at the inception of the atherosclerotic lesion, but at every stage in plaque development, including the events surrounding plaque rupture. Thus, NOD1 constitutes a potential therapeutic target not only for the blockade of atherosclerosis, but also for other diseases where the endothelium plays a key role such as the pathogenesis of sepsis and its sequelae. Future development of strategies aimed at local or arterial endothelial-specific neutralization of NOD1 may open new therapeutic approaches for the prevention and treatment of atherosclerosis either alone or in combination with standard treatments.

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AUTHOR CONTRIBUTIONS

S. González-Ramos designed the study, performed experiments, analyzed data, and wrote the manuscript; M. Paz-García, C. Rius, A. del Monte-Monge, C. Rodríguez, and V. Fernández-García performed experiments and analyzed data; V. Andrés provided intellectual input and funding, and revised the manuscript; J. Martínez-González provided clinical samples; M. A. Lasunción provided native LDL and oxLDL; P. Martín-Sanz provided intellectual input; O. Soehnlein performed experiments, analyzed data, wrote the manuscript, provided funding and intellectual input; and L. Boscá designed the study, provided funding, and wrote the manuscript.

REFERENCES

1. Brown, R. A., Shantsila, E., Varma, C., and Lip, G. Y. (2017) Current understanding of atherogenesis. *Am. J. Med.* **130**, 268–282
2. Schmidt, E. P., Kuebler, W. M., Lee, W. L., and Downey, G. P. (2016) Adhesion molecules: master controllers of the circulatory system. *Compr. Physiol.* **6**, 945–973
3. Glass, C. K., and Witztum, J. L. (2001) Atherosclerosis. the road ahead. *Cell* **104**, 503–516
4. Zimmer, S., Grebe, A., and Latz, E. (2015) Danger signaling in atherosclerosis. *Circ. Res.* **116**, 323–340
5. Girardin, S. E., Boneca, I. G., Carneiro, L. A., Antignac, A., Jéhanho, M., Viaja, J., Tadin, K., Taha, M. K., Labigne, A., Zähringer, U., Coyle, A. J., DiStefano, P. S., Bertin, J., Sansonetti, P. J., and Philpott, D. J. (2003) Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* **300**, 1584–1587
6. Kobayashi, K., Inohara, N., Hernandez, L. D., Galán, J. E., Núñez, G., Janeway, C. A., Medzhitov, R., and Flavell, R. A. (2002) RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* **416**, 194–199
7. Hasegawa, M., Fujimoto, Y., Lucas, P. C., Nakano, H., Fukase, K., Núñez, G., and Inohara, N. (2008) A critical role of RICK/Rip2 polyubiquitination in Nod-induced NF- κ B activation. *EMBO J.* **27**, 373–383
8. Viala, J., Chaput, C., Boneca, I. G., Cardona, A., Girardin, S. E., Moran, A. P., Athman, R., Mémet, S., Huerre, M. R., Coyle, A. J., DiStefano, P. S., Sansonetti, P. J., Labigne, A., Bertin, J., Philpott, D. J., and Ferrero, R. L. (2004) Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat. Immunol.* **5**, 1166–1174
9. Zhang, S. H., Reddick, R. L., Piedrahita, J. A., and Maeda, N. (1992) Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* **258**, 468–471

10. Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setälä, K., Walsh, A., Verstuyft, J. G., Rubin, E. M., and Breslow, J. L. (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* **71**, 343–353
11. Chamailard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M. A., Foster, S. J., Mak, T. W., Nuñez, G., and Inohara, N. (2003) An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* **4**, 702–707
12. Luque, A., Carpizo, D. R., and Iruela-Arispe, M. L. (2003) ADAMTS1/METH1 inhibits endothelial cell proliferation by direct binding and sequestration of VEGF165. *J. Biol. Chem.* **278**, 23656–23665
13. Fuster, J. J., González-Navarro, H., Vinué, A., Molina-Sánchez, P., Andrés-Manzano, M. J., Nakayama, K. I., Nakayama, K., Díez-Juan, A., Bernad, A., Rodríguez, C., Martínez-González, J., and Andrés, V. (2011) Deficient p27 phosphorylation at serine 10 increases macrophage foam cell formation and aggravates atherosclerosis through a proliferation-independent mechanism. *Arterioscler. Thromb. Vasc. Biol.* **31**, 2455–2463
14. Fueger, B. J., Czernin, J., Hildebrandt, I., Tran, C., Halpern, B. S., Stout, D., Phelps, M. E., and Weber, W. A. (2006) Impact of animal handling on the results of 18F-FDG PET studies in mice. *J. Nucl. Med.* **47**, 999–1006
15. Lichtenstein, L., Serhan, N., Espinosa-Delgado, S., Fabre, A., Annema, W., Tietge, U. J., Robaye, B., Boeynaems, J. M., Laffargue, M., Perret, B., and Martinez, L. O. (2015) Increased atherosclerosis in P2Y13/apolipoprotein E double-knockout mice: contribution of P2Y13 to reverse cholesterol transport. *Cardiovasc. Res.* **106**, 314–323
16. Rius, C., and Sanz, M. J. (2015) Intravital microscopy in the cremaster muscle microcirculation for endothelial dysfunction studies. *Methods Mol. Biol.* **1339**, 357–366
17. Swirski, F. K., and Robbins, C. S. (2013) Neutrophils usher monocytes into sites of inflammation. *Circ. Res.* **112**, 744–745
18. Jonsson, A. L., and Bäckhed, F. (2017) Role of gut microbiota in atherosclerosis. *Nat. Rev. Cardiol.* **14**, 79–87
19. Gimbrone, M. A., Jr., and García-Cardena, G. (2016) Endothelial cell dysfunction and the pathobiology of atherosclerosis. *Circ. Res.* **118**, 620–636
20. Cai, H., and Harrison, D. G. (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ. Res.* **87**, 840–844
21. Libby, P., Ridker, P. M., and Maseri, A. (2002) Inflammation and atherosclerosis. *Circulation* **105**, 1135–1143
22. Pirillo, A., Norata, G. D., and Catapano, A. L. (2013) LOX-1, OxLDL, and atherosclerosis. *Mediators Inflamm.* **2013**, 152786
23. Prieto, P., Vallejo-Cremades, M. T., Benito, G., González-Peramato, P., Francés, D., Agra, N., Terrón, V., González-Ramos, S., Delgado, C., Ruiz-Gayo, M., Pacheco, I., Velasco-Martín, J. P., Regadera, J., Martín-Sanz, P., López-Collazo, E., Boscá, L., and Fernández-Velasco, M. (2014) NOD1 receptor is up-regulated in diabetic human and murine myocardium. *Clin. Sci. (Lond.)* **127**, 665–677
24. Yang, H., Li, N., Song, L. N., Wang, L., Tian, C., Tang, C. S., Du, J., Li, H. H., Yu, X. H., and Wang, H. X. (2015) Activation of NOD1 by DAP contributes to myocardial ischemia/reperfusion injury via multiple signaling pathways. *Apoptosis* **20**, 512–522
25. Motomura, Y., Kanno, S., Asano, K., Tanaka, M., Hasegawa, Y., Katagiri, H., Saito, T., Hara, H., Nishio, H., Hara, T., and Yamasaki, S. (2015) Identification of pathogenic cardiac CD11c⁺ macrophages in Nod1-mediated acute coronary arteritis. *Arterioscler. Thromb. Vasc. Biol.* **35**, 1423–1433
26. Val-Blasco, A., Piedras, M. J. G. M., Ruiz-Hurtado, G., Suarez, N., Prieto, P., Gonzalez-Ramos, S., Gómez-Hurtado, N., Delgado, C., Pereira, L., Benito, G., Zaragoza, C., Domenech, N., Crespo-Leiro, M. G., Vasquez-Echeverri, D., Nuñez, G., Lopez-Collazo, E., Boscá, L., and Fernández-Velasco, M. (2017) Role of NOD1 in heart failure progression via regulation of Ca²⁺ handling. *J. Am. Coll. Cardiol.* **69**, 423–433
27. Kanno, S., Nishio, H., Tanaka, T., Motomura, Y., Murata, K., Ihara, K., Onimaru, M., Yamasaki, S., Kono, H., Sueishi, K., and Hara, T. (2015) Activation of an innate immune receptor, Nod1, accelerates atherogenesis in Apoe^{-/-} mice. *J. Immunol.* **194**, 773–780
28. Deanfield, J. E., Halcox, J. P., and Rabelink, T. J. (2007) Endothelial function and dysfunction: testing and clinical relevance. *Circulation* **115**, 1285–1295
29. Davignon, J., and Ganz, P. (2004) Role of endothelial dysfunction in atherosclerosis. *Circulation* **109** (23 Suppl 1), III27–III32
30. Martin, B. J., and Anderson, T. J. (2009) Risk prediction in cardiovascular disease: the prognostic significance of endothelial dysfunction. *Can. J. Cardiol.* (25 Suppl A), 15A–20A
31. Pober, J. S., Min, W., and Bradley, J. R. (2009) Mechanisms of endothelial dysfunction, injury, and death. *Annu. Rev. Pathol.* **4**, 71–95
32. Zeuke, S., Ulmer, A. J., Kusumoto, S., Katus, H. A., and Heine, H. (2002) TLR4-mediated inflammatory activation of human coronary artery endothelial cells by LPS. *Cardiovasc. Res.* **56**, 126–134
33. Ogawa, H., Rafiee, P., Heidemann, J., Fisher, P. J., Johnson, N. A., Otterson, M. F., Kalyanaraman, B., Pritchard, K. A., Jr., and Binion, D. G. (2003) Mechanisms of endotoxin tolerance in human intestinal microvascular endothelial cells. *J. Immunol.* **170**, 5956–5964
34. Lee, I. T., Lin, C. C., Hsu, C. K., Wu, M. Y., Cho, R. L., and Yang, C. M. (2014) Resveratrol inhibits Staphylococcus aureus-induced TLR2/MyD88/NF-κB-dependent VCAM-1 expression in human lung epithelial cells. *Clin. Sci. (Lond.)* **127**, 375–390
35. Liu, J., Wang, Y., and Ouyang, X. (2014) Beyond toll-like receptors: porphyromonas gingivalis induces IL-6, IL-8, and VCAM-1 expression through NOD-mediated NF-κB and ERK signaling pathways in periodontal fibroblasts. *Inflammation* **37**, 522–533
36. Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamaoka, S., and Nuñez, G. (2001) Nod2, a Nod1/Apa1 family member that is restricted to monocytes and activates NF-κB. *J. Biol. Chem.* **276**, 4812–4818
37. Lee, S., Chen, T. T., Barber, C. L., Jordan, M. C., Murdock, J., Desai, S., Ferrara, N., Nagy, A., Roos, K. P., and Iruela-Arispe, M. L. (2007) Autocrine VEGF signaling is required for vascular homeostasis. *Cell* **130**, 691–703
38. Kim, I., Moon, S. O., Kim, S. H., Kim, H. J., Koh, Y. S., and Koh, G. Y. (2001) Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-κB activation in endothelial cells. *J. Biol. Chem.* **276**, 7614–7620
39. Chiu, J. J., and Chien, S. (2011) Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. *Physiol. Rev.* **91**, 327–387
40. Aird, W. C. (2007) Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. *Circ. Res.* **100**, 174–190
41. Ortega-Gómez, A., Salvermoser, M., Rossaint, J., Pick, R., Brauner, J., Lemnitzer, P., Tilgner, J., de Jong, R. J., Megens, R. T. A., Jamsabji, J., Döring, Y., Pham, C. T., Scheiermann, C., Siess, W., Drechsler, M., Weber, C., Grommes, J., Zarbock, A., Walzog, B., and Soehnlein, O. (2016) Cathepsin G controls arterial but not venular myeloid cell recruitment. *Circulation* **134**, 1176–1188
42. Cid, M. C., Cebrián, M., Font, C., Coll-Vinent, B., Hernández-Rodríguez, J., Esparza, J., Urbano-Márquez, A., and Grau, J. M. (2000) Cell adhesion molecules in the development of inflammatory infiltrates in giant cell arteritis: inflammation-induced angiogenesis as the preferential site of leukocyte-endothelial cell interactions. *Arthritis Rheum.* **43**, 184–194
43. Lee, E. S., Van Spyk, E. N., Chun, K. C., Pitts, R. L., Wu, M. H., and Yuan, S. Y. (2012) Monocytic adhesion molecule expression and monocyte-endothelial cell dysfunction are increased in patients with peripheral vascular disease versus patients with abdominal aortic aneurysms. *J. Surg. Res.* **177**, 373–381
44. Gopal, K., Kumar, K., Nandini, R., Jahan, P., and Kumar, M. J. (2010) High fat diet containing cholesterol induce aortic aneurysm through recruitment and proliferation of circulating agranulocytes in apoE knock out mice model. *J. Thromb. Thrombolysis* **30**, 154–163

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Disruption of the CCL1-CCR8 axis inhibits vascular Treg recruitment and function and promotes atherosclerosis in mice



Marian Vila-Caballer^{a,b,1}, José M. González-Granado^{c,d,e,f,1}, Virginia Zorita^c, Yafa N. Abu Nabah^a, Carlos Silvestre-Roig^{c,g}, Alberto del Monte-Monge^{c,d}, Pedro Molina-Sánchez^{c,2}, Hafid Ait-Oufella^h, María J. Andrés-Manzano^{c,d}, María J. Sanzⁱ, Christian Weber^g, Leonor Kremer^j, Julio Gutiérrez^j, Ziad Mallat^{h,k}, Vicente Andrés^{c,d,*}

^a Instituto de Biomedicina de Valencia (IBV-CSIC), Valencia, Spain

^b Universidad Cardenal Herrera-CEU (CEU Universities), Valencia, Spain

^c Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain

^d CIBER de Enfermedades Cardiovasculares (CIBER-CV), Spain

^e LamImSys Laboratory, Instituto de Investigación Sanitaria Hospital 12 de Octubre (imas12), Madrid, Spain

^f Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid (UAM), Madrid, Spain

^g Institute for Cardiovascular Prevention (IPEK), Ludwig Maximilian University, Munich, Germany

^h Institut National de la Santé et de la Recherche Médicale (INSERM), Paris Cardiovascular Research Center, Paris, France

ⁱ Departamento de Farmacología, Universidad de Valencia and Instituto de Investigación Sanitaria-INCLIVA, Valencia, Spain

^j Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología-CSIC, Madrid, Spain

^k Division of Cardiovascular Medicine, University of Cambridge, Cambridge, UK

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ABSTRACT

The CC chemokine 1 (CCL1, also called I-309 or TCA3) is a potent chemoattractant for leukocytes that plays an important role in inflammatory processes and diseases through binding to its receptor CCR8. Here, we investigated the role of the CCL1-CCR8 axis in atherosclerosis. We found increased expression of CCL1 in the aortas of atherosclerosis-prone fat-fed apolipoprotein E (*Apoe*)-null mice; moreover, *in vitro* flow chamber assays and *in vivo* intravital microscopy demonstrated an essential role for CCL1 in leukocyte recruitment. Mice doubly deficient for CCL1 and *Apoe* exhibited enhanced atherosclerosis in aorta, which was associated with reduced plasma levels of the anti-inflammatory interleukin 10, an increased splenocyte Th1/Th2 ratio, and a reduced regulatory T cell (Treg) content in aorta and spleen. Reduced Treg recruitment and aggravated atherosclerosis were also detected in the aortas of fat-fed low-density lipoprotein receptor-null mice treated with CCR8 blocking antibodies. These findings demonstrate that disruption of the CCL1-CCR8 axis promotes atherosclerosis by inhibiting interleukin 10 production and Treg recruitment and function.

1. Introduction

Atherosclerotic disease is the main cause of mortality worldwide [1,2]. The disease features a robust inflammatory component that is characterized by lipid deposition and leukocyte recruitment in the artery wall during all disease phases [3]. The initiation and progression of atherosclerosis both involve chronic inflammatory responses to

lipoprotein deposition in the arterial intima. These inflammatory responses are caused by activation of both innate and adaptive immunity [4,5]. The innate immune response is triggered by the accumulation of certain lipid and protein components of apoB lipoproteins, which cause an inflammatory reaction that activates endothelial cells and stimulates the entry of bone-marrow-derived monocytes into the intima. Intimal Ly6C^{hi} monocytes differentiate into macrophages that acquire an

Abbreviations: *Apoe*, apolipoprotein E; *Apoe*-KO, apolipoprotein E-null mice; *Apoe/Ccl1*-DKO, apolipoprotein E-CCL1 doubly-deficient mice; BMdM, bone marrow-derived macrophage; CCL1, CC chemokine 1 (also named TCA3 and I-309); CCR8, C-C motif chemokine receptor 8; HFD, high-fat diet; IL-10, interleukin 10; *Ldlr*-KO, low-density lipoprotein receptor-null mice; RFP, red fluorescent protein; Th, helper T cell; Treg, regulatory T cell; VSMC, vascular smooth muscle cell

* Corresponding author.

E-mail address: vandres@cnic.es (V. Andrés).

¹ These authors contributed equally.

² The Tisch Cancer Institute at Mount Sinai, New York, USA.

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inflammatory phenotype, which promotes atherosclerosis development [4,5]. The adaptive immune response involves CD4⁺ T cells, which have been shown to promote atherosclerosis in fat-fed mice [6]. When naïve CD4⁺ T cells are activated after the recognition of an antigen presented by dendritic cells, they undergo a process of division and differentiation into several helper T (Th) cell subsets, including Th1, Th2, Th17, and regulatory T cells (Tregs) [7]. Several studies have shown that Th1 responses are pro-atherogenic in mice [8–11], contrasting with the atheroprotective effect of Tregs [12,13]. Regarding anti-inflammatory cytokines, both interleukin-10 (IL-10) [14,15] and transforming growth factor- β [16,17] have been shown to protect against atherosclerosis in mice.

Chemokines are key mediators of cell trafficking [18–20]. The C-C motif chemokine receptor 8 (CCR8) ligand CC chemokine 1 (CCL1, also called TCA3 and I-309) was initially described as a cytokine expressed by activated T lymphocytes [21]. CCL1 acts as a potent chemoattractant for monocytes and lymphocytes [22] and has been proposed to contribute to macrophage and lymphocyte recruitment and activation [23] in several inflammatory diseases, including type 1 diabetes [24], atopic asthma [25], atopic dermatitis [26,27], Kaposi sarcoma [28], septic peritonitis [29], liver fibrosis [30], and experimental autoimmune encephalomyelitis [31]. In addition, CCL1 may be involved in immune homeostasis, particularly through effects on Tregs [32]. In the context of the cardiovascular system, CCL1 is expressed in endothelial cells, macrophages, and the extracellular area of human atherosclerotic plaques [33]; moreover, CCL1 stimulates vascular smooth muscle cell (VSMC) migration [28] and activates endothelial cells in response to arterial wall injury [34]. However, the role of CCL1 in atherosclerosis remains unknown.

In the present study, we investigated whether the CCL1-CCR8 axis plays a role in mouse atherosclerosis. To this end, we generated atherosclerosis-prone mice deficient for CCL1 by crossing *Ccl1*-null mice with apolipoprotein E-null mice (*ApoE*-KO). Our results show that disruption of the CCL1-CCR8 axis impairs vascular Treg recruitment and function and promotes atherosclerosis.

2. Materials and methods

2.1. Mice and diets

Wild-type mice, *ApoE*-KO mice, and LDL receptor-null mice (*Ldlr*-KO, CD45.1 genotype) were obtained from Charles River Laboratories (Wilmington, Massachusetts, USA). Transgenic Foxp3-mRFP mice expressing red fluorescent protein in Tregs were a gift from Dr. Richard A. Flavell (Yale University School of Medicine, New Haven, Connecticut, USA) [35]. *Ccl1*-KO [36] and *ApoE*-KO mice were crossbred to generate *ApoE/Ccl1*-DKO mice. All mice were on a C57BL/6J genetic background. Mice for the flow chamber and intravital experiments were males. All other experiments were performed using littermates of both genders. After weaning, mice were maintained on a low-fat (control) standard diet (2.8% fat; Panlab, Barcelona, Spain) or placed on pro-atherogenic high-fat diet (HFD) for the indicated periods, starting at 2 months of age (10.8% total fat, 0.75% cholesterol, S4892-E010, Ssniff, Soest, Germany). Animal experiments were approved by the local ethics committee and the Spanish Ministry of Agriculture and Fisheries, Food and Environment. All animal procedures conformed to EU Directive 86/609/EEC and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

2.2. *Ccl1* mRNA expression studies

Femoral bone marrow-derived macrophages (BMDMs) and aortic VSMCs cultures were obtained from *ApoE*-KO mice as previously described [37]. Cultures were maintained at 37 °C in a humidified 5% CO₂

atmosphere with DMEM/10% FBS/10% macrophage-colony stimulating factor medium for BMDMs, and 20% FBS/DMEM/Fungizone medium for VSMCs. VSMC cultures were not used above passage 7, and purity was confirmed by immunohistochemistry with anti-SM α -actin monoclonal alkaline phosphatase conjugated antibody (1/20 dilution, clone 1A4, a-5691, Sigma, San Luis, Missouri, USA) and Fast Red substrate (Sigma). BMDMs and VSMCs were stimulated for 20 h with tumor necrosis factor α (TNF α , 10 or 50 ng/ml, Sigma), interferon γ (IFN γ , 20 or 200 U/ml, Peprotech, Rocky Hill, NJ, USA), or angiotensin II (AngII, 10⁻⁶ or 10⁻⁵ M, Calbiochem, Merck KGaA, Darmstadt, Germany) (all of mouse origin).

Total RNA was isolated from *ApoE*-KO mice aortas and cultured cells using TRIzol (Invitrogen, Carlsbad, California, USA). RNA purity and concentration were determined from the A260/280 ratio. Complementary DNA was obtained by reverse transcription using Superscript III First Strand Synthesis Supermix (Invitrogen) and amplified in TaqMan gene Expression Assays (TaqMan MGB probes, FAMTM dye-labeled): Mm00441236 (*Ccl1*), Mm99999115 (*Ccr8*), and Mm00607939 (*β -actin*, used as endogenous control). The relative *Ccl1* expression level was calculated from technical triplicates for each sample using the 2^{- $\Delta\Delta C_t$} formula and were expressed relative to *ApoE*-KO mice fed the control diet or untreated cells.

2.3. Metabolic measurements and circulating leukocyte analysis

Mouse blood was collected in EDTA tubes. For circulating lipid measurements (total cholesterol, HDL-cholesterol, and triglycerides), mice were fasted overnight and plasma was obtained by blood centrifugation. Lipids were measured using enzymatic procedures (WAKO, Richmond, VA, USA). HDL-cholesterol was determined after precipitation of the apoB-containing lipoproteins with dextran-sulphate/MgCl₂ (Sigma) [38].

Following lysis of erythrocytes, circulating T-cells (CD3⁺), B-cells (CD19⁺), and monocytes/granulocytes (CD11b⁺) were analyzed by flow cytometry after leukocytes were stained for rat anti-mouse cd11b-FITC IgG2b monoclonal antibody (1/100 dilution, clone M1/70, 557,396, Becton Dickinson, Franklin Lakes, New Jersey, USA), hamster anti-mouse CD3e chain-PE-Cy7 monoclonal antibody (1/10 dilution, clone 145-2C11, 552,774, Becton Dickinson), and rat anti-mouse CD19-FITC IgG2a monoclonal antibody (1/25 dilution, clone 6D5, MCA1439F, Serotec, Dusseldorf, Germany). Monocytes were distinguished from granulocytes by their granular content as reflected in low/high side scatter (SSC) [39].

2.4. Quantification of atherosclerosis burden and immunohistochemical characterization of atherosclerotic lesions

All studies were performed by an investigator blinded to genotype. Atherosclerosis was quantified in *ApoE*-KO and *ApoE/Ccl1*-DKO mice fed HFD for 2 months, and in γ -irradiated and bone marrow-transplanted *Ldlr*-KO mice fed HFD for 1 week. Aortic tissue was embedded in paraffin and cut into 5- μ m cross-sections. The extent of atherosclerosis was calculated as the intimal area in the aortic root and ascending aorta as previously described [37]. Five cross-sections separated 8–12 μ m were analyzed in each region. The distance between sections in the aortic root and ascending aorta was approximately 70 μ m.

Immunohistopathological examination of atheromas included the quantification of VSMCs, collagen, necrotic core, T lymphocytes, CCR8-immunoreactive cells, macrophages, proliferating cells, and apoptotic cells (see below). The content of lymphocytes and proliferating cells was expressed as cells/mm², whereas the content of VSMCs, collagen, necrotic core, CCR8-expressing cells and macrophages was expressed as both percentage (stained area normalized by total plaque area) and absolute content (mm² of plaque area stained for each marker).

VSMCs were identified with alkaline-phosphatase-conjugated mouse anti-SM α -actin monoclonal antibody (1/20 dilution, clone 1A4,

a-5691, Sigma) and Fast Red substrate (Sigma). Collagen was identified with Masson's trichrome stain. Necrotic cores were identified as hollows formed in preparations as a consequence of the deparaffinization protocol. T lymphocytes were identified with biotin-conjugated anti-CD4 antibody (0.5 mg/ml, clone L3 T3, BD-PharMingen) and streptavidin-conjugated Alexa-fluor 488 (1/300 dilution, S11223, Invitrogen) and were mounted after nuclear counterstaining using Topro-3 (1/1000 dilution, T3605, Invitrogen). CCR8+ area was identified with goat anti-CCR8 polyclonal antibody (1/200 dilution, ab1663, Abcam, Cambridge, United Kingdom). Macrophages were identified with rat anti-Mac3 monoclonal antibody (1/200 dilution, clone M3/84, sc-19,991, Santa Cruz Biotechnology). Specimens were counterstained with hematoxylin. Proliferating cells were identified with rabbit anti-Ki67 monoclonal antibody (prediluted, clone SP6, MAD-000319QD Master Diagnóstica, Granada, Spain) and apoptotic cells with rabbit anti-cleaved Caspase3 (Asp175) polyclonal antibody (1/10 dilution, 9661, Cell signalling, Danvers, Massachusetts, USA).

After incubation with primary antibodies and extensive washes, specimens were incubated with appropriate HRP-conjugated secondary antibodies: goat anti-rat-HRP (sc-2006) for Mac3, donkey anti-goat-HRP (sc-2042) for CCR8 and goat anti-rabbit-HRP (sc-2004) for Ki67 and Caspase3 (all from Santa Cruz Biotechnology, 1/300 dilution). Immunocomplexes were detected using streptavidin-HRP (TS-060-HR, Lab vision Corporation, USA) and DAB substrate (BUF021A, AbD Serotec). Images were captured with an Olympus CAMEDIA-C5060 wide zoom digital camera mounted on a stereomicroscope Axiolab (Carl Zeiss, Jena, Germany) except for T lymphocyte immunofluorescence, which was visualized with a TCS/SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany). All images were analyzed by computer-assisted morphometric analysis using Metamorph software (Molecular Devices, California, USA).

2.5. Flow chamber

Whole blood was collected from 5-month-old wild-type male mice (pooled from 6 to 8 mice for each replicate) and perfused in flow chambers containing vehicle-coated or CCL1-coated cover slips. Cover slips (Corning incorporation, Sigma) were coated with soluble mouse recombinant CCL1/TCA3 (5 mg/ml, R&D systems, Minneapolis, USA) and incubated at 4 °C for 18 h. To inhibit non-specific interactions with polystyrene, cover slips were previously incubated with 1% bovine serum albumin (Sigma) at 37 °C for 1 h. Subsequently, vehicle- and CCL1-coated cover slips were perfused with whole blood (diluted 10-fold and maintained at 37 °C) for 5 min at a constant shear rate of 0.75 dynes/cm², followed by perfusion for 20 min with Hanks' balanced salt solution (HBSS). The Glycotech flow chamber was assembled, and 10 random fields of interacting leukocytes were visualized and recorded on the vehicle-coated or CCL1-coated cover slip surface (20X objective, 10X eyepiece) by phase contrast microscopy.

2.6. Intravital microscopy in cremaster muscle

Male wild-type, *Apoe*-KO, and *Apoe/Ccl1-DKO* mice (approximately 24 weeks old) fed the HFD for 1 week were anesthetized by intraperitoneal injection with a mixture of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (200 mg/kg). A polyethylene catheter was placed in the jugular vein to permit intravenous administration of additional anesthetic if needed. The cremaster muscle was dissected free of tissues and exteriorized onto an optical clear viewing pedestal. The muscle was cut longitudinally with a cautery and held flat against the pedestal by attaching silk sutures to the corners of the tissue. The muscle was then perfused continuously at a rate of 1 ml/min with warmed bicarbonate-buffered saline (pH 7.4). The cremasteric microcirculation was then observed using an intravital microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, Netherlands) equipped with a 50× objective (Nikon SLDW, Badhoevedorp, The Netherlands) and a 10×

eyepiece. A video camera (Sony SSC-C350P, Koeln, Germany) mounted on the microscope projected the image onto a colour monitor and the images were video recorded for playback analysis and quantified as described [40].

2.7. Proliferation assays

CD4+ T cells, CD4 + CD25+ Tregs and CD4 + CD25- effector T cells were purified from spleens using magnetic cell separation (Miltenyi Biotech) as previously described [12]. To analyze T cell proliferation, CD4+ cells (1×10^5 cells/well) were co-cultured for 72 h at 37 °C with 10^4 CD11c+ dendritic cells and soluble anti-CD3 (1 µg/ml). Cells were pulsed with [³H]-thymidine for the last 18 h (1 µCi/well, Amersham, Little Chalfont, United Kingdom). Thymidine incorporation was assessed using a TopCount NXT scintillation counter (Perkin Elmer, Waltham, Massachusetts, USA).

To analyze Treg functions, effector T cells were cultured alone or co-cultured with purified Tregs (at 1:8, 1:4, 1:2 or 1:1 ratio) in round-bottom 96-well microplates (0.5×10^5 cells/well; total volume 200 µl/well). Cells were stimulated with purified soluble CD3-specific antibody (1 µg/ml, BD-PharMingen) in the presence of antigen-presenting cells purified on CD11c-coated magnetic beads (Miltenyi Biotech). Cells were cultured at 37 °C for 72 h and pulsed with [³H] thymidine for the last 18 h (1 µCi/well, Amersham). Thymidine incorporation was assessed using a TopCount NXT scintillation counter (Perkin Elmer).

2.8. IL-10 quantification

IL-10 was quantified from blood samples and culture medium with a specific ELISA (Bender MedSystems, Vienna, Austria and BD Biosciences, San Jose, California, USA). Blood samples of *Apoe*-KO and *Apoe/Ccl1-KO* mice fed the HFD for 1 week or 2 months were collected in EDTA tubes to obtain plasma by centrifugation. For detection of IL-10 production in culture medium, splenocytes (10^6 /ml) were stimulated with LPS (10 µg/ml) and IFN-γ (100 UI/ml) for 24 or 48 h. For detection in culture medium of untreated BMdMs, supernatants were concentrated with Amicon Ultra-15 3000 Nominal Molecular Weight Limit Centrifugal filters (Millipore, Burlington, Massachusetts, USA). The amount of IL-10 was normalized to total protein content.

2.9. Bone marrow transplantation and anti-CCR8 antibody treatment

CD45.1 *Ldlr*-KO recipient mice received 13 Gy of total body irradiation administered in two treatments from a ¹³⁷Cs source (MARK 1–68 A; Shepherd and Associates). Bone marrow cells from CD45.2 Foxp3-mRFP mice were transplanted into *Ldlr*-KO recipients by i.v. injection immediately after irradiation. Approximately four weeks after transplantation, the chimeric condition of the mice was assessed by flow cytometry analysis of blood cells stained with a combination of FITC-conjugated anti-CD45.1 and v450-conjugated anti-CD45.2 antibodies (both from Tonbo Bioscience, San Diego, California, USA), which confirmed that > 90% of the cells analyzed were derived from the transplanted bone marrow cells.

One month after transplant, irradiated *Ldlr*-KO mice were challenged with HFD and received i.p. injections of either anti-CCR8 antibody or isotype control every two days for 1 week (100 µg/mouse). Atherosclerosis burden was quantified in the aortic root by hematoxylin/eosin staining. Treg content in the abdominal aorta was estimated by qPCR quantification of mRFP mRNA levels. Total RNA was isolated with Qiazol Lysis Reagent (Qiagen, Hilden, Germany) and isopropanol precipitation or with the RNeasy Mini kit (Qiagen). RNA concentration and purity were assessed from the ratio of absorbances at 260 and 280 nm. Complementary DNA was synthesized from total RNA (0.1 to 1 µg per reaction) with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA) using random primers and RNase Inhibitor. Quantitative PCR was performed with the

ABI PRISM 7900HT Sequence Detection System using PCR Power SYBR Green PCR Master Mix (both from Applied Biosystems), with technical triplicates for each sample. Gene expression was analyzed by the comparative Ct method using the qBasePLUS software (Biogazelle, Zwijnaarde, Belgium) and the housekeeping gene β -actin as the internal control. The RT qPCR primer sequences were as follows: Mouse RFP-FW: 5'-ACTACAAGAAGCTGTCCTTCC-3'; Mouse RFP-RV: 5'-ATGTTTA AACTTACCCTCCAG-3'; Mouse β -actin-FW: 5'-GACGGCCAGGTCATC ACTATTG-3'; Mouse β -actin-RV: 5'-AGGAAGGCTGGAAAAGAGCC-3'.

2.10. Flow cytometry quantification of aortic and splenic immune cell content

Splenic immune cell content determination was performed as previously described [7,41,42]. Briefly, spleens from 11-month-old mice fed control diet were isolated and cellular content was obtained after mechanical processing. Cellular suspension was cultured in RPMI + 10%FCS and stimulated with phorbol 12 myristate 13 acetate (PMA, 10 ng/ml, Sigma-Aldrich) and ionomycin (1 μ M, Sigma-Aldrich) in the presence of brefeldin A (5 μ g/ml, Sigma-Aldrich) for 4 h at 37 °C. After washing, cells were fixed using Fixation/Permeabilization buffer (Invitrogen) for Th1 and Th2 determination and Foxp3 Fixation/Permeabilization buffer (BD-Pharmingen) for Treg staining. Aortic immune cell content quantification was performed as previously described [43,44]. Aortas from 11-month-old *Apoe*-KO and *Apoe/Ccl1*-DKO mice fed the HFD for 1 week were isolated and digested for 1 h at 37 °C with an enzyme cocktail containing collagenase I (450 units/ml), collagenase XI (250 units/ml), DNase I (120 units/ml), and hyaluronidase (120 units/ml). After washing, cells were incubated with RPMI + 10%FCS for 1 h at 37 °C to restore internalized surface antigens. Splenic or aortic isolated cells were then stained as indicated with rat anti-mouse CD4 (clone RM4-5), rat anti-mouse CD25 (clone PC61.5), rat anti-mouse FOXP3 (clone FJK-16 s), rat anti-mouse IFN γ (clone XMG1.2), and rat anti-mouse IL-4 (clone 11B11) (all from Invitrogen) and were analyzed with a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). Data were analyzed with FlowJo Software 10.1 (Flowjo LLC, Ashland, Oregon, USA).

2.11. Statistical analysis

Results are represented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed with Prism GraphPad Software (La Jolla, California, USA) and IBM SPSS (Armonk, New York, USA). Statistical significance was calculated by two-tailed Student's *t*-test or ANOVA with Bonferroni's post-hoc multiple comparison test, as appropriate. Data normality was examined using Shapiro-Wilk test, whereas homoscedasticity was confirmed by Levene's test. Non-parametric tests were applied when necessary. Results were considered statistically significant for *p*-values < .05.

3. Results

3.1. CCL1 ablation aggravates atherosclerosis in fat-fed *Apoe*-KO mice

We first performed qPCR studies to quantify aortic *Ccl1* mRNA expression in *Apoe*-KO mice fed either normal chow or a HFD for 1 week and 2 months, revealing a progressive induction of *Ccl1* in fat-fed mice (Fig. 1A). To investigate which cell types contribute to *Ccl1* upregulation under pro-inflammatory conditions in the artery wall, we performed experiments with primary cultures of VSMCs and BMDMs derived from *Apoe*-KO mice. Treatment of VSMCs with the proatherogenic agents TNF α and IFN γ , but not with Ang II, upregulated *Ccl1* mRNA expression level, with no significant effects on *Ccr8* mRNA levels (Fig. 1B, left). Compared with VSMCs, BMDMs exhibited lower level of *Ccl1* and *Ccr8* expression, which was not significantly affected by TNF α , IFN γ , and AngII (Fig. 1B, right). These results suggest that VSMCs

contribute significantly to *Ccl1* induction in the inflamed vessel wall of fat-fed *Apoe*-KO mice.

To assess whether CCL1 plays a role in atherosclerosis, we generated *Apoe/Ccl1*-DKO mice doubly deficient for *Apoe* and *Ccl1*. As expected, body weight in mice fed normal chow increased significantly during post-natal development and was higher in males than in age-matched females (Supplementary Fig. S1A). No between-genotype differences were seen in body weight except in the youngest male *Apoe/Ccl1*-DKO mice, which were slightly leaner than age-matched *Apoe*-KO controls (Supplementary Fig. S1A). Likewise, *Ccl1* deficiency did not affect plasma levels of total cholesterol, HDL-cholesterol, or triglycerides in mice fed either normal chow or challenged for 2 months with the HFD (Supplementary Fig. S1B). Fat feeding significantly increased total cholesterol and triglycerides while decreasing HDL-cholesterol in plasma of mice of both genotypes.

We also analyzed circulating leukocyte populations. Consistent with previous studies [45], challenging mice for 2 months with HFD significantly increased the percentage of granulocytes and reduced the percentage of B-cells, without significantly affecting the relative abundance of monocytes and T-cells (Supplementary Fig. S1C). *Ccl1* disruption did not affect the relative abundance of these circulating blood cells in either normal-diet-fed mice or HFD-fed mice (Supplementary Fig. S1C). Similarly, CD4 T-cell percentages among CD45+ cells were undistinguishable in aorta, spleen and blood of *Apoe*-KO and *Apoe/Ccl1*-DKO mice fed control diet, as revealed by flow cytometry (Supplementary Fig. S1D).

To assess the role of CCL1 in atherosclerosis, we quantified disease burden in aortic cross-sections of mice of both genotypes challenged with the HFD for 2 months. These studies revealed a significant increase in atheroma size in both the aortic root and ascending aorta of fat-fed *Apoe/Ccl1*-DKO mice compared with *Apoe*-KO counterparts (Fig. 2A). We also assessed the consequence of inactivating *Ccl1* on plaque composition. *Apoe/Ccl1*-DKO atheromas had lower percentage of area covered by macrophages, and this difference reached statistical significance in the aortic root (Fig. 2B). However, no between-genotype differences were found in absolute macrophage content (mm² of Mac3-immunoreactive plaque area, Supplementary Table S1). Moreover, other neointimal parameters measured in aortic root and ascending aorta of fat-fed *Apoe/Ccl1*-DKO mice did not show statistically-significant differences, neither when measured as percentages nor as absolute values, including necrotic core content and expression of CCR8, CD4 (T-cells), SM α -actin (VSMCs), collagen, Ki67 (cell proliferation marker), and caspase 3 (apoptosis marker) (Fig. 3, and Supplementary Table S1). Supplementary Fig. S2 shows representative images of all markers analyzed in aortic root cross-sections of *Apoe*-KO and *Apoe/Ccl1*-DKO mice.

3.2. CCL1 enhances leukocyte adhesion *in vitro* and *in vivo*

We next performed flow chamber experiments to investigate whether immobilized CCL1 can arrest peripheral leukocytes. Blood freshly collected from wild-type mice fed the control diet was perfused over CCL1-coated cover slips under flow conditions. Leukocyte adhesion was significantly higher in CCL1-coated cover slips than in vehicle-treated cover slips (Fig. 4A).

To investigate the role of CCL1 in *in vivo* leukocyte trafficking under pro-atherogenic conditions, we performed intravital microscopy in cremasteric arterioles of wild-type, *Apoe*-KO, and *Apoe/Ccl1*-DKO mice fed the HFD for 1 week. Leukocyte adhesion in arterioles was significantly higher in *Apoe*-KO mice than in wild-type controls and was abolished in *Apoe/Ccl1*-DKO mice (Fig. 4B). No between-genotype differences were observed in other hemodynamic parameters, such as arteriolar shear rate and diameter (Fig. 4B). Since *Ccl1* disruption did not affect the percentage of circulating leukocyte populations (Supplementary Fig. S1C), our results suggest that the effects of CCL1 deficiency on leukocyte adhesion were due to CCL1-mediated leukocyte-

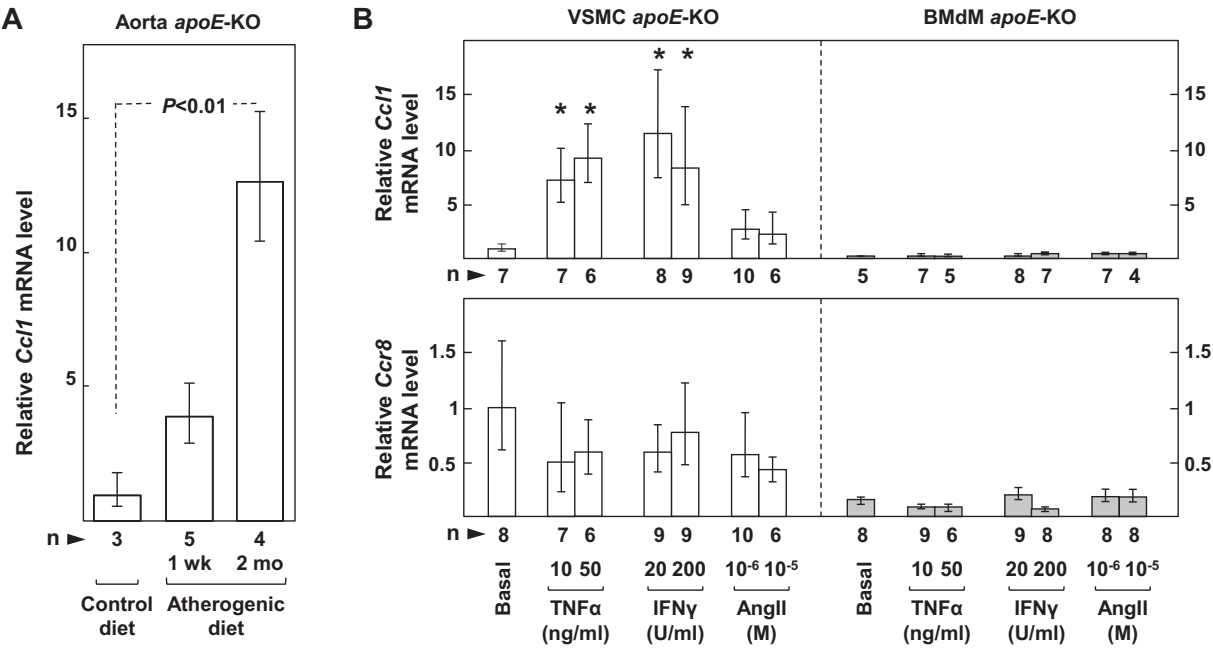


Fig. 1. Induction of *Ccl1* expression in aorta of fat-fed *ApoE-KO* mice, and *Ccl1* and *Ccr8* expression analysis in primary VSMCs and BMdMs. (A) RT-qPCR analysis of *Ccl1* expression in aorta of *ApoE-KO* mice fed a control diet or an atherogenic high-fat diet (HFD) for 1 week or 2 months. Results are expressed relative to *ApoE-KO* mice fed control diet. (B) RT-qPCR analysis of *Ccl1* and *Ccr8* expression in primary VSMCs and BMdMs derived from *ApoE-KO* mice. Cells were incubated during 20 h with the indicated doses of tumor necrosis factor α (TNFα), interferon γ (IFNγ) and angiotensin II (AngII). Results are expressed relative to basal expression in untreated VSMCs. *, $p < .05$ vs. basal condition. In A, n indicates the number of samples (each containing 2 aortas). In B, n indicates the number of culture plates (each containing VSMCs or BMdM from 3 mice).

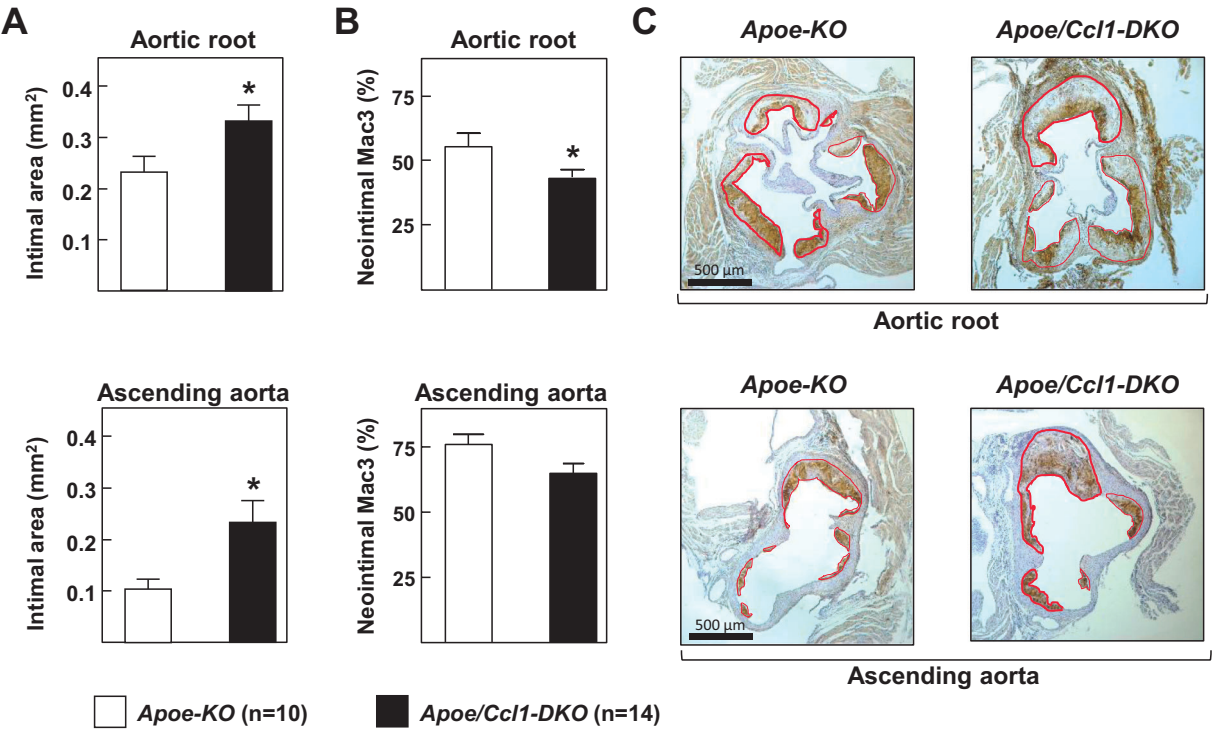


Fig. 2. *Ccl1* disruption aggravates diet-induced atherosclerosis. (A) Quantification of atherosclerosis burden measured as intimal area in aortic root and ascending aorta cross-sections of mice fed the HFD for 2 months. (B) Quantification of neointimal macrophage content in cross-sections of aortic root and ascending aorta of mice fed the high-fat diet for 2 months. Data are the % of the neointimal area covered by immunohistochemistry-detected Mac3-immunoreactive macrophages. Total macrophage content quantified as mm² of Mac3-immunoreactive areas is shown in Supplementary Table S1. (C) Representative specimens immunostained for Mac3 and counterstained with hematoxylin. The red line marks the boundaries of atherosclerotic lesions. Scale bar: 500 μm. n = number of mice. *, $p < .05$.

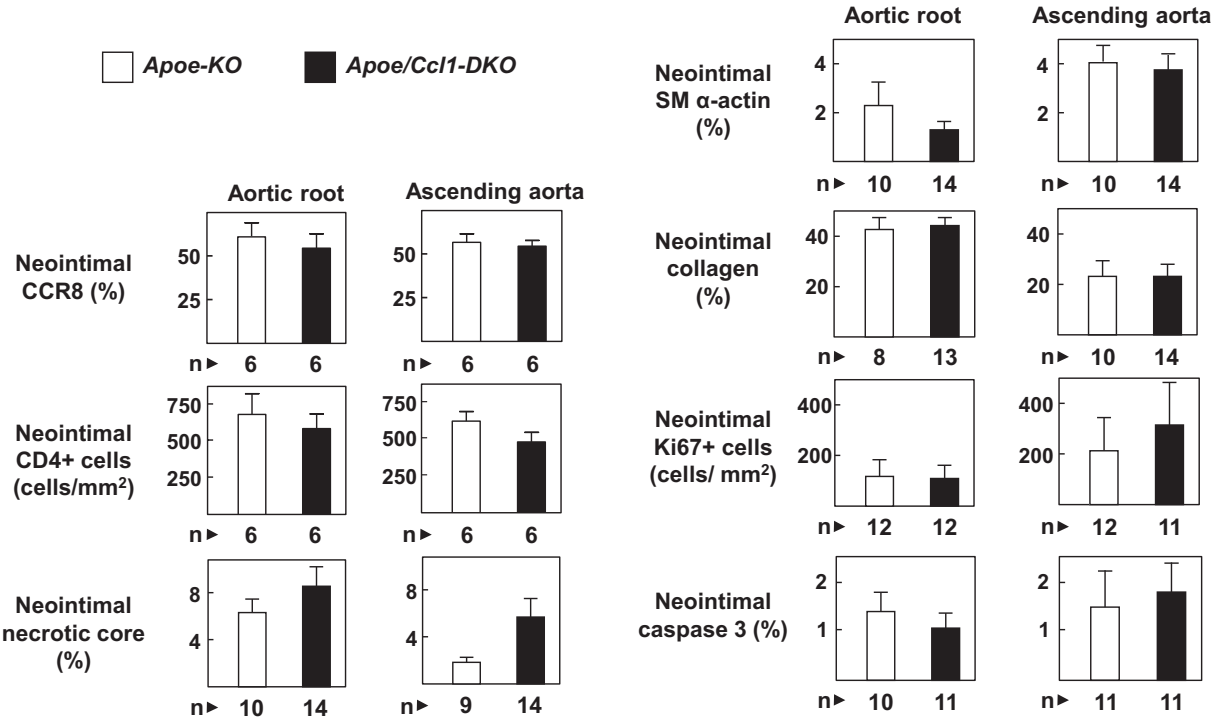


Fig. 3. *Ccl1* disruption does not affect aortic CCR8 expression or atherosclerotic plaque characteristics. Quantified immunohistopathological analysis of aortic root and ascending aorta from 2-month-HFD-fed *Apoe-KO* and *Apoe/CCL1-DKO* mice. Cross-sections were analyzed for neointimal content of CCR8, CD4+ T-cells, necrotic core, VSMCs (SMα-actin+), collagen (Masson's trichrome staining), proliferating cells (Ki67+), and apoptosis (caspase 3+). n = number of mice. Representative images are shown in Supplementary Fig. S2, and results expressed as total area are in Supplementary Table S1.

vessel wall interactions.

3.3. CCL1 regulates Treg accumulation and function, Th1/Th2 balance, and IL-10 production

CCL1 might regulate atherosclerosis development by affecting the recruitment, proliferation, and/or effector function of Th cells in atherosclerotic plaques. The observation that the percentage of CD4+ T-cells is unaltered in *Apoe/Ccl1-DKO* aorta, spleen, and blood (Supplementary Fig. S1D) suggested that CCL1 does not affect the recruitment or proliferation of this cell type. However, CCL1 disruption was accompanied by a lower percentage of Tregs in aorta and spleen, with no effect in blood (Fig. 5A and Supplementary Fig. S3A). To assess

whether CCL1 controls Treg function, we evaluated the ability of splenic Tregs (CD4 + CD25+ T-cells) to inhibit the proliferation of naïve CD4+CD25- T-cells that were activated with anti-CD3 plus anti-CD28 antibodies. CCL1 disruption did not affect the proliferative activity of CD4+ T-cells isolated from spleen and lymph nodes (Fig. 5B). However, CCL1 disruption reduced Treg suppressive activity on T effector cells (Fig. 5C). Since IL-10 has been shown to mediate Treg inhibitory functions [46], we analyzed the production of IL-10 by quantifying its concentration in the culture medium of LPS + IFNγ-stimulated splenocytes. These *in vitro* studies showed reduced IL-10 production in splenocytes of fat-fed *Apoe/Ccl1-DKO* mice (Fig. 5D). Likewise, *Ccl1* disruption was associated with reduced IL-10 production by BMdMs (Fig. 5E), and plasma IL-10 levels were significantly lower in

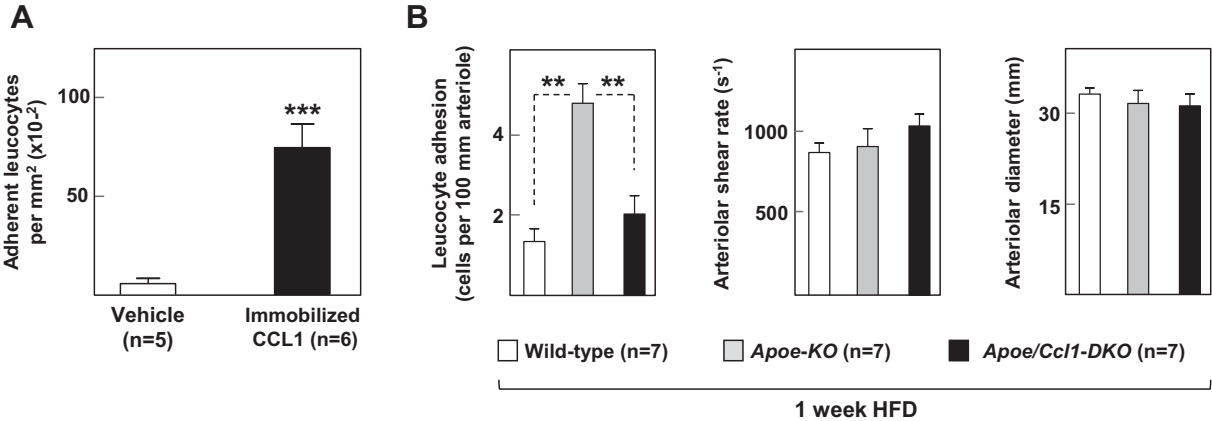


Fig. 4. *Ccl1* mediates leukocyte adhesion *in vitro* and *in vivo*. (A) *In vitro* leukocyte adhesion in flow chamber assays. Whole blood of wild-type mice was perfused in flow chambers containing vehicle-coated or CCL1-coated cover slips. n = number of cover slips. (B) *In vivo* intravital microscopy analysis of leukocyte adhesion, shear rate, and vessel diameter in cremaster arterioles of 1-week-HFD-fed wild-type, *Apoe-KO*, and *Apoe/Ccl1-DKO* mice. n = number of mice. **, *p* < .01; ***, *p* < .001.

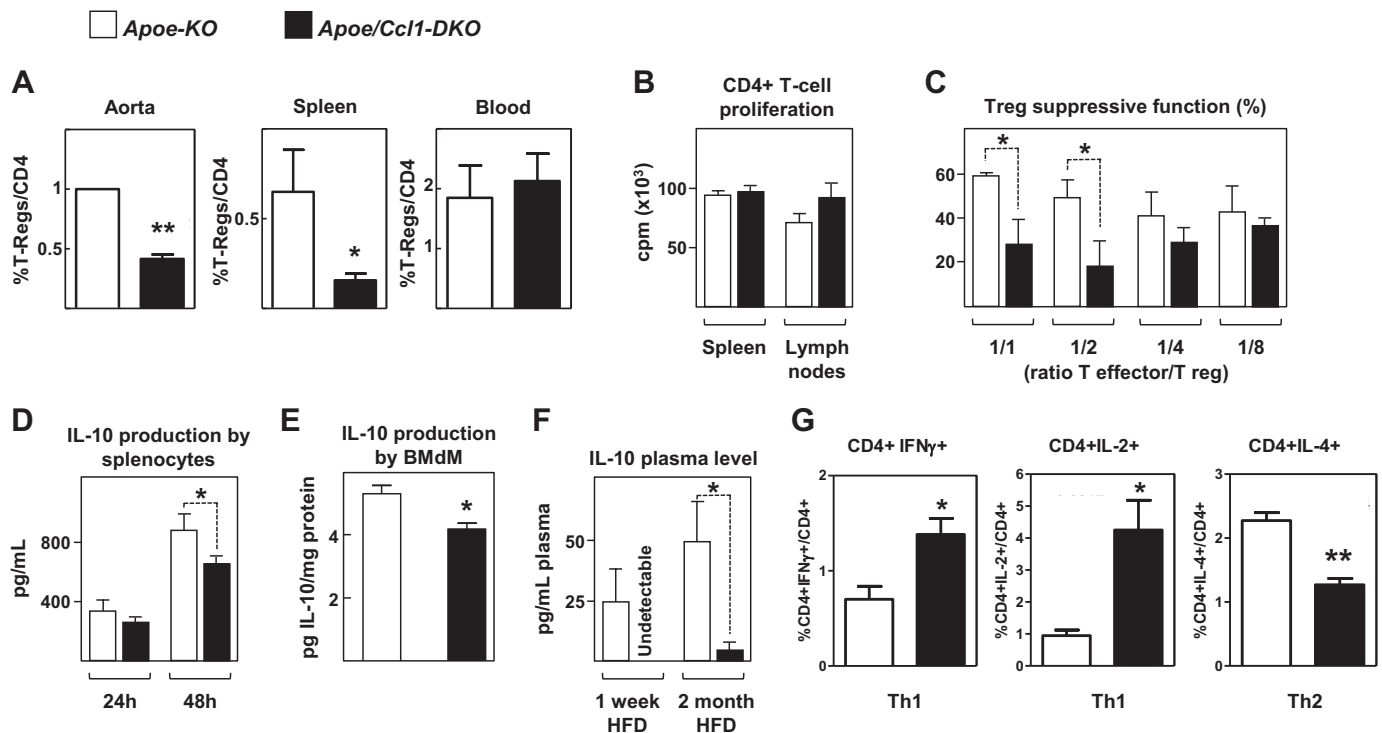


Fig. 5. *Ccl1* disruption increases the Th1:Th2 ratio, inhibits Treg recruitment and suppressive function, and reduces CD4⁺ T cell IL-10 production and plasma IL-10 levels. (A) Flow cytometry-determined content of Tregs (CD4⁺ CD25^{high} FOXP3⁺) as a percentage of CD4⁺ T-cells in aorta, spleen, and blood of 11-month-old control-diet-fed mice. Results are shown relative to *Apoe-KO* mice for aorta and as bare percentages for spleen and blood ($n = 7$ *Apoe-KO*; $n = 8$ *Apoe/Ccl1-DKO*). (B) Proliferation of CD4⁺ T cells isolated from spleen and lymph nodes of 11-month-old control-diet-fed *Apoe-KO* and *Apoe/Ccl1-DKO* mice (triplicates of 3 pooled mice per genotype). (C) Inhibition of wild-type effector T cell proliferation upon co-culture with splenic CD4⁺CD25⁺ Tregs isolated from 11-month-old control-diet-fed *Apoe-KO* and *Apoe/Ccl1-DKO* mice (triplicates of 3 pooled mice per genotype). The effector T cell:Treg ratio is shown in the horizontal axis. (D) IL-10 production in LPS- and IFN γ -stimulated splenocytes of mice fed the HFD for 2 months ($n = 9$ mice per genotype). (E) IL-10 production in BMdMs of *Apoe-KO* mice fed the HFD for 2 months ($n = 4$ culture plates, each containing BMdMs from 3 mice per genotype). (F) Plasma IL-10 in fat-fed mice ($n = 7$ *Apoe-KO* mice and $n = 5$ *Apoe/Ccl1-DKO* mice fed the HFD for 1 week; $n = 8$ *Apoe-KO* and *Apoe/Ccl1-DKO* mice fed the HFD for 2 months). (G) Flow cytometry-determined percentage of Th1 (CD4⁺IFN γ ⁺ and CD4⁺IL-2⁺) and Th2 (CD4⁺IL-4⁺) leukocytes in spleen of 11-month-old control-diet-fed mice. Results are expressed relative to total CD4⁺ leukocytes ($n = 3$ mice per genotype). *, $p < .05$; **, $p < .01$.

HFD-fed *Apoe/Ccl1-DKO* mice than in *Apoe-KO* controls (Fig. 5F). Consistent with lower percentage of Tregs in aorta and spleen and IL-10 levels upon *Ccl1* disruption, *Apoe/Ccl1-DKO* mice had a higher splenic Th1 content and a lower Th2 content than *Apoe-KO* controls (Fig. 5G and Supplementary Fig. S3B).

3.4. Antibody-mediated CCR8 blockade inhibits aortic Treg recruitment and promotes atherosclerosis

By interacting with its ligand CCL1, the receptor CCR8 plays a pivotal role in restraining immunity via Treg cell activation [31]. To further investigate the role of CCR8 in Treg function in the setting of atherosclerosis, we performed bone marrow transplant experiments in CD45.1 *Ldlr-KO* mice that were lethally-irradiated and reconstituted with bone marrow cells of Foxp3-mRFP knock-in mice, which express RFP in Tregs [35]. After recovery, transplanted mice were injected with CCR8 blocking antibody (or isotype control) and challenged with the HFD for 1 week. *Rfp* mRNA was then quantified by RT-PCR as a measure of aortic Treg infiltration. Compared with mice treated with isotype control antibody, animals injected with anti-CCR8 antibody had a lower content of RFP-expressing cells in the aorta (Fig. 6A), suggesting a role for CCR8 in the recruitment of Tregs to the inflamed vessel wall. Inhibition of Treg recruitment upon treatment with anti-CCR8 in transplanted *Ldlr-KO* mice was accompanied by an increased atherosclerosis burden in the aortic root (Fig. 6B).

4. Discussion

Chemokines and their receptors play key roles at all stages of atherosclerosis by regulating multiple processes involved in disease initiation, progression, and complications (eg, leukocyte and platelet recruitment and activation, mobilization of immune cells from bone marrow and recruitment into atherosclerotic plaques, and cell differentiation, proliferation, apoptosis, and survival) [18–20]. The chemokine CCL1 and its receptor CCR8 regulate leukocyte trafficking in several pathophysiological scenarios [30,47–50]. In the context of cardiovascular disease, previous studies showed CCL1 expression by endothelial cells in human arterial specimens and in injured mouse femoral arteries [28,33,51]. However, a direct role of the CCL1-CCR8 axis in atherosclerosis has not been demonstrated before. In this study, we establish for the first time a causal relationship between CCL1 expression and atherosclerosis development. We observed a progressive upregulation of CCL1 in the aorta of fat-fed *Apoe-KO* mice. Our studies with cultured cells stimulated with pro-inflammatory agents suggest that VSMCs, but not macrophages, contribute to CCL1 upregulation in the inflamed vessel wall. We also found that *Ccl1* disruption aggravates HFD-induced atherosclerosis in the aortic root and ascending aorta of *Apoe-KO* mice without affecting body weight, plasma lipid levels, or the abundance of circulating leukocytes. Likewise, *Ccl1* disruption did not affect important features of atherosclerotic plaques, such as the percentage of area occupied by T-cells, VSMCs, collagen, and necrotic cores, as well as the percentage of proliferating and apoptotic cells.

Immobilized CCL1 promoted leukocyte adhesion in *in vitro* flow

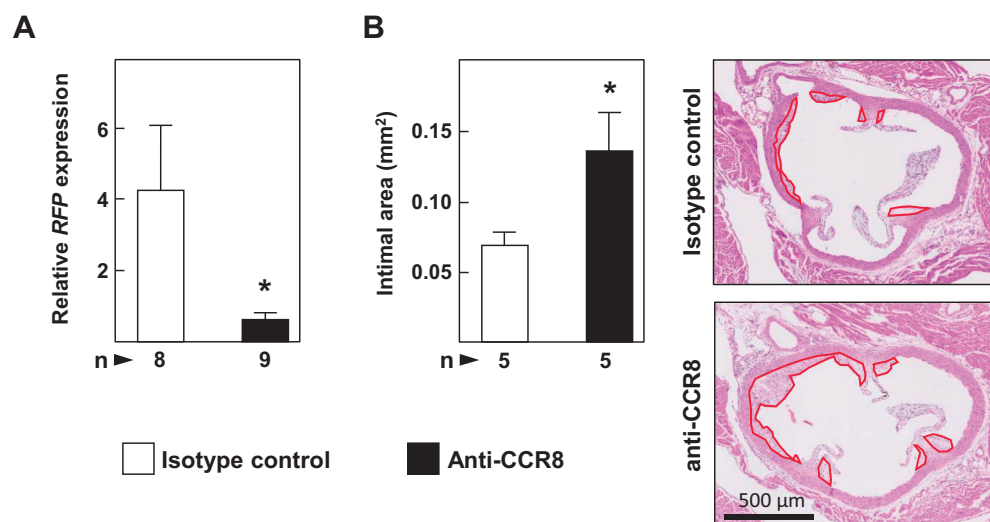


Fig. 6. Antibody-mediated CCR8 blockade inhibits aortic Treg recruitment and promotes atherosclerosis development. *Ldlr*-KO mice were gamma-irradiated and transplanted with bone marrow cells of Foxp3/mRFP mice (expressing RFP in Tregs). One month after irradiation and transplant, mice were challenged with the HFD and injected with either anti-CCR8 antibody or isotype control antibody every 2 days. Mice were sacrificed after 1 week. (A) Relative Treg accumulation in aorta quantified from qPCR-determined RFP expression. (B) Atherosclerosis burden determined as the intimal area in the aortic root of *Ldlr*-KO mice fed the HFD for 1 week. Representative hematoxylin/eosin-stained cross-sections are shown, with red line marking atherosclerotic lesion boundaries. n = number of mice. *, $p < .05$.

chamber assays, and CCL1 ablation was associated with reduced leukocyte adherence in the *in vivo* intravital microscopy studies. Although fat-fed *Apoe/Ccl1*-DKO mice had lower percentage of macrophages in atherosclerotic lesions in the aortic root (measured as percentage of Mac3-immunoreactive area), total content of neointimal macrophages (measured as Mac3-immunoreactive area in atheromata) was similar to that seen in *Apoe*-KO controls. Moreover, *Ccl1* disruption did not affect the relative and total macrophage content in atherosclerotic lesions in the ascending aorta. These findings suggest that macrophages do not contribute significantly to aggravated atherosclerosis upon *Ccl1* disruption. We therefore turned our attention to Tregs, which express CCR8 [52,53], and protect against atherosclerosis [12,13,32]. Clinical studies also support a role for Tregs in human atherosclerosis [54–57]. Treg cells, but not effector T cells, decline during atherosclerotic lesion development [58], possibly as a result of phenotypic plasticity and differences between Treg and effector T cells in migration into lesions, susceptibility to death, and proliferative activity [5,59]. Previous studies also showed the importance of CCL1 for Treg migration [60,61]. We therefore hypothesized that CCL1 might affect Treg recruitment and/or function in atheromas. Our results show reduced Treg content in the spleen and aorta of *Apoe/Ccl1*-DKO mice, without changes in blood, suggesting a role for CCL1 in Treg recruitment.

Tregs play critical roles in the inhibition of inflammatory responses and the regulation of autoimmunity [62,63]. In line with previous studies implicating the CCL1-CCR8 axis in Treg function [31], our *in vitro* studies reveal that *Ccl1* disruption in *Apoe/Ccl1*-DKO Tregs reduced their ability to inhibit T-effector cells, without affecting their proliferative activity. Tregs can reduce T effector functions through a variety of mechanisms, including direct contact with antigen-presenting cells, destruction of inflammatory cells, and production of immunosuppressive regulators such as IL-10 [64–66]. CCL1 potentiates human Treg function in part through inducing IL-10 expression [31], and IL-10 deficiency increases the number and activity of inflammatory cells, promoting pro-inflammatory cytokine production and accelerating atherosclerosis in mice [14]. In our analysis, *Ccl1* disruption impaired IL-10 production by BMdMs and LPS- and IFN γ -stimulated splenocytes obtained from mice fed the HFD for 2 months and markedly reduced plasma IL-10 in *Apoe/Ccl1*-DKO mice fed the HFD for 1 week or 2 months compared with *Apoe*-KO controls. These findings suggest that reduced IL-10 production contributes to increased atherosclerosis induced by *Ccl1* disruption. Further studies are required to assess whether *Ccl1* ablation affects the expression of other Treg-associated cytokines (eg., IL-35, TGF β).

CCR8 is expressed predominantly in Tregs and also in small fractions of Th2 cells, monocytes, and NK cells, but not in Th1 cells

[52,67–71]. Human CCR8 has four known ligands: CCL1, CCL8, CCL16, and CCL18 [72–74]. Of these, CCL1 is unique in potentiating the suppressive function of human Tregs [31]. CCL1, CCL8, and CCL16 have also been identified as mouse CCR8 ligands, with CCL1 being considered the only booster of the suppressive activities of mouse Tregs [31]. Our results show that treatment of fat-fed *Ldlr*-KO mice with CCR8-blocking antibodies reduces Treg content in aorta and enhances aortic atherosclerosis, consistent with a role for this receptor in Treg recruitment. Previous studies in Tregs have shown that CCL1 binding to CCR8 upregulates STAT3-dependent Foxp3 expression while inducing CD39, Granzyme B, and IL-10 [31]. Further studies are warranted to assess whether modulation of these factors in Tregs contributes to the regulation of atherosclerosis by the CCL1-CCR8 axis.

5. Conclusions

Our findings demonstrate increased atherosclerosis upon inactivation of the CCL1-CCR8 axis, which is mediated by reduced Treg recruitment into the inflamed artery wall and defective Treg function, possibly due to increases in both IL-10 production and Th1:Th2 ratio. Further studies are warranted to assess whether treatment with CCL1 offers a possible route to atherosclerosis inhibition.

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Author contributions

MVC and JMGG, designed and performed experiments, analyzed data, and prepared figures and the manuscript. VZ, YNAN, CSR, PMS, AdMM, HAO and MJAM, performed experiments and analyzed data. MJS, CW, LK and ZM, provided advice on the design and analysis of experiments and critically read and edited the manuscript. JG critically read and edited the manuscript. VA supervised the research, project planning, and data interpretation and prepared the manuscript. All authors read and approved the manuscript.

Conflicts of interest

The authors report no commercial or proprietary interest in any product or concept discussed in this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmcc.2019.05.009>.

References

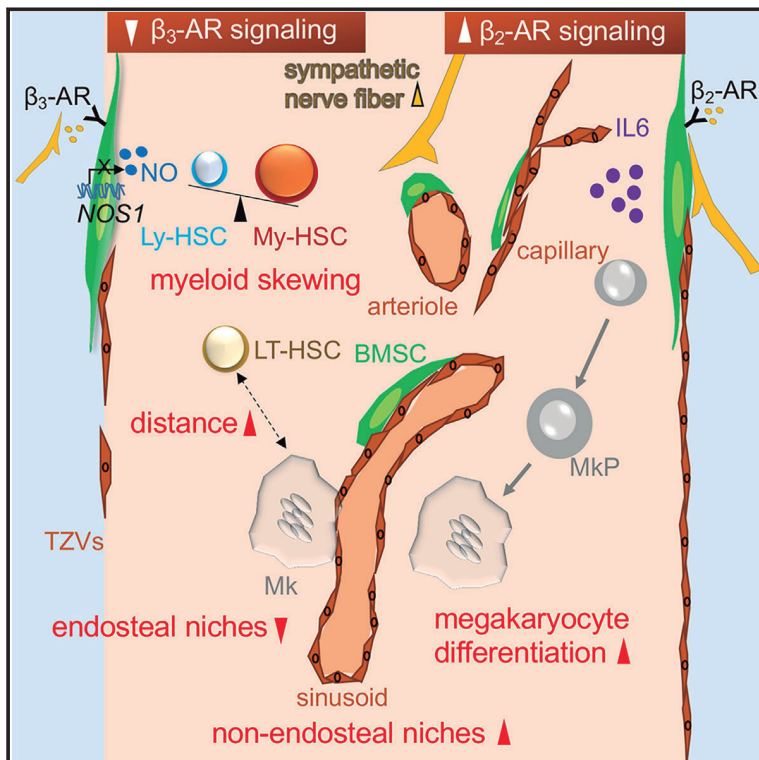
- [1] N. Savji, C.B. Rockman, A.H. Skolnick, Y. Guo, M.A. Adelman, T. Riles, et al., Association between advanced age and vascular disease in different arterial territories: a population database of over 3.6 million subjects, *J. Am. Coll. Cardiol.* 61 (2013) 1736–1743.
- [2] R.B. D'Agostino Sr., R.S. Vasan, M.J. Pencina, P.A. Wolf, M. Cobain, J.M. Massaro, et al., General cardiovascular risk profile for use in primary care: the Framingham heart study, *Circulation* 117 (2008) 743–753.
- [3] R. Ross, Atherosclerosis—an inflammatory disease, *N. Engl. J. Med.* 340 (1999) 115–126.
- [4] E. Galkina, K. Ley, Immune and inflammatory mechanisms of atherosclerosis (*), *Annu. Rev. Immunol.* 27 (2009) 165–197.
- [5] I. Tabas, A.H. Lichtman, Monocyte-macrophages and T cells in atherosclerosis, *Immunity* 47 (2017) 621–634.
- [6] E.E. Emeson, M.L. Shen, C.G. Bell, A. Qureshi, Inhibition of atherosclerosis in CD4 T-cell-ablated and nude (nu/nu) C57BL/6 hyperlipidemic mice, *Am. J. Pathol.* 149 (1996) 675–685.
- [7] S. Iborra, J.M. Gonzalez-Granado, In vitro differentiation of naive CD4(+) T cells: a tool for understanding the development of atherosclerosis, *Methods Mol. Biol.* 1339 (2015) 177–189.
- [8] R. Elhage, J. Jawien, M. Rudling, H.G. Ljungrgren, K. Takeda, S. Akira, et al., Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice, *Cardiovasc. Res.* 59 (2003) 234–240.
- [9] S. Gupta, A.M. Pablo, X. Jiang, N. Wang, A.R. Tall, C. Schindler, IFN-gamma potentiates atherosclerosis in ApoE knock-out mice, *J. Clin. Invest.* 99 (1997) 2752–2761.
- [10] C. Buono, C.E. Come, G. Stavrakis, G.F. Maguire, P.W. Connelly, A.H. Lichtman, Influence of interferon-gamma on the extent and phenotype of diet-induced atherosclerosis in the LDLR-deficient mouse, *Arterioscler. Thromb. Vasc. Biol.* 23 (2003) 454–460.
- [11] C. Buono, C.J. Binder, G. Stavrakis, J.L. Witztum, L.H. Glimcher, A.H. Lichtman, T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses, *Proc. Natl. Acad. Sci. U. S. A.* 102 (2005) 1596–1601.
- [12] H. Ait-Oufella, B.L. Salomon, S. Potteaux, A.K. Robertson, P. Gourdy, J. Zoll, et al., Natural regulatory T cells control the development of atherosclerosis in mice, *Nat. Med.* 12 (2006) 178–180.
- [13] R. Klingenberg, N. Gerdes, R.M. Badeau, A. Gistera, D. Strothoff, D.F. Ketelhuth, et al., Depletion of FOXP3+ regulatory T cells promotes hypercholesterolemia and atherosclerosis, *J. Clin. Invest.* 123 (2013) 1323–1334.
- [14] Z. Mallat, S. Besnard, M. Duriez, V. Deleuze, F. Emmanuel, M.F. Bureau, et al., Protective role of interleukin-10 in atherosclerosis, *Circ. Res.* 85 (1999) e17–e24.
- [15] L.J. Pinderski Oslund, C.C. Hedrick, T. Olvera, A. Hagenbaugh, M. Territo, J.A. Berliner, et al., Interleukin-10 blocks atherosclerotic events in vitro and in vivo, *Arterioscler. Thromb. Vasc. Biol.* 19 (1999) 2847–2853.
- [16] Z. Mallat, A. Gojova, C. Marchiol-Fournigault, B. Esposito, C. Kamate, R. Merval, et al., Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice, *Circ. Res.* 89 (2001) 930–934.
- [17] A.K. Robertson, M. Rudling, X. Zhou, L. Gorelik, R.A. Flavell, G.K. Hansson, Disruption of TGF-beta signaling in T cells accelerates atherosclerosis, *J. Clin. Invest.* 112 (2003) 1342–1350.
- [18] P. Libby, G.K. Hansson, Inflammation and immunity in diseases of the arterial tree: players and layers, *Circ. Res.* 116 (2015) 307–311.
- [19] D.P. Ramji, T.S. Davies, Cytokines in atherosclerosis: key players in all stages of disease and promising therapeutic targets, *Cytokine Growth Factor Rev.* 26 (2015) 673–685.
- [20] A. Zernecke, E. Shagdarsuren, C. Weber, Chemokines in atherosclerosis: an update, *Arterioscler. Thromb. Vasc. Biol.* 28 (2008) 1897–1908.
- [21] M.D. Miller, S. Hata, R. De Waal Malefyt, M.S. Krangel, A novel polypeptide secreted by activated human T lymphocytes, *J. Immunol.* 143 (1989) 2907–2916.
- [22] P.C. Harpel, N.S. Haque, Chemokine receptor-8: potential role in atherogenesis, *Isr. Med. Assoc. J.* 4 (2002) 1025–1027.
- [23] I.F. Charo, R.M. Ransohoff, The many roles of chemokines and chemokine receptors in inflammation, *N. Engl. J. Med.* 354 (2006) 610–621.
- [24] J. Cantor, K. Haskins, Recruitment and activation of macrophages by pathogenic CD4 T cells in type 1 diabetes: evidence for involvement of CCR8 and CCL1, *J. Immunol.* 179 (2007) 5760–5767.
- [25] R. Montes-Vizuet, A. Vega-Miranda, E. Valencia-Maqueda, M.C. Negrete-Garcia, J.R. Velasquez, L.M. Teran, CC chemokine ligand 1 is released into the airways of atopic asthmatics, *Eur. Respir. J.* 28 (2006) 59–67.
- [26] H.O. Kim, S.I. Cho, B.Y. Chung, H.K. Ahn, C.W. Park, C.H. Lee, Expression of CCL1 and CCL18 in atopic dermatitis and psoriasis, *Clin. Exp. Dermatol.* 37 (2012) 521–526.
- [27] M. Gombert, M.C. Dieu-Nosjean, F. Winterberg, E. Bunemann, R.C. Kubitz, L. Da Cunha, et al., CCL1-CCR8 interactions: an axis mediating the recruitment of T cells and Langerhans-type dendritic cells to sites of atopic skin inflammation, *J. Immunol.* 174 (2005) 5082–5091.
- [28] N.S. Haque, J.T. Fallon, J.J. Pan, M.B. Taubman, P.C. Harpel, Chemokine receptor-8 (CCR8) mediates human vascular smooth muscle cell chemotaxis and metalloproteinase-2 secretion, *Blood* 103 (2004) 1296–1304.
- [29] A. Matsukawa, S. Kudoh, G. Sano, T. Maeda, T. Ito, N.W. Lukacs, et al., Absence of CC chemokine receptor 8 enhances innate immunity during septic peritonitis, *FASEB J.* 20 (2006) 302–304.
- [30] F. Heymann, L. Hammerich, D. Storch, M. Bartneck, S. Huss, V. Russeler, et al., Hepatic macrophage migration and differentiation critical for liver fibrosis is mediated by the chemokine receptor C-C motif chemokine receptor 8 in mice, *Hepatology* 55 (2012) 898–909.
- [31] Y. Barshesht, G. Wildbaum, E. Levy, A. Vitenshtein, C. Akinseye, J. Griggs, et al., CCR8(+)FOXP3(+) Treg cells as master drivers of immune regulation, *Proc. Natl. Acad. Sci. U. S. A.* 114 (2017) 6086–6091.
- [32] C. Spitz, H. Winkels, C. Burger, C. Weber, E. Lutgens, G.K. Hansson, et al., Regulatory T cells in atherosclerosis: critical immune regulatory function and therapeutic potential, *Cell. Mol. Life Sci.* 73 (2016) 901–922.
- [33] N.S. Haque, X. Zhang, D.L. French, J. Li, M. Poon, J.T. Fallon, et al., CC chemokine I-309 is the principal monocyte chemoattractant induced by apolipoprotein(a) in human vascular endothelial cells, *Circulation* 102 (2000) 786–792.
- [34] G. Bernardini, G. Spinetti, D. Ribatti, G. Camarda, L. Morbidelli, M. Ziche, et al., I-309 binds to and activates endothelial cell functions and acts as an angiogenic molecule in vivo, *Blood* 96 (2000) 4039–4045.
- [35] Y.Y. Wan, R.A. Flavell, Identifying Foxp3-expressing suppressor T cells with a bi-cistronic reporter, *Proc. Natl. Acad. Sci. U. S. A.* 102 (2005) 5126–5131.
- [36] E. Mira, B. Leon, D.F. Barber, S. Jimenez-Baranda, I. Goya, L. Almonacid, et al., Statins induce regulatory T cell recruitment via a CCL1 dependent pathway, *J. Immunol.* 181 (2008) 3524–3534.
- [37] H. Gonzalez-Navarro, A. Vinue, M. Vila-Caballer, A. Fortuno, O. Belouqui, G. Zalba, et al., Molecular mechanisms of atherosclerosis in metabolic syndrome: role of reduced IRS2-dependent signaling, *Arterioscler. Thromb. Vasc. Biol.* 28 (2008) 2187–2194.
- [38] H. Gonzalez-Navarro, M. Vila-Caballer, M.F. Pastor, A. Vinue, M.F. White, D. Burks, et al., Plasma insulin levels predict the development of atherosclerosis when IRS2 deficiency is combined with severe hypercholesterolemia in apolipoprotein E-null mice, *Front. Biosci.* 12 (2007) 2291–2298.
- [39] C. Sunderkotter, T. Nikolic, M.J. Dillon, N. Van Rooijen, M. Stehling, D.A. Drevets, et al., Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response, *J. Immunol.* 172 (2004) 4410–4417.
- [40] C. Company, L. Piqueras, Y. Naim Abu Nabah, P. Escudero, J.I. Blanes, P.J. Jose, et al., Contributions of ACE and mast cell chymase to endogenous angiotensin II generation and leucocyte recruitment in vivo, *Cardiovasc. Res.* 92 (2011) 48–56.
- [41] R. Toribio-Fernandez, V. Zorita, V. Rocha-Perugini, S. Iborra, G. Martinez Del Hoyo, R. Chevre, et al., Lamin A/C augments Th1 differentiation and response against vaccinia virus and Leishmania major, *Cell Death Dis.* 9 (2018) 9.
- [42] R. Toribio-Fernandez, V. Zorita, B. Herrero-Fernandez, J.M. Gonzalez-Granado, An in vivo mouse model to measure naive CD4 T cell activation, proliferation and Th1 differentiation induced by bone marrow-derived dendritic cells, *J. Vis. Exp.* 138 (2018) e58118.
- [43] S. Taleb, O. Herbin, H. Ait-Oufella, V. Verreth, P. Gourdy, V. Barateau, et al., Defective leptin/leptin receptor signaling improves regulatory T cell immune response and protects mice from atherosclerosis, *Arterioscler. Thromb. Vasc. Biol.* 27 (2007) 2691–2698.
- [44] E. Galkina, A. Kadl, J. Sanders, D. Varughese, I.J. Sarembock, K. Ley, Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent, *J. Exp. Med.* 203 (2006) 1273–1282.
- [45] G. Tie, K.E. Messina, J. Yan, J.A. Messina, L.M. Messina, Hypercholesterolemia induces oxidant stress that accelerates the ageing of hematopoietic stem cells, *J. Am. Heart Assoc.* 3 (2014) e000241.
- [46] E.M. Shevach, A.M. Thornton, tTregs, pTregs, and iTregs: similarities and differences, *Immunol. Rev.* 259 (2014) 88–102.
- [47] Z. Mikhak, M. Fukui, A. Farsidjani, B.D. Medoff, A.M. Tager, A.D. Luster, Contribution of CCR4 and CCR8 to antigen-specific T(H)2 cell trafficking in allergic pulmonary inflammation, *J. Allergy Clin. Immunol.* 123 (2009) 67–73 (e3).
- [48] S. Das, E. Sarrou, S. Podgrabinska, M. Cassella, S.K. Mungamuri, N. Feirt, et al., Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses, *J. Exp. Med.* 210 (2013) 1509–1528.

- [49] Y. Zen, R. Liberal, Y. Nakanuma, N. Heaton, B. Portmann, Possible involvement of CCL1-CCR8 interaction in lymphocytic recruitment in IgG4-related sclerosing cholangitis, *J. Hepatol.* 59 (2013) 1059–1064.
- [50] J.M. Coghill, K.A. Fowler, M.L. West, L.M. Fulton, H. van Deventer, K.P. McKinnon, et al., CC chemokine receptor 8 potentiates donor Treg survival and is critical for the prevention of murine graft-versus-host disease, *Blood* 122 (2013) 825–836.
- [51] N.S. Haque, J.T. Fallon, M.B. Taubman, P.C. Harpel, The chemokine receptor CCR8 mediates human endothelial cell chemotaxis induced by I-309 and Kaposi sarcoma herpesvirus-encoded vMIP-I and by lipoprotein(a)-stimulated endothelial cell conditioned medium, *Blood* 97 (2001) 39–45.
- [52] C.M. Freeman, B.C. Chiu, V.R. Stolberg, J. Hu, K. Zeibecoglou, N.W. Lukacs, et al., CCR8 is expressed by antigen-elicited, IL-10-producing CD4+CD25+ T cells, which regulate Th2-mediated granuloma formation in mice, *J. Immunol.* 174 (2005) 1962–1970.
- [53] S. Sebastiani, P. Allavena, C. Albanesi, F. Nasorri, G. Bianchi, C. Traidl, et al., Chemokine receptor expression and function in CD4+ T lymphocytes with regulatory activity, *J. Immunol.* 166 (2001) 996–1002.
- [54] W.C. Zhang, J. Wang, Y.W. Shu, T.T. Tang, Z.F. Zhu, N. Xia, et al., Impaired thymic export and increased apoptosis account for regulatory T cell defects in patients with non-ST segment elevation acute coronary syndrome, *J. Biol. Chem.* 287 (2012) 34157–34166.
- [55] M. Wigren, H. Bjorkbacka, L. Andersson, I. Ljungcrantz, G.N. Fredrikson, M. Persson, et al., Low levels of circulating CD4+FoxP3+ T cells are associated with an increased risk for development of myocardial infarction but not for stroke, *Arterioscler. Thromb. Vasc. Biol.* 32 (2012) 2000–2004.
- [56] E. Ammirati, D. Cianflone, M. Banfi, V. Vecchio, A. Palini, M. De Metrio, et al., Circulating CD4+CD25hiCD127lo regulatory T-cell levels do not reflect the extent or severity of carotid and coronary atherosclerosis, *Arterioscler. Thromb. Vasc. Biol.* 30 (2010) 1832–1841.
- [57] A. Mor, G. Luboshits, D. Planer, G. Keren, J. George, Altered status of CD4(+)CD25(+) regulatory T cells in patients with acute coronary syndromes, *Eur. Heart J.* 27 (2006) 2530–2537.
- [58] E. Maganto-Garcia, M.L. Tarrio, N. Grabie, D.X. Bu, A.H. Lichtman, Dynamic changes in regulatory T cells are linked to levels of diet-induced hypercholesterolemia, *Circulation* 124 (2011) 185–195.
- [59] E.A. Heller, E. Liu, A.M. Tager, Q. Yuan, A.Y. Lin, N. Ahluwalia, et al., Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells, *Circulation* 113 (2006) 2301–2312.
- [60] L. Colantonio, A. Iellem, F. Sinigaglia, D. D'Ambrosio, Skin-homing CLA+ T cells and regulatory CD25+ T cells represent major subsets of human peripheral blood memory T cells migrating in response to CCL1/I-309, *Eur. J. Immunol.* 32 (2002) 3506–3514.
- [61] A. Iellem, M. Mariani, R. Lang, H. Recalde, P. Panina-Bordignon, F. Sinigaglia, et al., Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells, *J. Exp. Med.* 194 (2001) 847–853.
- [62] K. Wing, S. Sakaguchi, Regulatory T cells exert checks and balances on self tolerance and autoimmunity, *Nat. Immunol.* 11 (2010) 7–13.
- [63] E.M. Shevach, Mechanisms of foxp3+ T regulatory cell-mediated suppression, *Immunity* 30 (2009) 636–645.
- [64] T. Yamaguchi, J.B. Wing, S. Sakaguchi, Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions, *Semin. Immunol.* 23 (2011) 424–430.
- [65] M. Saraiva, A. O'Garra, The regulation of IL-10 production by immune cells, *Nat. Rev. Immunol.* 10 (2010) 170–181.
- [66] E.M. Shevach, R.A. DiPaolo, J. Andersson, D.M. Zhao, G.L. Stephens, A.M. Thornton, The lifestyle of naturally occurring CD4+CD25+ Foxp3+ regulatory T cells, *Immunol. Rev.* 212 (2006) 60–73.
- [67] D. D'Ambrosio, A. Iellem, R. Bonecchi, D. Mazzeo, S. Sozzani, A. Mantovani, et al., Selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells, *J. Immunol.* 161 (1998) 5111–5115.
- [68] A. Zingoni, H. Soto, J.A. Hedrick, A. Stoppacciaro, C.T. Storlazzi, F. Sinigaglia, et al., The chemokine receptor CCR8 is preferentially expressed in Th2 but not Th1 cells, *J. Immunol.* 161 (1998) 547–551.
- [69] M. Inngjerdingen, B. Damaj, A.A. Maghazachi, Expression and regulation of chemokine receptors in human natural killer cells, *Blood* 97 (2001) 367–375.
- [70] M. Inngjerdingen, B. Damaj, A.A. Maghazachi, Human NK cells express CC chemokine receptors 4 and 8 and respond to thymus and activation-regulated chemokine, macrophage-derived chemokine, and I-309, *J. Immunol.* 164 (2000) 4048–4054.
- [71] R.S. Roos, M. Loetscher, D.F. Legler, I. Clark-Lewis, M. Baggiolini, B. Moser, Identification of CCR8, the receptor for the human CC chemokine I-309, *J. Biol. Chem.* 272 (1997) 17251–17254.
- [72] S.A. Islam, D.S. Chang, R.A. Colvin, M.H. Byrne, M.L. McCully, B. Moser, et al., Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5+ T(H)2 cells, *Nat. Immunol.* 12 (2011) 167–177.
- [73] O.M. Howard, H.F. Dong, A.K. Shirakawa, J.J. Oppenheim, LEC induces chemotaxis and adhesion by interacting with CCR1 and CCR8, *Blood* 96 (2000) 840–845.
- [74] S.A. Islam, M.F. Ling, J. Leung, W.G. Shreffler, A.D. Luster, Identification of human CCR8 as a CCL18 receptor, *J. Exp. Med.* 210 (2013) 1889–1898.

Cell Stem Cell

Remodeling of Bone Marrow Hematopoietic Stem Cell Niches Promotes Myeloid Cell Expansion during Premature or Physiological Aging

Graphical Abstract



Authors

Ya-Hsuan Ho, Raquel del Toro, José Rivera-Torres, ..., Fawzia Louache, Vicente Andrés, Simón Méndez-Ferrer

Correspondence

sm2116@medschl.cam.ac.uk

In Brief

Recent studies have suggested a microenvironmental contribution to stem-cell aging, but the mechanisms are largely unexplored. Méndez-Ferrer et al. report anatomical remodeling of blood-stem-cell-supporting niches and functional switch of β adrenergic signals, leading to myeloid expansion during aging. Targeting the microenvironment can improve pathological, premature, niche-dependent hematopoietic aging in mice.

Highlights

- Reduction of endosteal BM and expansion of non-endosteal BM occurs with age
- β_2/β_3 -ARs exhibit opposite and niche-dependent regulation of myelopoiesis
- β_2 -AR overriding β_3 -AR promotes myeloid expansion during physiological aging
- Premature HSC aging in HGPS can be improved by targeting the microenvironment



Remodeling of Bone Marrow Hematopoietic Stem Cell Niches Promotes Myeloid Cell Expansion during Premature or Physiological Aging

Ya-Hsuan Ho,^{1,2,13} Raquel del Toro,^{3,4,11,13} José Rivera-Torres,^{3,4,12,13} Justyna Rak,^{1,2} Claudia Korn,^{1,2} Andrés García-García,^{1,2,3} David Macías,⁵ Cristina González-Gómez,^{3,4} Alberto del Monte,^{3,4} Monika Wittner,^{6,7} Amie K. Waller,^{1,2} Holly R. Foster,^{1,2} Carlos López-Otín,^{8,9} Randall S. Johnson,⁵ Claus Nerlov,¹⁰ Cedric Ghevaert,^{1,2} William Vainchenker,⁶ Fawzia Louache,^{6,7} Vicente Andrés,^{3,4} and Simón Méndez-Ferrer^{1,2,3,14,*}

¹Wellcome Trust-Medical Research Council Cambridge Stem Cell Institute and Department of Haematology, University of Cambridge, Cambridge CB2 0PT, UK

²National Health Service Blood and Transplant, Cambridge Biomedical Campus, Cambridge CB2 0PT, UK

³Centro Nacional de Investigaciones Cardiovasculares (CNIC), 28029 Madrid, Spain

⁴CIBER de Enfermedades Cardiovasculares (CIBER-CV), Spain

⁵Physiological Laboratory, Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3EG, UK

⁶INSERM (Institut National de la Santé et de la Recherche Médicale), Université Paris-Saclay, UMR1170, Gustave Roussy, 94805 Villejuif, France

⁷Université Paris-Saclay and CNRS GDR 3697 MicroNiT, Villejuif, France

⁸Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Instituto Universitario de Oncología, Universidad de Oviedo, 33006 Oviedo, Spain

⁹Centro de Investigación Biomédica en Red de Cáncer, CIBERONC, Madrid, Spain

¹⁰MRC Molecular Haematology Unit, MRC Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK

¹¹Present address: Instituto de Biomedicina de Sevilla-IBiS (Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla), 41013 Seville, Spain

¹²Present address: Universidad Europea de Madrid, Faculty of Biomedical and Health Sciences, Department of Pharmacy, Biotechnology, Nutrition, Optics and Optometry, 28670 Madrid, Spain

¹³These authors contributed equally

¹⁴Lead Contact

*Correspondence: sm2116@medschl.cam.ac.uk

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SUMMARY

Hematopoietic stem cells (HSCs) residing in the bone marrow (BM) accumulate during aging but are functionally impaired. However, the role of HSC-intrinsic and -extrinsic aging mechanisms remains debated. Megakaryocytes promote quiescence of neighboring HSCs. Nonetheless, whether megakaryocyte-HSC interactions change during pathological/natural aging is unclear. Premature aging in Hutchinson-Gilford progeria syndrome recapitulates physiological aging features, but whether these arise from altered stem or niche cells is unknown. Here, we show that the BM microenvironment promotes myelopoiesis in premature/physiological aging. During physiological aging, HSC-supporting niches decrease near bone but expand further from bone. Increased BM noradrenergic innervation promotes β_2 -adrenergic-receptor(AR)-interleukin-6-dependent megakaryopoiesis. Reduced β_3 -AR-Nos1 activity correlates with decreased endosteal niches and megakaryocyte apposition to sinusoids. However, chronic treatment of progeroid mice with β_3 -AR agonist decreases premature myeloid and HSC expansion and restores the proximal association of HSCs to megakaryocytes.

Therefore, normal/premature aging of BM niches promotes myeloid expansion and can be improved by targeting the microenvironment.

INTRODUCTION

Hematopoietic aging is characterized by expansion of hematopoietic stem cells (HSCs) with impaired function, such as reduced engraftment, quiescence, self-renewal, unfolded protein response, and lymphoid differentiation potential, leading to myeloid-biased output both in mice (Liang et al., 2005; Mohrin et al., 2015; Rossi et al., 2005; Sudo et al., 2000) and humans (Pang et al., 2011; Rundberg Nilsson et al., 2016). Myeloid malignancies are more frequent in the elderly, but whether changes in the aged HSCs and/or their microenvironment predispose to these malignancies remains unclear. HSC aging was initially considered to result only from intrinsic changes (Dykstra et al., 2011), such as epigenetic deregulation (Chambers et al., 2007), replication stress (Flach et al., 2014), deficient DNA repair (Rossi et al., 2007), transition from canonical to non-canonical Wnt signaling (Florian et al., 2013), and increased autophagy (Ho et al., 2017). However, aging also reduces HSC polarity and capacity to lodge near bone (Köhler et al., 2009), and microenvironmental alterations have been proposed to contribute to hematopoietic aging. For instance, myeloid bias can result from telomere dysfunction (Ju et al., 2007) or inflammatory cytokine production (Ergen et al., 2012) in the aged



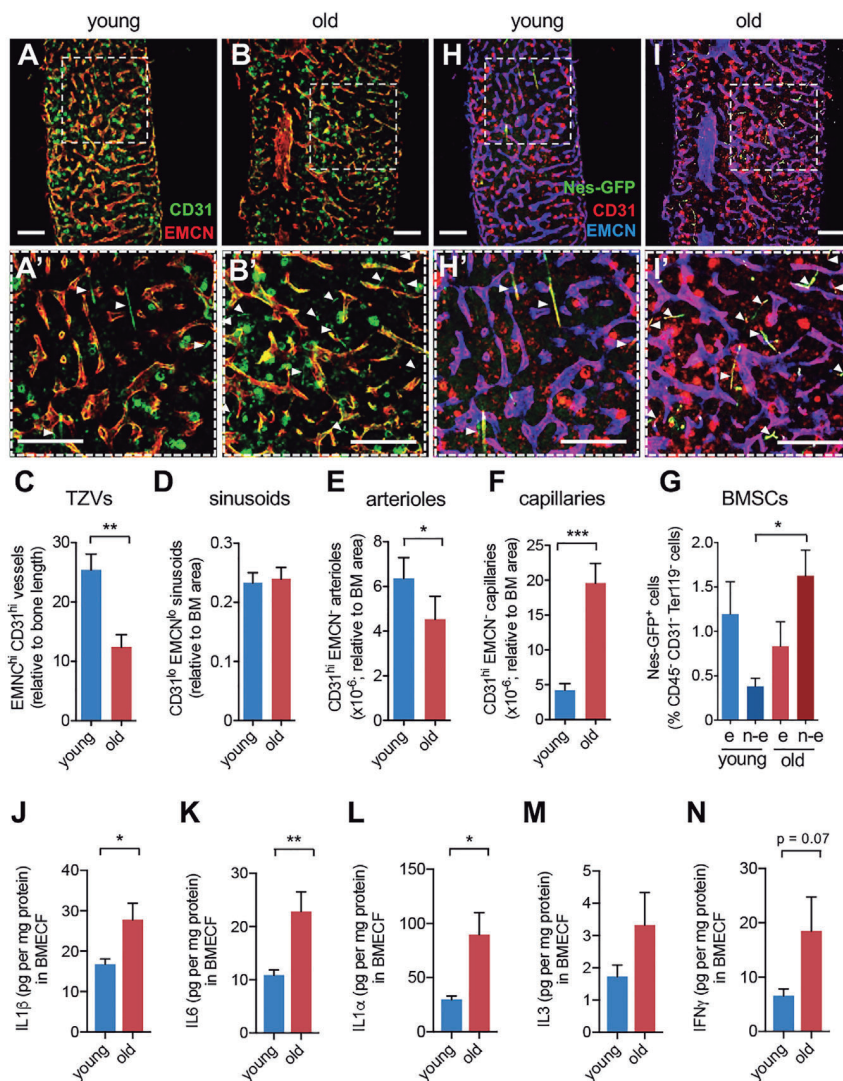


Figure 1. Reduction of Endosteal Niches and Expansion of Non-endosteal Niches during Aging

(A–B' and H–I') Representative whole-mount immunofluorescent staining of thick femoral sections for CD31 (A and B, green; H and I, red) and EMCN (A and B, red; H, I, blue) of young (8–30 weeks) and old (70–100 weeks) *Nes-gfp* mice with genetically labeled nestin⁺ cells (H and I, green). Arrowheads in insets (A', B', H', and I') depict CD31^{hi}EMCN[−] capillaries and their coverage by Nes-GFP⁺ cells.

(C–G) Quantification of (C) CD31^{hi}EMCN^{hi} transition zone vessels, (D) CD31^{lo}EMCN^{lo} sinusoids, (E) CD31^{hi}EMCN[−] arterioles with $\geq 6 \mu\text{m}$ diameter, and (F) CD31^{hi}EMCN[−] capillaries with $< 6 \mu\text{m}$ diameter. Scale bar, 200 μm (A, B, H, and I), 100 μm (A', B', H', and I'). (G) Frequency of endosteal and non-endosteal BM Nes-GFP⁺ cells from young adult (10–20 weeks, $n = 11$) and old mice (> 66 weeks, $n = 8$).

(J–N) Concentration of (J) IL-1 β , (K) IL-6, (L) IL-1 α , (M) IL-3, and (N) IFN γ in endosteal BM extracellular fluid (BMECF) from young WT mice ($n = 5$) and old WT mice ($n = 4$). Data are means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (C–F and J–N) Unpaired two-tailed t test. (G) One-way ANOVA and Bonferroni pairwise comparisons.

gated whether different BM microenvironments regulate myeloid differentiation and megakaryopoiesis during aging.

RESULTS

Reduction of Endosteal Niches and Expansion of Non-endosteal Neurovascular Niches during Aging

To characterize changes in vascular beds during aging, we performed whole-mount immunofluorescence staining of thick

hematopoietic microenvironment, and bone marrow (BM) adrenergic nerve degeneration has been recently proposed to drive HSC aging reversibly (Maryanovich et al., 2018). However, the relative contribution of intrinsic and extrinsic mechanisms to HSC aging remains debated. Myeloid-biased HSCs (Gekas and Graf, 2013) and more prominently platelet-primed HSCs (Sanjuan-Pla et al., 2013; Yamamoto et al., 2013) expand in aged mice (Grover et al., 2016). However, both platelet-primed and unprimed old HSCs exhibit myeloid bias (Grover et al., 2016), possibly suggesting a microenvironmental participation. The standard model of platelet generation (thrombopoiesis) suggests that megakaryocyte precursors migrate from endosteal BM (close to bone) to sinusoids (further from bone) for maturation (Eto and Kunishima, 2016), which can be promoted by cytokines like interleukin (IL)-1 α IL-1 β , IL-6, IL-3, and interferon-gamma (IFN γ) (Pietras, 2017). Whereas platelet-biased HSCs are located near sinusoids (Pinho et al., 2018), both megakaryocytes and their precursors have been recently found throughout BM, including the sinusoids where megakaryocyte maturation and thrombopoiesis take place (Plo et al., 2017; Stegner et al., 2017). In this study, we have investi-

gated whether different BM microenvironments regulate myeloid differentiation and megakaryopoiesis during aging. Consistent with previous findings (Kusumbe et al., 2016), endosteal vessels and TZVs were reduced in old mice; however, we also noted that, whereas sinusoidal areas appeared unchanged, arterioles were slightly reduced and capillaries located further from bone increased 4-fold in old mice (Figures 1A–1F). These vascular changes were paralleled by similar alterations of their associated perivascular cells. We used *Nes-gfp* transgenic mice, which carry HSC niche-forming perivascular BM mesenchymal stem or progenitor cells (BMSCs) labeled with GFP (Méndez-Ferrer et al., 2010b). Nes-GFP⁺ cells augmented 4-fold specifically in non-endosteal BM, mostly associated with the increased capillaries (Figures 1G–1I and S1A–S1D). These changes correlated with increased inflammatory cytokines that drive myeloid cell expansion (Pietras, 2017). The concentration of IL-1 α , IL-1 β , and IL-6 increased in the BM during aging, whereas IL-3 and IFN γ showed similar trends (Figures 1J–1N and S1M–S1Q).

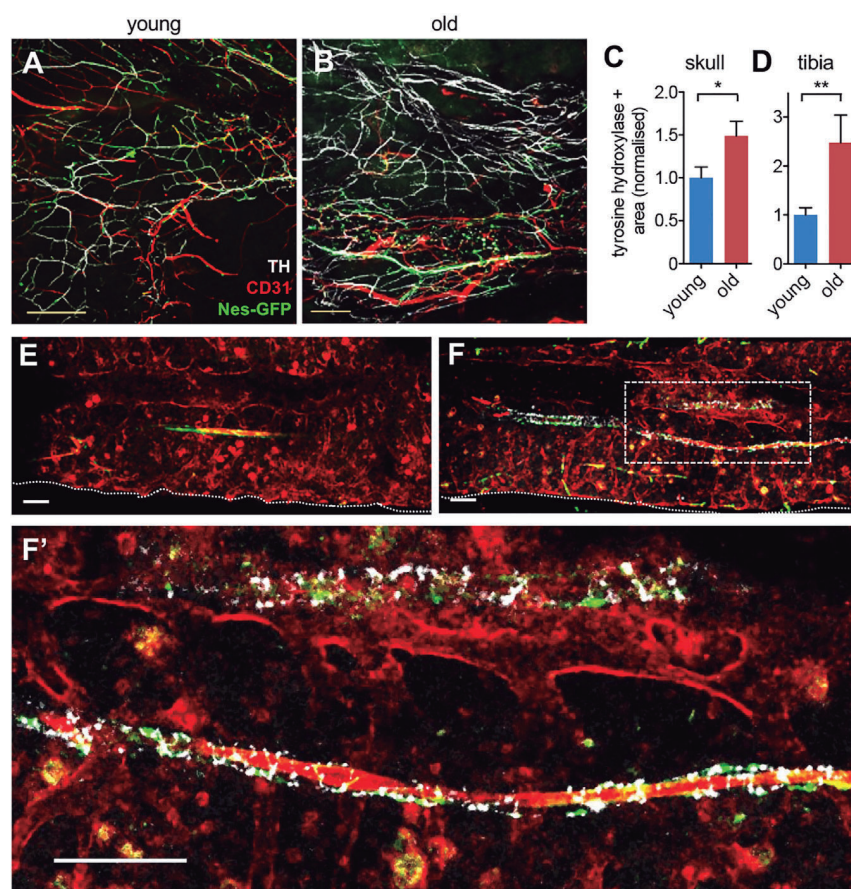


Figure 2. Increased Sympathetic Nerve Fibers during Aging

(A, B, E, and F) Immunofluorescence of tyrosine hydroxylase (TH)⁺ sympathetic noradrenergic nerve fibers (white), CD31⁺ endothelial cells (red), and GFP⁺ cells (green) in the skull (A and B) and tibial (E and F) BM of young (A and E) and old (B and F) *Nes-gfp* mice. Scale bar, 100 μ m. (C and D) Area covered by TH⁺ fibers in the (C) skull or (D) tibia of young ($n = 12$) and old ($n = 8$) *Nes-gfp* mice. Young mice were analyzed between 8–30 weeks of age, and old mice were 66–120 weeks old. Data are means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$ (unpaired two-tailed t test).

β -Adrenergic Signals Promote Megakaryopoiesis during Aging

To study the possible contribution of increased adrenergic innervation to aged hematopoiesis, we analyzed mice lacking β_2 -AR and β_3 -AR (*Adrb2*^{−/−} *Adrb3*^{−/−}), the main β -ARs that cooperate in HSC niche regulation (Méndez-Ferrer et al., 2010a). Importantly, myeloid and megakaryocyte progenitors expanded less in the endosteal BM of aged *Adrb2*^{−/−} *Adrb3*^{−/−} mice (Figures 3A, 3B, and S2A–S2F). Consequently, megakaryocytes did not increase in *Adrb2*^{−/−} *Adrb3*^{−/−} mice during aging (Figures 3C–3G), suggesting a functional role for increased noradrenergic signaling in aged hematopoiesis. Megakaryocytes

We have previously shown that sympathetic adrenergic signals regulate *Nes*-GFP⁺ cell proliferation (Méndez-Ferrer et al., 2010b) and are affected during age-related myeloproliferative neoplasms (Arranz et al., 2014). Additionally, increased sympathetic adrenergic activity has been previously described during aging (Hart and Charkoudian, 2014; Ng et al., 1993; Veith et al., 1986; Ziegler et al., 1976), chronic stress, and depression (Yirmiya et al., 2006), and might increase osteoporosis and fracture risk by restraining bone formation (Elefteriou et al., 2005; Takeda et al., 2002). However, the opposite (decreased BM adrenergic innervation) has been recently suggested as causative of HSC aging (Maryanovich et al., 2018). To clarify this, whole-mount preparations of skulls and thick tibial sections of *Nes-gfp* mice were immunostained for tyrosine hydroxylase (TH), to visualize sympathetic noradrenergic fibers and nestin⁺ cells in large 3D volumes. This study did not confirm reduced TH⁺ fibers in the aged BM (Maryanovich et al., 2018) but found these fibers increased by 50% in the skull of old mice (Figures 2A–2C) and augmented 2.5-fold in the aged tibial BM, compared with the young samples (Figures 2D–2F). In both cases, nestin⁺ cells were found in proximity of noradrenergic fibers (Figures S1E–S1L). Together, these results suggest contraction of endosteal (bone-associated) HSC niches and expansion of non-endosteal neurovascular HSC niches during aging.

were less frequently found in apposition to BM sinusoids and could form less protrusions (required for proplatelet formation) in aged *Adrb2*^{−/−} *Adrb3*^{−/−} mice (Figures 3H–3M). Accordingly, aged *Adrb2*^{−/−} *Adrb3*^{−/−} mice did not show the increased circulating platelets typically observed in aged WT mice (Figure 3N), whereas other circulating blood cell types remained unaffected (Figures S2G–S2J). These results suggest that increased β -adrenergic signaling in the BM promotes myeloid differentiation into platelets during aging.

β_2 -AR in the Microenvironment Promotes Megakaryocyte Differentiation

We analyzed single *Adrb2*^{−/−} mice and *Adrb3*^{−/−} mice to understand the role of each β -AR in myelopoeitic regulation. Resembling aged *Adrb2*^{−/−} *Adrb3*^{−/−} mice (Figure 3N), aged *Adrb2*^{−/−} mice did not exhibit increased circulating platelets (Figure 4A), suggesting that increased β_2 -adrenergic activity promotes thrombopoiesis during aging. Already at adulthood, *Adrb2*^{−/−} mice showed reduced frequency of myeloid and megakaryocyte progenitors, and this reduction persisted during aging (Figures 4B, 4C, S3A, and S3B). We generated BM chimeras using *Adrb2*^{−/−} mice or WT mice as donors/recipients to distinguish hematopoietic cell-autonomous from microenvironmental regulation. WT mice carrying β_2 -AR-deficient hematopoietic cells showed normal frequencies of myeloid and megakaryocyte progenitors (Figures 4B'–4C'

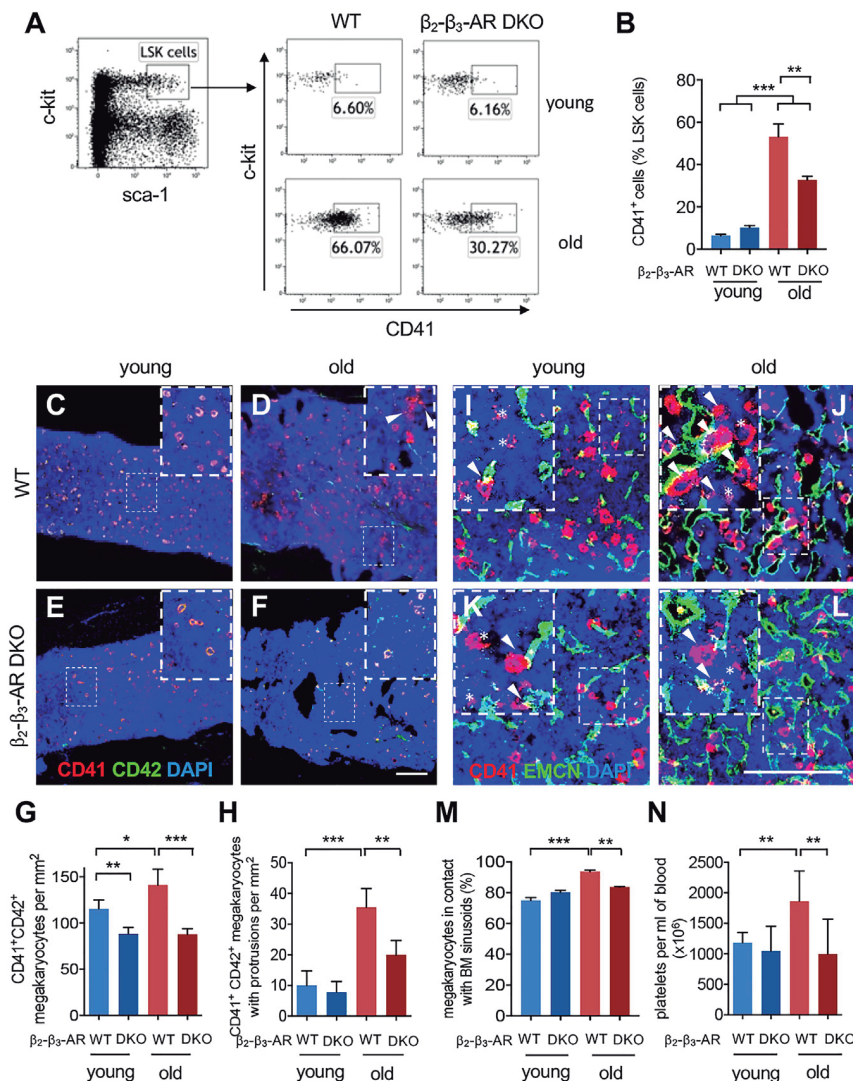


Figure 3. β -Adrenergic Signals Promote Megakaryopoiesis during Aging

(A and B) Representative flow chart (A) and quantification (B) of the frequency of CD41⁺ myeloid or megakaryocyte progenitors within lin[−]sca-1⁺c-kit⁺ (LSK) cells in endosteal BM from young WT mice (n = 7), young $\beta_2\beta_3$ -AR DKO mice (n = 7), old WT mice (n = 5), and old $\beta_2\beta_3$ -AR DKO mice (n = 4). (C–F) Representative immunofluorescence staining for CD41 (red) and CD42 (green) in femoral BM sections of young (C) or old (D) WT mice and young (E) or old (F) $\beta_2\beta_3$ -AR DKO mice. Arrowheads depict megakaryocytes with protrusions (CD41⁺CD42⁺ cells with cell body extensions). Scale bar, 250 μ m.

(G and H) Number of CD41⁺CD42⁺ megakaryocytes (G) forming protrusions (H) per BM area (n = 4 young WT; n = 5 young DKO; n = 3 old mice). (I–L) Representative immunofluorescence staining for CD41 (red) and EMCN (green) in femoral BM sections of young (I) or old (J) WT mice and young (K) or old (L) $\beta_2\beta_3$ -AR DKO mice, depicting CD41⁺ megakaryocytes adjacent (arrowheads) or nonadjacent (asterisks) to EMCN⁺ vasculature. Scale bar, 100 μ m.

(M) Frequency of CD41⁺ cells in contact with EMCN⁺ vasculature (n = 4 young WT; n = 4 young DKO; n = 4 old WT; n = 3 old DKO).

(N) Circulating platelets in young (n = 11) and old (n = 6) WT mice, compared with young (n = 9) and old (n = 5) $\beta_2\beta_3$ -AR DKO mice. Young mice were analyzed between 8–30 weeks of age, and old mice were 66–120 weeks old. Data are means $\pm$ SEM. *p < 0.05; **p < 0.01; ***p < 0.001 (one-way ANOVA followed by Bonferroni pairwise comparisons).

and S3A'–S3B'). In contrast, $\beta_2\beta_3$ -AR recipients of WT BM cells showed similar reductions as found in primary mice (Figures 4B'–4C' and S3A'–S3B'). These results suggest that β_2 -AR signals promote megakaryocyte differentiation through the microenvironment. To obtain mechanistic insight, we treated with selective β_2 -AR or β_3 -AR agonists the HSPC-like HPC-7 cell line (Pinto do O et al., 1998) cultured alone or co-cultured with MS-5 stromal cells, which resemble nestin⁺ BMSCs (Méndez-Ferrer et al., 2008, 2010b; Figures S3C and S3D). Whereas the selective β -AR agonists did not affect megakaryocyte differentiation of HPC-7 cells cultured alone (Figure S3E), β_2 -AR and β_3 -AR agonists had opposite stage-specific effects on megakaryocytic differentiation from HPC-7 cells co-cultured with MS-5 cells. Whereas β_3 -AR agonist increased the frequency of undifferentiated c-kit^{hi}CD41^{lo} cells and decreased the fraction of c-kit^{lo}CD41^{lo} megakaryocyte progenitors, β_2 -AR agonist did not affect early differentiation, but instead increased the frequency of CD41^{hi} megakaryocytic cells at a later differentiation stage (Figure S3F).

To test the potential significance of this regulation in the human setting, we treated human umbilical cord blood-derived hCD34⁺ HSPCs co-cultured with MS-5 cells with a selective β_2 -AR agonist (Figure 4D). Treatment with β_2 -AR agonist increased the frequency of human CD61⁺ megakaryocytic cells (Figures 4E–4G). These results suggest that β_2 -AR stimulation of stromal cells promotes megakaryocyte differentiation.

β_2 -AR Indirectly Promotes Megakaryocyte Differentiation through IL-6

To investigate the underlying mechanism, we measured several cytokines which regulate megakaryopoiesis. Among those, IL-6 concentration increased in WT BM during aging (Figure 1H) and was low in endosteal $\beta_2\beta_3$ -AR DKO BM (Figures 4H and S3G–S3I). Moreover, β_2 -AR agonist increased *Il6* mRNA 2-fold in MS-5 stromal cells; this effect was abrogated after blocking protein kinase A (Figure 4I) downstream of β_2 -AR signaling (Rosenbaum et al., 2009). Adult $\beta_2\beta_3$ -AR DKO mice and *Il6*^{−/−} mice showed similarly reduced BM megakaryocytes (Figures 4J–4M), confirming the role of β_2 -AR and IL-6 in megakaryopoiesis. To directly test the role of IL-6 downstream of β_2 -AR, we established primary BM cultures from *Il6*^{−/−} or control mice and treated them with

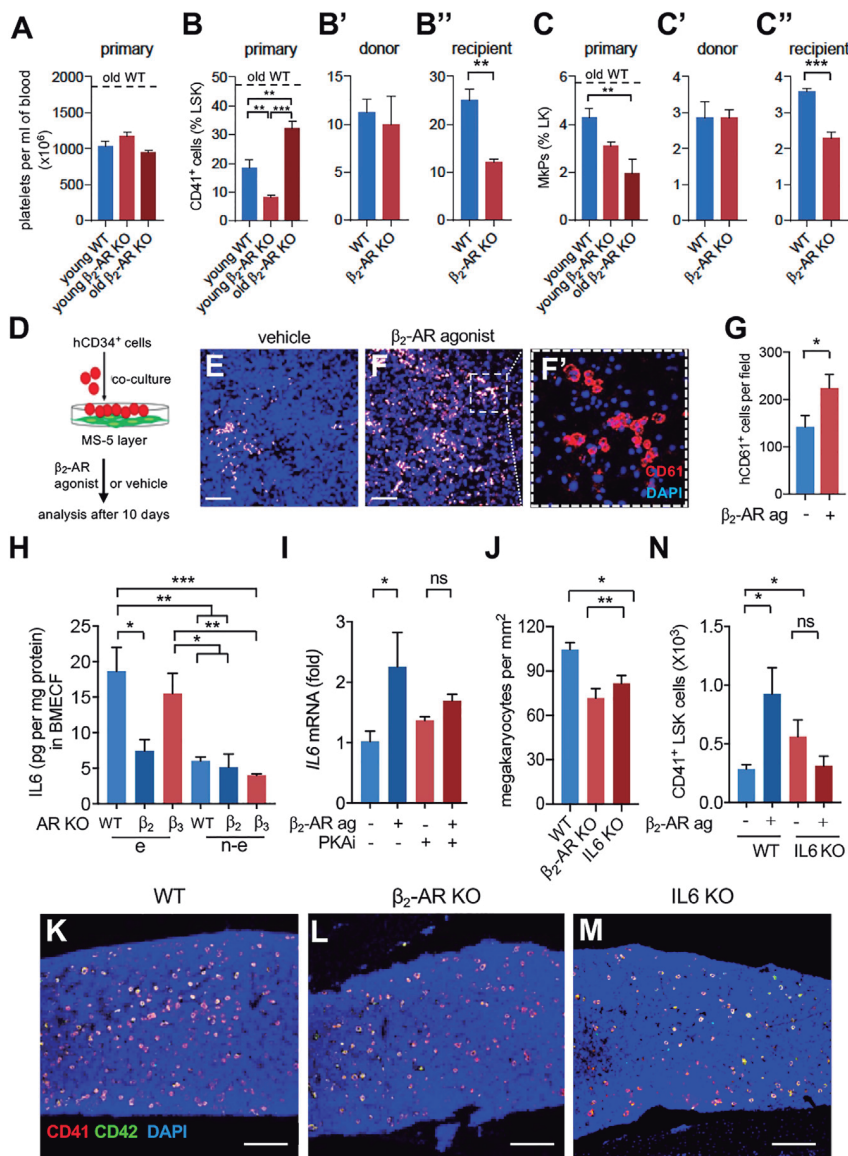


Figure 4. β_2 -AR Signaling in the Microenvironment Promotes Megakaryocyte Differentiation through IL-6

(A) Circulating platelets in young WT ($n = 11$), young *Adrb2*^{-/-} ($n = 3$), or old *Adrb2*^{-/-} ($n = 3$) mice. (B–C'') Frequency of (B, B', and B'') CD41⁺ myeloid or megakaryocyte progenitors within lin⁻sca-1⁺c-kit⁺ (LSK) cells or (C, C', and C'') CD150⁺CD41⁺ megakaryocyte progenitors (MKPs) within lin⁻c-kit⁺ (LK) cells in endosteal BM cells of the following mice: (B and C) young WT (B, $n = 6$; C, $n = 3$), young *Adrb2*^{-/-} (B, $n = 6$; C, $n = 8$) or old *Adrb2*^{-/-} ($n = 4$) mice; (B' and C') lethally irradiated WT recipients of WT ($n = 5$) or *Adrb2*^{-/-} ($n = 4$) BM cells; (B'' and C'') lethally irradiated WT recipients of WT BM cells.

(D) Scheme of human umbilical-cord-blood-derived CD34⁺ HSPCs cocultured with MS-5 stromal cells.

(E–G) Representative immunofluorescence (E and F) and number (G) of CD61⁺ (red) human megakaryocytes in cocultures treated with (E) vehicle or (F) β_2 -AR agonist (clenbuterol, 10 μ M) for 10 days ($n = 3$). Scale bar, 250 μ m. (F') Inset of (F).

(H) IL6 concentration in endosteal (e) or non-endosteal (n-e) BM supernatant from adult WT ($n = 6$), *Adrb2*^{-/-} ($n = 4$), or *Adrb3*^{-/-} ($n = 7$) mice. (I) *Il6* mRNA expression (fold change) in MS-5 stromal cells treated with β_2 -AR agonist (clenbuterol, 10 μ M), PKA inhibitor (H-89, 5 μ M), or vehicle for 2 days ($n = 3$).

(J–M) Quantification (J) and representative immunofluorescence (K–M) of CD41⁺ (red) CD42⁺ (green) megakaryocytes (yellow) in adult WT ($n = 5$), *Adrb2*^{-/-} ($n = 3$), or *Il6*^{-/-} ($n = 5$) mice. Scale bar, 250 μ m.

(N) Myeloid or megakaryocyte progenitors (CD41⁺ LSK cells) in primary BM culture from WT or *Il6*^{-/-} mice treated with β_2 -AR agonist (clenbuterol, 10 μ M) or vehicle for 4 days ($n = 4$). Data are means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (B', B'', C', C'', and G) Unpaired two-tailed t test. (A, B, C, H, I, J, and N) one-way ANOVA and Bonferroni pairwise comparisons.

β_2 -AR agonist or vehicle. β_2 -AR agonist tripled myeloid progenitors in WT samples, but not in *Il6*^{-/-} samples (Figure 4N). Altogether, these results suggest that β_2 -AR on stromal cells promotes megakaryocyte differentiation through IL-6.

β_3 -AR-Deficient Mice Exhibit Altered HSC Lineage Bias

Our co-culture experiments suggested that, contrasting β_2 -AR, β_3 -AR might inhibit megakaryocytic differentiation (Figures S3C–S3F). Additionally, β_3 -AR stimulation has been shown to promote HSC lymphoid skewing (Maryanovich et al., 2018). Therefore, we measured the frequency of immunophenotypically defined lymphoid-biased HSCs (Figure S4A; Yamamoto et al., 2013). Lymphoid-biased HSCs were reduced by one-third in adult endosteal *Adrb3*^{-/-} BM (Figures 5A and S4B). This phenotype was not observed in WT mice carrying β_3 -AR-deficient hematopoietic cells (Figures 5A' and S4B') but was reproduced in chimeric mice lacking β_3 -AR in the microenvironment (Figures

5A'' and S4B''), implying niche-mediated regulation. Opposite trends were observed for long-term HSCs (LT-HSCs) and myeloid-biased HSCs, with their frequencies tending to increase in endosteal *Adrb3*^{-/-} BM (Figures S4C–S4F). These results suggest accelerated lymphoid deficiency in the endosteal microenvironment lacking β_3 -AR.

We investigated possible mechanisms that might explain the intriguingly opposite effects of β_3 -AR and β_2 -AR signaling on lympho-myeloid lineage skewing. Whereas both β -ARs can activate G proteins (Rosenbaum et al., 2009), their differential cardiovascular effects have been attributed to β_3 -AR-dependent nitric oxide (NO) generation (Gauthier et al., 1998). Therefore, we measured nitrate concentration in the BM extracellular fluid and found it doubled in endosteal (compared with non-endosteal) WT BM and halved in *Adrb3*^{-/-} BM (Figure 5B). Among NO synthases, *Nos1* (but not *Nos2* or *Nos3*) showed higher mRNA expression in endosteal WT BM and was downregulated in

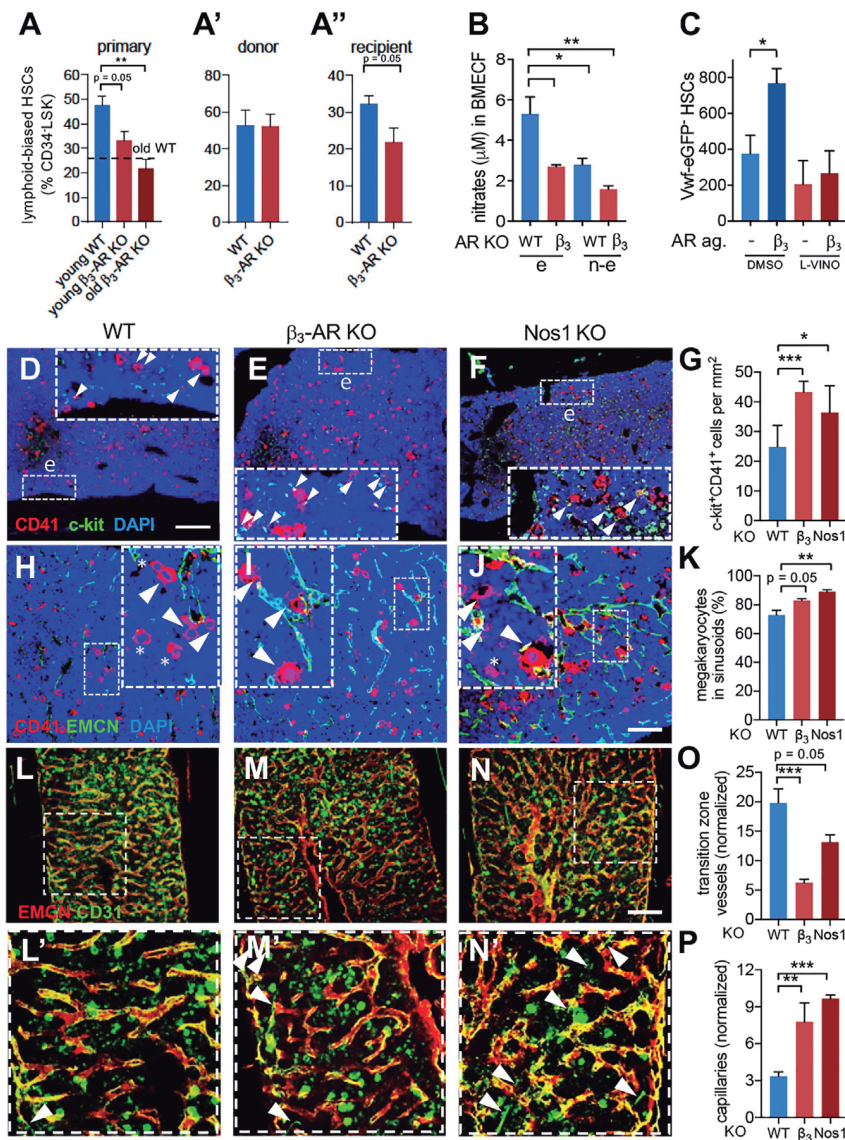


Figure 5. Lack of β_3 -AR and Nitric Oxide Synthase 1 (Nos1) Signaling in the Microenvironment Accelerates Aging

(A, A', and A'') Frequency of $CD150^{lo/-}CD41^{-}$ lymphoid-biased HSCs in endosteal BM $CD34^{-}LSK$ cells from the following mice: (A) young WT ($n = 7$), young $Adrb3^{-/-}$ ($n = 7$), or old $Adrb3^{-/-}$ ($n = 5$) mice; (A') lethally irradiated WT recipients of WT ($n = 5$) or $Adrb3^{-/-}$ ($n = 5$) BM cells; and (A'') lethally irradiated WT ($n = 4$) or $Adrb3^{-/-}$ ($n = 3$) recipients of WT BM cells.

(B) Nitrate concentration in BM extracellular fluid (BMECF) of young WT ($n = 6$) or $Adrb3^{-/-}$ ($n = 7$) mice.

(C) Number of $lin^{-}sca-1^{+}c-kit^{+}CD34^{-}CD48^{-}CD150^{+}Vwf-eGFP^{+}$ lymphoid-biased HSCs in primary BM culture from $Vwf-eGFP$ mice treated with β_3 -AR agonist (BRL37344, 10 μM), Nos1 inhibitor (L-VINO, 100 μM), or vehicle for 4 days ($n = 8$).

(D–G) Representative immunofluorescence (D–F) and quantification (G) of c-kit $^{+}$ (green) CD41 $^{+}$ (red) myeloid or megakaryocyte progenitors (arrowheads) in endosteal BM of WT ($n = 10$), $Adrb3^{-/-}$ ($n = 8$), or $Nos1^{-/-}$ ($n = 4$) mice. Endosteal BM is considered as regions within one-fifth marrow width from the bone surface. Scale bar, 300 μm . (H–K) Representative immunofluorescence (H–J) and quantification (K) of CD41 $^{+}$ (red) megakaryocytes adjacent (arrowheads) or nonadjacent (asterisks) to EMCN $^{+}$ (green) BM vasculature of WT ($n = 9$), $Adrb3^{-/-}$ ($n = 6$), or $Nos1^{-/-}$ ($n = 4$) mice. Scale bar, 250 μm .

(L–N') Representative whole-mount immunofluorescence of CD31 (green) and EMCN (red) in WT ($n = 7$), $Adrb3^{-/-}$ ($n = 5$), or $Nos1^{-/-}$ ($n = 4$) BM. Arrowheads in insets (L'), (M'), and (N') depict CD31 hi EMCN $^{-}$ capillaries.

(O and P) Quantification of (O) CD31 hi EMCN hi transition zone vessels and (P) CD31 hi EMCN $^{-}$ capillaries with $<6 \mu m$ diameter. Scale bar, 250 μm . Data are means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (A' and A'') Unpaired two-tailed t test. (A–C, G, K, O, and P) One-way ANOVA and Bonferroni pairwise comparisons.

$Adrb3^{-/-}$ BM (Figures S4G–S4I). To further study the role of β_3 -AR and Nos1 on HSC lineage bias, we established primary BM cultures from transgenic mice expressing GFP under the regulatory elements of Von Willebrand factor ($Vwf-eGFP$), which discriminate myeloid/platelet-biased ($Vwf-eGFP^{+}$) HSCs from lymphoid-biased ($Vwf-eGFP^{-}$) HSCs (Sanjuan-Pla et al., 2013). Treatment with β_3 -AR agonist did not affect platelet-biased HSCs (Figures S4J and S4K) but doubled lymphoid-biased HSCs in a Nos1-dependent manner (Figure 5C). Moreover, $Adrb3^{-/-}$ mice and $Nos1^{-/-}$ mice showed similar signs of premature hematopoietic aging; myeloid or megakaryocyte progenitors (Figures 5D–5G) and megakaryocyte apposition to blood vessels (Figures 5H–5K) similarly increased in adult $Adrb3^{-/-}$ mice and $Nos1^{-/-}$ mice. Consistently, transition zone vessels decreased (Figures 5L–5O) whereas capillaries expanded (Figure 5P) in both KO models. Despite the partial reduction of lymphoid-biased HSCs in endosteal $Adrb3^{-/-}$ BM, the frequencies of circu-

lating lymphocytes or myeloid cells appeared unchanged in 5-month-old $Adrb3^{-/-}$ mice (Figures S4L–S4N), contrasting previous findings (Maryanovich et al., 2018). Whereas adult $Adrb3^{-/-}$ mice did not yet show in our analysis premature hematopoietic aging in peripheral blood, adult $Nos1^{-/-}$ mice showed reduced lymphocytes and increased neutrophils and platelets in circulation (Figures 6A and 6B), suggesting that other pathways (besides β_3 -AR) contribute to Nos1-dependent regulation of hematopoiesis. Altogether, these results suggest that microenvironmental β_3 -AR contributes to balance HSC lineage-bias toward lymphoid production, which is at least partially dependent on Nos1-dependent NO production.

Premature Hematopoietic Aging in HGPS Is Not HSC-Autonomous

We next interrogated whether the BM microenvironment might promote pathological (and not only physiological) hematopoietic

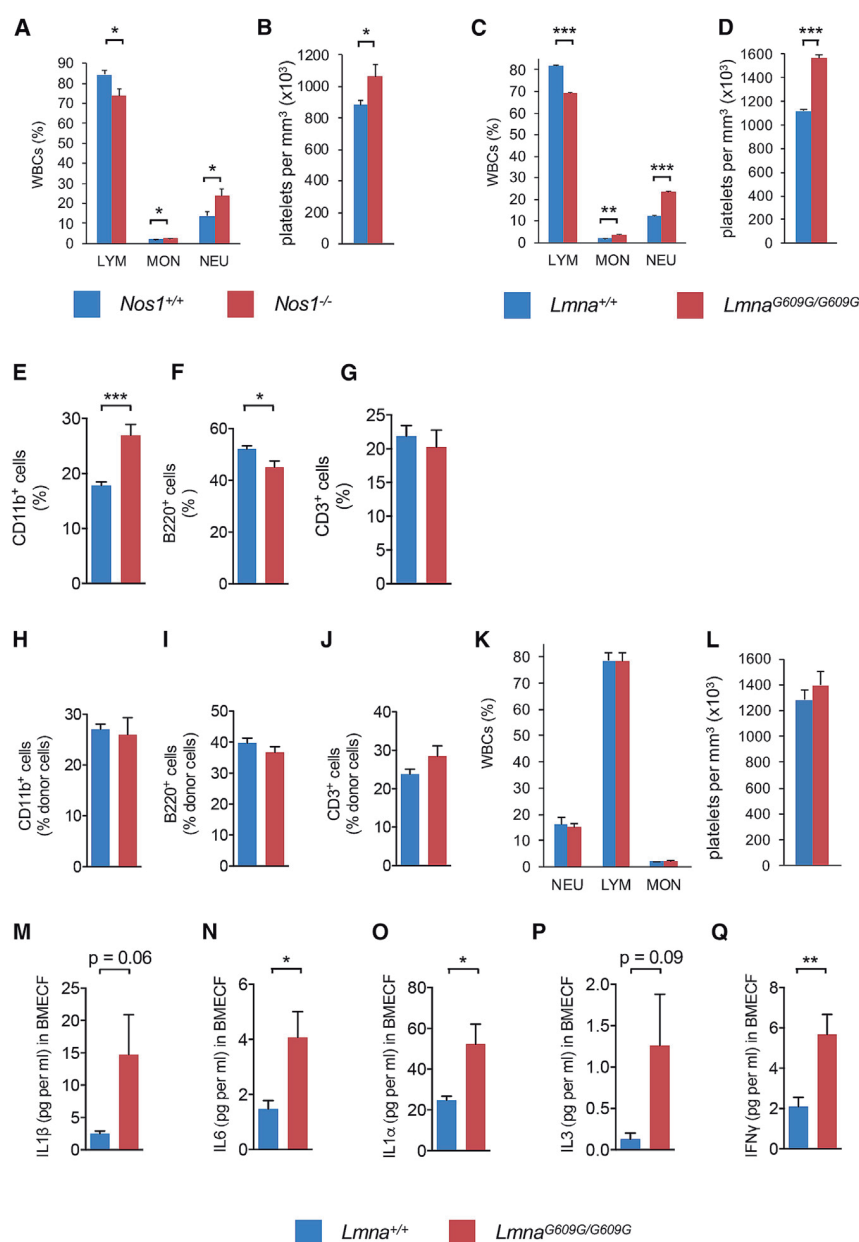


Figure 6. Premature Hematopoietic Aging in HGPS Is Not HSC-Autonomous

(A–D) Peripheral blood counts in adult (A and B) WT (*Nos1*^{+/+}; n = 15) or *Nos1*^{−/−} (n = 16), and (C and D) WT (*Lmna*^{+/+}; n = 22) or *Lmna*^{G609G/G609G} (n = 14) mice. (A, C, and K) Frequency of white blood cells (WBC); LYM, lymphocytes; MON, monocytes; NEU, neutrophils. (B, D, and L) Concentration of platelets.

(E–G) Frequency of (E) CD11b⁺ myeloid cells, (F) B220⁺ B cells, and (G) CD3⁺ T cells in circulating leukocytes.

(H–J) Frequency of (H) CD11b⁺ myeloid cells, (I) B220⁺ B cells, and (J) CD3⁺ T cells among donor-derived leukocytes 120 days after transplantation into WT mice (n = 11).

(K and L) Peripheral white blood cell (K) and platelet (L) counts in adult CD45.1 C57BL/6J mice 4 months after lethal irradiation and transplantation with CD45.2 BM cells from WT (n = 8) or *Lmna*^{G609G/G609G} (n = 6) mice.

(M–Q) Concentration of (M) IL-1β, (N) IL-6, (O) IL-1α, (P) IL-3, and (Q) IFNγ in the BM extracellular fluid (BMECF) of adult WT (*Lmna*^{+/+}; n = 9) and *Lmna*^{G609G/G609G} (n = 9) male mice. Data are means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 (unpaired two-tailed t test).

(Hamczyk et al., 2018; Osorio et al., 2011; Villa-Bellosta et al., 2013). Certain hallmarks of hematopoietic aging in mice, such as increased circulating platelets, have been observed in HGPS (Merideth et al., 2008). However, it remains unknown whether premature aging affects the overall hematopoietic system in HGPS and whether this is a consequence of the *LMNA* mutation in HSCs, other hematopoietic cells and/or their microenvironment.

We measured peripheral blood counts in progeroid mice and control mice. Remarkably, adult progeroid mice resembled adult *Nos1*^{−/−} mice (Figures 6A and 6B) in their premature myeloid skewing evidenced by decreased lym-

phocytes and increased neutrophils, monocytes, and platelets (Figures 6C and 6D), to a similar extent as normally aged mice (Dykstra et al., 2011; Rossi et al., 2005; Sudo et al., 2000). Flow cytometry analysis confirmed increased myeloid cells and decreased lymphoid cells in the peripheral blood of progeroid mice (Figures 6E–6G). To test HSC function, lethally irradiated CD45.1 recipients were transplanted with CD45.2 BM cells from *Lmna*^{G609G/G609G} mice or WT mice, together with competitor BM cells from congenic CD45.1 mice (Figure S5A). Long-term engraftment was lower for *Lmna*^{G609G/G609G} (compared with WT) hematopoietic cells (Figure S5B), as previously observed for normally aged HSCs (Liang et al., 2005), albeit to a lower extent. However, myeloid skewing was not observed in blood cells derived from

aging. Premature aging in Hutchinson-Gilford progeria syndrome (HGPS) is caused by the accumulation of a truncated prelamin A protein named “progerin,” which is produced via aberrant splicing resulting from a *de novo* synonymous c.1824C > T (p.G608G) point mutation in the *LMNA* gene (encoding lamin A and C) (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). This gene has been recently associated with changes in epigenetic and chromatin architecture in aged HSCs (Grigoryan et al., 2018). Progerin also accumulates during normal aging and HGPS displays most aging hallmarks, suggesting that HGPS can inform physiological aging (López-Otín et al., 2013). *Lmna*^{G609G} knockin mice exhibit key hallmarks of the human disease, including accelerated aging, shortened lifespan, and bone and cardiovascular defects

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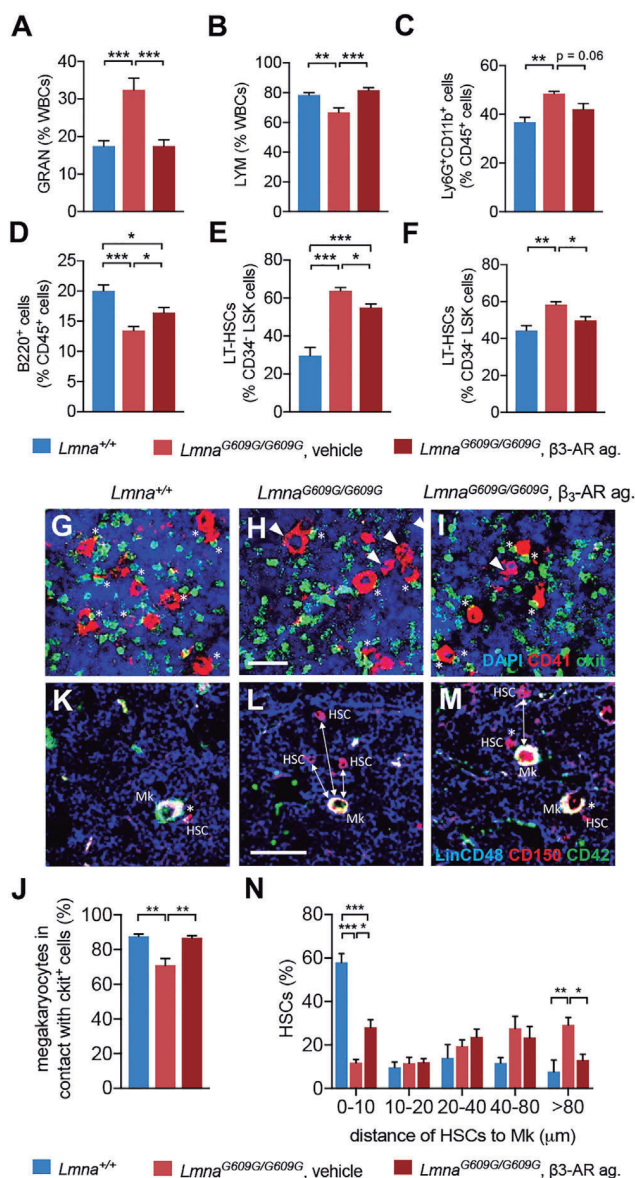


Figure 7. β₃-AR Agonist Improves Lineage Skewing, HSC Number, and Localization near Megakaryocytes in HGPS

(A and B) Frequency of (A) granulocytes and (B) lymphocytes in white blood cells (WBCs) of WT mice (n = 8) or *Lmna*^{G609G/G609G} mice (n = 6) treated with β₃-AR agonist (BRL37344, 2 mg/kg/day, intraperitoneally [i.p.]) or vehicle for 8 weeks.

(C–F) Frequency of BM (C) Ly6G⁺CD11b⁺ neutrophils, (D) B220⁺ B cells, endosteal (E), and non-endosteal (F) LT-HSCs in these mice (n = 6).

(G–J) Representative immunofluorescence (G–I) and quantification (J) of CD41⁺ megakaryocytes adjacent (asterisks) or nonadjacent (arrowheads) to c-kit⁺ (green) HSPCs in BM of WT mice (n = 3) and *Lmna*^{G609G/G609G} mice (n = 5) treated with β₃-AR agonist or vehicle. Scale bar, 50 μm.

(K–N) Representative immunofluorescence (K–M) and distribution (N) of BM CD150⁺ (red) HSCs (negative for mature hematopoietic lineage markers, blue) adjacent (asterisks), or nonadjacent (arrows) to CD42⁺ (green) megakaryocytes. Scale bar, 50 μm. Data are means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. (A–F and J) One-way ANOVA and Bonferroni pairwise comparisons. (N) Two-way ANOVA and Bonferroni pairwise comparisons.

Lmna^{G609G/G609G} HSCs (Figures 6H–6J), suggesting that myeloid skewing is a non-HSC-autonomous aging feature in HGPS. To assess this possibility, we transplanted CD45.2 BM cells from *Lmna*^{G609G/G609G} mice into lethally irradiated CD45.1 WT recipients. Sixteen weeks later, WT recipients carrying the *Lmna*^{G609G/G609G} mutation only in hematopoietic cells (Figures 6K and 6L) did not reproduce the myeloid skewing and increased platelet counts observed in constitutive *Lmna*^{G609G/G609G} mice (Figures 6C and 6D). Together, these results suggest that premature aging in HGPS affects the hematopoietic system but cannot be explained by HSC-autonomous alterations. Consistent with this idea, microenvironmental alterations (such as sinusoidal vasodilation) were found in *Lmna*^{G609G/G609G} BM (Figures S5D and S5E). Moreover, myelopoietic cytokines augmented in normally aged BM microenvironment (Figures 1J–1N) were also increased in adult progeroid mice (Figures 6M–6Q).

These results suggested that similar microenvironmental alterations might promote myeloid differentiation during physiological/pathological aging. Therefore, we tested whether targeting the microenvironment could impact hematopoiesis in HGPS. Progeroid mice were chronically treated with a β₃-AR agonist, which can rescue nestin⁺ niches in humans and mice with age-related myeloproliferative disorders (Arranz et al., 2014; Drexler et al., 2019) and has been recently suggested to rejuvenate normally aged HSCs (Maryanovich et al., 2018). Treatment with β₃-AR agonist over 2 months normalized circulating granulocytes and lymphocytes and BM neutrophils and partially rescued BM B cells (Figures 7A–7D). This effect correlated with decreased frequency of BM LT-HSCs (Figures 7E and 7F).

Megakaryocytes have been shown to regulate HSC proliferation (Bruns et al., 2014; Nakamura-Ishizu et al., 2015; Zhao et al., 2014) and their numbers were found to increase during normal/pathological aging in this study. Therefore, we examined the spatial relationships between megakaryocytes and HSPCs in the different models. Like in aged mice (Maryanovich et al., 2018), HSCs expanded but located further from megakaryocytes in adult *Adrb3*^{−/−} mice, as an additional premature aging feature in these mice (Figures S6A–S6D). Increased LT-HSC frequency in vehicle-treated progeroid mice (compared with WT mice) (Figures 7E and 7F) correlated with reduced co-localization of HSPCs and megakaryocytes, which was rescued by β₃-AR agonist (Figures 7G–7J). HSCs located more distantly from megakaryocytes in vehicle-treated progeroid mice (compared with WT mice) but this association was partially restored by β₃-AR agonist (Figures 7K–7N). These results suggest the possibility that decreased interactions between megakaryocytes and HSCs might reversibly regulate hematopoiesis during aging.

DISCUSSION

This study suggests that alterations of the BM microenvironment during pathological/physiological aging change hematopoiesis. Remodeling of BM HSC niches (namely reduced endosteal niches and increased non-endosteal neurovascular niches) is associated with the overproduction of pro-inflammatory cytokines which drive excessive myelopoiesis in normal/premature aging. However, the latter can be improved by modulating the microenvironment. Together, these results expand previous findings on the microenvironmental

contribution to hematopoietic aging to the role of different neurovascular beds in normal and pathological aging, and their potential therapeutic targeting.

Stem cell aging compromises tissue turnover and regeneration, but the contribution of the aged stem cell niche remains incompletely understood (López-Otín et al., 2013). In the hematopoietic system, HSCs expand during aging but exhibit impaired self-renewal and lymphoid differentiation potential, favoring myeloid output (Liang et al., 2005; Mohrin et al., 2015; Pang et al., 2011; Rossi et al., 2005; Rundberg Nilsson et al., 2016; Sudo et al., 2000). Moreover, myeloid malignancies are more frequent in the elderly, but whether changes in the aged HSCs and/or their microenvironment predispose to these malignancies has remained unclear. Although HSC aging was initially regarded as solely derived from intrinsic changes in HSCs (Chambers et al., 2007; Dykstra et al., 2011; Florian et al., 2013; Rossi et al., 2007), several alterations in the BM microenvironment or in its interaction with HSCs have been proposed to promote hematopoietic aging (Ergen et al., 2012; Ju et al., 2007; Köhler et al., 2009; Maryanovich et al., 2018). Our study supports this contention by demonstrating that changes in the microenvironment contribute to physiological hematopoietic aging and pathological premature aging (HGPS). In both settings, the aged microenvironment promotes myeloid differentiation through similar cytokines, such as IL-1 β and IL-6.

Progeroid mice exhibit several hallmarks of hematopoietic aging: expanded HSCs with reduced engraftment, lymphoid deficiency, and myeloid skewing toward platelets. However, chimeric mice carrying *Lmna*^{G609G/G609G} hematopoietic cells in a WT environment do not reproduce the hematopoietic aging of progeroid mice. Together with the altered vasculature and increased concentration of myelopoietic cytokines in progeroid BM, these results strongly suggest that the BM microenvironment causes premature hematopoietic aging in HGPS. In fact, chronic treatment of progeroid mice with β_3 -AR agonist (targeting the microenvironment) reduces HSCs and restores their proximity to megakaryocytes and their lympho-myeloid skewing.

During physiological aging, endosteal BM niches decrease, consistent with previous observations (Kusumbe et al., 2016), whereas non-endosteal neurovascular BM niches containing Nes-GFP⁺ stromal cells show a pronounced expansion. This microenvironmental remodeling might directly favor myeloid expansion during aging, because lymphoid niches have been found near bone (Morrison and Scadden, 2014), whereas myeloid cell expansion and thrombopoiesis mainly occurs in non-endosteal niches (Eto and Kunishima, 2016).

Recently, an age-related reduction of BM adrenergic nerve fibers has been reported to drive most hallmarks of HSC aging (Maryanovich et al., 2018). However, studies on BM innervation during aging have provided conflicting results, with BM adrenergic fibers reportedly been dramatically reduced (Maryanovich et al., 2018), unchanged, or increased (Chartier et al., 2018). The whole-mount or thick section imaging and 3D reconstruction of different bones in the present study supports the latter and demonstrates that these fibers are doubled in different bones during physiological aging. Moreover, these fibers are not reduced in adult progeroid mice (Figure S7), which exhibit premature hematopoietic aging. Our findings are consistent with the well-known increase in sympathetic activity in the elderly (Hart and

Charkoudian, 2014; Ng et al., 1993; Veith et al., 1986; Ziegler et al., 1976) and suggest a functional change of neurotransmission (β_2 -AR overriding β_3 -AR), rather than a general decline of BM innervation during aging. Actually, increased noradrenergic activity in the BM seems to account for augmented thrombopoiesis in aged mice because platelets are not elevated in aged *Adrb2*^{-/-} mice or *Adrb2*^{-/-}*Adrb3*^{-/-} mice. A previous study showed direct effects of α -ARs on mature megakaryocyte adhesion, proplatelet formation, and platelet release, whereas α -AR signaling on more primitive CD34⁺ progenitor cells did not affect lineage commitment (Chen et al., 2016). However, the present study indicates that HSPC lineage commitment is regulated by the microenvironment through β_2 -AR and β_3 -AR. Therefore, BM noradrenergic activity appears to regulate lineage commitment and megakaryopoiesis at different stages of maturation through distinct α - and β -ARs. Interestingly, β_2 -AR and β_3 -AR exhibit opposite roles on myeloid differentiation: whereas β_2 -AR signaling promotes megakaryopoiesis through stromal-cell-derived IL6 and becomes predominant during aging, β_3 -AR inhibits myelopoiesis. Lack of β_2 -AR in the microenvironment (but not in the hematopoietic system) halves myeloid and megakaryocyte progenitors in adult mice. Decreased frequency of megakaryocyte lineage cells is similarly found in adult *Adrb2*^{-/-} mice and aged *Adrb2*^{-/-}*Adrb3*^{-/-} mice (but not in WT chimeras carrying *Adrb2*^{-/-} hematopoietic cells), suggesting that predominant β_2 -AR activation promotes megakaryocyte differentiation during aging. Whereas adult *Adrb2*^{-/-} mice and *Adrb3*^{-/-} mice exhibit opposite deregulation of lymphoid/myeloid lineage output, only *Adrb2*^{-/-} mice retain this phenotype upon aging (Figures 4B, 4C, and 5A), further suggesting that β_2 -AR prevails over β_3 -AR in hematopoietic lineage regulation during aging. This regulation requires IL-6, a cytokine known to increase in elderly humans (Brunnahan et al., 2010) and to be similarly regulated in other cell types (Li et al., 2013). Supporting the role of IL6, megakaryocytes appear decreased in *Il6*^{-/-} BM. Moreover, β_2 -AR agonist specifically increased IL-6 expression in stromal cells co-cultured with human/murine HSPCs and consequently stimulates megakaryopoiesis in these co-cultures or in primary BM cultures from WT mice, but not *Il6*^{-/-} mice.

In contrast, microenvironmental (but not hematopoietic) lack of β_3 -AR partially reduces lymphoid-biased HSCs in adult mice, as previously reported (Maryanovich et al., 2018). However, the results here differ in that β_3 -AR loss does not appear overall sufficient to cause premature hematopoietic aging, because the frequencies of circulating lymphocytes and myeloid cells appeared unchanged in 5-month-old *Adrb3*^{-/-} mice. *In vitro*, β_3 -AR agonist-treated stromal cells decrease human and murine HSPC differentiation into megakaryocytes. This effect requires *Nos1*, because *Nos1*^{-/-} mice have high circulating platelets and granulocytes, but low circulating lymphocytes. Furthermore, β_3 -AR agonist specifically increases lymphoid-biased HSCs in primary BM cultures in a *Nos1*-dependent manner. Moreover, adult *Adrb3*^{-/-} or *Nos1*^{-/-} mice share premature microenvironmental aging features in the BM, such as reduced bone-forming transition zone vessels (Kusumbe et al., 2016) and increased BM capillaries, myeloid progenitors, and megakaryocyte apposition to blood vessels.

Megakaryocytes inhibit HSC proliferation (Bruns et al., 2014; Nakamura-Ishizu et al., 2015; Zhao et al., 2014). Whereas both

HSCs and megakaryocytes expand during aging, HSCs locate further from megakaryocytes in adult *Adrb3*^{-/-} mice and progeroid mice, suggesting that decreased HSC-megakaryocyte interactions might contribute to premature hematopoietic aging features in these mice. Supporting this possibility, chronic treatment of progeroid mice with β_3 -AR agonist decreased HSCs and corrected lineage skewing, correlated with normalized distribution of HSCs near megakaryocytes. Future studies will be required to investigate changes in the regulation of HSC proliferation by megakaryocytes during aging.

In summary, this study shows that the aged BM microenvironment promotes myeloid expansion during physiological aging and in premature aging. Normal murine aging concurs with the reduction of endosteal niches and the expansion of non-endosteal niches comprising capillaries and nestin⁺ cells associated with sympathetic noradrenergic fibers. Interestingly, β_2 -AR and β_3 -AR regulate myelopoiesis through opposite and stage-dependent effects on the hematopoietic microenvironment. During normal aging, increased β_2 -AR activity promotes IL6-dependent myeloid differentiation, whereas decreased β_3 -AR-Nos1-NO is associated with reduced endosteal niches and increased central niches. Adult Nos1 KO mice and progeroid mice display premature aging in peripheral blood, manifested as reduced lymphocytes and increased myeloid cells. Megakaryocytes move closer to BM sinusoids in *Adrb3*^{-/-} or progeroid mice, possibly explaining increased thrombopoiesis. Megakaryocytes and HSCs expand but separate from each other during normal aging and in adult *Adrb3*^{-/-} or progeroid mice. However, chronic treatment of progeroid mice with β_3 -AR agonist reduces HSCs and restores their proximity to megakaryocytes and their lympho-myeloid skewing. Therefore, normal or premature niche aging promotes normal myeloid expansion, which can be improved by targeting the microenvironment.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stem.2019.06.007>.

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AUTHOR CONTRIBUTIONS

Y.-H.H., R.d.T., J.R.-T., J.R., C.K., A.G.-G., D.M., A.d.M., and C.G.-G. designed and performed experiments and analyzed data. A.K.W. and H.R.F. assisted with co-culture experiments. M.W. and F.L. analyzed *Nos1* KO mice. C.L.-O. provided progeroid mice. R.S.J. facilitated nitrate measurements. C.N. provided *Vwf-eGFP* mice. W.V., C.G., and V.A. provided advice on the design, supervision, and analysis of experiments. Y.-H.H. and S.M.-F. prepared the figures and wrote the manuscript. S.M.-F. planned and supervised the overall study. All authors revised and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

- Arranz, L., Sánchez-Aguilera, A., Martín-Pérez, D., Isern, J., Langa, X., Tzankov, A., Lundberg, P., Muntión, S., Tzeng, Y.S., Lai, D.M., et al. (2014). Neuropathy of haematopoietic stem cell niche is essential for myeloproliferative neoplasms. *Nature* 512, 78–81.
- Bruns, I., Lucas, D., Pinho, S., Ahmed, J., Lambert, M.P., Kunisaki, Y., Scheiermann, C., Schiff, L., Poncz, M., Bergman, A., and Frenette, P.S. (2014). Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. *Nat. Med.* 20, 1315–1320.
- Brunnahan, S.K., McGuire, T.R., Jackson, J.D., Lane, J.T., Garvin, K.L., O’Kane, B.J., Berger, A.M., Tuljapourkar, S.R., Kessinger, M.A., and Sharp, J.G. (2010). Human blood and marrow side population stem cell and Stro-1 positive bone marrow stromal cell numbers decline with age, with an increase

in quality of surviving stem cells: correlation with cytokines. *Mech. Ageing Dev.* 131, 718–722.

Chambers, S.M., Shaw, C.A., Gatz, C., Fisk, C.J., Donehower, L.A., and Goodell, M.A. (2007). Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. *PLoS Biol.* 5, e201.

Chartier, S.R., Mitchell, S.A.T., Majuta, L.A., and Mantyh, P.W. (2018). The Changing Sensory and Sympathetic Innervation of the Young, Adult and Aging Mouse Femur. *Neuroscience* 387, 178–190.

Chen, S., Du, C., Shen, M., Zhao, G., Xu, Y., Yang, K., Wang, X., Li, F., Zeng, D., Chen, F., et al. (2016). Sympathetic stimulation facilitates thrombopoiesis by promoting megakaryocyte adhesion, migration, and proplatelet formation. *Blood* 127, 1024–1035.

Chruscinski, A.J., Rohrer, D.K., Schauble, E., Desai, K.H., Bernstein, D., and Kobilka, B.K. (1999). Targeted disruption of the beta2 adrenergic receptor gene. *J. Biol. Chem.* 274, 16694–16700.

De Sandre-Giovannoli, A., Bernard, R., Cau, P., Navarro, C., Amiel, J., Boccaccio, I., Lyonnet, S., Stewart, C.L., Munnich, A., Le Merrer, M., and Lévy, N. (2003). Lamin A truncation in Hutchinson-Gilford progeria. *Science* 300, 2055.

Drexler, B., Passweg, J.R., Tzankov, A., Bigler, M., Theodorides, A.P.A., Cantoni, N., Keller, P., Stussi, G., Ruefer, A., Benz, R., et al. (2019). The sympathomimetic agonist mirabegron did not lower JAK2-V617F allele burden, but restored nestin-positive cells and reduced reticulon fibrosis in patients with myeloproliferative neoplasms: results of phase 2 study SAKK 33/14. *Haematologica* 104, 710–716.

Dykstra, B., Olthof, S., Schreuder, J., Ritsema, M., and de Haan, G. (2011). Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *J. Exp. Med.* 208, 2691–2703.

Eleftheriou, F., Ahn, J.D., Takeda, S., Starbuck, M., Yang, X., Liu, X., Kondo, H., Richards, W.G., Bannon, T.W., Noda, M., et al. (2005). Leptin regulation of bone resorption by the sympathetic nervous system and CART. *Nature* 434, 514–520.

Ergen, A.V., Boles, N.C., and Goodell, M.A. (2012). Rantes/Ccl5 influences hematopoietic stem cell subtypes and causes myeloid skewing. *Blood* 119, 2500–2509.

Eriksson, M., Brown, W.T., Gordon, L.B., Glynn, M.W., Singer, J., Scott, L., Erdos, M.R., Robbins, C.M., Moses, T.Y., Berglund, P., et al. (2003). Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome. *Nature* 423, 293–298.

Eto, K., and Kunishima, S. (2016). Linkage between the mechanisms of thrombocytopenia and thrombopoiesis. *Blood* 127, 1234–1241.

Flach, J., Bakker, S.T., Mohrin, M., Conroy, P.C., Pietras, E.M., Reynaud, D., Alvarez, S., Diolaiti, M.E., Ugarte, F., Forsberg, E.C., et al. (2014). Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells. *Nature* 512, 198–202.

Florian, M.C., Nattamai, K.J., Dörr, K., Marka, G., Uberle, B., Vas, V., Eckl, C., Andrä, I., Schiemann, M., Oostendorp, R.A., et al. (2013). A canonical to non-canonical Wnt signalling switch in haematopoietic stem-cell ageing. *Nature* 503, 392–396.

Gauthier, C., Leblais, V., Kobzik, L., Trochu, J.N., Khoudoudi, N., Bril, A., Balligand, J.L., and Le Marec, H. (1998). The negative inotropic effect of beta3-adrenoceptor stimulation is mediated by activation of a nitric oxide synthase pathway in human ventricle. *J. Clin. Invest.* 102, 1377–1384.

Gekas, C., and Graf, T. (2013). CD41 expression marks myeloid-biased adult hematopoietic stem cells and increases with age. *Blood* 121, 4463–4472.

Grigoryan, A., Guidi, N., Senger, K., Liehr, T., Solter, K., Marka, G., Vollmer, A., Markaki, Y., Leonhardt, H., Buske, C., et al. (2018). LaminA/C regulates epigenetic and chromatin architecture changes upon aging of hematopoietic stem cells. *Genome Biol.* 19, 189.

Grover, A., Sanjuan-Pla, A., Thongjuea, S., Carrelha, J., Giustacchini, A., Gambardella, A., Macaulay, I., Mancini, E., Luis, T.C., Mead, A., et al. (2016). Single-cell RNA sequencing reveals molecular and functional platelet bias of aged haematopoietic stem cells. *Nat. Commun.* 7, 11075.

Hamczyk, M.R., Villa-Belostá, R., Gonzalo, P., Andrés-Manzano, M.J., Nogales, P., Bentzon, J.F., López-Otín, C., and Andrés, V. (2018). Vascular Smooth Muscle-Specific Progerin Expression Accelerates Atherosclerosis and Death in a Mouse Model of Hutchinson-Gilford Progeria Syndrome. *Circulation* 138, 266–282.

Hart, E.C., and Charkoudian, N. (2014). Sympathetic neural regulation of blood pressure: influences of sex and aging. *Physiology (Bethesda)* 29, 8–15.

Ho, T.T., Warr, M.R., Adelman, E.R., Lansinger, O.M., Flach, J., Verovskaya, E.V., Figueroa, M.E., and Passequé, E. (2017). Autophagy maintains the metabolism and function of young and old stem cells. *Nature* 543, 205–210.

Huang, P.L., Dawson, T.M., Bredt, D.S., Snyder, S.H., and Fishman, M.C. (1993). Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 75, 1273–1286.

Ierni, J., García-García, A., Martín, A.M., Arranz, L., Martín-Pérez, D., Torroja, C., Sánchez-Cabo, F., and Méndez-Ferrer, S. (2014). The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. *eLife* 3, e03696.

Ju, Z., Jiang, H., Jaworski, M., Rathinam, C., Gompf, A., Klein, C., Trumpp, A., and Rudolph, K.L. (2007). Telomere dysfunction induces environmental alterations limiting hematopoietic stem cell function and engraftment. *Nat. Med.* 13, 742–747.

Köhler, A., Schmithorst, V., Filippi, M.D., Ryan, M.A., Daria, D., Gunzer, M., and Geiger, H. (2009). Altered cellular dynamics and endosteal location of aged early hematopoietic progenitor cells revealed by time-lapse intravital imaging in long bones. *Blood* 114, 290–298.

Kopf, M., Baumann, H., Freer, G., Freudenberg, M., Lamers, M., Kishimoto, T., Zinkernagel, R., Bluethmann, H., and Köhler, G. (1994). Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* 368, 339–342.

Kusumbe, A.P., Ramasamy, S.K., Itkin, T., Mäe, M.A., Langen, U.H., Betsholtz, C., Lapidot, T., and Adams, R.H. (2016). Age-dependent modulation of vascular niches for haematopoietic stem cells. *Nature* 532, 380–384.

Li, W., Shi, X., Wang, L., Guo, T., Wei, T., Cheng, K., Rice, K.C., Kingery, W.S., and Clark, J.D. (2013). Epidermal adrenergic signaling contributes to inflammation and pain sensitization in a rat model of complex regional pain syndrome. *Pain* 154, 1224–1236.

Liang, Y., Van Zant, G., and Szilvassy, S.J. (2005). Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. *Blood* 106, 1479–1487.

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. *Cell* 153, 1194–1217.

Maryanovich, M., Zahalka, A.H., Pierce, H., Pinho, S., Nakahara, F., Asada, N., Wei, Q., Wang, X., Ciero, P., Xu, J., et al. (2018). Adrenergic nerve degeneration in bone marrow drives aging of the hematopoietic stem cell niche. *Nat. Med.* 24, 782–791.

Méndez-Ferrer, S., Lucas, D., Battista, M., and Frenette, P.S. (2008). Hematopoietic stem cell release is regulated by circadian oscillations. *Nature* 452, 442–447.

Méndez-Ferrer, S., Battista, M., and Frenette, P.S. (2010a). Cooperation of beta(2)- and beta(3)-adrenergic receptors in hematopoietic progenitor cell mobilization. *Ann. N Y Acad. Sci.* 1192, 139–144.

Méndez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., and Frenette, P.S. (2010b). Mesenchymal and hematopoietic stem cells form a unique bone marrow niche. *Nature* 466, 829–834.

Merideth, M.A., Gordon, L.B., Clauss, S., Sachdev, V., Smith, A.C., Perry, M.B., Brewer, C.C., Zalewski, C., Kim, H.J., Solomon, B., et al. (2008). Phenotype and course of Hutchinson-Gilford progeria syndrome. *N. Engl. J. Med.* 358, 592–604.

Mignone, J.L., Kukekov, V., Chiang, A.S., Steindler, D., and Enikolopov, G. (2004). Neural stem and progenitor cells in nestin-GFP transgenic mice. *J. Comp. Neurol.* 469, 311–324.

Mohrin, M., Shin, J., Liu, Y., Brown, K., Luo, H., Xi, Y., Haynes, C.M., and Chen, D. (2015). Stem cell aging. A mitochondrial UPR-mediated metabolic checkpoint regulates hematopoietic stem cell aging. *Science* 347, 1374–1377.

- Morrison, S.J., and Scadden, D.T. (2014). The bone marrow niche for haematopoietic stem cells. *Nature* 505, 327–334.
- Nakamura-Ishizu, A., Takubo, K., Kobayashi, H., Suzuki-Inoue, K., and Suda, T. (2015). CLEC-2 in megakaryocytes is critical for maintenance of hematopoietic stem cells in the bone marrow. *J. Exp. Med.* 212, 2133–2146.
- Ng, A.V., Callister, R., Johnson, D.G., and Seals, D.R. (1993). Age and gender influence muscle sympathetic nerve activity at rest in healthy humans. *Hypertension* 21, 498–503.
- Osorio, F.G., Navarro, C.L., Cadiñanos, J., López-Mejía, I.C., Quirós, P.M., Bartoli, C., Rivera, J., Tazi, J., Guzmán, G., Varela, I., et al. (2011). Splicing-directed therapy in a new mouse model of human accelerated aging. *Sci. Transl. Med.* 3, 106ra107.
- Pang, W.W., Price, E.A., Sahoo, D., Beerman, I., Maloney, W.J., Rossi, D.J., Schrier, S.L., and Weissman, I.L. (2011). Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proc. Natl. Acad. Sci. USA* 108, 20012–20017.
- Pietras, E.M. (2017). Inflammation: a key regulator of hematopoietic stem cell fate in health and disease. *Blood* 130, 1693–1698.
- Pinho, S., Marchand, T., Yang, E., Wei, Q., Nerlov, C., and Frenette, P.S. (2018). Lineage-Biased Hematopoietic Stem Cells Are Regulated by Distinct Niches. *Dev. Cell* 44, 634–641.
- Pinto do O, P., Kolterud, A., and Carlsson, L. (1998). Expression of the LIM-homeobox gene LH2 generates immortalized steel factor-dependent multipotent hematopoietic precursors. *EMBO J.* 17, 5744–5756.
- Plo, I., Bellanné-Chantelot, C., Mosca, M., Mazzi, S., Marty, C., and Vainchenker, W. (2017). Genetic Alterations of the Thrombopoietin/MPL/JAK2 Axis Impacting Megakaryopoiesis. *Front. Endocrinol. (Lausanne)* 8, 234.
- Rosenbaum, D.M., Rasmussen, S.G., and Kobilka, B.K. (2009). The structure and function of G-protein-coupled receptors. *Nature* 459, 356–363.
- Rossi, D.J., Bryder, D., Zahn, J.M., Ahlenius, H., Sonu, R., Wagers, A.J., and Weissman, I.L. (2005). Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proc. Natl. Acad. Sci. USA* 102, 9194–9199.
- Rossi, D.J., Bryder, D., Seita, J., Nussenzweig, A., Hoeijmakers, J., and Weissman, I.L. (2007). Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature* 447, 725–729.
- Rundberg Nilsson, A., Soneji, S., Adolfsson, S., Bryder, D., and Pronk, C.J. (2016). Human and Murine Hematopoietic Stem Cell Aging Is Associated with Functional Impairments and Intrinsic Megakaryocytic/Erythroid Bias. *PLoS ONE* 11, e0158369.
- Sanjuan-Pla, A., Macaulay, I.C., Jensen, C.T., Woll, P.S., Luis, T.C., Mead, A., Moore, S., Carella, C., Matsuoka, S., Bouriez Jones, T., et al. (2013). Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature* 502, 232–236.
- Stegner, D., vanEeuwijk, J.M.M., Angay, O., Gorelashvili, M.G., Semeniak, D., Pinnecker, J., Schmithausen, P., Meyer, I., Friedrich, M., Dütting, S., et al. (2017). Thrombopoiesis is spatially regulated by the bone marrow vasculature. *Nat. Commun.* 8, 127.
- Sudo, K., Ema, H., Morita, Y., and Nakauchi, H. (2000). Age-associated characteristics of murine hematopoietic stem cells. *J. Exp. Med.* 192, 1273–1280.
- Susulic, V.S., Frederick, R.C., Lawitts, J., Tozzo, E., Kahn, B.B., Harper, M.E., Himms-Hagen, J., Flier, J.S., and Lowell, B.B. (1995). Targeted disruption of the beta 3-adrenergic receptor gene. *J. Biol. Chem.* 270, 29483–29492.
- Takeda, S., Eleftheriou, F., Levasseur, R., Liu, X., Zhao, L., Parker, K.L., Armstrong, D., Ducey, P., and Karsenty, G. (2002). Leptin regulates bone formation via the sympathetic nervous system. *Cell* 111, 305–317.
- Veith, R.C., Featherstone, J.A., Linares, O.A., and Halter, J.B. (1986). Age differences in plasma norepinephrine kinetics in humans. *J. Gerontol.* 41, 319–324.
- Villa-Bellosta, R., Rivera-Torres, J., Osorio, F.G., Acín-Pérez, R., Enriquez, J.A., López-Otín, C., and Andrés, V. (2013). Defective extracellular pyrophosphate metabolism promotes vascular calcification in a mouse model of Hutchinson-Gilford progeria syndrome that is ameliorated on pyrophosphate treatment. *Circulation* 127, 2442–2451.
- Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K.L., Ema, H., and Nakauchi, H. (2013). Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell* 154, 1112–1126.
- Yirmiya, R., Goshen, I., Bajayo, A., Kreisel, T., Feldman, S., Tam, J., Trembovler, V., Csernus, V., Shohami, E., and Bab, I. (2006). Depression induces bone loss through stimulation of the sympathetic nervous system. *Proc. Natl. Acad. Sci. USA* 103, 16876–16881.
- Zhao, M., Perry, J.M., Marshall, H., Venkatraman, A., Qian, P., He, X.C., Ahamed, J., and Li, L. (2014). Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. *Nat. Med.* 20, 1321–1326.
- Ziegler, M.G., Lake, C.R., and Kopin, I.J. (1976). Plasma noradrenaline increases with age. *Nature* 261, 333–335.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD45.1 (A20)	BD Biosciences	Cat#560578; RRID:AB_1727488
CD45.2 (104)	TONBO Biosciences	Cat#20-0454-U100; RRID:AB_2621576
B220 (RA3-6B2)	BD Biosciences	Cat#553088; RRID:AB_394618
CD11b (M1/70)	BioLegend	Cat#101208; RRID:AB_312791
CD3 ϵ (145-2C11)	TONBO Biosciences	Cat#65-0031-U100; RRID:AB_2621872
Ly-6G (RB6-(C5)	BioLegend	Cat#127623; RRID:AB_10645331
CD90 (53-2.1)	BD Biosciences	Cat#553003; RRID:AB_394542
sca-1 (E13-161.7)	BioLegend	Cat#108128; RRID:AB_2563064
CD42d (1C2)	Fisher Scientific Ltd	Cat#17-0421-80; RRID:AB_1724073
biotinylated lineage antibodies (CD11b, Gr-1, Ter119, B220, CD3 ϵ)	BD Biosciences	Cat#559971; RRID:AB_10053179
c-kit (2B8)	Thermo Fisher Scientific	Cat#11-1171-81; RRID:AB_465185
CD150 (TC15-12F12.2)	BioLegend	Cat#115927; RRID:AB_11204248
CD34 (RAM34)	BD Biosciences	Cat#560238; RRID:AB_1645242
CD41 (MWRReg30)	BioLegend	Cat#133905; RRID:AB_2265179
Brilliant Violet 510 Streptavidin	BioLegend	Cat#405233
anti-CD45-biotinylated antibody	BD Biosciences	Cat#553078; RRID:AB_394608
anti-Ter119 –biotinylated antibody	BD Biosciences	Cat#553672; RRID:AB_394985
anti-CD31-APC antibody	BD Biosciences	Cat#551262; RRID:AB_398497
Streptavidin-APCCy7	BD Biosciences	Cat#554063; RRID:AB_10054651
anti-ckit-FITC antibody	BD PharMingen	Cat#553354; RRID:AB_394805
anti-CD41-PE antibody	BD PharMingen	Cat#558040; RRID:AB_397004
Human/Mouse CD117/c-kit Antibody (Polyclonal Goat IgG)	R&D	Cat#AF1356; RRID:AB_354750
rat anti-CD150 antibody	Biolegend	Cat#115902; RRID:AB_313681
CD34 Microbead kit	Miltenyi Biotec	Cat#130-046-702
rabbit anti-TH antibody	Millipore	Cat#AB152; RRID:AB_390204
Alexa Fluor 647 goat anti-rat IgG	Life Technologies	Cat#A21247; RRID:AB_141778
anti-rabbit biotinylated antibody	Jackson ImmunoResearch Labs	Cat#111-066-003; RRID:AB_2337966
Cy3-Tyramide secondary antibody	Perkin Elmer	Cat#SAT704A001EA
rat anti-EMCN antibody	Santa Cruz Biotechnology	Cat#sc-65495; RRID:AB_2100037
goat anti-CD31 antibody	R&D	Cat#AF3628; RRID:AB_2161028
Alexa Fluor 546 Donkey anti-rabbit IgG	Thermo Fisher Scientific	Cat#A10040; RRID:AB_2534016
Dylight650 donkey anti-rat IgG	Thermo Fisher Scientific	Cat#SA5-10029; RRID:AB_2556609
Cy3-donkey anti-goat IgG	Jackson ImmunoResearch Labs	Cat#705-165-147; RRID:AB_2307351
Alexa Fluor 647 donkey-anti-goat IgG	Life Technologies	Cat#A21447; RRID:AB_141844
Alexa Fluor 647 Goat-anti-armenian hamster IgG	Abcam	Cat#ab173004; RRID:AB_2732023
mouse-anti-human CD61 primary antibody	Serotec	Cat#MCA728; RRID:AB_321515
Alexa Fluor 546 donkey-anti-mouse IgG	Thermo Fisher Scientific	Cat#A10036; RRID:AB_2534012
Alexa Fluor 488 Streptavidin-conjugated antibody	Thermo Fisher Scientific	Cat#S32354; RRID:AB_2315383
Alexa Fluor 555 goat-anti-rat IgG	Thermo Fisher Scientific	Cat#A21434; RRID:AB_2535855

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Trizol Reagent	Sigma-Aldrich	Cat#T9424
α -MEM medium	StemCell Technologies	Cat#36450
MyeloCult M5300 medium	StemCell Technologies	Cat#05350
StemSpan H3000 medium	StemCell Technologies	Cat#09850
IMDM medium	ThermoFisher	Cat#12440053
Ammonium chloride	Sigma-Aldrich	Cat#254134
collagenase type I	Stem Cell Technologies	Cat#07902
DAPI	Sigma Aldrich	Cat#D9542
mSCF	PeProTech	Cat#250-03-100
TPO	PeProTech	Cat#AF-300-18
EPO	R&D	Cat#287-TC-500
IL-1 β	Cellgenix	Cat#1411-050
BRL37344	Sigma-Aldrich	Cat#B169
H-89	Sigma-Aldrich	Cat#B1427
Clenbuterol hydrochloride	Sigma-Aldrich	Cat#C5423
hydrocortisone	StemCell Technologies	Cat#07904
Avidin/biotin blocking kit	Vector Laboratories	Cat#SP-2001
Triton X-100	Sigma-Aldrich	Cat#T8787
TNB (0.1 M Tris-HCl, pH7.5, 0.15 M NaCl, 0.5% blocking reagent)	Perkin Elmer	Cat#FP1020
DPX Mountant for histology	Sigma-Aldrich	Cat#44581
donkey serum	Sigma-Aldrich	Cat#D9663
rat serum	Sigma-Aldrich	Cat#R9759
Dako Fluorescence Mounting Medium	Agilent	Cat#S3023
DMSO	Sigma-Aldrich	Cat#D5879
Critical Commercial Assays		
Lympholyte®-M Cell Separation Media	Cedarlane	Cat#CL5031
ABC amplification kit	Vector Labs	Cat#AK-5000
MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel	Merck Millipore	Cat#MCYTOMAG-70K
High-Capacity cDNA Reverse Transcription kit	Applied Biosystems	Cat#4368814
PowerUp SYBR Green Master Mix	Applied Biosystems	Cat#A25742
Experimental Models: Organisms/Strains		
<i>Nes-gfp</i>	Prof. Grigori N. Eikolopov, Stony Brook, USA	Mignone et al., 2004
<i>Adrb2^{-/-}</i>	Prof. Gerard Karsenty, Columbia University, New York, USA	Chruscinski et al., 1999
<i>FVB/N-Adrb3tm1Lowl/J</i>	The Jackson Laboratory	Stock#6402, backcrossed to C57BL/6J for 10 generations
<i>Lmnatm1.1Otin</i>	Prof. Carlos López-Otín, Oviedo University, Spain	Osorio et al., 2011
<i>Vwf-eGFP</i>	Prof. Claus Nerlov, University of Oxford, UK	Sanjuan-Pla et al., 2013
<i>Nos1tm1Plh/J</i>	The Jackson Laboratory	Stock#2986
<i>Il6tm1Kopf/J</i>	The Jackson Laboratory	Stock#2650
congenic CD45.1 C57BL/6	Charles River Laboratories	N/A
congenic CD45.2 C57BL/6	Charles River Laboratories	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Cell lines		
MS-5	DSMZ	ACC 441
HPC-7	Prof. Leif Carlsson, Umeå University, Sweden	N/A
Oligonucleotides		
NOS1-Fw: ACTGACACCCTGCACCT GAAGA	Sigma-Aldrich	N/A
NOS1-Rv: GTGCGGACATCTTCTGA CTTCC	Sigma-Aldrich	N/A
NOS2-Fw: CAGCTGGGCTGTACAAACCTT	Sigma-Aldrich	N/A
NOS2-Rv: CATTGGAAGTGAAGCGGTTCCG	Sigma-Aldrich	N/A
NOS3-Fw: CCTCGAGTAAAGAACTGG GAAGTG	Sigma-Aldrich	N/A
NOS3-Rv: AACCTCCTTGGAACAC CAGGG	Sigma-Aldrich	N/A
Gapdh-Fw: GCATGGCCTTCCGTGTTTC	Sigma-Aldrich	N/A
Gapdh-Rv: CTGCTTACCACCTTCTTGAT	Sigma-Aldrich	N/A

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be addressed to the Lead Contact, Dr. Simon Mendez-Ferrer, at sm2116@medschl.cam.ac.uk.

METHODS DETAILS**Mouse strains**

Young mice were analyzed between 8–30 weeks of age, and old mice were 66–120 weeks old. Mice were housed in specific pathogen free facilities. All experiments using mice followed protocols approved by the Animal Welfare Ethical Committees, according to EU and United Kingdom Home Office regulations (PPL 70/8406). *Nes-gfp* (Mignone et al., 2004), *Adrb2*^{−/−} (Chruscinski et al., 1999), *FVB/N-Adrb3tm1Lowl/J* (Susulic et al., 1995), *Lmna*^{tm1.1Otin} (Osorio et al., 2011), *Vwf-egfp* (Sanjuan-Pla et al., 2013), *B6.129S4-Nos1tm1Plh/J* (stock#2986) (Huang et al., 1993), *B6.129S2-Il6tm1Kopf/J* (stock#2650) (Kopf et al., 1994) (Jackson Laboratories) and congenic CD45.1 and CD45.2 C57BL/6 mice (Charles River Laboratories) were used in this study.

Mouse bone marrow transplantation and in vivo treatments

In *Lmna*^{G609G/G609G} mouse model, HSC activity was assessed by long-term competitive repopulation assay using the congenic CD45.1/CD45.2 isotypes. Recipient female CD45.1 C57BL/6 mice (8 week-old) were subjected to lethal irradiation (12 Gy whole body irradiation, split dose 6.0 + 6.0 Gy, 3 h apart). 10⁶ BM nucleated cells from (CD45.2) WT mice or *Lmna*^{G619G/G609G} mice were mixed with 10⁶ BM nucleated cells from CD45.1 C57BL/6 mice and i.v. transplanted in 200 μ L sterile PBS into lethally irradiated CD45.1 C57BL/6 mice. At various time points after transplantation (4, 8, 12 and 16 weeks), peripheral blood nucleated cells were collected from recipients. Cells were stained with fluorescent-conjugated antibodies against B220, Mac1 and CD90 antigens, and analyzed by fluorescence-activated cell sorting. The selective β 3-AR agonist BRL37344 (Sigma, St. Louis, MO) was administered at 2mg/kg through intraperitoneal (i.p.) injection once per day. Vehicle (saline solution) daily injections were performed in the same way. Treatment was initiated when *Lmna*^{G609G/G609G} mice were 7 weeks old and lasted for 8 weeks.

To generate chimeric mice carrying *Adrb2*^{−/−} or *Adrb3*^{−/−} BM cells, two-month-old C57BL/6 mice were lethally irradiated (12 Gy, two split doses) and i.v. transplanted with 2 X 10⁶ nucleated BM cells from two-month-old WT, *Adrb2*^{−/−} or *Adrb3*^{−/−} mice. To test microenvironment-dependent effects of β -adrenergic signaling, WT, *Adrb2*^{−/−} or *Adrb3*^{−/−} (CD45.2) mice were lethally irradiated (12 Gy, two split doses) and i.v. transplanted with 2 X 10⁶ nucleated BM cells from CD45.1 C57BL/6 mice. Hematopoietic cells in the BM were analyzed 4 months after transplantation in both settings.

BM cell extraction, flow cytometry and fluorescence-activated cell sorting

For BM hematopoietic cell isolation, bones were crushed in a mortar, filtered through a 40- μ m strainer to obtain single cell suspensions, and depleted of red blood cells by lysis in 0.15 M NH₄Cl for 10 min at 4°C. Blood samples were directly lysed. Cells were incubated with the appropriate dilution (2–5 μ g/ml) of fluorescent antibody conjugates and 4',6-diamidino-2-phenylindole (DAPI) for dead cell exclusion, and analyzed on LSRFortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ) equipped with FACSDiva Software

(BD Biosciences). The following antibodies were used: fluorescent CD45.1 (A20), CD45.2 (104), B220 (RA3-6B2), CD11b (M1/70), CD3 ϵ (145-2C11), Ly-6G (1A8), CD90 (53-2.1), sca-1 (E13-161.7), CD42d (1C2), biotinylated lineage antibodies (CD11b, Gr-1, Ter119, B220, CD3 ϵ) (BD Biosciences), c-kit (2B8) (eBioscience), CD150 (TC15-12F12.2), CD34 (RAM34) and CD41 (MWRReg30) (BioLegend). Biotinylated antibodies were detected with fluorochrome conjugated streptavidin (BD Biosciences).

To isolate endosteal and non-endosteal HSPCs, long bones were flushed gently to obtain hematopoietic cells less tightly associated with the bone and then flushed-bones were crushed in a mortar to obtain hematopoietic cells enriched in the endosteal compartment. Cells were stained with the above-mentioned antibodies and analyzed by flow cytometry or sorted (FACS Aria cell sorter, BD Bioscience). Long-term HSCs (LT-HSCs) were immunophenotypically defined as lin-sca-1+ckit+CD34-CD150+CD41- cells. Myeloid-biased HSCs were immunophenotypically defined as lin-sca-1+ckit+CD34-CD150+CD41+ cells. Lymphoid-biased HSCs were immunophenotypically defined as lin-sca-1+ckit+CD34-CD150-CD41- cells.

For analysis of Nes-GFP+ cell distribution in endosteal and non-endosteal BM fractions, the long bones were flushed with PBS and remaining endosteal part was crushed in a mortar. Both fractions were digested in 2ml of collagenase type I (Stem Cell Technologies, cat. No. 07902) for 30 min at 37°C with agitation. The enzyme was quenched by adding 18ml PBS/2%FCS. Cell suspensions were filtered, pelleted and red blood cell lysis was performed as stated above. Samples were stained with the following antibodies: 1:100 anti-CD45-biotinylated ab (BD Biosciences, cat. No. 553078), 1:100 anti-Ter119 –biotinylated ab (BD Biosciences, cat. No. 553672), 1:100 anti-CD31-APC ab (BD Biosciences, cat. No. 551262). Subsequently, cells were stained with Streptavidin-APCCy7 1:200 (BD Biosciences, cat. No. 554063). DAPI (Sigma Aldrich, cat.no. D9542) was added at 1:10 000 to discriminate dead cells. Samples were acquired on Gallios flow cytometer (Beckman Coulter) and a LSRFortessa cell analyzer (BD Biosciences) and were analyzed with Kaluza analysis software (Beckman Coulter).

Cell culture

HPC-7 cells were maintained in IMDM medium supplemented with 10% FCS and 100 ng/ml of mSCF (PeProTech 250-03).

MS-5 cells were maintained in α -MEM medium supplemented with 10% FCS. To induce megakaryocyte differentiation, HPC-7 cells were cultured with or without MS-5 stromal cells in StemSpan H3000 medium, supplemented with 50 ng/ml of TPO (PeProTech 300-18) and 2U/mL of EPO (R&D 287-TC-500) up to 4 days. 10 μ M of BRL37344 (β 3-AR agonist; Sigma B169), 10 μ M of clenbuterol (β 2-AR agonist; Sigma C5423), 5 μ M of H-89 (Protein kinase A inhibitor; Sigma B1427) and vehicle controls were added to the culture. To examine megakaryocyte differentiation, cells were incubated with anti-ckit-FITC ab (1:200, BD PharMingen 553354) and anti-CD41-PE ab (1:200, BD PharMingen 558040). DAPI was added at 1:10,000 to discriminate dead cells. Samples were analyzed by Gallios flow cytometer (Beckman Coulter).

Human umbilical cord blood CD34+ HSPCs were isolated using a CD34 Microbead kit (Miltenyi Biotec 130-046-702) following manufacturer's instructions, and were cultured with MS-5 stromal cells in Cellgro medium (CellGenix cat. no. 20802-0500), supplemented with 50 ng/ml of TPO (PeProTech 300-18) and 5 ng/ml of IL-1 β (Cellgenix 1411-050) for 7-10 days, during which vehicle, 10 μ M of BRL37344 (β 3-AR agonist; Sigma B169) or 10 μ M of clenbuterol (β 2-AR agonist; Sigma C5423) were added to the culture. Conditioned medium was refreshed at day3 and day7 of the coculture. To examine megakaryocyte differentiation, cells were fixed and stained with anti-human CD61 antibody (Serotec MCA 728).

For mouse bone marrow long-term culture, femurs and tibias were flushed gently to obtain bone marrow cells. Cells were seeded and cultured in MyeloCult M5300 medium (StemCell Technologies, cat. no. 05350) supplemented with 10⁻⁶ M hydrocortisone (Stem-Cell Technologies, cat. no. 07904) at 33°C for 14 days. Half of the medium was refreshed at day 7. At day 14 of culture, half of the medium was refreshed again and added with 50 ng/ml of TPO (PeProTech 300-18) for 4 days, during which vehicle, 10 μ M of BRL37344 (β 3-AR agonist; Sigma B169), 10 μ M of clenbuterol (β 2-AR agonist; Sigma C5423), or 100 μ M of L-VINO (Insight Biotechnology 728944-69-2) were added to the culture. At day 18 of culture, cells were collected and subjected to flow cytometry analysis.

Immunofluorescence staining

Immunofluorescence staining of cryostat sections was performed as previously described (Isern et al., 2014), with minor modifications. Briefly, tissues were permeabilized with 0.1% Triton X-100 (Sigma) for 10 min at RT and blocked with TNB buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent, Perkin Elmer) for 1 h at RT. Primary antibody incubations were conducted for 2 h at RT. Secondary antibody incubations were performed with PBS + 0.05% Triton X-100. Stained tissue sections were counterstained for 5 min with 5 μ M DAPI and rinsed with PBS. Slides were mounted in Vectashield Hardset mounting medium (Vector Labs) and sealed with nail polish. Skull bones were fixed with 2% PFA for 2h in 4°C, washed with PBS and cut through the sagittal suture. Each half was permeabilized with PBS-0.1% Triton with 20% goat serum (Thermo Scientific, cat. No. 9722) overnight at 4°C on the rocker. Endogenous biotin was blocked with the Avidin/biotin blocking kit (Vector Laboratories, cat. No. SP-2001) according to manufacturer's recommendation. Endogenous peroxidase was blocked with 0.4% peroxide (Sigma-Aldrich, cat. no. H1009) for 2h at RT. Subsequently, the skull bones were stained with rat anti-CD31 (BD Biosciences, cat. no. 551262) and rabbit anti-TH (Millipore, cat. no. AB152) in 0.1% Triton-20%goat serum-PBS (diluted 1:100 and 1:500 respectively) for 3 days, and washed over the next day with 0.05% Triton - PBS- at RT on a rocker. Samples were stained with secondary goat anti-rat-Alexa647 at 1:300 (Life Technologies, cat. no. A21247) and goat anti-rabbit biotinylated at 1:200 (Strattech Scientific, cat. no. 111-066-003) o/n at 4°C. Subsequently, the skull bones were treated with ABC amplification kit (Vector Labs) to detect Cy3-Tyrmide amplified signal from TH staining (Perkin Elmer, cat.no. SAT704A001EA). As a final step, the skull bones were stained with DAPI 1:1000 for 5 min. Images were acquired with Leica TCS SP5 confocal microscope with

10x objective. From each sample, 3 representative images were collected in different areas: frontal bone near bregma, central sinus of parietal bone and interparietal bone near lambda. The TH+ area was analyzed with ImageJ. For whole mount staining of long bones, tibia or femur BM thick sections were obtained with a cryostat and remaining OCT was removed by PBS washes. Samples were blocked and permeabilized in 0.1% Triton X-100 (Sigma) TNB (0.1 M Tris-HCl, pH7.5, 0.15 M NaCl, 0.5% blocking reagent, Perkin Elmer) overnight at 4°C. On the next day, samples were incubated with rabbit anti-TH (Merck AB152; 1:200), goat anti-CD31 (R&D AF3628 1:100), rat anti-EMCN (Insight Biotechnology sc-65495 1:100) or rat anti-CD31 (BD Biosciences Clone MEC13.3; 1:200) diluted in 0.1% Triton X-100 TNB overnight at 4°C. Samples were rinsed with 0.05% Triton X-100 PBS for 8 hours and incubated overnight with secondary antibodies including A546 Donkey anti-rabbit IgG (Invitrogen A10040; 1:200), Dylight650 donkey anti-rat IgG (Thermo Fisher SA5-10029; 1:300), Cy3-donkey anti-goat IgG (Jackson 705-165-147, 1:300) diluted in TNB. Samples were rinsed with 0.05% Triton X-100 PBS for 4 hours, washed with PBS and counterstained with DAPI (Sigma; 1:1000) to label cell nuclei. Images were acquired with Leica SP5 confocal microscope using 10x, 20x and 40x objectives and analyzed with ImageJ. At least 3 independent and randomly selected BM areas were imaged and analyzed per sample. To quantify neural fibers, total TH+ area was normalized per total BM area labeled with DAPI. For quantification of sinusoidal vessel area, EMCN+ area > 150 μ m distant to the bone surface was measured and normalized to BM area. Arteriolar (> 6 μ m diameter) and capillary (< 6 μ m diameter) CD31hiEMCN- vessel number was counted and normalized to BM area. To quantify transition zone vessels, CD31hiEMCNhi area < 150 μ m close to the bone surface was quantified and normalized to the bone surface length.

For staining of megakaryocyte and megakaryocyte progenitor (Figures 3C–3F, 4J–4L, 5D–5F, 7G–7I, and S2A–S2D), BM femur thin sections were blocked with TNB for 1h at RT. Samples were then incubated with primary antibodies for 2 h at RT or overnight at 4°C, followed by secondary antibodies incubation for 1h at RT. Finally, samples were counterstained with DAPI to label cell nuclei. Repetitive washes were performed with PBS. At least 3 independent and randomly selected BM areas were imaged and analyzed by ImageJ. The following primary antibodies were used: CD41 (1:200, PE conjugated rat monoclonal antibody, BD PharMingen 558040), CD42d (1:100, APC conjugated armenian hamster monoclonal antibody, eBioscience 17-0421-80) and ckit (1:100, goat polyclonal antibody, R&D AF1356). The following secondary antibodies were used: Alexa Fluor 647 Goat-anti-armenian hamster IgG (1:200, Abcam ab173004) and Alexa Fluor 647 donkey-anti-goat IgG (1:200, Life Technologies A21447).

For staining of megakaryocyte localization relative to sinusoids (Figures 3I–3L and 5H–5J), BM femur thin sections were blocked with 0.1% Triton X-100 TNB for 1h at RT. Samples were then incubated with rat anti-EMCN (Insight Biotechnology sc-65495 1:100) antibody diluted in 0.1% Triton X-100 TNB overnight at 4°C. On the next day, samples were incubated with Alexa Fluor 647 goat-anti-rat IgG (1:200, Life Technologies, cat. no. A21247) secondary antibody for 1h at RT, followed by rat serum blocking (1:10 rat serum in 0.05% Triton X-100 TNB) for 10 min. Samples were incubated with third antibody, CD41 (1:200, PE conjugated rat monoclonal antibody, BD PharMingen 558040) for 2h at RT. Finally, samples were counterstained with DAPI to label cell nuclei. Repetitive washes were performed with PBS or 0.05% Triton X-100 PBS. At least 3 independent and randomly selected BM areas were imaged and analyzed by ImageJ.

For HSCs and megakaryocytes staining (Figures 7, S5, and S6), BM femur thin sections were blocked with TNB for 1h at RT and followed by Avidin/biotin blocking. Samples were then incubated with rat anti-CD150 (1:50, Biolegend Cat. No. 115902) and armenian hamster anti-CD42d (1:100, eBioscience 17-0421-80) primary antibodies overnight at 4°C. On the next day, samples were incubated with Alexa Fluor 555 goat-anti-rat IgG (1:200, Life Technologies A21434) and Alexa Fluor 647 Goat-anti-armenian hamster IgG (1:200, Abcam ab173004) secondary antibodies for 1h at RT, followed by rat serum blocking (1:10 rat serum in TNB) for 10 min. Samples were re-stained with biotinylated lineage antibodies (1:100, BD Biosciences) for 2h at RT. Finally, samples were incubated with Alexa Fluor 488 Streptavidin-conjugated antibody (1:200, Invitrogen S32354) for 1h at RT and counterstained with DAPI to label cell nuclei. At least 3 independent and randomly selected BM areas were imaged and analyzed by ImageJ.

For staining of human cord blood CD34+ HSPC co-culture (Figures 4D and 4E), cells were fixed with 1% PFA for 20 mins at RT. After PBS washes, cells were blocked with 10% donkey serum (Sigma D9663) diluted in PBS for 1h at RT, followed by 2h incubation of mouse-anti-human CD61 primary antibody (1:500, Serotec MCA 728). After PBS washes, cells were incubated with Alexa Fluor 546 donkey-anti-mouse IgG (1:200, Life Technologies, cat. no. A10036) secondary antibody for 1h at RT. Finally, samples were counterstained with DAPI to label cell nuclei. 10 independent and randomly selected fields in culture wells were imaged and analyzed by ImageJ.

RNA isolation and qPCR

RNA isolation was performed using Trizol Reagent (Sigma T9424). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems 4368814), following the manufacturer's recommendations. qPCR was performed using the PowerUp SYBR Green Master Mix (Applied Biosystems A25742) and ABI PRISM® 7900HT Sequence Detection System. The expression level of each gene was determined by using the absolute quantification standard curve method. All values were normalized with *Gapdh* as endogenous housekeeping gene.

The following primers (mouse) were used:

NOS1-Fw: ACTGACACCCTGCACCTGAAGA
 NOS1-Rv: GTGCGGACATCTTCTGACTTCC
 NOS2-Fw: CAGCTGGGCTGTACAAACCTT
 NOS2-Rv: CATTGGAAGTGAAGCGGTTCG
 NOS3-Fw: CCTCGAGTAAAGAACTGGGAAGTG

NOS3-Rv: AACTTCCTTGGAACACCAGGG
Gapdh-Fw: GCATGGCCTTCCGTGTTC
Gapdh-Rv: CTGCTTCACCACCTTCTTGAT

ELISA

MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (MCYTOMAG-70K, Merck Millipore) was performed following the manufacture's protocol. Extracellular fluids of mouse long bones were collected from the supernatants following BM extraction and subjected to the mouse cytokine panel.

Measurement of nitrate concentration

NO(X) content was measured in freshly-thawed samples that had been kept at -80°C for less than 2 months using a nitric oxide analyzer (NOA) 280i (Siever, GE Healthcare) according to the manufacturer's instructions. Data were collected, processed and analyzed by using liquid software (Siever, GE Healthcare).

Statistical analyses

Statistical analyses and graphics were carried out with GraphPad Prism 5 software and Microsoft Excel.

DATA AND CODE AVAILABILITY

The published article includes all relevant datasets generated or analyzed during this study.